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Preface

The third international conference on bio-antioxidants was held in Nessebar, Bulgaria from 17 to 21 September, 2019 and chaired by Prof. Dr. Vessela D. Kancheva from the Institute of Organic Chemistry with Centre of Phytochemistry, Bulgarian Academy of Sciences. The total number of participants was 112, including 42 young scientists. The conference, once again gave the participants the opportunity to meet leading scientists from different countries, to discuss the latest trends in the development of new effective bio-antioxidants and their practical application. The strategic topics of BIO-ANTIOXIDANTS 2019 were:

A. Oxidative Stress and Human Health
B. Natural Bio-antioxidants
C. Synthetic Analogues of Natural Bio-antioxidants
D. Homogeneous and Heterogeneous Lipid Oxidation
E. Food Analysis, Food Additives and Food Supplements
F. Advanced Methods for Analysis of Bio-antioxidants

Within the frames of the conference, the 3rd Young Scientists School on Bio-antioxidants (YSSBA) was held. YSSBA gave the opportunity to young scientists (students, doctoral students, post-doctoral and students) to present their own results to the international audience, to gain new knowledge and exchange experience, ideas and contacts.

After a peer review process, 41 reports from the conference were chosen to be published in Bulgarian Chemical Communications, Special Issue D, vol. 52, 2020, guest edited by prof. Petko Denev from Bulgarian Academy of Sciences and prof. Ryzsard Amarowicz from the Polish Academy of Sciences.

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A. Oxidative Stress and Human Health

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Investigation of the plasmatic nitric oxide levels in women with preterm birth and women with symptoms of threatened preterm labor

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Prevention of preterm birth (PTB, birth before 37 gestational weeks) and threatened preterm labor (TPL) is a major undertaking in pregnant women health in prenatal care and identified as one of the main problems associated with redox imbalances in the reactive oxygen/nitrogen species (ROS/RNS) and increased nitrosative stress (NS) damages. The main goal of the herein reported study was to evaluate and compare plasmatic nitric oxide (•NO) radicals as a real time parameter of nitrosative stress in women with PTB and women with TPL symptoms, using spin-trapping EPR spectroscopy. Possible role of •NO radicals as a reliable marker for predicting PTB, TPL and for therapeutic purposes was also discussed. The •NO radical formations were measured in plasma specimens from 243 women divided into 3 groups: 1) n=73 pregnant women complicated by PTB; 2) n=30 pregnant women with symptoms of threatened preterm labor (TPL); and 3) n= 75, controls including singleton pregnant women in term. For the first time, •NO radical production during pregnancy complicated by PTB and TPL in Bulgarian women population was investigated in real time using the EPR spin-trapping method. It is important to emphasize that •NO radical production and oxidative/ nitrosative stress increases with advancing gestation during PTB and decrease in PTL groups. Based on previous studies and on our results, we argue that •NO radicals could be a reliable marker for predicting PTB, TPL and for therapeutic purposes.

Keywords: PTB, PTL, EPR, nitrosative stress

INTRODUCTION

Prevention of preterm birth (PTB) and threatened preterm labor (TPL) is a major undertaking in pregnant women health and in prenatal care. Preterm birth (birth before 37 gestational weeks) is identified as one of the main problems associated with advanced pregnancy and the cause of neonatal mortality. According to a WHO report from 2012, about 15 million premature babies were born each year in the world and in Europe, this number is around 500,000. The 1.1 million premature babies do not survive because of the complications related to premature birth. The premature born-children are with a high rate of mental, physical and neurological complications, [1, 2]. This leads to a number of socio-economic issues and a global problem for humanity. It was found that socio-economically poor families are at higher risk of premature birth [1, 2]. The preterm birth is observed in 12% of pregnancies and is associated with 50% long-term neurological consequences for the fetus [1, 2]. The etiological factors of preterm birth are: maternal stress, infection and inflammation, uterine distension, abnormal amounts of amniotic fluid, cervical insufficiency, and placental dysfunction. Early diagnosis of PTB symptoms is medically difficult. The PTB prediction is carried out considering obstetric history, but these methods are neither sensitive nor specific [3]. In early and middle normal pregnancy, the uterus is usually calm and inactive. As gestational age advances, maternal uterine activity and contractility increase and birth usually begins or is nearing. No precise mechanisms are known related to the initiation of normal, preterm (PTB), and threatened preterm labor (TPL), despite the studies described in the literature [4]. Several hypotheses identify redox imbalance in reactive oxygen/nitrogen (ROS/RNS) species and oxidative damages as a major cause of pathophysiological pregnancy, complicated by PTB and TPL. The increased oxidative stress and the destructive effects of free-radical formation are capable of leading to pathological processes during PTB and TPL. Different studies commented the antioxidant/ pro-oxidant imbalance in the intrauterine compartments and inflammation in the endothelial dysfunction [5, 6].

Nitric oxide is a highly active free radical (•NO) and a biological mediator, with short half-life time, that was synthesized by an enzyme group known as NO synthase (NOS). •NO is a vascular relaxation factor originating from the endothelium, with a potent inhibitory effect on smooth muscle contraction.

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Cells produce •NO radicals of three isoforms (endothelial NOS, inducible NOS and neural NOS) which have been identified and they catalyze the conversion of L-arginine to endothelial NO synthase and citrulline [7-9]. •NO metabolites, NO_3^-(nitrates) and NO_2^-(nitrites) are a factor in the feto-placentation process involved in the placental vascular reactivity regulation, bed resistance. Therefore, locally synthesized •NO can participate in physiological reproductive activities, including egg maturation, fertilization and embryonic progression, and can react with molecular oxygen and other ROS species. Numerous investigations reported results of •NO measured in maternal plasma, urine, and vaginal secretions [9, 10], and also of NOS activity in human pregnant myometrium, villous trophoblastic and fetal membranes [4, 11]. Studies on the pregnant women uterus and myometrium contractility demonstrated a declining level of NOS expression after birth, labor [4, 12, 13] and PTB complicated pregnancies.

However, there are conflicting data regarding the role of •NO radicals/ NOS system activity in control and prevention of the PTB and TPL during pregnancy. The clinical study of Rowlands *et al*., 1996 [14] demonstrated that •NO/ NO donors delay the preterm labor/ birth, which determines the •NO role in tocolysis and in PTB and TBP prevention [4]. Other clinical investigation describes that both endogenous and exogenous NO donors participate in uterine contractility to both ET-1 and AVP peptides, resulting in a local imbalance between narrowing and relaxing mediators [15]. Moreover, •NO radicals react with superoxide anion ($\bullet O_2$) radicals to formation of peroxynitrite (ONOO-) anions that suppress endothelial NOS activity [16, 17] and chronically elevated oxidative stress levels and immune disorders. Extreme ROS/ RNS generation leads to decreased antioxidant enzyme protection, impaired normal cellular responses and cellular growth in preterm injury [18]. Different experimental studies comment that RNS/•NO metabolites affect the normal function of the placenta [18] in PTB patients and at different preterm phases were able to be inhibited or increased [18-21]. Despite the serious number of experimental studies, the exact role of •NO radicals/ NOS system activity in control and prevention of the PTB and TPL during pregnancy has not yet been fully clarified.

The main goal of the herein reported study was to evaluate and compare plasmatic nitric oxide (•NO) radicals as a real time parameter of nitrosative stress (NS) in women with preterm birth and women with symptoms of threatening preterm labor, using spin-trapping EPR spectroscopy. Possible role of •NO radicals as a reliable marker for predicting PTB, TPL and for therapeutic purposes was also discussed.

EXPERIMENTAL

Ethics Statement

This work was conducted according to the Declaration of Helsinki, and approved by the Ethics Board, Clinic of "Obstetrics and Gynecology", UMHAT "Prof. St. Kirkovich" in Stara Zagora, Bulgaria. Written informed consent (*2017/2019 MF, TrU, Stara Zagora*) was obtained from the patients after hospitalization between June 2017 and September 2019.

Study design and subjects

The •NO radical formation was measured in plasma specimens from 243 women, 17-41 years old, including n=73 pregnant women complicated by PTB, n=30 pregnant women with symptoms of threatened preterm labor (TPL) and control group (CG; n= 75) including healthy singleton pregnant women in term (Table 1).

Gestational age was determined by an experienced sonographer, using transabdominal ultrasound (*Aloka, Prosound alpha 6*) when the patient's bladder was empty and the date of the last menstrual period was determined. Pregnant women with PTB symptoms have been detected in late preterm birth 32.1-36.2 weeks (n=73). The pregnant women with threatened preterm labor have been detected in late preterm birth 32.3-36.1 weeks (n=30). In the PTB and TPL groups, participants had no history of type 1 or 2 diabetes, gestational diabetes, high blood pressure, incompetent cervix, uterus anomaly, hypertension (n=5), cardiovascular and infectious diseases (n=6), maternal complications, fetal anomaly or amniotic fluid, pre-eclampsia.

The venous blood samples of patients with PTB and TPL were taken before the start of tocolytic therapy and before the onset of corticosteroid prophylaxis of neonatal respiratory distress (RDS). PTB and TPL women - smokers (n=14); patients (n= 6) with acute or chronic infection, and women with fetal asphyxia, fetal growth restriction, and placental problems were excluded from the experiment. A singleton pregnant group including healthy (17- 38 years old, n= 75) women delivered at term (after 38.2 weeks' gestation), without history of other pregnancies or family diseases was used as (CG).

Table 1.

Characteristics	CG (n= 75)	PTB (n=73)	TPL (n=30)	*p	**p
Age, years	28.8 ± 3.4	33.8 ± 1.4	34.8 ± 3.5	0.039	0.041
Family history of diabetes	15(27.3)	None	None	≤0.002	≤0.003
Birth weight, g	3,216.93± 50.91	1,670.45± 46.14	1,245.9 ± 96.14	≤0.002	≤0.003
Body mass index, kg/m^2	38.9 ± 2.7	16.7 ± 0.94	17.6 ± 0.99	0.53	0.55
Gestational age, weeks/ range	39.2 ± 2.52	36.1 ± 1.19	30.3± 1.78	- -	
Systolic blood pressure (SBP, mmHg)	119.8 ± 10.4	136.2 ± 3.1	138.7 ± 2.41	≤0.002	≤0.001
Diastolic blood pressure (DBP, mmHg)	69.1± 6.0	80.1± 4.0	83.3± 4.0	≤0.003	≤0.002
Mean arterial pressure (MAP, mmHg)	93.51 ± 2.2	93.42 ± 1.9	95.66 ± 2.1	≤0.002	≤0.003
Eclampsia	None	None	None	-	
pre-eclampsia	None	None	None	-	
incompetent cervix,	None	None	None	-	
uterus anomaly,	None	None	None	-	
cardiovascular diseases	None	None	(n=7)	-	
infectious diseases	-	-	(n=7)	-	
maternal complications,	None	None	None	-	
fetal anomaly	None	None	None	-	
amniotic fluid	None	None	None	-	
urine protein	None	None	None	-	
pregnancy parity	NA			-	
Pulse pressure	62.2 ± 8	79.7 ± 9.12	80.9 ± 8.34		
Chronic hypertension				<0.002	≤0.003
				≤0.002[a]	≤0.003[b]
Data presented as mean ± SD	0.36 %	0.16%	0.14%		
				≤0.051[a]	≤0.053[b]
	NA- not applicable				
				p*- comparison between CG and PTB groups	
				p-** comparison beween CG and TPL groups	

[a] p < 0.002 CG *vs* PTB, computed by LSD post hoc test (pulse / chronic)
[b] p < 0.003 CG *vs* TPL, computed by LSD post hoc test (pulse / chronic)
NA PTB *vs* TPL, computed by LSD post hoc test (birth weight, g)

Body weight, blood pressure, and urine protein concentrations (>170 mg for the last 24 h) were evaluated in the groups. The diagnosis of PTB and TPL cases was made by strict clinical criteria [22]: 1) Risk factors presence of preterm birth; 2) Cervical status determined by vaginal smear and trans-vaginal echography; 3) painful uterian contractions, documented for 1-1.5 h and regular contractions resulting in cervical changes in dilatation and effacement; 4) Uterine activity monitoring - anamnestic according to the data of the pregnant women and by cardio-tocography; 5) Traceability for genital bleeding - anamnestic and vaginal obstruction.

Blood collection and PTB and PTL registration

Venous peripheral blood (5 ml) was collected directly by venous puncture from the ante-cubital region, in the participants, when they were in the active phase of PTB and TPL at mean gestational ages of 36.1 and 30.3 weeks, respectively. The blood collected from the CG1 patients was at a mean gestational age of 39.2 weeks, at the day of birth. The blood samples from the four tested groups, containing EDTA anticoagulant, was collected into plastic tubes, and centrifuged at 4000 rpm for 10 min at 4°C. 1.3 ml of plasma samples

was separated and stored at –20°C until further assay was done.

The PTB and TPL were registered before the end of 37 gestational weeks. In the PTB and TPL patients was observed the appearance of regular contractions in every 5 min, each contraction lasting for about 42 s, and vaginal examination of the cervix was revealing that the cervix was centered, anterior, thin, short (1 cm long, and the cervical os dilated to 12 cm), and soft. In the absence of maternal contraindications, an attempt was made to stop the uterine contractions through tocolytic therapy in order to protect the fetus or at least to "take the time" to perform corticosteroid RDS prophylaxis - before the end of 34 gestational weeks. The births between 34 and 37 gestational weeks are associated with fewer complications for the newborn. In this regard, the preterm births are more problematic at <34 week gestation. In general, immaturity of the lungs, liver, digestive system, and immunity of the fetus lead to increased morbidity and mortality among premature infants [23]. The prematurity is the basis of 75% of perinatal infant mortality and of later psychiatric disorders (*27% of newborns weighing less than 1500 g die, while 50% of children die when weighing less than 1000 g).* A possible intrauterine retardation of the fetus further complicates the prognosis. Every earned day that prolongs pregnancy, brings 2% percent higher chance of fetus survival.

In vivo EPR study on the plasmatic •NO radical metabolism

We measured •NO radical metabolism in all tested groups by reduction and spin-adduct formation between Carboxy-Ptio.K and generated radical using the Yoshioka *et al.* [24] and Yokoyama *et al.* [25] methods. The EPR analysis was adapted for EMX^{micro}, X-band spectrometer/ standard resonator (Bruker, Germany; at 3505 G centerfield, 6.42 mW microwave power, 5 G modulation amplitude, 75 G sweep width, 2.5×10^2 gain, 40.96 ms time constant, 60.42 s sweep time, 1 scan per sample; 20-25 ºC) and results were calculated by double integration of the corresponding EPR spectra (arbitrary units).

Statistical analysis

Data analyses were performed using the Statistica 8.0, Stasoft, Inc., one-way ANOVA, to determine significant difference between data groups. To define witch groups are different from each other we have used LSD post hoc test. The results were expressed as means ± standard error (SE). A $p< 0.05$ value was considered statistically significant. The EPR spectral processing was performed using Bruker WIN-EPR and SimFonia software.

RESULTS AND DISCUSSION

Preterm birth and threatened preterm labor (PTB/ TPL) is an important perinatological problem that leads to various neonatal disorders, physical ailments and even to infant mortality [26]. Numerous studies related to increased risk of PTB/ TPL focused on the expression of fetal fibronectin in the cervicovaginal fluid, short cervix, infection in vaginal microflora and in gestational tissue [27]. On the other hand, other experiments were focused on the molecular mechanisms [27] and transvaginal sonography screening as predictive tests for spontaneous preterm birth (PTB) in symptomatic singleton pregnancy with threatened preterm labor (PTL) [28].

A lot of studies have also indicated that neuronal NOS, endothelial NOS, inducible NOS metabolites and indirect •NO radical production are informative PTB and TPL markers or predictors [4, 29, 30].

In our study, we emphasize on the importance of recorded •NO radicals real time formation in the maternal body, and thus emphasize on the potential functional and clinical significance of presented data for the prevention of preterm birth and threatened preterm labor.

Clinical characteristics

Clinical characteristics of 243 women included in the study are summarized in Table 1. The mean gestational age for PTB (33.2 ± 1.19 weeks, p=0.004, *t*-test) patients, and for TPL (32.8 ± 1.34 weeks p=0.004, t-test) patients, was significantly lower than the mean gestational age for the CG (38.1 ± 2.52 weeks) group of women in term, respectively (p<0.003).

Statistically significant differences were observed between CG and both PTB and TPL groups: for systolic blood pressure (p<0.002, *t*-test; p<0.001, *t*-test); for diastolic blood pressure (p<0.003, *t*-test; p<0.002, *t*-test); for maternal pulse pressure (p<0.002, *t*-test; p<0.003, *t*-test), and pregnancy parity (p<0.003, *t*-test; p<0.003, *t*-test). The statistically significant differences in age (p=0.039, *t*-test; p=0.041, *t*-test), body mass index (p<0.002, *t*-test; p<0.001, *t*-test) and registered secondary diseases between CG and both PTB and TPL groups were not recorded.

We did not observe statistically significant differences in systolic/ diastolic blood pressure, heart rate, and pregnancy parity between the PTB and TPL groups (p=0.003, *t*-test).

However, both PTB and TPB groups demonstrate elevated chronic hypertension values (p<0.0051, *t*-test; p<0.003, *t*-test) and pulse pressure (p<0.0034, *t*-test; p<0.002, *t*-test), statistically significant compared to CG groups. No statistically significant differences between PTB, TPL groups and controls were registered. There was a statistically significant difference between CG and PTB groups, and CG and TPL groups to the two measured factors, respectively (p<0.002, LSD; p<0.003, LSD). The birth weight of the babies in the TPL group (n=7; 1,245.93 ± 963.14 g; p<0.002, *t*-test) and in the PTB group (1,670.45 ± 467.14 g; p<0.002, *t*-test) was statistically significantly lower than those of the CG group (3,216.93 ± 500.91 g), respectively. No statistically significant differences between PTB and TPL groups in this measured factor were registered.

In vivo EPR analysis of the plasmatic •NO radicals

The EPR spectroscopy [31] is characterized by high sensitivity. It is easily scavenged and measures the levels of extremely unstable ROS and RNS free radicals, both in *in vitro* and *in vivo* systems - human blood and tissue. In the present study, the spin-trapping EPR method was used for investigation of the changes in the •NO radical levels during preterm birth and threatened preterm labor. The increase in the level of •NO radicals, measured in blood was characterized as a nitrosative stress (NS) factor. Typical •NO radical spectrum obtained in plasma gives rise to a characteristic 5-line with 1:2:3:2:1 intensity pattern.

The plasma •NO radical levels are illustrated in Figure 1.

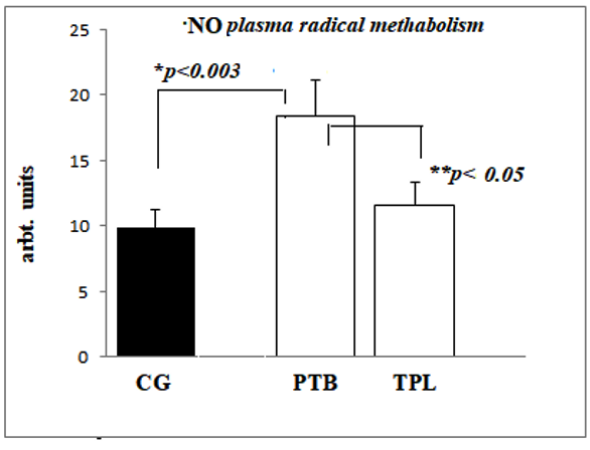

Figure 1. Plasma NO radical metabolism of pregnancy in CG group (n=75, birth in term), PTB group (n=73), (t-test) and TPL group (n=30), (t-test). *considered statistically significant PTB groups *vs* CG (p<0.003, t-test); **considered statistically significant PTB *vs* TPL groups (p<0.05).

The plasma •NO radical levels measured in the PTB group (18.34 ± 3.24 a.u., p<0.003, *t*-test) (Fig. 1) were significantly (two times) higher, compared to the pregnant in term (9.83 ± 2.11 a.u.). Our study was in support of others, showing that hypertensive disorders increased •NO radical levels, and NOx metabolites in patients with PTB increased and are associated with an increased risk of PTB or TPL-complicating pregnancy [18, 32, 33]. Increased endogenous metabolism of •NO radicals during preterm birth, a period of intense activity in pregnancy, could be associated with increased oxygen (O_2) intake, superoxide ($•O_2^-$) radicals synthesis, oxidative stress and increased free radicals generation [34, 35]. Nitric oxide radicals are powerful uterine smooth muscle relaxants that oxidize to NO metabolites containing NO_3^- and NO_2^- [33]. The NO metabolites are capable of relaxing the myometrium during pregnancy [35]. In addition, myometrial contractility (*pregnant, preterm or laboring*) could be enhanced by competing inhibitors of NO synthesis, such as NG-nitro-L-arginine and n-nitro-L-arginine methyl esters [35, 36]. In other studies, significantly increased NO metabolites production in PTB women [20, 32, 37] has been reported in inflammatory processes because of increased macrophage activity, activation of proinflammatory cytokines [4,27] and acidity.

Our results for measurement of the plasmatic •NO radical levels in the spontaneous TPL group (11.58 ± 1.39 a.u., p<0.002, *t*-test) (Fig. 2) were statistically significantly lower compared to the PTB group and almost comparable to the controls at term (9.83 ± 2.11 a.u.; p<0.05, *t*-test). These findings of a decreased •NO radical production in threatened preterm labor patients were in accordance with previous observations of Diejomaoha *et al*., and Ledingham [4, 34]. We suppose that decreased •NO radical production during threatened preterm labor could be explained with the decline of the •NO metabolism, possibly due to excessive oxidative/nitrosative stress. Probably, statistically significantly •NO decrease in TPL group, is recorded in processes that endeavored to convert plasmatic NO_2 to NO_3, to regulate oxidative/ nitrosative stress levels and redox - an imbalance in maternal-fetal placental blood flow [38]. Presumably, significantly reduced •NO levels in the women with threatened preterm labor stimulates uterine contraction, preterm ripening of the cervix and this is associated with a local, not systemic NO pathway activation [39]. The study of the levels of •NO metabolites in women with preterm birth and women with

symptoms of threatening preterm labor is forthcoming.

CONCLUSION

For the first time, nitric oxide (•NO) radical production during pregnancy complicated by preterm birth (PTB) and threatened preterm labor in Bulgarian women population was investigated in real time using the EPR spin-trapping method. It is important to emphasize that •NO radical production and oxidative/ nitrosative stress increases with advancing gestation during PTB and decrease in PTL groups. Based on previous studies, and on our results, we argue that •NO radicals could be a reliable marker for predicting PTB, TPL and for therapeutic purposes.

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Effects of pioglitazone on the hippocampal oxidative status of rats with prenatal valproic acid-induced autistic-like symptoms

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Valproic acid (VPA) is known as a potent teratogen for offspring of pregnant human females and is commonly used in animal models to environmental triggering of various behaviors, biochemical profiles and neuroanatomical features similar to those of patients with autistic spectrum disorder (ASD). The underlying molecular mechanisms often involve neuroinflammation and enhanced levels of oxidative stress. The hippocampus plays a key role in cognition, mood regulation, learning and memory functions, and is particularly vulnerable to oxidative damage. The aim of the current study was to investigate the effect of pioglitazone on the hippocampal oxidative status of rats with prenatal VPA-induced autistic-like symptoms. The male offspring of pregnant Wistar rats, treated or not with valproic acid during the pregnancy period, were separated from their mothers on the 23rd postnatal day and were divided into four groups: 1. Control; 2. Control, treated with pioglitazone; 3. With experimental autism; 4. With experimental autism, treated with pioglitazone. Our results demonstrated that the prenatal application of VPA is associated with alteration of the hippocampal reducing ability in the experimental groups. Treatment with pioglitazone demonstrated modulating activity on the oxidative status and beneficial effect on the proinflammatory parameters in this model of VPA-induced autistic-like features and its role may be suggested in the complex therapy of ASD.

Keywords: antioxidant capacity; valproic acid; autistic-like features; pioglitazone

INTRODUCTION

Autism spectrum disorder (ASD) is a complex and pervasive neurodevelopmental disorder, diagnosed by the age of three, upon clinical presentation of impaired social-communication skills and stereotypical, repetitive behaviors. In addition, most patients with ASD have attention deficit and cognitive, and intellectual challenges. Although genetic, environmental, inflammatory and metabolic conditions appear to interact as risk factors, the exact etiology of ASD remains unknown [1, 2].

Valproic acid (VPA) is an anti-epileptic drug, known as a potent teratogen for offspring of pregnant human females and is commonly used in animal models for environmental triggering of various behaviors, biochemical profile and neuroanatomical features, similar to patients with ASD [3, 4]. The offspring of VPA-treated rats is known to display disruption in their brain neuroplasticity (loss of neuronal cells, neuronal disorganization and structural connectivity alteration) [5, 6], as well as signs of neuroinflammation like reactive gliosis, elevation of inflammatory cytokines and altered immune responses [7, 8]

Oxidative stress is considered to play an important role in the etiopathogenesis of ASD [9, 10]. Studies reported participation of both central and peripheral markers of oxidative status disruption in the underlying molecular mechanisms of neuropsychiatric disorders [11, 12]. Also, there have been shown abnormal levels of detoxifying agents and antioxidants in ASD [9]. Since oxidative stress has been previously demonstrated as crucial in the neurodegeneration during development, it has been suggested as a possible molecular pathway by which VPA exerts teratogenicity [13]. Recent study has linked prenatal VPA exposure to impairment of cerebellum functions and dysregulation of motor, cognitive and social behavior in rats [14]. In addition, the hippocampus plays a key role in cognition and mood regulation, and is particularly vulnerable to oxidative damage [15].

Pioglitazone is an agonist of the peroxisome proliferator activated receptor gamma (PPAR-γ) and was initially designed as an anti-diabetic drug due to its insulin-sensitizing effect through changing expression of genes that influence carbohydrate and lipid metabolism [16].

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Consequently, PPAR-γ were found to be widely distributed in the central nervous system - in neurons, microglia, astrocytes, adipocytes, macrophages, etc. [17]. Pioglitazone has also been proved to possess anti-inflammatory and anti-proliferative effects [16, 18]. Strong evidence was demonstrated of pioglitazone neuroprotective activity against inflammation and oxidative stress in CNS and neuropsychiatric disorders [19-21]. Clinical studies have suggested the therapeutic potential of PPAR-γ agonists in ASD [22].

The aim of this study was to investigate the effect of pioglitazone on the hippocampal oxidative status of rats with prenatal VPA-induced autistic-like symptoms.

MATERIALS AND METHODS

The experiments were carried out in accordance with the Bulgarian regulations on animal welfare, in conformance with the European Communities Council Directive of 24 November 1986 (86/609/EEC) and with the approval of Bulgarian Food Safety Agency (BFSA license №188/13.11.2017).

Animals and VPA model

Pregnant Wistar rats (220±30 g body weight) were maintained under standard laboratory conditions (12 h light-dark cycle, temperature 20±0,5°C, humidity 65±1%). On gestational day 12.5 the rats were intraperitoneally treated with either physiological solution - control conditions or valproic acid (VPA) (600 mg/kg) to develop the experimental model of autism. The male offspring rats were separated from their mothers on the 23rd postnatal day and according to the treatment that followed they were divided into 4 groups (n=5): 1. Control (physiological solution, p.o.); 2. Pioglitazone "Pio" (pioglitazone 2 mg/kg, p.o.); 3. Experimental VPA model "VPA" (physiological solution, p.o.); 4. Experimental VPA model + pioglitazone "VPA+Pio" (pioglitazone 2 mg/kg, p.o.).

Substances and reagents

Sodium valproate, pioglitazone hydrochloride and rat IL-6 ELISA kit were of finest grade and were obtained from Sigma-Aldrich.

Pioglitazone was administered to the animals for 21 days (from 23rd to 43rd postnatal day). At the end of this period the animals were sacrificed and their blood and brains were collected. Blood sera were extracted and hippocampi were dissected for assessment of biochemical parameters.

Reagents for antioxidant capacity determination

Ferric chloride hexahydrate, 2,4,6-tri-(2-pyridil)-s-triazine (TPTZ), 2,2'-azinobis (3-ethylbenzotiazolin-6-sulfonic acid diammonium salt (ABTS) and potassium persulfate were obtained from Sigma-Aldrich. All other used chemicals were of reagent grade and were used without further purification.

Assessment of antioxidant capacity

For this part of the experimental work we used the ABTS test and the Ferric reducing ability assay. The former is based on the ability of the antioxidants in the studied samples to decrease the concentration of the preformed stable ABTS radical (estimating a decrease of the absorbance measured at 734 nm due to the capability of the sample antioxidant to eliminate the stable ABTS radical) and the other on the reduction of the ferric tripyridyltriazine (Fe^{3+}-TPTZ) complex to ferrous tripyridyltriazine (Fe^{2+}-TPTZ) complex, determined as increased absorbance at 593 nm. Both methods are characterized by operational simplicity and possibility to be automated.

ABTS assay

Experiments were done according to Re *et al.* [23], In order to obtain the stock ABTS radical solution 2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt and potassium persulfate were mixed in buffered distilled water to a concentration of 7mM and 2.45 mM, respectively. The obtained mixture was left overnight in the dark to form the stable ABTS cation radical (approximately 16 h before use). A working ABTS solution was prepared by diluting the stock until we obtained a solution with absorbance of 0.700 ± 0.005 at 734 nm. The ten times diluted brain homogenate supernatants were reacted with the ABTS radical working solution in a 96-well plate. Two groups of samples were prepared: controls where hippocampal homogenate was omitted and the samples contained the tested substance. The absorbance of the samples was measured using Epoch 2, BioTek reader at 734 nm after 60 min incubation in the dark. The radical scavenging activity of the tested samples was calculated on the base of the obtained results, using the following formula:

$$RSA\ \% = \left(\frac{A_{control} - A_{\ sample}}{A_{control}}\right).\ 100.$$

FRAP assay

The reducing ability of the biological samples was estimated by the Ferric reducing antioxidant power (FRAP) assay of Benzie and Strain [24]. The

FRAP reagent was prepared by mixing 25 ml of 300 mmol/L acetate buffer (pH 3,6), 2,5 ml of 10 mmol/L TPTZ (in 40 mM HCl) and 2,5 ml of 20 mmol/L $FeCl_3.6H_2O$ (proportion of 10:1:1) at 37°C. The homogenate supernatants were allowed to react with the FRAP solution in the dark for 30 min. The absorbance of the obtained blue colored product (ferrous tripyridyltriazine complex) was checked at 593 nm. Reagent blank reading was also taken at 593 nm.

Assessment of IL-6 levels

The estimation of serum interleukin-6 (IL-6) levels was performed by following the Sigma-Aldrich® Rat ELISA kit protocol: sandwich *in-vitro* enzyme-linked immunosorbent assay (ELISA) for the quantitative estimation of rat serum IL-6 on a 96-well plate reader at 450 nm. The values were expressed in pg/ml.

RESULTS

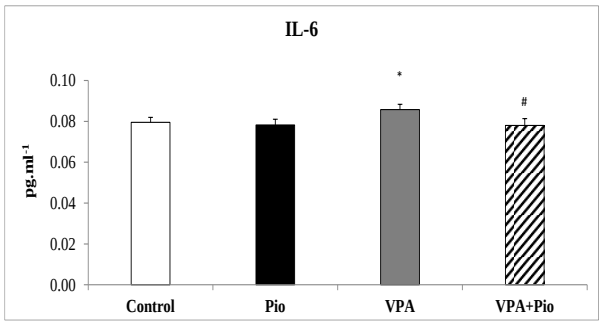

Figure 1. Effect of pioglitazone on serum IL-6 levels in experimental model of autistic like features. * $p<0.05$ *vs* control group; # $p<0.05$ *vs* VPA group.

Figure 1 demonstrates that the serum IL-6 levels in the "VPA" group are higher: 0.086±0.003 $pg.ml^{-1}$ in comparison to control group: 0.080±0.002 $pg.ml^{-1}$, $p<0.05$. This result shows that the VPA model induces elevation of pro-inflammatory cytokine and suggests peripheral inflammatory changes.

Administration of pioglitazone did not change serum level of IL-6 in the control condition but decreased it in the "VPA+Pio" group to a level comparable to the baseline: 0.078±0.003 $pg.ml^{-1}$, $p<0.05$, and indicated an anti-inflammatory effect of pioglitazone.

The results from the total antioxidant capacity (TAC) estimation assays are presented on Figure 2 for the FRAP assay and Figure 3 for the ABTS assay.

The FRAP assay established increased absorbance values from 0.424±0.023 to 0.502±0.032, $p<0,05$ in the samples containing hippocampal homogenate of the "VPA" group of animals in comparison to the control group. Application of pioglitazone attenuated the effect of VPA pretreatment on the absorbance values: 0.460±0.020.

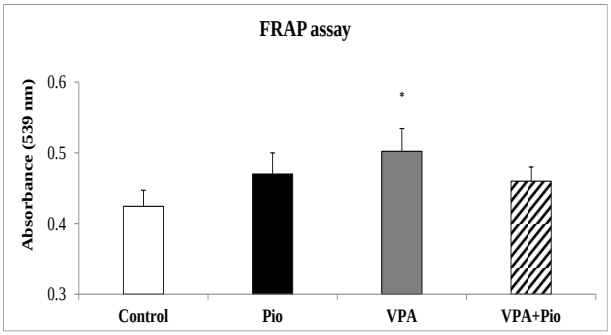

Figure 2. Absorbance values determined using FRAP assay in rat hippocampus homogenate. Each column represents the average ± SEM of five values corresponding to five animals, each value being the mean of triplicate assays. * $p<0.05$ *vs* control group.

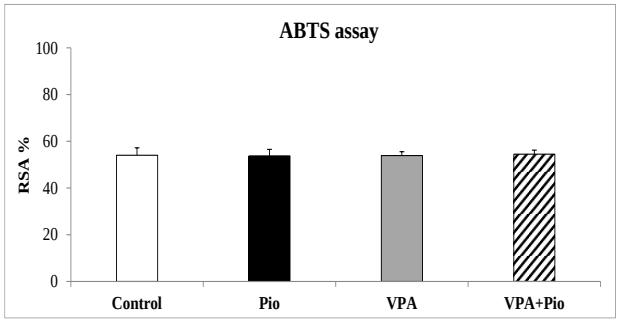

Figure 3. Radical scavenging activity (RSA) of hippocampal homogenates, estimated through the ABTS assay. Each column represents the average ± SEM of five values corresponding to five animals, each value being the mean of triplicate assays.

The ABTS assay did not demonstrate statistically significant changes in the absorbance values of the hippocampal supernatants of the studied groups. The calculated radical scavenging activities in the investigated groups were in the range from 53.73 to 54.50%.

DISCUSSION

Our present investigation demonstrates that prenatal VPA application induced increased serum IL-6 levels in the experimental animals, which suggests inflammatory changes. PPAR-γ agonist pioglitazone did not change the IL-6 levels in the animals that were not treated prenatally with VPA but decreased the IL-6 levels in the treated ones, thus proposing anti-inflammatory properties of pioglitazone in the model animals (Fig. 1).

This is in agreement with numerous studies for long-term neuroanatomical and biochemical abnormalities in the offspring after administration of VPA during pregnancy [25]. VPA exposure within the critical period for rodent brain organogenesis has

led to disrupted brain plasticity and neuroinflammatory changes, along with various behaviors, similar to autistic patients [26]. In addition, chronic neuroinflammation was shown to be present in ASD brains, including activation of microglia, increased levels of pro-inflammatory cytokines and other systemic inflammatory biomakers [27].

Recent clinical studies presented beneficial effects of pioglitazone on the stereotype behaviors and social interaction of autistic children [22, 28]. Furthermore, Kirsten *et al.* suggested that pioglitazone might eliminate autistic-like behavior through inhibition of IL-6 cascade in immune activation-based model of autism [21]. This finding was subsequently supported by experimental data for positive impact of pioglitazone on immune disruption in prenatal VPA-induced autistic features [26], and was confirmed by our present results.

Brain oxidative stress has been linked to autistic-like features in VPA-prenatal rodent models [29]. Oxidative status imbalance was associated with significantly elevated levels of oxidative stress markers of lipid peroxidation [30], as well as compromised antioxidant status - abnormal glutathione redox status and methionine cycle, and decreased activity of superoxide dismutase and catalase [31]. Oxidative stress due to excitotoxicity and deficiency of endogenic antioxidants was linked to abnormal brain development in cerebellum, frontal lobe, dorsal prefrontal cortex, orbitofrontal cortex, etc., that are key regions for social interaction, cognitive development, behavioral and communication functions, disrupted in ASD.

In consent with the abovementioned data, our results demonstrated imbalance in the hippocampal oxidative status, particularly the data from the FRAP assay concerning the rats prenatally treated with VPA. Various methods have been cited in the literature for estimation of TAC in biological samples and different approaches showed diverse values which many authors claimed to be because biological sample antioxidants comprised different reactivity against the indicators, used in the systems. The results from the present ABTS assay did not demonstrate statistically significant changes between the absorbance values of the hippocampal supernatants (Fig. 3). However, the FRAP assay established considerable differences between the hippocampal homogenates of the control and the "VPA" group of animals. Interestingly, increased ferric reducing activity in the supernatants of the VPA model rats was observed. Furthermore, application of pioglitazone diminished the effect of the VPA pretreatment on the redox imbalance in the hippocampal homogenates (Fig. 2). Several academic reports have also demonstrated contradictory results about the VPA impact on the oxidative status in animal models, as well as about antioxidant properties of some substances on the TAC of biological samples. There are no data for the hippocampal TAC, studied through a FRAP method in the present experimental model. On the other hand, there is inconclusive clinical and experimental evidence for the modulating activity of valproic acid on the endogenous antioxidant capacity [32].

One possible explanation of our data inconsistency is that the FRAP assay might be more sensitive at high altitude to the changes of the different types of antioxidants presented in the tested samples compared to the ABTS method. Previous investigation has established correlation between the results obtained in the ABTS system and the levels of endogenous glutathione whereas the measured activity in the FRAP assay was mainly associated with the levels of ascorbic acid, uric acid and α-tocoferol [33].

Moreover, the FRAP system is more biologically plausible. Iron is the most abundant metal in the body, and within the brain it shows an uneven distribution. Iron is essential for fundamental processes associated with the normal physiological brain functions like oxygen transportation, neurotransmitter synthesis and metabolism of myelin production. It is a well-known fact that brain tissue is highly vulnerable to oxidative stress. Reactive oxygen species (ROS) mediate iron toxicity *via* the iron-catalyzed Haber-Weiss reaction, and are known to interfere with cytochrome c- and caspase-3-dependent apoptotic pathways and subsequent neuronal damage.

Taken together, the literature data and our results suggest that treatment with pioglitazone of rats in an experimental model of autistic-like features reduced inflammatory changes *via* PPAR-gamma anti-inflammatory potential. Moreover, pioglitazone displayed modulating effects on the total antioxidant capacity and on the redox balance determined *via* the presented assays in this prenatal VPA-induced experimental model.

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Urinary total antioxidant capacity after unilateral nephrectomy in spontaneously hypertensive rats

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Hypertension is the most important risk factor contributing to cardiovascular disease while oxidative stress is one of the key players in its pathogenesis. The nephrectomy is a surgical manipulation in which one kidney or a part of it is removed. Total antioxidant capacity (TAC) of body fluids has been used for evaluation of disease progression, as well as for overall defense status against oxidative stress. The aim of the current study was to investigate the urinary TAC, determined by ABTS assay, after unilateral nephrectomy (UN) in the animal model of essential hypertension - spontaneously hypertensive rats (SHR). Experiments were performed on conscious, male, normotensive Wistar rats, (n=7) and SHR (n=7) before andfor after UN. The urine samples were collected in metabolic cages for 6 hours between 8.00 – 14.00 h AM. Urine flow rate was determined gravimetrically. Creatinine and urea concentrations were determined spectrophotometrically. Urinary TAC was determined by ABTS assay and expressed as μmol Trolox equivalent per μl. The excretion of creatinine and urea did not differ between Wistar and SHR and did not change during UN. We found that TAC in SHR was lower $p<0.01$ in comparison with normotensive rats and did not change in SHR after UN, unlike Wistar in which TAC was reduced in the first three days after the removal of the kidney. We suggest that the lack of response of urinary TAC to unilateral nephrectomy in SHR could be attributed to the reduced baseline total antioxidant activity, as well as to the altered antioxidant systems characteristics in hypertensive states.

Keywords: unilateral nephrectomy, antioxidant capacity, ABTS assay, SHR

INTRODUCTION

In the living organisms there is an established balance between the reactive oxygen/nitrogen species (ROS/RNS) generated due to exogenous or endogenous sources and the existing complex antioxidant system comprising enzymatic and non-enzymatic molecules. The antioxidant system possesses the capability to neutralize the generated ROS/RNS and to maintain their concentration in optimal ranges. Under certain conditions, when the antioxidant defense system is over-limited due to systemic exceed production of free radicals an abnormal condition of oxidative stress is established [1, 2]. The observed balance or imbalance between the generated ROS/RNS in the organism and its antioxidant defense system is defined as "antioxidant status" of the organism [3]. During the years many studies have been done in order to attempt to estimate the cumulative effect of all the antioxidants present in different types of biological samples. Several authors have used total antioxidant capacity (TAC) of body fluids and tissue homogenate as a parameter in their investigations in order to seek for correlation between patient state, disease progression, outcome and the overall defense status against oxidative stress [4].

Hypertension is a multifactorial disease in which different dynamic interactions between physiological, genetic and environmental factors are involved. The pathophysiological mechanism of hypertension includes activation of the sympathetic nervous system [5], abnormal G protein-coupled receptor signaling [6], immune factors [7] and upregulation of the renin-angiotensin-aldosterone system [8]. G protein-coupled receptors (GPCRs) represent the largest family of membrane receptors and are responsible for regulating a wide variety of physiological processes. G protein-coupled receptor kinases (GRKs), in concert with β-arrestins, classically desensitize receptor signal transduction, thus preventing hyperactivation of GPCR second-messenger cascades. Changes in GRK expression have featured prominently in many cardiovascular pathologies, including heart failure, myocardial infarction, hypertension, and cardiac hypertrophy. Available experimental data indicated that immune factors, altered mainly T-cells function, have a role for development of hypertension [7]. Oxidative stress is one of the important elements, common in all of these processes, which is a result from excess of ROS generation, decreased nitric oxide levels and reduced antioxidant capacity in the brain, vessels, heart and kidneys [9, 10].

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The increased production of ROS that have an important role in the homeostasis of the vascular wall, established in hypertension, is a result of action of different mechanisms: activation of enzymes, such as NADPH oxidase, xanthine oxidase, uncoupling eNOS, mitochondrial dysfunction as well as increased mechanical forces. Available experimental data indicated that activity of NADPH oxidase is under control of hormones such as angiotensin II, endothelin-1 and urotensin. Increased vascular superoxide as a result of activity of NADPH oxidase induced vasoconstriction and increased blood pressure [11]. In addition to control of homeostasis of the vascular wall the role of ROS has been established in the control of cardiac, renal function, as well as in hypertrophy, apoptosis, angiogenesis, hypertrophy, proliferation all of which are important processes involved in the development and establishment of hypertension.

Nephrectomy is the surgical removal of a kidney. The procedure is done to treat kidney cancer, as well as other kidney diseases and injuries. Nephrectomy is also done to remove a healthy kidney from a donor for transplantation. Despite the view that nephrectomy has minimal adverse effects on the overall health status, available data indicate that it provokes a complex physiological response, associated with increased oxidative stress, activation of different types of endocrine and immunologic mediators [12]. It was also established that nephrectomy caused an increased risk of developing hypertension [13]. Having the above mentioned in mind, it is essential to pay particular attention to changes of important blood pressure regulatory factors in patients with hypertension after nephrectomy.

Determination of total antioxidant capacity in various body fluids provides an opportunity to evaluate the antioxidant defense systems in patients with hypertension, acute and chronic kidney disease, and others. In comparison with other biological fluids, urine has a variable chemical composition which reflects the varying environment in the individual organism [14]. It is considered that the urinary antioxidant capacity reflects both renal and systemic antioxidant status [15]. In this regard the determination of TAC is a challenge. Nevertheless, experimental results show that ABTS assay provides a convenient marker for the antioxidant content in urine.

The aim of the present study was to estimate the urinary TAC after unilateral nephrectomy (UN) in the widely used animal model of essential hypertension - spontaneously hypertensive rats (SHR). In this investigation we followed up the changes in urinary total antioxidant capacity in SHR before and for 7 days after unilateral nephrectomy.

MATERIAL AND METHODS

Experimental animals

Experiments were performed on conscious, male normotensive Wistar rats, (n=7) and spontaneously hypertensive rats SHR (n=7) at an age of 12-14 weeks. The animals were housed under standard conditions: constant temperature 22 °C; 12/12 h light /dark cycle; free access to standard rat chow and tap water. Experiments were carried out in two periods: control period (before unilateral nephrectomy) and in the course of after unilateral nephrectomy (UN).

Surgical manipulation

The UN was performed under general anesthesia with pentobarbital sodium (Nembutal, Sigma) in a dose of 35 mg/kg b.w., applied intraperitoneally. Access to the right kidney was achieved by abdominal incision. Close to the hilum renale, a ligature was placed covering the ureter, renal vein and artery, and then the kidney was removed. The experiments were conducted in accordance with guidelines for the care and use of laboratory animals of the ethical standards at the Medical University - Sofia based on the Convention on Animal Protection and with the approval of Bulgarian Food Safety Agency (BFSA license №019/07.03.2017).

Investigated parameters

The urine samples from the experimental animals, needed for subsequent analysis, were collected in metabolic cages (Tecniplast, Italy) every day for 6 hours between 8.00 – 14.00 h AM. Urine flow rate was determined gravimetrically. The concentrations of urea and creatinine were measured spectrophotometrically using commercial kits (Giese diagnostics) according to the manufacturer's protocol. The urea and creatinine excretions were calculated.

Urine sample total antioxidant capacity estimation:

The assay was performed according to Re *et al*., [16]. The radical cation was pre-formed in buffer water by adding 14 mM ABTS stock solution to potassium persulfate (2.45 mM final concentration). The resulting mixture was allowed to stay overnight in the dark at 4 °C. When the reaction has stopped and a primary stock solution of the radical with stable absorbance was obtained, a working solution with absorbance 0.700 ± 0.005 at 734 nm of ABTS ●+ was prepared. The absorbance values were taken exactly 60 min after mixing 1 ml of the ABTS radical working solution with the tested urine samples. Calibration curve using the reference compound Trolox was prepared and on the base of the obtained

absorbance data the results were expressed as µmol Trolox equivalent (TE)/µl of urine.

All results were presented as mean ± SEM. Student's t-test was used for comparison between two means. Differences at a probability level of $p<0.05$ were considered significant.

RESULTS

We did not find differences in urine flow rate: 3.28±0.59 and 3.05±0.36 µl.min^{-1}.100 g b.w., excretions of creatinine: 141.83±11.44 and 144.97±13.95 ng.min^{-1}.100 g b.w.; and urea: 110.23±9.22 and 107.25±10.66 ng.min^{-1}.100 g b.w., between normotensive Wistar and spontaneously hypertensive rats under control conditions (Fig.1).

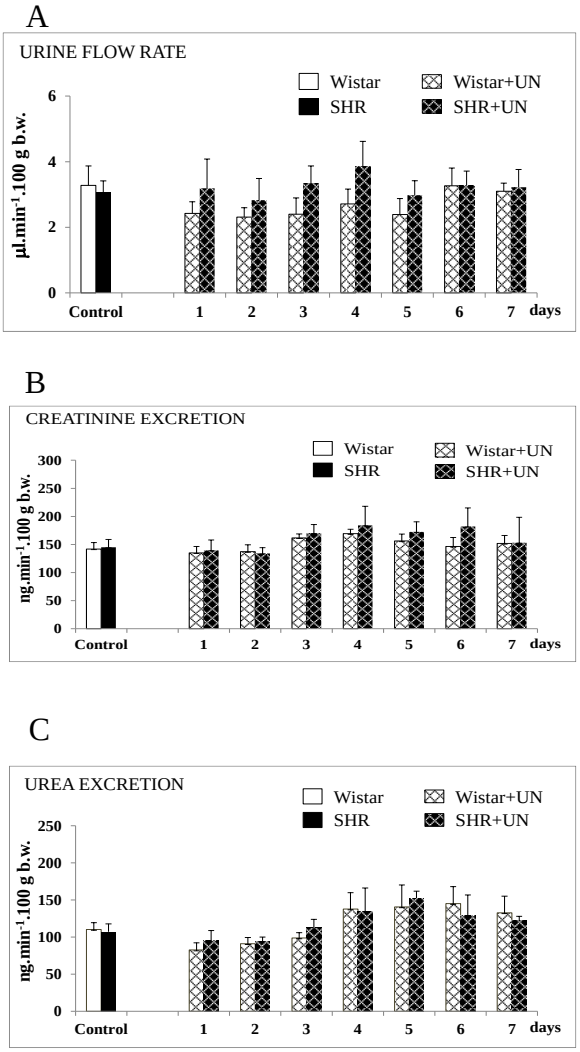

Figure 1. Urine flow rate (A), creatinine (B) and urea (C) excretion in normotensive Wistar rats (Wistar, n=7) and spontaneously hypertensive rats (SHR, n=7) in control period and for 7 days after unilateral nephrectomy (UN).

The unilateral nephrectomy did not cause changes in these parameters in either Wistar rats or SHR. On the first day after UN in Wistar rats, urine flow rate was 2.42±0.35 µl.min^{-1}.100 g b.w.; creatinine and urea excretion were 134.96±11.44 and 110.23±9.22 ng.min^{-1}.100 g b.w. respectively, and remained at this level until the end of the investigated period. The results obtained from the studied excretory parameters in SHR did not differ from those of Wistar rats and did not show statistically significant changes in the investigated period after the nephrectomy. On the first and seventh day after UN in SHR, the urine flow rate was: 3.19±0.89 and 3.22±0.54 µl.min^{-1}.100 g, creatinine and urea excretions were: 139.69±18.52 and 153.11±45.26; 95.99±12.81 and 121.79±6.32 ng.min^{-1}.100 g b.w.

The results concerning the estimation of the urinary total antioxidant capacity (TAC) before and after the unilateral nephrectomy in Wistar rats and SHR are presented on Fig. 2.

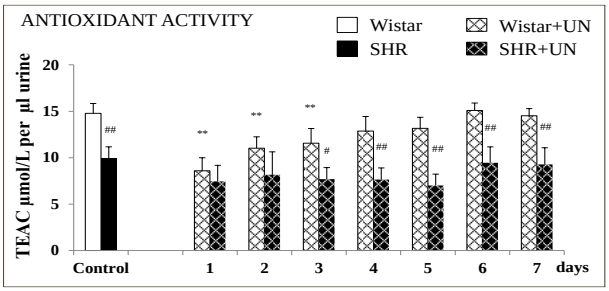

Figure 2. Total antioxidant capacity assessed using the ABTS model system in the urine of conscious, male normotensive Wistar rats, (n=7) and SHR (n=7) before (control period) and for 7 days after unilateral nephrectomy (UN). * ($p<0.05$); ** ($p<0.01$) - shows significant differences versus control; # ($p<0.05$); ## ($p<0.01$) - shows significant differences between Wistar rats and SHR.

We established differences between normotensive and spontaneously hypertensive rats in the control period of TAC, as well as alteration in dynamic changes of antioxidant potential after unilateral nephrectomy.

In the control period we observed lower TEAC in SHR: 9.90±1.27 µmol/L TE/µl, in comparison to Wistar rats: 14.77±1.07 µmol/L TE/µl, $p<0.01$.

In normotensive Wistar rats, the UN led to a statistically significant decrease of TEAC values in the first three days respectively: 8.58 ± 1.41; 11.02±1.22 and 11.56 ±1.59 µmol/L TE/µl urine, ($p<0.05$). For the rest of the tested period - day 4 do day 7 - the TEAC values were identical with the control. Differently from normotensive rats, in SHR the TEAC values were not affected by UN. The TEAC values vary from 7.45 ± 1.74 µmol/L TE/µl urine for day 1 to 9.26 ± 1.81 µmol/L TE/µl urine for day 7.

DISCUSSION

Our investigation concerns the follow-up of the effects of unilateral nephrectomy on urinary total antioxidant capacity (TAC) in normotensive and spontaneously hypertensive rats.

The first observation in our study was that in the control period, total antioxidant capacity was significantly (by more than 30%) lower in SHR, compared to Wistar rats. This fact is not surprising because several research studies demonstrated that essential hypertensive patients, as well as different animal models of hypertension produce excessive amount of ROS [17-20] and have abnormal levels of antioxidant status [17]. It has been demonstrated that increased ROS production in hypertension is accompanied by reduced nitric oxide level and antioxidants bioavailability [21]. The critical role of NO/NO-synthase (NOS) pathway in the regulation of blood pressure has been undoubtedly established [22]. Endothelial NOS catalyzes NO production from L-arginine and oxygen - a reaction that requires essential cofactor tetrahydrobiopterin (BH_4). The absence of BH_4 is associated with uncoupling of the L-arginine/NO pathway resulting in decreased formation of NO, and increased eNOS-mediated generation of superoxide. Superoxide leads to BH_4 oxidation, which provokes NO-uncoupling and increases ROS production. Its interaction with NO results in the formation of peroxynitrite [23]. In turn, peroxynitrite oxidizes and destabilizes eNOS which produces more superoxide [24, 25]. Decreased BH_4 and uncoupled NOS have been implicated in hypertension [26]. Decreased production of the fast and powerful vasodilator NO, as a result of absence of BH_4 and impaired L-arginine/NO pathway, led to endothelial dysfunction and to increase of blood pressure. It has been established that oxidative stress and hypertension in SHR were linked with the presence of functionally abnormal antioxidant enzymes [27]. On the other hand, the hypertension is associated with kidney redox imbalance resulting in enhanced reactive oxygen species (ROS) and enzymes-dependent phospholipid metabolism [28]. It is a possible that the reduced urine antioxidant capacity in SHR may also be due to established differences in their metabolic characteristics that can affect antioxidant defense system potency [29].

The decreased total antioxidant capacity in SHR, established in our study, by using ABTS assay in urine samples, confirms the abnormal level of antioxidant status found in other studies in various forms of hypertension in humans and in animal models [9, 10, 20].

In the next step of our experimental protocol we established an effect of unilateral nephrectomy on urinary total antioxidant capacity. Unilateral nephrectomy is a surgical procedure inducing renal mass reduction without direct pathological changes of the remaining kidney. We established that after UN the remained kidney in both experimental groups compensates the investigated major renal excretory functions – urine flow rate, creatinine and urea excretion. Despite this, several days are needed to restore the urinary antioxidant status in normotensive Wistar rats decreased after UN. Differently from Wistar rats, in SHR unilateral nephrectomy did not affect antioxidant capacity. The compensatory responses in the remaining kidney after unilateral nephrectomy are associated with a variety of factors such as reactive oxygen species, growth factors, and cytokines [30, 31]. The available experimental data demonstrated that after UN in mice superoxide formation in the remaining kidney significantly increased, peaking at 3 days and then gradually decreased over time [32]. Our results validate the need for 3 days for recovery of urine antioxidant capacity of normotensive Wistar rats after UN. In addition to our studies of "sham" operated normotensive rats, in which kidney was not removed, we tested the hypothesis for the possible effect of surgery on urinary antioxidant capacity. In this experiment, we detected a decrease in antioxidant capacity only on the first postoperative day. We hypothesize that the established extended period (3 days) of decrease of antioxidant capacity after nephrectomy is a result of dynamic compensation processes caused by renal removal which includes various factors responsible for maintaining homeostasis in the body. It has been found that nephrectomy changes the response of the kidney to Ang II but the mechanism of this effect has not been fully clarified [33]. The involvement of the renin angiotensin system in the compensatory responses of the kidney remaining after nephrectomy cannot be excluded, since the renin–angiotensin system (RAS) is responsible for preserving the fluid balance and vascular tone of the body. Available experimental data evidence the stimulating effect of Angiotensin II, which is an important component of the RAS, on NADPH oxidase (NOX), leading to an increase of ROS [34]. It is a possible, that the decreased in the first 3 days urinary total antioxidant capacity in Wistar rats, established in our study, may be a result of compensatory activated renin–angiotensin system, leading to an increase of ROS and decrease of TAC. It has been established that kidneys are rich sources of ROS, derived primarily from NADPH oxidase (NOX) [34].

Differently from normotensive Wistar rats, in SHR unilateral nephrectomy did not cause changes in total antioxidant capacity. There is substantial evidence suggesting that angiotensin II plays an important role in elevating blood pressure of spontaneously hypertensive rats, despite normal plasma renin activity [35]. We suggested that the lack of changes of TAC in SHR after nephrectomy, possibly mediated by RAS, is due to an inappropriately high Ang II–generating activity found in SHR [36], established up-regulated NOX, as well as increased ROS in hypertension [34, 37, 38]. These facts give us reason to believe that the reduced antioxidant capacity of SHR under control conditions is exhausted and does not have the capacity to a compensatory response to nephrectomy.

We suggest that the lack of response of urinary antioxidant activity to unilateral nephrectomy in SHR could be attributed to the baseline limited total antioxidant capacity as a result of uncoupling of the L-arginine/NO pathway, imbalance between Ang II levels and Ang II–generating activity, as well as to the altered metabolic and antioxidant systems characteristic for hypertensive states.

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B. Natural Bio-antioxidants

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Influence of enzymatic and ultrasonic extraction on phenolics content and antioxidant activity of *Hibiscus Sabdariffa* L. flowers

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The scientific support of the manifold benefits of phyto-molecules to human health has stimulated an increased consumption of natural products globally. Among phyto-molecules, polyphenols of intermediate and high molecular weights have been intensively studied because of their biological activities and uses. Flowers of roselle (*Hibiscus sabdariffa* L.) – a medicinal and culinary herb, contain compounds of polyphenolic structure, mainly anthocyanins, known for their antioxidant and anti-inflammatory activities. The extraction efficiency of biomolecules from various sources is tested through applying various technologies, conventional and non-conventional, in order to recover high amounts of such compounds and preserve their bioactivities. Consequently, an efficient approach is required to enhance the extractability of polyphenolic-based compounds from roselle flowers. The present study describes a combined technique of cellulase- and ultrasound-assisted extraction of *Hibiscus sabdariffa* phenolic compounds with enhanced yields. High amount of total anthocyanins (676.03±8.34 mg $100g^{-1}$ DM) and strong antioxidant activity of the crude acidified hydro-ethanolic extracts as measured by FRAP and DPPH assays were obtained in ultrasonic extracts pre-treated with cellulases. The optimum incubation time with the enzyme was 60 min, at which flavonoids and tannins were recovered in the highest amounts. The obtained results support the use of non-conventional environment-friendly extracting technologies to obtain an extract rich in antioxidants with potential uses in food, cosmetics and pharmaceutical industry

Keywords: *Hibiscus sabdariffa*, enzyme-assisted extraction, ultrasonication, phenolics, antioxidant activity.

INTRODUCTION

Roselle (*Hibiscus sabdariffa* L.) is a wild-grown or cultivated plant of the Malvaceae family, genus *Hibiscus*, widely used in tea production. Due to the particular content of micronutrients and antioxidant compounds (anthocyanins), the flowers of *Hibiscus sabdariffa* L. have been also used for medicinal and food purposes, as shown by Da-Costa-Rocha *et al.* [1] and Camelo-Méndez *et al.* [2]. Several health benefits of roselle have been reported, such as analgesic, anti-inflammatory, sedative, antimicrobial, immunomodulatory, as well as cardiovascular disease protection [3]. Although there are vast studies investigating the chromatic attributes of fresh petals of *Hibiscus sabdariffa* L., and their pharmacological properties [2], not the same could be noticed regarding the optimization of the extraction technologies, conventional and non-conventional. Testing the optimal extraction conditions using single or combined technologies is essential for high recovery of bioactive compounds, in particular polyphenols. Such biomolecules are efficiently extracted using polar organic solvents or acidified solvent mixtures which usually increase the extraction of anthocyanins due to the presence of the red flavylium cation as described by Giusti and Wrolstad [4]. The use of large quantities of organic solvents may generate a negative environmental impact and a few impurities in the final extract, leading to a time-consuming extraction. Ultrasound-assisted extraction (UAE) has been successfully used as a non-conventional technique for effective extraction of phenolic compounds from various plant materials through optimization of different experimental parameters [5]. The UAE of bioactive compounds is usually performed at frequencies ranging from 20 to 100 kHz and various ultrasonic amplitudes, applying direct and indirect irradiation through various devices. Other non-conventional extraction methods have been investigated with various yields, *e.g.* microwave-assisted extraction, pressurized liquid extraction, supercritical fluid extraction [6-8]. In recent years, mild "green" extraction of bioactive compounds with the aid of hydrolytic enzymes has emerged as a promising tool [9].

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Enzymes such as cellulases, pectinases or hemicellulases have been used for the disruption of plant cell walls and consequent liberation of increased amounts of targeted compounds. The extraction yield highly depends on the plant material, type of compounds/enzymes and process parameters. As the UAE reduces extraction time and increases extractability of phenolic compounds, it may provide efficiency in combination with the enzyme-assisted extraction.

This work aimed at evaluation of the optimal parameters of the combined enzyme- and pulsed ultrasound-assisted extraction of polyphenolic compounds from *Hibiscus sabdariffa* L. red petals. The following antioxidant compounds were targeted: anthocyanins, flavonoids, flavones and flavonols, phenolics and condensed tannins. The total antioxidant activities of the crude roselle extracts were determined as well.

EXPERIMENTAL

Plant material and chemical reagents

Commercially available *Hibiscus sabdariffa* L. petals were purchased from a local producer (Sibiu, Romania). Samples were ground and mixed using the Grindomix Retsch GM 200 mill and stored at -80 °C until analysis. The moisture content was determined using the moisture analyzer (MAC 210/NP Radwag, Poland).

All chemical reagents used for analysis were of analytical grade. Cellulase from *Aspergillus niger* with activity > 60,000 U/g (MP Biomedicals) was used.

Extraction procedure

The extraction approach consisted of the enzymatic technique combined with ultrasonication. The optimal UAE conditions previously described were applied [10]. Three incubation times of roselle samples with cellulase solution in acetate buffer of pH 4.8 were used (60 min, 120 min, 180 min) at 40 °C. An enzyme/substrate ratio of 0.166 (w/w) was used. After incubation, enzymes were inactivated at 100 °C for 5 min. Extraction was further performed by UAE using 70% ethanol acidified with 1% acetic acid. Control samples (without cellulase pre-treatment) were also investigated. UAE was performed using an ultrasonic device (Sonifier SLPe-150, Branson, USA) of 150 W power and 40 kHz frequency, equipped with a transducer.

The obtained extracts were centrifuged at 8000 rpm, at 4 °C for 10 min. The NF800R refrigerated centrifuge (Universal 320, Hettich, Germany) was used.

Determination of the total content of anthocyanins

The content of total anthocyanins was determined spectrophotometrically by the pH differential method [4]. The Specord 200Plus UV–Vis spectrophotometer (Analytik Jena, Germany) was used. The results were expressed as mg cyanidin-3-O-glucoside 100 g^{-1} DM.

Determination of the total content of phenolics

The phenolics content was determined according to Folin-Ciocalteu method [11]. The results were expressed as mg gallic acid equivalents GAE 100 g^{-1} DM.

Determination of the total content of flavonoids

The flavonoids content was determined spectrophotometrically as described in [12]. The results were expressed as mg quercetin 100 g^{-1} DM.

Determination of the total content of flavones and flavonols

The content of flavones and flavonols was determined spectrophotometrically as described in [13]. The results were expressed as mg rutin 100 g^{-1} DM.

Determination of the total content of tannins

The tannins content was determined spectrophotometrically as described in [14]. The results were expressed as mg catechin 100 g^{-1} DM.

Antioxidant activity assays

Ferric reducing antioxidant power (FRAP). The total antioxidant activity of enzyme ultrasonic extracts was determined by the ferric reducing ability assay [15]. The results were expressed as mg ascorbic acid 100 g^{-1} DM.

Radical scavenging activity (RSA) using 1, 1-diphenyl-2-picrylhydrazyl (DPPH). The RSA activity of enzyme ultrasonic extracts was determined by DPPH assay [16]. The results were expressed as inhibition percentage calculated according to the formula:

$$inhibition\ (\%) = 100 \times \frac{A_0 - A}{A_0}$$

where, A_0 is the absorbance at 515 nm of control and A is the absorbance at 515 nm of the sample.

Statistical analysis

The experimental parameters were analyzed in duplicate. Data were expressed as mean values±SD of duplicate experiments. The statistical analysis of the experimental data was carried out using the Systat v.10 software.

RESULTS AND DISCUSSION

Hybrid approach of enzyme- and ultrasound-assisted extraction of phenolic-based compounds from roselle

The UAE conditions that proved efficiency in extracting polyphenolic compounds from *Hibiscus sabdariffa* L. petals using 70% ethanol acidified with 1% acetic acid in a solvent/solid ratio of 40/1 were: 30 min extraction time at ultrasonic amplitude of 70%, as previously described by our group [10]. In order to further develop an efficient "green" extraction under mild conditions, we hereby report the development of a hybrid approach of enzymatic and ultrasonication technology. Three incubation times (60, 120 and 180 min) of samples with cellulase solution in acetate buffer of pH 4.8 were tested at 40 °C. The measured temperature of the mixtures after ultrasonication varied between 26.6 and 31.6 °C, depending on the experiment run.

The cellulolytic multi-enzyme complex used for extraction contains exo- and endo-β-1,4-D-glucanases which hydrolyze glucosidic bonds, and β-glucosidase which degrades small molecular weight cellulose hydrolysates.

The results regarding the content of total anthocyanins as a variation of incubation time are presented in Figure 1. The mean values of total anthocyanins contents were 676.03±8.34 mg 100 g^{-1} DM for enzyme ultrasonic extracts and 668.11±9.89 mg 100 g^{-1} DM for control, respectively. No statistically significant differences were found between combined method and control (single UAE). By increasing incubation time with cellulase, a decrease in total anthocyanins content was noticed, probably due to the time-dependent influence of acidic conditions given by the sample/solvent mixture on the enzyme denaturation. Based on these findings, evaluation of the other phenolic compounds was done after 60 min incubation of samples with cellulase followed by UAE. The results are shown in Table 1.

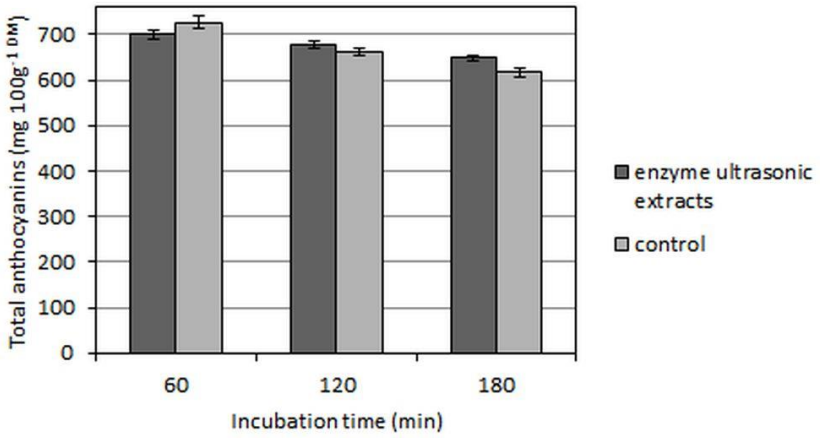

Fig. 1. The effect of incubation time on total anthocyanins content of enzyme ultrasonic extracts of roselle petals.

Table 1. The content of bioactive compounds of roselle extracts by enzymatic and ultrasonication combined technique.

Type of extraction / Bioactive compounds	Enzyme + Ultrasonication	Ultrasonication (Control)
Phenolics (mg GAE 100 g^{-1} DM)	1336.743±18.371	1338.169±21.584
Flavonoids (mg quercetin 100 g^{-1} DM)	2715.513±42.243	2533.792±33.104
Flavones and flavonols (mg rutin 100 g^{-1} DM)	1673.792±46.895	1671.569±40.784
Tannins (mg catechin 100 g^{-1} DM)	6561.118±80.559	6271.147±85.573

In experimental runs involving enzymatic pre-treatment, an increase in total content of flavonoids by 7% and tannins by about 5% was found. This might be explained by the enzymatic release of high-molecular weight tannins and pro-anthocyanidins from roselle samples, because such

compounds are usually associated with cell wall polysaccharides [17]. Our results showed that of total flavonoids, the contribution to their overall yield increase was not related to flavones and flavonols, but to other representatives of the class, mainly anthocyanins.

The effects of the improved material transfer determined by cavitation process during UAE as previously described by other authors [18] were further positively influenced by the cellulase pre-treatment of roselle samples for 60 min, resulting in high recovery of flavonoids/anthocyanins and tannins.

To our knowledge, no studies have been published on enzyme-assisted extraction, single or combined technology, of polyphenolic compounds from roselle. However, there are studies reporting a high recovery of anthocyanins from red and purple *Hibiscus sabdariffa* L. calyces under single UAE [19]. Regarding the use of enzymes to improve extraction of bioactive compounds, our findings are consistent with previous reports on other plant materials, mainly fruits, when various types of commercial hydrolytic enzymes have been involved [20].

In vitro antioxidant activities of enzyme ultrasonic extracts

The total antioxidant activity of the roselle enzyme ultrasonic extract was measured *in vitro* using different analytical assays, based on the measurement of ferric ion reducing capacity and on single electron transfer by DPPH. The results are presented in Figures 2 and 3.

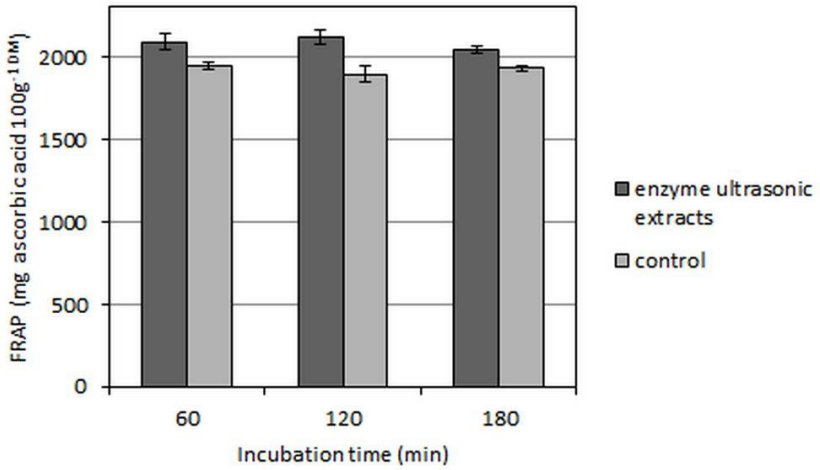

Fig. 2. The total antioxidant activity by FRAP of roselle enzyme ultrasonic extracts in relation to incubation time.

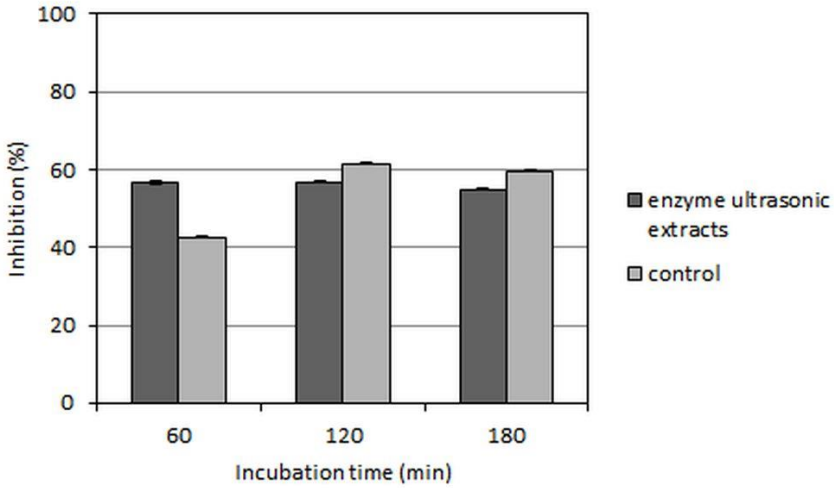

Fig. 3. Radical scavenging activity by DPPH of roselle enzyme ultrasonic extracts in relation to incubation time.

Regarding the antioxidant activity as measured by FRAP technique, the values obtained for the enzyme ultrasonic extracts were higher than those of the ultrasonic ones which did not undergo enzymatic pre-treatment (control). The mean FRAP values increased by 8.3% when cellulase pre-treatment was involved, compared to control samples.

The roselle crude extracts reduced the purple coloration of DPPH giving inhibition percentages > 50%, which confirm a good radical scavenging activity. The increase in RSA values was of about 4% when cellulase pre-treatment was involved, compared to control.

A study published in literature described the high antioxidant activity exhibited by extracts obtained through maceration of *Hibiscus sabdariffa* L. calyces in methanol, which was correlated to their high content of flavonoids [21].

CONCLUSIONS

Flowers of roselle (*Hibiscus sabdariffa* L.) contain high amounts of antioxidant compounds of polyphenolic structure.

An efficient enzyme-assisted extraction using cellulase combined with ultrasonication was developed. Lower incubation time (60 min) favored tannins and flavonoids extraction, improving their extractability by 5-7%.

The high antioxidant activity of acidified ethanol extracts, in terms of FRAP and radical scavenging activity by DPPH, was found in enzyme ultrasonic extracts.

The obtained results confirmed the efficacy of non-conventional extraction technologies with the development of "green" ones by using enzymes to formulate an extract rich in antioxidants with potential uses in food, cosmetics, textiles and pharmaceutical industry.

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Quantitative determination of ascorbic acid in *Callisia fragrans* under open-air hydroponic conditions

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The influence of growing media on the accumulation of ascorbic acid (AsA) in the medicinal raw material of *Callisia fragrans* (*C. fragrans*) was investigated for the first time. The plants were cultivated in open-air hydroponic conditions of Ararat Valley. Soil culture was used as a control. As a result of the experiments it was found that soilless culture had positive impact on the biosynthesis of AsA in the medicinal raw material: the content of AsA in the leaves and lateral sprouts of hydroponic plants was 20.3 and 29.4 mg/100 g, respectively, which, compared to the control was higher by about 40 and 30%, respectively.

Keywords*: C. fragrans*, ascorbic acid, hydroponics, soil culture, medicinal raw material.

INTRODUCTION

Growth, development and reproductive success of plants depend on numerous factors of environment such as temperature, amount of sunlight, water (humidity), nutrition, radiation, *etc*. These factors affect the intensity of physiological (photosynthesis, transpiration, respiration, *etc*.) and biochemical processes that occur in plants and sometimes are considered to be the limiting. That is why, plants cultivated under various growing conditions are differed with regularities of growth, development and metabolism. Having an opportunity to control the main factors in the root zone, it is possible to activate and/or direct the most important physiological and biochemical processes in plants.

Hydroponics (soilless culture) as an alternative way of plant production is becoming more and more popular in the modern world. As the construction of hydroponic stations is quite expensive, it is advisable to grow rare, endangered, expensive, valuable, small-tonnage plants, like medicinal plants [1].

C. fragrans (*Callisia fragrans* (Lindl.) Woodson) is a valuable medicinal plant of *Commelinaceae* family. The plant contains a number of bioactive substances such as phenolic compounds, amino acids, carbohydrates, flavonoids, coumarins, vitamins, *etc*. Due to its rich chemical composition, the medicinal plant has antioxidative, antiradical, stress-protective, immunomodulating, antiherpetic and other medical activities [2-5].

Years ago, *C. fragrans* (Fig. 1) was introduced into open-air hydroponic conditions of Ararat Valley for the first time by us.

According to the results of our experiments, in hydroponic conditions the plants were distinguished with high productivity of medicinal raw material. Due to the high yield of biomass, the output of bioactive substances from the medicinal raw material of hydroponic plants was also higher compared with the output from the soil plants. Moreover, the experiments also showed that hydroponic conditions had positive impact on the physiological activity of the leaves and the content of photosynthetic pigments in the leaves [6-8]. According to the literature review, the intensity of photosynthesis in plants is largely due to the presence of AsA (vitamin C), which is considered to be a cofactor of enzymes which are involved in the regulation of photosynthesis [9].

Fig. 1. *C. fragrans*

Based on the above, in the frame of this work the influence of growing soilless conditions (open-air hydroponics) of Ararat Valley on the content of AsA in the medicinal raw material of *C. fragrans* was studied.

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The present work is very important for understanding *C. fragrans* physiology and how the plants adapt to the growing conditions.

EXPERIMENTAL

The experiments were carried out at the vegetational experimental station of G. S. Davtyan Institute of Hydroponics Problems (National Academy of Sciences, Republic of Armenia).

Planting

For hydroponic growing of the plants special hydroponic vegetational pots (with 0.16 m^2 nutrient surface) were used. A mixture of gravel and volcanic red slag (1:1 volumetric ratio), with 3-15 mm diameter of particles, was used as a growing substrate, which was previously disinfected with 0.05% solution of $KMnO_4$. 12 plants were planted on 1 m^2 of nutrient surface. During the vegetation the plants were nourished twice a day with 0.5 N nutrient solution elaborated by academician G. S. Davtyan [10]. Soil culture was used as a control, where all the accepted agrotechnical rules (fertilization, weed cleaning, irrigation, *etc*.) were followed [1]. Both in hydroponics and in soil the plants were cultivated in open-air conditions of Ararat Valley and subjected to the influence of the same climatic factors.

Sampling

As an object of investigations fresh leaves (an average sample was taken from the top of the plant and its lateral sprouts) and lateral sprouts (with 9 and more brownish-purple interjointal spaces) of the plant were used, which were picked from the plant immediately before performing the analysis.

Determination of AsA

Quantitative determination of AsA in the leaves and sprouts was done by titration. The analytical sample (5 g) was homogenized in 20 mL of 1% solution of HCl, the obtained homogeneous mass was moved into a 100 mL volumetric flask and filled up with oxalic acid, then the solution was filtered and the content of AsA was determined in the filtrate [11].

Statistical analysis

The experiments were performed in 4-6 replications. For the statistical analysis of the obtained data GraphPad Prism 6 (GraphPad Software, Inc.) software package was used.

RESULTS AND DISCUSSION

It was found that the growing conditions had significant impact on the biosynthesis of AsA in the medicinal raw material of *C. fragrans*. According to the results of the biochemical analysis, the hydroponic conditions had positive influence on the accumulation of vitamin C. The content of AsA in the leaves and sprouts of hydroponic plants was 20.3 and 29.4 mg/100 g, respectively, which, compared to the soil ones was higher by about 40 and 30%, respectively. Making parallels between different upground parts of the plant, it is obvious, that regardless of the cultivation conditions the content of AsA in the sprouts was higher than in the leaves. The content of AsA in the sprouts of both soil and hydroponic plants exceeded that in the leaves 1.6 and 1.4 times, correspondingly (Figs. 2, 3).

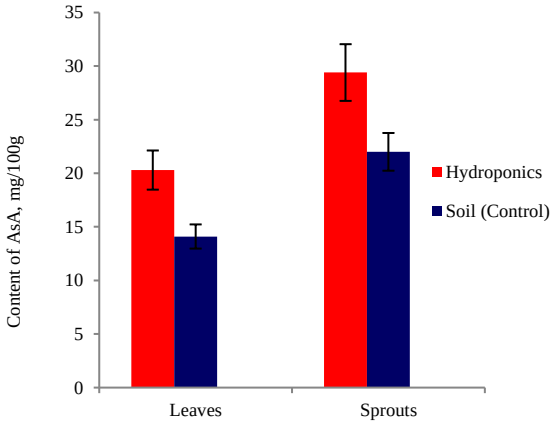

Fig. 2. Content of AsA in the medicinal raw material of *C. fragrans* depending on cultivation conditions.

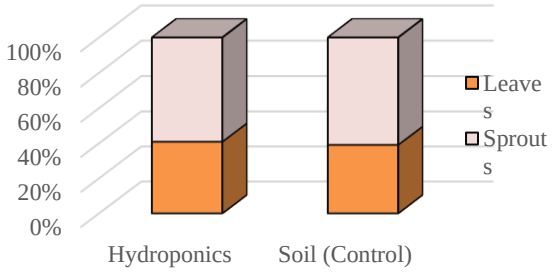

Fig. 3. Proportion of AsA in various upground parts of *C. fragrans* under hydroponic and soil culture conditions.

As a result of our experiments it is shown, that the medicinal raw material of *C. fragrans* obtained in soilless culture conditions is distinguished with high content of AsA compared to the soil ones. This can be explained by the fact that in soilless culture optimal air-water-nutrition conditions are created at the same time almost during the whole vegetation, which provides intensive physiological processes, while in soil culture the plants use the above

mentioned optimal factors only for a short-time period [1].

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In vitro Ultraviolet-B radiation mediated antioxidant response of Bulgarian Goldenrod (*Solidago virgaurea L.)* extract

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Damaging Ultraviolet B (UVB) radiation is an important part of the solar electromagnetic spectrum, which seasonal and long-term levels are likely to remain elevated for decades to come. Due to the high sensitivity of plants to UVB radiation, they need to react quickly to minimize surface reactive oxygen species (ROS) mediation. The increased levels of ROS, at cellular level, causes oxidation of proteins, lipids, and other biomolecules, thus jeopardizing enzymes and cell membranes functionality and integrity.

The present study for the first time reported the radical-scavenging, antioxidant-protective properties and stable structure of *Solidago virgaurea L.* extract before and after exposure to UVB radiation. The composition of the chemically pure (97 %) extract was established in the untreated and in the UVB-treated samples by RP-HPLC. By direct EPR spectroscopy, single symmetrical EPR signals were established in both untreated samples ($g = 2.0043 \pm 0.0003$) and UVB-treated samples ($g = 2.0054 \pm 0.0002$). The intensities of the EPR signals of both extracts demonstrated a possible formation of stable radical structures. It should be pointed out that stable radical structures were registered in the extract 2 and 6 months after the UVB-induced oxidation. Furthermore, both untreated and UVB-irradiated *S. virgaurea L.* extracts showed well-expressed radical-scavenging abilities and antioxidant-protective properties against reactive species such as nitric oxide (NO), reducing agents, non-enzymatic 2,2-azinobis (ABTS•+) structures, superoxide anion and DPPH stable radicals. The current results characterize the *S. virgaurea L.* extract as a promising ROS-scavenging antioxidant with UVB-protective properties, and outline its future applicability for the development of new photo-medications.

Keywords: *S. virgaurea L.*, RP-HPLC, EPR spectroscopy, antioxidant, UVB-protection

INTRODUCTION

Ultraviolet radiation (UVR; 100-400 nm) is an important part of the electromagnetic spectrum naturally emitted by the sunlight necessary for the normal physiological functions of medicinal plants [1]. Traditionally, the UVR is divided into three spectral regions: UVC (100-290 nm), UVB (290-320 nm) and UVA (320-400 nm) [1, 2]. The UVB light has received attention in the last two decades because the thinning of the ozone layer leads to an increase in the amount of UVB-irradiation of medicinal plants [3]. In general, many investigators have reported that UVB-irradiation has harmful effects on the growth, leaf expansion, physiology and productivity of different plants species [3, 4]. Moreover, UVB radiation has been found to increase plant membrane changes, protein destruction, hormone inactivation, biomass reductions and epidermal deformation [1, 2]. UVB induced generation of reactive oxygen (ROS) and nitrogen (RNS) species by direct and indirect pathological mechanisms through which the antioxidant defence systems decreased, and intracellular oxidative stress could generate plant cell damages [5]. The reactive species such as superoxide anion radicals (O_2^{-}), hydroxyl radicals (HO·), hydrogen peroxide (H_2O_2), peroxyl radicals (RCOO·) and nitric oxide (NO·/ $NOOO^{-}$) radicals are active components that are detoxified by the endogenous antioxidant system [6]. Sunlight, specifically UVB photons lead to the progression of the replication on the damaged DNA molecules and accumulation of proteins mutation and lipids peroxidation [5, 7]. More specifically, UVB light induces ROS by affecting the antioxidant enzymes, decreases protein kinase C (PKC) expression and increases nitric oxide synthase (NOS) synthesis [5, 7-9]. Studies indicate that synthesis of specific flavonoids and other phenolic compounds is regulated in response to UVB light [3, 10]. Flavonoids as medicinal plant antioxidants (e.g., B-ring substitution) lend beneficial place in the UV-oxidative stress protection and regulation [10]. Previous studies have shown that higher plants have independent photoprotective systems [3] and exposure to UVB radiation stimulated higher levels of flavonoids biosynthesis, and ROS removal [10, 11].

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Solidago virgaurea L. (Bulgarian Goldenrod) is an herbaceous perennial plant (family Asteraceae), which is widespread across Europe, North Africa and northern, central, and southwestern Asia. The aerial parts have been applied as spasmolytic, anti-inflammatory and diuretic agents in traditional medicine, as a urological remedy in kidney and bladder inflammation, urolithiasis, cystitis [12]. Studies proved that the plant extracts contain flavonoids (quercetin glycosides), salicylic acid derivatives, chlorogenic acid, caffeic acid, triterpene saponins, tannins, and essential oils. Besides, some of the proven bioactivities of the medicinal plant are due to synergic action of its bioactive substances [13].

In this regard, the aim of the present study was to determine the contents of catechins in *S. virgaurea* L. ethanol extract and to investigate and compare the EPR structural changes and *in vitro* antioxidant activity of untreated and UVB-irradiated *S. virgaurea* L. ethanol extracts.

EXPERIMENTAL

The study was conducted at the Electron Paramagnetic Resonance (EPR) Centre of the Medical Faculty, at the Bioorganic Chemistry Laboratory of the Faculty of Veterinary Medicine, the Central Scientific Laboratory, Trakia University, Stara Zagora, Bulgaria and at the laboratories of the Chemical Engineering Department, University of Chemical Technology and Metallurgy, Sofia, Bulgaria in 2019.

Reagents and standards

The following standards were used for the experimental studies: (+)-catechin hydrate (≥ 96.0 %, HPLC), (−)-epigallocatechin (analytical standard), quercetin (≥95%, HPLC) (Sigma-Aldrich). The reagents: acetonitrile (AcCN, ≥ 99.8%), orthophosphoric acid (H_3PO_4, 85%), methanol (MeOH, ≥ 99.9%), ethanol (EtOH, p.a. ≥ 99.8%), L-ascorbic acid, CH_3COOH and NaOH (p.a., HPLC) were supplied by Sigma-Aldrich. Milli-Q water was used to prepare the mobile phase.

Extraction procedure

The periodic extraction of *Solidago virgaurea* L. was conducted in a stirred batch extraction reactor at solid/liquid ratio $\xi = 0.03$ m^3 kg^{-1}, temperature T = 30ºC and solvent - 70% EtOH [14, 15]. The test results were obtained at an agitation rate n = 4 s^{-1}, which proved that the process was limited by internal diffusion (the external diffusion resistance was eliminated). A weight analysis with accuracy of 1.10^{-4} g was used to determine the contents of the extracts. A specified amount of extract was taken periodically and dried to constant weight in an oven at T = 70^{0}C.

RP-HPLC analyses

The concentrations of catechins (catechin, epigallocatechin) and quercetin in the ethanol extracts tested were determined by newly developed liquid chromatographic techniques specific for each antioxidant. A reverse-phase RP-HPLC system consisting of a Hypersil BDS C18 column (5 μM, 4.6 × 150 mm), a Surveyor LC Pump Plus pump, a PDA detector, and an Autosampler Plus autosampler (Thermo Fisher Scientific) were used. The mobile phase consisted of MeOH:AcCN:H_2O = 40:15:45 (v/v) (+1% CH_3COOH). To achieve better separation of the components and purification of the extracts, all analyses were performed with a precolumn. The analyses of all the samples tested were performed in triplicate and the mean values of the reported concentrations were presented. The experimental HPLC chromatograms of the test sample extracts were analysed with ChromQuestTM chromatography workstation software system Version 4.2 (Thermo Electron Corporation).

UVB treatment

All ethanol samples were irradiated at a distance from the light source of 25 to 35 cm and a quartz cover was used to allow UV transparency and to prevent extract evaporation and keeping it in a horizontal position on UVB–vis Transilluminator-4000, Stratagene/USA (emitting between 290 nm and 320 nm (peak 309 nm)). The experimental UVB intensity was calibrated in each experiment. To obtain the value of dose response about the UVB radiation effect, the samples were irradiated over a wide UVB radiation range (0 to 12 kJ m^{-2}) without visible ray, at less than 291-293 K. The UVB irradiated energy was controlled with exposure time of 120 min. Dark, fresh air and 46 % relative humidity was circulated in the illuminator throughout the irradiation course.

In vitro antioxidant activity

FRAP assay: The ferric reducing antioxidant power/electron donation potential of untreated and UVB-treated *S. virgaurea L.* extract samples, was conducted according to the previously reported method with a slight modification [16, 17]. 50 μg mL^{-1} before was firstly tested to determine the exhibited maximal donation potential. Further, 50 μg mL^{-1} concentration was UVB-irradiated and the *FRAP* potential was determined immediately post irradiation. The reaction mixture was left for 10 min

at 293 K temperature and the absorbance was measured at 700 nm.

DPPH assay: The ability of the *S. virgaurea L.* extract before and after UVB-treatment to scavenging 2,2-diphenyl-1-picrylhydrazyl (DPPH) stable radical was determined according to Cuendet *et al.* (1997) [18], with slight modifications [19]. Briefly, 1.0 ml of DPPH (100 μM) was added to 500 μl of 50 μg mL^{-1} volume concentration of the studied samples. Mixtures were incubated in the dark for 10 min and their absorbance at 517 nm was measured. Quercetin was used as a positive control.

$ABTS^{\bullet+}$ assay: The radical-scavenging activity of untreated and UVB-treated and *S. virgaurea* L. samples against 2,2-azinobis (3-ethylbenzothiazoline–6–sulfonic acid) was determined by Re *et al.,* (1999) [20] with modifications by Adhikari *et al*., (2012) [21]. The reaction mixtures were incubated at 297 K for 30 min and the chromogen intensity was measured at 734 nm. Antiradical activity of the examined samples was presented as the percentage of $ABTS^{\bullet+}$ radical-scavenging in μg/ml, conducted in triplicate and the results expressed as the mean ± standard deviation.

NO assay: The presence of nitrite potential, a stable oxidized product of nitric oxide ion scavenging of untreated and UVB-irradiated *S. virgaurea* L. samples (50 μg mL^{-1}) was determined according to the standard methodology described by Shirwaikar *et al.,* (2006) [22] with modification by Karamalakova *et al.,* (2017) [23]. The scavenging potential was evaluated as a decrease in % absorbance of the chromogen formed by diazotisation of nitrite with sulfanilamide and subsequent coupling with naphthylethylenediamine recorded at λ = 546 nm.

SOD-like activity assay: The superoxide dismutase (SOD) assay of *S. virgaurea L.* extract, before and after UVB irradiation was carried out according to the method of Sun *et al*., (1988) [24] as follows: The xanthine/xanthine oxidase system (Sigma Chemicals, USA) was used to generate the superoxide anion ($\cdot O_2^-$). Following 20 min of incubation, the superoxide anion reduces nitroblue tetrazolium (NBT; Sigma Chemicals, USA) to formazan. Absorbance was measured at λ = 560 nm and L-ascorbic acid was used as a standard. One unit (U) of enzymatic activity is defined as the amount of enzyme causing 50% inhibition of the NBT reduction to formazan.

To study the effect of UVB radiation on the antioxidant activity and free radical scavenging, the samples were stored at a temperature of 293 K in the dry air/dark and re-examined for 24 hours at the 2nd and 6th month post irradiation.

In vitro Electron Paramagnetic Resonance spectral analysis

Direct EPR analysis: The *S. virgaurea* L. ethanol extract was divided into two parts: 1) before irradiation and 2) after UVB irradiation for 2 h. Immediately after irradiation storage, the two extract samples were examined in triplicate on an X-band-EMX^{micro} spectrometer at a temperature of 293 K. The sample tubes were always positioned exactly in the centre of the cavity and *g-value* was calculated as first derivative of the EPR signal [25]. Spectral settings were as following: centre field 3514.00 G, sweep width 200.00 G, microwave power 0.635 mW, modulation amplitude 10.00 G, gain 1×105, time constant 1310.72 ms, sweep time 133.12 s, 1 scan per sample; and for UVB-irradiated samples - 1.00 G modulation amplitude.

DPPH• radical-scavenging activity: The ability to scavenge DPPH• radical was studied according to Santos *et al.,* (2009) [26] with modifications [27]. Untreated and UVB-irradiated *S. virgaurea* L. extract (50 μg mL^{-1}) was added to 250 μl of EtOH solution of DPPH (80 μmol L^{-1}). Following 10 min incubation (293 K, 10 min/dark) the samples were transferred into the EPR cavity and the scavenging ability was calculated as follows:

$$Scavenged\ DPPH\ radials\ (\%) = [(I_0 - I)/I_0]x100 \quad (1)$$

where I_0 is the integral intensity of the DPPH signal of the control sample; I - the integral intensity of the DPPH signal after addition of the tested sample to the control.

A solution containing 250 μl of DPPH and 50 μl of EtOH was used as a positive control. The settings were as follows: microwave power 3.232 mW, modulation amplitude 5.00 G, gain 5.02×10^3, time constant 163.84 ms, 1 scan per sample.

To investigate the UVB effect on free radical stability and structural changes, samples were stored at a temperature of 293±2 K in the dry air/ dark and re-examined for 24 hours, on 2nd and 6th months post irradiation.

Statistical analysis

The data obtained from the *in vitro* antioxidant activity study were expressed as means ± standard error (SE) with Statistica 7.1, StaSoft Inc., one-way ANOVA. The statistical significance was determined by Student's *t*-test as the post-hoc test. A value of $p < 0.05$ was considered as statistically

significant. The kinetic data were expressed as the average of two independent measurements, which were processed using the computer programmes Origin 6.1 and Microsoft Excel 2010. EPR spectral processing was performed with Bruker WIN-EPR and Sim-Fonia software (Germany, IRCF 2003).

RESULTS AND DISCUSSION

RP-HPLC analysis of S. virgaurea L. ethanol extract

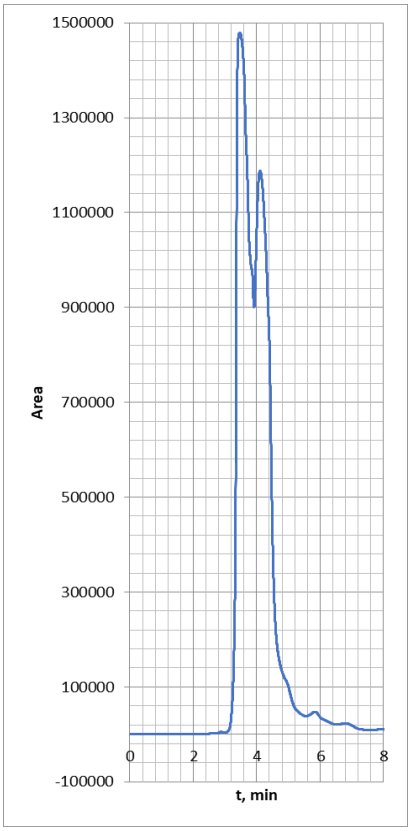
The concentrations of catechin, epigallocatechin and quercetin in the tested extract were determined by RP-HPLC on the basis of standard calibration curves of the corresponding three standards, which were characterized with high linearity ($R^2 > 0.998$).

The volume of all samples used in the RP-HPLC assays was 1.5 mL. The concentration of quercetin was determined at maximum wavelength λ = 360 nm, flow rate 0.5 ml min^{-1}, column temperature T = 30°C, detection time t = 5.8 min, volume of sample injected V = 20 µL. The catechin concentrations were determined at λ = 325 nm; flow rate: 0.5 mL/min, column temperature T = 30°C, detection time t = 3.5 min for epigallocatechin and t = 4.3 min for catechin, volume of sample injected V = 20 µL.

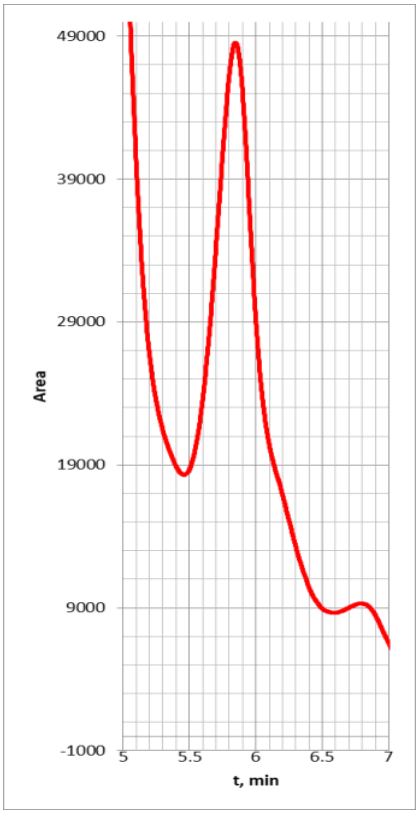
The concentrations of catechin, epigallocatechin and quercetin in the tested extract were 1384.23, 1427.04 and 18.9 µg mL^{-1}, respectively. The RP-HPLC chromatograms of the three biologically-active polyphenols are presented in Fig. 1.

In vitro antiradical activity promoted by UVB treatment

The photon energy of UVB radiation directly induces excessive production of ROS/RNS in medicinal plants, leading to the occurrence of oxidative stress damages. Plant are a potential source of natural antioxidants, which inhibit ROS/RNS activity by different mechanisms controlled by activation of the antioxidant enzymes system [28] and scavenging unstable free radicals such as superoxide, hydroxyl anion, nitric oxide and peroxynitrite. To identify whether free oxygen and nitrogen species function as signaling molecules in the UVB-provoked *S. virgaurea* L. extract response, different indirect methods were applied to evaluate the antioxidant activity. Previous studies have shown that the *S. virgaurea* L. extract possessed antioxidant activity [29]. In the present study, a maximal scavenging ability for untreated *S. virgaurea* L. extract and UVB-irradiated extract was observed at a concentration of 50 µg L^{-1}.



A.

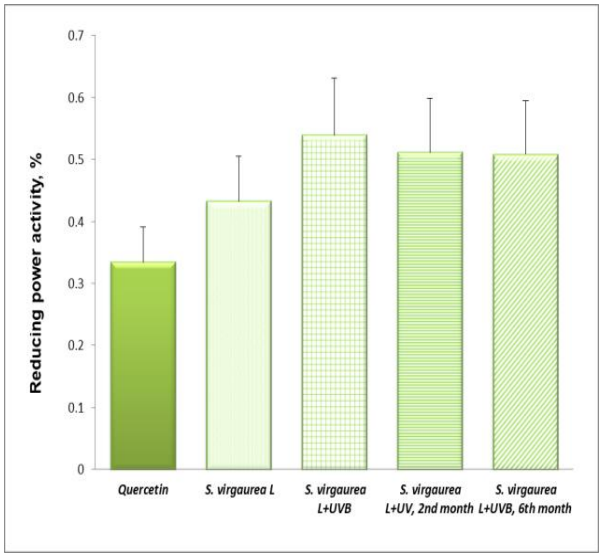


B.

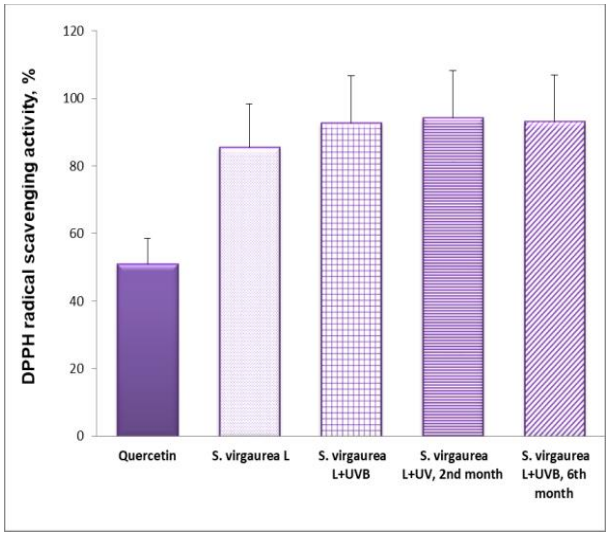
Fig. 1. RP-HPLC chromatograms of *S. virgaurea* L. extract of: **A.** catechin and epigallocatechin at λ = 325 nm; **B.** quercetin at λ = 360 nm.

FRAP assay (Fig. 2A) is generally associated with the presence of reductants in the untreated *S. virgaurea* L. extract and in the UVB-irradiated one, which exert antioxidant action by donating a hydrogen atom and reducing Fe^{3+} to Fe^{2+}. The ferric reducing activity for the UVB-irradaited *S. virgaurea L.* extract (0.539±0.01 %) was determined to be higher than that of untreated *S. virgaurea* L. (0.432±0.003 %) and the control sample (0.334±0.08 %, $p<0.005$). FRAP potential of the UVB-irradiated *S. virgaurea L.* was tested at different time intervals – on the 2nd and 6th months after treatment.

There were no significant differences ($p > 0.005$) in the FRAP activity between the post storage UVB-exposed samples compared to the immediate UVB-exposed ones, displaying a good restoring ability to the Fe^{3+} complex.



A.



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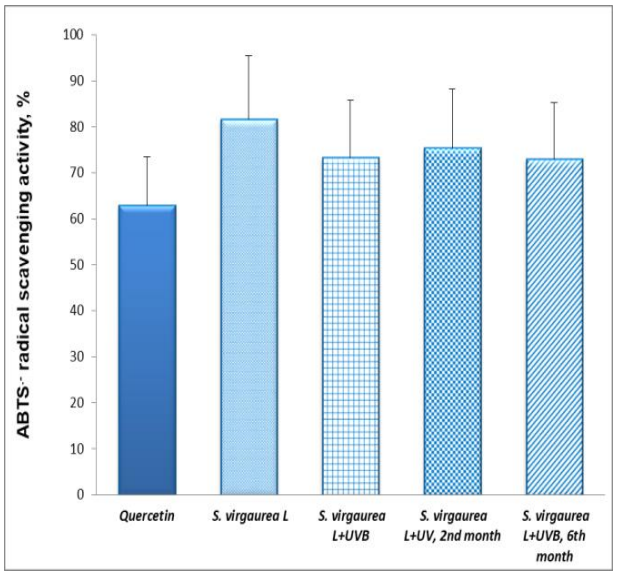

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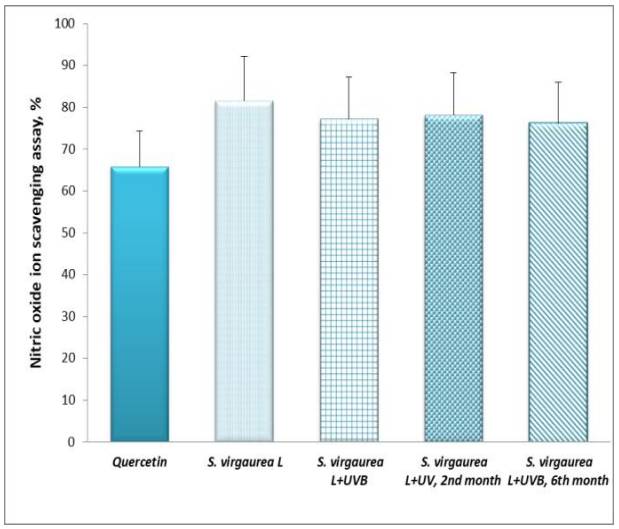

D.

Fig. 2. Effect of UVB irradiation on the *in vitro* antiradical activity of *S. virgaurea L.* extract: **A**. FRAP assay, **B**. DPPH radical scavenging activity, **C**. $ABTS^{\bullet+}$ radical scavenging activity, **D**. Nitric oxide ion scavenging assay.

UVB-exposure increases the load of iron in the cellular environment and leads to hemolysis processes. The reduction of Fe^{3+} into the less harmful Fe^{2+} might be assumed as a possible mode of action by which *S. virgaurea* L. extract [29] would exhibit protective efficacy to oxidative damages caused by UVB-radiation *in vitro* [23, 30].

The DPPH as a stable lipophilic free radical with high sensitivity was used for evaluating the antioxidant activity of *S. virgaurea* L. extracts [31]. The $DPPH^{\bullet}$ scavenging activity of untreated *S. virgaurea* L. extract (85.56±0.88%, $p < 0.05$) and of the UVB-exposed sample (92.78±1.37%, $p<0.003$)

were statistically insignificantly increased, compared to the standard (50.99±0.19%) (Fig. 2B). It should be noted that UVB-radiation increased the DPPH$^{\bullet}$ ability to scavenge free radicals in the *S. virgaurea* L. extract, compared to quercetin. Meanwhile, UVB-radiation enhanced the DPPH$^{\bullet}$ radical scavenging after 2 and 6 months of storage. The considerable increase in the scavenging effects of the *S. virgaurea* L. extract after UVB radiation could be explained by the stable compounds as flavonoids, phenolic acids, glucosides, polysaccharides, anthocyanins, tannins, and vitamins present in the extract. These results prove the suitability of *S. virgaurea* L. extracts as a natural antioxidant for food applications [29-32].

The proton-radical analysis (ABTS$^{\bullet+}$ method*)* is excellent to define the total antioxidant activity of hydrophilic plant antioxidants [33]. The ABTS$^{\bullet+}$ proton scavenging abilities in both the UVB-irradiated *S. virgaurea L.* sample (73.221±1.16%) and the untreated extract (81.556±0.97%, 0.00) (Fig. 2C) increased as compared to quercetin (62.8± 0.58%) - the standard antioxidant used in this study. Untreated *S. virgaurea* L. extract was more effective in quenching proton radicals in the system. The present study shows that after 2 (75.3±1.00%) and 6 months (72.89± 0.47%) after UVB-irradiation the *S. virgaurea* L. extract has accumulated active components and maintains the ABTS$^{\bullet+}$ reduction levels. These results suggest that UVB-irradiation probably stimulates the production of secondary metabolites (flavonoids, phenolic acids, anthocyanins), the antioxidant activity and, thus, the extract is able to adapt and to protect itself from UVB-intensity irradiation [34].

Nitric oxide (NO$^{\bullet}$) is considered as one of the important molecules, which is the regulator of physiological functions. In addition to its physiological actions, the ROS can affect overload of NO$^{\bullet}$ radicals leading to oxidative cell damages and to compromised cell NO-functionality [23, 35]. The NO$^{\bullet}$ scavenging ability (Fig. 2D) of unirradiated *S. virgaurea* L. extract (81.65±1.75%, $p < 0.05$) and the UVB-irradiated sample (77.3±0.39%) increased as compared to the control sample (65.81±0.9%). Probably, *S. virgaurea* L. contains hydrophilic scavengers able to regulate the NO$^{\bullet}$ production even in the UVB-irradiated sample. UVB-irradiation incubation of *S. virgaurea L.* samples for 2 (78.13±0.63%) and 6 months (76.11±1.23%) recorded stable NO$^{\bullet}$ radicals scavenging in comparison to the untreated extract and the UVB-treated sample. Investigations demonstrated that endogenous NO/NO$^{\bullet}$ production is able to modulate primary metabolism and oxidative stress signalling responses in plant molecules [36]. The latter suggests that flavonoids and other *S. virgaurea* L. active components act as potential photoprotectors and ROS/NO scavengers formed as a result of long-term UVB-stress exposure [8, 36].

To identify whether ROS function as signalling molecules in the UVB-provoked oxidative stress in the *S. virgaurea* L. extract, SOD-like activity non-enzymatic method [37] was applied. The SOD-like activity of 1 mL untreated *S. virgaurea* L. extract was 9 times higher than that of the standard antioxidant L-ascorbic acid (1 mg) (Fig. 3).

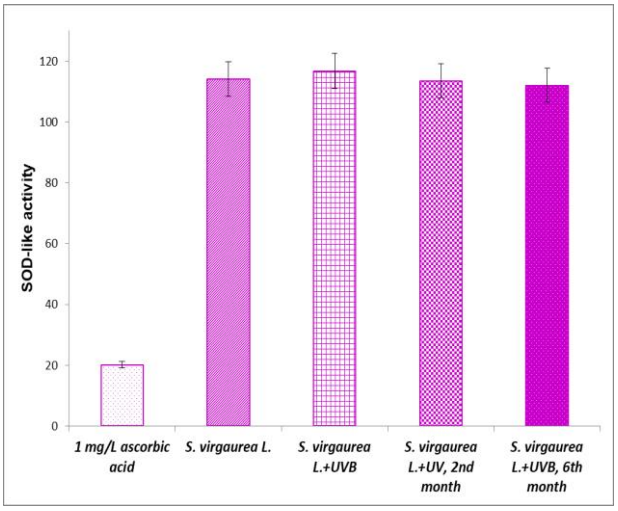

Fig. 3. Effect of UVB irradiation on the SOD-like activity of *S. virgaurea L.* extract.

Significantly slight increase in SOD-like activity in the UVB-treated extract in comparison with the non-treated sample was registered. In addition, the UVB-treated *S. virgaurea* L. registered 5 times higher SOD-like activity *vs* the standard, which was preserved for 2 and 6 months. The latter illustrates that abiotic stress of UVB-induced generation of superoxide radicals ($\bullet O_2^-$) was metabolically suppressed by the active compounds in the *S. virgaurea* L. extract into hydrogen peroxide (H_2O_2). Moreover, induced intracellular ROS were momentarily inhibited by the UVB-light-absorbing molecules of the *S. virgaurea* L. extract and converted into harmless molecules through SOD, H_2O_2, NAD (P) H oxidase and flavin oxidase [28].

EPR spectrum characterization before and after UVB-irradiation

An intensive symmetrical EPR singlet with g = 2.0043±0.0003 in the *S. virgaurea* L. sample (Fig. 4a) was recorded immediately after preparing the extract. A similar symmetrical signal with g-factor of 2.0054±0.0002 was registered in the UVB-irradiated extract (Fig. 4b) with almost commensurable singlet intensity. The g-factors of

the recorded spectra of *S. virgaurea* L. sample suggest the detection of *ortho*-semiquinone centred radical [38, 39] typical for the radical-scavenging of flavonoids, phenolic acids, glucosides, anthocyanins, tannins in the extract composition.

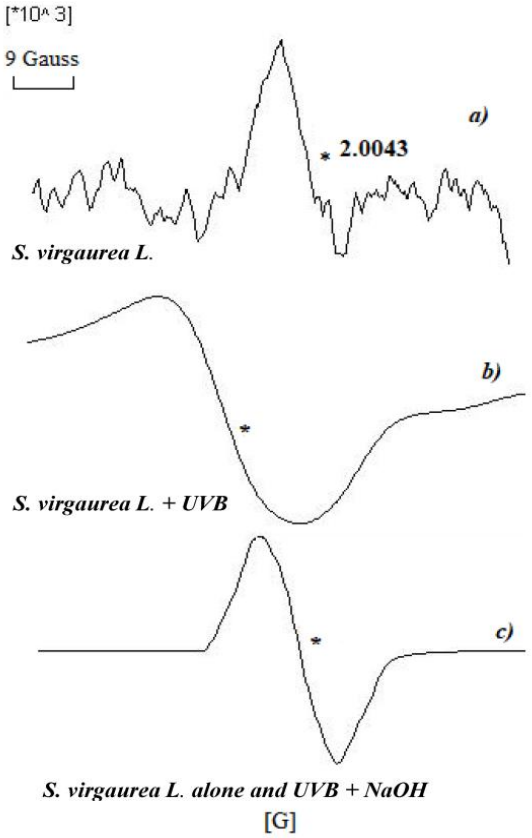

Fig. 4. EPR spectrum of *S. virgaurea* L. extract before and after UVB irradiation.

Considering that the EPR spectroscopy has the potential to verify the possibility of radical structures belonging to a semiquinone structure, we have studied the non-irradiated *S. virgaurea* L. extract and the UVB-irradiated sample after alkalization (Fig. 4c). Generating EPR radicals in both alkalized solutions resulted in intensive singlet signals of the *ortho*-semiquinone structures [40, 41] with equal g = 2.00456±0.0002 values.

Regarding the stable *ortho*-semiquinone structures in both samples for a more detailed analysis of the antioxidant capacity, we investigated the *in vitro* DPPH scavenging activity of the untreated *S. virgaurea L.* extract and of the UVB-irradiated sample. *S. virgaurea L.* extract (Fig. 5) displayed maximum radical-scavenging activity (89.11±7.45%). However, the UVB-treated sample was characterized with higher DPPH activity (91.37±9.71%), which was statistically insignificantly decreased on the 2nd (88.3 ± 5.71%) and 6th (85.2 ± 8.47%) months of UVB-endurance. The latter findings were in accordance with the results of Toiu *et al.*, (2019) [42]. The authors of the present study established that the flavonoids from *S. virgaurea* L. extract possess stronger antioxidant and DPPH radical-scavenging activity in comparison with vitamin C as a positive control.

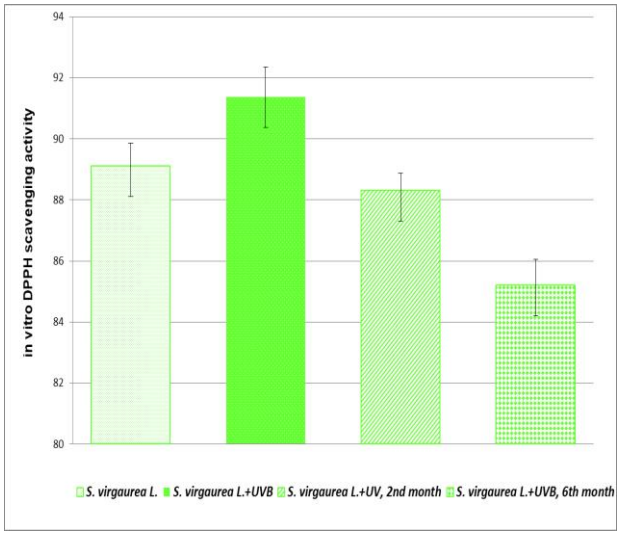

Fig. 5. *In vitro* DPPH scavenging activity of *S. virgaurea* L. extract before and after UVB irradiation.

Identical dependency was registered after ultra-sonication (150 gg cycle/20 sec) and dark incubation (at 297 K) of both extracts at different time intervals (the results are not presented).

CONCLUSIONS

In the present study the flavonoids content of *S. virgaurea* L. ethanol extract was determined by newly developed RP-HPLC methods. The concentrations of catechin, epigallocatechin and quercetin in the plant extract were 1384.23, 1427.04 and 18.9 μg mL^{-1}, respectively. It was established that the UVB-treated *S. virgaurea* L. extract demonstrated *in vitro* well-expressed DPPH radical-scavenging ability, SOD-like activity and antioxidant-protective capabilities due to the formation of stable *ortho*-semiquinone structures. The latter results prove that *S. virgaurea* L. extract is a promising ROS-scavenging antioxidant with UVB-protective properties and provoke future studies.

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Conflict of Interest: *The authors declare that they have no conflict of interest.*

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Quantification of catechin in *Acacia catechu* extract by non-derivative, first derivative UV/Vis spectrophotometry and FT-IR spectroscopy

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The aim of the present study was to quantify catechin in spray-dried extract of *Acacia catechu* by applying non-derivative (ND), first derivative (FD) UV/Vis spectrophotometry and FT-IR spectroscopy. The ND methodology at pH = 7.9 demonstrated to be the most sensitive, linear, precise, simple and accurate among all applied methods. Catechin content in two series of 12 *Acacia catechu* extract solutions (70% EtOH) at pH = 4.0 and pH = 7.9, respectively, was determined by the developed UV/Vis ND methods. The statistical analyses between the experimental data sets obtained by both techniques proved to be statistically significant. The highest catechin content in the non-diluted ethanol *Acacia catechu* extract was quantified as 169.88 mg/L at pH = 7.9 and 171.52 mg/L at pH = 4.0. The comparative analyses of the FT-IR spectra of pure catechin and *Acacia catechu* extract in powdered form and the insignificant bands width and intensity deviations proved undoubtedly the high content of the natural antioxidant in the plant extract. The latter conclusion was sustained by the established significant average percent recovery (97.17%) of catechin in the raw plant extract.

Keywords: *Acacia catechu*, catechin, UV/Vis, FT-IR

INTRODUCTION

Various natural antioxidants are being used to neutralize the harmful effects of reactive oxygen species (ROS) overproduced in diseased tissues and contaminated environments. Catechins are flavan-3-ols that are found widely in medicinal plants and are utilized for anti-inflammatory, antimicrobial, cardio protective, hepato-protective, neural protection, antimalarial [1] and other biomedical applications [2**],**

In view of the growing interest in bioflavanols, including catechins, scientific literature reports variety of methods for their extraction [3], separation and quantification in plant materials and food. Qualitative and quantitative determination of catechins has been significantly facilitated by HPLC techniques applying various detectors: UV, PDA [4], DAD, fluorescence, electrochemical, LC-MS, etc. [5,6]. Near-infrared spectroscopy, TLC and GC-MS have also been used for catechins quantification [7-10].

In this context, UV/Vis spectrophotometry is characterized as a simple technique with low operating costs giving fast and reliable results for polyphenols determination [11-13]. Among the main limitations, however, is the necessity of time consuming pre-separation techniques for interferences removal, as well as the low selectivity. In this respect, derivative UV/Vis spectrophotometry has been established as a vehicle to overcome such analytical problems encountered with conventional spectrophotometry, as it allows spectral interferences removal, increases assay selectivity and specificity, reduces noise and improves signal amplification [14]. Besides, the study of Zhou *et al.*, (2018) established the high feasibility of FT-IR spectroscopy for efficient detection of catechin monomers and caffeine in fresh tea leaves [15, 16]. In their study Park *et al*., (2015) assessed quantitatively the main extracted polyphenols from commonly consumed fruit and their antioxidant activities by FT-IR spectroscopy and proved the method suitability for bioactivity determination [17]. The results obtained by UV/Vis and FT-IR analyses on catechins/epicatechins contents and on the bactericidal activity of nanoparticles present in green tea extracts displayed high correlation, which indicated the applicability of both methods for polyphenols determination [13].

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In this respect, the present study was provoked by the valuable pharmacological properties and biomedical activities (antimicrobial, anti-inflammatory, antifungal, coagulant, vermifuge, antidiarrheal, etc.) of *Acacia catechu*, a plant indigenous in India, other Asian countries, and East Africa [18], and by the fact that currently there are no scientific reports on UV/Vis, derivative UV/Vis spectrophotometry and FT-IR methods for catechin quantification in *Acacia catechu* extract. Therefore, the aim was to quantify catechin in spray-dried extract of *Acacia catechu* by applying non-derivative (ND), first derivative (FD) UV/Vis spectrophotometry and FT-IR spectroscopy.

EXPERIMENTAL

Chemicals

(+)-Catechin hydrate (≥ 96.0 %, HPLC), C_2H_5OH (≥ 99.8 %, HPLC), CH_3COOH and NaOH (p.a., HPLC) were supplied by Sigma-Aldrich. *Acacia catechu* spray-dried extract was supplied from Northern India.

UV/Vis spectrophotometric analyzes

A catechin hydrate standard solution in 70% EtOH with initial concentration of 200 mg/L was prepared. Two series of 12 ethanolic catechin hydrate solutions each within the concentration range C_o 1 – 200 mg/L were prepared by the dilution method at pH = 4.0 and pH = 7.9, respectively. Two series of 12 ethanolic extract solutions at initial pH = 4.0 and pH = 7.9, respectively, were prepared by dissolving different quantities of the dried extract (1-20 μg) in 10 mL of 70% EtOH solutions. The pH of the standard and extract containing ethanol solutions was adjusted to pH = 4.0 by the addition of 2M CH_3COOH.

Catechin concentrations in EtOH were measured on a UV/Vis spectrophotometer DR 5000 Hach Lange (Germany), supplied with 10 mm quartz cells. All spectra were recorded in the UV region at λ = 281 nm at pH = 7.9 and pH = 4.0 with 2 nm slit width, 900 nm min^{-1} scan speed and very high smoothing.

FT-IR spectroscopy

FT-IR spectrum of the dry powdered *Catechu Acacia* extract was determined on Bruker Tensor 37 FT-IR spectrometer using KBr pellet technique. For the sample, 64 scans were collected at a resolution of 2 cm^{-1} over the 4000–400 cm^{-1} wavenumber region.

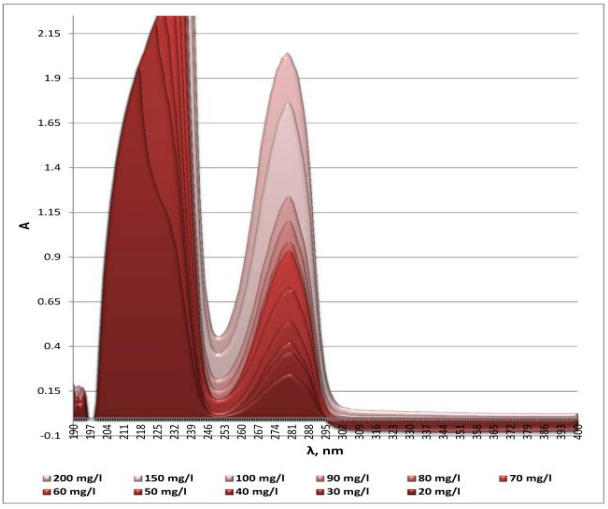
Statistical and Error Analysis

All experiments were carried out in triplicate, and the average values were taken to minimize random error. The values of the error criteria functions R^2, mean squared error (MSE), root mean square error (RMSE), mean absolute percentage error (MAPE), Durbin Watson (DW) function and Akaike information criterion (AIC) were determined by XLStat for Excel linear regression analyses.

RESULTS AND DISCUSSION

UV/Vis spectrophotometric analyses of standard (+)-catechin hydrate

The UV/Vis spectra of catechin hydrate ethanolic solutions in slightly alkaline medium (pH = 7.9) (Fig. 1) and acidic medium (pH = 4.0) (Fig. 2) were well resolved and displayed maximum absorbance in the UV region at λ 280 nm for the entire concentration range of 10 – 200 mg/L.



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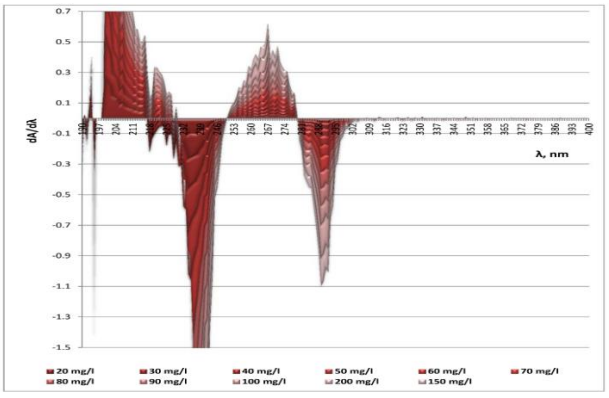

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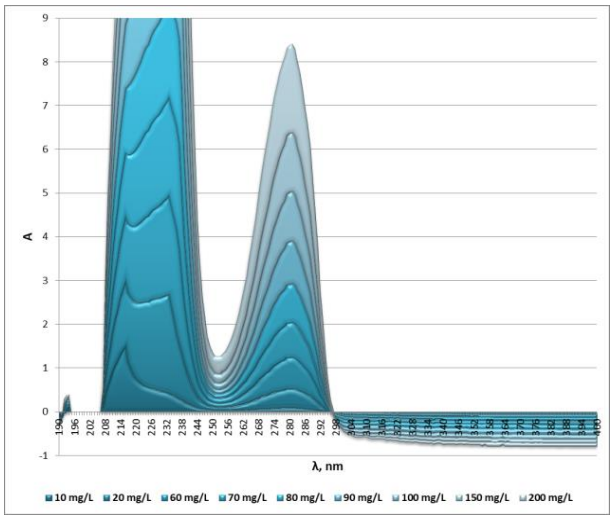
Fig. 1. **A**. Non-derivative and **B**. Derivative UV/Vis spectra of catechin hydrate solutions in 70% EtOH at pH = 7.9 (λ = 280 nm).



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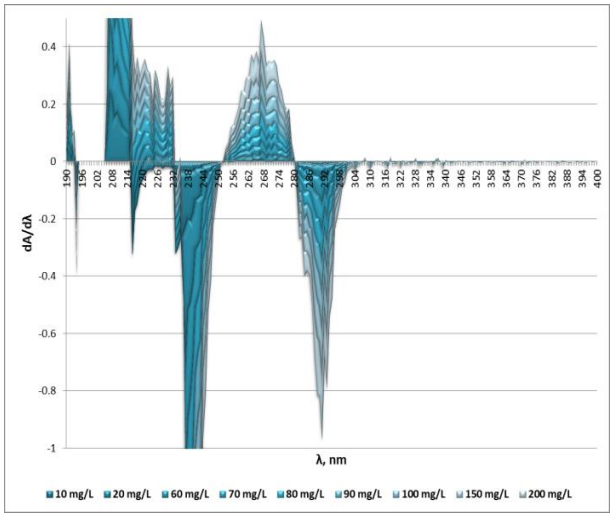
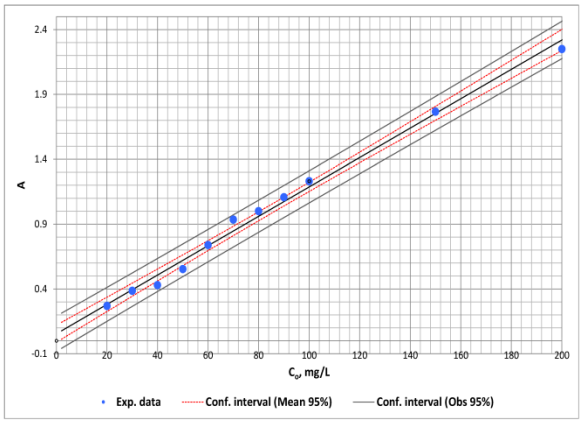


B.

Fig. 2. **A**. Non-derivative and **B**. Derivative UV/Vis spectra of catechin hydrate solutions in 70% EtOH at pH = 4.0 (λ = 280 nm).

The obtained standard curves of catechin hydrate (Fig. 3) are characterized with satisfactory linearity and accuracy as observed by the significantly low values of the regression coefficient (R^2), mean squared error (MSE), root mean square error (RMSE) and Akaike information criterion (AIC) (Table 1).

The Durbin Watson (DW) function is a test for autocorrelation in the residuals from a statistical regression analysis. The calculated DW value is within the interval (0; 2), which is indicative for positive autocorrelation between the concentrations data set. The mean absolute percentage error (MAPE) is a measure of prediction accuracy of a forecasting method in statistics. However, it could also be used as a loss function for regression problems in analytical chemistry. The MAPE value < 10 calculated in the present study is interpretative of the high accuracy of the analytical results.



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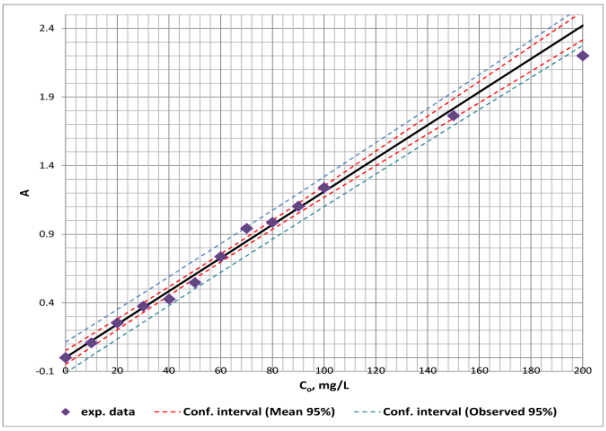


B.

Fig. 3. UV/Vis calibration curves of catechin hydrate at pH = 7.9 at λ = 280 nm: A. Non-derivative, **B**. Derivative UV/Vis spectrophotometry.

The developed UV/Vis methods at pH = 7.9 and pH = 4.0 employed standard catechin hydrate ethanol solutions. Based on the analyses of the experimental data it was established that the ND spectrophotometric techniques are characterized with lower LOD and LOQ values as compared to the FD methods, despite of the more favorable statistical error criteria analyses. Besides, the ND methodology at pH = 7.9 demonstrated to be the most sensitive, linear, precise, simple and accurate among all applied methods.

UV/Vis spectrophotometric analyses of Acacia catechu extract

To determine the concentration of catechin in *Acacia catechu* extract a stock solution was prepared by dissolving 20 mg of dry extract powder in 100 mL of 70% EtOH. Two series of 12 samples each were prepared by dilution of the stock extract-containing solution with 70% EtOH, at dilution ratios presented in Table 2, to a final volume of 10 mL.

Table 1. Values of error functions/criteria, lower limit of detection (LOD) and lower limit of quantification (LOQ) of catechin hydrate according to UV/Vis spectrophotometric analyses.

UV/Vis method	Non-derivative pH = 7.9	Non-derivative pH = 4.0	Derivative pH = 7.9	Derivative pH = 4.0
		Linear equation		
Error function	$A = 5.4055 \cdot 10^{-2} + 0.0113 \cdot C_o$	$A = 4.933 \cdot 10^{-3} + 9.8856 \cdot 10^{-3} \cdot C_o$	$A = 1.19 \cdot 10^{-3} + 1.2094 \cdot 10^{-2} \cdot C_o$	$A = 4.9632 \cdot 10^{-3} + 9.8175 \cdot 10^{-3} \cdot C_o$
R^2	0.993	0.982	0.993	0.983
MSE	0.003	0.006	0.002	0.006
RMSE	0.053	0.080	0.045	0.077
MAPE	5.180	13.728	5.789	13.462
DW	1.078	1.869	1.466	1.819
AIC	-68.532	-63.979	-72.602	-64.864
LOD (mg/L)	**6.368**	**11.903**	8.197	13.417
LOQ (mg/L)	**21.227**	**36.07**	24.84	40.657

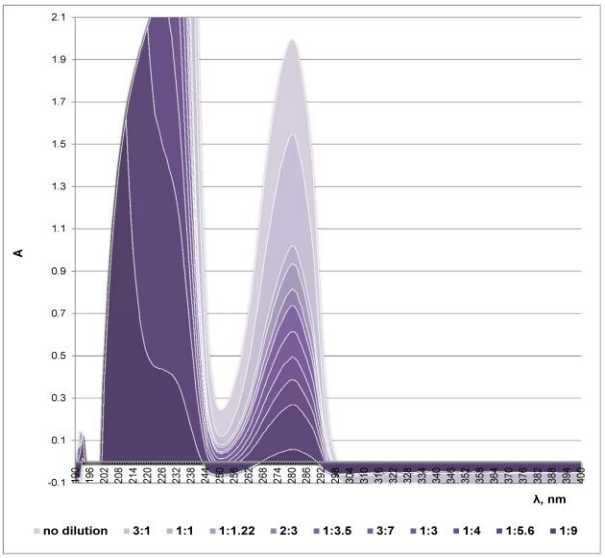
Table 2. Catechin contents and % recovery in *Acacia catechu* extract determined by non-derivative spectrophotometry.

Sample No.	Mixing ratio (v:v)	Extract concentration, mg/L	Abs	Catechin concentration, mg/L	Recovery, %	Abs	Catechin concentration, mg/L	Recovery, %
			pH = 7.9			pH = 4.0		
1	1:19	10	0.06	0.762	7.62	0.1055	10.170	101.70
2	1:9	20	0.158	7.812	39.06	0.237	23.472	117.36
3	1:5.6	30	0.268	17.724	59.08	0.334	33.285	110.95
4	1:4	40	0.385	28.549	71.37	0.3675	36.674	91.69
5	1:3	50	0.490	38.107	76.21	0.439	43.906	87.81
6	3:7	60	0.614	47.635	79.39	0.579	58.068	96.78
7	1:3.5	70	0.734	57.665	82.38	0.726	72.938	104.20
8	2:3	80	0.808	65.216	81.52	0.779	78.300	97.88
9	1:1.22	90	0.931	76.131	84.59	0.8775	88.264	98.07
10	1:1	100	1.015	83.240	83.24	0.9275	93.322	93.32
11	3:1	150	1.587	134.479	89.65	1.1985	120.735	80.49
12	no dilution	200	1.987	169.877	84.94	1.7005	171.516	85.76
					average 69.92%			average 97.17%

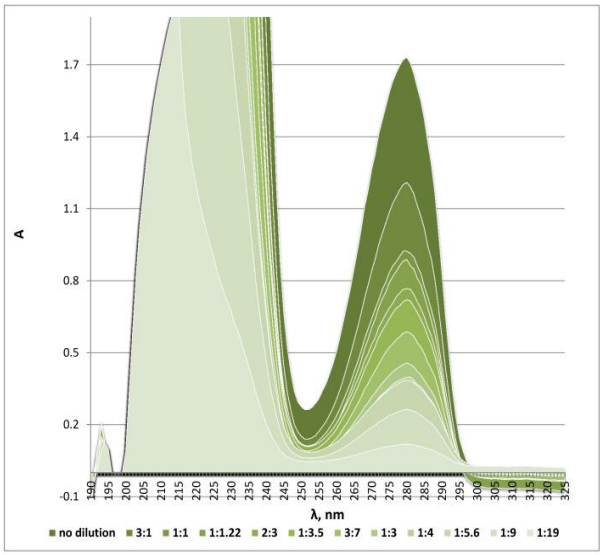
The concentrations of polyphenol in the extract series was determined by both ND UV/Vis methods. The UV/Vis spectra of catechin in slightly alkaline and acidic solutions (pH = 4.0) (Fig. 4 A, B) displayed maximum absorbance peaks in the UV region at λ 281 nm for the entire concentration range. The concentrations of the biologically active substance in the extracts and the percent recovery for both experimental series are presented in Table 2.

The highest catechin content in the non-diluted ethanolic *Acacia catechu* extract was quantified as 169.88 mg/L at pH = 7.9 and 171.52 at pH = 4.0. The recovery interval ranged from 80.49% to 117.36%, for the experimental series at pH = 4.0 and from 7.62% to 89.65% - at pH = 7.9. The recovery test measures the amount of the analyte present or added in the analytic portion of the test material that is recovered and could be quantified. The acceptable recovery intervals depend on the analytical complexity and the sample, and can range from 50 to 120% with precision up to ±15% [4, 19].

Obviously, the results obtained in an acidic medium are characterized with higher accuracy. Besides, the latter method is more suitable for scientific investigations with catechin, as it is well known that aqueous catechin solutions are unstable due to rapid oxidation/degradation, while ethanol solutions are characterized with higher stability, which is additionally increased by acidification of the solution [20, 21].



A.



B.

Fig. 4. UV/Vis spectra of *Acacia catechu* extracts at **A**. pH = 7.9 (λ = 281 nm); **B**. pH = 4.0 (λ = 281 nm).

Regarding the increasing number of investigations on plant polyphenols and their applicability for pharmaceutical, bio-medical and agricultural purposes in the last decade, as well as their encapsulation in various micro- and nano-matrices [22, 23], acetic acid is an appropriate acidifying agent [24]. Moreover, some methodologies such as encapsulation on natural mineral microparticles provoke pH increase during the incorporation process due to the alkaline nature of the supports, which in turn would provoke immediate photooxidation of catechin associated with unwanted color and structural changes influencing negatively the bioactivity of the natural polyphenol.

In this respect the developed accurate ND UV/Vis method in acidic medium will be valuable for quantification of catechin in various plant extracts and other studies subjected to catechin.

FT-IR study of Acacia catechu extract

The FT-IR spectra of catechin standard and *Acacia catechu* dry extract are presented on Fig. 5. The wavelengths of the characteristic bend assignments are given in Table 3.

Table 3. Characteristic bands on FT-IR spectra of catechin hydrate and *Acacia catechu* extract [13,25-27].

Bend assignments	Wavenumber, cm^{-1}	
	Catechin hydrate standard	*Acacia catechu* extract
C-H alkenes	965.3	970.16
-C-O alcohols	1020.1	1031.8
-OH aromatic	1144.1	1149.53
-C-O alcohols	1285.0	1245.97
Coordination bonding of -OH groups of aromatic ring	1364.9	1369.4
C-H bending vibration	1474.4	1475.49
C=C aromatic ring	1514.5	1523.71
C=C alkenes	1610.5	1618.22
coordination bonding of -OH groups of aromatic ring	1864.2	1865.1
methylene ($–CH_2$) C-H stretching	-	2923.9
O-H stretching	3412.0	3404.2; 3255.7

The comparative analyses of the FT-IR data outlined slight variations in the intensity and width of some peaks of the extract as compared to the standard, which could be explained as by the method of extraction so by the presence of a number of other polyphenols, tannins, carboxylic acids, etc. The broad bands at 3404.2 and 3255.7 cm^{-1} are corresponding to OH-groups. The absorption band at 2923.9 cm^{-1} indicates methyl ($-CH_3$) and methylene ($-CH_2$) stretching. The peak at 1523.71 cm^{-1} is indicative of C=C aromatic ring vibrations, while that at 1475.49 cm^{-1} is associated with alkane $-CH_2$ bending vibrations. The bands observed at 1865.1 and 1369.4 cm^{-1} outline coordination bonding of aromatic -OH groups. Peaks at 970.16, 1031.8 and 1245.9, 1149.5 cm^{-1} confirm the presence of alkene C-H, alcohol –C-O and aromatic –OH bending vibrations, respectively.

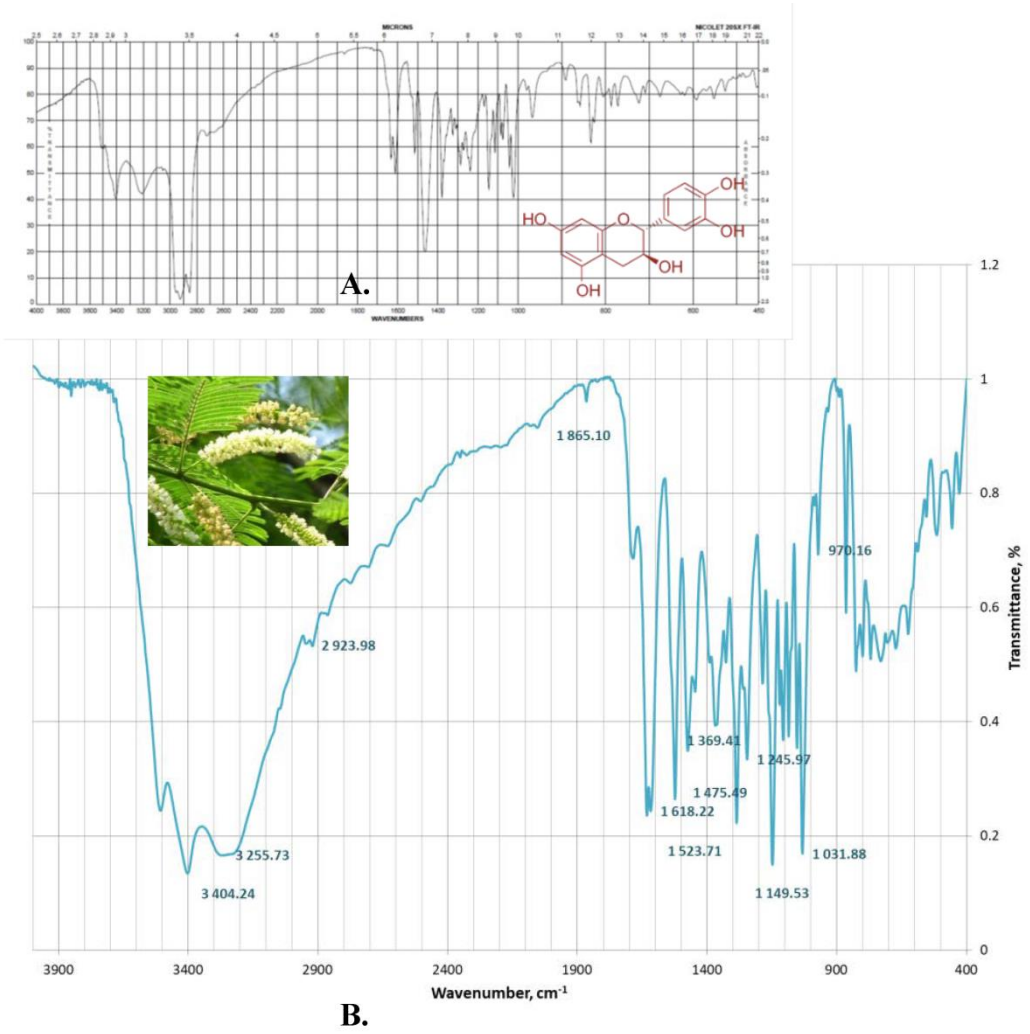


Fig. 5. FT-IR spectra of: **A**. catechin hydrate standard, **B**. Acacia catechu dry extract.

The comparative analyses of the FT-IR spectra of pure catechin and *Acacia catechu* extract in powdered form and the insignificant bands width and intensity deviations proved undoubtedly the high content of the natural antioxidant in the plant extract. The latter conclusion was sustained by the established significant average percent recovery (97.17%) of catechin in the raw plant extract.

CONCLUSIONS

In conclusion, based on the analyses of the experimental data it was established that the ND UV/Vis spectrophotometric techniques are characterized with lower LOD and LOQ values as compared to the FD methods, despite of the more favorable statistical error criteria analyses. Besides, the ND methodology at pH = 7.9 demonstrated to be the most sensitive, linear, precise, simple and accurate among all applied methods. The recovery interval of catechin in *Acacia catechu* extract determined by the ND technique at pH = 4.0 (80.49% - 117.36%) was within the acceptable values and proved the accuracy of the method. The applied UV/Vis and FT-IR assays can be successfully applied as quality and quantity control methods for determination of catechin in ethanol plant extracts.

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Conflict of Interest: *The authors declare that they have no conflict of interest.*

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Antioxidants in coffee: a DFT mechanistic study of the free radical scavenging activity

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Coffee is the single biggest source of antioxidants in our diet. Theoretical calculations at B3LYP/6-31+G(d,p) level were used for the evaluation of five reaction descriptors (thermodynamic quantities) – BDE, IP, PA, PDE and ETE. Those values are related to three possible antioxidant mechanisms: hydrogen atom transfer (HAT), single-electron transfer followed by proton transfer (SET-PT), and sequential proton loss electron transfer (SPLET) mechanisms. PCM implicit solvation model was used to simulate water environment for four coffee components - 5-O-caffeoylquinic acid, caffeic acid, cafestol and quinine. Both acids were the most potent antioxidants with similar activity for both mechanisms probable in water (SET-PT and SPLET), SPLET being the energetically favored one. Cafestol and quinine have weaker activity considering their aliphatic hydroxyl groups.

Keywords: Antioxidants, coffee, cafestol, caffeic acid, chlorogenic acid, quinine, DFT

INTRODUCTION

Coffee is one of the world's most popular beverages. We have estimated that worldwide people consume more than 2.9 billion cups of coffee every day [1, 2]. Drinking coffee stimulates us and provides an energy boost – that is why most people do it. Moreover, over the past few years, a series of studies have come out showing that drinking coffee has significant health benefits, such as a lower risk of: cancer (liver, colon and rectal cancer), type 2 diabetes, heart failure, premature ageing, cognitive decline, etc. [3-7]. Coffee is (potentially) identified as the biggest source of antioxidant power over the world: a new research indicates that taking 3-5 cups of coffee can serve up to 60 percent of daily phenolic antioxidant intake requirement. Coffee has more antioxidants than both green and black teas, red wine, dark chocolate…and even berry fruits [8]!

Although caffeine is not a particularly strong antioxidant [9], the beverage also contains a variety of other substances (phenolic and non-phenolic) with potential radical scavenging ability – caffeic acid (CA), chlorogenic acid (CGA), cafestol, trigonelline, hydroxycinnamic acid, quinine and melanoidins are only some of them. Some benefit the organism's natural antioxidant mechanisms [10]. The major phenolic compounds in coffee are chlorogenic acids [11]. Chlorogenic acids are a group of esters involving quinic acid (*(3R,5R)*-1,3,4,5-tetrahydroxycyclohexane-1-carboxylic acid) and caffeic, ferulic or *p*-coumaric acid. There are a few isomers due to quinic acid having a few hydroxyl sites available. The 5-O-caffeoylquinic acid is part of melanoidins. Cafestol (a natural diterpenoid) and quinine (a natural cinchona alkaloid that has been used for centuries in the prevention and therapy of malaria) are non-phenolic compounds. Non-phenolic terpenoids from three hydrocarbon classes – monoterpenes, sesquiterpenes and diterpenes) have been found capable of acting as C–H scavengers (*via* HAT mechanism dominant in the gas phase and SPLET mechanism in polar medium) [12]. Sugar alcohols have been shown to have oxy-radical scavenging abilities dependent on the number of aliphatic hydroxyl groups [13].

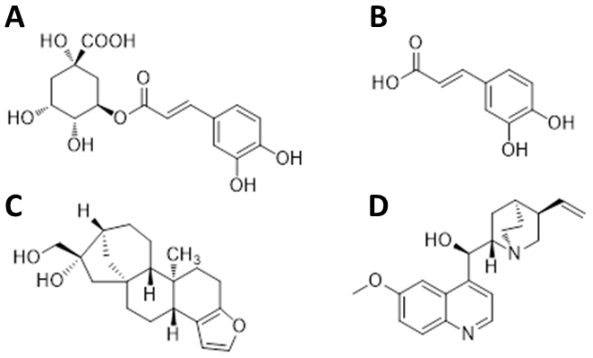

Figure 1. Chemical structures of chlorogenic acid (A), caffeic acid (B), cafestol (C) and quinine (D).

As there are many sorts of coffee beans and plenty of brewing methods, it is reasonable to mention the optimal variants for maximizing antioxidant concentrations. Current research shows that lightly roasted coffee beans combined with hot brew extraction methods show the best results [7, 14].

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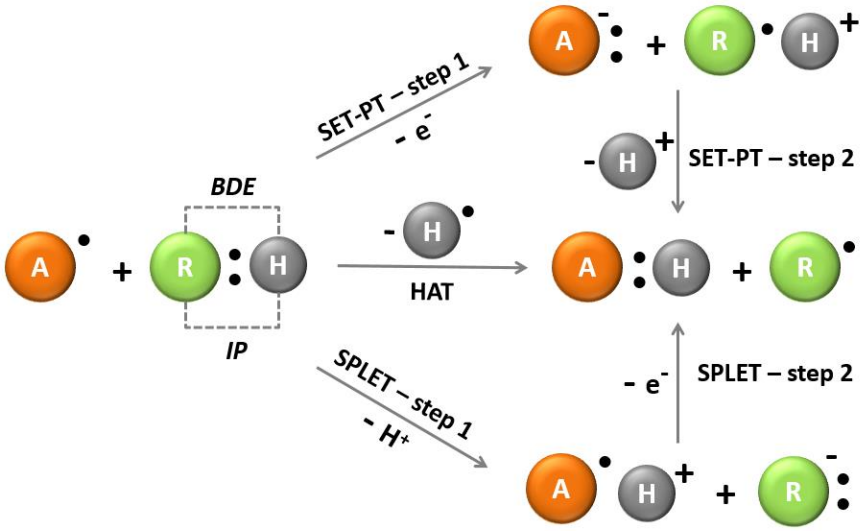
Roasting the beans has negative effect on subsequent caffeine and chlorogenic acid concentrations. Applying heat during the extraction process, however, is favorable. The quality of the coffee is also critical, as higher quality beans produce higher antioxidant concentrations [15].

The goal of our computational study is to shed light on the structure of some antioxidant active compounds in coffee and examine the mechanisms of their radical scavenging activity, including Hydrogen Atom Transfer (HAT), Sequential Proton Loss Electron Transfer (SPLET) and Sequential Electron Transfer – Proton Transfer (SET-PT) (shown in Scheme 1). The enthalpies of every step were calculated in water by means of Density Functional Theory (DFT) calculations.

Computational Details

Gaussian 16 [16] was used to perform quantum chemical calculations at the B3LYP/6-31+G(d,p) level of theory. B3LYP functional was chosen because it provides reliable geometries, frequencies, and bond lengths [17]. It has been reported that B3LYP used for evaluating the activity of phenolic hydroxyls complies well with experimental data [18, 19]. The 6-31+G(d,p) basis set was used by Koleva *et al.* to predict antioxidant activity for phenolic compounds [19]. Furthermore, increasing the basis set does not contribute to significant increase in accuracy [20]. An implicit solvation method (Polarizable Continuum Model, PCM [21]) was utilized to simulate solvation of the molecule species in water (ε=78). Geometry optimization was confirmed with frequency calculation at the same computational level to establish absence of negative frequencies. Possible intramolecular interactions (H-bonds) were taken into account in the initial geometries of the parent antioxidant structures. Radical scavenging activity was calculated as dissociation enthalpy for three distinct mechanisms, most often discussed in the literature [22, 23] and shown to occur in various conditions, illustrated below (Scheme 1).



Scheme 1. Possible mechanisms which describe antioxidant reactions (some mechanisms consist of two-step reactions). The free radical is designated with "A", the active molecule – with "R".

Radicals in non-singlet state are calculated using unrestricted B3LYP (UB3LYP). For every one of the studied molecules, enthalpies are calculated for the neutral molecule, radical, cation radical and anion states. Enthalpies for the hydrogen, proton and electron were calculated on the same level of theory. For the electron, a model proposed by Kumar *et al.* was used [24]. For the proton a standard solvation model was applied [25]. The obtained values are as follows: $H_{H^{\cdot}}$ = -312.45 kcal mol^{-1}, H_{H^+} = -236.00 kcal mol^{-1} and H_{e^-} = -48.70 kcal mol^{-1}.

Bond dissociation enthalpy (BDE) represents the reaction enthalpy of hydrogen atom abstraction or hydrogen atom transfer (HAT). Ionization potential (IP) is the enthalpy change from the loss of electron and formation of cation radical. Proton affinity (PA) is the enthalpy change for the dissociation of a proton and formation of an anion. Proton dissociation enthalpy (PDE) is the energy required for the cation radical to lose a proton during the SET-PT mechanism and lastly we have electron transfer enthalpy (ETE) when the anions become radicals in the SPLET mechanism.

The chlorogenic acid, modeled by us, is 5-O-caffeoylquinic acid. Cafestol and quinine were considered only as aliphatic O-H scavengers when discussing the SET-PT and SPLET mechanisms. BDEs of selected C-H bonds were calculated.

RESULTS AND DISCUSSION

The antioxidant active compounds selected for this study are chlorogenic acid, caffeic acid, cafestol and quinine (Fig. 1). The enthalpy of dissociation of their O−H bonds is calculated for three different mechanisms - HAT, SET-PT and SPLET (Scheme 1). The HAT mechanism only has a single step. For SET-PT and SPLET we have IP and PA as first step metrics, respectively.

An initial screening of the possible rotamers with H-bonds was performed for chlorogenic acid, caffeic acid and cafestol. Cafestol's rotamers are energetically identical in gas-phase calculations, so only one was considered. The energetically preferred rotamers of chlorogenic and caffeic acids and structures of cafestol and quinine are illustrated in Fig. 2.

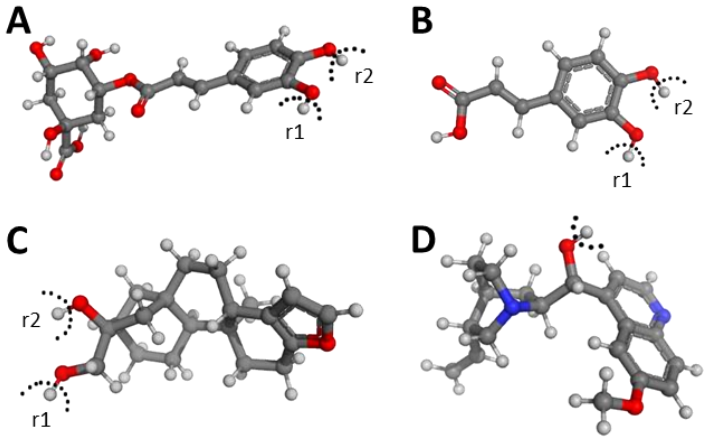


Figure 2. Optimized geometries of chlorogenic acid (A), caffeic acid (B), cafestol (C) and quinine (D). The dotted lines indicate the O-H bonds that participate most readily in antioxidant activity.

Table 1. Results obtained (in kcal mol^{-1}) for the BDE, the ionization potentials (IP), and the Proton Affinity (PA) of the neutral species, the Proton Dissociation Enthalpy (PDE) of the cation-radical and the Electron Transfer Enthalpy (ETE) of the anionic species.

	BDE	IP	PA	PDE	ETE
	ROH --> RO· + H·	ROH --> $ROH^{+\cdot}$ + e^-	ROH --> RO^- + H^+	$ROH^{+\cdot}$ --> RO· + H^+	RO^- --> RO· + e^-
CGA (r1)	76.77	86.98	48.68	17.55	48.93
CGA (r2)	78.71		50.79	19.48	48.75
Caffeic acid (r1)	76.90	87.34	48.55	17.31	49.19
Caffeic acid (r2)	78.82		50.59	19.23	51.11
Cafestol (r1)	100.09	83.17	68.74	44.67	52.19
Cafestol (r2)	99.15		69.86	43.73	50.13
Quinine	98.58	78.63	71.07	47.70	48.35

The calculated results for the different thermodynamic characteristics in water are collected in Table 1.

BDEs

CGA has the lowest BDE (76.77 kcal mol^{-1}) for r1 radical formation. It turns out that the BDEs calculated for CGA do not differ at all from those of parent caffeic acid (77.67 *vs.* 76.90 kcal mol^{-1} and 78.71 vs. 78.82 kcal mol^{-1}). The BDEs calculated for the hydroxyl groups of cafestol and quinine are high. According to the BDEs in water the ease by which OH-hydrogen atom abstraction from the compounds may be released approximately follows the order: CGA ~ caffeic acid > quinine > cafestol. When calculating C-H BDEs of tertiary carbon atoms of quinine and cafestol we get energies closer to the ones for hydroxyl groups of CA and CGA (Fig. 3). Despite this, when experimentally measuring antiradical activity, the values differ significantly. Caffeic acid (20 μg/ml) reduces 93.9% of DPPH [26] while the same amount of quinine reduces about 20% [27].

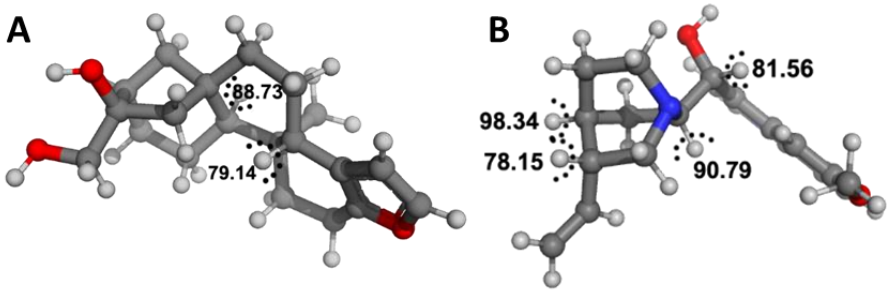


Figure 3. BDE of C-H bonds of tertiary carbon atoms (kcal/mol) of cafestol (a) and quinine (b).

A DFT study on the antioxidant activity of oleuropein (a phenylethanoid, a type of phenolic compound found in the olive leaf) suggests that since tertiary carbon atoms are usually tucked inside the molecule, the DPPH test is not a good method for measuring that activity [28].

IPs

The instability of cation-radicals is a reason for a higher IP value, and hence, for a lower reactivity. The most stable cation-radical is that of quinine. The IP of caffeic acid (87.34 kcal mol^{-1}) is the highest among all tested compounds, next is the IP of CGA. The order of decreasing the compounds' propensity to give up an electron is as follows: quinine > cafestol > CGA > caffeic acid. (The most powerful reducing agent in water is quinine (78.63 kcal mol^{-1}), and the most resistant to oxidation is caffeic acid (87.34 kcal mol^{-1})).

PDEs

The cation radicals lose their excess protons quite easily. The most stable once again prove to be quinine and cafestol. The caffeic and chlorogenic acids have similar values for both r1 and r2 radicals – very low at ~17 and ~19 kcal mol^{-1} respectively. This means that for the SET-PT mechanism the first step is the limiting one.

PAs

PA represents the reaction enthalpy of the protonic dissociation of the hydroxyl group from a neutral molecule, which is the first step in the SPLET mechanism. A reverse relation with IPs is observed with caffeic acid being the most reactive and quinine the most resistant. Caffeic acid and CGA have phenolic hydroxyl groups, so deprotonation is achieved more readily due to delocalization of the electron density.

ETEs

ETEs characterize the anions propensity for electron donation. With respect to ETE, the most active scavenger would be quinine (48.35 kcal mol^{-1}). Next come CGA (48.93 and 48.75 kcal mol^{-1}), followed by caffeic acid (49.19 and 51.11 kcal mol^{-1}). Least active toward radicals is cafestol (52.19 and 50.13 kcal mol^{-1}). The difference between all compounds is overall rather small.

CGA are a wide group of esters. The one we study is 5-O-caffeoylquinic acid. We focus on the phenolic hydroxyl groups, which are on the caffeic fragment of the ester (Fig. 2 A). Comparing all the studied values for CGA and CA, we see that they are very similar. The explanation is that the quinic acid fragment of CGA does not influence the radical scavenging properties of the caffeic fragment. Experiments show, however, that chlorogenic acids and caffeic acid have different antioxidant abilities [29]. We have to assume that those differences come from the quinic fragment of the ester.

The most readily occurring mechanism with similar values for both steps is SPLET, where CGA and caffeic acid are more reactive than cafestol and quinine. This is also valid when looking at the enthalpies of the HAT reaction. At physiological pH this mechanism would be even more favored. We are assuming therefore that out of the four compounds studied, those two are more important for direct radical scavenging abilities of coffee.

Quinine has an interesting reactivity. When looking at the steps that involve losing an electron – first step of SET-PT and second step of SPLET (IP and ETE values), we notice that it is energetically feasible, even when the other step of the mechanism might not be. In the case of SET-PT when the formation of the cation radical is the rate determining step, we cannot reject the possibility of the reaction going all the way.

CONCLUSIONS

Quantum chemical modelling was utilized to study 3 possible mechanisms (HAT, SPLET, SET-PT) of antioxidant activity for four compounds found in coffee (5-cafeoylquinic acid, caffeic acid, cafestol and quinine). Enthalpies for every step of the radical scavenging reaction were obtained (BDE, IP, PA, PDE, ETE). Of the two mechanisms most probable in water solution, SPLET is the more energetically feasible with 5-cafeoylquinic acid and caffeic acid being the best candidates with similar energy profiles, as also reported in literature [25, 30].

Cafestol's and quinine's O-H bonds are characterized with high BDEs in water (and the BDEs in non-polar environment are anticipated to be even higher), so the homolytic dissociation of the aliphatic OH group appears to be improbable. BDEs of C-H bonds of some of their tertiary carbons are comparable to those of caffeic acid. The O-H groups have ionization potentials with similar enthalpy, so SET-PT should still be considered a viable mechanism for them.

It is shown that even molecules in coffee without hydroxyl groups can positively influence the body's natural antioxidant abilities (trigonelline, caffeine) [4, 31]. While quinine and cafestol might not be as reactive as chlorogenic and caffeic acids, their effect as antioxidants cannot be overlooked. The wide variety of compounds with such properties in coffee and the variety of viable mechanisms they can exercise that activity with, makes the brew the best source of antioxidants accessible in everyday diet [8].

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Chemical composition, antioxidant and antimicrobial activity of essential oils from leaves and flowers of *Rosmarinus officinalis* L.

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The chemical composition, antioxidant and antimicrobial activity of essential oils obtained from leaves and flowers of rosemary (*Rosmarinus officinalis* L.) were examined. The essential oils were extracted by steam distillation and their chemical composition was determined by GC/MS. The antioxidant activity was studied by the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging assay. The main components of the essential oils were ρ-cymene, linalool, bornanone, α-pinene and β-pinene. Regarding the DPPH radical scavenging ability, the essential oil from leaves showed slightly higher activity than that obtained from flowers. The antimicrobial activity of the essential oils against pathogenic (*Staphylococcus aureus* NBIMCC 3703, *Salmonella* sp. (clinical isolate), *Pseudomonas aeruginosa* NBIMCC 1390, *Bacillus subtilis* NBIMCC 1208, *Escherichia coli* NBIMCC 3702) microorganisms was examined by the disc-diffusion method. Gram-positive bacteria were more sensitive to the oils (inhibition zones being between 12.00 and 12.50 mm) and the minimum inhibitory concentration was 60 ppm; Gram-negative bacteria were less sensitive. The results demonstrated that essential oils could be used as a biopreservative agent.

Keywords: rosemary (*Rosmarinus officinalis* L.), essential oil, antioxidant activity, chemical composition, antimicrobial activity

INTRODUCTION

Essential oils are commonly used as natural preservatives and fragrances in cosmetic products. More recently, mainly thanks to their antimicrobial properties, new applications as food preservatives, natural pesticides in organic agriculture and insecticides are emerging [1]. Rosemary (*Rosmarinus officinalis* L.), which belongs to mint family is widely spread and cultivated in the Mediterranean region. The chemical composition of the oil of rosemary from various geographical origins has been extensively studied. Rosemary essential oil includes phenolic constituents in its composition. Due to this composition which mainly involves monoterpenes like 1,8-cineole, α-pinene, camphor, camphene, rosemary essential oil has many therapeutical indications [2]. The effects of growth location, environmental characteristics, soil properties, micronutrients and vegetative stage have been pointed out. The essential oil and various extracts of rosemary have provoked interest as sources of natural products. They have been screened for their potential uses as alternative remedies for the treatment of many infectious diseases [3]. Particularly, the antimicrobial and antioxidant activities of plant oils and extracts have formed the basis of applications, including raw and processed food preservation, pharmaceuticals, alternative medicine and natural therapies. Because of the possible multiple resistances and side effects of the synthetic antimicrobial, increasing attention has been directed towards natural antimicrobial.

The purpose of the present study was to determine and compare the chemical composition, antioxidant and antimicrobial activity of essential oils obtained from leaves and flowers of *Rosmarinus officinalis* L. against some pathogens.

MATERIALS AND METHODS

Chemicals

All solvents and reagents were purchased from Sigma-Aldrich (Steinheim, Germany).

Essential oil extraction and chemical substances

Essential oils from leaves and flowers of *Rosmarinus officinalis* L. were used for the conduction of the experiments.

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The essential oil content was determined by steam distillation [4]. Steam distillation, the method used for essential oil extraction, takes advantage of both the volatility of a compound to evaporate when heated with steam and the hydrophobicity of the compound to separate into an oil phase during condensation.

Dried leaves and flowers of *Rosmarinus officinalis* L. were used for the extraction of essential oils, respectively. The extraction was carried out during 4 h from the first drop of distillate until the amount of essential oils stabilized. The oils were collected in glass tubes and were kept at 4°C till further use.

Gas chromatography/ mass spectrometry (GC/MS) analysis

The composition of the oils was determined by gas chromatography with mass selective detector (GC/MS) [5]. The GC/MS analyses were performed on a 7890A gas chromatograph (Agilent Technologies) coupled to an 5975C quadrupole mass spectrometer (Agilent Technologies). The analytes were separated on a HP-5MS capillary column (30 m×0.25 mm with a phase thickness of 0.25 μm). The split/splitless injector temperature was set at 250 °C and the temperature program was 60 °C for 3 min, 6 °C min^{-1} ramp rate to 250 °C and held constant for 3 min. The carrier gas was helium (99.999 %) at a 1 ml min^{-1} flow rate. In the SPME analysis, splitless injection (3 min) was used at 250 °C. The mass spectrometer was operated in the electron-impact mode (EI) at 70 eV. The identified components were arranged according to the retention time and their quantity is given in percentages.

The obtained mass spectra were analyzed using 2.64 AMDIS (Automated Mass Spectral Deconvolution and Identification System, National Institute of Standardization and Technology, NIST, Gaithersburg, MD, USA). The separated polar and nonpolar compounds were identified by comparison of their GC-MS spectra and Kovach retention index (RI) with referent compounds in the NIST 08 database (NIST Mass Spectral Database, PC-Version 5.0, 2008). The RIs of compounds were recorded with a standard *n*-hydrocarbon calibration mixture (C_{10}–C_{40}, Sigma-Aldrich) using the 2.64 AMDIS software.

DPPH radical scavenging assay

The ability of essential oils to scavenge free radicals was assayed with the use of a synthetic free radical scavenger compound 1,1-diphenyl-2-picrylhydrazyl (DPPH) (Sigma-Aldrich), according to the method employed in [6]. Briefly, essential oils were serially diluted (0.2, 0.4, 0.6, 0.8 and 1.0 mg/mL (w/v)) in methanol. A solution of DPPH (0.004% (w/v)) was prepared in the same solvent. Then 300 μL of each dilution were mixed with 2700 μL of DPPH solution. The reaction was performed at 37°C in darkness and the absorption at 517 nm was recorded after exactly 15 min against methanol. Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) (Sigma-Aldrich) was used as standard. Each test was performed in triplicate.

The antioxidant activity was calculated as follows:

$AA\% = [(Abs - Abs)/Abs] * 100$

AA: antioxidant activity

Abs: absorbance.

Determination of the antimicrobial activity against pathogenic microorganisms

- *Test microorganisms*: *Staphylococcus aureus* NBIMCC 3703, *Salmonella* sp. (clinical isolate), *Pseudomonas aeruginosa* NBIMCC 1390, *Bacillus subtilis* NBIMCC 1208, *Escherichia coli* NBIMCC 3702. All strains are deposited in the culture collection of the Institute of Food Preservation and Quality-Plovdiv, Agricultural Academy of Bulgaria.
- *Preparation of the suspensions of the test pathogenic microorganisms*: The test pathogenic microorganisms were cultured on LBG-agar (Luria Bertani Medium with glucose – agar medium, LB Broth, Miller-Novagen, Merck, Germany) at 37±1°C for 24-48 h. Using sterile loop biomass of the developed test pathogenic microorganisms were suspended in sterile saline solution in order to obtain suspensions of the test pathogenic microorganisms.
- *The antimicrobial activity was studied by the disc-diffusion method*: Agar disc-diffusion testing developed in 1940 [7], is the official method used in many clinical microbiology laboratories for routine antimicrobial susceptibility testing. Nowadays, many accepted and approved standards are published by the Clinical and Laboratory Standards Institute (CLSI) for bacteria and yeasts testing. In this well-known procedure, agar plates are inoculated with a standardized inoculum of the test microorganism. Then, filter paper discs (about 6 mm in diameter), containing the test compound at a desired concentration, are placed on the agar surface. The Petri dishes are incubated under suitable conditions. Generally, the antimicrobial agent diffuses into the agar and inhibits germination and growth of the test microorganism and then the diameters of inhibition growth zones are measured.

Sterile melted LBG-agar medium was poured in Petri dishes and after the hardening of the agar, the

dishes were spread-plated with suspensions of the test pathogenic microorganisms. Decimal dilutions of the essential oil in saline solution containing 1 % (v/v) Tween 80 were prepared. The experiments were conducted with dilutions of 1×, 10× and 100× in order to determine the MIC (minimum inhibitory concentration). The used paper discs were 6 mm in diameter. Six μL of the corresponding dilution were pipetted on the corresponding paper discs. Paper discs soaked in distilled water were used as blanks. The results were recorded as diameters of the clear zones around the paper discs, in millimeters, after 24-48 hours of incubation of the Petri dishes at optimal temperature for the growth of the corresponding test-microorganism 37°C [8]. The MIC was defined as the lowest concentration of the essential oil at which the microorganism does not demonstrate visible growth [9].

Statistical analysis

The experiments were performed in triplicate. The mean values and the standard deviations were calculated using MS Office Excel 2010. The MICs, in ppm, were calculated on the basis of the obtained results.

RESULTS AND DISCUSSION

Chemical composition

An essential oil is a concentrated hydrophobic liquid containing volatile chemical compounds from plants. Oil content in plants is a very small part (less than 5% of the dry matter content of the plant) and consists primarily of hydrocarbon terpenes [10]. Results obtained by GC/MS analysis of the essential oils of *Rosmarinus officinalis* L. are presented in Table 1.

Table 1. Chemical composition of *Rosmarinus officinalis* L. essential oils (leaves and flowers)

№	Compounds	RT	RI	Leaves	Flowers
				Area %	Area %
1	β-Thujene	5.62	928	0.47	0.21
2	α-Pinene	5.84	940	9.68	6.65
3	Camphene	6.27	955	4.44	3.24
4	Sabinene	6.78	967	0.16	0.16
5	β-Pinene	6.96	980	7.16	3.16
6	β-Myrcene	7.10	991	0.41	0.61
7	ρ-Cymene	8.16	1010	42.95	42.35
8	D-Limonene	8.26	1026	1.97	1.02
9	Eucalyptol	8.38	1029	4.27	4.31
10	cis-4-Thujanol	9.42	1031	0.11	-
11	Linalool	10.14	1037	6.48	20.40
12	L-Pinocarveol	11.50	1049	0.13	-
13	(+)-2-Bornanone	11.74	1068	10.40	6.40
14	δ-Terpineol	12.31	1086	0.29	2.19
15	endo-Borneol	12.40	1098	2.35	2.65
16	L-4-terpineneol	12.59	1155	0.64	0.66
17	α-Terpineol	13.00	1182	1.81	0.86
18	Myrtenal	13.11	1194	0.13	-
19	Bornyl acetate	15.40	1220	1.93	1.63
20	α-Copaene	17.83	1228	0.23	0.13
21	Caryophyllene	19.04	1230	1.40	2.20
22	Humulene	19.92	1238	0.21	-
23	γ-Muurolene	20.30	1246	0.20	0.23
24	Caryophyllene oxide	22.43	1253	1.55	0.56
25	Humulene-1,2-epoxide	22.89	1273	0.12	-
Total				99.49	99.62

*RT, retention time; RI, retention index (Kovach's index)

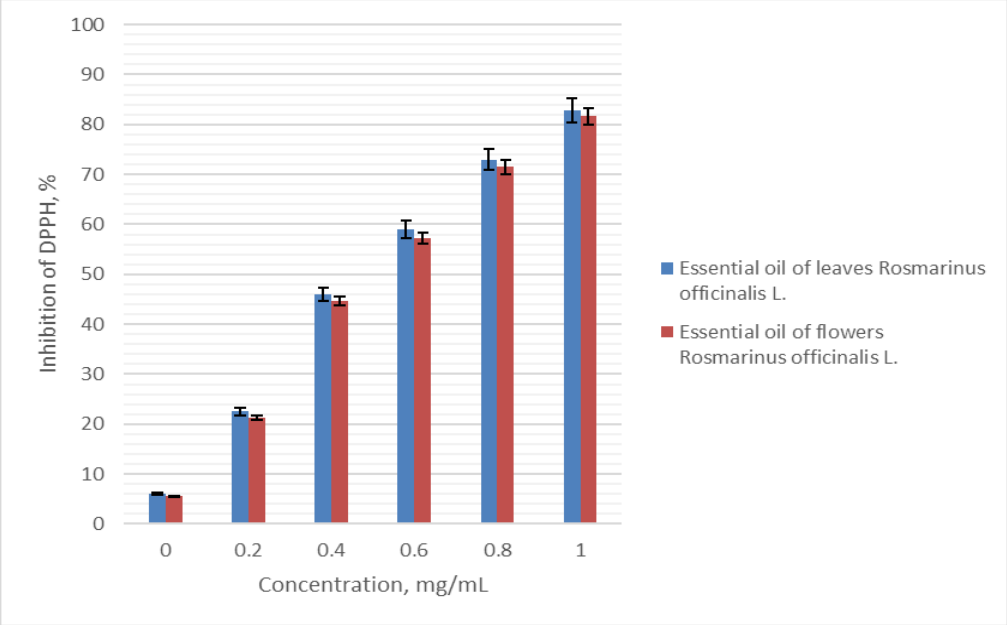

Fig. 1. Free radical-scavenging ability of *Rosmarinus officinalis* L. essential oils (leaves and flowers). The biological function of the chemical components of the *Rosmarinus officinalis* L. essential oil is not limited to their antioxidant and antimicrobial activity. Some of them also have antitumor (linalool, borneol), anti-inflammatory (pinene) and analgesic function [10, 16-18].

Twenty-five compounds were identified in the essential oil of leaves of *Rosmarinus officinalis* L. The main compounds were α-pinene (9.68%), β-pinene (7.16%), eucalyptol (4.27%), ρ-cymene (42.95%), camphene (4.44%), linalool (6.48%) and 2-bornanone (10.40%). Twenty compounds were identified in the essential oil of flowers of *Rosmarinus officinalis* L. The main compounds were the same, but linalool content was 20.40%. Linalool is contained in the flower of the aromatic plants, so the percentage in the essential oil of flowers of *Rosmarinus officinalis* L. is higher than the oil obtained from the leaves [11]. The chemical characterization by gas chromatography/ mass spectrometry (GC/MS) analysis of rosemary oils revealed the presence of many compounds, among them the most represented was ρ-cymene (42.95%) (leaves) and (42.35%) (flowers). This compound shows a variety of biological activities which include antioxidant, antinociceptive, anti-inflammatory, anxiolytic, anticancer and antimicrobial activities. Gema *et al.* [12] reported the presence of α-pinene, camphor and 1,8-cineole, constituting about 80% of the total *Rosmarinus officinalis* L. essential oil. Moreover, the major components α- and β-pinene, camphene, thimol, linalool were also reported to be in *Rosmarinus officinalis* L. essential oil [13]. The differences in chemical compositions of the *Rosmarinus officinalis* L. essential oils could be attributed to climatic effects on the plant.

These variations probably occur due to factors related to the oil extraction method, genetic characteristics of the species and environmental conditions in which they were grown.

Total antioxidant capacity based on the DPPH radical-scavenging assay

The antioxidant effect of rosemary is due to the polyphenols present in the leaves (mainly rosmarinic acid, carnosol and carnosic acid), which accumulate in the fatty membranes of cells where the antioxidant effect is required. One of the most significant aspects of the antioxidant activity of rosemary is the relationship between diterpenes and radical-scavenging activity. In this regard, the study by Munné-Bosch and Alegre [14] describes the antioxidant capacity of diterpenes in rosemary. The most important elements in the rosemary structure are the aromatic ring (C_{11}–C_{12}) in the catechol group together with the conjugation of the three basic rings. Fig. 1 shows the free radical-scavenging potential of different concentrations in the essential oils of leaves and flowers of *Rosmarinus officinalis* L., as determined by the DPPH assay. It could be seen from the results that, as the concentration of rosemary essential oils increased, the percentage of DPPH inhibition increased. The highest percentage of DPPH inhibition was for 1 mg/ml of leaves rosemary essential oil - 82.8% and of flowers rosemary essential oil - 81.7%. The antioxidant activity of both oils (leaves and flowers) is identical.

Wang *et al.* [15] reported that it is very difficult to attribute the antioxidant effect of a total essential oil to one or a few active principles (1,8-cineole, α-pinene and camphor), because an essential oil always contains a mixture of different chemical compounds. They also added that, in addition to the major compounds, also minor compounds may make a significant contribution to the oil activity. The results of antioxidant activity of *Rosmarinus officinalis* L. essential oil are needful by the food industry. *Rosmarinus officinalis* L. essential oil showed interesting results, being one of the best performing ones in terms of ability to neutralize free radicals.

Antimicrobial activity

Plant extracts and their secondary metabolites are rich sources of antimicrobial substances, including coumarins and psoralens, acetylenes, flavonoid and non-flavonoid polyphenols, and terpenes [19-23]. Monoterpenes (i.e., eucalyptol, borneol, camphor, bornylacetate, carvacrol, menthol, γ-terpinene, α-pinene, β-pinene, and p-cymene) are the most important constituents of essential oils produced through liquid extraction and steam distillation of edible and medicinal plants. In addition to the antibacterial and anti-biofilm activities of terpenes, in particular of p-cymene, an anti-inflammatory activity was also proved. It was concluded that limonene had an antibacterial effect weaker than the antifungal activity.

Table 2. Antimicrobial activity of *Rosmarinus officinalis* L. essential oils (leaves and flowers)

Test-microorganism	Leaves		Flowers	
	IZ, mm	MIC, ppm	IZ, mm	MIC, ppm
Staphylococcus aureus NBIMCC 3703	12.50±0.40	6	12.00±0.40	60
Pseudomonas aeruginosa NBIMCC 1390	9.00±0.47	600	9.00±0.47	600
Salmonella sp.	10.00±0.47	60	9.00±0.47	60
Escherichia coli NBIMCC 3702	9.00±0.47	>600	10.00±0.47	>600
Bacillus subtilis NBIMCC 1208	12.00±0.40	6	12.00±0.40	6

* IZ, inhibition zones; MIC, minimum inhibitory concentration

The results showed that both essential oils (from leaves and flowers of *Rosmarinus officinalis* L.) are active against all the pathogenic microorganisms (Table 2). Gram-positive bacteria were more sensitive to the activity of the essential oil, the measured zones of inhibition were 12.5 mm, the minimum inhibitory concentration was 60 ppm. The tested Gram-negative bacteria showed zones of inhibition between 9 and 10 mm, with a minimum inhibitory concentration of more than 600 ppm. The observed difference in the sensitivity of the different test microorganisms to the examined essential oil was due to the difference in the cell wall structure and composition of the two groups of bacteria. The inhibitory effect of rosemary is the result of the action of rosmarinic acid, rosmaridiphenol, carnosol, epirosmanol, carnosic acid, rosmanol and isorosmanol. They interact with the cell membrane, causing changes in genetic material and nutrients, altering the transport of electrons, leakage of cellular components and production changes in fatty acid. In addition, they also produced an interaction with the membrane of proteins that caused the loss of membrane functionality and its structure [12]. The presence of an outer membrane in Gram-negative bacteria hinders the diffusion of the essential oil through the membrane to the cytoplasm of the cell, making them more resistant to the action of the oil. Other investigations have shown the antibacterial activity of rosemary oil against *E. coli*, *Bacillus cereus*, *Staphylococcus aureus* [24], *Clostridium perfringens*, *Aeromonas hydrophila*, *Bacillus cereus* and *Salmonella choleraesuis*. Zaouali *et al.* [25] reported that, compared with *S. aureus*, the antimicrobial activity improves with the presence of α-pinene as a major component. This effect can be correlated with the fact that terpenes can disorganize the cell membrane, and therefore promote the lysis. The effectiveness of the essential oil of rosemary against *E. coli* is related to the combined action of the different minor components present in its volatile fraction and should not be associated with the action of any particular component, agreeing with the conclusions published by Zaouali *et al.* [25]. The results obtained for the different resistance of Gram-positive and Gram-negative bacteria to inhibitors of microbial growth were consistent with literature data [9, 26].

Antioxidant and antimicrobial activity of rosemary essential oil depend on the fruiting stage,

mode of extraction, presence of a synergistic effect with other components, and concentration of active components. If these aspects are taken into account, the application of this natural product can be complimented in different food systems such as sausages, vegetable, meat and fish canned food, chutneys, mayonnaise, ketchup, salad dressings, processed cheese and more. In view of its application, rosemary essential oil could be used in functional foods, pharmaceutical products, plant products and for food preservation.

CONCLUSIONS

Natural extracts can be obtained from leaves, flowers, peel and seeds. The studied rosemary essential oils (obtained from leaves and flowers of *Rosmarinus officinalis* L.) can act as antioxidant and antimicrobial agents in food products to replace synthetic additives. The chemical composition (determined by GC/MS), antioxidant ability (measured with 2,2-diphenyl-1-picrylhydrazyl (DPPH)) and their antimicrobial force (using a diffusion disk method with *Staphylococcus aureus* NBIMCC 3703, *Salmonella* sp., *Pseudomonas aeruginosa* NBIMCC 1390, *Bacillus subtilis* NBIMCC 1208, *Escherichia coli* NBIMCC 3702) were determined. The results show that both extracts are good antioxidant and antimicrobial agents *in vitro*, and their antioxidant and antimicrobial capacity can also be used in food products, acting as natural preservatives.

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Antiobesity, antioxidant and hepatoprotective properties of aqueous infusion of *Kochia scoparia* seeds in rats with diet-induced metabolic syndrome

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Metabolic syndrome (MS) is a disorder of energy homeostasis associated with oxidative stress. *Kochia scoparia* (KS) is used in traditional Chinese medicine to alleviate obesity, dyslipidemia, and other ailments. KS fruits contain triterpenoid and quercetin glycosides with anti-oxidant and anti-inflammatory activities. The seeds of KS are less widely studied.

The aim of this study was to determine the effects of aqueous infusion of KS seeds on energy metabolism and oxidative stress in rats with diet-induced MS. Fifty male Wistar rats were allocated into 5 groups: a control group, a MS group, and 3 groups of MS rats treated with an aqueous infusion of KS containing 1.5 g, 3 g, and 6 g seeds per 100 ml water (MS+1.5KS, MS+3KS, and MS+6KS, respectively). Control rats received regular rat chow diet, and all MS groups were fed high-fat high-fructose diet. KS-treated animals received the infusions as drinking water. After 12 weeks of dieting and treatment, the energy metabolism was evaluated, liver samples were examined histologically, and oxidative stress was determined by the levels of thiobarbituric acid reactive substances (TBARS). Rats from the MS group developed visceral adiposity, dyslipidemia, insulin resistance and TBARS elevation. The histological examination revealed liver steatosis and apoptotic bodies. KS treatment reduced visceral adiposity and improved dyslipidemia. TBARS levels were decreased in a dose-dependent manner. The highest strength of the infusion alleviated the histological liver impairment. The results demonstrate that the aqueous infusion of KS seeds possess antiobesity, anti-oxidant, and liver protective effects in rats with diet-induced metabolic syndrome.

Keywords: Rat, metabolic syndrome, oxidative stress, liver impairment, *Kochia scoparia* seeds, aqueous infusion

INTRODUCTION

Metabolic syndrome (MS) is a disorder of energy utilization and storage with high social impact. According to the International Diabetes Federation (IDF) it affects about 20–25% of the world adult population with increased prevalence in advanced ages [1]. At least one component of the syndrome is found in 26.9–41.2% of the adults at young age (18–30 years old) [2]. IDF defines MS as a cluster of metabolic abnormalities, including central obesity and at least two of the following: hypertriglyceridemia, reduced HDL-cholesterol, hypertension, and raised fasting plasma glucose. Insulin resistance and visceral adiposity are the major features in MS development [1]. In addition to the abnormalities of the glucose and lipid metabolism, MS is associated with increased level of oxidative stress [3]. According to some authors, oxidative stress can be considered as an additional main component of the syndrome [4]. MS is a risk factor for the development of cardiovascular diseases, type-2 diabetes, neuropsychiatric and other disorders. The rate of occurrence of MS and the severity of its complications determine the condition as one of the most important burdens to the healthcare system worldwide.

Kochia scoparia (KS) is a large annual plant of the family Amaranthaceae, native to Central and Eastern Europe and Asia. KS reproduces by seeds. The mature plant grows round and bushy. KS flowers are small, greenish, and inconspicuous at the end of branches. The fruits are star-shaped and clustered on the brunches. Each fruit contains a single teardrop-shaped brown to black seed. The commonly used name of KS is fireweed or burning bush because of the flaming red color that the plant takes on in autumn. KS is used as an attractive ornamental plant, as forage for cattle, sheep and horses, and for manufacture of brooms. In Japan, the seeds are used as a food garnish called Tonburi or field caviar. In traditional Chinese medicine they are used to treat a variety of diseases including obesity, dyslipidemia, and other ailments. In traditional Bulgarian medicine, the tea prepared from the seeds alleviates chronic liver disorders.

Regardless of the wide traditional use of KS, experimental data is scarce. There are several experimental studies finding beneficial effects of KS fruits in mouse models of dermatitis [5, 6], and asthma [7], as well as *in vitro* studies demonstrating

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anti-cancer activity in cell cultures [8, 9].

One experimental study demonstrated the antiobesity effect of an ethanol extract of KS fruits in high-fat fed mice [10]. KS fruits contain mainly the triterpenoid saponin, momordin Ic [11]. A number of flavone glycosides are also isolated from the fruits [12]. Triterpenoid saponins produce a variety of effects through their anti-inflammatory, antioxidant, hypocholesterolemic, and hypoglycemic properties [13].

The seeds of KS are less widely studied. A recently performed research reports on two phenolic compounds found in the seeds – hydroxytyrosol and morin hydrate, as well as several unknown compounds that should be determined further [14].

The aim of the current study was to determine the effects of aqueous infusion of KS seeds on the energy metabolism and oxidative stress in rats with diet-induced MS.

EXPERIMENTAL

Experimental animals

We used 50 male Wistar rats provided by the Vivarium of Varna Medical University. They were kept at a temperature of 20–25°C and 12-h light-dark cycle and had free access to food and drinking water. The study was ethically approved by the Bulgarian Food Safety Agency in the Ministry of Agriculture, Foods and Forestry. It was conducted in agreement with the national policies and the international guidelines (EU Directive 2010/63/EU for animal experiments).

Dieting and treatment

The animals were allocated into five experimental groups of 10 rats each: a control group (C), a metabolic syndrome group (MS), and 3 groups of MS rats treated with aqueous infusion of KS seeds (MS+1.5KS, MS+3KS, and MS+6KS).

Control rats received a standard rat chow diet and tap water. With each 100 g food consumed, the animals had a caloric intake of 279 kcal. According to the producer's recipe, the 100 g chow contains 20.48 g protein (29% kcal), 3 g fat (10% kcal), carbohydrates (61% kcal) in the form of starch (38.3 g) and sugars (4.32 g). This chow, utilized in the Vivarium for the normal animal feeding, was purchased from the Bulgarian forage producer TopMix. All other groups received a diet prepared by adding lard (20% w/w) and fructose (20% w/w) to the standard rat chow, and their caloric intake was 427 kcal/100 g food. The lard provided 42% kcal, and the added fructose accounted for 19% kcal as compared to 6% from simple sugars in the regular chow.

Throughout the experiment, the rats from the MS group were drinking tap water, while the rats from groups MS+1.5KS, MS+3KS and MS+6KS were drinking the corresponding aqueous infusions of KS seeds *ad libitum*. The infusions were prepared by soaking of 1.5 g, 3 g and 6 g seeds, respectively, in 100 ml boiling water for 15 min and straining after cooling down.

The duration of the study was 12 weeks.

Biological parameters

The food and water/infusions consumption per 5 rats in a cage was monitored daily and was calculated as a mean value per rat per day. The caloric intake was calculated. Body weight of animals was measured once weekly and weight gain was calculated. After animal sacrifice, the right retroperitoneal fat pads and the livers were dissected on ice and weighed. The corresponding organ indices were calculated as a ratio to the body weight $\times 10^3$. Liver samples were preserved in 10% neutral formalin for histopathological evaluation.

Insulin tolerance test (ITT)

ITT was performed in the last week of the experiment. After 6 hours of fasting, the animals were injected intraperitoneally with regular insulin (ActRapid), dissolved in saline, at a dose of 0.75 UI/kg. Blood sugar was measured by a glucometer (ACCU-CHEK Performa). Blood samples were taken by incision of the distal part of the tail [15] immediately before the injection of insulin (at time 0) and on the 30^{th}, 60^{th} and 90^{th} minute thereafter. The initial value of blood glucose level of each animal was referred to as fasting blood glucose.

Biochemical measurements

At the end of the experiment, blood was taken from the sublingual veins of the animals under ether anesthesia. The blood was centrifuged, and the serum samples were stored at -20°C for biochemical analyses. Serum triglyceride, cholesterol and alkaline phosphatase levels were measured on a spectrophotometer (AURIUS 2021, Cecil Instruments Ltd.) using colorimetric kits of BioMaxima S.A., Poland for the lipids and BioSystems S.A., Barcelona, Spain for alkaline phosphatase according to the instructions of the producers. Thiobarbituric acid reactive substances (TBARS) were determined as a marker of oxidative stress in serum. The method measures the color obtained as a result of the reaction of thiobarbituric acid with the lipid peroxides [16].

Histopathological examination

After 24-hour fixation, the samples were cut into appropriate portions and placed in embedding cassettes. The process continued with dehydration in progressively more concentrated ethanol baths, followed by a clearing agent (xylene) and finally molten paraffin wax infiltrated the samples. Next steps were to embed tissues into paraffin blocks, cut 5 μm slices and mount them on glass microscope slides. Ultimately, sections were stained with hematoxylin and eosin.

Statistical analysis

Results are presented as means ± SEM. The data were analyzed by one-way ANOVA followed by Dunnet's post-test to compare the MS group with all the other groups. The food and water consumption, the caloric intake, and the ITT were analyzed by two-way ANOVA with a column factor "animal treatment" and a row factor "time". Bonferroni's multiple comparison post-test was used to assess differences between groups. A level of $p < 0.05$ was considered significant. GraphPad Prism (GraphPad Software, Inc., La Jolla, CA, USA) statistical software was used.

RESULTS

Biological parameters

The consumption of food and water/KS infusion, as well as the caloric intake of the animals are presented in Table 1. The rats receiving high-calorie diet consumed significantly less amount of food and water than the rats on regular rat chow diet ($F(4,420)=48.92$, $p=0.0003$ for the food intake and $F(4,420)=8.104$, $p=0.0207$ for water/KS infusion intake). Despite of the lower food consumption of the MS groups, all of them had a higher intake of calories daily ($F(4,420)=27.37$, $p=0.0014$). The amount of the infusions drunk by groups MS+1.5KS, MS+3KS and MS+6KS did not differ from the amount of water drunk by group MS. KS infusions did not affect the food and caloric intake, either. In general, all the MS groups received similar amounts of food, liquids and calories.

Table 2 presents some of the biological measures. At the end of the study, the body weight did not differ significantly between the groups. However, the weight gain of the animals differed ($F(4,43)=4.709$, $p=0.0031$), and the rats from group MS had higher weight gain compared to the control group ($p<0.05$). There were no differences between the groups in respect to liver weight and liver index.

Lipid metabolism

The parameters characterizing visceral obesity are presented on Fig. 1. The weight of the retroperitoneal fad pads (Fig. 1A) was affected by the treatment ($F(4,43)=6.339$, $p=0.0004$), and the post-test demonstrated that it was significantly increased by the high-calorie diet in the group MS compared to group C ($p<0.001$) and reduced by the 3KS infusion compared to the MS rats ($p<0.05$). The fat index (Fig. 1B) was affected in the same way ($F(4,43)=7.118$, $p=0.0002$), and the post-test revealed the same effects of the diet and 3KS infusion ($p<0.001$ group MS vs. group C and $p<0.05$ group MS+3KS vs. group MS).

Table 1. Food and liquid consumption and caloric intake of experimental animals

	C	MS	MS+1.5KS	MS+3KS	MS+6KS
Food intake [g/rat/day]	22.79±0.20	18.58±0.19	16.88±0.18	18.6±0.12	18.49±0.16
Liquid intake [ml/rat/day]	34.35±0.27	29.03±0.32	26.87±0.28	29.72±0.27	27.49±0.28
Caloric intake [kcal/rat/day]	63.59±0.56	79.32±0.82	72.09±0.77	79.43±0.53	78.97±0.70

Table 2. Biological measures. ** $p<0.01$ (one-way ANOVA); $^{\#}$ $p<0.05$ *vs.* C (Dunnett's multiple comparison post-test)

Biological measures	C	MS	MS+1.5KS	MS+3KS	MS+6KS
Initial body weight [g]	241±4.42	239.8±5.97	239.2±4.48	239.4±4.24	240.2±8.24
Final body weight [g]	383.6±7.9	419.8±14.39	394.4±10.71	398.6±14.9	428.8±17.42
Weight gain [g] **	142.6±7.19	180±11.09$^{\#}$	146.7±8.14	146.4±19.41	188.6±14.3
Liver weight [g]	9.58±0.24	9.53±0.27	9.84±0.3	9.36±0.4	10.37±0.37
Liver index	2.602±0.06	2.394±0.06	2.55±0.07	2.39±0.05	2.502±0.07

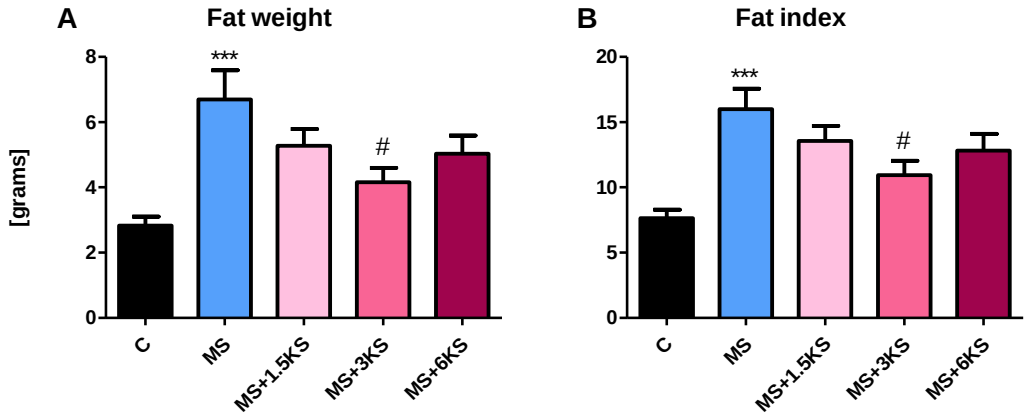

Fig. 1. Evaluation of visceral adiposity. Panel A: Weight of the right retroperitoneal fat pad. Panel B: Fat index. C – control rats; MS – rats receiving high-caloric diet; MS+1.5KS, MS+3KS and MS+6KS – rats receiving high-caloric diet and aqueous infusion of KS seeds of increasing strength (1.5KS, 3KS and 6KS, respectively); *** $p<0.001$ *vs*. C, # $p<0.05$ vs. MS (Dunnett's multiple comparison post-test)

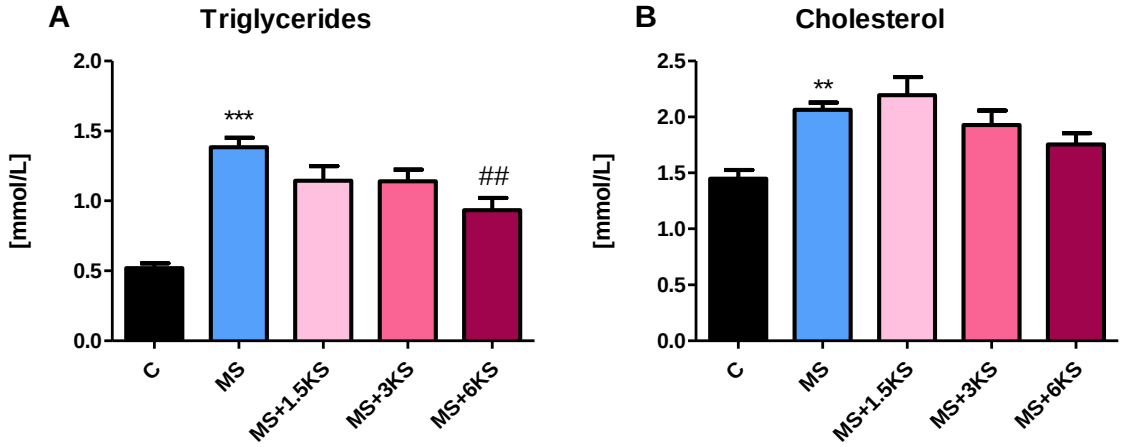

Fig. 2. Lipid profile. Panel A: Serum triglycerides. Panel B: Serum cholesterol. C – control rats; MS – rats receiving high-caloric diet; MS+1.5KS, MS+3KS and MS+6KS – rats receiving high-caloric diet and aqueous infusion of KS seeds of increasing strength (1.5KS, 3KS and 6KS, respectively); ** $p<0.01$, *** $p<0.001$ vs. C; ## $p<0.01$ vs. MS (Dunnett's multiple comparison post-test)

Table 3. Insulin tolerance test. * $p<0.05$ *vs*. C (Bonferroni post-test)

	30^{th} min [% of initial value]	60^{th} min [% of initial value]	90^{th} min [% of initial value]
C	59.83±2.02	44.29±3.04	43.86±2.36
MS	78.13±5.48*	56.13±5.38	52.88±5.29
MS+1.5KS	67.5±6.96	49.13±4.95	46.5±6.41
MS+3KS	89.75±4.50	57.75±2.56	53±3.85
MS+6KS	75.2±3.37	52.4±2.13	44.6±2.25

Serum lipids are presents on Fig. 2. The results from one-way ANOVA analyses showed a significant effect of the treatment on serum triglycerides ($F(4,42)=16.41$, $p<0.0001$) and cholesterol ($F(4,43)=5.864$, $p=0.0007$). The post-test confirmed that the rats from group MS had higher triglycerides ($p<0.001$) and cholesterol ($p<0.01$) compared to the control group, and that the strongest infusion reduced the serum concentration of triglycerides ($p<0.01$ vs. group MS).

Glucose metabolism

The results from the ITT are presented on Table 3. No differences were observed in the fasting blood glucose level between the groups. The ITT demonstrated lower sensitivity to insulin in the MS

group, significant at the 30th min after insulin administration, as revealed by the post-test following the two-way ANOVA analysis. KS infusions did not affect insulin resistance.

Oxidative stress

Serum concentrations of TBARS are presented on Fig. 3. One-way ANOVA revealed a significant difference between groups ($F(4,40)=2.918$, $p=0.0329$). The post-test showed an increased level of oxidative stress in group MS ($p<0.05$ *vs.* C). KS infusions demonstrated anti-oxidant properties in a dose-dependent manner with significant effect of the strongest infusion ($p<0.05$ *vs.* MS).

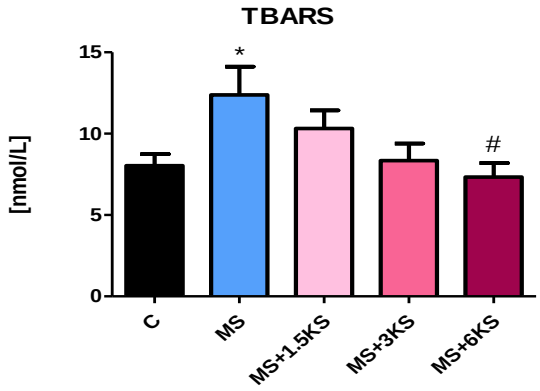

Fig. 3. Serum concentrations of thiobarbituric acid reactive substances (TBARS). C – control rats; MS – rats receiving high-caloric diet; MS+1.5KS, MS+3KS and MS+6KS – rats receiving high-caloric diet and aqueous infusion of KS seeds of increasing strength (1.5KS, 3KS and 6KS, respectively); * $p<0.05$ *vs.* C, # $p<0.05$ *vs.* MS (Dunnett's multiple comparison post-test.

Liver morphology and function

Fig. 4 presents the serum concentrations of alkaline phosphatase. One-way ANOVA revealed a significant difference between groups ($F(4,31)=5.838$, $p=0.0013$) and the post-test showed that alkaline phosphatase was elevated in rats from group MS ($p<0.05$ *vs.* C). The highest strength of KS infusion reduced alkaline phosphatase level, although insignificantly.

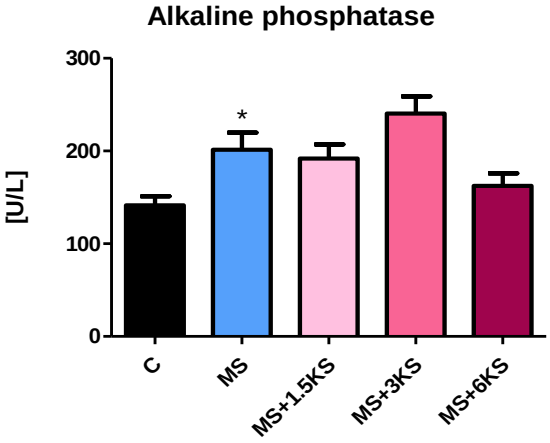

Fig. 4. Serum concentration of alkaline phosphatase. C – control rats; MS – rats receiving high-caloric diet; MS+1.5KS, MS+3KS and MS+6KS – rats receiving high-caloric diet and aqueous infusion of KS seeds of increasing strength (1.5KS, 3KS and 6KS respectively); *$p<0.05$ *vs.* C (Dunnett's multiple comparison post-test)

The histopathological examination of the liver samples is presented on Fig. 5. Compared to normal morphology of the liver samples of control rats, the liver tissue of the MS group displayed focal both macrovesicular and microvesicular steatosis in all rats. In most of the samples, there were apoptotic bodies and unspecific granulomas. The strongest KS infusion alleviated the signs of steatosis. In 4 of the animals from group MS+6KS the liver tissue displayed normal morphology. In the remaining 6 rats, there was only microvesicular steatosis.

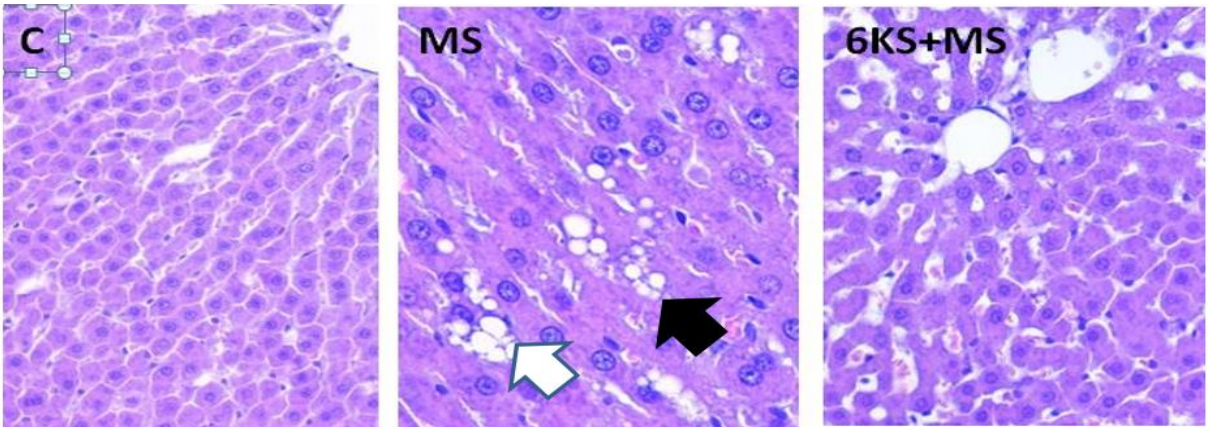

Fig. 5. Histopathological examination of liver tissue. The white arrow points to macrovesicular steatosis and the black arrow points to microvesicular steatosis (Hematoxylin-eosin, magnification ×100).

DISCUSSION

Experimental models of MS are a commonly used tool for studying the pathogenesis, prevention and therapy of the condition. In humans, obesity and MS usually result from non-rational nutrition with high caloric intake. Therefore, the dietary induced models, relying on increased intake of animal fats and simple sugars, are considered to mostly mimic the pathogenesis of the syndrome in humans. In our previous study, we demonstrated the ability of high-caloric diets to induce many features of MS in rats [17]. In the current study, we confirmed that the enrichment of rat chow with lard and fructose was able to produce visceral adiposity, hypertriglyceridemia, hypercholesterolemia, and insulin resistance, as shown by the experimental animals from group MS. In addition, they displayed the presence of oxidative stress, demonstrated by elevated serum concentration of TBARS, and signs of hepatosteatosis.

In the current study, the rats receiving high-fat high-fructose diet consumed less amount of food compared to control animals, probably as a result of the satiety effect due to the high lipid content of the food and/or because of stimulation of gut hormones [18]. The lower food intake in MS groups was expected as we have had already observed this previously [17]. Despite the lower food consumption of MS rats, all of the MS groups had higher daily intake of calories due to the higher calorie content of the diet. KS did not change food consumption, thus showing no effect on appetite. We measured the liquid intake during the experiment in order to monitor the consumption of KS infusions. The rats receiving high-caloric diet consumed less fluids daily compared to the control rats, an effect already described by Kaunitz *et al.* [19]. The type of liquid was not a factor determining the consumption: all rats given KS infusions drank similar amounts and these did not differ from the MS group.

Although KS infusion did not affect food and caloric intake, it did improve many of the symptoms of MS induced by the high fat high fructose diet. KS infusion prevented the diet-induced weight gain in the MS+1.5KS and MS+3KS groups. All strengths of KS infusion tended to reduce the retroperitoneal fat weight and the corresponding fat index, but this antiobesity effect was significant only in the MS+3KS group. Han *et al.* reported similar results [10]: they have treated mice subjected to high-fat diet with ethanol extract of KS fruits for 9 weeks and have observed reduction of fat storage and prevention of diet-induced weight gain. According to the authors, momordin Ic, which is the principal saponin constituent of KS fruits, is responsible for the antiobesity effect. This triterpenoid glycoside, as well as the total KS saponins inhibit the pancreatic lipase activity (an effect determined *in vitro*), thus reducing the intestinal absorption of dietary fat.

In our study, the action of KS on the visceral fat was accompanied by a beneficial effect on serum lipid profile. All KS-treated animals had lower serum triglyceride concentrations compared to the KS-untreated animals receiving the same diet. The effect was significant with the strongest KS infusion. This group also displayed the lowest cholesterol levels among the MS groups, although the effect was not statistically significant.

Increased level of oxidative stress is an important characteristic of MS. In diet-induced MS, increased levels of glucose and free fatty acids contribute to the generation of free radicals because of the enhanced oxidation of these energy substrates. On its turn, the increased level of reactive oxygen species induces cellular insulin resistance [20]. In addition to the increased production of free radicals, fructose-enriched diet is associated with reduced antioxidant defense [21]. It appears that oxidative stress is both a trigger and a consequence of obesity and metabolic syndrome. In addition, it serves as a link between the disturbances in energy metabolism and other conditions, such as cardiovascular diseases [3] or neuropsychiatric disorders [22]. In our study, all KS infusions reduced the serum concentration of TBARS. The antioxidant effect was dose-dependent but significant only at the strongest strength. The antioxidant capacity of KS has been studied by Wang *et al*. using a protein oxidation model [23]. The authors reported that fruit extracts of KS effectively scavenge different free radicals and related these antioxidant properties to the presence of momordin Ic in the extracts.

Nonalcoholic fatty liver disease is another disorder that is typically associated with obesity, insulin resistance and MS in humans and experimental models [24, 25]. In the current study, the liver weight and liver index did not differ between the groups. However, livers from the MS group displayed histopathological signs of focal macro- and micro-vesicular steatosis. The serum level of alkaline phosphatase was also increased thus demonstrating impaired liver function. Insulin resistance and increased amount of free fatty acids in hepatocytes play a key role in the origin and maintenance of hepatic steatosis creating the basis for triglyceride production. The excess of free fatty acids induces a rise in mitochondrial β-oxidation, production of reactive oxygen species and oxidative stress, as well as elevated lipid peroxidation [25]. In our experiment, the strongest infusion of KS entirely prevented the development of hepatic steatosis or alleviated the histopathological signs of the diet-induced liver injury. Alkaline phosphatase levels tended to be reduced by the 6KS infusion. The same strength of the infusion demonstrated the highest antioxidant activity, suggesting a possible mechanism for the liver protection. Kim *et al.* studied the hepatoprotective effect of momordin Ic and oleanolic acid obtained from KS fruits in a rat model of carbon tetrachloride-induced hepatotoxicity and concluded that the observed beneficial effects were produced by enhancing the hepatic antioxidant defense system [26].

Most of the published research on KS, including the above mentioned studies, focuses on the effects of the plant fruits. Momordin Ic, the principal glycoside isolated, is usually considered the major carrier of the activity. In all mentioned studies, the described antiobesity, antioxidant, and hepatoprotective effects of KS fruits are thought to be due to the actions of this triterpenoid saponin. As for the seeds of KS, however, their composition has not been fully established. Morin hydrate

and hydroxytyrosol have been found in KS seed extract, as well as five additional unidentified substances [14].

Morin hydrate is a polyphenolic compound with structure representing an isomeric form of quercetin, which is regarded as one of the flavonoids with the highest antioxidant potential. Morin acts as preventive antioxidant by inhibiting xanthine oxidase and as a curative antioxidant by scavenging reactive oxygen radicals [27]. Morin function is tested in many experimental settings [27-30]. In some of them, its antioxidant potential is evaluated by TBARS concentration [28, 29]. Thus, it is plausible to consider that morin could be responsible for the dose-dependent antioxidant effect of KS infusions demonstrated in our study. In addition to its antioxidant properties, morin hydrate has showed a hepatoprotective potential in different experimental models of hepatotoxicity, such as ischemia-reperfusion [27] and ethanol-induced liver damage [29]. These studies base the liver protective effects of morin on its antioxidant action.

Hydroxytyrosol is another potential carrier of the antiobesity, antioxidant and hepatoprotective activities of KS seeds. It is a phenolic alcohol with a strong antioxidant activity through free-radical scavenging. In addition, hydroxytyrosol increases the endogenous defense antioxidant systems by activating different cellular signaling pathways [31]. The antiobesity effect of hydroxytyrosol has been demonstrated in diet-induced experimental models of metabolic syndrome [32, 33]. It inhibits lipogenesis, suppresses the triglyceride accumulation and expression of adipogenesis-stimulating factors, and promotes lipolysis [34]. Hydroxytyrosol prevents liver steatosis in diet-induced obesity by reducing hepatic inflammation and oxidative stress [35, 36].

In this experiment, we demonstrated a parallel in the dose-dependent antioxidant and liver protective effect of KS infusion. A weakness of the study is the lack of information on the active principles present in the seed infusion that we used. Thus, it is not known, whether it is momordin Ic, morin, hydroxytyrosol, or any other component of the KS infusion, that is responsible for the observed benefits in the diet-induced metabolic and liver impairments. Such a tempting hypothesis remains speculative until it is proven. Therefore, an important future task is to investigate the composition of the aqueous infusion of KS, as it represents an easy to use approach to prevent or treat metabolic and liver disorders.

In conclusion, this study demonstrates that the aqueous infusion of KS seeds possesses antiobesity, antioxidant, and liver-protective effects in rats with diet-induced metabolic syndrome. In general, the strongest infusion exerted the highest beneficial effects – it improved the lipid profile, reduced the level of oxidative stress and alleviated the diet-induced liver impairment. The antioxidant effect was dose-dependent. The infusions of lower-to-moderate strength tended to prevent the diet-induced weight gain and reduced visceral obesity, but did not improve the dyslipidemia and liver steatosis.

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Effects of dwarf elder fruit infusion on nuclear factor kappa B and glutathione metabolism-related genes transcription in a model of lipopolysaccharides challenged macrophages

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Sambucus ebulus L. (SE) is shown to be implicated in combating oxidative stress in inflammation and cell death. We aimed to analyse the effect of SE fruit aqueous infusion (FAI) in a model of lipopolysaccharides (LPS)-induced cytotoxicity in J774A.1 mouse macrophages. Transcription levels of NF-κB, antioxidant enzymes glutamate-cysteine ligase, catalytic subunit (GCLc) and glutathione peroxidase (GPx) were analysed. Cell viability tests showed that LPS (25-200 ng/mL) caused gradual cell death by up to 14.5%, whereas SE FAI (0.625%-12.5% in culture media) was non-toxic. Salicylic acid (SA) as a positive control (25-200 μM) gradually induced cell proliferation by up to 15%. Real-Time PCR analysis revealed that SE FAI alone significantly up-regulated NF-κB, GPx and GCLc mRNA levels (3.8, 3.04 and 9.57-fold, respectively). Pre-treatment with SE FAI significantly reduced LPS (200 ng/mL)-stimulated transcription of NF-κB and GPx by 70%, and GCLc by 37%. The effect of SE FAI/+LPS was similar to the effect of SA/+LPS. NF-κB mRNA levels significantly correlated with those of GCLc (r=0.66), and GPx (r=0.79). We conclude that SE FAI may exhibit its anti-inflammatory and antioxidant effects by altering transcription of LPS-stimulated oxidative stress and inflammation related genes.

Keywords: *Sambucus ebulus*; NFκB; glutathione; glutathione peroxidase; lipopolysaccharides.

INTRODUCTION

Although being traditionally used as a medicinal plant, *Sambucus ebulus* L. (SE, dwarf elder) is a poorly studied species. Dwarf elder fruits aqueous infusion (FAI) is rich in polyphenols, flavonoids and anthocyanins [1-4]. Fruits also contain sugars, valeric acid, methyl salicylate, citronellal, methyl palmitate, ursolic acid, malic acid, tartaric acid, tannins, pectin, resins, vitamin C [4, 5]. Folk medicine prescribes fresh berries or decoctions in cases of haemorrhoids, gastric pain [6], tuberculosis [7] and rheumatoid arthritis [8].

Dwarf elder is a plant which fruit and flower infusions and extracts exhibit high antioxidant activity *in vitro* [2, 9] due to high polyphenol content, and can modulate antioxidant enzymes expression in cell cultures [1, 10]. However, the mechanism of boosted antioxidant defense is not clear. Polyphenols are known to increase glutathione (GSH) levels by activating expression of glutamate cysteine ligase (GCL) [11, 12]. The cells respond to oxidative stress by increasing the expression of GCL [13]. Another enzyme, which activity is related to GSH levels, is glutathione peroxidase (GPx), and its expression is also modulated by polyphenols [14].

One of the main signalling cascades involved in initiation of inflammation process acts *via* activation of transcription factor NF-κB [15]. NF-κB controls gene expression of many inflammation and oxidative stress related proteins [16-18]. Lipopolysaccharides (LPS) activate gene expression of inflammation related cytokines, adhesion molecules and enzymes by activating NF-κB-dependent signal pathway [19-24]. Plant polyphenols may reduce LPS – stimulated NF-κB activity [25].

Inflammatory response involving oxidative stress in macrophages could be provoked by different stimuli such as high levels of free fatty acid (obesity), high glucose levels (diabetes) and bacterial endotoxins (infections) [26]. Earlier we reported the effects of SE FAI on GCL catalytic subunit (GCLc) and GPx genes expression in a cell culture model of *tert*-butyl hydroperoxide (*t*-BuOOH)-induced oxidative stress [27]. To reveal the potential mechanisms of anti-inflammatory and antioxidant activity of SE fruits now we used a cell-culture model of LPS-stimulated inflammatory response exploring the effect of SE FAI on the expression of GCLc, GPx and NF-κB in J774A.1 macrophages.

MATERIALS AND METHODS

Plant material

Sambucus ebulus L. fruits were collected from North-Eastern Bulgaria in the period August – September, 2014 and were dried at room

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temperature. SE FAI was prepared from 150 mg finely ground dried fruits, vortex extracted three times with 3 mL of distilled H_2O for 3 min at room temperature. After centrifugation (5 min, 3500 rpm) the supernatants were collected and diluted to 15 mL with PBS buffer (pH=7.4).

Cell culture

J774A.1 mouse macrophage cells were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were cultured according to the manufacturer's requirements. Cell counts were prepared in quadruplicate by 0.4% trypan blue exclusion dye (Chemapol, Czech Republic) using a counting Burker-chamber.

Cell viability test

Viability of treated cells was evaluated using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay [28]. Cells were seeded and after 24 h were treated with culture media containing SE FAI (0.625% – 12.5% v/v), or SA (25 μM – 400 μM), or LPS (25 ng/mL – 200 ng/mL). After 20 h 100 μL MTT solution (2 mg/mL) (AppliChem, Germany) was added and cells were incubated in dark for additional 4 h. Formazan crystals were solubilized with dimethyl sulfoxide (Scharlau Chemie S.A., Spain). Absorbance was measured using a multiwell scanning spectrophotometer (ELISA reader-Synergy 2, BioTek) at 550 nm. Untreated cells were used as control. Cell viability (%) was calculated as [(mean absorbance of the sample/mean absorbance of the control) × 100]. Treatments were performed in triplicate. Results are presented as mean ±SD.

Experimental design

Experimental model involved macrophage cells seeded in 6-well plates (2×10^5 cells/well), and allowed to adhere overnight. Cells were pre-treated with SE FAI dissolved in different concentrations (2.5%, 5% and 10% v/v) in DMEM (with 4.5 g/L glucose, w/o phenol red and L-glutamine) supplemented with 10% FBS, 100 U/mL penicillin/streptomycin mixture and 2 mM L-glutamine. After 24 h cells were treated with 200 ng/mL LPS (*Escherichia coli* 026:B6, Sigma-Aldrich, Germany) for additional 24 h. After LPS stimulation the cells were lysed and total RNA was extracted using TRI reagent (Ambion, USA) according to the manufacturers requirement. Non-treated cells were used as control. Salicylic acid (SA) (Merck, Germany), in concentration of 100 μM was used as a positive control. Treatments were performed in triplicate.

Gene expression analysis

GCLc, GPx, NF-κB and β-actin (internal control) genes expression was analysed using Real-Time PCR as previously described [29]. Relative gene expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method [30]. The used primer sequences (Sigma-Aldrich, Germany) for each gene analysed are presented in Table 1. A denaturation step was added to the instrument's protocol to check for nonspecific amplification. Expression levels of mRNA are presented in relative units (RU) as compared to the control group, where the level of mRNA expression were considered to be equal to 1. Analyses were performed in triplicate.

Table 1. Primer sequences used in Real-Time PCR analysis.

Gene	Forward primer (5′–3′)	Reverse primer (5′–3′)
β-Actin	ACGGCCAGGTCATCACTATTG	CAAGAAGGAAGGCTGGAAAAG
GCLc	AATGGAGGCGATGTTCTTGAG	CAGAGGGTCGGATGGTTGG
GPx	CCCCACTGCGCTCATGA	GGCACACCGGAGACCAAA
NF-κB	ATGGCAGACGATGATCCCTAC	TGTTGACAGTGGTATTTCTGGTG

Statistical analysis

Graph Pad Prism 5.0 software was used to perform the statistical analyses (Student's *t*-tests). The values of $p<0.05$ were considered as significant.

RESULTS

Effects of SE FAI, LPS and SA on cell viability

SE infusion did not exert any cytotoxicity on macrophages cell culture when applied in increasing concentrations in the culture medium from 0.625% to 12.5% (Fig. 1A). What is more, a significant increase by 15% of cell viability was detected in the group treated with 1.25% SE FAI ($p<0.05$). The concentrations of 2.5%, 5% and 10% were selected for further testing of the SE FAI protective effect on J774A.1 macrophages in conditions of LPS-induced oxidative stress and inflammatory response.

The applied concentrations of SA from 25 μM to 400 μM showed no cytotoxic effect on treated macrophages (Fig. 1B). The presence of SA in the culture medium even induced cell proliferation. The

concentration of 200 μM induced cell proliferation in a statistically significant manner by almost 15% (p<0.001). SA in concentration of 100 μM in the culture medium was used in further cell culture treatments as a positive control.

A statistically significant decrease in macrophage cell viability by 14.5% (p<0.01) was observed upon treatment with 200 ng/mL LPS (Fig. 1C). In the range of 150-200 ng/mL LPS's effect on cell viability did not vary substantially, so the highest cytotoxic concentration applied, 200 ng/mL, was used for stimulation of macrophages in the next experiments.

The effect of SE FAI on gene expression of GCLc, GPx and NF-κB

GCLc mRNA levels were significantly induced by 2.5% and by 5% SE FAI up to 9.57 -fold(p<0.001) and 2 -fold(p<0.05), respectively, compared to untreated control cells (Fig. 2A). Pre-treatment with 2.5% and 5% SE FAI significantly reduced LPS-stimulated gene expression (3.68-fold, p<0.01 *vs*. control) of GCLc by 1.37 (p<0.05) and 0.68 (p<0.05)-fold, respectively. The effect of fruit infusion was similar to that of the positive control (100 μM SA), which reduced LPS-induced GCLc mRNA levels 2.12-fold (p<0.01)

Significant up-regulation of GPx mRNA levels was seen in groups treated with 2.5% and 5% SE FAI up to 3.04-fold (p<0.001) and 1.49-fold (p<0.05), respectively, as compared to untreated cells (Fig. 2B). LPS-induced enzyme gene expression (3.3-fold, p<0.01 *vs.* control) reduced by pre-treatment with 2.5% (1.4-fold, p<0.01), 5 % (2.33-fold, p<0.01) and 10% (1.75-fold, p<0.001) SE FAI in a culture medium (Fig. 2B). SA as a positive control significantly reduced (2.55-fold, p<0.001) GPx mRNA transcription, as compared to LPS group, similarly to the effect exerted by the fruit infusion.

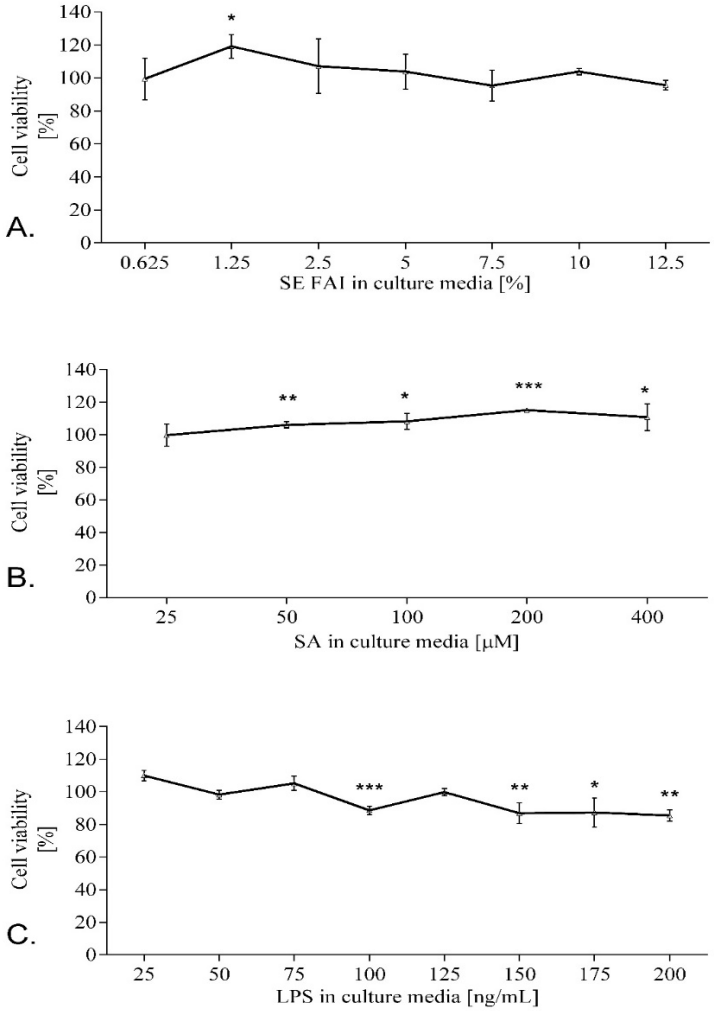

Figure 1. Effect of SE FAI (A), SA (B) and LPS (C) on J774A.1 cells viability. Data are presented as mean ±SD. *p<0.05, **p<0.01, ***p<0.001 *vs*. control (untreated cells).

Similar to the enzymes mentioned above, NF-κB transcription levels were up-regulated by 2.5%, 5% and 10% SE FAI treatments up to 3.8 (p<0.001), 2.3 (p<0.001) and 2.1-fold (p<0.01), respectively, as compared to untreated cells (Fig. 2C). Induced by LPS NF-κB transcription (3.7-fold, p<0.001 *vs.* control) was reduced by all applied SE FAI concentrations: 2.5% - 2.2-fold (p<0.001), 5 % - 2.6 –fold (p<0.01), and 10% - 2.2-fold (p<0.05). Expectedly, the effect of the fruits was comparable to that of the positive control, which significantly reduced (3.1-fold p<0.001) NF-κB mRNA levels as compared to LPS group.

Correlation analysis

Correlation analysis showed a highly significant linear dependence between mRNA levels of NF-κB and GCLc (r=0.66, p<0.05) and between NF-κB and GPx (r=0.79, p<0.01) in all treatment groups (Fig. 3).

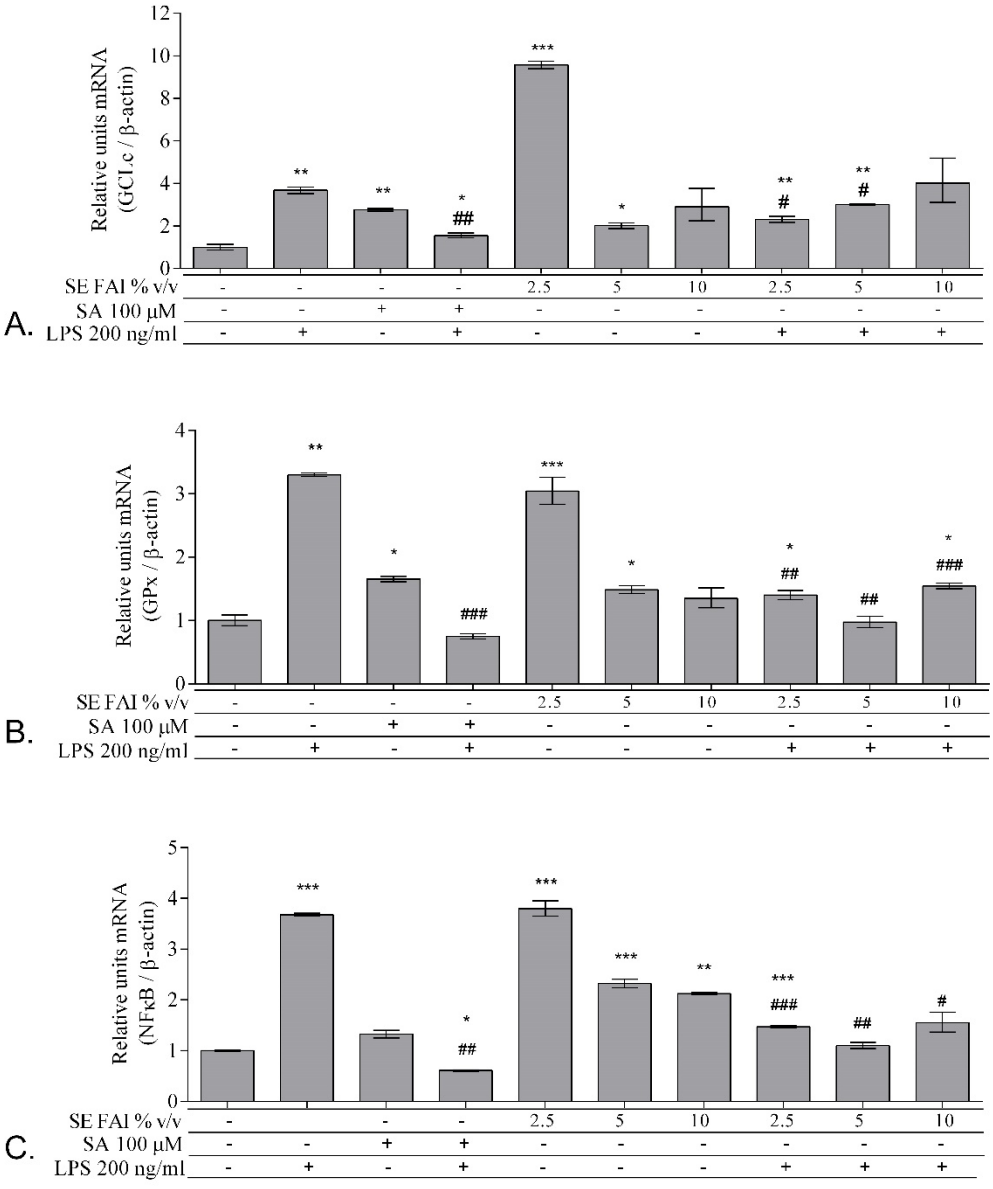

Figure 2. Changes of GCLc (A), GPx (B) and NF-κB (C) mRNA levels in J774A.1 cells pre-treated with increasing concentrations of SE FAI or SA, and stimulated with LPS. Data are presented as mean ±SEM. Legend: **C**-control (untreated cells); **SA –** 100 μM salicylic acid; **LPS –** 200 ng/mL lipopolysaccharides; **SE1 – 2.5**%, **SE2 – 5**%, **SE3 – 10**% SE FAI in culture medium. **p*<0.05, ***p*<0.01, ****p*<0.001 *vs.* **C**; #*p*<0.05, ##*p*<0.01, ###*p*<0.001 *vs*. **LPS**.

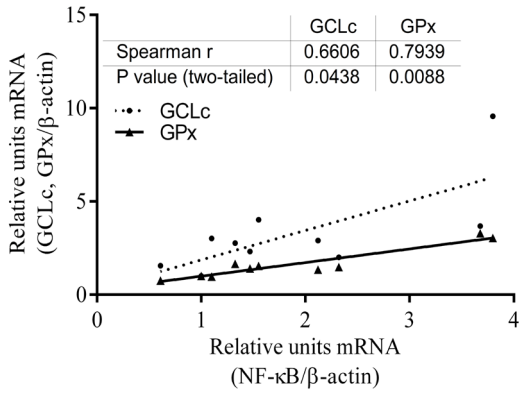

Figure 3. Correlation analysis between mRNA levels of NF-κB and GCLc, and of NF-κB and GPx in macrophages.

DISCUSSION

Whether a plant extract would have a cytotoxic or proliferative effect depends on the concentration administered. The type of extracting agent and substances contained in the extract, as well as the cell type are factors that may determine the effect on cell viability. In general, low concentrations stimulate the expression of genes associated with proliferation, and high concentrations activate caspase cascades that initiate apoptosis [31]. SE FAI did not exhibit any cytotoxicity in concentrations of 0.625% to 12.5% in the culture medium. Indeed, a significant increase in cell proliferation was established upon treatment of macrophages with 1.5% SE FAI. Similar results have also been reported for aqueous extracts of other medicinal plants [32]. Probably the biologically active substances contained in the Dwarf elder fruit aqueous extract affect the proliferation of the cells. Low concentrations of the aqueous extract had a cytoproliferative effect, whereas, with the increase in the concentration in the nutrient medium, cell vitality gradually declined.

Factors triggering cellular immune response, such as LPS, activate a respiratory burst that produces a large amount of reactive oxygen species (ROS) and develops oxidative stress [26]. Induced oxidative stress requires the activation of the endogenous antioxidant defence mechanisms, including the antioxidant enzymes. As a response to the increasing concentrations of free radicals in cells, in particular peroxides, in LPS-treated macrophages, the levels of the enzymes involved in the synthesis of the major cellular antioxidant glutathione (GCLc) and the enzyme reducing peroxides (GPx) increased significantly (p<0.01), (Fig. 2 A and B).

In RAW 264.7 macrophages, LPS treatment induced nitric oxide (NO) production [33], associated with production of peroxynitrites (ONOO.) [34, 35]. This enhances the oxidative stress in cells, thus explaining the activation of intracellular antioxidant defence, in particular, the enzymes GPx and GCLc.

In our experiments SE FAI alone induced significant gene expression of both enzymes GCLc (p<0.001) and GPx (p<0.001), as well as that of transcription factor NF-κB (p<0.001) in J774A.1 macrophages (Fig. 2). This effect could be attributed to the substances contained in the extract (e.g. polyphenols) activating redox-sensing transcription factors such as Nrf2 and NF-κB, which, on their turn, induce expression of cellular antioxidant enzymes and thus exhibit protective antioxidant and immunomodulatory action. Earlier we observed induction of GCLc and GPx genes expression in SE FAI-treated mouse 3T3-L1 preadipocytes [27]. Fruit extract rich in polyphenols such as quercetin and ellagic acid, can induce GCL transcription *in vitro* and *in vivo*, thus increasing GSH levels [11, 37]. The transcription of GCLc gene is controlled by response element binding sites including NF-kB and antioxidant response elements/electrophile response elements (AREs/EpREs) [11, 38]. Flavonoids, such as quercetin, established in SE fruits [4, 39, 40], activate GSH synthesis by AREs/EpREs in the promoter of the GCLc gene [11].

An oxidative stress associated transcription factor Nrf2 [41] is able to transfer to the nucleus and bind to AREs/EpREs element. Scientists claim that some quinones react with thiols [42], therefore, it can be assumed that quercetin oxidized to quinone by ROS, oxidizes thiols in the Keap1 protein, leading to Nrf2 release and to the activation of GCLc gene expression [10]. GPx is a second phase enzyme of xenobiotic metabolism, which expression, similarly to GCLc, is positively influenced by activation of the Nrf2 transcription factor [43, 44].

Cell glutathione levels and redox potential also increase as a result of NF-κB activation. As mentioned above, GCLc promotor contains NF-κB binding site [38]. We suggest that relationship exists between the activation of the NF-κB signaling pathway [45] and increased transcription levels of GCLc. These studies are in support to the observed high positive correlation between the transcriptional levels of NF-κB and those of the two enzymes GCLc and GPx (Fig. 3), indicating one of the possible mechanisms of GCLc and GPx induction by SE FAI itself.

In macrophages pretreated with SE FAI, there was a reduction in mRNA levels of GCLc and GPx enzymes, as compared to cells exposed to LPS only (Fig. 2 A and B). Similar effect was observed in SE

FAI-pre-treated mouse preadipocytes, in conditions of *t*-BuOOH-induced oxidative stress [27]. This effect may be explained by the *in vitro* antioxidant properties of the aqueous extract [1] in conditions of LPS stimulated oxidative stress. Flavonoids and anthocyanins found in SE fruits are able to bind and neutralize free radicals. On the other hand, SE FAI itself induces the gene expression of both enzymes from GSH metabolism, as discussed above. Thus, to a certain extent, SE FAI bioactive compounds compensate for the need to induce antioxidant protection as seen in cells pre-treated with SE FAI and subsequently stimulated with LPS.

NF-κB transcription factor plays a key role in the induction of inflammation and oxidative stress, for example in obesity and development of insulin resistance [26, 46, 47]. In LPS stimulated macrophages, transcriptional levels of NF-κB increased more than 3-fold, in contrast to cells where pre-treatment with SE FAI significantly reduced LPS-stimulated expression of the transcription factor (Fig. 2C).

In a model of LPS-induced inflammation in human monocytes, plants rich in anthocyanins have been shown to inhibit the activity of NF-κB [25]. Ursolic acid found in SE fruits [4] is known to exert anti-inflammatory action in various cell types inhibiting NF-κB activation [48]. Suppression of transcriptional levels of NF-κB might be a probable mechanism, by which SE fruits exhibit anti-inflammatory activity suggesting their use in the prevention of acute and chronic inflammation states.

In this aspect, quite convincing is the fact that the effect of SE FAI on gene expression in all experiments followed the same direction as the effect of SA (Fig. 2). Salicylates are well known inhibitors of NF-κB activation, thus inhibiting LPS-stimulated inflammatory response [48-50]. Probably the unique combination of active compounds is in the basis of the biological effects of SE fruits. Further experiments and detailed analysis of SE FAI immunoregulatory potential involving transcriptomics and proteomics in animal models of inflammation or human intervention studies may help to elucidate the mechanism of action.

CONCLUSIONS

These findings are in support to folk medicine traditions where the SE fruits are used for immunostimulation. Our study provides first scientific data on the effects of SE FAI in a model of LPS-stimulated cells. SE fruits alter GCLc and GPx expression levels in a model of oxidative stress and inflammation, possibly by modulating NF-κB mRNA expression. Data reveal the potential of SE fruits as a natural source for the development of new products for prevention of inflammation and oxidative stress related disease states.

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Conflict of Interest: *The authors declare that there are no conflicts of interest.*

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Effect of *Kochia scoparia* on carrageenan-induced paw edema in an experimental model of metabolic syndrome

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Metabolic syndrome (MS) is a global health issue affecting a significant part of the world population. Low-grade inflammation is one of the specific features of MS. *Kochia scoparia* is an annual plant used in the traditional Chinese medicine. The present study aimed to examine the effect of an aqueous infusion of *K. scoparia* seeds on the acute carrageenan-induced rat paw inflammation in an experimental model of MS. Forty male Wistar rats were allocated into four groups: MS, MS+1.5KS, MS+3.0KS and MS+6.0KS, all receiving a high-fat high-fructose diet. The drinking fluid of MS group was 10% fructose in water while the other three groups were drinking 10% fructose in aqueous *K. scoparia* (KS) seeds infusion of different strengths (prepared from 1.5, 3.0 and 6.0 g KS seeds in 100 ml of boiling water, respectively). At the end of the 10 weeks of dietary intervention, carrageenan (1 mg as 0.1 ml solution) was injected in the left hind paw. The paw edema was measured on the 30th, 60th, 120th, 180th, 240th and 300th minute after the injection. On the 30th minute, *K. scoparia* seeds infusion caused a significant dose-dependent decline in the paw edema. No further effect of the infusion was observed during the next time intervals. In conclusion, the results from the present study showed that the chronic administration of the aqueous *K. scoparia* seeds infusion decreased the carrageenan-induced paw inflammation in rats with metabolic syndrome but the anti-inflammatory effect appeared to be weak and short-lived.

Keywords: rats, carrageenan, paw edema, *Kochia scoparia*, metabolic syndrome

INTRODUCTION

Inflammation is an immune system response to harmful stimuli activated in order to enhance the healing process. Commonly triggered are the inflammatory NF-κB, MAPK and JAK-STAT pathways [1]. The primary inflammatory response is characterized by the production of mediators such as C-reactive protein (CRP), tumor necrosis factor-alpha (TNF-α), interleukins (IL-1, IL-6) and interferon-gamma (IFN-γ). On tissue level inflammation is characterized by swelling, redness, pain and impaired function.

Metabolic syndrome (MS) is a serious global problem affecting half quarter of the world's population. It is a constellation of impaired glucose tolerance, visceral adiposity, arterial hypertension and dyslipidemia (hypertriglyceridemia, increased non-esterified free fatty acids and decreased HDL-cholesterol) [2]. There is strong evidence that MS is accompanied by low-grade chronic inflammation [2]. Patients with MS have elevated CRP, interleukins, TNF-α and prostaglandins [3]. Furthermore, weight loss is accompanied by a decrease in the levels of the proinflammatory markers.

Kochia scoparia is an annual leafy ornamental plant. The fruits of *K. scoparia* are rich in triterpenoids, saponins and alkaloids [4, 5]. In China, Korea and Japan its fruits are used in the treatment of dysuria and skin diseases and the Bulgarian traditional medicine recommends the seed infusion in liver disease. An ethanol extract of *K. scoparia* fruits possesses anti-inflammatory and antiallergic effects [6, 7]. In MS, the triterpenoid momordin Ic, found as a major constituent in *K. scoparia* fruit, has shown anticancerogenic effect [8].

Carrageenan is a mucopolysaccharide extract, discovered by the British pharmacist Stanford in 1862. When injected subcutaneously, carageenan induces acute inflammatory response due to the release of bradykinin, histamine, tachykinins, reactive oxygen and nitrogen species [9]. This leads to edema due to extravasation of fluid and protein and neutrophil infiltration of the inflammatory site. Carrageenan-induced paw edema in rats is a broadly used model for testing the anti-inflammatory activity of various substances [10].

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The present study aimed to examine the effect of an aqueous infusion of *K. scoparia* seeds on the acute carrageenan-induced rat paw inflammation in an experimental model of MS.

EXPERIMENTAL

Animals and experimental protocol

Forty male Wistar rats (initial body weight 160-200 g) were included in the experiment. The animals were kept under a 12/12 hours light-dark cycle, an ambient temperature of 20-25°C and had free access to food and water. They were allocated into 4 groups (10 rats in a group): MS, MS+1.5KS, MS+3.0KS, MS+6.0KS. All rats received high-fat high-fructose (HFHF) diet which consisted of lard (17%), fructose (17%) and regular rat chow. The MS group serving as a control received 10% fructose in the drinking water while the remaining three groups received 10% fructose added to the aqueous infusion of *K. scoparia* seeds of different strengths. The infusions were prepared by soaking 1.5, 3.0 and 6.0 g of *K. scoparia* (KS) seeds, respectively, in 100 ml boiling water and subsequent percolation upon cooling down. The duration of the dietary intervention was 10 weeks. The experimental protocol is given in Table 1.

Table 1. Experimental protocol; MS – metabolic syndrome, HFHF – high-fat high-fructose, KS – *K. scoparia*

Groups	Diet	Drinking
MS	HFHF	10% fructose in the drinking water
MS+1.5KS	HFHF	10% fructose in KS seed infusion (1.5 g/100 ml)
MS+3.0KS	HFHF	10% fructose in KS seed infusion (3.0 g/100 ml)
MS+6.0KS	HFHF	10 % fructose in KS seed infusion (6.0 g/100 ml)

All procedures concerning animal treatment and experimentation were conducted in conformity with the national and international laws and policies (EU Directive 2010/63/EU for animal experiments) and were approved by the Bulgarian Food Safety Agency (Document 177/07.07.2017).

Induction of edema

At the end of the dieting period, carrageenan at a dose of 1 mg as 0.1 ml of freshly prepared solution in 0.9% saline was injected into the plantar surface of the left hind paw of the animals to induce acute inflammation. The paw volumes (ml) were measured before the injection and at the 30^{th}, 60^{th}, 120^{th}, 180^{th}, 240^{th}, and 300^{th} min after that. The digital plethysmometer LE7500 Panlab, Barcelona, was used to make the measurements. The difference between the paw volumes before and after the carrageenan injection was considered a marker of the intensity of the inflammation induced.

The paw edema (%) was calculated using the formula:

$$(V_s\text{-}V_0) \times 100,$$

where: V_s – the average paw volume measured at the six time intervals after the carrageenan injection, V_0 – the average initial paw volume.

Statistical analysis

The results were analyzed by one-way ANOVA and the post-test for linear trend. Graphpad Prism 5 Software was used. The data are presented as means ±SEM and $p<0.05$ was considered to indicate statistical significance.

RESULTS

The carrageenan injection induced acute local inflammation in the rats demonstrated by edema of the left hind paw. On the 30^{th} minute, the mean paw edema (%) of the rats from the control MS group was 12.15 % while the same parameter of the animals from MS+1.5KS, MS+3.0KS and MS+6.0KS groups were respectively 9.31, 7.37 and 5.28. The mean paw edema values in the subsequent time points are given in Table 2.

Table 2. Mean paw edema after carrageenan injection; MS – metabolic syndrome, KS – *K. scoparia*

Mean paw edema (%)	MS	MS+1.5KS	MS+3.0KS	MS+6.0KS	ANOVA	Post-test for linear trend
30^{th} min	12.15	9.31	7.37	5.28	p>0.05	p< 0.05
60^{th} min	12.60	12.54	13.43	9.88	p>0.05	p>0.05
120^{th} min	28.58	27.71	30.89	20.74	p>0.05	p>0.05
180^{th} min	31.34	38.24	34.44	35.65	p>0.05	p>0.05
240^{th} min	46.26	46.61	52.65	44.35	p>0.05	p>0.05
300^{th} min	45.42	47.47	43.69	36.30	p>0.05	p>0.05

DISCUSSION

In the present study we tested the potential of *K. scoparia* seed infusion to exert anti-inflammatory effect. On the 30th minute, a slight linear decline in paw edema from the MS group towards MS+6KS group was noted ($p<0.05$). During the next time periods there was only a non-significant reduction in the paw edema of the MS+6KS group. Our study demonstrated the presence of an early and weak, nevertheless dose-dependent anti-inflammatory effect of *K. scoparia* seed infusion. It occurred at the onset of the inflammatory process and gradually disappeared with time.

The results from our study are partially in line with a number of studies which indicate an anti-inflammatory potential of *K. scoparia*. In their study Jo *et al.* [11] found methanol extract of *K. scoparia* dried fruit to decrease ear swelling in a model of 1-fluoro-2,4-dinitrofluorobenzene (DNFB)-induced contact dermatitis in mice. They suggested that the anti-inflammatory effect is due to suppression of Th1 skewing reactions (decreased production of IL-6, IFN-γ). Another study [12] describes the anti-inflammatory and antiallergic effect of the *K. scoparia* ethanol extract on ovalbumin-induced murine asthma model, where attenuation in the levels of interleukins (IL-4 and IL-5) in a dose-dependent manner has been demonstrated. Similarly, Matsuda *et al*. [13] showed that 70% ethanol extract of *K. scoparia* fruits inhibits the rise of vascular permeability induced by acetic acid, prevents the increase of paw edema induced by carrageenan, histamine, serotonin or bradykinin and ear swelling induced by arachidonic acid and these have been translated into anti-nociceptive effects. Momordin Ic has also been shown to exhibit an inhibitory effect on carrageenan-induced edema. These results indicate that *Kochia* fruits exert a peripheral anti-nociceptive effect mediated by an anti-inflammatory action, and that these activities can be partially attributed to momordin Ic. Evidence that momordin Ic is the most potent anti-inflammatory ingredient in *Kochia scoparia* fruit, acting by inhibiting the production of inflammatory cytokines TNF-α and IL-6 and prostaglandin E_2 is presented in the work of Yoo *et al.* [14].

The majority of the studies focusing on the pharmacological activity of *K. scoparia* use alcohol extracts of the fruits of *Kochia*. More information is also available on the nature of the biologically active substances present in the fruits, but little or nothing is known about the phytochemical content of the *Kochia* seeds. Therefore, it is not surprising that the present results appear to be only a weak reminiscence of what has been found as activity from other parts of the plant. It is not unusual that different bioactive components reside in different anatomical parts of medicinal plants. We have, for example, found some other potentially beneficial effects of *Kochia* seed aqueous infusion (unpublished data).

Our study indicates, opposite to the expectations, a weak and short-lived anti-inflammatory potential of *K. scoparia* seed infusion on carrageenan-induced paw edema in the experimental model of MS. The lack of a more pronounced anti-inflammatory effect could be due to the use of a different form of extract originating from a different part of the Kochia plant compared to those cited in the literature. A further work is needed to identify the presence of phyto-active components in the *K. scoparia* seed infusion when trying to elucidate its potential health benefits.

In conclusion, the **c**hronic administration of the aqueous infusions of *K. scoparia* seeds decreased the paw edema early during the carrageenan-induced inflammation in the experimental rats with metabolic syndrome. However, the anti-inflammatory effect under the experimental circumstances in this study appeared to be weak and short-lived.

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Polyphenol-rich extracts from essential oil industry wastes

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The essential oil industry generates every year large amounts of waste, due to the low quantities of essential oils in the raw materials [8]. The wastes are usually discarded or used as biofertilizer, although they are rich in biologically active substances. Besides, these simple approaches are leading to ecological problems in the places where the wastes are dumped. Nowadays, the valorization of agricultural and food wastes became a priority and is a base for the successful implementation of circular economy. For this reason, the present study is focused on the utilization of essential oil industry waste as a source of polyphenolic compounds. Five residues from industrially processed raw materials (2018 harvest): one of *Lavandula angustifolia, Melissa officinalis* and *Ocimum basilicum*, and two of *Rosa alba* were collected and used for preparation of polyphenol-rich extracts. The extracts were analyzed for their polyphenol content (total and individual compounds), antioxidant activity, neutral sugars and uronic acids. The investigations suggested that the wastes were a rich source of polyphenols (flavonoids and phenolic acids) and could be used as supplements for increasing antioxidant activities in various food systems.

Keywords: Polyphenols, antioxidant activity, essential oil industrial waste, waste valorization.

INTRODUCTION

The essential rose oil industry is a key manufacturing field in Bulgaria. Besides the most important crops, such as *Rosa Damascena* and *Lavandula angustifolia* there are many others plants which are industrially processed. Because of the relatively low concentration of aroma compounds and pigments in the fresh plants, large quantities of wastes remain after the extraction or distillation of the important biologically active substances. The most common procedures for eliminating these wastes include simply throwing them away or composting. However, the waste could also serve as initial material for extraction of valuable by-products, such as polysaccharides, dietary polyphenols, aroma substances, etc. The beneficial economic effect of this approach has been recently outlined at the 8th World Congress on Polyphenols Applications [1] by the 5-Stages Universal Recovery Strategy of biologically active substances from waste biomass. The results of the previous studies on other industrial wastes: *Rosa damascena* [2] and *Calendula officinalis* [3] suggested that they could be successfully utilized as a source of polyphenols. Besides, investigation of the effect of lavender waste on the quality and safety of breads showed the potential of essential oil residues as natural bio preservatives [4]. Hence, the aim of the present study was to evaluate the potential of several industrial wastes of essential oil industry as a source of dietary polyphenols.

MATERIALS AND METHODS

Materials

The *Rosa alba* waste (steam distilled, RA_SD) was provided by Enio Bonchev distillery (Tarnichene, Bulgaria, 2018). The *Rosa alba* waste (obtained after supercritical CO_2-extracted fresh flowers, RA_CO2) was obtained from EKOMAAT distillery (Mirkovo, Bulgaria, 2018). The *Lavandula angustifolia* waste (after steam distillation, L_SD), the *Melissa officinalis* waste (after steam distillation, M_SD) and the *Ocimum basilicum* waste (after steam distillation, B_SD) were obtained from Zelenikovo distillery (Zelenikovo, Brezovo region, Bulgaria, 2018).

After treatment the steam distilled wastes were cooled down, inspected for elimination of impurities and dried under vacuum at 50°C. The CO_2-extracted waste was removed from the extraction cylinder and checked for impurities. Both wastes were stored at -18 °C until further treatment. All the solvents used were of analytical grade and purchased from local distributors.

Methods

The 70% ethanolic extracts from wastes were

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obtained according to [5] with small modifications: The dry wastes were ground and sieved (0.5 mm). 300 g of the dry residues were treated with 2000 mL of 70% ethanol for 1 h at 60°C, then left for 24 h at room temperature at constant stirring. The mass was filtered through nylon cloth (250 mesh), and the insoluble residue was extracted with additional 1000 mL of 70 % ethanol at the same conditions. The total polyphenol content of ethanolic extracts was determined using the method described by Singleton and Rossi [6]. The antioxidant activity was measured by ORAC and HORAC assays as described in [7] and the values were expressed as μmol Trolox equivalents per liter (μmolTE/L) and as μmol gallic acid equivalents (μmol GAE/L) per liter, respectively. The DPPH and FRAP analyses were performed according to the procedure described by Slavov *et al.* [2] and the results were expressed as μmolTE/L.

The individual phenolics and flavonoids were analyzed on Agilent 1220 HPLC system (Agilent Technology, USA), equipped with binary pump and UV-Vis detector. Wavelength of λ= 280 nm was used. Separation was performed using Agilent TC-C18 column (5 μm, 4.6 mm × 250 mm) at 25ºC. Mobile phases consisted of 0.5 % acetic acid (A) and 100% acetonitrile (B) at a flow rate of 0.8 mL/min. The gradient conditions started with 14% B, between 6 and 30 min linearly increased to 25% B, then to 50% B at 40 min. The standard compounds (gallic acid, 3,4-dihydroxy benzoic acid, chlorogenic acid, caffeic acid, *p*-coumaric acid, ferulic acid, ellagic acid, catechin, epicatechin, rutin, naringin, myricetin, quercetin, naringenin and kaempherol) were purchased from Sigma-Aldrich (Steinheim, Germany).

The amounts of acetaldehyde, ethyl acetate, methanol and higher alcohols were determined by GC-FID analysis by injecting 1μL on Shimadzu GC-17A (Shimadzu, Japan) equipped with capillary column TEKNOKROMA TRB-WAX (30 m; ϕ 0.32 mm; 0.25 μm thickness) and software GC Solution (Shimadzu, Japan). The injector and detector temperatures were 229 °C and 250 °C, respectively, the carrier gas pressure and speed were 32 κPa and 1 mL/min, respectively. The column temperature regimen was: starting at 40 °C, hold for 1 min, then increase with 5 °C/min until 100 °C, hold for 10 min, and finally increase with 15 °C/min until 220 °C.

The individual volatile compounds in the ethanolic extracts were determined according to the following procedure: 1.0 ml ethanolic extract was treated with 1.0 ml of dichloromethane (triple). The combined organic layers were dried under vacuum at 30 °C. To the dry residue 100 μL of dichloromethane was added. For analysis 1.0 μL from the solution was injected on the gas chromatograph Agilent GC 7890 with mass-selective detector Agilent MD 5975 and column HP-5ms. The following temperature regimen was used – initial temperature was 40 °C and then increase to 300 °C with 5 °C/min (hold for 10 min); injector and detector temperatures – 250 °C, helium was used as carrier gas at 1.0 ml/min. The scanning range of the mass-selective detector was m/z = 40 – 400 in splitless mode. The individual compounds were identified comparing the retention times and the relative indices (RI) with those of standard substances and mass-spectral data from libraries of The Golm Metabolome Database (http://csbdb.mpimp-golm.mpg.de/csbdb/gmd/gmd.html) and NIST'08 (National Institute of Standards and Technology, USA).

Statistical analysis

The analyses were run three times, and the data were given as mean values. Statistical significance was detected by analysis of variance (ANOVA) and Tukey's test; value of $p<0.05$ indicated statistical difference).

RESULTS AND DISCUSSION

Obtaining of ethanolic extracts from wastes

Treatment of the wastes with aqueous-ethanolic solutions is usually applied before extraction of polysaccharides from the raw materials. It aims at removing some low-molecular substances (pigments, sugars, etc.) which will hamper the future extraction. In our case it also aimed at obtaining of extracts rich in polyphenolic substances. In previous experiments we have investigated the influence of the ethanol concentration on the extractability of polyphenols and the subsequent polysaccharide extractions [2]. Our findings showed that extraction with 70% ethanol solutions gave the optimum results for possibilities of combined valorization of the waste materials of *Rosa damascena* and for this reason we have decided the treatment of the five residues after extraction or steam distillation to be performed with 70 % ethanol. The extracts obtained were subjected to preliminary analysis for their phenolic substances, polar aroma metabolites and antioxidant activity.

Total phenolic substances, individual phenolic acids and flavonoids, and antioxidant activity of ethanolic extracts from the wastes

The extracts were subjected to analysis for polyphenol content in order to assess the potential of each waste. This information, confronting the results of the two residues from industrial processing of *Rosa alba*, could also reveal the influence and extractability of different substances with different methods for essential oil crops treatment [8]. The results from the analysis are presented in Table 1. The highest polyphenol content exhibited the extract from RA_CO2 residue followed by the melissa waste (M_SD). These results suggested that supercritical CO_2 selectively extracts mostly the essential oil but not the polyphenols which are mostly solubilized when steam distillation is applied as method of processing. Nevertheless, the results for polyphenols, as well as the results for antioxidant capacity suggested that the wastes had potential to be used as a source of dietary antioxidants [9]. In the next experiments the individual substances – flavonoids and phenolic acids, contributing to the observed high antioxidant capacity, were determined by liquid chromatography (Tables 2 and 3). The RA_CO2 waste had a higher flavonoid content. This is the only residue obtained after supercritical CO_2 extraction process included in the study and these results suggested that this kind of extraction did not solubilize more hydrophilic compounds such as flavonoids. They remain in the waste and could be recovered as extracts in further treatment. In the subsequent experiments the amounts of major phenolic acids in the extracts were determined (Table 3).

Confronting the results for the phenolic acids the L_SD and RA_SD were found to be rich source of neochlorogenic acid, 3,4-dihydroxy benzoic acid, ferulic acid and gallic acid. In the B_SD extract the predominant phenolic acid was rosmarinic acid. Furthermore, the extracts were subjected to GC-MS analysis for content of polar volatile metabolites (Table 4).

Table 1. Polyphenol content and antioxidant capacity of ethanolic extracts from wastes

Waste	Polyphenols, mg/L	ORAC, µmolTE/L	HORAC, µmolGAE/L	FRAP, µmolTE/L	DPPH, µmolTE/L
RA_SD	2588±155^{d}	49203.9±1989.7^{c}	**23279.8±893.6^{a}**	1636.7±47.9^{c}	1176.7±48.5^{c}
RA_CO2	**11061±799^{a}**	**209214.4±4749.3^{a}**	**22361.4±1782.8^{a}**	**6000.0±43.7^{a}**	**5833.3±37.2^{a}**
L_SD	1453±91^{e}	48750.0±1480.1^{d}	16879.9±972.6^{b}	659.2±47.8^{d}	356.3±32.6^{d}
M_SD	**6630±292^{b}**	**225041.2±13683.3^{a}**	**24507.5±1117.8^{a}**	3331.3±33.9^{b}	2668.8±47.6^{b}
B_SD	3229±160^{c}	99635.3±5735.2^{b}	12834.7±1276.4^{c}	1745.0±58.3^{c}	1206.7±57.2^{c}

TE – Trolox® equivalents; GAE – Gallic acid equivalents; The results are presented as mean values of three replicates ± SD; $^{a, b, c, d, e}$ – different letters in a column mean statistical difference (one-way ANOVA, Tukey's test; $p<0.05$).

Table 2. Flavonoids (mg/L) in ethanolic extracts obtained from wastes

	RA_SD	RA_CO2	L_SD	M_SD	B_SD
Quercetin	170.67±2.48^{b}	185.37±1.93^{a}	37.41±1.86^{d}	53.26±1.99^{c}	nd
Quercetin-3-β-glucoside	157.14±1.90^{b}	**755.58±3.15^{a}**	49.28±2.24^{d}	102.79±1.83^{c}	154.52±2.59^{b}
Rutin	159.18±2.70^{d}	**1223.76±2.99^{a}**	nd	186.42±3.07^{c}	258.58±3.15^{b}
Myricetin	14.37±1.11^{e}	**114.19±1.39^{a}**	23.18±1.47^{d}	57.19±1.26^{b}	34.71±1.68^{c}
Kaempferol	**65.37±1.82^{a}**	28.68±1.34^{c}	12.46±1.27^{d}	34.12±1.56^{b}	25.19±1.47^{c}
Naringenin	nd	nd	nd	62.33±1.68^{a}	20.52±1.09^{b}
Apigenin	nd	nd	nd	153.99±2.17^{a}	20.56±1.67^{b}
Catechin	84.39±2.08^{e}	**1104.09±3.64^{a}**	413.14±2.84^{c}	439.18±2.73^{b}	204.78±3.64^{d}
Epicatechin	66.18±1.94^{c}	nd	**316.71±1.84^{b}**	**367.96±2.31^{a}**	nd

nd – not determined; The results are presented as mean values of three replicates ± SD; $^{a, b, c, d, e}$ – different letters in a row mean statistical difference (one-way ANOVA, Tukey's test; $p<0.05$).

Table 3. Phenolic acids (mg/L) in ethanolic extracts obtained from wastes

	RA_SD	RA_CO2	L_SD	M_SD	B_SD
Chlorogenic acid	nd	nd	nd	49.19±1.64	nd
Neochlorogenic acid	**162.92±2.10^{b}**	57.21±1.38^{c}	**197.66±2.17^{a}**	47.61±1.96^{d}	54.55±2.34^{c}
3,4-dihydroxy benzoic acid	**171.87±2.17^{b}**	76.22±2.08^{c}	**637.96±2.68^{a}**	57.08±1.84^{d}	30.39±2.07^{e}
Caffeic acid	32.27±1.59^{b}	nd	nd	49.19±1.60^{a}	nd
p-Coumaric acid	**42.42±2.08^{a}**	8.08±1.14^{c}	nd	9.68±1.10^{c}	12.01±1.06^{b}
Ferulic acid	**88.96±1.82^{b}**	8.32±1.17^{e}	**194.76±1.36^{a}**	28.32±1.27^{c}	24.35±1.48^{d}
Gallic acid	**159.39±1.67^{b}**	98.62±1.39^{c}	**365.56±2.43^{a}**	35.49±2.08^{e}	69.71±2.34^{d}
Rosmarinic acid	126.48±2.08^{c}	10.93±1.07^{e}	**154.83±1.29^{b}**	69.42±1.37^{d}	**1232.26±2.03^{a}**
Cinnamic acid	26.09±1.27^{b}	nd	nd	nd	43.38±1.56^{a}

nd – not determined; The results are presented as mean value of three replicates ± SD; $^{a, b, c, d, e}$ – different letters in a row mean statistical difference (one-way ANOVA, Tukey's test; $p<0.05$).

Table 4. Volatile metabolites (expressed as % of total ion current (TIC) in the extracts

Compound	RI	RA_SD	RA_CO2	L_SD	M_SD	B_SD
α-Pinene	940	0.45±0.07^{b}	0.48±0.06^{b}	0.25±0.06^{b}	0.36±0.06^{b}	0.68±0.07^{a}
β-pinene	980	0.32±0.05^{c}	0.33±0.05^{c}	1.54±0.08^{a}	0.91±0.09^{b}	0.31±0.04^{c}
β-Myrcene	991	0.18±0.04^{b}	0.19±0.04^{b}	1.19±0.09^{a}	0.25±0.08^{b}	nd
p-Cymene	1019	nd	nd	0.54±0.07^{a}	nd	0.72±0.06^{a}
Limonene	1025	nd	nd	**3.55±0.15^{a}**	**1.84±0.10^{b}**	0.22±0.05^{c}
Eucalyptol	1031	nd	nd	3.18±0.16^{a}	nd	2.91±0.10^{a}
cis-β-Ocimene	1040	nd	nd	**5.41±0.21^{a}**	**2.23±0.20^{b}**	0.21±0.06^{c}
trans-β-Ocimene	1050	nd	nd	3.37±0.19^{a}	1.65±0.15^{b}	0.35±0.10^{c}
γ-Terpinene	1062	0.47±0.07^{a}	0.50±0.07^{a}	0.38±0.06^{a}	nd	0.41±0.07^{a}
cis-Linalool oxide	1073	nd	nd	0.19±0.05^{a}	nd	0.16±0.04^{a}
trans-Linalool oxide	1078	nd	nd	0.29±0.05^{a}	nd	0.25±0.04^{a}
Terpinolene	1087	0.35±0.04^{a}	0.37±0.06^{a}	nd	nd	0.18±0.03^{b}
β-Linalool	1097	0.89±0.10^{c}	0.94±0.09^{c}	**18.91±0.15^{a}**	0.97±0.11^{c}	**5.92±0.12^{b}**
Phenethyl alcohol	1110	**8.70±0.18^{b}**	**11.10±0.16^{a}**	nd	nd	nd
cis-Rose oxide	1112	0.22±0.04^{a}	0.23±0.04^{a}	nd	0.33±0.05^{a}	nd
trans-Rose oxide	1127	0.15±0.04^{b}	0.16±0.04^{b}	nd	0.46±0.05^{a}	nd
Verbenol	1134	nd	nd	nd	0.33±0.06	nd
Camphor	1146	nd	nd	0.48±0.07	nd	nd
Citronellal	1151	nd	nd	nd	2.97±0.10	nd
Borneol	1169	nd	nd	0.58±0.10	nd	nd
Lavandulol	1171	nd	nd	**6.12±0.21**	nd	nd
Menthol	1173	nd	nd	nd	0.92±0.11	nd
Terpin-4-ol	1178	0.27±0.04^{c}	0.29±0.05^{c}	**3.10±0.11^{a}**	nd	0.77±0.09^{b}

Isomenthol	1180	nd	nd	nd	0.61±0.06	nd
Methyl chavicol	1182	nd	nd	nd	nd	2.79±0.10
α-Terpineol	1189	0.55±0.06^{c}	0.62±0.05^{b,c}	**3.13±0.09^{a}**	0.42±0.05^{c}	0.68±0.06^{b}
trans-Carveol	1195	nd	nd	nd	0.38±0.05^{a}	0.24±0.04^{a}
Myrtenol	1198	nd	nd	nd	1.33±0.09	nd
β-Citronellol	1228	**18.62±0.15^{b}**	**20.25±0.21^{a}**	nd	nd	nd
Nerol	1230	**3.81±0.11^{b}**	**4.02±0.12^{a}**	nd	2.65±0.12^{c}	nd
Neral	1240	nd	nd	nd	**15.78±0.21**	nd
Geraniol	1255	**8.51±0.14^{b}**	**10.37±0.16^{a}**	0.28±0.10^{d}	**3.43±0.09^{c}**	nd
Geranial	1270	nd	nd	nd	**18.11±0.23**	nd
Linalyl acetate, dihydro-	1275	nd	nd	**18.14±0.16**	nd	nd
(±)-Lavandulyl acetate	1290	nd	nd	**4.93±0.13**	nd	nd
Citronellyl acetate	1354	0.38±0.04^{a}	0.40±0.04^{a}	nd	0.42±0.05^{a}	0.15±0.03^{b}
Eugenol	1356	0.26±0.04^{a}	0.27±0.05^{a}	nd	nd	0.36±0.05^{a}
Neryl acetate	1364	0.55±0.09^{c}	0.58±0.08^{c}	0.95±0.08^{b}	**3.00±0.10^{a}**	nd
Geranyl acetate	1383	**4.51±0.15^{a}**	**3.68±0.13^{b}**	**2.94±0.11^{c}**	**4.42±0.16^{a}**	nd
Methyl eugenol	1401	nd	nd	nd	nd	0.61±0.03
β-Bourbonene	1383	0.28±0.07^{a}	0.30±0.08^{a}	0.20±0.09^{a}	nd	nd
β-Cubebene	1389	0.28±0.10^{a}	0.29±0.07^{a}	nd	nd	0.26±0.05^{a}
β-Elemene	1390	0.17±0.04^{b}	0.18±0.05^{b}	nd	nd	0.41±0.06^{a}
β-Caryophyllene	1419	2.66±0.10^{d}	**5.98±0.15^{c}**	**7.20±0.18^{b}**	**18.03±0.16^{a}**	2.07±0.16^{d}
α-Humulene (α-Caryophyllene)	1454	2.36±0.08^{b}	2.50±0.11^{b}	**5.06±0.12^{a}**	0.59±0.10^{c}	0.56±0.08^{c}
Germacrene D	1479	1.64±0.08^{c}	0.68±0.07^{d}	2.76±0.09^{b}	**4.33±0.11^{a}**	0.21±0.06^{e}
α-Farnesene	1508	0.63±0.05^{a}	0.66±0.05^{a}	0.27±0.04^{b}	nd	0.59±0.03^{a}
β-Bisabolene	1510	0.20±0.03^{a}	0.22±0.03^{a}	0.20±0.03^{a}	nd	nd
***trans*-Nerolidol**	1564	**2.90±0.12^{a}**	**3.06±0.15^{a}**	0.27±0.08^{b}	nd	nd
Spathulenol	1575	1.82±0.09^{a}	1.92±0.09^{a}	0.19±0.07^{b}	nd	nd
Caryophyllene oxide	1580	0.36±0.04^{c}	0.38±0.04^{c}	0.30±0.04^{c}	1.14±0.08^{a}	0.62±0.07^{b}
tau-Cadinol	1627	nd	nd	nd	1.16±0.09^{a}	0.31±0.06^{b}
tau-Muurolol	1629	nd	nd	nd	1.88±0.11^{a}	0.48±0.09^{b}
γ-Eudesmol	1631	0.33±0.04^{b}	0.35±0.04^{b}	0.42±0.05^{b}	1.17±0.08^{a}	nd
β-Eudesmol	1649	0.28±0.04^{b}	0.30±0.04^{b}	0.22±0.03^{b}	1.02±0.06^{a}	nd
α-Eudesmol	1651	0.99±0.06^{a}	1.04±0.06^{a}	0.34±0.05^{b}	nd	nd
α-Cadinol	1653	nd	nd	nd	**4.24±0.16^{a}**	1.64±0.10^{b}
Farnesol	1714	**4.37±0.09^{b}**	**4.62±0.11^{a}**	0.55±0.07^{d}	nd	1.51±0.08^{c}
n-Nonadecane	1901	0.14±0.06^{b}	0.15±0.05^{b}	0.17±0.04^{b}	0.81±0.07^{a}	0.28±0.06^{b}
n-Eicosane	2000	0.31±0.06^{b}	0.32±0.08^{b}	0.32±0.08^{b}	0.11±0.02^{c}	0.62±0.04^{a}

n-Heneicosane	2100	**12.80±0.16^{a}**	**7.10±0.21^{b}**	0.34±0.08^{d}	0.23±0.06^{d}	3.11±0.05^{c}
n-Docosane	2200	0.83±0.09^{b}	0.89±0.06^{b}	nd	nd	2.34±0.04^{a}
n-Tricosane	2300	**7.98±0.18^{a}**	**5.27±0.29^{b}**	nd	nd	2.10±0.16^{c}
n-Tetracosane	2400	3.15±0.12^{a}	2.87±0.13^{b}	nd	nd	1.31±0.10^{c}
n-Pentacosane	2500	1.95±0.16^{a}	2.06±0.10^{a}	nd	nd	1.63±0.09^{b}
n-Hexacosane	2600	2.69±0.14^{a}	2.84±0.17^{a}	nd	nd	1.84±0.13^{b}

nd – not determined; The results are presented as mean value of three replicates ± SD; $^{a, b, c, d}$ – different letters in a row mean statistical difference (one-way ANOVA, Tukey's test; $p<0.05$).

It is not surprising that the rose residues were rich in β-phenethyl alcohol, β-citronellol, geraniol and geranyl acetate. These compounds give the pleasant rose-like aroma of the extracts. The lavender waste was rich in β-linalool, linalyl acetate, lavandulol and lavandulyl acetate. The mellisa waste was among the richest in aroma metabolites waste – β-citral/neral, α-citral/ geranial, geranyl acetate, β-caryophyllene and germacrene D.

CONCLUSIONS

The results from the present study suggested that the wastes from the essential oil industry are rich in polyphenol and aroma substances. To the best of our knowledge, for the first time industrial wastes from *Rosa alba* (obtained from two different processing techniques) and *Ocimum basilicum* were investigated for their polyphenol and aroma substances. The obtained polyphenol- rich extracts could be purified and serve as source of dietary polyphenolic compounds. Besides, the extracts could additionally serve as a new type of aromatizing agents. The high amounts of polyphenolic compounds, known for their antimicrobial activity, also suggested that the wastes could successfully be used in the food industry as natural bio preservatives.

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Protective effect of gamma-irradiated extract of *Aronia Melanocarpa* L. in the gastro-intestinal tract of healthy mice models

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Over the last 20 years, antioxidant "food" therapy has been introduced as an effective method of reducing oxidative damage caused by various external factors. The antioxidants contained in *Aronia melanocarpa* L. berries can suppress oxidation reactions and regulate oxidative changes even in gastrointestinal tract diseases (GIT).

The aim of the study was to examine the influence of gamma ray irradiation at doses of 10 kGy and 25 kGy on the extract from dried berries of *A. Melanocarpa* L. on an experimental model of healthy mice, for its antioxidant activity and protective effect on the gastrointestinal tract. For this purpose, some markers of oxidative stress, such as reactive oxygen species (ROS) and nitric oxide NO· radicals were studied by EPR spectroscopy, before and after irradiation. The study was carried out with 24 mice divided in four groups: control group (n=6); 30% ethanol extract of non-irradiated *A. Melanocarpa* L. (n=6); 30% ethanol extract of 10 kGy *A. Melanocarpa* (n=6); and 30% ethanol extract of 25 kGy *A. Melanocarpa* L. (n=6). There was no statistically significant increase in the NO· levels ($p < 0.9$) between the groups treated with non-irradiated and irradiated with either 10kGy or 25kGy *A. Melanocarpa* L. The same dependence was observed in the results for ROS. There was no statistically significant difference ($p=0.1$) between the groups treated with non-irradiated and irradiated with 10 kGy and 25 kGy *A. Melanocarpa* L. Our results showed that irradiation with 10 kGy and 25 kGy does not impair the antioxidant and protective properties of the *A. Melanocarpa* L. extracts. Non-irradiated extract and irradiated extracts had a gastrointestinal protective effect.

Keywords: GIT, NO, ROS, oxidative stress, *Aronia Melanocarpa* L.

INTRODUCTION

Since the mid-20th century, there have been many researches related to the safety of irradiated food. Most provide only a small link in the evidence chain, but some provide key evidence concluding that pasteurization (with less harmful effects) may be an alternative method of food preservation [1]. It is well known that the dose of radiation applied to a food product is measured in kilogray (kGy). Under standard conditions for the storage of dried spices and herbs, a range of ^{60}Co irradiation is applied [2]. The gamma irradiation method is permitted for the decontamination of dried herbs and vegetable spices with a maximum total average absorbed dose of up to 10 kGy [3]. Exposure to gamma radiation at doses below 10 kGy has reduced the content of non-sporulating pathogens [1]. When the exposure to gamma irradiation is at doses above 10 kGy, it is effective for increasing food safety by reducing the pathogenic microorganisms and extending the shelf life of food by removing the microorganisms responsible for food spoilage. The gamma irradiation method is permitted for decontamination of dried aromatic herbs, spices and vegetable spices with a maximum total average absorbed dose of 10 kGy, but this limit has been raised by the FDA to doses of 30 kGy for these products [4]. Since every step of spices and herbs storage influences the final result of the products, the question is whether the irradiation of the starting material using ionizing radiation could possibly affect the composition of the plant material obtained and, therefore, its biological activities.

Aronia Melanocarpa L. fruits (*A. Melanocarpa; AM*) are widely used in the food industry for the production of juices, canned food, tinctures, fruit teas and nutritional supplements [5]. However, fresh, unprocessed *A. Melanocarpa* fruits are rarely consumed because of their bitter taste, as a result of the presence of a significant amount of polyphenols.

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In addition, the fruits are a rich source of anthocyanins, which include cyanidin 3-glucoside, 3-galactoside, 3-xyloside, 3-arabinoside, pelargonidine-3-galactoside and pelargonidine-3-arabinoside [3]. Polyphenols are biofactors that determine the high activity of *A. Melanocarpa*. Moreover, there is evidence that *A. Melanocarpa* fruits have the potential to inhibit the development of various cancers, and can suppress oxidation reactions and regulate oxidative changes even in gastrointestinal tract diseases (GIT) [3, 4].

The main goal of the herein reported study was to investigate whether gamma irradiation at doses of 10 kGy and 25 kGy influence the antioxidant activity of the extract from dried berries of *A. Melanocarpa L*. on an experimental model of healthy mice. For this purpose, we studied the oxidative stress biomarkers levels, reactive oxygen species (ROS), and nitric oxide NO· radicals by EPR spectroscopy, before and after irradiation. Possible protective effect of the extract on the gastrointestinal tract of the studied mice was also discussed.

EXPERIMENTAL

Materials

Carboxy PTIO.K potassium salt, dimethyl sulfoxide (DMSO), and other (HPLC grade) solvents and reagents were purchased from Sigma-Aldrich (Steinheim, Germany).

Animals

The study was carried out with 24 mice divided in four groups: Control group (n=6); 30% *A. melanocarpa* non-irradiated (n=6); 30% 10 kGy *A. melanocarpa* (n=6); and 30% 25 kGy *A. melanocarpa* (n=6).

Black chokeberry fruits

Black chokeberry fruits were supplied from Vitanea Ltd. (Plovdiv, Bulgaria) in the stage of full maturity, in 2017. Fresh fruits were frozen at -18˚C, lyophilized (Christ Alpha 1-4 LDplus, Martin Christ GmbH, Germany) and stored in a desiccator until use.

Gamma-irradiation of dried A. Melanocarpa fruits

Freeze dried berries were irradiated at a cobalt-60 source with 8200 Ci activity. The chosen absorbed dose was 10 kGy and 25 kGy. All gamma-irradiated samples and untreated controls were pulverized and 0.5 g was mixed with 40 ml of extract - 30% ethanol acidified with 0.5% formic acid.

Samples were extracted on a magnetic stirrer for 1 h at room temperature [6]. The total polyphenol content of the investigated samples varied between 6935 ± 79 mg/100 g DW and the total content of anthocyanins was in the range of 1192 ±mg/100 g DW, at 10 kGy, as reference [7].

For the experiment IRC/w non-inbred albino male mice (25±1.5 g) were used. The mice were housed in polycarbonate cages in controlled conditions (12 h light/dark cycles), at a temperature of 18-23ºC and humidity of 40-70%, with free access to tap water and standard laboratory chow at the Suppliers of Laboratory Animals in the Medical Faculty, Trakia University.

The animal study was approved with Directive 2010/63/EU/ Ethical Committee for Animals of BABH and Trakia University, Stara Zagora, Bulgaria (131/6000-0333/09.12.2016). The experimental animals were randomly assigned to four groups, each of 6 mice: control, non-irradiated 30% ethanol AM extract, 10 kGy 30% ethanol AM extract and 25 kGy 30% ethanol AM extract. The mice were treated orally in the acute experiment according to Eftimov *et al.* [8].

The control group was pretreated orally with saline solution (30 mL/kg) for 2 h. The non – irradiated group was treated with 30% ethanol AM extract. Both irradiated groups - 10 kGy and 25 kGy were pretreated with the respective extract at a dose of 30 mL/kg of 30% ethanol AM extract. The mice were anesthetized and euthanized 2 h after the treatment. The freshly isolated gastrointestinal tract was collected on ice and homogenized.

The electron paramagnetic (EPR) measurements were performed on an X-Band, Emx^{micro} spectrometer (Bruker, Germany). Spectral processing was performed using Bruker WIN-EPR and SimFonia software.

Levels of ROS

The levels of ROS were determined according to Shi *et al*. [9] modified by us. The levels of ROS were calculated as double integrated plots of EPR spectra and results were expressed in arbitrary units.

NO· radicals

Based on the methods published by Yoshioka *et al.* [10] and Yokoyama *et al.* [11] we developed and adapted the EPR method for estimation of the levels of NO· radicals. The levels of NO radicals were calculated as double integrated plots of EPR spectra and results were expressed in arbitrary units.

Statistical analysis

Statistical analysis was performed with Statistica 7, StaSoft, inc. and the results were expressed as means ± S.E. $p < 0.05$ was considered statistically significant. To define which groups are different from each other we have used LSD post hoc test.

RESULTS AND DISCUSSION

In recent years it has become very popular to use herbs as medicines in a significant proportion of cases for the treatment or prevention of digestive disorders. Regular consumption of fruits, herbs, vegetables and plant foods rich in antioxidants is associated with improving overall health and reducing the incidence of chronic diseases of the cardiovascular system, gastrointestinal tract and other [11, 12]. The plant antioxidants can scavenge the free radicals, unstable and reactive forms with an unpaired electron in the outer orbit. Free radicals can cause damage to the cells of the human body, inducing "oxidative stress" that leads to various chronic diseases. Some antioxidants have been able to provide hydrogen radicals and thus can act as scavengers of free radicals. Therefore, these compounds may prevent some crucial points in the development of the disorders [13]. The positive health effects from regular intake of plant foods are partly due to the presence of vitamins, minerals and fibers, but mostly due to the non-nutritional biologically active compounds such as polyphenols.

Phenolics are required for pigmentation, growth, resistance to pathogens and many other functions in plants. In the human body, phenols have a beneficial effect on health. Some of the reasons for the protection are their powerful antioxidant properties and free radical scavenging activity.

A. Melanocarpa berries are an extremely rich source of bioactive components, such as polyphenols, in particular anthocyanins and flavonoids, which are estimated to be 2-3 times higher than in comparable fruits, and considered as a very important nutritional antioxidant [14]. Phenolic compounds are a product of plant secondary metabolism. They are very different in structure and can be found in plants as simple molecules, such as phenolic acids, or as highly polymerized molecules, such as proanthocyanidins. A lot of studies indicated the high antiradical activity of chokeberry. Polyphenols have been shown to possess significant antioxidant properties by directly removing reactive oxygen species (ROS) and inducing cellular antioxidant systems to support in the fight against the oxidative damages.

In the present study, EPR methods were used to record free radicals in the samples tested. The results for the ROS levels (Fig. 1) in the studied groups showed a statistically significant difference only between the control group ($p=0.000$) and all groups treated with *A. Melanocarpa* (non–irradiated and irradiated at a dose of 10 kGy or 25 kGy).

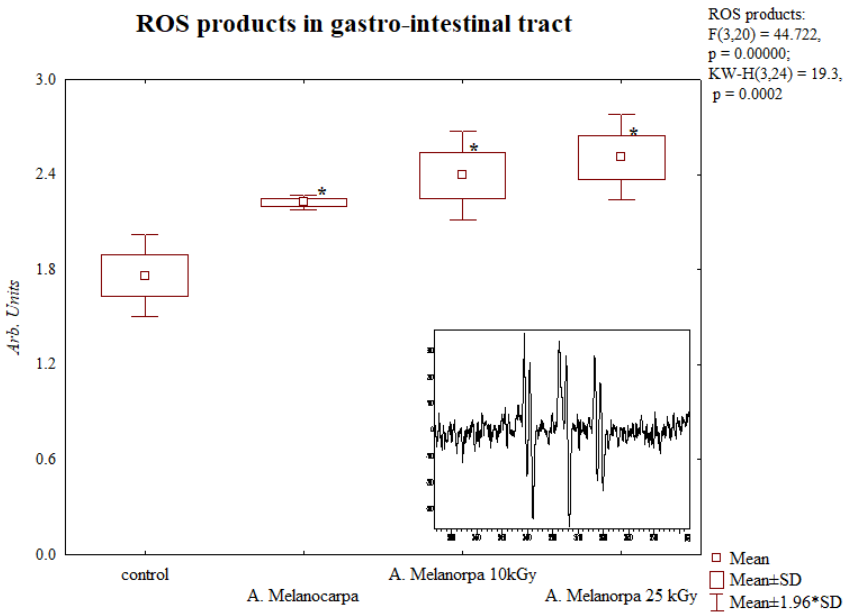

Fig. 1. ROS levels in the gastro-intestinal tract. Significant difference *$p < 0.05$ *vs* controls. The Scheffe post hoc test was used to determine the groups differing from each other.

Moreover, there was no statistically significant difference between the *A. Melanocarpa*-treated group (mean 2.20±0.05) and the groups treated with 10 kGy (mean 2.44 ± 0.07) and 25 kGy (mean 2.50 ± 0.06) *A. Melanocarpa* extracts.

Over the irradiation process, *A. Melanocarpa* is exposed to a controlled amount of radiation from a radioactive source, such as cobalt-60. Gamma rays penetrate the extracts evenly, quickly killing plant poisoning bacteria, harmful parasites and insects. The dose of radiation and the amount of radiation energy absorbed by black chokeberry are the most critical factors in the irradiation. Polyphenols without toxicity or side effects can help prevent and reduce the level of oxidative damage caused by free radicals and ionizing radiation. Plant polyphenols have been found to have significant radioprotective effects [3].

Despite many plants are being reported to have antioxidant potential, only a few of these antioxidant activities have been confirmed or investigated after a high dose of gamma irradiation [4]. In the presented study, we also evaluated reactive nitrogen species (RNS), such as the nitric oxide radicals (NO•). The results were the same as for the ROS levels (see Fig. 2), with a statistically significant difference between the control group (mean 2.28±0.08) and all groups treated with *A. Melanocarpa* extract (mean for non–irradiated A.M. 3.55 ± 0.07; mean for 10 kGy AM 3.64 ± 0.1; and mean for 25 kGy A.M. 3.69±0.2).

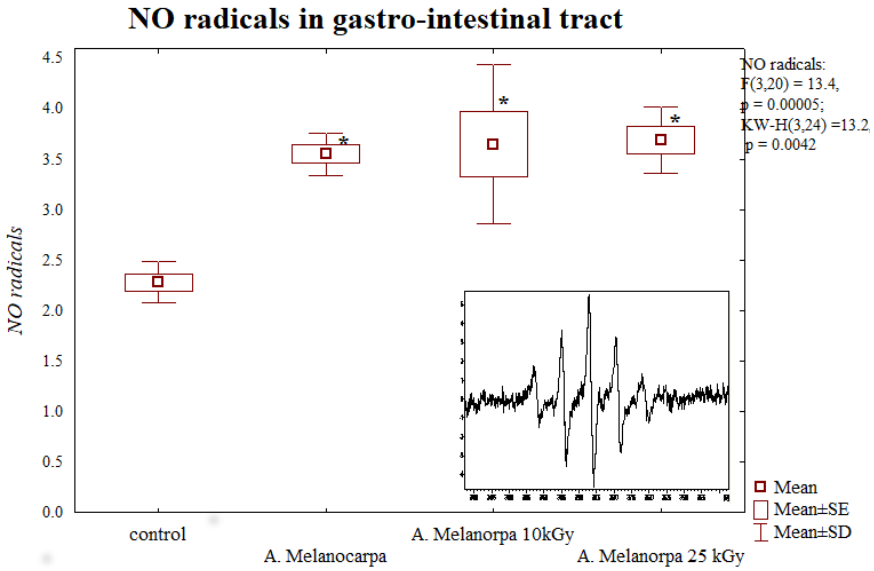

Fig. 2. NO radicals in the gastro-intestinal tract. Significant difference *p < 0.05 *vs* controls. The *Scheffe post hoc test* was used to determine the groups differing from each other.

Ionizing radiation induces cells to produce excessive ROS/RNS, leading to increased lipid peroxidation, disrupting redox homeostasis in cells and living tissues, and reducing the activity of enzymatic and non-enzymatic antioxidants *in vivo*. From the results presented in this study for levels of NO radicals and ROS, there were no statistically significant differences between the non-irradiated *A. Melanocarpa* extract and those irradiated with either 10 kGy or 25 KGy. These results of ours are supported by the study of Alloun *et al.* [3] who concluded that after irradiation at doses from 5 to 30 kGy the plant polyphenols had an anti-radiation and anti-oxidative stress effect by restoring the redox balance of the system.

The role of *A. Melanocarpa* antioxidants in gastrointestinal tract (GIT) diseases is important; the food (nutrition) contains various prooxidants, and increased the levels of lipid peroxides. Thus, prooxidants are likely to cause oxidative stress in the gastrointestinal tract, gastric ulcer and develop cancer of the stomach, colon and rectum [15].

In turn, antioxidants can suppress such oxidative stress and related diseases in the GIT before being absorbed. Moreover, chokeberry juice significantly and in a dose-dependent manner reduces the number and area of indomethacin-induced gastric ulcers in rats [3, 16]. In addition, administration of indomethacin resulted in extensive lipid peroxidation, indicating that ROS product and NO radicals are involved in the development of mucosal damage [17, 18]. The polyphenols from *A. Melanocarpa* are the least well absorbed. Aronia composition has significantly high concentrations of chlorogenic and non chlorogenic acids, but esterification impairs their intestinal absorption. At

the same time, there is considerable experimental evidence of the efficacy of chokeberry products in a wide range of pathological conditions mediated by uncontrolled oxidation processes [1, 19]. Furthermore, the first site of the antioxidant action of *A. Melanocarpa* polyphenols is the gastrointestinal tract, where proanthocyanidins and their metabolites might act as radical scavengers [20]. The mechanisms of the *in vivo* antioxidant activity of *A. Melanocarpa* polyphenols after absorption extend far beyond radical scavenging and include inhibition of ROS and RNS formation, prooxidant inhibition, and restoration of antioxidant enzymes and possibly cellular signaling to regulate the levels of enzyme and antioxidant compounds [1]. Moreover, it might be hypothesized that irradiation at 10kGy and 25kGy does not impair the antioxidant and protective properties of the *A. Melanocarpa* extract.

CONCLUSION

From the presented results we can conclude that irradiation at doses of 10 kGy and 25 kGy does not impair the antioxidant properties of the *A. Melanocarpa* extract. We suppose that non-irradiated extract *A. Melanocarpa* L. and irradiated at doses of 10 kGy and 25 kGy *A. Melanocarpa* L. extracts might be used as gastrointestinal tract protectors.

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Conflict of Interest: *The authors declare that they have no conflict of interest.*

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Amelioration of bleomycin-induced pulmonary toxicity in murine models by a semiquinone glucoside derivative isolated from *Bacillus sp.* INM-1

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The mechanism of action of bleomycin (BLM) is related to the ability to cause DNA fragmentation and myelo-depressive and immunosuppressive activity. In the present study, pulmonary protective effects of a semiquinone glucoside derivative (SQGD), a bacterial metabolite isolated from *Bacillus sp. INM-1*, were evaluated in a model of BLM-induced oxidative toxicity in IRC/white male mice. Mice were divided into four groups, i.e., (1) untreated controls; (2) SQGD-treated (40 mg/kg b. wt. i.p., every two days) mice; (3) BLM (0.34 U/kg b.wt., i.p., every two days); and (4) SQGD (40 mg/kg b.wt., i.p.) administered 2 h prior to BLM-administration (0.34 U/kg b.wt., i.p.). Till the end of the 28 experimental days of BLM administration, no mortality rate (-1) was observed in mice. Reactive oxygen species (ROS) and lung tissue homogenates of the treated animals were subjected to ascorbate radical estimation and ROS production and the level of three oxidative stress markers, i.e., the GSH ratio, and antioxidative capability of SOD and CAT were estimated. Treatment with SQGD protected BLM-induced pulmonary injury by suppressing oxidative stress with significant ($p< 0.05$) reduction of ascorbate radicals and ROS products in the lungs, and enhancement of the GSH ratio and SOD and CAT. Reduction in oxidative disorders was observed in healthy mice which were treated with SQGD only, compared to controls. Thus, it can be concluded that SQGD treatment alone and in combination SQGD+BLM neutralized BLM-induced pulmonary toxicity associated with oxidative damage caused by the anticancer drug not only by reducing lipid peroxidation but also by improving antioxidant status of lungs. Therefore, SQGD has a potential therapeutic effect as a strong radical-scavenger in the prevention and alleviation of pulmonary fibroses.

Key words: SQGD, BLM, lipid-peroxidation, pulmonary protection

INTRODUCTION

Bleomycin (a cytostatic-glycopeptidyl antibiotic) is a small peptide isolated from *Streptomyces verticillatus* in 1966 by Umezawa *et al.* and its mechanism of action is breaking the DNA-double binding region by the free-radical overproduction, which is oxygen and iron subordinate [1]. It has been shown to be an effective antitumor agent used for treatment of a variety of malignancies, predominantly germ cell tumors, testicular carcinoma and Hodgkin lymphoma [2, 3]. The drug caused partial marrow suppression, but pulmonary toxicity (PT) was a major adverse effect, because BLM damaged lung cells by inducing lipid peroxidation [4]. The serious limitation of BLM therapy is deposition in the pulmonary cells, activation of the anti-inflammatory cytokines IL-18 and IL-1 beta that result in BLM-induced lung injuries and idiopathic pulmonary fibrosis (called also fibrosing alveolitis) (IPF) [2, 3, 5]. Dempsey *et al.* specified IPF as a progressive disease characterized by lung alveoli (fibrotic) changes in the pulmonary structure that lead to decreased gas exchange and pulmonary cramps [6]. Also, Giri *et al.* [7] found that intratracheal BLM infusion in rodents gave a reliable and versatile therapeutic potential for the study of BIPF in animals and humans. Recently it has been reported that BLM induction in mice results in an explosive pulmonary inflammatory response in the alveolar wall, characterized by leukocyte infiltration, fibroblast proliferation and increased content of collagen and other matrix components [8, 9, 10]. In addition, the BLM-generated ROS in lung tissues were another factor partially responsible for induced cytotoxicity and carcinogenesis [11].

Given the mechanism of action of BLM and anti-inflammatory treatments, the use of antioxidant therapy may be a potential therapeutic strategy for clinical applications. Semiquinone glucoside derivative (SQGD) (deposited in NCBI gene bank with accession number EU 240544.1), isolated

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from a radio-resistant bacterium *Bacillus sp. INM-1* [12, 13], was used as anti-inflammatory and BLM injury protective agent in the present study. SQGD has already been characterized and evaluated for its antioxidant activity, immune-stimulatory properties and protective activities against gamma radiation-induced damages [12-17] and modulate immune-stimulatory cytokines [16, 17]. The present research was focused on the amelioration of the SQGD antioxidant potential to overcome BLM-induced pulmonary toxicity in terms of antioxidant enzymes (Glutathione (GSH); superoxide dismutase (SOD); catalase (CAT)) and intracellular ascorbate ($Asc^{\cdot}$) radicals and ROS production in lungs in male RCS/b mice.

EXPERIMENTAL

SQGD characterization

SQGD characterization (strong, single symmetrical signal, g= 2.0056 ± 0.0002 registered in powder) was carried out using electron paramagnetic resonance (EPR) spectroscopy [18] and other extraction procedures described previously [12, 13]. The type strain *Bacillus sp. INM-1, MTCC No. 1026, IBG-21* was deposited at the Institute of Microbial Technology, Chandigarh and INMAS, Delhi, India as reference.

Materials

The BLM solution (($C_{55}H_{84}N_{17}O_{21}S_3$ sulfate) EP 9041-93-4; dose 0.069U/ml); EDTA-containers (5 cm^3 Monovette, Germany); phosphate-buffered saline (PBS, pH 7.4); reduced GSH-ELISA commercial kit (Catalog No- CS0260, 2–8^{0}C); 5,5'-dithiobis (2-nitrobenzoic acid); nembutal; spin-trap dimethyl sulfoxide (DMSO) D4540-500 ml and N-*tert*-butyl-alpha-phenylnitrone (PBN) were purchased from Bristol-Myers Squibb Co., and Sigma Aldrich Company.

Instruments

The spectrophotometric measurements were performed on the UV–VIS spectrophotometer-400 (320 nm-750 nm), TERMO Sci., RS232C, Stratagene, USA. For the ELISA kit was used Urit-660 A spectrophotometer, Germany. The direct and spin-trapping EPR analyzes were carried out in triplicate on an EMX^{micro} spectrometer (Bruker, IFC-11007) Germany.

Animals used

Yung 24 male RCS/b mice (n=6; average weight 30 g; 8 weeks of age, specific pathogen-free, second line) were obtained from the Vivarium, Medical Faculty, Stara Zagora, Bulgaria and placed in polypropylene cages. Animals were fed with laboratory chow diet (standard conditions 20-22^{0}C and 12 h light/12 h dark; humidity of 40–60%). Food and tap water were provided *ad libitum*. The regulations and rules of the Research Ethics Commission of the Medical Faculty, Trakia University and the European directive 210/63/EU from 22.09.2010 were strictly followed during experiments.

Animal model of BLM-induced lung toxicity, SQGD administration and treatment protocol

Bleomycin-induced lung toxicity and co-treatment with SQGD: the male mice were once i.p. injected early in the morning with a BLM solution in cold saline of 250 μl (0.24 U/kg b.w.) continued on schedule from the 1st to the 28th day of the start of the experiment, into the lower part of the stomach by needle number 1, while breathing evenly. Pre-treatment with SQGD was given with i.p. injections at a concentration of 40 mg/ml (0.131 mg/kg bw) for 28 days, daily, in the early morning 2 h prior to BLM-injection, from the 1st to the 28th day.

Each of the mice was allowed to recover for 2 days under normal laboratory conditions, the body weight being measured every day during the first week and then weekly until they were killed on day 29 with lethal i.p. injection of nembutal (50 mg/kg). The toxicological symptoms, physiological status, and behavior (after 24 h) of the IRC/b mice were monitored daily. The freshly isolated lung tissue (un-extravasation with cold 0.9% saline) collected on ice were homogenized and after addition of solvents the samples were centrifuged at 4000 rpm for 10 min at 4°C and 300 μl of supernatant was prepared for biochemical analysis. The fresh blood (2 cm^3) was collected directly from the heart in cold EDTA-containers and centrifuged at 4000 rpm for 10 min at 4°C, and 200 μl of plasma from each group was placed at - 4^{0}C until used.

Experimental groups: The study was performed with 4 groups, with 6 animals per group: (1) control mice treated with 300 μL of cold PBS, pH=7.4; (2) animals that received SQGD before eating; (3) animals that received BLM before eating; (4) animals that received SQGD i.p. 2 h prior to BLM.

Superoxide dismutase assay

The activity of plasmatic and lung cellular SOD was assayed using Sun *et al*., 1988 method [19]. The absorbance was recorded at 420 nm for 3 min with 30 s intervals and calculated with the help of kinetics of the reaction mixture.

Catalase assay

CAT activity in the plasma and lung homogenate was assessed using Aebi method [20]. The absorbance was recorded at 240 nm for 1 min at 15 s intervals in both blank and test samples.

Glutathione assay

GSH levels in plasma and lung cells were estimated using the method of Akerboom and Sies, 1981 [21]. Reduced GSH was assessed by a continuous reduction of DTNB expressed as nanomoles of GSH per milliliter of protein. Blank without blood/ tissue homogenate was prepared similarly and the absorbance was recorded at 412 nm.

In vivo ascorbate levels

The method used according to Buettner & Jurkiewicz [22] was to evaluate the ascorbate levels ($Asc^{\bullet}$) and its protection against BLM-induced toxicity by SQGD pre-treatment. In brief, 200 mg of lung samples and 100 µl of plasma were homogenized in cold DMSO (10% w/v) and centrifuged at 4000×g for 10 min at 4°C. Supernatants were transferred into Eppendorf tubes, analyzed and EPR spectrum was registered in the lungs and blood plasma (*arbitrary units*). The spin-adduct formed between DMSO and generated $Asc^{\bullet}$ radicals was recorded in real time.

In vivo evaluation of ROS production

Lung tissue homogenates (100 mg) and 100 µl of plasma were homogenized with 900 µl of 50 mM spin-trap PBN dissolved in DMSO using ultrasound sonicator at one cycle for 1 min. After 5 min of incubation in cold, the suspension was centrifuged at 4000 rpm for 10 min at 4ºC and the EPR spectrum was registered (*arbitrary units*). The formation of ROS production in the supernatants was estimated as described earlier [23] with some modifications [18, 24].

Statistical analysis

The data obtained were processed by the statistical program Statistica Version 6.1 (StaSoft, Inc., USA) and presented as mean ± standard error (SE). Statistical analysis was performed using one-way ANOVA and Student t-test to determine significant differences among data groups. A value of $p<0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

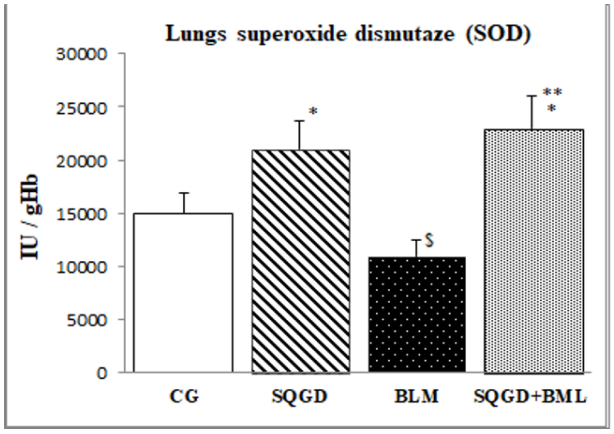
In this study, we demonstrated that i.p. SQGD inhibits effectively significant pulmonary changes following BLM-administration in mice. We also showed that SQGD regulated the antioxidant defense system and significantly decreased oxidative stress changes in lungs in combination SQGD + BLM- injured animals compared to BLM-administered alone. Furthermore, similarly un-limited intraperitoneal bioavailability of natural antioxidants parallels with previous findings in animals [25]. In his investigation Chandler, 1990 [26] reported that the ROS generated in the lung cells after BLM administration were probably responsible for its cytotoxic changes after cancer treatment. Therefore, the protective effects of bacterial metabolite on mortality and chronic inflammatory response were not surprising given the antioxidant ability to protect different tissues and organs of ROS adverse effects [12-18, 27]. In our study in the group treated with saline, and in the SQGD alone and SQGD + BML treated groups no deaths were recorded. 28 days of BLM-induction statistically insignificant, only 16% of the mice died, and death occurred 19 days after the start of the experiment possibly due to respiratory failure.

SQGD alone provided protection against 5-fluorouracil-induced immunosuppression and recovered the spleen, bone marrow and haematopoiesis [27]. In addition, SQGD has been shown to stimulate antioxidant production against radiation-induced ROS by enhancing antioxidant enzymes, and also provides increased antioxidant status at renal [17] and small intestine systems [15]. Significant increases in endogenous SOD activity were observed in lungs of mice treated with SQGD alone and in pretreatment with the antioxidant in the SQGD+BML group, compared to untreated controls ($p< 0.004$) and BML-administration ($p< 0.05$) at 28 days (Fig. 1a).

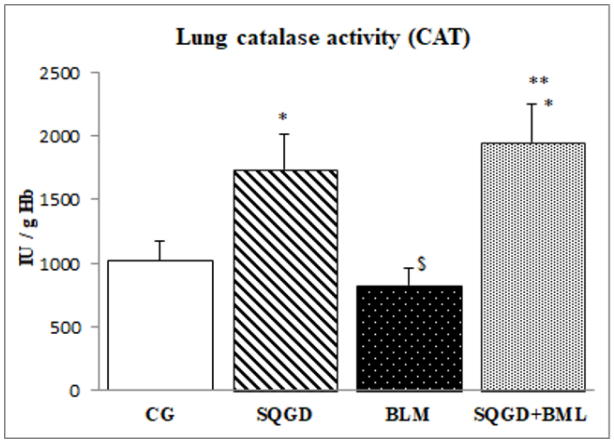
Maximal increase (23000.18 ± 408.52 UI/g of Hb) in SOD enzyme was observed at 28 days in lungs in the SQGD+BML group. The results demonstrated that SQGD significantly regulates SOD induction and alone does not induce oxidative stress changes by scavenging superoxide radicals. Moreover, SQGD accelerates SOD activity even after BLM pulmonary induction by its stable antioxidant properties [12, 17].

Hydrogen peroxide (H_2O_2) induces toxicity and increases oxidative disturbances in accumulation in the pulmonary cellular system. For the H_2O_2 decomposition to normal oxygen and water, the involvement of another endogenous enzyme - CAT is necessary. Results of the study demonstrated a slight increase in CAT activity (Fig. 1b) in lungs in mice treated with SQGD at 28 days of treatment (23.076±1.22 l mol/min/mg *vs.* 23.076±1.22), compared to controls. Though BLM-administered

animals showed significant decrease in CAT enzyme ($p<0.05$) as compared to controls (Fig. 1b), a significant increase in enzyme activity was evident in BLM mice pretreated by SQGD, as compared to BLM and control groups.



a)



b)

Figure 1. SQGD influence on SOD (a) and CAT activities against BLM-induced toxicity in lungs tissues. **1a)** *p< 0.003 SQGD treated *vs* CG; $p< 0.05 BLM-treatment vs control; **p< 0.05 SQGD+BLM group *vs* CG and BLM- group; **1b)** *p< 0.004 SQGD treated *vs* CG; $p< 0.05 BLM- treatment *vs* control; **p< 0.05 SQGD+BLM group *vs* CG and BLM- group.

We assume that the bacterial metabolite SQGD has the potential to increase the CAT activity levels in alveolar cells and to modulate the oxidative stress induced by BLM-induced toxicity. Earlier reports demonstrated that the SQGD intensity effect on pulmonary metabolic detoxifying enzymes (SOD and CAT activities) was due to the acceleration of the antioxidant protective mechanisms in a biological system [12, 17]. Concomitantly, the present study suggests that SQGD may inhibit BLM-induced pulmonary toxicity by immunosuppressive effects or by other unknown mechanism characteristic of the extremophilic action [17, 28, 29].

Meister, 1994 [30] draw attention to a tripeptide thiol GSH as an intracellular antioxidant and drug-toxicity protector against the oxidative changes caused by ROS. Significant increase in GSH levels in lungs ($p<0.05$) was observed at 28 days in the SQGD treated group and in SQGD+BLM, compared to controls (Fig. 2).

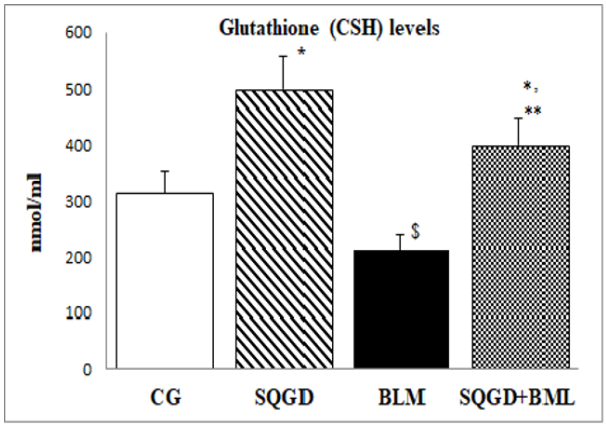


Figure 2. SQGD influence on CSH level against BLM-induced toxicity in lungs tissues. *p< 0.05 SQGD treated vs CG; $p< 0.05 BLM-treatment *vs* control; **p<0.05 SQGD+BLM group *vs* CG and BLM- group.

In contrary, a significant decrease ($p< 0.05$) in the GSH levels was observed in the BLM-administrated mice, compared to controls and SQGD groups. In accordance with our results, Mishra *et al.*, 2013 [17] commented that SQGD, as an excellent antioxidant increased organ GSH levels by free radical scavenging even at 10 Gy radiation exposure.

In addition to antioxidant status, the GSH function in the reduction of dehydroascorbate to ascorbate in tissues (lung, liver, kidney, brain, eye) was examined by different researchers in *in vitro/ ex vivo* animal systems [30, 31]. Buettner and Moseley, 1993, using EPR spin trapping investigated the ascorbate radicals presence, and found that BLM becomes a redox-inactive form incapable to break the DNA strand [32]. The EPR results of the ascorbate ($Asc^{\cdot}$) radicals assessment are shown in Fig. 3.

Twenty-eight days after BLM challenge, the lungs (0.218±0.06 a.u. *vs* 0.323±0.008 a.u.; $p<0.005$) and plasma samples (0.1988±0.0016 a.u. *vs* 0.299±0.01 a.u.; $p<0.004$) of the IRC/b mice showed statistically significant lower levels in $Asc^{\cdot}$, than the same in controls. In contrary, an increase in $Asc^{\cdot}$ expression in both lungs and plasma in the SQGD ($p<0.004$) and SQGD + BLM groups ($p<0.005$), was registered compared to untreated

controls and BLM-group. This study demonstrated that SQGD protects the reaction cycle of dehydroascorbate to ascorbate in alveolar tissues and blood against the BLM cytotoxicity in the lung by suppressing additional oxidative stress. Present findings were in complete agreement with the protective effect of the antioxidant fraction of SQGD alone and in combination in organs/ cells and in blood samples against lomustine-induced cytotoxicity [33] or in the regulation of stress-mediated signaling by transcriptional factor NF-kb and provided a gain support to the present research [14, 15].

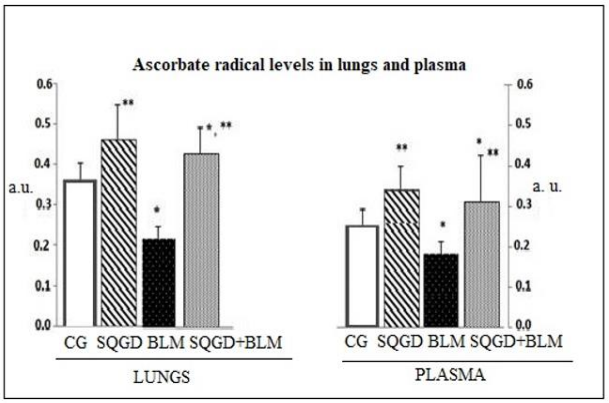


Figure 3. SQGD effect in BLM-treatment on ascorbate levels in the lungs and plasma. Mice pretreated with SQGD 2 h i.p. before BLM-treatment. All values shown are mean ± SE. Values significantly different: *p< 0.05 lungs and *p< 0.004 plasma BLM treated *vs* CG; $^{\$}$p< 0.05 SQGD *vs* BLM; **p< 0.05 SQGD+BLM group *vs* BLM-group.

Increased ROS generation, as well as changes in antioxidant/ prooxidant cells capabilities lead to tissue oxidative damage [34]. BLM administration stimulates the lipid macromolecules damages, resulting in excessive release of larger amounts of superoxide anion and nitric oxide from alveolar macrophages and oxidative burden imposed on pulmonary tissues [35]. The ameliorative SQGD activity on BLM-exposure in animals is shown in Fig. 4. In these analyses, the EPR method indicates a significant two-fold elevation in pulmonary ROS production/ lipid peroxidation in the BLM group, compared with controls and SQGD alone, in both lungs ($p < 0.05$) and blood ($p < 0.005$) samples. Moreover, better effects were observed in the SQGD + BLM groups, in both ($p < 0.004$). Our results are in accordance with Kara *et al*., 2007 other authors that have reported higher lipid peroxidation after BLM-exposure [36].

Earlier reports suggested that antioxidants possessed protective effect against pulmonary functional injuries, owing to direct antioxidant and free-radical scavenging mechanisms and ROS regulation [33-35].

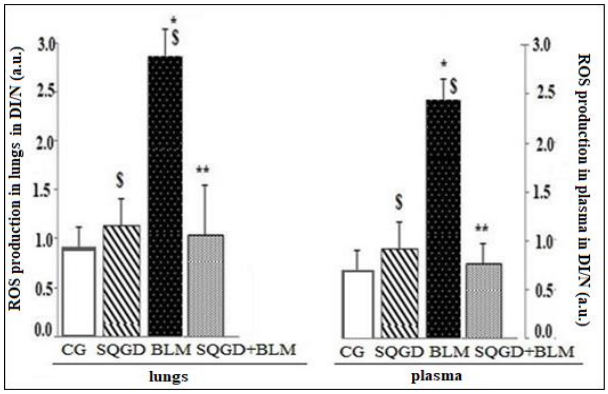


Figure 4. SQGD effect in BLM-treatment on ROS production in the lungs and plasma. Mice pretreated with SQGD 2 h i.p. before BLM-treatment. All values shown are mean ± SE. Values significantly different: *p< 0.05 lungs and *p< 0.004 plasma BLM treated *vs* CG; $^{\$}$p< 0.05 SQGD *vs* BLM; **p< 0.05 SQGD+BLM group *vs* BLM- group.

CONCLUSION

In conclusion, the study demonstrated that bacterial antioxidant SQGD significantly ameliorates the development of BLM-induced pulmonary toxicity and oxidative stress. The results suggest that SQGD pretreatment markedly suppresses the toxic exhibitions, by scavenging ascorbate radicals and ROS production and normalizing the enzyme protective system. However, further studies are needed to clarify the SQGD effect and its combinations in IPF therapy, applied to experimental animal models.

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Antioxidant effects and oxidative stress-modulatory activity of *Glycyrrhiza glabra* L. root extract against acute toxicity

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Glycyrrhiza glabra (G. glabra), Indian medicinal plant, exhibited good anti-inflammatory, anti-microbial, anti-oxidative, immunomodulatory, hepatoprotective, anticancer properties and excellent *ex vivo* radical-scavenging capacity against induced acute toxicity. In the present study, using *in vivo* methods, we investigated changes, therapeutic potential and antioxidant action against oxidative stress-induced conditions in tissues isolated from *G. glabra* extract-treated animals (80 mg/kg/ given 14 times/14 days) and in Bleomycin (dose 0.34 U/kg body) induced acute toxicity. After dissection of the liver, lung and spleen, the tissues were homogenates in cold PBS solution and studied by spin-trapping electron paramagnetic (EPR) spectroscopy. Tissue homogenates were prepared for determination of the ascorbate, nitric oxide radical levels and ROS products. It was established that in the *G. glabra* extract-treated animals the biomarker levels were close to the controls ($p<0.05$). Statistically significantly lower levels of nitrite and ascorbate radicals were measured only in spleen. Lipid peroxidation was significantly reduced in the *G. glabra*+BLM group, compared to the BLM-treated group ($p<0.004$). *In vivo* EPR study characterized *G. glabra* root extract as a good antioxidant scavenging free-radical formations with a possibility of neutralization of acute oxidative diseases.

Keywords: *G. glabra* root extract; BLM; ROS; acute oxidative stress

INTRODUCTION

Plant materials (leaves and roots) used in Hindu medicine (*Ayurveda*) are evaluated for antioxidant-pharmacological activities and characterized with low reactive oxygen species (ROS) levels [1, 2]. The roots of *Glycyrrhiza glabra L. (G. glabra, Fabaceae)*, commonly known as 'licorice', possess biologically active components as triterpenes, saponins, flavonoids, isoflavonoids, chalcones, and glycyrrhizic acid, as previously reported for the extract [3, 4]. *G. glabra*-extracted flavonoids have been widely investigated for ability to inhibit lipid peroxidation [5] and for hydroxyl groups scavenging activity [6-9]. Isoflavon derivatives (*glabridin, hispaglabridin A, hispaglabridin B*), isolated from *G. glabra*, were shown to be effective in protecting liver mitochondrial function against oxidative stress changes [10] and *in vitro* models displayed considerable antioxidant/protective effect against the human lipoprotein oxidative system [4, 11]. *G. glabra* extracts possess anti-asthmatic, anti-inflammatory, anti-viral, anti-microbial, anti-oxidative, anti-cancer, immunomodulatory, hepatoprotective and cardio-protective properties [3]. Because of its excellent antioxidant activity, the licorice aqueous extract is used for *in vivo* treatment of peptic ulcer, hepatitis C, and pulmonary diseases [3] and also exhibited anti-helminthic and anti-microbial activity. The cytotoxicity of the methanol extracts of *G. glabra* roots have been studied against immortal human keratinocyte, liver carcinoma (HepG2) and lung adenocarcinoma (A549) [12]. *G. glabra* roots aqueous extracts could be used as potent tools for a further development of cytoprotective preparations with anti-infectious potential [13]. Some researchers emphasized the anti-asthmatic and antioxidant properties of the licorice root, as well as its effectiveness against pulmonary (PF) and hepatic fibrosis (HF) in rats [3, 14, 15]. Ram *et al.* [16] reported that the presence of glycyrrhizin, as a main component in the structure of *G. glabra* mitigates the severity of asthma inflammation in animal models. The bioflavonoids presence (*glabridin*), multiply increases the anti-inflammatory and antioxidant activity of the *G. glabra* extract [17, 18]. Ghorashi *et al.* [19] revealed that the aqueous *G. glabra* root extracts caused reduction of collagen deposition and reduced the PF cells formation. For this purpose, need of stable natural compounds with lower organ toxicity, excellent antioxidant-modulatory activity and therapeutic effect against acute toxicity was felt.

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This study aims to investigate the antioxidant and oxidative stress protective effect of *G. glabra* root extract. For this reason, we studied: 1) the ascorbate and nitric oxide radical levels; 2) the ROS production as a real-time oxidative stress parameter; and 3) the antioxidant modulatory activity of *G. glabra* root extract against Bleomycin-induced acute toxicity in IRC mice.

MATERIALS AND METHODS

Plant extract and chemicals

The air-dried *G. glabra* roots were made into a coarse powder and after dissolution in 2 L of distilled water were subjected to a hot maceration process, with continuous stirring for 48 h. The aqueous extract was filtered through muslin cloth and the filtrate was concentrated by evaporation on a bath and then lyophilized. The extract was made and provided from INMAS, India as a reference. Dimethyl sulfoxide (DMSO), N-*tert*-butyl-alpha-phenylnitrone (PBN), 2-(4-carboxyphenyl)-4,4,5,5-tetra-methylimidazoline-1oxyl-3-oxide (carboxy-PTIO.K), bleomycin sulfate (C55H84N17O21S3, EP 9041-93-4) and PBS were purchased from Sigma Chemical Co, St. Louis, USA.

Animals

24 male ICR mice, aged 6–8 weeks, weighing 43-48±2.0 g were obtained from the Medical Faculty, Trakia University, Stara Zagora, Bulgaria. The animal procedures were in accordance with Directive 2010/63/EU on the protection of animals used for experimental and other scientific work (131/ 6000-0333/ 09.12.2016). Animals were housed in polypropylene cages at 18–23±2^{0}C and under a light/dark period of 12/12h daily. They were fed on a standard commercial feed, after 6 days of acclimatization on humidity 55% and free access to tap water. The lyophilized extract was dissolved in distilled H_2O and preserved at 4^{0}C until use. The animals were weighed every morning using a digital scale, and changes were recorded.

Bleomycin administration

Mice were divided into four groups (n=6) for a period of 14 days and administration of the drugs was through intraperitoneal (i.p.) injection. The groups were:

A) Control group (CG) (standard diet, tap fresh water);

B) Bleomycin sulfate administration (BLM) (0.069 U/ml; 0.321 U/kg body weight in saline (250μl) was given i.p. finishing on day 14);

C) *G. glabra* extract (80 mg/ml; 0.208 mg/kg body weight in distilled H_2O (250 μl) was given once daily i.p. continued on schedule till the 14. day);

D) *G. glabra* extract (80 mg/ml; 0.208 mg/kg body weight) + bleomycin (0.34 U/kg) (extract was injected once daily, 2 h prior to antibiotic, continued on schedule till the 14. day).

On day 14, mice were euthanized and their livers, lungs and spleens were removed in cold PBS solution and studied by direct and spin-trapping EPR spectroscopy.

Electron Paramagnetic Resonance (EPR) measurements

The measurements were performed on an X-Band, Emx^{micro} spectrometer (Bruker) with settings: center field 3505 G; sweep width 10-30 G; microwave power 12.70-12.83 mW; receiver gain 1 × 10^4 -1 × 10^6; mod. amplitude 5.00 G; 1-5 scans. Spectral processing was performed using Bruker WIN-EPR and SimFonia software.

EPR in vivo evaluation of ROS productions: The ROS production was studied according to Shi *et al.* [20]. Briefly, about 0.1 g of tissue samples were homogenized after addition of 1.0 ml of 50 mM solution of the spin-trapping agent PBN/ DMSO solution.

EPR in vivo evaluation of ascorbate ($Asc^{\cdot}$) radicals: The $Asc^{\cdot}$ levels in tissue homogenates were studied according to [21] with slight modifications. Tissue samples were weighed and homogenized in DMSO (10% w/v) and centrifuged at 4000 g, at 4^{0}C for 10 min. Supernatants were collected and $Asc^{\cdot}$ radicals were measured immediately in cold.

EPR in vivo evaluation of nitric (NO•) radicals: Tissue NO• radicals were studied according to methods [22, 23] adapted for EPR estimation of the spin-adduct formed between Carboxy-Ptio.K and generated radicals.

Statistical analysis

EPR spectral processing was performed using Bruker Win-EPR and SimFonia Software. Statistical analysis was performed with Statistica 8.0, Stasoft, Inc., one-way ANOVA, Student's t-test to determine significant differences between data groups. The results were expressed as means ± standard error (SE). A value of $p< 0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

Bleomycin (BLM) is used as an antibiotic possessed acute oxidative stress in animals. In the organs, BLM is rapidly metabolized by a complex of extracellular matrix deposition and oxidative changes [24], resulting in the generation of toxic products, which determine acute/general toxicity. Moreover, acute conditions reduce antioxidant capacity [9, 24]. Many authors have reported of active biomolecules from plant antioxidants against drug-possessed toxicity, as effective ROS inhibitors [25]. Kim *et al.* [18] reported that licorice (*G. glabra*) could prevent inflammatory processes, due to the presence of flavonoid structure - glabrin which modulates acute oxidative stress caused by tissue injuries. Over the last 15 years, the activity of aqueous/ alcoholic root herbal extracts and their components as ROS scavengers on acute toxicity have been studied and their protective effects have been investigated [26]. In this research, we showed that the application of *G. glabra* root extract, pointedly inhibited tissue free radical formation in acute model and reduced oxidative stress levels.

G. glabra root extract regulates body weight gain at acute toxicity

14 days after the start, the average weight gain of mice receiving BLM significantly decreased ($p<0.05$), compared to the CG. In contrast, administration of *G. glabra* extract and *G. glabra* + BLM significantly increased weight gain ($p<0.05$). No significant differences were observed regarding daily food consumption. In accordance with our results, Ghorashi *et al.* [19] found that animals receiving licorice extract + BLM showed a significant increase in weight, compared to the BLM group.

G. glabra root extract regulates ROS production and Asc· radicals at acute toxicity

Thermodynamically, ascorbic acid is the end product of a series of oxidized free radicals. This means that all oxidizing species which have a higher redox potential could be reduced and as a result, generate ascorbate radical [27]. Asc· radical has a relatively long half-life which makes it extremely convenient for direct detection by EPR spectroscopy, both *in vitro* and *in vivo* systems [21]. This property makes it the best non-toxic endogenous marker, proving toxic ROS in biological systems [28].

To investigate the effects of *G. glabra* root extract on acute toxicity, we measured ROS production (Fig. 1) in liver, lung and spleen from all tested groups. ROS products in liver (0.3249±0.08 *vs.* 0.4889 ±0.001, $p<0.00$), lung (0.559±0.02 *vs.* 0.759 ±0.001, $p<0.01$) and spleen (0.211±0.07 *vs.* 0.433 ±0.02, $p<0.00$) of *G. glabra* treated mice were statistically significantly lower, compared to CG. However, ROS production in the three organs significantly increased ($p< 0.05$) in BLM acute model, compared to CG. The combination with *G. glabra* root extract correspondingly reduced the increased tissue lipid peroxidation.

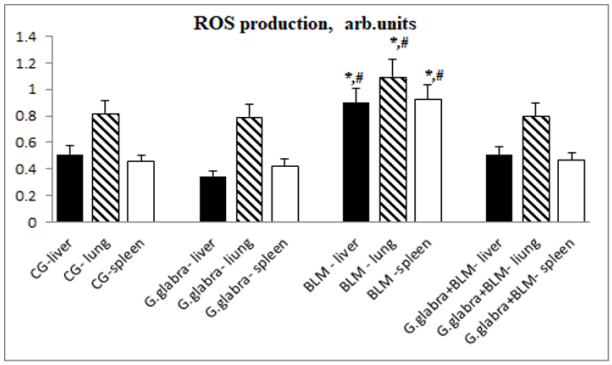

Figure 1. The ROS products in organ homogenates. *G. glabra root* extract normalizes lipid products accumulation in acute models. The experiments were repeated three times. * $p < 0.04$ *vs.* the CG group; #$p < 0.05$ vs. the *G. glabra* + BLM group ($n = 6$).

Based on the measured hyperfine splitting constants, the radicals trapped by PBN spin-adduct (consisting of six spectral lines) was identified as oxygen-centered lipid radicals (LO·) [29]. In this aspect, Sen *et al.* [29] found that treatment with licorice root extract containing glycyrrhizin, retain normal lipid peroxidation levels and oxidative stress parameters.

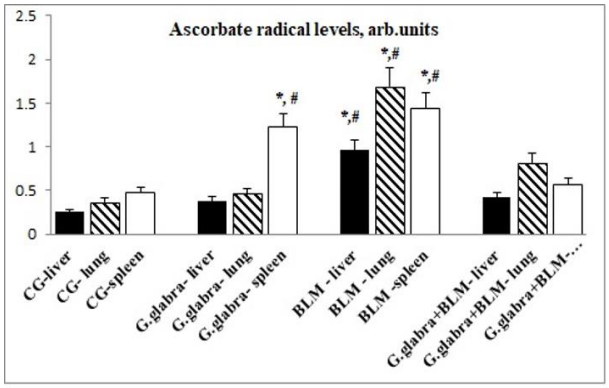

Figure 2. *In vivo* Asc• levels in organ homogenates. Results were calculated by double integration of the corresponding EPR spectrum immediately registered in plasma (expressed in arbitrary units/ *arbt. units*). The experiments were repeated three times. *$p < 0.05$ *vs.* the CG group; #$p < 0.05$ *vs.* the *G. glabra*+BLM group ($n = 6$).

Asc· levels in the tissues homogenates of *G. glabra* root extract mice, CG and in BLM combination are shown on Fig. 2. Asc• levels in liver and lung of *G. glabra* treated group were close to the CG (p=0.00). Interestingly, Asc• radicals in *G. glabra*-treated spleens were statistically significantly increased (almost three-fold), compared to the CG (1.226 ±0.048 *vs*. 0.472 ±0.014, p <0.002). In addition, the BLM-induced free ascorbate increase, recovered after treatment with plant extract (p<0.003).

Modern investigation showed that glycyrrhizic acid, *G. glabra* component, relieves drug-induced hepatic oxidative changes and pulmonary injuries in animals [30, 31]. Moreover, glycyrrhizin, a component of licorice root, reduces inflammatory cytokines (TNF-α, IL-1β) and as strong antioxidant and anti-inflammatory agent [32] could reduce acute oxidative stress in organs. However, reduced ROS products after *G. glabra* administration alone or in *G. glabra* + BLM combination, suggest that ascorbate and other endogenous antioxidant molecules were involved in the neutralization of lipid peroxidation. Our studies ascertained that the *G. glabra* treatment practically did not cause lipid peroxidation processes, but regulates those caused by acute oxidative stress. In addition, statistically higher ascorbic acid levels in spleen registered after *G. glabra* treatment were probably due to residual oxidative processes in the organ. Bonnet and Walsh [33], reported that *G. glabra* roots contain an antioxidant - licocalchone-c, inhibitor of oxidative stresses and inflammation processes.

G. glabra root extract regulates the in vivo nitric (NO•) radicals imbalance at acute toxicity

In vivo nitric oxide (NO•) is a free radical formed from its precursor, L-arginine [34] and was identified as an endogenous oxidative marker maintaining respiratory homeostasis [35-39]. To confirm the efficacy of *G. glabra* extract in neutralizing acute toxicity generated by BLM, NO• levels in liver, lung, spleen tissues (Fig. 3) were evaluated.

In *G. glabra* extract tested group insignificantly lower NO• levels (in all organs) were measured compared to the CG. The data demonstrated the BLM toxic effects, and showed an almost three-fold NO• radicals increase in lungs (16.55 ± 0.93 *vs* 8.557 ± 0.9 a.u., p<0.001), relative to the controls.

Fig. 3

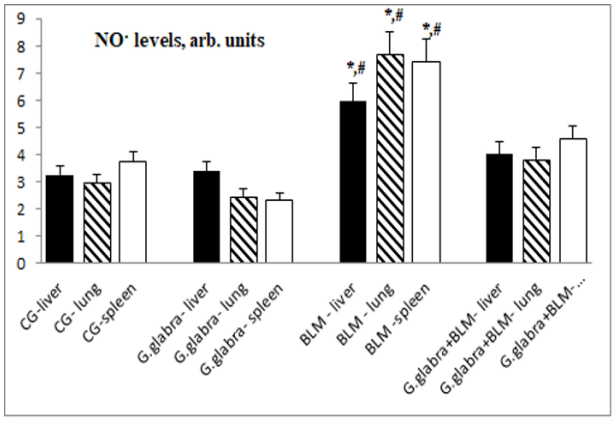

Figure 3. *In vivo* NO• free-radical formation in organs. Results were calculated by double integration of the corresponding EPR spectrum immediately registered in plasma (expressed in arbitrary units). The experiments were repeated three times. *p < 0.05 *vs.* the CG group; #p < 0.05 *vs.* the *G. glabra*+BLM group (n = 6).

However, *G. glabra* extract administration modulated the BLM acute toxicity in *G. glabra* + BLM- treated animals (9.331 ± 0.7 *vs* 16.55 ± 0.93 a.u., p<0.05). The antioxidant protective effect of the *G. glabra* extract is confirmed by the absence of residual oxidation processes, resulting in the scavenging effect against the accumulation of non-toxic NO levels, key factor to overcoming of BLM-induced acute oxidative stress.

CONCLUSION

Based on the present *in vivo* EPR spectroscopy investigations, we consider that *G. glabra* root extract exhibits antioxidant behavior and reduces lipid peroxidation and nitric oxide scavenging. Further experiments on the chemical content and the different biological properties of the plant extract can be added to the treatment of acute respiratory diseases associated with oxidative stress.

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Conflict of Interest*: The authors declare that they have no conflict of interest.*

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Chemical composition and cytoprotective and anti-inflammatory potential of *Sambucus ebulus* fruit ethyl acetate fraction

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Sambucus ebulus (SE) fruits are used in traditional medicine for immunostimulation and treatment of gastrointestinal disorders. We examined the anti-inflammatory potential of SE fruit ethyl acetate fraction (EAF) on a cell model of ethanol-induced cytotoxicity of 3T3-L1 preadipocytes. After fractionation the total polyphenol content (TPC) using Folin-Ciocalteu reagent and total antioxidant capacity (TAC) by $ABTS^+$ decolorization assay of SE fruit extract and fractions were measured. By thin-layer chromatography, the presence of selected polyphenols was analysed. Cell viability was tested using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide reduction assay. Cells were treated with SE EAF dissolved in ethanol or ethanol only. Transcription levels of tumor necrosis factor-alpha (TNF-α), interleukin-6 (IL-6), cyclooxygenase-2 (COX-2), inducible nitric oxide synthase (iNOS) and glutamate-cysteine ligase catalytic subunit (GCLc) were measured using qPCR. Relative transcription levels were calculated using $2^{-\Delta\Delta}$ Ct method.

SE EAF exhibited the highest TPC and TAC among the analysed extracts. For the first time the presence of hyperoside, isoquercetin, isorhamnetin-3-O-β-glucopyranoside and traces of rutin and of 3,5-dicaffeoylqunic acid are reported for the SE EAF. SE EAF showed a cytoprotective effect, by increasing the ethanol-suppressed cell viability up to 2.4 times ($p<0.001$). Treatment with SE EAF (0.02% w/v) decreased ethanol-induced iNOS, COX-2, TNF-α and GCLc gene expression by 63% ($p<0.01$), 54% ($p<0.001$), 64% ($p<0.01$) and 65% ($p<0.05$), respectively, indicating anti-inflammatory potential of the fruit extract. Lower concentration (0.01% w/v) of SE EAF decreased IL-6 and GCLc gene expression by 71% ($p<0.01$) and 45% ($p<0.05$), respectively. This study provides first scientific evidences about the cytoprotective and anti-inflammatory potential of SE fruit EAF.

Keywords: *Sambucus ebulus*, 3T3-L1, ethanol, flavonoids, cytoprotective, anti-inflammatory

INTRODUCTION

S. ebulus L. (SE), widely used as a medicinal plant, is in fact a poorly studied species in regard to its biological effects. Fruits are rich in polyphenols, flavonoids and anthocyanins [1–3], sugars, valerianic acid, malic acid, tartaric acid, tannins, pectin, resins, vitamin C [4, 5]. In traditional medicine fruits are used to treat inflammation-related gastrointestinal disorders [6], tuberculosis [7] and rheumatoid arthritis [8].

Depending on the different phytochemical composition, different parts of the herb possess diverse biological activities. In support to folk medicine, scientific researches revealed high antioxidant, anti-inflammatory, antinociceptive, antiarthritic, and antimicrobial activities for SE flowers, fruits, leaves and roots [2, 3, 9–19] in a variety of models and scientific approaches. A recent study on the activities of SE fruit ethyl acetate fraction (EAF) suggested also possible anticancer activity [20].

Plant extracts with anti-inflammatory properties may provide a new useful therapeutic targeting inflammation for prevention or treatment of various diseases. The aim of the current study was to analyse SE fruit EAF cytoprotective and anti-inflammatory properties using a human physiology-relevant model of ethanol-induced inflammatory response in 3T3-L1 mouse preadypocytes. Chronic ethanol consumption is known to increase the IL-6 and TNFα production, and it is associated with insulin resistance in rats [21]. Ethanol treatment induces production of the inflammatory enzymes iNOS and COX-2 in animal models [22, 23], as well as in cell cultures [24], including 3T3-L1 preadipocytes [25]. There are scientific studies considering ethanol consumption as a reason for development of inflammation in adipose tissue [26]. Elevated adipose tissue IL-6 and TNF-α levels correlate with ethanol induced liver injury [26].

MATERIAL AND METHODS

Plant material

Sambucus ebulus fruits were collected in Northern Bulgaria in the period from late August to the end of September. Plant material was dried in shade at room temperature.

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Extraction and fractionation

Five grams of powdered dry fruits were extracted for 30 min with 100 mL of methanol at room temperature in a ultrasound chamber. The extract was filtered and the plant material was extracted another two times using the same procedure. All filtrates were combined and the crude methanol extract was evaporated to dryness under vacuum. The residue was dissolved in 50 mL of distilled water and the solution was further extracted consecutively with petroleum ether, chloroform, ethyl acetate and butanol (Fig. 1).

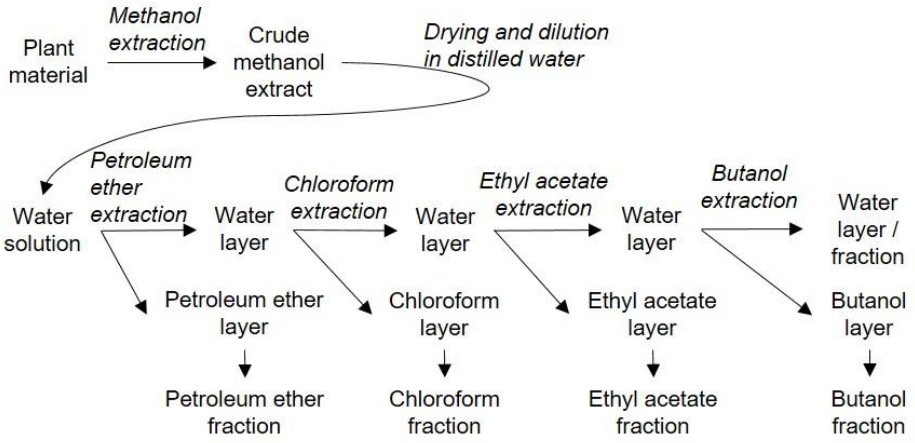

Figure 1. Extraction and fractionation procedure.

Measurement of total polyphenol content and total antioxidant capacity

Extracts and fractions were analysed immediately after their preparation. For the measurement of total polyphenol content (TPC) and total antioxidant capacity (TAC) 10 mg of fractionated material was dissolved in 1 mL of appropriate solvent: chloroform for petroleum ether and chloroform fraction; absolute ethanol for crude methanol extract, ethyl acetate and butanol fractions; distilled water for water fraction.

TPC was determined using Folin-Ciocalteu reagent [27]. Results were expressed as mmol/L quercetin equivalents (QE).

ABTS cation radical decolorization assay was performed to determine *in vitro* TAC of the extracts [28]. Results were expressed as mmol/L uric acid equivalents (UAE).

Thin-layer chromatography

Thin-layer chromatography (TLC) was performed using silica gel plates (Merck, Germany), $CH_3COOCH_2CH_3$: HCOOH: CH_3COOH: H_2O (100:11:11:26) and TLC spots were visualized with NP/PEG reagent at 366 nm [29].

Cell culture

3T3-L1 preadipocytes from American Type Culture Collection (ATCC, Manassas, VA, USA) were cultured in Dulbecco's modified Eagle media (DMEM) (LONZA, Belgium) supplemented with 10% foetal bovine serum (SAFC Biosciences ™, USA), and 1% antibiotic (100 U/mL penicillin, 100 U/mL streptomycin sulphate) (LONZA, Belgium) at 37 ˚C in a humidified 5% CO_2 incubator.

Treatment scheme

Solutions for cell treatment were prepared as follows: 20 mg of SE EAF was dissolved in 0.5 ml of absolute ethanol (Scharlau Chemie S.A., Spain) and diluted with 0.9% NaCl up to 2 mL; 0.5 mL of absolute ethanol was diluted with 0.9% NaCl up to 2 mL; control cells were treated with culture medium only.

For cytotoxicity analysis cells were treated with ethanol (0-0.625% v/v) and SE EAF (0-0.025% w/v) dissolved in ethanol, in increasing concentrations for 24 hours.

To study SE EAF cell protective activity, the cells were co-treated with two different concentrations of SE EAF (0.01% and 0.02% w/v) and ethanol (0.25% and 0.5% respectively), or only with ethanol (0.25% and 0.5%) as control treatment. The volumetric ethanol concentrations of 0.25% and 0.5% correspond to 42.8 mM and 85.6 mM ethanol, respectively, and are physiologically relevant *in vivo* [30].

Cell viability assessment

Viability of treated cells was evaluated using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay [31]. 3T3-L1 cells were seeded in six well plates (2×10^5 cells/well) and allowed to adhere overnight. After 24 h the culture media containing SE fruit EAF (0.005–0.025% w/v), and ethanol (0.125-0.625%) were

replaced to each well and the cells were incubated for 20 h. To each well 100 μL of MTT (1.5 mg/mL) (AppliChem, Germany) was added and the plates were incubated in dark for another 4 h. Formazan crystals were solubilized with dimethyl sulfoxide (Scharlau Chemie S.A., Spain). Absorbance was measured using a multiwell scanning spectrophotometer (ELISA reader-Synergy 2, BioTek) at 550 nm. Cell viability (%) was calculated as [(mean absorbance of the sample/mean absorbance of the control) × 100].

Gene expression analysis

Beta actin (β-actin), glutamate-cysteine ligase catalytic subunit (GCLc), inducible cyclooxygenase (COX-2), inducible nitric oxide synthase (iNOS), tumor necrosis factor-alpha (TNF-α), and interleukin 6 (IL-6) genes expression were analysed using Real Time quantitative polymerase chain reaction (qPCR). Relative mRNA levels were calculated using $2^{-\Delta\Delta Ct}$ method [32].

Total RNA was extracted using TRI Reagent (Ambion, USA), and quantified by spectrophotometry at 260 nm (M501 Single Beam UV/Vis, Camspec, UK). cDNA was synthesized from 0.02 μg of total RNA by using First Strand cDNA Synthesis Kit (Fermentas, Germany) according to the manufacturer's instructions. qPCR was performed using the PCR Master Mix with Eva Green dye (β-actin, GCLc, TNFα, COX-2, iNOS) (Genaxxon, Germany) and Probe/ROX qPCR Master Mix (Fermentas, Germany) (β-actin, IL-6) on an ABI Prism7500 Real-Time PCR System (Applied Biosystems, USA). As a reference β-actin gene was used in the comparative Ct method to determine the relative changes in the target samples. The primer sequences (Sigma-Aldrich, Germany) for each gene were: β-actin forward primer (FP) 5′ACGGCCAGGTCATCACTATTG3′, reverse primer (RP) 5′CAAGAAGGAAGGCTGGAAAAG3′, probe FAM5′ACGAGCGGTTCCGATGCCCTG3′TAMRA; IL-6 FP 5′CATCTGCTGGCCTTCTCCAA3′, RP 5′CAGGCTCTCTGGCTTCTG3′, probe FAM5′AGCTGCTCCCTGCCTCAGACCAGTG3′ TAMRA; GCLc FP 5′AATGGAGGCGATGTTCTTGAG3′, RP 5′CAGAGGGTCGGATGGTTGG3′; iNOS FP 5′GGCAGCCTGTGAGACCTTTG3′, RP 5′GCATTGGAAGTGAAGCGTTTC3′; COX-2 FP 5′TGAGCAACTATTCCAAACCAGC3′, RP 5′GCACGTAGTCTTCGATCACTATC3′; TNFα FP 5′CCCTCACACTCAGATCATCTTCT3′, RP 5′GCTACGACGTGGGCTACAG3′.

Statistical analysis

Results are presented as mean±SD for TAC, TPC and cell viability or ±SEM for relative units of mRNA. All measurements were performed in triplicate. GraphPad Prism 5.0 software was used to perform the statistical analyses. Differences between two groups were analysed applying two-tailed Student's *t*-tests. The values of $p<0.05$ were considered as significant.

RESULTS

TPC and TAC of SE fruit extract and fractions

TPC and TAC of different fractions isolated from SE fruit methanol extract are presented in Table 1. The correlation between TAC and TPC was significantly high (r=0.96) for all analysed extractions. Ethyl acetate fraction was with the highest TAC and TPC among the analysed extract and fractions.

Table 1. TPC and TAC of different fractions of *S. ebulus* fruit methanol extract. Data are presented as mean ±SD.

Extract/fraction	TPC [mmol/L QE]	TAC [mmol/L UAE]
Total methanol extract	1.24 ±0.05	6.58 ±0.64
Petroleum ether fraction	0.19 ±0.01	3.48 ±0.08
Chloroform fractions	0.36 ±0.01	0.04 ±0.001
Ethyl acetate fraction	6.04 ±0.22	29.31 ±0.69
Butanol fraction	4.12 ±0.20	17.41±0.50
Water fraction	3.14 ±0.10	8.91 ±0.09

TLC of SE fruit EAF

TLC comparison of SE fruit EAF with authentic standards confirmed the presence of hyperoside (quercetin-3-O-β-galactopyranoside), isoquercetin (quercetin-3-O-β-glucopyranoside), isorhamnetin-3-O-β-glucopyranoside and traces of rutin as well as 3,5-dicaffeoylqunic acid (Fig. 2).

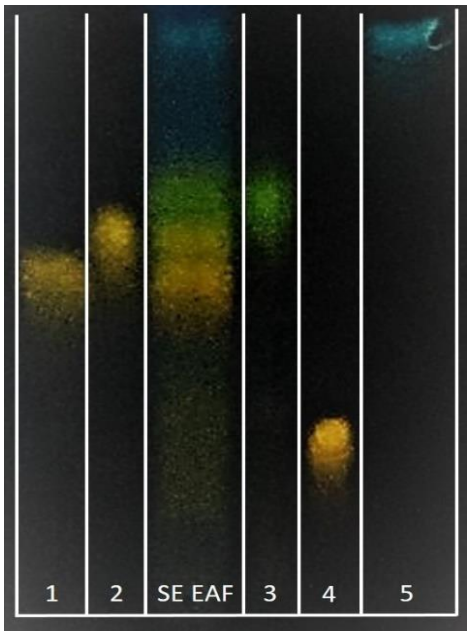

Figure 2. TLC of SE fruit EAF. Legend: standards 1 – hyperoside, 2 – isoquercetin, 3 – isorhamnetin-3-O-β-glucopyranoside, 4 – rutin, 5 – 3,5-dicaffeoylqunic acid.

Effect of SE fruit EAF on ethanol-induced cell death in 3T3-L1 preadipocytes

Using MTT assay we evaluated the cytoprotective effect of SE fruit EAF on ethanol-induced cytotoxicity in 3T3-L1 preadipocytes. Ethanol applied in final concentrations from 0.125 to 0.625% caused gradual decrease in cell viability, the latest causing 46% cell death (Fig. 3A). Co-treatment with 0.5% ethanol and 0.02% w/v SE fruit EAF significantly increased cell viability by 37% ($p<0.001$) *vs*. untreated cells and by 110% ($p<0.001$) *vs*. cells treated with 0.5% ethanol only (Fig. 3A). This effect was observed for all applied concentrations of SE fruit EAF when compared to respective ethanol control treatments, indicating its proliferative and cytoprotective effect on 3T3-L1 cells.

Effect of SE fruit EAF on ethanol-induced TNF-α, IL-6, iNOS, COX-2 and GCLc gene expression in 3T3-L1 preadipocytes

Ethanol applied in a concentration of 0.25% stimulated TNF-α (Fig. 3B) and IL-6 (Fig. 3C) transcription levels, vs. untreated cells by 1.2 ($p<0.05$) and 32.5 ($p<0.01$), respectively. Higher 0.5% ethanol concentration enhanced gene expression of TNF-α (Fig. 3B) and IL-6 (Fig. 3C), by 4.3 ($p<0.001$) and 38.3 ($p<0.001$) fold *vs.* untreated cells, respectively. The higher ethanol concentration induced also mRNA of COX-2 (Fig. 3D) and iNOS (Fig. 3E), by 6.8 ($p<0.05$) and 6.2 ($p<0.001$) fold *vs.* untreated cells, respectively. Treatment with 0.25% and 0.5% ethanol resulted in increased mRNA of GCLc, by 2.5 fold ($p<0.01$) and by 3.3 fold ($p<0.01$) *vs.* untreated cells, respectively, (Fig. 3F).

SE EAF treatment suppressed ethanol-induced expression of all analysed genes in 3T3-L1 mouse preadipocytes. Treatment with 0.02% w/v SE EAF decreased ethanol (0.5%) induced iNOS (Fig. 3E), COX-2 (Fig. 3D) and TNF-α (Fig. 3B) gene expression by 63% ($p<0.01$), 54% ($p<0.01$) and by 64% ($p<0.001$), respectively, indicating anti-inflammatory potential of the preparation. The lower concentration of 0.01% w/v SE EAF significantly decreased ethanol (0.25%)-induced IL-6 gene expression by 71% ($p<0.01$) (Fig. 3C). Both concentrations of the preparation 0.01% w/v and 0.02% w/v decreased ethanol-induced GCLc mRNA levels, by 44% ($p<0.05$) and 45% ($p<0.05$), respectively (Fig. 3F).

In all co-treated groups, we observed significant reduction in the transcription levels of analysed genes compared to ethanol controls, but still significantly higher compared to untreated cells (Figs. 3B, C, E and F), except those of COX-2 (Fig. 3D). This observation additionally confirms the strong inhibitory effect of SE fruit EAF on ethanol-induced COX-2 transcription.

DISCUSSION

Comparison of TPC and TAC of SE fruit extract and fractions

Fractionation procedure favours the comparison between different groups of plant-derived phytochemicals according to their biological activities. Measurement of TAC and TPC of the six different fractions indicated that the content of antioxidant substances was highest in the ethyl acetate fraction followed by butanol and water fractions (Table 1). Very high correlation ($r=0.96$) between TAC and TPC was established for all fractions. Results showed that polyphenols are mainly present in these three fractions. Considering these results we selected for further analysis the SE EAF as the one with the highest TAC and TPC.

Flavonoid glycosides in SE fruit EAF

We report for first time the presence of hyperoside, isoquercetin, isorhamnetin-3-O-β-glucopyranoside and traces of rutin and of 3,5-dicaffeoylqunic acid in the SE fruit EAF (Fig. 2). There are previous reports about the presence of hyperoside and rutin in ethanolic extracts of SE fruits [33], and about the presence of isorhamnetin-3-O-b-glucopyranoside and isoquercetin in SE leave methanol extract [34].

Cytoprotective effect of SE fruit EAF

We found that in 3T3-L1 cells SE EAF exerted cytoprotective effect in a cell model of ethanol-induced cytotoxicity (Fig. 2). Ethanol treatment in final concentrations of 0.125% up to 0.625% in the culture medium significantly decreased cell viability in a dose-dependent manner compared to untreated cells (Fig. 2). The highest ethanol concentration (0.625%) led to 46% cell death. In all co-treated groups SE EAF reversed ethanol-induced cell death, increasing proliferation up to 137%, as compared to untreated cells. In support to the cytoprotective effect of the extract, we detected a 2.4-fold increase in cell viability of SE EAF (0.025% w/v)- and ethanol (0.625%)- co-treated cells, compared to the control ethanol (0.625%) treatment. Cytotoxic or proliferative effects of the extracts are often concentration-related. Low concentrations often stimulate expression of genes to induce cell proliferation, while higher ones activate the caspase pathways initiating apoptosis [35]. This effect may also be dependent on the type of the extractor and on the type of compounds in the extracts, as well as on the type of the cell line.

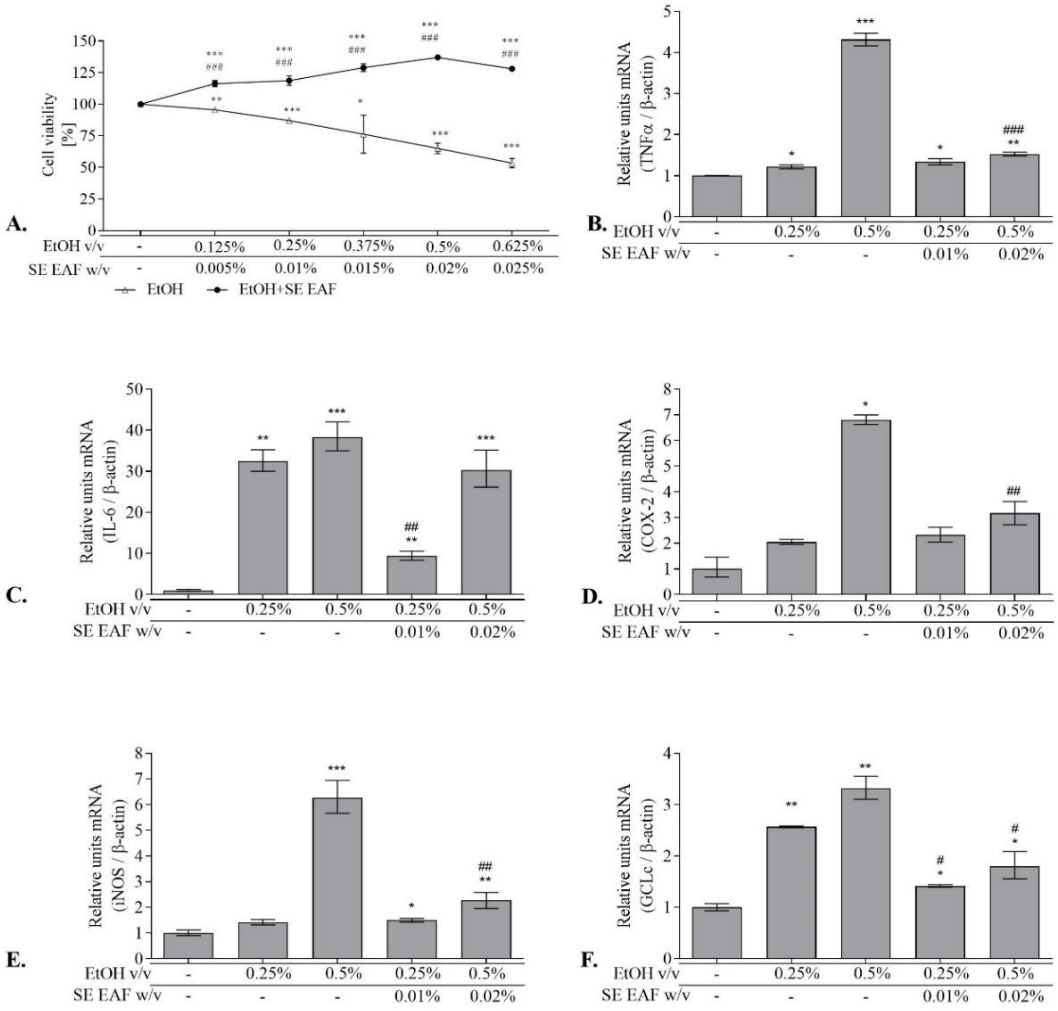

Figure 3. Viability of 3T3-L1 cells treated with different concentrations of ethanol and SE EAF dissolved in ethanol (data are presented as mean ±SD) (**A**) and changes in mRNA levels of TNFα (**B),** IL-6 **(C),** COX-2 **(D),** iNOS **(E)** and GCLc **(F**) in the cells upon treatment with increasing concentrations of ethanol and SE EAF+ethanol (data are presented as mean ±SEM; *p<0.05, **p<0.01, ***p<0.001 *vs.* untreated cells; #p<0.05, ##p<0.01, ###p<0.001 *vs*. respective treatment with ethanol). Legend: EtOH-ethanol.

Hyperoside and isoquercetin found in the SE fruit EAF by TLC are flavonoids known to suppress activation of caspase 3 cascade, thus increasing cell viability [36, 37] and their presence in the EAF could explain the established cytoprotective/proliferative effect of SE fruit extract, and specifically of its EAF.

SE fruit EAF alters ethanol-induced GCLc, TNF-α, IL-6, COX-2 and iNOS genes expression in 3T3-L1 preadipocytes

In individuals consuming alcohol in high doses [38] and even in these consuming lower amounts [39] the levels of cytochrome P450 2E1 are

increased. The high NADPH oxidase activity of P450 2E1 enzyme is associated with increased superoxide and H_2O_2 production. Since 1963 it is known that the oxidative stress is the main mechanism of ethanol toxicity [40] and thus we may assume that the cell model of ethanol induced cytotoxicity is also a model of ethanol-induced oxidative stress. We suggest that the availability of antioxidants of plant origin may reduce ethanol-induced oxidative stress and diminish the need for high supply with endogenous antioxidants, such as glutathione.

Glutathione consumption by glutathione peroxidase, responsible for H_2O_2 neutralization, drives up mechanisms responsible for recovery of reduced/oxidised glutathione ratio and *de novo* biosynthesis of glutathione. Transcription levels of the regulatory enzyme GCL of glutathione biosynthesis are stimulated by many phenolic compound. Plant extracts rich in flavonoids, including quercetin, activate gene promoter leading to an increase in glutathione levels in COS-1 and HepG2 cells [41]. Fruits rich in polyphenols and ellagic acid may also induce transcription levels of GCL in mouse [42]. In a previous study we showed that SE fruit extract rich in anthocyanins induces the gene expression of GCL in 3T3-L1 preadipocites [43]. The quercetin glycosides found in SE fruit EAF (fig. 2) are direct antioxidants [36] and modulators of GCL gene expression [43], GPx and catalase activity [36], thus having the potential to reduce ethanol-induced oxidative stress. We may suggest that reduction of ethanol-induced oxidative stress by the presence of plant-derived antioxidants, reduces the needs of more glutathione production, respectively, the GCLc transcription, as observed in the current study.

In response to ethanol treatment the expression levels of proinflammatory cytokines IL-6 and TNF-α, and enzymes iNOS and COX-2 are increased in rat and mouse models [22–24, 44]. There are few analyses of ethanol effect on adipose tissue cells, such as preadipocytes. Chronic ethanol intake *in vivo* in rats induces macrophage infiltration in adipose tissue, thus revealing its link to the development of insulin resistance [21]. The mRNA expression of COX-2, iNOS, TNFα and IL-6 increased significantly, probably also because of ethanol-induced oxidative stress. At the same time transcription levels of all above-mentioned proteins was significantly decreased in cells co-treated with SE EAF and ethanol vs. control ethanol treatment. The strong inhibitory effect of SE fruit EAF on ethanol-induced inflammatory response was additionally supported by the reduction of COX-2 transcription levels close to those of untreated cells.

Generation of oxidative stress leads to induction of inflammation including induction of related inflammatory proteins [45]. Production of peroxynitrite $ONOO^-$ as a result of iNOS induction additionally stimulates gene expression of COX-2 and production of prostaglandin E2 [46]. We could suggest that the antioxidant potential of SE fruit polyphenols to scavenge NO [16] and to neutralise free radicals such as $ONOO^-$, is one of the possible mechanisms by which the anti-inflammatory potential of SE fruit EAF is realised, altering the inflammation progression related to cytokines release.

Increased production of proinflammatory cytokines and chemokines such as, IL-6, TNFα, IL-1β and MCP-1 by the adipocytes is related to fat tissue low-grade inflammation and insulin resistance [47–49]. The suppression of TNFα activity improves insulin sensitivity in obese individuals and rat model of obesity [50, 51]. Since ethanol may additionally provoke inflammation and insulin resistance in adipose tissue [21-24, 44], an herb extract as SE fruit EAF able to suppress ethanol-induced transcription of proinflammatory proteins may have protective effects in such conditions.

It has been established that quercetin glycosides present in SE fruit EAF reduce the levels of proinflammatory cytokines by suppressing NF-κB activation [37]. Anthocyanin-rich plant consumption inhibits the activity of NF-κB transcription factor and reduces plasma concentrations of its target gene products such as IL-6 and CRP [52, 53].

CONCLUSIONS

A possible mechanism of the protective potential of SE fruits is suggested in support to its use by folk medicine as a therapeutic and preventive tool. SE fruit EAF may overcome ethanol toxicity by activating cell proliferation and reducing the mRNA expression of proinflammatory proteins. Additionally, it prevents ethanol-induced antioxidant GCLc gene expression, revealing a possible mechanism by improving cellular redox balance. This makes SE fruits a good potential source for the development of new therapeutic remedies for the prevention and control of oxidative stress and inflammation.

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Effect of mucus extract of *Helix aspersa* on scopolamine-induced cognitive impairment and oxidative stress in rat's brain

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Snail extracts are complex multicomponent mixtures comprising antibacterial, antiviral, immunomodulating, antioxidant and anti-inflammatory activities. Oxidative stress along with inflammatory and immune mechanisms are believed to be critical factors in the pathogenesis of neurodegenerative diseases. The aim of this pilot study was to evaluate the effect of snail (*Helix aspersa*) mucus extract on scopolamine-induced cognitive impairment and oxidative stress in rat brain cortex. The scopolamine (Sco) was applied i.p. (2 mg/kg) in male Wistar rats for 11 days, along with oral administration of snail mucus extract (0.5 mL/100 g). On the 1st, 5th and 12th day, the animals were subjected to Step-through behavioral test. On the 12th day, the cortex was isolated and oxidative stress parameters of lipid peroxidation (LP) and total glutathione (GSH), activities of catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GPx) were spectrophotometrically assayed. The results of the behavioral test showed a significant improving effect of snail extract on the learning and memory of Sco-treated animals. The Sco treatment provoked increase in LPO, CAT and GPx activities and decrease in tGSH and SOD activity. The application of snail mucus extract led to recovery of oxidative stress parameters close to the control group values. In conclusion, the snail extract demonstrated a protective effect against Sco-model of dementia, probably *via* an antioxidant mechanism. Further research is needed to evaluate the therapeutic potential of *Helix aspersa* mucus for treatment of neurodegenerative disorders.

Keywords*:* snail (*Helix aspersa*) mucus extract, oxidative stress, scopolamine dementia, rats

INTRODUCTION

Alzheimer's disease (AD) is a progressive neurodegenerative disorder of great social importance that has estimated prevalence of 10-30% in the population over the age of 65, with an incidence of 1-3% [1]. According to available forecasts worldwide, based on data from continuous surveys, by 2030 the total number of patients with AD in the world will reach 65.7 million, and by 2050 - 115.4 million people [2].

Oxidative stress is recognized as a common pathway of cellular injury in both acute and chronic neurological diseases [3]. The sources of ROS-mediated damage appear to be multi-faceted in AD, with interactions between abnormal mitochondria, redox transition metals and other factors [4]. Because of brain's high oxygen consumption, along with its abundant lipid content, lipid peroxidation was accepted as the primary mechanism for neuronal degeneration [5]. Reactive oxygen species (ROS) rapidly oxidize cellular lipids, resulting in the formation of numerous lipid peroxidation products, leading eventually to neuronal death. Increased activity of endogenous antioxidant enzymes (i.e. catalase, superoxide dismutase, glutathione peroxidase and glutathione reductase) have also been observed along with β-amyloid deposits in temporal regions (e.g. hippocampus) of the AD brain, reflecting a compensatory mechanism to counter oxidative stress [1].

Nowadays, the used treatments against AD give limited symptomatic improvements and cannot stop the progression of the disease. Since AD is a heterogeneous disorder, the efforts should be directed to examine multimodal strategies, including antioxidant therapy [6]. In the recent years, there are accumulating data about wide varieties of natural antioxidants from different biological sources, such as plants, fungi, bacteria, marine sponges and molluscs with neuroprotective effects [7].

It is known that terrestrial slugs and snails produce mucus which performs a variety of functions and the excreted biological fluid is a rich source of bioactive natural compounds that are used for the treatment of a number of skin ailments like wounds, burns, scars, psoriasis, acne, keratosis, wrinkles, and age and skin damage [8, 9]. In general, these properties are bound to the ability of the organism to counteract free radicals generation.

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The results from the antioxidant screening of *C. aspersum* mucus and its fractions showed that this naturally derived product, specifically the low-molecular weight fractions, possess the properties to counteract the formation of reactive free radicals [10].

Recently, some researchers have confirmed that the *Helix aspersa* mucus contains a large number of natural substances as allantoin and glycolic acid, with beneficial and therapeutic properties for human skin [11]. Moreover, according to Guskov *et al.* [12] the antioxidant potential of the mucus may be due to the allantoin, which has been shown to possess antioxidant properties. Other findings showed that allantoin has therapeutic potential for the cognitive dysfunctions observed in Alzheimer's disease [13]. However, to this date, there are scarce data and no conclusive evidence for the antioxidant potency of the compounds in snail species mucus, and this fact opens a variety of future prospective investigations in this aspect – eventual antioxidant properties in pathological conditions, including scopolamine-induced Alzheimer's type dementia. These facts determined the purpose of our pilot study: to evaluate the effect of snail (*Helix aspersa*) extract on cognitive impairment and oxidative stress in brain cortex of rats with experimental scopolamine-induced dementia.

EXPERIMENTAL

Materials and treatment

The main reagents (glutathione, riboflavin, methionin, 2-thiobarbituric acid, NADP+, reduced and oxidized NADPH) were obtained from Sigma-Aldrich (Germany). Scopolamine was purchased from ACROS Organics (Germany) (as Thiogamma Turbo-Set solution for injection 600 mg, 50 ml). All other used chemical substances were of the highest commercially available purity.

Crude mucus was collected from Bulgarian snail *Helix aspersa* and a fresh extract was purified using patented technology without suffering of any snail. We evaluated the snail extract (SE) effects in an experimental model of neurodegeneration - Alzheimer's disease (AD). AD type dementia was produced by scopolamine treatment (2 mg/kg, i. p., 11 days) of male Wistar rats (180-200 g). The animals were housed at 22°-25°C with free access to food and water, and a natural day/night light cycle. During scopolamine treatment, the animals received also snail extract (SE) (0.5 ml/100 g. p.o.) or saline for controls. The snail extract was administered for 16 days, 5 days before, and 11 days simultaneously with Sco. On the 12th day after last treatment, dynamics of changes in learning and memory performance in animals were evaluated behaviorally by a Step-through test. Immediately after the behavioral test was made and the brain cortex was isolated - dissected by the method of Valzelli and Garattini [14] and was spectrophotometrically assessed for the main oxidative stress markers. In 10% homogenates, centrifuged for 10 min at 3000 rpm, we measured the levels of lipid peroxidation and total glutathione, part of the post-nuclear homogenate was centrifuged for 20 min at 12,000 rpm. The post-mitochondrial supernatant was used for measuring the activities of antioxidant enzymes: catalase, Cu,Zn-superoxide dismutase and glutathione peroxidase.

All experiments were performed according to the "Principles of Laboratory Animal Care" (NIH publication No. 85-23, revised 1985), and the rules of the Ethics Committee of the Institute of Neurobiology, Bulgarian Academy of Sciences (registration FWA 00003059 by the US Department of Health and Human Services).

Behavioral Method

Using passive avoidance (Step-through test) [15] learning and memory performance of the rats were evaluated. Acquisition latency (initial latency (IL) time was measured before all treatments. The retention trial, where the interval between the placement in the illuminated chamber and the entry into the dark chamber was measured on the 12th day as step-through latency (STL). Behavioral observations were carried out from 9 a.m. to 12 a.m.

Analytical methods

Protein content was measured by the method of Lowry *et al.* [16]. Lipid peroxidation (LP) was determined by the amount of thiobarbituric acid reactive substances (TBARs) formed in fresh biological preparations according to Hunter *et al.* [17]. The values were expressed in nmoles malondialdehyde (MDA) per mg protein, with a molar absorption coefficient of $1.56 \times 10^5 M^{-1}cm^{-1}$.

Total glutathione (tGSH) levels were measured according to Tietze [18] and were expressed in ng/mg protein, with glutathione oxidized (GSSG) as a reference standard.

Catalase (CAT) activity was determined according to the method described by Aebi [19] and the enzyme activity was expressed as ΔA240/min/mg protein.

Cu,Zn-superoxide dismutase (SOD) activity was determined according to the method of Beauchamp and Fridovich [20], expressed in U/mg protein (one unit of SOD activity is the amount of the enzyme, producing a 50% inhibition of Nitroblue tetrazolium

reduction).

Glutathione peroxidase (GPX) activity was measured by the method of Günzler *et al*. [21], using a molar absorption coefficient of $6.22\times10^{6}M^{-1}cm^{-1,}$ and was expressed in nmoles NADPH oxidized per minute, per mg protein.

Statistical analysis

The results were statistically analysed by one-way ANOVA and Dunnett post-hoc test, with $p<0.05$ accepted as the minimum level of statistical significance of the established differences.

RESULTS AND DISCUSSION

After finishing the treatment of rats with scopolamine and snail extract, both the changes in the cognitive functions of the animals and the biochemical parameters were evaluated in all groups.

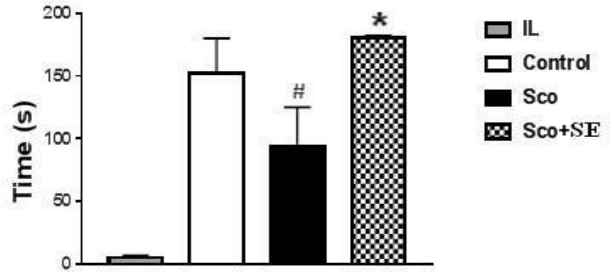

Fig. 1. Effects of SE on step-through latency (STL) in a single-trial passive avoidance test in rats; IL - initial-latency, *p< 0.05 Sco group *vs* Sco+SE group, # p< 0.05 Sco group *vs* control group.

The obtained results demonstrated statistically reliable changes in learning and memory in the Sco group – about 35% memory impairment compared to controls. Sco-SE group demonstrated significant improving effect on the learning and memory of animals treated simultaneously with Sco. SE recovered significantly (by 48%) the memory loss, in comparison to Sco-treated group.

The treatment with SE of Sco-animals also showed significant beneficiary effects upon all measured biochemical parameters. Significantly increased levels of LP were observed in the cerebral cortex of Sco-treated animals in comparison to the control rats (Fig. 2).

Scopolamine administration led to elevation of TBARs content in rat brain cortex by 25%. SE reduced the Sco-induced elevation of TBARs restoring to control levels.

As a result of lipid peroxidation, a great variety of aldehydes can be produced - malondialdehyde (MDA) is the most important indicator and excellent index of lipid peroxidation [22]. Our results correlated with several studies that reported elevated levels of LP in different brain regions (cortex, hippocampus, etc.) in AD [23, 24].

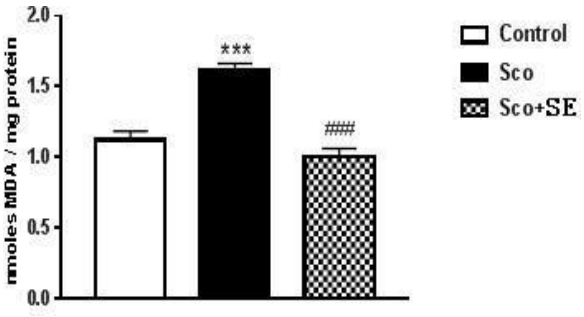

Fig. 2. Effects of SE on lipid peroxidation in rat brain cortex Data are expressed as the mean ± SEM, *p< 0.001 *vs* control group, # p< 0.05 *vs* Sco group

The treatment of the animals with Sco in comparison to the control group led to a decrease of tGSH levels in cortex by about 30% (Fig. 3).

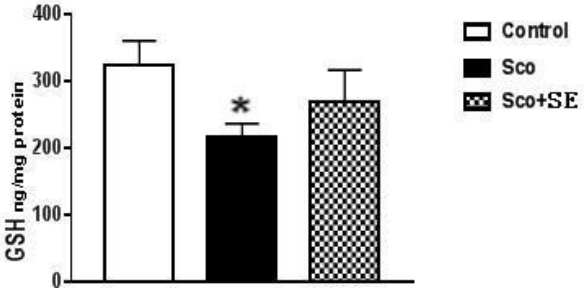

Fig. 3. Effects of SE on tGSH in brain cortex. Data are expressed as mean ± SEM *p< 0.05 *vs* control group.

The SE partly restored the Sco-induced reduction of tGSH levels in cortex and we indicated a preventing positive effect. In the literature, many scientists assessed and described the considerable role of GSH in AD onset and progression. AD-associated reductions in GSH levels have been documented *in vivo* in animal models of AD [25].

Cognitive impairment is accompanied with alterations in antioxidant enzymes in different brain regions and several laboratories have reported changes in the activities of SOD, CAT and GPx [26-28].

In our findings, there was an activation of antioxidant enzymes CAT and GPx in the co-group in cortex and decreased activity of SOD (Table 1).

Changes of CAT activity were observed - increase in the Sco-group about 50% compared to the control group. Evaluated CAT activity in the Sco-SE group was reduced by SE in cortex and values were lower by about 27% than those of healthy animals.

From the detected values of antioxidant enzymes' activities in cortex, this of the SOD was strongly pronounced – a significant decrease by about 50% in Sco-group compared to control group.

Table 1. Effects of snail (*Helix aspersa*) extract on the activities of antioxidant enzymes catalase, superoxide dismutase and glutathion peroxidase in cortex (controls, sco-treated and sco+snail in rats (mean±SEM).

Cortex	CAT [dA240/min/mg]	SOD [U/mg protein]	GPx [U/mg protein]
Controls	0.073 ± 0.02	10.11 ± 1.243	9.35 ± 2.025
Sco	0.110 ± 0.02	5.00 ± 0.986	13.84 ± 2.967
Sco + SE	0.056 ± 0.03	6.14 ± 2.276	10.86 ± 0.885

Differences were observed in Sco-SE treated animals – a slight increase in cortex by about 15% compared to that of Sco-treated. The obtained results for GRx activity demonstrated the following changes in the cortex – increased in Sco-treated rats by about 35% compared to control group. Snail extract had positive effect in cortex of SE-treated animals, recovering control values (statistically unproven). Our recent results support and extend numerous previous findings of enhanced oxidative stress in AD and also confirmed the ability of scopolamine to produce similar to AD-type dementia accompanied by oxidative stress. The changes of oxidative stress markers significantly correlated with reports about investigations in human post-mortem frontal cortex from individuals characterized as mild cognitive impairment, mild/moderate Alzheimer disease, and late-stage Alzheimer disease [28]. Tissue fraction had significant declines in antioxidants (glutathione, glutathione peroxidase, catalase, and superoxide dismutase) and levels of LPO. This effect was established by many other studies in the literature [29, 30]. It is well established that the neuroprotective effects of antioxidant compounds involved their radical scavenging and metal chelating activity and/or the regulation of antioxidant enzymes [31]. Moreover, molecular biology studies suggested that natural antioxidants were able to modulate the expression of genes that encode for apoptosis-related molecules [32]. There are different types of antioxidants, each of which has a slightly different role and provides guidance around their potential benefits in relation to dementia. This makes it difficult to examine 'antioxidants' as a general and single aspect in dementia risk. The molecular mechanisms of neuronal degeneration remain largely unknown and effective therapies are not currently available [33]. Trying to explain the proposed mechanisms of action, underlying the neuroprotective effects of the natural exogenous antioxidants, they are now being looked upon as persuasive therapeutics against neuronal loss, as they have capability to combat by neutralizing free radicals.

Unfortunately, for now, the outcomes of many clinical trials with different antioxidants demonstrated minimal therapeutic effects or have shown conflicting results, but there are lots of antioxidant supplements on the market containing bioactive compounds, claiming to have undeniable positive health benefits. Further investigations will bring us more clearly about the beneficial role of snail *(Helix aspersa)* extract and to clarify the mechanisms of neurodegenerative disorders, affecting learning and memory processes.

CONCLUSION

From our study, it could be assumed that the activation of antioxidant enzymes in response to Sco-induced OS is a cellular protective mechanism [34]. Although the obtained results could not provide clear understanding of the mechanism of action of the SE, whether acting directly or indirectly, it could be hypothesized that SE is able to affect positively the impaired brain cognitive functions. Owing to the obtained evidence, our suggestion is that snail extract acts as antioxidant and stimulates important stress-response pathways in cell affecting endogenous cellular antioxidant levels and diminishing the neurodegenerative processes and could help develop drugs for demented patients for controlling and manipulating memory, and may represent one of a new generation of bioactive drugs for improving brain functions.

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Comparative characteristics of polyphenols in extracts of wild and cultivated Bulgarian white oregano

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White oregano is a widespread aromatic plant with proven antioxidant properties due to its antioxidant content. The content of polyphenolic compounds in ethanol extracts of wild and cultivated Bulgarian white oregano was studied. It was found that wild oregano extracts are richer in polyphenolic compounds (2,78 – 4,17 mg/mL) than cultivated ones (1,44 – 1,78 mg/mL). Cinnamic acid derivatives are predominant in all white oregano extracts obtained with 70 % ethanol extractant. They occupy from 78.54 % to 84.57 % of the total phenolic content of wild oregano extracts and from 47.52 % to 59.92 % of cultivated oregano ones.

Keywords: *Origanum heracleoticum* L., ethanol extracts, phenolic acids, flavonoids, flavonoid glycosides

INTRODUCTION

Two species of the genus *Origanum* are disseminated in our country: the ordinary oregano (*Origanum vulgare* L.), which grows in mountainous and sub-mountainous areas throughout all the country and white oregano (*Origanum heracleoticum* L.), growing only on the rocky slopes of the Eastern Rhodopes, Belasitsa, South Struma Valley, Kresna Defile, Trakia Lowland, at an altitude of 250 to 700 m [1, 2]. In recent years, the interest in cultivating white oregano has been growing due to its widespread entry into the food industry [3].

Current nutrition trends are aimed at limiting the use of synthetic antioxidants and replacing them with natural ones. In this regard, a big part of the researches are focused on the use of the natural potential of herbs and spices [4-8].

Oregano extracts have been investigated to identify the antioxidant compounds contained in them. Solvents with increasing polarity and isolation of different fractions have been used. Their composition and effectiveness against free radicals and lipid oxidation have been determined [9]. The highest activity has been found in the fraction containing rosemary acid, which has significant antiradical properties and stronger activity than the synthetic antioxidants BHA, BHT, PG, OG, TBHQ [10-12].

Apigenin, eriodictyol dihydrokaempferol and dihydroquercetin have been identified by the study of flavonoid constituents in oregano extracts [9, 13].

It is considered the antioxidant activity of the essential oils of white oregano is due to the high content of volatile (carvacrol and thymol) and non-volatile (phenolic acids and flavonoids) compounds [9, 14-22]. It has been established that the effectiveness of the essential oils is higher than the activity of the individual components [23].

Oregano essential oil also exhibits strong antioxidant activity when added directly to chicken meat [24-26], beef [27], minced beef [28, 29], minced lamb [30] and fish fillets [31]. According to the authors, the oil can replace synthetic antioxidants in the meat products because it reduces lipid oxidation, improves the color stability of meat, and extends shelf life.

It is known that there is a good correlation between the total polyphenols content and the antioxidant activity of the plants. To our knowledge, there are no papers reporting the content of polyphenols in Bulgarian white oregano extracts. Such kind of information will be useful to food scientists and technologists for the development of functional foods rich in natural antioxidants. The results of the current study will enrich the national database of the white oregano, which is the aim of the research.

EXPERIMENTAL

Materials

The object of this research is Bulgarian white oregano (*Origanum heracleoticum* L.) – wild and cultivated. The wild oregano was collected at the end of July 2018 from the southern slopes of the Eastern Rhodopes (360 m altitude), the region of Ivaylovgrad, Haskovo. The cultivated oregano (dried crumbled leaf) was purchased from BulgarLuk Ltd. – Katunitsa, from the area of Parvomay, region Plovdiv, harvest year 2018. The raw material was in flowering phase.

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Preparation of extracts

The extraction was carried out with ethanol at a concentration of 70 % as a periodic process, without breaking, at two hydromodules (HM) - 1:8 and 1:10, 60 °C for 6 hours. The raw material was separated by filtration through a vacuum filter. A rotary vacuum evaporator with a water vacuum pump at a water bath temperature of 60-65 °C was used to separate the solvent. The resulting extracts were stored at 4 - 6 °C until analysis.

Determination of polyphenols

Sample preparation, μg/g. Dried samples were powdered by using laboratory homogenizer. Phenolic compounds were extracted from 0.5 g of powdered sample using 70 % methanol (Sigma) on a ultrasonic bath at 70 °C for 3 hours. The biomass was separated by filtration and then the extraction procedure was repeated another two times. The combined extract was evaporated to dryness on a rotary evaporator. The residue was dissolved in methanol and used for HPLC analyses after filtration with 0.45 μm syringe filter.

HPLC analysis. Qualitative and quantitative determinations of phenolic acids and flavonoids, were performed by using Waters 1525 Binary Pump HPLC systems (Waters, Milford, MA, USA), equipped with Waters 2484 dual absorbance detector (Waters, Milford, MA, USA) and Supelco Discovery HS C18 column (5 μm, 25 cm × 4,6 mm), operated under control of Breeze 3,30 software.

Analyses of phenolic acids

Gradient elution by using mobile phase of solvent A (2 % acetic acid) and solvent B (0.5 % acetic acid: acetonitrile (1:1)) was used. The gradients of the setup according to Marchev *et al.,* (2011) were as follows: 0-30 min solvent B increase from 5 % to 35 % at a flow rate of 0.8 mL/min; 30-45 min solvent B increase to 70 % at a flow rate of 0.4 mL/min; 45-50 solvent B increase to 80 % at a flow rate of 1.2 mL/min; 50-60 min solvent B increase to 100 % at the same flow rate; 60-65 min solvent B drop down to 5 % at a flow rate of 0.8 mL/min and hold on up to 70 min to equilibrate the column [32]. Gallic, protocatechuic, salicylic, chlorogenic, vanillic, caffeic, syringic, ferulic, sinapic, *p*-coumaric and cinnamic acids (Sigma) were used as standards to build calibration curves. The detection was carried out at 280 nm.

Analyses of flavonoids

Gradient elution by using mobile phase of solvent A (2 % acetic acid) and solvent B (methanol) was used.

The gradients of the setup according to Marchev *et al*., (2011) were as follows: 0-10 min solvent B increase from 30 % to 50 % at a flow rate of 1.0 mL/min; 10-15 min hold on at the same flow rate; 15-16 min solvent B increase to 52 % at a flow rate of 0.8 mL/min; 16-30 min solvent B increase to 80 % at the same flow rate; 30-35 min solvent B drop down to 30 % at a flow rate of 1.0 mL/min and hold on up to 40 min to equilibrate the column [32]. Myricetin, kaempferol, quercetin, hesperidine and apigenin (Sigma) were used as standards to build calibration curves. The detection was carried out at 380 nm.

The quercetin glycosides rutin and hyperoside were analyzed on the same HPLC system by using gradient elution by applying mobile phase of solvent A (2 % acetic acid) and solvent B (acetonitrile). The gradients of the setup according to Ivanov *et al*. (2014) were as follows: 0-15 min 20 % solvent B; 15-17 min 50 % solvent B; 17-20 min 20 % solvent B [33]. Rutin and hyperoside (Sigma-Aldrich) were used as standards to build calibration curves. The detection was carried out at 370 nm.

The reagents used were pure for analysis. All experiments were repeated three times. The data were expressed as means ± standard deviation (SD).

RESULTS AND DISCUSSION

Content of polyphenolic compounds in ethanol extracts of wild white oregano

The content of polyphenols was determined in the extracts obtained at 60 °C, duration 6 h, hydromodule 1:8 and 1:10 (Table 1).

In the analysis of the data it was found that the extracts obtained at HM 1:8 were richer in polyphenol compounds than those obtained at HM 1:10. Derivatives (mustard and ferulic acid in higher amounts, p-coumaric, caffeic, chlorogenic and cinnamic) predominated in the extracts of the phenolic acid, cinnamic acid. It is noteworthy that at HM 1:8 it is better to extract sinapic acid and at HM 1:10 - ferulic acid. 2-Hydroxy benzoic, vanilla, syringan and 3,4-dihydroxy benzoic acid derivatives were identified, with slightly higher amounts at HM 1:8. The flavonoids content in the extracts obtained at the two hydromodules was comparable. Flavone glycosides (hesperidin) and quercetin glycosides (rutin) predominated. Flavones (luteolin, apigenin) and flavonols (campferol, myricetin, quercetin) were identified.

Table 1. Content of polyphenolic compounds in ethanol extracts of wild white oregano

Type of structure	Compounds	Content, mg/mL (from the test sample)	
		Hydromodule 1:8	Hydromodule 1:10
Phenolic acids			
Derivatives of cinnamic acid	Caffeic acid	0,04±0,08	0,03±0,01
	Chlorogenic acid	0,03±0,06	0,02±0,08
	p- Coumaric acid	0,09±0,09	0,03±0,11
	Sinapic acid	2,98±0,11	0,81±0,03
	Ferulic acid	0,39±0,02	1,27±0,06
	Cinnamic acid	0,01±0,02	0,01±0,02
Derivatives of benzoic acid	3,4-dihydroxy Benzoic acid	0,01±0,04	0,01±0,11
	2- hydroxy Benzoic acid	0,07±0,05	0,05±0,01
	Vanillic acid	0,06±0,16	0,06±0,01
	Syringic acid	0,05±0,02	0,04±0,15
Flavonoids			
Flavonols	Myricetin	0,02±0,02	0,03±0,10
	Kaempferol	0,04±0,01	0,04±0,01
	Quercetin	0,01±0,06	0,01±0,02
Flavons	Apigenin	0,04±0,36	0,06±0,02
	Luteolin	0,06±0,08	0,06±0,03
Flavon glycosides	Hesperetin	0,18±0,04	0,17±0,68
Quercetin glycosides	Rutin	0,09±0,02	0,08±0,12
Total:		4,17	2,78

Data expressed as mean ± SD (n=3)

The content of polyphenols in ethanol extracts of wild white oregano cannot be compared with descriptions in the literature due to lack of data. The distribution of the polyphenol compounds by classes in the wild oregano extracts is shown in Fig. 1. For comparison of data, the amount of separate classes of polyphenols is recalculated as a percentage of the total polyphenols content of the relevant hydromodule.

In the extracts of the two variants, cinnamic acid derivatives predominated, their amount was higher in comparison with those obtained at HM 1:8 (84.57 %) compared to those at HM 1:10 (78.54 %). The amounts of the other classes of polyphenols were comparable in the extracts obtained at the two hydromodules: benzoic acid derivatives (4.88 % at HM 1:8 and 5.77 % at HM 1:10), flavonols (1.61 % at HM 1:8 and 2.51 % at HM 1:10), flavones (2.51 % at HM 1:8 and 4.16 % at HM 1:10), flavone glycosides (4.30 % at HM 1:8, and 6.22 % at HM 1:10), quercetin glycosides (2.14 % at HM 1:8 and 2.80 % at HM 1:10).

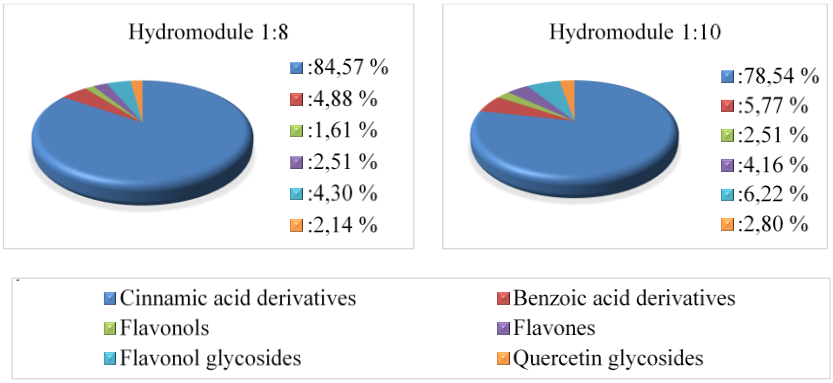

Figure 1. Classification of polyphenolic compounds in ethanol extracts of wild white oregano

Table 2. Content of polyphenolic compounds in ethanol extracts of cultivated white oregano

Type of structure	Compounds	Content, mg/mL (from the test sample)	
		Hydromodule 1:8	Hydromodule 1:10
Phenolic acids			
Derivatives of cinnamic acid	Caffeic acid	0,16±0,03	0,13±0,02
	Chlorogenic acid	0.04±0.01	0.03±0.05
	p- Coumaric acid	0.08±0.07	0.08±0.01
	Sinapic acid	0.37±0.11	0.34±0.07
	Ferulic acid	0.16±0.06	0.18±0.10
	Cinnamic acid	0.05±0.02	0.10±0.05
Derivatives of benzoic acid	2- hydroxy Benzoic acid	0.12±0.09	0.08±0.07
	Vanillic acid	0.08±0.10	0.07±0.04
	Syringic acid	0.04±0.05	0.04±0,09
Flavonoids			
Flavonols	Kaempferol	0.04±0.02	0.01±0.03
	Quercetin	–*	0.01±0.07
Flavons	Apigenin	0.06±0.08	0.04±0.11
	Luteolin	0.14±0.05	0.10±0.08
Flavon glycosides	Hesperetin	0.12±0.07	0.11±0.05
Quercetin glycosides	Rutin	0.13±0.02	0.12±0.07
	Hyperoside	0.19±0.01	–
Total:		1.78	1.44

* not identified; Data expressed as mean ± SD (n=3)

Content of polyphenolic compounds in ethanol extracts of cultivated white oregano

The content of polyphenols was determined in the extracts obtained at 60 ° C, duration 6 h, HM 1:8 and 1:10 (Table 2.).

Unlike the wild plant, in the cultivated one, relatively similar amounts of the polyphenolic compounds in the extracts, obtained at both hydromodules, were found. Cinnamic acid derivatives (mustard, caffeic, ferulic, p-coumaric, cinnamic and chlorogenic), predominated in the phenolic acids. The benzoic acid derivatives were represented by 2-hydroxy benzoic, vanillic and syringic acids. In cultivated white oregano extracts, unlike wild-growing, 3,4-dihydroxy benzoic acid was not detected. The flavonoids were dominated by quercetin glycosides (hyperoside – identified only in the extract obtained at HM 1:8) and rutin, and flavone glycosides (hesperitin). Flavones (luteolin and apigenin) and flavonols (caempferol and quercetin) were identified. The extracts of cultivated white oregano did not contain the flavonol myricetin that was found in the extract of the wild plant.

Polyphenols are a product of secondary metabolism. The richer polyphenolic composition in the wild white oregano is explained by the habitat of the plant and the lack of cultivation activities.

The correlation of the polyphenol compounds by classes in the cultivated white oregano extracts is shown in Fig. 2. For comparison of data, the amount of separate classes of polyphenols was recalculated as a percentage of the total polyphenols content of the relevant hydromodule.

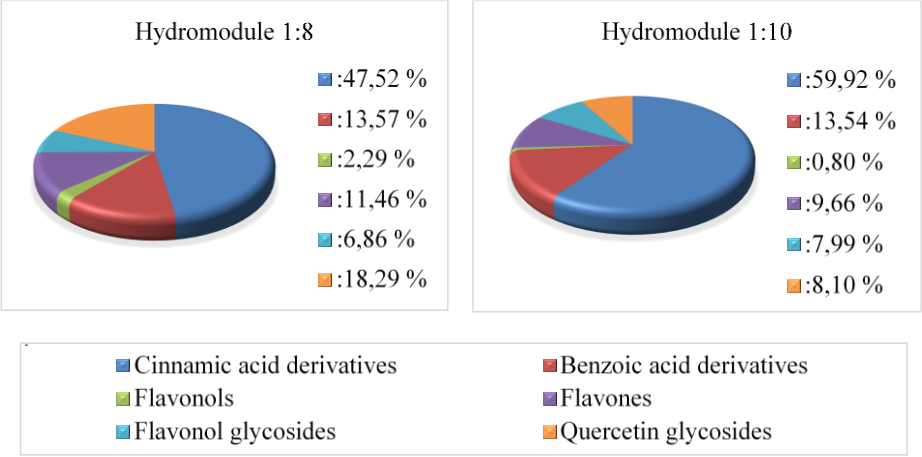

Figure 2. Classification of polyphenolic compounds in ethanol extracts of cultivated white oregano

Cinnamic acid derivatives also predominated in the cultivated white oregano extracts. Unlike the extracts obtained from the wild oregano, their amount is higher than those obtained at HM 1:10 (59.92 %), compared to obtained at HM 1:8 (47.52 %). Differences were observed also for quercetin glycosides (18.29 % at HM 1: 8 and 8.10 % at HM 1:10). The amounts of the other classes of polyphenols were comparable in the extracts obtained at both hydromodules: benzoic acid derivatives (13.57 % at HM 1:8 and 13.54 % at HM 1:10), flavonols (2.29 % at HM 1:8 and 0.80 % at HM 1:10), flavones (11.46 % at HM 1:8 and 9.66 % at HM 1:10), flavone glycosides (6.86 % at HM 1:8) and 7.99 % at HM 1:10.

The content of polyphenolic compounds in white oregano extracts is difficult to compare with the literature data because of the origin of the raw material, the various agro-meteorological conditions and the various methods of analysis.

CONCLUSIONS

In summary, the content of polyphenolic compounds in ethanol extracts of wild and cultivated Bulgarian white oregano was studied. The ethanol extracts of wild white oregano had higher contents of polyphenolic compounds. In both types of extracts cinnamic acid derivatives predominated. The content of polyphenols in Bulgarian white oregano is a prerequisite for antioxidant activity, which confirms scientific reports about white oregano of other origins.

Based on the proven relationship between the polyphenolic content and the antioxidant activity of Bulgarian herbs and spices, the results confirm that Bulgarian white oregano can be used as a natural source of antioxidants in food production. In addition, the effect of the antioxidant compounds in a food matrix (mixture) may be significantly different than the activity of a purified extract. This can be a subject to in-depth analysis in further research.

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An ethanol extract ability of cultivated white oregano (*Origanum heracleoticum* L.) of Bulgarian flora to attenuate oxidative stress effects formed under short-term UV-B radiation

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Ultraviolet (UV) radiation is a spectral part of the sun's rays, and plants have developed different mechanisms to cope with this potential oxidative stress factor. Due to their high sensitivity to UV-B exposure, the plant leaves need to react rapidly to minimize reactive oxygen species (ROS) generation. White oregano (*Origanum heracleoticum* L.) is an aromatic plant used as a spice and cultivated in the last years in Bulgaria, and its tolerance to short-term UV-B radiation remains unclear.

The aim of the present study was to determine for the first time the strong antioxidant activity towards DPPH stable spin-trap, *in vitro* direct and indirect EPR spectral characteristics and superoxide dismutase (SOD) content in Bulgarian *O. heracleoticum* leaves extract after UV-B exposure for 2 h (0 to 12 kJ/m). All measurements were performed with electron paramagnetic resonance (EPR) spectroscopy, twice: immediately and 6 months after the end of UV-B stress. Our data suggested that radical-scavenging and antioxidant abilities of the Bulgarian *O. heracleoticum* extract were not affected by short–term UV-B stress. Statistically significantly higher superoxide ($\bullet O_2^-$) scavenging activity (127.54±10.91U) (almost 6 times) after short-term UV-B exposure also supported the antioxidant properties of *O. heracleoticum* extract. Based on the EPR singlet signals with equal values of $g_\perp$=2.00456±0.0002 after alkalization we assumed that the *o*-semiquinone radical originates from the polyphenol substances present in high concentrations in Bulgarian white oregano extract. Our results indicated that Bulgarian *O. heracleoticum* extract has developed several antioxidant defense mechanisms and plasticity to dissipate excess UV-B energy and has the ability to remove free-radicals' stress.

Keywords: Bulgarian white oregano, EPR spectroscopy, antioxidant activity.

INTRODUCTION

Plants are a source of compounds with antioxidant activity; they use sunlight throughout their lifecycle for photosynthesis, in the processes of regulation and development. As a consequence, all plant species have evolved under the light environment, exposed to ultraviolet (UV) (UV-C, UV-A, and UV-B regions) radiation. UV radiation in the UV-B spectral region (280 - 320 nm) has received much attention in the last 20 years because rays from this region are known to damage various physiological plant processes including: DNA damage, direct photosynthetic damage, membrane changes, protein destruction, hormone inactivation, biomass reductions, and epidermal deformation [1, 2]. UV-B radiation exposure decreases plant height, leaf area and plant dry weight, increases auxiliary branching and leaf curling [3]. As reported by Jansen *et al.*, an excessive amount of UV-B radiation damages the plant cells [4]. Furthermore, reactive oxygen species (ROS), including hydrogen peroxide (H_2O_2) and superoxide anion ($\bullet O_2^-$) play a prominent role in different stages of plant pathogenesis. Free radicals directly damage plant cells by disrupting membrane phospholipids, proteins and nucleic acids [4, 5]. Allan and Fluhr [6] noted that cellular damage caused by H_2O_2 and $\bullet O_2^-$ free radicals is the result of conversion to even more reactive species as highly toxic hydroxyl radical (•OH). In addition, Pristov and co-workers [7], by using spin- trapping electron paramagnetic resonance (EPR) spectroscopy, have found that UV irradiation sets off constructive changes in plant cells and provokes the transformation of hydroxyl radical (•OH) into $\bullet O_2^-$ radicals on the cell wall polygalactic acid. There is evidence that *M. oleifera* plants, exposed to UV-B irradiation increase the content of pigments and malondialdehyde (MDA) concentration [8]. Moreover, the plant cells irradiated with UV-B rays induce ROS-overproduction by altering gene expression levels and reduction of the activity of endogenous enzymatic and non-enzymatic antioxidant systems [9]. Furthermore, Yokawa *et al.* commented that free radicals participate as signaling molecules in the effective regulation of cellular redox homeostasis [10].

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In contrast, many researchers have demonstrated that short-term UV-B rates stimulate protective and photomorphogenic responses [11, 12] that affect the resistance of medicinal plants and prevent plant cells from UV-B oxidative stress and other biotic stress types [13]. Several studies report that the effects of UV-B radiation on different parts of medicinal plants significantly increase the antioxidant activity and content of bioactive components [14, 15].

White oregano *(Origanum vulgare* subsp. *hirtum* (Link) Ietswaart), (Syn. *O. heracleoticum* L., *O. hirtum* L.) is an herbaceous, perennial plant with white flowers and pinnate leaves, belonging to the Lamiaceae family. It originates from Europe, the Caucasus, Southwest and Central Asia, Mediterranean regions and was cultivated in Greece, Italy, Spain and Bulgaria [16-18]. The flowers, stems, leaves and other parts of *O. heracleoticum* contain essential oils (carvacrol, thymol, *p*-cymene), phenolic acids (rosmarinic and caffeic acids), flavonoids (apigenin, luteolin, salvigenin, cirsimaritin, diosmetin), anthocyanins [16, 19]. The highest antioxidant activity of *O. heracleoticum*, comparable to the synthetic antioxidants, was associated with compounds as phenols, tannins, rosmarinic acid and carvacrol [16, 19]. Oregano essential oils containing carvacrol and phenolic compounds have been shown to possess antioxidant, antibacterial, antifungal, diaphoretic, antispasmodic, anticancer and analgesic properties [20, 21]. Many investigations have determined EPR antioxidant activity and free radical formation of teas, extracts and oils of cultivated oregano after gamma-radiation [22, 23] estimations. The scientific reports on the photo-related, UV-protecting activities of natural products and raw materials and the importance of prevention UV-exposure-connected damages have increased over the last decade.

Therefore, the aim of the study for the first time was focused on the effects of short-term UV-B radiation (2 h) on the changes in the character of the free radical species of the cultivated Bulgarian *O. heracleoticum* ethanol samples, using direct and indirect electron paramagnetic resonance (EPR) spectroscopy. Additionally, the antioxidant activity and radiomodulatory properties *in vitro* of irradiated oregano samples were determined by 1,1-diphenyl-2-picrylhydrazyl (DPPH) and superoxide dismutase (SOD) scavenging assays.

EXPERIMENTAL

Plant material and alcoholic extraction procedure

The object of this study was cultivated Bulgarian white oregano (*O. heracleoticum*). The raw material (dried leaves) was purchased from "BulgarLuk" OOD– Katunica (Parvomai region, Bulgaria), in 2018 and deposited in the Faculty of Technic and Technologies, Department of Food Technologies, Yambol, Bulgaria. Identification of the spice material was performed according to the requirements of the European Pharmacopoeia, *EC 1441/2007* [24], and the chemical composition was determined (Table 1).

Table 1. Chemical composition of Bulgarian white oregano

Components	Content, %
Moisture	5.98±0.03
Protein	12.56±0.07
Fat	3.96±0.08
Fibers	10.36 0.09
Ash	8.90±0.04
Essential oil, (v/w)	5.74±0.05
Tannins	13.84±0,09

In total, 100 g ofleaves material was weighed and extracted with 200 ml of 70% ethanol (Sigma-Aldrich Co.) on an ultrasonic bath at 60^0C and followed sonication. The *O. heracleoticum* raw material was separated from the resulting mycelium after filtration by vacuum filter and the residue was washed twice with ethanol. A rotary evaporator (Witeg Labortechnik GmbH) at a water temperature of 60-65^0C was used to separate the solvent. The sample was collected at 4^0C/dark in plastic bags till further analyses. The resulting ethanol extract was analyzed by gas chromatography (GC) and gas chromatography-mass spectrometry (GC/ MS) [25, 26]. Carvacrol (60,47%), thymol (8,55%), cymen-7-ol acetate, thymolhydroquinone, caryophyllene oxide, benzophenone, ethyl linoleate and other compounds were identified in the composition of *O. heracleoticum* extract according to ISO standards (ISO 11024-1:1998, ISO 11024-2:1998) [25, 26].

UV-B treatment

UV-B–vis Transilluminator-4000 capable of emitting between 290 nm and 320 nm (peak 309 nm) were purchased from *Stratagene/ USA*. The experimental UV-B intensity was calibrated in each experiment. To obtain the value of dose response about the UV-B radiation effect, the samples were irradiated over UV-B radiation wide range (0 to 12 kJ/m) without visible ray. The UV-B irradiated energy was controlled with short-time exposure - 120+2 min. Dark, fresh air and 40-41% relative humidity was circulated in the illuminator throughout the irradiation course. All samples were irradiated from 20 to 30 cm distance of the light source and a quartz cover was used to allow UV

transparency and to prevent the extract of evaporation and kept in a horizontal position.

Antioxidant activity on O. heracleoticum extracts by spectrophotometricall study

The superoxide dismutase (SOD) content of *O. vulgare* extracts, before and after UV-B exposure was determined according to Sun *et al.* [27] method. The xanthine/xanthine oxidase system (Sigma Chemicals, USA) was used to generate the superoxide anion ($\bullet O_2^-$). Superoxide anion reduces nitro blue tetrazolium (NBT; (Sigma Chemicals, USA)) to formazan after 20 min of incubation in dark. Absorbance was measured at λ = 560 nm and L-ascorbic acid was used as a standard. One unit (U) of enzymatic activity is defined as the amount of enzyme causing 50% inhibition of the NBT reduction to formazan.

Antioxidant activity on O. heracleoticum extracts by EPR spectroscopy analysis

The scavenging ability of DPPH• (Sigma Chemicals, USA) was studied according to Santos *et al.* [28]. Thus, 30 µl of standard or 30 µl of oregano samples (30 µg/ ml) before and after UV-B-radiation were mixed with 250 µl DPPH• ethanol (96%) solution (80 µmol/l) and incubated in dark (3 min). After incubation at 20-23^{0}C room temperature the samples were transferred into the EPR cavity (6 mm thin wall, Wilmad Lab Glass), to obtain the EPR spectra. The scavenging ability was calculated according to the following formula:

$$\text{Scavenged DPPH radicals (\%)} = [(I_0 - I)/I_0] \times 100,$$

where I_0 is the integral intensity of the DPPH• signal of the control sample and I is the integral intensity of the DPPH• signal after addition of the test sample to the control sample. The settings were as follows: center field 3516 00 G, sweep width 200.00 G, modulation amplitude 5.00 G. The percent obtained DPPH• scavenging is proportional to the antioxidant activity of *O. heracleoticum* extracts. IC_{50} values denote the concentration of the sample that is required to scavenge 50% of DPPH• free radicals.

Direct EPR characterization of O. heracleoticum extracts

The 70% *O. heracleoticum* ethanol extract was divided into two parts. First part of the extract was not irradiated while the second was short-term UV-B irradiated (2 h^{-1}). Immediately after irradiation storage, the two oregano extracts were examined by direct EPR spectroscopy. The same measurement was done after their 6-month storage. EPR study was made on a X-band- EMX ^{micro} spectrometer (Bruker, Germany) equipped with a standard resonator. Spectral processing (g-value calculation) was performed with Bruker WIN-EPR and Sim-Fonia software. The following EPR settings were used: center field 3513.50 G, microwave power 20.03 mW, modulation amplitude 10.00 G; gain 2×10^2; time constant 327.68 ms; sweep time 61.44 s.

EPR characterization of O. heracleoticum extracts after system alkalization

A 10 mM sodium hydroxide (NaOH (*Sigma Chemicals, USA*)) water solution was added to the 70% *O. heracleoticum* ethanol extract and to the UV-B irradiated (2 h^{-1}) oregano extract in a ratio of 1:1. The signal was detected after 5 min of incubation, and settings were the same as above. Deionized and distilled water was used for all experiments. Other chemicals used were of analytical or HPLC grade.

Statistical Analysis

The EPR spectral processing was performed using Bruker Win-EPR and Sim-fonia software. Statistical analysis was performed with Statistica 8.0, Stasoft, Inc., one-way ANOVA, Student-t-test to determine significant difference among data groups. The results were expressed as means ± standard error (SE). A value of $p<0.05$ was considered significant. The kinetic data were expressed as the average of two independent measurements which were processed using the computer programmes Origin 6.1 and Microsoft Excel 2010.

RESULTS AND DISCUSSION

Short-term UV-B radiation increase in antioxidant activity

Herbal antioxidants are molecules that mitigate free radicals generation, biotic and abiotic oxidative stress and protect oxidation-chain reactions from terminating *in vitro* [15]. Leyva-López *et al*. [29] reported that phenolic compounds and flavonoids from oregano species possess a high antioxidant activity. Phenolic compounds support plants to survive and to adapt to irregularities in the environment through physiological functions. On the other hand, flavonoids, and in particular dihydroxy B ring in the structure, have the ability to absorb UV-irradiation and to stimulate pigmentation, growth regulation, feeding insects, and oxidative disease resistance [30].

The most commonly used to determine the antioxidant activity of *O. heracleoticum* extracts *in vitro* towards DPPH stable radical, was EPR spectroscopy.

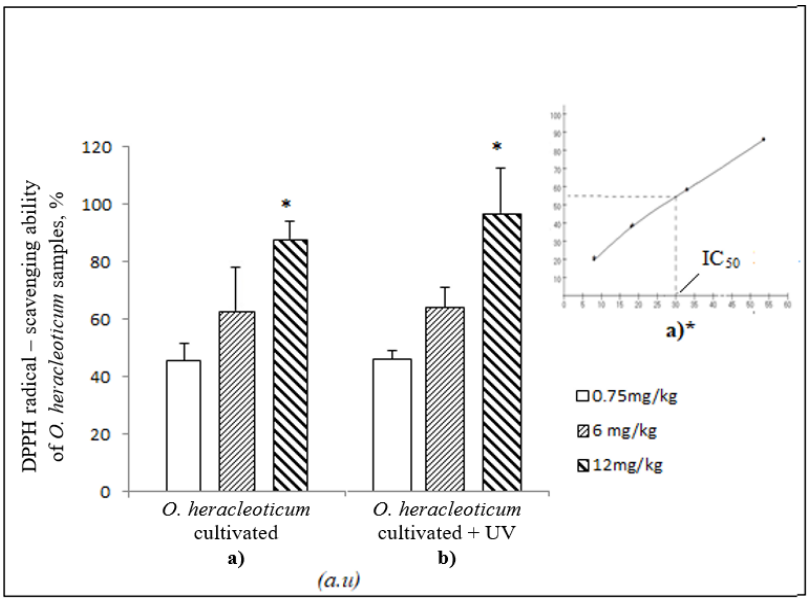

Fig. 1.

The untreated *O. heracleoticum* extract acted as a radical acceptor and significantly increased DPPH scavenging activity in a concentration-dependent manner in the EPR system. The maximum inhibition (88.755± 8.18%. $p<0.004$) (Fig. 1a) was obtained at a concentration of 12 mg/ml. Moreover, linear dependence of *O. heracleoticum* extracts concentration of 30.5±0.61 mM was defined as IC_{50} (Fig. 1a).

Our finding was in support of Amarowich *et al.* [31] and Shokrzadeh *et al.* [32]. The authors reported that ethanol *O. heracleoticum* extract possessed high scavenging capacity towards DPPH in a dose-depend manner, as opposed to thyme and marjoram extracts or to commercial antioxidant butylated hydroxytoluene (BHT). The effect of UV-B radiation on the oregano extract antioxidant activity is presented on Fig 1b. The radiation dose significantly enhanced the DPPH radical-scavenging abilities of the oregano extract (95.31±3.77 %, $p<0,005$) in a dose-dependent manner, compared to the DPPH standard.

The same dependency was established after ultra-sonication (150 gg cycle/ 10 sec) and incubation in the dark (at 25^0C) of both, untreated and irradiated samples at different time intervals (5 - 60 min). The 30-min incubation recorded a statistically significant maximum of EPR signal intensity (the results are not presented). UV-B high energy ROS mediation is particularly harmful to the DNA molecules whose nucleotides have absorption maximum near the border of the UV-B region waves [33, 10]. The well expressed DPPH-scavenging activity by the oregano extract is probably due to the development of effective mechanisms for UV-B radiation protection. Our results were sustained by investigations of Baranauskaite *et al.* who reported that the *O. heracleoticum* antioxidant activity is due to a variety of phenolic compounds and *carvacrol* potency [33].

Short-term UV-B radiation activate SOD-stress tolerance

UV radiation exposure accelerates the ROS overproduction across that of the normal oxygen metabolism, which leads to excessive oxidative stress effects, in relation to the different parts of plants. The superoxide radical generates a number of reactive and harmful secondary metabolites, such as alkoxyl radicals, H_2O_2, •OOH, ONOO−, •OH, lipid peroxyl that lead to damage to plant cells [34]. The adverse effects of $•O_2^-$ production could be reduced *in vivo* by the enzymatic activation of SOD, the levels of ascorbic acid, or anti-oxidant components in the plant cells, that increase the endogenous protection levels [35]. The reactivity of the 1 mg *O. heracleoticum* extract towards $•O_2^-$ radicals is shown on Fig. 2. It is seen that the SOD-like activity of *oregano* extract (111.93±11.34 U; $p < 0.003$) was 5 times higher than that of the used standard antioxidant L-ascorbic acid (1 mg).

Statistically significantly higher $•O_2^-$ scavenging activity (127.54±10.91U; $p<0.05$) (almost 6 times) after short-term UV-B radiation exposure also supported the antioxidant properties of the *O. heracleoticum* ethanolic extract. UV-B treated *O. heracleoticum* extract showed significant decrease and control of oxidation stress damages, plasticity and rapid response to environmental conditions. Our results indicate that the oregano plant has developed several antioxidant defense mechanisms to dissipate excess UV-B energy and free radical elimination capabilities [36].

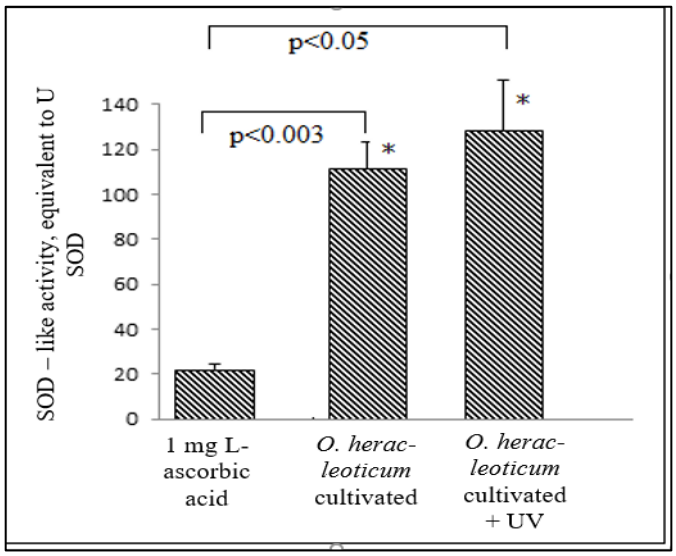

Fig. 2.

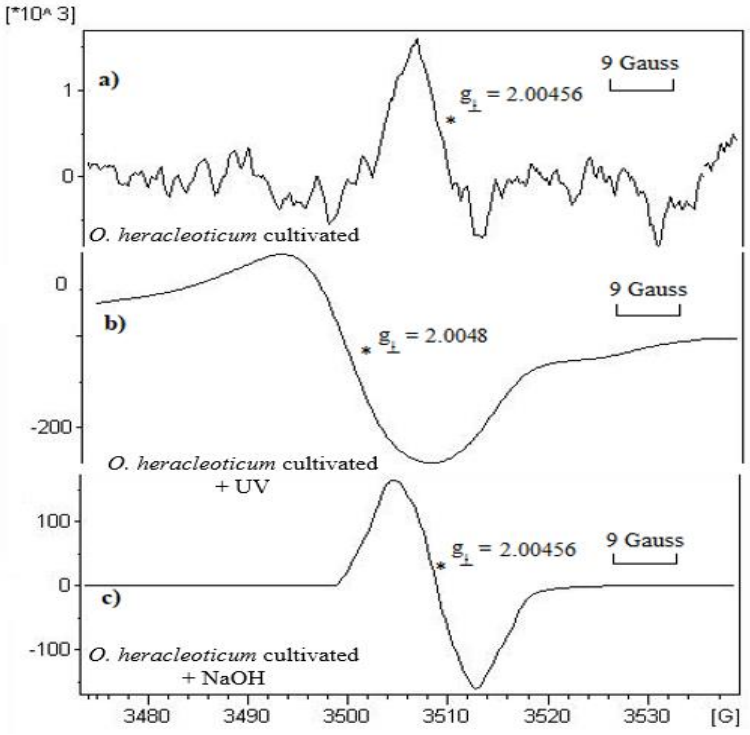

Fig. 3.

The acclimation to oxidative disorders is accomplished by initiating a series of enzymatic and non-enzymatic processes to prevent significant damage reaching the plasma membranes, chloroplasts or other plant organelles [37].

EPR spectral detection before and after short-term UV-B exposure

Moreover, gene expression could increase *oregano* plant tolerance to different stresses (UV-B irradiation), and many researchers invert attention to the potency of SOD-overexpression to increase oxidative stress tolerance [37, 38]. Importantly, the high concentration and synergism between carvacrol and thymol available in our oregano sample and the presence of other phenolic and no-phenolic components, also working as oxidation inhibitors, is positive for the high antioxidant activity [39, 40].

The detailed EPR spectral analysis (3513.50 G magnetic field) of the *O. heracleoticum* reference samples and of the short-term UV-B irradiated sample is depicted in Figure 3a. The *O. heracleoticum* non-irradiated spectrum was detected as a singlet almost symmetrical signal line characterized with a $g_{\perp}$=2.00456±0.0002 factor. The UV-B exposed (Fig. 3b) sample shows almost

commensurate singlet intensity, but with a slight change in the g value, characterized with $g_{\perp}$=2.0048±0.0002. The scavenged signal in both samples could be attributed to *o*-semiquinone radical structures produced by the oxidation of polyphenolic compounds present in plants, in accordance in previous investigations [41, 42].

Various EPR studies comment that flavonoids and phenolic compounds scavenging radicals effectively could form stable *o*-semiquinone structures in alkaline solution [43, 44]. To verify the possibility that the radicals registered in *O. heracleoticum* non-irradiated and UV-B treated extract belong to *o*-semiquinone class, their EPR spectra were evaluated in alkaline solution (1:1) (Fig. 3c). The application of alkalization on oregano samples led to EPR singlet signals with equal values of $g_{\perp}$=2.00456±0.0002. Based on earlier data [42-44] we assume that an *o*-semiquinone radical is practically not affected by short–term UV-B stress and originates from the polyphenol substances presented in high concentration in cultivated *O. heracleoticum* extract. Our EPR results complete the understanding that oregano extract acts as a strong reducing agent against ROS and in particular superoxide radical anion ($\bullet O_2^-$), and activates signaling pathways that prevent structural changes provoked by UV-B irradiation [45].

CONCLUSION

The current study for the first time demonstrates that the ROS inhibition and the negative oxidative stress effects caused by short-term UV-B exposure in cultivated Bulgarian *O. heracleoticum* extract lead to an increase in antioxidant activity. In conclusion, low doses of UV-B radiation induce protective effects based on signaling responses in plant cells and modulation in biological processes by activating enzyme activity tolerance and protecting constructive changes in plants compounds.

Author Contributions: *YK and SB worked equally on the article design and conducted and analyzed the experiments. YK, GN, KD and VG contributed to design of the experiments and composition of the manuscript.*

Conflict of Interest: *The authors declare that they have no conflict of interest.*

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Chlorogenic acid, gallic acid and ferulic acid prevent the development of hyperactivity and anxiety in olfactory bulbectomized rats

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Chlorogenic acid (CGA), gallic acid (GA) and ferulic acid (FA) are abundant biologically active polyphenols in human diet. The aim of this study was to investigate the effects of CGA, GA and FA on the behavior of rats subjected to bilateral olfactory bulbectomy (OB) using the elevated plus maze test. Experimental rats were divided into 5 groups (n=6): sham operated (SO), OB, OB+CGA, OB+GA and OB+FA. After a 15-day recovery period after the operation, rats were treated orally in the course of 14 days. SO and OB rats received saline, OB+CGA, OB+GA and OB+FA groups were treated with CGA, GA and FA (20 mg/kg), respectively. OB induced a state of hyperactivity and anxiety. CGA, GA and FA antagonized the behavioral changes induced by OB. GA and FA caused restoration of the measured indices to values that were significantly different from those of OB rats and did not differ from those of SO rats. The effect of CGA was even higher. It increased the open arms entries and open arms time, as well as the ratios open arms entries/total arms entries and open arms time/total arms time to values that were significantly higher not only from those of OB rats but also from those of SO rats. Similarly, the closed arms time of OB+CGA rats was lower than the respective time of both OB and SO rats. In conclusion, CGA, GA and FA prevented the development of hyperactivity and anxiety in OB rats. Most pronounced was the effect of CGA.

Keywords: chlorogenic acid, gallic acid, ferulic acid, hyperactivity, anxiety, olfactory bulbectomized rats

INTRODUCTION

Anxiety disorders are widespread psychiatric problems affecting human society [1]. They are often associated with depressive conditions or other mood disorders [2-5], and chronic illnesses [3]. The conventional treatment is accompanied by various side effects [6]. Natural products have been considered an alternative option for the treatment of these disorders with conceivably minimized adverse effects, and/or innovative mechanisms of action [7]. Plant polyphenols represent promising agents for treatment of central nervous system (CNS) diseases [8].

Phenolic acids are polyphenolic compounds of natural origin. Chlorogenic acid (CGA), gallic acid (GA) and ferulic acid (FA) are widespread biologically active phenolic acids in fruits, vegetables, nuts, coffee and tea, wine, whole grains [9-12]. It has been reported that CGA or its metabolites may cross the blood-brain barrier and exert neuroprotective effects on brain tissue [13, 14]. FA was found in rat brain approximately thirty min after its oral administration [15]. Some experiments have demonstrated an anxiolytic-like effect of CGA in mice [16]. FA and CGA have shown neuroprotective and cognition-enhancing effects in models of Alzheimer's disease [17-19]. In the study of Han *et al*., FA stimulated neural progenitor cell proliferation *in vitro* and *in vivo* [20]. GA treatment against trimethyltin-induced hippocampal degeneration ameliorated the depression-anxiety state in rats [21]. GA has been found to exert neuroprotective effects on amyloid β-mediated neurotoxicity [22].

The olfactory system in the rat forms a part of the limbic region, in which the amygdala and hippocampus contribute to the emotional and memory components of behavior. Bilateral removal of the olfactory bulbs in rodents induces behavioral deficits that reflect a dysfunction of the cortical-hippocampal-amygdala circuit. Olfactory bulbectomy (OB) in rats is associated with a variety of behavioral abnormalities and serves as a model of depression with comorbid anxiety, agitation, sexual and cognitive dysfunction [23-26]. Surgical removal of olfactory bulbs in experimental rodents is considered most suitable for studying the neurochemical mechanisms underlying the pathophysiology of these behavioral disorders.

Taking into consideration the above mentioned data, the aim of this study was to investigate the effects of CGA, GA and FA on the behavior of rats subjected to bilateral OB using the elevated plus maze test.

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EXPERIMENTAL

Animals and experimental substances

Male Wistar rats (weighing 200-220g) were used in this study. Experimental rats were divided into 5 groups of 6 animals each: sham operated (SO), OB, OB+CGA, OB+GA and OB+FA. They were housed in polypropylene boxes under a normal 12 h light to 12 h dark schedule (lights on at 6:00 am). All rats had free access to food and drinking water. Ambient temperature was maintained at 22-25°C. Animals were allowed to adjust to the housing conditions before experiments began. The behavioral test was conducted between 10:00 am and 1:00 pm. After the testing procedure, the rats were returned to their respective home cages.

All procedures concerning animal treatment and experimentation were conducted in compliance with the national laws and policies, in conformity with the international guidelines (EU Directive 2010/63/EU for animal experiments).

CGA, GA and FA were purchased from Sigma-Aldrich, Germany.

Surgical procedure: Bilateral olfactory bulbectomy (OB)

Bilateral OB was performed according to the method, described by Kelly et al. [23]. Rats were anesthetized (with intraperitoneal injections of Calypsol 50 mg/kg) and placed in a stereotaxic apparatus (Stoelting Co, USA). The coordinates of the olfactory bulbs were determined according to the stereotaxic atlas of Pellegrino and Cushman [27]. The head was shaven and 1.0 cm midline scalp sagittal incision was made. Then bilateral 2.0 mm burr holes were drilled (8.0 mm anterior to bregma and 2.0 mm from the midline). The bulbs were aspirated with a stainless needle attached to a water pump. The burr holes were then plugged with a hemostatic sponge (Gelaspon) to control the bleeding after the drilling.

After the surgery animals were treated daily with antibiotics – topically (with Nemybacin for 7 days) and intraperitoneally (with Gentamicin for 5 days). After the OB procedure, the rats were housed in groups of two and were handled daily during a 15-day recovery period. SO rats are treated similarly, except that the olfactory bulbs were left intact.

Verification: The extent of the lesion was assessed visually post-mortem.

Animal treatment

After a 15-day recovery period, rats were treated orally in the course of 14 days. SO and OB rats received saline (10 ml/kg), OB+CGA, OB+GA and OB+FA groups were treated respectively with CGA, GA and FA (20 mg/kg as a 10 ml/kg solution).

Behavioral experiment: Elevated plus maze (EPM) test

On the 14th day, 60 min after the last treatment, the animals were tested in the EPM, a frequently used test for studying anxiety in rodents and the anxiolytic activity of new drugs [28]. The EPM consisted of four arms, 50 cm long and 10 cm wide, elevated 50 cm above the ground. The apparatus was illuminated by a 40 W bulb positioned 50 cm above it.

Each rat was placed in the center of the maze facing one of the open arms. An arm entry was counted when the animal placed all four paws into the arm. The indices recorded during the 5-min test period were: number of entries into the open arms and time spent there, number of entries into the closed arms and time spent there, total number of arms entries, the ratio: number of open arms entries vs. total number of arms entries and the ratio: open arms time vs. total time in the arms. An increase in the number of entries into the open arms and the time spent there is regarded as a powerful marker for the anxiolytic effect of the tested substance [28,29]. After each assay, the EPM was carefully cleaned with 70% ethyl alcohol solution and dried to remove olfactory cues.

Statistical analysis

All analyses were performed using GraphPad Prism statistical software (GraphPad Software, Inc., La Jolla, CA, USA). Data were analyzed using the Students's *t*-test. All results are expressed as mean±S.E.M. A level of $p < 0.05$ was considered significant.

RESULTS

OB induced a state of hyperactivity demonstrated by a significant [$p < 0.001$] increase in the total number of arms entries of OB rats in comparison with SO rats (Fig. 1). The changes in the other indices demonstrated the development of a state of anxiety. Compared to SO rats, OB animals had a significantly lower number of entries into the open arms [$p < 0.05$] (Fig. 2A) and time spent there [$p < 0.01$] (Fig. 3A), significantly higher number of entries into the closed arms [$p < 0.001$] (Fig. 2B) and closed arms time [$p < 0.01$] (Fig. 3B), as well as significantly lower ratios open/total arms entries [$p < 0.001$] (Fig. 4A) and open/total arms time [$p < 0.01$] (Fig.4B).

Treatment of OB rats with CGA, GA and FA antagonized the behavioral changes induced by OB.

GA and FA caused restoration of the measured indices to values that were significantly different from those of OB rats and did not differ from those of SO rats (Figs. 1, 2 and 3). The effect of CGA was even higher. It increased the number of entries into the open arms and time spent there [$p < 0.001$] (Fig. 2A, 3A), as well as the ratios open arms entries/total entries [$p < 0.001$] (Fig. 4A) and open arms time/total time in the arms to values that were significantly higher [$p < 0.001$] (Fig. 4B) not only from those of OB rats but also from those of SO rats. Similarly, the closed arms time of OB+CGA rats was lower [$p < 0.001$] (Fig. 3B) than the respective time of both OB and SO rats.

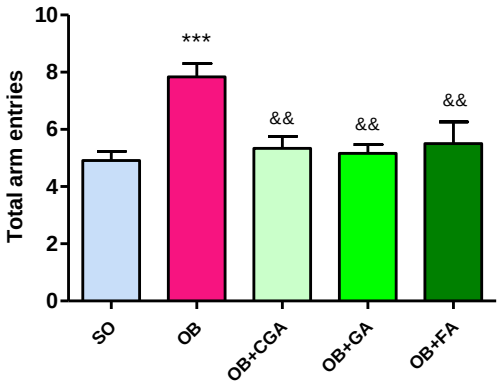

Fig. 1. Total number of arms entries in the elevated plus maze test in rats treated with chlorogenic acid (CGA), gallic acid (GA) and ferulic acid (FA). Results are presented as mean ± SEM; n=6; $^{***}p<0.001$ *vs.* sham operated (SO); $^{\&\&}p<0.01$, *vs.* olfactory bulbectomized (OB)

DISCUSSION

Removal of the olfactory bulbs in rats causes structural and functional alterations in brain regions that result in behavioral changes including anxiety-resembling behavior [24], exploratory hyperactivity, depressive mood, and irritability [30].

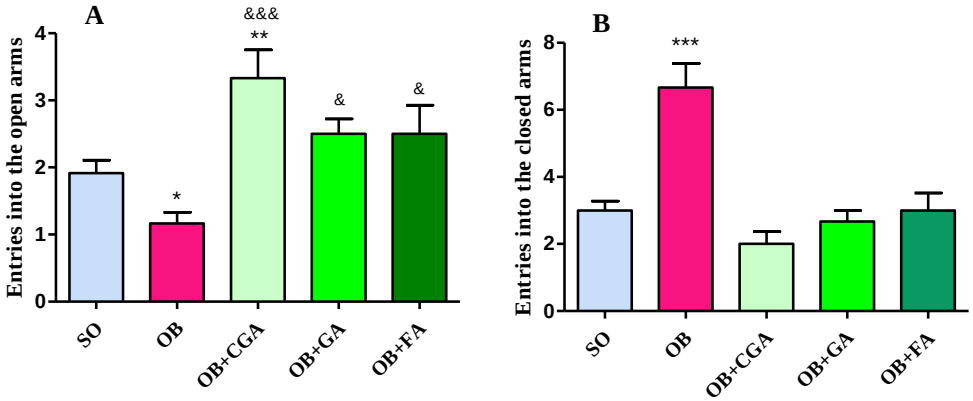

Fig. 2. Number of entries into the open arms (**A)** and number of entries into the closed arms (**B)** in the elevated plus maze test in rats treated with chlorogenic acid (CGA), gallic acid (GA) and ferulic acid (FA). Results are presented as mean ± SEM; n=6; $^{*}p<0.05$; $^{**}p<0.01$, $^{***}p<0.001$ *vs.* sham operated (SO); $^{\&}<0.05$, $^{\&\&\&}p<0.001$ *vs.* olfactory bulbectomized (OB)

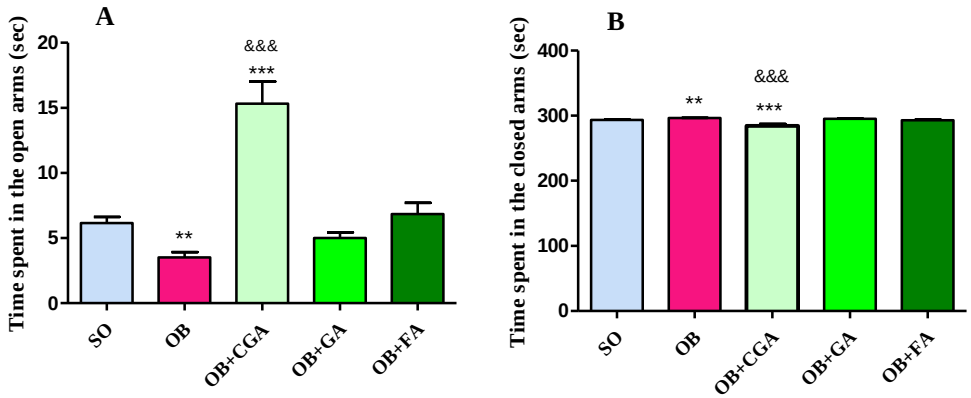

Fig. 3. Time spent in the open arms (sec) (**A)** and time spent in the closed arms (sec) (**B)** in the elevated plus maze test in rats treated with chlorogenic acid (CGA), gallic acid (GA) and ferulic acid (FA). Results are presented as mean ± SEM; n=6; $^{**}p<0.01$, $^{***}p<0.001$ *vs.* sham operated (SO); $^{\&\&\&}p<0.001$ *vs.* olfactory bulbectomized (OB)

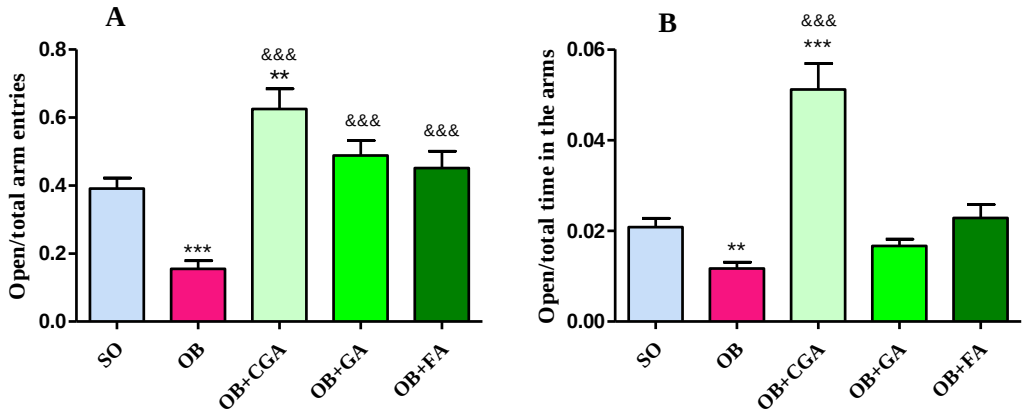

Fig. 4. Ratio of open arms entries vs. total entries in the arms (**A**) and ratio of open arms time vs. total time in the arms (**B**) in the elevated plus maze test in rats treated with chlorogenic acid (CGA), gallic acid (GA) and ferulic acid (FA). Results are presented as mean ± SEM; n=6; $^{**}p<0.01$, $^{***}p<0.001$ *vs.* sham operated (SO); $^{\&\&\&}p<0.001$ *vs.* olfactory bulbectomized (OB)

Bilateral olfactory increases the levels of reactive oxygen species (ROS), nitric oxide (NO) and pro-inflammatory cytokines (TNF-α, IL-6, IL-1β), and decreases glutathione and brain-derived neurotrophic factor (BDNF) in mammalian hippocampus [24,31,32].

In this experiment, OB resulted in hyperactivity demonstrated by the increase of the total number of arms entries. In other studies, such behavioral hyperactivity in OB rats was related to the increased glutamate level in the *striatum* [33] and *nucleus accumbens* [34]. In the present experiment, CGA, GA, FA decreased the hyperactivity of OB rats. Mikami at al. revealed that CGA reversed the glutamate-induced toxicity, as well as glutamate-induced death of primary cells isolated from mouse cortical neurons [35]. In another study, CGA and its metabolites reversed the glutamate-induced toxicity in primary cultures of rat cerebellar granule neurons [36].

Excessive glutamate concentration can induce oxidative stress by increasing the production of ROS, strongly related to the pathogenesis of anxiety behaviors. Literature data show that the pathophysiology of anxiety and related affective disorders is associated with a wide range of epigenetic changes: increased oxidative stress [37, 38], neuroinflammation [39], glutamatergic dysfunction [40], dysregulation of synaptic plasticity through alterations at the neurotrophin level and inhibition of signaling pathways [41]. The implication of oxidative stress in the pathogenesis of anxiety disorders (obsessive-compulsive disorder and panic disorder) was also suggested by Kuloglu *et al.* [42]. Another mechanism that might contribute to the pathogenesis of anxiety is the low level of BDNF [43]. Rinwa *et al*. revealed elevated levels of inflammatory cytokines (TNF-α) and caspase-3 accompanied by a marked reduction in BDNF in the brain of OB rats [44].

In this experiment, CGA, GA, FA showed an anxiolytic-like effect. Most of the biological actions of phenolic acids on the brain have been attributed to theirs anti-inflammatory and antioxidant properties [45]. A study of Gul *et al.* revealed a neuroprotective effect of CGA. That polyphenol attenuated the H_2O_2-induced increases in the levels of malondialdehyde and ROS in rat cortical slices [46]. Another experiment on primary cultures of rat cerebellar granule neurons revealed that CGA increased the protection against H_2O_2-induced proteasome inhibition and caspase-dependent intrinsic apoptosis [47]. In a study of Moghadas *et al.*, the mood stabilizing and neuroprotective effects of GA were attributed to the anti-oxidant activity and amelioration of cell density loss in the hippocampus [21]. Lenzi *et al.* showed that the effects of FA on the CNS were also coupled with its antioxidant activity, evidenced by increased superoxide dismutase and catalase activities, as well as low thiobarbituric acid reactive substances levels, found in hippocampus of treated mice [48]. In the study of Liu *et al*., FA increased the levels of BDNF in the prefrontal cortex and hippocampus, as well as inhibited microglia activation, pro-inflammatory cytokines expression, nuclear factor kappa B signaling [49]. The reduction of pro-inflammatory cytokines could contribute to the anxiolytic-like effects of phenolic acids. FA significantly inhibited the production of the TNF-α, IL-6, IL-1β, and NO, and reduced COX-2 and iNOS [50].

Receptor activation might also participate in the anxiolytic-like effects of phenolic acids. Bouayed *et al*. demonstrated an anxiolytic effect of CGA in mice

tested by EPM, light-dark test and free exploratory test [51]. In that study, the anxiolytic-like effect of CGA was reversed by the benzodiazepine antagonist flumazenil, and the authors suggested that CGA might act as a benzodiazepine receptor agonist [51]. 5-HT1A receptors are involved in the modulation of exploratory and fear-related behaviors, and reductions in 5-HT1A receptor density resulted in increased anxiety [52]. In the EPM tested rats, Mansouri and colleagues observed an anxiolytic-like activity of GA similar to the 5-HT1A receptor agonist buspiron [53].

In conclusion, chlorogenic acid, gallic acid and ferulic acid prevented the development of the state of hyperactivity and anxiety in olfactory bulbectomized rats. Most pronounced was the effect of chlorogenic acid.

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Hepatoprotective effects of *Tinospora cordifolia* extract against bleomycin-induced toxicity in mice

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Tinospora cordifolia (Willd.) Hook.f.&Thomson extract has previously been reported to alleviate appearance of liver alterations. The current study examined the antioxidant activity, therapeutic potential and action of the *T. cordifolia* extract to modulate and protect the liver alterations in bleomycin (BLM)-induced toxicity in ICR/w mice models. The hypothesis was that *T. cordifolia* extract would protect the liver alterations by inhibiting lipid peroxidation, lowering biochemical parameters, decreasing ROS production and reducing oxidative stress levels. Hepatocellular toxicity was induced by intraperitoneal injection of mice once daily with BLM (0.069 U/mL; 0.29 U/kg bw.) for a period of 4 weeks. The *T. cordifolia* was administered once a day for 4 weeks, 2 h prior at dose (80 mg/mL; 0.295 mg/kg/day). BLM intoxication produced oxidative stress in which the antioxidant system functioned incorrectly and ROS production significantly increased. The *T. cordifolia* extract provided significant hepatic protection against BLM toxicity by improving SOD, CAT ($p < 0.04$), MDA and total cholesterol (TC) levels and decreasing ROS in the group receiving BLM ($p < 0.05$), leading to reduced membrane lipid peroxidation. In conclusion, the *T. cordifolia* extract facilitated recovery from BLM-induced hepatic injury by suppressing oxidative stress damages. Therefore, the *T. cordifolia* stimulates antioxidant-scavenging activity and lipid peroxidation reduction in liver. Our results make it appropriate to propose the use of the *T. cordifolia* extract as a possible addition to the treatment of chronic liver alterations associated with BLM-induced toxicity.

Keywords: *T. cordifolia;* oxidative-scavenging imbalance; hepatotoxicity, mice.

INTRODUCTION

Tinospora cordifolia (Willd.) Hook.f. & Thomson. (*T. cordifolia, Guduchi*) belongs to the family Menispermaceae and is used as a protective antioxidant. Ayurveda, India's traditional health system, recommends that the whole plant be used for therapeutic purposes. The extract from *T. cordifolia* has various active components in the structure such as alkaloids, steroids, diterpenoid lactones, aliphatics, glycosides, etc [1]. Moreover, *T. cordifolia* inhibits lipid peroxidation [2], stimulates bile secretion, activates immune effector cells, e.g., differentiation of T cells and B cells [3] and has diuretic properties. Some experimental studies indicate that *T. cordifolia* extract significantly reduces chemotherapy–induced toxicity, cell membrane oxidation, and has a protective role against neurodegenerative changes in the rat hippocampus [4, 5]. In addition, Sharma and Padney [6] have determined that *T. cordifolia* extract has strong antioxidant, anti-inflammatory, anti-arthritic, anti-allergic, anti-diabetic, antimalarial, immunomodulatory, antineoplastic and hepatoprotective properties. There is also evidence that *T. cordifolia* extract reduces damage to cellular oxidative stress and has free radical scavenging activity against reactive oxygen and nitrogen species (ROS/RNS) [7]. In the study of Sangeetha *et al.* [7] the antioxidant activity of *T. cordifolia* was attributed to the presence of tannins and phenolic compounds. The presence of alkaloids such as choline, tinosporin, isocolumin, etc. in aqueous or alcoholic extract of *T. cordifolia* shows detoxification effects and protection against toxin-induced disorders in the mice kidneys [8]. Treatment with *T. cordifolia* extract effectively increases intestinal absorption, hepatoprotection and the regulated alcohol-induced multivitamin deficiency [9]. Phytochemical analysis has shown that *T. cordifolia* extract protects carbon tetrachloride hepatotoxicity in animals [10, 11], and alleviates cisplatin –induced nephrotoxicity *in vivo* [11, 12]. Although *T. cordifolia* extract has different medicinal properties, its protective role has not yet been evaluated against bleomycin-induced toxicity, if any.

Bleomycin (BLM), is an antitumor antibiotic that has been identified as a medicine that induces

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reactive oxygen species (ROS/ $\bullet O^{-2}$, H_2O_2, •OH, NO•); cell membrane instability, lipid and protein peroxidation, inflammatory responses in the lung (fibrosis) [13]; and as a result toxic products are generated [13, 14].

The present study attempts to elucidate the hepatoprotective efficacy of *T. cordifolia* extract in regulation against experimentally BLM-induced toxicity on free radical production and changes in oxidative stress in the liver cells of male IRC/ w mice.

MATERIALS AND METHODS

Chemicals and preparation of T. cordifolia extract

Bleomycin sulfate (EP 9041-93-4), Carboxy-Ptio.K, and other chemicals were purchased from Sigma Aldrich Co., USA, and were of analytical grade. *T. cordifolia* fine powder (ABC Limited, India; identified by a plant taxonomist) was kinetically extracted (for 48 h in 100% ethanol, v:v). The total filtrate was dried using a rotary evaporator (Buchi B-480, India) at 400c and was lyophilized (Iishin Lab Co. Ltd, USA) to crude extract. The *T. cordifolia* extract was stored in air-tight glass bottle at 8°C, and it was used as a practical approach to protect against BLM- intoxication.

Maintenance of animals

Male ICR/w mice weighing approximately 45-50 ± 3.0 g were obtained from the Medical Faculty, Trakia University, (Suppliers of Laboratory Animals), Stara Zagora, Bulgaria. The animal procedures were in accordance with Directive 2010/63/EU on the protection of animals used for experimental and other scientific work, and approved by the Ethical Committee for Animals of BFSA and Trakia University, Stara Zagora, Bulgaria (131/ 6000-0333/ 09.12.2016). The mice were housed in polypropylene cages at a temperature of 18–20°C and under a light/dark period of12/12 h daily. They were fed on a standard commercial feed (Indusrial, Bulgaria), after 10 days of acclimatization and free access to tap water. The essential cleanliness conditions were also maintained. The lyophilized *T. cordifolia* was dissolved in distilled H_2O and preserved at 4^0C until use.

Experimental protocol

Mice were divided into 4 groups (n=6) for a period of 29 days and drugs administration were through intraperitoneal (i.p.) injection as follows:

I) Group I (CG) (no treatment);

II) Group II (BLM) (BLM 0.069 U / ml; 0.34 U / kg body weight in saline (250 µl) was given i.p. and completed on day 16. After day 17 the animals were given BLM at a schedule up to the 28th day [15];

III) Group III (*T. cordifolia extract* 80 mg/ml; 0.295 mg/kg body weight in distilled H_2O (250 µl) was given once daily i.p. continued on schedule for up to 28 day);

IV) Group IV (*T. cordifolia* +BLM) *T. cordifolia* extract (80 mg/ml) + BLM (0.34 U/kg) (antioxidant was injected once daily 2 h before the antibiotic and continued on schedule for up to 28 days).

Additionally, the physiological status and behavior of animals were monitored daily. After the last drug administration, the mice were given rest and on the next day, they were sacrificed under anesthesia (Nembutal 50 mg/kg i.p.). The liver samples were removed, washed in phosphate buffer saline (pH=7.4, 4°C) homogenized and analyzed for biochemical parameters, and ROS production. The fresh blood (1.1-1.5 cm^3) was collected directly from the heart in cold plasma-containers (5 cm^3 Monovette, Germany). After centrifugation of blood samples at 4000 rpm, 4°C for 10 min, 200 µl of plasma from each group were investigated directly for TC estimation.

Biochemical analyses of hepatocellular antioxidant status

The liver tissue lipid peroxidation (malondialdehyde concentration (MDA)) was estimated by the method of Plaser *et al.*, 1966 [16], and the activities of superoxide dismutase (SOD) and catalase (CAT) were analysed using the method described by Sun *et al*., 1988 [17] and by Aebi, 1984 [18], respectively. The TC in blood was estimated using a commercially available diagnostic kit (AM-2035-KA, 2017). The biochemical analyses were performed on a UV–VIS spectrophotometer-400 (TERMO Sci., RS232C, Stratagene, USA).

Electron paramagnetic resonance (EPR) in vivo evaluation of ROS production

ROS production in the liver samples was investigated by *in vivo* EPR (X-Band, Emx^{micro} spectrometer, Bruker) method according to Shi *et al*. (2005) [19]. Briefly, to 100 µl plasma and 100 mg of spleen were added 900 µl of 50 mM N-t-butyl-alpha-phenylnitron (PBN) dissolved in dimethyl sulfoxide (DMSO) and centrifuged at 4000 rpm/ 10 min at 4ºC, with settings: 3505 g centerfield, 6.42 mw microwave power, 5 g modulated amplitude, 1-5 scans. All experiments were made in triplicate.

Statistical analysis

The processing of the spectra was performed using Bruker Win-EPR and Sim-fonia software. Statistical analysis was performed with Statistica 8.0, Stasoft, Inc., one-way ANOVA, Student- t- test to determine significant difference among data groups. The data were expressed as means ± standard error (SE). A value of $p< 0.05$ was considered significant.

RESULTS AND DISCUSSION

Changes in biochemical enzymes after exposure to BLM, both alone and in combination with *T. cordifolia* extract, showed a significant change in oxidative / pro-oxidative activity. BLM exposure (Fig. 1) produced statistically significant decrease in SOD (6.88±0.46 IU/gHb, $p<0.03$) and CAT (5.433±0.91n IU/gHb, $p<0.05$) activities, compared to CG (16.28±1.35 IU/gHb). In addition, *T. cordifolia* extract showed a statistically significant increase in the levels of both antioxidant enzymes (SOD: 18.49±3.16 IU/gHb; CAT: 14.83±1.21 IU/gHb,), compared to untreated CG ($p<0.05$) and to BLM treated ($p<0.05$) group. Moreover, administration of the *T. cordifolia* extract 2 h before BLM treatment showed a protective effect on the hepatic cells in SOD ($p<0.05$) and CAT ($p<0.003$) activities.

The BLM –induced toxicity increased oxidative stress disorders and inflammatory responses, due to the destructive free-oxygen production and leading to highly lipid peroxidation. Experimentally, BLM has been used to induce chronic toxicity in mice models at a dose of 0.069 U/mL; 0.29 U/kg bw dissolved in saline and to produce oxidative hepatocellular changes [21].

A number of studies have reported the isolation and protection of active biomolecules from plant antioxidants against chemotherapy-induced damage and toxicity [6, 20, 22] as effective inhibitors of ROS. There is evidence that plant extracts containing alkaloids and glycosidic compounds are potent inhibitors of various oxidative processes, exhibit significant antioxidant activity, prevent lipid peroxidation and restore SOD and CAT activity [23].

Superoxide dismutase (SOD) catalyses the dismutation of superoxide anion ($\bullet O_2^-$) to H_2O_2 and O_2, while catalase (CAT) reduces the H_2O_2 levels into H_2O molecules [24]. In our experiment, it was shown that SOD activity and CAT activity were statistically significantly decreased in the BLM group compared to controls ($p <0.05$ *vs*. CG).

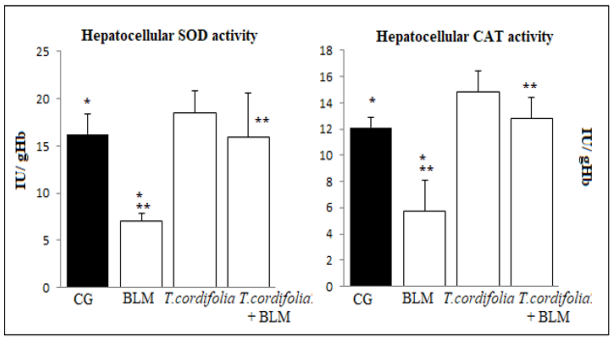

Figure 1. Levels of SOD and CAT activity in liver homogenates. *T. cordifolia extract* and its constituents, alkaloids and glycosides, regulate antioxidant biochemical enzymes in chronic BLM-model. Liver samples were collected from all sacrificed animals. The experiments were repeated three times. *$p < 0.05$ *vs*. the CG group; **$p < 0.05$ *vs*. the BLM group ($n = 6$).

The decrease in endogenous antioxidant enzymes is likely to be associated with increased oxidative damage that contributes to the inflammatory response of BLM administration. In addition, *T. cordifolia* extract contains alkaloids and glycosides and has the potential to reduce oxidative stress damage by inactivating H_2O_2 and by inhibiting inflammatory responses.

These results simultaneously support the claim that treatment with 80 mg / mL *T. cordifolia* provides protection against the effects of BLM-induced stress; and indicate the protective role of *T. cordifolia* in liver tissues [6, 25].

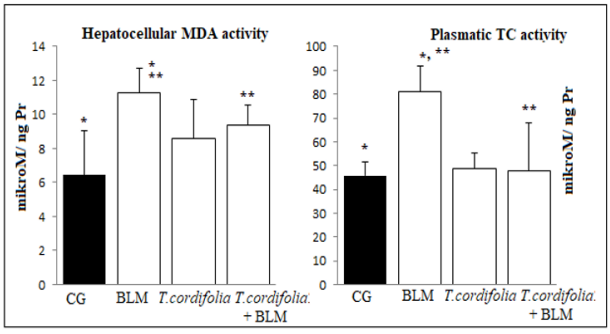

Figure 2. Levels of MDA in liver homogenates and levels of TC in plasma. *T. cordifolia extract* and its constituents normalize hepatocellular/ plasmatic levels of lipid accumulation in chronic BLM-model. Liver samples were collected from all sacrificed animals. *C longa* extract BIPF-model. The experiments were repeated three times. *$p < 0.003$ and $p < 0.05$ *vs*. the CG group, respectively; **$p < 0.05$ *vs*. the BLM group ($n = 6$).

Oxidative stress is associated with an imbalance between the production and purification of ROS products. ROS overproduction and BLM-induced oxidative damages contribute to hepatocyte injuries and these processes increase cell lipid damage and induce hepatic cell malformation [26, 27]. To investigate the effects of *T. cordifolia* extract on

hepatic lipid accumulation, we measured MDA levels in liver homogenates and total plasma cholesterol (TC) in all tested groups (Fig. 2).

However, MDA (11.6±4.16 μM/ng Pr *vs*. 6.16±1.03 μM/ng Pr *vs*; p< 0.003, t-test) and TC (80.56±11.12 μM/ng Pr *vs*. 45.7±7.03 μM/ng Pr; p< 0.05, t-test) levels all significantly increased in the BLM model, compared with the CG. The combination of *T. cordifolia* and BLM (80.56±11.12 μM/ng Pr *vs*. 45.7±7.03 μM/ng Pr; p< 0.05, t-test) correspondingly reduced the increased plasma lipid concentrations, in MDA (9.253±0.91 μM/ng Pr *vs*. 11.6±4.16 μM/ng Pr; p< 0.05, t-test) and TC (47.56±11.12 μM/ng Pr *vs*. 80.56±11.12 μM/ng Pr; p< 0.05, t-test), compared to BLM treatment. Consistent with these findings, we found comparable values in the plasmatic lipid peroxidation between the *T. cordifolia* extract and controls. In accordance with our results, other investigations report inhibition in the lipid peroxidation process, prevention of tissue damages thereby maintaining the membrane integrity and free-radicals reduction in chemo-induced toxicity, after *T. cordifolia* extract application [28-30].

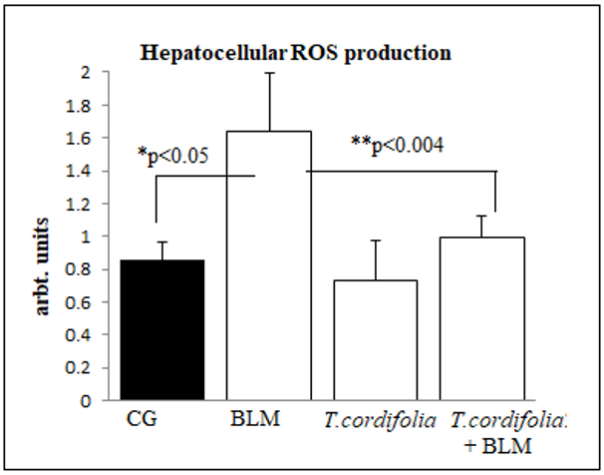

Figure 3. *In vivo* ROS radical production. Liver samples were collected from all sacrificed animals. Results were calculated by double integration of the corresponding EPR spectrum immediately registered in liver homogenates (expressed in arbitrary units/ *arbt. units*). The experiments were repeated three times. *p < 0.05 *vs.* the CG group; **p < 0.004 *vs.* the BLM group (n = 6).

Banerjee *et al*. [31] commented that intracellular, endogenous ROS expression in peripheral blood of patients suffering from persisting polyarthralgia post CHIK infection was significantly scavenged by *ex vivo* treatment with *T. cordifolia* leaf extract. To confirm the efficacy of *T. cordifolia* extract containing alkaloids and glycosides, reduction of the BLM-induced toxicity in liver homogenates was evaluated. Figure 3 shows the EPR spectra of ROS products in liver homogenate measured in arbitrary units.

The results demonstrate the highly toxic effects of BLM administration, and showed a statistically significant increase of ROS production in hepatic cells (1.72 ± 0.901 *vs* 0.858 ± 0.21 a.u., p<0.05, *t*-test), relative to the CG. However, the ROS products levels were close to that in CG in the group treated with *T. cordifolia* (0.739 ± 0.14 *vs*. 0.858 ± 0.21 a.u., t-test), or with a combination of *T. cordifolia* + BLM (0.997 ± 0.33 *vs*. 0.858 ± 0.21 a.u., t-test). The EPR method indicated an increased ROS concentration in hepatocytes. The statistically significant decrease in ROS production in hepatic cells was observed in *T. cordifolia* + BLM combination (0.923 ± 0.5 a.u. *vs* 1.72 ± 0.901, p<0.004, *t*-test), in comparison to the BLM administration. However, *T. cordifolia* extract administration completely ameliorated the ROS production and hepatic pro-oxidative effect in BLM-intoxicated mice (p < 0.05). Different investigations have suggested that the plant extract has a protective effect against damages in hepatic function due to direct antioxidant [32] and free radical scavenging mechanisms and regulation of ROS production [31-33]. Moreover, Baskaran *et al*. [25] reported that *T. cordifolia* extract regulates free radicals levels and lipid peroxidation by countering Cd-induced oxidative stress and by controlling enhanced ROS production effected over tissue glycoproteins in liver cells and hepatotoxicity.

CONCLUSION

Finally, our results indicated that *T. cordifolia* extract treatment stimulated endogenous antioxidant activity, reduced lipid peroxidation and scavenged ROS products. These results make it appropriate to propose the use of the *T. cordifolia* extract as a possible addition to the treatment of chronic hepatotoxicity associate with chemo-induced oxidative damages.

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Conflict of Interest: *The authors declare that they have no conflict of interest.*

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Use of silymarin for reducing nephrotoxicity caused by medicaments

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Seeds of *Silybum marianum (L) Gaertn (Carduus marianus L., Asteraceae)* have been used for more than 2 000 years to treat liver and gallbladder diseases, particularly in the treatment of hepatitis and cirrhosis. The growing interest in the plant is documented by the fact that nowadays the information platform PubMed has over 3 570 publications about it. There has been a steady increase in the number of publications on its use in diabetes, chemical weapons intoxication, its radio-protective effect. Silymarin, standardized extract of the plant is used as a chemoprotective and anticancer agent, especially as a protector against the toxic effects of some drugs used in oncology, as well as against the toxic action of antibiotics. The purpose of this publication is to examine silymarin's contribution to reducing nephrotoxicity induced by medicaments. We present some summarized examples of the nephroprotective effects of silymarin when applied with analgesics and non-steroid anti-inflammatory agents, with antibiotics, anti-tuberculosis agents, anticancer agents and immunosuppressive agents.

Keywords: silymarin, silybin, silibinin, nephrotoxicity

INTRODUCTION

The kidneys are often damaged by various toxic compounds - fungal poisons, heavy metals, organic solvents. Drug-induced nephrotoxicity (DIN) accounts for up to 60% of acute renal failures acquired at the hospital. Much effort is being made to reduce drug-induced renal impairment. However, DIN remains a problem that has a significant impact on patients and the health system. *Silybum marianum (L) Gaertn (Carduus marianus L., Asteraceae)* (milk thistle) is a medicinal plant which has been used for centuries in alternative and modern medicine for treatment of various diseases such as liver disorders and protecting the liver [1]. Silymarin is a standardized extract of *Silybum marianum* (milk thistle extract) consisting mainly of silybin, dehydrosilybin (DHSB), quercetin, toxifolin, silicristin, and a number of other compounds known to have numerous beneficial effects. The antioxidant, anti-inflammatory and anti-apoptotic properties of silymarin make it an interesting herb for medicines and these properties have included this agent as a potential renoprotective agent, similar to other plants [2, 3]. Silymarin exhibits significant protective effects against various toxic compounds. Whether the protective use of silymarin can be an effective clinical pharmacological strategy for preventing DIN is a question to be answered in clinical trials [1]. There is evidence of its role in reducing tumor growth, preventing liver toxicity, and protecting a number of organs against ischemic damage. A well-established fact is the hepatoprotective effect of silymarin, especially to prevent α-amanitin and alcohol intoxication causing liver damage. There is also strong evidence that silymarin has antimicrobial and anticancer effects [4]. The xanthine oxidase enzyme is involved in tissue oxidative damage after ischemia-reperfusion. The dehydrogenase/ oxidase ratio of homogenates in rats decreases during ischemia and reperfusion. Silymarin contains two flavonoids: quercetin and silybin, characterized as free radical scavengers and exerting a protective effect, preventing a decrease in the dehydrogenase / oxidase ratio during ischemia-reperfusion [5]. The purpose of this review is to discuss and summarize the information found in the literature regarding the potential for reducing the nephrotoxicity of various drugs using silymarin.

EXPOSITION

Paracetamol

Paracetamol (acetaminophen, N-acetyl-β-aminophenol, APAP) is the most widely used analgesic for acute pain and the most commonly used antipyretic agent [6]. Overdose with paracetamol causes severe damage to the liver and kidneys. Moreover, the APAP - induced liver

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damage is the most common cause of drug-induced liver failure [7].

Onolapo *et al.* [8] treated rats intraperitoneally (i.p.) at a dose of 800 mg/kg/day for 3 days.

Acetaminophen overdose leads to impaired motor activity, memory impairment, anxiety, impaired liver and kidney biochemistry, antioxidant balance, and histological changes in the liver, kidney, and cerebral cortex. Preliminary treatment with silymarin at a dose of 25 mg/kg per day for 14 days and then intraperitoneal (i.p.) administration of acetaminophen at 800 mg/kg per day for 3 days counteracted behavioral changes. This leads to improved biochemical indicators of liver and kidney damage and improves antioxidant activity.

In another study, acetaminophen overdose resulted in elevated levels of aspartate aminotransferase (AST), alanine transaminase (ALT), nitric blood nitrogen (BUN), and creatinine (SCr) in serum, as well as levels of nitric oxide in the liver and kidney. There are also significant histologic changes, including decreased body weight, hepatocyte edema, cellular infiltration, dilation and congestion, necrosis and apoptosis in the liver, and dilatation of Bowman's capsule space and glomerular capillaries, pale colored tubules, cellular infiltration and apoptosis in the kidney. Treatment with silymarin 1 hour after APAP injection for 7 days significantly normalized body weight, histologic lesions, serum ALT, AST, BUN, SCr and tissue NO levels. Silymarin has been hypothesized to improve the toxic effects of APAP-induced hepatotoxicity and nephrotoxicity in mice. The protective role of silymarin against AAPP-induced lesions may be due to its antioxidant and anti-inflammatory effects [9].

In an experimental model with Wistar albino rats, the nephroprotective effect of the ethanol extract of *Scrophularia hypericifolia* (stems, leaves), a plant grown in Saudi Arabia, was investigated. Toxic doses of paracetamol were used to induce renal toxicity, while the standard drug silymarin was used as a reference. Renal impairment was investigated by measuring serum urea, serum creatinine, and sodium and potassium levels [10].

Propacetamol is a medicine that is administered intravenously and is metabolized by the body to paracetamol. This suggests that if silymarin is protecting against the toxic effect of paracetamol, it is likely to have such an effect against propacetamol [11, 12].

Antibiotics and agents against tuberculosis

Gentamicin is used clinically against Gram negative bacteria because of its efficacy. However, it causes kidney damage. Silymarin could eventually reduce the kidney damage [13].

Drug-induced nephrotoxicity is an important cause of renal failure in dogs. Aminoglycoside antibiotics, such as gentamicin, can produce nephrotoxicity in dogs, due in part to imbalance of pro- and antioxidants (oxidative stress). Silymarin has potentially useful antioxidant properties. Dogs were given gentamicin by intramuscular injection at a dose of 20 mg/kg once daily for 9 days [14]. Renal function was evaluated using serum biochemical markers (creatinine and urea). Malondialdehyde (MDA) concentration is measured as a lipid peroxidation marker. The activity of total serum antioxidants (TSAO) is evaluated as a marker of antioxidant protections [15]. Concentrations of serum creatinine and urea increased significantly and TSAO decreased significantly due to the administration of gentamicin. Silymarin reduces gentamicin-induced nephrotoxicity in dogs [14]. In such experiments, rats were treated with gentamicin. Compared to rats in the control group, all rats injected with the antibiotic showed significantly elevated serum creatinine and urea levels, which was accompanied by an increase in renal relative weight, increased levels of reactive oxygen species (ROS), and MDA, and a decrease in the level of renal glutathione (GSH) and superoxide dismutase activity (SOD). Preliminary treatment with silymarin significantly lowers elevated serum urea and creatinine concentration, kidney weight, and kidney ROS and MDA levels. In addition, silymarin significantly increases the level of renal GSH and SOD activity [14]. According to Ghaznavi and co-workers [16] silymarin can reduce kidney damage in rats treated with gentamicin, possibly by reducing the level of ROS. Jedage and Manjunath [17] induced nephrotoxicity in male Wistar rats of gentamicin at 100 mg/kg/per day for 10 days, and silymarin (50 mg/kg, p.o.) was used as a lesion reducing drug. The renal biochemical markers creatinine, urea, uric acid, albumin, protein, and other parameters - kidney weight, body weight, and urine volume and kidney histopathology - were used to assess injuries. The results of the study suggest that gentamicin damages the kidneys, and silymarin reduces the extent of this damage.

Polymyxins were detected from different species of *Bacillus polymyxa*. Their efficacy against most Gram-negative bacteria has not been called into question, but their early use has been associated

with reports of adverse renal effects in a significant number of patients. This class of antibiotics consists of five chemically different compounds, polymyxin A, B, C, D, and E (colistin); but only polymyxins B and E have been used in clinical practice. After reports of its nephrotoxicity polymyxin E is discontinued. According to Hasan *et al*. [18] in a rat study, silybin had the potential to protect the kidney from polymyxin E. Rats were treated with polymyxin E and the other group was pretreated with silybin and the same antibiotic for 7 days. Histological, ultrastructural and morphometric analyzes were performed on rat kidney tissues. The results indicated that administration of silibin reduced the neomyotoxicity induced by polymyxin E in rat kidney.

In such a study [19], urine was examined. It was collected daily for 7 days to test for N-acetyl-beta-D-glucosaminase (NAG). Serum was collected after rat euthanasia on day 7 for a renal function test. The results indicate that polymyxin E affected the renal glomerulus and tubercles, as well as the possible protective effect of silybin against polymyxin E-induced nephrotoxicity.

The main drawback is the toxic side effect of isoniazid. Adverse reactions caused by the administration of INH (50 mg/kg) on haematological parameters, markers of oxidative status, markers of liver and renal function and their improvement were examined by administration of silymarin treated at a dose of 50 mg/kg for 1 hour with INH for 30 days in rats. The results showed that silymarin reduced the isoniazid toxicity [20].

In a similar study, rats were treated concomitantly with isoniazid and rifampicin (RIF) orally at a dose of 50 mg/kg/ per day for 28 days. Addition of silymarin at a dose of 25 mg/kg/ per day significantly reduced the toxic effects on the liver and kidneys. Treatment with INH and RIF resulted in a significant decrease in antioxidant levels and a significant increase in creatinine, urea and uric acid levels, which indicate impaired renal function. Silymarin treatment improved these effects. Moreover, histological studies of the kidney supported these findings and showed that the renal structure was almost normal [21]. Cecen *et al*. [22] conducted a study with doxorubicin at a single intraperitoneal dose of 10 mg/kg. Serum is secreted to determine SOD, GSH Px, CAT, MDA, NO, creatinine, urea, AST, ALT, lactate dehydrogenase (LDH) and creatine phosphokinase activity (CPK). Histopathological and electron microscopic examinations of the heart, kidneys and liver were performed. In the second group the rats were treated with a combination of doxorubicin and silymarin. The results indicated that doxorubicin caused a significant increase in serum NO levels compared to controls. This pointed out that silymarin significantly protected the renal and hepatic toxicity induced by doxorubicin in the rat, and suggested its use as a supportive treatment during anticancer treatment with doxorubicin.

Silymarin and renal toxicity of anticancer agents and immunosuppressants

There is a lot of evidence which considered that anticancer agents damaged the kidneys. Despite several preventive conditions, the nephrotoxicity of cisplatin remains a clinical problem. *In vitro* and *in vivo* studies addressed the protective effects of silymarin against the nephrotoxicity of cisplatin. Shahbazi *et al*. [23] evaluated the effect of silymarin on cisplatin nephrotoxicity as the first human study. During this pilot, randomized, double-blind, placebo-controlled clinical trial, the effect of oral silymarin 420 mg daily was studied in three divided doses beginning from 24 -48 hours prior to initiating the cisplatin infusion and continuing until the end of the 32nd day. Acute renal impairment associated with cisplatin was observed in 8% of patients; no side effects with silymarin have been reported. Prophylactic administration of a conventional form of silymarin tablets could not prevent cisplatin-induced impairment of renal function.

Ibrahim *et al.* [24] treated rats with cisplatin at a dose of 5 mg/kg for 5 days to cause acute renal failure. Silymarin was pretreated 6 hours before cisplatin. Functional kidney tests and histopathological examinations were performed. The results from the study showed a significant improvement in renal function tests and renal histopathology by using silymarin as a protective mechanism for cisplatin -induced acute renal failure.

Divya *et al.* [25] administered cisplatin once at 16 mg/ kg i.p. in Wistar rats. The dose is sufficient to cause nephrotoxicity. To some of the animals was given a methanolic extract of *Apodytes dimidiata* for 5 consecutive days before/after injection of cisplatin at a dose of 250 mg/kg. Blood and kidney parameters were analyzed. The results showed a significant protective effect of the extract on cisplatin –induced nephrotoxicity in the pretreated animals. Urea, creatinine and lipid peroxidation were reduced by 58.31%, 42.19% and 60%, respectively, and hemoglobin and leucocytes increased by 28.25% and 42.91%, respectively. GSH, GPx, SOD and catalase increased by 35.64%,

18.14%, 74.42% and 35.46% respectively. Tissue architecture of the kidneys is almost normal in animals treated with the extract. According to the authors, the results are comparable to the standard medicine, silymarin.

Prabhu *et al*. [26] previously administering 1,2-diazole alkaloid significantly reduced cisplatin-induced nephrotoxicity. Biochemical studies such as GPX, GSH and LPO levels, as well as urine volume, kidney weight, body weight, and histopathological studies confirmed that 1,2-diazole (10 mg/kg) possessed nephroprotective activity. These results were similar to those with the standard drug silymarin at a dose of 50 mg/kg. In addition, the results have shown that 1,2-diazole could be used as a neoprophylaxant in combination with silymarin.

Ninsontia *et al*. [27] also induced apoptosis and necrosis by cisplatin in NK-2 cells and caused cell viability to be reduced by ~ 40% and 60% at doses of 25 and 100 μM, respectively. Pretreatment with 25-200 μM silymarin significantly protected against cisplatin-induced cell death in a dose-dependent manner. Pretreatment of silymarin (25-100 μM) did not cause a significant change in cisplatin-induced cell death in H460 cells, but significantly enhanced cisplatin-induced apoptosis in G361 cells. These findings revealed the selectivity of silymarin in protecting kidney cells from cisplatin-induced cell death and might be useful for the development of the compound as a re-prophylactic agent. In a rat model, kidney damage was induced by a single dose of cisplatin (5 mg/kg). The protective effect of silibinin was studied in rats having received flavonoid at a dose of 200 mg/kg (i.v.) for 1 hour prior to the cisplatinate administration. Renal function was monitored by analyzing the urinary markers for glomerular and tubular function over a period of 11 days. The animals from a second identical treatment were sacrificed 4 days after drug administration to assess tubular microscopy light microscopy. Administration of cisplatin caused a reduction in renal function within one day of treatment. The observed symptoms were: a decrease in creatinine clearance and an increase in proteinuria. The effects of cisplatin on creatinine clearance and proteinuria were completely prevented by pretreatment of the animals with silibinin. Reduced damage to proximal tubular function. Silibinin itself did not affect renal function. Treatment with silibinin clearly reduced the morphologic changes seen in the S3-segment of the proximal tubule 4 days after administration of cisplatin. The effects of cisplatin on glomerular and proximal tubular function as well as proximal tubular morphology could be fully or partially improved by silibinin. In conclusion, silibinin might act as a non-prophylactic agent and it is believed that it may have an effect on the kidneys in the clinical setting [28]. Bokemeyer *et al.*, [29] conducted such study on an animal model in rats *in vitro* and in three human cancer cell lines of the testicular system. Cisplatin is one of the most active cytotoxic agents in the treatment of testicular cancer, but its clinical application is associated with side effects such as nephrotoxicity. The results show significant nephrotoxicity of cisplatin. Pre-infusion of silibinin decreases the toxicity of cisplatin. Silibinin alone did not affect renal function. The *in vitro* data excluded significant inhibition of the anti-tumor activity of the major nephrotoxic components, cisplatin and 4-hydroperoxyphosphamide, by co-administering silibinin in a human cell tumor cell line model of human germ cells.

In vitro experiments with kidney cells injured by acetaminophen, cisplatin and vincristine, showed that the administration of silibinin before or after chemically induced damage might reduce or avoid the nephrotoxic effects [30]. Single and multiple treatment with toxic doses of cyclophosphamide activates peroxidation of lipids in kidney cell membranes. Silymarin inhibits the cyclophosphamide prooxidant effect, suggesting additional antioxidant studies as a means of counteracting the adverse effects of cytostatic drugs [31].

Methotrexate is widely used in the treatment of various malignancies and non-cancer diseases, but its use is limited by its nephrotoxicity. A study was conducted to determine whether silymarin exhibited a protective effect against methotrexate-induced nephrotoxicity. The rats were injected with methotrexate at a dose of 20 mg/kg, i.p. single injection. Histopathological changes, including apoptotic changes in the kidneys, have been evaluated. Injection with methotrexate shows extended Bowman space, infiltration of inflammatory cells, glomerular and peritubular vascular congestion and edema of renal tubular epithelial cells. Apoptotic cell death was also markedly elevated in the renal tubules after methotrexate administration. Treatment with silymarin 300 mg/kg i.p. daily for 5 days resulted in a statistically significant improvement in histological changes and reduced the number of TUNEL –positive cells compared to methotrexate – treated rats ($p < 0.05$). In conclusion, treatment with silymarin resulted in a reduction in methotrexate – induced renal impairment in rats [32]. Adriamycin

is a potent anticancer agent, but its clinical use is limited due to pronounced cardiotoxicity and nephrotoxicity. El-Shitany *et al.* [33] performed rat studies with adriamycin and combination of adriamycin and silymarin. The first group of rats was treated with adriamycin at a dose of 10 mg/kg, the same dose of adriamycin was administered to the second group that was pretreated with silymarin at a dose of 50 mg/kg. On the third day after treatment was determined LDH, CPK, cholesterol and total lipids. Thirty days after injection creatinine and urea levels were determined. To evaluate the lipid peroxide and GSH content, frozen heart samples (72 h) and frozen kidney samples (30 days) were used. Histopathological examinations of cardiac and renal sites were also performed. Serious reduction in plasma CPK, LDH, creatinine and urea was observed in sildarin treated rats. On the other hand, silymarin treatment does not alter adriamycin-induced hyperlipidemia. Silymarin treatment significantly reduces myocardial MDA. In addition, the silymarin administration normalized the level of MDA and GSH in the kidney tissue. Histopathological examination of heart and kidney segments revealed that adriamycin caused only mild myocardial damage in silymarin–treated rats. Moreover, the silymarin administration inhibits adriamycin–induced renal tubular damage in rats [33]. The immunosuppressive drug cyclosporine A (CsA) is metabolised by cytochrome P-450 IIIA, and caused acute reversible and chronic irreversible nephrotoxic effects [34]. The effect is based on vasoconstriction of afferent and efferent glomerular arterioles, resulting in a reduction in glomerular plasma flow and glomerular filtration rate. Silibinin is the main ingredient of silymarin, and inhibits lipid peroxidation. The possibility of silibin to posses a protective effect due to its radical scavenging properties has also been investigated.

CONCLUSIONS

The investigated literature suggested that use of silymarin might reduce the nephrotoxicity of a number of medications. Silymarin appears to be one of the most promising nephroprotectors. Despite intensive studies, there are still a number of uncertainties. This requires more detailed studies of its mechanisms and effective doses to extend its practical application to prevent and treat the side effects of the above –mentioned drugs and these silymarin properties should be studied in greater detail and put into practice for treatment of intoxications.

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Sambucus ebulus extracts exhibit modulatory activity on inflammation and phagocytosis as revealed by changed gene expression in a model of LPS-treated J774A.1 macrophages

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Dwarf elder, also known as elderberry (*Sambucus ebulus* L., SE) is a popular herb in Bulgarian folk medicine, known for its antiseptic, anti-inflammatory and diuretic action. Despite its wide application as anti-inflammatory and immune stimulatory remedy, there is no data on the possible protective effect of SE total extract (TE) or anthocyanin fraction (AF) under conditions of induced inflammation. The health benefits of consuming *S. ebulus* fruits that are rich in polyphenols, especially anthocyanins, have been the focus of an *in vitro* investigation. The aim of the present study was to evaluate the effect of SE fruit total extract and anthocyanin fraction on the expression of genes associated with the inflammatory response and phagocytosis in a model of LPS-stimulated J774A.1 macrophages. TE and the purified AF from *S. ebulus* fruits exhibited anti-inflammatory activity by reducing LPS-induced expression of TLR-4, TNFα, IL-6, IL-1β, IL-1RN, CRP, COX-2 and iNOS. Stimulation of phagocytic enzymes NOX and MPO expression was also reduced by pretreatment with TE and AF. The results of the *in vitro* studies suggest a potential anti-inflammatory effect of the obtained fractions, which appear to modulate the expression of proteins directly related to control inflammatory processes: TLR-4, TNFα, IL-6, IL-1β, IL-1RN, CRP, COX-2, iNOS, as well as enzymes associated with phagocytosis NOX and MPO.

Keywords: *Sambucus ebulus* fruits, LPS, herbal extracts, antioxidants, anthocyanins

INTRODUCTION

Traditions of folk medicine in many countries around the world have preserved the millennial knowledge of the beneficial effects of herbs on health. Recent studies of the healing action of plants traditionally used in countries such as China, India, the countries of South America, the Mediterranean, etc., are numerous and usually the rich experience of folk medicine has served as a starting point for discovering new healing remedies [1-3]. Studying the molecular effects of medicinal plants and their biologically active phenolic compounds (proanthocyanidins, anthocyanins, flavonols, and phenolic acids) [4] and identifying new molecular targets for their action is essential for the development of nutrition, biotechnology and pharmacy science. Development of new foods and medicines is important for addressing public health and food safety challenges.

The immune system is a complex system of molecules, cells, and tissues that interact in concert to control and eliminate infectious agents, malignant and transformed cells, and other unwanted antigens. The immunomodulatory effects of medicinal plant extracts are most commonly associated with their ability to influence processes such as inflammation [4-8], phagocytosis [9, 10], activation of the complement system [11-13], etc. Inflammation is an immunological defense mechanism by which the body responds to damage or infection by sending *M.* cells of the immune system, which eliminate dead and/or dying cells of their own body, at the appropriate places. Cellular signaling pathways are responsible for appropriate tuning of the inflammatory process. However, in some pathological situations, chronic inflammation can lead to diseases such as rheumatoid arthritis, high fever, atherosclerosis, glomerulonephritis, gastroenteritis, etc., sometimes even promoting cancer progression [1, 14-16]. Inflammatory mediators, such as pro-inflammatory cytokines, can significantly stimulate progression of inflammation [17]. That is why counteracting inflammation is usually on the level of inflammatory mediators production and secretion [18, 19].

The expected inhibitory effects of plant extracts on the expression of inflammatory factors are described in detail in the literature [8, 20, 21]. However, there are few reports of a stimulatory effect on the expression and such studies are related to studies designed to demonstrate immune-stimulatory activity [5, 7, 22-28].

S. ebulus fruits contain organic acids and flavonols, glycosides, anthocyanins, phytosterols, phenols, triterpenes, tannins, iridoid glycosides, cardiac glycosides, derivatives of caffeic acid,

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chlorogenic acid, ursolic acid, lectins and many other compounds with biological activity [29, 30]. The anthocyanins present in elderberries are important for the beneficial health effects associated with their antioxidant properties. In ethnopharmacology *S. ebulus* is used in gastrointestinal inflammatory disorders [1] and in autumn-winter period for immune-stimulation against acute respiratory infections [1]. These applications are a prerequisite for the expectations for perspective pharmacological effects of *S. ebulus* extracts as an anti-inflammatory and immune modulatory remedy.

The aim of the present study was to evaluate the effect of SE fruit total extract and anthocyanin fraction on the expression of genes associated with the inflammatory response and phagocytosis in a model of LPS-stimulated J774A.1 macrophages.

MATERIALS AND METHODS

Plant material

Ripe *Sambucus ebulus* fruits were collected during maturity period (September-October 2017) from Shkorpilovtsi (Varna region, Bulgaria). After appropriate transportation in light-protected containers and careful removal of peduncles without disturbing the integrity of the fruits the collected fruits were frozen at -20ºC.

Extraction procedure

Three different extracts were obtained: total acetone extract (TE) (70% acetone); hydrophilic fraction (HF) obtained from TE by chloroform fractionation, and anthocyanin fraction (AF) obtained from HF by solid phase extraction. To remove sugars and organic acids from the total extract and to obtain purified anthocyanin fraction, solid phase extraction was used. Immature (C18) solid phase extraction columns, Hypersep C18 / 500 mg (Thermo Scentific, USA), were selected to retain the hydrophobic organic compounds. At each step dry residues were obtained by vacuum evaporation below ≤40ºC with a rotary vacuum evaporator (Genevac SP Scientific, United Kingdom). The resulting dry material from each step was dissolved either in dH_2O for chemical analyses or in cell culture media DMEM for cell culture experiments.

The results of the three spectrophotometric methods revealed the content of total polyphenols in the TE (522.8 ± 4.83 mg/L), the content of total flavonoids in the extracts and the obtained fractions (630.2 ± 7.25 mg /L), and the content of total monomeric anthocyanin pigments (161.97 ± 0.80 mg/L), which decreased by 25% in the purification process. With the UPLC-UV-MS method developed, we found 0.84 mg/100 g fresh weight (FW) of epicatechin, 0.15 mg/100 g FW of quercetin and 0.05 mg/100 g FW of campherol in the total fruit extract of *S. ebulus*.

Cells culture

J774A.1 mouse macrophage cell line was obtained from the American Type Culture Collection (ATCC). Cells were cultured in 75 cm^3 flasks at 37°C in a humidified chamber containing 5% of CO_2 in DMEM (Sigma-Aldrich) with 4.5 g/L of glucose, L-glutamine and supplemented with fetal bovine serum (FBS, Sigma-Aldrich) to a final concentration of 10% and penicillin/streptomycin mixture to a final concentration of 100U/ml of each. Cells were sub-cultivated until 80% confluence was achieved.

Experimental procedure

The experimental procedure is based on a model of pretreatment with SE extracts, followed by LPS stimulation. Cells (J774A.1 macrophages) were seeded in 6-well flasks with a density of 2×10^5 cells/well. After overnight incubation, the cells were pretreated with a medium containing TE and AF. The pre-treatment media contained established final concentrations of 5, 15 and 135 μg/mL. Prior to treatment, the extracts were filtered through a 0.2 μm filter to eliminate bacterial contamination. After 24 h of incubation with extracts containing the medium the latter was removed and replaced with a medium with 100 ng/mL of LPS (*Escherichia coli*, 026: B6). Using this model, the following experimental groups were obtained: untreated control; TE pretreatment (5, 15 and 135 μg/mL) with LPS treatment; AF pretreatment (5, 15 and 135 μg/mL) with LPS treatment.

Determination of levels of gene expression

To determine the level of gene expression of selected genes in the cell cultures, two-step quantitative Real-Time PCR was used. Gene expression values were calculated by the $2^{-\Delta\Delta Ct}$ method and were presented in relative units as compared to the untreated control at which the expression level of the analyzed gene was considered to be 1. The results were presented as the mean (n=3) of the relative units ± standard error of mean (SEM). Expression of the following genes was studied: COX2 - F: TGAGCAACTATTCCAAACCAGC; R: GCACGTAGTCTTCGATCACTATC; MCP-1 – F: GGCTCAGCCAGATGCAGTTAA; R:CCTACTCATTGGGATCATCTTG; IL – 6 – F: CTGCAAGAGACTTCC; R:

GAAGTAGGGAAGGCC; TNFα - F: CCCTCACACTCAGAT CATCTTCT; R: GCTACGACGTGGGCTACAG; CRP - F: GTCTGCTACGGGGATTGTAGA R: GCACCTTGGGTTTCC CATCAA; IL - 1β – F: TTCAGGCAGGCACTA; R: CCACGGGAAAGACAC; IL – 1RN – F: GCTCATTGCTGGGTACTTACAA; R: CCAGACTTGGCACAAGACAGG; TLR4 - F: AGGCACATGCTCTAGCACTAA; R: AGGCTCCCCAGTTTAACTCTG; iNOS – F: GGCAGCCTGTGAGACCTTTG; R: GCATTGGAAGTGAAGCGTTTC; NOX – F: AGAGGAGAGCCCTTATCCCAACC; R: TGTCCAGAATTTCTTGAGCCTTG; MPO – F: GACATGCCCACCGAATGACAA; R: CAGGCAACCAGCGTACAAAG. β-actin (F: CAAGAAGGAAGGCTGGAAAAG; R: ACGGCCAGGTGATCACTATTG) served as an endogenous control.

Statistical processing and graphical presentation of the results

The values obtained were represented as the average of a minimum of three measurements ± SEM. The data were statistically treated with one-way ANOVA, at a confidence level $p<0.05$ and compared with Student's *t*-test. Data processing was performed using the statistical software product Graph Pad Prism (Ver. 5.0 Graph Pad Software, Inc.).

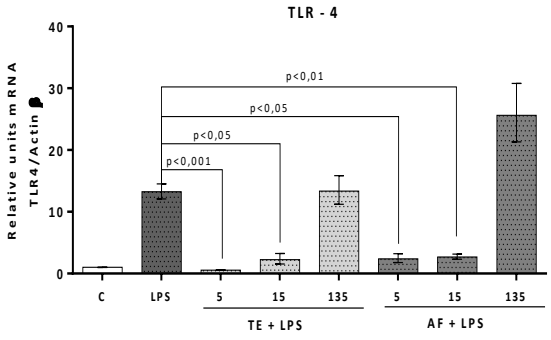
RESULTS AND DISCUSSION

Expression of genes associated with inflammatory response

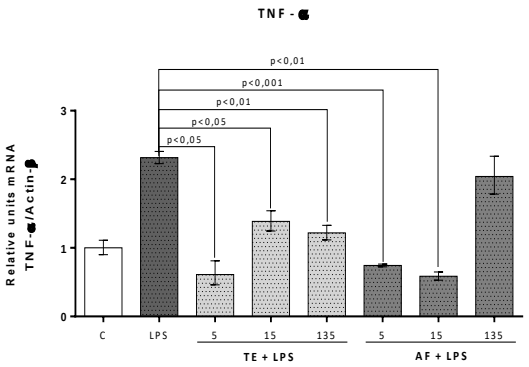
Inflammation is characterized with increased plasma levels of some inflammatory factors, such as TNFα, IL-6, MCP-1 and others. Macrophages play an important role in innate and adaptive immune responses by the release of various factors, such as proinflammatory cytokines, reactive oxygen forms, nitrogen species.

LPS treatment induces gene expression by increasing both the cytosolic protein levels of cytokines (IL-1β and IL-6) and pro-inflammatory enzymes (iNOS) by activating the NF-*κ*B transcription factor [31]. TLR4 activates the translocation of NF-*κ*B and AP-1 to the nucleus, followed by binding to inflammatory regulators of cytokines and molecules, including TNFα, IL-6, NO and COX-2, responsible for targeted eicosanoids synthesis during inflammation. LPS can activate several extracellular signaling pathways, including NF-*κ*B and MAPKs [32, 33]. NF-*κ*B is a key transcriptional regulator of the inflammatory response and plays an important role in the development of inflammatory process and cellular damage. It is activated in response to various extracellular stimuli, including oxidative stress, LPS and cytokines [33-35]. TLR4 pathway in macrophages is a key target in studies of the potential properties of multiple phytochemicals exhibiting anti-inflammatory properties [36].

In support of this was the result obtained by us, under LPS treatment, in which the expression of all of the studied inflammation-related genes (TLR4, TNFα, IL-6, IL-1β, MCP-1, iNOS, COX-2 and CRP) was expectedly higher in comparison to the untreated control group of cells (Fig. 1) [37]. In order to investigate possible protective activity of *S. ebulus* fruit preparations in LPS-induced inflammatory response models we performed a 24 h pretreatment of the cells with 5, 15 and 135 μg/mL extract/fraction in culture media (Fig. 1).



A



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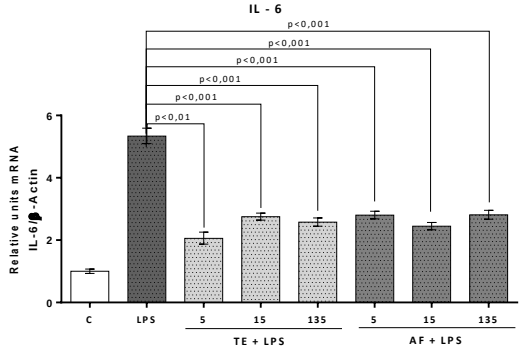

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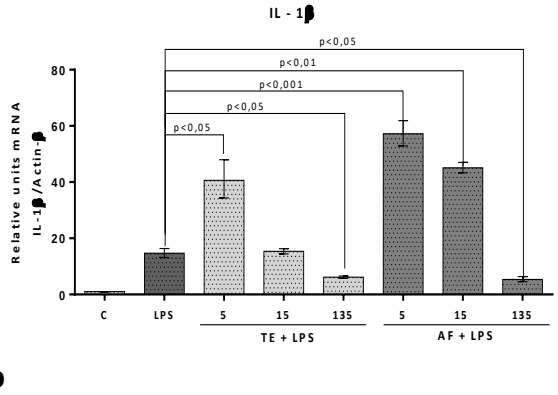

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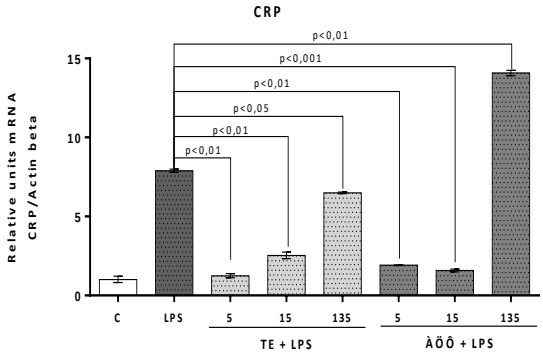

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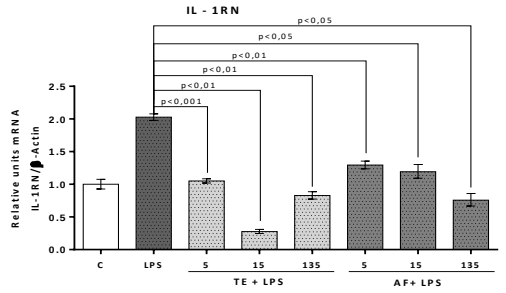

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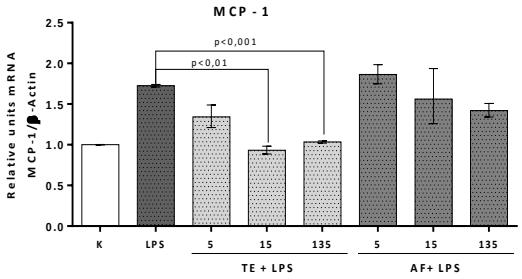

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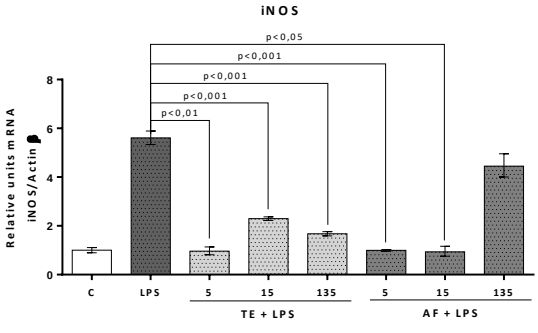

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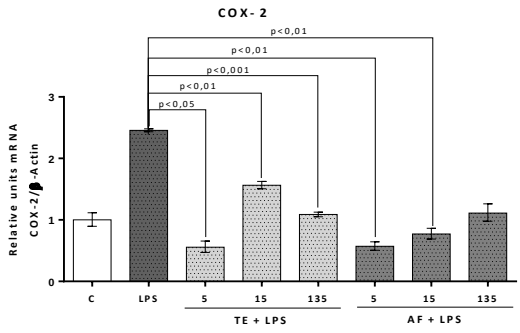

H

Figure 1. Inflammation-related genes expression levels in J774A.1 macrophages treated with 5, 15 and 135 μg/mL *S.ebulus* total extract (TE) and anthocyanin fraction (AF) in presence of 100 ng/mL LPS. (A) TLR-4, (B) TNF-α, (C) IL-6, (D) IL-1β, (E) IL-1RN, (F) MCP-1, (G) iNOS, (H) COX-2, (J) CRP.

Pretreatment of macrophages with TE and AF significantly reduced the upregulating effect of LPS on the expression of IL-6 and IL1-RN in all applied concentrations of TE and AF. Similar was the effect on TLR-4, iNOS, TNFα, COX-2 and CRP, with the exception of the highest AF concentration applied (135 μg/mL).

For the experiment we have selected 5, 15 and 135 μg/mL concentrations of polyphenols in the TE and AF extracts. These concentrations were selected on the basis of previous non-published data about TE and AF cytotoxicity using a MTT test. Results of the test represented slight proliferative and strong proliferative activity for 5 μg/mL and 15 μg/mL, respectively and cytotoxic effect of 135 μg/mL for both TE and AF. The expected inhibitory effect of 135 μg/mL on the gene expression of these gens in LPS stimulated macrophages might be due to its cytotoxic activity, which interferes and predominates over AF anti-inflammatory potential. AF had no significant lowering effect on LPS-induced MCP-1 expression.

The overall assumption is that herbal extracts and natural products can stimulate the immune system in conditions of tumor and infectious diseases [5, 20, 38] and to positively suppress it in conditions of autoimmune diseases [39, 40]. There are a lot of data demonstrating a possible immune stimulatory activity of plant preparations and they refer predominantly to unstimulated cells, e.g., for various preparations containing *Echinacea purpurea* on RAW264.7 macrophage cell culture [19]. *Uncaria tomentosa* extract has the same effect in experiments with primary rat macrophages [20], increasing IL-6 expression under the influence of the extract, both in non-stimulated and LPS-treated cells. Aqueous

extract of *Platycodon grandiflorum* stimulates the expression of IL-6 in murine peritoneal macrophages [21], and aqueous extract of *Prunella vulgaris* - the expression of IL-6 in RAW264.7 macrophages [5]. Our studies have also found induction expression of this cytokine as a result of the treatment of macrophages (J774A.1) with a plant extract from *Agrimonia eupatoria* [7]. Concerning the *in vivo* effects, the data are contradictory - from inhibitory [22, 23] to activatory [24, 25].

The potential of phytochemicals and/or plant extracts to decrease the effect of various stimuli on inflammation has been established by a number of studies. For example, some anthocyanins show an inhibitory effect on COX-2 by inhibiting C/EBP, AP-1, and NF-κB [29], as well as by suppressing iNOS enzyme and mRNA expression in LPS-stimulated RAW 264 cells [20, 30]. The anthocyanin-rich red raspberry fraction exhibits anti-inflammatory properties by suppression of the expression of iNOS and COX-2, as well as the activity of IKK in LPS/IFN-γ stimulated RAW264.7 macrophages [8]. Anthocyanins in raspberry extracts suppress LPS-induced NF-κB activation and COX-2 production. Subsequently, suppression of COX-2 gene expression may reduce the production of pro-inflammatory cytokines IL-1β, IL-6, IL-8 and TNFα [31, 32].

Namely, suppression of above mentioned pathways may be a possible mechanism for the anti-inflammatory effects of *S. ebulus* extracts. Aqueous and ethanol extracts from *S. ebulus* have been previously established to inhibit NF-κB transcription in cells [36].

Expression of phagocytosis-associated genes

In pro-inflammatory activation, phagocytic cells, such as macrophages and monocytes, produce a large amount of ROS, mainly in the form of superoxide anion and subsequent formation of radicals that accompanies the "respiratory burst" [39]. The process of high oxygen consumption and production of the superoxide anion that accompanies the respiratory burst is controlled by the phagocytic NADPH oxidase (NOX enzyme) [40]. Activated phagocytic oxidase releases a superoxide anion within the phagosome, which then undergoes disruption in H_2O_2 by superoxide dismutase (SOD). Myeloperoxidase catalyzes the next reaction of formation of hypochlorous acid. In our study we analyzed the expression levels of two phagocytosis related genes: NOX and MPO.

In LPS-stimulated macrophages, the NOX and MPO expression levels were significantly increased over the untreated control ($p < 0.001$) (Fig. 2). In this study, we show that TE and AF of *S. ebulus* fruit inhibit the MPO and NOX expression in LPS-stimulated macrophages.

TE and AF from *S. ebulus* fruits inhibited in a concentration-dependent manner the expression of both MPO and NOX, which explains their anti-inflammatory effect in LPS-stimulated macrophages. MPO stimulates macrophages to produce active oxygen forms (AOF), which in turn enhances the expression of TNFα and other proinflammatory cytokines [41, 42]. Our findings are in line with reports by other authors [43]. The result obtained in our study is confirmed by other authors in studies done on cells treated with strawberry extracts before incubation with LPS [44]. These results suggest that TE and AF of *S. ebulus* fruits can reduce LPS-induced inflammation indirectly by inhibiting MPO-related release and inflammation. Pre-treatment of J774A.1 macrophages with TE and AF from fruits of *S. ebulus* decreases intracellular AOF levels, attenuating apoptosis in LPS-treated cells. This makes it possible to claim that berry extracts counteract LPS-mediated inflammation by modulating signaling pathways and reducing AOF.

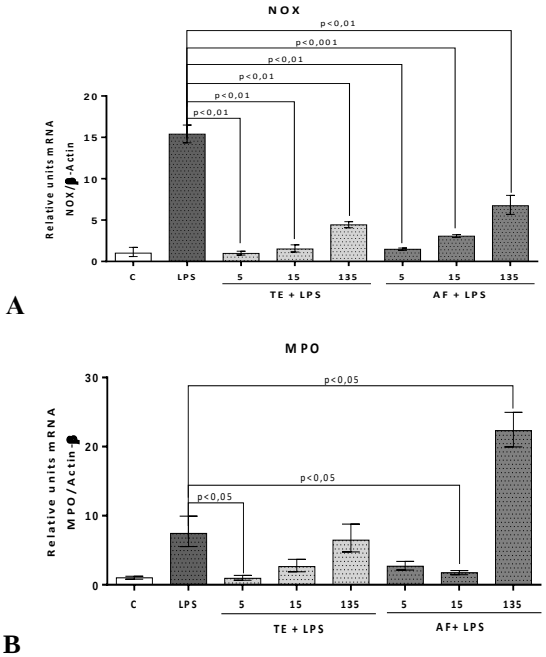

Figure 2. Phagocytosis-related genes expression levels in J774A.1 macrophages treated with 5, 15 and 135 μg/mL *S. ebulus* total extract (TE) and anthocyanin fraction (AF) in presence of 100 ng/mL LPS (A) NOX, (B) MPO.

Fruits of *S. ebulus are* rich in various polyphenol compounds, and it is difficult to identify a single component responsible for the observed effect. In future studies, it is necessary to perform additional

studies of the structure-activity relationship or to perform biocontrolled isolation of phenolic fractions.

CONCLUSIONS

The investigated TE and ACF have an effect on processes related to immune defense - inflammation and phagocytosis. Under conditions of induced inflammatory response, the test extracts administered as pretreatment mitigate the stimulatory action of LPS by lowering the expression levels of all the genes involved in inflammation (TLR-4, TNFα, IL-6, IL-1β, IL-1RN, CRP, COX-2; iNOS) and phagocytosis (NOX, MPO). The TE and the AF exhibit a pronounced modulatory effect on the expression of inflammation- and phagocytosis-related proteins. This finding supports the indications of the folk medicine about the use of *S. ebulus* fruits for improvement of immune response against infections.

Additional *in vivo* and analytical studies are needed to further disclose the mechanisms of action of the fruit extracts of *S. ebulus* in order to obtain more data on its feasibility as a feedstock in functional foods and curative remedies development and application as supportive and complementary therapy.

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Antioxidant activity and chemical composition of crude extracts from different tobaccos and tobacco blends

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The aim of this study was to investigate the antioxidant activity by FRAP-method and the chemical composition of crude tobacco extracts obtained with different solvents - 60 % methanol, ethanol and water. Bulgarian varieties of Oriental tobaccos, Virginia tobacco, American and Virginia blends from cigarettes were used. The content of polyphenols, nicotine, carbohydrates and antioxidant activity of the extracts was obtained. The extracts, obtained with 60% methanol were characterized with high content of polyphenols and reduced nicotine and carbohydrates content. They had high antioxidant activity. The lowest levels of antioxidant activity of polyphenols, nicotine and carbohydrates in ethanol extracts were reported. Extraction with water produced insoluble complexes. The yields of polyphenols, nicotine and carbohydrates, as well as the antioxidant activity varied widely. The extracts obtained from Virginia tobacco had a lower content of polyphenols compared to the Oriental tobaccos extracts, but the antioxidant activity was higher. The results showed that the antioxidant activity, except for the content of polyphenols, is probably related to the synergistic or antagonistic effect of the other compounds present in the samples.

Key words: Tobaccos, tobacco blends, tobacco extracts, FRAP-method, chemical composition.

INTRODUCTION

Tobacco (*Nicotina tobaccum* L.) is grown in different parts of the world. The main use of tobacco is for the production of tobacco products for smoking and less for chewing, snuffing, etc. On the other hand, tobacco is a plant containing a huge number of substances. It can be considered as a raw material that is more widely used in addition to its traditional uses [1, 2]. Tobacco as a medicinal plant is knowns since 15th century. After the isolation of nicotine from tobacco leaves in 1828, the medical world became yet more mistrustful of tobacco as a general treatment, because the plant contained the alkaloid nicotine [3].

Currently, the interest in the investigation of phytochemicals in tobacco has increased. The metabolites isolated from leaves, flowers and other parts of the plant include alkaloids, terpenoids, polyphenols, isoprenoids and many other classes of chemicals [1, 4]. More than 15 polyphenols have been identified in tobacco. The major polyphenols in tobacco are chlorogenic acid, neochlorogenic acid, 4-*O*-caffeoylquinic acid, rutin and kaempferol-3-rutinoside [5-7].

Polyphenols are secondary metabolites isolated from plants. They possess a wide variety of activities such as antimicrobial, antioxidant, anti-cancer, anti-inflammatory and wound healing [8]. Polyphenols are recognized as antioxidant and scavenging agents against free radicals related to oxidative damage [9, 10]. The antioxidant compounds are capable of neutralizing free radicals and may play a major role in the prevention of certain diseases such as cancer, cataracts, cerebral pathologies and rheumatoid arthritis properties [11].

The aim of this study was to investigate the antioxidant activity by FRAP-assay and the chemical composition of crude tobacco extracts obtained with different solvents – methanol, ethanol, water and their mixtures.

MATERIALS AND METHODS

Plant material

Bulgarian oriental tobacco varieties Djebel basma 1, Basma 79, Srednogorska yaka, Myumunovo seme and Virginia tobacco Linia 543 were used for analysis. The plants were grown on the experimental fields of the Tobacco and Tobacco Products Institute, Plovdiv, under identical agro-ecological and meteorological conditions. Packs of the Virginia blend and American blend cigarettes were purchased from the local shops in Plovdiv city.

Preparation of extracts

Dry tobacco powder (0.5 g) was extracted with 10 ml of 60% (v/v) MeOH, 100 % ethanol and water for 30 min on a mechanical shaker. The extract was filtered. An aliquot of the obtained extracts was subjected to analysis for content of nicotine, sugars, polyphenols and antioxidant activity by FRAP-assay.

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Ferric reducing antioxidant power (FRAP-assay)

The method measures the ferric reducing ability in which a ferric-tripyridyltriazine (Fe^{3+}-TPTZ) complex was reduced to ferrous (Fe^{2+}-TPTZ) form. The FRAP assay was conducted according to the method reported by Benzie and Strain [12]. The FRAP reagent was freshly prepared before analysis by mixing 0.3 M acetate buffer (pH 3.6), 0.01 M 2,4,6-tripyridyl-triazine (TPTZ) in 10 ml 0.04 M HCl and 0.02 M $FeCl_3.6H_2O$ in distilled water in a ratio of 10:1:1. For the assay 0.05 ml extracts and 0.15 ml distilled water were mixed with 1.5 ml FRAP reagent. Absorbance against a blank sample was measured at 593 nm with a UV–Visible spectrophotometer Pharo 300, Merck after 15 min in dark at room temperature. $FeSO_4.7H_2O$ at a concentration between 0.1 M and 1 M was used for calibration. The calibration curve was generated from detection of samples containing known amounts of the standard $FeSO_4.7H_2O$; $R^2 = 0.9969$. The results were expressed as mM Fe^{2+}/g DM.

Determination of polyphenols in tobaccos and tobacco extracts

One hundred milligrams (0.1 g) of tobacco powder was sonicated for 30 min with 5 ml of 60% (v/v) CH_3OH. The extract was filtered under vacuum. The polyphenols were purified by passing the solution through a C18 cartridge according to the validated method described by Dagnon and Edreva [5] and subjected to HPLC analysis.

Determination of nicotine in tobaccos and tobacco extracts

Content of nicotine in tobaccos and tobacco extracts was determined by continuous-flow analysis method according to ISO 15152:2003 [13].

Determination of carbohydrates in tobaccos and tobacco extracts

Content of carbohydrates in tobaccos and tobacco extracts was determined by continuous-flow analysis method according to ISO 15154:2003 [14].

Statistics

All experimental procedures were done in triplicate. The quantitative data were expressed as mean ± standard deviation.

RESULTS AND DISCUSSION

Tobacco samples

Tobacco is a rich source of phytochemicals – alkaloids, polyphenols, terpenes and other [4, 15].

The main components of tobacco – alkaloids as nicotine and carbohydrates, which are related to the quality of tobacco and the polyphenols that are associated with the antioxidant activity of tobacco are presented in Table 1. The tobaccos were selected from low to high content of polyphenols, nicotine and sugars. Oriental tobaccos had higher polyphenols (average 34.23 mg/g) compared to the Virginia tobacco (19.83±1.56 mg/g). The highest content of polyphenols was reported in Myumunovo seme 50.79±3.99 mg/g, and Basma 79 - 35.78±2.81 mg/g. Polyphenols in Djebel basma 1 (27.62±2.17 mg/g) and Srednogorska yaka (22.75±1.79 mg/g) were approximately equal. The content of polyphenols in these varieties is lower than that in Myumunovo seme. Tobacco blends had a lower content of polyphenols (average 14.5 mg/g), which was related to the different qualitative and quantitative composition of the blends tobacco and the presence of additives [6].

At the moment, there is a lot of published data on the content of polyphenols of tobaccos [6, 7, 16]. The total content of polyphenols in Oriental tobacco ranged from 10 mg/g and 30 mg/g, while in Virginia tobacco it may exceed 30 mg/g [5]. Comparing our investigations with previous ones, the data for polyphenols in Oriental tobacco Djebel Basma 1 (24 mg/g) and Virginia tobacco Virginia 454 (15 mg/g) appear to be close [6, 16].

Table 1. Polyphenols, nicotine and sugars in tobaccos and tobacco blends, mg/g

Sample	Varieties	Polyphenols	Nicotine	Carbohydrates
Oriental tobaccos	Djebel basma 1	27.62±2.17	2.0±0.6	193±8
	Basma 79	35.78±2.81	4.3±0.1	161±6
	Srednogorska yaka	22.75±1.79	4.1±0.1	188±8
	Myumunovo seme	50.79±3.99	6.8±0.2	133±5
Virginia tobacco	Linia 543	19.83±1.56	17.2±0.5	128±5
Tobacco blends	Virginia blend	15.57±1.22	16.0±0.05	121±5
	American blend	13.52±1.06	17.5±0.05	108±4

Tobacco, as a source of a polyphenols, can be used for preparation of extracts containing polyphenols [7, 17-20]. Extraction of tobacco and tobacco blends with solvents of different polarity presumes the extraction of different groups of polar substances, which would affect the chemical composition of the extracts and their antioxidant activity [9].

Extracts obtained with 60 % methanol

The chemical composition of crude tobacco extracts is presented in Table 2. Full recovery of polyphenols was achieved with 60% methanol [17]. At the same time, the content of nicotine and sugars in tobacco extracts was lower than that in tobaccos. The yield of nicotine varied between 49% and 59%, except for the extract from Djebel basma 1 – 70%. The amount of sugars in tobaccos was proportional to the content of sugars in tobacco extracts. An average yield of 57% was reported.

Table 2. Chemical composition of crude tobacco extracts, obtained with 60 % methanol

Sample	Varieties	Extract, mg/g	Nicotine, mg/g	Carbohydrates, mg/g
Tobaccos	Djebel basma 1	370±10	0.9±0.03	123±5
	Basma 79	360 ±10	2.2±0.07	99±4
	Srednogorska yaka	370±10	2.6±0.08	108±4
	Myumunovo seme	380 ±10	3.6±0.11	65±3
	Linia 543	370 ±10	10.8±0.32	70±3
Tobacco blends	Virginia blend	340 ±10	9.0±0.27	81±3
	American blend	360±10	8.8±0.26	56±2

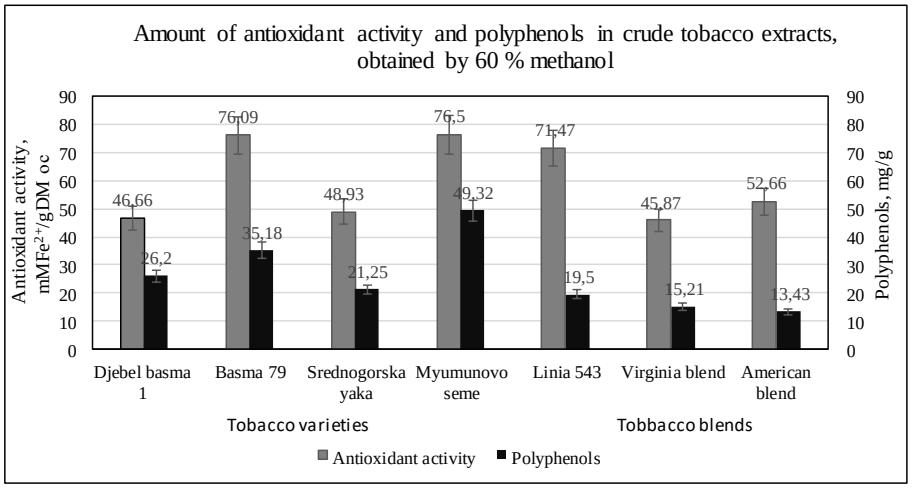

Fig. 1. Antioxidant activity and polyphenols in crude tobacco extracts obtained with 60 % methanol

Antioxidant activity of tobacco extracts can be separated in two groups – extracts with higher antioxidant activity - Myumunovo seme (76.50±7.12 mMFe^{2+}/g DM), Basma 79 (76.09±7.32 mMFe^{2+}/g DM) and Linia 543 (71.47±7.16mMFe^{2+}/g DM) and extracts with lower antioxidant activity - Djebel basma 1 (46.66±4.23 mMFe^{2+}/g DM) and Srednogorska yaka (48.93±4.81 mMFe^{2+}/g DM). The antioxidant activity of tobacco blends ranged from 45.87±4.62 mMFe^{2+}/g DM (Virginia Blend) to 52.66±5.29 mMFe^{2+}/g DM (American Blend) and was close to the antioxidant activity of extracts obtained from Djebel Basma 1 and Srednogorska yaka.

A correlation (R^2=0.7434) between antioxidant activity and content of polyphenols was obtained only in Oriental tobaccos. The extract obtained from Virginia tobacco Linia 543 had half the content of polyphenols compared to Myumunovo seme, but the antioxidant activity was close to that of Myumunovo seme – Fig. 1.

It was reported that the FRAP value strongly correlated with the flavonoids and polyphenols ($R^2 = 0.951$, $R^2 = 0.953$). This is highlighted by the

observation that the total flavonoid contents showed a strong correlation with FRAP values, whereas rutin and chlorogenic acid concentrations showed a weaker correlation with the FRAP values [21].

The differences in the antioxidant activity of the extracts were related to the different qualitative and quantitative composition of the tobacco varieties and accordingly, the different composition of the extracts. In addition, the large amounts of extracts (340±10 mg/g - 380±10 mg/g) indicated the presence of many other substances besides the investigated ones, that affected antioxidant activity.

Tawaha *et al.* (2007) determined the antioxidant activity and polyphenols content of Jordan's plant species, and estimated that a polyphenol content higher than 20 mg/g extract can be considered to be high. On the basis of this result, tobacco extracts, obtained with 60 % methanol must be considered to be promising sources of polyphenols [22].

Extracts obtained with water

Chemical composition of aqueous extracts is presented on Table 3. The amounts of tobacco extracts obtained with water were relatively high - 350±10 mg/g - 360±10 mg/g, and were close to the extracts obtained with 60% methanol (Table 2). This indicated the presence of many substances in the extracts. In aqueous extracts a precipitate was observed. There were differences in the type and amount of precipitate in the different extracts. The content of nicotine (0.1±0.003 mg/g - 4.2±0.13 mg/g) and carbohydrates (0.14±0.01 mg/g - 11.1±0.44 mg/g) varied significantly (Table 3).

The antioxidant activity of the extracts varied widely – Fig. 2. The highest antioxidant activity is manifested by the extract obtained from Basma 79 - 60.38±6.08 mMFe^{2+}/g DM. The lowest activity was recorded in Djebel Basma 1 extract - 3.36±0.33 mMFe^{2+}/g DM, where a large amount of precipitate was reported.

Tawaha *et al*. (2007) established that there was no significant difference between aqueous and methanolic extracts for antioxidant activity or total phenolic content testing 51 species, while there was a difference in tobacco extracts [22].

Table 3. Chemical composition of crude tobacco extracts, obtained with water

Sample	Varieties	Extract, mg/g	Nicotine, mg/g	Carbohydrates, mg/g
Tobaccos	Djebel basma 1	350±10	0.1±0.003	11.1±0.44
	Basma 79	360±10	1.4±0.04	5.2±0.21
	Srednogorska yaka	350±10	2.3±0.07	10.0±0.40
	Myumunovo seme	360±10	2.4±0.07	0.14±0.01
	Linia 543	350±10	4.2±0.13	0.63±0.02
Tobacco blends	Virginia blend	360±10	3.8±0.11	2.8±0.11
	American blend	360±10	3.5±0.10	3.9±0.16

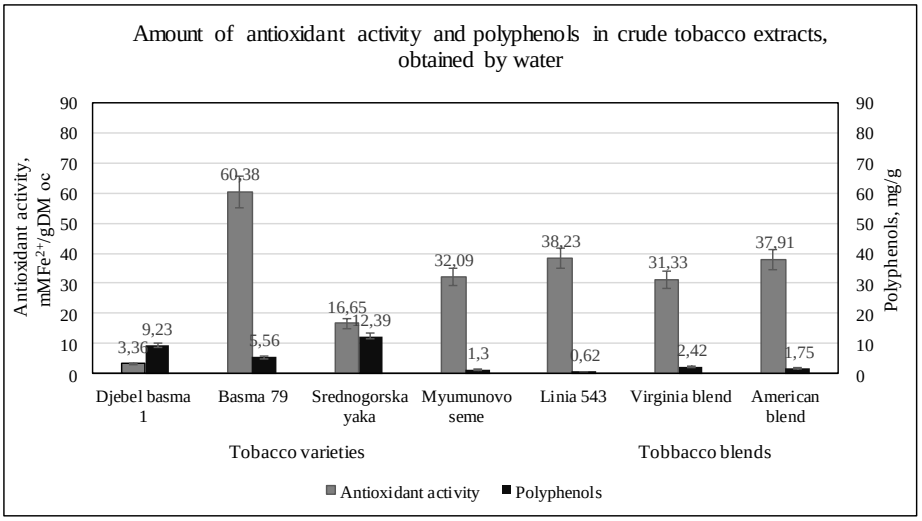

Fig. 2. Antioxidant activity and polyphenols in crude tobacco extracts obtained with water

Extracts obtained with ethanol

By extraction with the least polar solvent 100% ethanol, the most purified extracts were obtained. The average amount of ethanol extracts was 110 mg/g and was about three times lower than with 60 % methanolic and aqueous extracts – Table 2 and Table 3. The content of nicotine in oriental tobacco extracts was lower than 0.35 mg/g, while in the extracts obtained from Virginia tobacco and tobacco blends it was about 1.30 mg/g. A relatively low carbohydrates content of the extracts was reported - an average of 2.23 mg/g – Table 4. The amount of nicotine and carbohydrates in the extracts was proportional to that of tobaccos– Table 1. The polyphenols contents in the tobacco extracts were between 5.09±0.40 mg/g (Myumunovo seme) and 1.35±0.11 mg/g (Linia 543), while in tobacco blends extracts they were equal – 0.96 mg/g, Fig. 3. Polyphenols in ethanolic extracts were 7 and 15 times less than those in tobaccos and in 60 % methanolic extracts and were proportional to those in tobaccos (Table 1).

Tobacco extract obtained from Myumunovo seme, characterized with the high content of polyphenols – 5.09±0.40 mg/g, had the highest antioxidant activity 17.14±1.72 mMFe^{2+}/g DM. The other extracts had approximately the same antioxidant activity – average 9.79 mMFe^{2+}/g DM (Fig. 3). It is important to note that the Virginia tobacco extract Line 543 had twice as low polyphenols content in comparison with the Djebel Basma 1 extract, but has higher antioxidant activity – Fig. 3. The lowest antioxidant activity in Virginia blend and American blend extracts (4.82±0.41 mMFe^{2+}/g DM and 4.25±0.46 mMFe^{2+}/g DM) and the lowest polyphenols content was reported – Fig. 3.

Table 4. Chemical composition of crude tobacco extracts, obtained with ethanol

Sample	Varieties	Extract, mg/g	Nicotine, mg/g	Carbohydrates, mg/g
Tobaccos	Djebel basma 1	100±3	-	3.9±0.16
	Basma 79	100±3	-	2.10±0.08
	Srednogorska yaka	120±3	0.35±0.01	3.30±0.13
	Myumunovo seme	120±3	0.29±0.01	2.26±0.09
	Linia 543	110±3	1.25±0.04	1.55±0.06
Tobacco blends	Virginia blend	100±3	1.31±0.04	1.50±0.06
	American blend	90±3	1.32±0.04	1.01±0.04

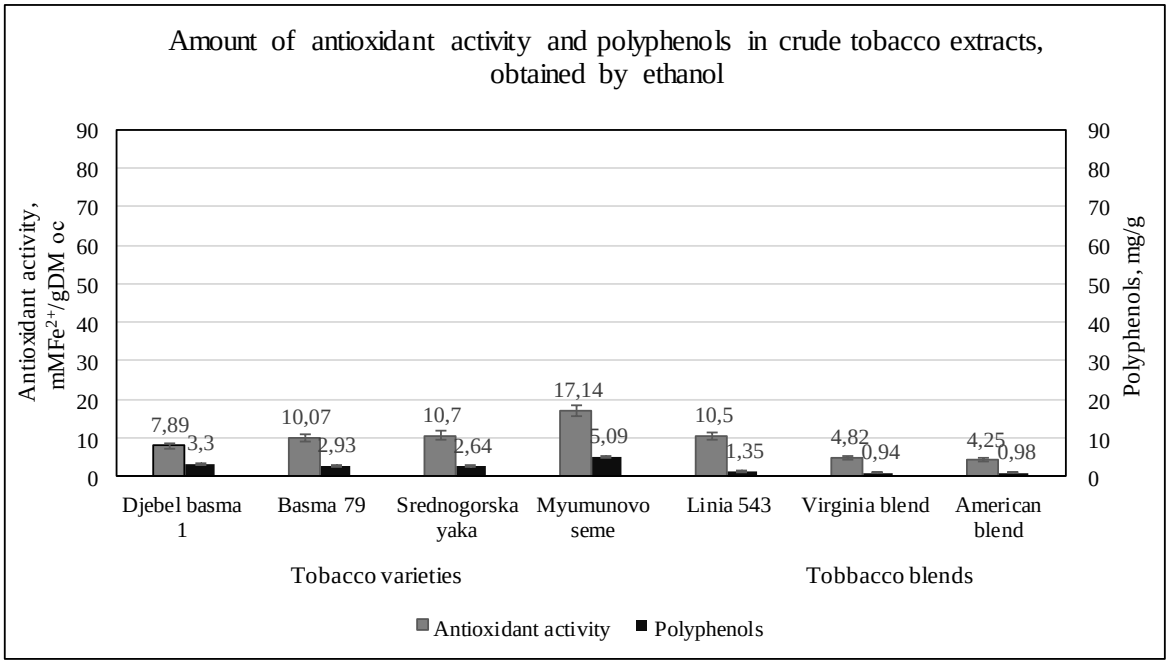

Fig. 3. Antioxidant activity and polyphenols in crude tobacco extracts obtained with ethanol

The average antioxidant activity of ethanol extracts was 6.4 times lower than that of 60 % methanolic extracts – Fig. 1. Thus, ethanol was the most inappropriate solvent for producing tobacco extracts with high antioxidant activity, but the extracts were of highest purity. The reducing ability of the extract obtained from Indian medicinal herb known as wedelia (*Sphagneticola trilobata sp.*) was in the range of 0.172 to 0.630 Mm Fe^{2+}/g and was lower than that of tobacco extracts [23].

CONCLUSION

Extracts from Bulgarian oriental tobacco varieties Djebel basma 1, Basma 79, Srednogorska yaka, Myumunovo seme, Virginia tobacco Linia 543, Virginia blend cigarettes and American blend cigarettes with different solvents were obtained. The extracts obtained by extraction with 60% methanol had the highest antioxidant activity, followed by aqueous extracts, an exception being the extract obtained from Djebel Basma 1. Ethanol extracts exhibited the lowest activity by FRAP-assay, but they were of the highest purity. The content of polyphenols, nicotine and carbohydrates in the extracts was lower than that in tobaccos. The extracts obtained from Virginia tobacco Linia 543 had lower content of polyphenols in comparison with the extracts obtained from Oriental tobaccos, but the antioxidant activity was close to or higher than that of extracts from Oriental tobaccos. The extracts obtained from Oriental tobaccos Basma 79, Myumunovo seme and Virginia tobacco Line 543 obtained with all tested solvents exhibited relatively high antioxidant activity. The differences in the antioxidant activity of the extracts were associated with the different qualitative and quantitative composition of the tobacco varieties and the different composition of the extracts, respectively. Tobacco blends extracts had lower antioxidant activity than tobaccos. The results showed that the antioxidant activity, except for the content of polyphenols, is probably related to the synergistic or antagonistic effect of the other compounds present in the samples.

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Determination of total phenol content and antioxidant activity of five medicinal plants growing in Bulgaria

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Interest in natural compounds with antioxidant activity as alternatives to commercial antioxidants has increased in recent years. Herbal extracts are well recognized sources of antioxidants. The phenols contained therein, including flavonoids, have been increasingly identified by many researchers as important dietary antioxidant factors. Studies have shown that there are differences in the content of bioactive substances in plants collected from different geographical regions. About 750 species in the Bulgarian flora are medicinal and have not yet been sufficiently studied. Because of this, in the present paper the total polyphenol content and antioxidant activity of some populations of *Equisetum arvense* L., *Equisetum telmateia* Ehrh., *Juniperus communis* L., *Lavandula angustifolia* Mill. and *Rosmarinus officinalis* L. were evaluated. In *Juniperus communis* the highest total phenol content of 946 mg GAE.kg^{-1} DM and antioxidant activity of 58.5 mmol TE.kg^{-1} DM were measured.

Keywords: total phenol content, antioxidant activity, *Equisetum, Juniperus communis, Lavandula angustifolia, Rosmarinus officinalis*

INTRODUCTION

Interest in natural compounds with antioxidant activity as alternatives to commercial antioxidants has increased in recent years. Herbal extracts are well recognized sources of antioxidants. The phenols contained therein have been increasingly identified by many researchers as important antioxidant factors. About 750 species in the Bulgarian flora are medicinal and have not yet been sufficiently studied. The subject of this study are five species: *Equisetum arvense* L., *Equisetum telmateia* Ehrh., *Juniperus communis* L., *Lavandula angustifolia* Mill. and *Rosmarinus officinalis* L. The most widely known phytochemical compounds of *E. arvense* are flavonoids, phenolic acids, alkaloids, phytosterols, tannins, and triterpenoids [1] and its extract exhibits significant antioxidant, anticancer, antimicrobial and many other effects [2, 3]. Studies have shown that there are differences in the content of bioactive substances in *E. arvense* plants collected in different geographical areas [4]. According to Radojevic *et al.* [5] *E. telmateia* has anti-inflammatory and antioxidant activity. The ethyl acetate fraction of needles from *J. communis* possesses high antioxidant and hepatoprotective properties [6] and the essential oil from them has strong disinfectant properties [7, 8]. The essential oil of *L. angustifolia* contains over 300 chemical compounds [9] and it has antibacterial [10], antimicrobial [11], antifungal [12] and antioxidant [13, 14] properties. The essential oil also has an antispasmodic effect [15] and analgesic activity [16]. According to the review by Andrade *et al.* [17], *R. officinalis* has a great pharmacological potential. Its essential oil has various pharmacological activities such as antibacterial [18], antidiabetic [19], anti- inflammatory [20, 21], antitumor [22-24] and antioxidant [25].

The plant species selected in the present study have long been known for their health potential and have been used in folk medicine and in the pharmaceutical and food industries. It is not yet clear, however, which fractions their useful properties are due to. This study aims to determine the amounts of total phenols and the corresponding antioxidant activity.

MATERIALS AND METHODS

Plant material and extract preparation

Plant parts of the studied species were collected from June to September in the 2018 growing season. The location of the plant populations is indicated in Table 1. To determine the total phenol content and antioxidant activity sterile non-reproductive stems of *E. arvense* and *E. telmateia*; leaves, unripe and ripe berries of *J. communis*; leaves of *R. officinalis* and flowers of *L. angustifolia* were used. Voucher specimens from the studied populations are kept in the herbarium of the Agricultural University in Plovdiv (SOA). They were dried in shade at 20 - 24 °C, ground in a mechanical grinder (final powder size less than 400 μm) and stored at 18 - 20 °C. The extractions were performed by maceration of 1 g of powdered plant material in 10 ml of methanol at room temperature for 7 days.

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Table 1. Location of the plant populations

Plant population	Location	North	East	Elev., m a. s. l.
E. arvense	Eastern Balkan Range, Sinite Kamani Natural Park, Karandila area – near the Karandila bakery	42°42.852’	26°22.654’	915
E. telmateia	Eastern Balkan Range, Sinite Kamani Natural Park, Karandila area – near the Karandila bakery	42°42.582’	26°22.284’	915
J. communis	Eastern Balkan Range, Sinite Kamani Natural Park, Upper lift station	42°43.100’	26°21.619’	1015
L. angustifolia	Thracian Plain, Chirpan, Tselina village	42°07.497’	25°26.126’	153
R. officinalis	Thracian Plain, Stara Zagora, Trakia University	42°24.027’	25°34.192’	275

After filtration, the residue was washed up in triplicate. Finally, the extracts were adjusted to a concentration of 1 mg.ml^{-1} calculated on dry matter (DM).

Determination of total phenol content

The experimental procedure described by Anesini *et al*. [26] was applied for determination of total phenol content. Briefly, 1 ml of the methanolic extract was mixed in separate tubes with 5.0 ml of Folin-Ciocalteu’s reagent (1:10 dilution with water of the commercial reagent). Then, 4 ml of 7.5 % Na_2CO_3 aq (w/v) were added and the tubes were left at room temperature for one hour. The absorbance at 765 nm was measured against water. Each sample was analyzed in triplicate. Gallic acid (Sigma-Aldrich, St. Louis, MO) solutions in methanol ranging from 2 to 60 μg.ml^{-1} were used for calibration curve (R^2 = 0.9987). TPC of each sample was expressed as mmol GAE in 1 kg DM of plant extract.

Determination of antioxidant activity by DPPH method

The method described by Serpen *et al.* [27] was applied to measure the radical-scavenging potential of methanolic extracts obtained from the selected plants. To 2 ml of 100 μM solution of DPPH in methanol 20 μl of methanolic extract was added. Absorption at 517 nm was measured 30 min later. Since the composition of the extracts is complex, the results for their radical-binding capacity were compared with that of Trolox (water-soluble analogue of vitamin E) and calculated by regression analysis from the linear dependence between concentration of Trolox and absorption at 517 nm. Trolox standard was purchased from Sigma-Aldrich (St. Louis, MO). Standard solutions in methanol ranging from 1 to 50 μmol.l^{-1} were used for calibration curve (R^2 = 0.9989). The results were expressed as mmol Trolox equivalent in 1 kg DM of plant material.

Statistical data analysis

The statistical analyses were performed using Statistica 6 for Windows. All analytical determinations were performed in triplicate and the mean values ± standard deviation (SD) were reported.

RESULTS AND DISCUSSION

Determination of the total phenol content

Total phenol content (TPC) in the studied plants varies within wide range: from 92± 8 to 946 ± 76 milligrams of gallic acid equivalents (GAE) in 1 g of dry matter (DM) of methanolic extract (Table 2). In *E. arvense* and *E. telmateia* the values are very close – 151± 12 and 148 ± 11 mg GAE.g^{-1} DM.

The highest total phenolic content is in *J. communis* leaves. In the various parts of the plant it varies within a wide range – from 170 ± 15 in ripe berries to 946 ± 76 mg GAE.g^{-1} DM in leaves – 5.6 times more compared to ripe berries and 2.5 times more compared to unripe berries. Compared to the other plants, the highest total phenol content is in juniper except for the ripe berries only. High phenol content was found in the *R. officinalis* leaves, too (365 ± 33 mg GAE.g^{-1} DM) 2.4 times more than the two horsetail species and 4 times more than *L. angustifolia.* The lowest total phenol content was found in *L. angustifolia* flowers – 92 ± 8 mg GAE.g^{-1} DM.

Table 2. Total phenol content and antioxidant activity of the tested plants, (n = 3)

Plant	mg GAE.g^{-1} DM	mmol TE.kg^{-1} DM
Equisetum arvense sterile stems	151 ± 12	18.9 ± 1.7
Equisetum telmateia sterile stems	148 ± 11	18.2 ± 1.7
Juniperus communis leaf	946 ± 76	58.5 ± 5.5
Juniperus communis ripe berry	170 ± 15	25.1 ± 2.3
Juniperus communis unripe berry	373 ± 34	46.3 ± 4.6
Rosmarinus officinalis leaf	365 ± 33	46.6 ± 4.3
Lavandula angustifolia flower	92 ± 8	13.4 ± 1.2

Kukrić *et al.* [2] reported that total phenol content in alcohol extracts of field horsetail is high 355.80 ± 17.8 mg GAE.g^{-1} of the dried extract. Quantitative and qualitative variations in the content of some phenol compounds present in *E. arvense* are possible owing to ecological and geographical factors. The highest concentration of phenol compounds in plant extracts was obtained by means of high-polarity solvents. Total phenol content is high in all *E. telmateia* extracts, among which methanolic extract – 262.7 mg GAE.g^{-1}, acetone extract – 145 mg GAE.g^{-1}, ethyl acetate extract – 159 mg GAE.g^{-1}. According to Radojevic *et al.* [5] methanol appears to be the best solvent for extracting phenol compounds from *E. telmateia*. The results obtained in the present study are lower than those reported in [2] and [5].

It has been found that total polyphenol content in various *J. communis* leaf extracts (aqueous fraction, hexane fraction, ethanol extract and ethyl acetate fraction) varies from 189.65 to 315.33 mg GAE.g^{-1}. Maximum phenol amount is found in the ethyl acetate fraction [6]. In methanolic extracts of branches of five *Juniperus* species from Turkey, the total polyphenol content varies from 170.43 ± 2.13 mg GAE.g^{-1} to 253.29 ± 3.16 mg GAE.g^{-1} extract. In their water extracts the content is lower and varies from 98.74 ± 0.49 mg GAE.g^{-1} to 212.88 ± 2.95 mg GAE.g^{-1} extract [28, 29]. According to Živić *et al.* [30] and other authors, the highest polyphenol concentrations are found in alcohol extracts. In studying ethanol extracts of *J. oxycedrus* and *J. communis* berries, 58.73 ± 0.14 and 189.82 ± 0.27 mg GAE.g^{-1} were obtained, respectively. Ethyl acetate and chloroform extracts showed significantly lower total phenol content compared to ethanol extracts. Total polyphenol content in methanolic extracts of ripe berries of the two *J. oxycedrus* subspecies from Turkey is between 5.14 ± 0.06 and 17.89 ± 0.23 mg GAE.g^{-1} extract [31]. The results obtained by us about the polyphenol content in methanolic extracts of ripe *J. communis* berry are close to those in [31], but the values for unripe berries and leaves are higher.

Ethanolic extracts of rosemary leaves were produced by maceration and percolation and different ethanol concentrations (30, 40, 50, 60, 70, 80, 90 and 96 %) were used for extraction. The most potent solvent concentration was 50 % for the evaluation of total phenols 47.39 ± 0.21 mg/ml rosemarinic acid equivalents. After maceration and stirring the total phenol content increases up to 212.5 ± 0.05 and 219.45 ± 0.05 mg/ml RAE [32]. Tawaha *et al.* [33] reported *R. officinalis* to be a good source of polyphenol compounds varying from 2.8 to 70.3 mg GAE.g^{-1} methanolic extract. Due to the positive linear relationship found between antioxidant activity and total phenol content for methanolic extracts, they established that phenolic compounds were the predominant antioxidant components in the studied plant species. Pérez *et al.* [34] confirmed that rosemary extracts could serve as electron donors and reacted with free radicals transforming them into more stable products, thus terminating radical chain reactions. Solvents considerably affect total phenolic concentration in extracts. Methanolic extracts show higher antioxidant activity and higher phenolic content regardless whether irradiated with gamma rays or not. Therefore, methanol is the most efficient solvent for extracting phenolic compounds from rosemary leaves. Ünver *et al.* [35] reported high polyphenol content in methanolic extracts of rosemary – 214.21 ± 1.14 mg GAE.g^{-1}. High polyphenol content of methanolic extracts from leaves of species from Lamiaceae family, in particular rosemary and lavender, have been found by Spiridon *et al.* [36]. Comparing these results with the results from the present study: 365 mg GAE.g^{-1} for extract from *R. officinalis* leaves, the species from Bulgaria demonstrates higher TPC values.

Ethanolic extracts of plant cell cultures of lavender contain 85.6 ± 5.3 mg GAE.g^{-1} total

phenols [37]. Total polyphenol content in alcohol extracts from the flowers of 5 lavender species from Romania has been established by Robu *et al.* [38]. The values vary from 74.98 to 89.88 mg.g^{-1} dry extract. The TPC results obtained in the present study are very close to theirs. Lower phenol content in the above-ground parts of *L. stricta* in Sothern Iran has been reported by Alizadeh *et al.* [39]. Total phenolic content varies from 61.05 to 64.45 mg GAE.g^{-1} DM.

Some authors have found higher phenolic and flavonoid content in lavender leaves compared to flowers. Due to the existing positive correlation of total phenolic content with the antioxidant activity (AA), leaves demonstrate higher AA than flowers [40, 41].

Determination of the antioxidant activity

Antioxidant activity (AA) of the plant species included in this study was expressed in mmol of Trolox equivalents (TE) in 1 kg DM of the methanolic extract. It ranged from 13.4 ± 1.2 to 58.5 ± 5.5 mmol TE.kg^{-1} DM (Table 2). From the studied plants the highest antioxidant activity was found for the extracts from *J. communis* leaves – 58.5 ± 5.5 mmol TE.kg^{-1} DM. Twice lower is AA of the ripe berries. Unripe berries of *J. communis* showed AA closer to that of leaves. *R. officinalis* leaf extracts also showed high AA (46.6 ± 4.3 mmol TE.kg^{-1} DM) – almost the same as that determined for the *J. communis* unripe berries.

L. angustifolia flower extracts showed the lowest AA – 13.4 ± 1.2 mmol TE.kg^{-1} DM. The difference between the AA values of *E. arvense* and *E. telmateia* is insignificant – 18.9 ± 1.7 and 18.2 ± 1.7 mmol TE.kg^{-1} DM.

E. arvense ethanolic extract belongs to the group of strong antioxidants due to the stable DPPH radical [2]. The greatest capacity for neutralizing DPPH radicals found by [5] for three different *E. telmateia* extracts was measured in its methanolic extract, which neutralizes 50 % of the free radicals in a very low concentration (33.4 µg/ml).

The results obtained about a number of plant species, [33], indicate *R. officinalis* to be one of the best sources of compounds removing free radicals.

According to Živić *et al.* [30] *J. communis* and *J. oxycedrus* berry extracts reveal significant AA. Compared to ethyl acetate and chloroform extracts, AA of the alcoholic extracts is the highest. Phenol-rich ethyl acetate fraction of the ethanolic extract of the leaves of *J. communis* has high AA, which determines its hepatoprotective potential [6].

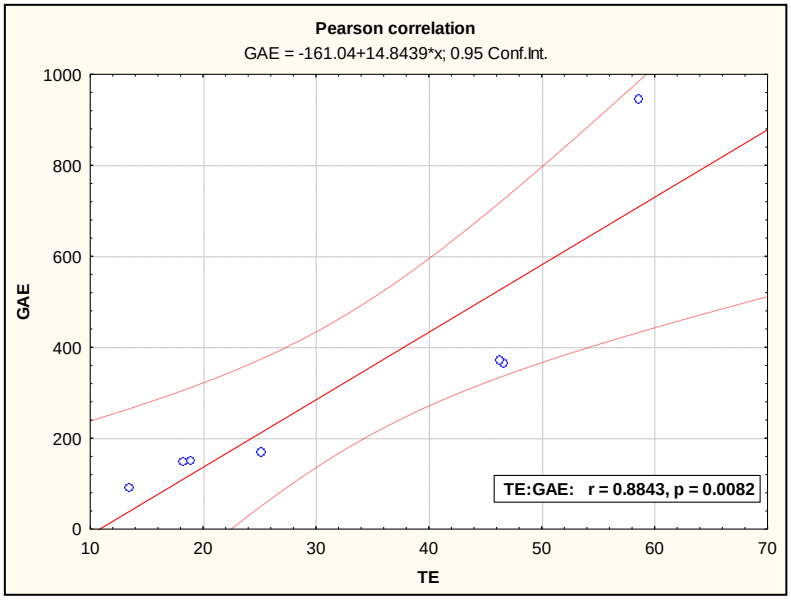

Figure 1. Pearson correlation between TPC and antioxidant activity, P ≤ 0.01 (2-tailed)

The present study has found a positive linear dependence between the AA and TPC values (Figure 1). Pearson correlation between the total phenol content and the antioxidant activity of the methanolic extracts was observed with a high positive coefficient r = 0.8843 (p ≤ 0.01), so these compounds are responsible for the antioxidant activity of the metanolic extracts of the tested plants. Most authors cited in this study, including [2, 5, 30, 33, 41, 42, etc.] also established such correlation.

CONCLUSION

In the present study the total phenol content and the antioxidant activity were measured by determination of the radical scavenging potential, thus bringing some clarity to the properties of the polar methanol extractions prepared from the tested medicinal plants.

The highest total phenol content and antioxidant activity were measured in *J. communis* leaves and unripe berries and *R. officinalis* leaves. The correlation between TPC and radical scavenging potential was found to be positive with high a correlation coefficient. Thus, although plant species from different families were tested, a positive relationship between the two determined parameters was confirmed.

The medicinal plants from populations in Bulgaria included in this study demonstrate high phenol content and antioxidant activity, therefore they can be used as substitutes of synthetic antioxidants in food products and additives used for people and animals and pharmaceuticals.

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Chemical composition, antioxidant activity and total phenol content of six vascular medicinal plants

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The Bulgarian flora is rich in medicinal plants, the annually collected and exported herbs are used on the Bulgarian and international markets as a raw material for a number of medicinal, cosmetic and other objectives. Despite the exceptional biodiversity and significant resources, the antioxidant potential of Bulgarian medicinal plants is still insufficiently explored. Data on the chemical composition of a number of medicinal wild plants are not complete. The aim of this study was to determine the chemical composition, antioxidant activity and total phenol content of the aerial parts of *Artemisia annua* L. (sweet wormwood), *Artemisia vulgaris* L. (common mugwort), *Prunus laurocerasus* L. (cherry laurel), *Tanacetum vulgare* L. (common tansy), *Urtica dioica* L. (common nettle) and *Verbascum densiflorum* Bertol. (denseflower mullein) from their populations in the Thracian Lowland. The Weende method was used to determine crude protein, crude fat, crude fiber, ash, and nitrogen free extracts (NFE). The antioxidant activity was tested by determining the radical scavenging capacity of the selected species by the DPPH method and the total phenol content - by using Folin-Ciocalteu reagent and gallic acid as a standard.

Keywords: total phenol content, antioxidant activity, chemical composition, *Artemisia, Prunus laurocerasus, Tanacetum vulgare, Urtica dioica, Verbascum densiflorum*

INTRODUCTION

The Bulgarian flora is rich in medicinal plants, the annually collected and exported herbs are used on the Bulgarian and international markets as a raw material for a number of medicinal, cosmetic and other objectives. Despite the exceptional biodiversity and significant resources, the antioxidant potential of Bulgarian medicinal plants is still insufficiently explored. Data on the chemical composition of a number of medicinal wild plants in Bulgarian flora are not complete.

Artemisia annua L. (Asteraceae) is spread throughout the country from sea level up to 1000 m above sea level. Various researchers reported that the species have several biological activities such as antioxidant, antispasmodic, antimicrobial, insecticidal, anticancer, antifungal, cytotoxic [1-8]. According to Ćavar *et al.* [9] variability of chemical composition of essential oil of *A. annua* depends on the geographical origin and stage of plant development.

Artemisia vulgaris L. (Asteraceae) is widespread in Bulgaria from 0 to 1000 m above sea level. Aerial parts of the species contain polysaccharides which are employed to treat numerous diseases and carbohydrates extracted from this plant exhibit several beneficial properties [10]. However, the main polysaccharide in the infusion is inulin-type fructan [11]. According to Temraz and El-Tantawy [12] *A. vulgaris* extract possesses antioxidant activity which might be helpful in preventing or slowing the progress of various oxidative stress-related diseases.

Prunus laurocerasus L. (Rosaceae) is naturally distributed in the Central and Eastern Balkan range and the Strandja. It is cultivated for landscaping parks and gardens throughout the country. The fruits of the species contain vitamins (A, C and D) with high antioxidant activity and abundant phenolic compounds (phenolic acids, flavonoids, flavonols, anthocyanin, tannins and lignin) [13-15]. Fatty acids in seeds [16] and essential oil constituents in leaves and fruits [17] have been determined.

In Bulgaria, *Tanacetum vulgare L.* (Asteraceae) grows in all phytogeographical areas from 0 to 2000 m above sea level. It is often grown as an ornamental plant, too. The essential oils of the species find application as cardial, stomach remedies and are used as a food preservative and containing bitter substances and sesquiterpene lactones exhibit cytotoxicity, antimicrobial activity, and regulate growth [18]. In Bulgaria, the dry leaves and flowers of *T. vulgare* are used as spasmodic, antiseptic means and for protection against dandruff [19]. The extract from the aerial parts of the species has been reported to exhibit antitumor [20], anti-inflammatory [21] and antioxidant [22, 23] properties.

Urtica dioica L. (Urticaceae) is widespread in ruderalized terrains throughout the country from 0 to 1700 m. Its leaves have high levels of protein,

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vitamins, nine carotenoids [24-28]. The antimicrobial and antioxidant activities have been studied [29-31]. But, to our knowledge, there is no data available on the antioxidant activities of *U. urens* from Bulgaria.

Verbascum densiflorum Bertol. (Scrophulariaceae) is widespread in Bulgaria from 0 to 2000 m above sea level. The flowers of the species are used for treatment of sore throat, chills, phlegm congestion [32]. Both flowers and leaves possess mildly demulcent, expectorant, and astringent properties [32]. Phytochemical investigations of the flowers have shown the presence of flavonoids, iridoids, phenolic acids, saponins, amino acids and free sugars [33-35].

The aim of this study was to determine the chemical composition, antioxidant activity and total phenol content of the aerial parts of *Artemisia annua* L., *Artemisia vulgaris* L., *Prunus laurocerasus* L., *Tanacetum vulgare* L., *Urtica dioi*ca L. and *Verbascum desniflorum* Bertol. from their populations in the Thracian Lowland.

MATERIALS AND METHODS

Plant material and extract preparation

Plant parts of the studied species were collected in the 2018 growing season from their natural populations. They were dried in shade at 20 - 24 °C, ground in a mechanical grinder (final powder size less than 400 μm) and stored at 18 - 20 °C. The extractions were performed by maceration of 1 g of powdered plant material in 10 ml of methanol at room temperature for 7 days. After filtration, the residue was washed up in triplicate. The collected methanol extracts were concentrated to a final volume of ca. 7 ml by a rotary evaporator under vacuum at 30 °C. The dry matter (DM) of these methanol extracts was determined gravimetrically by drying 1 ml of each extract at 120 °C for 6 hours. Finally, the extracts were adjusted to 1 $mg.ml^{-1}$ calculated on DM by diluting of the concentrated extracts.

Chemical composition, g kg^{-1} DM, determined by the Weende method, includes the following determinations: crude protein – by Kjeldahl method [36]; crude fat – by Soxhlet method [37]; crude fiber [38]; ash [39]; and NFE was calculated by the formula: 1000 – (crude protein + crude fat + crude fibre + ash).

Determination of total phenol content

The experimental procedure described by Anesini *et al.* [40] was applied for determination of total phenol content (TPC). Briefly, 1 ml of the methanolic extract was mixed in separate tubes with 5.0 ml of Folin-Ciocalteu's reagent (1:10 dilution with water of the commercial reagent). Then, 4 ml of 7.5 % Na_2CO_3 aq (w/v) was added and the tubes were left at room temperature for one hour. The absorbance at 765 nm was measured against water. Each sample was analyzed in triplicate. Gallic acid (Sigma-Aldrich, St. Louis, MO) solutions in methanol ranging from 2 to 60 $\mu g.ml^{-1}$ were used for a calibration curve (R^2 = 0.9987). Total phenol content (TPC) of each sample was expressed as mg gallic acid equivalents (GAE) in 1 g DM of plant extract.

Determination of antioxidant activity by DPPH method

The method described by Serpen *et al.* [41] was applied to measure the radical-scavenging potential of methanolic extracts obtained from the tested plant species. Briefly, to 2 ml of 100 μM solution of DPPH in methanol was added 20 μl of methanolic extract. Absorption at 517 nm was measured 30 minutes later. Since the composition of the extracts is complex, the results for their radical-binding capacity were compared with those of Trolox (water-soluble analogue of Vitamin E) and calculated by regression analysis from the linear dependence between concentration of Trolox and absorption at 517 nm. Trolox standard was purchased from Sigma-Aldrich (St. Louis, MO). Standard solutions in methanol ranging from 1 to 50 μmol l^{-1} were used for a calibration curve (R^2 = 0.9989). The results were expressed as mmol of Trolox equivalents (TE) in 1 kg DM of the methanolic extract.

Statistical data analysis

The statistical analyses were performed using Statistica 6 for Windows. All analytical determinations were performed in triplicate and the mean values ± standard deviation (SD) were reported.

RESULTS AND DISCUSSION

Chemical composition in the above-ground biomass of the tested plants

The results from the complete chemical analysis show that in the studied six plants nitrogen free extracts (NFE) predominate (Table 1). No significant differences were observed between the NFE values in the various plants. The highest values were determined in *P. laurocerasus, T. vulgare* and *V. densiflorum* flower (604.38; 594.71; 592.67 g kg^{-1} DM, respectively).

Table 1. Chemical composition of the tested plants, g kg^{-1} DM

Plant	Crude protein, g kg^{-1}	Crude fat, g kg^{-1}	Crude fiber, g kg^{-1}	Ash, g kg^{-1}	NFE, g kg^{-1}
Artemisia annua	180.94 ± 15	32.26 ± 3	125.29 ± 10	110.13 ± 9	551.38 ± 52
Artemisia vulgaris	183.42 ± 16	44.46 ±	131.58 ± 10	99.56 ± 7	540.98 ± 52
Prunus laurocerasus	103.27 ± 9	34.99 ± 3	146.81 ± 12	110.56 ± 9	604.38 ± 58
Tanacetum vulgare	106.97 ± 9	27.34 ± 2	186.72 ± 14	84.27 ± 7	594.71 ± 58
Urtica dioica	160.73 ± 13	22.99 ± 2	81.71 ± 6	166.88 ± 13	567.68 ± 53
Verbascum densiflorum leaf	125.32 ± 9	9.82 ± 1	242.29 ± 20	57.75 ± 5	564.82 ± 53
Verbascum densiflorum flower	110.10 ± 7	16.58 ± 1	231.35 ± 18	49.31 ± 5	592.67 ± 57

In the other plants NFE vary within a close range – from 567.68 to 540.98 g kg^{-1} DM. In the *V. densiflorum* leaves lower NFE content was found, but higher crude protein, crude fiber and ash content compared to flowers. The two wormwood species demonstrated close NFE values – 551.38 and 540.98 g kg^{-1} DM.

Crude protein content varies from 103.27 to 183.42 g kg^{-1} DM. High protein values are typical of *A. vulgaris*, *A. annua* and *U. dioica* – 183.42; 180.94; 160.73 g kg^{-1} DM, respectively. The two horsetail species exhibit almost the same crude protein content. The protein content is lower in *V. densiflorum* leaf – 125.32 g kg^{-1} DM. In *P. laurocerasus, T. vulgare, V. densiflorum* flower low crude protein values were established – 103.27; 106.97 and 110.10 g kg^{-1} DM.The highest crude fiber content was found in *V. densiflorum* – 242.29 g kg^{-1} DM in leaves and 231.35 g kg^{-1} DM in flowers. Medium is the position of *T. vulgare* and *P. laurocerasus* (186.72; 146.81 g kg^{-1} DM) followed by the two wormwood species (131.58; 125.29 g kg^{-1} DM). In *U. dioica* the lowest crude fiber value was recorded – 81.71 g kg^{-1} DM.

Mineral substances (ash) values range from 49.31 to 166.88 g kg^{-1} DM. The highest ash content is shown by *U. dioica.* Similar are the values of *P. laurocerasus, A. annua, A. vulgaris* and *T. vulgare* – 110.56; 110.13; 99.56; 84.27 g kg^{-1} DM, respectively. The lowest values were recorded in *V. densiflorum* flower – 49.31 g kg^{-1} DM, and slightly higher – in *Verbascum densiflorum* leaf – 57.75 g kg^{-1} DM.

The chemical composition of the 6 plant species included in this study is characterized by the lowest crude fat content. Higher values were found in *A. vulgaris, P. laurocerasus* and *A. annua* (32.26; 44.46; 34.99 g kg^{-1} DM). The lowest crude fat content is observed in *V. densiflorum* – 16.58 g kg^{-1} for flowers and 9.82 g kg^{-1} DM for leaves. With values of 27.34 and 22.99 g kg^{-1} DM, *T. vulgare* and *U. dioica* occupy medium position by that indicator.

For the two wormwood species included in the study, *A. vulgaris* and *A. Annua*, close values of the tested chemical composition parameters were found. The obtained crude protein and ash values of *A. annua* are higher than those published by Iqbal *et al.* [46] and lower for crude fat and fiber.

Various researchers report high protein content in nettle leaves. In addition to the higher protein level, nettle has a better amino acid profile than most other leaf vegetables [57]. According to Sidaoui *et al.* [30] protein content is 15.75 %. The results from the chemical analysis by Adhikari *et al.* [58] show 33.8 % crude protein, as well as high ash (16.2 %), crude fat (3.6 %) and crude fiber (9.1 %) content. The crude protein values obtained in our study are lower compared to [58] and similar to [30]; for ash, crude fiber and fat they are close and higher for NFE compared to [58].

Total phenol content and antioxidant activity of the tsted plants

Total phenol content (TPC) varies from 152 ± 11 to 591 ± 56 mg GAE.g^{-1} DM of methanolic extracts in the plants studied (Table 2). The values for *A. annua* and *A. vulgaris* are similar: 270 ± 22 and 282 ± 25 mg GAE.g^{-1} DM.

Table 2. Total phenol content and antioxidant activity of the tested plants, (n = 3)

Plant	mg GAE.g^{-1} DM	mmol TE.kg^{-1} DM
Artemisia annua stems	270 ± 22	27 ± 3
Artemisia vulgaris stems	282 ± 25	39 ± 3
Prunus laurocerasus leaves	324 ± 28	47 ± 4
Tanacetum vulgare leaves	525 ± 44	62 ± 5
Tanacetum vulgare flowers	291 ± 27	61 ± 5
Urtica dioica leaves	591 ± 56	45 ± 5
Verbascum densiflorum leaves	330 ± 29	35 ± 3
Verbascum densiflorum flowers	152 ± 11	14 ± 2

Differences in the phenol content were found in the different parts of *T. vulgare* and *V. densiflorum*. Higher TPC content is typical for the leaves of these plants – 525 ± 44 and 330 ± 29 mg GAE.g^{-1} DM. In flowers TPC is twice lower. From the plants included in the study, the total phenol content is the highest in the *U. dioica* leaves – 591 ± 56 mg GAE.g^{-1} DM. Over 500 mg GAE.g^{-1} DM were found in *T. vulgare* leaves as well. In the other plants TPC vary from 270 ± 22 to 330 ± 29 mg GAE.g^{-1} DM – from 2.2 to 1.6 times lower compared to *U. dioica* and *T. vulgare.* With TPC value of 152 ± 11 mg GAE.g^{-1} DM, *V. densiflorum* flowers are determined to have the lowest TPC – from 3.4 to 3.9 times lower.

Antioxidant activity (AA) varies from 14 ± 2 to 62 ± 5 mmol TE.kg^{-1} DM (Table 2). *T. vulgare* leaf and flower extracts demonstrate the highest AA. The AA values for the different plant parts are very close – 61 ± 5; 62 ± 5 mmol TE.kg^{-1} DM. With regard to TPC big differences between leaf and flower were observed, with leaf values being 1.8 times higher. High AA is demonstrated by the methanolic extracts of *P. laurocerasus* and *U. dioica* – 47 ± 4 and 45 ± 5 mmol TE.kg^{-1} DM. Due to the established highest phenol content for nettle leaves, it is expected it to reveal the highest AA compared to the other plants included in the study. Probably for nettle AA phenol content is primarily accountable, while in *T. vulgare* and *P. laurocerasus* other biologically active substances also have an effect.

Lower AA values were determined in *A. annua, V. densiflorum* leaves and *A. vulgaris.* AA found for *A. annua* is lower than that of *A. vulgaris*, which corresponds to the lower TPC of that plant. The lowest AA is exhibited by the *V. densiflorum* flower extracts – 2.5 times lower than that of the leaves of the same plant.

Phenols are plant ingredients with important significance for antioxidant activity. There is a strong positive relation between total phenols and antioxidant activity of many plant species due to the high reactivity of the phenol group that participates in the following reaction:

$$\text{Ar-OH} + \text{R.} \rightarrow \text{Ar-O.} + \text{RH}$$

Single electron delocalization makes this reaction thermodynamically favorable. A reaction turns the phenolic group into a stable quinone structure [42]. According to some authors, what makes phenol compounds good antioxidants is that they are efficient hydrogen donors [43]. Temraz and El-Tantawy [12] found that total phenol content in *A. vulgaris* water extract is 19 ± 0.16 mg GAE.g^{-1} plant extract. The extraction method and the solvent used play a key role in extracting phenols from the plant material. According to Skowyra *et al.* [44] the ethanol extract of *A. annua* leaves contains 23.36 ± 0.92 mg GAE.g^{-1} DM. In another study about methanolic and acetone extracts from *A. annua* leaves [45] TPC values of 384.1 ± 6.7 and 521.2 ± 5.4 mg GAE.100 g^{-1} DM, respectively, have been determined. Higher values have been reported by [46]. In different extracts TPC values vary from 90.12 to 134.50 mg GAE.g^{-1} DM with the highest ones having been reported for the methanolic extract. The TPC values obtained in the present study (270 ± 22 mg GAE.g^{-1} DM) are higher that the cited ones.

Antioxidant activity of *A. vulgaris* can be useful for preventing or delaying the development of various diseases related to oxidative stress [12]. *A. annua* extract can be used as a substitute of synthetic antioxidants [44].

A number of authors report TPC and AA in *P. laurocerasus* fruit [13-15]. For *P. laurocerasus* leaves, depending on the extraction technique, Karabegović *et al.* [47] found a fluctuation of TPC from 119.4 to 85.4 mg GAE.g^{-1} DM, and of AA – from 124.5 to 108.1 μg/ml. They noted down a high correlation among TPC, AA and total flavonoid content with 0.945 and 0.985 ratio. The TPC values

determined in the present study are higher compared to the results presented in [47].

Methanolic extract from the above-ground parts of *T. vulgare* shows AA with a value of 37 ± 1.2 μg.ml^{-1}. The revealed strong antioxidant activity of the extract and the isolated active ingredients come to support the traditional medicinal applications of the plant in healing wounds, rheumatoid arthritis and other inflammatory conditions [48].

According to Bączek *et al.* [49] *T. vulgare* and *T. balsamita* extracts have antioxidant potential values established by the DPPH method of 13.59 and 13.86 μmol TE.g^{-1} DM, respectively, and can be used in the pharmaceutical and food industries as antiseptics and preservers. Tansy extracts can be effectively used as an antioxidant in rapeseed oil [22, 50]. Ivănescu *et al.* [51] report values of *T. vulgare* TPC from Romania 26.37 mg GAE.g^{-1} DM and AA 242.8 μg.ml^{-1}. In the present study extracts from both *T. vulgare* leaves and flowers exhibit the highest AA compared to the other plants.

Alan *et al.* [52] used various extract solvents in order to determine antioxidant activity of three *Verbascum* species. According to them, methanol and water extracts exhibit greater antioxidant activity compared to the other extracts. The study by Saltan *et al.* [53] also shows that methanolic extracts from various *Verbascum* species reveal good antioxidant activity. AA of *Verbascum* species is mainly determined by the secondary metabolite verbascoside, and methanolic extracts show the strongest antioxidant activity in various *in vitro* methods [54].

The results from the study by Sidaoui *et al.* [30] reveal that the phenol content is 11.62 mg GAE.g^{-1} DM, whereas AA is 8.11 mM.g^{-1} DM. In a publication by Mzid *et al.* [31] TPC values of 31.41 mg GAE.g^{-1} DM and of AA 560 mmol Trolox.g^{-1} of DM in *U. dioica* alcohol extract have been obtained. According to Biesiada *et al.* [55] from Poland the average TPC content in the methanolic extract of nettle leaves is 14.47 mg.g^{-1} DM and AA is 26.5 μM TE.g^{-1} DM. The values stated by Ozkan *et al.* [56] about TPC and AA in methanolic extracts of nettle leaves from Turkey are 332.19 mg GAE.g^{-1} DM and 40.59 mM TE.g^{-1} DM, respectively. Total phenol content in nettle extracts from Serbia amounts to 208.37 mg GAE.g^{-1} DM, while antioxidant activity measured by using the DPPH and ABTS methods has IC_{50} values of 31.38 and 23.55 μg mL^{-1}, respectively [27]. The TPC values of 591 ± 56 mg GAE.g^{-1} DM obtained in the present study are higher than the cited ones.

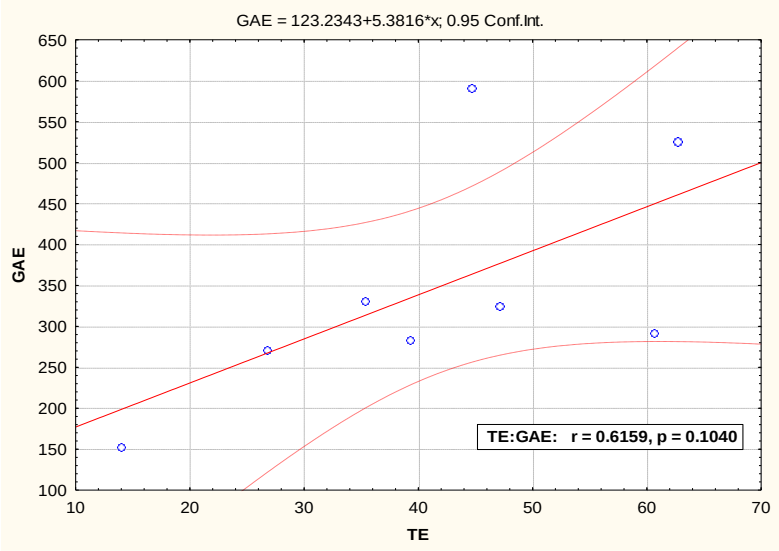

Figure 1. Pearson correlation between TPC and antioxidant activity, P ≤ 0.05 (2-tailed)

In the present study a positive linear dependence was found between the antioxidant activity and total phenol content (Figure 1). Pearson correlation measures the linear correlation between two variables. In the present study, the correlation between the values of TPC and the values of AA of the tested methanolic extracts was observed. The calculated correlation coefficient was positive with a value of 0.6159 (p ≤ 0.05). A conclusion could be drawn that these compounds are responsible for the antioxidant activity of the metanolic extracts of the tested plants.

CONCLUSION

The highest crude protein content was found in *A. vulgaris* and *A. annua*, crude fat – in *A. vulgaris*, crude fiber – in *V. densiflorum*, ash – in *U. dioica* and nitrogen free extracts – in *P. laurocerasus*.

The highest total phenol content was measured in *U. dioica* leaves and antioxidant activity – in *T. vulgare* leaves and flowers.

Although Pearson correlation was evaluated between total phenol content and radical scavenging potential measured at extracts from different plant species, the correlation coefficient observed had a large, positive value. This once again confirmed the strong influence and a high contribution of the total phenolic content to the antioxidant activity of methanolic plant extracts.

Referring to the results obtained in the present study, the tested plant species can be used in further works of researching of their qualities as health-promoting additives or preservatives in the pharma-; food- and cosmetic industries.

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Phenolic content and antioxidant capacity of *Inula britannica* from different habitats in Bulgaria

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The aim of this study was to evaluate the total phenolic and flavonoid contents and antioxidant capacity of the methanol extracts of 11 samples of *I. britannica* from different habitats and to identify the potent antioxidant compounds. The values of phenolics ranged from 85.35±7.64 to 141.01±4.97 mg GAE/g DE, while those of flavonoids – from 19.66±0.75 to 36.80±0.56 mg CE/g DE. Antioxidant capacity of the studied extracts measured by the DPPH method (0.229±0.015 to 0.620±0.001 mM TE/g DE) correlated well with the total phenolic and flavonoid content, while that determined by $ABTS^{\bullet+}$ assay (0.420±0.010 - 0.550±0.003 mM TE/g DE) showed a moderate correlation with total phenolic content only. HPLC analysis revealed that chlorogenic (5-CQA), 1,5- and 3,5-dicafeoylquinic (DCQA) acids were the major components in all samples, while the amounts of the other two positional isomers 4,5-, and 3,4-DCQA were significantly lower. Principal component analysis (PCA) was used to investigate the variations in the chemical content within *I. britannica* populations.

Keywords: *Inula britannica*; Asteraceae; phenolics; flavonoids; DPPH and ABTS assays; caffeoylquinic acids

INTRODUCTION

Inula britannica L. is widely distributed in Western Europe and Turkey, extending eastward to China through Iran and Pakistan [1, 2]. The plant is an important plant species used in Traditional Chinese Medicine (TCM) and Kampo Medicines as antibacterial, carminative, diuretic, laxative, stomach, tonic remedies, and for treating asthma, hepatitis and tumours [3]. *I. britannica* L. has shown to possess various biological activities – anti-inflammatory, antitumor, antibacterial, antitussive, antiproliferative, antioxidant, hepatoprotective, etc., which were attributed to the abundance of bioactive components mainly sesquiterpene lactones, phenolic acids, and flavonoids [3-5]. As a part of the ongoing project, we have recently studied *I. britannica* L. growing in Bulgaria and five sesquiterpene lactones, three triterpenoids, three flavonoids, and 1,5-dicaffeoylquinic acid were isolated and identified by spectral methods [6]. Considering the use of the species as herbal medicine, we have decided to expand the investigation on *I. britannica* from different populations in Bulgaria to investigate the qualitative and quantitative differences in the main constituents and their impact on biological activity. In this study, the results of the comparative study of the phenolic constituents and antioxidant capacity of *I. britannica* from 11 natural habitats in Bulgaria were described.

EXPERIMENTAL

Plant material

Aerial parts from wild growing *I. britannica* were collected in full flowering stage in July 2018 from 11 different natural habitats in Bulgaria: **1** – Berkovitsa (43°15'1.75"N 23° 9'30.78"E), **2** - Gavril Genovo village (43°23'54.35"N 23° 3'43.08"E), **3** – G. Brestniza village (42°16'49.0"N 22°37'33.4"E), **4** - Vetren village (44°08'24.6"N 27°01'49.7"E), **5** - Gabrovo village (41°53'50.45"N 22°56'36.22"E), **6** - Aglen village (43°12'56.1"N 24°19'38.5"E), **7** - Chervenata stena, Rhodopes Mts (41°51'52.34"N 24°56'38.09"E), **8** - Kraishte village (41°54'23.93"N 23°35'11.98"E), **9** - Belimel village (43°25'46.47"N 22°57'36.06"E), **10** - Brezhani village (41°52'4.69"N 23°12'2.70"E), 1**1** - Slavyanka Mts. (41°24'31.54"N 23°33'51.34"E). The plant was identified by Dr. Ina Aneva (Institute of Biodiversity and Ecosystem Research, BAS, Sofia).

Extraction of plant material

Air-dried and powdered plant material (1 g) was initially defatted by extraction with chloroform (20 mL, 3 times) followed by extraction with CH_3OH (20 mL, in triplicate).

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Each extraction was performed for 12 hours at room temperature. After filtration, the solvent was evaporated under vacuum and the resulting methanol extracts were used for HPLC analysis, determination of total phenolic and flavonoid content, as well as for antioxidant activity assays.

Determination of total phenolic content

Total phenolic content (TPC) was measured using Folin–Ciocalteu method [7]. The concentration was calculated using gallic acid as a standard and the results were expressed as milligrams (mg) gallic acid equivalents (GAE) per 1 g of dry extract (mg GAE/g DE).

Determination of total flavonoid content

The total flavonoid content (TFC) was measured using a previously developed colorimetric assay [8]. The concentration was calculated using a calibration curve of (+)-catechin (in the range of 2 μg/ml to 80 μg/ml). The result was expressed as milligrams of catechin equivalent per gram of dry plant extract (mg CE/g DE).

Determination of DPPH radical scavenging activity

The 1,1-diphenyl-2-picrylhydrazyl radical (DPPH) scavenging activity assay was performed according to the procedure described by Thaipong *et al*. [9]. The antioxidant activity was expressed as mM Trolox equivalents per gram dry extract (mM TE/g DE), using a calibration curve of Trolox dissolved in methanol at different concentrations (0.1- 0.5 mM).

Determination of $ABTS^{\bullet+}$ scavenging activity

The procedure was previously described by Re *et al.* [10]. Results were expressed as Trolox equivalents antioxidant capacity (mM Trolox equivalents per gram dry extract), using a calibration curve of different concentrations of Trolox in methanol (0.1-0.5 mM).

High-Performance Thin Layer Chromatography (HPTLC)

HPTLC analysis was done with pre-coated HPTLC glass plates (20 × 10 cm, Si G60 F_{254}, Merck) using a Camag HPTLC system (Switzerland). Toluene: ethyl acetate: formic acid: water (5:100:10:10 v/v/v/v) was used as a mobile phase. The chromatographic spots were visualized by UV light at 366 nm.

HPLC Analysis of mono- and dicaffeoyl esters of quinic acid

The HPLC equipment was a Waters HPLC system (Waters 2795) with a Waters binary pump, an auto-sampler, a column oven, and a Waters 2487 Dual wavelength absorbance detector. The LiChrospher 100 RP-18 column (5 μm, Merck) was used with a guard column that was filled with the same stationary phase. Solvent A (20% CH_3OH in H_2O) and solvent B (CH_3OH) were used as the mobile phase under gradient conditions (0 min, 0% A; 30 min, 0% A; 65 min, 20% A; 70 min, 0% A) to analyse the samples. The analysis was carried out at a flow rate of 0.8 mL/min. The detection wavelength was set at 327 nm and the sample injection volume was 10 μL. The peak identification was based on the retention time of the standard compounds (t_R) as follows: chlorogenic acid (7.1 min), 3,4-dicaffeoylquinic acid (49.5 min), 3,5-dicaffeoylquinic acid (53.0 min), 1,5-dicaffeoylquinic acid (56.4 min), and 4,5-dicaffeoylquinic acid (63.2 min). The correlation coefficients (R^2) were higher than 0.99 (five concentrations in three replicates each) and the relative standard deviations (% RSD) were < 5% confirming the linearity and repeatability of the method for each compound. All samples were run in triplicate and quantification was carried out using external standards. The content of each compound was calculated and expressed as mg/g of dry extract (DE).

Statistical analysis

All data were reported as means ± standard deviation (SD) using three independent measurements. Data were analysed using Student's t-test and differences were considered as significant at $p<0.05$. Analysis of variance with a confidence interval of 95% was performed using MS Excel software. Principal component analysis (PCA) was performed using the PAST 4.0 software.

RESULTS AND DISCUSSION

The total phenolic (TPC) and flavonoid (TFC) contents of the methanol extracts obtained from the aerial parts of eleven *I. britannica* native populations were analysed using spectrophotometric methods and were expressed as mg GAE/g DE and mg CE/g DE, respectively (Table 1). The values of phenolics varied from 85.35±7.64 mg GAE/g DE (sample **6**) to 141.01±4.97 mg GAE/g DE (sample **1**). The performed one-way ANOVA analysis indicated that the total phenolic contents in all 11 studied populations were significantly different at the 95%

confidence level. However, TPC in samples **2**, **4**, **5**, **7** and **10**, as well as those in samples **3, 7** and **9,** did not differ significantly from each other (p>0.05, t-test). Therefore, two main groups are formed according to the amount of phenolics: the first group (samples **1**, **2**, **4**, **5**, **10** and **11**) with TPC more than 110 mg GAE/g DE, and the second one (samples **3**, **6, 8** and **9**), in which TPC was less than 110 mg GAE/g DE. Sample **7** occupied an intermediate position.

Samples **1** and **11** contained approximately the same amount of flavonoids (35.72±0.42 and 36.80±0.56 mg CE/g DE, respectively) and were the richest in this type of compounds (Table 1). Sample **3** was the poorest in flavonoids and their amount was 19.66±0.75 mg CE/g DE only. Statistical analysis (one-way ANOVA) indicated that there were also significant differences (p<0.05) among populations. Nevertheless, TFC in samples **5**, **6**, **7** and **8** (> 30 mg CE/g DE), as well as in samples **2**, **4**, **9** and **10** (< 30 mg CE/g DE) were not significantly different from each other (p>0.05, t-test). Among the studied populations, the samples **1** and **11** were found to be the richest in both classes of compounds - phenolics and flavonoids.

The amounts of TPC and TFC in the 11 *I. britannica* samples were found to be higher from that in the flower methanol extract of *I. britannica* from another Bulgarian population (79.41 mg GAE/g DE and 19.94 mg CE/g DE, respectively) [6] and lower from that reported for the water extract of *I. britannica* var. *chinensis* (318.10 and 335.87 mg CE/g DE, respectively) of South Korean origin [11]. In another recent study, the water extract of *I. britannica* herb from South Korea was found to be richer in TPC than the ethanol extract (50.8 *vs* 42.1 mg GAE/g solid) [12]. On the contrary, flavonoids dominated in the ethanol extract (225.7 mg QE/g solid), while their quantity in the water extract was 51.6 mg QE/g solid only [12].

Table 1. Total phenolic content (TPC), total flavonoid content (TFC) and antioxidant capacity (DPPH and ABTS assay) from *I. britannica* from different habitats in Bulgaria

Sample	TPC* [mg GAE/gDE]	TFC* [mg CE/gDE]	Antioxidant capacity* [mM TE/g DE]	
			DPPH	ABTS
1	141.01±4.97	35.72±0.42^{a}	0.620±0.010^{a}	0.550±0.003^{a}
2	115.84±3.03^{a}	25.47±0.81^{b}	0.365±0.005^{b}	0.501±0.004^{a,b}
3	97.93±2.79^{b}	19.66±0.75	0.229±0.015^{c}	0.420±0.013^{c}
4	118.95±5.44^{a,c}	24.34±0.41^{b,c}	0.350±0.007^{b,d}	0.430±0.004^{c,d}
5	120.02±6.14^{a,c,d}	30.66±0.44^{d}	0.430±0.009	0.430±0.009^{c,d,e}
6	85.35±7.64	32.89±0.22^{a,e}	0.340±0.033^{b,d,e}	0.440±0.011^{c,d,f}
7	101.73±10.40^{a,b,e,f}	33.05±1.29^{a,d,e,f}	0.380±0.024^{b,d,e,f}	0.420±0.021^{c,d,g}
8	104.47±4.89$^{b,e,f.g}$	31.73±1.21^{d,e,f}	0.354±0.031$^{b.d,e,f}$	0.462±0.010^{b}
9	100.80±4.78^{b,e,f,g}	25.12±3.42^{b,c,d,e,g}	0.240±0.021^{c}	0.430±0.007^{c,d,e,f,g}
10	114.14±2.07^{a,c,d,f}	27.11±1.02^{b,c,g}	0.300±0.010	0.540±0.008^{a,b}
11	126.24±5.89^{d}	36.80±0.56^{a}	0.600±0.020^{a}	0.490±0.007^{b}

*Values are means ± SD (n=3). Means in the columns with the same letter are not significantly different from each other (p>0.05) (t-test)

Further, DPPH and ABTS assays were used to estimate free radical scavenging properties of the studied extracts and the obtained results were expressed as mM Trolox equivalents per gram of dry extracts (mM TE/g DE (Table 1). The antioxidant capacity of the studied extracts measured by the DPPH method ranged from 0.229±0.015 to 0.620±0.001 mM TE/g DE. Samples **1** and **11** were the most active DPPH$^{\bullet}$ scavengers, while samples **3** and **9** showed the lowest values. A good correlation was observed between antioxidant capacity assessed with the DPPH test and TPC and TFC in the studied extracts (Pearson, r = 0.721 and 0.786, respectively). The antioxidant capacity determined by ABTS$^{\bullet+}$ assay showed values between 0.420±0.010 and

0.550±0.003 mM TE/g DE. The highest $ABTS^{\bullet+}$ scavenging activity was found for samples **1** and **10**, while the other samples exhibited activity in comparable values. The antioxidant capacity assessed with the ABTS test showed a moderate correlation with TPC (r = 0.652) and a weak correlation with TFC (r = 0.334). The different antioxidant activity levels obtained from both assays is probably due to the difference in the ability of antioxidant compounds in the extracts to quench ABTS and DPPH free radicals in *in vitro* systems. DPPH and ABTS activities of the studied *I. britannica* methanol extracts were comparable with those found for another Bulgarian sample (0.376 and 0. 403 mM TE/g DE) [6].

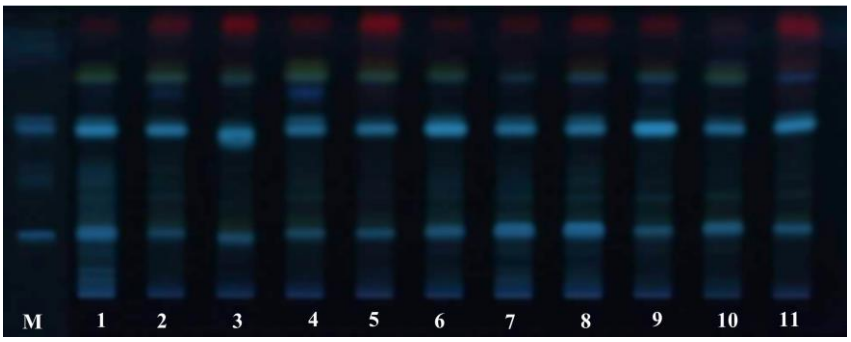
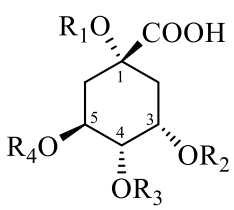


Fig. 1. HPTLC of a model mixture (**M**, R_f 0.23 (5-CQA), 0.42 (4,5-DCQA), 0.44 (3,4-DCQA), 0.58 (1,5-DCQA) and 0.62 (3,5-DCQA) and *I. britannica* methanol extracts (**1**-**11**)



	R_1	R_2	R_3	R_4
5-CQA	H	H	H	Caff
1,5-DCQA	Caff	H	H	Caff
3,4-DCQA	H	Caff	Caff	H
4,5-DCQA	H	H	Caff	Caff
3,5-DCQA	H	Caff	H	Caff

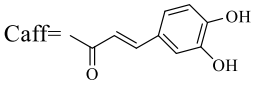


Fig. 2. Structures of mono- and dicaffeoyl esters of quinic acid

Table 2. Content of individual compounds in *I. britannica* methanol extracts [mg/g DE]

Sample	CQA*	3,4-DCQA*	3,5-DCQA*	1,5-DCQA*	4,5-DCQA*
1	23.72±0.82^{a}	3.96±0.14^{a}	18.68±0.27	62.76±1.31	8.37±0.24
2	18.02±0.25^{b}	4.75±0.17	11.52±0.16	48.51±1.01^{a}	5.63±0.17^{a}
3	20.20±0.69^{b}	3.68±0.13^{a}	7.09±0.10^{a}	40.46±0.85^{b}	3.32±0.10^{b}
4	14.99±0.54^{c}	5.23±0.19	14.21±0.20	30.75±0.64	5.81±0.16^{a}
5	24.69±1.34^{a,d}	0.83±0.03	22.07±0.32	47.28±0.99^{a}	7.56±0.22
6	20.57±0.28^{b}	0.17±0.01	7.54±0.11^{a,b}	39.13±0.82^{b,c}	3.21±0.10^{b,c}
7	26.37±0.91^{d,e}	1.86±0.07^{b}	7.04±0.10^{a,c}	27.63±0.58	2.61±0.07^{d}
8	27.68±1.50^{e,f}	1.44±0.05^{c}	7.65±0.11^{b}	31.33±0.65	2.80±0.08^{d,e}
9	16.02±0.55^{b,c}	1.73±0.06^{b}	5.78±0.08	54.33±1.14^{d}	3.30±0.10^{b,c}
10	27.36±0.19^{f}	1.31±0.05^{c}	6.84±0.10^{a,c}	37.88±0.79^{b,c}	2.70±0.08^{d,e}
11	51.41±0.72	2.44±0.09	8.80±0.13	53.02±1.11^{d}	2.28±0.07

*Values are means ± SD (n=3). Means in the columns with the same letter are not significantly different from each other (p>0.05) (t-test)

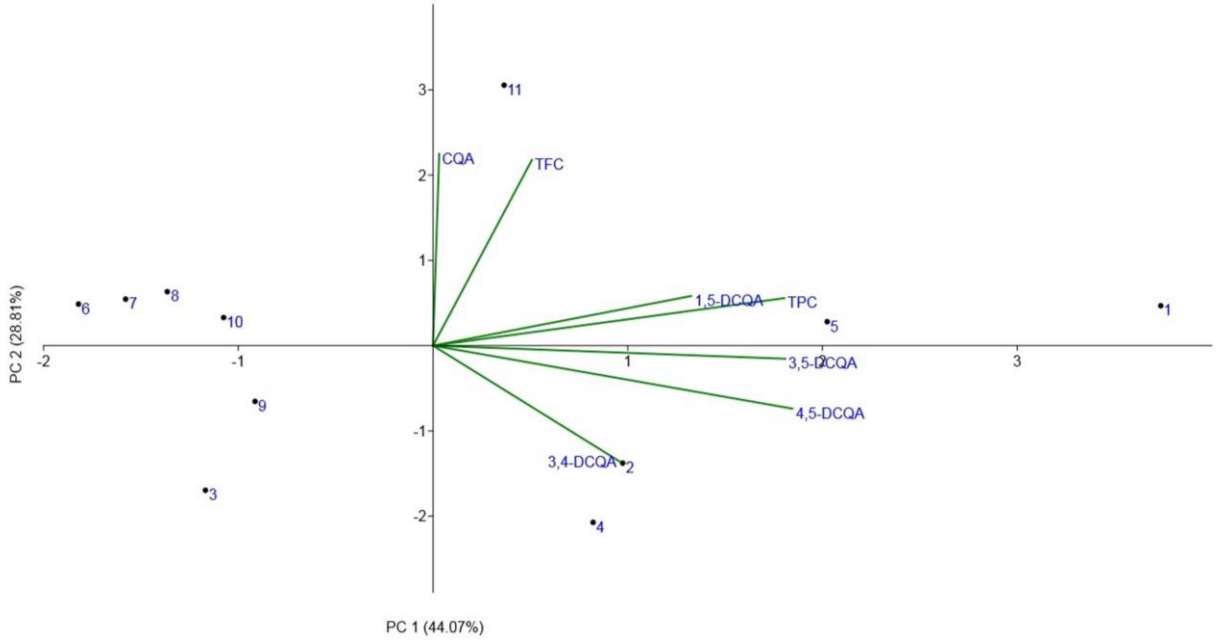


Fig. 3. Biplot (PCA) carried out on TPC, TFC and the content of individual acids of each sample of *I. britannica* (**1** – **11**)

It is difficult to compare the obtained results with those published for other *Inula* species, because of different methods of testing and presentation of the results.

Preliminary HPTLC analysis (Fig. 1) of the methanol extracts of *I. britannica* has shown that they contained chlorogenic (5-CQA) and dicaffeoyl esters of quinic acid (DCQA) (Fig. 2). They were recognized by the characteristic blue fluorescence at 366 nm in the presence of commercially available standards. As can be seen, chlorogenic (5-CQA) and 1,5-dicafeoylquinic (DCQA) acids were the major components in all samples, while the amount of the other three positional isomers 4,5- 3,5- and 3,4-DCQA was lower. This observation was further confirmed by HPLC analysis which was used for their quantitative determination. As can be seen from Table 2, the amount of 5-CQA varied from 14.99±0.54 (sample **4**) to 51.41±0.72 mg/g DE (sample **11**) and 1,5-DCQA (27.63±0.58-62.76±1.31 mg/g DE) was the most abundant dicaffeoyl ester of quinic acid followed by 3,5-DCQA (5.78±0.08 - 18.68±0.27 mg/g DE), 4,5-DCQA (2.28±0.07 - 8.37±0.24 mg/g DE) and 3,4-DCQA (0.17±0.01– 5.23±0.19 mg/g DE). Sample **1** was found to be the richest in 1,5- and 4,5-DCQA, sample **5** - in 3,5-DCQA, and sample **4** – in 3,4-DCQA.

Literature survey showed only several reports on the content of 5-CQA and DCQA isomers in *I. britannica*, but it was difficult to compare their quantity as the data referred to their isolation, LC/MS or TLC detection, but not their real quantity. Thus, 5-CQA has been detected in ray and disk florets of the plant from Hungary [13] and the herb from South Korea [12], while 1,5-DCQA was isolated from another Bulgarian population [6]. The average contents of 5-CQA in 33 samples of *I. britannica* flowers, stems and aerial parts from China were found to be 323, 146 and 302 μg/g DM, respectively [14]. Finally, principal component analysis (PCA) was applied to study variability between different populations of *I. britannica*. The PCA performed on TPC, TFC, the content of 5-CQA, 1,5-, 3,5-, 4,5- and 3,4-DCQA of each sample showed that the first two principal components (PC) accounted for 72.88 % of the total variations (Fig. 3). As can be seen, PC1 (44.07 %) had a strong positive correlation with all variables, while PC2 (28.81 %) was positively related to TPC, TFC, 5-CQA, and 1,5-DCQA and negatively related to 3,5- DCQA, 3,4-DCQA, and 4,5-DCQA. The samples **1** and **11** occupied the most distant positions because of the highest amounts of phenolics (TPC) and 1,5-DCQA in sample **1** and TFC and 5-CQA in sample **11**. Sample **5** was situated at the positive sites of PC1 and PC2 and was associated with 3,5-DCQA. Samples **2** and **4** were settled at the negative side of PC2 and were connected with 4,5- and 3,4-DCQA. In fact, these compounds were detected in the highest concentration in the respective samples (Table 2).

Samples **3** and **9** were depleted in flavonoids (TFC) therefore they were located at the negative sites of PC1 and PC2. Similarly, samples **6** – **8** and **10** were settled at the positive side of PC2 because of the relatively low content of 4,5-DCQA.

CONCLUSION

The results of this study revealed significant variability in the contents of total phenolics, total flavonoids and individual compounds (chlorogenic, 1,5-, 3,5-, 4,5-, and 3,4- dicafeoylquinic acids) within the investigated populations of *I. britannica*, which reflected on their antioxidant capacity too. The samples containing the highest amounts of phenolics and flavonoids were found to be the best DPPH and ABTS radical scavengers. The obtained results could be used in the selection of the prospective populations of *I. britannica* providing a greater concentration of active components and, consequently, a higher biological activity.

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Agrimonia eupatoria tea intake has the potential to change oxidative and inflammatory response of human pbmc to *ex vivo* lps stimulation – an example of phenotypic flexibility modulation

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Agrimonia eupatoria L. (AE) is an herb widely used in the Bulgarian traditional medicine. Current study aims to assess the anti-inflammatory and antioxidant potential of the herb in an intervention study involving healthy volunteers. A model of bacterial lipopolysaccharide (LPS) *ex vivo* stimulation of peripheral blood mononuclear cells (PBMC) was used to analyse changes in expression of two pro-inflammatory (IL-1β and IL-6) and two antioxidant genes (GCLc and SOD1) after supplementation with agrimony tea for 25 days. The effect of BMI was also taken into consideration. LPS stimulation before intervention (Day 0) significantly stimulated IL-1β and IL-6 both in normal weight (NW) (3.7- and 14-fold, respectively, $p<0.001$) and overweight (OW) (2.8- and 2.5-fold, respectively, $p<0.05$) groups and of GCLc in NW (5 fold, $p<0.001$). After the AE intervention (Day 25) LPS stimulation significantly increased IL-6 (3-fold, $p<0.05$) and IL-1β (3-fold, $p<0.001$) mRNA levels only in OW, while in the NW such effect was not observed. GCLc and SOD1 mRNA levels were not elevated at Day 25 both in NW and OW groups. We established that AE consumption resulted in significant decrease in LPS stimulated expression of IL-6 (7.6-fold, $p<0.001$), IL-1β (3.8-fold, $p<0.001$) and GCLc (3-fold, $p<0.05$) gene expression only in NW.

All these results confirm the anti-inflammatory potential of the herb. They also highlight the capability of NW subjects for a better adaptation after the agrimony intake since their PBMC manifested a better phenotypic flexibility in comparison to the OW subjects in *ex vivo* inflammatory conditions.

Keywords: PBMC, LPS, cytokines, antioxidant enzymes, agrimony

INTRODUCTION

Realization that one's nutrition-related health status is a result of the interaction of individual's genome and life-long dietary exposure has led to the estimation of nutrition as a gene-environment interaction science [1]. A complex regulatory system, affected by environmental parameters, main constituent of which is nutrition, controls expression at all levels [2]. A healthy subject is known to be more adaptable to the constantly changing living conditions, which is a modern explanation of the concept for 'health'. This adaptation process is described as phenotypic flexibility of the individual [3]. Experts in nutrigenomics define the phenotypic flexibility as interaction between all processes involved in the metabolic adaptation. Therefore, the main goal is to select a wide range of biomarkers from genetics, transcriptomics, proteomics, metabolomics fields, behavioral changes and others to evaluate individual's adaptation capacity and by that – his/her health status [4–6].

One of the main goals of recent nutrition studies is to identify and develop standardized methods and techniques to study the changes in the phenotypic flexibility in response to nutrition, lifestyle, physical activity, obesity and other factors. The ability of the organism to regain homeostasis, after its balance has been disturbed by external factors, can be used as an indicator for metabolic health. Stress tests are constructed with the aim to temporarily disturb the homeostasis of the body. In response to such stress tests, the system will aim to restore the balance usually within hours [7].

Bacterial lipopolysaccharides (LPS) have been widely used in models studying inflammation *in vitro* and *in vivo* [8–11] or the mechanisms of anti-inflammatory action of a variety of compounds or plants [12–15]. Treating with LPS can modulate the gene expression by raising the cytosolic protein levels of cytokines (such as IL-1β and IL-6) and pro-inflammatory enzymes (e.g. iNOS) *via* activation of NF-κB transcription factor [13, 16–18]. Thus, application of LPS stimuli appears to be a useful tool to trigger inflammatory response in cell culture and *in vivo*. White blood cells circulate over the whole body and respond to various endogenous and exogenous stimuli. Recent studies, especially transcriptome analyses, show that peripheral blood mononuclear cells (PBMC) are a

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valuable source of data and representative target tissue in intervention studies. Priorities in applying PBMC are: accessibility of the blood samples, easy isolation of PBMC from whole blood, potent complex tissue in studying challenges and complex responses to different stimuli [9–11, 15].

In our study, we approached to include application of LPS stimulation that can induce inflammatory and oxidative stress response in PBMC freshly isolated from healthy subjects, which is thought to be representative as an example of stress test. Changes in gene expression in these cells can be informative about their susceptibility to such stimuli and differences in this response after an intervention can be used as an indicator about altered phenotypic response. We included intake of infusion from the herb *Agrimonia eupatoria* (AE) by healthy subjects as a possible source of compounds with a potential for counteraction to the inflammation stimuli. In addition, we checked whether the response will depend on Body Mass Index (BMI). Expression levels of antioxidant defense and inflammation related genes were analysed.

Agrimonia eupatoria L. (AE) is an herbal remedy used in Bulgarian folk medicine. Because of its high polyphenol and flavonoid content, it is established that the herb is a valuable source of antioxidants and possesses anti-inflammatory properties [19–21].

It is usually applied in prevention and treatment of liver, kidney and gall bladder diseases, conditions like mild diarrhea, pulmonary and gastrointestinal inflammatory diseases, ulcers, bleeding gums, rheumatism, hemorrhoids, and even in diabetes or obesity [22]. Enrollment of AE tea in an intervention study with the application of the PBMC LPS stimulation model would provide data about its healing properties based on antioxidant and/or anti-inflammatory activities.

MATERIALS AND METHODS

Plant material and infusion procedure

For implementing the intervention we used dry aerial parts of the plant, readily available in the drugstores. The procedure for preparing the infusion was following the traditional agrimony tea recipe: 2.5 g of the plant material was infused with 200 mL of boiling water for 10 min. The tea was prepared in the Department of biochemistry, molecular medicine and nutrigenomics, Medical University – Varna, Bulgaria and volunteers consumed it on the spot.

Intervention and volunteers

Prior to intervention an approval from the local ethics committee was received (Protocol №27/21.02.2013). Each one of the volunteers who responded to the invitation and joined the intervention was first interviewed about their lifestyle habits, health status and tea consumption frequency and habits. They all signed informed consent prior to the start of the intervention.

The intervention included 40 clinically healthy volunteers, aged between 20 and 60 years. They were divided in two groups regarding their Body Mass Index (BMI) – 23 subjects with BMI<25 – normal weight (NW), and 17 with BMI≥25 overweight subjects (OW). They consumed 200 mL/day of agrimony tea prepared as described above for a period of 25 days.

PBMC collection and separation

Fasting blood samples were collected before start of the intervention (Day 0) and at the end of the intervention period (Day 25) using lithium heparin vacutainer tubes. Whole blood was used to continue further with the isolation of PBMC. For that purpose we used LeucoSep™ centrifuge separation tubes (by GreinerBioOne, Austria) containing a porous barrier which enables cell separation by means of density gradient centrifugation following the manufacturer's instruction.

Cell cultivation and experimental design

Cell yield was determined using standard trypan blue staining method and seeded in density of 1×10^6 cells/well in RPMI 1640 (Sigma-Aldrich, Germany), supplemented with 0.01M HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), 2 mM α-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, and 10% heat-inactivated FBS (Sigma-Aldrich, Germany).

Stimulation of the cells was performed with bacterial LPS (*Escherichia coli* 026:B6, Sigma-Aldrich, USA), diluted in saline solution (0.9 % NaCl). Final concentration used in PBMC treatment was 100 ng/mL.

Both at Day 0 and Day 25 there were two treatment groups – control, cultivated only with RPMI medium and the test group, cultivated in culture medium containing 100 ng/mL LPS. Flasks were incubated for 4 h at 37°C in a humidified chamber with 5% CO_2 atmosphere. Each treatment was performed in duplicate.

RNA isolation and cDNA synthesis

After the incubation period, total RNA was extracted from the cells with Tri reagent (Ambion®, Life Technologies, USA). RNA was subsequently DNase treated (RiboPure™ – Blood Kit; Sigma-Aldrich, USA). First strand cDNA synthesis was performed with 0.8 μg of total RNA using Thermo Scientific M-MuLV reverse transcriptase (USA) following the steps of manufacturer's instructions.

Real-Time PCR

Quantitative gene expression analysis was performed using two-step real-time qPCR. Each reaction was amplified in a reaction mix containing SYBR Green qPCR 1 × Master Mix with ROX (KAPA SYBR FAST qPCR Kit, KAPA BIOSYSTEMS, USA) and 0.3 μM of each primer.

Primer sequences used for the Real-Time PCR were as follows: RPL37A (Bioneer, USA) Forward ATTGAAATCAGCCAGCACGC; Reverse AGGAACCACAGTGCCAGATCC; IL-1β (Alpha DNA, Canada) Forward TCCCCAGCCCTTTTGTTGA, Reverse TTAGAACCAAATGTGGCCGTG; IL-6 (Bineer, USA) Forward AAACAACCTGAACCTTCCAAAGA, Reverse GCAAGTCTCCTCATTGAATCCA; GCLc (Bioneer, USA) Forward GGAGGAAACCAAGCGCCAT, Reverse CTTGACGGCGTGGTAGATGT; SOD1 (Invitrogen, USA) Forward GTGCAGGTCCTCACTTTAAT, Reverse CTTTGTCAGCAGTCACATTG. Analysis was performed on AppliedBiosystems® 7500 Real-Time PCR instrument (USA). The amount of mRNA of each gene of interest was normalized according to the amount of mRNA encoding RPL37A. Gene expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method [23]. Each sample was analyzed at least in triplicate. The results are presented as relative units mRNA±SEM.

Statistical analysis

GraphPad Prism 7.0 software (USA) was used for statistical analysis and graphics. Student's *t* test was used for column srtatistics. A *p* value of less than 0.05 was considered statistically significant.

RESULTS AND DISCUSSION

This study was undertaken in order to test wherther an *ex vivo* stimulation of isolated human PBMC with LPS would detect changes in the response, expected after 25 day intake of an herbal remedy – *Agrimonia eupatori*a tea. We examined the effect on expression of four selected genes – antioxidant defense related (glutamate-cysteine ligase, GCLc and superoxide dismutase 1, SOD1) and inflammation related (IL-1β and IL-6). We compared the response to LPS stimulation before Day 0 to the response after Day 25 of the intervention period. We also determined the effect of AE tea intake on respective genes expression in non-stimulated cells again before and after the intervention. In addition we compared the response of PBMC from normal weight (NW) subjects to the response of overweight (OW).

Effect of ex vivo LPS stimulation on PBMC gene expression – LPS Day 0 vs. untreated Day 0 or verification of the model for stimulation of genes expression under LPS treatment

Various studies prove the potential application of PBMC in diagnostics of diseases like myeloid leukemia, atherosclerosis, autoimmune disease, etc. which are characterized with a specific gene expression profile of the PBMC [24–28].

Lipopolysaccharides (LPS) are the most abundant component within the cell wall of Gram-negative bacteria. They can stimulate the release of inflammatory cytokines in various cell types, leading to an acute inflammatory response toward pathogens [29]. Acute inflammation is characterized by increased blood flow and vascular permeability, accumulation of fluid, leukocytes, and inflammatory mediators like cytokines. The cytokines are the main inflammatory mediators which orchestrate the inflammatory response on the level of cell activation and infiltration, as well as the systemic responses to inflammation. Cytokines involved in acute inflammation are IL-1, TNF-α, IL-6, IL-11, IL-8 and others. IL-1 and TNF-α are the primary cytokines that mediate acute inflammation induced in animals by intradermal injection of bacterial LPS [30]. Main source of IL-1α and IL-1β are the mononuclear phagocytes, fibroblasts, keratinocytes and T and B lymphocytes. Both cytokines play a role in the fever induction. They activate cyclooxygenase (COX) and increase the prostaglandins synthesis [29]. They also stimulate the T cell proliferation. There are data from *in vitro* and *in vivo* studies which prove that IL-1α and IL-1β also induce the synthesis of C-reactive protein (CRP) which is a protein from the acute phase of inflammation [29]. In the acute phase of inflammation IL-6 acts as a growth factor for mature B cells and stimulates their transformation into antibody-producing plasma cells. Up-regulation of IL-6 production is established also in a variety of chronic

inflammatory and autoimmune disorders like thyroiditis, type I diabetes, rheumatoid arthritis, etc. [31, 32].

It is known that oxidative stress and production of reactive oxygen species (ROS) is provoking inflammatory processes and production of chemokines and cytokines [33]. On the other hand, bacterial endotoxins that bind to TLR4 and activate NF-κB pathway also stimulate the NOX4 complex, which in turn generates ROS [34, 35]. This in turn raises the question about the effect on the antioxidant enzymes which are responsible to combat the action of ROS after such inflammatory stimuli. Gamma glutamate-cysteine ligase (GCL) is the rate-limiting enzyme in *de novo* biosynthesis of glutathione which is the most abundant endogenous antioxidant in the cell. Superoxide dismutase (SOD) catalyzes the transformation of the superoxide anion to hydrogen peroxide. Compounds or conditions that increase the production of the superoxide anion induce the activity of different SOD isoforms.

According to literature, the peak of the cytokine release after LPS stimulation in cell culture is reached until the 4th hour and this is the reason why we chose this time period for LPS stimulation [10].

We detected whether the *ex vivo* LPS stimulation is effective and analyzed the response of PBMC to the LPS stimuli by measuring the changes in gene expression levels of IL-6, IL-1β, GCLc and SOD1 in both groups of NW and OW.

Before the intervention period, Day 0, four hour incubation of *ex vivo* cultured PBMC with 100 ng/mL LPS resulted in a significant increase in IL-1β and IL-6 both in NW ($p<0.001$) and OW ($p<0.05$) groups, and also of GCLc in NW group ($p<0.001$) (figure 1).

Treating the PBMC of NW subjects with LPS on Day 0 increased mRNA levels for IL-6 approximately 14 times ($p<0.001$), and in the PBMC of OW group – nearly 2.5 times ($p<0.05$). Similarly, treating with LPS preceding the intervention led to elevation in the levels of IL-1β of both groups – in NW group it was by 3.7 times ($p<0.001$) and in OW – by 2.8 times ($p<0.05$). Notably before the intervention with agrimony intake treating of PBMC with LPS significantly stimulates transcription of GCLc in NW subjects by 5 times ($p<0.001$), while the induction in OW group is visible, but not statistically significant. We established no significant changes for the levels of SOD1 gene expression in both groups (Figure 1).

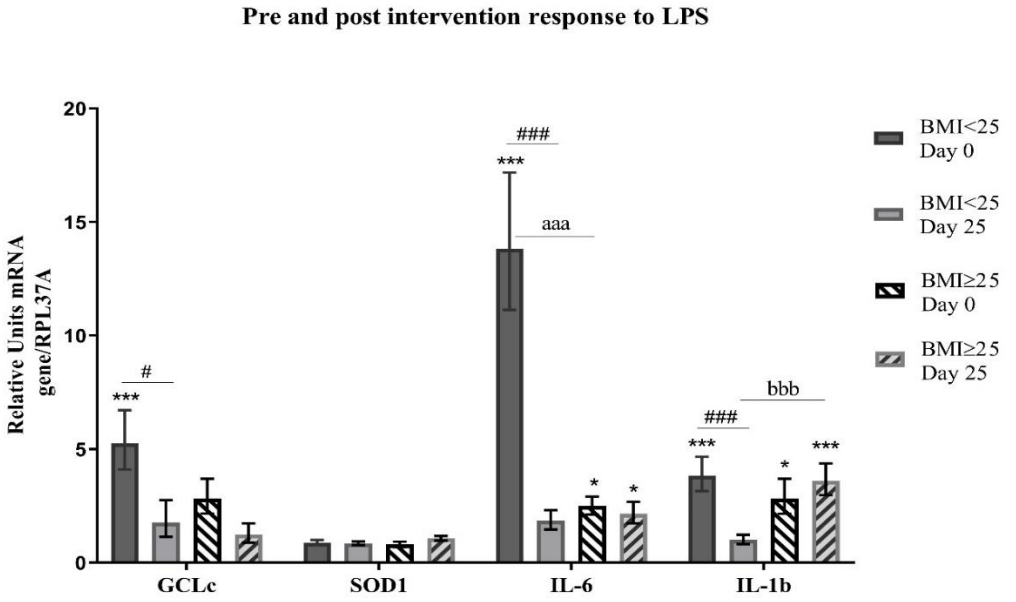

Figure 1. Changes in gene expression levels as a response of PBMC to LPS stimulation vs. untreated cells for both groups of volunteers – NW (BMI<25) and OW (BMI≥25). Gene expression is presented as relative units mRNA ±SEM normalized to RPL37A as endogenous control gene. Legend: *p<0.05, ***p<0.001 vs. untreated cells; #p<0.05, ###p<0.001 Day 25 vs. Day 0; ^{aaa}p<0.001 Day 0 BMI<25 vs. BMI≥25; ^{bbb}p<0.001 Day 25 BMI<25 vs. BMI≥25.

As seen on Figure 1, on Day 0 before the intervention period treatment with bacterial LPS induces several times and statistically significant the mRNA levels of both cytokines, which proves the effectively induced inflammatory response in these cells. It is known that the oxidative stress and free radicals production is linked to inflammation conditions and cytokines production [33]. The

observed induction of the gene for GCLc in the NW group ($p<0.001$) is probably linked to stimulation of glutathione synthesis, presumably evoked due to the need of an antioxidant agent to combat an eventual oxidative stress during the application of inflammatory stimuli.

Effect of ex vivo LPS stimulation on PBMC gene expression after the AE tea intake – LPS Day 25 vs. untreated Day 25 or what is PBMC reactivity after the intervention

Intervention with AE tea for a period of 25 days resulted in a lower effect of the same treatment conditions on cultured PBMC from the NW group. On Day 25, treatment with LPS in the NW group did not induce the gene expression of IL-1β anymore, while the OW group still remained sensitive to the inflammatory stimuli by an increase in the expression of this gene by 3 times ($p<0.001$) compared to the non-treated cells (Fig. 1). Similarly, after 25 days of intervention, gene expression of IL-6 in the PBMC of NW group was not affected by LPS stimulation, but the levels in the OW group were approximately 3 times increased ($p<0.05$). No significant changes about the mRNA levels of GCLc and SOD1 were established after application of LPS on Day 25 both in NW and OW groups. This might be interpreted as an increased sustainability of NW subjects' PBMC to inflammatory stimuli after the 25 days agrimony intake period and could be perceived as an improved phenotypic flexibility profile. Considering the BMI as a factor with a high impact on the inflammatory profile outlook, it is known that overweight and obesity are characterized by a higher mass of adipose tissue, which is known to be accompanied by a low-grade inflammation in a different degree [36]. Presumably, this could be a reason for the sensitivity of PBMC of OW subjects to an additional inflammatory stimulation.

Effect of AE tea intake on the response to LPS treatment – LPS Day 25 vs. LPS Day 0 or is there any difference between gene expression levels in LPS provoked cells before and after intervention

Significantly lower mRNA levels of IL-6, IL-1β and GCLc ($p<0.001$, $p<0.001$ and $p<0.05$, respectively) were established after the intervention period in LPS treated cells of the NW group only. There were no significant differences in gene expression of the analyzed cytokines and antioxidant enzymes in LPS treated PBMC of OW group (Fig. 1).

Comparing the levels of IL-1β and IL-6 mRNA in cells treated with LPS on Day 0 and Day 25 we see that there are significantly lower levels (approximately 3.8-fold for IL-1β and 7.6-fold for IL-6, $p<0.001$) after the intervention in the NW group. This confirms the reformed response to LPS stimuli probably manifesting an improved phenotypic flexibility of these cells. However, we did not observe such changes in the OW group as they remain as sensitive to the inflammatory stimulation as they were before the intervention period. Conditions like diabetes, metabolic syndrome, and diseases caused or accompanied by chronic low-grade inflammation are considered to appear in cases where metabolic homeostasis and therefore phenotypic flexibility is disturbed. Our results probably confirm the reduced capacity of PBMC from OW subjects to manifest phenotypic flexibility of some degree, which might be explained by the metabolic changes they probably already bear. Similarly, in the NW group mRNA levels of GCLc are significantly lower (3-fold, $p<0.05$) in conditions of LPS stimuli application after the intervention period. After the 25 days of the intervention period the inflammatory and oxidative response of PBMC from the NW group is significantly weaker when treated with LPS. An intake did not result in a difference in SOD1 mRNA levels in LPS treated cells on Day 0 and Day 25 in both NW and OW groups.

Difference between NW and OW groups in regard to their response to LPS stimulation before and after the AE tea intake – LPS Day 0 NW vs. LPS Day 0 OW and LPS Day 25 NW vs. LPS Day 25 OW or how the BMI affects the LPS response

Gene expression of IL-6 in response to LPS stimuli on Day 0 was significantly lower in the OW group when compared to NW (about 5.5-fold, $p<0.001$), while there was no significant difference for Day 25 (Fig. 1). With reference to IL-1β we observed no significant difference when comparing Day 0 of LPS response of both groups of volunteers whereas a significantly higher level of gene expression for LPS stimualated cells on Day 25 was estimated for OW (3.6-fold, $p<0.001$). There was no significant difference in the changes of antioxidant enzymes gene expression in response to LPS stimulation between both groups.

It is known that adiposity is characterized with increased infiltration of macrophages in the adipose tissue [37]. It is considered that these macrophages are the main source of inflammatory mediators such as TNF-α and IL-6 which disturb the normal function of adipose tissue by inducing the inflammatory profile and suppressing the insulin potency in the tissue [38–40].

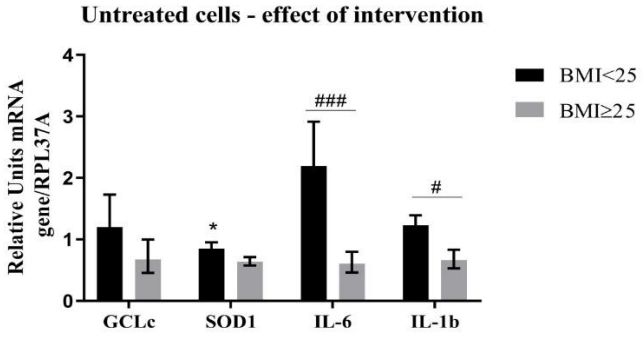

Figure 2. Changes in gene expression levels in untreated PBMC as a result of the 25 days of AE intervention. Gene expression is presented as relative units mRNA ±SEM normalized to RPL37A as endogenous control gene. Legend: *p<0.05, Day 25 vs. Day 0; #p<0.05, ###p<0.001 BMI<25 vs. BMI≥25. Each column represents changes in levels of gene expression in untreated control cells after the AE intervention (Day 25) compared to the expression on Day 0 as a control.

Presumably constant low-grade inflammation and cytokine activity in obese subjects leads to lower susceptibility to inflammatory stimuli represented as significantly lower IL-6 levels at the starting point (Day 0) in comparison to NW, while levels of IL-1β are visibly but not significantly lower.

After the tea intake period (Day 25) we observed a drastic drop in the levels of cytokines expression for the NW subjects since they might be with increased stability towards the LPS stimuli. When comparing the response between them and OW in Day 25 it is visible that IL-1β is expressed siginifcantly higher in OW (3.6-fold, p<0.001) (Fig. 1). The latter group manifests similar levels of both cytokines expression on Day 0 and Day 25 apparently regardless of herbal intervention. This in contrast is not the case in the NW group and they display significantly lower IL-1β levels than the OW group.

Effect of AE tea intake on gene expression in non-stimulated cell – control Day 0 vs. control Day 25 or how AE tea intake changes inflammatory/redox status in non-compromised individuals

Data indicate that AE tea intake does not influence inflammatory/redox status in healthy, non-compromised individuals, as represented by the lack of difference in studied genes expression before (Day 0) and after (Day 25) the intervention. The only statistically significant difference after the 25 days of AE infusion intake is observed in the NW group, where levels of SOD1 gene expression are significantly decreased compared to the levels of mRNA before the intervention (by 15%, p<0.05). Presumably, by being rich in polyphenols and a powerful antioxidant itself, the agrimony herbal infusion is compensating the need for the antioxidant enzyme SOD1 and therefore reducing its gene expression by mechanisms which need further investigation to be established.

When comparing the differences in the expression levels of studied genes between the NW and OW groups we established a stronger change in cytokines expression levels in the NW (approximately 4-fold for IL-6 and 2-fold for IL-1β) (Fig. 2). We may speculate that this is a possible immunostimulatory effect of the herb itself where the OW subjects are expected to already possess higher cytokine expression because of the suggested low-grade inflammation and probably the tea intake doesn't affect them with the similar intensity. This, in turn, is also observed and confirmed by the lower response to LPS stimulation of these subjects (Fig. 1).

In literature, there are *in vitro* studies investigating the biological effect of different herbs on PBMC gene expression and response to inflammatory stimuli [13, 14, 41, 42]. However, intervention studies comparing the *in vivo* effect by *ex vivo* LPS stimulation and response of PBMC from individuals with varying BMI are difficult to find [43, 44]. Yet, interventions applying this approach are limited but still gaining popularity because of its multilateral informative potential [45–47].

CONCLUSION

The main aim in our study was to establish a well-working model for induced inflammation in PBMC isolated from human whole blood by application of bacterial LPS. Changes in the transcription levels of IL-6 and IL-1β in PBMC cells are informative about their susceptibility to LPS stimuli, which can be used as a stress test in studying phenotypic response and assessment of the

phenotypic flexibility under different conditions. Furthermore, this outline can be used for examining anti-inflammatory and antioxidant properties of the tested herbal extracts, fractions or isolated compounds. Our study also demonstrates differences in the metabolic and phenotypic flexibility of subjects varying in their BMI, as well as differences in their potential to counteract to environmental challenges and stimuli. AE tea intake leads to decreased response to LPS stimulation in NW subjects, which could be an explanation of its possible preventive anti-inflammatory properties. In addition to unchanged reactivity to LPS treatment as a result of AE intake, OW individuals demonstrate no ability of their PBMC to respond to agrimony intake with a change in studied genes, with the exception of SOD1.

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Chemical composition and antioxidant activity of partially defatted milk thistle (*Silybum marianum* L.) seeds

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Milk thistle (*Silybum marianum* L.) is a herbaceous plant of the Asteraceae family that is widely grown in Bulgaria. It has been used in Egypt and Europe for over 2000 years as a medicinal plant for protecting, detoxifying and regenerating liver, gallbladder, spleen, and the entire body. The hepatoprotective effect of milk thistle is due to a mixture of flavonolignans contained in its seed, collectively called silymarin. Some producers of milk thistle process its seeds to obtain seed oil, then discarding the silymarin-rich residues. In our study, we investigated the chemical composition and antioxidant activity of partially defatted *S. marianum* seeds obtained after cold oil pressing. We found the milk thistle seed pressings to be a very rich source of silymarin – 3050 mg/100 g, expressed as silibinin. The total polyphenol content of seed pressings was 2796.4 mg/100 g, whereas their antioxidant activity measured by ORAC and HORAC methods was 1425.8 μmol TE/g and 192.6 μmol GAE/g, respectively. The investigated material was a very rich source of protein - 20.5 g/100 g and contained 10.8 g/100 g residual oil. The total carbohydrate content was 42.2 g/100 g embracing 39.7 g/100 g polysaccharides and 0.9 g/100 g reducing sugars. Free sugars in milk thistle seed pressings included fructose, glucose, galactose, rhamnose, xylose, and sucrose. Based on these results, it can be concluded that the residual pressings from *S. marianum* seeds after oil extraction are a rich source of protein, lipids, and carbohydrates. Moreover, they contain a substantial amount of silymarin and could be used as a hepatoprotective functional food or food supplement.

Keywords: Milk thistle (*Silybum marianum* L.); oil pressings; silymarin, chemical composition, antioxidant activity

INTRODUCTION

Milk thistle (*Silybum marianum*, Asteraceae) is an annual or biennial plant, native to the Mediterranean region, which grows in many warm and dry regions, mainly in Europe, Asia, and North America [1]. It has been used for centuries in Egypt and Europe as a medicinal plant for protecting, detoxifying and regenerating liver, gallbladder, spleen and the entire body. In recent years, several studies have indicated that milk thistle has antioxidant, antiatherosclerotic, antihypertensive, anti-obesity, anti-diabetic, anti-inflammatory, and anti-carcinogenic effects [2]. The hepatoprotective effect of *S. marianum* seeds is due to a mixture of flavonolignans collectively called silymarin [3]. It is mainly composed of silibinin, silychristin, silidianin, and isosilybin. Silymarin is found in the whole plant of milk thistle, including fruits, seeds, roots, stems, and leaves, but it is the most abundant in the seeds [4]. The anticancer activity of silymarin, as well as of silibinin was demonstrated against various cancer cells, such as breast, skin, colon, cervix, ovary, prostate, lung and hepatocellular cancers [5-8]. Silymarin has been shown to reduce the plasma levels of cholesterol, thus having a potential as a hypocholesterplaemic agent [9]. Apart from silymarin, milk thistle seeds contain a high amount of oil, exceeding 20%, as well as numerous beneficial components such as essential amino acids, carbohydrates, minerals, and phytochemicals with antioxidant or antimicrobial effects [10]. Due to the complex and expensive processing on the extraction and purification of silymarin, some producers of *S. marianum* use its seeds to obtain milk thistle oil, then discarding the silymarin-rich residues. The oil has a high concentration of unsaturated fatty acids, especially linoleic (omega-6) and oleic (omega-9) acids, which are beneficial to human health in preventing atherosclerosis, diabetes, and cancer [11]. Milk thistle seed oil has been recommended as a potential source of natural antioxidants, since it is rich in vitamin E [12]. Therefore, the present study aimed to investigate the chemical composition and antioxidant activity of partially defatted *S. marianum* seeds, obtained after cold oil pressing, in order to assess their potential to serve as a silymarin-rich food supplement.

MATERIALS AND METHODS

Chemicals

All solvents (HPLC grade) and reagents were purchased from Sigma-Aldrich (Steinheim, Germany).

Sugar standards (D-(+)-Fru, D-(+)-Glc, D-(+)-Gal, D-(+)-Xyl, Suc) were purchased from Sigma-

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Aldrich and Sigma Life Sci. L-Rhamnose (Alfa Aesar, Germany, 98+%) was purchased from VWR Prolabo.

Milk thistle seeds

Partially defatted milk thistle seeds, obtained after cold pressing, were supplied by Phytoviv Ltd. (Veliko Tarnovo, Bulgaria).

Extraction of carbohydrates

The extraction of carbohydrates was performed according to Denev *et al.* [13] with some modifications. Briefly, approximately one gram of partially defatted *S. marianum* seeds was accurately weighed and subjected to extraction with 30 mL of distilled water for 1 h at 30°C on a shaking thermostatic water-bath (NÜVE, Turkey). After that, the samples were centrifuged (6000×g) and the supernatants were used for HPLC analysis of sugars.

Extraction of silymarin and other phenolic compounds

Extraction of silymarin and other phenolic compounds was performed according to the procedure described for milk thistle seeds in the British pharmacopeia, 2013 [14]. Briefly, 5 g of partially defatted milk thistle seeds were placed in a continuous-extraction Soxhlet apparatus and 100 mL of light petroleum was added. Extraction was performed at 70°C in a water-bath for 8 h. The solid residue was separated and dried at room temperature. Petroleum ether was evaporated *in vacuo* and the residual lipid extract was weighed in order to determine the lipid content of the partially defatted seeds.

The defatted seeds were placed in a continuous-extraction Soxhlet apparatus with 100 mL of methanol and extracted at 60°C in a water-bath for 5 h. The methanolic extract was reduced *in vacuo* to a volume of about 30 mL, filtered into a 50 mL volumetric flask, by rinsing the extraction flask and the filter, and diluted to 50 mL with methanol. Five mL of this solution was further diluted to 50 mL with methanol and used for the determination of silymarin, total polyphenols, ORAC and HORAC analyses.

Determination of protein content

The protein content of milk thistle seeds was evaluated by the micro-Kjeldahl method [15]. Acetylacetone-formaldehyde colorimetric method, using ammonium sulfate as a standard, was used for the determination of nitrogen expressed as ammonia content of the digested sample [16]. The results were calculated using 6.25 as a conversion factor.

Determination of total carbohydrate content

The total carbohydrate content of *S. marianum* seeds was determined by the phenol-sulfuric acid method, using glucose for the calibration curve construction [17]. Firstly, the defatted plant material was incubated at 50 °C for 1 h with 70% (v/v) aqueous ethanol and then the solids were separated by centrifugation (14,000×g, 10 min, 5 °C). The same procedure was repeated 3 times. Finally, the residue was washed 2 times with acetone at room temperature and vacuum-dried to obtain alcohol-insoluble solids (AIS). Before analysis, the AIS samples were solubilized with 72% (w/w) H_2SO_4 for 1 h at 30 °C, followed by a hydrolysis step with 1 M H_2SO_4 for 3 h at 100 °C [18]. The obtained hydrolyzates were used for the determination of total carbohydrate content. The absorbance was measured at 490 nm.

Determination of reducing sugar content

The reducing sugar content of the samples was estimated by the method of Miller with the 3,5-dinitrosalicylic acid reagent, using glucose for the calibration curve construction [19]. The extraction of soluble sugars was performed according to Denev *et al*. [18]. In brief, approximately 1 g of the defatted milk thistle seeds were weighed and subjected to extraction with 30 mL of distilled water for 1 h at 30 °C on a magnetic stirrer. Before analysis, the mixture was centrifuged (6000×g) and the supernatant was used after an appropriate dilution for analysis of reducing sugars.

Total polyphenol compound analysis

The total polyphenols were determined according to the method of Singleton & Rossi with the Folin-Ciocalteu's reagent [20]. Gallic acid was used for the calibration curve and results were expressed as gallic acid equivalents (GAE) per 100 g dry weight (DW).

High-Performance Liquid Chromatography (HPLC) analysis of free sugars

The HPLC determination of free sugars was performed on an HPLC system Agilent 1220 (Agilent Technology, USA), with a binary pump and a refractive index detector (Agilent Technology, USA). The column was Zorbax Carbohydrate (150 × 4.6 mm, 5 μm, Agilent), connected to a guard column Zorbax Reliance Cartridge (Agilent), and as eluent was used 80% acetonitrile in water at a flow rate of 1.0 mL/min and temperature 25°C. The quantification of free sugars was done by comparison of areas of the peaks with those of the corresponding standards. Results were expressed as mg/100 g DW.

HPLC analysis of silymarin

HPLC analysis of silymarin was performed according to the procedure described in the British pharmacopeia, 2013, for milk thistle seeds [14] on a HPLC system Agilent 1220 (Agilent Technology, USA), with a binary pump and UV-Vis detector (Agilent Technology, USA). Separation was performed on an Agilent TC-C18 column (5 μm, 4.6 mm × 250 mm) at 25ºC and a wavelength of 288 nm was used. The following mobile phases were used: (A) phosphoric acid, methanol, water (0.5:35:65 V/V/V) and (B) 100% acetonitrile at a flow rate of 0.8 ml/min. The gradient elution started with 0 % A, between 0 min and 28 min linearly increased to 100% B, the same ratio was maintained until 35 min, and then until 36 min A linearly increased to 100%. Silicristin, silidianin, silibinin A, silibinin B, isosilibinin A and isosilibinin B were identified by comparison with the chromatogram obtained with *S. marianum* standardized dry extract CRS. The percentage content of silymarin, calculated as silibinin, was determined using the following expression:

$$\frac{(A1 + A2 + A3 + A4 + A5 + A6) \times m_1 \times p \times 1000}{(A7 + A8) \times m_2 \times (100 - d)}$$

where:

A1 = area of the peak due to silicristin in the chromatogram;

A2 = area of the peak due to silidianin in the chromatogram;

A3 = area of the peak due to silibinin A in the chromatogram;

A4 = area of the peak due to silibinin B in the chromatogram;

A5 = area of the peak due to isosilibinin A in the chromatogram;

A6 = area of the peak due to isosilibinin B in the chromatogram;

A7 = area of the peak due to silibinin A in the chromatogram;

A8 = area of the peak due to silibinin B in the chromatogram;

m_1 = mass of milk thistle standardized dry extract CRS used to prepare the reference solution, in grams;

m_2 = mass of the drug to be examined, in grams;

p = combined percent content of silibinin A and silibinin B in milk thistle standardized dry extract CRS;

d = percent loss on drying of the drug.

Oxygen Radical Absorbance Capacity (ORAC) assay

ORAC was measured according to the method of Ou *et al.* [21] with some modifications, described in details by Denev *et al*. [22]. Solutions of AAPH, fluorescein (FL), and Trolox were prepared in phosphate buffer (75 mM, pH 7.4). Samples were diluted in the phosphate buffer as well. Reaction mixture (total volume 200 μL) contained FL (170 μL, final concentration 5.36×10^{-8} mol/L), AAPH (20 μL, final concentration 51.51 mM), and sample – 10 μL. The FL solution and sample were incubated at 37°C for 20 min directly in a microplate reader, and AAPH (dissolved in buffer at 37°C) was added. The mixture was incubated for 30 s before the initial fluorescence was measured. After that, the fluorescence readings were taken at the end of every cycle (1 min) after shaking. For the blank, 10 μL of phosphate buffer was used instead of the extract. The antioxidant activity was expressed in micromole Trolox equivalents (μmol TE) per liter of extract. Trolox solutions (6.25; 12.5; 25 and 50 μmol/L) were used for defining the standard curve. ORAC and HORAC analyses were carried out using a FLUOstar OPTIMA plate reader (BMG Labtech, Germany), an excitation wavelength of 485 nm, and an emission wavelength of 520 nm were used. The results were expressed in micromole Trolox equivalents (μmol TE) per g DW.

Hydroxyl Radical Averting Capacity (HORAC) assay

The HORAC assay measures the metal-chelating activity of antioxidants in the conditions of Fenton-like reactions employing a Co(II) complex and hence, the protecting ability against the formation of hydroxyl radicals [23]. Hydrogen peroxide solution of 0.55 M was prepared in distilled water. 4.6 mM Co(II) solution was prepared as follows: 15.7 mg of $CoF_2.4H_2O$ and 20 mg of picolinic acid were dissolved in 20 mL of distilled water. Fluorescein - 170 μL (60 nM final concentration) and 10 μL of the sample were incubated at 37°C for 10 min directly in the FLUOstar plate reader. After incubation,10 μL of H_2O_2 (27.5 mM final concentration) and 10 μL of Co(II) (230 μM final concentration) solutions were added. The initial fluorescence was measured, after which the readings were taken every minute after shaking. For the blank sample, phosphate buffer solution was used and 100, 200, 600, 800 and 1000 μM gallic acid solutions (in phosphate buffer 75 mM, pH = 7.4) were used for building the standard curve.

The area under the curves was calculated as it was done for the ORAC. The results were expressed in micromole gallic acid equivalents (μmol GAE) per gram DW.

Statistical analysis

The processing was repeated two times and the analyses were performed in duplicate or triplicate. The results were expressed as mean values ± standard deviations. One-way analysis of variance (ANOVA) and Student's t-test were used to evaluate the differences of the mean between groups. P values less than 0.05 were considered to be significant.

RESULTS AND DISCUSSION

Protein, lipid and carbohydrate contents of partially defatted milk thistle seeds

The total amount of protein, lipids and carbohydrates in partially defatted milk thistle seeds was 73.5%, as shown in Table 1.

Table 1. Protein, lipid, carbohydrate, polysaccharide and reducing sugar contents of partially defatted milk thistle seeds

	Partially defatted milk thistle seeds
Total lipids, g/100 g	10.8
Total protein, g/100 g	20.5
Total carbohydrate, g/100 g	42.2
Reducing sugars, g/100 g	0.9
Polysaccharides, g/100 g	39.7

The total carbohydrate content was 42.2 g/100 g, embracing 39.7 g/100 g polysaccharides. Although *S. marianum* seeds are a rich source of carbohydrates, the amount of reducing sugars is very low - 0.9 g/100 g, indicating the presence of a substantial quantity of polysaccharides. It has been found that milk thistle residues contain different bioactive heteropolysaccharides, which express *in vitro* antioxidant, hemolysis inhibitory, *α*-amylase and *α*-glucosidase inhibitory activities [24]. The investigated material was a very rich source of protein - 20.5 g/100 g and it also contained 10.8 g/100 g residual oil. Compared with the data found in literature, the partially defatted milk thistle seed used in this work contained less oil, due to the preliminary cold pressing. Dabbour *et al.* reported that oil content of *S. marianum* seeds grown in Jordan is 26.90 g/100 g [25], whereas Fathi-Achachlouei and Azadmard-Damirchi reported oil content from 26 to 31 g/100 g in four varieties of *S. marianum* planted in Iran [26]. Additionally, Wallace *et al.* and Abenavoli *et al.* found that the plant seeds contained 15 – 30 % lipids and about 30 % protein [27, 28].

It was of interest to investigate the individual sugars present in the studied raw material. The results from the HPLC analysis are shown in Table 2. The predominant free sugar in milk thistle seeds was sucrose (1643.3 mg/100 g), but fructose, glucose, galactose, rhamnose, and xylose were also found. Quantitative data on the free sugar composition in *S. marianum* seeds were not found in the available literature.

Table 2. Free sugar content (mg/100 g) and composition of partially defatted milk thistle seeds

	Partially defatted milk thistle seeds
Fructose	113.9
Glucose	168.2
Galactose	143.9
Rhamnose	162.5
Xylose	134.2
Sucrose	1643.3

However, Barnes *et al.* have reviewed that the seeds contain arabinose, rhamnose, xylose and glucose [29]. Furthermore, Ghafor *et al.* determined high carbohydrate content in the stems, especially fructose [30].

Polyphenol and silymarin contents, and antioxidant activity of partially defatted milk thistle seeds

The total polyphenol and silymarin contents of the studied partially defatted milk thistle seeds are shown in Figure 1.

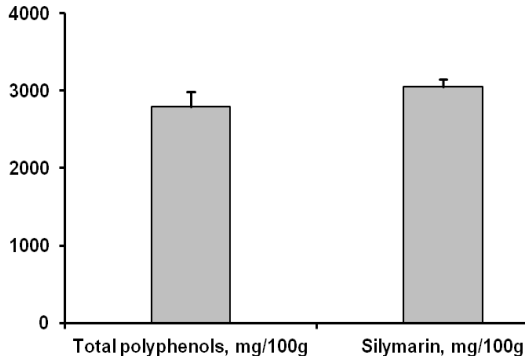

Figure 1. Silymarin and total polyphenol contents of partially defatted milk thistle seeds

The polyphenol content of the investigated material was 2796 mg GAE/100 g and its silymarin content was significant - 3050 mg/100 g. As already mentioned, the hepatoprotective properties of milk thistle are due to its silymarin content, and therefore the studied milk thistle oil pressings (or by-product) could be used as a hepatoprotective functional food or food supplement. It is known that silymarin content and composition strongly depend on *S.*

marianum cultivar, geographical location of cultivation (soil physical properties, weather), and agronomic conditions (time of sowing, fertilizing, irrigation, time of harvest, maturity of seeds) [31-33]. The current study showed that silymarin content is slightly higher than the results proposed by Stancheva *et al.* [34]. However, considerably high content of silymarin has been reported for fruits of *S. marianum* by Hasanloo *et al.* [35]. Hasanloo *et al.* [36] obtained a higher silymarin content in cell cultures of *S. marianum* kept in dark. Our results for the total polyphenol content in the partially defatted milk thistle were comparable to those reported by Mhamdi *et al*. (29 mg GAE/g DW) [37]. On the contrary, Lucini *et al*. [38] indicated a considerably lower total phenolic content in *S. marianum* (3.6 mg GAE/g DW). This difference might be due to both genotypic and environmental factors, extraction conditions and further analysis. Mhamdi *et al*. [37] have also observed that *S. marianum* seed extract contains different phenolic acids and flavoligans. Eight phenolic compounds have been successfully identified in the seeds, and the major were silybin A (12.3%), silybin B (17.6%), isosilybin A (21.9%), isosilybin B (12.8%), silychristin (7.9%) and silydianin (7.5%) [37]. These compounds are known as some of the most active phytochemicals and are largely responsible for the claimed benefit of the silymarin complex.

The antioxidant properties of the studied residual pressings from *S. marianum* seeds were assayed by ORAC and HORAC methods and to the best of our knowledge, this is the first report on the evaluation of ORAC and HORAC antioxidant activity of milk thistle seeds. ORAC method measures the ability of an antioxidant to scavenge peroxyl radicals, whereas HORAC method is an indicator for its metal-chelating properties, thus reflecting its ability to act as a preventive antioxidant [22]. The high amount of silymarin resulted in a very high ORAC value - 1426 μmol TE/g, whereas HORAC result was lower - 192 μmol GAE/g (Figure 2).

Flavonolignans have shown radical scavenging properties and protective effects against the damage of lipid membranes [39] and oxidation of low-density lipoproteins [40]. The antioxidant effect is due to the modulation of pathways such as cell growth, apoptosis, and differentiation [41]. Silymarin can scavenge free radicals, it also has been found to increase the production of glutathione in hepatocytes and the activity of superoxide dismutase in erythrocytes [42]. In another *in vitro* study, free radical scavenging activity and antioxidant properties of silymarin (>200 μM) were shown by four different assays [43].

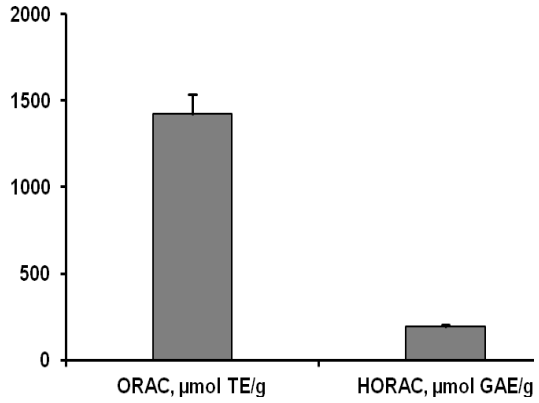

Figure 2. ORAC and HORAC antioxidant activity of partially defatted milk thistle seeds

It is worth mentioning that the free radical scavenging activity of pure individual compounds of silymarin has been reported to vary considerably, with silydianin and silychristin being 2-10-fold more active than silibinin and on a dry mass basis, silymarin is shown to be about 8 times more potent than silibinin as a free radical scavenger [44]. In our study, silymarin ability to scavenge peroxyl radicals was demonstrated by the high ORAC value of the investigated partially defatted milk thistle seeds.

CONCLUSION

Based on our results, it can be concluded that residual pressings from milk thistle seeds, after oil extraction, are a rich source of protein, lipids, and carbohydrates. Additionally, seed pressings contain a substantial amount of silymarin and could be used as a hepatoprotective functional food or food supplement.

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C. Synthetic Analogues of Natural Bio-antioxidants

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Antioxidant activity evaluation of new compounds - hydrazones of bexarotene

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Retinoids are compounds related to vitamin A. The role of vitamin A as an antioxidant has long been known. Vitamin A and its natural and synthetic analogues play an important role in the human body and are implicated in several biological functions. Bexarotene is a third-generation synthetic retinoid used in the treatment of cutaneous T-cell lymphoma. The present paper reports the evaluation of new compounds – hydrazones of bexarotene – about their free radical scavenging activity. The tested substances were synthesized in our previous work and were analyzed by infrared spectroscopy. The antioxidant potential of hydrazones was determined by three different approaches. We used classical 1,1-diphenyl-2-picryl-hydrazyl (DPPH) and 2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid (ABTS) tests. DPPH is a stable free radical which has an unpaired valence electron at one atom of the nitrogen bridge. Scavenging of DPPH radical is the basis of the popular DPPH antioxidant assay. Another approach by which we investigated the antioxidant properties of the new compounds was an electrochemical method. The experimental methodology involves the recording of a voltammogram of cathodes electro-oxygen reduction.

Keywords: retinoids, hydrazones, bexarotene, antioxidant activity

INTRODUCTION

The term retinoids describes the entire set of compounds including both natural and synthetic vitamin A analogs. The antioxidant effects of retinoids and carotenoids are known for decades. Their lipid nature and the localization within the lipophilic compartment of membranes and lipoproteins make retinoids effective in reducing lipid peroxidation by acting as chain-breaking antioxidants [1, 2].

Oxidation reactions are crucial for the maintenance of life. However, oxidative stress can also be damaging and cause different pathological states. Currently, scores antioxidants have been either synthesized or extracted from naturally occurring resources such as fruits, plants, and marine animals. Many of them exhibit good antioxidative activity against DPPH, ABTS, and hydroxyl radical [3].

A number of studies have examined the antioxidant potential of hydrazone derivatives. Hydrazone derivatives synthesized by Musad et al. (2011) are reported to have radical scavenging activity at the concentration of 10 μg/mL. Abdel-Wahab et al. (2011) evaluated imidazoline-based hydrazones 4 by 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid (ABTS) assay and reported them to have promising antioxidant activity [4, 5].

Bexarotene is a synthetic third-generation retinoid compound that exerts its biological action through selective binding and activation of the three retinoid X receptors (RXRs). When the retinoid receptors are activated, they function as transcription factors that regulate cellular differentiation and proliferation. *In vitro*, bexarotene inhibits the growth of tumor cell lines, *in vivo* it causes tumors regression in some animal models and prevents tumor induction in others. However, the exact mechanism of action of bexarotene in the treatment of cutaneous T-cell lymphoma (CTCL) is unknown [6].

There is evidence that some non-phenol hydrazones have the potential to maintain a moderate DPPH neutralization capacity that can be can be explained to the involvement of the -NH bond to antiradical capacity [7].

The present study aims to investigate the antioxidant potential of hydrazones of bexarotene by three different methods.

EXPERIMENTAL

Five newly synthesized bexarotene hydrazones derivatives were tested to determine their antioxidant potential.

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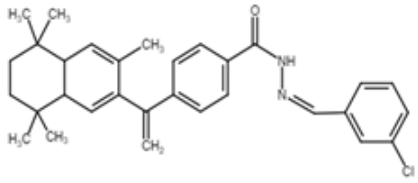
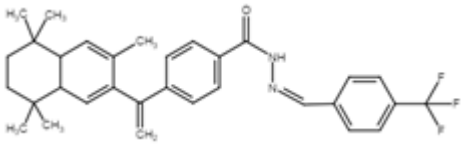
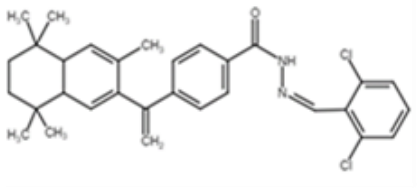
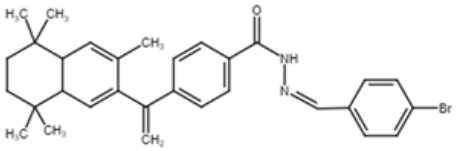
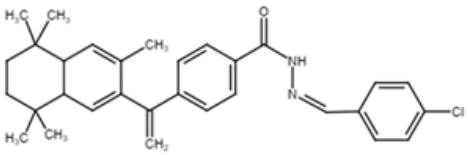
Table 1. Some characteristics of bexarotene hydrazones derivatives

Compound	Chemical structure	Chemical name	Molecular mass	Physical properties
V_1		3-Chlorophenyl-methylidene-4- [1-(3,5,5,8,8-pentamethyl-6,7-dihydronaphthalen-2-yl) ethenyl] benzohydrazide	485	Pale yellow crystalline substance, slightly soluble in water, soluble in methanol and DMSO
V_2		4- (3-fluoromethyl) phenyl-methylidene-4- [1- (3,5,5,8,8-pentamethyl-6,7-dihydronaphthalen-2-yl) ethenyl] benzohydrazide	519	Pale yellow crystalline substance, slightly soluble in water, soluble in methanol and DMSO
V_3		2,6-Dichlorophenyl-methylidene-4- [1-(3,5,5,8,8-pentamethyl-6,7-dihydronaphthalen-2-yl) ethenyl] benzohydrazide	519	Pale yellow crystalline substance, slightly soluble in water, soluble in methanol and DMSO
V_4		4-Bromophenyl-methylidene-4- [1-(3,5,5,8,8-pentamethyl-6,7-dihydronaphthalen-2-yl) ethenyl] benzohydrazide	529	Pale yellow crystalline substance, slightly soluble in water, soluble in methanol and DMSO
V_5		4-Chlorophenyl-methylidene-4- [1-(3,5,5,8,8-pentamethyl-6,7-dihydronaphthalen-2-yl) ethenyl] benzohydrazide	485	Yellow crystalline substance, slightly soluble in water, soluble in methanol and DMSO

For the synthesis of the target hydrazones, bexarotene hydrazide reacts with aldehydes, forming the hydrazones presented in Table 1.

Three different approaches were used to determine the antioxidant potential of the compounds.

DPPH analysis is an approach for evaluating the antioxidant potential of compounds, plant extracts, etc. DPPH assay is characterized a as rapid and accessible method in determining the antioxidant activity of various compounds. The antioxidant activity (AOA) of hydrazone compounds was also determined by the ABTS [2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid)] cation radical decolorization method with modifications.

These assays (ABTS • + and DPPH) are widely used for evaluating the antioxidant capacity of natural products. Both approaches are spectrophotometric techniques based on the extinction of stable color radicals. They exhibit the ability to detect antioxidants even when present in complex biological mixtures such as plant or food extracts.

In addition to the two approaches mentioned above, an electrochemical method was used to determine the antioxidant capacity.

DPPH assay

DPPH assay is a rapid and accessible method in determining the antioxidant activity of various compounds.

The activity rate is calculated by the formula:

$$Ab - Aa \times 100 \quad (1)$$

where: Ab is the absorption of DPPH, Aa- absorption of each sample.

The result is expressed as a percent inhibition of DPPH radical.

To determine the antioxidant potential of our test substances, 4 mg of DPPH dissolved in 1 ml of methanol were used. Transfer the resulting solution quantitatively into a 50 ml round-bottom flask and make up to the mark with methanol. As a result, a solution with a concentration of 1 mM is obtained.

The DPPH concentration of the radical was determined using Trolox as a standard. For this purpose, standard Trolox solutions in methanol were prepared at concentrations of 50, 25, 12.5, 6.25 and 3.125 μM. The solutions thus prepared were stirred on a vortex mixer for 1 min and then incubated at room temperature for 30 min in the dark. Absorption was measured at 517 nm on a Synergy 2 multifunction reader (BioTek). Based on the reported results, the calibration graph presented in Figure 1 was constructed.

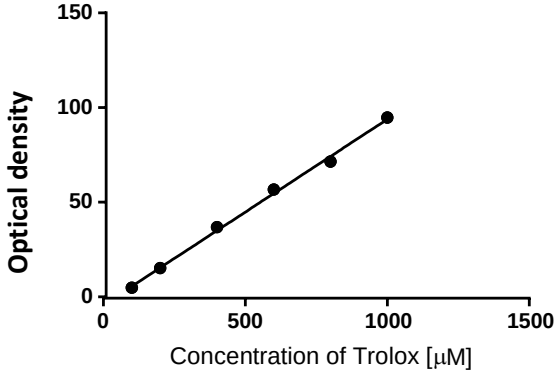


Fig. 1. Calibration graph of Trolox

To prepare the working solutions of the analytes, methanol was again used as the solvent. Solutions with appropriate concentrations were obtained: 1 mg / ml, 0.50 mg / ml, 0.250 mg / ml, 0.125 mg / ml.

The calibration graph was constructed using the obtained data, with the abscissa showing the concentration of the standard in μM and the ordinate the optical density. The resulting graph is presented in Figure 2.

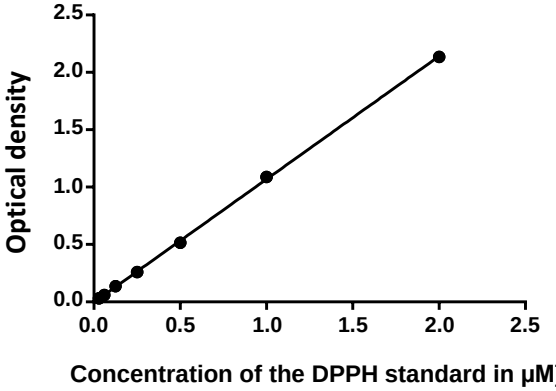


Fig. 2. Concentration of the DPPH standard graph

The decrease in absorption is linearly dependent on the antioxidant concentration. The percentage inhibition of DPPH radical is determined according to the following formula:

$$\% \text{ DPPH} = 1 - (\text{Sample} - \text{Blank} / \text{Control} - \text{Blank}) \times 100, \quad (2)$$

where: Sample - sample absorption (sample + DPPH), Blank - Blank Absorption (Methanol + DPPH), Control - the absorption of pure methanol.

ABTS test

To conduct the ABTS test, 10 μl of the analyte was added to 1000 μl of ABTS + solution in phosphate buffer (pH 7.4). The absorbance of the solution was previously adjusted to values of 0.700 ± 0.02 and read immediately before (0 min) and 6 min after the addition of the sample. Phosphate buffer (pH 7.4) was used as a blank.

Sampling absorption was calculated by the following formula:

$$A = (A \text{ sample } 0 \text{ min} - A \text{ sample } 6 \text{ min}) - (A \text{ blank sample } 0 \text{ min} - A \text{ blank sample } 6 \text{ min}) \quad (2)$$

where: A sample 0 min - the reported absorbance of the sample at the start of analysis; A sample for 6 min - the reported absorption of the sample after 6 min; A blank 0 min - the recorded blank absorbance at the start of the analysis; A blank for 6 min - reported blank absorption 6 min after the start of the analysis.

The AOA of the solution was determined by the calibration line constructed from the absorbance values of the standard solutions presented in Figure 3.

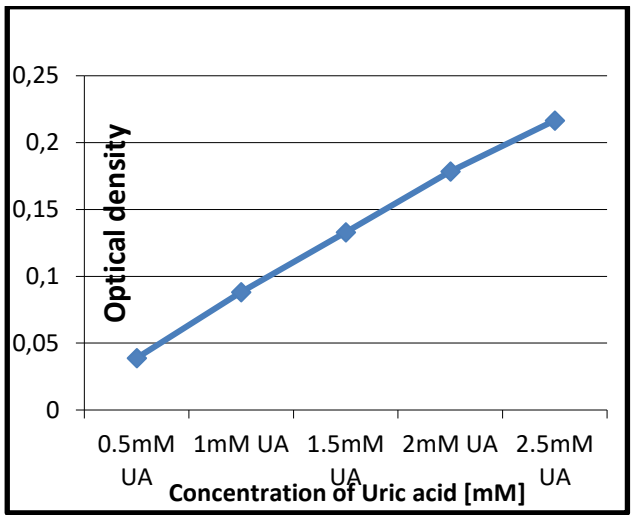


Fig. 3. Calibration line constructed from the absorbance values of the standard solutions

Determination of antioxidant activity by an electrochemical method

The methodology of the experiment is to register a voltammogram of cathodes oxygen reduction using an AOA Analyzer (RU.C.31.113.A N28715) connected to a personal computer. The electrochemical cell is a glass cup with a background electrolyte solution and a working glass carbon electrode and a comparative silver chloride electrode immersed therein. A 0.1 M ethanol solution of $NaClO_4$ with a volume of 10 ml was selected as the background solution.

The antioxidant activity of the samples tested was estimated by the kinetic criterion K (μmol / l.min) which reflects the amount of oxygen reacted with the sample over time and is determined by the following formula:

$$K = \frac{Co_2}{t}\left(1 - \frac{I_i}{I_0}\right) \quad (4)$$

where: CO_2 is the concentration of oxygen in the stock solution, μmol/l; Ii - magnitude of the current of oxygen reduction, μA; Io is the magnitude of the current of electro-reduction of oxygen in the absence of a substance in solution, μA; t - process time, min.

The antioxidant activity of the sample is compared to the antioxidant activity of Trolox by the following formula:

$$AOA = Trial / Krolox \quad (5)$$

where: Sample - the activity of the sample according to the kinetic criterion calculated by the formula, Krolox - Trolox activity according to the kinetic criterion calculated by the formula.

RESULTS AND DISCUSSION

Three different methods for the determination of free radicals scavenging activity were applied in this paper as an attempt to establish the possible antioxidant effects demonstrated by the newly synthesized hydrazones.

All five newly synthesized compounds were tested for their interaction with the stable free radical DPPH and this interaction, in turn, indicated their radical scavenging activity.

Based on the obtained results, we can conclude that the chemical structural features of the hydrazone derivatives of bexarotene do not lead to a significant antioxidant potential.

These results can be explained by the lack of free hydroxyl groups in the skeleton of the compounds obtained. It is apparent that structural change against retinol leads to a change in their potential for action as antioxidants. Structural modification of the molecules does not lead to the formation of antioxidant potential, and this is without exception manifested in all newly synthesized hydrazones.

The results obtained at the DPPH assay of the antioxidant potential of hydrazones are presented in table 2.

Table 2. DPPH assay of antioxidant potential of hydrazones

Compounds	% DPPH neutralization
Bexarotene	0
Bexarotene methyl ester	12
V_1	2
V_2	0
V_3	0
V_4	0
V_5	4

Table 3. ABTS - test to evaluate the antioxidant potential of bexarotenes hydrazone derivatives.

Compounds	C mM/UA
Bexarotene	0
Bexarotene methyl ester	0
V_1	0
V_2	0
V_3	0
V_4	0
V_5	0

Table 4. Antioxidant potential of bexarotenes hydrazone derivatives determined by electrochemical method

Compounds	C, μg/ml	K, μmol/l.min±SD	AOA
Bexarotene	100	17.817±0.993	1.729
Bexarotene methyl ester	100	13.625±0.823	1.322
V1	100	12.053±0.642	1.169
V2	100	11.791±0.322	1.144
V3	100	10.328±0.226	1.002
V4	100	13.014±0.774	1.263
V5	100	12.994±0.628	1.261
Trolox	100	10.306±0.113	1.000

The data obtained from the ABTS test for bexarotene hydrazone derivatives are presented in Table 3. The electrochemical method allows us to determine the overall antioxidant potential of our samples. The analysis shows that the highest antioxidant potential is displayed by bexarotene and subsequent modifications in its structure has led to a decrease in the antioxidant effect. Thus, for compound V3, the value calculated for the antioxidant effect by an electrochemical method of analysis almost coincides with the value of Trolox, namely 1.002 AOA for V3 and 1.000 for Trolox.

The data obtained on the antioxidant potential of bexarotene hydrazone derivatives are presented in Table 4.

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Structure-activity relationship of *in vitro* radical-scavenging activity of 2-(hydroxyphenyl) benzothiazole derivatives

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The introduction of the lipophilic benzothiazole group increases the oxidant stability of the phenols and modifies their antioxidant activity. Finding a suitable method for detecting these modifications will afford studying of the structure-activity relationship. The aim of this study is to examine the antioxidant activity of 2-(hydroxyphenyl) benzothiazole derivatives obtained in high yields (56%–94%) by one-pot *α*-amidoalkylation reactions of benzothiazole, alkyl chloroformates with various phenols. Synthetic series of 2-(4-hydroxyphenyl), 2-(dihydroxyphenyl) and 2-(trihydroxyphenyl) benzothiazole derivatives were evaluated *in vitro* for their DPPH and ABTS free radical scavenging activities and compared to the radical scavenging activity of natural compounds – rutin, quercetin, gallic acid, catechol, resorcinol, hydroquinone and pyrogallol, defined under the same conditions. The radical scavenging activity of 2-(hydroxyphenyl) benzothiazole derivatives was analysed by taking rutin as positive standard and compared their IC_{50} values. Antioxidant activity mainly depends on the number and position of phenolic hydroxyl groups. The benzothiazole compounds **6ac**, **7bd** containing pyrogallol and resorcinol fragment demonstrated similar activity as the natural antioxidant - quercetin. The results obtained using the ABTS method showed possibility for studying the structure - activity relationship of the tested examples.

Keywords: Antioxidant activity, free radical scavenging potential, phenols, structure-activity relationship, benzothiazole derivatives, DPPH and ABTS assays.

INTRODUCTION

Antioxidant activity is one of the important characteristics for estimating the medicinal properties of polyphenols and various synthetic analogues. The definition of antioxidants as substances that can efficiently reduce pro-oxidants with concomitant formation of products without any or low toxicity is well known [1]. Conventional definition of antioxidant was suggested by Halliwell and Gutteridge [2] as "any substance that when present at low concentrations, compared to those of an oxidizable substrate, significantly delays or prevents oxidation of that substrate". The presence of free radical-scavenging activity is necessary but not sufficient condition for the manifested antioxidant capacity of a compound [3].

The activity of antioxidants depends not only on their structure, but also on the concentration in which they are present, the properties of the oxidized substrate, the conditions of oxidation, including the environment and temperature [4]. From the tested set of flavonoids in the literature, quercetin exhibits the highest free radical-scavenging and antioxidant activity explained by the presence of the structural fragments according to the criteria [5].

It is possible for one compound to play a role as a free radical scavenger and as lipid oxidation inhibitor, but quite often the strongest radical traps appear as weak inhibitors (antioxidants) and *vice versa* [6].

Phenols and their derivatives show various ranges of biological activity due to their ability to inhibit various enzymes. They are widely used in medicine as antioxidants and preservatives in the food industry [7]. Catechol and pyrogallol are allelochemicals which belong to the phenolic compounds synthesized in plants [8].

Benzothiazole and its derivatives are an important class of heterocyclic compounds which are a common feature of many natural products and pharmaceutical agents. 2-Aryl benzothiazoles show a wide variety of chemotherapeutic activities including their use as antitumor and antibacterial agents. In recent years, the discovery of new methods for synthesis of 2-substituted benzothiazoles plays an important role in organic synthesis [9].

In earlies studies we synthesized new benzothiazole derivatives *via* one-pot approach of *α*-amidoalkylation. This method was successfully applied for obtaining various 2-(hydroxyphenyl) benzothiazoles as well [10, 11].

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It is known that introduction of the lipophilic benzothiazole group increases the oxidant stability of the phenols and modifies their antioxidant activity. Finding a suitable method for detecting these modifications will enable the subsequent studying of the structure-activity relationship. In this regard, nowadays many of the antioxidants are synthetic modifications of naturally occurring compounds [12, 13].

The aim of this study was to investigate the radical scavenging activity of 2-(hydroxyphenyl) benzothiazole derivatives obtained in high yields (56%–94%) *via* one-pot *α*-amidoalkylation of various phenols with benzothiazole and alkyl chloroformates.

MATERIALS AND METHODS

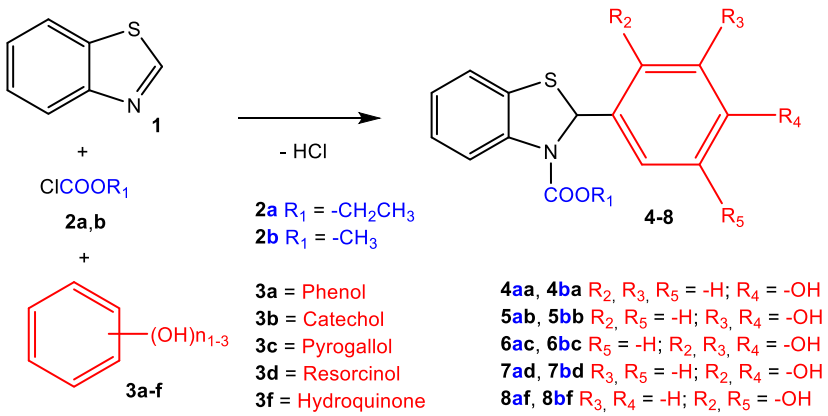
Reagents and equipment

All reagents and solvents as 2,2-diphenyl-1-picrylhydrazyl (CAS Number 1898-66-4), 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (CAS number: 30931-67-0), methyl alcohol (Cas. No 67-56-1), potassium persulfate (CAS No 7727-21-1), quercetin (CAS No 117-39-5), rutin (CAS No 153-18-4), gallic acid (CAS No 149-91-7), pyrogallol (CAS No 87-66-1), catechol (CAS No 120-80-9), resorcinol (CAS No 108-46-3), hydroquinone (CAS No 123-31-9) were purchased from Sigma-Aldrich, USA.

The absorbance of free radical scavenging assay was measured by a Spectroquant Pharo 300, UV/Vis spectrophotometer.

Materials

2-(Hydroxyphenyl) benzothiazole derivatives were synthesized by one-pot *α*-amidoalkylation according to Stremski *et al*. [10, 11] (Scheme 1). The obtained products were purified by column chromatography and characterized by IR, ^{1}H- ^{13}C-NMR and ESI-MS analyses.



Scheme 1. Synthesis of 2-(hydroxyphenyl) benzothiazole derivatives by one-pot α-amidoalkylation reactions

Methods

DPPH free radical scavenging assay. The DPPH free radical (Figure 1) scavenging activities were measured as previously reported by Docheva *et al*. 2014 [14]: 0.12 mM DPPH was dissolved in methanol. The absorbance change was measured at 515 nm on a UV-Vis spectrophotometer within 30 min. The total DPPH radical scavenging activity within 30 min was measured in triplicate in the absence of light. The blank sample was prepared as above by replacing the test sample with equivalent methanol. The radical scavenging activity (RSA%) was calculated. IC_{50} value determined the effective concentration at which 50% of DPPH radicals were scavenged and it is obtained by interpolation from linear regression analysis. Lower IC_{50} value indicates a higher antioxidant activity.

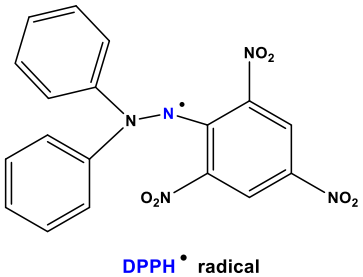


Figure 1. DPPH free radicals

ABTS free radical scavenging assay.

The ABTS free radical (Figure 2) was prepared by the method of Re *et al*., 1999 [15] with some modification. ABTS radical cation ($ABTS^{\bullet+}$) was produced by 7 mM ABTS and 2.45 mM $K_2S_2O_8$ (the mixture stayed in the dark at room temperature for 12–16 h before use) dissolved in deionized H_2O. Mixture of the reagents as 1:1 (v/v) $ABTS^{\bullet+}$ solution was diluted with methanol to an absorbance of

0.70±0.02 at 734 nm. The ABTS radical-scavenging activity within 10 min was measured in triplicate in the absence of light at room temperature. The percentage inhibition (%) of radical scavenging activity was calculated according to the corresponding equation of method.

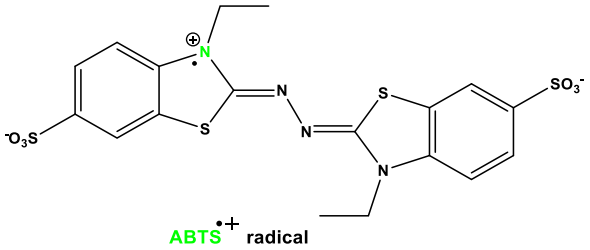


Figure 2. ABTS free radical

The stock solution of all compounds was prepared in concentration of 1 mg/ml. The working solutions were prepared by dissolving aliquot parts of the stock solution with methanol.

Statistical analysis

Experimental results were presented as the mean ± standard deviation (SD) of three parallel measurements.

RESULTS AND DISCUSSIONS

A series of synthetic 2-(4-hydroxyphenyl), 2-(dihydroxyphenyl) and 2-(trihydroxyphenyl) benzothiazole derivatives were evaluated for their DPPH and ABTS free radical-scavenging activities and compared to the radical-scavenging activity of natural compounds - rutin, quercetin, gallic acid, catechol, resorcinol, hydroquinone and pyrogallol, defined under the same conditions.

The DPPH method was related with a colour change from violet to yellow (Figure 3). The results were presented as IC_{50} for every compound in μM. The antioxidant activity of phenols – pyrogallol (IC_{50} = 5.71±0.45 μM) and catechol (IC_{50} = 5.72±0.45 μM) using DPPH method was similar to the antioxidant activity of natural antioxidant rutin (IC_{50} = 5.02±0.35 μM) and quercetin (IC_{50} = 4.60±0.30 μM) (Table 1).

DPPH •

$$2\ ABTS + S_2O_8^{2-} \longrightarrow 2\ ABTS^{\bullet+} + 2\ SO_4^{2-}$$

Figure 3. DPPH and ABTS assays

The final results for IC_{50} of rutin and quercetin confirmed those established by Docheva *et al.* (2014) [13]. The free radical scavenging activity of the benzothiazole derivatives varied between of IC_{50} = 3.66±0.21 μM – 38.22±2.19 μM, lower than the values defined for natural compounds - rutin, quercetin, pyrogallol and catechol (Table 1).

The results obtained for IC_{50} using ABTS method were associated with colour change – from blue-green to colourless (Figure 3). As a result, high activity of the natural compounds gallic acid (IC_{50} = 37.7±1.25 μM) and quercetin (IC_{50} = 48.01±4.36 μM) was observed (Table 1). The highest free radical-scavenging activity of phenols determined by ABTS the method possessed pyrogalol (IC_{50} = 22.7±1.45 μM), lower activity for resorcinol (IC_{50} = 48.2±4.93 μM), hydroquinone (IC_{50} = 88.9±5.59 μM) and the lowest - catechol (IC_{50} = 111.2±0.5 μM).

The free radical scavenging activity of the benzothiazole derivatives containing catechol **5ab**, **5bb**, resorcinol **7ad**, **7bd**, hydroquinone **8af**, **8bf** and pyrogallol **6ac**, **6bc** fragment, varied between of IC_{50} = 45.9±2.09 μM – 129.0±3.8 μM (Figure 4). The compound **4aa** with one phenolic hydroxyl group showed low antioxidant activity in concentration > 1000 μM. We suggest that the difference of activity is associated with the number and position of phenolic groups in their structures.

The difference in IC_{50} values between rutin (IC_{50} = 95.3±4.45 μM) and quercetin (IC_{50} = 48.01±4.36 μM), obtained by the ABTS method was significant, while that was not observed in the DPPH method.

Compounds **6ac**, **7ad** and **7bd** showed activity similar to quercetin and higher than the other 2-aryl benzothiazoles. This is explained by the fact that various substituents on "*o*-" and "*p*-" position in the phenolic fragment, form a resonance-stable radical (Kancheva *et al.* 2009) [12]. The results are presented in Table 1. The reported results (Table 1) are also illustrated by diagrams (Figures 4 and 5).

Table 1. DPPH and ABTS assay – free radical scavenging activities.

Compound	MW	DPPH Method	ABTS Method
		IC_{50}(μM)	IC_{50}(μM)
Quercetin	302.24	4.60±0.30	48.01±4.36
Rutin	610.52	5.02±0.35	95.3±4.45
Gallic acid	170.12	-	37.7±1.25
Pyrogallol	126.11	5.71±0.45	22.7±1.45
Catechol	110.11	5.72±0.45	111.2±0.5
Resorcinol	110.11	-	48.2±4.93
Hydroquinone	110.11	-	88.9±5.59
4aa	301.36	38.22±2.19	>1000
5ab	317.36	13.58±0.80	102±4.75
5bb	303.33	13.58±0.81	116.2±1.0
6ac	333.36	3.66±0.21	45.9±2.09
6bc	319.33	18.10±0.85	72.9±6.82
7ad	317.36	-	55.6±0.51
7bd	303.33	-	46.3±6.21
8af	317.36	14.00±0.82	111±11.2
8bf	303.33	-	129±3.8

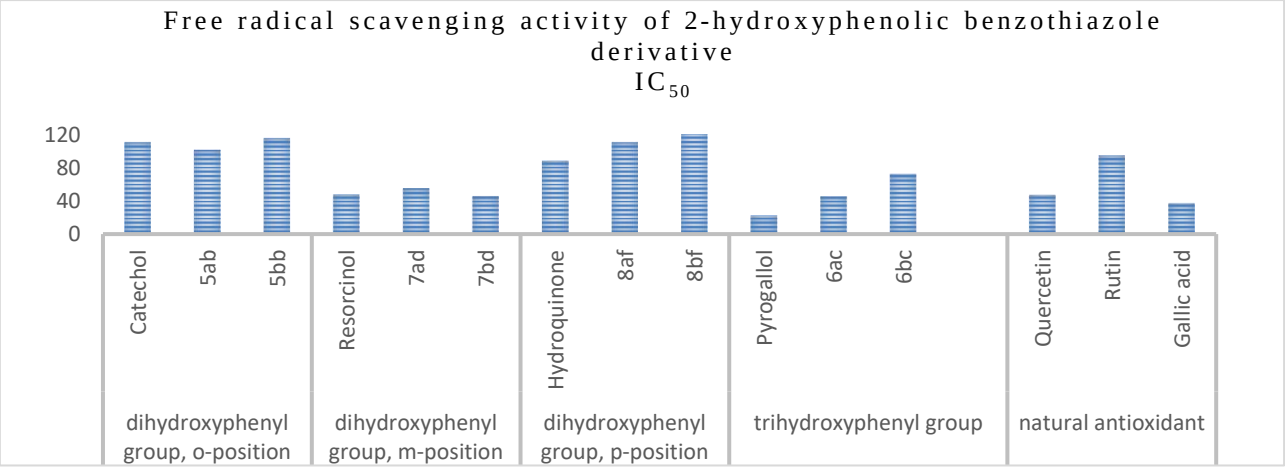


Figure 4. Free radical scavenging activity using ABTS-method (μM)

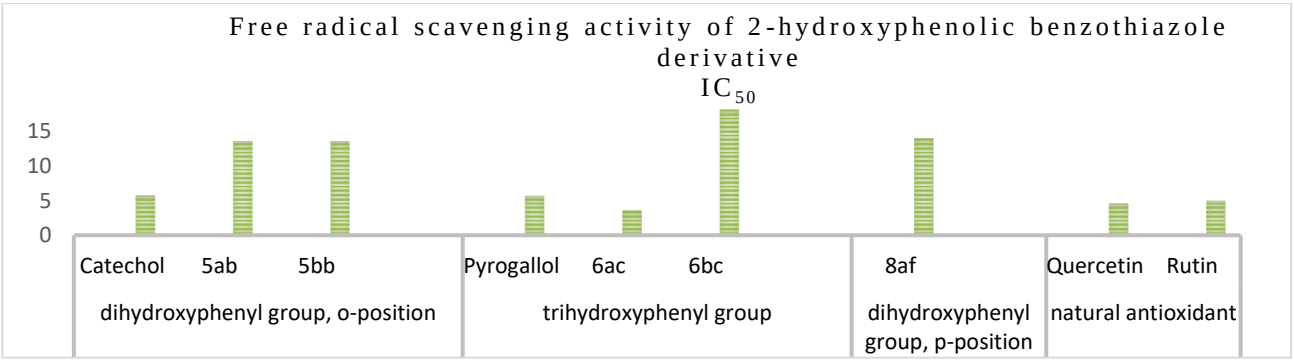


Figure 5. Free radical scavenging activity using DPPH-method (μM)

Antioxidant activity mainly depends on the number and position of phenolic hydroxyl groups. The benzothiazole compounds containing pyrogallol **6ac** (IC_{50} = 45.9±6.21 μM) and resorcinol - **7ad** (IC_{50} = 55.6±0.51 μM), **7bd** (IC_{50} = 46.3±6.21 μM) fragment demonstrated similar activity as quercetin, gallic acid and resorcinol examined *via* ABTS method (Table 1). The results obtained using ABTS method showed a possibility for studying of the structure-activity relationship of the tested examples.

CONCLUSION

A series of synthetic 2-(4-hydroxyphenyl), 2-(dihydroxyphenyl) and 2-(trihydroxyphenyl) benzothiazole derivatives were evaluated for their DPPH and ABTS free radical scavenging activity and compared to natural compounds - rutin, quercetin, gallic acid, catechol, resorcinol, hydroquinone and pyrogallol, defined under the same conditions. The results obtained for IC_{50} values showed a high radical scavenging activity for flavonoids - rutin (IC_{50} = 5.02±0.35 μM) and quercetin (IC_{50} = 4.60±0.30 μM), followed by phenols - pyrogallol and catechol (IC_{50} = 5.7±0.45 μM) using DPPH method. The free radical scavenging activity of the benzothiazole derivatives varied in the range of IC_{50} = 3.66±0.21 μM – 38.22±2.19 μM. The obtained IC_{50} values for ABTS method showed high activity for pyrogallol (IC_{50} = 22.7±1.45 μM), gallic acid (IC_{50} = 37.7±1.25 μM) and quercetin (IC_{50} = 48.01 ± 4.36 μM). The determined free radical scavenging activity of the benzothiazole derivatives was IC_{50} = 45.9±2.09 μM – 129±3.8 μM. The compound **4aa** with one phenolic hydroxyl group showed low antioxidant activity in concentration > 1000 μM. It was proved that antioxidant activity mainly depends on the number and position of phenolic hydroxyl groups. The benzothiazole compounds containing pyrogallol **6ac** (IC_{50} = 45.9±6.21 μM) and resorcinol **7ad** (IC_{50} = 55.6±0.51 μM), **7bd** (IC_{50} = 46.3±6.21 μM) fragment demonstrated similar activity as the natural antioxidants quercetin, gallic acid and resorcinol examined via ABTS method. The results obtained using ABTS method showed possibility for studying of the structure-activity relationship of the tested examples.

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D. Homogeneous and Heterogeneous Lipid Oxidation

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Interfacial concentrations of catechin in corn oil-in-water emulsions: effects of surfactant concentration, oil-to-water ratio and temperature

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Control of the oxidation of lipids in food-grade emulsions by antioxidants (AOs) is challenging because AOs should be present at the site of reaction with the lipid radicals (the interfacial region) in a concentration high enough to make the rate of the inhibition reaction equal to, or higher than, the rate of propagation of lipid radicals. Here we investigated the effects of increasing surfactant volume fraction (Φ_I), oil-to-water ratio, O/W, and temperature (T) on the aqueous and interfacial concentrations of catechin (CAT) in stripped corn oil-in-water emulsions. CAT only distributes between the aqueous and the interfacial region of emulsions and its efficiency depends on the effective concentration in the interfacial region. The partition constant P_W^I values are independent of Φ_I and of the O/W ratio, but incorporation of CAT into the interfacial region increases upon increasing temperature. However, the effective interfacial concentration of CAT decreases upon increasing Φ_I (constant T and O/W) and slightly increases upon increasing T and O/W ratio at constant Φ_I.

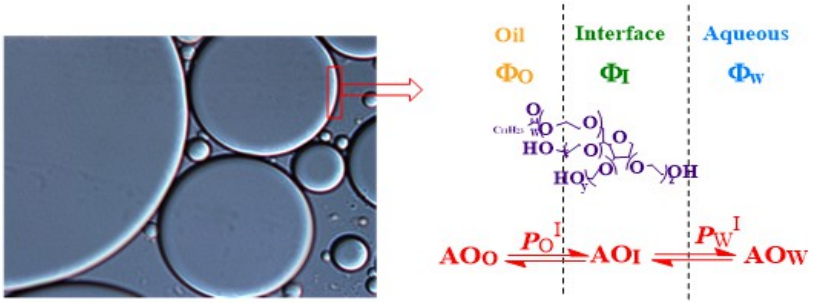
Keywords: Catechin, partitioning, emulsions, interfacial concentrations, corn oil.

INTRODUCTION

Lipids are mostly present in foods as oil-in-water emulsions, and addition of antioxidants (AOs) is one of the main practical technologies employed in the food industry to minimize their oxidation, a radical reaction of great concern because of its close relationship to food quality deterioration and health complications [1-4]. AOs react with lipid peroxyl radicals present in the system, equation (1), yielding a lipid hydroperoxide (LOOH) and a radical antioxidant AO$^\bullet$ much less reactive than the lipid peroxyl radical LOO$^\bullet$[5-7]. AOs are effective in inhibiting the lipid oxidation reaction when the rate of production of peroxyl radicals, r_p, is lower than the rate of the inhibition reaction, r_{inh} [8].

$$LOO^\bullet + AOH \rightarrow LOOH + AO^\bullet \quad (1)$$

Most lipids in foods exist in the form of oil-in-water (O/W) emulsions, consisting of small spherical oil droplets surrounded by an aqueous solution and kinetically stabilized by addition of surfactants, Scheme 1.



Scheme 1. Microphotograph of an oil-in-water emulsion that, conceptually, is divided into three distinct regions, the continuous (aqueous) region, the oil interior and the interfacial region. The scheme on the right shows the partitioning of an antioxidant, AO, of moderate hydrophobicity between those regions is also shown.

The unsaturated components of the lipids are prone to oxidation, and added antioxidants partition between the oil (O), water (W) and interfacial (I) regions of emulsions. Therefore, their efficiency in minimizing lipid oxidation depends not only on the rate constant (k) of the chemical reaction between the AO (AOH in equation (2)) and the peroxyl lipid radical but also on their concentrations at the reaction site, equation 2.

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$$v_{inh} = k_w(LOO^{\bullet}_W)(AOH_W) + k_O(LOO^{\bullet}_O)(AOH_O) + k_I(LOO^{\bullet}_I)(AOH_I) \quad (2)$$

We have recently demonstrated that the interfacial region of the emulsions is the main region where the inhibition reaction takes place [9-12], and thus, we can safely drop off the aqueous and oil contributions to the overall rate of inhibition in equation (2). This means that, once we have chosen an antioxidant of interest, its efficiency depends on its interfacial concentration, the higher the interfacial concentration, the higher the inhibition rate and thus its efficiency.

However, prediction of the interfacial concentrations of antioxidants is not a simple task because the partitioning of the antioxidant strongly depends on the hydrogen-bond ability of the antioxidant and the solvent properties of the various regions. In recent works [9-12], we showed that the interfacial concentration of antioxidants does not correlate with their hydrophobicity, increasing upon increasing the hydrophobicity up to a maximum after which, a further increase in the AO hydrophobicity results in a decrease in its interfacial concentration. This parabolic-like variation is known as the "cut-off" effect, Figure 1, and is a consequence of the differential solubility of the antioxidant in the oil and interfacial regions of the emulsion. This parabolic variation for the interfacial concentration of antioxidants, however, correlates with their antioxidant efficiency.

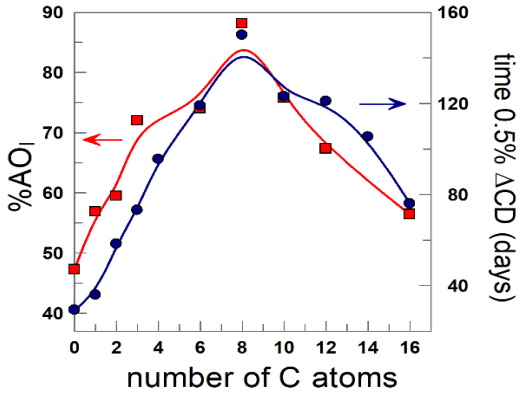
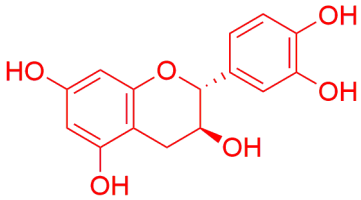


Figure 1. A parallel and parabolic variation in interfacial concentrations of hydroxytyrosol derivatives and in their antioxidant efficiencies in 4:6 olive oil-in-water emulsions. Adapted from [11], copyright Americal Chemical Society.

Thus, proper understanding on how antioxidants are distributed in emulsified systems is important to predict their efficiency and to the food industry for improving the quality and shelf-life of lipid-based products [13].

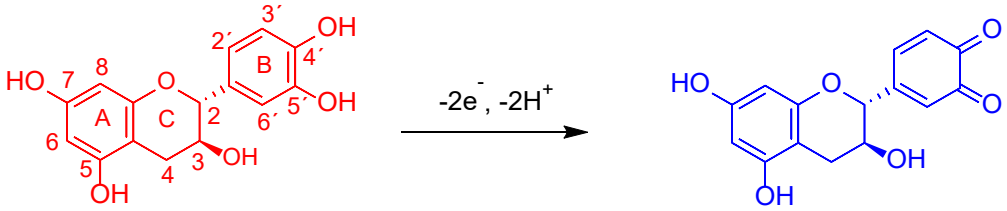
However, the physical impossibility of separating the interfacial region from the oil or aqueous regions makes the prediction of the distribution of antioxidants in emulsified systems a challenge [14]. In general, two partition constants are needed to describe the distribution of antioxidant, that between the oil-interfacial (P_O^I) and that between the aqueous-interfacial regions (P_W^I). These partition constants can not be measured independently by isolating and analyzing the concentration of antioxidant in each region because of the emulsion breakdown and thus, determining antioxidant distribution in emulsified systems requires determining partition constants in the intact emulsion [14]. Here we have employed our kinetic methodology to investigate the effects of surfactant concentration (Φ_I), oil-to-water ratio (O/W) and temperature (T) on the interfacial concentrations of (+) catechin, CAT, Scheme 2. The health benefits of the dietary intake of flavonoids make catechins (flavan-3-ol derivatives) the center of many nutritional studies because of their antioxidant properties [15-17].



(+)-catechin (CAT)

Scheme 2. Chemical structure of catechin.

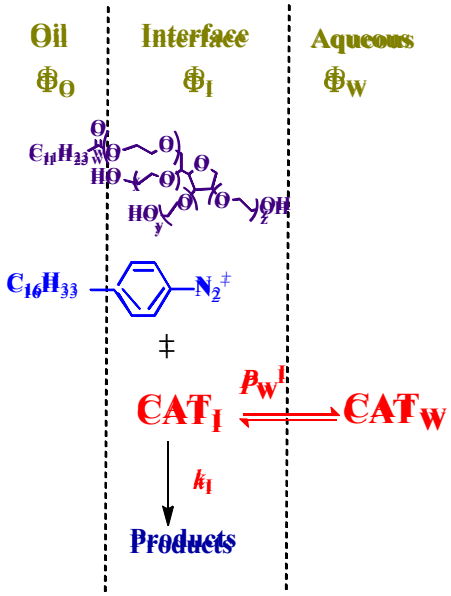
The specific mechanism by which CAT reacts with lipid radicals has not been fully stablished so far. It is currently believed that antioxidant properties of catechin arise from the stability of the stable quinone formed in the catechol (3,4-dihydroxybenzene) group on the B ring, Scheme 3, which participates in the delocalization and stabilization of the corresponding antioxidant radical [18]. The -OH group on the C ring and those located on the A ring (resorcinol group), do not contribute substantially to their antioxidant efficiency but only increase its water solubility by adding polar groups to the molecule.



Scheme 3. Oxidation mechanism of CAT to yield a stable quinone [19].

Determining the partition constants of catechin: application of the pseudophase kinetic model.

Previous solubility experiments [20] showed that CAT is oil-insoluble and thus, only distributes between the aqueous and interfacial regions of emulsions, Scheme 4. The solvent properties of the interfacial region are different from those of the aqueous region and, consequently, their solubility in those regions is different. Determining the solubility of CAT in the aqueous region is a relatively simple task by employing a number of analytical methods [20, 21]. However, determining the solubility in the interfacial region is quite complex if not impossible because the interfacial region cannot be isolated from the aqueous and oil regions whithout disrupting the exisiting equilibria, and because the interfacial region is a highly anisotropic region whose exact composition is unkown.



Scheme 4. Illustration of the partitioning of CAT between the interfacial and aqueous regions of an emulsion. Φ_I indicates the volume fraction of the surfactant, P_W^I, is the partition constant between aqueous and interfacial region and k_I is the rate constant for reaction between 16-ArN_2^+ and CAT in the interfacial region.

Rather than determining its solubility, we determined the partition constant P_w^I (i.e., its distribution, equation (3) by employing a well-established kinetic method, based on the reaction between a hydrophobic arenediazonium ion, 16-ArN_2^+, and the antioxidant in the intact emulsion. The method is described in detail elsewhere and only a brief description will be given here [14]. Experimentally, we determine the variation of the observed rate constant, k_{obs}, with the surfactant volume fraction Φ_I (defined as $\Phi_I = V_{surf}/V_{emulsion}$), and the relationship between P_W^I and the observed rate constant k_{obs} can be established on the grounds of the pseudophase kinetic model, equation (4). In brief, for a bimolecular reaction in an emulsion, the observed rate, v, is the sum of the rates in each region of the macroemulsion[14]. The reaction between 16-ArN_2^+ and CAT takes place exclusively in the interfacial region of the emulsion because 16-ArN_2^+ is water insoluble due to its long hydrophobic tail, and is oil insoluble because of its cationic headgroup, Scheme 4.

The mathematical relationship between k_{obs} and P_W^I, equation (4), has been derived elsewhere[14]. The reciprocal form of equation (4), equation (5), predicts that plots of 1/ k_{obs} *vs* Φ_I should be linear with positive intercepts, from where P_W^I values can be obtained.

$$P_W^I = \frac{(CAT_I)}{(CAT_W)} \tag{3}$$

$$k_{obs} = \frac{k_I [CAT_T] P_W^I}{\Phi_I P_W^I + \Phi_W} \tag{4}$$

$$\frac{1}{k_{obs}} = \frac{\Phi_W}{k_I [CAT_T] P_W^I} + \frac{\Phi_I}{k_I [CAT_T]} \tag{5}$$

Once P_W^I is known, determining the percentage of the antioxidant the water and interfacial regions is straightforward by employing equations (6) and (7).

$$\%CAT_W = \frac{100\Phi_W}{\Phi_I P_W^I + \Phi_W} \tag{6}$$

$$\%CAT_I = \frac{100\Phi_I P_W^I}{\Phi_I P_W^I + \Phi_W} \quad (7)$$

The aqueous and interfacial concentrations of CAT can be obtained from equations (8) and (9), respectively.

$$(CAT_W) = \frac{\%CAT_W [CAT_T]}{\Phi_W} \quad (8)$$

$$(CAT_I) = \frac{\%CAT_I [CAT_T]}{\Phi_I} \quad (9)$$

EXPERIMENTAL

Materials

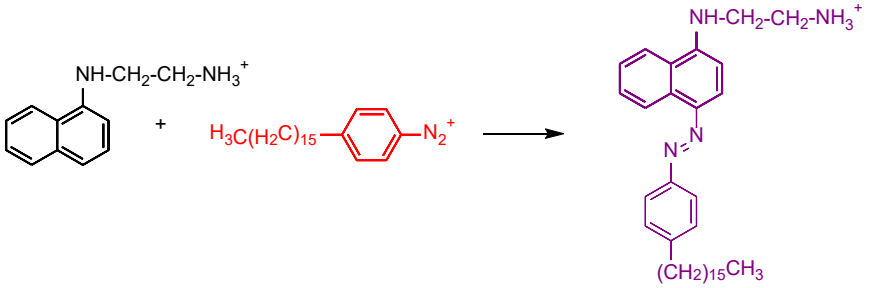
Catechin (Aldrich), the polyoxyethylene (20) sorbitan monolaurate emulsifier (Tween 20, Fluka) and the corn oil (Across Organics, d = 0,918 g/mL) stripped from endogenous antioxidants were of the highest purity available and used as received. 4-Hexadecylbenzene diazonium tetrafluoroborate, 16-ArN_2BF_4, was prepared as described elsewhere [14] and was stored in the dark at low temperature to minimize its descomposition. All aqueous acid solutions were prepared by employing citric acid-sodium citrate buffer (pH = 2.14, 0.04 M). Solutions of the coupling reagent N-(1-naphthyl) ethylenediamine (NED, Aldrich) were prepared in a 50:50 (v/v) BuOH:EtOH mixture to finally give [NED] = 0.02 M.

Emulsion preparation

Corn oil-in-water emulsions were prepared by mixing 4 mL of stripped corn oil and 6 mL of a citric-citrate buffer solution (pH 2.14) containing a weighed amount of non-ionic surfactant volume fraction of emulsifier, Φ_I. The volume fraction of emulsifier was varied from $\Phi_I = 0.005$ to $\Phi_I = 0.04$. CAT was dissolved in the buffered aqueous solution employed to prepare the emulsions. The stoichiometric concentration of CAT in the emulsion was $[CAT_T] = 4$ mM. The oil and aqueous mixture was stirred with a high-speed rotor (Polytron PT 1600 E) for 1 minute and transferred to a continuously stirred thermostated cell. No phase separation was visually (naked eye) observed within 3-4 hours, a time much higher than that required to monitor the reaction between 16-ArN_2^+ and CAT for more than 3-4 half-lives.

Methods

Determining k_{obs} at different surfactant volume fractions. The observed rate constants, k_{obs}, for the reaction between the chemical probe 16-ArN_2^+ and CAT were determined as in previous work by employing a derivatization method [9-11, 14] that exploits the rapid reaction between 16-ArN_2^+ and the coupling agent N-(1-naphthyl)ethylenediamine, NED. The reaction leads to the formation of a stable azo dye, Scheme 5, whose absorbance, Figure 2, can be measured by UV-Vis spectroscopy. Details can be found in detail elsewhere [22].



Scheme 5. Reaction between the coupling agent N-(1-naphthyl) ethylenediamine, NED, with 4-hexadecylbenzenediazonium ions, leading to the formation of an azo dye.

In a typical experiment, the reaction between the 16-ArN_2^+ and the catechin was initiated by adding an aliquot (16 µL) of a 0.17M stock 16-ArN_2^+ solution in acetonitrile to a thermostated and continuously stirred emulsion. At selected times, aliquots (200 µL) of emulsion were transferred to vessels containing 2.5 mL of a 0.02M alcoholic solution 50:50 (v:v) BuOH/EtOH- mixture of NED. Under our experimental conditions, 16-ArN_2^+ reacts with NED much faster than with CAT so that the reaction between 16-ArN_2^+ and CAT is effectively quenched. Values of k_{obs} were determined from the variations of the absorbance at λ= 572 nm with time by fitting the pairs of data (absorbance, time) to the first-order equation (10) by employing a nonlinear least-squares method provided by a commercial computer program (GraFit 5.0.5). In equation (10), A_t, A_o and A_∞ are measured absorbance at any time, at t = 0 and at infinite time.

$$\ln(A_t - A_\infty) = -k_{obs}t + \ln(A_0 - A_\infty) \quad (10)$$

Figure 2 is illustrative and shows a typical variation of the absorbance of the azo dye with time and the corresponding linear plot according to equation (10).

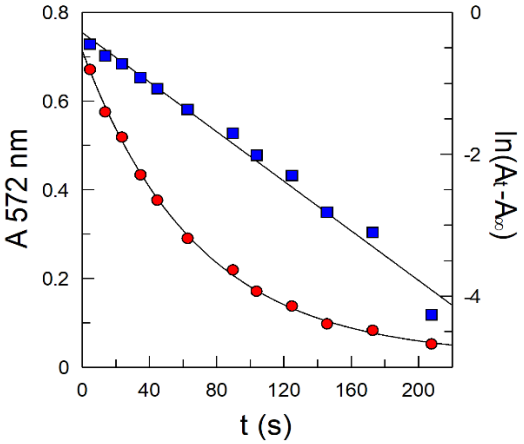


Figure 2. Variation in absorbance of the formed azo dye (-●-) and ln[A_t-A_∞] (-■-) plots for the reaction of 16-ArN_2^+ with CAT in 4:6 (O/W) corn oil-in-water emulsions (pH 2.14) and Tween 20. [CAT] ≈ 4 mM, [16-ArN_2^+] = 0.29 mM, [NED] = 0.02M, T = 25ºC.

Statistical analysis

Duplicate or triplicate experiments gave k_{obs} values within ± 7-9 %. Data are displayed as means ± standard deviation of the measurements.

RESULTS AND DISCUSSION

Partition constants of CAT in corn oil-in-water emulsions: effects of temperature and determination of thermodynamic parameters

The partition constants of CAT were determined, as indicated before, from the variation of k_{obs} with Φ_I at different temperatures (T = 15 - 35ºC) in 4:6 corn oil-in-water emulsions (pH = 2.14), Figure 3, by fitting the experimental data to equations (4)-(5). Figure 3 is representative and shows that, at T = 15º and 25 ºC, k_{obs} decreases 1-3-fold on going from Φ_I = 0.005 to Φ_I = 0.04, consistent with the predictions of equation (4). These variations are quite similar to those found when analysing the behaviour of other antioxidants such as caffeic acid or hydroxytyrosol [9, 11].

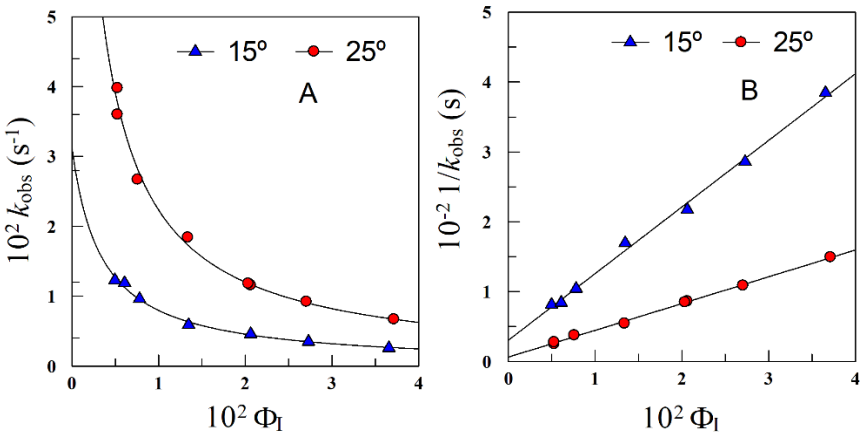


Figure 3. Effects of increasing Φ_I on k_{obs} **(A)** and on $1/k_{obs}$ **(B)** for the reaction of 16-ArN_2^+ with catechin at different temperatures. The solid lines are the theoretical curves obtained by fitting the experimental (k_{obs}, Φ_I) or ($1/k_{obs}$, Φ_I) pairs of data to equations 4 and 5, respectively. Experimental conditions: 4:6 corn oil-in-water emulsions stabilized with Tween 20. [16-ArN_2^+] = 0.29 mM, [CAT] = 4mM, pH 2.14 (citric-citrate buffer 0.04M).

The straight lines shown in Fig. 3B were used to obtain the partition constant P_W^I. Table 1 shows that P_W^I increases 1.6 - 4.3-fold upon increasing T from 15 to 35 ºC.

As we have shown in previous works [23, 24], the transfer of molecules between the different regions of fluid emulsions is not rate limiting and thus, the system is in dynamic equilibrium. This means that the chemical potential of CAT in each region, defined by equations (11)-(12), should be identical. In equations (11)-(12), $\mu_{CAT}^{0,W}$ and $\mu_{CAT}^{0,I}$ are the standard chemical potential, and X_{CAT}^{W} and X_{CAT}^{I} are the mole fractions of catechin in the aqueous and interfacial regions, respectively.

Table 1. Values of the partition constant P_W^I for CAT in corn oil-in-water emulsions at different temperatures.

T(ºC)	P_W^I
15	190
20	310
25	360
35	804

$$\mu_{CAT}^{W} = \mu_{CAT}^{0,W} + RT \ln X_{CAT}^{W} \text{ (aqueous)} \quad (11)$$

$$\mu_{CAT}^{I} = \mu_{CAT}^{0,I} + RT \ln X_{CAT}^{I} \text{ (interfacial)} \quad (12)$$

The Gibbs free energy, $\Delta G_T^{0,W\to I}$, for the transfer of 1 mol of catechin from the aqueous to the interfacial region, is given by equation (13), where V_m^W and V_m^I are the molar volumes of water and emulsifier. Values for the molar volumes can be obtained from literature density values and we assume that they are essentially constant over the relatively small temperature ranges employed (T ≈ 290 - 310 K). Thus, $\Delta G_T^{0,W\to I}$ is an easily accessible parameter that can be determined at a given temperature from the partition constant values in Table 1.

$$\Delta G_T^{0W\to I} = \mu_{CAT}^{0,I} - \mu_{CAT}^{0,W} = RT \ln \frac{V_m^W}{P_W^I V_m^I} \quad (13)$$

$$\Delta H_T^{0,W\to I} = R \left[\frac{\partial \left(\ln P_W^I \right)}{\partial \left(\frac{1}{T} \right)} \right]_P \quad (14)$$

The values of P_W^I in Table 1 can be also employed to obtain the enthalpy of transfer, $\Delta H_T^{0,W\to I}$, by using the van't Hoff, equation (14) which predicts that a plot of ln P_W^I with 1/T should be a straight line. Figure 4 shows that this prediction is fulfilled, and from the slope of the straight line, a value for $\Delta H_T^{0,W\to I}$ can be obtained. The entropy for the transfer of CAT from the aqueous to the interfacial region, $\Delta S_T^{0,W\to I}$, can be obtained by using the Gibbs equation (15).

$$\Delta S_T^{0,W\to I} = \frac{\Delta H_T^{0,W\to I} - \Delta G_T^{0,W\to I}}{T} \quad (15)$$

The thermodynamic parameters for CAT transfer were obtained from equations (13)-(15) and they are listed in Table 2.

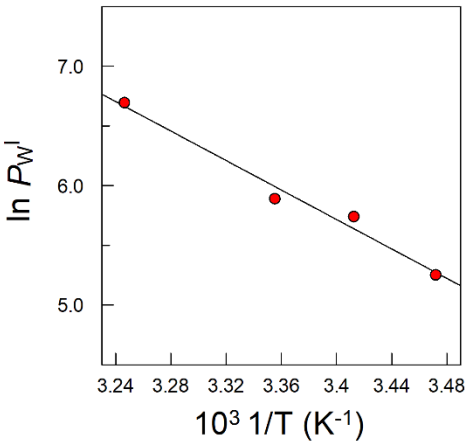


Figure 4. Plot of the variation of ln P_W^I *versus* 1/T according to the van´t Hoff equation (11). Data extracted from Table 1.

Table 2. Thermodynamic values (Gibbs free energy, enthalpy and entropy) for the transfer of 1 mol of CAT from the aqueous to the interfacial region of corn oil-in-water emulsions.

$\Delta G_T^{0W\to I}$ (kJ/mol)	$\Delta H_T^{0W\to I}$ (kJ/mol)	$\Delta S_T^{0W\to I}$ (kJ/mol K)
-24.81	-51.29	-0.09

Results suggest that the transfer of CAT from the aqueous to the interfacial region is spontaneous at any T because $\Delta G_T^{0W\to I}$ is negative. $\Delta S_T^{0W\to I}$ is also negative, suggesting that there is not a net increase in disorder of the transfer process of CAT from the aqueous region to the interfacial region of the emulsions. The $-T\Delta S_T^{0W\to I}$ contribution is thus positive, however, the negative entalphy contribution is much higher than the $-T\Delta S_T^{0W\to I}$ contribution, therefore suggesting that the transfer of catechin from aqueous to interfacial region is essentially an entalphy-driven process.

Interfacial concentrations of CAT in corn oil emulsions: effects of temperature, surfactant concentration and oil-to-water ratio

The determined P_W^I values, Table 1, are much higher than the unit, ranging 190 – 804, suggesting that catechin is mostly located in the interfacial region of the emulsion. The value at T = 25 ºC, P_W^I = 360, is much higher than those obtained for hydrophilic catecholics such as hydroxytyrosol [11] (P_W^I = 120) but similar to that of caffeic acid [9] (P_W^I = 370). All together, these results show that the partition constants of catecholics cannot be predicted exclusively on the basis of their polarity [9-11] and need to be determined for each species

in the intact emulsions. Moreover, the negative $\Delta H_T^{0W\to I}$ value shows that van der Waals interactions and hydrogen bonds between -OH groups of catechin and polyoxyethylene groups of the surfactant may play a major role in the the transfer of catechin from the aqueous to the interfacial region. This also may have important consequences on the effective concentration of CAT whose values in the aqueous and interfacial concentrations can be determined by employing equations (8) and (9), respectively. The results also suggest that the presence of polyoxiethylene sorbitan fatty acid esters (Tween 20) and polar fatty acid of corn oil in the interfacial region contribute to enhance the interactions with -OH group of catechin at high temperatures.

Figure 5 shows the variation of the local aqueous and interfacial concentrations of catechin with the surfactant volume fraction in the T = 15 – 35 ºC range.

At any given temperature, the effective interfacial concentrations of CAT are 20 -200 times higher than the stoichiometric concentration ($[CAT_T] = 4\times10^{-3}$ M), depending on the temperature and, mainly, on the surfactant concentration. On the contrary, the effective aqueous concentration of CAT is much lower than the stoichiometric concentration. This means that the interfacial region of the emulsion acts as a very efficient "microreactor" concentrating the reactant (in this case, the antioxidant) and thus increasing notably its efficiency in inhibiting the oxidation of lipids with respect to a bulk system. Note that an increase in the surfactant volume fraction Φ_I decreases the effective interfacial concentration because of the increase in the interfacial volume.

Results shown before suggested that the transfer of CAT from aqueous to interfacial region is entalphy driven. Enthalpic contributions are usually associated to the strength of hydrogen bonds and this contribution may change in a different extent in the aqueous and interfacial regions upon increasing temperature because of the increase in the thermal motion of the catechin. Thus, upon increasing the temperature, the local concentrations of CAT can change.

Figure 6 shows the effects of increasing temperature on the effective concentrations of CAT in the aqueous and interfacial region of the corn emulsions at selected surfactant volume fractions. Upon increasing T, the effective concentration of CAT decreases in the aqueous region, increasing concomitantly that in the interfacial region. Note the effects of the surfactant volume fraction. At low $\Phi_I = 0.005$ the effective interfacial concentrations increases a modest 1.5-fold, meanwhile at $\Phi_I = 0.04$ the increase is negligible.

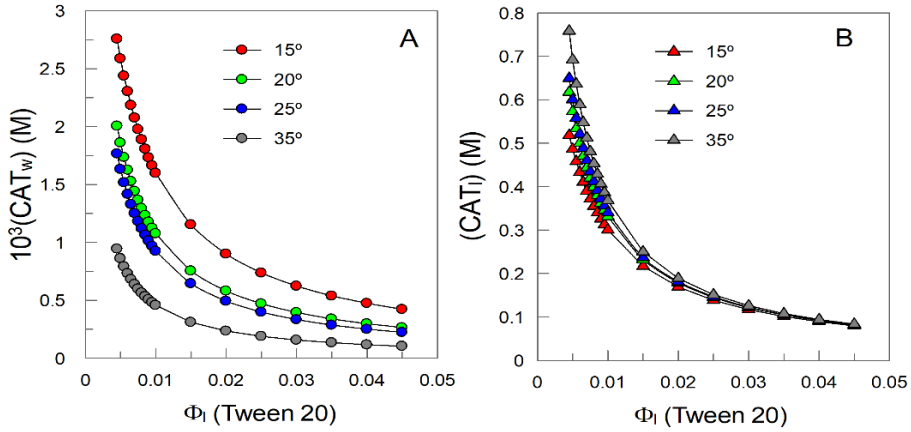


Figure 5. Variation of the effective concentration of CAT in the aqueous **(A)** and interfacial **(B)** regions of 4:6 corn oil emulsions as a function of temperature and emulsifier concentration. The effective concentrations (in parentheses) are expressed as moles of antioxidant per volume of the particular region, meanwhile stoichiometric concentrations are defined in terms of moles of the antioxidant per volume of the emulsion.

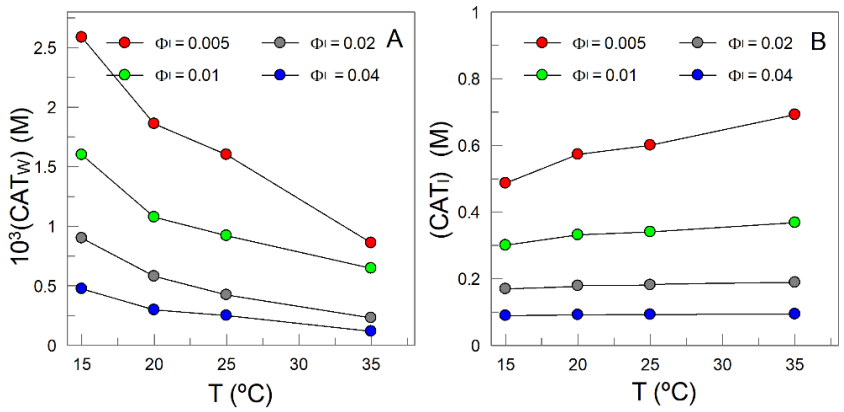


Figure 6. Effect of temperature on the concentration of catechin in the aqueous **(A)** and interfacial **(B)** regions of 4:6 corn oil-in-water emulsions at selected Tween 20 volume fractions.

Figure 6 shows that the compostion of the interfacial region alters the balance of the various intra- and intermolecular forces that define the actual distribution of the antioxidant. Thus, one could also expect that the oil-to-water ratio may also affect the effective concentrations of the antioxidant. Figure 7 shows the variation in the effective aqueous and interfacial concentrations of CAT upon increasing the oil volume fraction Φ_O (defined as $\Phi_O = V_{oil} / V_{emulsion}$). At any temperature, the interfacial concentrations of CAT increase upon increasing Φ_O, suggesting that its solubility is higher in the interfacial region than in the aqueous region.

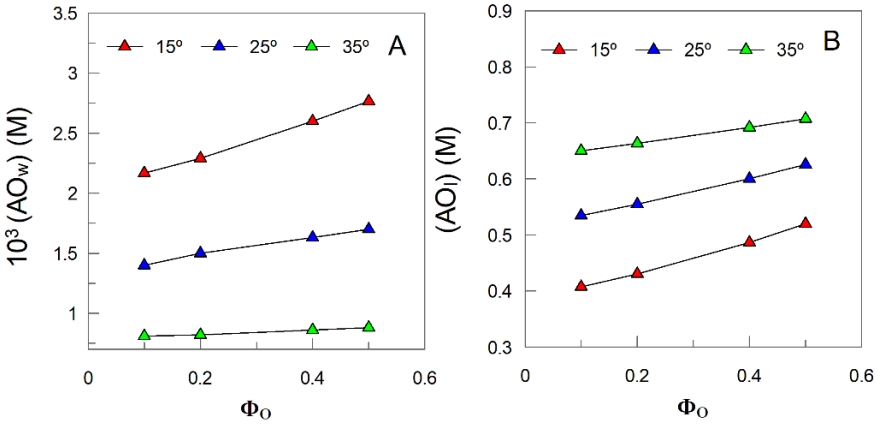


Figure 7. Effect of oil volume fraction (Φ_O) on the concentration of catechin in the aqueous **(A)** and interfacial **(B)** regions of 4:6 corn oil-in-water emulsions at different temperatures.

CONCLUSIONS

We have evaluated the effects of temperature, oil- to-water ratio and surfactant concentration on the effective concentrations of a model flavonoid in the aqueous and interfacial regions regions of corn oil-in-water emulsions. The Gibbs free energy, enthalpy and entropy for the transfer of 1 mol of CAT from the aqueous to the interfacial region of the emulsions were also evaluated.

P_W^I values were obtained by employing a kinetic method, and the good straight line obtained for the variation of ln P_W^I with 1/T (Van´t Hoff equation), Figure 4, demonstrates the feasibility of our methodology to determine valuable thermodynamic parameters for the transfer of antioxidants from the aqueous (or oil) regions to the interfacial region of emulsions. The method also allows determining the effective concentrations of the antioxidants in those regions, which are basic to rationalize the antioxidant efficiency of emulsions in multiphasic systems. At present, no other methodology allows estimations of these parameters because reported methods require the rupture of the emulsion, disrupting the existing equilibria.

Results show that the transfer of CAT from the aqueous to the interfacial region is spontaneous and enthalpy driven. The sensitivity of the changes in the P_W^I values with T is consistent with the significant changes in the solvation properties on going from an aqueous to interfacial region. The changes in P_W^I values with T contrast strongly with

the modest dependence of P_W^I obtained for other hydrophilic antioxidants such as gallic or caffeic acid [23, 24].

The effects of surfactant concentration, oil-to-water ratio and temperature on the concentration of CAT in the aqueous and interfacial regions of corn oil-in-water emulsions were evaluated. Our results show that the larger variations in the interfacial concentrations are obtained when changing the surfactant concentration, meanwhile changes in the O/W ratio and T (other things being equal) only have a modest effect.

Finally, we would like to stress the importance of determining the effective concentrations of antioxidants in the interfacial region of emulsions because, in general, the antioxidant efficiency depends on such values, the higher the interfacial concentration, the higher is the antioxidant efficiency.

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E. Food Analysis, Food Additives and Food Supplements

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In vitro antioxidant activity of *Physalis peruviana* L. fruits

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Physalis peruviana L. (Cape gooseberry) is recognized as a valuable source of nutrients and antioxidants, and is extensively cultivated nowadays in many countries of the tropical and sub-tropical zone. To the best of our knowledge, there are no previous reports about the antioxidant capacity of *P. peruviana* fruits originating from Bulgaria. We hypothesized that the local environmental, variety, production and other factors would have an impact on the antioxidant properties, the polyphenol and flavonoids content of the fruits. Thus, the aim of the current study was to evaluate the antioxidant activity of different extracts from two genotypes of locally produced *P. peruviana* fruits (BF and BP) and to compare it with that of the imported fruit available on the market (CM). The extracts with acetone (AEPP), water (WEPP), 95% ethanol (EEPP), and 50% methanol (MEPP) were obtained from the whole berries and the pulp fraction. The total phenolic content was the highest in EEPP and AEPP from the pulp fraction, varying from 23.98 to 30.60 GAE/100 g FW (EEPP) and from 14.99 to 26.06 GAE/100 g FW (AEPP). Similarly, EEPP and AEPP from the pulp fraction were with a high total flavonoids content. There was an origin-related differentiation; the fruits from Colombia (CM) were the richest in phenolics and flavonoids, followed by the fruits of Bulgarian origin (BF and BP). All extracts demonstrated antioxidant activity, which generally was well expressed in the EEPP from fruit pulp. DPPH activity was the highest in CM (176.99 mM TE/100 g FW). The same tendency was observed in other assays. The maximal antioxidant activity values were: ABTS - 384.20 mM TE/100 g FW (CM), FRAP - 170.94 mM TE/100 g FW (BP), and CUPRAC - 588.36 mM TE/100 g FW (CM). The results showed positive linear correlations between antioxidant activities and total phenolic and flavonoids content. According to this study, Cape gooseberry from Bulgaria possesses radical scavenging and metal chelating properties that are not inferior to those of the varieties produced worldwide.

Key words: *Physalis peruviana* L., ABTS, FRAP, CUPRAC, DPPH, antioxidant activity, flavonoids, polyphenols.

INTRODUCTION

Physalis peruviana L. is the commercially most important fruit among the over 100 species of the genus *Physalis* (family Solanaceae) [1, 2]. The plant originates from the Andean region (Peruvian and Ecuadorian Andes), spreading throughout South America in pre-Incan and Incan times. Nowadays, its cultivation extends to many countries of the tropics and sub-tropics, Central and South Europe, the United States, and Asia [3]. *P. peruviana*, also known as Cape gooseberry, Inca berry, golden berry or Peruvian ground cherry, is an herbaceous, semi-shrub plant, annual in the temperate zones and perennial in the tropics and sub-tropics, well-adapted to different altitudes, soils and climatic conditions [4]. As in all *Physalis* species, the fruit is a berry completely covered by an inflated balloon or lantern-like protective calyx (husk), formed by the strongly grown sepals. The berries are small, with a diameter between 1.25 and 2.50 cm and weigh between 4 and 10 g, oval, containing approx. 100 to 300 small seeds. The ripe erries are bright yellow to orange in color, shiny, with a tender and juicy texture, rich in flavor (sweet and sour, with a hint of citrus). The berries are consumed mostly fresh, but a substantial part of the annual production is also dehydrated or processed into jams, jellies, juices, dressings and other products [1, 5-7]. The largest producer and exporter of fresh or dehydrated fruit is Colombia, followed by South Africa [2-4].

P. peruviana fruit has a long history of ethnomedical purposes worldwide, as an antimycobacterial, antileukemic, antipyretic and diuretic agent [2] and it was used in the treatment of cancer, hepatitis, asthma, malaria, dermatitis, rheumatism, hyperglycemia, fevers, and many other conditions [3, 8, 9]. The pursuit of functional foods has provoked intensive scientific research on Cape gooseberry fruit in the last two decades, revealing the presence of various classes of metabolites. The fruit contains vitamins [10-13], minerals [5, 6], polysaccharides [6, 10], protein [14], fatty acids and phytosterols [2, 3, 9], polyphenols [6, 7, 12-15], and many other functional nutrients [1, 11, 16-18]. The phytochemical composition of Cape gooseberry fruit outlines its antimicrobial, antiviral, antioxidant, anti-inflammatory, immunomodulatory hepato-protective, anti-diabetic, antitumor and other properties [3, 10, 19-21], making it valuable

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for the nutraceutical and pharmaceutical industries [1, 17, 18].

Many biochemical assays have been introduced to quantify the antioxidant activity (AOA) or antioxidant capacity (AOC) of food and other biological samples, but none of them is currently accepted as a single versatile assay, unanimously applied to different matrices [22]. In general, most of AOA assays are associated with single electron transfer (SET) or hydrogen atom transfer (HAT) reaction kinetics, although there is no distinct boundary between them [22-24].

Along with other exotic fruits, such as goji berry, Acai berry, Maqui berry, lychee or pitaya, Cape gooseberry is recently being promoted as one of the "superfruits" [16]. Cape gooseberry fruit is supported by numerous data for the high levels of antioxidants such as phenolic acids, flavonoids, flavanols, proanthocyanidins, coumarins, tannins, carotenoids, and anthocyanins, and the evidence for its medicinal benefits. Total polyphonic content in plant samples of the same species is affected by factors such as environmental conditions, phenological stage, genotype, etc., but the qualitative polyphenol profiles are more stable and species-specific [7, 13, 25, 26]. Although *P. peruviana* and some of the other cultivated *Physalis* species (*P. philadelphica* Lam., *P. ixocarpa* Brot., *P. pubescens* L.) are gaining popularity worldwide, Cape gooseberry remains considerably unknown in Bulgaria. A brief overview of Cape gooseberry status in Bulgaria reveals that fresh fruit imported from Colombia is occasionally available in some of the biggest supermarkets, while packed dry berries (or mixes with other dry fruits) are distributed by a number of bio food suppliers, often under the label of "exotic superfruits". The most popular use of fresh fruit remains that of an exotic decoration to the various dishes and desserts in gourmet restaurants. There is fragmented public information about some recent endeavors in Cape gooseberry cultivation of a few organic farms in Bulgaria. However, practically no data are available about yields, quality or market success. In the period between 1996 and 2001 the only Bulgarian variety of *P. peruviana* named "Plovdiv" has been selected at the Department of Horticulture at the Agricultural University of Plovdiv, and in 2006 it has been registered in the Official Variety List by the Executive Agency for Variety Testing, Field Inspection and Seed Control [27]. The ripe fruits of the local variety are described as having a typical strawberry-vanilla flavor and a pleasant, sweet to slightly sour taste. They contained 35.45 mg% vitamin C, 10.72% total sugar, 1.03% pectin, 1.03% total acids, and 0.51% flavonoids [27]. Several studies afterwards reported data on the fruit yield, fruit post-harvest ripening dynamics, the possibilities of extended market supply with locally-produced fruit, and on other details of the experimental production of "Plovdiv" variety in Bulgaria [28-34].

To the best of our knowledge, this is the first report about the antioxidant capacity of *P. peruviana* fruits originating from Bulgaria. We hypothesized that local environmental, variety, production and other factors would have an impact on the antioxidant properties, the polyphenol and flavonoid content of the fruit, and that there would be some variation of the data available for fruit of other origin. Therefore, the aim of this study was to evaluate the antioxidant activity of different extracts from locally produced *P. peruviana* fruits and to compare them with that of the imported Colombian fruit available on the Bulgarian market.

EXPERIMENTAL

Plant material

The ripe fruits of Cape gooseberry (*P. peruviana* L.) from three different origins were studied. Two of them represented Cape gooseberry genotypes grown under the environmental conditions in Bulgaria. The first of these genotypes was the only locally selected variety named "Plovdiv" (BP), and was produced in the experimental fields of the Agricultural University, located in Plovdiv, Central Southern Bulgaria. The second sample (BF) consisted of fruit of an introduced Cape gooseberry variety, produced and purchased from a certified organic farm (Versol Bio-farm, Lik village, municipality of Mezdra, North-West Bulgaria). These were compared to the fruits imported from Colombia (produced by C.I. FRUTIREYES S.A.S., Bogotá DC, Colombia; imported by KM Delivery EOOD), purchased from a local supermarket (CM). The fruit calyces were removed. A portion of the berries was further processed to obtain fruit pulp (without the seeds), which was analyzed individually in order to examine the influence of the different parts of the berry. The fruit samples were kept in a refrigerator at a temperature of –18°C until analysis.

Methods

Extraction procedure: The extracts with four different polarity solvents: acetone (AEPP), water (WEPP), 95% ethanol (EEPP), and 50% methanol (MEPP), were obtained from whole berries and the pulp fraction of *P. peruviana*. The extraction procedure was performed in an ultrasonic bath

(SIEL, Gabrovo, Bulgaria), operating at a frequency of 35 kHz and power of 300 W for 20 min, at 75 °C and a solid-to-solvent ratio of 1:10 (w/v). The obtained extracts were filtered, and the extraction was performed in duplicate. The combined extracts were used for further analyses.

Total phenolic compounds: Total phenolic content (TPC) was measured by a slight modification of the Folin-Ciocalteu method. One ml of Folin-Ciocalteu reagent (diluted five times) was mixed with 0.2 ml of the extracts and then 0.8 ml of 7.5% Na_2CO_3 were added. After a reaction time of 20 min at room temperature (20±2°C) the absorbance of the solution was read at 765 nm against the blank. The results were expressed as milligram equivalents of gallic acid (GAE) per gram fresh weight (FW) [35].

Total flavonoids: The total flavonoids (TF) content was analyzed according to the spectrophotometric method with 10% $Al(NO_3)_3$ reagent previously described [36]. The absorbance of the reaction mixture was measured after 40 min at 415 nm against the blank. The results were presented as milligram equivalents of quercetin (QE) per gram fresh weight (FW) [35].

2,2-diphenyl-1-picrylhydrazyl (DPPH) assay: A portion of the extracts (0.15 ml) was mixed with 2.85 ml of freshly prepared 0.1 M solution of DPPH in methanol. The sample was incubated for 15 min at 37°C in the dark. The reduction of absorbance was measured at 517 nm, in a parallel to the blank containing methanol [35].

2,2'-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid (ABTS) assay: The ABTS radical was generated by mixing aliquot parts of 7.0 mM ABTS in the distilled water and 2.45 mM $K_2S_2O_8$ (Merck) in double-distilled water. The reaction was performed for 16 h at room temperature (20±2°C) in the dark. Before analysis, 2.0 ml of the stock solution with the generated ABTS radicals was diluted with methanol at a proportion of 1:30 (v/v), in order to adjust the absorbance of the working solution to 1.0 ÷ 1.1 at 734 nm. Working solution (2.85 ml) was mixed with 0.15 ml of plant extracts. After incubation for 15 min at 37°C in the dark the absorbance was measured at 734 nm against methanol [35].

Ferric reducing antioxidant power (FRAP) assay: The assay was performed according to [37] with a slight modification. The FRAP reagent was freshly prepared by mixing 10 parts of 0.3 M acetate buffer (pH 3.6), 1 part of 10 mM 2,4,6-tripyridyl-s-triazine (TPTZ) (Fluka) in 40 mM HCl (Merck) and 1 part of 20 mM $FeCl_3.6H_2O$ (Merck) in double-distilled water. FRAP reagent (3.0 ml) was mixed with 0.1 ml of the investigated extracts. After 10 min at 37 °C in darkness the absorbance was measured at 593 nm relative to a reagent blank prepared with solvent instead of an extract.

Cupric reducing antioxidant capacity (CUPRAC) assay: The reaction mixture contained 0.1 ml of the analyzed extract mixed with 1 ml of $CuCl_2.2H_2O$, 1 ml of Neocuproine (7.5 ml in methanol), 1 ml of 0.1 M ammonium acetate buffer and 1 ml of distilled water. The solution was incubated at 50°C for 20 min in darkness and the absorbance was measured at 450 nm [38].

All assays for measuring the antioxidant activity of the extracts were performed in triplicate and the results (mean ± SD) were expressed as mM Trolox equivalents (mM TE) per 100 g by fresh weight.

RESULTS AND DISCUSSION

Data about the total phenolic content and total flavonoids content in the analyzed extracts of Cape gooseberry fruit are presented in Table 1. As hypothesized, the results showed certain trends of variation of the respective chemical indices, depending on the solvent, the genotype and the fraction of Cape gooseberry fruit.

The highest total phenolic content was in the ethanol and acetone extracts, regardless of fruit genotype or berry fraction (whole berries or pulp). Significantly less phenolic compounds were extracted with 50% methanol and water. These results were obviously determined by the extracting potential of the solvent with regard to total phenolic substances. The results were in compliance with the previous findings [39], stating that the yields of polyphenols are strongly influenced by the solvent (the percentage of the organic solvent in the extraction mixture). A partial exception was BP genotype, producing MEPP with higher phenolic content than the EEPP or AEPP - 10.34 GAE/100 g FW and 15.87 GAE/100 g FW, respectively, for MEPP from the whole berries and the pulp fraction. In all of the studied fruit genotypes the total phenolic content was higher in the extracts obtained from the pulp fraction, compared to the respective value in the intact berries. The total phenolic content in this category of extracts varied in the range from 23.98 to 30.60 GAE/100 g FW (EEPP) and from 14.99 to 26.06 GAE/100 g FW (AEPP). The differences were obviously connected to the nature of the studied plant matrices. The isolation of seeds to obtain the pulp fractions resulted in an increase of the total phenolic content relative to the fresh weight of the sample.

Table 1. Total phenolic content and total flavonoid content in extracts from different *P. peruviana* L. fruit

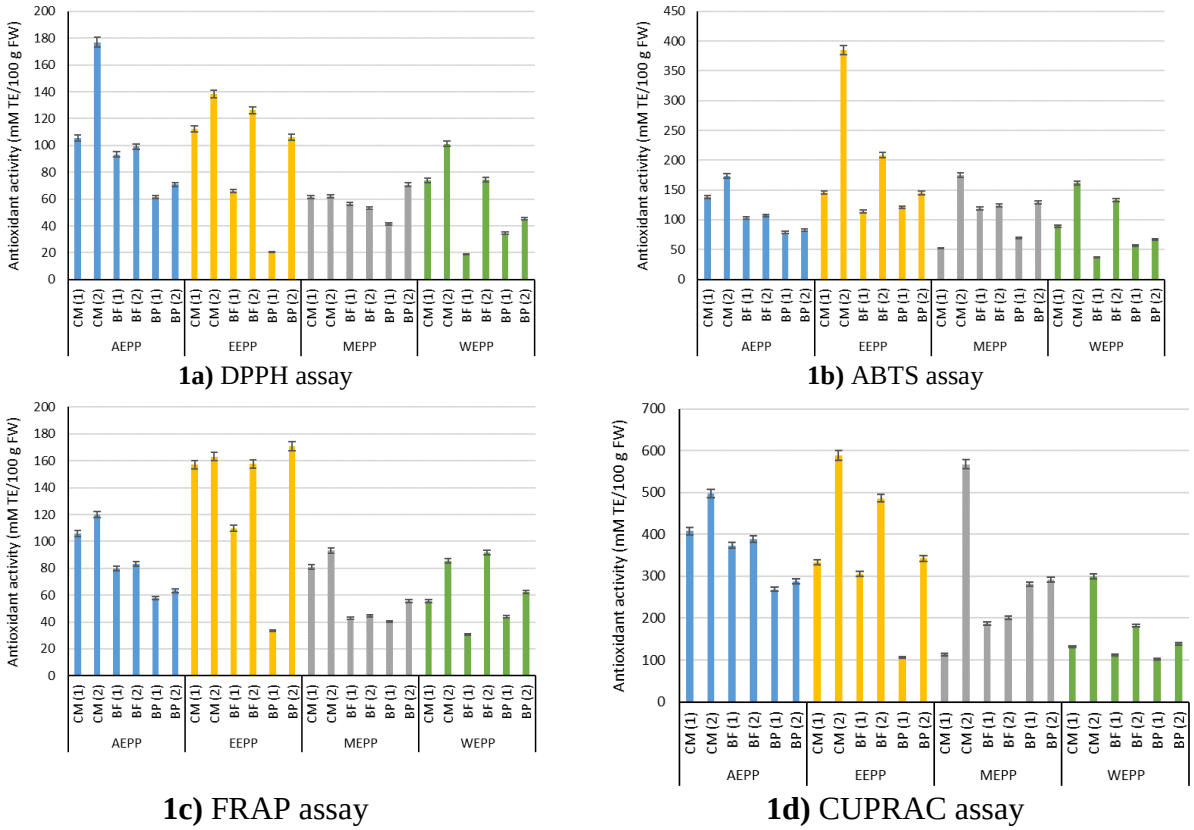
Fruit sample	Extract	Total phenolic content (mg GAE/100 g FW)	Total flavonoids (mg QE/100 g FW)
CM (1) [a]	Acetone	22.59±0.31	17.49±1.31
	95% Ethanol	22.29±0.55	19.63±0.54
	50% Methanol	15.14±0.85	0.73±0.12
	Water	13.76±0.23	nd[d]
CM (2) [a]	Acetone	26.08±0.21	17.88±0.21
	95% Ethanol	25.48±0.45	28.06±0.34
	50% Methanol	16.50±0.32	1.58±0.54
	Water	16.16±1.11	nd
BF (1) [b]	Acetone	18.67±0.85	17.35±0.43
	95% Ethanol	18.15±0.34	10.98±0.11
	50% Methanol	5.15±0.12	1.51±0.30
	Water	3.82±0.81	nd
BF (2) [b]	Acetone	23.06±0.80	18.00±0.13
	95% Ethanol	30.60±0.34	11.08±0.31
	50% Methanol	12.97±0.12	1.22±0.43
	Water	10.16±0.65	nd
BP (1) [c]	Acetone	14.53±0.11	9.48±0.30
	95% Ethanol	5.61±0.12	4.65±0.21
	50% Methanol	10.34±0.81	0.80±0.11
	Water	6.81±0.11	nd
BP (2)[c]	Acetone	14.99±0.21	12.87±0.19
	95% Ethanol	23.98±0.22	4.58±0.28
	50% Methanol	15.87±0.43	1.11±0.35
	Water	13.28±0.54	nd

[a] CM – origin Colombia, as supplied from the market; [b] BF – origin Bulgaria, from Bio-farm "Versol", Lik village; [c] BP – origin Bulgaria, variety "Plovdiv", from the Agricultural University, Plovdiv; [d] nd – not detected; (1) whole berries; (2) berries without seeds

Similarly, the total flavonoids content in the pulp fraction was relatively higher – between 4.58 and 28.08 mg QE/100 g FW (EEPP) and between 12.87 and 18.00 mg QE/100 g FW (AEPP). None of the WEPP extracts were found to contain flavonoids, while MEPP were with very low total flavonoids values (between 0.73 and 1.58 mg QE/100 g FW). As these results suggest, there was a more significant variation between the extracts with regard to their flavonoid content compared to that of total phenolic content.

These results revealed that Cape gooseberry fruits were rich in the phenolic compounds, which are a group of bioactive agents with expressed antioxidant activity. As suggested, there were some interesting origin-related differences, both in total phenolic and in total flavonoid content. In general, the imported Colombian fruits (CM) were the richest in phenolics and flavonoids, followed by the fruits of local origin, the farm (BF) and variety "Plovdiv" (BP) genotypes, although some variation also existed, for example in BF pulp (AEPP and EEPP) and BP pulp (EEPP) extracts. These results were probably due to the effect of genetic, environmental, ripeness stage and other factors on the accumulation of phenolic metabolites and their individual composition. The data suggest that fruit origin and genotype is a significant aspect that has to be considered when the biologically active or beneficial properties of Cape gooseberry are discussed, and provide fields for future studies on the chemical composition of the fruit from the different origins and on the respective influencing factors [40].

The observations in the present study were supported by previous findings, although a direct comparison of data was not always possible, due to the variation of the applied solvents, the extraction procedures and analytical methods used in different studies. The values of total phenolic content were very close to those reported by [40-47] (24.91-77.42 mg GAE/100 g), and those of the total flavonoid content – to the values of other authors [15].



1a) DPPH assay **1b)** ABTS assay

1c) FRAP assay **1d)** CUPRAC assay

Fig. 1. The antioxidant activity of acetone (AEPP), ethanol (EEPP), methanol (MEPP) and water (WEPP) extracts from *P. peruviana* L. fruit: CM – origin Colombia, from the market; BF – origin Bulgaria, Bio-farm; BP – origin Bulgaria, variety "Plovdiv"; (1) whole berries; (2) berry pulp.

All previous studies, as well as the current study, unanimously revealed that Cape gooseberry fruit is a rich source of polyphenols and other biologically active constituents determining the nutritional quality.

The results of the antioxidant activity assays for Cape gooseberry fruit of different origin are presented in Figure 1. All extracts demonstrated antioxidant activity which was generally well expressed in the EEPP from fruit pulp. In these extracts, the DPPH radical scavenging activity was the highest in CM (176.99 mM TE/100 g FW), followed by BF (126.38 mM TE/100 g FW) and BP (106.11 mM TE/100 g FW). The same tendency in the antioxidant potential was observed in other assays. ABTS activity values were 384.20 mM TE/100 g FW for CM, 208.38 mM TE/100 g FW for BF and 145.20 mM TE/100 g FW for BP, respectively. The maximal FRAP values were 163.12 mM TE/100 g FW in CM, 157.58 mM TE/100 g FW in BF and 170.94 mM TE/100 g FW in BP. CUPRAC results were in the ranges from 588.36 mM TE/100 g FW (CM) to 486.60 (BF) and 342.76 mM TE/100 g FW (BP). These results were in accordance with previous findings about Cape gooseberry antioxidant activity determined by different assays [7, 10, 13, 42-44, 46-50]. Although the antioxidant activity of the extracts from locally produced fruit was generally weaker than that of the imported Colombian fruit, the parallel to the available data from the studies cited above revealed that both BF and BP were in no way inferior to those of the varieties produced worldwide. Our results reflected correspondingly the known differences between the respective SET antioxidant activity assays, as well as the correlations between them [51]. Several studies reported that Cape gooseberry antioxidant activity was lower than that of cranberries, blueberries and other small fruit, but higher or close compared with that of apples, pears, cherries, plums, red grape, pitaya, etc. [23, 45, 47, 51]. Our results were in accordance with these studies, as well. One very important aspect in the interpretation of results about the antioxidant properties of plant extracts is their intended use. Their applicability depends on the safety of the solvent [39]. In this context the high values of the antioxidant activity of the ethanol extracts in this study, as well as the radical scavenging and metal chelating activity demonstrated by the water extracts can be assumed as promising results, as these extracts are fully applicable in food, beverage or cosmetics production [47, 52].

The correlations between the values of DPPH, FRAP, ABTS and CUPRAC activity and total phenolic contents were also evaluated (Table 2).

Table 2. Correlation coefficient (r^2) between total phenolic content, total flavonoids and the antioxidant activities (DPPH, ABTS, FRAP and CUPRAC) of *P. peruviana* fruit

Index	DPPH	ABTS	FRAP	CUPRAC
Total phenols	0.8811	0.6006	0.8566	0.8000
Total flavonoids	0.7240	0.5515	0.5690	0.5904

Positive linear correlations between the total antioxidant activities, total phenolic contents and the total flavonoids content were found. Therefore, according to the current study, the phenolic and flavonoid compounds of Cape gooseberry fruit have antioxidant, radical scavenging and metal chelating properties. The correlations between the total antioxidant activities and the total phenolic content were better defined (coefficient of correlation r^2=0.88 and 0.86 for DPPH and FRAP values, respectively), than for the total flavonoid content, suggesting that mainly total phenols in Cape gooseberry provided antioxidant activity. Several studies have indicated a positive correlation between phenolic contents and the antioxidant power of plant extracts. Our results were in good agreement with the findings by [23] about the strong positive correlation of the antioxidant capacity of the different fruits, vegetables and beverages with total phenolic content (r^2=0.946 for ABTS and 0.897 for DPPH, respectively) and the moderate correlation with total flavonoid content (0.718 for ABTS, 0.708 for DPPH, respectively). Similar results on total phenolics basis were obtained by [51] (r^2=0.7569 for DPPH, 0.8447 for FRAP and 0.8025 for ABTS, respectively), [45] (r^2=0.9871 for DPPH), [47] (r^2 values between 0.87 and 0.78 for DPPH, FRAP, ABTS and CUPRAC) and others [53]. It should be considered that the antioxidant power depends not only on the overall quantity of these classes of phytochemicals, but also on their individual composition and proportions, and moreover, on the synergistic effect of other antioxidants of different chemical nature existing in the extracts [7, 10, 13, 18, 25, 46, 47, 50, 52]. Therefore, future studies on the phenolic profiles and on other phytochemicals with antioxidant properties would be relevant in order to characterize in more detail the antioxidant potential of Cape gooseberry genotypes from Bulgaria.

CONCLUSIONS

To the best of our knowledge, this study presented for the first time results about the antioxidant activity (determined by DPPH, ABTS, FRAP, and CUPRAC assays) of *P. peruviana* fruit originating from Bulgaria (in comparison with imported fruit of Colombian origin), and about the total phenolics and total flavonoids content in different extracts obtained from them. The Cape gooseberry fruits were a rich source of total phenols and flavonoids, with the highest concentration of the bioactive compounds achieved in the ethanol extracts of fruit pulp fraction (23.98-30.60 GAE/100 g FW and 4.58-28.08 mg QE/100 g FW, respectively for the total phenolic content and total flavonoid content). All extracts demonstrated antioxidant activity (DPPH, FRAP, ABTS and CUPRAC), which was generally well expressed in the EEPP from fruit pulp. Although the antioxidant activity of the extracts of the locally produced fruit was generally weaker than that of the imported Colombian fruit, the study revealed that the two local genotypes were in no way inferior to those of the varieties produced worldwide. There were positive linear correlations between total antioxidant activities, total phenolic contents and the total flavonoids, therefore, according to the current study, phenolic and flavonoid compounds of Cape gooseberry fruit have antioxidant, radical scavenging and metal-chelating properties. The results of the study make relevant a further investigation on Cape gooseberry fruit originating from Bulgaria, aimed at a more detailed characterization of their composition, health benefits and potential for use.

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Impact of the duration of ultrasound-assisted extraction on total phenolics content and antioxidant activity of lupin seeds

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The influence of the duration of ultrasound-assisted extraction on antioxidant activity and total phenolics content of lupin seeds (*Lupinus angustifolius* L. cultivar 'Boregine') was investigated for the first time. Lupin seeds were with German origin but introduced in Bulgaria. They were extracted for 10, 20 and 30 min with absolute methanol and the obtained extracts were evaluated for total phenolic content and antioxidant activity. The polyphenol content of the extracts was found to be in the range of 1.65 – 2.03 mg gallic acid equivalents (GAE)/g dry weight sample, depending on the duration of the extraction process. The antioxidant activity was estimated by ABTS•+ (2,2′azinobis-(3-ethylbenzthiazoline-6-sulfonic acid)) (2.28 – 2.89 mmol Trolox Equivalent (TE)/g dw), DPPH• (1,1-diphenyl-2-picrylhydrazyl radical) (2.01 – 2.71 mmol TE/g dw), FRAP (ferric reducing/antioxidant power) (3.76 – 4.36 mmol TE/g dw) and CUPRAC (cupric ion reducing antioxidant capacity) (3.07 – 4.69 mmol TE/g dw) methods. Generally, methanol extracts with 30 min of extraction displayed the highest total phenolic contents, while 10 min of extraction time was the least efficient ultrasound-assisted method. On the other hand, antioxidant activity of the extracts was highest in 20 min of extraction apart from CUPRAC method where 30 min of extraction depicted more antioxidant capacity of the methanol extract.

Keywords: Lupin seeds, antioxidant activity, total phenolic content, extraction duration.

INTRODUCTION

Phenolic compounds are found in all plant species and represent secondary metabolites which may possess resistance to oxidation processes [1, 2]. They have antioxidative, antiallergic, anti-inflammatory, and anticarcinogenic activities. Phenolic compounds can also protect against cell damage and prevent the risk of degenerative diseases [3-5].

There are several methods for extracting the phenolic compounds from different parts of the plants including hot continuous extraction method (Soxhlet), liquid-liquid extraction, solid-liquid extraction, supercritical fluid extraction, solid-phase micro extraction, microwave extraction, sonication, etc. [6]. Ultrasound-assisted extraction is often performed in order to enhance the molecular interaction and to reduce the extraction time of polyphenols. On the other hand, many authors report that the total polyphenol amounts from the same plant and its antioxidant activity may vary widely, depending on the applied extraction conditions – type of solvents and duration of extraction [1, 7].

Due to their biologically active molecules many leguminous plants (in particular lupin) are considered to have antioxidant activity. Lupin, which belong to family Fabaceae, is a good source of valuable nutrients such as proteins, lipids, dietary fibres, minerals and vitamins, as well as phytochemicals (polyphenols, mainly tannins and flavonoids) which possess antioxidant capacity [8-11]. Few researchers reported that lupin seeds are a source of polyphenols and exhibit antioxidant activity [11-13]. On the other hand, there is scarce information about the impact of the extraction time on antioxidant activity and total phenolic content of lupin seeds. For that reason, the aim of the present study is to examine the total phenolic content and antioxidant activity of lupin seeds (*Lupinus angustifolius* L. cultivar 'Boregine') using ultrasound-assisted extraction performed with absolute methanol for different time intervals (10, 20 and 30 min).

MATERIAL AND METHODS

Samples

Lupin (*Lupinus angustifolius* L. cultivar 'Boregine') is a German variety introduced in the southern part of Bulgaria. The plant was harvested in June 2018, the seeds were removed, air-dried and used for the subsequent analysis.

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Chemicals and reagents

Chromatographic grade methanol was used for the analyses (VWR, Austria). Ammonium acetate, copper(II) chloride, gallic acid, glacial acetic acid, sodium acetate trihydrate, ferric chloride hexahydrate, hydrochloric acid, 6-hydroxy-2,5,7,8-tetramethychroman-2-carboxylic acid (Trolox,) and reagents 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), neocuproine (2,9-dimethyl-1,10-phenanthroline), 2,4,6-tris(2-pyridyl)-*s*-triazine (TPTZ), Folin–Ciocalteu reagent, phosphate buffered saline, pH 7.4, (PBS), were purchased from Sigma-Aldrich.

Preparation of extracts

The milled plant material was weighed with 0.0001 g precision and 2 g were used for analysis. The initial milled material was extracted *via* ultrasonication for 10, 20 and 30 min with 40 mL of methanol at room temperature. All samples were filtered under vacuum. The extraction was repeated three times. The supernatants were combined and evaporated under vacuum at a temperature of the water bath 40°C. The volume of all samples was adjusted to 60 mL and passed through a membrane filter with pore size of 0.45 μm prior to analysis.

Total phenolic content (TPC)

TPC in the extracts was determined by a colorimetric method using Folin-Ciocalteau's reagent [14] with slight modifications. Calibration curve was obtained using as standard an ethanolic solution of gallic acid at concentrations between 25 and 1000 μg/mL. Briefly, 100 μL of extract or gallic acid standard was mixed with 2.4 mL of distilled water, 500 μL of 0.2 M Folin-Ciocalteu's reagent and 2 mL of 7.5 % sodium carbonate solution. The tested samples were incubated for 2 h in dark at room temperature. The absorbance of the samples was measured at 765 nm with a spectrophotometer Camspec M508, England, using a blank sample. The total phenolics content was expressed as mg gallic acid equivalent per gram of dry weight (mg GAE/g dw) based on the calibration curve.

Trolox Equivalent Antioxidant Capacity (TEAC)

ABTS method. The Trolox Equivalent Antioxidant Capacity (TEAC) was determined by the colorimetric method reported by Re *et al*. (1999) [15]. For this assay, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) cation radical (ABTS•+) solution was prepared by dissolving 7 mM of ABTS in 2.45 mM $K_2S_2O_8$. This mixture was shaken for 12 – 16 h at ambient temperature in the dark until obtaining a stable oxidative state. For the study of the extracts, the ABTS•+ stock solution was diluted with PBS until absorbance became 0.70±0.02 at 734 nm. Sample analysis was performed as follows: 2 mL of ABTS solution and 20 μL of sample or standard were mixed. Absorbance of sample was measured at 734 nm with a spectrophotometer Camspec M508, England after samples incubation at 25°C for 5 min. The calibration curve was plotted by using 6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox) as a standard. The results were expressed as mmol Trolox equivalents per g of dry weight (mmol TE/g dw).

Cupric ion reducing antioxidant capacity (CUPRAC) method

The lupin seeds extracts were investigated by cupric ion reducing antioxidant capacity (CUPRAC) method described by Apak *et al*. (2006) [16]. In test tubes were mixed 1 mL copper(II) chloride solution (10 mM), 1 mL neocuproine alcoholic solution (7.5 mM) and 1M ammonium acetate buffer solution (pH = 7), 0.2 mL tested extract or Trolox and 0.9 mL water (final volume, 4.1 mL). Absorbance against a blank sample was measured at 450 nm with a spectrophotometer Camspec M508, England after 30 min in dark at room temperature. Calibration curve was achieved using Trolox as a standard ethanolic solution at concentration ranges between 0.045 and 1.5 mM. Trolox equivalent antioxidant capacity was plotted as mmol Trolox equivalents per g of dry weight (mmol TE/g dw).

Ferric reducing antioxidant power (FRAP) method

All samples were investigated by ferric reducing antioxidant power (FRAP) method described by Benzie and Strain (1996) [17]. The FRAP reagent was freshly prepared before analyzes by mixing 0.3 M acetate buffer (pH 3.6), 10 mM 2,4,6- tripyridyl-*s*-triazine (TPTZ) in 40 mM HCl and 20 mM $FeCl_3.6H_2O$ in dd H_2O in a ratio 10:1:1. In test tubes were mixed 0.15 mL tested extract or standard Trolox and 2.85 mL FRAP reagent. Absorbance against a blank sample was measured at 593 nm with a spectrophotometer Camspec M508, England after 15 min in dark at room temperature. Calibration curve was achieved using Trolox as a standard ethanolic solution at concentration ranges between 0.045 and 1.5 mM. Trolox equivalent antioxidant capacity was plotted as mmol Trolox equivalents per g of dry weight (mmol TE/g dw).

DPPH method

Antioxidant activity was measured according to Brand-Williams *et al*. (1995) [18] procedure. In the test tubes were mixed 150 μL of extract or Trolox and 2.85 mL of 0.12 mM DPPH (2,2-diphenyl-1-picrylhydrazyl) reagent, which was prepared with 4.8 mg DPPH dissolved in 100 mL CH_3OH). The mixtures were shaken and then incubated for 30 min at room temperature. The absorbance was recorded at 517 nm with a spectrophotometer Camspec M508, England. To quantify the antioxidant activity a standard Trolox curve was used in the concentration range from 0.045 to 1.5 mmol Trolox. The results were expressed as mmol TE/g dw.

Statistical analysis

All measurements were performed in triplicate (n = 3) and the results were presented as mean value with the corresponding standard deviation (SD). Significant differences were determined by analysis of variance (Duncan test) with a significance level $p<0.05$ using IBM SPSS Statistics 19.

RESULTS AND DISCUSSION

Plant extracts were obtained by ultrasound-assisted solvent extraction with methanol for 10, 20 and 30 min and were subjected to estimation for their total phenolic content and antioxidant activity.

Total phenolic content (TPC) of the lupin seeds extracts is shown in Figure 1.

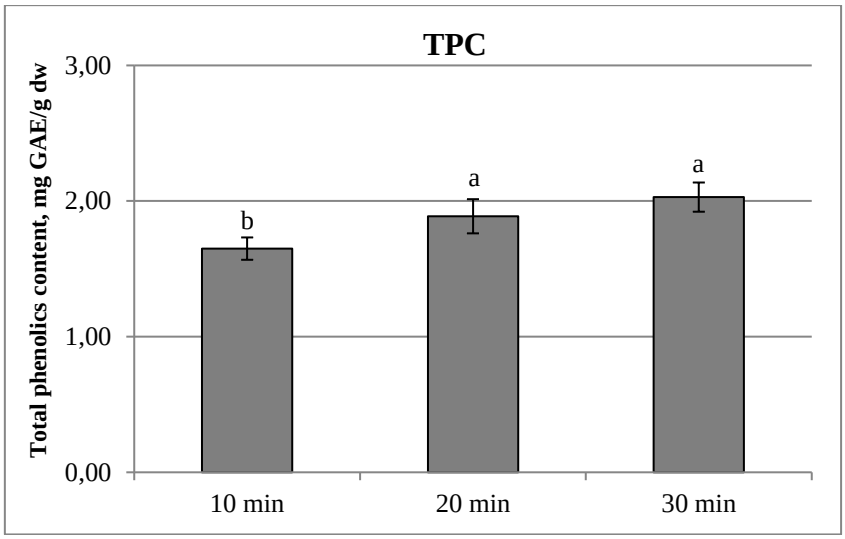

Fig. 1. Total phenolic contents (TPC) of extracts from lupin seeds. * Different letters indicate significant difference at $p < 0.05$ levels by Duncan's Multiple Range Test

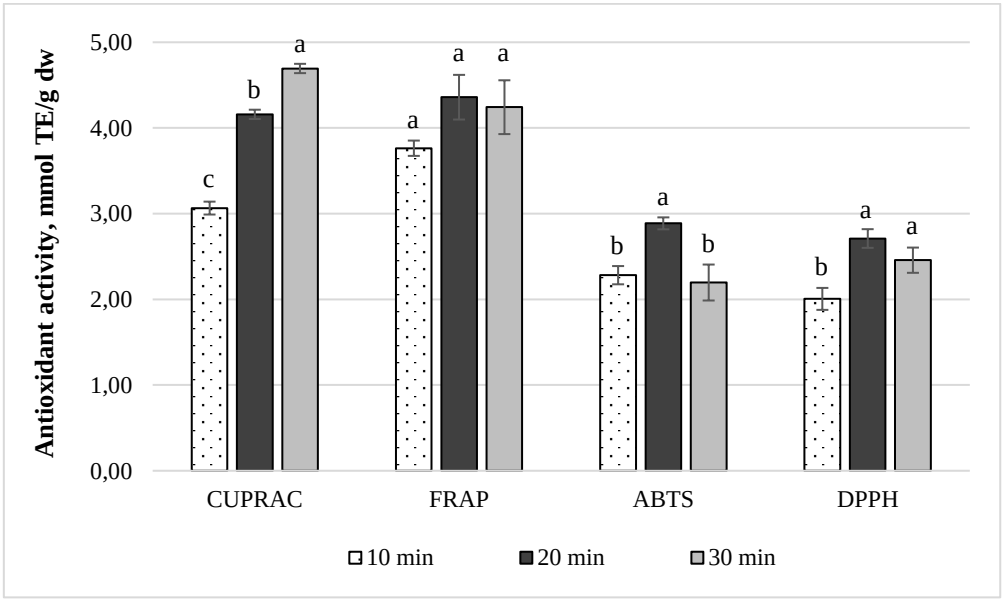

Fig. 2. Antioxidant activity of methanol extracts of lupin seeds using CUPRAC, FRAP, ABTS and DPPH methods. *Different letters for the same method indicate significant difference at $p < 0.05$ levels by Duncan's Multiple Range Test

TPC of the samples ranged from 1.65 to 2.03 mg GAE/g dw. It was observed that the content of the total phenolics increased with increasing the time of the extraction. The analysis of variance using Duncan test (with a significance level $p < 0.05$) revealed that there were significant differences in total phenolics in 10 min and the other extraction times. On the other hand, the differences between 20 min and 30 min of extraction were not significant ($p > 0.05$). Similar results were obtained by Martínez-Villaluenga *et al.* (2009) [8] and Fernandez-Orozco *et al.* (2006) [19] who reported the total phenolics content to range from 1.8 to 2.5 mg ferrulic acid equiv/g and from 1.43 to 3.55 mg (+) catechin/g, respectively. Karamać *et al*. (2018) [20] observed that total polyphenols in different wild and cultivated *Lupinus albus* L. seeds were slightly higher (from 4.36 to 7.24 mg GAE/g) than the reported in the present study. Significantly higher results for eight *L. angustifolius* genotypes grown in western Canada were reported by Oomah *et al*. (2006) [11] (from 12.75 to 14.65 mg (+) catechin/g).

Four different tests were used for the analysis of the antioxidant capacity of methanol extracts of the examined seeds. The scavenging activity of the extracts towards DPPH, ABTS, FRAP and CUPRAC was expressed in mmol TE/g dw.

The CUPRAC values for the lupin seeds ranged from 3.07 to 4.69 mmol TE/g dw, where the lowest value was for the shortest time of extraction and the highest was observed for 30 min of extraction. There were significant differences between the antioxidant activities of the extracts for all durations, which signified that the best extraction time for lupin seeds using CUPRAC method was 30 min.

The FRAP values ranged between 3.76 – 4.36 mmol TE/g dw, but the analysis of variance using Duncan test (with a significance level $p < 0.05$) revealed that there were not significant differences of the antioxidant activity of lupin seeds extracted for 10, 20 and even 30 min. These results were much higher than those reported for lupin seeds by Karamać *et al*. (2018) [20].

The antioxidant capacity by ABTS radical cation of the methanol extracts of lupin seeds ranged from 2.20 (30 min) to 2.89 mmol TE/g dw (20 min). No significant differences between the ABTS values for lupin seeds extracted for 10 and 30 min were noticed, but they were significantly different for 20 min of extraction where the highest value was observed. The results obtained were much higher than those reported by Martínez-Villaluenga *et al*. (2009) [8] who established that the antioxidant capacity of raw seeds of *L. angustifolius* cv. Troll, *L. angustifolius* cv. Emir and *L. albus* cv. Multolupa, measured by TEAC assay, were as follows: 47.9, 47.0 and 71.4 μmol Trolox/g d.m., respectively.

The DPPH radical scavenging activity is used for estimation of the antioxidant capacity of extracts against oxidation caused by free radicals [21] and the values for the lupin extracts ranged from 2.01 (10 min) to 2.71 mmol TE/g dw (20 min). It was observed that the antioxidant activity of lupin seeds using DPPH assay were not significantly different for 20 and 30 min of extraction, even though the value in 30 min was lower. The results from the present study were much higher than those reported by Martínez-Villaluenga *et al.* (2009) [8] who established that the antioxidant capacity of raw seeds of *L. angustifolius* cv. Troll, *L. angustifolius* cv. Emir and *L. albus* cv. Multolupa were as follows: 3.09, 3.06 and 2.83 μmol Trolox/g dw, respectively.

As it is observed, CUPRAC and FRAP antioxidant capacity assay of lupin seed extracts showed higher values than ABTS and DPPH methods.

It is well known that the antioxidant activity is also related to the content of polyphenols [22]. The results from the present study confirmed this statement – the highest total phenolic content was observed in the lupin seeds extracted for 20 and 30 min and the highest values for antioxidant capacity were noticed in the seeds for the same duration of extraction. The analysis of variance using Duncan test depicted that the duration of the ultrasound-assisted extraction did influence the total polyphenol content and the antioxidant activity of lupin seeds when they were extracted for 20 and 30 min in absolute methanol.

CONCLUSION

The impact of the duration of ultrasound-assisted extraction on antioxidant activity and total phenolic content of lupin seeds (*Lupinus angustifolius* L. cultivar 'Boregine') was examined for the first time. The phenolic contents of the seeds during 30 min of extraction did not differ significantly from those during 20 min of extraction. The same tendency was observed in the antioxidant activity of the extracts determined by FRAP and DPPH methods. The methanol extracts of the seeds exhibited higher antioxidant activity in 30 min using CUPRAC method which was significantly different from the extraction for 10 and 20 min. The extraction for 20 min showed higher antioxidant activity for ABTS method, while those for 10 and 30 min they were not significantly different.

Overall, the best methods for evaluating the antioxidant capacity of lupin seeds were CUPRAC and FRAP, and the most suitable time for extraction was 20 min.

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Antioxidant effect of an aqueous extract of alga *Cystoseira stricta* during the frozen storage of Atlantic Chub mackerel (*Scomber colias*)

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An aqueous extract of alga *Cystoseira stricta* was included in the glazing medium employed during the frozen storage of Atlantic Chub mackerel (*Scomber colias*). Rancidity stability of frozen fish muscle was determined throughout a 9-month storage at –18 ºC. An inhibitory effect on the development of lipid oxidation (assessment of peroxides, thiobarbituric acid and fluorescence indices) was observed as a result of the alga extract presence in the glazing system; thus, a marked retention of polyunsaturated fatty acids and alpha-tocopherol contents was achieved. Furthermore, an inhibitory effect on the lipid hydrolysis development and trimethylamine formation was implied as a result of the alga extract presence. Interestingly, enhancement of rancidity stability in frozen mackerel was found stronger by increasing the concentration of the alga extract in the glazing medium. A preservative effect of aqueous alga extract is established, this effect being attributed to the presence of potential active compounds able to stabilise radicals responsible for the lipid oxidation development.

Keywords: *Cystoseira stricta*; aqueous extract; frozen fish; glazing; rancidity; quality.

INTRODUCTION

The presence in fish species of a highly unsaturated lipid composition and the high content of pro-oxidant molecules have been reported as the most decisive factors influencing the shelf-life of frozen fatty fish products [1]. To retard lipid oxidation as long as possible and, accordingly, extend the shelf life, a wide number of advanced and traditional strategies to be combined to freezing and frozen storage have been tested such as hydrostatic high pressure, vacuum packaging, glazing, preservatives addition and active and intelligent packaging [2-5].

Marine algae have widely been consumed as food in Asian countries for centuries. As photosynthetic organisms, algae are known to be exposed to a combination of light and high oxygen concentration. The lack of structural damage in their organs has led to consider that their protection against oxidation would arise from their natural content on antioxidant substances [6, 7]. Consequently, marine algae are receiving an increasing attention as a source of bioactive compounds (i.e., polyphenols, carotenoids, etc.) able to inhibit lipid oxidation development during food processing [8-10].

Among brown macroalgae, *Cystoseira* genus has shown to be widely distributed in temperate regions of the Northern hemisphere such as the Mediterranean Sea, and the Indic and Pacific Oceans. Interestingly, several *in vitro* studies reported the preservative effect of certain bioactive compounds present in such algae. Thus, an antioxidant effect of *C. tamarisfolia* [11], *C. compressa* [12] and *C. hakodatensis* [13] extracts has been reported, this effect being explained by the presence of potential active compounds such as pholoroglucinol, manitol, fatty acids, fucosterol and polyphenols in general.

The current research focused on *C. stricta*. Thus, previous research showed the presence in this alga of profitable chemical constituents such as polyunsaturated fatty acids (PUFA), macro- and micro-mineral elements, as well as a relevant alpha-tocopherol content [14]. In this study, an aqueous extract of this alga was included in the glazing medium employed during the frozen storage of Atlantic Chub mackerel (*Scomber colias*). Its effect on lipid damage development was measured throughout a 9-month frozen storage (–18 ºC) in mackerel muscle.

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MATERIALS AND METHODS

Preparation of algae extracts and glazing systems

Fresh *C. stricta* was collected in May 2016 on the Oran coast, Western Algeria (35° 44′ 29.72″ N and 0° 50′ 14.21″ W). Species identification was made in the Laboratory of Aquaculture and Bioremediation by employing the Algabase site (www.algabase.com) [15]. Upon arrival to the laboratory, alga samples were washed thoroughly with running water to remove salts, sand and epiphytes. Biomass was then washed with distilled water and dried at room temperature (23 ± 2 °C) for 72 h in the dark. Then, the dried material was milled, powdered and stored at −20 °C until further analysis.

To prepare the glazing systems, 100 g of dried alga were mixed with distilled water (1,600 mL), stirred for 30 s, centrifuged at 3,500 rpm for 10 min at 4 ºC and the supernatant recovered. This procedure was carried out two more times. The collected extracts were pooled together and adjusted to 5 L with distilled water.

Three different concentrations of the alga extract were tested as glazing medium. For it, 385, 1,153 and 3,461 mL (corresponding to 7.7, 23.1 and 69.2 g of dried alga, respectively) of the alga extract were adjusted to 11 L by addition of distilled water, respectively. The resulting solutions were employed as glazing systems and labelled as G-1, G-2 and G-3 conditions, respectively.

Fish material, processing and sampling

Fresh Atlantic Chub mackerel (153 specimens) were caught near the Galician Atlantic coast (North-Western Spain) and transported in ice to the laboratory. The length and weight of the fish specimens ranged from 24.5 to 27.5 cm and from 105 to 123 g, respectively.

Upon arrival to the laboratory, nine specimens (three groups of three individuals each) were separated and analysed independently (n = 3). The remaining fish specimens were divided into four batches (36 individuals in each batch) that were immediately frozen at –40 ºC. After 48 hours at –40 ºC, one batch was immersed in water, while the three others were immersed in the G-1, G-2 and G-3 systems, respectively. In all cases, fish specimens were immersed for 30 s at 0 ºC, allowed to drain for 15 s, packaged in polyethylene bags (three pieces per bag) and stored at –18 ºC. Sampling was undertaken at months 1, 3, 6 and 9 of frozen storage at –18 ºC. At each time and for each condition, nine individuals were taken, that were divided into three groups (three individuals per group) and studied separately. Analysis of frozen material was undertaken after thawing; thawing was carried out by overnight storage in a cool room (4 ºC).

All solvents and chemical reagents used were of reagent grade (Merck, Darmstadt, Germany).

Chemical analyses related to quality loss

Lipids were extracted from the mackerel white muscle by the Bligh and Dyer [16] method, which employs a single-phase solubilisation of the lipids using a chloroform-methanol (1:1) mixture.

Peroxide value (PV) was determined spectrophotometrically (520 nm) (Beckman Coulter DU 640 spectrophotometer) on the lipid extract of the fish muscle by peroxide reduction with ferric thiocyanate, according to Chapman and McKay [17]. The results were calculated as meq. active oxygen·kg^{-1} lipids.

Thiobarbituric acid index (TBA-i) was determined according to Vyncke [18]. This method is based on the reaction between a trichloroacetic acid extract of the fish muscle and thiobarbituric acid (TBA). Content of thiobarbituric acid reactive substances (TBARS) was spectrophotometrically measured at 532 nm and calculated from a standard curve using 1,1,3,3-tetraethoxy-propane (TEP). Results were calculated as mg malondialdehyde·kg^{-1} muscle.

Fluorescent compounds formation (fluorimeter LS 45; Perkin Elmer España; Tres Cantos, Madrid, Spain) was measured in the aqueous phase obtained during the lipid extraction [16] of the fish muscle. As described previously [19], fluorescence was measured at excitation/emission of 393/463 and 327/415 nm. The relative fluorescence (RF) was calculated as follows: $RF = F/F_{st}$, where F is the fluorescence measured at each excitation/emission wavelength pair and F_{st} is the fluorescence intensity of a quinine sulphate solution (1 μg·mL^{-1} in 0.05 M H_2SO_4) at the corresponding wavelength pair. The fluorescence ratio (FR) was calculated as the ratio between the two RF values: $FR = RF_{393/463\ nm}/RF_{327/415\ nm}$.

Lipid extracts were converted into fatty acid methyl esters (FAME) by using acetyl chloride, being then analysed by gas–liquid chromatography (Perkin Elmer 8700 chromatograph, Madrid, Spain) [20]. Peaks corresponding to FAME were identified by comparison of their retention times with those of standard mixtures (Qualmix Fish, Larodan, Malmo, Sweden; FAME Mix, Supelco, Inc.). Peak areas were automatically integrated; C19:0 was used as the internal standard for quantitative purposes. The polyene index (PI) was calculated as the following

fatty acids contents ratio: (C20:5ω3 + C22:6ω3)/C16:0.

The profile of tocopherol compounds was analysed according to the method of Cabrini *et al*. [21]. For this, mackerel muscle was extracted with hexane, which was eliminated under nitrogen flux. The resulting alga extracts were then dissolved in isopropanol and injected into an HPLC system (ODS column, 15 cm × 0.46 cm i.d.); detection was achieved at 280 nm. The presence of different tocopherol compounds (alpha, beta, gamma and delta) was checked, their content being calculated with calibration curves prepared from the corresponding commercial tocopherols. For each tocopherol compound, results were expressed as $mg \cdot kg^{-1}$ fish muscle.

Free fatty acid (FFA) content was determined using the lipid extract of the fish muscle by the Lowry and Tinsley [22] method, which is based on complex formation with cupric acetate-pyridine followed by spectrophotometric (715 nm) assessment. The results were expressed as mg $FFA \cdot kg^{-1}$ muscle.

Trimethylamine-nitrogen (TMA-N) values were determined using the picrate colorimetric method, as previously described by Tozawa *et al*. [23]. This method involved the preparation of a 5% trichloroacetic acid extract of fish muscle (10 g/25 mL). Results were calculated as mg $TMA\text{-}N \cdot kg^{-1}$ muscle.

Statistical analysis

Data obtained from all chemical analyses were subjected to the ANOVA method to explore differences resulting from the effect of the glazing system. The comparison of means was performed using the least-squares difference (LSD) method. In all cases, analyses were carried out using the PASW Statistics 18 software for Windows (SPSS Inc., Chicago, IL, USA); differences among glazing batches were considered significant for a confidence interval at the 95 % level ($p < 0.05$) in all cases.

RESULTS AND DISCUSSION

Lipid oxidation development

This damage mechanism was measured by assessing the primary (peroxides), secondary (TBARS) and tertiary (fluorescence ratio) lipid oxidation compounds, as well as by the determination of the polyene index.

Peroxides formation showed a progressive increase with frozen storage time in all kinds of samples (Table 1). Comparison among batches revealed an inhibitory effect on primary oxidation as a result of the presence of the alga extract in the glazing system. Thus, lower average values could be observed in fish corresponding to alga-glazing batches when compared to their counterparts from the Control throughout the 3-9-month period. Differences were found significant ($p < 0.05$) at months 3 and 9 (G-3 batch) and at month 9 (G-2 batch).

Formation of TBARS also showed a progressive increase with time in all batches (Table 1).

Lower average values were obtained during the 3-9-month period for fish corresponding to G-2 and G-3 batches when compared to their counterparts from Control and G-1 batches; however, scarce significant differences ($p > 0.05$) could be obtained.

A slight increasing tendency of fluorescent compounds was observed in all kinds of batches under study (Table 1). Interestingly, lower average values throughout the 1-9-month period were implied for fish samples belonging to G-2 and G-3 batches when compared to their counterparts from Control and G-1; differences were found significant ($p < 0.05$) at months 3 and 6 (G-3 batch) and month 1 (G-2 batch). As for primary and secondary lipid oxidation compounds, no significant differences ($p > 0.05$) could be observed between G-2 and G-3 batches.

Finally, the PI assessment showed a general loss in all batches with storage time (Table 2), which agrees to the above-mentioned progressive increase of the content of all kinds of oxidation compounds. Also in agreement to such data, higher average values were observed in samples including any of the concentrations tested of the alga extract in the glazing system when compared to the Control; such differences were found significant ($p < 0.05$) for G-3 (3-9-month period) and G-2 (month 3) batches.

Frozen storage is known to be associated with fish lipid oxidation processes where different kinds of endogenous enzymes may be involved [24]. Inhibition of lipid oxidation found in the current research can be explained by the presence of antioxidant compounds in the aqueous extract of the current alga. Thus, although alcoholic solvents have been preferred as being the most accurate to obtain a high total phenolic content [6, 8], water extraction of algae has been reported to produce in most cases the highest yields, this including the majority of water-soluble compounds with preservative effects such as polysaccharides, proteins, glycosides and peptides [7, 9, 10].

Table 1. Assessment of lipid damage* in frozen mackerel glazed under different conditions**

Quality index	Storage time (months)	Glazing medium			
		Control	G-1	G-2	G-3
Peroxide value (meq. active oxygen·kg^{-1} lipids)	Initial	2.93 (1.15)			
	1	5.53 a (0.99)	4.42 a (1.53)	5.25 a (1.02)	5.61 a (1.29)
	3	9.20 b (0.47)	9.19 ab (3.94)	8.19 ab (1.51)	7.15 a (1.29)
	6	9.71 a (1.18)	9.05 a (1.70)	8.19 a (1.51)	6.98 a (1.77)
	9	19.92 b (2.62)	19.26 b (1.15)	12.30 a (0.96)	9.19 a (1.85)
Thiobarbituric acid index (mg malondialdehyde kg^{-1} muscle)	Initial	0.27 (0.07)			
	1	0.49 a (0.07)	0.50 a (0.03)	0.60 a (0.07)	0.56 a (0.12)
	3	0.86 b (0.18)	0.74 ab (0.14)	0.58 a (0.10)	0.64 ab (0.04)
	6	0.87 ab (0.23)	0.92 b (0.18)	0.54 ab (0.20)	0.52 a (0.15)
	9	1.22 a (0.02)	1.36 a (0.22)	1.12 a (0.11)	0.91 a (0.27)
Fluorescence ratio	Initial	0.49 (0.07)			
	1	0.81 b (0.16)	0.73 b (0.06)	0.58 a (0.02)	0.61 ab (0.09)
	3	0.82 b (0.01)	0.82 b (0.14)	0.77 ab (0.04)	0.75 a (0.01)
	6	0.91 b (0.05)	0.93 b (0.07)	0.80 ab (0.18)	0.79 a (0.04)
	9	1.32 a (0.10)	1.24 a (0.12)	1.23 a (0.11)	1.19 a (0.14)

* Average values of three replicates (n = 3); standard deviations are indicated in brackets. For each frozen storage time, values accompanied by different letters (a, b) indicate significant differences ($p < 0.05$); ** Glazing conditions: Control (water glazing, without alga extract), G-1 (low content of alga extract), G-2 (medium content of alga extract) and G-3 (high content of alga extract) in agreement to the Material and Methods section.

In agreement with the present study, previous related research accounts for lipid oxidation inhibition in canned fish by including a water extract of *Durvillaea Antarctica*, *Pyropia columbina* and *Ulva lactuca* [25] and of *Fucus spiralis* and *U. lactuca* [26] in the packaging medium. Related to fresh fish, the presence of a combined aqueous-ethanolic extract of *Fucus spiralis* in the icing medium employed during chilling storage of European hake (*Merlucccius merlucius*) led to an increased rancidity inhibition [27]. Furthermore, the presence of Pollock (*Theragra chalcogramma*) skin hydrolysates in frozen (4 months at –35 ºC) pink salmon [3] led to a lower TBARS formation, while the inclusion of aqueous/ethanolic extracts of *F. spiralis* and *Bifurcaria bifurcata* in the glazing system led to a lower development of rancid odour and taste in frozen (8 months at –18 ºC) mackerel (*S. colias*) [5]. Concerning the analysis of tocopherol compounds, only alpha-tocopherol was detected in mackerel muscle in the present study. Such result agrees to previous studies on wild fish species [26]. Contrary, farmed fish species have shown the presence of different kinds of tocopherol compounds, according to the composition of their diet [28]. In the current study, a marked loss of alpha-tocopherol was evident as a result of the frozen storage (Table 2). However, this loss was partially inhibited by the presence in the glazing system of the alga extract. Thus, higher average values were observed throughout the whole storage period for any of the alga-glazing batches when compared to the Control; however, significant differences ($p < 0.05$) were only obtained at month 3 for fish corresponding to the G-2 batch.

Table 2. Assessment of the polyene index and alpha-tocopherol content* in frozen mackerel glazed under different conditions**

Quality index	Storage time (months)	Glazing medium			
		Control	G-1	G-2	G-3
Polyene index	Initial	2.83 (0.12)			
	1	2.68 a (0.23)	2.68 a (0.12)	2.92 a (0.16)	2.86 a (0.07)
	3	2.56 a (0.08)	2.69 a (0.08)	2.75 b (0.07)	2.74 ab (0.14)
	6	2.38 a (0.10)	2.43 a (0.07)	2.48 a (0.07)	2.56 a (0.07)
	9	2.23 a (0.19)	2.41 a (0.21)	2.48 a (0.17)	2.51 a (0.09)
Alpha-tocopherol content ($mg \cdot kg^{-1}$ fish muscle)	Initial	39.59 (5.79)			
	1	24.05 a (3.80)	30.53 a (6.42)	28.98 a (1.66)	31.68 a (7.91)
	3	27.87 a (1.38)	30.01 ab (3.38)	30.43 b (0.83)	35.46 b (5.45)
	6	22.44 a (3.55)	23.48 ab (5.26)	28.63 ab (5.18)	29.75 b (3.02)
	9	12.61 a (4.59)	18.83 ab (5.66)	19.94 ab (3.21)	26.81 b (0.63)

* Average values of three replicates (n = 3); standard deviations are indicated in brackets. For each frozen storage time, values accompanied by different letters (a, b) indicate significant differences ($p < 0.05$). ** Glazing conditions as expressed in Table 1.

Table 3. Evolution of free fatty acids (FFA) and trimethylamine (TMA) formation* in frozen mackerel glazed under different conditions**

Quality index	Storage time (months)	Glazing medium			
		Control	G-1	G-2	G-3
FFA ($mg \cdot kg^{-1}$ muscle)	Initial	40.27 (0.19)			
	1	315.95 a (16.26)	336.19 a (9.86)	347.00 a (29.21)	325.17 a (4.79)
	3	484.4 b (15.40)	464.15 ab (6.20)	458.01 ab (11.41)	447.32 a (9.29)
	6	722.05 b (7.73)	658.06 a (4.79)	657.03 a (12.03)	641.86 a (4.73)
	9	1,081.41 d (12.87)	983.23 c (11.68)	905.66 b (12.42)	848.49 a (6.72)
TMA value (mg TMA-N$\cdot kg^{-1}$ muscle)	Initial	1.75 (0.13)			
	1	5.94 b (1.28)	3.94 ab (0.47)	4.42 b (0.44)	3.53 a (0.18)
	3	7.25 b (0.78)	6.08 a (0.10)	7.01 b (0.65)	6.76 b (0.21)
	6	10.67 a (1.77)	8.73 a (0.71)	8.41 a (1.21)	9.26 a (0.58)
	9	14.21 b (0.22)	12.89 ab (1.25)	11.87 a (0.18)	11.90 a (1.28)

* Average values of three replicates (n = 3); standard deviations are indicated in brackets. For each frozen storage time, values accompanied by different letters (a, b, c, d) indicate significant differences ($p < 0.05$). ** Glazing conditions as expressed in Table 1.

It is concluded that a preservative effect on the alpha-tocopherol content has been produced during the frozen storage time as a result of the presence of the antioxidant compounds present in the alga extract. This result agrees with the above-mentioned lower levels in oxidation indices previously described. A protective effect on alpha-tocopherol content was also observed in canned fish by the presence of a water extract of different kinds of algae such as *Durvillaea Antarctica, Pyropia columbina* and *U. lactuca* [25] and of *F. spiralis* and *U. lactuca* [26] in the packaging medium.

Formation of FFA and TMA

A very strong formation of FFA was implied throughout the frozen storage in all kinds of samples (Table 3).

However, the presence in the glazing system of the alga extract led in all cases to lower average values in the 3-9-month period when compared to the Control; interestingly, a significant effect ($p < 0.05$) was implied in the 3-9-month period (G-3 batch) and in the 6-9-month period (G-2 and G-1 batches). At the end of the study, fish corresponding to the G-3 batch showed lower ($p < 0.05$) FFA values than any other batch, so that a positive effect of the alga extract content in the glazing medium could be concluded.

Lipid hydrolysis development has been recognised as a most important event during the frozen storage of fish species [19, 28]. This degradative pathway has been signalled during the frozen storage as the result of an increased hydrolytic endogenous enzyme (lipases, phospholipases) activity by lipases release from liposomes in muscle, which then facilitates closer proximity between enzyme and substrate [24].

A marked formation of TMA was detected in all samples throughout the whole study (Table 3). At all sampling times, fish corresponding to Control condition provided higher average values than their counterparts of the different alga-glazing batches. Differences were found significant ($p < 0.05$) at months 1 and 9 (G-3 batch), month 9 (G-2 batch) and month 3 (G-1 batch). Consequently, an inhibitory effect on the formation of this amine could be concluded by the presence of an extract of the current alga in the glazing system.

During fish processing in general, TMA formation has been explained on the basis of microbial activity (i.e., breakdown of trimethylamine N-oxide, TMAO) [29] and protein degradation [30]. Since a frozen storage process is encountered in the current study, microbial activity ought to be minimised so that protein breakdown should be the main pathway for TMA formation. According to the lower TMA formation in the current study, it could be implied that a protective effect on protein degradation has been produced by the presence in the glazing medium of *C. stricta* extract.

CONCLUSIONS

An increase of rancidity stability in frozen mackerel was observed on the basis of the determination of lipid oxidation indices (peroxides, TBA and fluorescence indices), as well as by the protective effect observed on PUFA and alpha-tocopherol contents. Furthermore, an inhibitory effect on lipid hydrolysis development and TMA formation was implied as a result of the alga extract presence in the glazing system. Interestingly, this preservative effect was found stronger by increasing the concentration of alga extract in the glazing medium. A profitable effect of the aqueous extract of the current alga *C. stricta* is concluded. Further research is envisaged to analyse molecules involved in this preservative action.

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Quality increase of refrigerated fish by employment of a gelatine biofilm including a protein hydrolysate obtained from alga *Fucus spiralis*

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This study focusses on the employment of a gelatine-based biofilm during fish refrigeration. In it, a *Fucus spiralis* protein hydrolysate (PH) is included in the biofilm and used as packaging system in European hake (*Merluccius merluccius*) slices during the refrigerated storage (up to 6 days at 4 ºC). A progressive quality loss can be observed in hake slices by increasing the storage time. However, comparison with control fish shows a partial inhibitory effect on microbial activity development (counts assessment of aerobes, psychrotrophs, *Enterobacteriaceae*, proteolytics, lipolytics and anaerobes) and lipid oxidation (determination of thiobarbituric acid and fluorescence indices) and hydrolysis (free fatty acids formation) in hake samples packaged in the alga-gelatine condition. A preservative effect resulting from the PH presence in the gelatine-based biofilm is concluded, this showing a quality and safety enhancement and a potential commercial value increase. Further research focused on the optimisation of the current biofilm preparation ought to be addressed.

Keywords: gelatine film; *Fucus spiralis*; protein hydrolysate; refrigerated fish; lipid damage; microbial activity

INTRODUCTION

Marine foods deteriorate rapidly *post-mortem* as a consequence of a variety of biochemical and microbial breakdown mechanisms [1]. According to the increasing demand for high-quality fresh products, different strategies have been tested to delay fish damage as long as possible [2]. One strategy has been the use of packaging films containing preservative compounds (i.e., antimicrobials and antioxidants) so that a marine product with an increased shelf-life time is attained [3, 4]. Among preservative compounds, adverse health problems resulting of persistent consumption of synthetic antioxidants have recommended the use of natural antioxidants as an alternative to synthetic ones [5]. Therefore, the identification and isolation of novel natural antioxidants from aquatic and terrestrial sources is receiving an increasing interest.

Marine macroalgae have been reported to include a great variety of chemical constituents with potential antioxidant [6] and antimicrobial [7] activities susceptible to be applied during seafood processing. Thus, a wide number of biopreservative molecules such as polyphenols, phlorotannins, terpenes, chlorophylls, carotenoids, etc., have been isolated from such kind of algae. Among them, *Fucus spiralis*, a brown macroalga living in the littoral shore of the Atlantic coasts of Europe and North America, has shown promising preserving properties in recent studies [8, 9].

This work was focused on the development of novel active packaging methods based on natural compounds. Accordingly, its main objective was to investigate the potential preservative effects that the inclusion of a protein hydrolysate (PH) obtained from *F. spiralis* in a gelatine-based film may have on the preservation of hake (*Merluccius merluccius*) slices kept under refrigerated (4 ºC) conditions. For it, microbiological and chemical analyses related to quality loss were monitored throughout a 6-day storage.

MATERIALS AND METHODS

Film systems preparation

Lyophilised alga (*F. spiralis*) was provided by Porto-Muiños (Cerceda, A Coruña, Spain). A PH from this lyophilised alga was prepared following the procedure described by Benelhadj *et al*. [10] and later on incorporated into a cross-linked bovine gelatine film. For it, films were prepared by casting from their film-forming solutions (FFS). Thus, 50 g of alga PH:gelatine (1:24) powder were dissolved in 500 mL of 0.01 M NaOH and stirred for 20 min at 40 ºC. Oxidised sodium alginate (2.5 g; 5 % wt) and glycerol (7.5 g; 30 % wt) were then incorporated into the FFS as crosslinking agent and plasticiser, respectively. The suspension was stirred at 40 ºC for 120 min.

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Then, the FFS were cast onto teflon-coated trays and dried at 40 ºC in a convection oven for 48 h. The films were conditioned during 48 h in a chamber at 4±1 ºC prior to use. The resulting biofilm was referred to as FS-GE packaging condition.

A control gelatine biofilm was prepared in the same way as the FS-GE batch, but without *F. spiralis* PH (GE-CT packaging condition; gelatine control).

All solvents and chemical reagents used throughout the study were of reagent grade (Merck, Darmstadt, Germany).

Fish material, processing and sampling

Fresh hake (*Merluccius merluccius*) (9 specimens; 3.7-4.2 kg each) were caught near the Galician Atlantic coast (North-western Spain) and transported to the laboratory. Throughout this process (10 h), the fish were maintained in ice.

Upon arrival to the laboratory, specimens were cut in order to obtain 63 slices of 115-125 g each. Nine of the slices were distributed into 3 batches (three slices per group) that were analysed separately as initial material (n = 3). Meantime, 18 slices were placed in open air in a refrigerated room (4 ºC) and were considered as the Control batch (CONT condition). The remaining 36 slices were divided into two groups (18 slices per group) and were sealed-packaged individually in the two above mentioned packaging conditions (GE-CT and FS-GE), respectively. Packaged samples were placed in the above-mentioned refrigerated room (4 ºC). Fish samples from all batches were stored under such condition for a 6-day period, being sampled and analysed on days 1, 4 and 6. At each sampling time, 6 slices were taken from each batch for analysis and divided into three groups (two slices in each group) that were studied independently (n = 3).

Analysis of microbial development

Portions of 10 g of fish muscle were dissected aseptically from refrigerated fish specimens, mixed with 90 mL of 0.1 % peptone water (Merck, Darmstadt, Germany) and homogenised in sterilised Stomacher bags (AES, Combourg, France) as previously described [11, 12]. Serial dilutions from the microbial extracts were prepared in 0.1 % peptone water in all cases.

Total aerobes were determined on plate count agar (PCA, Oxoid Ltd., London, UK) after incubation at 30 ºC for 48 h. Anaerobes were investigated in the same manner, except that an anaerobic atmosphere kit was placed, together with the plates, inside the anaerobiosis jar. Psychrotrophs were determined in PCA, after incubation at 7-8 ºC for 7 days. *Enterobacteriaceae* were investigated in Violet Red Bile Agar (VRBA) (Merck, Darmstadt, Germany) after incubation at 37±0.5 ºC for 24 h. Microorganisms exhibiting proteolytic or lipolytic phenotypes were investigated in casein-agar or tributyrine-agar, respectively, after incubation at 30 ºC for 48 h, as previously reported [13].

In all cases, bacterial counts were transformed into log $CFU \cdot g^{-1}$ muscle units before undergoing statistical analysis. All analyses were conducted in triplicate.

Analysis of lipid damage development

Lipids were extracted from the hake white muscle by the Bligh and Dyer [14] method, which employs a single-phase solubilisation of the lipids using a chloroform-methanol (1:1) mixture. The results were calculated as g $lipid \cdot kg^{-1}$ muscle.

Free fatty acid (FFA) content was determined using the lipid extract of the fish muscle by the Lowry and Tinsley [15] method, which is based on complex formation with cupric acetate-pyridine followed by spectrophotometric (715 nm) assessment (Beckman Coulter DU 640 spectrophotometer). The results were expressed as g $FFA \cdot kg^{-1}$ muscle.

Thiobarbituric acid index (TBA-i) was determined according to Vyncke [16]. This method is based on the reaction between a trichloroacetic acid extract of the fish muscle and thiobarbituric acid. Content of thiobarbituric acid reactive substances (TBARS) was spectrophotometrically measured at 532 nm and calculated from a standard curve using 1,1,3,3-tetraethoxy-propane (TEP). Results were calculated as mg $malondialdehyde \cdot kg^{-1}$ muscle.

Fluorescent compounds formation (fluorimeter LS 45; Perkin Elmer España; Tres Cantos, Madrid, Spain) was measured in the aqueous fraction obtained from the lipid extraction [14]. As described previously [17], fluorescence was measured at excitation/emission of 393/463 and 327/415 nm. The relative fluorescence (RF) was calculated as follows: $RF = F/F_{st}$, where F is the fluorescence measured at each excitation/emission wavelength pair and F_{st} is the fluorescence intensity of a quinine sulphate solution (1 $\mu g \cdot mL^{-1}$ in 0.05 M H_2SO_4) at the corresponding wavelength pair. The fluorescence ratio (FR) was calculated as the ratio between the two RF values: $FR = RF_{393/463\ nm}/RF_{327/415\ nm}$.

Statistical analysis

Data obtained from all microbiological and chemical analyses were subjected to the ANOVA method to explore differences resulting from the effect of the packaging system. The comparison of means was performed using the least-squares difference (LSD) method. In all cases, analyses were carried out using the PASW Statistics 18 software for Windows (SPSS Inc., Chicago, IL, USA); differences among packaging batches were considered significant for a confidence interval at the 95 % level ($p < 0.05$) in all cases.

RESULTS AND DISCUSSION

Microbial development

Analysis of aerobe counts showed an increase with storage time in most cases (Table 1). However, in all batches under study, values obtained can be considered as acceptable from a safety point of view as being below 6 log units even after 6 days of storage. Comparison among batches showed lower average values in fish corresponding to the alga-treated batch when compared with their counterparts from both control conditions; interestingly, significant differences ($p < 0.05$) could be observed at day 4 when compared with fish from CONT condition.

Table 1. Development of aerobes, psychrotrophs and *Enterobacteriaceae* counts (log CFU·g^{-1} muscle)* in refrigerated hake stored under different packaging conditions**

Microbial group	Packaging condition	Refrigeration time (days)			
		Initial	1	4	6
Aerobes mesophiles	CONT	2.50 (0.46)	3.09 a (0.76)	3.89 b (0.27)	4.13 a (0.68)
	GE-CT		2.43 a (0.23)	3.81 ab (0.57)	3.53 a (0.68)
	FS-GE		2.20 a (0.17)	3.10 a (0.17)	3.50 a (0.17)
Psychrotrophs	CONT	2.36 (0.32)	2.70 a (0.20)	3.86 b (0.06)	4.50 ab (1.06)
	GE-CT		2.46 a (0.28)	3.22 ab (0.59)	3.95 b (0.10)
	FS-GE		2.40 a (0.17)	2.36 a (0.32)	3.30 a (0.30)
Enterobacteriaceae	CONT	BDL***	1.74 b (0.52)	1.07 a (0.12)	1.34 a (0.24)
	GE-CT		BDL a	1.39 a (0.35)	1.23 a (0.24)
	FS-GE		BDL a	1.07 a (0.12)	1.17 a (0.15)

* Average values of three replicates (n=3); standard deviations are indicated in brackets. For each refrigeration time, values accompanied by different letters (a, b) indicate significant differences (p<0.05); ** Packaging conditions: CONT (not packaged; control), GE-CT (biofilm including gelatine; gelatine control) and FS-GE (biofilm including gelatine and alga protein hydrolysate) in agreement to the Material and Methods section;***BDL: microbial numbers were below the limit of detection of the technique (1 log CFU·g^{-1} muscle).

Psychrotrophs counts revealed a progressive increase in fish corresponding to CONT and GE-CT batches (Table 1); however, fish belonging to the batch corresponding to the alga-treated fish only provided a marked increase at the end of the experiment. Comparison among batches showed higher average values for fish belonging to both control batches (CONT and GE-CT); a significant inhibitory effect ($p < 0.05$) of the alga PH presence in the gelatine film was obtained at days 4 and 6.

The assessment of *Enterobacteriaceae* counts did not provide a general trend along storage time (Table 1). No marked evolution and development of this microbial group was observed at any of the packaging conditions. However, comparison among batches showed lower average values when alga hydrolysate was present in the packaging system.

Development of proteolytic and lipolytic microorganisms showed a similar pattern throughout the storage time (Table 2); in most cases, a progressive formation could be observed in all batches. An inhibitory effect of the alga PH presence in the gelatine film was implied since lower average values were obtained in all cases in

fish corresponding to the FS-GE batch; interestingly, differences were found significant ($p < 0.05$) for lipolytic counts at day 1.

Anaerobes counts assessment showed a progressive formation with time in all kinds of samples (Table 2). Average values corresponding to alga-treated fish were found lower throughout the whole study when compared with their counterparts corresponding to both controls; differences were found significant ($p < 0.05$) at day 1.

A partial microbial activity inhibition can be implied as a result of the presence in the packaging system of the PH of the current alga. An inhibitory effect on microbial activity development of *Fucus* spp. and other kinds of macroalgae has been reported to be caused by the presence of terpenes and polyphenols, among other compounds [7]. In a previous study, lyophilised *F. spiralis* was included in a polylactic-based film, this leading to a reduced microbial development in megrim (*Lepidorhombus whiffiagonis*) fillets kept under refrigerated (4 ºC) condition for 11 days [9].

The inclusion of other macroalgae extracts in biofilms has also provided antimicrobial activity. This is the case of the red *Gelidium corneum*, whose presence in an edible film also including persimmon peel and grape fruit seed extracts, improved the physical properties and provided antimicrobial activity [18]. Furthermore, the presence of polyhydroxybutyrate and phenolic compounds in microalga *Spirulina platensis* protein isolates led to a marked inhibitory effect when included in an edible packaging system [19, 20].

Lipid oxidation development

A marked and progressive formation of secondary lipid oxidation compounds was observed in all kinds of samples throughout the storage time (Table 3). Surprisingly, a higher ($p < 0.05$) level was determined in fish corresponding to the FS-GE batch at day 1. However, lower average values were detected for the 4-6-day period for alga-treated fish when compared with samples belonging to both control batches.

A great formation of fluorescent compounds was observed at day 1 in all kinds of samples (Table 3); after that time, a slight decrease in their content was implied till the end of the experiment in most cases. Comparison among samples provided lower average values in all cases for fish samples corresponding to the batch including the alga PH in the gelatine-packaging system. Interestingly, differences with both controls were found significant ($p < 0.05$) at day 6; at that time, a lower ($p < 0.05$) value was obtained for the gelatine control when compared with the CONT batch.

In agreement to the results obtained, a partial antioxidant behaviour can be accorded to the PH obtained from alga *F. spiralis* when included in a gelatine-film system. An antioxidant effect of *F. spiralis* extract has already been proved in different *in-vitro* tests [21], showing a marked content on polyphenols [8] and α-tocopherol [22]. Furthermore, the presence of lyophilised *F. spiralis* in a polylactic acid packaging film also showed a marked antioxidant effect during the refrigerated storage (11 days at 4 ºC) of megrim (*Lepidorhombus whiffiagonis*) fillets [9].

Previous research also accounts for an antioxidant effect as a result of the inclusion in biofilms of extracts obtained from other macroalgae. Thus, alginate-based films prepared from a red macroalga (*Sargassum fulvellum*) provided antioxidant properties (ABTS and DPPH assays) to a biofilm also including black chokeberry [23]. Moreover, Carissimi *et al*. [24] reported the antioxidant properties of a starch-based film including an ethanolic extract of microalgae *Heterochlorella luteoviridis* and *Dunaliella tertiolecta*; this effect, determined as a thiobarbituric acid index decrease, was observed in salmon fillets stored at 6±2 ºC for 6 days.

Lipid hydrolysis formation

FFA content showed a marked and progressive increase throughout the whole study for all kinds of samples (Table 3). Comparison among batches showed lower values ($p < 0.05$) in fish samples that were packaged in gelatine films including the alga PH presence during the 4-6-day period.

Both endogenous enzyme activity and microbial activity have been signalled as responsible for FFA formation during the refrigerated storage of fish [2, 25]. Before the end of the microbial lag phase, endogenous enzyme activity should be predominant; after that time, microbial activity should gain importance and be mostly responsible for the development of lipid hydrolysis. Current results obtained on FFA content evolution, i.e., lower formation as a result of the alga PH presence, can be explained on the basis of the above-mentioned inhibitory effect on lipolytic counts development (Table 2) observed throughout the whole study.

Table 2. Development of proteolytics, lipolytics and anaerobes counts (log CFU·g^{-1} muscle)* in refrigerated hake stored under different packaging conditions**

Microbial group	Packaging condition	Refrigeration time (days)			
		Initial	1	4	6
Proteolytics	CONT	2.26 (0.24)	2.76 b (0.15)	3.43 a (0.51)	3.50 a (0.70)
	GE-CT		2.43 ab (0.51)	3.32 a (0.55)	3.37 a (0.47)
	FS-GE		2.10 a (0.17)	3.20 a (0.17)	2.69 a (0.36)
Lipolytics	CONT	BDL***	2.91 c (0.23)	2.72 a (0.39)	3.47 a (0.77)
	GE-CT		2.39 b (0.18)	2.36 a (0.32)	2.89 a (0.11)
	FS-GE		BDL a	2.36 a (0.39)	2.56 a (0.24)
Anaerobes	CONT	BDL***	2.39 b (0.35)	2.60 a (0.30)	3.46 a (0.15)
	GE-CT		2.20 b (0.17)	2.36 a (0.32)	3.20 a (0.17)
	FS-GE		BDL a	2.16 a (0.28)	2.85 a (0.55)

* Average values of three replicates (n = 3); standard deviations are indicated in brackets. For each refrigeration time, values accompanied by different letters (a, b) indicate significant differences ($p < 0.05$). ** Packaging conditions as expressed in Table 1. ***BDL: microbial numbers were below the limit of detection of the technique (2 log CFU·g^{-1} muscle).

Table 3. Assessment of lipid damage* in refrigerated hake stored under different packaging conditions**

Chemical quality index	Packaging condition	Refrigeration time (days)			
		Initial	1	4	6
Thiobarbituric acid index **(mg malondi-aldehyde· kg^{-1} muscle)	CONT	0.09 (0.10)	0.12 a (0.01)	0.79 b (0.10)	1.48 a (0.98)
	GE-CT		0.22 b (0.01)	0.60 ab (0.07)	0.96 a (0.29)
	FS-GE		0.38 c (0.05)	0.43 a (0.08)	0.61 a (0.34)
Fluorescence ratio	CONT	1.25 (0.87)	5.76 b (0.57)	5.08 a (0.37)	5.00 c (0.24)
	GE-CT		4.92 ab (0.59)	5.18 a (0.48)	4.61 b (0.18)
	FS-GE		4.69 a (0.36)	4.37 a (0.50)	3.91 a (0.14)
Free fatty acids content (g·kg^{-1} muscle)	CONT	51.99 (4.95)	132.73 b (15.48)	338.00 b (58.95)	390.70 b (16.27)
	GE-CT		106.26 ab (10.47)	312.27 b (13.29)	381.96 b (14.43)
	FS-GE		77.30 a (21.14)	264.36 a (23.28)	339.53 a (14.21)

* Average values of three replicates (n = 3); standard deviations are indicated in brackets. For each refrigeration time, values accompanied by different letters (a, b, c) indicate significant differences ($p < 0.05$). ** Packaging conditions as expressed in Table 1.

Previous studies on the effects of algae extracts or any other alga-derivate can be considered as scarce. Thus, Taghavi Takyar *et al*. [26] reported the inhibitory effect of ethanol extracts of *S.*

platensis on lipid hydrolysis (FFA formation); such extract was added to rainbow trout (*Oncorhynchus mykiss*) fillets that were packaged in polyethylene bags and kept at 4 ºC up to 16 days. Related to gelatine-film systems, an inhibitory effect on lipid hydrolysis during fish products storage has also been reported. Thus, a chitosan-gelatine coating slowed down FFA formation in rainbow trout (*O. mykiss*) fillets [27] and Belanger's croaker (*Johnius balangerii*) fillets [28] during refrigerated storage (4 ± 1 ºC). Interestingly, a marked direct effect of FFA formation has been reported to be exerted on lipid oxidation, this effect being explained on the basis of a lower oxidative stability in FFA than in their corresponding methyl esters and triacylglycerides [29].

CONCLUSIONS

A novel gelatine-based film including a *F. spiralis* PH was tested as packaging method for the preservation under refrigerated conditions of hake slices. In it, a progressive quality loss could be observed in hake muscle by increasing the storage time. However, comparison with control fish showed a partial inhibitory effect on microbial activity development (counts assessment of aerobes, psychrotrophs, *Enterobacteriaceae*, proteolytics, lipolytics and anaerobes) and lipid oxidation (determination of thiobarbituric acid and fluorescence indexes) and hydrolysis (free fatty acids formation) evolution. A preservative effect resulting from the PH presence in the gelatine-based biofilm is concluded, this showing a quality and safety enhancement and a potential commercial value increase. Further research focused on the optimisation of the current biofilm preparation ought to be addressed.

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Gamma radiation effects on antioxidant activity of black chokeberry fruits (*Aronia Melanocarpa L.*) in mice models

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Food irradiation is a process of food exposing to ionizing radiation. The maximum dose absorbed by the food should not exceed 10 kGy unless it is necessary to achieve a legitimate technological purpose. The black chokeberry fruit *(Aronia Melanocarpa)* is a specific berry with high polyphenol content and possesses one of the highest *in vitro* antioxidant activities among other fruits. We aimed to investigate the *in vivo* effect on the plasmatic and hepatic oxidative status of experimental mice after treatment with non-irradiated and 10 kGy γ-irradiated samples of *A. Melanocarpa* (samples were pulverized to powder, 0.5 g of which was mixed with 40 ml of extract (30% ethanol solution). For this purpose, we studied the oxidative stress biomarkers levels -ascorbate radicals (·Asc), reactive oxygen species (ROS) products and nitric oxide NO· radicals by EPR spectroscopy. The obtained results show that the antioxidant defense system in the plasma of treated mice fails to overcome the induced oxidative stress after treatment of *A. Melanocarpa* extract with 10 kGy.

Keywords: oxidative stress, *Aronia Melanocarpa*, gamma radiation, ROS products, NO radicals, Ascorbate radicals

INTRODUCTION

Spices and herbs are always contaminated with microorganisms. Therefore, irradiation is currently used as the main method of decontamination of dry plant materials such as spices and herbs [1]. General standards for food irradiation state that the minimum absorbed dose should be sufficient to achieve the technological objective and the maximum absorbed dose should be less than that which would compose consumer safety. The maximum dose absorbed by the food should not exceed 10 kGy unless it is necessary to achieve a legitimate technological purpose [2].

Black chokeberry fruits (*Aronia Melanocarpa L.; A. Melanocarpa*; Michx Elliott) have a high content of B vitamins and trace elements as manganese and iron. *A. Melanocarpa* fruits are extremely rich in polyphenol substances – their content varies between 40 and 70 mg/g dm, 50 % of which are anthocyanins [3]. Recently, particular attention is given to natural products which are characterized by high antioxidant capacity due to the radical scavenging effects of their substituents on reactive oxygen species (ROS) and reactive nitrogen species (RNS) which occurs in the body due to metabolic reactions, as well as in the case of inflammation and neutralization of xenobiotics. The elevation in temperature of plant tissue and UV irradiation cause changes in polyphenol content, the range of which depends on the power and, sometimes, on the operating time of abiotic stress factors [4, 5]. The abiotic stress factors as UV-radiation, electromagnetic field, microwave radiation, and ultrasound, at different operation times, caused differentiation in the contents of anthocyanins, phenolic acids, flavonols, and flavan-3-ols in chokeberry [6].

The study aim was the *in vivo* examination of the effect on the plasmatic and hepatic oxidative status of experimental mice after treatment with non-irradiated and 10 kGy γ-irradiated 30% *A. Melanocarpa* ethanol extract. To realize the aim of the current research the levels of the oxidative stress biomarkers such as ascorbate radicals (·Asc), ROS products and NO· radicals were studied by direct and spin-trapping EPR spectroscopy.

EXPERIMENTAL

Carboxy.PTIO.K potassium salt, dimethyl sulfoxide (DMSO), and other (HPLC grade) solvents and reagents were purchased from Sigma-Aldrich (Steinheim, Germany). Black chokeberry

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fruits were supplied from Vitanea Ltd. (Plovdiv, Bulgaria) in the stage of full maturity in August 2017.

Fresh fruits were frozen at -18˚C, lyophilized (Christ Alpha 1-4 LDplus, Martin Christ GmbH, Germany) and stored in a desiccator until use.

Gamma-irradiation of dried Aronia Melanocarpa fruits

Freeze dried berries were irradiated at a ^{60}Co source with 8200 Ci activity. The chosen absorbed dose was 10 kGy. All gamma irradiated samples and untreated controls were pulverized to powder, 0.5 g of which were mixed with 40 ml of extract - 30% ethanol solution acidified with 0.5% formic acid. Samples were extracted on a magnetic stirrer for 1 h at room temperature [7]. The total polyphenol content of the investigated samples varied between 6935 ± 79 mg/100 g DW and the total content of anthocyanins was in the range of 1192 ±mg/100 g DW, at 10 kGy, as reference [8].

For the experiment, IRC/w non-inbred albino male mice (25±1.5 g) were used. The mice were housed in polycarbonate cages in controlled conditions (12 h light/dark cycles), at a temperature of 18-23°C and humidity of 40-70%, with free access to tap water and standard laboratory chow at Suppliers of Laboratory Animals of the Medical Faculty, Trakia University. The animal study was approved with Directive 2010/63/EU/ Ethical Committee for Animals of BABH and Trakia University, Stara Zagora, Bulgaria (131/6000-0333/09.12.2016). The experimental animals were randomly assigned to three groups, each of 6 mice: control, 30% ethanol solution *A. Melanocarpa* extract, 10 kGy 30% ethanol solution *A. Melanocarpa* extract. The mice were treated orally in the acute experiment according to Eftimov *et al.* [9]. The control group was pretreated orally with saline (30 mL/kg) for 2 h. The *A. Melanocarpa* and 10 kGy *A. Melanocarpa* groups were pretreated with the respective extract at a dose of 30 mL/kg.

The mice were anesthetized and euthanized 2 h after the treatment. The fresh blood (1.3-2 ml) was collected directly from the heart in cold EDTA-containers. After centrifugation the plasma samples were immediately studied by EPR spectroscopy for their radical scavenging abilities. The freshly isolated liver was collected on ice and homogenized. The electron paramagnetic (EPR) measurements were performed on an X-Band, Emx^{micro} Spectrometer (Bruker, Germany). Spectral processing was performed using Bruker WIN-EPR and SimFonia software.

Levels of ascorbate radicals

The plasma and liver homogenates from the three groups were prepared according to Bailey *et al*. [10]. The levels of ascorbate radicals were calculated as double integrated plots of EPR spectra and results were expressed in arbitrary units.

Levels of ROS products

The levels of ROS products were determined according to Shi *et al.* [11] modified by us. The levels of ROS products were calculated as double integrated plots of EPR spectra and results were expressed in arbitrary units.

NO· radicals

Based on the methods published by Yoshioka *et al.* [12] and Yokoyama *et al*. [13] we developed and adapted the EPR method for estimation of the levels of NO· radicals. The levels of NO· radicals were calculated as double integrated plots of EPR spectra and results were expressed in arbitrary units.

Statistical analysis

Statistical analysis was performed with Statistica 7, StaSoft, inc. and the results were expressed as means ± S.E. $p < 0.05$ was considered statistically significant. To define which groups are different from each other we have used LSD post hoc test.

RESULTS AND DISCUSSION

Phenolic compounds have a perfect chemical structure to scavenge and neutralize free radicals because they have phenolic OH groups that are ready to donate H^+ or an electron to a free radical and an expanded conjugated aromatic system to unblock the unpaired electron [14]. To evaluate the extractability of phenolic compounds in methanol/water extract of *G. biloba*, Pereira and coauthors have studied non-irradiated and irradiated with 10 kGy extract samples. They found that irradiated with 10 kGy methanol/water extract and infusion preparation gave the highest content of phenolic compounds and concluded that this dose of irradiation ensures the disinfection and decontamination of the products from microbes and contributes to increasing the extractability of the phenolic compounds [15]. All mentioned studies led us to assess *ex vivo* the effect of 10kGy radiation on the radical scavenging capabilities of an extract isolated from *A. Melanocarpa* fruits. Results from ascorbic radicals' levels measured by direct EPR spectroscopy in the plasma of control mice and mice treated with non-irradiated and irradiated *A. Melanocarpa* extract are given in Fig. 1.

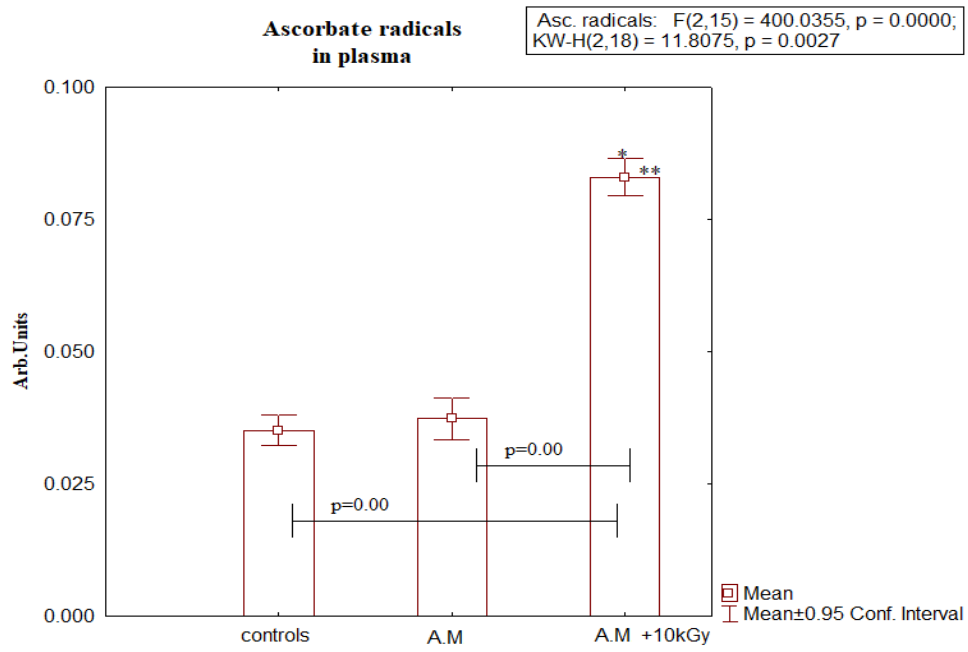

Fig. 1. ·Asc radicals levels measured in plasma of mice treated with non-irradiated and irradiated *A. Melanocarpa*, and compared with the controls. Significant difference *p < 0.05 vs controls. **p < 0.05 vs 10 kGy irradiated A. *Melanocarpa*. The LSD post hoc test was used to determine the groups differing from each other.

In the order of the standard one-electronic reduction potentials, the ascorbate ranks at the end of the series of oxidative free radicals such as hydroxyl (·OH); alkylperoxyl (ROO·); lipidperoxyl (LOO·); tocoperoxyl (·TO); $\cdot O_2^-/HO_2$.

All of these radicals have higher redox potential and can be reduced by ascorbic acid, and as a result, ascorbate radicals are generated [16]:

$$AscH^- + R\cdot = \cdot Asc^- + RH$$

Ascorbate radical has a relatively long half-life and can be directly confirmed by EPR spectroscopy [17]. This property turns the ascorbate radical into the best endogenous non-toxic biomarker for proving the generation of toxic reactive radicals in biological systems [18, 19].

The group treated with irradiated extract exhibits statistically significant higher levels of ·Asc radicals compared to controls (mean 0.083±0.001 *vs* mean 0.035±0.001, according to the LSD post hoc test p=0.00), and mice treated with non-irradiated *A. Melanocarpa* extract (mean 0.083±0.001 *vs* mean 0.037±0.001, according to the LSD post hoc test p=0.00). The group of mice with non-treated *A. Melanocarpa* extract shows values close to the controls (mean 0.037±0.001 *vs* mean 0.035±0.001, according to the LSD post hoc test p=0.03), while 10 kGy irradiation causes a 2.4- fold increase in plasma ·Asc levels comparing to controls and mice treated with the non-irradiated extract.

As is seen (Fig. 2) the levels of ROS products in plasma of mice treated with 10 kGy irradiated *A. Melanocarpa* extract were statistically significantly higher compared to healthy controls (mean 7.01±0.2 *vs* mean 2.05±0.1, according to the LSD post hoc test p=0.000) and the group treated with non-irradiated samples of *A. Melanocarpa* (mean 7.01±0.2 *vs* mean 2.42±0.1, according to the LSD post hoc test p=0.000). Moreover, ROS plasma values of mice treated with non-irradiated *A. Melanocarpa* were close to controls (mean 2.42±0.2 *vs* mean 2.05±0.1, according to the LSD post hoc test p=0.01).

As is shown (Figs. 1 and 2), the levels of the registered ·Asc and ROS products in the plasma of the mice treated with non-irradiated *A. Melanocarpa* extract were commensurable with the control group. This means that the non-irradiated extract does not induce ROS generation in the plasma of treated mice. The significant increase in ·Asc levels registered after treatment with 10 kGy irradiated extract shows that the antioxidant non-enzymatic defense system (in particular ascorbic acid) is involved in neutralizing the generated oxidative radicals to overcome the induced oxidative stress in mice plasma, but apparently it failed. This finding is additionally supported by the results obtained for the levels of ROS registered in mice plasmas after treatment with irradiated extract (see Fig. 2).

Results obtained for NO· in plasma are given in Fig 3. Plasma of mice treated with 10kGy irradiated *A. Melanocarpa* extract showed (Fig.3) statistically higher NO· values compared to controls (mean 6.84±0.1 *vs* mean 2.33±0.1, according to the LSD post hoc test p=0.00), as well as to those of mice treated with non-irradiated *A. Melanocarpa* (mean 6.84±0.1 *vs* mean 3.34±0.1, according to the LSD post hoc test p=0.00).

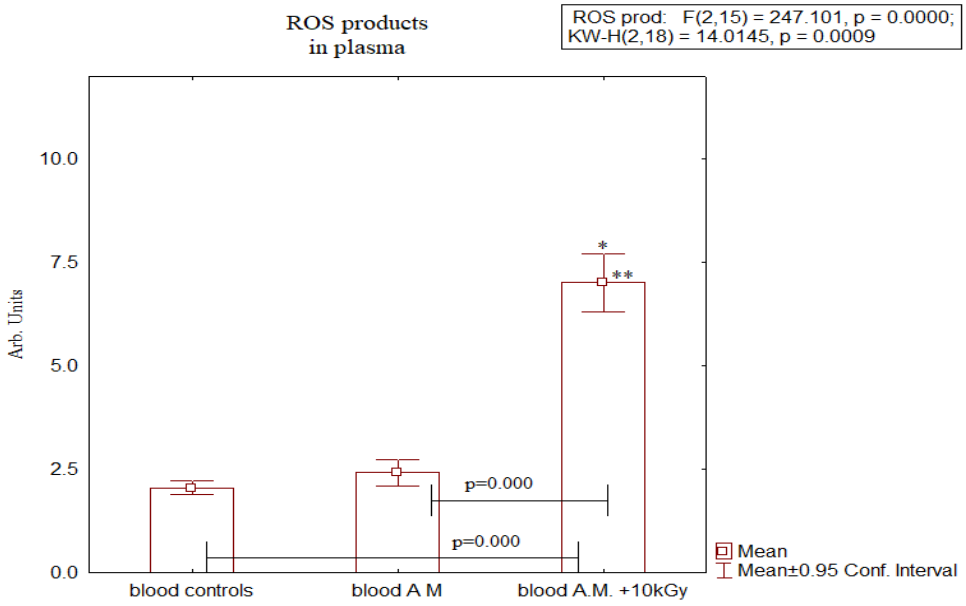

Fig. 2. Levels of ROS products measured in plasma of mice treated with non-irradiated and irradiated A. Melanocarpa extract, and compared to the controls. Significant difference *p < 0.05 vs controls. **p < 0.05 vs 10 kGy irradiated A. Melanocarpa. The LSD post hoc test was used to determine the groups differing from each other.

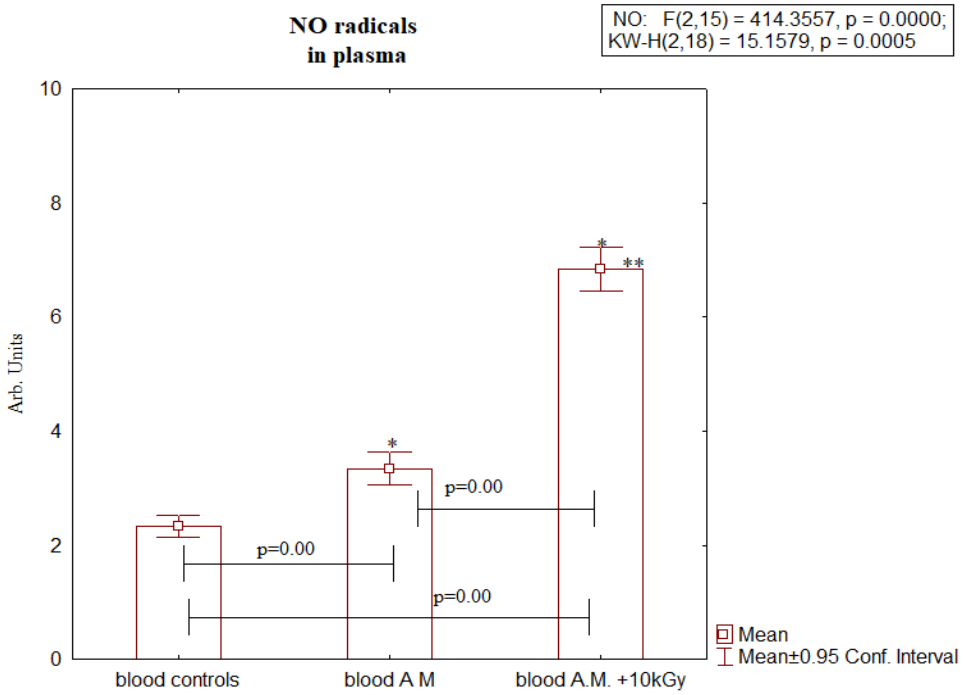

Fig. 3. Levels of NO· measured in plasma of mice treated wit*h non-irradiated and irradiated A. Melanocarpa, and compared to the control mice. Significant difference *p < 0.05 vs controls. **p < 0.05 vs 10 kGy irradiated A. Melanocarpa. The LSD post hoc test was used to determine the groups differing from each other.

It should be mentioned that significantly higher NO· levels were also found for the group of mice treated with non-irradiated extract compared to the control group (mean 3.34±0.1 *vs* mean 2.33±0.1, according to the LSD post hoc test p=0.00).

It is of interest to note that in the plasma of mice treated with 10 kGy irradiated *A. Melanocarpa*

extract, about 2.3-fold increase in ·Asc levels was observed in comparison with both groups: mice treated with non-irradiated extract and control group. On the other hand, from 3 to 3.5-fold increase in the ROS levels was also found compared to the same groups. This result unambiguously suggests that the antioxidant system in mice blood plasma fails to protect from the oxidative stress induced by the treatment with 10 kGy irradiated extract. It might also be supposed that the dose of 10 kGy irradiation provokes changes in polyphenol structure responsible for *A. Melanocarpa* extract antioxidant activity.

It is a well-known fact that a critical target of $\cdot O_2^-$ may be NO· produced by endothelium macrophages, neutrophils, etc. Moreover, $\cdot O_2^-$ and NO· are known to rapidly react to form the stable peroxide anion (ONOO-). After protonation $ONOO^-$ can decompose and generate a strong oxidant such as HO·[20]. As can be seen from Figs. 2 and 3, after treating the mice with 10 kGy irradiated extract, the levels of ROS products and NO· in the plasma are 3.5 times and 3 times higher than the controls, respectively. We hypothesize that these increased amounts of NO· and ROS (in particular $\cdot O_2^-$) registered in mice plasma might produce $ONOO^-$, and ultimately generate the highly toxic HO·. We also accept that the probability of generating $ONOO^-$ and subsequently the highly toxic HO· is significantly lower in the plasma of mice treated with non-irradiated extract due to the fact that their ROS plasma levels were commensurate with controls and furthermore, their plasma NO· levels were about 2 times lower than those found in mice treated with 10 kGy irradiated extract.

The mice liver (Fig. 4) treated with 10 kGy irradiated *A. Melanocarpa* extract showed levels of ·Asc radicals, close to those in the group treated with non-irradiated *A. Melanocarpa* (mean 0.035±0.001 *vs* mean 0.033±0.001, according to the LSD post hoc test p=0.09). Both groups treated with *A. Melanocarpa* extract showed statistically significant increased levels compared to controls, namely mean 0.033±0.001 *vs* mean 0.029±0.001, according to the LSD post hoc test p=0.000, for mice treated with non-irradiated extract and mean 0.035±0.001 *vs* mean 0.029±0.001, according to the LSD post hoc test p=0.000, for mice treated with 10 kGy irradiated extract.

As shown in Fig. 5, the ROS products levels in the liver homogenates of mice treated with irradiated extract were significantly lower than those registered in the controls (mean 1.78±0.07 *vs* mean 2.05±0.02, according to the LSD post hoc test p=0.006) and the mice treated with non-irradiated *A. Melanocarpa* extract (mean 1.78±0.07 *vs* mean 2.01±0.02, according to the LSD post hoc test p=0.001). The values of ROS products in the group treated with non-irradiated *A. Melanocarpa* are close to those in the controls (mean 2.05±0.02 *vs* mean 2.01±0.02, according to the LSD post hoc test p=0.4).

NO· radicals registered in liver homogenates (Fig. 6) of mice treated with 10kGy *A. Melanocarpa* are statistically significantly higher than in controls (mean 8.53±0.2 *vs* mean 5.93 ±0.03, according to the LSD post hoc test p=0.000), and to mice treated with the non-irradiated extract (mean 8.53±0.2 *vs* mean 6.22±0.1, according to the LSD post hoc test p=0.000). No statistically significant increase was found in NO· levels in the liver homogenates of mice treated with non-irradiated *A. Melanocarpa* extract, compared to the controls (mean 6.22±0.1 *vs* mean 5.93±0.03, according to the LSD post hoc test p=0.09).

Bearing in mind that the ascorbate radical is a *real-time* biomarker for the oxidative processes, the about 1.2-fold increase in ascorbate radicals in the mice liver after treatment with either non-irradiated or 10 kGy irradiated *A. Melanocarpa* extract indicates that ascorbate is actively involved in neutralizing oxidative radicals generated in liver [21, 22]. This finding was also supported by the fact that the registered ROS level in mice liver treated with non-irradiated extract was the same as in the control group, while in the mice liver treated with 10 kGy irradiated extract, it was even lower than in controls. Given these results, we assume that hepatic antioxidant protection in mice is more effective in neutralizing oxidative toxic species than blood plasma. This assumption is additionally supported by the findings that: 1) NO· levels (see Figs. 3 and 6) measured in livers of both groups of mice treated with non–irradiated and 10 kGy irradiated *A. Melanocarpa* extracts were found to be lower than in the plasmas of the same groups; 2) NO· level in livers of mice treated with non-irradiated extract were close to the controls, while the NO· levels in the plasma of mice treated with non-irradiated extract were about 1.4 times higher than controls.

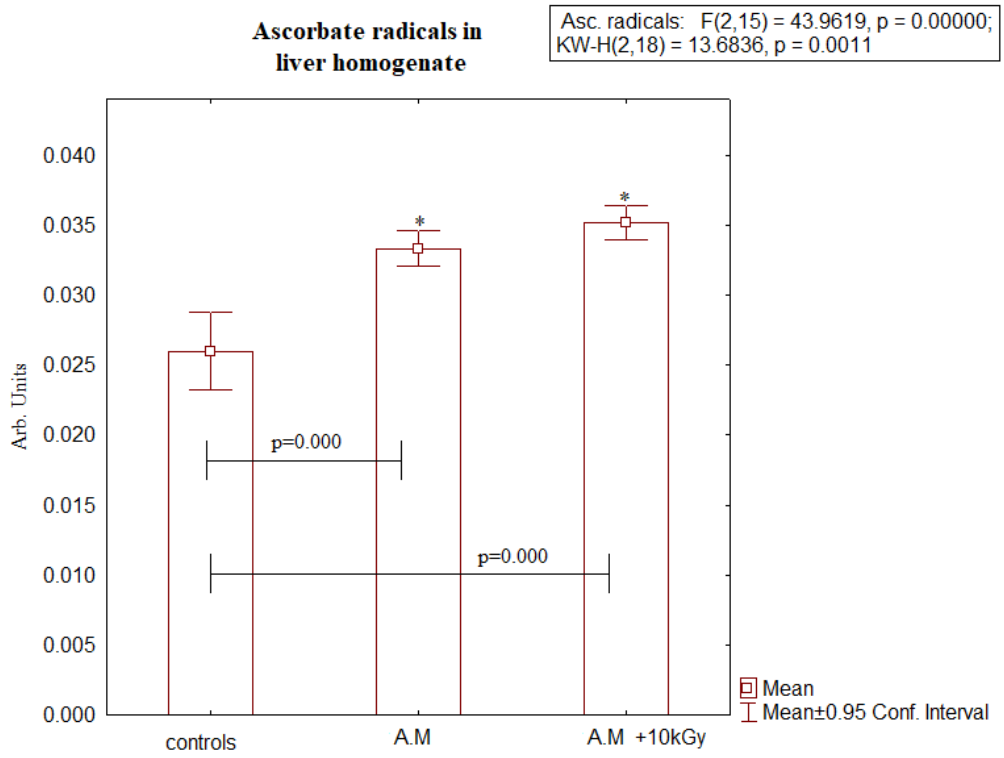

Fig. 4. ·Asc radicals levels measured in liver of mice treated with non-irradiated and 10 kGy irradiated *A. Melanocarpa*, and compared with the control mice. Significant difference *p < 0.05 vs controls. **p < 0.05 vs 10 kGy irradiated A. *Melanocarpa*. The LSD post hoc test was used to determine the groups differing from each other.

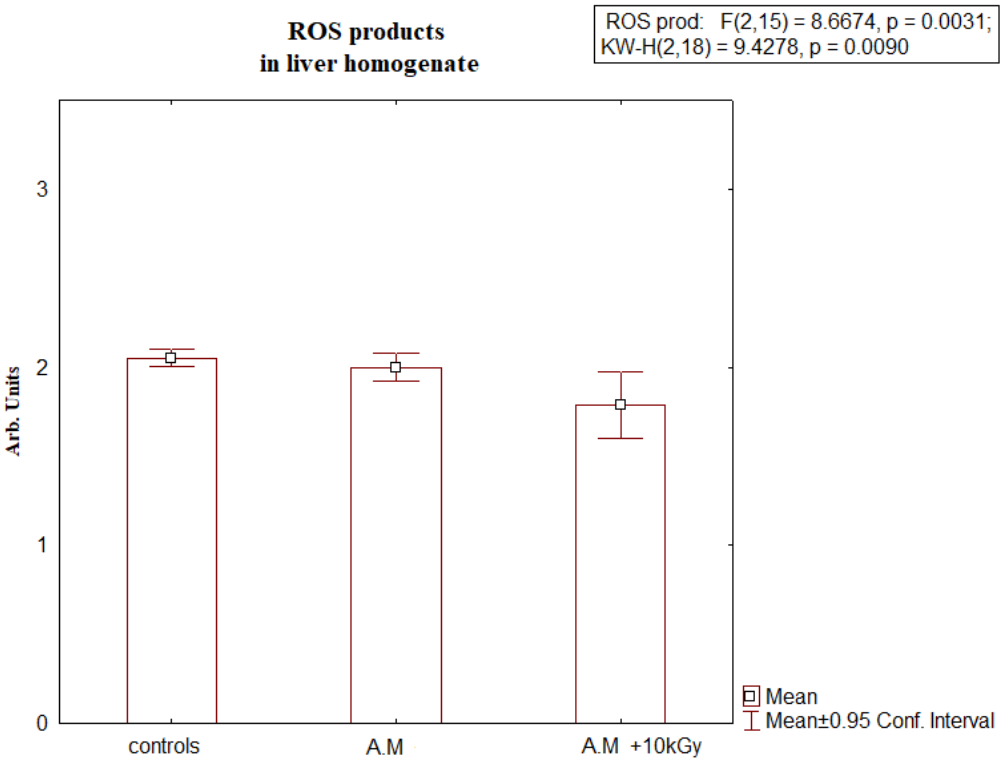

Fig. 5. ROS products levels measured in mice liver of treated with non-irradiated and 10kGy irradiated *A. Melanocarpa* and compared to the control mice. The LSD post hoc test was used to determine the groups differing from each other.

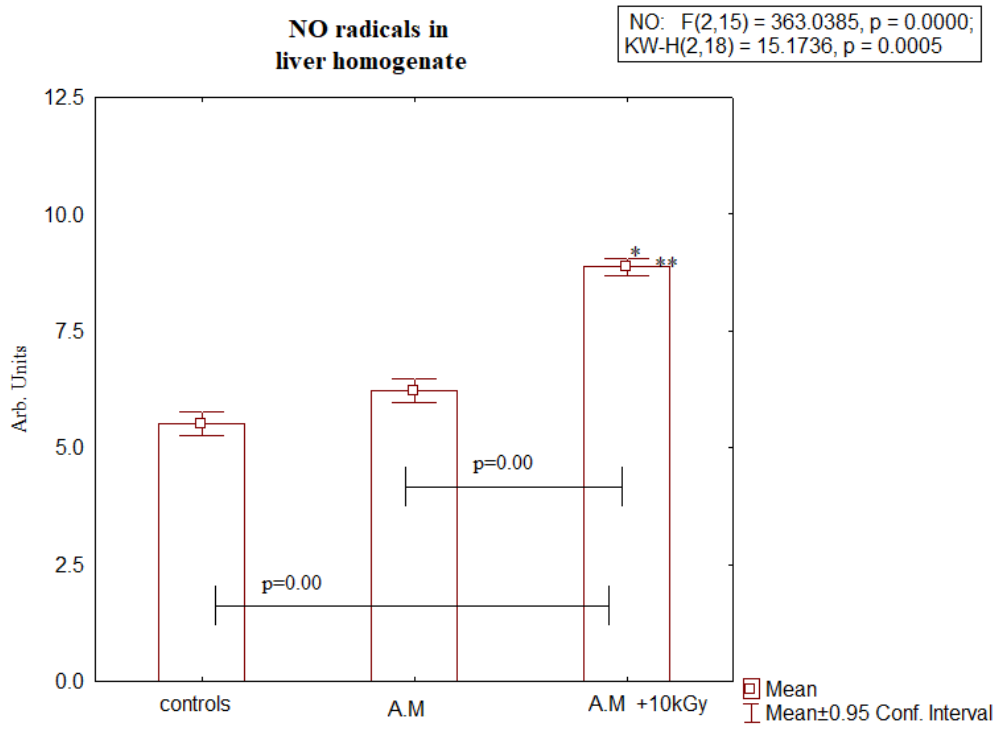

Fig. 6. NO· radical levels measured in liver of mice treated with non-irradiated and 10kGy irradiated *A. Melanocarpa*, and compared to the control mice. Significant difference *p < 0.05 vs controls; **p < 0.05 vs 10 kGy irradiated A. *Melanocarpa*. The LSD post hoc test was used to determine the groups differing from each other.

NO· radicals registered in liver homogenates (Fig. 6) of mice treated with 10kGy *A. Melanocarpa* are statistically significantly higher than in controls (mean 8.53±0.2 *vs* mean 5.93 ±0.03, according to the LSD post hoc test p=0.000), and to mice treated with the non-irradiated extract (mean 8.53±0.2 *vs* mean 6.22±0.1, according to the LSD post hoc test p=0.000). No statistically significant increase was found in NO· levels in the liver homogenates of mice treated with non-irradiated *A. Melanocarpa* extract, compared to the controls (mean 6.22±0.1 *vs* mean 5.93±0.03, according to the LSD post hoc test p=0.09).

Bearing in mind that the ascorbate radical is a *real-time* biomarker for the oxidative processes, the about 1.2-fold increase in ascorbate radicals in the mice liver after treatment with either non-irradiated or 10 kGy irradiated *A. Melanocarpa* extract indicates that ascorbate is actively involved in neutralizing oxidative radicals generated in liver [21, 22]. This finding was also supported by the fact that the registered ROS level in mice liver treated with non-irradiated extract was the same as in the control group, while in the mice liver treated with 10 kGy irradiated extract, it was even lower than in controls. Given these results, we assume that hepatic antioxidant protection in mice is more effective in neutralizing oxidative toxic species than blood plasma. This assumption is additionally supported by the findings that: 1) NO· levels (see Figs. 3 and 6) measured in livers of both groups of mice treated with non–irradiated and 10 kGy irradiated *A. Melanocarpa* extracts were found to be lower than in the plasmas of the same groups; 2) NO· level in livers of mice treated with non-irradiated extract were close to the controls, while the NO· levels in the plasma of mice treated with non-irradiated extract were about 1.4 times higher than controls.

Later studies have shown that elevated temperature and irradiation cause changes in the plant polyphenol content, the range of which depends on the power and, sometimes, on the operating time of abiotic stress factors [23]. The first EPR studies on the effect of 10 kGy irradiation of *A. Melanocarpa* extract in relation to the oxidative state in mice are somewhat in support of the research of Cebulak and collaborators [6]. The same authors established that among the abiotic stress factors employed, such as UV-C radiation, electromagnetic field, microwave radiation, and ultrasound, at different operation times, the statistically significant decrease in the content of all polyphenol compounds determined in chokeberry

fruits has been found only in the case after their exposure to 5-MFL (electromagnetic field) agent.

Based on the present EPR spectroscopy results it is very likely the observed decrease in antioxidant protection in the plasma of mice treated with *A. Melanocarpa* extract irradiated with a dose of 10 kGy to be indirectly caused by a reduction in the number of polyphenols in the extract after irradiation. It is not excluded this dose of irradiation to induce some changes in the polyphenols structures responsible for the *A. Melanocarpa* extract antioxidant activity.

CONCLUSION

For the first time, the effect of 10 kGy irradiated *A. Melanocarpa* extract on the level of oxidative stress induced in mice plasma and liver was assessed using direct and spin-trapping EPR spectroscopy. The results obtained show that the antioxidant defense system in the plasma of the treated mice fails to overcome the induced oxidative stress after treatment of *A. Melanocapra* extract with 10 kGy. In order to establish the reasons provoking oxidative stress in the blood plasma of experimental mice treated with a dose of 10 kGy we believe that detailed studies need to be made to clarify the changes occurring in the amount and structure of the constituents (in particular polyphenols) of the *A. Melanocarpa* extract responsible for the antioxidant activity of the latter. Therefore, further studies should be undertaken to broaden the current state of knowledge about the stimulating effect of various abiotic stress agents on the increased content of polyphenol compounds in the fruit.

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Flavonoids content in fresh and processed vegetable foods

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Flavonols and flavones are classes of flavonoids with antioxidant activity. Dietary flavonoids are assumed to have protective effect against different degenerative diseases; therefore, it is important to have reliable information about their content in various foods. Data for flavonoids composition in plant foods are compiled in several databases, however, data for specific processed traditional foods, which are substantial part of our diet, are missing. The aim of the study is to present new analytical data for flavonols and flavones content of 6 and 10 vegetable food varieties, widely consumed in Bulgaria. Flavonols – quercetin and kaempferol and flavones – luteolin and apigenin were determined by HPLC analysis with UV detection after acid hydrolysis with 1.2M hydrochloric acid. The results show that in lutenitsa (traditional vegetable spread) the level of quercetin is 1.42 mg/100g and of luteolin is 2.65 mg/100 g, very close to their level in the row ingredients. We can conclude that flavonoids are stable after food technological processing and in contrast to other antioxidants they do not degrade in canned foods.

Keywords: Flavonols, Flavones, HPLC, Processed foods, Food composition

INTRODUCTION

Flavonoids are polyphenolic antioxidants, ubiquous in plant foods. In the 1990s, flavonoids triumphantly returned to the scientific literature on nutrition due to their potent antioxidant activity [1, 2]. Dietary flavonoids have protective effect against the development of different degenerative diseases like cardiovascular, cancer, diabetes mellitus, and Alzheimer diseases [3-7]. Therefore, it is important to have reliable information about their content in various foods. Food composition information is the core of all nutritional programs. The development of nutrition science poses increasing requirements on the quality of food composition data to meet the needs in establishing the link food-nutrients-dietary intake-health. Knowledge of the flavonoid content of foods will allow a more precise and comprehensive evaluation of their quality, for elaboration of databases necessary for establishing relevant food policy and data exchange on national and international level.

Data for flavonoids content in plant foods are compiled in several international databases like USDA Database for the Flavonoid Content of Selected Food and Phenol-Explorer [8-10] but data for processed foods is scattered, moreover, for some specific traditional foods from different parts of the world such data are missing. The technological processing of food can affect the content of flavonoids, thereby reflecting on dietary intake assessment. According to the National Statistical Institute, Bulgarians consume yearly about 20 kg of tomatoes, 8 kg of peppers and 10 kg of vegetable canned food *per capita*, suggesting that processed vegetable foods also are a considerable dietary source of flavonoids [10]. Taking into consideration the rich biological activity of flavonoids, many epidemiological studies have shown their association with suppression of a large range of pathophysiological processes in the human body. The information in Bulgarian food composition database is constantly updated and a need for new original quantitative data for flavonoids is demanded.

The aim of the study is to present new analytical data for the content of flavonoids from the group of flavonols and flavones of fresh and processed vegetable food varieties, typical for Bulgarian diet and widely consumed in Bulgaria.

EXPERIMENTAL

Food sampling plan

The need for representative data places the development of a sampling plan as a key and starting point in the food analysis process, which has its specificity, according to the purpose of the study. In this work, following the current scientific approaches in the field [12, 13] a detailed sampling plan was developed.

Each individual sample of fresh vegetables is a composite sample of three single samples, which were purchased from three different items on the same day. The quantity of single samples was not less than 1 kg for fresh vegetables. For each single sample, a sampling protocol was filled up, reflecting the origin and identification of the samples.

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The samples were transported to the laboratory where they were mixed into combined samples – 3 kg. After removing the non-edible food parts, an average sample of 1 kg was prepared by random selection of vegetables, followed by freeze-drying. The freeze-dried sample was stored at -18^{0}C. Before analysis the average sample was finely ground and homogenized and analytical sample of 0.500 – 1.500 g was taken for determination of flavonoids. For processed foods three samples from three different producers were analyzed. Each composite sample consisted of three single samples of the respective product (packages, jars, cans) with different production dates, which were stored at 4^{0}C or -18^{0}C (for frozen foods). Before analysis an average sample was prepared and well homogenized using a blender. For flavonoids analysis an analytical sample of 10.00 g was taken.

Fresh vegetable samples

In the present study 7 vegetable food items from 3 botanical plant families were included. The samples were selected according to their wide consumption in Bulgaria. From *Solanaceae* family tomatoes and peppers were included; from *Papilonaceae* family - green beans and from *Umbiliferae* family – carrots. Samples were collected randomly from open markets or supermarkets in Sofia during a 2-year period. In Bulgaria the vegetable production is season-determined and correspondingly they were collected during their ripening stage and presence in the market. Carrots are present throughout the year and were purchased in May, August and December. Tomatoes were purchased in August and September (field production) and in May (greenhouse production). The general requirement for plant species that differ in color to be present separately in the sample is reflected [12]. For example, pepper was purchased from two different types: "Kapia" variety within their green and red rape stage; two types of green beans: yellow and green variety.

Processed food samples

In the present study we have analyzed 6 processed vegetable products produced in Bulgaria, in which, according to preliminary data, we expected to find flavonols and flavones, and which are widely consumed in Bulgaria. We have selected the following processed vegetable products: tomato puree, canned tomatoes, ketchup, lutenitsa, green beans frozen and green beans canned. Lutenitsa is a typical for Bulgaria product, which is a vegetable spread produced from tomatoes, red peppers, carrots and condiments.

Analytical method for determination of flavonols and flavones

Extraction and hydrolysis. The analytical sample was weighed in a 200 ml Erlenmayer flask with stopper. 0.500 – 1.500 g of analytical freeze-dried sample (10 g for processed foods) was taken and then 25.0 ml of TBHQ, 19 ml of water and 6 ml of HCl were added. Internal standard morine was used so that the final concentration in the sample to be 2.5 μg/ml. In this study extraction and hydrolysis of flavonoids was performed at 1.2 M HCl in 50% MeOH in a water bath at 90^{0}C refluxing for 2 h. At the end of the hydrolysis, the sample was left to cool for about 5 min and then, to ensure stability of aglycones, one ml of ascorbic acid solution (1 mg/ml) was added. The sample was transferred to a 100 ml volumetric flask and the volume was brought to the mark with MeOH. The sample was placed in an ultrasonic bath for 3 min and then adjusted to the mark if necessary. The extract was homogenized and an aliquot of 2 ml was ultracentrifuged for 5 min at 14000 rpm. The supernatant was filtered through a membrane filter (HV-Millipore) with a pore size of 0.45μm and then 50 μl were injected into the liquid chromatograph.

HPLC analysis. The chromatographic separation of flavonols – quercetin, kaempferol and myricetin and flavones – luteolin and apigenin was performed on Alltima (100 × 4.6 mm i.d., 3 μm) C18 column attached to a pre-column Alltima (4 × 4.6 mm i.d., 3 μm) C18. Isocratic elution with 53% MeOH in 2% acetic acid with a flow rate of 0.8 ml/min was used, resulting in operational pressure of 18.0-18.5 MPa. Ultraviolet detection at a fixed wavelength = 365 nm was used to determine flavonols and flavones. For quantitative determination the method of internal standard was used. The results were expressed in mg/100 g f.w. (fresh weight). Applying the present analytical methodology, the following parameters of the method were obtained: limit of detection – 0.03 mg/100 g f.w; limit of determination – 0.09 mg/100 g f.w.

Statistical analysis

A Student's t-test was applied to compare two arithmetic mean values. All statistical values were calculated using SPSS 11.0 for Windows.

RESULTS AND DISCUSSION

The results for flavonols and flavones content of the analyzed fresh and processed vegetable foods are presented in Table 1.

Table 1. Flavonoids in selected fresh and processed vegetable foods

Food Products	n	Flavonoids	Mean value	SD	Min	Max
			mg/100 g f.w.			
Tomatoes	7	Quercetin	1.42	0.55	0.78	2.59
	7	Kaempferol	0.19	0.44	0.07	0.44
Tomatoes, greenhouse	3	Quercetin	0.51	0.12	0.43	0.65
Tomato puree	3	Quercetin	4.10	2.16	1.67	5.64
	3	Kaempferol	0.08	0.13	0.06	0.15
Tomato, canned	3	Quercetin	0.32	0.26	0.24	0.63
Tomato, ketchup	3	Quercetin	0.45	0.09	1.67	5.64
	3	Luteolin	2.48	1.15	1.53	3.76
Pepper, red	5	Quercetin	1.49	0.91	0.71	3.03
	5	Luteolin	0.79	0.36	0.23	1.38
Pepper, green	5	Quercetin	10.27	5.92	4.92	19.49
	5	Luteolin	2.79	1.66	1.19	5.42
Carrots	5	Luteolin	0.88	0.47	0.31	1.43
Lutenitsa	3	Quercetin	1.42	0.43	0.93	1.70
	3	Luteolin	2.65	1.42	1.75	4.29
Green beans, green	5	Quercetin	2.13	0.83	1.11	3.05
Green beans, yellow	5	Quercetin	2.29	0.60	1.96	3.05
Green beans, frozen	3	Quercetin	1.95	0.38	1.56	2.32
Green beans, canned	3	Quercetin	0.84	0.16	0.66	0.96

n – number of samples; f.w. – fresh weight

The results are expressed in mg/100 g fresh weight or mg/100 g processed food. Data below limit of detection are not included in the table. Tomatoes and peppers are the most widely consumed vegetables in our country and are the basic ingredient of various canned vegetable product that are present at the table during the winter. The results show that the mean value of quercetin in tomatoes is 1.42 ± 0.55 mg/100 g (n=7). This value is statistically significantly higher than the results of Hertog *et al*., 1992 (0.80 ± 0.31 mg/100 g, n=4), of Crozier *et al.*, 1997 (0.59 ± 0.31 mg/100 g, n=8) and Stewart *et al.*, 2000, (0.67 ± 0.44 mg/100 g, n=13); that in USDA Database for the flavonoids content of selected foods 3.2 (2015) is a combined value for row red ripe tomatoes: 0.58 ± 0.98 mg/100 g, n=96 and is comparable to the data for quercetin in tomatoes presented by Justesen *et al.*, 1998 (1.40 ± 0.8 mg/100 g, n=5) [13-15, 8, 16]. Since flavonoids biosynthesis is a light-dependent process, it is suggested that tomatoes produced in direct sunlight have higher flavonol content than tomatoes grown in greenhouses. The influence of cultivation conditions was examined by Stewart *et al.*, 2000, according to which the flavonols content of tomatoes produced in Spain and South Africa is 4-5 times higher than those grown in the UK, where their production is almost entirely concentrated in greenhouses [15]. Our results confirm the cited relation. The content of quercetin in the early greenhouse tomatoes we examined (n=3) purchased in May is 0.51 mg/100 g, and kaempferol was not detected. It should be noted that Bulgarian tomatoes have very high values for quercetin and kaempferol, in some cases close to data for Cherry tomato presented in USDA database [8]. It seems that the cultivated tomato Bulgarian varieties in combination of microclimatic conditions in our country are favorable for accumulation of high amounts of flavonoids.

In Table 1 the results for the content of quercetin in pepper of two different types - “Kapia” green and red are presented. Kapia peppers in their technological maturity are dark green in color, and in their botanical maturity are red. In our country the most widely consumed peppers are Kapia, both in their red and green color. The results for Kapia sweet pepper show that in red pepper the mean value for quercetin is 1.49 mg/100 g, while the green peppers Kapia are extremely rich in quercetin – 10.27 mg/100 g. This fact can be explained by the general tendency for flavonoids to be synthesized in large quantities in the early stage of development and their quantity decreases during ripening with different rate for different plant species. The results for the

flavone luteolin show higher amount in green Kapia variety. We could compare our data for red and green pepper with USDA data, where quercetin in red sweet pepper has a mean value of 0.23 mg/100 g (n=7) as in both cases the USDA data [8] were statistically significantly lower than our results. As the consumption of green pepper Kapia is significant in Bulgaria, we can assume that it is one of the most important sources of flavonoids in the Bulgarian diet.

Carrots contain no flavonols. In our study, we detected luteolin in all carrot samples, ranging from 0.31 to 1.43 mg/100 g with a mean value of 0.88 mg/100 g (n=5). In the USDA study, luteolin in carrots has a mean value of 0.11 mg/100 g (n=7).

The study includes some processed vegetable foods that take part in different national dishes or are widely consumed as ready-to-eat foods in various combinations, such as tomato puree, ketchup and lutenitsa. Tomato puree is a rich source of quercetin with a mean value of 4.10 mg/100 g (n=3). Our results are similar to data from USDA database, where high levels of quercetin in tomato puree were found (4.12 mg/100 g, n=9) [8]. This can be explained by the effect of concentrating the starting material (fresh tomatoes) during the production of tomato paste and the stability of flavonols during heat treatment.

Three ketchup samples prepared from tomato paste, modified starch and various spices were analyzed. In consistence with the lower amount of tomatoes in this product, the results for quarcetin content in different types of ketchup are not high (0.45 mg/100 g) and vary within a narrow range (SD = 0.09 mg/100 g). These results are higher than the data presented in Phenol-Explorer [9, 17], obtained from compilation of 26 samples from 2 literature sources (4.23×10^{-3} mg/100 g quercetin).

The amount of flavonols in three different samples of lutenitsa was determined, which, according to product label is made from a mixture of pepper, tomato puree, carrots, modified starch and various condiments. For the first time original analytical data for lutenitsa (national typical vegetable spread) are presented, showing that quercetin content is 1.42 mg/100 g and luteolin content is 2.65 mg/100 g. Taking into consideration the traditionally high consumption of lutenitsa in the Bulgarian diet, it is an important source of qurecetin, especially during the winter season. Luteolin is relatively high in lutenitsa and ketchup – 2.65 mg/100 g and 2.48 mg/100 g respectively, which might be due to the presence of carrots or different spices.

Leguminous crop and especially green beans, have been extensively studied for the content of myricetin, quercetin and kaempferol by many research groups in the field of food science, as they are staple food in many regions of the world. This study presents data on the amount of quercetin in two types of green beans – flat green peppers pods and flat yellow peppers pods. The results show that myricetin, kaempferol and flavones are not detected in the samples. The level of quercetin in green beans is not affected by the color of peppers (2.13 mg in green beans and 2.29 in yellow beans / 100 g). the mean values for quercetin in green beans published in the USDA database are close to our results – 2.73 mg and 3.03 mg/100 g for green and yellow beans, respectively [8].

The three samples of frozen green beans consisted of green cylindrical pods. Two of the canned green beans were flat pods and one of them was cylindrical pod. Frozen green beans are characterized by a slight decrease in quercetin level (1.95 mg/100 g), whereas in classical preservation in cans the amount of quercetin is less (0.84 mg/100 g) due to the passage of flavonols into the aqueous environment of the finished food product. It is important to note that in beans, flavonols were found only in green beans but not in mature white beans (USDA) [8], which is confirmed by our study.

CONCLUSION

Flavonoids are present in the studied fresh vegetables and in their processed products, which is a proof of their stability at different production temperatures, assuming that the tested products retain the antioxidant activity of the fresh ones. A specific characteristic of the flavonoid composition in both target groups is the high content of quercetin. The present data can be used to fill the gaps in the Bulgarian food composition database and to be integrated into international networks for data exchange and scientific information. This study proves the need to preserve traditional foods in Bulgarian diet, due to the high amount of bioactive antioxidant compounds.

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Preliminary study of phenolic content in farmed *Mytilus galloprovincialis* from the Black Sea coast

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Marine bivalves constitute a rich source of nutrients and antioxidants, essential for providing a balanced diet. There are numerous studies devoted to the nutritional quality of farmed black mussels (*Mytilus galloprovincialis*) which reported the presence of health-beneficial components such as polyunsaturated fatty acids, fat-soluble vitamins and carotenoids. However, data about the phenolic content of mussels from the Bulgarian Black Sea waters is limited. The aim of this study was to determine and compare the total phenolic contents and phenolic composition of farmed black mussels (*M. galloprovincialis*) cultured in the Black Sea. Mussel tissue was extracted with five solvent systems: methanol, acetone:water, ethanol:water, hot water and ethyl acetate. Total phenolic content (TPC) of each extract was determined by Folin-Ciocalteu method. All extracts were further subjected to RP-HPLC/UV to analyze individual phenolic acids (4-hydroxybenzoic, gallic, caffeic, p-coumaric and cinnamic acid) and quercetin. The highest TPC of *M. galloprovincialis* was shown in methanol (84.5±7.1 μgGAE.g^{-1} ww) and ethanol:water (66.7±4.8 μgGAE.g^{-1} ww). The chromatographic analysis confirmed the presence of phenolic compounds in all mussel extracts, revealing that farmed black mussels (*M. galloprovincialis*) from the Black Sea could be a good source of phenolic compounds. Further studies are needed to explore the antioxidant potential of this commercially important species.

Keywords: Black mussels (*Mytilus galloprovincialis),* phenolic acids, quercetin, HPLC/UV, total phenolic content

INTRODUCTION

Over the past decades, there has been a growing interest in marine bivalves as inexpensive and easily accessible source of high-quality proteins, lipids and secondary metabolites. The black mussel (*Mytilus galloprovincialis*) is the main species in marine aquaculture and the most consumed shellfish in Bulgaria. A number of studies devoted to the nutritional quality of *M. galloprovincialis* farmed along the Bulgarian Black Sea coast reported fatty acid composition, vitamin and protein content of mussels meat [1-3]. Moreover, health-beneficial potential of Black Sea mussels is being complemented by the functional properties of their tissue extracts [4-7].

Marine organisms are exposed to a variety of exogenous and endogenous oxidants, consequently they produce a number of secondary metabolites with antioxidant activity – peptides, polysaccharides, carotenoids, etc. In addition, marine shellfish could be a good source of other natural antioxidants, such as flavonoids and phenolic acids. The main sources of phenolic compounds are plants and plant-derived foods, but polyphenols and their metabolites are also found in animal tissues and fluids [8]. The data about the phenolic content and antioxidant activity of marine bivalves is limited. Few studies investigated the total phenolic content (TPC) of green mussel (*Perna veridis*) [9, 10] and Moncheva *et al*. suggested that polyphenols in Black Sea *M. galloprovincialis* extracts play an important role for their antioxidant capacities [5]. However, data about the phenolic content and individual phenolic components in mussels from the Bulgarian Black Sea waters is scarce. Therefore, the aim of this study was to determine and compare the total phenolic contents and individual phenolic compounds in different extracts (methanol, acetone:water, ethanol:water, hot water and ethyl acetate) from farmed *M. galloprovincialis* from the Black Sea.

MATERIALS AND METHODS

Chemicals

All solvents and standards were of HPLC grade. The five phenolic acids: 4-hydroxybenzoic acid (4HBA), gallic acid monohydrate (GA), 3,4-dihydroxycinnamic acid (CA) and *trans*-cinnamic acid (CiA) were purchased from Acros Organics, New Jersey, USA; *trans*-4-hydroxycinnamic acid (p-coumaric acid, pCoA) – from Alfa Aesar, Thermo Fisher, Germany; and quercetin (Q) – from Fluorochem, Hadfield, UK. The solvents (water (W), methanol (Me), ethanol (E), acetone (Ac) and ethyl acetate (EAc)) were purchased from Fisher Chemicals, Thermo Fisher, Germany.

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Mussels sampling and pre-treatment

Live mussels (*M. galloprovincialis*) were purchased in the summer of 2019 from a mussel farm near Sozopol, Bulgaria. Samples were transported to the laboratory in iceboxes. Individual shell length of one hundred mussels was measured using a digital calliper and only mussels of a mean size (45.0±5.9 mm) were chosen for analysis. Mussels shells were thoroughly brushed, washed with distilled water and steamed for 6 min at 90±5 ºC.

Extraction

Three pools (n=20) of steamed mussels were homogenized using a blender. A three-step extraction procedure was applied for each extractant [11, 12]. The method includes triple liquid extraction of homogenized tissue with a portion of the following solvents – 100% methanol (Me), 70% acetone (AcW), 50% ethanol (EW), 100% ethyl acetate (EAc) and hot water (80 ºC). Combined extracts were centrifuged and filtered through 0.45 µm PTFE filter.

Total polyphenols determination

Total phenolic content of each mussel extract was determined by Folin-Ciocalteu method [13, 14]. The spectrophotometric analysis was performed using UV-Vis spectrometer Evolution 220 (Termo Fisher Scientific, USA). The absorbance was measured at 746 nm. Gallic acid was used as calibration standard and results were expressed in microgram gallic acid equivalents per gram wet weight (µgGAE.g^{-1} ww) as mean values (n=3) ± standard deviation.

HPLC analysis of phenolic acids and quercetin

The five phenolic acids – 4HBA, GA, CA, pCoA, CiA and Q in mussel extracts were analyzed by an HPLC/UV/FL system (Termo Fisher Scientific, USA), coupled with reverse phase column Acclaim™ Phenyl-1 Dionex Bonded Silica (C18, 120Å, 3µm, 250×3.0 mm, Termo Fisher Scientific, Waltham, MA, USA). The chromatographic elution of analytes was performed using a gradient program by the method of Özturk *et al*. [15]. Solvent A consisted of methanol:water:formic acid = 10:88:2 (v/v) and solvent B – methanol:water:formic acid = 45:53:2 (v/v). The chromatographic system used the following gradient program: from 0 to 27 min – 100% A, from 28 to 65 min – 100% B, then returned to 100% A. The flow rate of the mobile phase was 0.4 ml/min from 0 to 27 min and 0.5 ml/min from 28 to 65 min and the column temperature was 40 ºC. Gallic acid, CA, pCoA, CiA and Q were detected at 280 nm, and 4HBA – at 255 nm. Results were expressed in micrograms per gram wet weight (µg.g^{-1} ww) as mean values (n=3) ± standard deviation.

RESULTS AND DISCUSSION

Total phenolic content

It is widely accepted that polyphenols are most abundant in plants and plant-derived foods. However, the filter-feeding nature of marine shellfish suggests that the primary sources of polyphenols in mussels are algae and phytoplankton, comprising a major part in their diet. In the cited literature, most of the proposed methods for antioxidant determination of mussels as potential biomarkers are based on the water-soluble enzymes. The approach of extracted polyphenols as the main antioxidants has not been used for these purposes. The results for TPC in *M. galloprovincialis*, presented on Fig. 1 show that all prepared extracts could exhibit antioxidant potential. Methanolic extracts showed the highest TPC (84.5 µg GAE.g^{-1} ww), followed by EW and HW extracts. Lowest TPC was measured for AcW extract (36.3 µg GAE.g^{-1} ww).

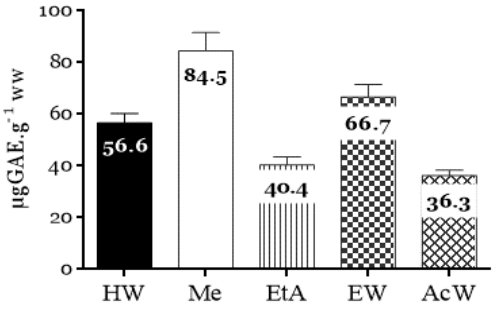

Fig. 1. Total phenolic content in *M. galloprovincialis* extracts

Gorinstein *et al*. [4] reported significant differences in TPC (varying from 391.8±35.8 to 892.7±76.9 µg GAE.100 g^{-1} DW) of methanolic extracts of boiled *M. galloprovincialis* collected from two regions of the Black Sea coast – Cape Galata and the area of Port Varna. Mussels from ecologically clean regions (Cape Galata) presented lower TPC than samples from the more polluted region of Port Varna [4]. Since *M. galloprovincialis* is a filter-feeding species the total phenolic content found in their tissues could be self-defense reaction against various pathogens or pollutants in their habitats. Our results for the methanolic extracts of farmed *M. galloprovincialis* from Sozopol were significantly higher than those reported by Gorinstein *et al*. [4] and Moncheva *et al*. [5]. On the other hand, aqueous extracts of Indian fresh-water pearl mussel (*Lamellidens marginalis*) showed higher TPC (82.81±0.75 µg GAE.mg^{-1} DW)

compared to our results [16]. Microwave assisted extraction and the use of protease inhibitors have been applied to facilitate the extraction of total polyphenols from the green mussel (*Perna viridis*) with water, methanol and ethanol. Ethanolic extracts showed the highest TPC (13.5±5.8 mg GAE.g^{-1}) compared to methanol and water extracts [9]. The discrepancies in the results published by other authors are most probably species-specific, related to environmental (geographical distribution, food availability) and extraction conditions (solvents, temperature and duration). The diverse nature of mussel species, extraction methods and results representation makes the comparison of data rather complicated.

Phenolic acids and quercetin content

Qualitative and quantitative results for the major individual phenolic acids and quercetin in *M. galloprovincialis* extracts are presented in Table 1.

Table 1. Individual phenolic acids and quercetin content in *M. galloprovincialis* extracts

	HW	Me	EtA	EW	AcW
4HBA	40.8±5.1	25.9±1.9	1.6±0.2	2.7±0.3	2.8±0.3
GA	10.5±0.9	5.5±0.6	nd	6.6±0.5	2.2±0.3
CA	3.1±0.4	1.7±0.2	nd	1.6±0.1	1.8±0.2
pCoA	2.3±0.3	0.34±0.02	0.35±0.02	0.41±0.02	1.0±0.09
CiA	1.1±0.08	0.4±0.01	<LOQ	0.08±0.01	1.1±0.08
Q	nd	0.32±0.01	0.3±0.01	0.47±0.02	<LOQ

LOQ – limit of quantification; nd – not detected

Five phenolic acids and quercetin were detected in *M. galloprovincialis* extracts. Several previous studies only examined the TPC of bivalves [4, 5, 9, 10, 16] but did not progress to further phenolic profiling. This study identified 4HBA and GA as the major phenolic acids, regardless of the extraction solvent used. Ethyl acetate and acetone:water extracts yielded the lowest TPC and phenolic compounds, while methanol and hot water extracts of *M. galloprovincialis* gave the highest phenolic content. Not surprisingly, quercetin was detected only in Me, EtA and EW extracts, since it has a low-polarity structure and is commonly extracted from plants with ethanol or aqueous-based ethanol and methanol solutions [17].

CONCLUSIONS

This preliminary study reveals that farmed black mussels (*M. galloprovincialis*) from the Black Sea could be an interesting source of phenolic compounds. Further qualitative and quantitative analyses explaining the relationship between total phenolic contents and total antioxidant capacity could be helpful to explore the antioxidant potential of this commercially important species.

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Cytotoxicity of water from five Bulgarian wetlands contaminated by toxigenic cyanobacteria and cyanotoxins

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Microscopic photosynthetic cyanoprokaryotes/cyanobacteria, or blue-green algae, produce various numbers of bioactive compounds, including different cyanotoxins which are hazardous for the ecosystem and human health. Cyanoprokaryotes are widely spread on the Earth and in Bulgaria specifically, where during the last two decades their toxins were found in different wetlands. However, only few studies conducted in Bulgaria mention cytotoxic effects of waters contaminated with cyanotoxins and up-to-now only three types of cell lines were used in the tests. Therefore, the present study was focused on the cytotoxic effect of waters from five chosen Bulgarian wetlands (two reservoirs and three lakes) with proved development of toxigenic cyanoprokaryotes. Moreover, for the first time in the country, the cytotoxicity was tested on the Hs27 human skin cells line. MTT test was performed to measure the cell viability upon exposure to increasing concentrations of water samples in culture medium. During the study three important results, which generally correspond to the cyanoprokaryote composition, biomass and detected cyanotoxins, were obtained: 1) applied water samples exhibited their effect after 24 hours of exposure; 2) at the lowest concentration of 1% cytotoxic effects were not observed; 3) at concentration of 8% in the culture medium, all water samples decreased cell viability by more than 50% compared to non-treated cells. These results allow to suppose the strong adverse effect of cyanoprokaryotes and their metabolites (mainly cyanotoxins) which should be considered as a risk factor for animal and human health in the studied water bodies.

Keywords: cyanobacteria, cyanoprokaryotes, cyanotoxins, health risk, toxigenic algae

INTRODUCTION

Cyanoprokaryotes/Cyanobacteria (known also as blue-green algae) are photosynthetic prokaryotic organisms, which develop as single cells, colonies or filaments rapidly growing in all types of aquatic, aeroterrestrial and extremophilic habitats. During the last decades the expansion of cyanoprokaryote growth at high blooming densities is increasing because of human activities, growing human population, globalization and climatic changes leading to global warming [1]. Cyanoprokaryotes form a high number of bioactive molecules, and certain species produce cyanotoxins as defense mechanisms against different ambient stress factors [2, 3]. Currently more than 120 different cyanotoxins are known, classified in three major groups by their chemical structure (alkaloids, cyclic peptides and lipopolysaccharides) or in three main groups according to the main target of their activity (hepatotoxins, neurotoxins and dermatotoxins) [2, 4]. The cyclic heptapeptides microcystins are the most widespread hepatoxins in water blooms and therefore are best known and commonly studied [5]. Nowadays another cyanotoxin – the neurotoxin cylindrospermopsin – attracts the attention of the research community because of its extracellular character and diverse multiple effects [2].

Humans' intoxication with cyanotoxins is possible *via* different pathways such as bathing and recreational activities with contaminated water, aerosolization or consumption of contaminated food [2, 6-9]. In the period 1960-2016, cyanotoxin poisonings of animals and humans were registered in different world's regions (Australia, Brazil, Canada, China, Namibia, Portugal, Serbia, Sri Lanka, Sweden, UK, and USA) [5]. Besides direct acute cases, some of which lethal [8, 9], experiments demonstrated that chronic exposures to low concentrations of cyanotoxins, and of microcystins in particular, could increase the risk for carcinogenesis because of their potential long-term adverse effects, and the International Agency for Research on Cancer classified them as a possible human carcinogen [10]. The effects of microcystins on different types of cell cultures were

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investigated and the observed mechanisms of cytotoxicity were summarized [11].

Among them, for example, was the ability of microcystins to cause apoptosis or necrosis in varying concentrations applied to different cell types in *in vitro* cell culture studies [12, 13]. However, it is important to recall that the harmful effects of cyanoprokaryotes cannot be attributed only to the known cyanotoxins and in this respect different *in vitro* tests for cytotoxicity of complex water samples are useful [2].

In Bulgaria, a pilot assessment of cyanotoxins as potential risk factor for cancer was made [14] following the survey on studies on cyanoprokaryotes conducted during 15 years (2000-2015) in 120 wetlands which demonstrated occurrence of blooms, toxigenic species and cyanotoxins [15]. The presence of cyanotoxins (microcystins LR, LA, RR, YR, nodularins, and saxitoxins) in 16 of these 120 wetlands was proved by using ELISA, HPLC or *in vitro* cytological tests with the latter method applied only in studies of six, mainly small, reservoirs [15]. Up-to-now three types of cell lines were used for *in vitro* detection of water toxicity in Bulgarian water bodies: HeLa (human cervical epithelial adenocarcinoma), 3T3 (mouse embryonic fibroblasts) and FL (normal amniotic human cells) (for details and references see [15]) and no studies on dermatotoxicity had been made. Therefore, the aim of the present study was to apply for the first time in Bulgaria a cell line of human skin fibroblasts (Hs27) for *in vitro* measurement of the changes of cell viability in respect to increasing concentrations of water samples collected from five different wetlands (2 reservoirs and 3 lakes) in which toxigenic cyanoprokaryote species and cyanotoxins were found [16-18].

MATERIALS AND METHODS

Water samples

Water samples were collected in June 2018 from the reservoirs Mandra and Sinyata Reka, and from the lakes Durankulak, Vaya and Uzungeren after application of a drone for finding of spots of blooming algae (for sampling details see [16]) – Table 1. Additional samples from Durankulak and Mandra were collected in the same way and from the same places in August 2019, and were labelled with Arabic number 2. All studied wetlands were chosen because of their different classification types in the Inventory of Bulgarian wetlands (IBW) and their biodiversity [19], their diferent usage and conservation importance (except Sinyata Reka, for details see [19]) and because of proved presence of harmful cyanoprokaryotes and their cyanotoxin metabolites by different methods (light microscopy, chemical analyses and molecular-genetic studies) [16-18]. For this study, identification of phytoplanktonic cyanoprokaryotes in all samples was done using conventional light microscopy according to standard taxonomic manuals and toxigenic genera were identified after [2].

Table 1. Main types and usage of the studied wetlands according to the Inventory of Bulgarian wetlands [19], where IBW is the relevant number in [19].

Wetland	IBW	Type	Position	Usage
Durankulak (DRK)	IBW0216	freshwater lake	coastal lowland in North-East Bulgaria	irrigation, recreation, sport fishing, industrial yield of crayfish
Mandra (MND)	IBW1720	large reservoir	coastal lowland in South-East Bulgaria	irrigation, fishing
Vaya (VA)	IBW0191	lake with varying halinity	coastal lowland in South-East Bulgaria	recreation, sport fishing
Uzungeren (UZNG)	IBW0710	lake with varying halinity	coastal lowland in South-East Bulgaria	irrigation, recreation, sport fishing
Sinyata Reka (SNR)	IBW1793	small reservoir	Inland kettle in Central Bulgaria	fish-breeding; irrigation

Cell line

Human skin cell fibroblasts (Hs27) were obtained from the American Type Culture Collection (ATCC). Cells were raised in 75 cm^2 flasks at 37°C in a humidified chamber with 5% CO_2 atmosphere. Complete nutrient medium comprised phenol of red-containing Dulbecco's Modified Eagle's medium (DMEM, Lonza) with

4.5 g L^{-1} glucose, L-glutamine and supplemented with fetal bovine serum (FBS, Sigma-Aldrich) to a 10% final concentration and penicillin/streptomycin mixture to final concentrations of 1%.

Experimental procedure

All water samples were filtered through 0.2 μm filter. Each water sample was added to the cell growing medium without any supplements to reach following concentrations: 1 v/v %; 2 v/v %; 4 v/v %; 8 v/v %; 16 v/v %. Hs27 human cells were collected and seeded in 12 well flasks at a density of 6.5×10^4 cells per well. Each water sample at all five concentrations was separately applied to Hs27 cell line after overnight incubation in two replicates.

Viability of treated cells was estimated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (commonly abbreviated as MTT assay) [20, 21]. The assay is based on the ability of viable cells to reduce the yellow MTT to purple insoluble formazan [20, 21]. At each well 100 μL of MTT solution in phosphate buffer saline (pH=7.4) at a concentration of 2 mg mL^{-1} was added 20 hours after the start of water treatment. After a 4-h incubation the medium was removed and 1 mL of dimethyl sulfoxide was added to each well for cell lysis. After thorough mixing, 100 μL were transferred to 96-well plates and the absorbance was measured at 550 nm wavelength. Afterwards Synergy 2 plate reader (BioTek) was used. Viability of treated cells was presented in percentage of the viability of the non-treated cells, which is considered 100%. All the treatments were performed in duplicate.

Statistical analyses were performed using Microsoft Excel Office software with calculated standard deviation (SD) and probability threshold (*p*) value less than 0.05 considered as significant.

RESULTS AND DISCUSSION

All results obtained during this study are represented on Fig. 1 as mean values and show the general decrease of the viability of the studied Hs27 cells with increase of the sample concentrations. The calculated SD was less than 0.5-1% of the measured values and therefore is not specifically indicated on the presented graph. The statistically significant changes ($p<0.05$) in the cell viability were recorded in 20 (or 57%) from 35 studied samples and are shown in Table 2. The lowest applied concentration of 1% did not induce changes in the cell viability, which remained 100% (except for the cell treated with water from Vaya, where the viability had fallen to 95%) indicating that at this concentration there were no cytotoxic effects. However, the double increase of the sample concentration (up to 2%) reduced cell viability below 83% compared to non-treated cells, with statistically significant slight decrease to 95% only in the samples treated by water from Uzungeren. At 4% concentration statistically significant decrease of cell viability was detected only for samples treated by water from Vaya, Mandra 1 and Mandra 2. The decrease of the Hs27 cell viability compared to non-treated cells was much better pronounced when the applied concentrations increased to 8%: the cell viability significantly decreased by more than 50% (68-53%) in all studied samples. It decreased to the lowest values when Hs27 cells were treated with the highest concentration of 16% with samples from Durankulak 1, Sinyata Reka and Uzungeren – 25, 36 and 37%, respectively, where Durankulak 1 shows the strongest cytotoxicity. According to these results it is possible to state that the effective concentration causing 50% inhibition (IC_{50}) was 8-16% and the lowest observable effect level (LOEL) was at 2-4% concentrations.

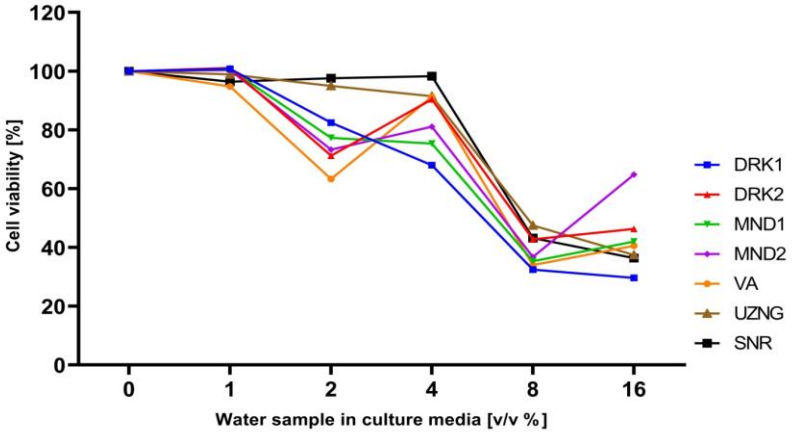

Fig. 1. Cell viability of HS27 fibroblasts treated with water collected from wetlands in Bulgaria. For each sample mean values with standard deviation are represented. Legend: DRK1 – Durankulak 1, DRK2 – Durankulak 2, MND1 - Mandra 1, MND2 - Mandra 2, VA – Vaya, UZNG – Uzungeren, SNR – Sinyata Reka.

Table 2. Statistically significant ($p<0.05$) changes of cell viability after application of water samples from five studied water bodies in different concentrations (from 0 to 16%) vs. untreated control cells. Legend: DRK1 – Durankulak 1, DRK2 – Durankulak 2, MND1 - Mandra 1, MND2 - Mandra 2, VA – Vaya, UZNG – Uzungeren, SNR – Sinyata Reka.

	DRK 1	DRK 2	MND 1	MND 2	VA	UZNG	SNR
1%	0.870304	0.360103	0.499897	0.676256	**0.000970**	0.503676	**0.013847**
2%	0.182717	0.141699	0.100993	0.122090	**0.025245**	**0.000490**	0.238410
4%	0.067736	0.144963	**0.000003**	**0.002156**	**0.000362**	0.101237	0.360264
8%	**0.000009**	**0.000017**	**0.000013**	**0.000787**	**0.000030**	**0.000113**	**0.000124**
16%	**0.000559**	**0.000001**	**0.000713**	0.126381	**0.000225**	**0.000111**	**0.000039**

In addition to the evaluation of the toxic effects, the exposure is important for the risk assessment [2]. In our study, we tested the effect of water samples in condition of 24 hours of exposure. At first glimpse, this result is in disagreement with another study of Bulgarian small reservoirs, where slight cytotoxic effect was recorded only after 48 hours of treatment [22], or with a study on the cytotoxicity of the most dangerous known microcystin MC-LR on cultured cells, where the effect occurred after 72-96 hours [23]. However, considering the differences in the types of the studied cell lines, of the water bodies, their algal biodiversity and detected cyanotoxins, these "discrepancies" are logical and easily explainable. Moreover, it is well-known that different strains of the same cyanoprokaryote species have different biochemical properties and the gene expression of toxic genes can vary depending on the environment [2]. In the same time, our results are in accordance with the detected cytotoxic effects at 24 hours of exposure reported by other authors [24].

Observations from this study concerning exposure time and concentrations correspond well with data available from *in vitro* investigations of other types of cell cultures [2]. For example, low dose of MC-LR after 24 h exposure did not induce apoptosis in the cell line of cultured Chinese hamster ovary, while the application of higher MC-LR concentrations induced apoptosis in a concentration-dependent manner [25].

Although in different amounts, cyanoprokaryotes were found in all five studied wetlands ([16-18], this study - Table 3) and this strongly corresponds to the general result from the present study, which demonstrated cytotoxic effects of the water collected from all of them. However, some differences in the cytotoxic effects of the applied water samples were observed. This result is logical when data on their biomass, algal and cyanotoxin composition are compared (Table 3)

For example, the highest number of toxigenic cyanoprokaryotes (12, among which was the dominant genus) was found in Vaya (Table 3) and this explains the best pronounced effect of the water of this wetland applied in different concentrations (Table 2). By contrast, the number of toxigenic genera was low (3) in the reservoir Sinyata Reka, but there cyanoprokaryotes from one toxigenic genus (*Microcystis*) were dominating the phytoplankton and this is in accordance with the strong effect of significant decrease of the cell viability with the increased sample concentrations (Table 2). Toxins, proved by ELISA and HPLC in these water bodies were also different, with saxitoxins found only in Durankulak 1 and cylindrospermopsin found in Mandra 1 and Vaya, and microcystins proved in Durankulak 1 and Sinyata Reka [16] – Table 3. Here we have to recall that saxitoxins are not specific for cyanoprokaryotes only, but are produced by algae from other taxonomic groups, like dinoflagellates, and therefore the identification of their producers is more complicated [2]. In the samples from Durankulak, collected in both years, such algae were found and their role in the water cytotoxicity is yet to be explored. In the samples from Durankulak 2 and Mandra 2, collected in 2019, microcystins were not found and checking for other cyanotoxins is in progress (V. Pavlova, M. Mitreva – pers. comm.). However, for the same samples the presence of toxigenic cyanoprokaryotes was proved by molecular-genetic methods (M. Radkova, K. Stefanova – pers. comm.). In Table 3 we indicate all recorded cyanoprokaryote genera, including those for which toxicity was not yet proved or was not searched for at global scale, in order to obtain a complete picture of the biodiversity and with the idea to consider them in future investigations. Last but not least, we would like to note that our findings could be related also with the presence of other cyanotoxins than those checked by us

Table 3. Quantitative distribution of cyanoprokaryote genera in the studied Bulgarian wetlands**.** Legend: DRK1 – Durankulak 1, DRK2 – Durankulak 2, MND1 - Mandra 1, MND2 - Mandra 2, VA – Vaya, UZNG – Uzungeren, SNR – Sinyata Reka, TPB – total phytoplankton biomass; d – dominant (>10-25% of TPB), c – common and abundant (5-10% of TPB), o – scarcely occurring (5-0.5% of TPB), r – rare species (<0.5% of TPB); MC – microcystins, SXT – saxitoxins, CYN – cylindrospermopsin, ndm – not detected microcystins. Toxigenic genera are provided after [2], with asterisk (*) are labelled genera published in [17, 18] and cyanotoxins published in [16].

	DRK1	DRK2	MND1	MND2	VA	UZNG	SNR
Cyanoprokaryota as % from the total biomass	25%	24%	40%	25%	42%	12%	90%
Toxigenic genera							
Anabaena					o		
Anabaenopsis					c		r
Aphanizomenon	d	o	o	r		o	r
Aphanocapsa		r	r		r		
Aphanothece		r					
Coelosphaerium			r		r		
Cuspidothrix		r			o	o	
Dolichospermum		o			c		
Gomphosphaeria	r				r		
Limnothrix			r			o	
Merismopedia	o	o			r	r	
*Microcystis**	d	d	c		c		d
Oscillatoria					r		
Phormidium		r		r			
Planktothrix			r		d		
Pseudanabaena	r	o	r				o
*Raphidiopsis**					c	d	
Trichodesmium		r					
Woronichinia		o					
picoplancton				d			
Total toxigenic genera	5	11	7	3	12	6	4
Non-toxigenic genera							
Borzia			r				
Chroococcus	r	c			r		
Coelomoron	r						
Cyanobium		r					
Cyanodictyon		o					
Pannus	r	r	r		o		
Planktolyngbya	r	r		r		d	
Romeria					r		
Snowella	r	r					
Synechocystis					r	r	
Total non-toxigenic genera	5	6	2	1	4	2	0
Total genera	10	17	9	4	16	8	4
CYANOTOXINS	MC*, SXT*	ndm	CYN*	ndm	CYN*	ndm	MC*

(microcystins LR, YR, and RR, saxitoxins and cylindrospermopsin), or by different products of cyanoprokaryote metabolism in the tested water samples.

CONCLUSION

Our results on the cytotoxic effects are in general accordance with the previous data obtained by chemical, molecular-genetic and conventional microscopic studies, which showed the presence of

cyanotoxins and cyanoprokaryotic toxin-producers in the studied samples [16-18]. There was a strong agreement between the cyanoprokaryote composition and biomass, with the presence of various toxigenic species, and detected effects on cell viability. The obtained results clearly demonstrated the applicability of human cell line Hs27 for *in vitro* cytotoxic measurements of water samples. This is valid especially for the cases when cyanotoxins have not been chemically proved due to their extreme diversity and impossibility to check all of them during conventional monitoring studies which cover just a small part of all cyanotoxins. Moreover, our results showed that cytotoxic effects occur fast, after 24 hours, even in quite low sample concentration (1-4%) and that 50% decrease of cell viability could be achieved at 8% concentration of the contaminated water. These results inevitably indicate the presence of a serious risk for ecosystem and human health in all five investigated water bodies which are used for recreation, sport fishing, fish-production and irrigation.

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