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Beneficial effects of *Clinopodium vulgare* water extract on spontaneously hypertensive rats

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Clinopodium vulgare L. (Lamiaceae) is a perennial herbaceous plant widespread in Bulgaria. Aerial parts are used in Bulgarian folk medicine for treatment of diabetes, gastric ulcers and cancer. The herbal drug alleviates symptoms associated with mastitis, prostatitis, skin irritation and swelling but until now there is no information about its effects on cardio-vascular system. Hypertension (HTN) is the most important cardiovascular risk factor, leading to coronary and cerebrovascular diseases. Along with antihypertensive medications, plant-based treatment has been thought to be effective for the prevention and control of HTN. *Clinopodium vulgare* aqueous extract (CVE) was analyzed by ultra high-performace liquid chromatography coupled with high resolution mass spectrometry (UHPLC-HRMS). The effects of CVE were evaluated in spontaneously hypertensive rats (SHR). Animals were treated with CVE (100 mg/kg, oral gavage) for 14 days. Reduced glutathione (GSH), malonedialdehyde (MDA), catalase (CAT), glutathione peroxidase (GPx) and superoxide dismutase (SOD) activities were measured in liver, kidney, and brain homogenate. Based on the MS/MS spectra and comparison with reference standards, the major compound in CVE - rosmarinic acid was identified. Administration of CVE slightly decreased the systolic blood pressure by 20% ($p < 0.05$) and MDA level by approximately 20% ($p < 0.05$) in all investigated organs compared to controls. CVE increased the antioxidant capacity of the liver, kidney, and brain evidenced by the increased activities of CAT, GPx and SOD, and preserved the GSH depletion.

Keywords: *Clinopodium vulgare*, Oxidative stress, Hypertension

INTRODUCTION

Plants of the Lamiaceae family are highly regarded by some cultures for their medicinal properties, including anti-inflammatory and antioxidant activities. Wild basil *(Clinopodium vulgare* L.*)* (Lamiaceae) is a perennial herbaceous plant widespread in Bulgaria. Aerial parts are used in Bulgarian folk medicine for treatment of diabetes and gastric ulcers. The herbal drug alleviates symptoms associated with mastitis, prostatitis, skin irritation and swelling. Previous investigations revealed that *C. vulgare* water extract presented the highest free radical scavenging activity (DPPH and ABTS) and reducing potential (FRAP and CUPRAC) compared to methanol and acetone extracts [1]. Moreover, acetone, ethanol, and ethyl acetate extracts demonstrated antibacterial activity and synergetic effect with some antibiotics [2]. Anti-inflammatory and antitumor activities of *C. vulgare* extract were also determined [3].

Hypertension (HTN) is the most important cardiovascular risk factor, contributing to coronary heart disease and cerebrovascular diseases [4]. Oxidative stress is an independent risk factor in the development of hypertension in human and experimental animal models, as spontaneously hypertensive rats (SHRs). If oxidative stress is indeed a cause of hypertension, then antioxidants should have beneficial effects on hypertension control. Reduction of oxidative damage should result in a reduction in blood pressure. The essential oil [5], as well as the water extract [1] of *C. vulgare*, have shown antioxidant activity.

In the present study, the effects of *Clinopodium vulgare* aqueous extract (CVE) were evaluated in spontaneously hypertensive rats (SHR). The biomarkers of oxidative stress: reduced glutathione (GSH), malonedialdehyde (MDA), superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase (CAT) in liver, kidney, and brain from SHRs, treated with CVE were measured. Moreover, the effect of CVE on the animal's blood pressure was assessed.

EXPERIMENTAL

Plant material

C. vulgare L. aerial parts were collected in July 2017 from region of German village near Sofia, Bulgaria (voucher specimen SO 107606).

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Chemicals and reagents

Bovine serum albumin (fraction V), beta–nicotinamide adenine dinucleotide 2`-phosphate reduced tetrasodium salt (NADPH), reduced glutathione (GSH), oxidized glutathione (GSSG), glutathione reductase (GR) enzyme, and cumene hydroperoxide were purchased from Sigma Chemical Co. (Taufkirchen, Germany). 2,2- Dinitro-5,5 dithiodibenzoic acid (DTNB) was obtained from Merck (Darmstadt, Germany). Rosmarinic acid was obtained from Phytolab (Germany). All solvents were LC-MS grade and were purchased from Ficher Scientific (Waltham, USA). CVE was analyzed by UHPLC-HRMS using a quaternary pump and a hybrid quadrupole - "Orbitrap" high resolution "Q-Exactive" mass spectrometer coupled with a HESI (heated electrospray ionization) probe. The chromatographic separation was performed on RP (reversed phase) "Poroshell" C18 3 x 150 mm 2.7 µm column with gradient of 10÷95% acetonitrile in 0.08% formic acid as mobile phase at flow rate 250 µl/min, and on Silica-HILIC (hydrophilic interaction liquid chromatography) on 150 x 3 mm 2.6 µm "Kinetex" column with 5÷50% gradient of water in acetonitrile with 10 mM ammonium formate pH 4.6 at flow rate 450 µl/min. HESI worked at 250°C, spry voltage 3 kV, ion transfer tube at 300°C, sheath gas pressure 45 Psi and mass tolerance of 5 ppm. Data acquisition and processing were carried out with Xcalibur 3.0 software (Thermo Fisher Scientific Inc., USA).

Animals

Experiments were performed with 12 male SHRs (initial body weight 180-230 g). The animals were housed in Plexiglas cages (3 per cage) at 20 ± 2 °C and 12/12 h light/dark cycle. Food and water were provided *ad libitum*. All procedures were approved by the Bulgarian Agency of Food Safety (№ of permission 169) and performed strictly following the principles stated in the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes (ETS 123).

Blood pressure measurement

The blood pressure was measured in conscious animals using an automated tail-cuff device (CODA non-invasive blood pressure system, Kent Scientific Corporation, USA). Before the experimental period, the rats were conditioned to restraining cylinders and were pre-warmed for 10 min using a temperature-controlled warming holder (37°C) to facilitate tail blood flow before their blood pressure was measured. The mean of three tail-cuff readings was used as the systolic and diastolic blood pressure value. SHRs with highest blood pressure values were taken for the *in vivo* experiment.

Design of the in vivo experiment

The rats were randomly divided into two groups (n=6) as follows:

Group 1: control SHRs, treated with the saline vehicle, administered by gavage at 5 mL/kg body weight/ day, 14 days.

Group 2: SHRs treated with CVE alone at 100 mg/kg bw/ day, 14 days, administered by oral gavage at the dose volume of 5 mL/kg b.w.

On the 15th day from the beginning of the experiment, all animals were sacrificed. Brains, livers and kidneys were taken for assessment of parameters of antioxidant status. The excised organs were perfused with cold saline solution (0.9% NaCl), blotted dry, weighed, and homogenized with corresponding buffers.

Markers of oxidative stress

Reduced glutathione (GSH) was assessed by measuring non-protein sulfhydryls after precipitation of proteins with 5% trichloroacetic acid (TCA), using the method described by Bump *et al.* [6]. A total of 10% homogenates were prepared in 0.05M phosphate buffer (pH 7.4) and centrifuged at 7 000 × g and the supernatant was used for antioxidant enzymes assay. Glutathione peroxidase (GPx) was measured by NADPH oxidation, using a coupled reaction system consisting of GSH, GR, and cumene hydroperoxide [7]. Catalase (CAT) activity was determined by measuring the decrease in absorbance at 240 nm of a reaction mixture consisting of H_2O_2 in phosphate buffer, pH 7.0, and requisite volume of supernatant sample. The molar extinction coefficient of 43.6 M cm^{-1} was used to determine catalase activity. The specific activity was calculated and was expressed as nmol/min/mg of total protein [8]. Superoxide dismutase activity (SOD) was measured according to the method of Misura and Fridovich [9], following spectrophotometrically the autoxidation of epinephrine at pH=10.4, 30°C, using the molar extinction coefficient of 4.02 mM^{-1} cm^{-1}. Lipid peroxidation was determined by measuring the rate of production of thiobarbituric acid reactive substances (TBARS) (expressed as malondialdehyde (MDA) equivalents) described by Polizio and Peña [10] with slight modifications. One volume of homogenate was mixed with 1 mL of 25% trichloroacetic acid (TCA) and 1 mL of 0.67% thiobarbituric acid (TBA). Samples were then mixed thoroughly, heated for 20 min in a boiling water bath, cooled and centrifuged at 4000 rpm for 20 min. The absorbance of supernatant was measured at

535 nm against a blank that contained all the reagents except the tissue homogenate. MDA concentration was calculated using a molar extinction coefficient of 1.56 10^5 M^{-1} cm^{-1} and expressed in nmol/g wet tissue.

Statistical analysis

Statistics were performed with the 'MEDCALC' statistical programme. The results were expressed as mean ± SEM for six rats in each group. The significance of the data was assessed using the nonparametric Mann–Whitney test. For both statistical methods, values of $p \leq 0.05$ were considered statistically significant.

RESULTS

UHPLC-HRMS analysis revealed that the major compound in CVE was rosmarinic acid. The structural elucidation of the compound was based on comparison of retention times (t_R), and MS/MS-fragmentation patterns with a reference standard. The $[M-H]^-$ ion at *m/z* 359.0775 of rosmarimic acid produced the fragments at *m/z* 161.0229 $[M\text{-}H\text{-}198]^-$ (100%) and *m/z* 197.0445 $[M\text{-}H\text{-}162]^-$ (23%) corresponding to the cleavage of *a* bond. Furthermore, the compound also produced the fragment ion at *m/z* 179.0337 $[M\text{-}H\text{-}180]^-$ (18%), corresponding to the cleavage of *b* bond (Figure 1).

Blood pressure values are shown on Figure 2. After 14-day oral administration of CVE, systolic and diastolic blood pressure were decreased by 20% ($p < 0.05$) and by 19% respectively, compared to control values.

The changes in oxidative stress markers MDA quantity and GSH level are presented in Table 1. Administration of CVE at dose 100 mg/kg for 14 days decreased statistically significantly the quantity of MDA, a marker of lipid peroxidation in liver, kidney and brain by 18% ($p < 0.05$), by 22% ($p < 0.05$), and by 20% ($p < 0.05$) respectively.

The changes in the activity of antioxidant enzymes in the liver, kidney and brain of experimental animals are presented on Table 2.

After administration of CVE for two weeks the catalase activity augmented in statistically significant manner by 26% ($p < 0.05$) in the liver and kidney and by 34% ($p < 0.05$) in the brain, compared to the controls. SOD activity was also significantly increased by 22% ($p < 0.05$) in the liver, by 28% ($p < 0.05$) in the kidney and by 20 % ($p < 0.05$) in the brain. GPx augmented in the liver by 18% ($p < 0.05$), in the kidney by 26% ($p < 0.05$) and in the brain by 21% ($p < 0.05$), compared to the controls.

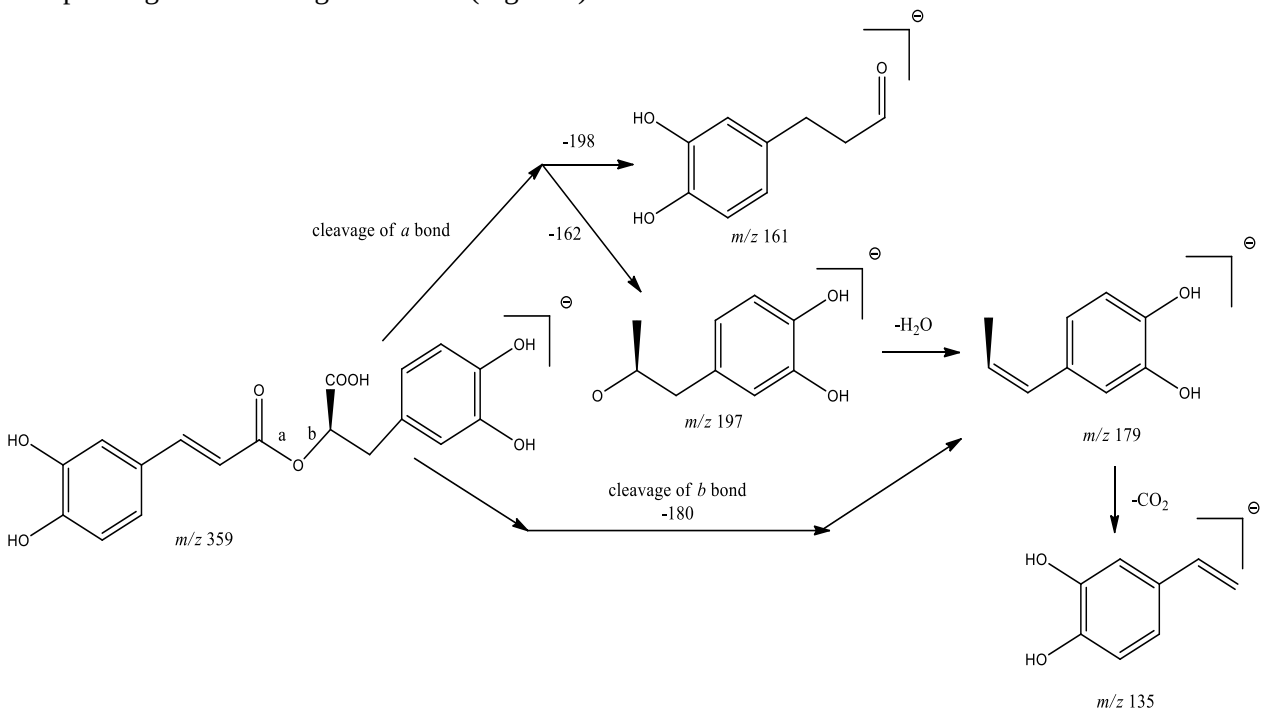

Figure 1. MS/MS fragmentation pathway of rosmarinic acid.

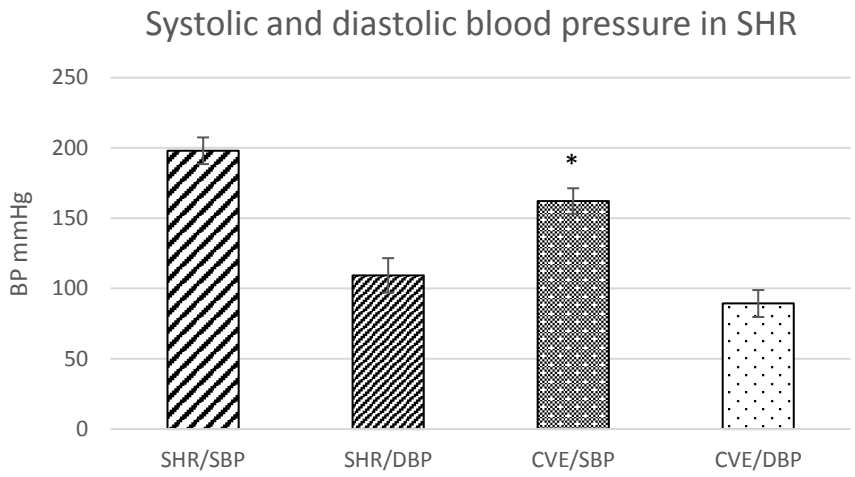

Figure 2. Blood pressure values SHR/SBP – systolic pressure of SHR group; SHR/DBP – diastolic pressure of SHR group; CVE/SBP – systolic pressure of SHR group treated with CVE; CVE/DBP – diastolic pressure of SHR group treated with CVE. *p < 0.05 vs SHR/SBP.

Table 1. Markers of oxidative stress

Parameters	**MDA (nmol/ g wet tissue)**		**GSH (nmol/ g wet tissue)**	
Organs	SHR control	SHR - CVE	SHR control	SHR - CVE
Liver	6.28 ± 0.32	5.19 ± 0.18*	5.22 ± 0.15	6.62 ± 0.38*
Kidney	5.29 ± 0.23	4.16 ± 0.13*	3.55 ± 0.21	4.46 ± 0.29*
Brain	7.09 ± 0.27	5.68 ± 0.15*	1.11 ± 0.10	1.52 ± 0.25*

*p < 0.05 vs control

GSH level was increased after CVE administration by 26% (p < 0.05) in the liver and kidney and by 37% (p < 0.05) in the brain.

Table 2. Activities of antioxidant enzymes CAT, SOD and GPx

Parameters	**CAT (nmol/ min/mg)**		**SOD (µmol/min/mg)**		**GPx (µmol/min/mg)**	
Organs	SHR control	SHR - CVE	SHR control	SHR -CVE	SHR control	SHR-CVE
Liver	33.38±2.19	42.30±1.70*	0.23±0.01	0.28±0.01*	0.34±0.02	0.40±0.01*
Kidney	12.93±0.49	16.33±1.02*	0.18±0.02	0.23±0.01*	0.19±0.01	0.24±0.01*
Brain	21.15±0.87	28.25±2.06*	0.25±0.02	0.30±0.01*	0.28±0.02	0.34±0.04*

*p < 0.05 *vs* control group

DISCUSSION

Oxidative stress occurs in the case of imbalance between free radical production and the antioxidant capacity of non-enzymatic and enzymatic substances present in the tissues. The living organism is equipped with a battery of antioxidants, some of them enzymatic, such as: superoxide dismutase, catalase and GPx, and other non-enzymatic, such as GSH that serve to counterbalance the effect of the reactive oxygen species (ROS). In addition to the endogenous system, antioxidant status can be improved by exogenous antioxidant food supplementation. Oxidative injury is one of the main causes of HTN.

Hypertension is associated with greater than normal lipoperoxidation and an imbalance in antioxidant status, suggesting that oxidative stress is significant in the pathogenesis of this disease [11].

SHRs are a suitable model for evaluation and examination of oxidative stress. There is recent evidence that increased microvascular oxidative stress is present in SHR [12]. The hepatic level of MDA, an indirect measure of tissue lipid peroxides level, is higher in SHR than in normotensive controls [13]. Long lasting untreated hypertension increases the risk of chronic organ damage and cardiovascular complications. One of the common mechanisms suggested in the pathogenesis of this disease is the formation of ROS that induce endothelial oxidative stress [14].

In the recent years, an increased interest in the mechanisms of ROS generation and their role in the development and complications of hypertension, is seen. Along with antihypertensive medications, plant-based treatment has been thought to be effective for the prevention and control of

hypertension [15]. On the basis of this assumption, the aim of the current study was to investigate the possible antihypertensive and antioxidant activity of the CVE, applying a model of spontaneously hypertensive rats, strain Okamoto-Aoki.

The treatment of the SHR with CVE decreased in a statistically significant manner both systolic and diastolic blood pressure by approximately 20%. CVE ameliorated antioxidant status in SHRs by increasing the level of GSH and activity of antioxidant enzymes CAT, SOD and GPx and by decreasing the formation of MDA, a marker of lipid peroxidation. In the view of the fact that oxidative stress is implicated in the pathogenesis and/or maintenance of hypertension in SHR, the reduction of blood pressure elicited by CVE might be due to its antioxidant effects. The antioxidant effects observed in our study could be attributed to many phytochemicals in the CVE. Based on the MS and MS/MS spectra, comparison with reference standards and literature data, rosmarinic acid (RA) was the major compound in CVE. Abundance of literature data proved the antioxidant activity of RA both *in vitro* and *in vivo* studies [16-18]. RA is known to possess marked antioxidant properties as a scavenger of ROS [19, 20].

RA supplementation elevated SOD, CAT, and GSH-Px activity and reduced MDA production in liver and kidney of aging mice [21]. These findings support our results regarding the antioxidant effects of CVE in SHRs. Furthermore, it was found that RA decreased blood pressure in hypertensive animals by reduction of angiotensin converting enzyme (ACE) activity [22]. Therefore, the antihypertensive effect of CVE we observed after 14-day oral administration to SHRs, could be due not only to antioxidant effects of RA as a main constituent in CVE, but also to its ACE inhibitory action.

Further examinations to elucidate the exact mechanism of antioxidant activity and antihypertensive effect of *Clinopodium vulgare* should be performed in order to precise its use as a supplementation in pathologies, caused by increased oxidative stress.

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