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Phytochemical composition and biological activity of *Echium italicum* L. plant extracts

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The aim of this study was to assess the biological activity of five different extracts of the plant *Echium italicum* L. and to determine their phytochemical composition. The chloroform, ethyl-acetate, ethanol, acetone and petroleum ether extracts of the plant were examined. The ethanol extract of the plant *E. italicum* had the highest content of total phenolics and flavonoids, while the chloroform and acetone extracts had the highest tannin content. Several different methods were used to determine the antioxidant activity of the tested extracts, and the ethanol and acetone extracts of the plant displayed the best antioxidant activity. HPLC analysis showed that the main phenolic compounds in the tested extracts were rosmarinic acid, chlorogenic acid, *p*-hydroxybenzoic acid and rutin. Evaluation of the antimicrobial activity of plant extracts was conducted by to the microdilution method. The results of MIC ranged from 3.91 to 500 μg/ml. Determinations of cytotoxic activity were done according to the MTT assay on human rhabdomyosarcom cells (RD), a cell line derived from human cervix carcinoma (Hep2c) and a cell line derived from mouse fibroblast carcinoma (L2OB). This study suggests that the examined extracts of the plant *E. italicum* L. may serve as sources of antioxidants and antibiotic agents.

Keywords: *Echium italicum* L., phytochemical composition, biological activity.

INTRODUCTION

Throughout history and across the globe, the plant kingdom has provided a variety of medicines. From ancient times, traditional medicinal plants have been known to possess diverse biological activities as antimicrobial, analgetic, anticancer, antipyrexial, and antihypertensive activity and to be an important source of many biological active compounds [1]. Medicinal plants have been used extensively for their health care and remedy of diseases during 2000 years and a high degree of correlation between traditional medicinal plant uses and laboratory analysis has been revealed [2]. Today, using the most modern instrumental methods, detailed qualitative and quantitative analysis, isolation and testing of even traces of substances present in plant tissue is possible [3]. The most interesting area of application of medicinal plant extracts is the inhibition of growth and reduction in the number of serious pathogens [4, 5], and a great deal of efforts is focused on using available experimental techniques to identify natural antioxidants from plants. In the search for sources of natural antioxidants, in recent years some medicinal plants have been extensively studied for their antioxidant activity and radical-scavenging activity [6, 7]. Also, a number of clinical trials have shown that various secondary metabolites of plants can be used in the treatment of different cancer types [8].

The family *Boraginaceae* is known as medicinal plants classified as dicotyledones. Many members of the *Boraginaceae* family produce secondary metabolites such as alkaloids, naphthoquinones, polyphenols, phytosterols and terpenoids [9, 10]. Polyphenols, including flavonoids and phenolic acids, produced by the family *Boraginaceae*, have a wide range of pharmaceutical activities, including antiinflammatory, antiviral and antibacterial activities [11, 12]. *Echium italicum* is a perennial, shrub-like plant, inhabiting thermophilic, sandy grounds of the submediterranian area [13]. Its leaves are used as seasoning; apiarists use the plant to make uniquely flavoured honey [14]; flowers are used as an „anti-stress“, tranquilizer, and energizer drink, fighting common cold and bronchitis. In Turkish folk medicine, roots of *Echium italicum* and *Echium vulgare* are used externally for healing wounds [15-18]. Phytochemical analysis of the *E. italicum* plant has shown that the contents of condensed tannins and gallotannins were 21.49 mg Ga/g and 28.85 mg GA/g, while the total antioxidant capacity of the plant extract was 112.92 μg AA/g [19]. Albreht [20] identified in the root of *E. italicum* L. nine shikonin pigments: shikonin, acetylshikonin, propionylshikonin, isobutyl-shikonin, tigla shikonin, 3,3-dimethylacrylshikonin, angelylshikonin, 2-methyl-*n*-butyrylshikonin and isovalerylshikonin. The *E. italicum* oil exhibited

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concentration-dependent antimicrobial activity on all microorganisms tested [21]. The aim of this study was to assess *in vitro* the biological activity of five different extracts of *Echium italicum* L. and to determine their phytochemical composition.

EXPERIMENTAL

Preparation of the plant extracts

The plant material was collected from Brdjanska gorge near Gornji Milanovac in June 2013, in the flowering stage. The above-ground parts of the plant were crushed using a cylindrical crusher. The plant material was extracted in a Soxhlet extractor. The plant material was degreased by extraction with petroleum ether (40°C), followed by extraction with a series of solvents. The resulting solutions were allowed to cool and after 24 h their pairing in a rotary vacuum evaporator at a temperature of 40°C was performed. Investigations were carried out in the chemical and microbiological laboratories of the Faculty of Agronomy in Čačak.

Determination of total phenolic content

Total phenols were estimated according to the Folin-Ciocalteu method [22]. The extracts were diluted to a concentration of 1 mg/ml, and aliquots of 0.5 ml were mixed with 2.5 ml of Folin-Ciocalteu reagent (previously diluted 10-fold with distilled water) and 2 ml of $NaHCO_3$ (7.5%). After 15 min of staying at 45˚C, the absorbance was measured against a blank sample at 765 nm.

Determination of flavonoid content

Total flavonoids were determined according to Brightene *et al.* [23]. A total of 0.5 ml of 2% aluminium chloride ($AlCl_3$) in methanol was mixed with the same volume of methanol solution of plant extract. After 1 h of staying at room temperature, the absorbance was measured at 415 nm on a spectrophotometer against a blank sample.

Determination of tannin content

The method for determination of condensed tannins relies on the precipitation of proanthocyanidins with formaldehyde [24]. First, total phenolics were measured using the Folin-Ciocalteu reagent as described above. A 0.5 mol equivalent of phloroglucinol was added for every gallic acid equivalent in the extract. An aliquot of 2 ml of the extract dissolved in methanol was mixed with a calculated amount of phloroglucinol, followed by 1 ml of 2:5 HCl/H_2O solution and 1 ml of formaldehyde solution (13 ml of 37% formaldehyde diluted to 100 ml with water). After overnight incubation at room temperature, unprecipitated phenols were determined in the supernatant by the Folin-Ciocalteu method.

Determination of total antioxidant activity

The total antioxidant activity of *E. italicum* extracts was evaluated using the phosphomolybdenum method [25]. This assay is based on the reduction of Mo (VI) to Mo (V) by antioxidant compounds and subsequent formation of a green phosphate/Mo (V) complex at acidic pH. A total of 0.3 ml of sample extract was combined with 3 ml of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The tubes containing the reaction solutions were incubated at 95°C for 90 min. After staying at room temperature, the absorbance of the solutions was measured at 695 nm against a blank sample. Methanol (0.3 ml) was used as the blank.

Determination of DPPH free radical scavenging activity

The method used by Takao *at al.* [26] was adopted with suitable modifications from Kumarasamy *et al.* [27]. DPPH (8 mg) was dissolved in MeOH (100 ml) to obtain a concentration of 80 μg/ml. Serial dilutions were carried out with the stock solution (1 mg/ml) of the extract. Solutions (2 ml each) were then mixed with DPPH (2 ml) and allowed to stay for 30 min to allow any reaction to occur, and the absorbance was measured at 517 nm. Ascorbic acid (AA), gallic acid and BHT were used as reference standards and were dissolved in methanol to prepare stock solutions with the same concentrations (1 mg/ml). Control samples were prepared containing the same volume without test compounds or reference antioxidants.

Determination of inhibitory activity against lipid peroxidation

Antioxidant activity was determined by the thiocyanate method [28]. Serial dilutions were carried out with stock solutions (1 mg/ml) of the extracts, and 0.5 ml of each solution was added to a linoleic acid emulsion (2.5 ml, 40 mM, pH 7.0). The linoleic acid emulsion was prepared by mixing 0.2804 g of linoleic acid and 0.2804 g of Tween-20 as an emulsifier in 50 ml of 40 mM phosphate buffer and the mixture was homogenised. The final volume was adjusted to 5 ml with 40 mM phosphate buffer, pH 7.0. After incubation at 37°C in the dark for 72 h, 0.1 ml aliquot of the reaction solution was mixed with 4.7 ml of ethanol (75%), 0.1 ml of $FeCl_2$ (20 mM) and 0.1 ml of ammonium thiocyanate (30%). The mixture was stirred for 3

min and the absorbance was measured at 500 nm. Ascorbic acid, gallic acid, α-tocopherol and BHT were used as reference compounds.

Determination of hydroxyl radical scavenging activity

The ability of *E. italicum* extracts to inhibit non-sitespecific hydroxyl radical-mediated peroxidation was carried out according to Hinneburg *et al.* [29]. The reaction mixture contained 100 µl of extract dissolved in water, 500 µl of 5.6 mM 2-deoxy-D-ribose in KH_2PO_4– NaOH buffer (50 mM, pH 7.4), 200 µl of premixed 100 µM $FeCl_3$, 104 mM EDTA (1:1 v/v) solution, 100 µl of 1.0 mM H_2O_2 and 100 µl of 1.0 mM aqueous ascorbic acid. Tubes were vortexed and incubated at 50ºC for 30 min. Thereafter, 1 ml of 2.8% TCA and 1 ml of 1.0% TBA were added to each tube. The samples were vortexed and then heated in a water bath at 50^0C for 30 min. The extent of oxidation of 2-deoxyribose was estimated from the absorbance of the solution at 532 nm. The percentage inhibition was calculated from the absorbances of the controls (Ac) and the samples (As), where the controls contained all reaction reagents except the extract or positive control substance. The values are presented as the means of triplicate analyses. Spectrophotometric measurements were performed using a UV–Vis spectrophotometer MA9523-Spekol 211 (ISKRA, Horjul, Slovenia).

HPLC analysis of E. italicum plant extracts

Determination of polyphenol components in the tested extracts was done on the HPLC Agilent 1200 Series instrument (Agilent Technologies, USA) with UV-Vis DAD for multiwavelength detection. After injecting 5 µl of sample, the separation was performed in an Agilent-Eclipse XDB C-18 4.6·150 mm column. The column was thermostated at 25 ^{0}C. Two solvents were used for the gradient elution: A - (H_2O+2% HCOOH) and B - (80% ACN+2% HCOOH+H_2O). The elution program used was as follows: from 0 to 10 min 0% B, from 10 to 28 min gradual increase 0-25% B, from 28 to 30 min 25% B, from 30 to 35 min gradual increase 25-50% B, from 35 to 40 min gradual increase 50-80% B, and finally for the last 5 min gradually decrease 80-0% B. All identifications of individual compounds were based on the retention times of the original standards, where available, and spectral data.

Measurement of minimum inhibitory concentration (MIC and MBC) of E. italicum extracts

MIC of the crude extracts was determined by the microdilution method using 96-multi-well microtiter plates [30]. In the experiment were included pure cultures of the following bacteria: *Listeria ivanovii* ATCC 19119, *Listeria inocuu* ATCC 33090, *Enterococcus faecalis* ATCC 29212, *Listeria monocytogenes* ATCC 19112, *Bacillus spieizenii* ATCC 6633, *Enterococcus faecium* ATCC 6057, *Staphylococcus aureus* ATCC 25923, *Staphylococcus saprophiticus* ATCC 15035, *Klebsiella pneumoniae* ATCC 13883, *Escherichia coli* ATCC 25922, *Proteus vulgaris* ATCC 13315, *Proteus mirabilis* ATCC 14153, *Salmonella enteritidis* ATCC 13076, *Enterobacter aerogenes* ATCC 13048, *Citrobacter freundii* ATCC 43864, *Salmonella Typhimurium* ATCC 14028, *Pseudomonas aeruginosa* ATCC 27853 and yeast *Candida albicans* ATCC 10231 and *Aspergillus niger* ATCC 16404, obtained from the Microbiological laboratory of the Faculty of Agronomy in Čačak. All tests of bacterial and yeast cultures were performed in Mueller–Hinton broth and Sabouraud dextrose broth, respectively. In the first row of the plate 100 µl of a stock solution of *E. italicum* extracts (200 µg/ml) and a solution of cirsimarin (2 mg/ml) in 10 % DMSO were applied. In the other wells 50 µl of Mueller Hinton or Sabouraud dextrose broth containing Tween 80 to a total concentration of 0.5 % (v/v) was added for analysis of the extracts. From the first row test wells a volume of 50 µl was transferred into the second row wells. Thereafter, from the second to the twelfth well a volume of 50 µl of scalar dilution was transferred. Then, to each well 10 µl of indicator solution (prepared by dissolving resazurin in sterile distilled water) and 30 µl of nutrient broth was added. Finally, to each well 10 µl of bacterial suspension (106 CFU/ml) and yeast spore suspension (3×104 CFU/ml) were added. Amracin (tetracycline hydrochloride) and ketoconazole were used as positive control for the test bacteria and yeast, respectively. The plates were wrapped loosely with cling film to ensure that bacteria did not become dehydrated. The plates were prepared in triplicate and incubated at 37 °C for 24 h for the bacteria and 48 h for the yeast. Mean MIC (n = 3) for the test extracts and standard drugs were taken. For the determination of MBC, a portion of liquid (5 µl) from each plate well that exhibited no growth was taken and then incubated at 37 ^{0}C for 24 h. The lowest concentration that revealed no visible bacterial growth after subculturing was taken as MBC.

Measurement of cytotoxic activity by MTT assay

Determinations of cytotoxic activity were done according to the MTT assay (3-[4,5-

dimethylthiazol-2-yl-2,5 diphenyl tetrazolium bromide test). The following cell lines were used: Hep2c (cell line derived from human cervix carcinom), RD (cell line derived from human rhabdomyosarcom), and L2OB (cell line derived from mouse fibroblast carcinom). Using 96-well cell culture plates, the cells were seeded in nutrient medium (minimum essential medium—MEM) and grown at 37 ^{0} C in humidified atmosphere for 24 h. After completing 24 h of incubation the medium was replaced with 100 ml of medium with different concentrations of extracts of the test plant (25, 50, 100, 250, 500, 750 and 1000 µg/ml). Control cells were added to fresh medium without the extract. After incubation of the cell extracts MTT was added (to a final concentration of 5 mg/ml PBS) to each well, and the plate was incubated for 24 h at 37° C. Colored formazan crystals generated were dissolved with 150 ml of DMSO. The absorbance was measured at 570 nm on a microplate reader. The percentage of viable cells was determined as the ratio of absorbance of treated cells and control cells multiplied by 100. Experiments as those using MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] are based on the ability of the viable cells to dissolve the tetrazolium salt. IC_{50} concentration was defined as the concentration of an agent inhibiting cell survival by 50%, compared with a vehicle-treated control. The results of the measurements were expressed as the percentage of positive control growth taking the Cis-DDP used in positive control wells as 100 % growth [31-33]. All experiments were done in triplicate.

Statistical analysis

The results were subjected to a one-factor analysis of variance (extracts), and the significance of differences was computed by the LSD test. The results of antioxidant activity are presented as means ± standard deviations of three analytical determinations.

RESULTS

The results of total phenolics, flavonoids and tannin content of various extracts of the plant *E. italicum* are presented in Table 1. The phenolic contents in the ethanol (109.15±0.51 mg GA/g), chloroform (105.22±0.07 mg GA/g) and acetone extract (104.39±0.18 mg GA/g) were higher than the phenolic contents in the ethyl acetate and petroleum extract. Statistically significant differences in the concentrations of total phenolics are between chloroform and ethanol extracts in comparison to the ethyl acetate and petroleum ether extracts. The highest flavonoid contents were in the ethanol (25.16±0.19 mg RU/g) and petroleum ether extracts (25.03±0.29 mg RU/g). Statistically significant differences in the concentrations of flavonoids were between petroleum ether and ethanol extracts in comparison to the chloroform extract. The tannin contents ranged from 75.44±0.26 to 79.60±0.18 mg GA/g in the chloroform extract (79.60±0.18 mg GA/g) and the acetone extract (79.43±0.20 mg GA/g) had the highest value of tannins. Statistically significant differences in the concentrations of tannins were between chloroform and acetone extracts in comparison to the other examined extracts.

Table 1. Total phenolics, flavonoids and tannins content in the various extracts of the plant *E. italicum*

Extracts of *E. italicum*	T. phenolics (mgGA/g)	Flavonoids (mgRU/g)	Tannins (mgGA/g)
Chloroform	105.22±0.07b	23.20±0.51b	79.60±0.18a
Et. acetate	97.10±0.25c	24.09±0.60ab	78.18±0.38b
Ethanol	109.15±0.51a	25.16±0.19a	75.97±0.34c
Acetone	104.39±0.18b	24.11±0.06ab	79.43±0.20a
Petr. ether	93.02±0.38d	25.03±0.29a	75.44±0.26c

Statistically significant difference for the level of 0.05% is shown by the letter next to the concentration value (compared to extract employed). With the same letters that difference is not significant. If the letters are different, the difference is significant. Several methods were used to determine the antioxidant activity of the tested extracts - Table 2. The values of total antioxidant capacity ranged from 86.09±1.40 μg AA/g to 97.31±0.69 μg AA/g, and ethanol extract (97.31±0.69 μg AA/g) and chloroform extract (95.22±0.78 μg AA/g) had the highest values of total antioxidant capacity. The acetone extract (42.54±1.13 µg/ml IC_{50}) and chloroform extract (43.29±1.20 µg/ml IC_{50}) displayed the highest lipid peroxidation inhibition. The ethanol extract (61.55±0.79 µg/ml IC_{50}) and the acetone extract (62.15±0.27 µg/ml IC_{50}) had the highest hydroxyl radical scavenging activity. The acetone extract had the highest DPPH scavenging activity (62.46±0.66 µg/ml IC_{50}).

HPLC analysis of the phenolic components from various extract of the plant *E. italicum* enabled the identification of 9 compounds (Table 3). The petroleum ether extract had the highest number of identified compounds, followed by the acetone extract, the ethanol extract, the ethyl acetate extract and the chloroform extract. Among the identified constituents, rosmarinic acid, chlorogenic acid, *p*-hydroxybenzoic acid and rutin were found as major components in the tested extracts.

Table 2. Total antioxidant capacity, inhibitory activity against lipid peroxidation, hydroxyl radical and DPPH scavenging activity of the plant *E. italicum*

Extracts of *E. italicum*	Total antioxidant capacity (μg AA/g)	Inhibition activity against lipid peroxidation (μg/ml)	Hydroxyl radical scavenging activity (μg/ml)	DPPH (μg/ml)
Chloroform	95.22±0.78	43.29±1.20	63.00±0.98	65.07±1.02
Et. acetate	93.12±0.55	47.26±1.12	65.37±0.68	66.00±0.92
Ethanol	97.31±0.69	44.56±1.29	61.55±0.79	63.59±0.48
Acetone	94.15±0.78	42.54±1.13	62.15±0.27	62.46±0.66
Petr. ether	86.09±1.40	49.36±1.10	70.16±0.78	71.79±0.89
Gallic acid	-	255.43±11.68	59.14±1.10	3.79±0.69
Ascorb.acid	-	> 1000	160.55±2.31	6.05±0.34
BHT	-	1.00±0.23	33.92±0.79	15.61±1.26
α-Tocopherol	-	0.48±0.05	-	-

Table 3. HPLC analysis of the plant *E. italicum*

Compound/ Sample	Petroleum ether (mg/g)	Chloroform (mg/g)	Acetone (mg/g)	Ethyl acetate (mg/g)	Ethanol (mg/g)
Protocatehuic acid					
p-Hydroxybenzoic acid	9.143	1.468	4.865		1.930
Caffeic acid					
Vanillic acid					
Chlorogenic acid	10.776		1.915		1.293
Syringic acid					
p-Coumaric acid					
Ferulic acid	0.928	0.408	0.519		0.346
Synapic acid					
Rutin	6.024	6.080	4.926	9.091	10.739
Luteolin-glycine	2.542		0.748		0.626
Apigenin-glycine					
Rosmarinic acid	44.134	1.632	12.131	2.494	8.452
Quercetin	0.887	0.581	1.006	0.879	0.902
Luteolin					
Naringenin		0.244	0.371		0.411
Caempherol	0.729	0.287	0.985	0.919	0.927
Apigenin					
Σ	75.163	10.700	27.466	13.383	25.626

Antimicrobial activity was tested using the broth dilution procedure for determination of minimum inhibitory concentration (MIC). MICs were determined against seventeen strains of bacteria, and the antifungal activity was tested against *Aspergillus niger* and *Candida albicans* - Table 4. The tested extracts showed strong antimicrobial activity against Gramm-positive, Gramm-negative bacteria and fungi. MIC of the ethyl acetate extract for *Escherichia coli* ATCC 8739, *Proteus vulgaris* ATCC 13315, *Citrobacter freundii* ATCC 43864 were 3.91 μg/ ml, and for *Listeria inocuu* ATCC 33090, *Listeria monocytogenes* ATCC 19112, *Enterococcus faecium* ATCC 6057 and *Aspergillus niger* ATCC 16404 were 7.81 μg/ ml. MICs of the petroleum ether extract were 3.91 μg/ ml for *Proteus mirabilis* ATCC 35659, *Enterobacter aerogenes* ATCC 13048 and *Aspergillus niger* ATCC 16404, and MIC for *Listeria ivanovii* ATCC 19119, *Enterococcus faecalis* ATCC 29212 and *Staphylococcus saprophiticus* ATCC 15035 were 7.81 μg/ml. MIC of the acetone extract were 3.91 μg/ml for *Salmonella enteritidis* ATCC 13076, and 7.81 μg/ml for *Escherichia coli* ATCC 8739, *Klebsiella pneumoniae* ATCC 13883, *Proteus vulgaris* ATCC 13315, *Enterococcus faecium* ATCC 6057 and *Candida albicans* ATCC 10231. MIC of ethanol extract were 3.91 μg/ml for *Salmonella Typhimurium* ATCC 14028 and *Staphylococcus aureus* ATCC 25923, and for

Table 4. MIC/MBC values (μg/ ml) of *E. italicum* plant extracts

Bacteria	MIC/MBC (μg/ ml)						
	Ethanol extract	Ethyl acetate extract	Chloroform extract	Petroleum ether extract	Acetone extract	Amracin	Ketoconazole
Proteus mirabilis	250.00/500.00	125.00/250.00	7.81/15.625	3.91/7.82	62.5/125.00	0.49	-
Escherichia coli	62.5/125.00	3.91/7.82	15.625/31.25	31.25/62.5	7.81/15.625	0.97	-
Klebsiella pneumoniae	125.00/250.00	31.25/62.5	3.91/7.82	62.5/125.00	7.81/15.625	0.49	-
Proteus vulgaris	250.00/500.00	3.91/7.82	125.00/250.00	62.5/125.00	7.81/15.625	0.49	-
Salmonella enteritidis	7.81//15.625	31.25/62.5	62.5/125.00	125.00/250.00	3.91/7.82	0.49	-
Enterobacter aerogenes	15.625/31.25	62.5/125.00	31.25/62.5	3.91/7.82	125.00/250.00	0.97	-
Citrobacter freundii	31.25/62.5	3.91/7.82	125.00/250.00	62.5/125.00	500.00/1000	0.49	-
Salmonella Typhimurium	3.91/7.82	125.00/250.00	7.81/15.625	31.25/62.5	125.00/250.00	0.49	-
Pseudomonas aeruginosa	7.81/15.625	62.5/125.00	3.91/7.82	125.00/250.00	250.00/500.00	0.97	-
Listeria ivanovii	125.00/250.00	31.25/62.5	250.00/500.00	7.81/15.625	62.5/125.00	0.97	-
Listeria inocu	31.25/62.5	7.81/15.625	125.00/250.00	500.00/1000	62.5/125.00	0.49	-
Enterococcus faecalis	125.00/250.00	31.25/62.5	250.00/500.00	7.81/15.625	62.5/125.00	0.97	-
Listeria monocytogenes	31.25/62.5	7.81/15.625	125.00/250.00	500.00/1000	62.5/125.00	0.97	-
Bacillus spieizenii	7.81/15.625	31.25/62.5	125.00/250.00	62.5/125.00	31.25/62.5	0.97	-
Enterococcus faecium	62.5/125.00	7.81/15.625	31.25/62.5	15.625/31.25	7.81/15.625	0.49	-
Staphylococcus aureus	3.91/7.82	62.5/125.00	7.81/15.625	125.00/250.00	15.625/31.25	0.97	-
Staphylococcus saprophiticus	62.5/125.00	125.00/250.00	31.25/62.5	7.81/15.625	31.25/62.5	0.97	-
Aspergillus niger	31.25/62.5	7.81/15.625	15.625/31.25	3.91/7.82	62.5/125.00	-	0.97
Candida albicans	15.625/31.25	31.25/62.5	500.00/1000	31.25/62.5	7.81/15.625	-	1.95

Salmonella enteritidis ATCC 13076, *Pseudomonas aeruginosa* ATCC 27853, *Bacillus spieizenii* ATCC 6633 MIC values were 7.81 μg/ml. MIC of chloroform extract were 3.91 μg/ml for *Klebsiella pneumoniae* ATCC 13883 and *Pseudomonas aeruginosa* ATCC 27853, and 7.81 μg/ml for *Proteus mirabilis* ATCC 35659, *Salmonella Typhimurium* ATCC 14028 and *Staphylococcus aureus* ATCC 25923. The results of MBC are shown in Table 4. The MBC values ranged from 7.82 μg/ml to 1000 μg/ml. The lowest MBC values 7.82 μg/ml had the ethyl acetate extract for *Escherichia coli* ATCC 8739, *Proteus vulgaris* ATCC 13315, *Citrobacter freundii* ATCC 43864, the petroleum ether extract for *Proteus mirabilis* ATCC 35659, *Enterobacter aerogenes* ATCC 13048 and *Aspergillus niger* ATCC 16404, the acetone extract for *Salmonella enteritidis* ATCC 13076, the ethanol extract for *Salmonella Typhimurium* ATCC 14028 and *Staphylococcus aureus* ATCC 25923, and chloroform extract for *Klebsiella pneumoniae* ATCC 13883 and *Pseudomonas aeruginosa* ATCC 27853.

Determinations of cytotoxic activity were done according to the MTT assay on the three cell lines: cell line derived from human rhabdomyosarcom (RD), cell line derived from human cervix carcinom (Hep2c) and cell line from mouse fibroblast carcinom (L2OB) - Table 5. The IC_{50} values of cytotoxic activity of the tested extracts ranged from 87.30 ±4.09 μg/ml to 172.52±2.44 μg/ml. The chloroform (87.30±4.09 μg/ml) and acetone extracts of plant (91.56±2.31 μg/ml) showed the best cytotoxic activity on L2OB cells. The ethanol and acetone plant extracts significantly influenced Hep 2c, the petroleum ether extract significantly influenced RD cells and the chloroform and acetone extracts significantly influenced L2OB cells.

Table 5. Cytotoxic activities (μg/ml) of *Echium italicum* plant extracts

Extract	Hep 2c	RD	L2OB
Chloroform	129.70±2.04b	161.56±0.43b	87.30±4.09c
Et. acetate	158.42±0.36a	173.61±1.30a	137.36±0.53b
Ethanol	121.48±0.85c	172.52±2.44a	133.20±0.04b
Acetone	122.37±1.47c	162.35±0.28b	91.56±2.31c
Petr. ether	162.80±2.14a	106.59±1.21c	146.33±0.76a

DISCUSSION

Phenolic compounds are a major class of plant secondary metabolites. These compounds represent an important component of human diets and exhibit a wide range of biological effects, including antioxidant, antimutagenic, and anticarcinogenic effects. The phenolic contents in the ethanol, chloroform and acetone extracts were higher than in the ethyl acetate and petroleum extracts. The highest total phenolic contents of the plant *E. italicum* were found in the ethanol extract. The highest flavonoid content was in the ethanol extract and the highest tannin contents in the chloroform and acetone extracts. The study of total phenolic and flavonoid contents obtained from the ethanol extract of roots and herbs of *E. italicum* L. [34] showed a lower phenolics content and a higher flavonoid content than our study. This can be related to the type of solvent and the period of collecting the plants (in June), which is consistent with the results obtained by Meddini [35] that at the flowering stage the plant has a higher level of phenolic compounds than at the vegetative stage. Bano *et al*. [36] have shown that the amount of polyphenols in plants and their antioxidant activities depend on both biological factors (genotype, organ andontogeny) and edaphic and environmental (temperature, salinity, water stress and light intensity) conditions. The solubility of phenolic compounds is governed by the type of solvent (polarity) used, the degree of polymerization of phenols and their interaction [37]. The content of total phenolics is in positive correlation with the total antioxidant capacity. These results were consistent with the findings of various research groups, who reported positive correlations between total phenolic content and antioxidant activity [38, 39].

The acetone extract displayed the highest lipid peroxidation inhibition, followed by the chloroform and ethanol extracts. The results of hydroxyl radical scavenging activity of the various extracts of the plant *E. italicum* showed that the ethanol extract had the highest IC_{50} value, followed by the acetone extract and the chloroform extract. The DPPH scavenging activity in the various extracts of the plant *E. italicum* showed that the acetone extract had the highest activity IC_{50}, followed by the ethanol extract and chloroform extract. Previous studies showed that concentration-response is related to the DPPH scavenging activity, and an increase in the concentration of extract is synonymous with an increase in scavenging capacity [34]. Many researchers reported an influence of different extraction techniques on the content of natural antioxidants in the extracts [40, 41]. Efficiency of solvents and methods is strongly dependent on the plant matrix used [41-43]. Solvents such as methanol, ethanol, acetone, propanol and ethyl acetate have been commonly used for the extraction of phenolics from fresh products [42, 43].

HPLC analysis of the phenolic components from various extracts of the plant *E. italicum* enabled the identification of 9 compounds. Among the identified constituents, rosmarinic acid, chlorogenic acid, *p*-hydroxybenzoic acid and rutin were determined as major components in the tested extracts. Rosmarinic acid and rutin have been reported to have strong antioxidant properties and also antidiabetic, antithrombotic, antiinflammatory and anticarcinogenic activity [44, 45]. Bouayed *et al*. [46] have found that chlorogenic acid has anxiolytic and antioxidant activity.

The antibacterial activity of plants is continuously attracting global attention [47]. The antimicrobial activity may be due to the presence of antioxidants in the extracts that have the potential to prevent the activity of free radicals and reactive oxygen species thus helping in fighting diseases caused by bacteria and other pathogens [48, 49]. All extracts expressed a certain level of antimicrobial activity with MIC values in the range from 3.91 µg/ml to 500 µg/ml, which is considered to be very good compared to standard antibiotics amracin (for bacteria) and ketoconazole (for fungi). The ethyl acetate extract dieplayed the greatest antimicrobial activity against *Escherichia coli*, *Proteus vulgaris*, *Citrobacter freundii*, the petroleum ether extract - against *Proteus mirabilis*, *Enterobacter aerogenes* and *Aspergillus niger*, the acetone extract - against *Salmonella enteritidis*, the ethanol extract - against *Salmonella Typhimurium* and *Staphylococcus aureus*, the chloroform extract - against *Klebsiella pneumoniae* and *Pseudomonas aeruginosa*. Overall, the antibacterial activity of the tested samples was noticeably higher against the growth of Gram-negative bacteria strains compared to Gram-positive bacteria strains. The antimicrobial activity of the *E. italicum* oil was studied against *Bacillus subtilis*, *Staphylococcus aureus*, *Escherichia coli*, *Salmonella Typhimurium*, *Pseudomonas aeruginosa*, *Aspergillus niger* and *Candida albicans* and showed a pronounced concentration dependence on all microorganisms tested [21]. The MBC values ranged from 7.82 µg/ml to 1000 µg/ml. Antimicrobial activity of the plant extracts might be explained by the synergistic or additive effects of several phytochemicals rather than arising from a

single compound. Different bioactive compounds in a mixture can interact to provide a combined effect which is similar to the sum of the effects of the individual components (additive), or greater than the sum of the individual components (synergistic). The mechanisms of action of each phenolic compound against various bacteria are also very complicated [50, 51]. Therefore, it is necessary to further investigate and understand the relationship between antibacterial activity and chemical structure of each phenolic compound in the tested extracts.

Cancer development, a dynamic and long-term process, involves many complex factors with stepwise progression ultimately leading to an uncontrolled spreading and growth of cancerous cells throughout the body, called metastasis. Laboratory research has further demonstrated that a number of bioactive dietary components, collectively referred to as natural products, have the ability to prevent cancer [52] and other chronic diseases [53, 54]. Results obtained in the present study show that all examined samples exhibit *in vitro* cytotoxic activity against the target cells. Phytochemical analysis of plant extracts of *Echium italicum* showed the presence of a large number of bioactive phenolic components (chlorogenic acid, *p*-hydroxybenzoic acid, naringenin, kaempferol, lutein), and numerous literature data suggest their anticancer effect [55-63]. Biological activity of the plant extracts from the family *Boraginaceae* growing in Serbia has been confirmed by other authors [64, 65].

CONCLUSIONS

The results of this investigation which determined the phytochemical composition and biological activity of five different extracts *of E. italicum* L., demonstrate that these might be proposed as antioxidant dietary supplements for the prevention and/or treatment of conditions that occur due to oxidative damage and can protect DNA damage by hydroxyl radicals. The plant has got a broad spectrum of antimicrobial and biological activity and could be a potential alternative for treating various diseases.

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ФИТО-ХИМИЧЕН СЪСТАВ И БИОЛОГИЧНА АКТИВНОСТ НА ЕКСТРАКТИ ОТ Echium italicum L.

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(Резюме)

Целта на тази работа е да се оцени биологичната активност на пет различни екстракта от растението Echium italicum L. и да се определи тяхния фито-химичен състав. Изпитани са хлроформ, етилацетат, етанол, ацетон и петролев етер като разтворители. Спиртният екстракт от E. italicum е с най-високо съдържание на общи феноли и флавоноиди, докато екстрактите в хлороформ и ацетон имат най-много танинови вещества. Няколко различни метода са използвани за определянето на анти-оксидантна активност на изпитваните екстракти, като тези в етанол и ацетон показват най-висока активност. HPLC-анализът показва, че основните фенолни съединения в изпитваните екстракти са розмаринова, хлорогенова, p-хидроксибензоена киселини и рутин. Определянето на анти-микробната им активност е извършено по метода на микроразрежданията. Резултатите от от микроразрежданията са в граници от 3.91 до 500 μg/ml. Определянето на цито-токсичната активност са извършено по метода MTT върху човешки клетки на рабдомиосаркома (RD), клетъчна линия от човешки карцином на маточната шийка (Hep2c) и клетъчна линия от карцином на миши фибропласти (L2OB). Изследването внушава, че изследваните екстракти от растението E. italicum L. Може да послужат като източници на антиоксиданти и антибиотични агенти.