A Study on the Ethanolic Extract of *Onosma aucheriana*
Biological and toxicological evaluation

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This research studies the antioxidant activity and efficacy of the ethanolic extract of the plant species Onosma aucheriana DC. This plant growing wild in Serbia, in inhibiting the development of selected fungi and bacteria. The most sensitive to the ethanolic extracts were bacteria B. subtilis and S. aureus (MIC = 15.62 μg/mL), while the fungi, A. niger (MIC = 15.62 μg/mL) showed the highest susceptibility. Total phenolic, flavonoid, condensed tannin and gallotannin contents were 90.26 mg GA/g, 35.24 mg RU/g, 74.65 mg GA/g and 31.74 mg GA/g, respectively. Phenolic compounds are found as dominant in the extract of rosmarinic acid. Total antioxidant capacity was 78.45 μg AA/g. IC_{50} values were determined for each measurement: 21.45 μg/mL for DPPH free radical scavenging activity, 36.46 μg/mL for inhibitory activity against lipid peroxidation, 99.11 μg/mL for hydroxyl radical scavenging activity and 45.91 μg/mL for chelating ability. A potent inhibitor of cell growth to all three cell lines (Hep2c, RD, L2OB) is the ethanol extract of plant species O. aucheriana . Results of Allium anaphase-telophase genotoxicity assay revealed that the ethanolic extract of O. aucheriana at concentrations of 62.5 μg/mL does not produce toxic or genotoxic effects.

Keywords: antimicrobial activity, antioxidant activity, genotoxic examination, cytotoxic activity, Onosma aucheriana DC., HPLC analysis, phenolic compounds

For the treatment of many diseases but also for other reasons people use medicinal plant. Plants contain many biologically active natural products. This products are all biodegradable and renewable. Screening less-studied plant species for the presence of substances with potential medicinal significance is important. With their environment, plants communicate by producing a diverse range of chemicals. A common feature of specific plants and plant families are these secondary metabolites. Many plant extracts and secondary metabolites have antimicrobial properties. These antimicrobial properties make plant products successful in the treatment of fungal, bacterial and viral infections [1]. A number of diseases can be treated effectively by using different parts of plants. Syntethic antioxidants like butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA), that are known to terminate the chain raction of lipid peroxidation, have also been proven to cause liver damage and that they are carcinogenic. Bacterial resistance to a large number of antibiotics and the ability of the plants to synthesize biologically active substances are the cause of the increasing importance given tot, he use of plant-derived products in bacterial control. Plant use in the food industry has increased significantly over the last years to replace synthetic preservatives, antioxidants or other food additives [2]. Because of the content of phenolic compounds many herb species are active antioxidants[3-5]. Phenoloic compounds are pervasive in plants. For normal plant development and growth and defence against infection and injury, flavonoids and other plant phenolics, such as phenolic acids, stilbenes, tannins, lignans, and lignins are very important. Secondary metabolites such as alkaloids, polyphenols, naphtho-quinones, phytosterols and terpenoids are produced by many members of the Boraginaceae family [6, 7]. Anti-inflammatory, anti-viral and anti-bacterial activities of polyphenols, including flavonoids and phenolic acids [8, 9], among other pharmaceutical activities found in Boraginaceae, make them interesting for further research. With it's cosmopolitan distrubution, the Boraginaceae family consists of 117 genera and about 2400 species. *Onosma* L. includes about 150 species distributed in Asia, Eurasia, Mediterranean regions and Europe. *Onosma aucheriana* DC., is a plant with woody branching rhizome, and whitish leaves covered with dense bristly hairs [10-12]. Studies on the biological activity or chemical constituents of *O. aucheriana* have been reported in the [13]. Aqueous, extracts from Auchers golden-drop (*Onosma aucheriana*) from Lebanon were investigated for their *in vitro* immunomodulatory and antileishmanial activities as compared to their toxicity against human cells and exhibited interesting antileishmanial activities on the intracellular amastigote form of the parasite (IC_{50}=5.1 μg/mL; SI>49), while several extracts were shown to induce

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nitrous oxide (NO) production by human macrophages[14]. These studies determined the content of phenol, flavonoid, condensed tannin and gallotanins contents of *O. aucheriana*, growing wild in Serbia and their antioxidant, cytotoxic, genotoxic and antimicrobial activities.

Experimental part

Materials and methods

Chemicals used

All standards for HPLC analysis were of analytical grade and were purchased from Sigma Chemical Co. (St Louis, MQ, USA) and Alfa Aesar (Karlsruhe, Germany). Acetonitrile and phosphoric acid were of HPLC grade (Tedia Company, USA). Ethanol was of analytical grade (Aldrich Chemical Co., Steinheim, Germany).

Spectrophotometric measurements

Spectrophotometric measurements were performed using a UV-VIS spectrophotometer MA9523-SPEKOL 211 (ISKRA, Horjul, Slovenia).

Plant material

Aerial parts of O. aucheriana were collected at the foot of Mt. Kablar, Ovcar Banja (Central Serbia) in May/June 2008. Voucher specimens (16436 BEOU, Lakusic Dmitar) were confirmed and deposited at the Herbarium of the Department of Botany, Faculty of Biology, University of Belgrade.

Extract preparation

By a cylindrical crusher the air-dried aerial parts of the plant (90 g) were broken into small pieces, and extracted with ethanol (99.8%) using a Soxhlet apparatus. Through filter paper the ethanolic extract was filtered (Whatman, No.1) and concentrated to dry mass (6.51 g). The residues were stored in a dark glass bottle for further processing.

Minimum inhibitory concentration (MIC)

By microdilution method in 96 multi-well microtiter plates [15], minimum inhibitory concentrations (MIC) of the extract and cirsimarin against the test bacteria were determined. All tests were performed in Muller–Hinton broth (MHB) with the exception of the yeast, in which case Sabouraud dextrose broth was used. A volume of 100 µL stock solutions of oil (in methanol, 200 µL/mL) and cirsimarin (in 10 % DMSO, 2 mg/mL) was pipetted into the first row of the plate. Fifty µL of Mueller Hinton or Sabouraud dextrose broth (supplemented with Tween 80 at a final concentration of 0.5% (v/v) for analysis of oil) were added to the other wells. A volume of 50 µL from the first test wells were pipetted into the second well of each microtiter line, and then 50 µL of scalar dilution were transferred from the second to the twelfth well. Ten µL of resazurin indicator solution (prepared by dissolution of a 270-mg tablet in 40 mL of sterile distilled water) and 30 µL of nutrient broth were added to each well. Finally, 10 µL of bacterial suspension (10^6 CFU/mL) and yeast spore suspension (3×10^4 CFU /mL) were added to each well. The growth conditions and the sterility of the medium were checked, for each strain. To control the sensitivity of the tested bacteria was used standard antibiotic amracin. Nystatin was used as control against the tested yeast. Plates were wrapped loosely with cling film to ensure that bacteria did not become dehydrated and prepared in triplicate, and then they were placed in an incubator at 37 °C for 24 h for the bacteria and at 28 °C for 48 h for the yeast and color change was assessed visually. Any color change from purple to pink or colorless was recorded as positive. The lowest concentration at which color change occurred was taken as the MIC value. The average of 3 values was calculated, and the obtained value was taken as the MIC for the tested compounds and standard drug.

Determination of total phenolic and flavonoid content

According to the Folin-Ciocalteu method were estimated total phenols [16]. The absorbance was measured at 765 nm using a spectrophotometer against a blank sample. As gallic acid equivalents (mg GA/g extract), were determined total phenols. Total flavonoids were determined according to Brighente method [17]. As rutin equivalents (mg RU/g dry extract) were determined total flavonoides.

Determination of condensed tannins and gallotannins

The method for determination of condensed tannins relies on the precipitation of proanthocyanidins with formaldehyde[18]. The concentration of condensed tannins was calculated as residuum of the total phenolic and unprecipitated phenol concentrations, and expressed as gallic acid equivalents. Gallotannins are hydrosoluble tannins containing a gallic acid residue esterified to a polyol. Gallotannins can be detected quantitatively by the potassium iodate assay. On the reaction of potassium iodate (KIO_3) with galloyl esters [18], which will form a red intermediate and ultimately a yellow compound, is based this assay. The concentration of the red intermediate can be measured spectrophotometrically at 550 nm. Using gallic acid as standard was determined gallotannin content.

Determination of total antioxidant capacity

By the phosphomolybdenum method [19] was evaluated the total antioxidant activity of the ethanolic extract of *O. aucheriana.* Then, the absorbance of the solution was measured at 695 nm using spectrophotometer against the blank after cooling to room temperature. As the blank, was used methanol. As the standard and total antioxidant capacity was used ascorbic acid (AA).

Determination of DPPH free radical scavenging activity

The method used by [20] was adopted with suitable modifications from [21]. As reference standards were used: ascorbic acid (AA), gallic acid (GA) and butylated hydroxytoluene (BHT) and dissolved in methanol to make the stock solution with the same concentration. Control sample was prepared containing the same volume without test compounds or reference antioxidants. Ninety-five percent methanol was used as a blank. The DPPH free radical scavenging activity (%) was calculated.

The IC_{50} value, defined as the concentration of the test material that leads to 50% reduction in the free radical concentration, was calculated as µg/mL through a sigmoidal dose-response curve.

Determination of inhibitory activity against lipid peroxidation

By the thiocyanate method [22] was determined antioxidant activity. Ascorbic acid, gallic acid, α-tocopherol and BHT were used as reference compounds. To eliminate the solvent effect, the control sample, which contained the same amount of solvent added to the linoleic acid emulsion in the test sample and reference compound, was used. Inhibition percent of linoleic acid peroxidation was calculated using the following same formula as that for DPPH free radical scavenging activity..

Determination of hydroxyl radical scavenging activity

The ability of *O. aucheriana* to inhibit non site-specific hydroxyl radical-mediated peroxidation was carried out according to the method described by [23]. The extent of oxidation of 2-deoxyribose was estimated from the absorbance of the solution at 532 nm. The percentage inhibition values were calculated from the absorbance of the control (Ac) and of the sample (As), where the controls contained all the reaction reagents except the extract or positive control substance. As the means of triplicate analyse are presented the values.

Measurement of cytotoxic activity by MTT assay

By MTT assay, the influence of *O. aucheriana* extract on growth of malignantly transformed cell lines was evaluated. The following cell lines have been used: RD (cell line derived from human rhabdomyosarcoma), Hep2c (cell line derived from human cervix carcinoma - HeLa derivative) and L2OB (cell line derived from murine fibroblast). Cells were seeded (2 x 10^5 cell/mL; 100 μL/well) in 96-well cell culture plates (NUNC) in nutrient medium (MEM Eagle supplemented with 5% (for Hep2c) or 10% (for RD and L2OB) FCS) and grown at 37°C in humidified atmosphere for 24 h. Then, corresponding extract (stock solution: 5 mg of extract dissolved in 1 mL of absolute ethanol) or absolute ethanol (control) diluted with nutrient medium to desired concentrations were added (100 μL/well) and cells were grown at 37°C in humidified atmosphere for 48 h. Positive control for each cell line were wells where 100 μL of pure nutrient medium were added. After incubation period, supernatants were discarded, MTT (stock solution: 5 mg/mL in PBS) dissolved in D-MEM medium to final concentration 500 μg/mL was added in each well (100 μL/well) and plates were incubated at 37°C in humidified atmosphere for 4h. Reactions were halted by the addition of 10% SDS/10 mM HCl (100 μL/well). After overnight incubation at 37°C, absorbances were measured at 580 nm using a spectrophotometer (Ascent 6-384 [Suomi], MTX Lab Systems Inc., Vienna, VA 22182, USA). The number of viable cells per well (NVC) was calculated from a standard curve drawn as cell numbers plotted against A_{580}. As standards were used, corresponding cells (grown in flasks), after cell count by haemocytometer. Standard suspensions were plated in serial dilution, centrifuged at 800 rpm for 10 min and then treated with MTT/D-MEM and 10% SDS/10 mM HCl solutions in the same way as the experimental wells (*ut supra*). The number of viable cells in each well was proportional to the intensity of the absorbance of light, which was then read in an ELISA plate reader at 580 nm. Absorbance (A) at 580 nm was measured 24 h later. To get cell survival (%), A of a sample with cells grown in the presence of various concentrations of the investigated extracts was divided with control optical density (the A of control cells grown only in nutrient medium), and multiplied by 100. It was implied that A of the blank was always subtracted from A of the corresponding sample with target cells. 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 are expressed as the percentage of positive control growth takingthe cis-diamminedichloroplatinum (cis-DDP) determined in posi-tive control wells as the 100% growth [24-26]. In triplicate werw done all experiments.

Genotoxic examination

According to the procedure of Fiskesjo [27], as modified by Rank and Nielsen [28], all samples werw investigated and recognized as the *Allium* anaphase-telophase genotoxicity assay. For the assay were used Onion bulbs of *Allium cepa*, weighing 2-4 grams, obtained from local companies. Prior to placement of the onions into the samples the yellow shallows and the dry centre of the primordia were cautiously removed. General toxicity and genotoxicity are two parts of the test procedure. The following concentrations of 500, 250, 125 and 62.5 μg/mL of ethanolic extracts of *O. aucheriana* samples (I, II, III and IV) were evaluated respectively. With the aim to see how the presence of different concentrations of ethanol influence test results, the same test procedure was independently applied for different concentrations of ethanol used as solvent for plant extracts (Ie - 0.250%, IIe - 0.125%, IIIe - 0.063% and IVe - 0.032%). For each sample, as well as the positive and negative controls, twelve onions were placed on test tubes filled with test liquids. Methyl methanesulfonate - MMS (final concentration 10 μg/L) was used as the positive control and commercial bottled water as the negative. Each day fresh test solution was added. For the first 24 h the onions were grown in fresh commercial bottled water and subsequently exposed for two days to the test samples. To evaluate toxicity, roots were cut off on the fourth day and the length of each root was measured to the nearest mm in all groups. Statistical significance of differences in root length was assessed using Tukey HSD for unequal N (Statistica 6.0). For genotoxicity evaluation, root tips were hydrolyzed in 1N HCl at 60 °C for 12 min. From each onion five apical parts of the root tips were placed on a slide, stained with 2% orcein and squashed in 45% acetic acid. The slides were coded and examined blind. Chromosome aberrations were scored only on slides with a mitotic index higher than 1%. One hundred or more mitoses per slide (only anaphase and telophase stages) were examined for five slides in each group. The following aberrations were scored: bridges, vagrant chromosomes, multipolarity, fragments and c-mitoses. A 2x2 contingency X^2 statistical test (Statistics 6.0) was used to determine the significance of differences between the analyzed groups and controls.

HPLC analysis

Quantification of individual phenolic compounds were performed by reversed phase HPLC analysis, using a modified method of Misan et al. [29]. HPLC analysis was performed by using a liquid chromatograph (Agilent 1200 series), equipped with a diode array detector (DAD), Chemstation Software (Agilent Technologies), a binary pump, an online vacuum degasser, an autosampler and a thermostated column compartment, on an Agilent, Zorbax Eclipse Plus-C18, 1.8μm, 600 bar, 2.1×50 mm column, at a fow-rate of 0.8 mL/min. Gradient elution was performed by varying the proportion of solvent A (methanol) to solvent B (1% formic acid in water (v/v)) as follows: initial 0-2 min, 100% B; 2-4 min, 100-98% B; 4-6 min, 98-95% B; 6-7 min, 95-73% B; 7-10 min, 75-48% B; 10-12 min 48% B; 12-20 min, 48-40% B. The total running time and post-running time were 21 and 5 min, respectively. The column temperature was 30°C. The injected volume of samples and standards was 5 μL and it was done automatically using autosampler. The spectra were acquired in the range 210–400 nm and chromatograms plotted at 280, 330 and 350 nm with a bandwidth of 4 nm, and with reference wavelength/bandwidth of 500/100 nm.

Statistical analysis

The results are presented as mean ± standard deviations of three determinations. Statistical analyses were performed using Student´s t-test and one way analysis of

variance. Multiple comparisons of means were done by LSD (least significant difference) test. A probability value of 0.05 was considered significant. All computations were made by employing the statistical software (SPSS, version 11.0). IC_{50} values were calculated by nonlinear regression analysis from the sigmoidal dose-response inhibition curve.

Results and discussions

Total phenolics, flavonoids, condensed tannins, gallotannins and total antioxidant capacity of the ethanolic extract of O. aucheriana

Phenolic compounds and flavonoids have been reported to be associated with antioxidant action in biological systems, mainly due to their red-ox properties, which can play an important role in absorbing and neutralizing free radicals, quenching singlet and triplet oxygen, or decomposing peroxides [30]. One of the more prominent properties of flavonoids is their excellent radical scavenging ability, which makes them valuable for therapeutic and prophylactic applications, e.g., after infection, inflammation, burns, or radiation injury [31]. The activity of crude methanol extracts is due to the presence of flavonoid monomers and polymers (condensed tannins), hydrolyzable tannins, and phenolics. Recently, polyphenolic compounds from plants such as condensed and hydrolyzable tannins have been shown to be powerful antioxidants [32]. Furthermore, tannins are reported to be 15-30 times more effective in quenching peroxyl radicals than simple phenolics. Therefore, tannins should be considered as important biological antioxidants [33]. The results on total phenolics, flavonoids, condensed tannin and gallotannin, and total antioxidant capacity are given in table 1. Total phenolic, flavonoid, condensed tannin and gallotannin contents were 90.26±0.69 mg GA/g, 35.24±0.55 mg RU/g, 74.65±0.75 mg GA/g and 31.74±1.05 mg GA/g, respectively. The results showed that the ethanolic extract of *O. aucheriana* possesses antioxidant activity, with total antioxidant capacity being 78.45±0.98µg AA/g. In research [13] following results were obtained: total phenolic 111.32 mg GA/g, flavonoids constituted almost half of the total phenols, condensed tannin 44.29 mg GAE/g, gallotannin 30.32 mg GAE/g. Ethanol extract of plant species *O. aucheriana* has less of quantity phenols and flavonoids, while the content of tannin and galotanina the ethanolic extract greater than in compared to the research [13].

Antioxidant activity

In edible and nonedible plants are commonly found phenolic compounds. They have been reported to have multiple biological effects, including antioxidant activity [34]. Various investigations implied that total phenolic compounds are closely related to antioxidant activity [35], with flavonoids and tannins being major plant compounds having antioxidant activity ([36]. IC_{50} values were determined for each measurement: 21.45±1.55 µg/mL for DPPH free radical scavenging, 36.46±1.68 µg/mL for inhibitory activity against lipid peroxidation, 99.11±0.23 µg/mL for hydroxyl radical scavenging activity and 45.91±0.88 µg/mL for chelating ability (table 2). Comparing the results with the research [13] can conclude that a higher DPPH free radical scavenging, hydroxyl radical scavenging activity and inhibitory activity against lipid peroxidation, has a water extract of plant species *O. aucheriana*. Generally, higher antioxidant activity has aqueous extract , compared to ethanolic extract of plant species *O. aucheriana*.

HPLC analysis of phenols

Characteristic phenolic component of the Boraginaceae family [37], are verified in rosmarinic acid. Therefore, we expected to identify primarily rosmarinic acid in the tested sample. HPLC method was used for identification and quantification of dominant metabolites in this plant. HPLC analysis showed rosmarinic acid (t_R = 14.17 min) to be the dominant component of the extract, its proportion being 4.561 mg/g extract. Besides rosmarinic acid, nine phenolic compounds (gallic acid **1**, protocatechuic acid **2**, p-hydroxybenzoic acid **3**, caffeic acid **4**, chlorogenic acid **5**, syringic acid **6**, p-coumaric acid **7**, ferulic acid **8** and quercetin **10**) were also identified in the extract by comparison of their retention times to those of standards (fig. 1).

A lower content was observed for caffeic acid (t_R = 3.250 min) and gallic acid (t_R =0.889), 2.862 mg/g extract and 1.96 mg/g extract, respectively (table 3). Rosmarinic acid has many biological activities, some of them including: adstringent, antioxidant, anti-inflammatory, antimutagen, antibacterial and antiviral activities [37].

Phenolic compounds such as rosmarinic acid can provide protection against cancer, and rosmarinic acid

Table 1

TOTAL PHENOLICS, FLAVONOIDS, CONDENSED TANNINS, GALLOTANNINS AND TOTAL ANTIOXIDANT CAPACITY OF THE ETHANOLIC EXTRACT OF *O. AUCHERIANA*

Total phenolics (mg GA/g)	Flavonoids (mg RU/g)	Condensed tannins (mg GA/g)	Gallotanins (mg GA/g)	Total antioxidant capacity (µg AA/g)
90.26±0.69	35.24±0.55	74.65±0.75	31.74±1.05	78.45±0.98

Table 2

ANTIOXIDANT ACTIVITY OF THE ETHANOLIC EXTRACT OF *O. AUCHERIANA*

Sample	*IC50 (µg/mL)			
	DPPH scavenging activity	Inhibitory activity against lipid peroxidation	Metal chelating activity	Hydroxyl radical scavenging activity
Onosma aucheriana	21.45±1.55*	36.46±1.68	45.91±0.88	99.11±0.23
Gallic acid	3.79±0.69	255.43±11.68	-	59.14±1.10
Ascorbic acid	6.05±0.34	> 1000	-	160.55±2.31
BHT	15.61±1.26	1.00±0.23	-	33.92±0.79
α-Tocopherol	-	0.48±0.05	-	-

*Results are mean values ± SD from three experiments

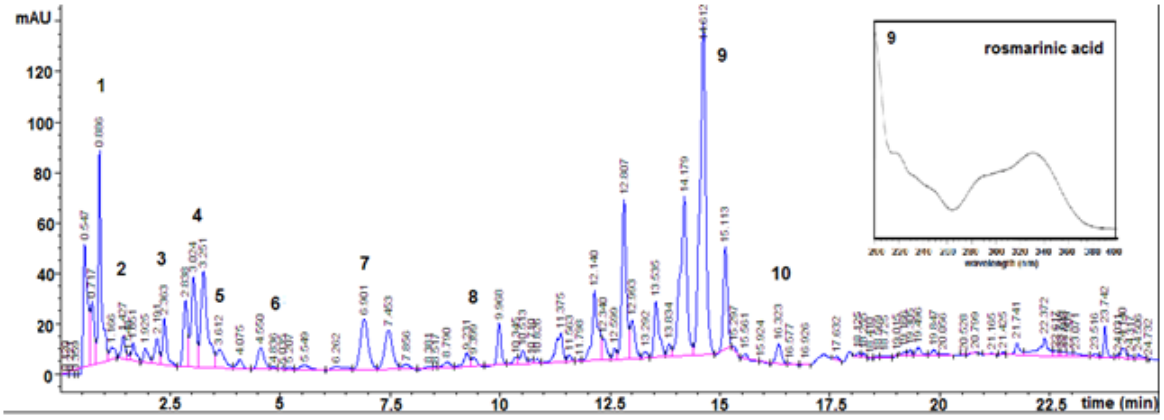

Fig. 1. HPLC chromatogram of the ethanolic extract of *Onosma aucheriana* DC. Peaks numbers corresponding to: 1, gallic acid, 2, protocatechuic acid, 3, p-hydroxybenzoic acid, 4, caffeic acid, 5, chlorogenic acid, 6, syringic acid, 7, p-coumaric acid, 8, ferulic acid, 9, rosmarinic acid and 10, quercetin

Table 3

RETENTION TIME (t_R) AND CONCENTRATION OF TEN STANDARDS PHENOLIC ACIDS AND FLAVONOID IN *O. AUCHERIANA* ETHANOLIC EXTRACT

Sample	Component Number	Component	t_R (min)	Concentration mg/g extract
Onosma aucheriana	1	Gallic acid	0.889	1.96
	2	Protocatechuic acid	1.428	0.2243
	3	p-Hydroxybenzoic acid	2.364	0.452
	4	Caffeic acid	3.250	2.862
	5	Chlorogenic acid	3.614	0.255
	6	Syringic acid	4.550	0.023
	7	p-Coumaric acid	6.901	0.282
	8	Ferulic acid	9.398	0.101
	9	Rosmarinic acid	14.172	4.561
	10	Quercetine	16.323	0.259

Table 4

MINIMUM INHIBITORY CONCENTRATIONS (MIC) OF THE ETHANOLIC EXTRACT OF *O. AUCHERIANA*

	MIC µg/mL		
Microbial strains	*Onosma aucheriana*	Amracin	Nystatin
Staphylococcus aureus ATCC 25923	15.62	0.97	/
Klebsiella pneumoniae ATCC 13883	31.25	0.49	/
Escherichia coli ATCC 25922	31.25	0.97	/
Proteus vulgaris ATCC 13315	62.50	0.49	/
Proteus mirabilis ATCC 14153	62.50	0.49	/
Bacillus subtilis ATCC 6633	15.62	0.24	/
Candida albicans ATCC 10231	31.25	/	1.95
Aspergillus niger ATCC 16404	15.62	/	0.97

contributes to the antioxidant activity of plants used in the cosmetic industry [37].

Anti-microbial activity

By the dilution method are given in table 4, obtained the results on antimicrobial activity. For 8 selected indicator strains were determined minimum inhibitory concentrations. The results presented in table 4 reveal antimicrobial activity of the ethanolic extract of O. aucheriana within the concentration range of 15.62 µg/mL to 62.50 µg/mL. The highest susceptibility to the ethanolic extract of O. aucheriana among the bacteria tested was exhibited by B. subtilis ATCC 6633 and S. aureus ATCC 25923 (MIC = 15.62 µg/mL), followed by strains of E. coli ATCC 25922 and K. pneumoniae ATCC 13883 (MIC = 31.25 µg/mL), and P. vulgaris ATCC 13315 and P. mirabilis ATCC 14153 (MIC = 62.50 µg/mL). Among the fungi, A. niger ATCC 16404 (MIC = 15.62 µg/mL) showed the highest susceptibility, and C. albicans ATCC 10231 (MIC = 31.25 µg/mL) the lowest.

Genotoxicity and general toxicity

The degree of toxicity of the analyzed samples was assessed from the mean root lengths expressed as a percentage of the mean root length of the negative control (fig. 2). Significant inhibition of growth compared to the negative control was observed only for plant extract sample I ($p<0.02$). Statistical analysis of aberration frequencies scored in anaphase and telophase showed that all samples differed significantly from the positive control. All samples, excepting plant extract sample IV significantly differ ($P<0.001$) from the negative control (fig. 3). This show that plant extract with lowest concentration does not have genotoxic potential. The relative number of total chromosome aberrations was the highest for plant extract sample I (12.89%) and the lowest for sample IV (table 5). Overall, the most frequent single aberrations in plant extracts were cells with multipolarity (39.1%),

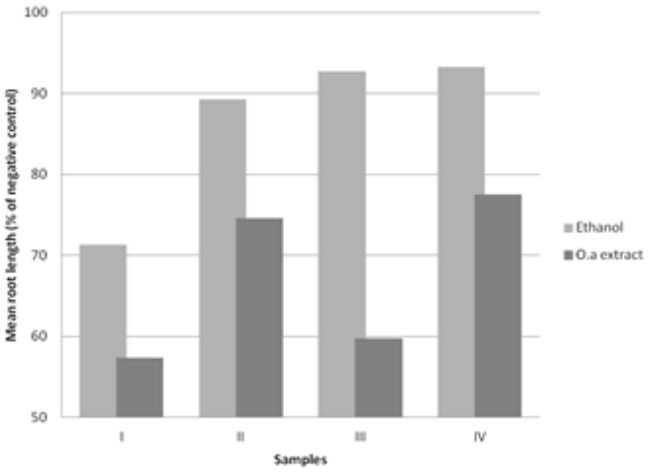

Fig. 2. Average mean root length in the test samples expressed as percentage of negative control

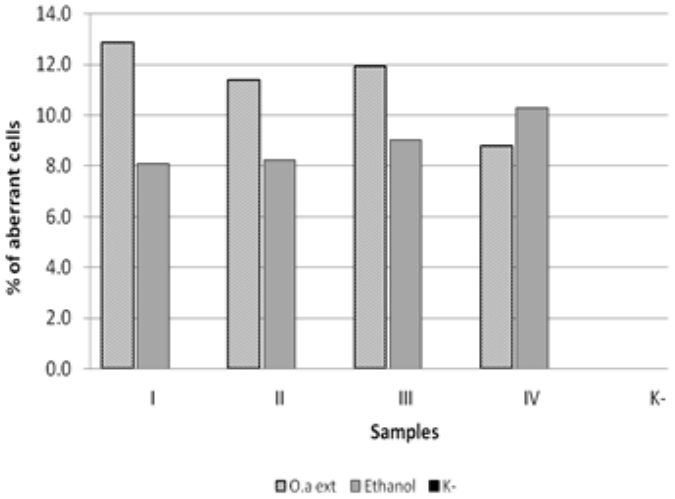

Fig. 3. Differences in frequency of aberrant cells in all analysed samples and negative control

followed by bridges (28.6.7%), and vagrant chromosomes (24.4%), while fragments and C-mitosis were found in less than 1.5% of aberrant cells. Multiple aberrations were found in 11.8% of aberrant cells. Two groups of aberrations were detected in the analyses. The first type was produced by spindle disturbance and it included vagrant chromosomes, multipolar configurations and C-mitoses, whereas the second type was produced by action on the chromosomes and it covered bridges and fragments. The first group of aberrations was much frequent among aberrant cells (63.5%), indicating that the tested samples were predominantly acting by disturbing the spindle. In tested samples of ethanol solutions the most frequent aberrations were bridges (32.8%), while fragments were present in less than 1%. In general, it could be concluded that ethanol in higher concentrations increase toxicity, while in all concentrations it slightly influence genotoxicity.

Cytotoxic activity

The tested extracts manifested a strong cytotoxic activity against target cells in vitro. The parameter used to compare the cytotoxic activity was the inhibition concentration at 50% inhibition (IC_{50}). A lower IC_{50} meant better cytotoxic activity. A significant inhibitory effect of the ethanolic extract of O. aucheriana on the growth/survival of the test cells was observed at concentrations above 25 μg/mL. RD cells were the least susceptible to the O. aucheriana extract, which led to 50% inhibition of their growth only at a concentration of 50 μg/mL. As regards Hep2c and L2OB cells, a reduction in cell number by more than 50% compared to the corresponding positive controls was observed after treatment with the ethanolic extract of O. aucheriana at concentrations above 41 and 20 μg/mL. The extract proved to be a potent growth inhibitor of all three cell lines. Microscopic analysis showed a marked cytolytic effect of the ethanol extract of this plant used at somewhat higher concentrations (>100μg/mL) on the three test cell lines, with the IC_{50} value being < 30 μg/mL. According to the American National Cancer Institute (NCI), the criterion of cytotoxic activity for plant extracts is IC_{50} < 30 μg/mL[38]. Table 6. gives IC_{50} (μM) for 48 h activity of the tested extracts on Hep2c, RD and L2OB cells, as determined by MTT assay.

Table 5

NUMBER OF DIFFERENT TYPES OF ABERRATIONS IN ROOT TIP CELLS OF ONIONS EXPOSED TO SAMPLES OF PLANT EXTRACT AT DIFFERENT CONCENTRATIONS (I-IV), ETHANOL SAMPLES OF DIFFERENT PERCENT (Ie-IVe), NEGATIVE (K-) AND POSITIVE CONTROL (K+). BR – BRIDGES; FR – FRAGMENTS; Vch – VAGRANT CHROMOSOMES; MP – MULTIPOLARITY; Cm – C-MITOSIS; MA – CELLS WITH MULTIPLE ABERRATIONS

Sample	BR	FR	Vch	MP	Cm	MA	Total number of analyzed cells	Aberrant cells (%)
I	17	3	21	29	1	3	574	12.89
II	21	-	15	21	-	11	589	11.40
III	27	-	18	19	-	6	586	11.95
IV	10	-	10	20	-	11	580	8.79
Ie	14	1	18	11	-	4	583	8.23
IIe	22	1	14	13	-	3	585	9.01
IIIe	22	-	17	13	-	6	562	10.30
IVe	9	-	11	25		1	568	8.10
K-	17	-	7	8	-	4	564	6.38
K+	21	3	14	32	3	50	538	22.86

Table 6

IC_{50} (μM) FOR 48 h ACTIVITY OF THE TESTED EXTRACTS ON Hep2c, *RD* AND *L2OB* CELLS, AS DETERMINED BY MTT ASSAY

Cell line	IC_{50} values ($\mu g/mL$)	
	Onosma aucheriana	cis-DDP***
Hep2c cells[a]	38.96 ± 0.38	0.94 ± 0.55
RD cells[b]	41.22 ± 0.84	1.4 ± 0.97
L2OB cells[c]	29.09 ± 0.53	0.72 ± 0.64

** Mean value ±2SD.
***Cis-diammine dichloroplatinum.
[a]Cell line derived from human cervix carcinoma.
[b]Cell line derived from human rhabdomyosarcoma.
[c]Cell line derived from murine fibroblast.

Conclusions

Of great interest in both fundamental science and alternative medicine are antioxidant and antimicrobial properties of various extracts of many plants, since their potential use as natural extracts has emerged from a growing tendency to replace synthetic antioxidants by natural ones. This research confirmed the antimicrobial and antioxidant activities of the ethanolic extract of O. aucheriana, growing wild in Serbia. Determination of polyphenolic components by HPLC analysis revealed the presence of high amounts of rosmarinic acid responsible for the reported antimicrobial activity of O. aucheriana. The extract of O. aucheriana shows antimicrobial activity under in vitro conditions against the tested bacteria and fungi as well as antioxidant activity relative to the control antioxidants. Genotoxicity assay revealed that the ethanolic extract of O. aucheriana at concentrations of 62.5 μg/mL does not produce toxic or genotoxic effects meaning that all concentrations below are safe for use. By in vitro tests per-formed on human rhabdomyosarcoma cells (RD), cell line derivedfrom human cervix carcinoma Hep2c (HeLa) and cell line derived from murine fibroblast (L2OB), with IC_{50} values for Hep2c, RD and L2OB 40.34, 50.57 and 25.54 μg/mL, respectively, were confirmed antitumor properties of O. aucheriana extracts. Research demonstrated that O. aucheriana extract has great benefits and could find application in the food industry.

References

1. GUPTA. D., DUBEY. J., KUMAR. M.,(2016): Phytochemical analysis and antimicrobial activity of some medicinal plants against selected common human pathogenic microorganisms. Asian Pacific Journal of Tropical disease, pages 15-20

2. HINTZ. T., MATIHEWS. K., DI. R. (2015): The use of plant antimicrobial compounds for food preservation. BioMedResearch International Vol.2015, p1-12,.

3. SHAHIDI, F., JANITHA, P. K., WANASUNDARA, P. D., 1992. Phenolic antioxidants. Crit. Rev. Food Sci. Nutr. 32(1), 67-103

4. PIETTA, P. G., 1998. Flavonoids in medicinal plants, in: Rice-Evans, C. A., Packer, L., Eds., Flavonoids in Health and Disease. Dekker, New York, pp. 61-110.

5. ZEUTHEN, P., BOGH-SORENSEN, L., 2003. Food preservation techniques. Woodhead Publishing Limited, Cambridge

6. GOTTSCHLING, M., HILGER, H.H., WOLF, M., DIANE, N., 2001. Secondary structure of the ITS1 transcript and its application in a reconstruction of the phylogeny of Boraginales. Plant Biol. 3, 629-636.

7. ZHOU, R.H., DUAN, J.A., 2005. Plant Chemotaxonomy. Shanghai scientific & Technical Publishers, Shanghai

8. ZENG, Z-B., 1994 Precision mapping of quantitative trait loci. Genetics 136: 1457–1468

9. IQBAL, K., NAWAZ, S.A., MALIK, A., RIAZ, N., MUKHTAR, N., MOHAMMAD, P., CHOUDHARY, M.I., 2005. Isolation and lipoxygenase-inhibition studies of phenolic constituents from Ehretia obtusifolia. Chem. Biodi. 2, 104-111.

10. JOSIFOVIC, M., STJEPANOVIC, L., JANKOVIC, M., GAJIC, M., KOJIC, M., DIKLIC, N., 1977. The Flora of SR Serbia VI. Serbian Academy of Sciences and Arts, Belgrade.

11. KUMAR, N., KUMAR, R., KISHORE K. 2013. Onosma L.: A review of phytochemistry and ethnopharmacology. Pharmacogn Rev. 7(14): 140–151.

12. DIMOPOULOS P., RAUS TH., BERGMEIER E., CONSTANTINIDIS TH., IATROU G., KOKKINI S., STRID A. & TZANOUDAKIS D. 2013: Vascular plants of Greece: An annotated checklist. – Ånglera 31, 372 pages. – Berlin: Botanic Garden and Botanical Museum Berlin-Dahlem; Athens: Hellenic Botanical Society.

13. MASKOVIC. P., DIAMANTO. L., VUJIC. J., CVETANOVIC. A., RADOJKOVIC. M., GADZURIC. S., ZENGIN. G. (2015): Onosma aucheriana: A source of biologically active moleculs for novel food ingredients and pharmaceuticals. Journal of Functional foods 19, 479-486

14. DI GIORGIO C., DELMAS F., TUENI M., CHEBLE E., KHALIL T., BALANSARD G. 2008. Alternative and Complementary Antileishmanial Treatments: Assessment of the Antileishmanial Activity of 27 Lebanese Plants, Including 11 Endemic Species. The Journal of Alternative and Complementary Medicine. March, 14(2): 157-162

15. SATYAJIT, D., SARKER, L.N., KUMARASAMY, Y., 2007. Microtitre plate based antibacterial assay incorporating resazurin as indicator of cell growth, and its application in the in vitro antibacterial screening of phytochemicals. Methods. 42(4), 321-324.

16. SINGLETON, V., ORTHOFER, R., LAMUELA-RAVENTOS, R.M., 1999. Analysis of total phenols and other oxidation substrates and antioxidants by means of Folin-Ciocalteu reagent. Methods Enzymol. 299, 152-175

17. BRIGHENTE, I.M.C., DIAS, M., VERDI, L.G., PIZZOLATTI, M.G., 2007. Antioxidant activity and total phenolic content of some Brazilian species. Pharm. Biol. 45, 156-161

18. VERRMERIS, W., NICHOLSON, R., 2006. Phenolic compound biochemistry. Springer, Netherlands.

19. PRIETO, P., PINEDA, M., AGUILAR, M., 1999. Spectrophotometric quantitation of antioxidant capacity through the formation of a phosphomolybdenum complex: specific application to the determination of vitamin E. Anal. Biochem. 269-337, 341

20. TAKAO, T., WATANABE, N., YAGI, I., SAKATA, K., 1994. A simple screening method for antioxidants and isolation of several antioxidants produced by marine bacteria from fish and shellfish. Biosci. Biotechnol. Biochem. 58, 1780-1783.

21. KUMARASAMY, Y., BYRES, M., COX, P.J., JASPARS, M., NAHAR, L., SARKER, S.D., 2007. Screening seeds of some Scottish plants for free-radical scavenging activity. Phytother. Res. 21, 615-621

22. HSU, C.K., CHIANG, B.H., CHEN, Y.S., YANG, J.H., LIU, C.L., 2008. Improving the antioxidant activity of buckwheat (Fagopyrum tataricm Gaertn) sprout with trace element water. Food Chem. 108, 633-641.

23. HINNEBURG, I., DORMAN, H.J.D., HILTUNEN, R., 2006. Antioxidant activities of extracts from selected culinary herbs and spices. Food Chem. 97, 122-129.

24. MOSMANN, T., 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J. Immunol. Meth 65, 55–63

25. DIGHE, R.D., SHIRADKARA, M.R., ROHOMB, S.S., DIGHE, P.D., 2011. Synthesis and SAR of methyl linked cyclohexyl thiophenyl triazoles for their Anti-Alzheimer activity. Der Chemica Sinica 2(3), 70-87.

26.BAVISKAR, B.A., KHADABADIA, S.S., DEORE, S.L., SHIRADKAR, M.R., 2012. Synthesis of clubbed Triazolyl Indeno [1,2-C] Isoquinolines as an Novel Anticancer Agent. Der Pharmacia Sinica 3(1), 24-30.
27. FISKESJO, G., 1985. The Allium test as a standard in environmental monitoring. Hereditas 102, 99-112.
28. RANK, J., NIELSEN, M.H., 1993. A modified Allium test as a tool in the screening of genotoxicity of complex mixtures. Hereditas 118, 49-53
29. MISAN, A.E., MIMICA-DUKIC, N.M., MANDIÆ, A.I., SAKAE, M.B., MILOVANOVIC, I.LJ., SEDEJ, I.J., 2011. Development of a rapid resolution HPLC method for the separation and determination of 17 phenolic compounds in crude plant extracts. Cent. Eur. J. Chem., 9(1), 133-142
30. SAHA, M.R., HASAN, S.M.R., AKTER, R., HOSSAIN, M.M., ALAM, M.S., ALAM, M.A., MAZUMDER, M.E.H., 2008. In vitro free radical scavenging activity of methanol extract of the leaves of Mimusops elengi Linn. Bangl. J. Vet. Med. 6(2), 197-202.
31. HAVSTEEN, B.H., 2002. The biochemistry and medical significance of the flavonoids. Pharmacol. Ther. 96(2-3), 67-202.
32. BENINGER, C.W., HOSFIELD, G.L., 2003. Antioxidant activity of extracts, condensed tannin fractions, and pure flavonoids from Phaseolus vulgaris L. seed coat color genotypes. J. Agric. Food Chem. 51, 7879-7883.
33. AMAROWICZ, R., NACZK, M., ZADERNOWSKI, R., SHAHIDI, F., 2000. Antioxidant activity of condensed tannins of beach pea, canola hulls, evening primrose, and faba bean. J. Food Lipids. 7, 195-205.
34. KAHKONEN, M.P., HOPIA, A.I., VUORELA, H.J., RAUHA, J.P., PIHLAJA, K., KUJALA, T.S., HEINONEN, M., 1999. Antioxidant Activity of Plant Extracts Containing Phenolic Compounds J. Agric. Food Chem. 47(10), 3954-3962.
35. DUH, P.D., YED, G.C., 1997. Antioxidative activity of three herbal water extracts. Food Chem. 60(4), 639-645.
36. FRANKE, A.A., COONEY, R.V., CUSTER, L.J., MORDAN, L.J., TANAKA, Y., 1998. Inhibition of neoplastic transformation and bioavailability of dietary flavonoid agents. Adv Exp Med Biol. 439, 237-248
37. PETERSEN, M., SIMMONDS, M.S.J., 2003. Rosmarinic acid. Phytochem. 62, 121-125.
38. ITHARAT, A., HOUGHTON, P., ENO-AMOOGUAYE, E., BURKE, P., SAMPSON, J., RAMAN, A., 2004. In vitro cytotoxic activity of Thai medicinal plant used traditionally to treat cancer. Journal of Ethopharmacology 90, 33-38.

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