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Original Scientific Paper

Evaluation of bioactivities and phenolic composition of extracts of *Salvia officinalis* L. (Lamiaceae) collected in Montenegro

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ABSTRACT: Sage (*Salvia officinalis*) is the best-known species of the genus *Salvia*, due to its medicinal and flavouring properties. This research was conducted on samples of *S. officinalis* collected from Potoci (SOP) in the continental part of Montenegro and from Valdanos (SOV) and Luštica (SOL) in the country´s coastal region. Extracts prepared using 96% ethanol, 50% ethanol and hot distilled water were examined for phenolic composition, as well as for their antioxidant, enzyme-inhibiting and cytotoxic activities. The HPLC-DAD method was employed for quantitative-qualitative analysis of phenolic compounds in extracts, which confirmed the presence of rosmarinic and caffeic acids and luteolin-7-*O*-glucoside. Total phenolic and flavonoid contents, antioxidant activity, and inhibition of acetylcholinesterase and tyrosinase were measured spectrophotometrically. In the applied antioxidant tests (DPPH, FRAP and β-carotene/linoleic acid tests) and tests of inhibition of the enzymes acetylcholinesterase and tyrosinase, the SOP extracts showed stronger antioxidant and enzyme-inhibiting activities compared to SOV and SOL, while the most efficient solvent was 50% ethanol. The highest cytotoxic activity in the MTT test was recorded for 96% ethanol extracts, especially in the case of the SOL sample, against the A375 cell line. All of the tested bioactivities were more strongly correlated with total phenolic content than with flavonoid content. Differences in the tested bioactivities of extracts obtained from plants collected at ecologically different localities and with different extraction solvents could be explained by variations in total phenolic and flavonoid contents.

Keywords: sage, phenolics, antioxidant activity, antineurodegenerative activity, cytotoxic activity

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INTRODUCTION

Salvia officinalis L., a perennial subshrub that originated from the northern Mediterranean coast, is widely cultivated around the world (Hedge 1972) due to its broad use in traditional medicine and cooking. Numerous scientific studies have confirmed the healing properties of sage and revealed a range of pharmacological activities (Ghorbani & Esmaeilizadeh 2017).

Free radicals cause an oxidative stress which is damaging for various cell structures, playing an essential role in the development of diverse diseases, including diabetes, cardiovascular disease, atherosclerosis, cancer, rheumatoid arthritis, inflammation, neurological disor-

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ders and afflictions associated with ageing (Pham-Huy *et al.* 2008). Oxidative damage has a central role in the occurrence and progress of neurodegenerative diseases, including Alzheimer's and Parkinson's diseases. In Alzheimer's disease (AD), neuronal degeneration causes a loss of memory, decrease of cognition and disturbing emotional behaviour, while Parkinson's disease (PD) is characterised by motor disturbances and non-motor symptoms (Jiang *et al.* 2016).

Salvia officinalis has been shown to be a promising antioxidant agent of natural origin (Lu & Foo 2001; Koşar *et al.* 2010; Roby *et al.* 2013; Martins *et al.* 2015; Duletić-Laušević *et al.* 2016). It also possesses the ability to enhance brain function, improve memory, elevate mood and delay age-related cognitive problems (Ferreira *et al.* 2006; Kennedy *et al.* 2006), which makes it a potential therapeutic agent for neurodegenerative disorders. Cytotoxic effects of *S. officinalis* extracts are well known (Xavier *et al.* 2009; Kontogianni *et al.* 2013; Ghorbani & Esmaeilizadeh 2017). Antioxidants of natural origin attract great attention of scientists and consumers since researchers have suggested that industrial synthetic antioxidants could be harmful for users (Shebis *et al.* 2013).

It has been demonstrated that the composition and bioactivities of *S. officinalis* extracts can be influenced by the collection locality, season, phenological phase and solvent used for extraction (Ben Farhat *et al.* 2009, 2013, 2014; Duletić-Laušević *et al.* 2016).

The aim of this study was to determine the antioxidant, antineurodegenerative and cytotoxic activities of different extracts obtained from *S. officinalis* plant material collected from continental and coastal natural habitats in Montenegro.

MATERIAL AND METHODS

Plant material. Aerial parts of *S. officinalis* from Potoci (SOP) in the continental region and from Valdanos (SOV) and Luštica (SOL) in the coastal region of Montenegro were collected in June and July of 2014 during the flowering phase. The plant material was air-dried and kept in shade at room temperature for further processing. Voucher specimens were deposited in the herbarium of the Institute of Botany and Botanical Garden "Jevremovac", Faculty of Biology, University of Belgrade (BEOU). Information about the origin of plant material and voucher specimens is given in Table 1.

Chemicals and reagents. Methanol, ethanol, glacial acetic acid, hydrochloric acid and chloroform were purchased from Zorka Pharma, Šabac (Serbia). Gallic acid, quercetin, ascorbic acid, 2(3)-t-butyl-4-hydroxyanisole (BHA), 3,5-di-tert-butyl-4 hydroxytoluene (BHT), 2,2-dyphenyl-1-picrylhydrazyl (DPPH), 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid diammonium salt (ABTS), 2,4,6-tripyridyl-s-triazine (TPTZ), potassium acetate ($C_2H_3KO_2$), potassium-persulphate ($K_2S_2O_8$), dimethylsulphoxide (DMSO), sodium carbonate anhydrous (Na_2CO_3), aluminum nitrate nonahydrate [$Al(NO_3)_3 \times 9H_2O$], sodium acetate ($C_2H_3NaO_2$), iron (III) chloride ($FeCl_3$), iron (II)-sulphate heptahydrate ($FeSO_4 \times 7H_2O$), β-carotene, Folin-Ciocalteu phenol reagent, sodium phosphate monobasic (NaH_2PO_4), sodium phosphate dibasic (Na_2HPO_4), 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), acetylcholinesterase from *Electrophorus electricus* (electric eel) (AChE), acetylcholine iodide, galanthamine hydrobromide from *Lycoris* sp., kojic acid, tyrosinase from mushroom, 3,4-dihydroxy-L-phenylalanine (L-DOPA), rosmarinic acid, caffeic acid and luteolin-7-O-glucoside were purchased from Sigma Chemicals Co. (USA), while Tween 40 and linoleic acid were purchased from Acros Organics (Belgium).

Preparation of plant extracts. Dry plant material was ground up into small pieces (2-4 mm) in a laboratory crusher (Waring laboratory blender, No. 8010ES). Plant material (10 g) was extracted with 100 mL of solvent (96% ethanol, 50% ethanol or hot distilled water) for 24 h at room temperature including use of an ultrasound bath in the first and last hour of the extraction process, as described before (Alimpić *et al.* 2017a, b). The extracts were filtered through filter paper (Whatman No.1), evaporated under reduced pressure (using a Buchi rotavapor R-114) and stored at +4°C for further experiments. The yields of obtained extracts are presented in Table 1.

Determination of total phenolic and flavonoid contents. The total phenolic content (TPC) and total flavonoid content (TFC) were quantified using a Perkin Elmer LAMBDA BIO UV/Vis spectrophotometer according to the previously described procedure (Alimpić *et al.* 2017a, b) at a concentration of 500 μg/mL. The total phenolic content of extracts was calculated from the gallic acid curve formula and expressed as gallic acid equivalents (mg GAE/g of dry extract). Flavonoid content of extracts was calculated from the quercetin curve formula and expressed as quercetin equivalents (mg QE/g of dry extract). Values are presented as the mean ± standard deviation averaged from three measurements.

HPLC analysis. Phenolic compounds in the tested extracts were determined by comparing the retention times and absorption spectra (200-400 nm) of unknown peaks with the reference standards. The HPLC-DAD analysis was performed using an Agilent 1200 Series HPLC (Agilent Technologies, Palo Alto, CA, USA) equipped with a Lichrospher® 100 RP 18e column (5 μm, 250 x 4 mm). Mobile phase A was formic acid in water (0.17%), while mobile phase B was acetonitrile. The injection volume was 10 μL, the flow rate was 0.8 mL/min and the gra-

Table 1. Populations of *S. officinalis* used in this study.

Acronym of Population	Origin of plant material	Voucher
SOP	Montenegro, Morača Canyon, Potoci, leg.: Lakušić, D., 2.6.2014.	BEOU-17294
SOV	Montenegro, Valdanos, leg.: Lakušić, D., 3.6.2014.	BEOU-17295
SOL	Montenegro, Luštica, leg.: Lakušić, D., 31.7.2014.	BEOU-17296

dient program was 0–53 min (0–100% B). Stop time of the analysis was 55 min. The investigated samples were analysed in triplicate.

Evaluation of antioxidant activity. For testing of antioxidant activity, extracts were dissolved in the appropriate solvent in concentrations of 100, 200 and 500 μg/mL before experiments. Absorbances were recorded using a Perkin Elmer LAMBDA BIO UV/Vis spectrophotometer. As positive controls (standards), we used BHA, BHT and ascorbic acid in concentrations of 100 μg/mL.

DPPH assay. The DPPH free radical-scavenging assay was performed according to the previously described experimental protocol (Alimpić *et al.* 2017a, b). The decrease of absorption of DPPH radicals at 517 nm was calculated using the formula: $[(A_C\text{-}A_S)/A_C] \times 100\%$, where A_C is absorbance of the control (without the test sample) and A_S is absorbance of the test samples at different concentrations. Results are presented as the percentage of inhibition averaged from three measurements.

FRAP assay. The FRAP assay, evaluating the sample´s total antioxidant power, was performed according to Alimpić *et al.* (2017a, b). The FRAP values of samples were calculated from the standard curve formula of $FeSO_4 \times 7H_2O$ and expressed as μmol of Fe(II)/g of dry extract. Results are presented as the mean ± standard deviation averaged from three measurements.

β-Carotene bleaching (β-CB) assay. The β-carotene bleaching (β-CB) assay was performed according to Alimpić *et al.* (2017a, b). The antioxidant activity of extracts was evaluated on the basis of β-carotene bleaching using the following formula: $[(A_{120}\text{-}C_{120})/(C_0\text{-}C_{120})] \times 100\%$, where A_{120} and C_{120} are the absorbance values measured at 120 min for the sample and the control, respectively, while C_0 is absorbance of the control at 0 min.

Evaluation of cytotoxic activity

Cell cultures. Human melanoma cells (A375), human lung carcinoma cells (A549) and human fetal lung fibroblast cells (MRC-5) were maintained as a monolayer culture in the Roswell Park Memorial Institute (RPMI) 1640 nutrient medium (Sigma Chemicals Co., USA). The RPMI 1640 nutrient medium was prepared in sterile deionised water, supplemented with penicillin (100 IU/ml), gentamicin (50 μg/mL), 4-(2-hydroxyethyl) piperazine-1-ethanesulphonic acid (HEPES) (25 mM), L-glutamine (3 mM) and 10% heat-inactivated fetal calf serum (FCS) (pH 7.2). The cells were grown at 37°C in 5% CO_2 and a humidified air atmosphere.

Cytotoxicity assay. The cytotoxic activity of extracts was investigated against three cell lines using the MTT assay (Plumb 2004). Cells were seeded in 96-well cell culture plates (ThermoFisher) in the appropriate density and grown for 24 h. Stock solutions of extracts were made in DMSO in a concentration of 50 mg/mL and further diluted with nutrient medium to the concentrations desired for treatment (up to 500 μg/mL). Stock solutions of aqueous extracts were centrifuged and the supernatant was further diluted with the medium. Solutions of 96% ethanol extracts in the nutrient medium were further filtered through a non-pyrogenic sterile filter (0.2 μm) (Sarstedt, Germany). Following continual treatment for 72 h, the medium was removed and a fresh batch of it was added, after which the MTT assay was continued as described previously (Gligorijević *et al.* 2012). The IC_{50} concentration was defined as the concentration of extract producing 50% inhibition of cell survival and was determined from the representative cell survival diagrams. Cisplatin (CDDP) was used as a standard cytotoxic agent (in a range of concentrations of up to 30 μg/mL).

Evaluation of antineurodegenerative activity. Acethylcholinesterase (AChE)- and tyrosinase (TYR)-inhibiting activity assays were performed according to the spectrophotometric method using 96-well plates as described before (Alimpić *et al.* 2017a, b). The applied concentrations of extracts and standards (galanthamine and kojic acid) were 100, 200 and 500 μg/mL. Absorbances were measured using a Tecan Sunrise SN microplate reader equipped with XFluor4 software. The results are expressed as percents of inhibition of samples compared to controls.

Statistical analysis. All measurements were carried out in triplicate and expressed as the mean ± standard deviation. Analysis of variance (two-way ANOVA) and Tukey's post-hoc test were performed to test the significance

of differences among mean values. Pearson's correlation coefficients were calculated between the content of phenolic components and values obtained in different bioactivity assays and interpreted as described in previous papers (Alimpić *et al.* 2017a, b). All calculations were performed using MS Office Excel (2007) and Past Paleontological Statistics 3.21 (Hammer *et al.* 2001).

RESULTS AND DISCUSSION

Extract yields, total phenolic content and total flavonoid content. The yields of *S. officinalis* extracts ranged from 6.95 to 14.15% (Table 2). The yields of SOL extracts were higher compared to the other localities, while 50% ethanol was a more efficient extraction solvent compared to the others tested (Table 2). The authors of previous studies also obtained different results for sage yields depending on the locality and solvent used (Ben Farhat *et al.* 2009; Cvetkovikj *et al.* 2013; Duletić-Laušević *et al.* 2016), or on the phase of plant development (Ben Farhat *et al.* 2014).

All of the tested extracts showed a broad range of TPC (42.28-124.91 mg of GAE/g) and TFC (7.39-45.82 mg of QE/g). In general, higher TPC was obtained in SOP and SOV than in SOL, while the most efficient solvent was 50% ethanol (Table 2). The collection localities and extraction solvents used significantly affect TPC and TFC of the tested extracts ($p<0.05$) (Table 2).

The results obtained in the present study correspond to previous results for *S. officinalis* (Duletić-Laušević *et al.* 2016), with similar values for plants originating from Luštica. However, Schnitzler *et al.* (2008) obtained higher TPC in aqueous extracts than in ethanol extracts of *S. officinalis* samples. Martins *et al.* (2015) compared aqueous and methanol extracts and observed that the highest concentration of phenolic compounds was in the aqueous extract prepared by a decoction of *S. officinalis* leaves. Extracts of SOL showed the highest TFC (45.82 mg of QE/g), where the most efficient solvent proved to be 50% ethanol. Abdelkader *et al.* (2014) studied the chemical composition and bioactivities of Algerian *S. officinalis* and obtained TPC and TFC in amounts of 31.15 mg of GAE and 18.46 mg of QE per 100 g of dw, respectively. Several researchers studied various extracts of *S. officinalis* and concluded that total phenolic and flavonoid contents are influenced by the locality and solvent used (Kontogianni *et al.* 2013; Ben Farhat *et al.* 2014; Duletić-Laušević *et al.* 2016).

HPLC analysis. Presented in Table 3, the results obtained by HPLC analysis revealed the presence of rosmarinic and caffeic acids and luteolin-7-*O*-glucoside as the most abundant components in the tested extracts. The amount of rosmarinic acid (9.99-102.15 μg/mg) was higher in the 50% ethanol and aqueous extracts than in the 96% ethanol extract. Caffeic acid was present in the range of 0.29-6.76 μg/mg, with its highest abundance in the aqueous extracts, while the content of luteolin-7-*O*-glucoside (22.14-87.62 μg/mg) was greatest in the 50% ethanolic extracts. The extraction solvents used and sampling localities significantly affected the amount of examined polyphenolic compounds in the tested extracts (Table 3).

Caffeic and rosmarinic acids have been frequently reported for *S. officinalis* extracts (Lu & Foo 1999, 2000, 2001; Hossain *et al.* 2010; Roby *et al.* 2013; Martins *et al.* 2015; Garcia *et al.* 2016). They are considered to be potent antioxidant molecules (Khan *et al.* 2016; Nicolai *et al.* 2016; Adomako-Bonsu *et al.* 2017) that also exhibit antimutagenic, anticarcinogenic, antiinflammatory and antiallergenic activities (Konishi *et al.* 2005). One of the most common flavonoids was luteolin, which (along with its glycosides) is widely distributed in plants, being abundantly represented in the family Lamiaceae (Lopez-Lazaro 2009). Luteolin-7-*O*-glucoside has been previously identified in *S. officinalis* (Lu & Foo 2000; Hossain *et al.* 2010; Cvetkovikj *et al.* 2013; Martins *et al.* 2015) and shown to possess antioxidant activity (Hu & Kitts 2003). In the study of Schnitzler *et al.* (2008), aqueous extracts of sage showed a larger amount of luteolin-7-*O*-glucoside than ethanol extracts with different ethanol/water ratios, while in the present study the 50% ethanol extract was richest in the mentioned compound.

Table 2. Yields of extracts, total phenolic content (TPC) and total flavonoid content (TFC) of *S. officinalis* extracts.

	Yield (%)			TPC (mg GAE/g)			TFC (mg QE/g)		
Extracts	**SOP**	**SOV**	**SOL**	**SOP**	**SOV**	**SOL**	**SOP**	**SOV**	**SOL**
96% ETOH	7.26	8.72	7.58	98.24±3.32	102.12±0.93	83.58±0.76^{c}	19.17±0.16	15.81±1.03	7.39±0.28
50% ETOH	11.14	9.70	11.71	124.00±2.16	77.44±2.35	124.91±1.14	40.07±0.93	22.73±0.86	45.82±1.26
aqueous	6.95	8.72	14.15	78.84±1.42	97.07±1.97	42.28±2.00	21.48±1.04	34.36±1.41	9.38±0.41

For yield, n = 1. For TPC and TFC, values are presented as means ± SD (n = 3). Means without superscript letters are significantly different (two-way ANOVA, Tukey's post hoc; $P < 0.05$).

Table 3. HPLC quantification of main phenolic components of *S. officinalis* extracts.

Extracts	Components (μg/mg dry extract)								
	Rosmarinic acid			Caffeic acid			Luteolin-7-*O*-glucoside		
	SOP	SOV	SOL	SOP	SOV	SOL	SOP	SOV	SOL
96% ETOH	9.99±0.23	11.87±0.38	5.17±0.11	1.26±0.01	0.69±0.00	0.57±0.00	57.89±0.89	55.56±0.72	22.14±0.21
50% ETOH	72.72±2.15	61.71±1.84	102.15±4.12	0.29±0.02	0.58±0.00	0.30±0.00	87.62±2.01	77.66±1.75	74.76±1.95
aqueous	66.04±1.54	43.29±1.01	88.65±2.89	1.03±0.02	4.23±0.05	6.76±0.15	71.35±2.01	40.41±0.67	50.80±0.87

Values are presented as means ± SD (n = 3). For each component, means without superscript letters are significantly different (two-way ANOVA, Tukey's post hoc; $P < 0.05$).

Antioxidant activity of *S. officinalis* extracts. In all applied assays, the extracts of *S. officinalis* showed concentration–dependent antioxidant activity which was comparable with those obtained for the standards BHA, BHT and vitamin C. Collection localities and solvents used did not significantly influence the antioxidant activity of extracts except in the case of the β-carotene/linoleic acid test, where extracts of plants from different localities showed significant differences. In addition, aqueous extracts were significantly different from ethanol extracts (Table 4). In the DPPH test, extracts of SOP showed the strongest activity in all of the applied solvents (Table 4). Ethanol extracts showed similar results, but the best results (20.76-92.55% inhibition) were achieved using 96% ethanol.

Ollanketo *et al.* (2002) studied DPPH activity of *S. officinalis* aerial parts applying various solvents, among which water gave the best results. In the study of Lima *et al.* (2007), the methanol extract had higher antiradical activity against DPPH than the aqueous extract. Duletić-Laušević *et al.* (2016) showed that the ethanol extract of *S. officinalis* originating from Pleš had higher DPPH activity compared to the synthetic antioxidants BHA and BHT, while extracts of plants originating from Luštica had activity similar to that of BHT.

The values obtained for FRAP antioxidant activities are presented in Table 4. The highest activity was recorded for 50% ethanol extracts of SOL and SOP, while SOV showed the best results using 96% ethanol as the solvent. The results obtained in the present study are in accordance with previously published results of Duletić-Laušević *et al.* (2016) for plants originating from Luštica and Pleš collected in winter and summer, stronger activity being recorded by those investigators for ethanol extracts of summer samples. Ben Farhat *et al.* (2009) obtained a high antioxidant capacity of methanol extracts of *S. officinalis* cultivated in Tunisia's coastal region. Wojdylo *et al.* (2007) studied the antioxidant activity of methanol extracts of 32 plant species, and *S. officinalis* had a FRAP activity of 167 μM/100 g. Ben Farhat *et al.* (2013) compared the FRAP activity of methanol extracts obtained from four *Salvia* species, among which those from *S. officinalis* had the highest value [178.65-197.33 mM Fe (II)/mg].

Table 4 presents results of the β-carotene/linoleic acid test. The highest percentage of inhibition was recorded for extracts of SOP. The best activity was measured in the cases of 50% ethanol and aqueous extracts, with the best result (76.13%) recorded for a 50% ethanol extract of SOP. All extracts showed stronger activity than that of vitamin C at corresponding concentrations. The results obtained in the present study are in agreement with previous research of Alimpić *et al.* (2017a, b), where good results were obtained with ethanol extracts of *S. jurisicii* and *S. amplexicaulis*. In the β-carotene/linoleic acid test, extracts of plants from different sampling localities were significantly different. In addition, aqueous extracts differed from ethanol extracts (Table 4).

Cytotoxic activity. Results of 72-h treatment of three cell lines with different *S. officinalis* extracts showed significant differences of cytotoxicity related to the solvents used for extraction. Extracts of SOP and SOL were similar in tests with A375 and A549, while SOV and SOL showed no differences in the test with MRC-5 (Table 5). The obtained results showed that 96% ethanol extracts of *S. officinalis* from all localities exhibited higher cytotoxic activity on tumour cell lines (A375 and A549) than did 50% ethanol extracts. The aqueous extracts did not reach IC_{50} values on tumour cells in the investigated range of concentrations (up to 500 μg/mL). The A375 cell line was more sensitive than the A549 cell line for ethanol extracts from plants originating from all of the investigated localities. Treatment of normal human fetal lung fibroblast cells (MRC-5) showed that 50% ethanol extracts of *S. officinalis* exhibited higher cytotoxic activity than 96% ethanol extracts, opposite to the results obtained for tumour cell lines. The IC_{50} values for cisplatin in the case of 48-h treatment of the three analysed cell lines were up to 7.2 μg/mL (6.92 μg/mL for A375 cells,

Table 4. Antioxidant activity of *S. officinalis* extracts.

Extracts/ standards	Conc.(µg/mL)	DPPH assay (% inhibition)			FRAP assay (µmol Fe (II)/g)			β-carotene/linoleic acid assay (% inhibition)		
		SOP	SOV	SOL	SOP	SOV	SOL	SOP	SOV	SOL
96% ETOH	100	25.90±4.03^{xy}	28.58±0.85^{xy}	20.76±0.07^{xy}	185.97±15.57^{xy}	225.82±4.37^{xy}	144.87±5.62^{xy}	56.16±2.95^{y}	49.77±3.85^{y}	43.92±3.51^{y}
	200	57.69±2.40^{xy}	42.16±2.38^{xy}	51.27±3.12^{xy}	427.15±5.75^{xy}	403.90±4.37^{xy}	271.07±17.43^{xy}	58.93±4.09^{y}	51.73±3.50^{y}	44.37±0.57^{y}
	500	92.55±0.07^{xy}	88.62±3.38^{xy}	91.42±1.64^{xy}	933.17±36.74^{xy}	912.00±29.84^{xy}	751.76±17.43^{xy}	63.14±6.05^{y}	63.36±3.38^{y}	48.57±3.64^{y}
50% ETOH	100	34.67±1.36^{xy}	13.69±2.04^{xy}	32.06±0.79^{xy}	230.39±5.75^{xy}	187.63±9.96^{xy}	259.44±14.33^{xy}	54.95±2.72^{y}	46.92±1.80^{y}	40.39±1.95^{y}
	200	73.72±2.60^{xy}	47.18±0.07^{xy}	60.34±0.17^{xy}	501.45±6.23^{xy}	341.64±6.86^{xy}	555.83±3.13^{xy}	59.91±2.04^{y}	56.08±2.41^{y}	50.38±2.53^{y}
	500	92.40 ±0.20^{xy}	91.15±0.23^{xy}	91.53±0.13^{xy}	1134.08±38.61^{xy}	814.86±3.13^{xy}	1261.10±39.54^{xy}	76.13±1.06^{y}	61.11±2.93^{y}	55.86±1.32^{y}
Aque-ous	100	14.18±2.33^{xy}	12.93±1.87^{xy}	12.14±1.19^{xy}	242.42±7.47^{xy}	157.74±12.27^{xy}	212.95±9.35^{xy}	43.09±0.45	36.34±0.68	10.89±0.40
	200	56.45±3.53^{xy}	29.45±1.36^{xy}	27.75±1.20^{xy}	471.98±28.57^{xy}	264.84±6.23^{xy}	259.44±9.35^{xy}	60.21±5.18	43.69±4.09	17.57±0.70
	500	85.86±4.39^{xy}	78.11±2.10^{xy}	72.63±3.61^{xy}	993.36±22.42^{xy}	709.01±6.86^{xy}	562.47±8.72^{xy}	73.57±3.86	57.81±2.26	41.52±2.90
BHA	100	43.33±0.87			572.85±5.71			57.70±1.91		
BHT	100	34.31±0.43			413.03±3.13			56.29±1.44		
Vita-min C	100	91.65±0.21			576.17±7.61			2.99±2.13		

Values are presented as means ± SD (n = 3). For each assay, means with different superscript letters within the same row $^{(x)}$ and column $^{(y)}$ differ significantly (two-way ANOVA, Tukey's post hoc; $P < 0.05$).

Table 5. Cytotoxic activity of *S. officinalis* extracts presented as IC_{50} (μg/mL).

Ex-tracts	Cell lines								
	A375 (melanoma cells)			A549 (lung carcinoma)			MRC-5 (normal cells)		
	SOP	SOV	SOL	SOP	SOV	SOL	SOP	SOV	SOL
96% ETOH	118.90±6.98^{x}	151.13±1.10	107.52±2.00^{x}	183.85±8.82^{x}	168.28±9.28	172.34±4.38^{x}	207.25±11.37	159.73±0.12^{x}	186.00±5.00^{x}
50% ETOH	167.60±1.64^{x}	175.51±7.92	166.15±5.52^{x}	250.15±4.52^{x}	200.40±5.47	272.52±7.57^{x}	136.07±10.32	109.79±15.43^{x}	122.24±4.51^{x}
Aqueous	>500	>500	>500	>500	>500	>500	493.74±5.52	477.84±2.47^{x}	433.47±5.38^{x}

Values are presented as means ± SD (n = 3). For each cell line, means with different superscript letters within the same row $^{(x)}$ and column $^{(y)}$ differ significantly (two-way ANOVA, Tukey's post hoc; $P < 0.05$).

7.24 μg/mL for A549 cells and 6.56 μg/mL for MRC-5 cells).

Salvia officinalis extracts proved to be efficient in suppression of the growth of tumour cells (Xavier *et al.* 2009; Garcia *et al.* 2016). Garcia *et al.* (2016) used an 80% ethanol extract and obtained a high level of cytotoxicity for A-549, while the aqueous extract achieved the best result for the A-375 cell line. Xavier *et al.* (2009) found the aqueous extract of *S. officinalis* to be an effective inhibitor of proliferation and induced apoptosis in a concentration-dependent manner in the colon carcinoma-derived cell line HCT15. *Salvia officinalis* hexane and ethyl acetate extracts were effective at concentrations 50-100 μl/mg on RINm5F rat insulinomas, and also induced their apoptosis (Kontogianni *et al.* 2013).

Rosmarinic acid (Xavier *et al.* 2009; Oliveira *et al.* 2013), caffeic acid (Matejczyk *et al.* 2018) and luteolin 7-*O*-glucoside (Hu & Kitts 2003) were previously proved to possess cytotoxic activity.

Antineurodegenerative activity of *S. officinalis* extracts. The results of AChE inhibition are presented in Table 6. The highest percentage of inhibition was obtained for extracts of SOP (12.07-21.62%). Samples from the three examined localities showed significantly different inhibition of AChE. All extracts obtained using 50% ethanol showed significantly lower inhibition than those obtained using 96% ethanol and water. The tested extracts exhibited activity considerably weaker than that of galanthamine.

Vladimir-Knežević *et al.* (2014) studied AChE-inhibitory activity of the ethanol extracts of several Lamiaceae species at a concentration of 1 mg/mL. Extracts of *S. officinalis* achieved inhibition rates above 75%. Ferreira *et al.* (2006) obtained 16.4% inhibition of AChE by the ethanol extract of *S. officinalis* at a concentration of 0.5 mg/mL, while decoction at a concentration of 5 mg/mL caused inhibition of 57.2%. Previous results of ours showed differences among applied solvents, i.e., the ethanol extract of aerial parts of *S. amplexicaulis* showed better results in AChE inhibition (Alimpić *et al.* 2017a), while the aqueous extract of aerial parts of *S. jurisicii* was more efficient (Alimpić *et al.* 2017b).

The results of testing for TYR inhibition are presented in Table 6. The highest percentage of inhibition was obtained for extracts of SOP (14.29-42.12%) and the lowest for extracts of SOL (11.72-27.84%), while SOV and SOL exhibited similar rates of TYR inhibition. It turned out that 96% ethanol was the most efficient solvent (13.92-42.12%). Both 96% and 50% ethanol extracts were significantly stronger TYR inhibitors than the aqueous ones. The tested extracts exhibited activity weaker than that of kojic acid.

In our previous analysis of *S. amplexicaulis* and *S. jurisicii* extracts, ethanol extracts were more potent than aqueous extracts in TYR inhibition, both exhib-

Table 6. Activity of *S. officinalis* extracts in inhibition of AChE and TYR.

Extracts/ standards	Conc. (μg/mL)	AChE inhibition assay (% inhibition)			TYR inhibition assay (% inhibition)		
		SOP	SOV	SOL	SOP	SOV	SOL
96% ETOH	100	19.32±1.90^{y}	18.27±1.86^{y}	11.97±1.00^{y}	40.29±3.36^{y}	29.67±1.90^{xy}	23.08±2.20^{xy}
	200	20.21±3.23^{y}	16.85±2.71^{y}	12.84±0.77^{y}	42.12±1.27^{y}	14.29±1.10^{xy}	33.33±1.68^{xy}
	500	18.11±2.86^{y}	16.26±2.39^{y}	11.50±0.54^{y}	37.36±3.96^{y}	13.92±1.68^{xy}	15.38±3.30^{xy}
50% ETOH	100	12.47±0.25	9.97±1.33	4.63±1.75	36.63±3.36^{y}	23.08±1.90^{xy}	27.84±2.29^{xy}
	200	15.18±0.89	12.46±1.02	7.24±1.60	30.04±2.29^{y}	18.32±1.68^{xy}	16.48±1.10^{xy}
	500	12.07±1.16	11.87±1.74	4.69±1.61	32.23±2.77^{y}	13.92±1.68^{xy}	18.68±1.10^{xy}
aqueous	100	17.62±1.25^{y}	14.55±0.35^{y}	7.63±1.69^{y}	13.55±1.68	12.82±1.68^{x}	11.72±1.68^{x}
	200	21.62±1.85^{y}	13.78±1.12^{y}	8.89±3.04^{y}	17.22±1.68	17.95±1.68^{x}	15.75±2.29^{x}
	500	19.54±1.71^{y}	16.96±1.16^{y}	16.67±2.97^{y}	14.29±2.20	18.68±1.10^{x}	21.25±3.36^{x}
Galam-thamine	50		50.56±0.51		-		
	100		57.11±1.67		-		
	200		62.59±0.53		-		
Kojic acid	50		-		33.93±5.35		
	100		-		51.81±3.61		
	200		-		87.91±7.91		

Values are presented as means ± SD, n = 3. For each assay, means with different superscript letters within the same row $^{(x)}$ and column $^{(y)}$ differ significantly (two-way ANOVA, Tukey's post hoc; $P < 0.05$).

iting stronger inhibition (52.24-64.92%) than kojic acid (Alimpić *et al.* 2017a, b). Ethyl-acetate and methanol extracts of 16 *Salvia* species showed weak TYR inhibition in the study of Orhan *et al.* (2012). Compared to other *Salvia* species, *S. officinalis* in the present research showed stronger activity than ethanol extracts of *S. cryptantha* and *S. cyanescens* (Süntar *et al.* 2011).

Correlation of TPC, TFC and tested activities. Table 7 presents Pearson's coefficients of correlation of TPC, TFC and individual phenolics in the assays employed for testing of *S. officinalis* extracts. In general, a stronger correlation was established for TPC in the applied antioxidant tests compared to TFC. The content of luteolin-7-*O*-glucoside exhibited a significant correlation with antioxidant activity in the FRAP and β-carotene/linoleic acid assays. Weak and/or negative correlations were obtained for antineurodegenerative and other parameters. The content of caffeic acid was strongly correlated with cytotoxicity on the tested cell lines (Table 7).

Influence of extract concentration, solvent type and sampling locality on observed traits. The influence of sampling locality, extract concentration and solvent type on all of the observed traits was investigated using multifactorial ANOVA and interpreted in terms of *p*-values. All of the examined factors proved to have a significant impact on the analysed traits (chemical composition and tested biological activities of the extracts). An exception was the impact of extract concentration on TYR inhibition. Testing of interaction among the three analysed factors also showed the existence of a significant effect (except in the case of AChE inhibition). The results of ANOVA analysis confirmed the importance of these investigations. By selecting a suitable sampling locality and appropriate extraction parameters, the yield of polyphenols and the pharmacological potential of the extract can be maximised.

Table 7. Correlation of TPC, TFC and individual phenolics in assays employed for testing bioactivities of *S. officinalis* extracts.

		TPC	TFC	Caffeic acid	Rosmarinic acid	Luteolin-7-O-glucoside
Antioxidant assays	DPPH assay	0.66	0.25	**-0.96**	-0.29	0.34
	FRAP assay	**0.83**	**0.71**	**-0.74**	0.29	**0.67**
	β-carotene/linoleic acid assay	0.56	0.42	-0.60	-0.02	0.66
Cytotoxic assay	A375 cells	-0.53	-0.07	**0.74**	0.40	-0.10
	A549 cells	-0.44	0.05	**0.72**	0.49	-0.06
	MRC-5 cells	-0.53	-0.22	**0.70**	0.14	-0.30
Enzyme inhibition assays	AChE inhibition assay	-0.48	-0.53	0.41	-0.40	-0.23
	TYR inhibition assay	0.30	0.24	0.01	-0.07	0.12

According to Taylor (1990): $r \leq 0.35$ weak correlation; $0.36 < r < 0.67$ moderate correlation; $0.68 < r < 1$ strong correlation; R values written in bold are statistically significant ($P < 0.05$).

CONCLUSIONS

The highest TPC and TFC were obtained in 50% ethanolic extracts, especially for SOP and SOV samples. All samples contained rosmarinic and caffeic acids and luteolin-7-*O*-glucoside. SOP demonstrated the highest antioxidant activities in applied antioxidant tests with the highest values obtained for 50% ethanol extract. The antioxidant potential is more strongly correlated to TPC comparing to TFC. The strongest cytotoxic activity in MTT test was recorded for 96% ethanolic extracts, especially for the SOL sample. Caffeic acid demonstrated a strong correlation to cytotoxic effects on tested cell lines. In the antineurodegenerative tests the best results were obtained for SOP. AChE inhibition was better for water and 96% ethanolic extracts, while TYR inhibition was the best with 96% ethanolic extracts. Correlation among enzyme inhibition and other parameters was weak and/or negative. This confirms that *S. officinalis* is rich in polyphenols, which are responsible for biological activities. Future investigation should be focused on identification of polyphenols and determination of other bioactivities of *S. officinalis*, especially from the area of Potoci.

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Botanica SERBICA

REZIME

Procena bioaktivnosti i sastava fenolnih jedinjenja ekstrakata žalfije iz Crne Gore

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Žalfija (*Salvia officinalis* L.) je najpoznatija vrsta roda *Salvia* zahvaljujući svojim lekovitim i aromatičnim svojstvima. Ovo istraživanje je obuhvatilo uzorke *S. officinalis* sakupljene sa lokaliteta Potoci (SOP) u kontinentalnom delu i sa Valdanosa (SOV) i Luštice (SOL) u priobalju Crne Gore. Ekstrakti su pripremljeni pomoću sledećih rastvarača: 96% etanol, 50% etanol i vrela destilovana voda, a zatim je analiziran sadržaj ukupnih fenola, kao i njihovo antioksidativno i citotoksično dejstvo i aktivnost u inhibiciji enzima. Kvantitativno-kvalitativna analiza fenolnih jedinjenja u ekstraktima je izvršena pomoću HPLC-DAD, čime je potvrđeno prisustvo ruzmarinske kiseline, kafene kiseline i luteolin-7-*O*-glukozida. Sadržaj ukupnih fenola i flavonoida, antioksidativna aktivnost i inhibicija acetilholinesteraze i tirozinaze su mereni spektrofotometrijski. U primenjenim testovima (DPPH, FRAP i β-karoten/linolna kiselina) i u testovima inhibicije enzima acetilholinesteraze i tirozinaze, SOP ekstrakti su pokazali jače antioksidativno dejstvo i bolju inhibiciju enzima, u poređenju sa SOV i SOL, dok je najefikasniji rastvarač bio 50% etanol. Najviša citotoksična aktivnost u MTT testu je zabeležena za 96% etanolne ekstrakte, posebno za SOL na ćelijskoj liniji A375. Sve testirane bioaktivnosti su bile jače korelisane sa sadržajem ukupnih fenola u poređenju sa sadržajem flavonoida. Razlike u testiranim bioaktivnostima ekstrakata dobijenih iz biljaka sakupljenih na ekološki različitim lokalitetima i ekstrahovanih pomoću različitih rastvarača bi mogle biti objašnjene varijacijama sadržaja ukupnih fenola i flavonoida.

Ključne reči: žalfija, fenoli, antioksidativna, antineurodegenerativna, citotoksična aktivnost.