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[1]University of Belgrade, Faculty of Biology, Institute of Botany and Botanical Garden "Jevremovac", Belgrade, Serbia
[2]Institute for Medicinal Plant Research "Dr. Josif Pančić", Belgrade, Serbia

Antineurodegenerative, antioxidant and antibacterial activities and phenolic components of *Origanum majorana* L. (Lamiaceae) extracts

Sonja Duletić-Laušević[1], Ana Alimpić Aradski[1*], Stoimir Kolarević[1], Branka Vuković-Gačić[1], Mariana Oalđe[1], Jelena Živković[2], Katarina Šavikin[2], Petar D. Marin[1]

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Summary

The aim of this study was to examine chemical composition, as well as antineurodegenerative, antioxidant and antibacterial activity of aqueous and ethanolic extracts of *Origanum majorana* L. (Lamiaceae) originating from Serbia, Greece, Egypt and Libya. Total phenolics and flavonoids, antioxidant activities, and acetylcholinesterase and tyrosinase inhibitory activities were measured spectrophotometrically. Determination of phenolic compounds in extracts was done using HPLC-DAD technique. Antibacterial activity included determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) using the microdilution method. The highest phenolic and flavonoid contents were recorded in the ethanolic extract of the Egyptian sample and in aqueous extract of Serbian sample. The HPLC analysis showed high content of rosmarinic acid, with the highest amount found in the ethanolic extract of the plants from Egypt. Water extracts showed prevalently better antioxidant and antineurodegenerative activity in applied tests than the ethanolic extracts. Gram-positive bacterial strains showed higher sensitivity to tested extracts. According to the obtained results, sweet marjoram samples from Serbia and Egypt can be marked as more promising, due to the highest content of total phenolics and flavonoids and the best antioxidant, antibacterial and tyrosinase inhibitory activity.

Keywords: *Origanum majorana*, extracts, chemical composition, antioxidant activity, antibacterial activity, antineurodegenerative activity

List of abbreviations

AAE - ascorbic acid equivalent; ABTS - 2,2-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt; AChE - acetylcholinesterase; AD - Alzheimer disease; BHA - 2(3)-*t*-butyl-4-hydroxyanisole; BHI - Brain-Heart infusion; BHT - 3,5-di-*tert*-butyl-4-hydroxytoluene; DMSO - dimethylsulfoxide; DPPH - 2,2-dyphenyl-1-pikrylhydrazyl (DPPH); DTNB - 5,5'-dithiobis(2-nitrobenzoic acid); FRAP - ferric reducing ability of plasma; GAE - gallic acid equivalent; L-DOPA - 3,4-dihydroxy-L-phenylalanine; MBC - minimal bactericidal concentration; MHB - Mueller-Hinton broth; MIC - minimal inhibitory concentration; QE - quercetin equivalent; TFC - total flavonoid content; TPC - total phenolic content; TPTZ - 2,4,6-tripyridil-*s*-triazin; TYR - tyrosinase; β-CB assay - β-Carotene Bleaching assay

Introduction

The mint family (Lamiaceae) includes aromatic plants widely used for culinary, medicinal, cosmetic and ornamental purposes, such as basil, rosemary, sage, oregano, lavender, thyme and mint (Raja, 2012). The genus *Origanum* is composed of 42 species and 18 hybrids widely distributed in Eurasia and North Africa (Ietswaart, 1980), being native to the mountainous areas of Mediterranean and Asia (Chishti et al., 2013). Species belonging to the genus *Origanum* are used since the ancient times as spices, medicinal, aromatic and ornamental plants (Meyers, 2005). *In vitro* pharmacological investigations showed their antibacterial, antifungal, antioxidant, antispasmodic, antimutagenic, antitumoral, analgesic, antithrombin and antihyperglycaemic activities (Chishti et al., 2013).

Origanum majorana L. (sweet marjoram) is herbaceous perennial shrub, inhabiting dry slopes and rocky places, native to Cyprus and Eastern Mediterranean area (Ietswaart, 1980). It is used as a medicinal plant, against cold, as a spasmolytic, antirheumatic, diuretic, and antiasthmatic drug, and as a culinary herb, for flavouring sausages, sauces, soups and condiments (Chishti et al., 2013).

The essential oil of marjoram of different origin was previously analyzed for the composition and biological activities (Teixeira et al., 2013; Hajlaoui et al., 2016). Various extracts were studied for antioxidant (Jun et al., 2001; Vági et al., 2005a; El-Maati et al., 2012; Roby et al., 2013; Ayari et al., 2013; Benchikha et al., 2013; Vasudeva et al., 2014; Afifi et al., 2014; Erenler et al., 2015) and antimicrobial activities (Vági et al., 2005b; Leeja and Thoppil, 2007; Abdel-Massih et al., 2010).

In the presented manuscript for the first time the marjoram extracts were tested for the antineurodegenerative effects, which is of growing scientific and public interest. The marjoram spices used in the analyses were produced by respectable companies for culinary herbs and spices, while Libyan marjoram was purchased at the local market. In several papers the commercially purchased marjoram material was subjected to various analyses (Mossa et al., 2015; Wahby et al., 2015; Fernandes et al., 2016), because of importance of testing the commercial, widely used spices in daily diet for possible benefits for consumers health. Considering the insufficiency of data on medicinal properties of *O. majorana* extracts, the goals of this study were chemical analysis as well as investigation of antibacterial, antioxidant and antineurodegenerative activity of marjoram aqueous and ethanolic extracts obtained from plants cultivated in Serbia and three Mediterranean countries (Greece, Egypt, Libya).

Material and methods

Chemicals and reagents

Methanol, ethanol, distilled water, 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-pikrylhydrazyl (DPPH), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), 2,4,6-tripyridil-*s*-triazin (TPTZ), potassium acetate ($C_2H_3KO_2$), potassium-persulfate ($K_2S_2O_8$), dimethylsulfoxide (DMSO), sodium carbonate anhydrous (Na_2CO_3), aluminium nitrate nonahydrate ($Al(NO_3)_39H_2O$), sodium acetate ($C_2H_3NaO_2$), iron(III) chloride ($FeCl_3$), iron(II)-sulfate heptahydrate ($FeSO_4 \cdot 7H_2O$), β-carotene, Folin-Ciocalteu phenol reagent, sodium phosphate monobasic (NaH_2PO_4), sodium phosphate

* Corresponding author

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 and 3,4-dihydroxy-L-phenylalanine (L-DOPA), streptomycin, rifampicin and resazurin sodium salt (>90% (LC) were purchased from Sigma Chemicals Co., St. Louis, MO (USA), while Tween 40 and linoleic acid were purchased from Acros Organics (Belgium). Brain-Heart infusion (BHI) and Mueller-Hinton broth (MHB) were purchased from Himedia (India).

Plant material

Dried and crushed plant material of *O. majorana,* which was used in the experimental procedure, originating from Serbia, Greece, Egypt and Libya, was commercially purchased. Marjoram is cultivated at appropriate field and collected, stored and controlled by the respectable companies. The plant material from Serbia is a product of Institute for Medicinal Plant Research "Dr Josif Pančić", the sample from Greece is produced by "Kagia" company, from Egypt is a product of "Kotanyi" company, while the plant material from Libya was purchased from the local market.

Preparation of extracts

Grinded plant material (50 g) was extracted during 24 h at room temperature (5% w/v) using two solvents, hot water and 96% ethanol. The mixture was exposed to ultrasound 1 h before and after 24 h to improve extraction process. Subsequently, extracts were filtered through filter paper (Whatman No.1) and evaporated under reduced pressure (Buchi rotavapor R-114). During the process of concentration of ethanolic extracts high temperatures were not used in order to prevent the loss of analytes and artifact formation. The obtained crude extracts were stored in a refrigerator at +4 °C for further experiments.

Determination of total phenolic and flavonoid contents

The total phenolic (TPC) and flavonoid contents (TFC) were measured using JENWAY 6305UV/Vis spectrophotometer as described before (Alimpić et al., 2015, 2017). Phenolic content of extracts was calculated from gallic acid curve equation and expressed as gallic acid equivalents (mg GAE/g dry extract). Flavonoid content of extracts was calculated from quercetin curve equation and expressed as quercetin equivalents (mg QE/g dry extract). Values were presented as 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. HPLC-DAD analysis was performed on an Agilent 1200 Series HPLC (Agilent Technologies, Palo Alto, CA, USA) equipped with Lichrospher® 100 RP 18e column (5 μm, 250 × 4 mm). Mobile phase A was formic acid in water (0.17%), while mobile phase B was acetonitrile. The injection volume was 10 μL, and flow rate 0.8 mL/min with gradient program (0-53 min 0-100% B). Stop time of the analysis was 55 min. The investigated samples were analyzed in triplicate.

Evaluation of antioxidant activity

DPPH free radical scavenging assay was performed according to previously described experimental protocol (Alimpić et al., 2015, 2017). BHA, BHT and ascorbic acid were used as antioxidant standards. Results were expressed as IC_{50} values (μg/mL) averaged from three measurements.

ABTS free radical scavenging assay was performed according to Alimpić et al. (2015, 2017). The extracts and antioxidant standards BHA and BHT were tested in concentration of 1 mg/mL and 0.1 mg/mL, respectively. ABTS activity was calculated from ascorbic acid calibration curve and expressed as ascorbic acid equivalents per gram of dry extract (mg AAE/g), averaged from three measurements.

FRAP assay (ferric reducing ability of plasma), evaluating total antioxidant power of the sample, was performed according to Alimpić et al. (2015, 2017). The extracts were tested in concentration of 0.5 mg/mL. BHA, BHT and ascorbic acid (0.1 mg/mL) were used as reference antioxidants. FRAP values of samples were calculated from standard curve equation and expressed as μmol $FeSO_4$ × 7 H_2O/g dry extract and presented as mean ± standard deviation averaged from three measurements.

β-carotene bleaching (β-CB) assay was performed according to Alimpić et al. (2017). Crude extracts and standards BHA, BHT and ascorbic acid were tested in concentration of 0.5 mg/mL. The absorbances were measured using Tecan Sunrise SN microplate reader. The antioxidant activity of the extracts was evaluated in term of β-carotene bleaching using the following formula: $[(A_{120}-C_{120})/(C_0-C_{120})] \times 100\%$, where A_{120} and C_{120} were the absorbance values measured at 120 minutes for sample and control, respectively, while C_0 is absorbance of control at 0 minutes.

Antibacterial assay

The antibacterial activity of extracts and standard antibiotics streptomycin and rifampicin was tested against four gram-negative (*Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 15442, *Salmonella enteritidis* ATCC 12076, *Shigella flexneri* ATCC 9199) and four Gram-positive bacterial strains (*Enterococcus fecalis* ATCC 29212, *Staphylococcus aureus* ATCC 25923, *Bacillus subtilis* ATCC 6633 and *Listeria innocua* ATCC 33090). The lowest concentrations without visible growth, i.e. minimal inhibitory concentrations (MICs) were determined according to Kolarević et al. (2016).

Antineurodegenerative activities

Acethylcholinesterase (AChE) and tyrosinase (TYR) inhibitory activity assays were performed according to spectrophotometric method using 96-well plates as described before (Alimpić et al., 2017). The applied concentrations of extracts and standards (galanthamine and kojic acid) were 25, 50 and 100 μg/mL. The absorbances were measured using Tecan Sunrise SN microplate reader equipped by XFluor4 software. The results are expressed as percents of inhibition of samples comparing to controls.

Statistical analysis

Differences between the group means and their significance were verified by one-way ANOVA using the Software package STATISTICA v.7.0. The significance of differences was evaluated using Bonferroni's test and statistical significance was set at $p<0.05$. Pearson's correlation coefficients were calculated between content of phenolic components and antioxidant assays and interpreted according to Taylor (1990). Calculations and constructing of the charts were performed using MS Office Excel (2013).

Results and discussion

Yield of extracts, total phenolic and flavonoid contents

The yields of extracts varied depending on the plant origin and applied solvent (Tab. 1), with higher yields in aqueous extracts. Among aqueous extracts, the extract of the Egyptian *O. majorana* had the highest yield (29.51%), while the lowest yield was obtained for the

Greek sample (16.47%). The highest yield among ethanolic extracts was recorded for the Libyan sample (13.49%), and the lowest for the Serbian plants (8.51%). In the study of VÁGI et al. (2005a) the yield of ethanolic extract of plants from Egypt was higher compared to our results (28.99% and 11.91%, respectively). TRIANTAPHYLLOU et al. (2001), applied different extraction methods and obtained a yield as low as 0.88%, in the water extract of Greek *O. majorana*. In the study of BENCHIKHA et al. (2013) ethanolic extract of Algerian *O. majorana* yielded 8.16%, which is close to the Serbian sample in this study.

Total phenolic and flavonoid contents of extracts were measured spectrophotometrically and presented as gallic acid (mg GAE/g of dry extract) and quercetin (mg QE/g of dry extract) equivalents, respectively (Tab.1). All tested aqueous extracts of *O. majorana*, except for the one originating from Egypt, showed higher phenolic content comparing to ethanolic extracts (Tab. 1). The highest content of phenolics was found in aqueous extracts of the Serbian *O. majorana* (122.71 mg GAE/g dry extract), and in ethanolic extracts of the Egyptian plant (139.98 mg GAE/g dry extract). VÁGI et al. (2005a) obtained higher percentage of phenolics in ethanolic extracts of *O. majorana* from Egypt compared to the plants from Hungary. They explained the results by influence of different climate conditions, while many more sunny days in Egypt could cause higher production of self-defense compounds against radiation and microbes. Ethanolic extract of Egyptian marjoram showed higher amount of total phenolic and flavonoid compounds, similar to the results of EL-MAATI et al. (2012), who analyzed extracts of five Egyptian medicinal plants and obtained higher amounts of phenolics and flavonoids in ethanolic extracts of *O. majorana* compared to aqueous extracts. BENCHIKHA et al. (2013) studied ethanolic extracts of Algerian oregano species and obtained higher content of phenolics in *O. majorana* than in *O. vulgare* extract.

Flavonoids were detected in higher amounts in all tested ethanolic extracts compared to the aqueous extracts (Tab. 1). Ethanolic extract of the plant originating from Egypt was the most abundant in flavonoids (20.05 mg QE/g dry extract) compared to other samples. EL-MAATI et al. (2012) also obtained significantly higher amounts of flavonoids in ethanolic extracts of the Egyptian *O. majorana* than in the aqueous extract. When comparing the percentage of flavonoids in ethanolic extracts of Hungarian and Egyptian *O. majorana* VÁGI et al. (2005a) obtained lower values in ethanolic extracts of *O. majorana* from Egypt. BENCHIKHA et al. (2013) found a high phenolic content in the ethanolic extract of *O. majorana* from Algeria (266.86 mg GAE /g), but the extract was poor in flavonoids.

Tab. 1: Yields, total phenolic and flavonoid content of *O. majorana* extracts

Sample origin	Yield (%)	TPC (mg GAE/g)	TFC (mg QE/g)
Aqueous extracts			
Serbia	22.54	122.71±2.68^{a}	15.64±0.52^{ad}
Greece	16.47	111.10±1.32^{b}	15.13±0.55^{abd}
Egypt	29.51	95.33±4.61^{c}	13.51±0.42^{b}
Libya	24.81	84.76±1.84^{d}	11.03±0.46^{c}
Ethanol extracts			
Serbia	8.51	84.38±2.23^{d}	16.33±0.44^{d}
Greece	10.36	83.25±0.85^{d}	18.85±0.74^{e}
Egypt	11.91	139.98±1.14^{e}	20.05±1.11^{e}
Libya	13.49	58.62±0.51^{f}	14.87±0.29^{abd}

Means followed with different letters in the same column are significantly different at $p<0.05$.

HPLC analysis of the extracts

Chemical composition of *O. majorana* extracts was determined using HPLC-DAD (Tab. 2) in order to determine components responsible for certain types of bioactivities. Among phenolic acids rosmarinic acid was present in a significant amount, especially in ethanolic extracts of all tested samples. The ethanolic extract of the Egyptian plant contained the highest amount of rosmarinic acid (41.54 mg/g). Rosmarinic acid is restricted to the subfamily Nepetoideae of the Lamiaceae family (PETERSEN and SIMMONDS, 2003). This acid was previously reported as a dominant phenolic acid of the Lamiaceae (WANG et al., 2004; LEE, 2010; EMBUSCADO, 2015), that has a range of biological activities (AL-DHABI et al., 2014). In the comparative study of rosmarinic and caffeic acids contents of 76 Lamiaceae species using TLC-densitometric method, JANICSAK et al. (1999) have found low content of rosmarinic acid (0.87-2.40 mg/g) and caffeic acid (0.09-0.27 mg/g) in tested *Origanum* species.

ERENLER et al. (2015) studied the phytochemical content of the Turkish *O. majorana* water-soluble ethyl acetate extract and also detected rosmarinic acid and arbutin. In our samples, arbutin was, generally, more abundant in ethanolic extracts with the higest con-

Tab. 2: Phenolic components identified in *O. majorana* extracts

Sample origin	Content of phenolic components (mg/g dry extract)				
	Rosmarinic acid	Caffeic acid	Chlorogenic acid	Arbutin	Luteolin-7-*O*-glucoside
Aqueous extracts					
Serbia	20.09±0.81^{a}	1.02±0.03^{a}	4.52±0.15^{a}	14.73±0.52^{a}	0.99±0.02^{a}
Greece	9.37±0.19^{b}	0.54±0.02^{e}	3.91±0.10^{c}	2.22±0.06^{e}	3.82±0.13^{d}
Egypt	23.98±0.79^{e}	0.90±0.02^{f}	4.42±0.17^{a}	7.47±0.21^{f}	5.57±0.19^{e}
Libya	8.06±0.30^{b}	1.88±0.07^{c}	3.82±0.14^{c}	3.33±0.09^{c}	1.66±0.07^{b}
Ethanol extracts					
Serbia	22.47±0.75^{ae}	0.13±0.00^{b}	0.04±0.00^{bd}	10.02±0.38^{b}	1.10±0.03^{a}
Greece	27.71±1.02^{d}	0.49±0.01^{e}	0.34±0.01^{be}	15.46±0.49^{a}	0.65±0.02^{c}
Egypt	41.54±1.42^{f}	0.26±0.01^{d}	0.62±0.02^{e}	25.67±1.03^{g}	1.90±0.06^{b}
Libya	13.11±0.26^{c}	0.32±0.01^{d}	0.03±0.00^{d}	13.73±0.42^{d}	0.40±0.01^{c}

Means followed with different letters in the same column are significantly different at $p<0.05$.

tent in Egyptian sample. On the contrary, Serbian sample contained greater amount of arbutin in water extract. Arbutin has been shown to have antityrosinase (YE et al., 2010), antimicrobial (KUNDAKOVIĆ et al., 2014), antioxidant, antihyperglycaemic and antihyperlipidemic activities (SHAHABODDIN et al., 2011).

Water extracts of analyzed samples contained much more chlorogenic acid compared to ethanolic extracts. Chlorogenic acid showed several beneficial health effects, such as antioxidant, neuroprotective, antiinflamatory, hypoglycemic, antifungal, etc. (UPADHYAY and RAO, 2013). This acid is an ester of caffeic and quinic acids, but caffeic acid showed stronger antioxidant activity when compared to chlorogenic acid in *in vitro* and *in vivo* experiments (SATO et al., 2011).

Flavone luteolin and its glycosides have been reported to possess a wide range of bioactivities involved in prevention and treatment of various diseases (LOPEZ-LAZARO, 2009). Among flavonoids, luteolin-7-*O*-glucoside was detected in all extracts with the highest content in aqueous extract of Egyptian sample.

After comparison of HPLC-DAD chromatograms of water and ethanolic extracts at 280 and 330 nm we have not detected any unusual peak that might suggest formation of artifacts as a result of using ethanol as extraction solvent.

Evaluation of antioxidant activity of extracts

Considering the facts that oxidative stress has been involved in the development of different human diseases and occurrence of various side effects of synthetic antioxidants, researchers have focused on the beneficial health effects of phytochemicals (EMBUSCADO, 2015), including those isolated from *Origanum* species.

Antioxidant activity of *O. majorana* extracts was measured using four assays (Tab. 3). Tested aqueous extracts showed better activity against DPPH radicals compared to ethanolic extracts. Among aqueous extracts, efficiency varied from the Serbian sample which showed the best activity (28.25 μg/mL) to the Libyan sample with low activity (51.84 μg/mL). Among ethanolic extracts the best activity was obtained for extract of the Egyptian plants (54.15 μg/mL) which was also the most abundant in phenolics content, while the Libyan extract showed the lowest efficiency (114.47 μg/mL).

In the study of VÁGI et al. (2005a) the ethanolic extract of the Egyptian herb showed stronger antioxidant properties when compared to the Hungarian one, exhibiting antioxidant power comparable to that of BHT, probably caused by differences in the amounts of phenolic compounds. RAMADAN et al. (2014) obtained a higher percentage of inhibition of DPPH radicals for ethanol than methanol extract of Egyptian *O. majorana* at analyzed concentrations. Our results showed the weakest activity of Libyan extracts, while BENCHIKHA et al. (2013) obtained significant results for the geographically close Algerian *O. majorana* and *O. vulgare* ethanolic extracts, where all samples had better antioxidant activity than used controls (BHA and α-tocopherol), with better results for the extract of *O. majorana*.

Antioxidant activity of tested *O. majorana* extracts was also determined using ABTS radical decolorization assay and expressed as ascorbic acid equivalents (Tab. 3). The tested aqueous extracts in ABTS assay, with exception of the Egyptian sample, demonstrated better activity than the ethanolic ones (Tab. 3). The aqueous extract of the Serbian sample showed the best activity (2.06 mg AAE/g), and the ethanolic extract of the Egyptian plant was the most powerful against ABTS radicals (2.25 mg AAE/g), while the lowest results for both extracts were obtained for the Libyan sample. EL-MAATI et al. (2012) obtained better ABTS activity using ethanolic extracts compared to aqueous extracts of *O. majorana* from Egypt (81.1 and 33.3%, respectively), which is in accordance with our results for the Egyptian plant. RAMADAN et al. (2014) also studied Egyptian marjoram and obtained high ABTS scavenging capacity of ethanolic extract (854 μm trolox/g). PALANISWAMY and PADMA (2011) obtained high percentage of inhibition of ABTS radicals for aqueous leaf extract, but lower than the methanolic extract (78.31% and 88.96%, respectively).

In the FRAP assay tested aqueous extracts showed much better activity than the ethanolic ones, except for the Egyptian plant extract (Tab. 3). Among aqueous extracts, the Serbian sample showed the strongest activity (826.45 μmol Fe (II)/g), while the Egyptian plants showed the lowest activity (554.66 μmol Fe (II)/g). However, similarly to the results recorded in DPPH and ABTS assays, among ethanolic extracts, the strongest activity was obtained for the Egyptian plants (796.25 μmol Fe (II)/g), while extract of the Libyan plants showed the lowest activity (260.32 μmol Fe (II)/g). This assay was previously applied to *O. vulgare*, which revealed various results, better activity of the aqueous extracts of *O. vulgare* compared to the ethanolic ones in Portuguese samples (TEIXEIRA et al., 2013), or

Tab. 3: Antioxidant activities of *O. majorana* extracts

Sample origin	DPPH (IC_{50}, μg/mL)	ABTS (mg AAE/g)	FRAP (μmol Fe(II)/g)	β-CB assay (% inhibition)
Aqueous extracts				
Serbia	28.25±0.56^{a}	2.06±0.03^{a}	826.45±8.14^{a}	83.24±2.40^{a}
Greece	35.27±0.81^{a}	1.88±0.09^{b}	754.59±6.32^{b}	46.54±0.81^{b}
Egypt	50.90±1.54^{b}	1.52±0.06^{c}	554.66±3.50^{c}	83.24±2.48^{a}
Libya	51.84±1.33^{bc}	1.31±0.04^{d}	602.06±9.55^{d}	71.28±2.97^{c}
Ethanol extracts				
Serbia	59.07±0.60^{c}	1.52±0.07^{c}	362.00±7.64^{e}	61.70±2.98^{df}
Greece	74.27±0.77^{d}	1.52±0.06^{c}	288.23±6.39^{f}	63.30±1.60^{df}
Egypt	54.15±3.60^{cb}	2.25±0.02^{e}	796.25±7.52^{g}	60.90±1.99^{def}
Libya	114.47±6.15^{e}	1.30±0.04^{d}	260.32±7.64^{h}	66.49±2.79^{dc}
Standards				
BHT	17.94±0.17^{f}	2.78±0.02^{f}	445.34±5.77^{i}	53.72±2.26^{ebf}
BHA	13.37±0.43^{f}	2.82±0.01^{f}	583.72±5.26^{d}	57.71±3.39^{f}
Ascorbic acid	5.11±0.14^{g}	-	180.81±8.61^{j}	17.82±1.13^{g}

Means followed with different letters in the same column are significantly different at $p<0.05$.

on the contrary, lower activity of the aqueous extracts of *O. vulgare* from Tunisia than the ethanolic ones (BÉJAOUI et al., 2013).

Similar to the results obtained in the other applied tests, in the β-carotene bleaching assay most of the aqueous extracts showed a higher activity than ethanolic ones, except for the extracts of the plants originating from Greece (Tab. 3). Among aqueous extracts, the strongest activity was shown by the Egyptian and Serbian samples (83.24%), while the lowest activity was recorded for the Greek plants (46.54%). The strongest activity among ethanolic extracts showed the Libyan plants (66.49%), while the etanolic extract of marjoram originating from Egypt showed the lowest activity (60.90%). During the analysis of Egyptian *O. majorana* extracts, EL-MAATI et al. (2012) found a relatively low percentage of β-carotene inhibition, obtaining a better result for the ethanolic extract.

The ethanolic extract of the Egyptian plants showed considerable antioxidant activity in applied tests, probably due to the high content of rosmarinic acid, which is considered as a component with significant antioxidant activity (TEPE, 2008; ERENLER et al., 2015).

Correlation between antioxidant activity and phenolic components content

Correlation between results obtained for antioxidant activity and phenolics content is presented as correlation coefficient (Tab. 4). Antioxidant activity was more strongly correlated to total phenolic than total flavonoid content which is in accordance to WOJDYŁO et al. (2007). Chlorogenic acid content was strongly correlated to DPPH and FRAP values, rosmarinic acid and arbutin to ABTS values, while caffeic and chlorogenic acids were correlated to β-carroten bleaching assay. Chlorogenic, caffeic and rosmarinic acids could be responsible for the antioxidant activity of tested extracts. Antioxidant properties of these phenolic acids were also proved by RAUDONIS et al. (2008), SATO et al. (2011) and UPADHYAY and RAO (2013). Strong correlation is established between DPPH and FRAP as well as ABTS and FRAP assays, while correlations between other assays are assessed as moderate to weak. Results obtained in DPPH and ABTS assays showed moderate negative correlation between each other as it was previously found for Brasilian spices by BARROS MARIUTTI et al. (2008). In this study, ABTS and FRAP assays showed strong correlation which is consistent with HOSSAIN et al. (2008).

Antibacterial activity of extracts

Search for natural antimicrobials is growing in current studies because of the undesirable health impact of synthetic antimicrobial food preservatives and the occurrence of pathogenic microorganisms resistant to pharmaceuticals (TAJKARIMI et al., 2010). In addition to the flavoring effect, *Origanum* species are proved to have antimicrobial activity on human and plant pathogens (VÁGI et al., 2005b; LEEJA and THOPPIL, 2007; ASHRAF et al., 2011; JABER et al., 2012; CHISHTI et al., 2013; TEIXEIRA et al., 2013).

The results of antibacterial activity of extracts against Gram-positive bacteria are presented in Tab. 5. The most sensitive strains among Gram-positive bacteria were *B. subtilis* and *L. innocua*, both for aqueous and ethanolic extracts, especially for *O. majorana* originating from Serbia and Egypt (Tab. 5). Marjoram extracts showed almost no activity on tested concentrations against Gram-negative bacterial strains, except aqueous extract of Libyan sample which produced bacteriostatic and bactericidal effect on *S. flexneri*.

Gram-positive bacterial strains showed higher sensitivity against tested extracts compared to the Gram-negative ones, which has also been proved by AYARI et al. (2013). They found that some of the tested bacterial strains showed higher sensitivity towards methanolic extracts at lower values than antibiotics used as positive controls, as in the case of *B. subtilis*, *L. innocua* and *S. aureus*. Antibacterial effect of extracts of other *Origanum* species was analyzed by several authors. ASHRAF et al. (2011) conducted the comparative analysis of antibacterial activity of *O. vulgare* chloroform, methanol and aqueous extracts, using agar well diffusion method and obtained promising results for chloroform and methanol extracts, but aqueous extract was not active against most of the studied strains. JABER et al. (2012) compared the antibacterial activity of aqueous, ethanolic and methanolic extracts of *O. vulgare* against three Gram-positive and three Gram-negative bacterial strains, using the well diffusion method. The ethanolic extract had higher activity than the aqueous one. In determination of the minimum inhibitory concentration the best activity against *B. subtilis* was obtained using the ethanolic extract, followed by the methanolic and aqueous extracts. TEIXEIRA et al. (2013) also reported on higher ability of the *O. vulgare* ethanolic extract for the inhibition of growth of seven bacterial strains compared to the hot and cold water extracts, which showed almost no inhibition.

Antineurodegenerative activities of extracts

Cholinesterase inhibition is a commonly used approach for treating the symptoms of Alzheimer disease (AD), which is a widely distributed neurological disorder. Various plants could be employed as a new strategy in the treatment of neurodegenerative diseases, inclu-

Tab. 4: Correlation between antioxidant assays and content of phenolic components in *O. majorana* extracts

	DPPH assay	ABTS assay	FRAP assay	β-CB assay
Rosmarinic acid	-0.03[a]	0.56[b]	0.12[a]	0.07[a]
Caffeic acid	-0.36[b]	-0.26[a]	0.31[a]	0.48[b]
Chlorogenic acid	-0.72[c]	0.16[a]	0.65[b]	0.41[b]
Arbutin	0.23[a]	0.53[b]	0.05[a]	0.07[a]
Luteolin-7-*O*-glucoside	-0.44[b]	0.10[a]	0.37[b]	0.09[a]
Total phenolic content	-0,74[c]	0.95[c]	0.89[c]	-0.03[a]
Flavonoid content	0,08[a]	0.61[b]	0.03[a]	-0.35[a]
DPPH assay	1.00	-0.59[b]	-0.82[c]	-0.10[a]
ABTS assay		1.00	0.79[c]	-0.13[a]
FRAP assay			100	0.06[a]
β-CB assay				1.00

According to Taylor (1990): [a]$r \leq 0.35$ weak correlation; [b]$0.36 < r < 0,67$ moderate correlation; [c]$0.68 < r < 1$ strong correlation.

Tab. 5: Antibacterial activity of *O. majorana* extracts presented as MIC (mg/mL) and MBC (mg/mL)

		Gram-positive bacteria						Gram-negative bacteria							
Sample origin	Extracts	*B. subtilis*		*E. faecalis*		*L. innocua*		*S. aureus*		*E. coli*		*P. aeruginosa*		*S. flexneri*		*S. enteritidis*	
		MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
Serbia	Aqueous	≤ 0.625	0.625	≤ 5	5	≤ 2.5	2.5	-	-	> 5	> 5	> 5	> 5	≤ 2.5	2.5	≤ 5	5
	Ethanolic	≤ 0.625	0.625	≤ 5	5	0.625	1.25	0.625	1.25	> 5	> 5	> 5	> 5	> 5	> 5	> 5	> 5
Greece	Aqueous	≤ 1.25	1.25	5	≥ 5	> 5	> 5	≤ 5	5	> 5	> 5	> 5	> 5	≤ 2.5	2.5	≤ 5	5
	Ethanolic	≤ 2.5	2.5	> 5	> 5	1.25	2.5	1.25	2.5	> 5	> 5	> 5	> 5	5	≥ 5	> 5	> 5
Egypt	Aqueous	≤ 1.25	1.25	-	-	0.625	1.25	≤ 5	5	> 5	> 5	> 5	> 5	2.5	≥ 2.5	≤ 5	5
	Ethanolic	≤ 2.5	2.5	> 5	> 5	1.25	2.5	0.625	1.25	> 5	> 5	> 5	> 5	5	≥ 5	> 5	> 5
Libya	Aqueous	≤ 1.25	1.25	≤ 2.5	2.5	0.625	1.25	1.25	2.5	> 5	> 5	> 5	> 5	1.25	2.5	≤ 5	5
	Ethanolic	≤ 2.5	2.5	> 5	> 5	1.25	2.5	1.25	2.5	> 5	> 5	> 5	> 5	5	≥ 5	> 5	> 5
Streptomycin*		≤ 3.125	3.125	≤ 200	200	≤ 25	25	≤ 1.532	3.125	≤ 12.5	12.5	25	50	≤ 1.562	1.562	≤ 3.125	3.125
Rifampicin*		-	-	≤ 3.125	50	≤ 3.906	7.812	-	-	-	-	-	-	-	-	-	-

* MIC and MBC of antibiotics were presented as μg/mL.

ding several Lamiaceae species (Perry et al., 2000; Orhan et al., 2010, 2012).

O. majorana extracts were tested for the inhibition of acetylcholinesterase and tyrosinase (Tab. 6 and 7). Analyzed aqueous extracts showed higher percentage of acethylcholinesterase inhibition than the ethanolic ones, with the exception of aqueous and ethanolic extracts of Serbian marjoram (Tab. 6). Aqueous and ethanolic extracts of the Greek plant showed the highest percentage of AChE inhibition (22.80-37.29%), but lower than galanthamine (42.38-57.11%). The obtained results were not concentration dependent, contrary to used standard. The Egyptian (Mossa and Nawwar, 2010) and Tunisian (Hajlaoui et al., 2016) marjoram essential oils showed significant antiacethylcholinesterase activity, but extracts were rarely examined. Chung et al. (2001) in screening of 139 spices have obtained the highest inhibitory effect of marjoram ethanol extract, finding the ursolic acid as an active component and potent AChE inhibitor. Ali-Shtayeh et al. (2014) detected much higher inhibition of AChE for ethanolic extract of Palestinian marjoram, while methanolic extract of Iranian *O. majorana* showed significantly lower inhibition of AChE (Gholamhoseinian and Razmi, 2012), when compared to our results.

Several studies presented results for antiacethylcholinesterase activity of *O. vulgare* extracts. Vladimir-Knežević et al. (2014) tested ethanolic extracts of 26 Croatian medicinal plants for their inhibitory activity against AChE, including *O. vulgare* which showed around 30% inhibition of enzyme activity at the concentration of 1 mg/mL. Orhan et al. (2010) found 35.84% inhibition at 100 μg/mL for the acetone extract of Algerian *O. vulgare* var. *glandulosum*, while at 25 and 50 μg/mL no activity was recorded.

Tyrosinase inhibitors are of great concern due to the role of tyrosinase in mammalian melanogenesis, fruit browning, and also in production of dopamine quinone derivatives involved in the degeneration of nigrostriatal dopaminergic neurons in Parkinson's disease (Hasegawa, 2010).

The ethanolic extracts of marjoram had higher activity than the aqueous ones in testing tyrosinase inhibitory activity (Tab. 7). Among aqueous extracts, sample from Serbia showed the highest percentage of tyrosinase inhibition (7.77-16.38%), while among ethanolic extracts the highest activity was exibited by the Egyptian herb (15.11-23.09%). Mentioned extracts with the highest activities contained the highest level of arbutin, which is used as strong tyrosinase inhibitor and whitening agent in cosmetic products (Lim et al., 2009; Sarikurkcu et al., 2015). The obtained results showed dependence on the concentration of extracts. The used standard, kojic acid, showed stronger activity than the examined extracts (33.93-51.81%).

There are very few literature data on the tyrosinase inhibitory activity of *Origanum* extracts and essential oils. Lin et al. (2011) found that antityrosinase activities were 21.8% for *O. majorana* and 4.7% for *O. vulgare* from China. Significant tyrosinase inhibition activity of the methanolic extract and origanol A of the Indian *O. vulgare* was recorded by Rao et al. (2011).

Sarikurkcu et al. (2015) examined essential oils of two *O. vulgare* subspecies and obtained significantly better results for *O. vulgare* subsp. *hirtum* when compared to *O. vulgare* subsp. *vulgare*, probably due to high linalool content. Essential oil of Italian *O. vulgare* significantly inhibited mushroom tyrosinase in a dose-depended manner (Fiocco et al., 2016).

Conclusion

Literature data related to *O. majorana* bioactivities are mostly limited to essential oils, but this study showed that water and ethanolic extracts could also be promising natural sources of bioactive compounds. The aqueous extracts generally showed better antioxidant, antimicrobial and antineurodegenerative activity than the ethano-

Tab. 6: Acetylcholinesterase inhibitory activity of *O. majorana* extracts and galanthamine (standard) presented as percentage of AChE inhibition (%)

Sample origin	Aqueous extracts			Ethanolic extracts		
	25 μg/mL	50 μg/mL	100 μg/mL	25 μg/mL	50 μg/mL	100 μg/mL
Serbia	6.32±0.38^{a}	30.33±2.83^{a}	8.60±0.78^{a}	31.26±1.60^{a}	18.70±3.30^{a}	5.96±0.35^{a}
Greece	34.18±2.45^{b}	35.22±0.79^{a}	33.12±2.20^{b}	22.80±3.20^{a}	27.67±0.93^{b}	37.29±1.79^{b}
Egypt	6.43±0.09^{a}	33.33±2.17^{a}	7.64±0.41^{a}	30.07±4.91^{a}	5.31±0.33^{c}	6.44±0.18^{a}
Libya	29.03±4.13^{b}	32.02±4.66^{a}	30.77±3.57^{b}	6.53±0.48^{b}	30.18±2.15^{b}	34.88±2.84^{b}
Galanthamine	42.38±0.74^{c}	50.56±0.51^{b}	57.11±1.68^{c}			

Means followed with different letters in the same column are significantly different at $p<0.05$.

Tab. 7: Tyrosinase inhibitory activity of *O. majorana* extracts and kojic acid (standard) presented as percentage of tyrosinase inhibition (%)

Sample origin	Aqueous extracts			Ethanolic extracts		
	25 μg/mL	50 μg/mL	100 μg/mL	25 μg/mL	50 μg/mL	100 μg/mL
Serbia	16.38±2.23^{a}	15.53±0.80^{a}	7.77±1.15^{a}	16.28±3.21^{a}	15.74±0.55^{a}	7.87±0.66^{a}
Greece	15.53±1.76^{a}	13.19±3.04^{a}	7.66±1.21^{a}	17.45±3.70^{a}	15.96±2.56^{a}	9.89±1.21^{a}
Egypt	14.04±2.44^{a}	11.17±1.44^{a}	7.87±1.21^{a}	23.09±1.99^{a}	18.83±2.71^{a}	15.11±1.69^{b}
Libya	15.64±2.17^{a}	8.94±1.29^{a}	6.17±0.96^{a}	21.38±1.81^{a}	14.79±2.78^{a}	11.38±1.95^{a}
Kojic acid	35.73±5.46^{b}	33.93±3.78^{b}	51.81±2.55^{b}			

Means followed with different letters in the same column are significantly different at $p<0.05$.

lic ones, with the exception of the Egyptian sample in some tests. Among tested marjoram samples originated from Serbia, Greece, Egypt and Libya, the most promising were plants from Serbia and Egypt, which contained high level of rosmarinic acid. This acid was found to be the predominant constituent in investigated extracts and presumably had a substantial influence on their antioxidant and antineurodegenerative activities.

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Address of the corresponding author:
E-mail: alimpic.ana@bio.bg.ac.rs

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