Total phenolic content, antioxidant capacity, and antimicrobial activity of *Origanum heracleoticum* L., extracted with different solvents

Jovana D. Ickovski[1*], Ivan R. Palić[1], Aleksandra S. Đorđević[1], Vesna P. Stankov Jovanović[1], Violeta D. Mitić[1], Gordana S. Stojanović[1]

1- University of Niš, Faculty of Sciences and Mathematics, Department of Chemistry, Višegradska 33, 18000 Niš, Serbia

ABSTRACT

This research was based on the examination of the total phenolic content, antioxidant and antimicrobial activities of hexane, diethyl ether, ethyl acetate, and methanol extracts of *Origanum heracleoticum* L. grown in Serbia. The antimicrobial activity was determined against five bacteria and two fungi using the disk diffusion method. The total phenolic content of *O. heracleoticum* solvent extracts was determined and five different tests were used for screening of the antioxidant capacity. The highest total phenolic content was found in ethyl acetate extract (848.48 µg GAE/mg dry extract) and methanol extract (733.43 µg GAE/mg dry extract). The examination of antioxidant activity showed that methanol and ethyl acetate extracts had the strongest activity. The highest correlation was found between DPPH and FRAP ($R^2 = 0.99$), as well as DPPH and CUPRAC ($R^2 = 0.96$) assays. The ABTS test was highly correlated with the FRAP test ($R^2 = 0.95$). The antimicrobial assay proved that each extract had an effect against all bacteria and fungi, except against the bacterium *Pseudomonas aeruginosa*. The highest antibacterial activities were found for methanol extract and ethyl acetate extract against *Staphylococcus aureus*. The highest antifungal activity was observed for the ethyl acetate extract against both *Candida albicans* and *Aspergillus brasiliensis*.

Keywords: Origanum heracleoticum, total phenolic content, antioxidant capacity, antimicrobial activity

[*] Corresponding author: jovana.ickovski@pmf.edu.rs

Introduction

One of the members of the Lamiaceae (Labiatae) family of plants is the genus *Origanum. Origanum* has been largely used in traditional medicine. A strong interest is devoted to it, due to the scientific results, which demonstrate its effectiveness as a source of antioxidant and antimicrobial principles (Carrubba & Calabrese, 1998). The most commonly found oregano species belong to the botanical genus *Origanum*. One of the representatives of this genus is a species *Origanum heracleoticum* L. (syn.: *O. hirtum* L., *O. creticum* Sieber and Bentham, *O. vulgare* L. subsp. *hirtum* (Link) Ietswaart) (Bocchini et al., 1998; Exarchou et al., 2002; Ozcan & Akgul, 1995). *O. heracleoticum* is indigenous to Mediterranean Europe from Spain to northern Balkan and Asia and it is used worldwide in large quantities as a condiment (Lawrence et al., 1974). This subspecies was systematically and widely cultivated in Greece, and it is also known by the vernacular name Greek oregano (Vokou et al., 1993). Oregano has demonstrated antioxidant activity in lipid substrates. Extracts of this herb can retard both the initiation and the rate of lipid oxidation during storage (Tsimogiannis et al., 2006). The essential oil obtained from *O. heracleoticum* plants is characterized by a high phenolic content. The large quantities of carvacrol, thymol, and their precursors, *γ*-terpinene and *p*-cymene (Poulose & Croteau, 1978) characterized all “oregano” types of essential oils (Kokkini et al., 1997). Essential oil is mainly used in the pharmaceutical and food industries (Lawless, 1996). It is also applied in aromatherapy for asthma, bronchitis, rheumatism, and digestive problems. This oil is considered to be cytotoxic (Sivropolou et al., 1996). Chemical analysis of the oregano essential oil revealed the presence of several ingredients, most of which have important antioxidant, antibacterial, and antifungal properties (De Martino et al., 2009; De Souza et al., 2009; Tsimogiannis et al., 2006; Zheng et al., 2009). Very high carvacrol content that was found in a biotype of *O. heracleoticum* is particularly relevant, since this species is known – according to the USA Cancer Chemotherapy National Service Center – to have high antitumoral activity (Baricevic, 1996). Up to now, the *O. heracleoticum* essential oil was the subject of many studies because of all above-mentioned characteristics, while there are only hints of working with Greek oregano extracts. Lamaison et al. (1990, 1993) extensively studied the antioxidant activity of members of Lamiaceae and reported that the content of rosmarinic acid and of total hydroxycinnamic derivatives in hydroalcoholic extracts of *O. vulgare* ssp. *hirtum* was only partly correlated with their antioxidant effect, estimated by measuring the

free radical scavenger effect on DPPH. The antimicrobial test results showed that the essential oil of *O. heracleoticum* has great potential of antimicrobial activity against bacteria, fungi, and yeast species and therefore can be used as a natural preservative ingredient in the food and/or pharmaceutical industry (Biondi et al., 1993). Ethanol extracts were the subject of some studies which were primarily related to the antioxidant activity of these extracts (Baycheva et al., 2020; Conforti et al., 2011) of Bulgarian flora to attenuate oxidative stress effects formed under short-term UV-B radiation.). To the best of our knowledge, this is the first report regarding the total phenolic content, antioxidant and antimicrobial activities of hexane, diethyl ether, ethyl acetate, and methanol extracts of *Origanum heracleoticum* L.

Experimental

Chemicals and reagents

Folin-Ciocalteu's phenol reagent, ABTS, 2,2-diphenyl-1-picrylhydrazyl (DPPH), $FeCl_3$, ascorbic acid, gallic acid, neocuproine, hexane, diethyl ether, ethyl acetate, and methanol were purchased from Sigma Co. (St. Louis, Missouri, USA). 6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) was purchased from Acros Organics (Morris Plains, New Jersey, USA). TPTZ (2,4,6-tripyridyl-*s*-triazine), $K_3(Fe(CN)_6)$, phosphate buffer (NaH_2PO_4-Na_2HPO_4; pH 6.6), trichloroacetic acid, HCl, acetate buffers (pH 3.6 and pH 7), Na_2CO_3, $K_2S_2O_8$, $CuCl_2x2H_2O$, and $FeSO_4x7H_2O$, were purchased from Merck (Darmstadt, Germany). All the chemicals and reagents were of analytical purity.

Plant material and plant material extraction

The plant material (*Origanum heracleoticum* L.) was collected at bloom stage on August 2013 on location Berilovac (surroundings of the town of Pirot, Serbia); a voucher specimen 7299 has been deposited in the Herbarium Moesiacum Niš (HMN), Department of Biology and Ecology, Faculty of Science and Mathematics, University of Niš, Serbia. Aerial parts of the herb were used for the experiment. Plants were dried at room temperature and then milled. The ground plant material was packed in a paper bag and kept in a dark, dry, and cool place. The dry plant material (40 g) was extracted with hexane, diethyl ether, ethyl acetate, and methanol (400 mL), by the method of maceration. The resulting mixture was kept for 72 hours in the dark, at room

temperature, with occasional shaking. The resulting extracts were filtered and concentrated on a rotary vacuum evaporator to dryness. The solutions of specified concentrations were made from dried residues for the determination of antioxidant and antimicrobial activity.

Determination of antioxidant activity

Dry extracts were dissolved in methanol (1:1) and the resulting solutions were used for testing the antioxidant activity. All spectrophotometric measurements of the antioxidative potential of *O. heracleoticum* L. extracts were performed on a UV-visible spectrophotometer Perkin Elmer lambda 15 (Massachusetts, USA). Experiments for all assays listed below were performed in three independent repetitions. The mean values of the measurements are presented.

Determination of total phenolic content (TPC)

The total phenolic content of the extracts was determined spectrophotometrically using Folin–Ciocalteu reagent according to the method of Singleton (Singleton et al., 1999). A specified volume of the extract was mixed with 0.625 mL of Folin–Ciocalteu reagent and 2.5 ml of sodium carbonate solution (20%) and the reaction mixture was diluted with distilled water to a total volume of 10 mL. The mixture was shaken and stored in the dark for 30 min. Subsequently, the coloration of the samples was measured at an absorbance of 760 nm using a spectrophotometer. Gallic acid was used as the standard for the calibration curve. The total phenolic content was expressed as gallic acid equivalent per milligram of dry extract weight (μg GAE/mg dw).

2,2-Diphenyl-1-picrylhydrazyl radical scavenging activity (DPPH)

Free radical scavenging activity was determined using the stable 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical according to the method of Hatano (Hatano et al., 1988). The appropriate amounts of extracts were mixed with 2.5 mL of DPPH solution and methanol was added to a total volume of 10 mL. The mixtures were stirred and left to stand in the dark for 1h. The absorbance of the solution was measured at 515 nm. Trolox was used as standard. The free radical capacity toward DPPH radical was determined based on the Trolox calibration curve and the results were expressed as micrograms of Trolox equivalents per milligram of dry extract weight (μg TE/mg dw).

ABTS$^{•+}$ radical cation decolorization assay (ABTS)

The method used for the determination of the antioxidant free radical capacity was the ABTS$^{•+}$ (radical cation) decolorization assay (Re et al., 1999). A stock solution of ABTS$^{•+}$radical cation was prepared by mixing ABTS solution and potassium persulfate solution (1:1). The mixture was maintained in the dark at room temperature for 12 h before use. The working ABTS$^{•+}$solution was produced by dilution of the stock solution in methanol to achieve an absorbance value of 0.7 ±0.02 at 734 nm. An aliquot of diluted extract was poured into ABTS$^{•+}$ working solution (1.8 mL) and methanol was added to a volume of 5 mL. Methanol was used as a referent solution and for the standard calibration curve, Trolox solution was utilized. Absorbance was measured using UV-visible spectrophotometer at 734 nm immediately after addition and mixing and then every minute for 6 min. The radical scavenging capacity of extracts was quantified as micrograms of Trolox equivalents per milligram of dry extract weight (μg TE/mg dw).

Ferric reducing antioxidant power (FRAP)

The ferric reducing antioxidant power (FRAP) assay was based on the methodology of Benzie and Strain (Benzie & Strain, 1996). FRAP reagent was prepared by combining TPTZ with ferric chloride and acetate buffer (1:1:10). The samples were mixed with FRAP reagent (1.0 mL) and water was added to a total volume of 5 mL. Solutions stood for 5 min at 37°C and the absorbance was determined at 595 nm, using methanol as a referent solution. Ferrous ion solution was used as a standard for the calibration curve and FRAP values were expressed as micromoles Fe^{2+} per mg of dry extract weight (μmol Fe/mg dw).

Total reducing power (TRP)

The capacity of extracts to reduce iron (III) to iron (II) was determined according to the method of Oyaizu (Oyaizu, 1986). Reducing power of the extracts was expressed by reducing power of ascorbic acid as a standard antioxidant (AEAC- Ascorbate Equivalent Antioxidant Capacity). The sample (1 mL) was mixed with 2.5 mL of potassium hexacyanoferrate III (1%) and 2.5 mL of phosphate buffer (pH 6.6). After 30 min of incubation at 50 ºC in the dark, 2.5 mL trichloroacetic acid (10%) and 1.5 mL ferric chloride was added. The total volume with the addition of water was 10 mL. The absorbance was measured at 700 nm in a spectrophotometer. Negative (methanol) and positive (ascorbic acid) control reactions were performed, to plot the

absorbance of ascorbic acid against concentration. The results were quantified as mg of ascorbic acid per mg of dry extract weight (mg AAE/mg dw).

Cupric reducing antioxidant capacity assay (CUPRAC)

Cupric reducing antioxidant capacity assay was used for determining antioxidants and hydroxyl radical scavengers, according to the method of Apak (Apak et al., 2004). The reaction mixture was prepared by mixing a specified volume of the extract, 1 mL of neocuproine solution, 1 mL of ammonium acetate buffer (pH 7), 1 mL of copper (II) chloride solution, and distilled water to a total volume of 5 mL. After staying at room temperature for 30 min, the absorbance of the solution was measured at a wavelength of 450 nm. In the same manner, the absorbance of a series of Trolox solutions was determined and the obtained results were used for drafting the calibration curve. The results of the CUPRAC method were expressed as Trolox equivalent per milligram of dry extract weight (mg TE/mg dw).

Antimicrobial activity

The *in vitro* antimicrobial activities of hexane, diethyl ether, ethyl acetate, and methanol extracts of *O. heracleoticum* L. were tested against a panel of laboratory control strains belonging to the American Type Culture Collection (ATCC; Gaithersburg, Maryland, USA) except *S. abony*, belonging to National Collection of Type Cultures (NCTC, Public Health England, London, United Kingdom). Antibacterial activity was evaluated against two gram-positive and three gram-negative bacteria. The gram-positive bacteria used were *Bacillus subtilis* (ATCC 6633) and *Staphylococcus aureus* (ATCC 6538). The gram-negative bacteria utilized in the assay were: *Escherichia coli* (ATCC 8739), *Pseudomonas aeruginosa* (ATCC 9027), and *Salmonella abony* (NCTC 6017). The antifungal activity was tested against two fungal organisms *Aspergillus brasiliensis* (ATCC 16404) and *Candida albicans* (ATCC 10231). Fresh suspensions of microorganisms were prepared from cultures of microorganisms that were kept on agar slants. Extract solutions were made by dissolving 100 mg of dry extract in 1 ml of methanol and the resulting solutions were stored in sealed vials.

Disc-diffusion method

A disc-diffusion method was employed for the determination of the antimicrobial activity of the extracts, according to National Committee for Clinical Laboratory Standards (NCCLS, 1997). The inoculates of the bacterial and fungal strains were prepared from overnight broth cultures and suspensions were adjusted to 0.5 McFarland standard turbidity. A volume of 100 µL of the suspension containing 1.0×10^8 CFU/mL of bacteria and 1.0×10^4 CFU/mL of fungal spores spread on Mueller-Hinton agar (Torlak, Serbia) and Sabouraud dextrose agar (Torlak, Serbia) respectively, in sterilized Petri dishes (90 mm in diameter) making the 4 mm layer. The discs (12.7 mm in diameter, "Antibiotica Test Blattchen"-Schleicher and Schull, Dassel, Germany) were impregnated with 50 µL of extracts (conc. 100 mg/mL) and placed on the inoculated agar. Negative controls were prepared using methanol. Chloramphenicol (30 µg, Torlak), cefalexin (30 µg, Torlak), and Nystatin (30 µg, Torlak) were used as positive reference standards to determine the sensitivity of a strain of each tested microbial species. The inoculated plates were kept at 4°C for 2 h and incubated at 37°C (24 h) for bacterial strains and at 28°C (48 h) for fungal strains. The antimicrobial activity was evaluated by measuring (in millimeters) the zone of inhibition against the test microorganisms using the appliance "Fisher-Lilly Antibiotic Zone Reder" (Fisher Scientific Co., USA). All microorganisms were completely insusceptible to the control discs imbued with methanol (negative control). The antimicrobial assay was performed in triplicate and the mean values were reported.

Statistical analysis

To examine the interrelationships between the investigated samples, the results were mutually correlated using cluster and linear regression analyses. Cluster Analyses (CA) were carried out with the data of total phenolic content and antioxidant activity to identify various groups. The CA produced a dendrogram using Ward´s method of hierarchical clustering, based on the Euclidean distance between assays. All computations were done using the Statistica 8 software (StatSoft, Tulsa, Oklahoma, USA).

Results and Discussion

The total phenolic content of hexane, diethyl ether, ethyl acetate, and methanolic extracts, calculated from the calibration curve (R^2 = 0.9951), expressed as µg gallic acid equivalents/mg is presented in Table 1. The highest total phenolic content was in diethyl ether and methanolic extract (848.5 µg GAE/mg and 733.4 µg GAE/mg, respectively). Higher phenolic content in those extracts, compared to those of hexane and diethyl ether extracts can be a consequence of the different polarity of the extraction solvent (Zhou & Yu, 2004). The analysis of the antioxidant activity of *O. heracleoticum* was performed using five methods: DPPH, ABTS, TRP, FRAP, and CUPRAC, which are distinguished by their mechanism of action. The results of the analysis, represented in Table 1, can vary as a result of the solvent nature and particularly methods of analysis. Results of *in vitro* antioxidant tests give the basic impression of the relative antioxidant activity of *O. heracleoticum* extracts. In general, methanolic and ethyl acetate extracts have higher antioxidant activity than hexane and diethyl ether extracts. This could be the result of a higher total phenolic content in those extracts.

Table 1. Total phenolic (TPC) content, and antioxidant activity (DPPH, ABTS, TRP, FRAP and CUPRAC assays) of *O. heracleoticum* solvent extracts

Extract	**TPC** (µg GAE/mg)	**DPPH** (µg TE/mg)	**ABTS** (µg TE/mg)	**TRP** (mg AAE/mg)	**FRAP** (µmol Fe/mg)	**CUPRAC** (mg TE/mg)
Hexane	532.10	23.40	104.77	49.04	110.32	125.26
Diethyl ether	568.05	37.59	105.13	49.53	151.58	147.28
Ethyl acetate	848.48	47.60	105.48	50.03	189.47	155.92
Methanol	733.43	53.44	106.56	59.44	225.68	155.32

Statistical analysis

Results of the correlation between the total phenolic content and antioxidant activities of *O. heracleoticum* extracts are presented in Table 2. The highest correlation was found between DPPH and FRAP (R^2 = 0.99), as well as DPPH and CUPRAC (R^2 = 0.96) assays and consequently between FRAP and CUPRAC (R^2 = 0.90) tests. The ABTS test was highly correlated with the FRAP test (R^2 = 0.95). The ABTS method also showed a high correlation with the DPPH test (R^2 = 0.89), as well as with the TRP assay (R^2 = 0.95). Such a high correlation may be explained by the fact that all four assays (DPPH, FRAP, ABTS, and CUPRAC) are electron transfer methods. Minimum interdependence was observed between the total phenolic content and the total reducing power assay (R^2 = 0.35), which can be explained by the fact that phenols are not the only class of

compounds that may affect the total reduction potential of the examined extracts. The correlation coefficients of TPC with DPPH and CUPRAC assays are 0.82 and 0.80, respectively, suggesting that phenolic compounds are likely to contribute to the radical scavenging activity of the plant.

Table 2. Correlation coefficients between TPC and the results of antioxidant assays (DPPH, ABTS, TRP, FRAP, CUPRAC)

	TPC	DPPH	ABTS	FRAP	TRP	CUPRAC
TPC	1.00	0.82	0.59	0.78	0.35	0.80
DPPH		1.00	0.89	0.99	0.71	0.96
ABTS			1.00	0.95	0.95	0.73
FRAP				1.00	0.81	0.90
TRP					1.00	0.51
CUPRAC						1.00

Cluster analysis was used to classify *O. heracleoticum* extracts (objects) based on a set of measured antioxidant activities (variables) into several different groups (clusters). In the case of agglomerative hierarchical cluster analysis, a dissimilarity matrix was used. The elements of this matrix were the Euclidean distances as the measure of similarity/dissimilarity and complete linkage as the agglomerative method. To obtain clusters, Ward's method was used. This agglomerative method considers each sample as a separate cluster, there are as many clusters as samples and then combines the clusters sequentially, reducing the number of clusters at each step until only one cluster is left. The clusters are linked at increasing levels of dissimilarity (Rencher, 2003). The linkage distance is shown as D_{link}/D_{max}, which represents the quotient between the linkage distances for a particular case divided by the maximal linkage distance.

The dendrogram of the AHC analysis, with two statistically different classes, is given in Fig. 1. According to this, FRAP, CUPRAC, and ABTS (the same cluster) are mutually similar while TRP and DPPH form separate classes. The results of the correlation analysis to some extent coincide with the results of the AHC analysis in the part of good correlation between FRAP and ABTS, that is, FRAP and CUPRAC tests, while there are noticeable discrepancies between these two statistical methods in the part of the correlation of ABTS and CUPRAC, as well as DPPH and TRP methods.

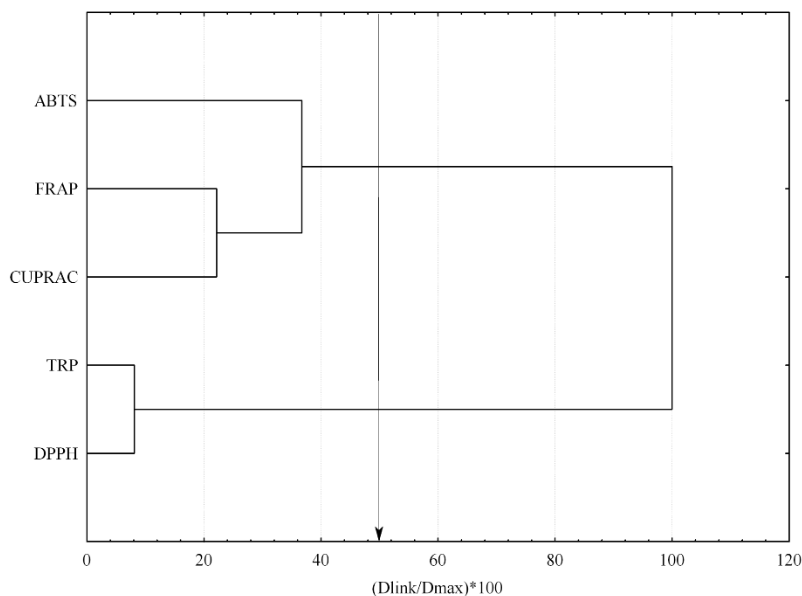

Figure 1. Dendrogram obtained by agglomerative hierarchical clustering of antioxidant potential (determined by five different methods: DPPH, ABTS, TRP, FRAP, and CUPRAC) of four *O. heracleoticum* solvent extracts

The antimicrobial activity of the solvent extracts against five bacteria and two fungi species was assessed using the disc-diffusion method. The results are presented in Table 3. The studied samples showed good antimicrobial potential, none of the samples exhibited very low activity against tested microorganisms, except against *P. aeruginosa*; none of the extracts showed any activity against *P. aeruginosa*. All samples showed almost equal activity against *E. coli*. The same was observed with extracts against *S. abony*, as well. Only the ethyl acetate extract was slightly more active against *S. abony* in comparison to other samples. In the case of Gram-positive bacteria, all examined extracts exhibited strong activity against *S. aureus*, methanol extract and ethyl acetate extract evinced the highest activity. All tested samples (5 mg of each) showed different diameters of inhibition zone against *B. subtilis*. The highest value was measured for methanol extract. Anti-*C. albicans* activity of the extracts was very high, the highest was for ethyl acetate extract. Also, all tested samples, except hexane extract, showed significantly high anti-*A. brasiliensis* activity; ethyl acetate extract was again the most active one.

Table 3. The results of the antimicrobial activity testing (inhibition zones presented in millimeters including disk diameter)

Extract	Gram-negative bacteria			Gram-positive bacteria		Fungi	
	E. coli	*S. abony*	*P. aeruginosa*	*S. aureus*	*B. subtilis*	*C. albicans*	*A. brasiliensis*
Hexane extract	21	19	/	50	25	36	17
Diethyl ether extract	21	20	/	60	27	38	35
Ethyl acetate extract	20	25	/	65	31	50	47
Methanol extract	24	19	/	65	35	42	44
Methanol	/	/	/	/	/	/	/
Cephalexin	19	18	/	36	39	nt	nt
Chloramphenicol	22	25	12	30	29	nt	nt
Nystatin	nt	nt	nt	nt	nt	18	17

/ - not detected, nt - not tested

Conclusion

The highest total phenolic content was found in ethyl acetate extract and methanol extract. Higher phenolic content in those extracts, compared to those of hexane and diethyl ether extracts is a consequence of different solvent polarity. The examination of antioxidant activity showed that methanol and ethyl acetate extracts had the strongest activity, and this is the result of a higher total phenolic content in those extracts, than in hexane and diethyl ether extracts. Anti-*C. albicans* activity of the extracts was very high, the highest was for ethyl acetate extract. All tested samples, except hexane extract, showed significantly high anti-*A. brasiliensis* activity. To the best of our knowledge, this is the first report regarding total phenolic content, antioxidant and antimicrobial activities of hexane, diethyl ether, ethyl acetate, and methanol extracts of *Origanum heracleoticum* L.

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