

FACTA UNIVERSITATIS

Series: Physics, Chemistry and Technology Vol. 12, N° 1, 2014, pp. 47-54

DOI: 10.2298/FUPCT1401047V

TOTAL PHENOL, FLAVONOID AND HEAVY METAL CONTENT AND ANTIOXIDANT ACTIVITY OF SOLVENT EXTRACTS OF *ORIGANUM VULGARE L.*

UDC 635.714 :615.074

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Abstract. *Water, ethanol and water-ethanol (1:1, v/v) extracts of the (ethno)pharmacologically valued plant species *Origanum vulgare L.* were studied for the total phenol (TP), flavonoid (TF) and heavy metal (HM; Zn, Fe, Cu, and Mn) content. All three prepared extracts contained a rather high amount of phenolics (23.6-75.2 mg gallic acid equivalents/g) and a low amount of flavonoid compounds (1.0-2.1 mg quercetin equivalents/g). In addition, the content of (toxic) metals (Zn, Fe, Cu, and Mn) was low (0.4-153 mg/kg). Antioxidant potential of the extracts was determined using a DPPH assay and correlated with TP, TF and HM. According to the obtained results, the high observed radical scavenging activity (75.1-93.8%) is mostly related to the presence of flavonoid and other phenolic compounds.*

Key words: *Origanum vulgare L., phenols, flavonoids, heavy metals, antioxidant activity*

1. INTRODUCTION

Scientific interest toward medicinal plants has increased in the last few decades, even in the well-developed European countries and America. The pharmaceutical industry still largely relies on medicinal herbs or compounds isolated from them [1]. Polyphenolic plant secondary metabolites have been shown to possess a number of beneficial pharmacological properties, one of the most important being antioxidant activity. It is known that free radicals attack the highly unsaturated fatty acid membrane systems (especially that in the spinal cord and brain) and induce lipid peroxidation, which is a key process in many pathological conditions and one of the reactions that cause oxidative stress. Moreover, brain contains significant amounts of transitional pro-oxidant metals and consumes enormous

Received May 13th, 2014; revised June 27th, 2014; accepted August 8th, 2014.

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quantity of oxygen. These features facilitate the formation of oxygen radicals involved in the aging processes, Alzheimer's and Parkinson's diseases, ischemic heart damage, arthritis, myocardial infarction, arteriosclerosis and cancer. Phenolic antioxidants (e.g. flavonoids) stop free oxygen radicals and free radicals formed from the substrate by donating hydrogen atoms or electrons. Investigations of plant polyphenolic secondary metabolites intensified after discovery that some commercial synthetic antioxidants have toxic, mutagenic and carcinogenic properties [2]. Many aromatic plantspecies have been screened for antioxidant and antiradical potential [3].

Plant species belonging to plant family Lamiaceae, among which is oregano (*Origanum vulgare* L.), are known to possess a number of beneficial therapeutic properties (diaphoretic, carminative, antispasmodic, antiseptic, tonic) and are widely used in traditional medicine in numerous countries [4-7]. In addition, they are extensively exploited in agricultural, pharmaceutical and cosmetic industries, as a culinary herb, flavoring substances in food products, alcoholic beverages and in perfumery [8]. It has been proven that the solvent extracts of oregano have rather high antioxidant potential [9]. Its antioxidant properties are mainly attributed to the presence of rosmarinic and caffeic acids [10-12].

Nonetheless, despite beneficial medicinal properties of a number of plants' metabolites, prolonged intake of certain botanical drugs can cause health problems. These are often due to the presence of elevated amounts of heavy metals [13-17]. Plant species can be easily contaminated with heavy metals during cultivation or plant material processing, and, unfortunately, they can easily accumulate them. On the other hand, some of these elements are necessary for the normal growth and functioning of human body, and thus, they have to be included in everyday diet.

Having all mentioned above in mind, and knowing that chemical composition of botanical drugs can significantly vary (different chemotypes, populations, environmental factors, storage conditions, etc.), we have decided to determine content of total phenols, flavonoids and heavy metals (Zn, Fe, Cu, and Mn) in different plant extracts prepared from dry flowers of *O. vulgare* collected in SE Serbia. In addition, we decided to investigate antioxidant properties of mentioned extract and try to correlate them with plant's chemical composition (total phenols, flavonoids and heavy metals).

2. MATERIALS AND METHODS

2.1. Chemicals and reagents

DPPH (1,1-diphenyl-2-picrylhydrazyl), quercetin and aluminium chloride were purchased from Sigma Aldrich (St. Louis, MO, USA). Folin-Ciocalteu's phenol reagent and sodium carbonate, sodium chlorate buffer (pH 1.0) and acetate buffer (pH 4.5) were purchased from Merck (Darmstadt, Germany). All used chemicals, including solvents, were of analytical grade. The working solutions were prepared immediately before the analysis from the basic stock solution (concentration 1000 mg/L for all metals). For the preparation of standard solutions high purity Milli-Q water was used. The glassware and polyethylene containers used for analysis were washed with tap water, then soaked over the night in 6 M HNO₃ solution and rinsed several times with ultra-pure water to eliminate possible errors due to absorbance of detergent molecules.

2.2. Plant material

Plant material originating from a wild-growing *Origanum vulgare* L. (Lamiaceae) population (flowering phase) was collected in July, 2013, in the rural area in Southeast Serbia (vicinity of the city of Niš). Sample sites were selected in accordance with the methods used in the European moss monitoring project [18]: a minimal distance to major roads and larger settlements was 300 m, a minimal distance to minor roads and houses was 100 m and a minimal distance to forest roads was 5 m.

2.3. Preparation of herbal extracts

Three batches of dried *O. vulgare* (2 g each) flowers, cut into small pieces, were extracted with water, ethanol or water/ethanol (50:50, v/v) in an ultrasonic bath. The extraction was carried out three times, using 30/20/20 mL of appropriate solvent. The duration of a single extraction run was 15 min. The extracts were filtered off, transferred into a volumetric 100 mL flask and the same solvent was added to the mark.

2.4. Determination of heavy metals

The determination of content of heavy metals in dry plant material was determined according to the previously published procedure [19]. Accurately weighed dried plant material sample (2 g) was transferred into a silica crucible and kept in a muffle furnace for ashing at 450 °C for 3 h. After that, 5 mL of 6 M HCl was added to the crucible. Care was taken to ensure that all ash came into contact with acid. The crucible containing acid solution was kept on a hot plate and digested to obtain a clear solution. The final residue was dissolved in 0.1 M HNO₃ solution and made up to 50.0 mL. Working standard solutions were prepared by diluting the stock solution with 0.1 M nitric acid in order to check the linearity.

Atomic absorption measurements were made using a Varian Spectra AA 10 with background correction and hollow cathode lamps. Air-acetylene flame was used for determination of all the elements. The calibration interval, wavelength, slit, and detection level are given in Table 1. The same method (AAS) was also used to determine the contents of selected metals in studied plant extracts.

Table 1 Working conditions of AAS apparatus

Metal	Calibration range (mg/L)	Limit of detection (LOD) (mg/L)	Working wave length (nm)	Slit (nm)	Acetylene flow rate (L/min)
Fe	0.00-10.00	0.015	248.3	0.2	2.0
Cu	0.00-1.00	0.007	324.8	1.0	1.8
Zn	0.00-5.00	0.021	213.9	0.5	2.0
Pb	0.00-1.00	0.002	217.0	1.0	1.6
Cd	0.00-1.00	0.003	228.8	0.5	2.0
Mn	0.00-2.00	0.005	279.5	0.2	1.8
Ni	0.00-1.00	0.002	232.0	0.2	2.0

2.5. Determination of the total phenols

The total phenols were determined by the modified Folin-Ciocalteu method [20]. An aliquot of the extract (1 mL) was mixed with 0.5 mL of Folin-Ciocalteu reagent and 1.5

mL of sodium carbonate solution (20%). Tubes were shaken for 15 s and allowed to stand at 40.0 °C for 30 min in order to develop the color. Absorbance was measured at 765 nm using Hewlett Packard UV-VIS spectrophotometer. Total phenol content was expressed as mg of gallic acid equivalent (GAE) per g of dry sample. The result of each assay was obtained from three parallel determinations.

2.6. Determination of the total flavonoid content

The total flavonoids were determined spectrophotometrically, using method based on the formation of yellow colored complex of flavonoids with aluminum(III) chloride [21]. A volume of 0.5 mL of 2% AlCl₃ methanol solution was added to 0.5 mL of sample solution. The mixture was kept one hour at room temperature. After that, the absorbance was measured at 420 nm. Total flavonoid content was expressed as concentration of quercetin (mg QE/g); the calculation was based on the equation derived from the calibration curve.

2.7. Free radical scavenging activity

The free radical scavenging activity of the plant extracts was determined using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) method [22, 23]. The antioxidant assay is based on the change of absorbance at 517 nm, caused by the reaction of DPPH with the tested sample. The reaction was monitored using a UV-VIS spectrophotometer. Appropriate plant extract solution (0.2 mL) and 1.8 mL of freshly prepared DPPH solution in methanol (20 mg/dm³) were placed into a cuvette. After 20 min of incubation at room temperature, the absorbance at 517 nm was read against a blank probe. All measurements were performed in triplicates. Radical scavenging activity (RSC;%) of each of the studied plant extracts was calculated using following equation:

$$\text{RSC}(\%) = \frac{1 - A_s}{A_b} \cdot 100 \quad (1)$$

Where A_b is the absorbance of control reaction, and A_s is the absorbance of the tested sample.

2.3. Statistical analysis

The experimental results were expressed as mean value ± standard error of mean value of three replicates. In order to estimate if there were statistically significant difference among mean values, where applicable, the data were subjected to a one-way analysis of variance (ANOVA test) [24].

3. RESULTS AND DISCUSSION

In this study we have investigated the content of total phenols, flavonoids and heavy metals (Fe, Zn, Cu, and Mn), and antioxidant activities of the *O. vulgare* population from Southeast Serbia. The obtained results are summarized in Table 2.

Table 2 Total phenols, flavonoids, heavy metals and antioxidant activities of *Origanum vulgare* L. extracts

Extract	Total phenols content ^a	Flavonoid content ^b	Antioxidant activity	
			RSC (%)	RSC ^c
Water	74.3±1.2	2.1±0.0	75.1±0.4	6.0±0.1
Ethanol	23.6±0.4	1.0±0.0	93.8±0.4	0.8±0.1
Ethanol-water (50/50, v/v)	75.2±0.6	2.0±0.0	87.2±1.1	2.6±0.0
Extract	Fe (mg/kg)	Zn (mg/kg)	Cu (mg/kg)	Mn (mg/kg)
Plant	152±3	49.6±1.0	23.9±0.5	21.8±0.4
Water	-	29.1±0.6	4.5±0.1	-
Ethanol	113±2	22.3±0.4	3.8±0.1	16.3±0.3
Ethanol-water (50/50, v/v)	-	22.0±0.4	0.4±0.1	-

^aExpressed as mg of gallic acid per g of dry sample (mg GAE/g); ^bExpressed as mg of quercetin per g of dry sample (mg QE/g); ^cExpressed as mg of quercetin per g of dry sample (mg QE/g).

The content of the total phenols in *O. vulgare* water, ethanol and water/ethanol extracts was determined using Folin-Ciocalteu method and was expressed as mg GAE/g of dry sample. Total phenols content ranged from 23.6 mg to 75.2 mg GAE/g of dry sample. The highest content of phenols was present in water/ethanol extracts, Table 2 (75.2 mg GAE/g of dry sample). Pharmacologically speaking, the total phenol content of a plant species is its important property. In the case of medicinal and feeding herbs, the concentration of phenols is usually in the range from 0.23 to 17.51 mg GAE/g of dry plant sample. According to the previously published literature, species from the genus *Origanum* are contributed with the rather high content of total phenols (around 20 mg GAE/g dry sample) and good capacity of the absorption of oxygen radicals [25]. Nonetheless, here in presented results suggests even higher content of phenolic compounds in oregano. This is not completely surprising, taking into account the results of numerous previous studies, which revealed that the content of polyphenols depends on genotype, soil conditions and the difference in plants' ripening [26]. Also, outdoor conditions, like altitude, light, temperature, the content of feeding materials in soil can have an effect on the phenylpropanoids metabolism [27].

The content of total flavonoids, expressed in the form of mg of quercetin equivalent (QE) per gram of dry sample, in the studied *O. vulgare* extracts was much lower than the phenol content, Table 2. The content of flavonoids was comparable in water and water/ethanol extracts. The lowest flavonoid content was found in ethanol extract. This is not surprising, taking into account high solubility of phenols, flavonoids and their glycosides in water.

Antioxidant activity of *O. vulgare* extracts was determined using DPPH method. The results are given in percent and as quercetin-equivalents (QE) (as concentration of solution of quercetin, which shows identical activity to that of the sample in question). As expected, all of the studied extracts had strong scavenging activity, ranging from 75.1 to 93.8%. DPPH radical scavenging abilities of the investigated extract confirmed that these contain molecules with proton-donating ability and may serve as free radical inhibitors or scavenger (possible primary antioxidants). Herein obtained results regarding content of total phenols, flavonoids and antioxidant activity of the investigated *O. vulgare* extracts are in general agreement with the previous ones.

The presence of metals in plants is result of metals transfers from soil, water and atmospheric precipitation during growing. The presence of heavy metals in the plant (organic) solvent extracts can be explained by the possible formation of appropriate complexes between metal ions and plant metabolites. Contents of metals in extracts are generally lower compared

to that in plant tissue and depend on solvent used for extract preparation. This is in agreement with the data summarized in Table 2.

Among the studied metals, *O. vulgare* samples had the highest content of iron (152.0 mg/kg), Table 2. Iron is an essential element for humans and animals, because it is an important component of hemoglobin. Also, a lack of iron can cause gastrointestinal problems. However, large amounts of iron in the body cause damage to the intestinal tract, vomiting, diarrhea, liver damage, abdominal and joint pain, loss of body weight, fatigue, feeling of thirst and hunger, cancer, heart disorders, arthritis, osteoporosis, diabetes and various psychiatric disorders, liver cirrhosis, excessive skin pigmentation, body weakness. High concentrations of iron in plants, water and soil are result of absorption of metals from the environment.

Concentrations of Cu, Zn, and Mn were remarkably lower than that of Fe (between 21.8 and 49.6 mg/kg). Their quantities were highest in ethanol extract. Although copper is an essential element, it can be toxic for humans and animals if the concentration exceeds the permitted level. High concentrations of copper cause thyroid gland disorders de-pigmentation of skin and hair, dermatitis, upper respiratory tract damage, etc. Zinc is another essential element for humans, animals and plants. It participates in over 200 enzymatic reactions, and it is also required for the structure and normal functioning of cell membranes. Zinc takes part in the creation of connective tissue, teeth, bones, nails, hair and skin. Manganese (II) ions function as cofactors for a great number of enzymes in higher organisms, where they are essential in detoxification of superoxide free radicals. The element is a required trace mineral for all known living organisms. In larger amounts, and apparently with far greater activity by inhalation, manganese can cause a poisoning syndrome in mammals, with neurological damage which is sometimes irreversible [13, 14]. Ions of Cd, Pb and Cr were not detected in any of the investigated extracts.

The results regarding of content heavy metals in *O. vulgare*, summarized in Table 2, are in good agreement with those previously published. The content of copper in Palestinian plants varied from 7.06 to 19.19 mg/kg, and of zinc from 17.38 to 65.85 mg/kg [14]. The copper content in the black tea originating from the region of south India varies between 15.9 and 32.2 mg/kg [15]. In the black tea samples originating from the region of Iran, the copper concentration is within the range from 17.59 to 32.80 mg/kg, and in water extracts from 1.15 to 1.65 mg/kg [28]. Concentration of Fe in medicinal plant from Turkey ranges from 2.45 to 107.4 mg/kg, zinc from 3.90 to 18.00 mg/kg, and copper from 2.45 to 8.10 mg/kg [29].

In order to test for the possible correlation between the content of total phenols, flavonoids and heavy metals and antioxidant activity of the investigated extracts, we have determined corresponding correlation coefficients (Table 3).

Table 3 Correlation coefficients between the content of total phenols, flavonoids and heavy metals and antioxidant activity of *O. vulgare* extracts

	Total phenols	Total flavonoids	RSC	Fe	Zn	Cu	Mn
Total phenols	1.0	1.0	0.6	1.0	0.2	0.1	1.0
Total flavonoids	-	1.0	0.7	1.0	0.3	0.1	1.0
RSC	-	-	1.0	0.6	0.9	0.1	0.6
Fe	-	-	-	1.0	0.2	0.1	1.0
Zn	-	-	-	-	1.0	0.4	0.2
Cu	-	-	-	-	-	1.0	0.1
Mn	-	-	-	-	-	-	1.0

Generally speaking, if correlation coefficient has the value of 0.8 or higher, correlation is considered to be high; for correlation coefficient of a value between 0.5 and 0.79, correlation is substantial. Thus, content of total phenols and flavonoids have a substantial correlation with RSC. Interestingly, there is a high correlation between content of Fe and Mn and content of total phenols and flavonoids.

3. CONCLUSIONS

In this study we have shown that water, ethanol and water/ethanol extracts of flowers of oregano (*O. vulgare*; population from SE Serbia) contained high quantity of polyphenolic compounds and showed good antioxidant activities. With the exception of Fe, the contents of studied heavy metals were rather low. As expected, metal content in extracts was lower than that in plant material. There was relative strong correlation between content of total phenols/flavonoids and radical scavenging activity. Interestingly content of total phenols/flavonoids strongly correlated with content of Mn and Fe.

Herein presented results suggest investigated *O. vulgare* plant population, is suitable for the preparation of teas, spices and herbal extracts. Rather low heavy metal content and good antioxidant activity implies corresponding botanical drugs would be safe to use and would have beneficial pharmacological effects.

Acknowledgement. This research was supported by Ministry of Education, Science and Technological Development of Republic Serbia (Project 172044).

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SADRŽAJ UKUPNIH FENOLA, FLAVONOIDA I TEŠKIH METALA, KAO I ANTIOKSIDATIVNA AKTIVNOST EKSTRAKATA BILJNE VRSTE *ORIGANUM VULGARE* L.

U ovom radu su izloženi rezultati ispitivanja sadržaja ukupnih fenola (TP), flavonoida (TF) i teških metala (HM; Zn, Fe, Cu i Mn) u vodenom, etanolnom i vodenom-etanolnom (1:1, v/v) ekstraktu (etno)farmakološki cenjene biljne vrste Origanum vulgare L. U svim ispitivanim ekstraktima je utvrđeno prisustvo relativno visoke koncentracije fenolnih jedinjenja (23,6-75,2 mg ekvivalenata galne kiseline/g) i niske koncentracije flavonoida (1,0-2,1 mg ekvivalenata kvercetina/g). Pored toga, sadržaj (toksičnih) metala (Zn, Fe, Cu i Mn) je bio relativno nizak (0,4-153 mg/kg). Antioksidativni potencijal ispitivanih ekstrakata je određen DPPH metodom i korelisan sa TP, TF i HM. Na osnovu dobijenih rezultata, jaka antioksidativna aktivnost (75,1-93,8%) ekstrakata je uglavnom posledica prisustva flavonoida i drugih fenolnih jedinjenja.

Ključne reči: *Origanum vulgare* L., fenoli, flavonoidi, teški metali, antioksidativna aktivnost