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TOTAL PHENOLIC CONTENT, FLAVONOID CONCENTRATION AND ANTIOXIDANT ACTIVITY OF *Marrubium peregrinum* L. EXTRACTS

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ABSTRACT. In this study, *in vitro* antioxidant activity, total phenolic content and concentration of flavonoids of five different extracts, from the whole herb of *Marrubium peregrinum* L. (Lamiaceae) were determined using spectrophotometric methods. Antioxidant activity of extracts were expressed as percentage of DPPH radicals inhibition and IC_{50} values (µg/ml). Values in percentage ranged from 27.26 to 89.78%. The total phenolic content ranged from 27.26 to 89.78 mg/g of dry weight of extract, expressed as gallic acid equivalents. The total flavonoid concentrations varied from 18.72 to 54.77 mg/g, expressed as rutin equivalents. Methanolic exstract of *M. peregrinum* showed the highest phenolic and flavonoid concentration and strong antioxidant activity. The significant linear correlation was confirmed between the values for the total phenolic content and antioxidant activity of plant extracts. The high contents of phenolic compounds indicated that these compounds contribute to the antioxidant activity. The *M. peregrinum* can be regarded as promising candidates for natural plant sources of antioxidants with high value.

Key words: Antioxidant activity, flavonoids, *Marrubium peregrinum* L.*,* phenols

INTRODUCTION

Horehound, *Marrubium peregrinum* L. (Lamiaceae) is a perennial plant with a rectangular stem, branched in the upper part, up to 100 cm high. Rhizomes of this species are ligneous, leaves oblong, flowers grouped in loose inflorescence. It inhabits dry rocky meadows and arid sandy substrates in Europe and Middle Asia and belongs to the Pontic-Mediterranean floristic element (JOSIFOVIĆ, 1974).

Due to their notable pharmacological effects, some species of genus *Marrubium* are widely used in traditional and modern medicine for preparation of bitter tonics, treatment of digestive disorders, loss of appetite and dyspepsia (BLUMENTHAL *et al.*, 2000), easing bloating, and as nervous system stimulant. Medicinal substances from plant species of genus *Marrubium* exhibit antinociceptive (DE JESUS *et al.*, 2000), antihypertensive (EL BARDAI *et al.*, 2003), antispasmodic (RIGANO *et al.*, 2009), antioedematogenic (STULZER *et al.*, 2006), analgetic (MEYRE-SILVA *et al.*, 2005), insecticidal (PAVELA, 2004), antiinflammatory (SAHPAZ *et al.*, 2002a), antimicrobial (RIGANO *et al.*, 2006; QUAVE *et al.*, 2008; WARDA *et al.*, 2009),

especially antihelicobacter activity (RAMADAN and SAFWAT, 2009), cytoprotective (MARTIN-NIZARD *et al*., 2003) and significant antileukemic activity (ALKHATIB *et al*., 2010).

Active substances of species from genus *Marrubium* are labdane-structured bitter materials (TELEK *et al*., 1997), furanic labdane diterpene, marubin (BLUMENTHAL *et al*., 2000) and marrubenol (EL BARDAI *et al*., 2003), phenylethanoid glycoside, marruboside, phenylpropanoid esters (SAHPAZ *et al*., 2002b), methoxylated flavones (ALKHATIB *et al*., 2010), caffeic acid derivatives, sterols, and flavonoids (KHANAVI *et al*., 2005; LAZARI *et al*., 1999). In *M. peregrinum* have been proven many phenolic compounds and diterpenoids (HENNEBELLEA *et al*., 2006), diterpene alcohol peregrinol, diterpene hydroxylactone peregrin (SALEI *et al*., 1966), methoxylated flavones, ladanein, scutellarein-5,7,4'-trimethyl ether, and scutellarein-5,6,7,4'-tetramethyl ether (ALKHATIB *et al*., 2010). In previous research, sesquiterpene hydrocarbons (β-caryophyllene, bicyclogermacrene and germacree-D) and oxygenated sesquterpenes (spathulenol and caryophyllen oxide) have been proven in essential oil of *M. peregrinum* as active compounds and good antioxidant activity of essential oil has shown (KAURINOVIĆ *et al*., 2010).

Secondary metabolites from plants have important biological and pharmacological activities, such as anti-oxidative, anti-allergic, antibiotic, hypoglycemic and anti-carcinogenic (BORNEO *et al*., 2008; KATALINIĆ *et al*., 2004; MULABAGAL and TSAY, 2004).

Many disorders in human organism such as atherosclerosis, arthritis, Alzheimer disease, cancer etc., may be the result of increased concentrations of free radicals in an organism. Reactive oxygen species (ROS) and nitrogen (RNS) species, as the most frequent pro-oxidants, either originate from normal metabolism or are induced by UV radiation and different pollutants. Harmful effects of disturbed antioxidant-prooxidant balance can be largely prevented by intake of antioxidant substances (GHOSH *et al*., 2008; OGNJANOVIĆ *et al*., 2008).

Antioxidants have already been found in plant materials and supplements. Due to their natural origin, the antioxidants obtained from plants are of greater benefit in comparison to synthetic ones (ROHMAN *et al*., 2010; ZHENG and WANG, 2001). The use of natural antioxidants from plants does not induce side effects, while synthetic antioxidants were found to have genotoxic effect (CHEN *et al*., 1992; KAHL and KAPPUS, 1993). Therefore, the investigations of biological activity and chemical composition of medicinal plants as a potential source of natural antioxidants are numerous.

The basic aim of the research was to determine the total phenolic content and concentrations of flavonoids in various extracts of the species *M. peregrinum* using spectrophotometric methods, as well as to examine antioxidant activity of plant extracts using *in vitro* model system.

MATERIALS AND METHODS

Chemicals

Acetone, methanol, petroleum ether, ethyl acetate and sodium hydrogen carbonate were purchased from "Zorka pharma" Šabac, Serbia. Standards of phenolic acids (gallic acid) and of flavonoids (rutin hydrate), chlorogenic acid and 2,2-dyphenyl-1-picrylhydrazyl (DPPH) were obtained from Sigma Chemicals Co., St Louis, MO, USA. The Folin-Ciocalteu's phenol reagent, 3-tert-butyl-4-hydroxyanisole (BHA) and aluminium chloride ($AlCl_3$) were from Fluka Chemie AG, Buchs, Switzerland. All other solvents and chemicals were of analytical grade.

Plant material

Marrubium peregrinum was collected in July 2009, from the region of Suva Planina Mt. in eastern Serbia. The voucher specimen of *M. peregrinum* was confirmed and deposited in Herbarium at the Department of Biology and Ecology, Faculty of Science, University of

Kragujevac. The collected plant material was air-dried in darkness at room temperature (20 ^{o}C). Dried plant parts were cut up and stored in tight-seal dark containers until needed.

Preparation of plant extracts

Plant extracts were prepared according to a standard protocol. Prepared plant material (10 g) was transferred to dark-coloured flasks and mixed with 200 ml of solvents with different polarities (water, methanol, ethyl-acetate, acetone, petroleum ether) respectively and stored at room temperature. After 24 h, infusions were filtered through Whatman No. 1 filter paper and residue was re-extracted with equal volume of solvents. After 48 h, the process was repeated. Combined supernatants were evaporated to dryness under vacuum at 40 ^{o}C using Rotary evaporator. The obtained extracts were kept in sterile sample tubes and stored in a refrigerator at 4 ^{o}C.

Determination of total phenolic contents in the plant extracts

The concentration of phenolics in plant extracts was determined using spectrophotometric method (SINGLETON *et al*., 1999). Methanolic solution of the extract in the concentration of 1 mg/ml was used in the analysis. The reaction mixture was prepared by mixing 0.5 ml of methanolic solution of extract, 2.5 ml of 10% Folin-Ciocalteu's reagent dissolved in water and 2.5 ml 7.5% $NaHCO_3$. Blank was concomitantly prepared**,** containing 0.5 ml methanol, 2.5 ml 10% Folin-Ciocalteu's reagent dissolved in water and 2.5 ml of 7.5% of $NaHCO_3$. The samples were thereafter incubated in a thermostat at 45 ^{o}C for 45 min. The absorbance was determined using spectrophotometer at λ_{max} = 765 nm. The samples were prepared in triplicate for each analysis and the mean value of absorbance was obtained. The same procedure was repeated for the standard solution of gallic acid and the calibration line was construed. Based on the measured absorbance, the concentration of phenolics was read (mg/ml) from the calibration line; then the content of phenolics in extracts was expressed in terms of gallic acid equivalent (mg of GA/g of extract).

Determination of flavonoid concentrations in the plant extracts

The content of flavonoids in the examined plant extracts was determined using spectrophotometric method (QUETTIER *et al*., 2000). The sample contained 1 ml of methanol solution of the extract in the concentration of 1 mg/ml and 1 ml of 2% $AlCl_3$ solution dissolved in methanol. The samples were incubated for an hour at room temperature. The absorbance was determined using spectrophotometer at λ_{max} = 415 nm. The samples were prepared in triplicate for each analysis and the mean value of absorbance was obtained. The same procedure was repeated for the standard solution of rutin and the calibration line was construed. Based on the measured absorbance, the concentration of flavonoids was read (mg/ml) on the calibration line; then, the content of flavonoids in extracts was expressed in terms of rutin equivalent (mg of RU/g of extract).

Evaluation of antioxidant activity

The ability of the plant extract to scavenge DPPH free radicals was assessed by the standard method (Tekao et al., 1994), adopted with suitable modifications (KUMARASAMY *et al*., 2007). The stock solution of extracts were prepared in methanol to achieve the concentration of 1 mg/ml. Dilutions were made to obtain concentrations of 500, 250, 125, 62.5, 31.25, 15.62, 7.81, 3.90, 1.99 and 0.97 µg/ml. Diluted solutions (1 ml each) were mixed with 1 ml of methanolic solution of DPPH in concentration of 1 mg/ml. After 30 min incubation in darkness at room temperature (23 ^{o}C), the absorbance was recorded at 517 nm. Control simple contained all the reagents except the extract. Percentage inhibition was calculated using equation 1, whilst IC_{50} values were estimated from the % inhibition versus concentration plot, using a non-linear regression algorithm. The data were presented as mean values ± standard deviation (n = 3).

$$\% \, inhibition = \left(\frac{A \text{ of control} - A \text{ of sample}}{A \text{ of control}} \right) x \, 100 \qquad (1)$$

Statistical analysis

All experimental measurements were carried out in triplicate and are expressed as average of three analyses ± standard deviation.

The magnitude of correlation between variables was done using a SPSS (Chicago, IL) statistical software package (SPSS for Windows, ver. XII, 2004).

RESULTS AND DISCUSSION

Aqueous, methanol, ethyl acetate, acetone and petroleum ether extracts were prepared to examine the total phenolic content, flavonoid concentration and antioxidant activity. The yield of extract obtained from 10 g of dry plant material was measured for each extract (Table 1). The highest yield of solid residue was obtained using water or methanol as extraction solvents.

Table 1. - The yields of solid residue after extraction and evaporation from 10 g dried plant parts

extract	yields (g)
Methanol	1.98 ± 0.082 [1]
Water	1.54 ± 0.094
Ethyl acetate	0.49 ± 0.021
Acetone	0.32 ± 0.047
Petroleum ether	0.15 ± 0.014

[1]Each value is the average of three measurements ± standard deviation.

The total phenolic contents in the examined plant extracts using the Folin-Ciocalteu's reagent is expressed in terms of gallic acid equivalent (the standard curve equation: $y = 7.026x - 0.0191$, $r^2 = 0.999$). The values obtained for the concentration of total phenols are expressed as mg of GA/g of extract (Table 2).

The total phenolic contents in the examined extracts ranged from 27.44 to 49.27 mg GA/g. The highest concentration of phenols was measured in methanolic, acetone and water extracts. Ethyl acetate and petroleum ether extracts contains considerably smaller concentration of phenols. The total phenolic contents in plant extracts of the species *M. peregrinum* depends on the type of extract, i.e. the polarity of solvent used in extraction. High solubility of phenols in polar solvents provides high concentration of these compounds in the extracts obtained using polar solvents for the extraction (MOHSEN and AMMAR, 2008; ZHOU and YU, 2004).

Table 2. - Total phenolic contents in the plant extracts expressed in terms of gallic acid equivalent (mg of GA/g of extract)

extract	mg of GA/g of extract
Methanol	49.27 ± 0.815 [1]
Water	46.78 ± 0.258
Ethyl acetate	33.51 ± 0.616
Acetone	48.72 ± 0.407
Petroleum ether	27.44 ± 0.556

[1]Each value is the average of three analyses ± standard deviation.

The concentration of flavonoids in various plant extracts of the species *M. peregrinum* was determined using spectrophotometric method with aluminum chloride. The content of flavonoids was expressed in terms of rutin equivalent (the standard curve equation: y = 17.231x – 0.0591, r^2 = 0.999), mg of RU/g of extract (Table 3). The concentration of flavonoids in plant extracts from *M. peregrinum* ranged from 18.72 to 54.77 mg/g. Metanolic, acetone and ethyl acetate extracts contains the highest flavonoid concentration. The concentration of flavonoids in methanol extract was 54.77 mg RU/g, which was very similar to the value of acetone extract concentration. The lowest flavonoid concentration was measured in petroleum ether and water extract. The concentration of flavonoids in plant extracts depends on the polarity of solvents used in the extract preparation (MIN and CHUN-ZHAO, 2005).

Table 3. - Concentrations of flavonoids in the plant extracts expressed in terms of rutin equivalent (mg of RU/g of extract)

extract	mg of RU/g of extract
Methanol	54.77 ± 0.598[1]
Water	18.72 ± 0.417
Ethyl acetate	51.33 ± 0.793
Acetone	53.47 ± 0.940
Petroleum ether	22.92 ± 0.386

[1]Each value is the average of three analyses ± standard deviation.

The antioxidant activity of different plant extracts from *M. peregrinum* was determined using a methanol solution of DPPH reagent. DPPH is very stable free radical. Unlike *in vitro* generated free radicals such as the hydroxyl radical and superoxide anion, DPPH has the advantage of being unaffected by certain side reactions, such as metal ion chelation and enzyme inhibition. A freshly prepared DPPH solution exhibits a deep purple colour with an absorption maximum at 517 nm. This purple colour generally fades when antioxidant molecules quench DPPH free radicals (i.e. by providing hydrogen atoms or by electron donation, conceivably via a free-radical attack on the DPPH molecule) and convert them into a colourless-/bleached product (i.e. 2,2-diphenyl-1-hydrazine, or a substituted analogous hydrazine), resulting in a decrease in absorbance at 517 nm band (AMAROWICZ *et al*., 2003).

The antioxidant activity of five different extracts from the species *M. peregrinum* is expressed in terms of percentage of inhibition (%) and IC_{50} values (μg/ml) (Fig. 1). Parallel to examination of the antioxidant activity of plant extracts, the values for two standard compounds were obtained and compared to the values of the antioxidant activity. The standard substances were BHA and chlorogenic acid.

The examination of antioxidant activities of plant extracts from *M. peregrinum* showed different values. The obtained values varied from 27.26% to 89.78%. The largest capacity to neutralize DPPH radicals was found for methanolic extract, which neutralized 50% of free radicals at the concentration of 187.41 μg/ml. A moderate activity was found for acetone, aqueous and ethyl acetate extracts. The minutest capacity to inhibit DPPH radicals was determined for petroleum ether extract. Due to low activity of petroleum ether extract, IC_{50} are not calculated for it. In comparison to IC_{50} values of BHA and chlorogenic acid, methanolic extract from *M. peregrinum* manifested the strongest capacity for neutralization of DPPH radicals.

The extraction of antioxidant substances of different chemical structure, was achieved using solvents of different polarity. Numerous investigations of qualitative composition of plant extracts revealed the presence of high concentrations of phenols in the extracts obtained

using polar solvents (ČANADANOVIĆ-BRUNET *et al*., 2008). The extracts that perform the highest antioxidant activity (Figure 1) have the highest concentration of phenols (Table 2). Phenols are very important plant constituents because of their scavenging ability on free radicals due to their hydroxyl groups. Therefore, the phenolic content of plants may contribute directly to their antioxidant action (TOSUN *et al*., 2009). A significant linear correlation was found between the values for the concentration of phenolic compounds (Table 2) and the antioxidant activity of extracts from *M. peregrinum*. Between the values for the concentration of phenolic compounds (Table 2) and antioxidant activity of different plant extracts of *M. peregrinum* has been proved a significant linear correlation (Figure 2). Numerous investigations of the antioxidant activity of plant extracts have confirmed a high linear correlation between the values of phenol concentration and antioxidant activity (BORNEO *et al*., 2008; KATALINIĆ *et al*., 2004).

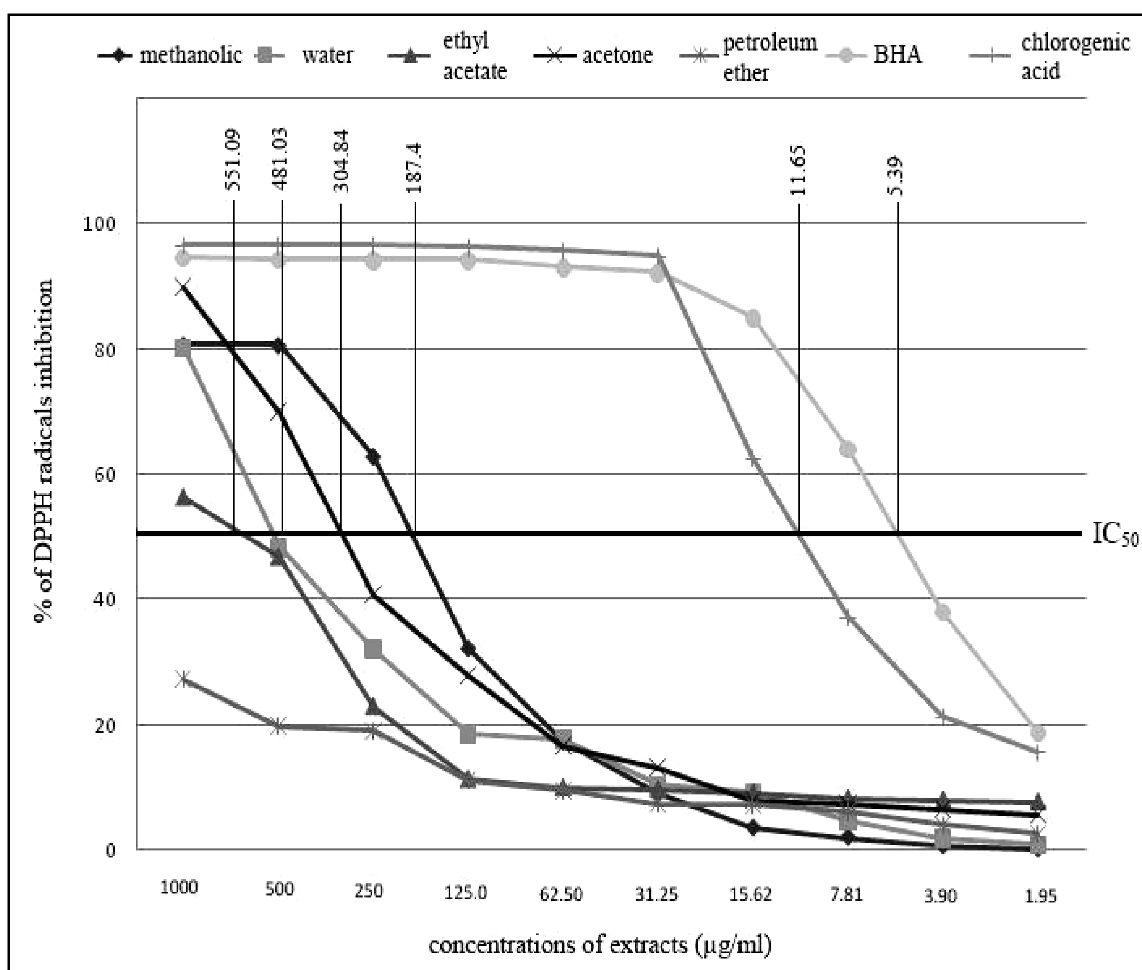

Figure 1. - Antioxidant (DPPH scavenging) activity of investigated plant extracts presented as percentage of DPPH radicals inhibition and IC_{50} values (µg/ml).

Flavonoids are class of secondary plant metabolites with significant antioxidant and chelating properties. Antioxidant activity of flavonoids depends on the structure and substitution pattern of hydroxyl groups (SHARIFIFAR *et al*., 2008). Methanolic and acetone extracts from *M. peregrinum* have high concentration of total phenols (Table 2) and flavonoids (Table 3), which is in correlation with intense antioxidant activity of these extracts (Figure 1).

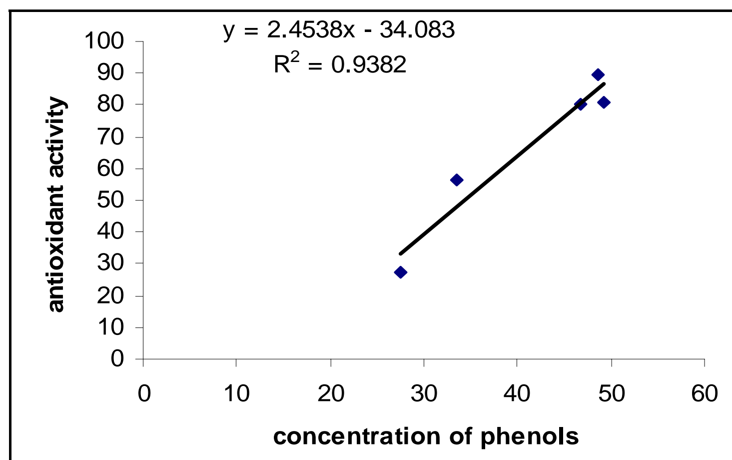

Figure 2. - Linear correlation between the amount of total phenols and antioxidant activity.
Corelation coefficient r = 0.969, coefficient of determination (R^2) = 0.9382.
Correlation is significant at the 0.01 level (2-tailed).

CONCLUSIONS

Results of our study suggest the great value of the species *M. peregrinum* for use in pharmacy and phytotherapy. Based on this information, it could be concluded that this plant is natural sources of antioxidant substances of high importance.

It is noticed that the highest concentration of phenolic compounds in the extracts were obtained using solvents of high polarity; the methanolic extract manifested greater power of extraction for phenolic compounds from *M. peregrinum*.

The high contents of phenolic compounds and significant linear correlation between the values of the concentration of phenolic compounds and antioxidant activity indicated that these compounds contribute to the strong antioxidant activity.

Further studies of this plant species should be directed to carry out *in vivo* studies of its medicinal active components in order to prepare a natural pharmaceutical products of high value.

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