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Evaluation of antioxidant activity of medicinal plants containing polyphenol compounds. Comparison of two extraction systems

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This study investigates the influence of extraction system on the extractability of polyphenol compounds and antioxidant activity of various medicinal plants. Oxygen radical absorbance capacity (ORAC) and total polyphenol content of 25 Bulgarian medicinal plants subjected to water or 80% acetone extractions were investigated and compared. The type of extragent significantly influenced the efficiency of the polyphenol extraction and the antioxidant activity. In all cases ORAC results and total polyphenol content were higher for acetone extraction than for water extraction. The acetone extract of peppermint had the highest ORAC value — 2917 μmol Trolox equivalent (TE)/g dry weight (DW) and polyphenol content — 20216 mg/100 g DW. For water extraction thyme exhibited the highest ORAC antioxidant activity — 1434 μmol TE/g DW. There was a significant linear correlation between the concentration of total polyphenols and ORAC in the investigated medicinal plants. It can be concluded that the solvent used affects significantly the polyphenol content and the antioxidant activity of the extract and therefore it is recommended to use more than one extraction system for better assessment of the antioxidant activity of natural products. Several of the investigated herbs contain substantial amounts of free radical scavengers and can serve as a potential source of natural antioxidants for medicinal and commercial uses.

Keywords: medicinal plants, ORAC, polyphenols

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INTRODUCTION

A growing amount of evidence indicates a role of reactive oxygen species (ROS) such as peroxyl radicals ($ROO^{\bullet}$), hydroxyl radical ($HO^{\bullet}$), superoxide anion ($O_2^{\bullet-}$) and singlet oxygen ($^1O_2$) in the pathophysiology of aging and different degenerative diseases such as cancer, cardiovascular diseases, Alzheimer's disease and Parkinson's disease (Davies, 2000; Fenkel & Holbrook, 2000). Living cells possess a protective system of antioxidants which prevents excessive formation and enables the inactivation of ROS. The antioxidants protect from the potentially damaging oxidative stress, which is a result of an imbalance between the formation of ROS and the body antioxidant defense. Antioxidants have also been used in food industry to prevent deterioration, nutritional losses and off-flavoring in various foods, especially those containing polyunsaturated fatty acids. Recently, interest has increased considerably in finding naturally occurring antioxidants for use in foods because of their potential in health promotion and disease prevention, and their high safety and consumer acceptability (Gorinstein *et al.*, 2003).

In search of novel sources of antioxidants in the last years, medicinal plants have been extensively studied for their antioxidant activity. From ancient times, herbs have been used in many areas, including nutrition, medicine, flavoring, beverages, cosmetics, etc. The ingestion of fresh fruit, vegetables and tea rich in natural antioxidants has been associated with prevention of cancer and cardiovascular diseases (Willcox *et al.*, 2004). The higher intake of plant foods correlates with lower risk of mortality from these diseases (Johnson, 2001). Approximately 60% of the commercially available anti-tumoral and anti-infective agents are of natural origin (Cragg *et al.*, 1997).

Polyphenols are the most significant compounds for the antioxidant properties of plant raw materials. The antioxidant activity of polyphenols is mainly due to their redox properties, which allow them to act as reducing agents, hydrogen donors, singlet oxygen quenchers, metal chelators and reductants of ferryl hemoglobin (Rice-Evans *et al.*, 1995; 1997; Prior *et al.*, 2005; Lopez *et al.*, 2007; Ciz *et al.*, 2008; Gebicka & Banasiak, 2009).

Investigation of natural products is a research field with great potential and is especially important in countries possessing great biodiversity, like Bulgaria. About 600 plant species from the Bulgarian flora are recognized as medicinal and are traditionally used in ethnopharmacology and phytotherapy (Dimkov, 1979; Petkov, 1982). There are many reports in the literature about the antioxidant properties of medicinal plants (Zheng & Wang, 2001; Djeridane *et al.*, 2006; Katalinic *et al.*, 2006; Wojdylo *et al.*, 2007), but there are only few papers reporting data about the antioxidant properties of Bulgarian herbs using methods such as DPPH and ABTS (Ivanova *et al.*, 2005; Kiselova *et al.*, 2006). The current study employs the oxygen radical absorbance capacity (ORAC) method, which has been found to be the most relevant one for biologic samples (Wang *et al.*, 2004; Huang *et al.*, 2005; Prior *et al.*, 2005**)**. Different extraction systems were used to extract antioxidant components from the plant material and often it is difficult to compare the results for the antioxidant properties even for the same plant material. Water (Zheng & Wang, 2001; Ivanova *et al.*, 2005; Katalinic *et al.*, 2006; Kiselova *et al.*, 2006), metha-

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Abbreviations: AAPH, 2,2-azobis-(2-amidino-propane)dihydrochloride; ABTS, 2,2'azinobis(3-ethylbenzothiazoline-6-sulfonic acid; AUC, area under the curve; SD, standard deviation; DW, dry weight; FL, fluorescein; ORAC, oxygen radical absorbance capacity; TE, Trolox equivalents.

Table 1. Medicinal plants commonly used in traditional medicine

Botanical name	Family	Common name	Part of plant used	Medical use
Achillea millefolium	*Asteraceae*	Yarrow	Flowers	Antiseptic, anti-inflammatory, stomach ulcer, gastrointestinal disorders, liver diseases
Arctium lappa	*Asteraceae*	Greater burdock	Roots	Diuretic, kidney stones, rheumatism, gastritis, stomach ulcer, gout
Betula pendula	*Betulaceae*	Birch	Leaves	Diuretic, kidney disorders, bladder disorders
Calendula officinalis	*Asteraceae*	Marigold	Flowers	Anti-inflammatory, pain-relieving, local treatment of wounds, duodenum and stomach ulcer, gastrointestinal disorders
Cichohrium intybus	*Asteraceae*	Chicory	Aerial parts	Digestive, chologogue, liver diseases
Clinopodium vulgare	*Labiatae*	Wild basil	Leaves	Immunostimulant, cardio-tonic, verruca.
Crataegus monogyna	*Rosaceae*	Hawthorn	Flowers, leaves	Promotes capillary formation and heart microcirculation, cardiovascular diseases, ischemia
Glycyrrhiza glabra	*Fabaceae*	Liquorice	Roots	Adaptogen, anticancer
Humulus lupulus	*Cannabaceae*	Hop	Flowers	Sedative, digestive, menstrual disorders
Hypericum perforatum	*Hypericaceae*	St. John's wort	Aerial parts	Anti-inflammatory, astringent, antibacterial, diuretic, ulcer, colitis, gastritis
Laurus nobilis	*Lauraceae*	Laurel leaves	Leaves	Immunostimulant, antidiabetic, stomatitis, sinusitis
Matricaria chamomilla	*Asteraceae*	Chamomile	Flowers	Anti-inflammatory, antiseptic, sedative, throat and mouth inflammations, gastrointestinal disorders, influenza, pharyngitis, laryngitis
Melissa officinalis	*Labiatae*	Common balm	Leaves	Sedative, gastrointestinal disorders
Mentha piperita	*Labiatae*	Peppermint	Leaves	Spasmolytic, antiseptic, gastric disorders, indigestion, neuralgia, myalgia, antivomiting
Mentha spicata	*Labiatae*	Spearmint	Leaves	Hormone regulating, spasmolytic, antiseptic, gastric disorders, indigestion, neuralgia, myalgia
Ocimum basilicum	*Labiatae*	Basil	Leaves	Antiseptic, spasmolytic, expectorant gastrointestinal diseases, antitussive
Rubus idaeus	*Rosaceae*	Raspberry	Leaves	Anti-inflammatory, antiseptic, antidiarrheic, gastrointestinal disorders
Salvia officinalis	*Labiatae*	Sage	Leaves	Anti-inflammatory, antiseptic, inflammations of throat and mouth
Sideritis scardica	*Labiatae*	Mountain tea	Aerial parts	Expectorant, antitussive, bronchitis, cough
Taraxacum officinale	*Asteraceae*	Dandelion	Aerial parts	Diuretic, cholagogue, appetizer
Thymus vulgaris	*Labiatae*	Thyme	Aerial parts	Expectorant, spasmolytic, antibacterial, antitusive, asthma, emphysema, whooping-cough, diseases of respiratory tract
Tilia cordata	*Tiliaceae*	Lime	Flowers	Anti-inflammatory, expectorant
Tribulus terrestris	*Zygophyllaceae*	Caltrop	Aerial parts	Hormone regulating, sperm promoting, prevents ovary cysts
Trigonella foenum-graecum	*Fabaceae*	Fenugreek	Seeds	Anticancer, metabolic syndrome and diabetes
Urtica dioica	*Urticaceae*	Nettle	Leaves	Hormone regulating, prostate cancer prevention, podagra, diabetes, allergies, anaemia

nol (Shan *et al.*, 2005; Wojdylo *et al.*, 2007) and ethanol (Djeridane *et al.*, 2006) have been widely used. In very few cases only, more than one extragent or sequential multi-solvent extractions were preferred (Su *et al.*, 2007; Wojcikowski *et al.*, 2007). It has been recognized that the extraction solvent may significantly alter the antioxidant activity estimation (Zhou & Yu, 2004). In the present work, two extragents were used for the extraction of plant antioxidants — water and 80% acetone. Plant extracts made with water are nutritionally more relevant, moreover, herbs are traditionally ingested as hot-water infusions. On the other hand, acetone is preferred for more exhaustive extraction of polyphenol compounds and it was of particular interest to compare the polyphenol content and ORAC antioxidant activity in water infusions and acetone extracts.

The objective of the current study was to investigate the influence of the extraction agent on the extractability of polyphenol components and the antioxidant activity of 25 Bulgarian medicinal plants. These two parameters were evaluated in water and 80% acetone extracts of plants. Results from this study will lead to a better characterization of the antioxidant properties of the medici-

nal plants investigated and will reveal which of them are the best sources of dietary antioxidants.

MATERIALS AND METHODS

Chemicals. Fluorescein disodium salt, 2,2-azobis-(2-amidino-propane)dihydrochloride (AAPH), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), and gallic acid were obtained from Sigma-Aldrich (Steinheim, Germany). Folin-Ciocalteu's phenol reagent was purchased from Merck (Darmstadt, Germany). All other solvents used were of analytical grade and purchased from local distributors.

Plants. All medicinal plants used were either obtained from local pharmacies (Plovdiv, Bulgaria) or collected from nature in 2008. The choice of the plants investigated was based on their use in the traditional medicine. In total, 25 medicinal plants were investigated (Table 1).

Plants were dried, packed in paper bags and stored at ambient temperature prior to the analysis.

Extraction. All plant materials were subjected to extractions with acetone and with water. For the acetone extraction, 10 g of the plant material was powdered in a laboratory mill, then 0.5 g of the powder was transferred into extraction tubes and mixed with 20 ml of the extragent (80% acetone in 0.2% formic acid). Extraction was conducted on an orbital shaker at room temp. for one hour. After that, the samples were centrifuged (6000×*g*) and supernatants were removed. The solid residue was subjected to the second extraction under the same conditions. Both supernatants were combined and analyzed for antioxidant activity and total polyphenol content.

Water infusions were prepared in compliance with the traditional preparation which is close to home conditions. For that purpose 5 g of the herb powder was added to 200 ml water (90°C). Aerial parts of the plants were incubated for 15 min, whereas roots were incubated for 45 min. The slurry was centrifuged (6000×*g*) and supernatants were used for further analysis.

ORAC assay. ORAC was measured according to the method of Ou *et al.* (2001) with some modifications (Ciz *et al.*, 2010). The method measures the antioxidant scavenging activity against peroxyl radical generated by thermal decomposition of AAPH at 37°C. Fluorescein (FL) was used as the fluorescent probe. The loss of fluorescence of FL was an indication of the extent of damage from its reaction with the peroxyl radical. The protective effect of an antioxidant was measured by assessing the area under the fluorescence decay curve (AUC) relative to that of a blank in which no antioxidant has present. Solutions of AAPH, fluorescein and Trolox were prepared in a phosphate buffer (75 mmol/l, pH 7.4). Samples were diluted in the phosphate buffer as well. Reaction mixture (total volume 200 µl) contained FL — (170 µl, final concentration 5.36×10^{-8} mol/l), AAPH — (20 µl, final concentration 51.51 mmol/l), and sample — 10 µl. The FL solution and sample were incubated at 37°C for 20 min directly in a microplate reader, and AAPH (dissolved in buffer at 37°C) was added. The mixture was incubated for 30 s before the initial fluorescence was measured. After that, the fluorescence readings were taken at the end of every cycle (1 min) after shaking. For the blank, 10 µl of phosphate buffer was used instead of the extract. The antioxidant activity was expressed in micromole Trolox equivalents per gram of dry weight (DW). Trolox solutions (6.25, 12.5, 25, 50 and 100 µmol/l) were used for defining the standard curve.

Table 2. Antioxidant activity of 25 medicinal plants.
Comparison between 80% acetone (ac) and water (w) extraction. Results are presented as mean ± S.D.

Medicinal plant	$ORAC_{ac}$ µmol TE/g	$ORAC_{w}$ µmol TE/g	Ratio $ORAC_{w}/ORAC_{ac}$ %
Peppermint	2917±52	1409±62	48.3
Hawthorn	2163±89	364±28	16.8
Thyme	1637±59	1434±54	87.6
Wild basil	1437±60	844±41	58.7
Birch	1185±73	142±18	12.0
Raspberry	1156±80	608±35	52.6
St. John's wort	1141±93	629±41	55.1
Common balm	1121±60	996±26	88.8
Lime	1020±88	97±11	9.5
Sage	966±69	609±54	63.0
Yarrow	842±80	394±30	46.8
Laurel leaves	837±81	170±12	20.3
Caltrop	819±56	272±16	33.2
Camomile	814±72	469±25	57.6
Mountain tea	778±77	294±21	37.8
Hop	749±62	260±19	34.7
Spearmint	748±57	598±31	79.9
Liquorice	670±48	213±12	31.8
Marigold	407±57	247±17	60.7
Basil	402±40	271±18	67.4
Chicory	398±22	132±14	33.2
Dandelion	381±16	193±16	50.7
Greater burdock	365±31	323±20	88.5
Fenugreek	327±28	320±12	97.9
Nettle	162±11	141±10	87.0

Total polyphenol compounds analysis. Total polyphenols were determined according to the method of Singleton and Rossi (1965) with Folin-Ciocalteu's reagent. Gallic acid was employed as calibration standard and results were expressed as gallic acid equivalents (GAE) per 100 g DW.

RESULTS AND DISCUSSION

It is of particular interest to investigate the antioxidant properties of medicinal plants, especially those traditionally used in folk medicine. More than one extraction system is recommendable for detailed assessment of the antioxidant properties of medicinal plants. It was found in a recent study by Su *et al.* (2007) that the ORAC values of acetone extracts were higher than those for methanolic extracts for several herbs. Therefore, aiming at the maximum extractability of the polyphenol compounds, we chose to extract raw materials with acetone. On the other hand, the traditional ingestion of medicinal plants and their clinical usage usually requires their extraction with water. Table 2 shows the ORAC antioxidant activity of the investigated medicinal plants extracted by acetone and water ($ORAC_{ac}$ and $ORAC_{w}$, respectively).

Table 3. Polyphenol content of 25 medicinal plants.
Comparison between 80% acetone (ac) and water (w) extraction. Results are presented as mean ± S.D.

Medicinal plant	Polyphenols$_{ac}$ mg/100g	Polyphenols$_{w}$ mg/100g	Ratio PF$_{w}$/ PF$_{ac}$ %
Peppermint	20216 ± 359	9356 ± 204	46.3
Hawthorn	7104 ± 111	1903 ± 181	26.8
Thyme	11409 ± 171	8583 ± 241	75.2
Wild basil	9468 ± 128	4645 ± 201	49.1
Birch	5542 ± 201	1197 ± 124	21.6
Raspberry	7759 ± 216	4932 ± 164	63.6
St. John's wort	11283 ± 74	6428 ± 152	57.0
Common balm	11885 ± 109	8240 ± 207	69.3
Lime	9296 ± 427	787 ± 43	8.5
Sage	5295 ± 148	3845 ± 65	72.6
Yarrow	5728 ± 232	1968 ± 84	34.4
Laurel leaves	7081 ± 299	1766 ± 52	24.9
Caltrop	5681 ± 200	2790 ± 101	49.1
Camomile	4665 ± 137	1790 ± 45	38.4
Mountain tea	3984 ± 201	2044 ± 21	51.3
Hop	5728 ± 262	1697 ± 25	29.6
Spearmint	4522 ± 102	3713 ± 46	82.1
Liquorice	3452 ± 98	1548 ± 72	44.8
Marigold	2141 ± 115	1537 ± 33	71.8
Basil	2391 ± 38	1816 ± 52	76.0
Chicory	1821 ± 63	786 ± 46	43.2
Dandelion	2206 ± 58	1577 ± 51	71.5
Greater burdock	2742 ± 112	2531 ± 68	92.3
Fenugreek	1692 ± 105	1445 ± 41	85.4
Nettle	958 ± 43	776 ± 43	81.0

Since polyphenols significantly contribute to the overall antioxidant activity, it was reasonable to determine their total amount in the selected medicinal plants. The total polyphenol content in the medicinal plants is shown in Table 3. It is evident that in all cases the ORAC values and total polyphenol content obtained with acetone extraction were higher than the respective results for water extraction. The observed differences could be explained by the different polarity of the polyphenol compounds present in the investigated medicinal herbs. This observation complies with the findings of Wojcikowski *et al.* (2007) who used sequential three-solvent extraction for herb polyphenols. The antioxidant activity of the samples varied significantly for both acetone and water extracts in our study. The greatest ORAC$_{ac}$ value was found in peppermint, while the highest ORAC$_{w}$ value was found in thyme, followed by peppermint. Since the ORAC method is preferred for the measurement of the antioxidant activity of foods and biological samples, it is surprising that in the literature there are ORAC data just for several of the medicinal plants investigated in the current study. Ninfali *et al.* (2005) performed a comprehensive evaluation of different foods and spices using the ORAC method. On the basis of fresh weight, they reported ORAC values for thyme (274.26 μmol TE/g), sage (320.04 μmol TE/g) and common balm (59.97 μmol TE/g). As the reported data are based on fresh weight, it is difficult to compare them with our results. Zheng and Wang (2001) determined ORAC of five herbs from the current study, but again the results were expressed on the basis of fresh weight. Moreover, they used R-phycoerythrin as a fluorescent agent, which could significantly alter the ORAC results in their study (Ou *et al.*, 2001). In another recent study, Wojcikowski *et al.* (2007) investigated the ORAC antioxidant activity of 55 medicinal plants after sequential three-solvent extraction. Since this presumes very exhaustive extraction, it can explain the higher ORAC values obtained by them for several herbs from our study: liquorice — 1029 μmol TE/g (670 μmol TE/g in our study), basil — 524.7 μmol TE/g compared with 402 μmol TE/g, and nettle — 430.4 μmol TE/g against 162 μmol TE/g. Despite the more exhaustive sequential extraction, three herbs in our work showed ORAC values several times higher

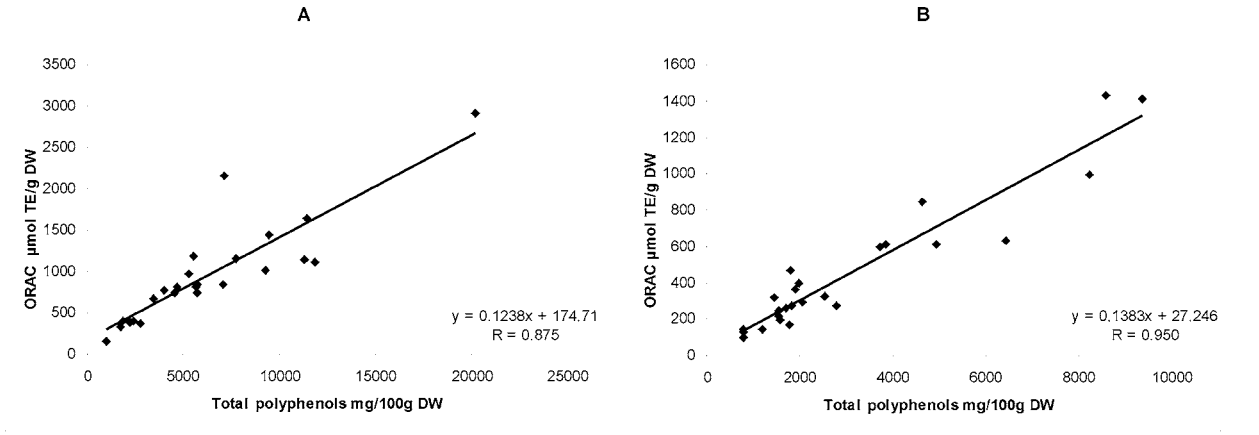

Figure 1. Correlation between total phenolic content and ORAC antioxidant activity in acetone (A) and water (B) extracts obtained from 25 medicinal plants.

than the ones in the Wojcikowski's study. For example, our results for yarrow — 842 μmol TE/g, sage — 966 μmol TE/g and dandelion — 381 μmol TE/g were 3.2-fold, 2.7-fold and 3.9-fold higher, respectively, than the ORAC values for the same herbs in the above-mentioned paper. The differences in the antioxidant activity between the same materials can be attributed to some environmental factors such as climate, location and temperature which can significantly affect the accumulation of the antioxidant components in plant material. From the investigated 55 herbs in the same study, the root of black cohosh (*Cimisifuga racemosa*) showed the highest ORAC value of 1264.9 μmol TE/g. In our study, four medicinal plants (peppermint, hawthorn, thyme and wild basil) revealed higher antioxidant activity, and another four (birch, raspberry, St. John's wort and common balm) showed comparable ORAC values to that result. Our study reports the ORAC values of antioxidant capacity of several plants for the first time — those for wild basil (*Clinopodium vulgare*) leaves, birch (*Betula pendula*) leaves, caltrop (*Tribulus terrestris*) aerial parts, mountain tea (*Sideritis scardica*) aerial parts, hop (*Humulus lupulus*) flowers, marigold (*Calendula officinalis*) flowers and greater burdock (*Arctium lappa*) roots.

Several studies have investigated the relationship between the antioxidant activity and the content of polyphenol compounds in herbs. Some authors have reported good linear correlation between these two parameters (Zheng & Wang 2001; Shan *et al.*, 2005; Djeridane *et al.*, 2006; Katalinic *et al.*, 2006), whereas others have not observed such correlation (Kahkonen *et al.*, 1999). Figure 1 depicts the correlation between the total polyphenols and the ORAC values of the medicinal plants investigated in our study. The correlation coefficient between ORAC and total polyphenol content was $R = 0.875$ for acetone extracts and $R = 0.950$ for water extracts. These correlations suggest that the ORAC antioxidant activity could be attributed to the polyphenol compounds. However, there are several discrepancies in the correlation. Such an example is hawthorn whose high ORAC value does not match its low polyphenol content. Several explanations could be used to account for that. First, it has been reported that polyphenol compounds differ significantly in their antioxidant properties which are determined by several structural features of the polyphenol molecule (Ou *et al.*, 2002). Second, the investigated medicinal plants probably contain other substances with antioxidant effect apart from the polyphenols. Moreover, the amount of polyphenols does not represent the potential synergism or antagonism between the individual compounds in the samples, which depends on their structure and mutual interactions.

A recent study by Prior *et al.* (2007) demonstrated that the consumption of certain foods was associated with increased plasma ORAC in the postprandial state, while the consumption of an energy source of macronutrients containing no antioxidants was associated with a decline in the plasma antioxidant capacity. The authors estimated that according to the energy intake of the diet, 5000–15000 μmol TE are necessary to supply daily human antioxidant needs. The ORAC values reported in the current study are several times higher than the ORAC values of many fruits and vegetables (Ou *et al.*, 2002; Wu *et al.*, 2004; Ciz *et al.*, 2010). This means that the studied medicinal herbs exhibited a higher antioxidant activity and contained more polyphenols than the common vegetables and fruit. In the search for natural antioxidants, herbs turned out to be a suitable source of dietary antioxidants. The differences between the ORAC values and polyphenol content obtained after acetone and water extractions indicate that the traditional way of ingestion of herbs does not fully utilize the available antioxidants in the plant material. These antioxidant compounds could be isolated and then used as antioxidant functional foods (Grajek *et al.*, 2005).

CONCLUSION

It can be concluded that the extracting solvent affects significantly the polyphenol compound content and the antioxidant activity measured and therefore it is recommended to use more than one extraction system for better assessment of the antioxidant activity of natural products. Several of the Bulgarian medicinal plants tested are rich sources of polyphenol compounds and free radical scavengers. Some medicinal plants thus can be considered as promising sources of natural antioxidants for medicinal and commercial uses.

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