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NATURAL DRUGS

ANTIOXIDANT ACTIVITY OF EXTRACTS AND FLAVONOIDS FROM *BIDENS TRIPARTITA*

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Abstract: Extracts from herb and flowers of *Bidens tripartita* L. (Asteraceae), obtained using solvents of different polarity, were studied for their radical scavenging effects. Antioxidant activities of pure flavonoids: flavanomarein (isookanin 7-O-glucoside), cynaroside (luteolin 7-O-glucoside) and luteolin, which had been isolated from this plant, were also evaluated. Radical-scavenging activity was measured by electron paramagnetic resonance (EPR) spectroscopy using stable 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical. The content of flavonoids in flower heads is half of that found in the herb; however, the extract from flowers showed that the antioxidant activity was almost two times higher there. Some extracts (*n*-BuOH fraction) showed long lasting radical scavenging activity and the EPR spectra were recorded in time to follow the reaction kinetics. Scavenging of DPPH showed second-order kinetics at the beginning of the assay period and later the first-order one. Different kinetics suggested the presence of polymerized and/or less active antioxidants with different scavenging mechanisms for particular polyphenolic compounds. Bur-marigold extracts are a potential source of natural antioxidants that may be used in pharmaceutical or food industry.

Keywords: *Bidens tripartita,* antioxidants, flavonoids, radical-scavenging, DPPH, EPR

Flavonoids are a group of polyphenolic compounds widely distributed as secondary metabolites in the plant kingdom. Numerous epidemiological studies showed that a high intake of plant flavonoids (consumption of fruits and vegetables) is inversely related to the risk of developing cardiovascular diseases, stroke or some cancers (1, 2). The preventive effects of flavonoids are mainly due to their antioxidant activity and their ability to scavenge free radicals (3, 4). Antioxidant activities of numerous pure flavonoids and phenolic acids had been determined earlier (5). Recent research focuses more on plant extracts and natural formulations.

In this paper we have examined the antioxidant activity of the extracts and the main flavonoids isolated from bur-marigold *Bidens tripartita* L. (Asteraceae). It is a perennial, branched herb, widely distributed throughout Europe (6-8). The herb of bur-marigold has been used in folk medicine as a diuretic, sudorific and anti-inflammatory agent. It is also used in the treatment of fevers, skin diseases, bladder and kidney troubles, and as a stimulant of the immunological system (9-11). From interviews, which were carried out by Sezik et al. (12) in Uzbekistan area, the bur-marigold was reported to be effective treating allergic itching in children. The methylene chloride extract of *B. tripartita* demonstrated high activity in the inhibition of cancer L1210 (mouse leukemia) cells and against thrombin (13). Phytochemical studies on bur-marigold herb showed the presence of flavones, flavanones, chalcones and aurones (14-18), coumarins (19), small amounts of vitamin C, carotenoids and a volatile oil (20). The green parts of *B. tripartita* contain polyacetylenic compounds, linoleic acid and ocimene, whereas in the flower heads thiophene, traces of cosmene and eugenol were identified (21). To our knowledge, radical scavenging activity of the *B. tripartita* extracts has not been reported so far. In this report, the extracts of different polarity from *B. tripartita* were prepared and studied for their antioxidant activity. Three flavonoid compounds (Figure 1) that had been isolated from that plant were also evaluated. One of them (flavanomarein) is rather rare in the plant kingdom and its radical scavenging activity was not previously established.

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EXPERIMENTAL

Material and Methods

Chemicals

1,1-Diphenyl-2-picrylhydrazyl (DPPH) radical was purchased from Sigma-Aldrich. The purified flavonoids: isoookanin 7-O-glucoside, luteolin 7-O-glucoside and luteolin were isolated from the herb of *B. tripartita* according to the described procedures (18, 22). All solvents, of analytical and HPLC grade, were purchased from POCH (Gliwice, Poland). Milli-Q Plus (Millipore, USA) treated water (18.2 MΩ cm) was used.

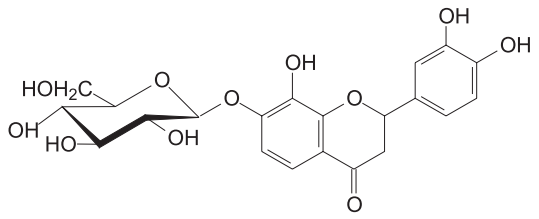
Plant material and preparation of extracts

The plant materials were collected on August 2001 in Bielsk Podlaski area (Poland). The voucher specimen was deposited in the Herbarium of Department of Pharmacognosy, Medical University of Białystok, Poland (No. BT97005).

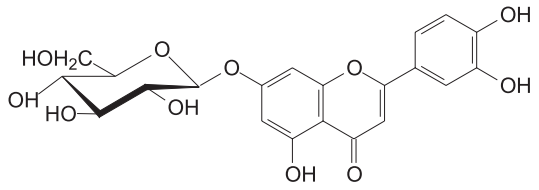
The air-dried and powdered *B. tripartita* flowers (BF) and herb (BH) were subjected to several extraction procedures: water, methanol/water, acetone/water and methanol extraction. In all the cases, 15 g samples of plant material were used for extraction. For water extraction, the samples were treated with 100 mL of boiling distilled water. After cooling, the aqueous extracts were left at room temperature for 20 min, filtered off, and then evaporated to dryness on a rotary vacuum evaporator to afford BF1 and BH1.

Methanol/water extracts were obtained by soaking powdered flowers (or herb) in 400 mL of 1:1, v/v methanol/water with agitation and heating under reflux for 2 h. After filtration, the residue was extracted again with the same solvent. The combined extract was evaporated to dryness *in vacuo* affording BF2; the same extraction procedure performed with herb afforded BH2. Acetone/water (1:1) extracts were obtained by heating the samples in that solvent (400 mL) under reflux for 2 h. The extraction was repeated and the combined extract BF3 (BH3) was evaporated to dryness.

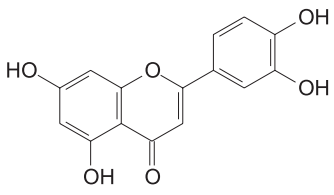
Prior to methanol extraction, dried plant materials (flowers or herb) were treated with petrol and chloroform in a Soxhlet apparatus. The plant residue was further extracted with 3 × 500 mL of methanol and the combined extracts were evaporated under reduced pressure. The residue redissolved in 50 mL of water was successively partitioned between diethyl ether (fractions BTF1 and BTH1), ethyl acetate (fractions BTF2, BTH2) and *n*-butanol (fractions BTF3, BTH3).



Flavanomarein (isookanin 7-O-glucoside)



Cynaroside (luteolin 7-O-glucoside)



Luteolin

Figure 1. Chemical structures of flavonoids isolated from *B. tripartita*.

Determination of the content of antioxidant compounds

The total flavonoid content in flowers and herb of *B. tripartita* was determined by the Christ-Müller's method (23) with modifications according to Polish Pharmacopoeia VI (24). The absorption was measured at 425 nm using a SPECORD 40 spectrophotometer (Analytik Jena, Germany). The content of flavonoids was expressed as quercetin (or hyperoside) equivalents/100 g of dry weight (in %) (see Table 1).

The quantitative determination of some flavonoids in the samples of flowers and herb of bur-marigold was performed on a Waters HPLC system (Milford, MA, USA) equipped with a Waters 600E pump, a 600 Controller and a 996 PDA detector scanning between 190 to 400 nm. The data were collected and analyzed with Millennium Chromatography Manager V4.0 Software. Separation was carried out using Symmetry C_{18}, particle size: 5 μm; 3.9 × 150 mm column (Waters Corp., USA). The

flavonoids were detected at 350 nm, corresponding to the λ_{max} of the analyzed compounds in methanol solution. Solvent gradients were formed by the dual pumping, varying the proportion of solvent A (methanol) to solvent B (0.5% (v/v) *ortho*-phosphoric acid in water). The elution profile was as follows: 0 min 40% A in B, 0-0.5 min 40% to 60% A in B, 0.5-2.5 min 65% A in B, 2.5-6.0 min 65% to 45% A, 6.0-8.0 min 40% A in B. All gradients were linear. The mobile phase was delivered at a flow rate of 1.0 mL/min and samples of 20 µL were injected into the HPLC system. For each sample, three replicate assays were performed (n = 3). The retention times for the flavanomarein (isookanin 7-O-glucoside), cynaroside (luteolin 7-O-glucoside) and luteolin were 1.88, 5.36 and 7.97 min, respectively. The content of each flavonoid (see Table 1) in the analyzed extracts was calculated on the basis of the calibration curve (determined for pure standards).

Sample preparation for HPLC analysis

Dried and pulverized plant material, 2 g (BF, BH) was successively extracted with petrol (5 × 50 mL) and chloroform (5 × 50 mL). The plant residues were then heated for 2 h under reflux with 6 × 50 mL of 70% methanol. The combined extracts were evaporated to dryness and redissolved in 20 mL of 40% methanol with 0.5% *ortho*-phosphoric acid. A 5 mL portion of the solution was filtered through a Chromafil O-45/25 (PTFE 25 mm, 0.45 µm, Macherey-Nagel, Germany) and an aliquot (20 µl) was injected into the HPLC column.

DPPH assays

The extract solutions were colored, and for spectrophotometric UV-vis measurements the corrections for background absorbances were necessary. Therefore, radical scavenging activity was estimated by electron paramagnetic resonance (EPR) spectroscopy. Measurements were performed at ambient temperature (298K) on a Bruker ELEXSYS E 500 spectrometer at 9,4508 GHz, operating in continuous wave mode. SHQE – Super High Q cavity was employed.

The scavenging effect on DPPH radical was determined as described by Hatano et al. (25). The decay of DPPH signal was monitored and compared with the control sample; stock solution of the radical in methanol (0.0025 mol/dm^3) was used. EPR spectra were recorded at 298K with the spectrometer settings: receiver gain – 55 dB, power – 20 mW, center field – 3370 G, sweep width – 200 G, sweep time – 83.89 s, modulation frequency – 100 kHz, modulation amplitude -1 G. Methanol solution (25 µL) of plant extract (or a flavonoid) was added to 0.5 mL of DPPH solution, mixed, and a volume of 25 µL was transferred into the quartz EPR cell.

DPPH radical scavenging activities of the extracts were calculated according to the equation:

$$\text{scavenged DPPH (\%)} = [(I_0^I - I^I)/I_0^I] \times 100$$

where: I_0^I – integral intensity of DPPH signal for control sample; I^I – integral intensity measured after addition of the scavenger. Signal intensity was registered as a function of magnetic induction and time. The spectra were recorded every 42.6 s. Non-linear regression analyses were carried out for n = 15 scans. The experiment was carried out in triplicate and total error (RSD) of integral intensity for I_0^I was less than 2%.

RESULTS AND DISCUSSION

The flavonoid compounds are distributed in both herb and flower heads of bur-marigold. As shown in Table 1, the content of flavonoids in flower heads is half of that found in the herb. The main flavonoid constituents of the extracts are 7-O-glucosides of isookanin and luteolin. Flavanomarein dominates in flowers and cynaroside in green parts of this plant. The content of flavonoids, according to the Christ-Müller's method, was calculated as quercetin and as hyperoside. Flavonoid content, based on the Christ-Muller's method, was higher for the bur-marigold herb than for flowers. The results obtained using the Christ-Muller's method for hyperoside varied to 1.85% in the bur-marigold herb and to 0.92% in the bur-marigold flowers. The results according to this method calculated for quercetin were much lower and varied for all sources to 1.23%. The extracts of *B. tripartita* were analyzed by RP-HPLC coupled with diode-array detector, using gradient elution of methanol and 0.50% *ortho*-phosphoric acid in water. The major phenolics were found to be: flavone such as luteolin and its derivative and flavanone such as 7-O-glucoside isookanin. The values obtained for total flavonoid content determined by HPLC were lower than those obtained using the Christ-Müller's method. The differences in flavonoid patterns observed between both methods showed a significant error in the spectrophotometric method and were caused by a possible reaction between aluminum chloride and non-flavonoid compounds present in the investigated samples (26).

All the extracts, prepared by using solvents of different polarity, were examined for their radical scavenging activity. It is known that chemical com-

Table 1. Contents (in % of dry weight) of total flavonoids and of isookanin 7-O-glucoside, luteolin 7-O-glucoside and luteolin in the extracts from flowers and herb of *B. tripartita*.

Extract/compound	UV-vis method		HPLC method	
	(as quercetin equivalent)	(as hyperoside equivalent)	flowers	herb
BF, flowers	0.649	0.928	–	–
BH, herb	1.230	1.854	–	–
isookanin 7-O-glucoside (flavanomarein)	–	–	0.229	0.157
luteolin 7-O-glucoside (cynaroside)	–	–	0.116	0.179
luteolin	–	–	0.047	0.031

Table 2. DPPH radical scavenging activities (%) of extracts from flowers and herb of *B. tripartita* and of pure compounds isolated from this plant material.

Extract	Flowers		Herb		Pure compounds
water	BF1	36	BH1	35	–
methanol:water	BF2	68 (95[a])	BH2	34	–
acetone:water	BF3	66	BH3	56 (82[a])	–
Flavonoids					
luteolin	–	–	–	–	41
cynaroside	–	–	–	–	25
flavanomarein	–	–	–	–	32

[a] measured after 20 min, all other results after 2 min.

position of the mixture and molecular structure of the major antioxidants influence the pathway of radical reactions (27). The number and arrangement of hydroxyl groups and glycosylation pattern determine the antioxidant activity of polyphenolic compounds. However, other factors, such as pH of the solution (28) and the properties of the solvent may be important as well (29, 30). The rate of scavenging of DPPH radicals by twelve commonly occurring flavonoids (flavonols, flavanols, flavanones and flavones) was determined by EPR. Flavonols showed a significantly higher activity (I_{EPR} values of 0.20-0.39) than other compounds, but for flavonoids not belonging to this group, luteolin had the highest antioxidative activity. Luteolin is a flavone with four hydroxyl groups, its reactivity towards radicals can be ascribed mainly to the reducing power of the *o*-dihydroxy structure in the B ring (31). Luteolin was examined previously using spectrophotometric assay and EC_{50} = 85 g_{antiox}/kg_{DPPH} in methanol was found (32), whereas the value established by us amounts to 65. The difference may result from the difficulties of background corrections in the UV-vis spectra. The antioxidant activity of two glucosides: flavanomarein (isookanin 7-O-glucoside) and cynaroside (luteolin 7-O-glucoside) was evaluated. The data (summarized in Table 2) for glucosides may be compared with the activity of luteolin.

All three pure flavonoids, with the same 3',4'-dihydroxyl arrangement in the B ring (Figure 1), were effective radical scavengers. The glycosylation in the 7 position diminished the antioxidant activity, as it was shown previously (3) when the values of TEAC (Trolox equivalent antioxidant capacity, mM) were compared for luteolin (2.1) and its 3',7-diglucoside (0.79) (5). Pure flavonoid compounds were less effective scavengers when compared to the extracts. Higher activity of the extracts suggests that other active compounds are also present in the isolated mixtures and that antioxidative synergism may occur. Water extracts from flowers and herb showed similar activity, of approximately 36%, and were the least effective radical scavengers. Methanol and acetone extracts were considerably more active, reducing ca. 66% of the radicals. The extract from flowers exhibited almost twice as high activity as that from the herb (especially for the methanol-water fraction), which may be somewhat

surprising taking into account the smaller content of total flavonoids (see Table 1). The diethyl ether and ethyl acetate fractions, obtained by successive extraction of water extract, displayed the highest content of effective antioxidants. Both extracts scavenged DPPH radicals almost completely and reacted very fast. The activity of butanol fraction was weak at the beginning but the reaction continued for another 20 min or even longer. Radical scavenging activities of these extracts (summarized in Table 3) are also based on the changes in integral intensities of EPR signals after 2 or 20 min.

Kinetic parameters of the reaction between DPPH radical and the scavengers may provide additional information about the antioxidants from *B. tripartita*. Usually, the reaction of DPPH with an efficient flavonoid antioxidant proceeds very fast (within seconds). The kinetics of this reaction for flavonoids in methanol was investigated using stopped-flow absorption spectroscopy (30); the majority of the rate data were in seconds, only the reactions with hesperetin, naringenin and chrysin were slow ($t_{1/2}$ >1000 s). The slow reaction might be expected in the presence of very weak antioxidants (with small number of OH groups) or polymerized ones and exhibiting some steric hindrances (that prevent closer contact between the DPPH radical and hydroxyl groups).

In our assays, the reactions were completed after 2-5 min, and the measurements performed for additional 30 min showed no changes in signal intensity. However, some extracts showed long lasting scavenging activity: after 2 min. BH3 extract depleted 56% of the radical, while after 20 min – up to 82%. It seemed worth comparing all the extracts that reacted longer than 2-5 min, namely: BF2, BH3, BTF3 and BTH3. Those samples were dissolved in methanol, added to the radical solution and the decays of DPPH signal were recorded in time. The 2D-EPR spectrum for the BF2 extract is illustrated in Figure 2, and the time-dependent scavenging effect of various extracts is compared in Figure 3.

At the beginning, the reaction proceeds according to the second-order kinetics and is very fast (the first points match second-order kinetic equation). This may be due to the reaction of effective, low molecular weight antioxidants. After about 5 min the reactions slow down and show the pseudo first-order kinetics. Less active scavengers as well as secondary radical products slowly decrease the amount of DPPH. The pseudo first-order kinetic equation was fitted using non-linear regression analysis (carried out for n = 15):

$$I(t) = a + b \times \exp(-k_{obs} \times t)$$

where: I(t) – EPR signal intensity as a function of time; a, b – regression parameters; k_{obs} – pseudo first-order rate constant, t – time.

The calculated pseudo first-order rate constants are presented in Table 4. Different kinetics suggested the presence of different radical scavenging mechanisms by polyphenolic compounds, as reported earlier (33); in the reaction mixture small, active flavonoids and larger polymeric products (e.g. oligomeric catechins) are probably present.

Table 3. DPPH radical scavenging activities (%) of extracts after successive extraction, obtained from flowers and herb of *B. tripartita* using solvents of different polarity.

Extract	Flowers		Herb	
diethyl ether	BTF1	97	BTH1	92
ethyl acetate	BTF2	98	BTH2	89
n-BuOH	BTF3	22 (36[a])	BTH3	65 (84[a])

[a] measured after 20 min, all other results after 2 min.

Table 4. The pseudo first-order rate constants k_{obs} [10^{-3} 1/s] for the reaction with DPPH radical.

Extract	$k_{obs} \times 10^{-3}$ [1/s]
BF2	3.48 ± 0.17
BH3	2.13 ± 0.25
BTF3	1.66 ± 0.41
BTH3	2.35 ± 0.25

CONCLUSIONS

Flowers and herb of *B. tripartita* are a source of antioxidants that exhibit radical scavenging properties. Although the total content of phenolics in herbal extracts was higher, flower extracts scavenged more effectively than herbal ones. This proves that not only the content of total polyphenols, but also the composition of the extract and other factors (solvent, antioxidative synergism) may play an important role in predicting antioxidant activity. The rates of reaction with DPPH radical may characterize more or less active antioxidant fractions. However, the complex mixture of compounds exhibiting radical scavenging properties makes it difficult to reach any conclusions about the reaction mechanism. A detailed analysis of components and characteristics of their reactivity against radicals is required. Extracts from *B. tripartita* are a promising source of antioxidants that can be used for protection of foods and pharmaceuticals.

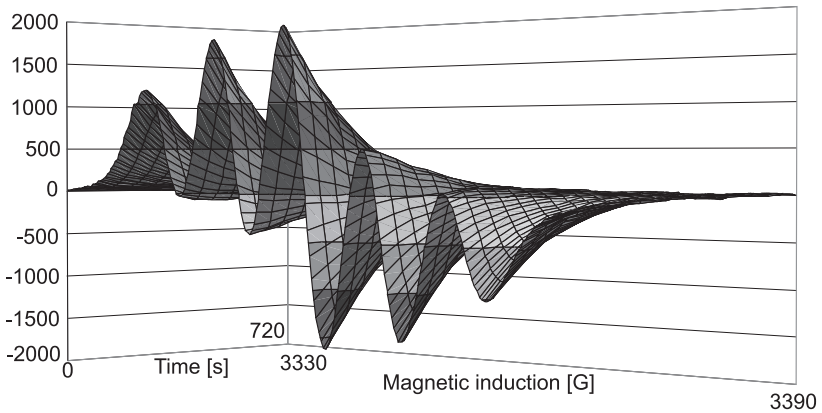


Figure 2. 2D EPR spectrum of DPPH measured in time upon addition of BF2 extract.

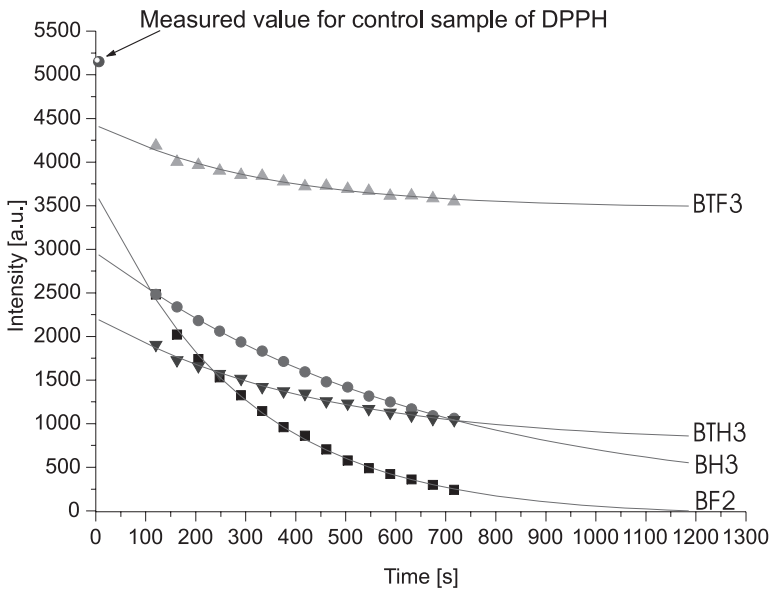


Figure 3. Time-dependent scavenging effect of various extracts of bur-marigold on DPPH; the fitted curves were obtained by non-linear regression, using the equation for a first-order reaction. The value for DPPH without a scavenger for t = 0 was obtained from direct measurement of the control sample. Other intensities for t = 0 come from fitted curves.

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