

1-1-2012

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### Recommended Citation

DIMITROVA, DIMITRINA ZHELEVA and BALABANOVA, VESSELA (2012) "Antioxidant and acetylcholinesterase inhibitory potential of *Arnica montana* cultivated in Bulgaria," *Turkish Journal of Biology*. Vol. 36: No. 6, Article 15. <https://doi.org/10.3906/biy-1204-70>  
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## Antioxidant and acetylcholinesterase inhibitory potential of *Arnica montana* cultivated in Bulgaria

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Received: 29.04.2012 • Accepted: 30.07.2012

**Abstract:** The antioxidant and acetylcholinesterase inhibitory potential of methanol extract from *Arnica montana* cultivated in Bulgaria was evaluated. For the determination of antioxidant activity 1,1-diphenyl-2-picrylhydrazyl (DPPH), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) di-ammonium salt (ABTS) free radicals, and ferric reducing antioxidant power (FRAP) assay were used. Modified Ellman's colorimetric method was used for quantitative assessment of acetylcholinesterase inhibition potential. Butylated hydroxytoluene (BHT) and galantamine hydrobromide were used as positive controls, respectively. In addition, total polyphenols and tannins, flavonoids, hydroxycinnamic derivatives, and sesquiterpene lactones were determined using Folin-Ciocalteu reagent,  $AlCl_3$ ,  $Na_2MoO_4$ , and m-dinitrobenzene, respectively. The results demonstrated that *Arnica* extract has strong antioxidant activity and moderate ability to inhibit acetylcholinesterase. Total polyphenols, tannins, flavonoids, hydroxycinnamic derivatives, and sesquiterpene lactones were:  $468.46 \pm 4.03 \text{ mg g}^{-1}$ ,  $362.09 \pm 2.81 \text{ mg g}^{-1}$ ,  $25.48 \pm 1.44 \text{ mg g}^{-1}$ ,  $76.76 \pm 6.58 \text{ mg g}^{-1}$ , and  $14.69 \pm 0.76 \text{ mg g}^{-1}$  respectively. Therefore, *A. montana* is a powerful natural source of antioxidants and acetylcholinesterase inhibitors and could be useful in therapy for free radical pathologies and neurodegenerative disorders.

**Key words:** *Arnica montana*, antioxidant activity, acetylcholinesterase inhibition, phenolic compounds

### Introduction

*Arnica montana* L. (Asteraceae) is a valuable medicinal plant, and it has been used for centuries for its strong anti-inflammatory activity (1,2). Its plant substance (*Arnicae flos*) contains sesquiterpene lactones, volatile oils, and thymol derivatives as well as phenolic compounds such as flavonoids and phenolic acids, among others (3,4). The main application of *Arnica* is for treatment of injuries such as sprains, bruises, and hematomas (5). The oxidative damage caused by reactive oxygen species (ROS) has been associated with the pathogenesis of various conditions in the human body such as aging, arthritis, cancer, inflammation, and heart disease (6). In recent decades there has been an increasing interest in finding naturally

occurring antioxidants for use in foods, cosmetics, and/or medicinal materials (7). Acetylcholinesterase (AChE) inhibitors are widely used for the symptomatic treatment of Alzheimer's disease (AD) to enhance central cholinergic transmission (8). Some potent AChE inhibitors are derived from natural sources (e.g., galanthamine and huperzine A) and are already used for the treatment of different forms of dementia. Most of these secondary metabolites belong to the chemical class of alkaloids and are clinically known to have serious side effects connected with the peripheral effects of these highly bioactive compounds. From this perspective, there is a great need for improved AChE inhibitors that show low toxicity, good brain penetration, and high bioavailability (9).

However, the use of synthetic antioxidants such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT), which are widely used in processed food products, is now in doubt due to concerns about their potential toxicity and unwanted side effects (10,11). Thus, attention is increasingly focused on the development and utilization of natural sources of antioxidants (12).

Oxidative stress is directly related to neurodegenerative diseases; therefore, the antioxidant and AChE inhibitory potentials of various plant extracts can be helpful in providing neuroprotection (13).

The dichloromethane extracts of *Arnica montana* flower heads have shown anti-inflammatory effects and have also produced allergenic and cytotoxic reactions (14). Seeds of *A. montana* and *A. chamissonis* have been investigated as potential sources of natural antioxidants because samples are rich in lipophilic and hydrophilic compounds (15). Recent investigations show strong antioxidant, lipoxygenase, and xanthine oxidase inhibitory activity of *A. montana* and *A. chamissonis* tinctures (6), and methanol extracts of *A. chamissonis* subsp. *foliosa* demonstrated selective inhibition against AChE (8). In order to discover new sources of natural compounds with both antioxidant and acetylcholinesterase inhibitory potential for the treatment of neurodegenerative disorders, the methanol extract from *Arnica montana* flower heads grown on plantation in Bulgaria was investigated.

#### Experimental plant material

Seeds of a native *Arnica* strain collected from the Carpathians, Ukraine, were used. The plant material was cultivated in the experimental field on Mt Vitosha, Zlatni mostove locality. Flower heads of 3-year-old *Arnica montana* (*Arnicae* flos) were collected during the flowering in June 2011. The voucher specimen was confirmed by Assist Prof Vessela Balabanova and deposited in the herbarium (SOM) at the Institute of Biodiversity and Ecosystem Research (IBER), Bulgarian Academy of Science (BAS), Sofia, Bulgaria.

#### Chemicals and reagents

From Sigma-Aldrich, 2,2'-diphenyl-1-picrylhydrazyl (DPPH); 2,2'-azinobis-(3-ethylbenzothiazine-6-sulfonic acid) (ABTS); sulfanilamide; 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid

(Trolox); 2,4,6-tripyridyl-s-triazine (TPTZ), ferric chloride.6H<sub>2</sub>O; sodium acetate; potassium persulphate; acetylcholinesterase (AChE) type VI-S from electric eel, 349 U mg<sup>-1</sup> solid, 411 U mg<sup>-1</sup> protein; acetylthiocholine iodide (AChI); and 5,5'-dithiobis [2-nitrobenzoic acid] (DTNB) were purchased. All other chemicals, including the solvents, were of analytical grade.

#### Determination of total polyphenols and tannins content

The determination of total polyphenols and tannins was performed according to the European Pharmacopoeia (16), with Folin-Ciocalteu reagent and pyrogallol as standards. Analyses were carried out at 760 nm, and measurements were carried out using a Shimadzu spectrophotometer (Japan). The content of total polyphenols and tannins was calculated as pyrogallol equivalent (PE) in milligrams per gram of dry extract (de). All determinations were performed in triplicate (n = 3).

#### Determination of total flavonoids content

Flavonoid content was spectrophotometrically determined at 430 nm by creating a complex with AlCl<sub>3</sub> according to the European Pharmacopoeia (17). Flavonoid content was calculated as hyperoside equivalent (HE) in milligrams per gram of dry extract. The measurements were carried out using a Shimadzu UV-VIS spectrophotometer (Japan). All determinations were performed in triplicate (n = 3).

#### Determination of total hydroxycinnamic derivatives content

The amount of total phenolic acids was determined following the European Pharmacopoeia 7.0 method (18) at 505 nm using a Shimadzu spectrophotometer (Japan). Hydroxycinnamic derivatives content was expressed as rosmarinic acid equivalent (RAE) in milligrams per gram of dry extract. All determinations were performed in triplicate (n = 3).

#### Determination of total sesquiterpene lactones content

The amount of sesquiterpene lactones was spectrophotometrically determined at 535 nm using m-dinitrobenzene and KOH (19). The content of sesquiterpene lactones was calculated as dihydrohelenalinacetate equivalent (DE) in

milligrams per gram of dry extract. The measurements were carried out using a Shimadzu UV-VIS spectrophotometer (Japan). All determinations were performed in triplicate (n = 3).

### Measurement of antioxidant activity

#### DPPH radical scavenging activity

Free radical scavenging activity was measured by DPPH method (20). Different concentrations (1 mL) of dry extract in MeOH was added to 1 mL methanolic solution of 2,2'-diphenyl-1-picrylhydrazyl (DPPH) (2 mg mL<sup>-1</sup>). The absorbance was measured at 517 nm after 30 min. Results were evaluated as percentage scavenging of radical: DPPH radical scavenging activity

$$(\%) = \frac{\text{Abs}_{\text{control}} - \text{Abs}_{\text{sample}}}{\text{Abs}_{\text{control}}} 100,$$

where Abs<sub>control</sub> is the absorbance of DPPH radical in MeOH, and Abs<sub>sample</sub> is the absorbance of DPPH radical solution mixed with sample. The IC<sub>50</sub> value of the sample (concentration of sample where absorbance of DPPH decreases 50% with respect to absorbance of blank) was determined. BHT was used as positive control. All determinations were performed in triplicate (n = 3).

#### ABTS radical scavenging assay

For the ABTS assay Arnao et al. (21) was followed with some modifications. Stock solutions included 7 mM ABTS solution and 2.4 mM potassium persulphate solution. The working solution was prepared by mixing the 2 stock solutions in equal quantities and allowing them to react for 14 h at room temperature in the dark. The solution was then diluted by mixing 2 mL of ABTS solution with 50 mL of methanol to obtain an absorbance of 0.305 ± 0.01 units at 734 nm by spectrophotometer. A fresh ABTS solution was prepared for each assay. Different concentrations (1 mL) of dry extract were allowed to react with 2 mL of ABTS solution, and the absorbance was taken at 734 nm after 5 min. The ABTS scavenging capacity of the compound was compared with that of BHT, and percentage inhibition was calculated as:

ABTS radical scavenging activity,

$$(\%) = \frac{\text{Abs}_{\text{control}} - \text{Abs}_{\text{sample}}}{\text{Abs}_{\text{control}}} 100,$$

where Abs<sub>control</sub> is the absorbance of ABTS radical in methanol, and Abs<sub>sample</sub> is the absorbance of ABTS radical solution mixed with sample. The IC<sub>50</sub> value of the sample (concentration of sample where absorbance of ABTS decreases 50% with respect to absorbance of blank) was determined. BHT was used as positive control. All determinations were performed in triplicate (n = 3).

#### Ferric reducing/antioxidant power (FRAP)

The FRAP assay was performed according to Benzie and Strain (22) with some modifications. The stock solutions included 300 mM acetate buffer (3.1 g C<sub>2</sub>H<sub>3</sub>NaO<sub>2</sub>·3H<sub>2</sub>O and 16 mL C<sub>2</sub>H<sub>4</sub>O<sub>2</sub>); pH 3.6, 10 mM TPTZ solution in 40 mM HCl; and 20 mM FeCl<sub>3</sub>·6H<sub>2</sub>O solution. Fresh working solution was prepared by mixing 25 mL of acetate buffer, 2.5 mL of TPTZ solution, and 2.5 mL of FeCl<sub>3</sub>·6H<sub>2</sub>O solution and warming at 37 °C before use. Then 0.15 mL of compound in MeOH was allowed to react with 2.8 mL FRAP solution for 30 min in the dark. Readings of the colored product (ferrous tripyridyltriazine complex) were then taken at 593 nm. Results are expressed in µg Trolox equivalent (µg TE mg<sup>-1</sup> de), and BHT was used as reference. All determinations were performed in triplicate (n = 3).

#### AChE inhibition assay

The enzyme inhibition activities for AChE were evaluated according to the spectrophotometric method of Ellman et al. (23) with minor modifications. (24). The plant extracts were tested in a concentration range of 60 to 1000 µg mL<sup>-1</sup>. Galanthamine hydrobromide was used as positive control; it was tested in a concentration range between 0.01 and 100 µg mL<sup>-1</sup>. By this method, 1500 µL of phosphate buffer (pH 8), 200 µL of AChE solution (0.3 U mL<sup>-1</sup>), 200 µL of test sample, and 1000 µL of DTNB (3 mM) were mixed and incubated at 37 °C for 15 min in a 1 cm path length glass cell. Then 200 µL of ATCI (15 mM) was added to the reaction mixture, and the absorbance of the yellow 5-thio-2-nitrobenzoate anion produced was measured at a wavelength of 412 nm using a

Shimadzu spectrophotometer (Japan) every 10 s for 10 min. A control mixture was created with the addition of methanol instead of extract. Results were expressed as the average of triplicates. The enzyme inhibition (%) was calculated from the rate of absorbance change over time ( $V = \text{Abs}/t$ ) data as follows: inhibition

$$(\%) = \frac{V_{\text{control}} - V_{\text{sample}}}{V_{\text{control}}} 100,$$

where  $V_{\text{control}}$  is the change of control absorbance, and  $V_{\text{sample}}$  is the change of sample absorbance. Data are expressed as mean  $\pm$  standard error (SEM), and the results were taken from at least 3 independent experiments performed in duplicate. The  $IC_{50}$  values (the concentration of test compounds that inhibits hydrolysis of substrates by 50%) were determined by spectrophotometric measurement of the effect of increasing test compound concentrations (plant extracts and positive controls) on AChE activity. The  $IC_{50}$  values were obtained from dose-effect curves by linear regression. Galanthamine hydrobromide was used as positive control. All determinations were performed in triplicate ( $n = 3$ ).

## Results and discussion

The amount of total polyphenols and tannins expressed as pyrogallol equivalent (by Folin–Ciocalteu method) was  $468.46 \pm 4.03 \text{ mg g}^{-1}$  dry extract (de) and  $362.09 \pm 2.81 \text{ mg g}^{-1}$  de, respectively (Figure). Total flavonoids and hydroxycinnamic

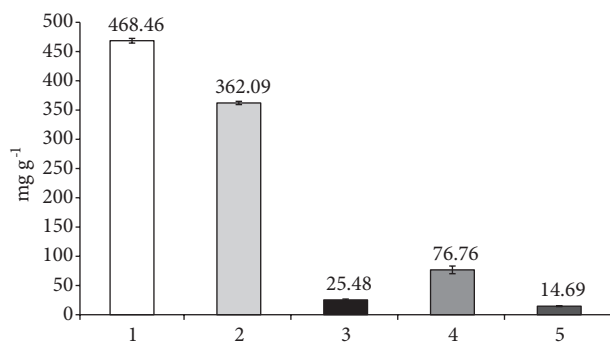


Figure. Total polyphenols, tannins, flavonoids, hydroxycinnamic derivatives, and sesquiterpene lactones in methanol extract from *Arnica montana* flower heads ( $\text{mg g}^{-1}$  de). 1: total polyphenols; 2: tannins; 3: total flavonoids; 4: total hydroxycinnamic derivatives; 5: total sesquiterpene lactones.

derivatives contents were expressed as milligrams HE and milligrams RAE and were  $25.48 \pm 1.44 \text{ mg g}^{-1}$  de and  $76.76 \pm 6.58 \text{ mg g}^{-1}$  de. Previous investigations revealed significantly lower levels of total polyphenols [ $116.90 \pm 1.00 \text{ GAE mg g}^{-1}$  dry weight (dw)] and a higher quantity of flavonoids ( $113.22 \pm 8.72 \text{ QE mg g}^{-1}$  dw) in *A. montana* tincture (15). However, the content of total phenolic acids ( $73.14 \pm 2.33 \text{ CAE mg g}^{-1}$  dw) was similar to that of total hydroxycinnamic derivatives ( $76.76 \pm 6.58 \text{ mg g}^{-1}$  de) in our study. Variations in quoted and current data are probably due to differences in methods used for extraction and quantification.

Spitaler et al. (25) investigated altitudinal variation in the phenolic content of flowering heads of cultivated *Arnica montana*. The total amount of phenol compounds increased with elevation, and this corresponded to an increase in radical scavenging potential of extracts from plants grown at different altitudes. Temperature is the key to the altitudinal variation of phenolics in *Arnica montana* cv. ARBO. The increased UV-B radiation did not affect phenolic metabolites in *Arnica*, but a  $5 \text{ }^\circ\text{C}$  decrease in temperature affected the ratio of B-ring ortho-diphenolic (quercetin) compared to B-ring monophenolic (kaempferol) flavonols (26).

For determination of sesquiterpene lactones in *Arnica* flower head, a color reaction with m-dinitrobenzene (DNB) was used. DNB only reacts with cyclopenten-4-on- and 2a-hydroxycyclopentan-4-on (arnifolines) structures, but not with those of the chamissonolides (19). The studied methanol extract contained  $14.69 \pm 0.76 \text{ mg g}^{-1}$  de (0.49% dw) sesquiterpene lactones. This is in accordance with the requirements published in the European Pharmacopoeia (more than 0.4% m/m expressed as 11,13-dihydrohelenalin tiglite) (27).

Numerous studies revealed antioxidants and extract activities of well known medicinal plants such as *Cichorium intybus* (28), *Salvia officinalis* (29), and *Artemisia* (30) as well as the acetylcholinesterase inhibitory potential of *Arnebia densiflora* extracts (31). However, no detailed evaluations of antioxidant and acetylcholinesterase capacity using different in vitro methods in *A. montana* extracts have been undertaken so far.



Table. DPPH, ABTS-radical scavenging, FRAP, and AChE inhibitory activities of methanol extract from *Arnica montana* flower heads.

Sample	DPPH IC <sub>50</sub> [µg mL <sup>-1</sup> ]	ABTS IC <sub>50</sub> [µg mL <sup>-1</sup> ]	FRAP [µg TE mg <sup>-1</sup> de]	AChE IC <sub>50</sub> [µg mL <sup>-1</sup> ]
<i>Arnica montana</i>	44.65	9.87	158.59 ± 33.92	311.51
BHT	64.76	17.70	30.50 ± 0.24	-
Galanthamine hydrobromide	-	-	-	0.16

Results are represented as means ± standard deviation, n = 3.

DPPH and ABTS assays are based on the ability to scavenge synthetic free radicals using a variety of radical-generating systems and methods for detection of the oxidation end-point. ABTS or DPPH radical scavenging methods are common spectrophotometric procedures for determining the antioxidant capacities of components. The radical scavenging and total antioxidant activities of methanol extract from *A. montana* flower heads cultivated in Bulgaria were compared with those of BHT and expressed as IC<sub>50</sub> µg mL<sup>-1</sup> of inhibition against DPPH, ABTS, and mM TE mg<sup>-1</sup>, respectively (Table). The scavenging effect of *A. montana* was stronger than the control, with corresponding IC<sub>50</sub> values of 44.65 µg mL<sup>-1</sup> (DPPH) and 9.87 µg mL<sup>-1</sup> (ABTS). A recent study showed significantly lower DPPH radical activity (20.00 mg mL<sup>-1</sup>) in *A. montana* tincture (15). This fact unambiguously confirms the importance of polyphenol and tannin content, particularly for the radical scavenging activity of plants. In FRAP assay the reduction of ferric tripyridyl triazine (Fe III TPTZ) complex to ferrous form (which has an intense blue color) at low pH can be monitored by measuring the change in absorbance at 593 nm. The change in absorbance is, therefore, directly related to the combined or total reducing power of the electron donating the antioxidants present in the reaction mixture. The *A. montana* extract manifests higher FRAP activity (158.59 ± 33.92 µg TE mg<sup>-1</sup> de) than BHT (30.50 ± 0.24 µg TE mg<sup>-1</sup> de).

In the present study methanol extract of *Arnica* flower heads exhibited moderate AChE inhibitory

potential with an IC<sub>50</sub> of 311.51 µg mL<sup>-1</sup>. Wszelaki et al. (8) reported AChE inhibitory activity of methanol and hexane extracts of *Arnica chamissonis* subsp. *foliosa* flower heads as IC<sub>50</sub> 43 µg mL<sup>-1</sup> and 29 µg mL<sup>-1</sup>, respectively; on the other hand, no reports on the activity of *Arnica montana* methanol extract against AChE inhibition have been published.

## Conclusion

The extract of *Arnica montana* flower heads cultivated in Bulgaria has great potential and should be considered for further studies to identify the constituents responsible for antioxidant and AChE inhibitory activity, which may eventually be utilized in the treatment of neurodegenerative disorders.

## Acknowledgments

The authors are grateful to Assist Prof Reneta Gevrenova, Department of Pharmacognosy, Faculty of Pharmacy, Medical University, Sofia.

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