Turk J Biol
35 (2011) 111-115

doi:10.3906/biy-0812-25

Evaluation of the antioxidant and acetylcholinesterase inhibitory activities of *Arnebia densiflora* Ledeb.

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Received: 19.12.2008

Abstract: *Arnebia densiflora* Ledeb. (Boraginaceae), a medicinal plant growing in Turkey, has been reported to contain a well-known red pigment, shikonin, a naphthoquinone derivative. In the current study, the acetylcholinesterase (AChE) inhibitory and antioxidant activities of the chloroform, ethyl acetate, methanol, and water extracts of the root, stem, and flowers of the plant, as well as shikonin, were investigated. AChE inhibition was tested using an ELISA microplate reader at 250, 500, 1000, and 2000 μg mL^{-1}. Antioxidant activity was determined by the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging test and the Fe^{+2}-ferrozine test system for metal chelating power. The results indicated that the methanol and water extracts of the roots had a moderate DPPH radical scavenging effect at 2000 μg mL^{-1}, while the extracts exerted a better ferrous ion chelating effect as compared to the reference, butylated hydroxyanisole. Only the root chloroform extract showed mild inhibition against AChE at 62.5 μg mL^{-1} (49.6 ± 1.69%). Shikonin was inactive in all of the assays performed.

Key words: *Arnebia densiflora*, Boraginaceae, shikonin, antioxidant activity, acetylcholinesterase inhibition

Arnebia densiflora Ledeb.'in antioksidan ve asetilkolinesteraz inhibe edici aktivitelerinin değerlendirilmesi

Özet: Türkiye'de yetişen tıbbi bir bitki olan *Arnebia densiflora*'nın Ledeb. (Boraginaceae), iyi bilinen naftakinon türevi kırmızı bir pigment olan şikonin içerdiği bildirilmiştir. Bu çalışmada bitkinin kök, gövde ve çiçeklerinin kloroform, etil asetat, metanol ve sulu ekstreleri ile şikonin'in asetilkolinesteraz (AChE) inhibitör ve antioksidan aktiviteleri incelenmiştir. Asetilkolinesteraz inhibisyonu ELISA mikroplak okuyucu kullanılarak 250, 500, 1000 ve 2000 μg mL^{-1}'de test edilmiştir. Antioksidan aktivite 2,2-difenil-1-pikrilhidrazil (DPPH) radikal süpürme testi ve Fe^{+2}-ferrozin test sistemi ile metal bağlama gücü ile tespit edilmiştir. Sonuçlar kök metanol ve sulu ekstrelerinin 2000 μg mL^{-1} de orta derecede DPPH radikal süpürücü etki gösterirken, ekstrelerin referans olan bütil hidroksi anisola oranla daha iyi demir-iyonu bağlayıcı etkiye sahip olduğunu göstermiştir. Sadece kök kloroform ekstresi 62,5 μg mL^{-1} de AChE'a karşı orta derecede (49,6 ± 1,69%) inhibisyon göstermiştir. Şikonin yapılan tüm testlerde inaktif bulunmuştur.

Anahtar sözcükler: *Arnebia densiflora*, Boraginaceae, şikonin, antioksidan aktivite, asetilkolinesteraz inhibisyonu

Introduction

Arnebia Forssk. (Boraginaceae) is a genus that contains 4 species of Turkish flora (1,2). Among them, *A. densiflora* (syn. *Lithospermum densiflorum* Ledeb. ex Nordm., *A. cephatoles* A.DC., *Munbya cephalotes* Boiss., *M. densiflora* Boiss., and *Macrotomia cephalotes* Boiss.), endemic to Turkey and Greece, is a well-known species for its medicinal properties and is quite popular due to its rich naphthoquinone content. The naphthoquinones in *A. densiflora* are alkannin derivatives, and alkannin and shikonin (β-alkannin) are the most famous among them (Figure) (3). Shikonin and some other naphthoquinones are widely used as red/purple colorants in foods, drugs, cosmetics, and the textile industry (3).

Another *Arnebia* species, *A. hispidissima* DC., was reported to be used as a stimulant and tonic (4). This information prompted us to investigate the acetylcholinesterase (AChE) inhibitory activity of *A. densiflora*, which is a well-known medicinal species, and its major component, shikonin. AChE is a key enzyme in the pathogenesis of Alzheimer's disease (AD), and inhibition of AChE has been accepted as one of the most effective approaches currently available for the treatment of AD (5). Since AD is also associated with oxidative stress, the antioxidant activity of *A. densiflora*, as well as shikonin, was examined by 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging (250, 500, 1000, and 2000 μg mL^{-1}) and ferrous ion chelating power tests (500 and 1000 μg mL^{-1}). The AChE inhibitory effect was evaluated by the spectrophotometric method of Ellman (6) using an ELISA microplate reader at 250, 500, 1000, and 2000 μg mL^{-1} concentrations.

Materials and methods

Plant material

The plant sample of *A. densiflora* was collected in May 2007 from Sivrihisar, Eskişehir, Turkey. The plant was identified by Prof. Dr. Osman Ketenoğlu (Department of Botany, Faculty of Science, Ankara University), and the voucher specimen was preserved in the herbarium of the Faculty of Pharmacy of Gazi University (GUE 2601).

Extraction

The plant material was dried in the shade, dissected, and powdered, and a sample (10 g) of each part (root, stem, and flowers) was soaked by shaking it successively in chloroform, ethyl acetate, methanol, and finally water for 3 days. The organic layers were filtered and evaporated in vacuo to give the chloroform, ethyl acetate, methanol, and water extracts. The corresponding extract yields are as follows: for the root, 1.00, 1.50, 1.5, and 1.8 g; for the stem, 0.40, 0.50, 2.00, and 5.5 g; and for the flowers, 0.65, 0.95, 2.60, and 4.90 g, respectively.

Shikonin

Shikonin, used as a standard in the thin layer chromatography (TLC) analysis and also employed in the activity tests, was purchased from Chromadex®, CA, USA.

DPPH radical scavenging activity test

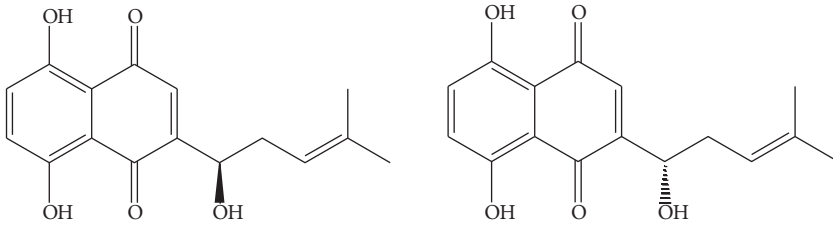
The stable DPPH radical scavenging activity was determined by Blois's method (7). The samples and references, dissolved in ethanol (75%), were mixed with DPPH solution (1.5×10^{-4} M). The remaining DPPH amount was measured at 520 nm using a Unico 4802 UV-visible double beam spectrophotometer (Dayton, NJ, USA). Gallic acid and butylated hydroxyanisole (BHA) were employed as the references. Inhibition of DPPH as a percentage (I%) was calculated as follows:

$$I\% = [(A_{blank} - A_{sample})/A_{blank}] \times 100,$$

where A_{blank} is the absorbance of the control reaction (containing all reagents except the test sample) and A_{sample} is the absorbance of the extracts/reference.

Fe^{+2}-ferrozine test system for metal chelating

The ferrous ion chelating effect of the extracts was estimated with the Fe^{+2}-ferrozine test system (8). Briefly, 740 μL of methanol and the samples were incubated with 20 μL of 2 mM $FeCl_2$ solution. The reaction was initiated by the addition of 5 mM ferrozine into the mixture and was left standing at ambient temperature for 10 min. The absorbance of the reaction mixture was measured at 562 nm. The ratio of inhibition of the ferrozine-Fe^{2+} complex formation was calculated as follows:



(A) (B)

Figure. Chemical structure of shikonin (A) and alkannin (B).

% Inhibition = [(absorbance of control - absorbance of test sample)/absorbance of control] × 100. The control contained only $FeCl_2$ and ferrozine. Analyses were run in 4 replicates and expressed as mean values with standard errors of the means (SEM). BHA was employed as the reference in this test.

AChE inhibitory activity

AChE inhibition was assayed according to the spectrophotometric method of Ellman (6). Electric eel AChE (Type-VI-S, EC 3.1.1.7, Sigma, St. Louis, MO, USA) was employed as the enzyme source, while acetylthiocholine iodide (Sigma) as a substrate and 5,5´-dithio-bis(2-nitrobenzoic)acid (DTNB) were also used in the anti-AChE activity determination. All reagents and conditions were the same as those reported previously (9,10). The experiments were performed in 4 parallel sets. Galanthamine purchased from Sigma was the reference in this study.

Results and discussion

The results obtained with the chloroform, ethyl acetate, methanol, and water extracts of the different plant parts (root, stem, and flowers) of *A. densiflora*, shikonin, and the references in the antioxidant activity tests are given in Tables 1 and 2. The AChE inhibitory effects of the extracts and shikonin were also tested in vitro. Neither the extracts nor shikonin showed inhibition against AChE, except for the root chloroform extract, which displayed 49.6 ± 1.69% of inhibition at 62.5 μg mL^{-1}. However, a drastic decrease in the AChE inhibitory effect of the extract was observed at increasing concentrations (125 and 250 μg mL^{-1}, 12.3 ± 1.14% and 11.9 ± 0.51%, respectively), which did not allow us to measure its absorbance. Otherwise, it might be speculated that the extracts and shikonin would have been effective in the AChE inhibitory assay, as the root chloroform extract had significant inhibition (49.6 ± 1.69%) even at 62.5 μg mL^{-1}.

On the other hand, the extracts and shikonin did not possess an appreciable DPPH radical scavenging effect at the tested concentrations. Only the methanol and water extracts of the roots displayed mild scavenging effects over 50% at 2000 μg mL^{-1} (56.1% and 56.2%, respectively), while the methanol extracts of the stem and flowers had an approximately 50% scavenging effect (49.5% and 48.6%, respectively). Only the chloroform and ethyl acetate extracts of the stem had relatively higher abilities (73.5% and 81.7%, respectively) to chelate ferrous ions as compared with the reference, butylated hydroxyanisole (BHA), at 1000 μg mL^{-1}.

During the literature survey, several reports were identified on the antioxidant activity of shikonin or shikonin-containing plants. For instance, alkannin and shikonin were found to inhibit lipid peroxidation in vitro (11). Shikonin had a strong, nonreductive, scavenging effect towards 1,1-diphenyl-2-picrylhydrazyl radical measured by electron spin resonance (ESR) spectrometry (12). The antioxidant activity of 3 compounds, β,β-dimethyl-acrylshikonin, acetylshikonin, and shikonin, isolated from *Lithospermum erythrorhizon* Siebold & Zucc. was measured by the oxidative stability instrument (OSI) in lard, and it was concluded that these compounds had an antioxidant effect in that assay (13). Similarly, antioxidant effects of alkannin and shikonin isolated

Table 1. DPPH radical scavenging activity (inhibition % ± SEM) of the extracts of *A. densiflora* and shikonin.

Extracts	Inhibition % ± SEM[a] against DPPH radical			
	250 µg mL^{-1}	500 µg mL^{-1}	1000 µg mL^{-1}	2000 µg mL^{-1}
Root-$CHCl_3$	-b	-	-	-
Root-EtOAc	-	-	-	-
Root-MeOH	14.4 ± 1.38	23.0 ± 1.04	40.1 ± 1.35	56.1 ± 1.83
Root-H_2O	9.2 ± 0.29	18.6 ± 0.09	35.1 ± 0.55	56.2 ± 1.74
Stem-$CHCl_3$	-	-	-	3.9 ± 0.48
Stem-EtOAc	-	-	-	4.6 ± 0.04
Stem-MeOH	7.0 ± 0.30	12.7 ± 0.74	24.6 ± 0.39	49.5 ± 1.12
Stem-H_2O	4.2 ± 0.32	5.2 ± 0.16	8.5 ± 1.09	14.8 ± 0.49
Flower-$CHCl_3$	-	-	-	6.4 ± 0.24
Flower-EtOAc	-	-	-	5.3 ± 0.29
Flower-MeOH	8.6 ± 0.09	15.8 ± 0.50	27.2 ± 1.33	48.6 ± 1.26
Flower-H_2O	-	6.6 ± 0.19	14.5 ± 0.68	23.1 ± 1.03
Shikonin	-	-	-	-
References				
BHA	--c	78.0 ± 0.48	81.6 ± 1.67	82.9 ± 0.68
Gallic acid	--	91.6 ± 0.06	92.6 ± 0.10	93.2 ± 0.10

[a]SEM = Standard error of the mean, [b]- = No activity

Table 2. Ferrous ion chelating % ± SEM of the extracts of *A. densiflora* and shikonin.

Extracts	Ferrous ion chelating % ± SEM[a]	
	500 µg mL^{-1}	1000 µg mL^{-1}
Root-$CHCl_3$	37.3 ± 0.01	56.5 ± 1.16
Root-EtOA	-[b]	7.7 ± 1.31
Root-MeOH	-	10.4 ± 0.41
Root-H_2O	-	17.9 ± 1.59
Stem-$CHCl_3$	61.1 ± 1.21	73.5 ± 1.62
Stem-EtOAc	71.7 ± 0.96	81.7 ± 0.63
Stem-MeOH	-	2.5 ± 0.21
Stem-H_2O	-	13.5 ± 1.20
Flower-$CHCl_3$	22.2 ± 1.15	41.1 ± 0.11
Flower-EtOAc	31.4 ± 1.04	38.3 ± 1.27
Flower-MeOH	-	6.0 ± 1.16
Flower-H_2O	16.5 ± 0.63	38.6 ± 1.18
Shikonin	9.5±1.04	17.6 ± 1.19
Reference		
BHA	21.7 ± 1.10	26.9 ± 1.48

[a]SEM = Standard error mean, [b]- = No activity

from *Alkanna tinctoria* (L.) Tausch roots in oily substrates were also reported; the antioxidant activity of alkannin was low in corn oil, whereas shikonin, combined with citric acid, exhibited a moderate antioxidant activity in sunflower oil (14).

In this study, TLC analysis of *A. densiflora* extracts using a solvent system of toluene and acetic acid (99:1) revealed that shikonin was clearly present in all of the root extracts. However, neither the extracts nor shikonin exhibited an antioxidant effect in these experiments.

In conclusion, this is the first study examining the antioxidant and AChE inhibitory activity of different extracts from various parts of *A. densiflora* as well as the AChE inhibitory activity of shikonin.

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