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Anti-Acetylcholinesterase and Antioxidant Appraisal of the Bulb Extracts of Five *Sternbergia* Species

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Abstract: In the current study, we examined anti-acetylcholinesterase (AChE) and antioxidant activities of the ethyl acetate, methanol, and water extracts from the bulbs of Turkish *Sternbergia* Waldst. & Kit. (Amaryllidaceae) species; *S. candida*, *S. clusiana*, *S. fisheriana*, *S. lutea* subsp. *lutea*, and *S. lutea* subsp. *sicula*. Anti-AChE activity was tested by spectrophotometric method of Ellman using ELISA microplate reader at 50, 100, and 200 µg mL^{-1} concentrations. Antioxidant activity of the extracts was evaluated by DPPH radical scavenging activity, ferrous ion-chelating capacity, ferric-reducing antioxidant power, and beta-carotene bleaching assays at 500, 1000, and 2000 µg mL^{-1}. Total phenol and flavonoid contents of the extracts were determined *via* Folin-Ciocalteau's and $AlCl_3$ reagents, respectively. The ethyl acetate extract of *S. fischeriana* was the most active in anti-AChE assay (90.94% and 98.02% of inhibitions at 100 and 200 µg mL^{-1} concentrations, respectively. Antioxidant activity of the extracts was found to be not significant.

Keywords: *Sternbergia*; Amaryllidaceae; lycorine; acetylcholinesterase; antioxidant

1. Introduction

Herbal medicines are also in great stipulate in the world for health care purposes because of their efficacy and safety. They also offer therapeutics for age-related disorders like Alzheimer's disease (AD), osteoporosis, immune disorders, etc. for which a limited number of modern medicine is accessible. The genus *Sternbergia* Waldst. & Kit. (Amaryllidaceae) is represented by 6 taxa in Turkey; *S. lutea* Waldts. A. Kit., *S. sicula* Tineo ex Guss., *S. colchiciflora* Waldst. & Kit., *S. fischeriana* (Herert) Rupr., *S. candida* Mathew & T. Baytop, and *S. clusiana* Ker Ggawl.) Ker Gawl. ex Sprengel [1]. Among them, *S. candida* is endemic to Turkey, which is considered to be the possible gene center for this genus. Bulbs and leaves of Amaryllidaceae species were reported to be used as poultices and decoctions for treating sores and digestive disorders by Africans [2]. As other members of Amaryllidaceae family, *Sternbergia* species, known as imperative medicinal plants, are also known to

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be affluent in alkaloid content [3, 4, 5, 6]. Lycorine, dominant in *Sternbergia* genus, and galanthamine are the alkaloids having the same chemical skeleton, which are so-called "Amaryllidaceae alkaloids". Galanthamine, as an acetylcholinesterase (AChE) enzyme inhibitor of herbal origin, is currently a licensed drug (Reminyl®) accessible in the market for the treatment of Alzheimer's disease (AD) [7]. AChE inhibitors, which make higher level of acetylcholine available in the brain, have been recently accepted as one of the most effectual treatment approaches towards AD. However, it is still in need to discover new anti-AChE agents since there is no cure to impede moderate and severe types of the disease.

Hence, in this study, we aimed to inspect anti-AChE and antioxidant activities of the ethyl acetate, methanol, and water extracts prepared from the bulbs of *S. candida*, *S. clusiana*, *S. fisheriana*, *S. lutea* subsp. *lutea*, and *S. lutea* subsp. *sicula* for discovery of possible AChE inhibitors.

2. Materials and Methods

2.1. Plant Materials

The bulbs of *S. candida*, *S. clusiana*, *S. fischeriana*, *S. lutea* subsp. *lutea*, and *S. lutea* subsp. *sicula* growing in Turkey were collected from Fethiye town (Mugla province), Göksun district (Kahramanmaraş province), Yayladag district (Hatay province), Torbali town (Izmir province), and Marmaris district (Mugla province), respectively, during 2004-2006. The voucher specimens are deposited at the Herbarium belonging to Faculty of Pharmacy of Ankara University with the corresponding herbarium numbers; AEF 23794, AEF, 23697, AEF 22913, AEF 23694, and AEF 23695, respectively.

2.2. Preparation of the Extracts

Air-dried and powdered materials of the bulbs of the *Sternbergia* species used in this work were weighed accurately and then, extracted sequentially with ethyl acetate, methanol, and water. The extracts were prepared by maceration 50 g of each plant powder in 300 ml of ethyl acetate (EtOAc), methanol (MeOH), and distilled water (H_2O) for 8 hours, respectively. The macerates obtained with ethyl acetate and methanol were evaporated until dryness and the water macerate was lyophilized.

2.3. AChE Inhibitory Activity Assay

AChE inhibitory activity was measured by slightly modifying the spectrophotometric method developed by Ellman et al. [8] Electric eel AChE (Type-VI-S, EC 3.1.1.7, Sigma) was used, while acetylthiocholine iodide (Sigma, St. Louis, MO, USA) was employed as substrates of the reaction. 5,5´-Dithio-bis(2-nitrobenzoic) acid (DTNB, Sigma, St. Louis, MO, USA) was used for the measurement of anti-AChE activity. All the other reagents and conditions were same as described previously [9]. In brief, 140 µL of 0.1 mM sodium phosphate buffer (pH 8.0), 20 µL of DTNB, 20 µL of test solution and 20 µL of AChE solution were added by multichannel automatic pipette (Eppendorf, Germany) in a 96-well microplate and incubated for 15 min at 25 °C. The reaction was then initiated with the addition of 10 µL of acetylthiocholine iodide. The hydrolysis of acetylthiocholine iodide was monitored by the formation of the yellow 5-thio-2-nitrobenzoate anion as a result of the reaction of DTNB with thiocholines, catalyzed by enzymes at a wavelength of 412 nm utilizing a 96-well microplate reader (VersaMax Molecular Devices, USA). The measurements and calculations were evaluated by using Softmax PRO 4.3.2.LS software. % Inhibition of AChE was determined by comparison of rates of reaction of samples relative to blank sample (ethanol in phosphate buffer pH=8) using the formula *(E-S)/E x 100*, where *E* is the activity of enzyme without test sample and *S* is the activity of enzyme with test sample. The experiments were done in triplicate. Galanthamine was purchased from Sigma (St. Louis, MO, USA) and used as reference.

2.4. Determination of Total Phenol and Flavonoid Contents of the Extracts

Phenolic compounds were determined using Folin-Ciocalteau's reagent according to Singleton and Rossi's method [10]. In brief; the samples were mixed with 750 µL of Folin-Ciocalteau's reagent and 600 µL of sodium carbonate in test tubes. The tubes were then vortexed and incubated at 40°C for 30 min. Afterward absorption was measured at 760 nm at a Unico 4802 UV-visible double beam spectrophotometer (USA). Total flavonoid content was calculated by aluminum chloride colorimetric method [11]. To sum up, a number of dilutions of rutin were obtained to prepare a calibration curve. Then, the same dilutions from the sample were also prepared and separately mixed with 95% ethanol, 10% aluminum chloride, 1 M sodium acetate as well as 2.8 mL of distilled water. Following incubation for 30 minutes at room temperature, absorbance of the reaction mixture was measured at wavelength of 415 nm with a Unico 4802 UV-visible double beam spectrophotometer (USA). The total phenolic and flavonoid contents of the extracts were expressed as gallic acid and rutin equivalents (mg/g extract), respectively.

2.5. Antioxidant Assays

2.5.1. DPPH Radical Scavenging Activity

The stable 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity was determined by Blois's method [12]. The samples and references dissolved in ethanol (75%) were mixed with DPPH solution (1.5×10^{-4} M). Remaining DPPH amount was measured at 520 nm using a Unico 4802 UV-visible double beam spectrophotometer (USA). Gallic acid and butylated hydroxyanisol (BHA) were employed as the references. Inhibition of DPPH in percent (I%) was calculated as given below:
$I\% = [(A_{blank}-A_{sample}) / A_{blank}] \times 100$, 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. Analyses were run in triplicates and the results were expressed as average values with S.E.M. (Standard error mean).

2.5.2. Ferrous Ion-Chelating Effect

The ferrous ion-chelating effect of the samples was estimated by the method of Chua et al [13]. Briefly, 740 µL of methanol and the samples were incubated with 2 mM $FeCl_2$ solution. The reaction was initiated by the addition of 5 mM ferrozine into the mixture and left standing at ambient temperature for 10 min. The absorbance of the reaction mixture was measured at 562 nm. The ratio of inhibition of ferrozine-Fe^{2+} complex formation was calculated as follows:
$I\% = [(A_{blank}-A_{sample}) / A_{blank}] \times 100$, where A_{blank} is the absorbance of the control reaction (containing only $FeCl_2$ and ferrozine), and A_{sample} is the absorbance of the extracts/reference. Analyses were run in duplicates and the results were expressed as average values with S.E.M. (Standard error mean).

2.5.3. Ferric-Reducing Antioxidant Power Assay (FRAP)

The ferric-reducing power (FRAP) of the samples was tested using the assay of Oyaizu [14]. 1 mL of different concentrations of the samples as well as chlorogenic acid (reference for comparative purposes) was added to phosphate buffer (0.1 M, pH 6.6) and potassium ferricyanide (1%, w/v). Later, the mixtures were incubated at 50°C for 20 min and then 10% trichloroacetic acid (TCA) was added. After the mixtures were shaken vigorously, 2.5 mL of the solutions were mixed with 2.5 mL of distilled water and $FeCl_3$ (0.1%, w/v). After 30 min-incubation, absorbances were read at the wavelength of 700 nm. Analyses were achieved in duplicate. Increased absorbance of the reaction meant increased reducing power.

2.5.4. Beta-Carotene Bleaching Microplate Assay

A modified method of Dapkevicious et al. was used to test bleaching ability of the extracts against beta-carotene [15]. Briefly, 1 mg of beta-carotene was dissolved in dichloromethane and then, linoleic acid and Tween 40 were added to this mixture. After dichloromethane was removed *in vacuo* using a rotary evaporator at 40 °C, oxygenated pure water (50 ml) was added and vigorously shaken by hand. An aliquot of 250 µL of the beta carotene-linoleic acid emulsion was applied to each well of the 96-well microplate using micropipette (Eppendorf). 30 µL of the test solutions were then added in each well in triplicate, where ethanol was used as blank. The microtiter plates were incubated at 55 °C and their absorbances were determined at the wavelength of 492 nm using an ELISA microplate reader (VersaMax Molecular Devices, USA). Reading of all samples was carried out at the start (t=0) and after 105 min of incubation. The Antioxidant Activity Coefficient (ACC) was calculated according to the formula given below:

$ACC = [(A_{A105} - A_{B105}) / (A_{B0} - A_{B105})]$

Where A_{A105} and A_{B105} are the absorbance of the test and blank samples at 105 min, respectively, and A_{B0} is the absorbance belonging to blank sample at the start (t=0).

2.6. Statistical Analysis of data

Data obtained from *in vitro* experiments were expressed as standard error mean (±SEM).

3. Results and Discussion

In the light of our evidence as tabulated in Table 1, the ethyl acetate extract of *S. fischeriana* was revealed to be very effective towards AChE having 90.94 and 98.02% of inhibitions at 100 and 200 µg mL^{-1} concentrations, respectively, followed by the ethyl acetate of *S. candida*, water and ethyl acetate extracts of *S. clusiana*, and the methanol extract of *S. candida*, which showed inhibition over 70% at 200 µg mL^{-1} concentrations. Nevertheless, none of the extracts belonging to *S. lutea* subsp. *lutea* and *S. lutea* subsp. *sicula* exerted inhibitory effect in this assay.

The *Sternbergia* extracts were found to exert insignificant antioxidant effect in all assays used (Tables 2 and 3). Total phenol contents of the extracts were calculated according to the equation ($y=0.0007x+0.0185$, $r^2=0.9744$) as gallic acid equivalent (GAE, mg g^{-1} extract), whilst their total flavonoid contents were determined in accordance with the equation ($y=0.6497x+0.3094$, $r^2=0.9852$) obtained by calibration curves as rutin equivalent (RUE, mg g^{-1} extract) (Figure 1). The richest phenol content was found for the water extracts of *S. lutea* subsp. *sicula* as 248.58 mg g^{-1} extract and for *S. candida* 232.50 mg g^{-1} extract as GAE, while the ethyl acetate extract of *S. lutea* subsp. *lutea* (3.53 mg g^{-1} extract) had the richest total flavonoid content as RUE (Table 2).

Table 1. Acetylcholinesterase (AChE) inhibitory activity of the extracts of the *Sternbergia* species

Extracts	Percentage of inhibition±S.E.M[a] against AChE		
	50 µg mL^{-1}	100 µg mL^{-1}	200 µg mL^{-1}
S. candida (EtOAc)	31.52±0.98	46.54±1.29	80.52±1.35
S. candida (MeOH)	43.57±0.87	57.30±1.09	70.56±1.23
S. candida (H_2O)	-[b]	32.58±0.60	36.53±0.95
S. clusiana (EtOAc)	-	18.27±1.45	73.24±0.68
S. clusiana (MeOH)	-	11.27±1.29	55.03±0.77
S. clusiana (H_2O)	13.38±1.00	51.31±0.54	79.35±1.43
S. fischeriana (EtOAc)	-	90.94±0.03	98.02±1.36
S. fischeriana (MeOH)	-	32.47±0.32	59.39±1.02
S. fischeriana (H_2O)	-	-	-
S. lutea subsp. *lutea* (EtOAc)	-	-	-
S. lutea subsp. *lutea* (MeOH)	-	-	-
S. lutea subsp. *lutea* (H_2O)	-	-	-
S. lutea subsp. *sicula* (EtOAc)	-	-	-
S. lutea subsp. *sicula* (MeOH)	-	-	-
S. lutea subsp. *sicula* (H_2O)	-	-	-
Lycorine	55.99±1.25	83.53±1.46	ND[c]
Galanthamine (Reference)	90.45±0.83	98.97±0.24	ND

[a]Standard error mean; [b]No activity; [c]Not determined

Most of the extracts prepared from *Sternbergia* species studied herein showed promising inhibitory effects against AChE at tested concentrations. *Sternbergia* species used in this study were previously investigated for their alkaloid contents and existence of lycorine, homolycorine, haemanthidine, haemanthamine, 6α- and 6β-hydroxy-haemanthamine, and tazettine was reported [4, 5, 6, 16]. In one of these studies [16], quantity of lycorine was reported to be the highest in the methanol extracts of *S. lutea* subsp. *lutea* bulbs (1.069%) and *S. lutea* subsp. *sicula* (1.007%). However, the extracts of these two subspecies were found to be completely ineffective in our AChE inhibitory activity tests (Table 1). Thus, this may lead a commentary that lycorine might be one of the contributing alkaloid to anti-AChE effect of the extracts, but, apparently, it is not directly responsible for the mentioned activity. This means that some other alkaloids might be potentiating AChE inhibitory activity of the active extracts of *S. candida*, *S. clusiana*, and *S. fischeriana*. In fact, some previous studies suggested anticholinesterase activity of the plant extracts from Amaryllidaceae family is correlated with their galanthamine content [17, 18], while some other reports came to conclusion that lycorine and hamaine were the most active alkaloids in *Crinum* species [19], which is evident to our data to some extent. In another study [20], twenty-three Amaryllidaceae alkaloids from various species of the genus *Narcissus* were screened for their AChE inhibitory potentials and only seven alkaloids having to galanthamine and lycorine skeleton types were found to be significantly effective. Besides, all of the *Narcissus* extracts having high inhibition against AChE contained galanthamine as the major alkaloid except for only *N. assoanus* bearing a lycorine-type alkaloid. A literature survey revealed that antioxidant activity of *Sternbergia* species has not been investigated so far. Therefore, our results have constituted the first data on antioxidant activity of this genus. However, the extracts of *Sternbergia* species screened herein displayed a low profile of antioxidant activity in the above-mentioned test models, which might be considered to be related to their low total phenol and flavonoid contents. Because antioxidant activity of herbs is usually linked to their phenolic contents. On the other hand, iron is one of the most vital transition metals involved in the formation of oxygen-free radicals, owing to its interaction with hydrogen peroxide through Fenton chemistry. Since iron dysregulation has been reported to be associated with AD as it accumulates in brain during aging [21]. Therefore, it would be quite advantageous for any drug candidate against AD to have both anti-AChE activity as well as iron-chelating effect. However, only the methanol extract of *S. candida* displayed a moderate chelating effect.

Table 2. DPPH radical scavenging activity and ferrous ion-chelating capacity (inhibition %±S.E.M.), total phenol and total flavonoid contents of the extracts the *Sternbergia* species

Extracts	Total phenol content[a]	Total flavonoid content[b]	A) Percentage of inhibition±S.E.M.[c] against DPPH radical B) Ferrous ion-chelating percentage±S.E.M.					
			500 µg mL^{-1}		1000 µg mL^{-1}		2000 µg mL^{-1}	
			A	B	A	B	A	B
S. candida (EtOAc)	38.93±0.01	2.54±0.64	-[d]	-	-	-	4.56±0.20	-
S. candida (MeOH)	35.36±1.23	0.78±0.08	-	-	-	-	-	52.73±0.13
S. candida (H_2O)	232.5±0.89	0.55±0.12	-	-	-	-	-	-
S. clusiana (EtOAc)	23.22±1.04	2.06±0.04	-	-	-	-	-	-
S. clusiana (MeOH)	37.15±1.55	0.95±0.01	-	-	-	-	6.80±0.61	-
S. clusiana (H_2O)	96.07±1.09	0.60±0.04	-	-	-	-	-	-
S. fischeriana (EtOAc)	26.79±0.99	1.41±0.06	-	-	-	-	-	-
S. fischeriana (MeOH)	28.93±1.06	1.37±0.96	-	-	-	-	-	-
S. fischeriana (H_2O)	30.36±1.87	3.11±1.42	-	-	-	-	-	-
S. lutea subsp. *lutea* (EtOAc)	53.57±0.51	3.53±0.23	-	-	6.64±0.46	-	12.61±1.03	-
S. lutea subsp. *lutea* (MeOH)	82.14±1.26	2.03±0.38	4.68±0.17	-	8.02±0.52	-	20.13±1.54	-
S. lutea subsp. *lutea* (H_2O)	52.50±0.97	2.19±0.56	-	-	-	-	-	-
S. lutea subsp. *sicula* (EtOAc)	36.43±1.56	1.96±0.22	-	-	-	-	6.67±1.52	-
S. lutea subsp. *sicula* (MeOH)	86.07±1.07	1.17±0.05	5.25±0.22	-	9.22±0.82	-	17.52±1.29	-
S. lutea subsp. *sicula* (H_2O)	248.58±1.73	3.09±1.03	-	-	-	-	6.93±1.57	-
Lycorine	ND[e]	ND	-	44.86±1.11	-	79.75±1.51	7.88±1.22	83.42±1.12
Butylated hydroxyanisol (Reference)	ND	ND	77.99±0.48	21.71±1.10	81.60±1.67	26.94±1.48	82.94±0.68	32.05±1.62

[a]Data expressed in mg equivalent of gallic acid (GAE) to 1 g of extract; [b] Data expressed in mg equivalent of rutin to 1 g of extract;[c] Standard error mean;
[d] No activity; [e] Not determined

Table 3. Ferric-reducing antioxidant power (FRAP, absorbance at 700 nm ±S.E.M.) values of the *Sternbergia* extracts

Extracts	A) Ferric-reducing antioxidant power±S.E.M[a] B) Antioxidant activity coefficient (ACC) for beta-carotene bleaching assay±S.E.M					
	500 µg mL^{-1}		1000 µg mL^{-1}		2000 µg mL^{-1}	
	A	**B**	**A**	**B**	**A**	**B**
S. candida (EtOAc)	0.179±0.01	-[b]	0.357±0.02	-	0.668±0.03	-
S. candida (MeOH)	0.101±0.02	-	0.126±0.01	-	0.290±0.11	-
S. candida (H_2O)	0.054±0.01	-	0.066±0.01	-	0.163±0.09	-
S. clusiana (EtOAc)	0.155±0.02	-	0.263±0.01	-	0.483±0.03	-
S. clusiana (MeOH)	0.134±0.02	-	0.193±0.01	-	0.386±0.03	0.46
S. clusiana (H_2O)	0.071±0.01		0.110±0.01		0.215±0.01	
S. fischeriana (EtOAc)	0.151±0.01	-	0.299±0.03	-	0.585±0.03	-
S. fischeriana (MeOH)	0.072±0.01	-	0.110±0.01	-	0.151±0.01	-
S. fischeriana (H_2O)	0.074±0.01	-	0.080±0.01	-	0.145±0.01	-
S. lutea subsp. *lutea* (EtOAc)	0.257±0.01	-	0.430±0.01	-	0.740±0.06	-
S. lutea subsp. *lutea* (MeOH)	0.238±0.01	-	0.423±0.01	-	0.760±0.01	-
S. lutea subsp. *lutea* (H_2O)	0.091±0.01	-	0.144±0.01	-	0.287±0.03	-
S. lutea subsp. *sicula* (EtOAc)	0.184±0.02	-	0.333±0.01	-	0.660±0.07	-
S. lutea subsp. *sicula* (MeOH)	0.265±0.01	-	0.484±0.02	-	1.026±0.06	-
S. lutea subsp. *sicula* (H_2O)	0.123±0.02	4.0±1.41	0.187±0.02	4.0±1.82	0.433±0.03	12.33±1.52
Lycorine	0.145±0.02	-	0.155±0.02	-	0.331±0.02	-
Chlorogenic acid (Reference)	2.525±0.21	-	3.547±0.06	2.25±1.30	3.618±0.01	2.75±1.25

[a]Standard error mean;[b] No activity

In conclusion, AChE inhibitory and antioxidant activities, related to AD pathogenesis, of five *Sternbergia* species were screened herein. *S. candida*, *S. clusiana*, and *S. fischeriana* extracts showed promising AChE inhibition and therefore, these extracts deserve further studies for their anti-AChE activity. To the best of our knowledge, we report the first study about AChE inhibitory and antioxidant activity of *Sternbergia* species.

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