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RESEARCH ARTICLE

Anticholinesterase, antioxidant, analgesic and anti-inflammatory activity assessment of *Xeranthemum annuum* L. and isolation of two cyanogenic compounds

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ABSTRACT

Context: *Xeranthemum annuum* L. (Asteraceae) (XA) is an ornamental and medicinal species with limited bioactivity and phytochemical data.

Objective: Identification of anticholinesterase, antioxidant, anti-inflammatory and analgesic effects of the flower and root–stem (R-S) extracts of XA.

Materials and methods: Anticholinesterase (at 100 μg mL^{-1}) and antioxidant (at 1000 μg mL^{-1}) effects of various extracts were evaluated via microtiter assays, while anti-inflammatory and analgesic effects of the R-S extracts were tested using carrageenan-induced hind paw oedema (100 and 200 mg kg^{-1}) and *p*-benzoquinone (PBQ) writhing models (200 mg kg^{-1}) in male Swiss albino mice. The R-S ethanol extract of XA was subjected to isolation studies using conventional chromatographic methods.

Results: Most of the extracts showed inhibition over 85% against butyrylcholinesterase and no inhibition towards acetylcholinesterase. The flower chloroform and the R-S ethyl acetate extracts were most effective (97.85 ± 0.94% and 96.89 ± 1.09%, respectively). The R-S ethanol extract displayed a remarkable scavenging activity against DPPH (77.33 ± 1.99%) and in FRAP assay, while the hexane extract of the R-S parts possessed the highest metal-chelating capacity (72.79 ± 0.33%). The chloroform extract of the R-S caused a significant analgesic effect (24.4%) in PBQ writhing model. No anti-inflammatory effect was observed. Isolation of zierin and zierin xyloside, which were inactive in anticholinesterase assays, was achieved from the R-S ethanol extract.

Discussion and conclusion: This is the first report of anticholinesterase, antioxidant, analgesic and anti-inflammatory activities and isolation of zierin and zierin xyloside from XA. Therefore, XA seems to contain antioxidant and BChE-inhibiting compounds.

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Asteraceae; cholinesterase inhibition; zierin; zierin xyloside

Introduction

Xeranthemum annuum L. (XA) (syn. *Xeranthemum squarrosum* Boiss.) (Asteraceae) (Picture 1), known as 'common immortelle or everlasting flower', is an annual herb distributed only around southeastern parts of Europe, Caucasus, Turkey, Lebanon, Syria and Iran (Heywood et al. 2007). The plant contains typical botanical characteristics of Asteraceae family; it is a popular ornamental species, due to its attractive everlasting purple flowers, and is endangered in some countries such as the Czech Republic and Slovakia. In addition, XA is a medicinal plant reported to have various ethnobotanical and traditional uses in some European countries (Vogl-Lukasser & Vogl 2004; Watson & Preedy 2008). XA was reported to cure burn pains and is used against toothache by mixing with tobacco in Turkish folk medicine (Ozaydin et al. 2006; Tuzlaci & Dogan 2010; Altundag & Ozturk 2011). However, only very limited biological activity and phytochemical data are available on this plant according to our extensive literature survey. Since we have so far screened some species of Asteraceae family during our ongoing studies on finding new inhibitors of acetylcholinesterase (AChE, EC 3.1.1.7) and butyrylcholinesterase (also called pseudocholinesterase, BChE, EC 3.1.1.8) herbal origin, we now investigate cholinesterase inhibitory potential of XA from the

Picture 1. *Xeranthemum annuum* L. (photo taken by Ilkay Erdogan Orhan).

same family. Actually, AChE and BChE are the sister enzymes related to pathogenesis of Alzheimer's disease (AD), which is a progressive neurodegenerative disorder and the most common form of dementia affecting mostly elder population over the age of 65 (Schneider et al. 2014). According to the cholinergic

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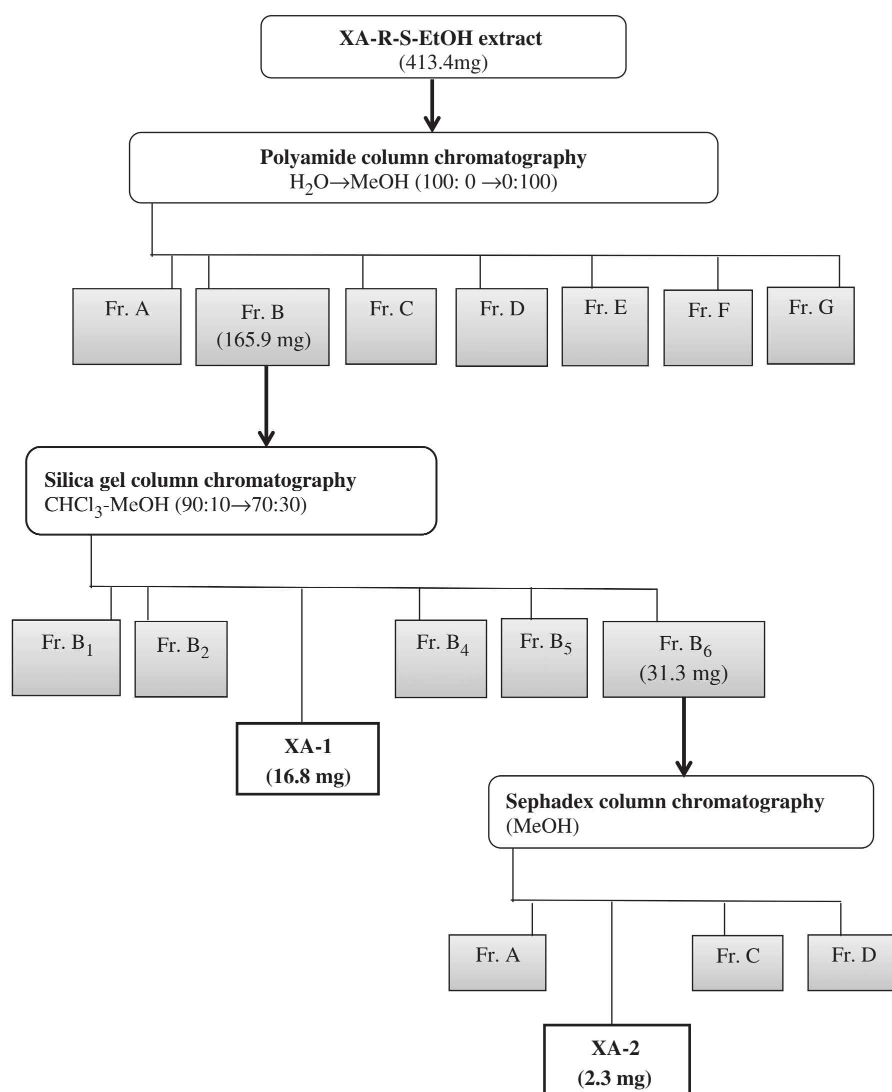

Figure 1. Isolation scheme of compounds XA-1 and XA-2.

hypothesis suggested for pathogenesis of the disease, deficiency in acetylcholine (ACh) level has been found in the brains of AD patients. Since ACh is hydrolysed by AChE, inhibition of this enzyme is a modern treatment strategy against AD (Orhan et al. 2011). Besides, BChE, the sister enzyme of AChE, has the ability to break down ACh and replace it with AChE when its level is depleted (Nordberg et al. 2013). As a multi-factorial disease, AD is strongly associated with metal ion dyshomeostasis and oxidative stress, which cause a serious damage to neurons (Lee et al. 2014). Consequently, antioxidant and radical scavenging activities of the extracts from the flower and root–stem (R-S) parts of XA were also tested. Memory-enhancing effect of various extracts of the flower and R–S parts of XA was investigated via their AChE and BChE inhibitory activities, as well as antioxidant activity, using high-throughput screening technique through ELISA microplate reader.

Neuroinflammation has been also reported to be involved in the complex pathologic cascade of AD supported by a considerable amount of pathological and clinical evidence (Akiyama et al. 2000; Lonskaya et al. 2015). Hence, anti-inflammatory effect of the plant was also searched in the current work based on its traditional use against burns and pain, anti-inflammatory and analgesic effects of the plant.

Materials and methods

Plant material

The plant sample was collected from the roadside between Mardin and Batman provinces in Turkey in June 2009. Identification of the plant was done by Prof. Dr. Hayri Duman from Department of Biology, Faculty of Science, Gazi University (Ankara, Turkey). The voucher specimen is preserved at the Herbarium of Faculty of Pharmacy, Gazi University (Ankara, Turkey).

Extraction and isolation

The dried and powdered flowers (F) and R-S parts of XA were subjected to maceration subsequently with *n*-hexane, chloroform ($CHCl_3$), ethyl acetate (EtOAc), ethanol (80%) (EtOH) and distilled water (H_2O) at room temperature. The organic solvents were independently evaporated *in vacuo* until dryness and each extract was prepared in the same manner. The yields (%, w/w) of the extracts were as follows: XA-F-hexane: 5.14%, XA-F-$CHCl_3$: 7.14%, XA-F-EtOAc: 1.14%, XA-F-EtOH: 3.71%, XA-F-H_2O: 14.29%; XA-R-S-hexane: 2.06%, XA-R-S-$CHCl_3$:

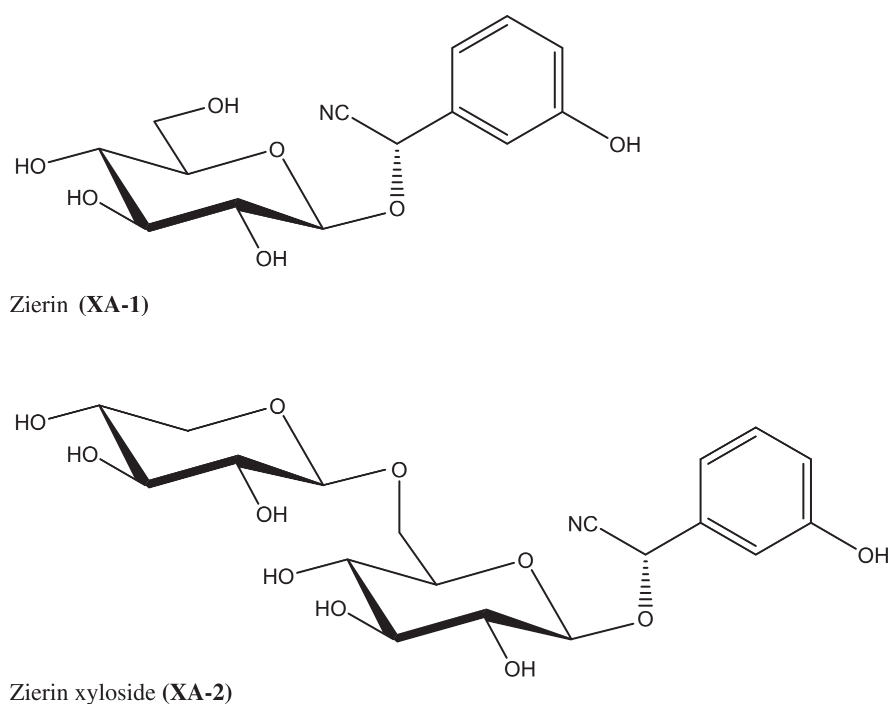

Figure 2. Structures of zierin (XA-1) and zierin xyloside (XA-2).

Table 1. ^{1}H and ^{13}C NMR data for zierin (XA-1) and zierin xyloside (XA-2).

		Zierin (XA-1)		Zierin xyloside (XA-2)	
		δ_C (ppm)	δ_H (ppm), J (Hz)	δ_C (ppm)	δ_H (ppm), J (Hz)
C/H atom					
Aglycon					
1	C	118.6		118.7	
2	CH	69.9	5.97 s	68.3	5.89 s
3	C	133.6		132.9	
4	CH	117.1	6.63 ϕ	116.7	6.65 ϕ
5	C	160.3		160.4	
6	CH	120.4	6.56 ϕ	121.4	6.59 ϕ
7	CH	127.2	7.38 ϕ	126.2	7.43 ϕ
8	CH	128.8	7.43 ϕ	128.9	7.47 ϕ
Glucose					
1′	CH	104.3	4.66 d (7.9)	104.1	4.51 d (7.9)
2′	CH	75.7	3.00–3.43 ϕ	76.3	3.00–3.45 ϕ
3′	CH	76.8	3.41 ϕ	77.1	3.45 ϕ
4′	CH	70.7	3.00–3.30 ϕ	70.4	3.00–3.30 ϕ
5′	CH	76.2	3.17 ϕ	75.9	3.25 ϕ
6′	CH_2	62.5	3.55 ϕ	69.3	3.56 ϕ
			3.95 ϕ		3.84 ϕ
Xylose					
1″	CH			106.5	4.39 d (7.6)
2″	CH			75.7	3.01 dd (7.6/9.0)
3″	CH			78.0	3.25 ϕ
4″	CH			70.9	3.36 m
5″	CH_2			67.0	3.74 ϕ
					3.09 ϕ

ϕUnclear splitting for this peak.

1.75%, XA-R-S-EtOAc: 0.32%, XA-R-S-EtOH: 5.01%, XA-R-S-H_2O: 15.89%.

All of the F and R-S extracts were tested for their anticholinesterase and antioxidant activities, while only the R-S extracts were evaluated in anti-inflammatory and analgesic activity experiments due to very little amount of the F extracts. For isolation studies, the XA-R-S-EtOH extract (413.4 mg) was employed, which was dissolved in H_2O and the water-soluble portion was fractionated over a polyamide column (ICN, VLC, 80 g), eluted with H_2O, followed by increasing concentrations of methanol (MeOH) to afford seven main fractions (Frs. A–G) (Figure 1). Purification of fraction B (165.9 mg) by silica gel column chromatography [Merck Co., Darmstadt, Germany, Co., Darmstadt, Germany, 230–400 mesh, 25 g, $CHCl_3$/MeOH (90:10 → 70:30)] furnished XA-1 (16.8 mg) and Frs. $B_{1–6}$. Then, Fr. B_6 (31.3 mg) was re-chromatographed on a Sephadex LH-20 column (15 g) eluted with MeOH to yield XA-2 (2.3 mg).

Structure elucidation of compounds XA-1 and XA-2

Structure elucidation of the isolated compounds XA-1 and XA-2 was carried out by the ^{1}H and ^{13}C NMR spectral techniques along with the detailed data, which was compared with the relevant literature (Schwind et al. 1990; Nahrstedt & Schwind 1992). XA-1 and XA-2 were identified as the known cyanogenic compounds zierin (XA-1) and zierin xyloside (XA-2), respectively, whose chemical structures are given in Figure 2. The NMR data for both compounds are given in Table 1.

AChE and BChE inhibitory activity assays

AChE and BChE inhibitory activity of the samples was determined by spectrophotometric method of Ellman et al. (1961). Electric eel AChE (Type-VI-S, EC 3.1.1.7, Sigma, St. Louis, MO) and horse serum BChE (EC 3.1.1.8, Sigma) were used as the enzyme sources, while acetylthiocholine iodide and butyrylthiocholine chloride (Sigma) were employed as substrates of the reaction. 5,5′-Dithio-bis(2-nitrobenzoic) acid (DTNB, Sigma) was used for the measurement of the cholinesterase activity. All the other reagents and conditions were modified as described previously (Orhan et al. 2010). In brief, 140 µL of 0.1 mM sodium phosphate buffer (pH 8.0), 20 µL of 0.2 M DTNB, 20 µL of sample solutions and 20 µL of 0.2 M AChE/BChE solution were added by

multichannel automatic pipette (Gilson S.A.S., Villiers le Bel, France) 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 0.2 M acetylthiocholine iodide/butyrylthiocholine chloride. The hydrolysis of acetylthiocholine iodide/butyrylthiocholine chloride was monitored by the formation of the yellow 5-thio-2-nitrobenzoate anion as a result of the reaction of DTNB with thiocholines, catalysed by enzymes at a wavelength of 412 nm utilizing a 96-well microplate reader (VersaMax Molecular Devices, Sunnyvale, CA). Galanthamine, the anticholinesterase alkaloid-type of drug isolated from the bulbs of snowdrop [*Galanthus nivalis* L. (Amaryllidaceae)], was purchased from Sigma and was employed as the reference drug.

Data processing for enzyme inhibition assays

The measurements and calculations were evaluated by using Softmax PRO 4.3.2.LS software (Molecular Devices Corporation, Sunnyvale, CA). Percentage inhibition of AChE/BChE and TYR was determined by comparison of rates of reaction of test samples relative to blank sample [ethanol in phosphate buffer pH 8 for AChE/BChE and DMSO (50%) for TYR]. Extent of the enzymatic reaction was calculated based on the following equation: $E = (C - T)/C \times 100$, where E is the activity of the enzyme. E value expresses the effect of the test sample or the positive control on the enzyme activity articulated as the percentage of the remaining activity in the presence of test sample or positive control. C value is the absorbance of the control solvent (blank) in the presence of enzyme, where T is the absorbance of the tested sample (plant extract or positive control in the solvent) in the presence of enzyme.

Data are expressed as average inhibition ± standard error mean (SEM) and the results were taken from at least three independent experiments performed in triplicate.

Chemical-based antioxidant activity assays

DPPH radical scavenging assay

The hydrogen atom or electron donation capacity of the corresponding samples was computed from the bleaching property of the purple-coloured methanol solution of 2,2′-diphenyl-1-picrylhydrazyl (DPPH). The stable DPPH radical scavenging activity of the extracts was determined by the method of Blois (1958). The samples (2700 μL) dissolved in ethanol (75%) were mixed with 300 μL of DPPH solution (1.5×10^{-4} M). Remaining DPPH amount was measured at 520 nm using a Unico 4802 UV–visible double beam spectrophotometer (Dayton, NJ). The results were compared to that of gallic acid employed as the reference.

DMPD radical scavenging assay

Principal of the assay is based on reduction of the purple-coloured radical $DMPD^{+}$ (*N*, *N*-dimethyl-*p*-phenylenediamine) (Schlesier et al. 2002). According to the method, a reagent comprising of 100 mM DMPD, 0.1 M acetate buffer (pH 5.25), and 0.05 M ferric chloride solution, which led to formation of DMPD radical, was freshly prepared and the reagent was equilibrated to an absorbance of 0.900 ± 0.100 at 505 nm. Then, the reagent (1000 μL) was mixed with 50 μL of the sample dilutions dissolved in ethanol (75%) and absorbance was taken at 505 nm using a Unico 4802 UV–visible double beam spectrophotometer. Quercetin was employed as the reference; the experiments were done in triplicate.

Super oxide radical scavenging activity

Super oxide (SO) radical scavenging activity of the corresponding samples was determined by the method of Nishimiki et al. (1972). Nitro blue tetrazolium solution (156 μM) and 468 μM β-nicotinamide adenine dinucleotide dissolved in 100 mM phosphate buffer (pH 7.4) were mixed with 100 μL of the extracts dissolved in ethanol (75%) at various dilutions. The reaction was initiated by addition of 60 μM phenazine methosulphate prepared in 100 mM phosphate buffer (pH 7.4). The reaction mixture was incubated at 25 °C for 5 min, then absorbance was measured against control samples, which contained all reagents except the test samples, using a Unico 4802 UV–visible double beam spectrophotometer.

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

The metal-chelating effect of the samples by Fe^{2+}-ferrozine test system was estimated in consistent with the method of Chua et al. (2008). Accordingly, 740 μL of ethanol and 200 μL of the samples dissolved in ethanol (75%) were incubated with 2 mM $FeCl_2$ solution. The reaction was initiated by the addition of 40 μL of 5 mM ferrozine solution into the mixture, shaken vigorously 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 given in '*Data processing for antioxidant activity assays*' and ethylenediaminetetraacetic acid (EDTA) was employed as the reference in this assay.

Ferric-reducing antioxidant power assay

The ferric-reducing antioxidant power (FRAP) of the corresponding samples was tested using the assay of Oyaizu (1986) based on the reduction of Fe^{3+} to Fe^{2+}. Different concentrations of the samples dissolved in ethanol (75%) were added into 2500 μL of phosphate buffer (pH 6.6) and 2500 μL of potassium ferricyanide $[K_3Fe(CN)_6]$ (1%, w/v). Later, the mixture was incubated at 50 °C for 20 min and then 2500 μL of trichloroacetic acid (10%) was added. After the mixture was shaken vigorously, this solution was mixed with 2500 μL of distilled water and $FeCl_3$ (100 μL, 0.1%, w/v). After 30 min incubation, absorbance was read at 700 nm using a Unico 4802 UV–visible double beam spectrophotometer. Analyses were achieved in triplicate. Chlorogenic acid was the reference in this assay.

Phosphomolibdenum-reducing antioxidant power assay

In order to perform phosphomolibdenum-reducing antioxidant power (PRAP) assays on the extracts, each dilution of the samples was mixed with 10% phosphomolybdic acid solution in ethanol (w/v) (Falcioni et al. 2002). The solution was subsequently subjected to incubation at 80 °C for 30 min and the absorbance was read at 600 nm using a Unico 4802 UV–visible double beam spectrophotometer and compared to that of quercetin as the reference.

Data processing for antioxidant activity assays

Inhibition of DPPH, DMPD and metal-chelation capacity was calculated as given in accordance with their corresponding literature

and the results were expressed as percent inhibition (*I*%): $I\% = [(A_{blank} - A_{sample})/A_{blank}] \times 100$, 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. Analyses were run in triplicate and the results were expressed as average values with SEM.

For the FRAP and PRAP assays, the analyses were also achieved in triplicate and increased absorbance of the reaction meant increased reducing power in both assays.

Determination of total phenol and flavonoid contents in the extracts

Phenolic amount of the extracts was determined in accordance with Folin-Ciocalteau's method (Singleton & Rossi 1965). In brief, a number of dilutions of gallic acid dissolved in ethanol (75%) were obtained to prepare a calibration curve. The extracts and gallic acid dilutions 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. Afterwards, absorption was measured at 760 nm at a Unico 4802 UV–visible double beam spectrophotometer. Total flavonoid content of the extracts was calculated by aluminium chloride colorimetric method (Woisky & Salatino 1998). To sum up, a number of dilutions of quercetin dissolved in ethanol (75%) were obtained to prepare a calibration curve. Then, the extracts and quercetin dilutions were mixed with 95% ethanol, aluminium chloride reagent, 100 µL of sodium acetate as well as distilled water. Following incubation for 30 min at room temperature, absorbance of the reaction mixtures was measured at wavelength of 415 nm with a Unico 4802 UV–visible double beam spectrophotometer. The total phenol and flavonoid contents of the extracts were expressed as gallic acid and quercetin equivalents (mg g^{-1} extract), respectively.

Anti-inflammatory and analgesic activity experiments

Animals

Male Swiss albino mice (20–25 g) were purchased from the animal breeding laboratories of Experimental Animal Research Center of Gazi University (GÜDAM) (Ankara, Turkey). The animals left 3 days for acclimatization to animal room conditions were maintained on standard pellet diet and water *ad libitum*. The food was withdrawn on the day prior to the experiment, but was allowed free access of water. A minimum of six animals was used in each group. Throughout the experiments, animals were processed according to the suggested European ethical guidelines for the care of laboratory animals.

This study was permitted by the Institutional Animal Ethics Committee of Gazi University Ethical Council (Ankara, Turkey) (Protocol code: G.U. ET-05.004).

Analgesic effect in p-benzoquinone-induced writhing model

In accordance with the method of Okun et al. (1963), 60 min after the oral administration of test samples, the mice were intraperitoneally injected with 0.1 mL 10 g^{-1} body weight of 2.5% (v/v) *p*-benzoquinone (PBQ; Merck) solution in distilled water. Control animals received an appropriate volume of dosing vehicle. The mice were then kept individually for observation and the total number of abdominal contractions (writhing movements) was counted for the next 15 min, starting on the 5th min after the PBQ injection. The data represent average of the total number of writhes observed. The antinociceptive activity was expressed as the percentage change from writhing controls. Aspirin (ASA) at 100 mg kg^{-1} was used as the reference drug.

Anti-inflammatory effect in carrageenan-induced hind paw oedema model

As described previously (Kupeli et al. 2007), 60 min after the oral administration of test sample or dosing vehicle, each mouse was injected with freshly prepared suspension of carrageenan (0.5 mg 25 μL^{-1}) (Sigma) in physiological saline (154 nM NaCl) into subplantar tissue of the right hind paw. As to the control, 25 µL saline solution was injected into that of the left hind paw. Paw oedema was measured in every 90 min during 6 h after induction of inflammation. The difference in footpad thickness was measured by a gauge calipers (Ozaki Co., Tokyo, Japan). Mean values of treated groups were compared with mean values of a control group and analysed using statistical methods. Indomethacin (10 mg kg^{-1}) was used as a reference drug.

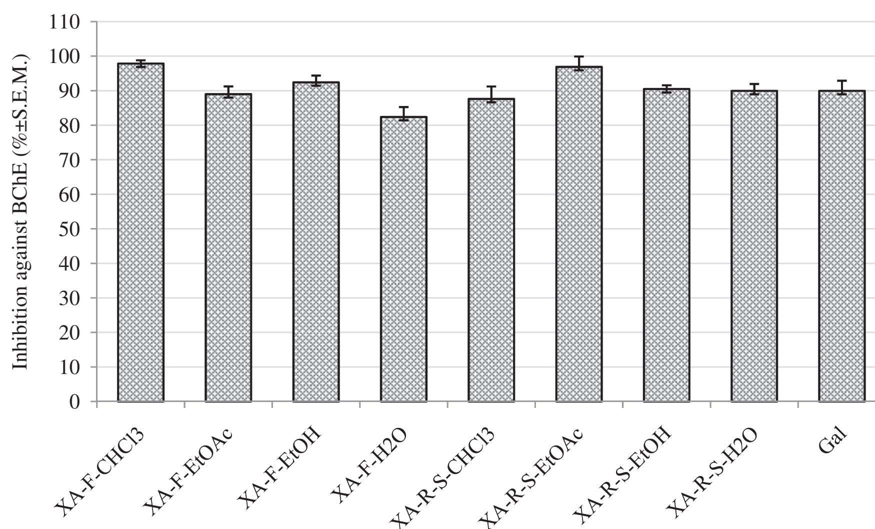

Figure 3. Inhibition (%±SEM) of the extracts of *Xeranthemum annuum* (XA) against BChE at 100 µg mL^{-1} (F, flower, R-S, root and stem; Gal, galanthamine – reference).

Table 2. Total phenol and flavonoid amounts, DPPH radical scavenging and FRAP activities of the extracts of XA at 1000 µg mL−1.

Extracts	Total phenol contenta±SEMb	Total flavonoid contentc±SEM	Radical scavenging effect (%±SEM) against DPPH	FRAP (absorbance at 700 nmd±SEM)
XA-F-hexane	NTe	NT	NT	NT
XA-F-$CHCl_3$	–f	9.16 ± 2.98	1.33 ± 0.21****	0.339 ± 0.006****
XA-F-EtOAc	14.36 ± 1.17	6.14 ± 0.52	3.84 ± 1.05****	0.312 ± 0.077****
XA-F-EtOH	71.27 ± 0.01	14.55 ± 1.03	13.37 ± 0.31****	0.565 ± 0.022****
XA-F-H_2O	68.38 ± 0.58	12.36 ± 0.26	19.58 ± 3.66****	0.276 ± 0.005****
XA-R-S-hexane	5.28 ± 0.02	25.08 ± 4.12	2.74±±0.93****	0.402 ± 0.029****
XA-R-S-$CHCl_3$	76.63 ± 1.08	62.23 ± 1.68	2.66 ± 0.42****	0.325 ± 0.026****
XA-R-S-EtOAc	46.52 ± 3.00	40.36 ± 6.99	17.06 ± 0.52****	0.585 ± 0.033****
XA-R-S-EtOH	145.51 ± 3.67	30.84 ± 1.56	77.33 ± 1.99**	1.126 ± 0.021***
XA-R-S-H_2O	173.56 ± 2.16	1.37 ± 0.02	23.93 ± 0.42****	0.745 ± 0.031***
Gallic acid (reference for DPPH scavenging activity)			92.88 ± 0.19	
Chlorogenic acid (reference for FRAP)				3.618 ± 0.01

**$p < 0.01$;
***$p < 0.001$,
****$p < 0.0001$.
[a]Data expressed in mg equivalent of gallic acid to 1 g of extract.
[b]Standard error mean ($n = 3$).
[c]Data expressed in mg equivalent of quercetin to 1 g of extract.
[d]Higher absorbance indicated the greater antioxidant activity.
[e]Not tested.
[f]Not able to calculate to very low absorbance.

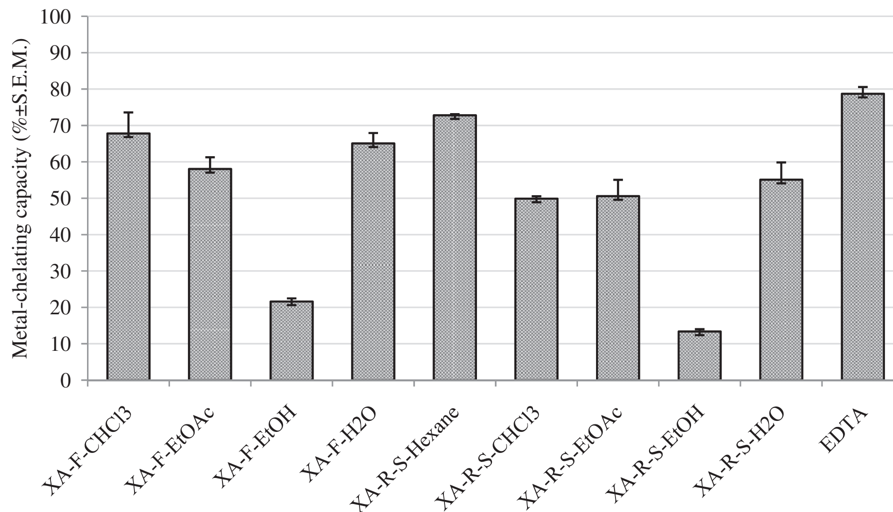

Figure 4. Metal-chelating capacity (%±SEM) of the extracts of *Xeranthemum annuum* (XA) at 100 µg mL^{-1} (F, flower, R-S, root and stem; EDTA, ethylenediaminetetraacetic acid – reference).

Statistical analysis of data

Data obtained from *in vitro* enzyme inhibition and antioxidant experiments as well as the animal experiments were expressed as the mean standard error (±SEM). Statistical differences between the reference and the sample groups were evaluated by ANOVA (one way). Dunnett's multiple comparison tests were used as post hoc tests. $p< 0.05$ was considered to be significant (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$, ****$p < 0.0001$).

Considering the animal experiments in this study, statistical differences between the treatments and the control were evaluated by ANOVA and Students–Newman–Keuls post hoc tests. $p < 0.05$ was considered to be significant [*p <0.05; **p <0.01; ***p <0.001].

Results

Cholinesterase inhibitory effect

The *n*-hexane, EtOAc, $CHCl_3$, EtOH and H_2O extracts from the flower (F) and R-S parts of XA were subjected to AChE and BChE enzyme inhibition assays at 100 µg mL^{-1}. According to our results, none of the extracts was active against AChE, whereas all of them (except XA-F-hexane that was not tested due to the small amount) displayed a selectively high level of BChE inhibition varying from 87.62 ± 3.01% to 97.85 ± 0.94% (Figure 3). Due to their scarce amounts, zierin (XA-1) and zierin xyloside (XA-2) isolated herein were only tested against AChE and BChE at 100 µg mL^{-1} and shown to be ineffective.

Chemical-based antioxidant activity and total phenol and flavonoid contents

When the extracts were tested in six different assays for their possible antioxidant activity, only the XA-R-S-EtOH extract had a marked DPPH radical scavenging effect (77.33 ± 1.99%), while rest of them showed a low activity against this radical (Table 2). Occurrence of scavenging activity against DMPD radical was observed with only two extracts, namely XA-R-S-EtOH (39.05 ± 0.25%) and H_2O (23.10 ± 1.40%) at 50 µg mL^{-1}, while only the XA-R-S-$CHCl_3$ extract showed a SO radical scavenging

Table 3. Analgesic effect of the R-S extracts of XA in PBQ-induced writhing model in mice.

Extracts	Dose (mg kg^{-1})	Writhing number ± SEM[a]	Inhibition rate (%)	Ulceration rate
Control		41.7 ± 4.2		
XA-R-S-hexane	100	36.9 ± 4.5	11.5	0/6
	200	32.8 ± 3.1	21.3	0/6
XA-R-S-$CHCl_3$	100	33.4 ± 3.6	19.9	0/6
	200	31.1 ± 2.9	25.4*[b]	1/6
XA-R-S-EtOAc	100	42.6 ± 6.0	–[c]	2/6
	200	39.5 ± 4.5	5.3	3/6
XA-R-S-EtOH	100	43.2 ± 5.1	–	0/6
	200	35.8 ± 2.7	14.1	0/6
XA-R-S-H_2O	100	38.5 ± 3.2	7.7	0/6
	200	45.2 ± 4.6	–	0/6
ASA[d]	100	21.5 ± 2.1	48.4***	4/6
	200	19.6 ± 1.7	52.9***	6/6

[a]Standard error mean.
[b]*$p < 0.05$,
***$p < 0.001$ significant from the control.
[c]No activity.
[d]Acetyl salicylic acid (reference).

Table 4. Anti-inflammatory effect of the R-S extracts of XA in carrageenan-induced paw oedema model in mice.

		Swelling thickness (×10^{-2} mm)±SEM[a] (inhibition %)			
Extracts	Dose (mg kg^{-1})	90 min	180 min	270 min	360 min
Control		53.1 ± 5.3	60.4 ± 6.1	57.6 ± 5.6	66.3 ± 4.7
XA-R-S-hexane	200	49.9 ± 4.5 (6.0)	54.8 ± 5.7 (9.3)	59.6 ± 6.1	67.3 ± 6.0
XA-R-S-$CHCl_3$	200	45.1 ± 3.7 (15.1)	49.6 ± 4.2 (17.9)	51.8 ± 4.6 (10.1)	57.1 ± 4.0 (13.9)
XA-R-S-EtOAc	200	57.5 ± 3.6	62.1 ± 3.9	65.2 ± 4.8	69.6 ± 5.3
XA-R-S-EtOH	200	54.4 ± 3.9	59.5 ± 3.6 (1.5)	55.8 ± 4.0 (3.1)	62.5 ± 5.2 (5.7)
XA-R-S-H_2O	200	56.8 ± 6.4	65.1 ± 5.3	68.7 ± 5.9	72.5 ± 5.8
Indomethacin	10	40.2 ± 3.3 (24.3)*	44.1 ± 3.1 (26.9)*	39.7±±3.4 (31.1)**	40.8 ± 3.0 (38.5)**

[a]Standard error mean.
[b]*$p < 0.05$,
**$p < 0.01$, significant from the control.

effect at a very low level (11.41 ± 3.92% at 500 µg mL^{-1}). However, the highest FRAP value was obtained with the XA-R-S-EtOH extract, whereas all of the extracts were found to be inactive in PRAP assay (Table 2). The XA-R-S-hexane extract possessed the best metal-chelation capacity (72.79 ± 0.33%), followed by the XA-F-$CHCl_3$ extract (Figure 4). Relevantly, total phenol amounts were higher in the XA-R-S-EtOH and H_2O extracts, while the richest total flavonoid content was determined in the XA-R-S-$CHCl_3$ extract (Table 2). In general, total flavonoid amounts of all extracts were lower as compared to their total phenol quantities.

Anti-inflammatory and analgesic effect

Among the tested R-S extracts in PBQ-induced writhing model in mice for evaluation of analgesic effect, the only active one was found to be the XA-R-S-$CHCl_3$ extract having 25.4% inhibition of the writhing numbers, which was statistically comparable to that of the reference drug (ASA, 52.9%) (Table 3). Nevertheless, none of the R-S extracts exerted a statistically significant anti-inflammatory effect in carrageenan-induced paw oedema model in mice as illustrated in Table 4.

Discussion

Our literature survey on XA pointed out to the fact that a very little phytochemical and biological activity studies are available on this plant. For instance, only a few studies have been reported on fatty acids, essential oil, α-amyrin type of triterpenes and flavonoids of this species (Powell et al. 1967; Zemtsova & Molchanova 1980; Skaltsa et al. 2000). However, the presence of some cyanogenic compounds such as zierin (XA-1), zierin xyloside (XA-2), sambunigrin and epilucumin was identified only in another *Xeranthemum* species, for example *X. cylindraceum* Sm. (Huebel et al. 1982; Schwind et al. 1990, Nahrstedt & Schwind 1992). Nevertheless, in the current study, it should be noted that we have also isolated zierin (XA-1) and zierin xyloside (XA-2) for the first time from this species (XA) (Figures 1 and 2).

The cyanogenic compounds [zierin (XA-1) and zierin xyloside (XA-2)] isolated from XA were tested in our study against AChE and BChE and did not show any inhibition. Thus, we may speculate which compounds reported to be present in this species could be responsible for the important BChE-inhibitory property of the plant. Therefore, the cyanogenic compounds are definitely not responsible for BChE inhibitory effect of the plant.

In this regard, ursolic acid, earlier isolated from *Micromeria cilicica* Hausskn. Ex P.H. Davis and *Salvia nipponica* Miq. var. *formosana* (Hayata) Kudo (Lamiaceae), is among the triterpenes found in XA, which was previously reported to exert a potent BChE inhibition (IC_{50} = 46.70 µM), while it was ineffective towards AChE (Chan et al. 2011; Ozturk et al. 2011). Since the presence of ursolic acid has been already reported in XA, we can speculate that ursolic acid also possibly present in our XA extracts, may contribute to the high BChE inhibitory effect of the plant. However, not only ursolic acid, but flavonoids such as luteolin and quercetin isolated from this plant might also be donating to its anti-BChE effect in connection with our earlier report on elucidation of the moderate BChE-inhibiting property of quercetin in a competitive manner (Khan et al. 2009). Katalinić et al. (2010) reported the marked BChE-inhibiting effect of luteolin, another flavonoid derivative isolated from XA. Luteolin was shown to make hydrogen bonds with the following amino acids: Asp70, Gly115, Glu197, Trp82 and Tyr332 located at the active

gorge of this enzyme, where quercetin also made hydrogen bonds with the same amino acid units for BChE inhibition. Consequently, BChE inhibitory activity of the XA extracts might be possibly associated with interaction of the enzyme with its flavonoids and triterpenes. According to a very recent report (Dekić et al. 2015), isolation of guaianolide xerantholide and its 11,13-dihydro derivative as well as two sesquiterpene lactones, an eudesmanolide, for example 11,13-dihydroisoalantolactone, a pseudoguaianolide, and confertin was achieved from the diethylether extract of XA. Although no report on cholinesterase inhibitory effect of xerantholide is available to date, guaianolides have been revealed to exert a high potential to inhibit BChE such as the guaianolides from *Amberboa ramosa* (Khan et al. 2005; Ibrahim et al. 2013). Hence, guaianolides may also contribute to the marked BChE inhibitory effect of the plant.

Many researchers reported that phenol content is well known to correlate with higher antioxidant activity in plant extracts (Alali et al. 2007). Hence, more substantial amount of total phenols found in the XA-R-S-EtOH and H_2O extracts could be suggested to cause a higher antioxidant activity in DPPH and DMPD radical scavenging and FRAP assays in these two extracts. Doubtlessly, level of antioxidant activity relies on the mechanisms of action such as scavenging free radicals, chelating various metal ions, inhibiting lipid peroxidation and possessing reducing power, which also correlate with type of phytochemical content of plant extracts. Conversely, the less polar extracts including the XA-F-$CHCl_3$ and XA-R-S-hexane displayed higher metal-chelating capacity in our study (Figure 4). Metal-chelating capacity is important since metal accumulation is a negative factor that triggers development of AD (Bush 2013). Since hydroxyl groups in flavonoids are known to facilitate their antioxidant activity by either scavenging free radicals and/or by chelating metal ions (Kumar & Pandey 2013), one can speculate that flavonoids might be one of the major contributors to the metal-chelating capacity of XA extracts. However, Stankovic et al. (2011) studied DPPH radical scavenging effect of various extracts of XA growing in Serbia and found that the acetone ($IC_{50} = 59.25 \pm 0.93$ µg mL^{-1}) and methanol ($IC_{50} = 91.31 \pm 1.32$ µg mL^{-1}) extracts had low to moderate effect as compared to that of chlorogenic acid ($IC_{50} = 11.65 \pm 0.52$ µg mL^{-1}) used as the reference, which seems somewhat similar to our results. Since the part of the plant used was not specified in Stankovic et al.'s (2011) study, it might be assumed to use whole plant, whereas we divided the plant into two parts as the flowers per se (F) and R-S together, because we determined a great DPPH radical scavenging activity in the R-S-EtOH extract (77.33 ± 1.99%, Table 1). Stankovic et al. (2011) also suggested a direct correlation between total phenol content and DPPH radical scavenging activity as we have already emphasized above.

Based on the traditional knowledge on utilization of XA in Turkish folk medicine to treat pain, we investigated its analgesic effect in an *in vivo* model herein and found out that only the XA-R-S-$CHCl_3$ extract had a moderate level of analgesic effect (25.4%) in PBQ-induced writhing model in mice in comparison to the efficacy of ASA (48.4%), the reference used in the same experiment. This effect might be again related to triterpenes especially ursolic acid, which was reported to produce analgesic action in acetic acid-induced abdominal contractions in mice acting on TRPV1 receptors (Verano et al. 2013). For the analgesic activity, the guaianolide sesquiterpene lactones reported earlier to be present in XA might be considered enhance activity, to some extent, as analgesic effect of some guaianolides, that is lactucin, lactucopicrin and 11β,13-dihydrolactucin from *Lactuca virosa* L. (wild lettuce) and *Cichorium intybus* L. (chicory), both Asteraceae, were demonstrated (Wesołowska et al. 2006).

However, the plant did not exhibit anti-inflammatory effect in the present study in carrageenan-induced paw oedema model in mice, although it has been recorded to be used against burnt pain, which may be presumably acting on a different mechanism.

Conclusions

According to the bioactivity and phytochemical work performed in the current study, we herein disclose the first report on the cholinesterase inhibitory, antioxidant, analgesic and anti-inflammatory activities of this species along with isolation of zierin (XA-1) and zierin xyloside (XA-2) from XA for the first time. The present study also revealed that this plant has a potential to develop a memory-enhancing and antioxidative agent, particularly against BChE.

Disclosure statement

The authors declare that there are no conflicts of interest. This study is the major part of the M.Sc. Thesis of Fulya Gulyurdu completed at the Institute of Health Sciences, Gazi University (Ankara, Turkey) in 2011.

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