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Antinociceptive Effects of Turkish Endemic *Eryngium kotschyi* Boiss. Roots by Bioactivity Guided Fractionation

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Abstract: *Eryngium* species (Apiaceae) are well known plants in ethnobotanical culture throughout world and also in Turkey. They are used as antitussive, diuretic as well as for analgesic and antiinflammatory purposes in traditional medicine. This study aimed to evaluate the antinociceptive activity of endemic *Eryngium kotschyi* Boiss. root extracts by bioguided fractionation. The antinociceptive activity of the extracts/fractions/compound was studied in mice using acetic acid induced writhing test and and hot plate test. The methanolic extract was sequentially partitioned with hexane, dichloromethane and water saturated *n*-butanol. Among the fractions, the *n*-BuOH fraction showed the most significant results in both hot plate test (*n*-butanol 18,83±1.81 s $p<0,05$ versus control: 8.33±0.67 s) and acetic acid induced writhing test (*n*-butanol 19,17±2,41 $p<0,005$ versus control 32,67±2,23) and was chosen for further bioguided purifications by column chromatography and MPLC yielding a pure known triterpene saponin which was characterized as a derivative of A1-barrigenol, showing a moderate antinociceptive activity by hot-plate test (14,33 ± 0,33 s, $P < 0.05$ versus control). These findings contribute to the justification of the ethnobotanical use and relevance of *Eryngium* species. The results suggest there may be a synergy of compounds in the selected activity and support the usage of bioguided fractionation in the search for responsible compounds in plants for antinociceptive activity. Further studies are needed to elucidate the mechanism for action and the structure of compounds which might be responsible of the effect.

Keywords: *Eryngium kotschyi*; Apiaceae; antinociceptive; fractionation; triterpene saponin.

1. Introduction

The genus *Eryngium* is an important taxon within the Apiaceae and represented with 317 taxa in Eurasia, North Africa, North and South America and Australia [1–2]. In Turkey, *Eryngium* is represented by 26 taxa of which 10 are endemic [2–5]. They are well known plants in ethnobotanical

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culture in the world and also in Turkey. *Eryngium campestre* is used for muscular-skeletal and digestive disorders in Spain [6]. In Cyprus underground parts of *E. maritimum* are used for the reabsorbtion of oedema and pain relief [7]. *E. foetidum* has a number of usages in several parts of world: Decoction of the aerial parts is used for anti-inflammatory purposes in French Antilles and the decoction from leaves is used for the treatment of pain [8]. Root extracts of different *Eryngium* species are used in folk medicine for the treatment of low-back pain [9]. In Turkey, *Eryngium campestre*, *E. maritimum* and related taxa are known as boğadikeni, deveelması, devecidikeni, göz dikeni, tengeldikeni and used as antitussive, diuretic, appetizer, stimulant, aphrodisiac as well as to recover scorpion stings [10–11]. Although they have a wide usage in traditional medicine there are only a number of studies concerning biological activity of *Eryngium* species to confirm their usage [7, 11–13].

In our previous study, 8 *Eryngium* species were found to possess antinociceptive and anti-inflammatory activities in which underground parts of *E. kotschyi* was found as one of the most active fraction [11]. Furthermore previous studies of our research group resulted with the isolation of 5 new triterpene saponins from *E. campestre* [14–15]. Based on these results, the present study is aimed to find compounds which might be responsible for the antinociceptive activity of the underground parts of *E. kotschyi* by assessing methanol fractions obtained by bioactivity guided fractionation on two nociception models.

2. Materials and Methods

2.1. General Experimental Procedures

The 1D and 2D NMR spectra (^{1}H-^{1}H COSY, TOCSY, HSQC and HMBC) were performed using a UNITY-600 spectrometer at an operating frequency of 600 MHz on a Varian INOVA 600 instrument equipped with a SUN 4 L-X computer system (600 MHz for ^{1}H and 150 MHz for ^{13}C spectra). The carbon type (CH_3, CH_2 and CH) was determined by DEPT experiments. All chemical shifts (δ) are given in ppm, and the samples were dissolved in pyridine-d_5 (δ_C 149.3, 135.8, 123.5). FAB-MS (negative-ion mode) were conducted in negative-ion mode on a JEOL SX 102 mass spectrometer. TLC (Merck, Silycycle) and HPTLC (Merck) were performed on precoated silica gel plates $60F_{254}$. For medium-pressure liquid chromatographic (MPLC) separations a Master Flex L/S Digital Economy Drive MPLC pump equipped with Spectra/Chrom LC column (110 x 250 mm) connected with Foxy 200 Fraction collector and Gilson Pump 305 with Büchi glass column (460 x 15 mm and 230 x 150 mm), Büchi pre-column (110 x 15 mm) were used. Open column chromatography was performed on Silica gel 60 (15-40 µm). Komarowsky reagent (*p*-hydroxybenzaldehyde - 2% in MeOH) and aqueous H_2SO_4 (30%) were used to monitor the TLC chromatograms.

2.2. Drugs

During the experiments following drugs were used; Morphine HCl (Sigma, USA), acetylsalicylic acid (ASA; Sigma, USA) and acetic acid (Merck, USA). The drugs were dissolved in saline and intraperitoneally administered. Dilutions from stock solution were performed on a daily basis. Fractions/subfractions/compound were given orally with a gauge needle. Both the drugs and the fractions were given in a volume of 0.1 mL/10 g of body weight.

2.3. Plant Material

Eryngium kotschyi Boiss. roots were collected in August 2005, from Hadim-Konya Turkey and identified by Prof.Dr. Hayri Duman (Department of Biology, Faculty of Sciences, Gazi University, Ankara). A voucher specimen (AEF 25381) was deposited in the herbarium of the Faculty of Pharmacy (AEF), Ankara University, Ankara, Turkey.

2.4. Animals

Swiss-Albino male mice weighing 25–35 grams from Hacettepe University Laboratory Animals Unit were used for antinociception experiments. All mice were kept in a room where temperature (24±2 °C) and relative humidity (55±15%) were kept within stable limits. A light–dark cycle of 12 h dark/12 h light was applied. Animals had free access to food and water, except during the time of experiments. Drugs were applied to mice only once and those mice weren't used again. Mice were allowed to live in the Animal Unit of Hacettepe University Faculty of Medicine Department of Pharmacology after the experiments. Six animals were used in each group in order to perform statistical comparisons. The Guiding Principles in the Care and Use of Laboratory Animals together with The Recommendations from the Declaration of Helsinki were strictly adhered to the execution of all the procedures described within this study.

2.4. Extraction and Isolation

The dried, powdered roots of *E. kotschyi* (400 g) were extracted with metanol using soxhlet apparatus for 17 hours. After evaporating the methanol under reduced presssure, 90.7 g of dry extract was obtained. This extract was then dissolved in 300 mL of H_2O and successively partitioned against hexane (1100 mL), dichloromethane (955 mL), H_2O-saturated *n*-BuOH and the remaining H_2O extract respectively to give after evaporation hexane (2.4 g), dichloromethane (1.3 g), H_2O-saturated *n*-BuOH (46.2 g) and the remaining H_2O (30.3 g) fractions.
The *n*-BuOH fraction was applied to open column chromatography using silica gel 60 (63-200 µm). Elution was carried out using a gradient system (CH_2Cl_2-MeOH-H_2O, 80:20:2, 70:30:3, 50:50:5 and MeOH) to afford 7 main subfractions (sFr.1-7) according to the TLC results. Among them, sFr. 7 has shown significant efficiency in antinociceptive bioassays. It showed the presence of 6 saponins by TLC on silica gel in the solvent system $CHCl_3$-MeOH-H_2O 60:32:7) in the 0.3-0.7 rf range. Then, sFr.7 was applied to MPLC on normal-phase silica gel, eluted with $CHCl_3$-MeOH- H_2O, (80:20:2 →60:30:7) to give 10 subfractions (ssFr.7A-J). SsFr.7D (37.8 mg), ssFr.7E (20.1 mg) and ssFr.7F (97.5 mg) were subjected to MPLC on reversed-phase silica gel eluted with MeOH-H_2O [(50:50→80:20), (40:60→70:30), (50:50→80:20)] respectively to afford one major pure compound **1** (26.5 mg) (see S1 and S2 in supporting information). Its structural elucidation is done by using spectroscopic methods including 1D and 2D NMR experiments and mass spectrometry, and by comparison of the NMR data with literature values (see S3-S8 in supporting information).

2.5. Hot Plate Test

Hot plate test was chosen as thermal pain model and a hot plate system with a surface temperature of 52°C was used [16]. A thermal pain model was performed under constant temperature conditions aided with a contact thermostat. Hind leg retraction, licking or jumping behaviour was considered as cognition of pain and recorded in seconds. Lengthening of reaction time is accepted as decrease in nociception. Measurements with hot plate system were performed for 80 minutes at each 10 minute intervals. To avoid any tissue damage in mice cut-off time was set as 30 seconds. Six animals were used in each group in order to perform statistical comparisons. The results were evaluated by calculating the mean seconds for each group. Morphine (10 mg/kg) was used as reference drug.

2.6. Writhing Test

An intraperitonally applied 0.6% acetic acid solution in saline was used for induction of nociceptive activity as previously described [17]. Number of writhings was counted between 5^{th} and 15^{th} minutes after acetic acid injection. Six animals were used in each group in order to perform statistical comparisons. The results were evaluated by calculating the mean writhings per group and

the percentage of inhibition of writhings. Acetylsalicylic acid (ASA; 100 mg/kg) was used as reference drug.

2.7. Toxicity

No deaths or complications were recorded during experiments and in the following week. Furthermore, animals treated with drugs were tested with a rotarod device with a rotation speed of 12 r.p.m in order to eliminate the possibility of neurotoxicity. Performance of mice on rotarod device was observed and outcomes of this test were used for the assessment of locomotor activity of mice. Any attenuation from the control was accepted as a loss of locomotor capacity because of the chemicals applied.

2.8. Statistical Analysis

All data are expressed as the mean±SEM. Data were analysed with Student's t-test, Mann–Whitney U-test and one way repeated analysis of variance (ANOVA) followed by a post hoc Bonferroni test. Results were assigned as significant when $P < 0.05$. Graphics and statistics were prepared at Graph Pad Software Prism 3.0 and Microsoft Excel.

Table 1. Antinociceptive activity of *Eryngium kotschyi* root fractions in hot plate test.

Material	Dose (mg/kg)	Hind-leg retraction time (s) mean± SEM
Control	-	8.33 ± 0.67
Hexane	200	9.00 ± 0.63
Dichloromethane	200	10.67 ± 1.31
n-Butanol	200	18.83 ± 1.81*
Water	200	16.17 ± 2.09*
Morphine	10	30.00 ± 0**

*: p<0.05, **: p<0.005 compared to control. Data are expressed as mean±SEM, n=6

Table 2. Antinociceptive effects of *Eryngium kotschyi* root fractions in writhing test.

Material	Dose (mg/kg body wt.)	Number of writhings ± SEM	% Inhibition
Control	-	32.67 ± 2.23	
Hexane	200	29.17 ± 0.87	10.71
Dichlorometane	200	14.83 ± 1.82**	54.61
n-Butanol	200	19.17 ± 2.41**	41.32
Water	200	16.17 ± 2.21**	50.51
ASA	100	18.17 ± 1.14**	49.71

*: p<0.05, **: p<0.005 compared to control. Values are expressed as mean ± SEM, n=6; ASA: Acetylsalicylic acid

3. Results and Discussion

Eryngium species are well known plants in ethnobotanical culture in the world and also in Turkey. They are used as antitussive, diuretic as well as for analgesic and antiinflammatory purposes [6–7, 10, 18].

Antinociceptive activity of fractions obtained by liquid-liquid partition from the root MeOH extract is tested with hot plate and writhing tests. Results of hot plate test for main extracts are presented in Table 1. The hexane, dichloromethane, *n*-BuOH and water fractions at 200 mg/kg, all increased the hind-leg retraction time from 9.00 to 18.83 s compared with the control group (8.33 s),

but this increase was significant only for the *n*-BuOH and water fraction ($p<0.05$). In the writhing test, the dichloromethane, *n*-BuOH and water fractions reduced significantly the number of writhings in comparison with the control ($P<0.005$) (Table 2). The % inhibition of constrictions was calculated as 54.61 % (dichloromethane fraction, 200 mg/kg *p.o.*), 41.32 % (BuOH fraction, 200 mg/kg *p.o*) and 50.51 % (water fraction, 200 mg/kg *p.o*).

Seven subfractions (sFr1-sFr7) gathered from normal-phase silica gel column chromatography eluting with gradient system from CH_2Cl_2:MeOH:H_2O (80:20:2) to MeOH. Fractions were evaluated and combined using TLC. Subfractions were tested both with hot plate test and writhing test. In the hot plate test (Table 3), all tested fractions significantly prolonged the hind-leg retraction time from 13.00 s to 20.67 s versus control (8.33 s) with sFr1-sFr4 $p<0.05$ and sFr5-sFr7 $p<0.005$. In the writhing test (Table 4), all the fractions excepted sFr.4 and sFr.6 significantly decreased the number of writhings induced by acetic acid compared to control ($p<0.005$). sFr 5 and sFr 7 had higher activity compared to the remaining fractions in both tests and the subfraction sFr 7 (37.7 % inhibition in the writhing test, and 20.67 s ± 2.59 s in the hind-leg retraction time, 200 mg/kg) revealed the highest significance ($P<0,005$) compared to control. Therefore in a first stage, sFr.7 was submitted to several chromatographic steps by MPLC on normal-phase and reversed-phase silica gel yielding one major pure compound **1**.

Compound 1, a white amorphous powder (26.5 mg), showed a quasi-molecular ion peak at *m/z* 1041 $[M-H]^-$, indicating a molecular weight of 1042. Among the other fragment ion peaks *m/z* 909 $[(M-H)-132]^-$ and *m/z* 879 $[(M-H)-162]^-$, revealing the elimination of one terminal pentosyl and one hexosyl moiety respectively [14, 19]. The ^{1}H-NMR spectrum of the compound 1 showed signals for seven methyl groups as singlets , (Supporting Information; S4); δ_H 0,74 (3H, s, H-25), 0,97 (3H, s, H-29), 0,98 (3H, s, H-26), 1,04 (3H, s, H-24), 1,16 (3H, s, H-23), 1,19 (3H, s, H-30), 1,76 (3H, s, H-27) ; one oxymethylene group at δ_H 3,54 ve 3,68 (2H, H-28) ; four oxymethine groups at δ_H 3,17 (1H, H-3), 4,22 (1H, H-15), 4,44 (1H, H-16), 6,04 (1H, H-22) and an ethylene group at δ_H 5,46 (1H, H-12). Cross peaks observed in NOESY spectrum between δ_H 4,44 (H-16) and δ_H 3,54 ; 3,70 (H-28) and 4,22 (H-15) are determiner signals of the two secondary alcohol groups at C-15 and C-16 (Supporting Information; S5). This founding is also confirmed with the correlation between δ_H 1,76 (H-27) and δ_C 67,1 (C-15). Crosspeaks observed in NOESY spectrum between δ_H 3,17 (H-3) ile 0,74 (H-1) and 1,16 (H-23) (Spectrum 3.18); and correlations at δ_H 0,74 (H-25) with δ_C 46,8 (H-9); δ_H 1,04 (H-24) with δ_C 55,3 (C-5), 89,7 (C-3), 39,2 (C-4), 27,6 (C-23), 26,2 (C-2); δ_H 1,76 (H-27) ile δ_C 145,6 (C-13) in HMBC (Supporting Information; S7) and when considering with allover datas the main structure is determined to be A1-barrigenol [14-15, 20]. The ^{1}H NMR spectrum of the Compound 1displayed signals for three anomeric protons at δ_H 4,79 (1H, d, *J*= 7,14 Hz, H-1′), 5,17 (1H, d, *J*= 7,39 Hz, H-1‴) and 5,40 (1H, H-1″), revealing that the molecule is triglycosidic (Supporting Information; S8). These signals gave correlations with anomeric carbon signals at δ_C 104,7 (C-1′), 104,3 (C-1‴) and 103,8 (C-1″), respectively, in HSQC spectrum. The other protons of sugar molecules are determined starting from the determined anomeric signals, using COSY, TOCSY, HSQC and HMBC spectrums. In consideration of these datas, β-glucurunopyranose (C′; H′), β-galactopyranose (C″; H″) and α-arabinopyranose (C‴; H‴) has been determined as the sugar molecules of the Compound 1.

When evaluated together with ^{1}H and ^{13}C, 2D NMR (COSY, HSQC, HMBC, NOESY, TOCSY) ve FAB-MS spectrum datas and comparison with literature data; its molecular formula is determined as $C_{52}H_{82}O_{21}$ (molecular weight = 1042); and its structure is determined as 3-*O*-β-D-galactopyranosyl-(1→2)-[α-L-arabinopyranosyl-(1→3)]-β-D-glucuronopyranosyl-22-*O*-*α*-angeloyl-A1-barrigenol which is previously reported as pittangretoside C (Figure 1) from *Pittosporum angustifolium* [19].

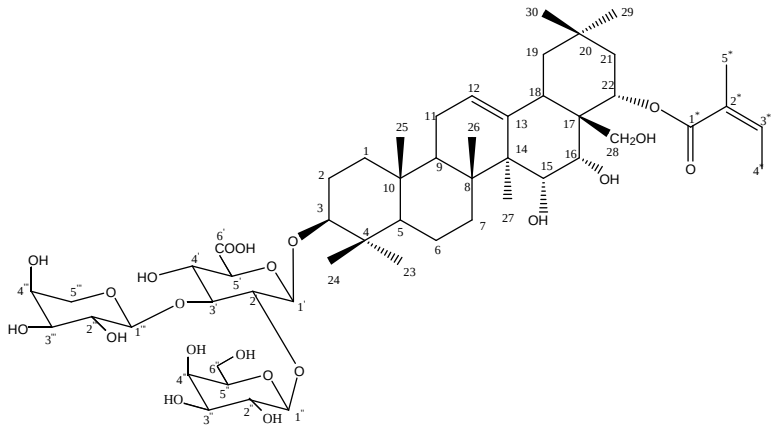

Figure 1. Pittangretoside C.

In the literature, phytochemical investigations on *Eryngium* species were focused mainly on triterpenic saponins [14–15, 20, 22–24], but the antinociceptive activity of such compounds has not previously been reported underlining the interest of the present work. However, the activity of compound **1** in the hot plate test (hind-leg retraction time 14.33 ± 0,33 s versus control 8.33 ± 0.67 s, $P<0.05$; Table 3) was found to be lower than that of the main *n*-BuOH fraction and its subfraction sFr 7. This finding implies that the antinociceptive activity observed in sFr 7 might be related with a possible synergistic activity with other compounds whose phytochemical study is currently in progress.

Table 3. Antinociceptive activity of n-BuOH subfractions and Compound 1 in hot plate test

Material	Dose (mg/kg)	Hind-leg retraction time (s) ± SEM
Control	-	8.33 ± 0.67
sFr. 1 [CH_2Cl_2:MeOH:H_2O (8:2:0.2)]	200	13.00 ± 1.51*
sFr. 2 [CH_2Cl_2:MeOH:H_2O (8:2:0.2)]	200	12.83 ± 2.09*
sFr. 3 [$CHCl_3$:MeOH:H_2O (8:2:0.2)]	200	13.67 ± 1.78*
sFr. 4 [$CHCl_3$:MeOH:H_2O (7:3:0.3)]	200	13.33 ± 2.11*
sFr. 5 [$CHCl_3$:MeOH:H_2O (6:4:0.4)]	200	19.67 ± 1.12**
sFr. 6 [$CHCl_3$:MeOH:H_2O (5:5:0.5)]	200	19.50 ± 1.65**
sFr. 7 [100 % MeOH]	200	20.67 ± 2.59**
Compound 1	200	14.33 ± 0.33*
Morphine	10	30.00 ± 0**

*: $p<0.05$, **: $p<0.005$ compared to control. Values are expressed as mean ± SEM, n=6

Table 4. Antinociceptive effects of *n*-BuOH subfractions in writhing test

Material	Dose (mg/kg)	Number of writhings, mean± SEM	% Inhibition
Control	-	32.67 ± 2.23	
sFr. 1 [CH_2Cl_2:MeOH:H_2O (8:2:0.2)]	200	19.60 ± 1.75**	40.01
sFr. 2 [CH_2Cl_2:MeOH:H_2O (8:2:0.2)]	200	18.83 ± 1.25**	42.36
sFr. 3 [$CHCl_3$:MeOH:H_2O (8:2:0.2)]	200	20.83 ± 1.62**	36.24
sFr. 4 [$CHCl_3$:MeOH:H_2O (7:3:0.3)]	200	28.17 ± 2.41	13.77
sFr. 5 [$CHCl_3$:MeOH:H_2O (6:4:0.4)]	200	21.00 ± 2.25**	35.72
sFr. 6 [$CHCl_3$:MeOH:H_2O (5:5:0.5)]	200	26.17 ± 1.08	19.90
sFr. 7 [100 % MeOH]	200	20.33 ± 0.99**	37.77
ASA	100	18.17 ± 1.14	44.38

*: $p<0.05$, **: $p<0.005$ compared to control. Values are expressed as mean ± SEM, n=6; ASA: Acetylsalicylic acid

In conclusion, the results of this study confirmed the ethnobotanical use and relevance of the plants belonging to the genus *Eryngium*. The *n*-butanol fraction was chosen as the starting material in bioactivity guided fractionation. Several fractions, one of them rich in saponins, showed a significant antinociceptive effect, but the isolated pure triterpene saponin showed only a moderate effect. Therefore further studies will be required to elucidate the exact mechanism of action and the principles responsible of the antinociceptive activity.

Supporting Information

Supporting Information accompanies this paper on http://www.acgpubs.org/RNP

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