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RESEARCH ARTICLE

Analgesic and antioxidant activity of some *Echium* species wild growing in Turkey

Nuraniye ERUYGUR[*], Gülderen YILMAZ[**], Osman ÜSTÜN[**°]

Analgesic and antioxidant activity of some Echium species wild growing in Turkey

Summary

The roots of E. italicum L. E. vulgare L. and E. angustifolium Miller locally known as "Kızılcık dikeni, Engerek otu", are used for healing wounds in Turkey. The aim of the present study was to investigate the antioxidant and possible analgesic activities of the ethanol extracts from the roots and herbs of Echium species, to support its use in folk medicine and screen the major phytochemical constituents of these extracts. Antioxidant activity was assessed by DPPH free-radical scavenging, Fe2+- chelating ability, total phenolic contents and total flavonoid contents methods. Analgesic activity of the ethanol extracts was estimated with acetic acid-induced writhing and tail flick methods. The analgesic effect of root extracts of E. italicum, E. angustifolium and E. vulgare (0,5 mg/g) was comparable with the standard drugs, Aspirin and Morphine. These findings imply the involvement of both peripheral and central antinociceptive mechanisms. The present report demonstrates the analgesic and antioxidant properties of 4 Echium species and validates its use in Turkish traditional medicine. Thus, further studies can be recommended.

Key Words: *E. italicum L. E. vulgare L. E.angustifolium Miller, E. parviflorum Moench; antioxidant, analgesic activity, in-vitro, in-vivo*

Türkiye'de yetişen bazı Echium türlerinin analjezik ve antioksidan aktivitesinin araştırılması

Özet

Türkiye'de yetişen "kızılcık dikeni, engerek otu" gibi isimlerle bilinen E. italicum L. E. vulgare L. ve E. angustifolium Miller kökleri yara iyileştirici olarak kullanılmaktadır. Çalışmamızın amacı, Echium türlerinin kök ve toprak üstü kısımlarından hazırlanan etanol ekstresinin antioksidan ve olası analjezik aktivitesini araştırmak, major fitokimyasal bileşenleri tespit etmek ve halk arasındaki kullanımına bilimsel yönden destek sağlamaktır. Ekstrelerin antioksidan aktivitesi DPPH radikal süpürücü etki, Demir iyonu şelasyon etki, total fenolik miktarı ve total flavonoit miktar tayini gibi yöntemler kullanılarak, analjezik aktivitesi için asetik asitle indüklenen kıvranma tesi ve kuyruk çekme yöntemleri kullanarak tespit edilmiştir. E. italicum L. E. vulgare L. ve E. angustifolium Miller köklerinden hazırlanan etanol ekstrelerinin analjezik aktivitesi referans olarak kullanılan Aspirin ve Morfin karşılaştırabilir seviyede çıkmıştır. Bu rapor Echium türlerinin antioksidan ve analjezik aktiviteye sahip olduklarını göstermekte ve halk arasındaki kullanımı doğrulamakta, o yüzden daha ileriki çalışmaların sürdürülmesi öngörülmektedir.

Anahtar kelimeler: *E. italicum L. E.vulgare L. E. angustifolium Miller, E.parviflorum Moench; antioksidan, analjezik aktivite, in-vitro, in-vivo*

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INTRODUCTION

The roots of *E. italicum* L., *E. vulgare* L., *E. angustifolium* Miller (Boraginaceae), locally known as "Kızılcık dikeni, Engerek otu" in Turkey, are widely used in folk medicine because of their reputed wound healing effects (1). Previous studies on *Echium* species describe shikonin derivatives, flavonoids, phenolic acids, pyrrolizidine alkaloids and fatty acids as main chemical constituents (2-7) In folk medicine, various parts of *Echium* species (herbs, petals, roots and root barks) have been used for rheumatic pain, wound healing, demulcent, diuretic, sedative and antioxidants (8-11). In Turkish folk medicine, roots of *Echium italicum* and *Echium vulgare* are used externally for healing wounds (12-15). In scientific studies, antibacterial, antiinflammatory, antiproliferative, antidepressant, antioxidant, antiviral, anxiolytic and cytotoxic properties were found in some species of *Echium* (11, 16-27). The aim of this study is the validation of the medicinal use of *Echium* species roots and

[*] Gazi University, Faculty of Pharmacy, Department of Pharmacognosy, Etiler-06330 Ankara, Turkey
[**] Ankara University, Faculty of Pharmacy, Department of Pharmaceutical Botany, Beşevler-06100 Ankara, Turkey
[°]Corresponding author: E-mail: oustun@yahoo.com, Phone: +90 312 202 31 82, Fax: +90 312 223 50 18

searching for analgesic and antioxidant activities in the crude drug. Pain and inflammation are strictly related to wound healing and free radicals increase during all these pathological process, extending inflammation and influencing wound healing (28). Some chronic disorders including diabetes, and cardiovascular abnormalities, can be obviously induced by free radicals. The radical scavenging ability is often used to investigate antioxidant compounds' activities (29, 30).

The present investigation was undertaken to establish the analgesic and antioxidant effects of some Turkish samples of *Echium* species through *in-vivo* analgesic and *in vitro* antioxidant of extracts. Despite the publications on other biological activities of *Echium* species, there is no scientific evidence on activities related to traditional use. Therefore, it is considered important to study the analgesic and antioxidant activity of ethanol extract from the herbs and roots of *Echium* species as well as determining its main constituents to provide scientific support to the safe use of the plants.

MATERIALS AND METHODS

Chemicals

DPPH, Rutin, Gallic acid, Quercetin, EDTA, sodium hydrogen carbonate were obtained from Sigma Chemicals Co., St Louis, MO, USA. The Folin-Ciocalteu's phenol reagent, $AlCl_3$ and Ferrozine were from Fluka. All other solvents and chemicals were of analytical grade.

Ethical considerations

Experimental procedures and protocols used in this study were approved by the Ethics Committee of GUDAM, Turkey. The animals were left for one week for acclimatization to the animal room conditions, and were maintained on standard pellet diet and water ad libitum. The food was withdrawn on the day before the experiment, but free access was allowed for water. Minimum of six animals were used in each group. Throughout the experiments, animals were processed according to the suggested ethical guidelines for the care of laboratory animals (Gazi University Ethical Council Project Number: G.U. ET-11.023).

Plant materials

Plant materials were collected in different regions of Turkey. The species were identified and the voucher specimens were deposited at the Department of Pharmacognosy, Faculty of Pharmacy, University of Gazi (GÜEF) and Faculty of Pharmacy, University of Ankara (AEF). Details on the selected plants are given in Table 1.

Preparation of the plant extracts

The air-dried aerial parts and roots of the plants were ground using a cylindrical crusher, and extracted with ethanol (96%) using maceration techniques. Extracts were then filtered through a paper filter and combined supernatants were evaporated to dryness under vacuum at 40°C using a rotary evaporator. The extracts obtained were kept in sterile sample tubes and stored in a refrigerator at 4°C until the bioassays time.

Table 1. Detailed information about the *Echium* species collected from various regions of Turkey

Plant species	Plant part used	Traditional use	Locality	Voucher No.	Yield (%)
E. italicum	Aerial parts	Wound healing, diaphoretic, emollient, diuretic (31)	North of Ankara, entry of Kazan, 52.km	GUE 2991	3.23
	Roots	Wound healing, ulcer, rheumatic pain, blister, treat bruises (15, 32)			4.61
E. vulgare	Aerial parts	Diuretic (33)	North of Ankara, entry of Kazan, 56.km	GUE 2992	5.68
	Roots	Wound healing, ulcer (34)			2.67
E. angustifolium	Aerial parts	-	Beside the sea, Side, Antalya province	AEF 26023	3.25
	Roots	Wound healing, ulcer (33)			5.08
E. parviflorum	Aerial parts	-	Beside the sea, Side, Antalya province	AEF 26024	8.08
	Roots	-			2.63

Antioxidant activity

Antioxidant activities of the extracts were evaluated by DPPH radical scavenging and ferrous ion-chelating capacity tests. Gallic acid, a natural phenolic-type antioxidant, a widely used synthetic antioxidant, was employed as references in the tests.

DPPH Free Radical Scavenging Assay

The stable 2,2-diphenyl-2-picrylhydrazyl ($DPPH^{\cdot}$) radical scavenging activity was determined by Blois's method (35). The samples and references dissolved in ethanol (75%) were mixed with $DPPH^{\cdot}$ solution (1.5×10^{-4} M). Remaining amount of DPPH was measured at 520 nm using a Unico 4802 UV–Vis double beam spectrophotometer (USA). Gallic acid and Quercetin were employed as references. Inhibition of $DPPH^{\cdot}$ in percent (%) was calculated as given below:

$$\text{Inhibition } \% = A_{Control} - A_{Sample} / A_{Control} \times 100$$

Where $A_{Control}$ is the absorbance of the control reaction (containing all reagents except for the test sample), and A_{Sample} is the absorbance of the extracts/reference.

Fe^{2+}-Ferrozine Test System for Iron Chelating

The ferrous ion-chelating effect of the extracts by Fe^{2+}-ferrozine test system was estimated by the Fe^{2+}-Ferrozine Test System (36). Briefly, 740µL of methanol and the samples were incubated with 2 mM $FeCl_2$ solution. The reaction was initiated by adding 40 µL of ferrozine solution into the mixture, then 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: % Inhibition = $A_{Control} - A_{Sample} / A_{Control} \times 100$

The control contained only $FeCl_2$ and ferrozine. Analyses were run in three replicates and expressed as average values with SD.

Total Flavonoid Content

The flavonoid content was determined by aluminium trichloride method (37) using quercetin as a reference compound with slight modification. This method based on the formation of a complex flavonoid-aluminium having the absorption maximum at 415 nm, then remained react at room temperature for 30 min. Briefly, 0.5 mL of each extract (1:10 g/mL) in methanol was separately mixed with 1.5 mL of methanol, 0.1 mL of 10% aluminium chloride, 0.1 mL of 1 M sodium acetate and 2.8 mL of distilled water. The calibration curve was prepared by preparing quercetin solutions at different concentrations from 12.5 to 100 g/mL in methanol. The samples were prepared in triplicate for each analysis and the mean value of absorbance was obtained. The same procedure was repeated for the standard solution of quercetin and the calibration line was construed. Based on the measured absorbance, the concentration of flavonoids was read (mg/mL) on the calibration line; then, the content of flavonoids in extracts was expressed in terms of quercetine equivalent (mg of QE/g of extract).

Total Polyphenol Content

The amount of total polyphenol in the extracts was determined using modified Folin-Ciocalteu colorimetric method (38). Stock solution of sample extracts (25 µl each) were dissolved in methanol and further dilutions were performed to obtain readings within the standard curve made with gallic acid. The extracts were oxidized by the Folin-Ciocalteu reagent (100 µl) and the neutralization was made with 80 µl $NaHCO_3$ (7.5 %), after 5 minutes. For standard solutions, 10mg gallic acid was dissolved in 100mL of distilled water and used as a stock solution (100 $\mu g mL^{-1}$) to make serial dilutions and to obtain the standard solution at the concentration of 100, 50, 25, 12.5, and 6.25 $\mu g mL^{-1}$. The absorbance was measured at 750 nm after 90 minute in the dark, at room temperature. The results were expressed as milligram of gallic acid per gram extract (mg GAE/g extract).

Analgesic Activity

Animals

Male Albino Swiss mice (25–30 g) were used for the experimental study. The animals were maintained under standard husbandry conditions in polypropylene cages with water ad libitum and food until 3 days before the experiment. All the procedures were approved by the Ethics Committee for Animal Use of GUADEK.

Acetic Acid-Induced Writhing Test

The test was carried out using the technique described by Koster (39). Mice were divided into eleven groups of six each. Group 1 was injected 0.5% carboxy methyl cellulose as a negative control group by intraperitonally. Group 2-3 were injected with Acetylsalicylic acid (100 mg/kg) and Morphine (10 mg/kg) as a positive control. Groups 4-11 were injected ethanol (96 %) extract of root and arial part of *E. vulgare, E. italicum, E. angustifolium, E. parviflorum* (500 mg/kg) respectively. 20 minutes after receiving the plant extracts, reference substance or solvent, each mouse received a 0.8% aqueous solution of acetic acid intraperitoneally (10 mL/kg body weight). Immediately after the acetic acid injec-

tion, each animal was placed in a transparent observation cage and the number of writhes per mice was counted for 20 min.

Tail-Flick Method

The selected animals were divided into 10 groups of six mice each (40). Each animal each group received one of the following extracts (500 mg/kg), Acetylsalicylic acid (100 mg/kg) and Morphine (10 ml/kg) in 0.5% w/v of CMC intraperitoneally. Analgesia was assessed with a tail flick apparatus (Analgesiometer). The basal reaction time was measured initially and another set of four measurements were taken at 20, 40, 60 and 80 minutes intervals and the reaction of the animals were considered as the post-drug reaction time. A cut-off period of 10sec. was observed to prevent tissue damage of the tails of the animals.

Statistical analysis

All experimental measurements were carried out in triplicate and values were expressed as means ± S.D. A statistical analysis was performed by using one-way analysis of variance of (ANOVA) followed by Dunnett's Multiple Comparison Test. $p < 0.05$ was considered as significant from the control.

RESULTS AND DISCUSSIONS

Antioxidant and analgesic effects of the ethanol extracts from herbs and roots of *E. italicum*, *E. vulgare*, *E. angustifolium* and *E. parviflorum* were tested in the present study. The experimental results were presented in Table 2-5 and Figure 1. Samples were analyzed for antioxidant components viz. polyphenols and flavonoids. For the antioxidant activity assessment, *in vitro* DPPH free radical scavenging activity and ferrous ion chelating ability test results were used. For analgesic activity assessment, *in vivo* tail-flick and acetic acid-induced writhing methods were used.

Total phenolic content and total flavonoid content were determined from the calibration curves of gallic acid ($y=0.0146 x + 0.0464$, $r^2=0.9994$), and quercetin ($y=0.5418x+ 0.0105$, $r^2=0.9983$), respectively. The total phenolic and total flavonoid contents among the different extracts of four *Echium* species are presented in Table 2. The results showed that ethanol extract of roots of *E. angustifolium* possessed the highest phenolic [(38.86±0.008) mg GAE/g of dry material] and flavonoid components [(56.12±0.01) mg QE/g of dry material], followed by the *E.italicum* and *E. vulgare* extract, while the *E. parviflorum* extract contained lower polyphenolic compounds.

Table 2. Total phenolic content and total flavonoid content of the hydro alcoholic extracts using Folin-Ciocalteu method and aluminium trichloride colorimetric method separately

Alcoholic extract	Plant part used	Total phenolic content (mg GA/g)[a]± S.D	Total flavonoit content (mg Quercetin /g)[b]± S.D
E. italicum	herbs	11.46 ± 0.08	49.42 ± 0.02
	roots	19.97 ± 0.01	47.11 ± 0.01
E. vulgare	herbs	9.71 ± 0.03	46.43 ± 0.03
	roots	16.82 ± 0.01	35.98 ± 0.03
E. angustifolium	herbs	12.62 ± 0.03	65.35 ± 0.09
	roots	38.86 ± 0.01	56.12 ± 0.01
E. parviflorum	herbs	9.69 ± 0.05	48.03 ± 0.06
	roots	11.97 ± 0.02	33.11 ± 0.03

[a] Values expressed as gallic acid equivalents mg /g of extract.

[b] Values expressed as quercetin equivalents mg /g of extract.

Table 3. DPPH Free Radical Scavenging Activity of Ethanol Extracts of *Echium* Species

No	Ethanol extracts of the plants	DPPH Free Radical Scavenging Activity (Scavenging activity%± S.D*)			
		50 µg/ml	100 µg/ml	500 µg/ml	1000 µg/ml
1	*E. italicum* herbs	3.18 ± 0.02	16.49 ± 0.01	25.90 ± 0.06	33.44 ± 0.03
2	*E. italicum* roots	11.93± 0.01	13.83 ± 0.02	57.24 ± 0.01	81.43 ± 0.01
3	*E. vulgare* herbs	-	-	15.71 ± 0.01	43.36 ± 0.37
4	*E. vulgare* roots	18.85 ± 0.02	27.02 ± 0.06	69.99 ± 0.01	71.2 ± 0.005
5	*E. angustifolium* herbs	-	5.45 ± 0.02	20.41 ± 0.01	45.32 ± 0.03
6	*E. angustifolium* roots	13.24 ± 0.01	20.87 ± 0.03	69.12 ± 0.04	75.49 ± 0.11
7	*E. parviflorum* herbs	2.61 ± 0.01	7.41 ± 0.01	43.93 ± 0.01	68.84 ± 0.03
8	*E. parviflorum* roots	2.75 ± 0.01	3.34 ± 0.02	30.65 ± 0.06	44.7 ± 0.07
References					
9	Quercetine	48.95 ± 0.02	72.97 ± 0.08	90.38 ± 0.01	92.61 ± 0.01
10	Gallic acid	96.62 ± 0.01	96.29 ± 0.01	96.68 ± 0.01	97.98 ± 0.01

*: Results are expressed as Means ± Standard Deviation, n=3

-: have not detected

Table 4. Ferrous Ion Chelating Activity of the Ethanol Extracts of *Echium* Species

No	Ethanol extracts of the species	Ferrous Ion Chelating activity (Chelating activity%± S.D.*)		
		50 µg/ml	200 µg/ml	400 µg/ml
1	*E. italicum* herbs	-	5.93 ± 0.04	7.26 ± 0.06
2	*E. italicum* roots	20.97 ± 0.02	25.93 ± 0.02	32.0 ± 0.06
3	*E. vulgare* herbs	2.62 ± 0.03	6.90 ± 0.04	17.10 ± 0.04
4	*E. vulgare* roots	10.48 ± 0.03	25.10 ± 0.06	34.34 ± 0.01
5	*E. angustifolium* herbs	-	1.24 ± 0.03	22.34 ± 0.03
6	*E. angustifolium* roots	-	17.52 ± 0.04	23.72 ± 0.01
7	*E. parviflorum* herbs	19.45 ± 0.05	27.17 ± 0.02	35.72 ± 0.01
8	*E. parviflorum* roots	27.59 ± 0.04	34.34 ± 0.03	48.69 ± 0.04
References				

9	Concentration	20 µM	100 µM	200 µM	500 µM	1000 µM
	EDTA	8.05 ± 0.07	39.59 ± 0.11	67.13 ± 0.02	97.29 ± 0.02	97.98 ± 0.01

*: Results are expressed as Means ± Standard Deviation, n=3

-: have not detected

Table 5. Effect of the extracts on tail flick test in mice

Treatment	Plant parts used	Dose mg/ kg, i.p	Time after injection (mean ± S.D.)				
			0 min (basal)	20 min	40 min	60 min	80 min
Vehicle (CMC)			3.20 ± 0.29	3.51±0.41	4.17±0.20	5.10±0.18	4.92± 0.39
Morphine		10 mg	4.18 ± 0.62	9.64±0.57	11.64±0.49	12.29±0.37	11.77± 0.62
E. italicum Ethanol extract	herbs	500 mg	6.32 ± 1.46	7.82±1.58	7.27±1.14	7.50±0.59	7.67± 0.68
	roots	500 mg	5.60 ± 0.94	9.73 ±0.72	10.57±1.09	10.98 ±1.02	11.18 ±1.36
E. vulgare Ethanol extract	herbs	500 mg	4.98 ± 1.43	7.00 ± 1.43	7.05 ±1.00	7.47 ± 1.26	7.03 ± 1.15
	roots	500 mg	5.35 ± 1.14	9.13 ±1.64	9.07 ±1.34	10.87±1.29	10.17± 1.66
E. angustifolium Ethanol extract	herbs	500 mg	4.80 ± 0.55	5.65 ±0.98	7.23 ± 1.46	7.42 ± 1.10	6.97 ± 1.60
	roots	500 mg	4.70 ± 1.03	9.22 ± 1.47	10.58± 1.50	11.03 ±1.00	11.0 ± 1.37
E. parviflorum Ethanol extract	herbs	500 mg	4.67 ± 0.50	7.37 ± 1.62	6.62 ± 1.59	7.23 ± 1.71	6.50 ± 1.12
	roots	500 mg	5.48 ± 1.43	6.92 ± 1.42	7.00 ± 1.60	7.50 ± 0.90	8.47 ± 1.48

n = 6 animals; CMC: Carboxymethyl cellulose; S.D.: standard deviation

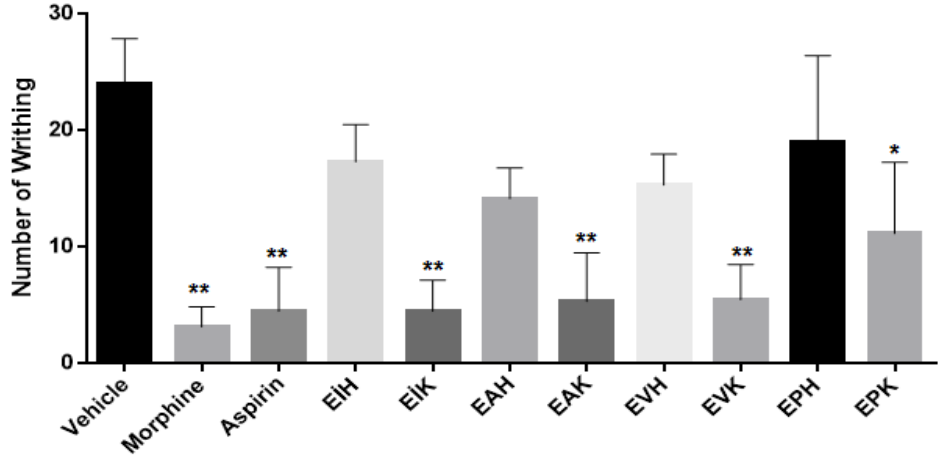

Figure 1. Effect of *Echium* species ethanol extracts on acetic acid-induced writhing in mice.

Note: EİH: *E. italicum* L. herbs; **EİK**: *E. italicum* roots; **EVH**: *E. vulgare* herbs; **EVK**: *E. vulgare* roots; **EAH**: *E. angustifolium* herbs; **EAK**: *E. angustifolium* roots; **EPH:** *E. parviflorum* herbs; **EPK:** *E. parviflorum* roots. Ethanol extracts (500 mg/kg) or aspirin (100 mg/kg), Morphine (10 mg/kg) or vehicle (carboxymethyl cellulose) was administered 20 min prior to acetic acid injection. Data are represented as mean ± S.D. of 6 animals of each group.

All extracts showed a concentration-response relationship in DPPH scavenging activity. An increase in the concentration is synonymous with an increase in scavenging capacity. But root extracts of the four *Echium* species shows more potent DPPH scavenging activity than the herb extracts. Among the extracts, *E. italicum* root extract showed high scavenging activity with 81.43± 0.01%, followed by *E. angustifolium* and *E. vulgare* root extract with 75.49 ± 0.11%and 71.20±0.01 % and *E. parviflorum* herb extract with 68.84±0.03 % in 1000 µg/ml concentration (Table 3).

All the extracts interfered with the formation of ferrous and ferrozine complex suggesting that the extracts have chelating activities. The chelating activities of the extracts increased with the concentration. The highest iron chelating activity was shown by *E. parviflorum* root extract with 48.69 ± 0.04%, followed by *E. vulgare*, *E.italicum, E. angustifolium* root extracts with 34.34 ± 0.01%, 32.0 ± 0.06%, 23.72 ± 0.01 % respectively at 400 µg/ml concentration (Table 4).

Considering the common dosages used among people, we prepared the extracts. *In vivo* test results of its analgesic activity are given in Tables 5 and Figure 1. As

shown in Table 5, i.p. administration of the different extracts of *Echium* species at a dose of 500 mg/kg inhibited tail flick response at the 20th min. in mice. This response was rapid and durable similar to that observed with morphine. The analgesic activity, although decreased, was detected 80 minutes after.

The acetic acid induced abdominal constriction method is widely used in evaluation of peripheral antinociceptive activity (41). It is very sensitive and is able to detect antinociceptive effects of compounds at dose levels that may appear inactive in other methods, like the tail flick test (42, 43). When ethanol extracts from root and herbs of four *Echium* species were used in writhing test, *E. italicum* root extract was found to have the strongest analgesic activity (Figure 1). Once again, its efficacy was very close to morphine. The other extracts attenuated the number of acetic acid induced abdominal writhes were between observed with aspirin and morphine.

Tail-flick test is considered as a specific model for compounds producing central antinociceptive activity (44). These results indicate that in the both tail-flick and acetic acid-induced writhing method, 95% ethanol extracts of *E. italicum*, *E. vulgare* and *E. angustifolium* roots and standard drug showed significant activity as compared to the control group.

CONCLUSION

The ethnobotanical inquiry revealed that the species studied are used in the local folk medicine in inflammatory-based diseases or related conditions, such as wound healing. The results obtained support the validity of the traditional uses of these species against inflammatory disorders. However, more pharmacological and toxicological experiments are needed for the use of this plant as an official herbal drug for clinical use. Based on the results of this study, it is clearly indicated that the three species: *E. italicum*, *E. vulgare* and *E. angustifolium* roots have powerful antioxidant activity against various oxidative systems *in vitro*. Moreover, the *Echium* roots can be used as an accessible source of natural antioxidants and as possible food supplement or in pharmaceutical industry. The various antioxidant mechanisms of the three *Echium* species roots may be attributed to strong hydrogen donating ability, a metal chelating ability and their effectiveness as scavengers of hydrogen peroxide, superoxide, and free radicals. Phenolic compounds appear to be responsible for the antioxidant activity of these species.

In conclusion, the present study clearly demonstrated that the roots of *E. angustifolium*, *E. italicum* and *E. vulgare* have shown to possess potent analgesic activities in all nonciceptic models, at the doses tested, signifying that they possess both central and peripherally mediated activities. Flavonoids, saponins, polyphenols and tannins have been shown to exert analgesic effects on acetic acid induced writhing test. This is the first report demonstrating the analgesic activities of *E. angustifolium in vivo*; however, further studies will be necessary to isolate the active compounds which are responsible for the analgesic effects and in order to be able to understand the exact mechanisms of these activities.

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