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Wound-Healing Activity of Some Species of *Euphorbia* L.

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Abstract: Some species of *Euphorbia* have been used as medicinal plants to treat wounds, and skin diseases, around the world. The solvents *n*-hexane, ethyl acetate, and methanol were used successively to prepare extracts of the aerial parts of *E. characias* subsp. *wulfenii, E. helioscopia, E. macroclada*, *E. seguieriana* subsp. *seguieriana,* and *E. virgata.* Linear incision, circular excision wound models and the hydroxyproline assay method were used to assess the wound-healing activity. The inhibition of the increase in capillary permeability induced by acetic acid was used to assay the anti-inflammatory activity. The methanol extract of the aerial parts of *E. characias* subsp. *wulfenii* showed statistically significant wound-healing activity with 43.03% tensile strength for the linear incision wound model and a 65.24% reduction in the area of the wound by day 10 for the circular excision model. The tissue treated with this extract was found to contain 35.47 µg/mg of hydroxyproline. The methanol extract of *E. characias* subsp. *wulfenii* inhibited inflammation induced by acetic acid with a value of 34.74%. The results showed that the aerial parts of *E. characias* subsp. *wulfenii* possess wound-healing and anti-inflammatory activities on different models.

Keywords: *Euphorbia*; anti-inflammatory; excision; incision; wound-healing.

1. Introduction

The *Euphorbia* genus is the largest of the Euphorbiaceae plant family of about 2000 species, ranging from annuals to trees. *Euphorbia* species contain latex and have very different flower states. An important part of these are mostly originated from Africa or Madagascar. Ninety-one species of *Euphorbia* grow in Turkey [1]. Some species of *Euphorbia* which are commonly known as "sütleğen" in Turkish or "spurge" in English are used in traditional medicine to treat skin diseases, wounds, warts, gonorrhea, migraines, and intestinal parasites in Turkey and other parts of the world [2]. The latex of *Euphorbia armena* Prokh., and *Euphorbia seguieriana* subsp. *seguieriana* Necker, is used to treat wounds and warts on the skin. The flowers of *Euphorbia virgata* Waldst. & Kit. are used on eczema [3-5], *Euphorbia fusiformis* Buch.-Ham. ex. D.Don, *Euphorbia helioscopia* L., *Euphorbia peplus* L. *Euphorbia hirta* L., and *Euphorbia characias* L. are used as antimicrobial and antifungal agents [6-10]. Ethnobotanical studies have shown that other species of this genus, such as *Euphorbia macroclada* L.

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and *Euphorbia coniosperma* Boiss., were used to enhence the healing of wound and also against scorpion and snake bites [4,11].

Plants of the genus *Euphorbia* have been found to contain diterpenoids with the basic skeletons of jatrophane, lathyrane, and myrsinane [12]; ingenane, daphnane and tigliane [13]; paraliane, pepluane, and segetane. The sesquiterpenoids clovandiol, euphanginol, euphorbioside A, and euphorbioside B have also been reported [14]. The flavonoids kaempferol, myricetin, rutin, quercetin, and their derivatives and phenolic compounds have been found [15], along with the volatile compounds α-terpineol, β-caryophyllene, α-humulene, linalool, terpinene, and germacrene-D [16]. Tannins (euphorbins), triterpenoids (lupeol, lupeol acetate, betulin, and β-amyrin) and phytosterols such as β-sitosterol have also been reported [17].

Euphorbia species have an increasingly prevalent due to chemical compounds which have different skeletal structure and their therapeutic importance. The results showed that *Euphorbia* species have cytotoxic, antitumor, antibacterial, anti-inflammatory and anti-HIV activities [18]. *Euphorbia* species also show analgesic, antidiorrheal, antifeedant, antimicrobial, antiproliferative, antipyretic, and molluscicidal activities and the ability to modulate multidrug resistance [13-14].

The existing ethnobotanical studies covering traditional uses of *Euphorbia* species do not provide scientific proof of their effectiveness. The aim of the present study is to investigate the *in vivo* wound-healing and anti-inflammatory potentials of five *Euphorbia* species, some of which have been used in traditional medicine. The species *E. characias* subsp. *wulfenii* (Hoppe ex W. Koch) A. R. Smith, *E. helioscopia*, *E. macroclada*, *E. seguieriana* subsp. *seguieriana*, and *E. virgata* were chosen. Extracts of the aerial parts of the selected species were prepared by using *n*-hexane, ethyl acetate, and methanol. Linear incision, circular excision wound models and the hydroxyproline assay method were used to assess the wound-healing activity. The inhibition of the increase in capillary permeability induced by acetic acid method was used to assay the anti-inflammatory activity.

2. Materials and Methods

2.1. Plant Material

Plants of various species of *Euphorbia* were collected from different regions of Anatolia, Turkey (Table 1). Taxonomic identification of the plants was confirmed by Prof. Dr. Hayri Duman at Department of Biology, Faculty of Sciences, Gazi University, and Assoc. Prof. Mehmet Tekin at Department of Pharmaceutical Botany, Faculty of Pharmacy, Trakya University. Voucher specimens have been deposited in the herbarium at the Faculty of Pharmacy of Ankara University (AEF) and in the herbarium at the Faculty of Science of Cumhuriyet University (CUFH). The localities where the plant materials were collected and the dates when they were collected are shown along with the assigned herbarium numbers in Table 1.

Table 1. Locality of the plants sample

Plant species	Locality	Altitude	Date	Herbarium No
E. macroclada	Çayyolu village, Yenimahalle, Ankara	900 m	10.07.2012	AEF 26268
E. helioscopia	Çomaklı village, Korkuteli, Antalya	1000 m	14.05.2012	AEF 26269
E. characias subsp. *wulfenii*	Yazır village, Korkuteli, Antalya	950 m	15.05.2012	AEF 26270
E. virgata	Tödürge Lake, Zara, Sivas	1250 m	16.09.2012	M 1326
E. seguieriana subsp. *seguieriana*	İğneada, Kırklareli	250 m	21.08.2012	M 1321

2.1.1. Extraction

Air-dried and powdered aerial parts of each plant (80 g) were extracted with n-hexane (900mL x 4) at room temperature during 8 h/day for 4 days with continuous stirring. The residues were dried and extracted with ethyl acetate (900 mL x 4) at room temperature during 8 h/day for 4 days with continuous stirring. These residues were dried and extracted with methanol (900 mL x 4) at room

temperature during 8 h/day for 4 days with continuous stirring. The extracts were filtered and the solvent was removed to dryness at 50 °C under reduced pressure to yield the crude extracts.

2.2. Biological Activity Tests

2.2.1. Animals

Male Sprague Dawley rats (160–180 g) and Swiss albino mice (20–25 g) were purchased from Laboratory of Experimental Animals, Kobay, Ankara, Turkey. The animals were acclimated for 3 days in room conditions and fed a standard pellet diet and water ad libitum. Six animals from each group were used to evaluate the activity tests. Throughout the experiments, the animals were processed according to the suggested European ethical guidelines for the care of laboratory animals. The study was performed according to the international rules covering animal experiments and biodiversity rights (Gazi University Ethical Council Project Number: G.U.ET-12.049).

2.2.2. Preparation of the Test Samples

The test materials in an ointment base were applied topically to the wounded area of the test animal to evaluate the wound-healing activity using the incision and excision wound models. The extracts were mixed thoroughly in a mortar with a mixture of glycol stearate: propylene glycol and liquid paraffin (3:6:1) to form an ointment with a concentration of 1%. The animals allocated to the control group were treated topically with the blank vehicle, while the animals in the negative control group were not treated with anything. Madecassol® (Bayer) (0.5 g) was used topically as a reference drug. Madecassol® contains a 1% extract of *Centella asiatica* L. [19].

Test samples suspended in a mixture of distilled water and 0.5% sodium carboxy methyl cellulose (CMC) were given to the test animals orally to evaluate anti-inflammatory activity. Animals in the control group received only the vehicle [20]. Indomethacin (10 mg/kg) in 0.5% CMC was used as a reference drug [21].

2.2.3. Wound Healing Activity

2.2.3.1. Linear Incision Wound Model

Ketasol® (Richterpharma) with a dose of 0.15 cc were used to anesthetize the animals. The dorsal sides of the rats were shaved and disinfected with 70% ethanol. Two linear-paravertebral incisions, each 5 cm long and 1.5 cm from the dorsal midline were made through the shaved skin using a sterile blade. Three surgical sutures were each placed 1 cm apart.

The test ointments, the reference drug, or the ointment base (vehicle) was applied topically on the wounds once a day for 9 days. The sutures were removed on day ten and the tensile strengths of the injured and treated skin were measured with a tensiometer (Zwick/Roell Z 0.5, Germany) [22-23].

2.2.3.2. Circular Excision Wound Model

The circular excision wound model was used to evaluate the wound contraction and wound closure time. Each animal group was anesthetized using 0.01 cc of Ketasol® (Richterpharma). After the dorsal parts of the animals had been shaved, a circular wound was created on application area of each mouse by excising the skin with a 5 mm biopsy punch (Nopa instruments, Germany) and nothing was applied on the wounds [24]. The test samples, the reference drug (Madecassol®, Bayer), and the vehicle ointments were applied topically once a day until the wound was completely healed. The wound areas were photographed every day using a camera (Fuji, S20 Pro, Japan). The wound area was evaluated using the program AutoCAD. The wound contraction was calculated as a percentage of the reduction in the area covered by the wound [23,25].

2.2.3.3. Estimation of Hydroxyproline

Isolated tissues were dried at 60-70 °C in a hot-air oven until a consistent weight was achieved. Each sample was hydrolyzed with 6 N HCl for 3 h at 130 °C. The solution was adjusted to pH 7 and

subjected to chloramin T oxidation. Absorbance of the colored adduct-product formed with Ehrlich reagent at 60 °C was read at 557 nm using a Beckmann Dual Spectrometer (Beckman, Fullerton, CA, USA). Standard hydroxyproline was also run and values reported as µg/mg dry weight of tissue [26, 27].

2.2.4. Anti-inflammatory Activity

The effects of the test samples on the increased vascular permeability induced by acetic acid in mice was determined according to the Whittle method [28]. Test samples were orally administered with 0.2 mL/20 g body weight doses. 0.1 mL of 4% Evans blue in saline solution was injected to the tails of each animal thirty minutes later. After 10 min 0.4 mL of 0.5% (v/v) acetic acid was injected intraperitoneally. The mice were sacrificed 20 min later by dislocation of the neck, and the viscera was washed with distilled water, then poured into 10 mL volumetric flasks from glass wool. Each flask was made up to 10 mL with distilled water, 0.1 mL of 0.1 N NaOH solution was added, and the absorption of the final solution was measured at 590 nm using a Beckmann Dual Spectrometer (Beckman, Fullerton, CA, USA). The control group was given a mixture of oral water and 0.5% CMC.

2.3. Statistical Analysis of the Data

One-way analysis of variance (ANOVA) and Students-Newman-Keuls post hoc tests were used to analyze the data. Values of $p < 0.05$ were considered statistically significant.

3. Results and Discussion

Wound-healing and anti-inflammatory activity results of n-hexane (Hex), ethyl acetate (EtOAc), and methanol (MeOH) extracts prepared from the aerial parts of *E. characias* subsp. *wulfenii*, *E. helioscopia*, *E. macroclada*, *E. seguieriana* subsp. *seguieriana*, and *E. virgata*, are shown in the tables.

Table 2. Effects of the test materials on the linear incision wound model and the hydroxyproline content of each extract

Material	Extract	Tensile strength of wound ± SEM (tensile strength %)	Hydroxyproline (µg/mg) ± SEM
Vehicle		13.34±1.79 (2.30)	12.47±2.15
Negative control		13.04±1.87 (-)	9.66±2.51
E. virgata	Hex	14.07±1.38 (5.47)	13.01±2.70
	EtOAc	14.61±1.42 (9.52)	12.36±2.02
	MeOH	16.42±1.56 (23.09)	17.85±2.17
E. macroclada	Hex	13.08±1.41 (-)	14.78±2.12
	EtOAc	13.87±1.29 (3.97)	11.06±2.33
	MeOH	17.35±1.17 **(30.06**)**	**28.37±1.59****
E. seguieriana subsp. *seguieriana*	Hex	14.02±1.46 (5.09)	10.28±2.19
	EtOAc	14.58±1.15 (9.30)	10.92±2.26
	MeOH	16.50±1.39 (23.69)	20.74±1.99
E. characias subsp. *wulfenii*	Hex	14.17±1.52 (6.22)	9.67±2.13
	EtOAc	16.11±1.79 (20.76)	20.62±2.01
	MeOH	19.08±1.47 **(43.03**)**	**35.47±1.38*****
E. helioscopia	Hex	14.27±1.58 (6.97)	11.73±2.07
	EtOAc	15.12±1.70 (13.34)	15.08±2.11
	MeOH	18.35±1.84 **(37.56**)**	**30.64±1.44****
Madecassol®		21.41±1.17 **(60.49***)**	**47.61±1.13*****

SEM: Standard Error of the Mean; Hex- hexane extracts; EtOAc- ethyl acetate extracts; MeOH- methanol extracts;
* : $p < 0.05$;
** : $p < 0.01$;
*** : $p < 0.001$

The methanol extract of *E. characias* subsp. *wulfenii* showed more wound-healing activity than the other extracts, with a 43.03% tensile strength value for the linear incision wound model (Table 2) and a 65.24% reduction of the wound area at day 10 for the circular excision wound model (Table 3). The tissue treated with the methanol extract of *E. characias* subsp. *wulfenii* was found to contain 35.47 µg/mg of hydroxyproline (Table 2).

Table 3. Effects of the test materials on the circular excision wound model

Material	Extract	Wound area (mm^2) ± SEM (Contraction %)					
	Day	0	2	4	6	8	10
Vehicle		19.89±2.31	17.32±2.37 -	15.29±2.42 -	12.71±1.96 (1.93)	6.49±1.49 (8.46)	3.05±1.25 (10.03)
Negative Control		19.21±2.19	17.15±2.24	15.21±2.30	12.96±1.77	7.09±1.57	3.39±1.82
E. virgata	Hex	19.33±2.02	17.48±1.84 -	5.02±1.47 (1.77)	13.02±1.38 -	6.19±1.03 (4.62)	2.95±0.41 (3.28)
	EtOAc	19.53±2.11	16.91±1.96 (2.37)	14.78±1.29 (3.34)	12.82±1.26 -	5.82±1.07 (10.32)	2.98±0.66 (2.29)
	MeOH	19.38±2.25	15.88±1.82 (8.31)	14.51±1.25 (5.10)	11.91±1.17 (6.29)	5.74±0.98 (11.56)	2.23±0.71 (26.89)
E. macroclada	Hex	19.62±2.28	16.34±1.61 (5.66)	14.92±2.10 (2.42)	12.85±1.40 -	6.04±1.51 (6.93)	2.97±0.98 (2.62)
	EtOAc	19.45±2.17	15.01±1.45 (13.34)	13.95±1.98 (8.76)	11.72±1.36 (7.79)	5.72±0.60 (11.86)	2.47±0.49 (19.02)
	MeOH	19.47±2.26	14.37±1.58 (17.03)	12.99±1.79 (15.04)	10.87±1.44 (14.48)	4.44±0.35 **(31.59)***	1.71±0.77 **(43.93)****
E. seguieriana subsp. *seguieriana*	Hex	19.49±2.14	17.42±1.79 -	15.04±1.59 (1.64)	11.24±1.42 (11.57)	5.84±1.12 (10.01)	2.86±0.81 (6.23)
	EtOAc	20.02±2.03	16.49±1.81 (4.79)	16.42±1.10 -	11.32±1.45 (10.94)	5.76±1.13 (11.25)	2.59±0.89 (15.08)
	MeOH	19.99±2.22	16.32±1.85 (5.77)	15.44±1.47 -	11.43±1.55 (10.07)	4.89±1.08 (24.65)	2.46±0.92 (19.34)
E. characias subsp. *wulfenii*	Hex	19.32±2.01	16.85±1.43 (2.71)	14.18±1.12 (7.26)	10.17±1.39 (19.98)	5.74±1.22 (11.56)	2.72±0.49 (10.82)
	EtOAc	19.27±2.10	16.39±1.59 (5.37)	13.41±1.19 (12.29)	9.99±1.43 (21.40)	4.52±0.89 (30.35)	1.95±0.58 **(36.07)***
	MeOH	21.03±2.11	15.11±1.34 (12.76)	12.88±1.23 (15.76)	9.90±1.57 (22.11)	4.36±0.92 **(32.82)***	1.06±0.39 **(65.24)****
E. helioscopia	Hex	19.57±2.04	17.25±1.28 (0.40)	14.25±1.31 (6.80)	11.52±1.37 (9.36)	5.87±0.97 (9.55)	3.22±0.71 -
	EtOAc	19.31±2.07	16.81±1.19 (2.94)	14.06±1.43 (17.20)	10.84±1.30 (16.28)	4.92±0.81 (24.19)	2.35±0.65 (22.95)
	MeOH	20.10±2.31	15.71±1.23 (9.29)	14.01±1.25 (8.37)	10.79±1.53 (15.11)	4.02±0.74 **(38.06)***	1.72±0.32 **(43.61)****
Madecassol®		19.81±2.05	14.28±1.30 (17.55)	12.20±1.37 (20.21)	6.82±1.24 **(46.34)****	1.85±0.62 **(71.49)****	0.00±0.00 **(100.00)*****

SEM: Standard Error of the Mean; Hex- hexane extracts; EtOAc- ethyl acetate extracts; MeOH- methanol extracts
* : $p < 0.05$
** : $p < 0.01$
*** : $p < 0.001$

Anti-inflammatory activity test results showed that, the methanol extracts of *E. characias* subsp. *wulfenii*, and *E. macroclada* inhibited inflammation by 34.74% and 38.81%, respectively (Table 4). The

wound-healing and anti-inflammatory activity tests were consistent. The methanol extract of *E. characias* subsp. *wulfenii* demostrated remarkable bioactivity in both the wound-healing and acute inflammation models.

Table 4. Inhibitory effect of the test materials on the acetic acid-induced increase in capillary permeability

Material	Extract	Dose (mg/kg)	Evans Blue Concentration (µg/mL) ± SEM	Inhibition (%)
Control			12.29±1.25	
E. virgata	Hex	100	12.41±1.02	-
	EtOAc	100	11.85±1.27	3.58
	MeOH	100	10.32±1.21	16.03
E. macroclada	Hex	100	12.75±1.33	-
	EtOAc	100	11.47±1.39	6.67
	MeOH	100	7.52±0.93	**38.81*****
E. seguieriana subsp. *seguieriana*	Hex	100	13.04±1.76	-
	EtOAc	100	11.31±1.49	7.97
	MeOH	100	10.75±1.05	12.53
E. characias subsp. *wulfenii*	Hex	100	11.95±1.34	2.77
	EtOAc	100	9.24±0.85	24.82
	MeOH	100	8.02±0.79	**34.74****
E. helioscopia	Hex	100	10.99±0.91	10.58
	EtOAc	100	9.91±0.74	19.37
	MeOH	100	9.45±1.02	23.11
Indomethacin		10.0	6.81±0.37	**44.59*****

SEM: Standard Error of the Mean; Hex- hexane extracts; EtOAc- ethyl acetate extracts; MeOH- methanol extracts
* : $p < 0.05$
** : $p < 0.01$
*** : $p < 0.001$

Collagen is the major protein of the extracellular matrix. It makes the hydroxyproline and its peptides free up [29]. The rate of collagen synthesis and the maturation process where covalent binding of collagen fibrils are deterministic for a tensile strength of a wound [30]. In the present study, the tensile strength of the new tissue is better than the treated groups and the signs of infection were shown at least on level. The tensile strength is the resistance to breakage shown against stretching. This shows how much the repaired tissue is resistanted against breaking. For this purpose, the newly repaired tissue was removed and the tensile strength was measured [22]. Large amounts of hdyroxyproline in tissues indicate the presence of collagen and accelerated healing [31]. The amount of hyroxyproline indicated by the wound tensile strength parallels the results of incision wound model in this study.

The first phase of healing is inflammation. However, an elongated inflammatory response delays healing. For this purpose, anti-inflammatory activity of the extracts was evaluated by using the Whittle method. This model of inflammation determines the effectiveness of a test material against the increased capillary permeability induced by acetic acid [32]. In the present study, the methanol extracts of *E. characias* subsp. *wulfenii*, and *E. macroclada* significantly inhibited this inflammation more than other groups.

Anti-inflammatory activity has been reported for *Euphorbia australis* Boiss., *E. drummondii* Boiss., *E. heyneana* Spreng, *E. hirta*, *E. kansui* T.N. Liou ex. T.P. Wang, and *E. royleana* Boiss [33-37]. *E. fusiformis*, *E. helioscopia*, and *E. segetalis* L. have shown antimicrobial activity [8,38,39]. Antioxidant activity has been attributed *to E. helioscopia*, *E. hirta*, *E. macroclada*, and *E. rigida* M. Bieb. [40-42], and wound-healing activity to *E. caducifolia* Haines., *E. hirta*, and *E. neriifolia* L. [43,44]. The results obtained are similar to the wound-healing potential of the latex of *E. caducifolia* which accelerated closure of the wound with greater contents of fibroblasts and collagen in the treated animals [45]. The same type of wound-healing effect has also been observed for *E. neriifolia* [46]. Ahmed et al. [47] have suggested that the topical administration of ethyl acetate and methanol extracts of *E.*

consobrina N.E.Br., ethyl acetate extract of *E. inarticulata* Schweinf., and methanol extracts of *E. balsamifera* Aiton, and *E. schimperi* C. Presl. have significant therapeutic effects on the various phases involved in the process of wound contraction and healing. Badgujar et al. [48] have reported that the latex of *E. nivulia* Buch.-Ham. which included alkaloids, cynogenic glycosides, phenolics, saponins, and tannins significantly reduced the bleeding and whole-blood clotting times. Results of the ethnobotanical studies show that *Euphorbia* species have been used such as analgesic, anti-inflammatory, antifungal, antiviral, cytotoxic, diuretic, laxative, and wound healer agents in traditional medicine. However, there is not enough satisfactory studies which provide scientific evidence to prove the effectiveness of these uses.

The aerial parts and latex of *Euphorbia* species are reputed to have various biological effects that make them useful in traditional medicines. Giordiani et al. [7] have reported that plants of the *Euphorbia* genus showed significant antimicrobial activity against Gram-positive and Gram-negative bacteria. Such antimicrobial activity is particularly important because of it prevents infection of the wounded area during the healing process. Free radicals are known to induce cell damage by lipid peroxidation. Antioxidant activity therefore also contributes to the healing process. The antioxidant activities of the *Euphorbia* species, *E. heyneana*, *E. hirta*, *E. macroclada*, and *E. rigida* have been investigated [34, 40, 49]. Phytochemicals such as alkaloids, triterpenoids, tannins, phenolic compounds and flavonoids are also known to support healing process, especially because of antimicrobial and astringent properties. Flavonoids and their derivatives have been known to prevent or slow down the progression of cell necrosis by increasing vascular formation of the tissues. Flavonoids are also thought to be effective compounds responsible for wound contraction and epithelialisation.[50-54]. The genus *Euphorbia* has been reported to be a rich source of sesquiterpenoids, glycerols, cerebrosides, phloracetophenones, steroids, phenolic compounds and flavonoids [14]. In our study, significant wound-healing and anti-inflammatory effects of the extracts are attributed to flavonoids and quercetin glycosides. Flavonoid and flavonoid derivative contents of our selected species are consistent with studies in literature. Previously quercetin-3-*O*-glucoside, kaempferol, kaempferol-3-*O*-glucoside, kaempferol-3-rutinoside and rutin were isolated from *E. virgata* [13]. Ertaş et al. [55] have reported that *E. macroclada* contained rutin, hyperoside, quercetin, apigenin, kaempferol, myricetin, naringenin and hesperetin. In biological activity study report published by Pisano et al. [56], researchers have been showed phytochemical profile of *E. characias* by using LC-MS/MS. Results of the study have been showed that *E. characias* contain flavonoids namely quercetin-3-*O*-glucoside, quercetin-3-*O*-rhamnoside, quercetin-3-*O*-arabinoside and quercetin-3-*O*-xyloside, which have antioxidant and antimicrobial activities.

In the present study, results have shown that the methanol extract proposed from the aerial parts of *E. characias* subsp. *wulfenii* possesses the best wound-healing activity of any of the five species of *Euphorbia* we tested using three different solvents for extraction and it also outperformed the control groups. This is attributed to the combined effect of the constituents present in extracts, especially the flavonoids (see supporting information). The present study provides evidence to support the traditional use of the aerial parts of *E. characias* subsp. *wulfenii* for wound healing.

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Supporting Information

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