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Daphne oleoides Schreber ssp. *oleoides* Exhibits Potent Wound Healing Effect: Isolation of the Active Components and Elucidation of the Activity Mechanism

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Abstract: Ethnobotanical surveys revealed that *Daphne oleoides* Schreber has been used against rheumatic pain and for wound healing in Turkish folk medicine. The aim of the present study is to verify the folkloric assertion of *D. oleoides* ssp. *oleoides* (DOO) by bioassay-guided fractionation procedures leading to determination of the active component(s) and to elucidate the activity mechanisms of the isolated compounds. The wound healing activity of the methanol extract, its subextracts, fractions and isolates was evaluated by using two different *in vivo* wound healing experimental techniques. Anti-inflammatory and antioxidant activities of the test materials were also evaluated. For the determination of the activity mechanisms, the isolated compounds were screened for hyaluronidase, collagenase and elastase enzyme inhibitory activities. The methanolic extract of DOO was found to possess potent wound healing activity. This extract was then subjected to successive solvent extractions with *n*-hexane, dichloromethane, ethyl acetate (EtOAc) and *n*-butanol. EtOAc subextract yielded three compounds, quercetin 3-*O*-glucoside, triumbellin and rutarensin by using chromatographic separation techniques. The experimental study revealed that *D. oleoides* subsp. *oleoides* methanolic extract possesses significant wound healing effect and quercetin 3-*O*-glucoside was determined as the active component responsible from the activity.

Keywords: Anti-inflammatory; Daphne; Excision; Incision; Thymelaeaceae; Wound healing.

1. Introduction

The *Daphne* L. genus belongs to the family Thymelaeaceae and there are seven species distributed in Turkey [1]. However, among them only *D. oleoides*, *D. pontica* and *D. mezereum* have been used in Turkish folk medicine [2]. The stem bark of *D. pontica* has been used against diarrhea

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[3], while *D. mezereum* is used as a purgative in Anatolia [4]. The mashed roots of *D. oleoides* have been used to treat malaria and the poultice prepared from the aerial parts and roots was used against rheumatism, lumbago, to reduce fever and for wound healing [2]. There are two subspecies of *D. oleoides* in Turkey, e.g., ssp. *kurdica* and ssp. *oleoides*, and both are grown in the same district. However, there is no discrimination in the folkloric reports, whether former or later subspecies was more active.

In a previous report, to evaluate the effect of aerial parts of *D. oleoides* ssp. *oleoides* on rheumatism and other inflammatory disorders, *in vitro* effects of the EtOAc extract and its fractions on interleukin-1 (IL-1α, IL-1β) and tumor necrosis factor-alpha (TNF-α) biosynthesis were investigated. Among the seventeen compounds isolated from the EtOAc extract through activity-guided procedures, two diterpenoids, genkwadaphnin and 1,2- dehydrodaphnetoxin and a coumarin derivative, daphnetin were isolated as potent inhibitors of these cytokines, while rest demonstrated moderate to mild effects [5]. Later, a study was conducted aiming at investigation of wound healing potential of both *D. oleoides* subspecies comparatively. Recently, the wound healing potential of the aerials parts of *D. oleoides* ssp. *kurdica* has been reported [6]. From the active EtOAc extract of this plant, daphnetin was isolated as one of the potential active ingredients supporting the previous findings [5]. However, another constituent, luteolin 7-*O*-glucoside in the active fraction exerted more potent anti-inflammatory and wound healing activity.

The aim of the present study was to evaluate the wound healing activity of the methanolic extract from the aerial parts of *D. oleoides* ssp. *oleoides* and to isolate and define the active constituent(s) through bioassay-guided fractionation procedures and to find out the activity mechanisms.

2. Materials and Methods

2.1. Plant material

Flowering aerial parts of *Daphne oleoides* Schreber ssp. *oleoides* (DOO) were collected from Meydan Plateau (Bolkar Mountains), Adana, Turkey in 2009. The plant was authenticated by Prof. Dr. Hayri Duman from Gazi University, Department of Biology, Faculty of Science and Art, Ankara, and a voucher specimen (GUE 2976) was deposited in the Herbarium of Faculty of Pharmacy, Gazi University, Ankara, Turkey.

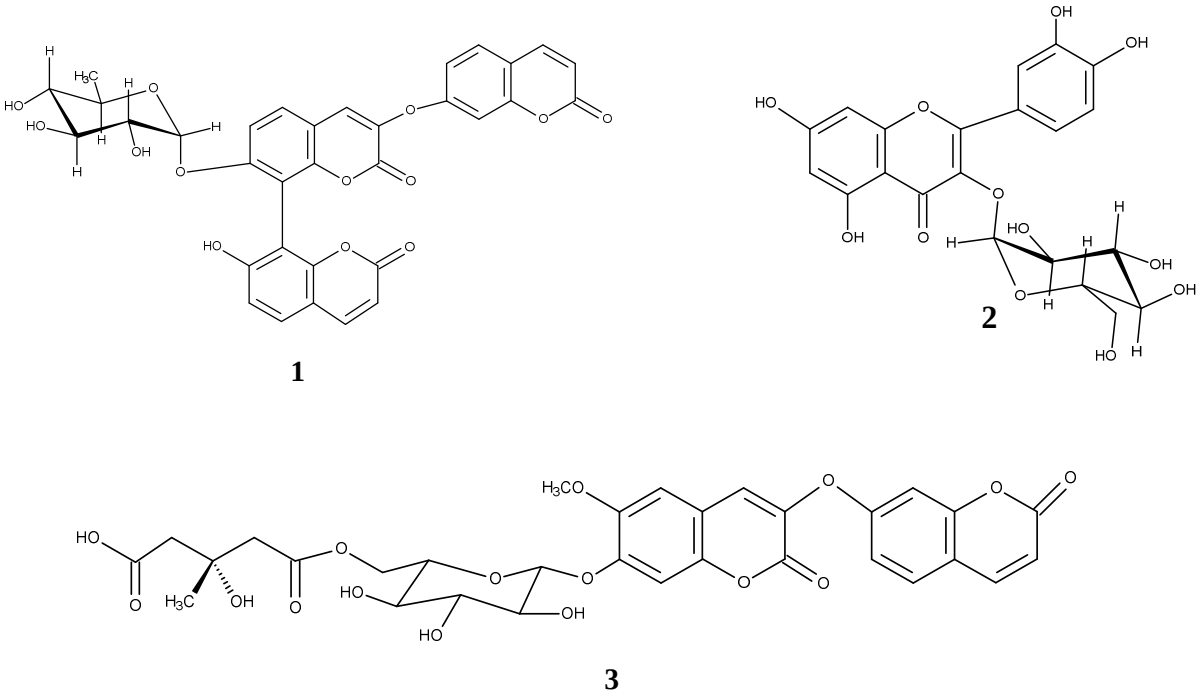
2.2. Extraction, fractionation and isolation procedures for the bioassays

The plant material was extracted and fractionated as previously described [6]. Consequently "DOO-MeOH", "DOO-*n*-Hexane", "DOO-CH_2Cl_2", "DOO-EtOAc", "DOO-*n*-BuOH" and "DOO-R-H_2O" were obtained in 18.53, 4.20, 12.08, 6.48, 22.24 and 23.80% yields, respectively.

2.2.1. Chromatographic separation and isolation of the active constituents

2 g of DOO-EtOAc was applied to silica gel column using $CHCl_3$; $CHCl_3$:MeOH (95:5) and $CHCl_3$:MeOH (90:10), successively, as eluent systems. The eluents were combined into four fractions according to TLC behavior as follows: Fr. (1-6) (77.7 mg), Fr. (7-18) (178.2 mg), Fr. (19-27) (1181.9 mg) and Fr. (28-33) (476.4 mg).

After biological activity assays, fraction Fr. (19-27) was further subjected to high pressure liquid chromatographic separation (Dionex Ultimate 3000 HPLC) to yield compounds **1** (23.8 mg), **2** (177.2 mg) and **3** (28.3 mg) (Figure 1). The following chromatography conditions and systems were used; PN: 5722.0025 used with Phenomenex column, Luna C_{18} (5µ, 150x10 mm). As mobile phase, solvent A: HPLC grade water (H_2O); solvent B: methanol (MeOH); mode: gradient, increasing the organic phase (MeOH) from 30 to 100% over 30 min; flow rate: 2 mL/min; injection volume: 200 µL. Detection wavelength of 220 nm on Channel A, 254 nm on Channel B, 280 nm on Channel C and 331 nm on Channel D.



1 **2** **3**

Figure 1. Chemical structures of Triumbellin (**1**), Quercetin-3-*O*-glucoside (**2**) and Rutarensin (**3**)

2.2.2. Determination of the chemical structures of the isolated compounds

For the identification of the bioactive compounds Nuclear Magnetic Resonance (^{1}H and ^{13}C NMR) and Mass Spectral (MS) techniques were employed. NMR spectra were recorded on a Bruker spectrometer (400 MHz for ^{1}H NMR and 100 MHz for ^{13}C NMR) instrument, and using MeOD as solvent. A Finnigan spectrometer was used for FT-MS analyses. The isolated compounds were elucidated as triumbellin (**1**), quercetin *3-O-glucoside* (**2**) and rutarensin (**3**), respectively, by comparing their spectroscopic data with those of published literatures [7,8].

2.3. Pharmacological procedures

2.3.1. In vivo biological activity tests
2.3.1.1. Animals

Male Sprague–Dawley rats weighing 160–180 g and Swiss albino mice weighing 20–25 g were obtained from a local animal breeding laboratory (Saki Yenilli, Ankara, Turkey). All animals were maintained in accordance with the directions of Guide for the Care and Use of Laboratory Animals, and the experiment was approved by the Experimental Animal Ethics Committee of Gazi University (G.U.ET-10.027). Prior to the experiments the animals were left 3 days for acclimatization at 21-24°C, 40-45% humidity, and light-controlled (12 hours light/12 hours dark) conditions and were given *ad libitum* access to food and water. A minimum of six animals were used in each experimental group.

2.3.1.2. Preparation of test samples for bioassay

The test samples were prepared either in an ointment base (for the assessment of wound healing activity), or as a suspension (for the evaluation of anti-inflammatory activity) as described previously [6].

2.3.1.3. Wound healing activity

2.3.1.3.1. Linear incision wound model

Linear incision wound model was applied as described in Süntar et al. [6]. In this *in vivo* experimental model, two linear full thickness incisions were created on the dorsal part of the rat. Tensile strength of the treated skin was measured with a tensiometer (Zwick/Roell Z0.5, Germany) [9,10].

2.3.1.3.2. Circular excision wound model

The circular wound was created on the dorsal interscapular region of each animal by excising the skin. After the treatment, wound contraction was calculated as percentage of the reduction in wounded area. A specimen sample of tissue was removed for the histopathological analyses [6, 11, 12].

2.3.1.3.3. Histopathology

At the end of the experiment the skin specimens were removed, stained and histopathologically examined as explained previously [6].

2.3.1.3.4. Hydroxyproline estimation

Tissues were dried in hot air oven, hydrolyzed and subjected to chloramin T oxidation. The colored adduct formed with Ehrlich reagent was read at 557 nm [6, 13].

2.3.1.4. Anti-inflammatory activity: acetic acid-induced increase in capillary permeability

Effect of the test samples on the increased vascular permeability induced by acetic acid in mice was determined according to Whittle method [14] with some modifications [15, 6].

2.3.2. In vitro biological activity tests

2.3.2.1. Determination of antioxidant activity and total phenolics

The antioxidant activity of the extracts was determined according to the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay [16]. Total phenolic contents of the methanolic extract and its subextracts were performed employing the methods involving Folin-Ciocalteu reagent and gallic acid as a standard [17].

2.3.2.2. Determination of hyaluronidase, collagenase and elastase inhibitory activity

The inhibitiory effects of the isolated compounds on hyaluronidase [18, 19], collagenase [20] and elastase enzymes [21] were assessed according to the procedures described in Süntar et al. [6].

2.3.3. Statistical analysis of the data

The data on percentage anti-inflammatory and wound healing was statistically analyzed using one-way analysis of variance (ANOVA). The values of $p \leq 0.05$ were considered statistically significant. Students-Newman-Keuls posthoc was used from the active extract and fractions. Histopathologic data were considered to be nonparametric; therefore, no statistical tests were performed.

3. Results and Discussion

In the present study wound healing activity of the 85% methanolic extract prepared from the aerial parts of DOO was evaluated by using linear incision and circular excision wound models. In linear incision wound model, tensile strength values of the healed tissues were determined. Tensile strength indicates the collagen concentration and stabilization of the fibers [9]. In circular excision wound model, the percentage of wound contraction was calculated which is an indication of re-epithelialization [22]. The tissues, both isolated from incised and excised wounds were then also evaluated histopathologically. Tissues treated with the test ointments were further assessed for their hydroxyproline content as an evidence of increased collagen concentration in the healed tissues. Collagen is an important structural protein of the connective tissue which has a great importance in wound healing [123,24]. Besides all these investigations, antioxidant and anti-inflammatory effects of the extract and subextracts of DOO were determined.

In the wound healing process, inflammation is the first response which starts with hemostasis and subsequently infiltration of the inflammatory cells to the wounded area. Although the inflammatory cells act as a defense mechanism in the wounded area against infections, long duration of the inflammation in the wounded part causes some undesirable effects such as edema and functional loss. Therefore, agents having anti-inflammatory activity may help to accelerate the wound healing process by inhibiting inflammation [25].

Likewise, antioxidant activity is also a necessity in the wound healing process. Particularly, free radicals are known to cause cell damage due to lipid peroxidation [26,27]. Therefore, the total phenolic content of the extract and subextracts of DOO were also evaluated. The total phenolic content of the EtOAc subextract of DOO was found to be the highest compared to the other subextracts tested. In the antioxidant activity assay, IC_{50} values of the MeOH extract, EtOAc subextract, DOO-Fr.19-27 and quercetin-3-*O*-glucoside were found to be quite comparable to that of the reference (quercetin) (Table 1).

Table 1. The DPPH scavenging activity and total phenolic content of the extract and sub-extracts from *D. oleoides* subsp. *oleoides*

Extracts & Sub-extracts	**DPPH assay IC_{50} (µg/mL)**	**Total phenolics (mg GA/g ± S.E.M.)**
DOO-MeOH	230.62	119.57 ± 0.98
DOO-*n*-Hexane	183.41	4.57 ± 1.22
DOO-CH_2Cl_2	131.30	88.86 ± 0.92
DOO-EtOAc	89.53	169.57 ± 1.03
DOO-*n*-BuOH	371.77	114.57 ± 1.19
DOO-R-H_2O	924.00	123.86 ± 1.42
DOO-Fr. (1-6)	57.65	
DOO-Fr. (7-18)	68.54	
DOO-Fr. (19-27)	43.92	
DOO-Fr. (28-33)	51.17	-
Triumbellin	26.33	
Quercetin-3-*O*-glucoside	20.15	
Reference (Quercetin)	2.14	

S.E.M.:Standard error of the mean

The results of wound healing activity studies have shown that 1% ointment formulation prepared from 85% methanolic extract of DOO exerts wound healing activity in the wound models, as well as anti-inflammatory and antioxidant activities. Therefore, DOO-MeOH was subjected to successive solvent extraction. The subextracts were then applied to the bioassay tests by using the same experimental models and EtOAc subextract was found to be the most active one with the values of 30.92% in linear incision (Table 2); and 57.36% in circular excision wound models (Table 3). For the evaluation of the dose-dependent effect of the EtOAc subextract, 3% and 5% formulations were also tested along with 1% ointment formulation. However, the activity did not change dose-dependently, therefore, the further studies were preceded with 1% dose of this fraction.

Table 2. Effects of the extract and sub-extracts from *D. oleoides* subsp. *oleoides* on linear incision wound model

Material	Dose (%)	Tensile strength ± S.E.M.	(%Tensile strength)
Vehicle		13.10 ± 1.98	9.72
Negative Control		11.94 ± 1.59	-
DOO-MeOH	1	17.58 ± 2.31	**34.19***
DOO-*n*-Hexane	1	14.92 ± 2.13	13.89
DOO-CH_2Cl_2	1	15.39± 1.53	17.48
DOO-EtOAc	1	17.15 ± 1.84	**30.92***
	3	17.72 ± 1.56	**35.27***
	5	17.48 ± 1.49	**33.44***
DOO-*n*-BuOH	1	16.69 ± 1.75	**27.40***
DOO-R-H_2O	1	15.20 ± 2.18	16.03
Madecassol®	1	20.80 ± 1.61	**58.78*****

* : $p < 0.05$; ** : $p < 0.01$; *** : $p < 0.001$; S.E.M.: Standard error of the mean

The most active fraction (DOO-EtOAc) was then applied to silica gel column. As shown in Table 4, among the fractions obtained from the silica gel column chromatography, DOO-Fr.19-27 showed the best wound healing effect with the values of 29.48% in linear incision (Table 4) and 43.31% in circular excision wound models (Table 5). By using reverse phase-HPLC method, triumbellin (**1**), quercetin 3-*O*-glucoside (**2**) and rutarensin (**3**) were isolated from the active fraction DOO-Fr.19-27. Since, triumbellin and quercetin-3-*O*-glucoside were commercially available; the purchased pure compounds were tested in biological activity assays. Quercetin 3-*O*-glucoside (**2**) exerted significant wound healing activity with the values of 32.19% in linear incision (Table 6); and 65.29% in circular excision wound models (Table 7).

Table 3. Effects of the extract and sub-extracts from *D. oleoides* subsp. *oleoides* on circular excision wound model

Material	Dose (%)	Wound area (mm^2) ± S.E.M. (Contraction%)						
		Day 0	**Day 2**	**Day 4**	**Day 6**	**Day 8**	**Day 10**	**Day 12**
Vehicle		20.02±2.51	17.59±2.50 (3.51)	14.93±2.09 (2.03)	12.98±1.74 (9.67)	8.33±1.61 (6.09)	5.83±1.14 (7.31)	3.26±0.29 (8.43)
Negative Control		19.98±2.11	18.23±2.15	15.24±2.04	14.37±1.51	8.87±1.10	6.29±1.84	3.56±0.67
DOO-MeOH	1	19.84±2.28	16.08±2.74 (8.58)	13.40±1.84 (10.25)	10.43±0.93 (19.65)	6.51±1.54 (21.85)	2.62±0.50 **(55.06)****	0.95±0.44 **(70.86)*****
DOO-*n*-Hexane	1	19.51±1.96	16.65±1.94 (5.34)	14.05±2.17 (5.89)	12.54±1.22 (3.39)	8.35±1.50 -	5.35±1.04 (8.23)	2.76±0.66 (15.34)
DOO-CH_2Cl_2	1	19.16±1.76	16.15±1.81 (8.19)	13.33±1.95 (10.72)	12.68±1.78 (2.31)	7.97±1.39 (4.32)	5.29±0.96 (9.26)	2.83±0.52 (13.19)
DOO-EtOAc	1	19.85 ± 1.85	16.09±1.69 (8.53)	13.22±1.48 (11.45)	10.57±1.67 (18.57)	6.84±1.40 (17.89)	3.58±0.82 **(38.59)***	1.39±0.09 **(57.36)****
	3	19.29 ± 1.72	16.63±1.84 (5.46)	13.79±1.76 (7.64)	10.95±1.39 (15.64)	5.88±0.73 **(29.41)***	3.42±0.51 **(41.34)****	1.64 ±0.11 **(49.69)***
	5	19.44±1.93	15.51±1.53 (11.82)	12.02±2.13 (19.49)	10.53±1.18 (18.88)	6.14±0.95 (26.29)	3.76±0.73 **(35.51)***	1.57±0.03 **(51.84)****
DOO-*n*-BuOH	1	20.10±2.13	14.93±1.99 (15.12)	12.29±1.72 (17.68)	10.58±1.37 (18.49)	6.59±1.17 (20.89)	3.55±0.19 **(39.11)****	1.70±0.25 **(47.85)****
DOO-R-H_2O	1	19.98±1.41	15.87±2.04 (9.78)	13.65±1.29 (8.57)	11.52±1.55 (11.25)	7.47±1.43 (10.32)	5.04±0.62 (13.55)	2.88±0.83 (11.66)
Madecassol®	1	20.23±2.25	14.18±1.86 (19.39)	11.88±1.46 (20.43)	7.59±1.03 **(41.53)***	2.04±0.26 **(75.51)****	1.09±0.11 **(81.30)*****	0.00±0.00 **(100.00)*****

* : $p < 0.05$; ** : $p < 0.01$; *** : $p < 0.001$; S.E.M.: Standard error of the mean

Table 4. Effects of the fractions from DOO-EtOAc on linear incision wound model

Material	Tensile strength ± S.E.M.	(%Tensile strength)
Vehicle	12.28 ± 2.19	2.59
Negative Control	11.97 ± 2.30	-
DOO-Fr. (1-6)	12.74 ± 2.17	3.75
DOO-Fr. (7-18)	14.14 ± 1.79	15.15
DOO-Fr. (19-27)	15.90 ± 1.58	**29.48****
DOO-Fr. (28-33)	13.71 ± 1.83	11.64
Madecassol®	17.66 ± 1.42	**43.81*****

* : p < 0.05; ** : p < 0.01; *** : p < 0.001; S.E.M.: Standard error of the mean

Table 5. Effects of the fractions from DOO-EtOAc on circular excision wound model

Material	Wound area (mm^2) ± S.E.M. (Contraction%)						
	Day 0	Day 2	Day 4	Day 6	Day 8	Day 10	Day 12
Vehicle	19.14±2.30	18.15±1.80 (0.77)	16.08±1.70 (8.43)	13.44±1.72 (2.82)	8.42±1.39 (3.66)	4.36±0.20 (13.15)	3.14±0.51 (8.99)
Negative Control	19.39±2.12	18.29±1.69	17.56±1.48	13.83±1.31	8.74±1.18	5.02±0.56	3.45±0.81
DOO-Fr. (1-6)	19.42±2.18	17.13±1.26 (5.62)	15.17±1.31 (5.66)	12.38±1.43 (7.89)	7.36±1.28 (12.59)	3.92±0.62 (10.09)	2.96±0.56 (5.73)
DOO-Fr. (7-18)	19.57±2.22	16.85±1.31 (7.16)	15.10±1.05 (6.09)	11.89±1.23 (11.53)	6.94±0.26 (17.58)	4.02±0.41 (7.79)	2.83±0.34 (9.87)
DOO-Fr. (19-27)	19.09±2.07	18.02±1.34 (0.72)	14.26±1.14 (11.32)	11.45±1.90 (14.81)	5.67±1.15 **(32.66)***	2.63±0.48 **(39.68)***	1.78±0.22 **(43.31)****
DOO-Fr. (28-33)	19.51±1.89	17.21±1.18 (5.18)	15.34±1.06 (4.60)	12.31±1.08 (8.41)	6.86±0.67 (18.53)	3.96±0.29 (9.17)	2.82±0.70 (10.19)
Madecassol®	19.35±1.96	16.43±1.36 (9.48)	13.24±1.39 (17.66)	9.15±0.58 **(31.92)***	4.48±0.42 **(46.79)****	1.56±0.12 **(64.22)****	0.00±0.00 **(100.00)*****

* : p < 0.05; ** : p < 0.01; *** : p < 0.001; S.E.M.: Standard error of the mean

Table 6. Effects of the compounds from DOO-Fr. (19-27) on linear incision wound model

Material	Tensile strength ± S.E.M.	(%Tensile strength)
Vehicle	10.16 ± 2.40	1.80
Negative Control	9.98 ± 2.25	-
Triumbellin	10.91 ± 1.74	7.38
Quercetin-3-*O*-glucoside	13.43 ± 1.21	**32.19****
Madecassol®	14.79 ± 0.76	**45.57*****

* : p < 0.05; ** : p < 0.01; *** : p < 0.001; S.E.M.: Standard error of the mean

Percentage of the tensile strength values: The vehicle group was compared to the negative control group; The extracts and the reference material were compared to vehicle group

Table 7. Effects of the compounds from DOO-Fr. (19-27) on circular excision wound model

Material	Wound area (mm^2) ± S.E.M. (Contraction%)						
	Day 0	Day 2	Day 4	Day 6	Day 8	Day 10	Day 12
Vehicle	19.38±2.15	17.88±1.70 (2.40)	15.92±1.63 (0.69)	12.85±1.52 (8.61)	7.79±1.21 (2.38)	4.02±0.87 (12.42)	3.28±0.43 (12.53)
Negative Control	19.23±2.09	18.32±1.76	16.03±1.65	14.06±1.04	7.98±1.25	4.59±0.83	3.75±0.61
Triumbellin	19.40±2.11	16.37±1.14 (8.45)	14.65±1.22 (7.98)	11.79±1.13 (8.25)	7.01±1.07 (10.01)	3.24±0.79 (19.40)	2.51±0.34 (23.48)
Quercetin-3-*O*-glucoside	20.06±2.19	16.26±1.47 (9.06)	13.82±1.17 (13.19)	10.72±1.15 (16.58)	5.10±1.03 **(34.53)***	2.18±0.26 **(45.77)***	1.14±0.18 **(65.29)*****
Madecassol®	19.42±1.87	15.31±1.18 (14.37)	13.05±1.26 (18.03)	8.06±0.41 **(37.28)***	3.18±0.52 **(59.18)****	1.25±0.09 **(68.91)****	0.00±0.00 **(100.00)*****

* : p < 0.05; ** : p < 0.01; *** : p < 0.001; S.E.M.: Standard error of the mean

Following the *in vivo* experiments, the tissue sections were removed from each experimental animal was subjected to histopathological examination. Inflammation, proliferation, and remodeling phases of the healing process of the treated tissues were observed and recorded. The histopathological results supported the outcome of the wound healing activity models. The ointments prepared with the methanolic extract of DOO, EtOAc subextract, DOO-Fr.19-27 and quercetin-3-*O*-glucoside provided complete healing by the enhancement of re-epithelialization. On the other hand, wound healing process was shown to be delayed in the vehicle and negative control group animals (Table 8; Figures 2-4).

Table 8. Wound healing processes and healing phases of the experimental groups

Groups	**Wound Healing Processes**							
	S	**U**	**RE**	**FP**	**CD**	**MN**	**PM**	**NV**
Vehicle	++	+	+/++	++/+++	+	++/+++	++/+++	++
Negative Control	+/++	+	+/++	++/+++	+	++	++	++
DOO-MeOH	++	-	+/++	++	++	++	+	+/++
DOO-*n*-Hexane	+/++	-	++	++/+++	+	++/+++	++	++/+++
DOO-CH_2Cl_2	++	-	++	++/+++	++	++	+/++	+/++
DOO-EtOAc	++	-	++	++	++	++	+	+/++
DOO-*n*-BuOH	++/+++	-	++	++/+++	++/+++	++	+/++	++
DOO-R-H_2O	++	-/+	++	++	++	+/++	+	+/++
DOO-Fr. (1-6)	++	-	++	++/+++	++	++	++	++
DOO-Fr. (7-18)	++	-	++	++/+++	++	++	+/++	++
DOO-Fr. (19-27)	+	-	++	++/+++	++/+++	++	+/++	+/++
DOO-Fr. (28-33)	+	-	++	++/+++	++	++	+	+/++
Triumbellin	++/+++	+/++	-/+	++/+++	++	++	++	++
Quercetin-3-*O*-glucoside	++/+++	+	+	++/+++	+++	++/+++	++	++
Madecassol®	+	-	++/+++	++	+++	+/++	-/+	+/++

HE and VG stained sections were scored as mild (+), moderate (++) and severe (+++) for epidermal and/or dermal re-modeling. S: Scab, U: Ulcus, RE: Re-epithelization, FP: Fibroblast proliferation, CD: Collagen depositions, MNC: Mononuclear cells, PMN: Polymorphonuclear cells, NV: Neovascularization

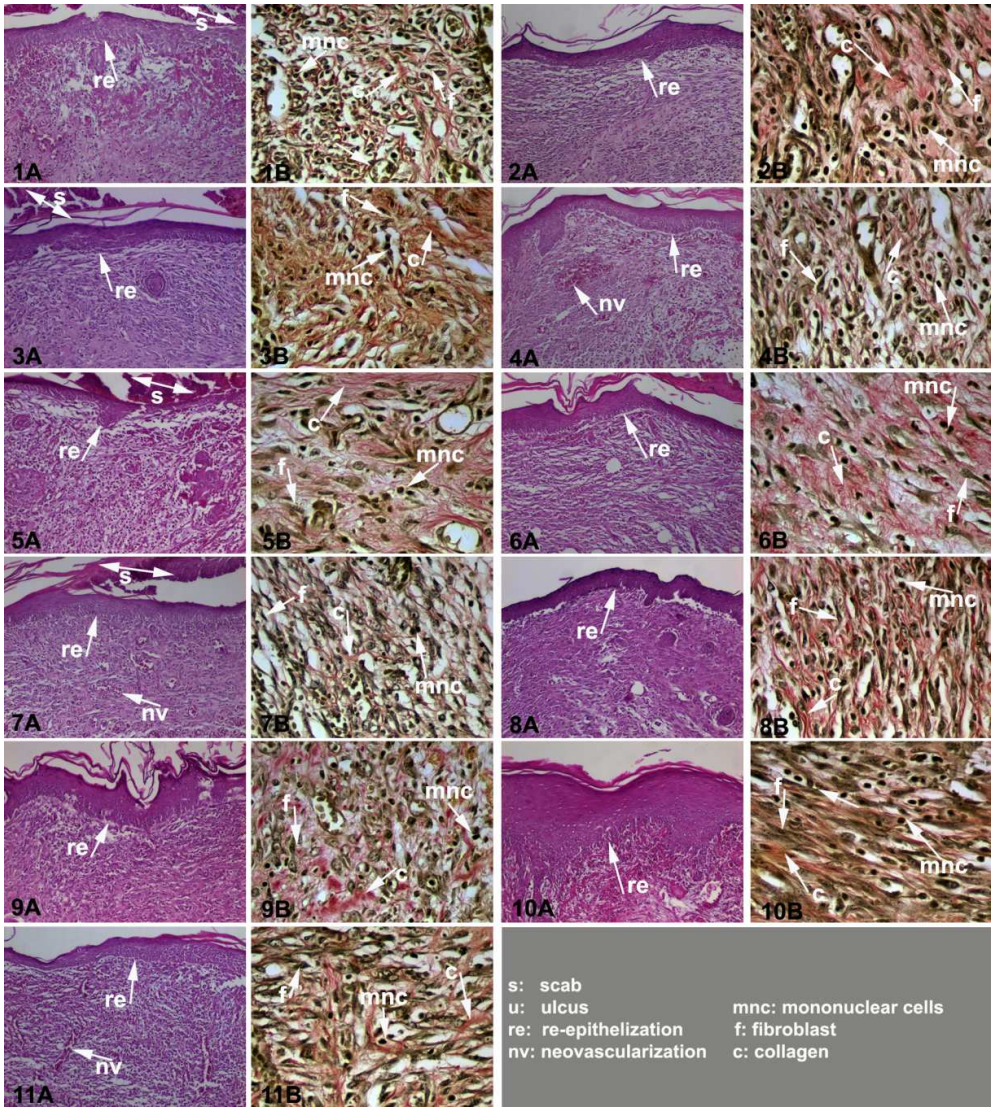


Figure 2. Histopathological view of wound healing and epidermal/dermal re-modeling in the vehicle, negative control, extract, sub-extracts and reference ointment Madecassol® administered animals.

Skin sections show the hematoxylin & eosin (HE) stained epidermis and dermis in A, and the dermis stained with Van Gieson (VG) in B. The original magnification was x 100 and the scale bars represent 120 µm for figures in A, and the original magnification was x 400 and the scale bars represent 40 µm for B. Data are representative of 6 animal per group. 1. Vehichle, 2. Negative Control, 3. DOO-MeOH, 4. DOO-*n*-Hekzan, 5. DOO-CH_2Cl_2, 6. DOO-EtOAc (%1), 7. DOO-EtOAc (%3), 8. DOO-EtOAc (%5), 9. DOO-*n*-BuOH, 10. DOO-R-H_2O, 11. Madecassol

Arrows pointing events during wound healing; s: scab, u: ulcus, re: re-epithelization, f: fibroblast, c: collagen, mnc: mononuclear cells, pmn: polymorphonuclear cells, nv: neovascularization

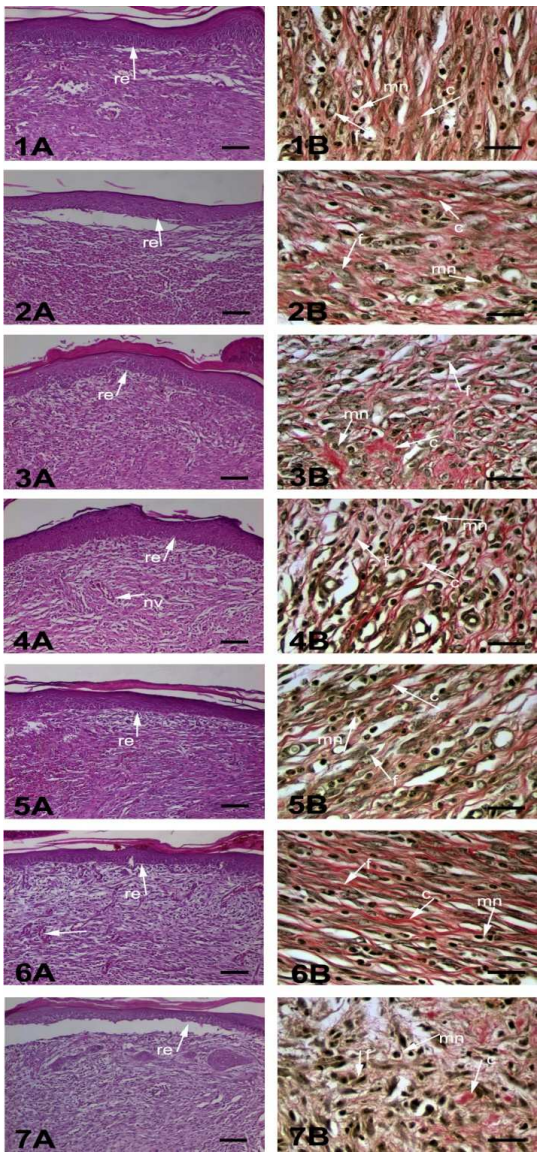


Figure 3. Histopathological view of wound healing and epidermal/dermal re-modeling in the vehicle, negative control, fractions and reference ointment Madecassol® administered animals.

Skin sections show the hematoxylin & eosin (HE) stained epidermis and dermis in A, and the dermis stained with Van Gieson (VG) in B. The original magnification was x 100 and the scale bars represent 120 µm for figures in A, and the original magnification was x 400 and the scale bars represent 40 µm for B. Data are representative of 6 animal per group.1. Vehicle, 2. Negative Control, 3. DOO-Fr. (1-6), 4. DOO-Fr. (7-18), 5. DOO-Fr. (19-27), 6. DOO-Fr. (28-33), 7. Madecassol

Arrows pointing events during wound healing; s: scab, u: ulcus, re: re-epithelization, f: fibroblast, c: collagen, mnc: mononuclear cells, pmn: polymorphonuclear cells, nv: neovascularization

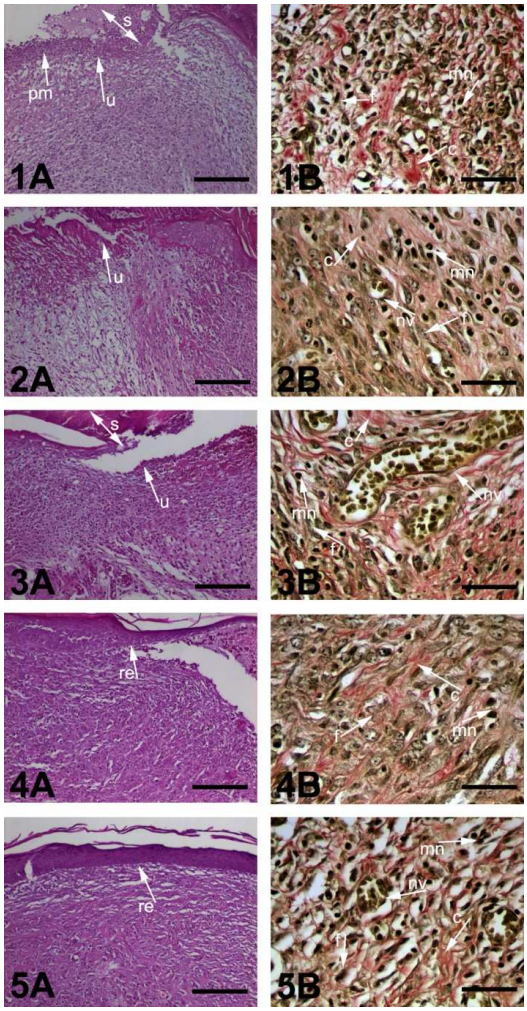


Figure 4. Histopathological view of wound healing and epidermal/dermal re-modeling in the vehicle, negative control, isolated compounds and reference ointment Madecassol® administered animals.

Skin sections show the hematoxylin & eosin (HE) stained epidermis and dermis in A, and the dermis stained with Van Gieson (VG) in B. The original magnification was x 100 and the scale bars represent 120 µm for figures in A, and the original magnification was x 400 and the scale bars represent 40 µm for B. Data are representative of 6 animal per group. 1. Vehicle, 2. Negative Control, 3. Triumbellin, 4. Quercetin-3-*O*-glucoside, 5. Madecassol;
Arrows pointing events during wound healing; s: scab, u: ulcus, re: re-epithelization, f: fibroblast, c: collagen, mnc: mononuclear cells, pmn: polymorphonuclear cells, nv: neovascularization

According to the results of hydroxyproline analysis, a similar activity pattern was obtained. Tissues treated with the methanolic extract of DOO, EtOAc subextract, DOO-Fr.19-27 and quercetin-3-*O*-glucoside ointments were found to possess higher hydroxyproline content, respectively (Tables 9-11), indicating that quercetin 3-*O*-glucoside (**2**) was the active component of the DOO methanolic extract.

In the anti-inflammatory activity screening test, DOO-EtOAc was found to be the most active subextract showing 27.15% inhibitory effect at the dose of 200 mg/kg dose in Whittle method (Table 12). DOO-Fr.19-27 and quercetin-3-*O*-glucoside also exerted high and significant anti-inflammatory activity in the same experiments (Tables 13 and 14). Eventually, the data obtained from the anti-inflammatory and antioxidant assays have also evidenced the results of wound healing activity experiments.

In order to elucidate the activity mechanism of the compounds isolated through bioassay-guided procedures, effects of the isolated active components were investigated for hyaluronidase, collagenase and elastase enzyme inhibitory activities. Due to their degenerative effects on the structural proteins,

these metalloproteinases are expected to be inhibited especially in chronic wounds [28] and therefore agents having inhibitory activity on metalloproteinases may contribute the wound healing process. *In vitro* enzyme inhibitory activity assays revealed that the active wound healing component quercetin-3-*O*-glucoside to possess significant hyaluronidase and collagenase enzyme inhibitory activities with the values of 63.02% and 57.10%, respectively, while did not show a noteworthy inhibitory effect on elastase enzyme when compared to reference compound (Tables 15 and 16).

Table 9. Effects of the test ointments prepared from the extract and sub-extracts of *D. oleoides* subsp. *oleoides* on hydroxyproline content

Material	**Hydroxyproline (µg/mg) ± S.E.M.**
Vehicle	10.43 ± 1.84
Negative Control	9.32 ± 1.37
DOO-MeOH	**26.75 ± 1.24****
DOO-*n*-Hexane	17.96 ± 1.40
DOO-CH_2Cl_2	9.21 ± 1.06
DOO-EtOAc	**29.13 ± 0.85****
DOO-*n*-BuOH	15.69 ± 1.43
DOO-R-H_2O	12.62 ± 1.95
Madecassol®	**45.24 ± 0.48*****

* : p < 0,05; ** : p < 0,01; *** : p < 0,001; S.E.M.: Standard error of the mean

Table 10. Effects of the test ointments prepared from the fractions of DOO-EtOAc on hydroxyproline content

Material	**Hydroxyproline (µg/mg) ± S.E.M.**
Vehicle	8.42 ± 2.25
Negative Control	13.85 ± 1.86
DOO-Fr. (1-6)	15.16 ± 2.01
DOO-Fr. (7-18)	12.76 ±1.96
DOO-Fr. (19-27)	**21.13 ± 1.10****
DOO-Fr. (28-33)	10.69 ± 2.17
Madecassol®	**48.23 ± 0.94*****

* : p < 0,05; ** : p < 0,01; *** : p < 0,001; S.E.M.: Standard error of the mean

Table 11. Effects of the test ointments prepared from the compounds of DOO-Fr. (19-27) on hydroxyproline content

Material	**Hydroxyproline (µg/mg) ± S.E.M.**
Vehicle	11.74 ± 1.93
Negative Control	5.18 ± 1.80
Triumbellin	18.50 ± 2.42
Quercetin-3-*O*-glucoside	**32.73 ± 1.20*****
Madecassol®	**44.63 ± 1.08*****

* : p < 0,05; ** : p < 0,01; *** : p < 0,001; S.E.M.: Standard error of the mean

Daphne genus has importance due to its diverse biological effects such as antimicrobial, antioxidant, antinociceptive, anti-inflammatory, anti-ulcerogenic and haemostatic [29-34] as well as phytochemical content such as terpenoids, steroids, coumarins, lignans and flavonoids [35-37]. The present study demonstrated the wound healing activity potential of *Daphne oleoides* ssp. *oleoides*, and the activity was mainly attributed to the flavonoid type compound quercetin-3-*O*-glucoside.

Table 12. Effects of the extract and sub-extracts from *D. oleoides* subsp. *oleoides* on Whittle Method

Material	Dose (mg/kg)	Evans blue concentration (µg/mL) ± S.E.M.	Inhibition (%)
Control		10.24 ± 0.38	
DOO-MeOH	100	9.06 ± 0.28	11.52
	200	7.83 ± 0.11	**23.53***
DOO-*n*-Hexane	100	10.31± 0.77	-
	200	9.65 ± 0.85	5.76
DOO-CH_2Cl_2	100	10.89 ± 0.69	-
	200	9.18 ± 0.42	10.35
DOO-EtOAc	100	8.46 ± 0.91	17.38
	200	7.46 ± 0.15	**27.15****
DOO-*n*-BuOH	100	8.58 ± 0.71	16.21
	200	7.95 ± 0.25	22.36
DOO-R-H_2O	100	10.94 ± 0.89	-
	200	8.69 ± 0.57	15.14
Indomethacin	10.0	5.65 ± 0.28	**44.82*****

* : p < 0.05; ** : p < 0,01; *** : p < 0,001; S.E.M.: Standard error of the mean

Table 13. Effects of the fractions from DOO-EtOAc on Whittle Method

Material	Dose (mg/kg)	Evans blue concentration (µg/mL) ± S.E.M.	Inhibition (%)
Control		11.77 ± 0.98	
DOO-Fr. (1-6)	200	10.91 ± 0.79	7.31
DOO-Fr. (7-18)	200	9.50 ± 0.84	19.29
DOO-Fr. (19-27)	200	7.85 ± 0.57	**33.31****
DOO-Fr. (28-33)	200	11.95 ± 0.91	-
Indomethacin	10.0	6.17 ± 0.23	**47.58*****

* : p < 0.05; ** : p < 0,01; *** : p < 0,001; S.E.M.: Standard error of the mean

Table 14. Effects of the compounds from DOO-Fr. (19-27) on Whittle Method

Material	Dose (mg/kg)	Evans blue concentration (µg/mL) ± S.E.M.	Inhibition (%)
Control		10.91 ± 0.63	
Triumbellin	200	9.26 ± 0.56	15.12
Quercetin-3-*O*-glucoside	200	7.43 ± 0.47	**31.89****
Indomethacin	10.0	6.42 ± 0.18	**41.15*****

* : p < 0.05; ** : p < 0,01; *** : p < 0,001; S.E.M.: Standard error of the mean

Table 15. Hyaluronidase enzyme inhibitory activity of the isolated compounds from *D. oleoides* subsp. *oleoides*

Material	Concentration (µg/mL)	Inhibition (%) ± S.E.M.
Triumbellin	50	20.01 ± 1.16
	100	25.23 ± 1.09
Quercetin-3-*O*-glucoside	50	**38.16 ± 0.76***
	100	**63.02 ± 0.51****
Tannic acid	100	**89.38 ± 0.43*****

* : p < 0.05; ** : p < 0.01; *** : p < 0.001; S.E.M.: Standard error of the mean

Table 16. Collagenase and elastase enzyme inhibitory activities of the isolated compounds from *D. oleoides* subsp. *oleoides*

Material	Concentration (µg/mL)	Collagenase inhibition (%) ± S.E.M.	Elastase inhibition (%) ± S.E.M.
Triumbellin	50	10.29 ± 1.84	6.13 ± 1.19
	100	13.24 ± 1.19	8.11 ± 1.23
Quercetin-3-*O*-glucoside	50	**33.53 ± 0.96***	18.43 ± 1.21
	100	**57.10 ± 0.92****	39.08 ± 1.36
Epigallocatechin gallate	100	**42.07 ± 0.80****	**81.01 ± 1.01*****

* : $p < 0.05$; ** : $p < 0.01$; *** : $p < 0.001$; S.E.M.: Standard error of the mean

In the previous reports, quercetin and its glucosides were found to have antioxidant [38], anti-inflammatory [39,40] and anticancerogenic [41] activities. In our previous study, we reported that the methanolic extract of *Sambucus ebulus* was shown to possess wound healing potential, and through bioassay-guided procedures quercetin 3-*O*-glucoside was isolated as the major active component in the active fraction [42]. Previous studies have also revealed that quercetin and its glucosides to possess metalloproteinase enzyme inhibitory effect [43,44] and the results of the present study were in accordance with those of previous reports.

The other components isolated from the active fraction were coumarin type compounds "triumbellin" and "rutarensin". However, triumbellin (**1**) was found ineffective in wound healing process. In fact triumbellin impeded the wound healing process possibly due to its anticoagulant effect. Therefore, flavonoid type component in the active fraction, quercetin 3-*O*-glucoside, was the solely wound healing constituent of DOO.

The aerial parts of *Daphne oleoides* subspecies, e.g., ssp. *oleoides* and ssp. *kurdica*, growing wild in the same vicinity have been used as wound healing remedy without discrimination in Turkish folk medicine. In a previous study, we investigated the wound healing potential of ssp. *kurdica* [6]. Although a similar activity pattern was observed, from the active ethylacetate extract of ssp.*kurdica* two coumarin derivatives, daphnetin and demethyldaphnoretin 7-*O*-glucoside, and a flavonoid, luteolin-7-*O*-glucoside, were isolated and the latter (flavonoid) was shown to exert highest wound healing activity.

In the present study, however, the other subspecies ssp. *oleoides* also yielded two coumarin derivatives, triumbellin (**1**) and rutarensin (**3**), and a flavonoid glycoside, quercetin 3-*O*-glucoside (**2**), from the active EtOAc subextract through bioassay-guided procedures and the flavonoid glycoside again exerted a significant wound healing activity. However, the structures of active constituents were chemically different. It is noteworthy that daphnetin was previously isolated from the roots of DOO and was shown to possess significant inhibitory effects on inflammatory cytokines; interleukin 1-alpha, interleukin 1-beta and tumour necrosis factor-alpha [5,45].

As conclusion, quercetin-3-*O*-glucoside was isolated as the main active wound healing constituent from the aerial parts of *Daphne oleoides* ssp. *oleoides*. In addition to our previous study [42], the activity potential of quercetin-3-*O*-glucoside was tested by both *in vivo* and *in vitro* methods. According to the results of the present study, the wound healing activity of this compound was found be due to its hyaluronidase and collagenase enzyme inhibitory activities. Furthermore, significant anti-inflammatory and antioxidant effects of this compound might have a contributory role in the wound healing effect.

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Supporting Information

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

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