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ORIGINAL ARTICLE / ÖZGÜN MAKALE

SECONDARY METABOLITES OF ENDEMIC *CENTAUREA APHRODISEA* BOISS.

ENDEMİK CENTAUREA APHRODISEA BOISS.'İN SEKONDER METABOLİTLERİ

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ABSTRACT

Objective: *This study aimed to isolate secondary metabolites from the aerial parts of endemic Centaurea aphrodisea Boiss. using several chromatographic methods and elucidate the structure of the compounds by using spectroscopic methods.*
Material and Method: *Aerial parts of the endemic C. aphrodisea were collected from Bozdağ (Ödemiş, İzmir) and n-hexane, chloroform and metanol extracts were prepared. The chloroform extract was investigated by using various chromatographic methods, and the structures of the isolated compounds were determined using spectroscopic methods (1D-2D NMR and LC-MS).*
Result and Discussion: *One elemane type sesquiterpene (methyl 8α,6α,15-trihydroxyelema-1,3,11(13)-trien-12-oate) and four flavone derivatives (cirsimaritin, 3'-O-methyl eupatorin, eupatorin and salvigenin) were isolated and identified. In addition, the presence of a phenylpropanoid glycoside (syringin) was determined in a fraction by comparison with a referance compound using TLC technique. These compounds are reported for the first time from C. aphrodisea with this study.*
Keywords: *Centaurea aphrodisea, elemane, flavone, secondary metabolite, sesquiterpene*

ÖZ

Amaç: *Bu çalışmada endemik Centaurea aphrodisea Boiss. bitkisinin toprak üstü kısımlarında bulunan sekonder metabolitlerin, kromatografik yöntemlerle saflaştırılması ve spektroskopik yöntemlerlede yapılarının aydınlatılması amaçlanmıştır.*
Gereç ve Yöntem: *Bu çalışmada, endemik C. aphrodisea'nın toprak üstü kısımları Bozdağ'dan (Ödemiş, İzmir) toplanmış ve n-hekzan, kloroform ve metanol ekstreleri hazırlanmıştır. Kloroform ekstresi çeşitli kromatografik yöntemler kullanılarak incelenmiş ve izole edilen bileşiklerin yapıları spektroskopik yöntemler (1D-2D NMR ve LC-MS) kullanılarak aydınlatılmıştır.*
Sonuç ve Tartışma: *Bir eleman tip seskiterpen (metil 8α,6α,15-trihidroksielema-1,3,11(13)-trien-12-oat) ve dört flavon türevi (sirsimaritin, 3'-O-metil öpatorin, öpatorin ve salvigenin) izole edilerek yapıları aydınlatılmıştır. Ayrıca İTK tekniği ve şahit bileşikler kullanılarak bir fraksiyonda fenilpropanoit glikozitinin (siringin) varlığı saptanmıştır. Bu bileşikler C. aphrodisea'dan tarafımızca ilk kez rapor edilmektedir.*
Anahtar Kelimeler: *Centaurea aphrodisea, elemane, flavon, sekonder metabolit, seskiterpen*

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INTRODUCTION

The genus *Centaurea* L. is the fourth largest genus in the Asteraceae family, and there are approximately 700 species in the world [1]. The majority of the species are distributed in Western Asia and the Mediterranean Region. In the flora of Turkey, the *Centaurea* genus is represented by 213 taxa, 125 of which are endemic [2].

Various species of *Centaurea* are used as herbal remedies for stomach upset, abdominal pain, expectorant and antipyretic in common colds and against inflammatory conditions, such as abscesses in Anatolian traditional medicine [3,4]. Bioactivity studies generally revealed that *Centaurea* species have antiinflammatory, cytotoxic, antioxidant, antimicrobial and antiulserogenic properties [5].

Previous phytochemical and bioactivity studies showed that the pharmacological effect of *Centaurea* species are generally related to sesquiterpene lactones (germacranolide, eudesmanolide, elemanolide and guaianolide type) and flavonoids as their main secondary metabolites [6,7].

In a previous study, methanol (MeOH) extract of *C. ensiformis* was reported to have strong antioxidant activity with 86.2% FRSA (Free radical scavenging activity) and the total phenolic content of the extract was determined as 59.33 ± 1.76 GAE (Gallic acid equivalent) mg/l. 11 phenolic compounds were isolated from the MeOH extract, and strong DPPH radical scavenging activities were observed with protocatechuic acid (IC_{50}:6.47 µM), tacioside (IC_{50}:22.87 µM) and dihydrodehydrodiconiferyl alcohol 4-*O*-β-glucopyranoside (IC_{50}:27.7 µM) [8,9].

In another study, three guaianolide type sesquiterpene derivatives (13-acetyl solstitialin A, solstitialin A and chlorojanerin) were isolated from the chloroform ($CHCl_3$) extract of *C. solstitialis* ssp. *solstitialis*. Chlorojanerin (59.2 mg/kg), and a mixture of 13-acetyl solstitialin A (95%) and solstitialin A (5%) (179 mg/kg) were reported to exhibit antiulcerogenic activities in various ulcer models in rats and mice [10].

Demiröz et al., evaluated the *in-vivo* anti-inflammatory activities of *C. calolepis* $CHCl_3$ extract and cnicin (a germacrenolide type sesquiterpene), against edema induced by *Macrovipera lebetina obtusa* and *Montivipera xanthina* venoms in a rat model. The $CHCl_3$ extract demonstrated strong inhibition on edema at all doses and hours against both venoms. Cnicin (27.31% edema increase at 2.5 mg/kg) was more effective than the extract (36.43% edema increase at 25 mg/kg) against rat paw edema induced by *M. lebetina obtusa* venom [11].

C. aphrodisea Boiss. is one of the endemic species naturally distributed in Western and South-Western parts of Anatolia including Aydın, Denizli and Izmir. It is a perennial plant, with rose-purple florets and tomentose leaves. It differs from the other *Centaurea* species with large appendages with distinct hyaline border ending with a terminal mucro, and light brown central parts [12].

In our previous study, composition of the essential oil of *C. aphrodisea* was investigated, and the major metabolites were detected as spathulenol (8.1%), hexahydrofarnesyl acetone (7.8%), tridecanal (5.4%), and heptacosane (4.5%) [13]. *In vitro* cytotoxic and anti-inflammatory activity of different extracts of the plant were also reported. Among the tested extracts, chloroform extract exhibited cytotoxic activity against SK-MEL (malignant melanoma), KB (oral epidermal carcinoma), BT-549 (breast ductal carcinoma), SK-OV-3 (ovarian carcinoma) cancer cell lines. It was also found that chloroform extract showed anti-inflammatory activity by inhibiting NF-κB (Nuclear factor kappa B) and iNOS (inducible nitric oxide synthase) activation (IC_{50}:21 µg/ml and 23 µg/ml, respectively) [5].

In our continuous search on *Centaurea* species, we aimed to isolate the secondary metabolites of the chloroform extract of endemic *C. aphrodisea* by chromatographic techniques and to elucidate their structures by spectroscopic methods.

MATERIAL AND METHOD

General Experimental Procedures

Column chromatography was carried out on silica gel, RP-C_{18} (Merck), and Sephadex LH-20 (GE Healthcare) using analytical grade purity solvents [*n*-hexane, ethylacetate (EtOAc), chloroform ($CHCl_3$), methanol (MeOH) and acetonitrile (ACN); Merck]. Isolation procedure was monitored by thin layer chromatography (TLC, Silica gel 60 F_{254} plates, Merck), and for detection of the metabolites UV

light (254, 366 nm) and vanillin/H_2SO_4 (V/S) reagent were used. 1D and 2D NMR spectra were recorded on Varian Oxford AS-400 spectrometer, and Thermo-Scientific TSQ Quantum Access Max LC-MS/MS was used for mass analysis.

Plant Material

C. aphrodisea was collected from İzmir-Ödemiş-Bozdağ (1700 m) during the flowering period (June 2010) (38019'06.10"N 28004'53.63"E). Prof. Dr. Serdar Gokhan Senol (Section of Botany, Department of Biology, Faculty of Science, Ege University) confirmed the plant material and a voucher specimen was recorded in the IZEF Herbarium of Ege University (IZEF-5915).

Extraction and Isolation

Aerial parts of *C. aphrodisea* (1.5 kg) were dried and finely crushed. The plant material was extracted sequentially with *n*-hexane, $CHCl_3$ and MeOH (3 x 2l, 24 h each) with ultrasonic water bath. The extracts were filtered and separately concentrated to dryness with an evaporator at 40°C and yielded 14.65 g *n*-hexane, 20.68 g $CHCl_3$ and 73.04 g MeOH extracts.

$CHCl_3$ extract (17.8 g) was chromatographed over RP-C_{18} column (400 g) using H_2O:MeOH mixtures (80:20 to 0:100; 10%, 1l each) and 12 main fractions were obtained according to TLC profiles. Three main fractions (A1: Fr.13-18; A2: Fr. 23-28, and A3: Fr.5-9) were selected for further purification steps.

Fr. A1 (2.2 g) was chromatographed using silica-gel column (30 g) and eluted with EtOAc:MeOH:H_2O (100:5:1, isocratic, 1l) and afforded 38 subfractions. Fr.A1/25-36 (402 mg) was chromatographed using silica-gel (15 g) and $CHCl_3$:MeOH:H_2O (90:10:0.5, isocratic, 500 ml), and subfractions 25-26 were combined to afford compound **1** (73 mg).

Fr.A2 (2.5 g) was fractionated using sephadex LH-20 column (75 g) with MeOH, and Fr.A2/30-56 (256 mg) was chromatographed over silica-gel (15 g) ($CHCl_3$:MeOH; 100:0 and 98:2, 200 ml each). Subfraction 1-10 (184 mg) was chromatographed using silica-gel (20 g) with $CHCl_3$:MeOH (100:0, 99:1 and 98:2, 300 ml each) and afforded 144 subfractions. Subfractions 45-81 were combined to yield compound **4** (64 mg).

Fr.A2/30-56/1-10/116-end (22 mg) was chromatographed using RP-C_{18} (10 g) with an elution system of H_2O:ACN (65:35 and 60:40, 100 ml each). Compound **2** (9 mg) was isolated from subfractions 10-33 (19 mg) using silica-gel (7 g) and $CHCl_3$ (100%, 100 ml) as mobile phase.

Fr.A2/30-56/1-10/26-44 (49 mg) was fractionated using RP-C_{18} (20 g) column and H_2O:ACN mixtures (70:30 to 55:45, 5% decreasing polarity, 200 ml each) and afforded compounds **3** (12 mg) and **5** (4 mg).

Fr. A3 (1.5 g) was chromatographed over sephadex column (60 g) using MeOH. Among the 51 subfractions, Fr.A3/13-19 (600 mg) was chromatographed over silica-gel (50 g) using EtOAc:MeOH mixtures (100:0 to 50:50, 10%, 300 ml each) and the presence of compound **6** was detected in Fr.A3/13-19/9 (51 mg) by TLC comparison with an authenthic sample of syringin, which was previously isolated from *C. polyclada* (Figure 1) [11].

RESULT AND DISCUSSION

Previous phytochemical studies on *Centaurea* species have generally resulted in the isolation of sesquiterpene lactones (germacranolide, eudesmanolide, elemanolide and guaianolide type) and flavonoids as main metabolites.

In this study, $CHCl_3$ extract of *C. aphrodisea* was evaluated with a series of column chromatographic separation steps. Isolation studies yielded one elemane type sesquiterpene; methyl 6α,8α,15-trihydroxyelema-1,3,11(13)-trien-12-oate (**1**) [14,15], and four methoxyflavone derivatives; cirsimaritin (**2**), 3'-methoxy eupatorin (**3**), eupatorin (**4**), and salvigenin (**5**) [16], in accordance with previous studies. The structures of the isolated compounds were determined by comparing their NMR data with those of previously reported metabolites. ^1H and ^{13}C NMR data of compounds **2-5** are presented in Tables 1 and 2. Additionally, the presence of a phenylpropanoid glucoside; syringin (**6**)

was detected in a fraction by TLC comparison with an authenthic sample, which was previously isolated from *C. polyclada* (Figure 1) [15]. Structures of the compounds **1-6** are given in Figure 2.

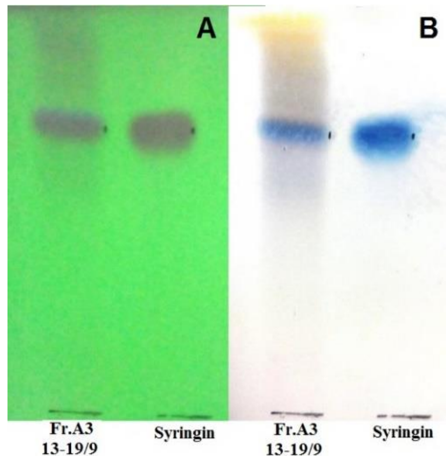

Figure 1. TLC comparison of syringin and Fr.A3/13-19/9. Silica gel 60 F_{254} TLC plate, $CHCl_3$:MeOH:H_2O-70:30:3, A: TLC plate under UV-254 nm, B: TLC plate sprayed with V/S reagent

Figure 2. Structures of compounds **1-6**

Methyl 6α, 8α,15-trihydroxyelema-1,3,11(13)-trien-12-oate (**1**): ^{1}H NMR (400 MHz, CD_3OD), δ 5.75 (dd, *J* = 18/10 Hz, H-1), 4.93 (d, *J* = 10.4 Hz, H2a), 4.92 (d, *J* = 18 Hz, H2b), 5.33 (brs, H3a), 4.93 (brs, H3b), 1.85 (d, *J* = 10.8 Hz, H5), 4.12 (t, *J* = 10.4 Hz, H6), 2.31 (t, *J* = 10.4 Hz, H7), 4.10 (td, *J* = 10.8/4.4 Hz, H8), 1.73 (dd, *J* = 12.4/4.4 Hz, H9a), 1.49 (dd, *J* = 12.4/12 Hz, H9b), 6.34 (d, *J* = 1.6Hz,

H13a), 5.74 (d, J = 1.6 Hz, H13b), 1.12 (s, 3H, H14), 3.99 (d, J = 14.8 Hz, H15a), 3.89 (d, J = 14.8 Hz, H15b) and 3.75 (s, 3H, $-OCH_3$); LC-MS (ESI), *m/z*= 297.13 $[M+H]^+$ and 319.12 $[M+Na]^+$

Cirsimaritin (**2**): ^{1}H NMR and ^{13}C NMR data: see Tables 1 and 2, LC-MS (ESI), *m/z*= 315.02 $[M+H]^+$ and *m/z*= 313.13 $[M-H]^-$

3'-methoxy eupatorin (**3**): ^{1}H NMR and ^{13}C NMR data: Tables 1 and 2, LC-MS (ESI), *m/z*= 359.06 $[M+H]^+$

Eupatorin (**4**): ^{1}H NMR and ^{13}C NMR data: Tables 1 and 2, LC-MS (ESI), *m/z*= 345.08 $[M+H]^+$

Salvigenin (**5**): ^{1}H NMR and ^{13}C NMR data: Tables 1 and 2, LC-MS (ESI), *m/z*= 329.08 $[M+H]^+$

Table 1. ^{1}H NMR data of compounds **2-5** [400 MHz, DMSO-d_6, δ_H (J in Hz)]

Position	2	3	4	5*
3	6.81, s	6.80, s	6.71, s	6.50, s
8	6.92, s	6.91, s	6.79, s	6.48, s
2'	7.94, dd (8.8)	7.55, d (1.6)	7.41, d (2.4)	7.76, d (8.8)
3'	6.90, d (8.4)	-	-	6.95, d (8.8)
5'	6.90, d (8.4)	7.11, d (8.8)	7.02, d (8.4)	6.95, d (8.8)
6'	7.94, dd (8.8)	7.67, dd (8.8/1.6)	7.48, dd (8.4/2.4)	7.76, d (8.8)
6-OCH3	3.73, s	3.72, s	3.71, s	3.89, s
7-OCH3	3.92, s	3.88, s	3.88, s	3.93, s
3'-OCH3	-	3.91, s	-	-
4'-OCH3	-	3.85, s	3.84, s	3.85, s

* in $CDCl_3$

Table 2. ^{13}C NMR data of compounds **2-5** (100 MHz)

Position	2[a]	3[b]	4[a]	5[b]
2	164.2	163.9	164.2	163.9
3	102.3	104.4	103.7	103.9
4	182.1	182.6	182.5	182.5
5	152.0	153.2	152.5	153.1
6	131.8	132.7	132.3	132.6
7	158.5	158.7	159.0	158.7
8	91.5	90.6	91.8	90.5
9	152.6	153.0	153.0	153.0
10	105.0	106.1	105.5	106.0
1'	120.3	123.8	123.3	123.4
2'	128.5	108.9	113.5	127.9
3'	116.1	149.4	147.2	114.4
4'	162.2	152.3	151.6	162.6
5'	116.1	111.2	112.4	114.4
6'	128.5	120.1	119.1	127.9
6-OCH3	60.0	60.8	60.4	60.8
7-OCH3	56.4	56.1	56.8	56.2
3'-OCH3	-	56.1	-	-
4'-OCH3	-	56.3	56.2	55.5

[a] in DMSO-d_6
[b] in $CDCl_3$

In our previous study, chloroform extract exhibited cytotoxic activity against SK-MEL (IC_{50}=65 μg/ml), KB (IC_{50}=60 μg/ml), BT-549 (IC_{50}=86 μg/ml), SK-OV-3 (IC_{50}=60 μg/ml) cell lines, and non-cancerous LLC-PK1 cells (kidney epithelial cells) (IC_{50}=58 μg/ml) [5]. The elemane derivative, compound **1,** was previously reported from *Centaurea aspera* var. *subinermis* [14], *C. polyclada* [15],

Onopordum acaulon [17], and *O. cynarocephalum* [18], and was reported to exhibit cytotoxic activity against A375 (human melanoma) cell line (IC_{50}: 9.2 µM) [18]. Among the methoxyflavone derivatives, cirsimaritin was reported to have cytotoxic activity against HL-60 (human leukemia), COLO-205 (human colon carcinoma), MCF-7 (human breast adenocarcinoma), and NCI-H520 (lung squamous carcinoma) cell lines (IC_{50}: 61 and 13.1, 59.86, and 23.29 µM respectively) [19-21]. *In vivo* studies have also shown that, salvigenin exhibited antitumor effects in drug-resistant HCC (hepatocellular carcinoma) cell lines by increasing cell sensitivity and decreasing the IC_{50} of 5-fuorouracil, and suppressing MCF-7 tumor growth [22,23].

Best to our knowledge, all compounds are reported for the first time from Turkish endemic *C. aphrodisea*. When the molecules obtained during this phytochemical study and the literature data are evaluated together, it may be thought that the isolated molecules may be responsible for the cytotoxic activity of the plant or may contribute to this activity.

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AUTHOR CONTRIBUTIONS

Concept: F.G., C.K.; Design: F.G., C.K.; Control: S.D., C.K.; Sources: F.G., C.K.; Materials: F.G., S.D., S.B.O., C.K.; Data Collection and/or Processing: F.G., S.D., C.K.; Analysis and/or Interpretation: F.G., S.D., S.B.O., C.K.; Literature Review: F.G., S.D., S.B.O., C.K.; Manuscript Writing: F.G., S.D., S.B.O., C.K.; Critical Review: S.B.O., C.K.; Other: -

CONFLICT OF INTEREST

The authors declare that there is no real, potential, or perceived conflict of interest for this article.

ETHICS COMMITTEE APPROVAL

The authors declare that the ethics committee approval is not required for this study.

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