

Specialized metabolites from the aerial parts of *Centaurea polyclada* DC.



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ABSTRACT

The genus *Centaurea* L. (Asteraceae) is represented by 200 taxa in the flora of Turkey and several *Centaurea* species are used as herbal remedies against different conditions. Previous phytochemical investigations on this genus generally revealed the isolation of sesquiterpene lactones and flavonoid derivatives. In our continuous search on *Centaurea* genus, a phytochemical study was performed on *Centaurea polyclada* DC., an endemic of West Anatolia.

Previously undescribed two sesquiterpene-amino acid conjugates, an elemene and an eudesmane derivative were isolated from the aerial parts of *Centaurea polyclada*, together with eight known compounds; two elemene derivatives, three flavonoids, a lignan, a phenolic glucoside and a phenylpropanoid glucoside. Structural elucidation of the compounds was based on spectroscopic evidence, including 1D and 2D NMR and high-resolution mass spectrometry, chemical degradation results and reference data comparison. Sesquiterpene-amino acid conjugates are representatives of an unusual group of sesquiterpenes, and elemene-amino acid conjugates are herein reported for the first time in nature.

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1. Introduction

Centaurea polyclada DC. (Asteraceae) is an endemic, profusely branched, perennial plant and naturally distributed in West Anatolia (Uysal, 2012; Wagenitz, 1975). The genus *Centaurea* L. is represented by 200 taxa in the flora of Turkey and several *Centaurea* species are used as herbal remedies against inflammatory conditions, such as abscesses, asthma, hemorrhoids, peptic ulcer, malaria, common colds, stomach upset and abdominal pain (Akkol et al., 2009; Ozcelik et al., 2009). Previous studies have confirmed that this genus is a source of novel bioactive compounds. Phytochemical investigations on this genus generally revealed the isolation of sesquiterpene lactones, flavonoids, phenyl propanoids, lignans and phenolic compounds (Astari et al., 2013; Baykan-Erel et al., 2010; Bruno et al., 2013; Cardona et al., 1997, 1991; Erel et al., 2011; Gulcernal et al., 2010; Karamenderes et al., 2007a, 2007b; Skaltsa et al., 1999). Composition of the essential oil of *Centaurea polyclada* was previously reported (Erel et al., 2013). In our continuous search on *Centaurea* species, a phytochemical study was performed on *Centaurea polyclada* DC.

Fractionation of CHCl₃ and *n*-BuOH extracts from the aerial parts of *C. polyclada* led to the isolation of four previously undescribed specialized metabolites (1–4) together with eight known compounds (5–12). Herein, the isolation and structural elucidation of compounds 1–4 are described. ¹H and ¹³C-NMR data of compounds 5 and 6 are also provided (Tables 1 and 2).

2. Results and discussion

CHCl₃ and *n*-BuOH extracts from the aerial parts of *C. polyclada* was subjected to a series of column chromatographic separation steps. Purification studies led to the isolation of previously undescribed two elemene sesquiterpene-amino acid conjugates (1 and 2) together with an elemene derivative (3) and an eudesmane type sesquiterpene (4). The known compounds; two elemene derivatives; 6 α ,8 α ,15-trihydroxyelemene-1,3,11(13)-trien-12-oic acid (5) (Cardona et al., 1992) and its methyl ester (6) (Cardona et al., 1992), three flavonoids; salvigenin (7) (Salan et al., 2001), eupatorin (8) and 3'-methoxy eupatorin (9) (Salan et al., 2001), a lignan; arctiin (10) (Shoeb et al., 2004), a phenolic glucoside; tachioside (11) (Liu et al., 2013) and a phenylpropanoid glucoside; syringin (12) (Konuklugil and Bahadir, 2004) were identified by comparison of their spectroscopic data with literature values (Fig. 1). Herein, the

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Table 1
¹H NMR data of compounds **1–6** [400 MHz, CD₃OD, δ_H (J in Hz)].^a

Position	1	2	3	4	5	6
1	5.84 dd (17.2/10.8)	5.73 dd (17.2/11.2)	5.77 dd (18/10.8)	3.28 dd (11.2/4.4)	5.76 dd (18/10.4)	5.76 dd (18/10.4)
2	5.01 d (10.8)	4.87 d (11.2) ^b	4.94 d (10.8)	1.77 m	4.92 d (10.4)	4.93 d (10.4)
	4.99 d (17.2)	4.89 d (17.2)	4.92 d (18.0)	1.67 ddd (12.8/4.4)	4.91 d (18)	4.92 d (18)
3	5.39 brs	5.34 brs	5.35 brs	1.51 m ^b	5.33 brs	5.34 brs
	4.98 brs	4.96 brs	4.97 brs	1.55 m ^b	4.94 brs	4.93 brs
4				2.19 m ^b		
5	2.34 d (12)	1.89 d (10.8)	1.92 d (10.8)	1.52 m ^b	1.87 d (10.8)	1.85 d (10.8)
6	4.56 dd (11.6/10.8)	3.90 m ^b	4.26 t (10.4)	3.87 t (10.4)	4.10 dd (10.4/10)	4.13 dd (10.8/10.4)
7	2.03 ddd (11.6/10.8)	1.80 m	2.64 t (10.8)	2.23 t (10.4)	2.33 dd (10.4/10)	2.32 t (10.4)
8	4.08 td (10.8/4.4)	3.93 m ^b	5.53 td (11.2/4.4)	4.07 td (10.8/4.8)	4.08 td (10.4/4)	4.11 td (10.8/4.4)
9	1.80 dd (13.2/4.4)	1.69 dd (12.4/4)	1.86 dd (12.4/4.4)	2.27 dd (12.4/4.8)	1.73 dd (12.4/4)	1.74 dd (12.8/4.4)
	1.61 dd (13.2/10.8)	1.50 dd (12.4/12)	1.62 dd (12/11.6)	1.09 dd (12.4/11.6)	1.50 t (12.0)	1.50 t (12)
10						
11	3.30 m ^b	3.25 m ^b				
12						
13	3.66 dd (13.2/5.2)	3.88 m ^b	6.28 brs	6.33 d (1.2)	6.22 brs	6.34 d (1.6)
	3.44 dd (13.2/8)	3.10 m ^b	5.70 brs	5.70 d (1.2)	5.62 brs	5.75 d (1.6)
14	1.13 s	1.05 s	1.21 s	0.95 s	1.11 s	1.13 s
15	4.03 d (15.2)	3.98 d (14.4)	4.01 d (14.8)	9.31 d (5.2)	3.99 d (14.8)	3.99 d (14.4)
	3.93 d (15.2)	3.88 d (14.4) ^b	3.91 d (14.8)		3.89 d (14.8)	3.89 d (14.4)
1'						
2'	3.99 dd (9.2/4)	3.87 m ^b				
3'	2.44 m	2.40 m	6.30 qd (7.2/1.2)			
	2.26 m	2.20 m				
4'	2.12 m	2.10 m	1.97 dd (7.2/1.2)			
	1.93 m	1.97 m				
5'	3.78 m	3.75 m	4.16 dd (13.6/1.2)			
	3.24 m ^b	3.10 m ^b	4.10 dd (13.6/1.2)			
-OCH ₃				3.74 s		3.76 s

^a Assignments of ¹H and ¹³C NMR data are based on ¹H-¹H COSY, HSQC and HMBC experiments.^b Overlapping signals.**Table 2**
¹³C NMR data of compounds **1–6** [100 MHz, CD₃OD, δ_C (ppm)].^a

Position	Type	1	2	3	4	5	6
1	CH	148.4	149.1	148.5	78.3	149.2	149.0
2	CH ₂	112.5	111.3	111.8	28.8	111.3	111.4
3	CH ₂	112.9	112.4	112.0	25.4	11.9	112.0
4	C	146.2	148.5	148.1	49.9 (CH)	148.5	148.3
5	CH	51.6	57.1	56.6	53.0	56.7	56.7
6	CH	80.7	70.5	72.0	71.4	72.5	71.6
7	CH	58.0	55.1	56.9	61.6	59.7	60.6
8	CH	67.6	67.4	72.0	68.1	68.9	68.2
9	CH ₂	49.8	49.8	44.8	46.5	49.8	49.6
10	C	43.0	41.0	41.2	39.8	41.0	41.0
11	C	44.5 (CH)	41.3 (CH)	140.8	139.8	142.5	140.2
12	C	176.8	180.3	170.4	168.5	166.8	168.7
13	CH ₂	56.6	57.3	128.7	130.0	125.4	129.4
14	CH ₃	19.1	19.4	19.1	12.6	19.4	19.4
15	CH ₂	66.8	67.7	67.6	204.9 (CH)	67.7	67.6
1'	C	174.0	173.7	167.5			
2'	CH	72.0	72.1	133.6 (C)			
3'	CH ₂	30.5	30.1	139.1 (CH)			
4'	CH ₂	24.8	24.6	15.7 (CH ₃)			
5'	CH ₂	57.1	53.6	63.9			
-OCH ₃					52.0		52.1

^a Assignments of ¹H and ¹³C NMR data are based on ¹H-¹H COSY, HSQC and HMBC experiments.isolation and structural elucidation of compounds **1–4** are described. NMR data for compounds **5** and **6** were also provided.Compound **1** was obtained as a yellowish gum. QTOF-MS analysis displayed protonated and deprotonated molecular ion peaks at 380.2028 [M+H]⁺ and 378.1921 [M-H]⁻ for positive and negative modes, suggesting a molecular formula of C₂₀H₂₉NO₆. Of the seven degrees of unsaturation deduced from the molecular formula, four were present as multiple bonds including two carbonyl carbons (δ_C 176.8, 174.0, 148.4, 146.2, 112.9 and 112.5). Initial inspection of the¹H, ¹³C-NMR and HSQC spectra of **1** revealed a mono-substituted olefinic system (δ_H 5.84, 5.01 and 4.99; δ_C 148.4 and 112.5) suggesting an isolated ethenyl group, a pair of broad singlet protons (δ_H 5.39 and 4.98, each 1H; δ_C 112.9) implying an exocyclic double bond, two geminal oxymethylene protons as AB system doublets (δ_H 4.03 and 3.93; δ_C 66.8), two oxymethine groups (δ_H 4.56 and 4.08; δ_C 80.7 and 67.6) and a tertiary methyl signal (δ_H 1.13, 3H; δ_C 19.1) (Fig. 1, Tables 1 and 2). The covalent connectivity of **1** was established by thorough analysis of the COSY and HSQC spectra. The association of partial structures and assignment of quaternary carbon atoms were completed by means of long-range correlations in the HMBC spectrum.Based on the COSY and HMBC correlations (Fig. 2), an isolated framework was readily deduced that was consistent with proline moiety (δ_H 3.99, dd, J = 9.2, 4.0 Hz, H-2'; 2.44 and 2.26, m, H₂-3'; 2.12 and 1.93, m, H₂-4'; 3.78 and 3.24, m, H₂-5'; δ_C 174.0, C-1'; 72.0, C-2'; 30.5, C-3'; 24.8, C-4' and 57.1, C-5') (Matsuda et al., 2000). After subtracting 5 carbon resonances of the proline residue and two degrees of unsaturation, the remaining 15 signals were attributable to a bicyclic sesquiterpene skeleton with three multiple bonds.A detailed examination of the ¹H NMR spectrum of **1** (δ_H 5.84, dd, J = 17.2, 10.8 Hz, H-1; 5.01, d, J = 10.8 Hz and 4.99, d, J = 17.2 Hz, H₂-2; 5.39 and 4.98, each br s, H₂-3; 4.03 and 3.93, d, J = 15.2 Hz, H₂-15 and 1.13, s, H₃-14) displayed characteristic low-field and high-field signals of an elemene-type framework, which is a common sesquiterpene skeleton encountered in *Centaurea* genus (Cardona et al., 1997; Karamenderes et al., 2007b). The splitting patterns of the AB system doublets deriving from the hydroxymethyl residue (δ_H 4.03 and 3.93, J_{AB} = 15.2, H₂-15) and the methyl signal (δ_H 1.13, s, H₃-14) suggested their attachments to quaternary carbons, whereas the ²J_{C-H} correlations between H₂-15 and C-4 (δ_C 146.2), and H₃-14 and C-10 (δ_C 43.0) in the HMBC spectrum put

forward the positions of this two groups. Also the allylic coupling between exomethylene protons at δ_{H} 5.39 and 4.98 (H₂-3) with H₂-15 in the COSY spectrum, and the long-range $^3J_{\text{C-H}}$ correlations between C-3/H-5 and H-15; C-15/H-3 and H-5; C-5/H-3, H-7 and H₃-14 confirmed their positions in accordance with the elemene structure.

The α -methylene- γ -lactone system is a common structural feature for the sesquiterpenes isolated from the genus *Centaurea* (Bruno et al., 2013). In the case of compound **1**, comparison of the ^1H and ^{13}C NMR spectra with previously reported spectroscopic data of related metabolites revealed that the low-field shift of the oxymethine proton assigned to H-6 (δ_{H} 4.56) indicated an acylation at this position, compatible with the 6,12- γ -lactone moiety. On the other hand, the high-field shift of the carbon resonance assigned to C-11 (δ_{C} 44.5) and the COSY correlations of H-7 \rightarrow H-11 \rightarrow H₂-13 implied the saturation of the characteristic exocyclic double bond extending from lactone ring (C-11) (Matsuda et al., 2000). This prediction was subsequently confirmed by the heteronuclear long-range correlations of the methine (δ_{C} 58.0 and 44.5, C-7 and C-11, respectively) and carbonyl (δ_{C} 176.8, C-12) resonances with the methylene protons at δ_{H} 3.66 and 3.44 assigned to H₂-13. Chemical shift of H₂-13 also suggested its connection to an electron withdrawing group. In the HMBC spectrum, the long-range correlations between C-2'/C-5' (δ_{C} 72.0 and 57.1, respectively) and H₂-13 protons not only verified the presence of abovementioned electronegative group but also linkage site of the proline moiety.

The relative stereochemistry of **1** was proposed by analyzing the coupling constants and comparison of the spectroscopic data with previously reported similar metabolites. Evaluation of the other naturally occurring elemene-type sesquiterpenes reported from the genus *Centaurea*, H-7 was readily assigned with α -configuration (Bruno et al., 2013; Karamenderes et al., 2007b). Based on this postulation, H-6 must be β -oriented as the coupling constant suggested an antiperiplanar orientation ($J_{6,7} = 11.6$ Hz). Moreover, the coupling constant between H-6 and H-5 protons was also large ($J_{5,6} = 12$ Hz), indicating that H-5 and H-6 were trans-diaxially oriented. These results suggested that **1** had H-5(α)/H-6(β)/H-7(α)/H-8(β) configurations. On the basis of these findings, the structure of **1** was established as 13-*N*-proline-8 α ,15-dihydroxy-elema-1,3-diene-6 α ,12-olide, and named as 13-*N*-proline melitensin in accordance with previously reported secondary metabolite melitensin (Bruno et al., 2013).

Compound **2** was isolated as a colorless gum. QTOF-MS spectra of **2** exhibited protonated and deprotonated molecular ion peaks for $[\text{M}+\text{H}]^+$ and $[\text{M}-\text{H}]^-$ at m/z 398.1655 and 396.2018, respectively, in agreement with the molecular formula $\text{C}_{20}\text{H}_{31}\text{NO}_7$, which was 18 amu greater than compound **1**. The ^1H and ^{13}C NMR spectra of **2** revealed the similarity of structures when compared to those of **1**. The spectroscopic assignment was completed comprehensively by examining COSY, HSQC and HMBC spectra. In the case of compound **2** the only difference observed was the high-field shift of a methine proton (ca. 0.7 ppm) and a carbon (ca. 10 ppm) signal assigned to C(H)-6 (δ_{H} 3.90 and δ_{C} 70.5), and these observations suggested a free hydroxyl group at C-6. Additionally, 6 degrees of unsaturation deduced from the molecular formula revealed the monocyclic nature of the structure. All these data clearly proved the absence of 6,12- γ -lactone ring in accordance with the aforementioned 18 amu difference. The relative stereochemistry of compound **2** was based on 2D-NOESY data. Particularly, the strong NOESY correlation between H-6 and H-11 allowed us to locate H-11 at β face of the molecule.

Consequently, the structure of **2** was established as 13-*N*-proline-6 α ,8 α ,15-trihydroxy elema-1,3-diene-12-oic acid.

To determine the absolute configuration of proline, compound **2** was taken into acidic hydrolysis study because of its higher

quantity compared to **1**. Treatment of **2** with 1% aqueous hydrochloric acid (HCl) at room temperature liberated **6** and proline. Optical rotation measurement of purified proline made clear that its absolute configuration was L ($[\alpha]_{\text{D}}^{20} -85$, c. 0.001, MeOH) (Convention, 2005). Compound **1**'s proline residue was assumed as L by way of analogy with **2**, whereas the configuration of H-11 in **1** was also proposed to be β by means of same inference.

Molecular formula of **3** was determined as $\text{C}_{20}\text{H}_{28}\text{O}_7$ due to the sodium adduct and deprotonated ion peaks at m/z 403.1726 $[\text{M}+\text{Na}]^+$ and 379.1590 $[\text{M}-\text{H}]^-$ obtained by QTOF-MS analyses. Close examination of the ^1H , ^{13}C -NMR and HSQC spectra of **3** once again suggested an elemene-type structure (δ_{H} 5.77, dd, $J = 18$, 10.8 Hz, H-1; 4.94, d, $J = 10.8$ Hz and 4.92, d, $J = 18$ Hz, H₂-2; 5.35 and 4.97, each br s, H₂-3; 4.01 and 3.91, d, $J = 14.8$ Hz, H₂-15 and 1.21, s, H₃-14). When compared to **1** and **2**, characteristic exocyclic methylene (α -methylene) protons were present as a pair of broad singlet signals at δ_{H} 6.28 and 5.70 (H₂-13) in the ^1H NMR spectrum. On the other hand loss of a methine group and presence of an olefinic carbon resonance referred to C-11 (δ_{C} 140.8) were notable. The location of the exocyclic double bond was straightforwardly located by the long-range correlations between H₂-13 protons and C-7, C-11 and C-12 (δ_{C} 56.9, 140.8 and 170.4, respectively).

Subtraction of the 15 carbon resonances and proton signals referring to the elemene framework revealed additional resonances including an olefinic proton (δ_{H} 6.30; δ_{C} 139.1; C-3'), a secondary methyl signal (δ_{H} 1.97; δ_{C} 15.7; C-4'), an oxymethylene group (δ_{H} 4.16 and 4.10; δ_{C} 63.9; C-5') and two carbon resonances related to these groups (a carbonyl and an olefinic at δ_{C} 167.5 and 133.6; C-1' and C-2', respectively). The correlations between H-3' and H-4' ($J_{3',4'} = 7.2$ Hz) in the COSY spectrum and the allylic couplings observed for $J_{3',5'}$ and $J_{4',5'}$ (each 1.2 Hz) were evident for the presence of (*E*)-2-hydroxymethyl-but-2-enoyl (*syn*. sarraceny) (Gil et al., 1992) moiety, a common side chain encountered in *Centaurea* sesquiterpenes (Youssef, 1998).

When compared to **1** and **2**, the low-field shift (ca 1.5 ppm) of the proton at δ_{H} 5.53 ($J = 11.2$ and 4.4 Hz) assigned to the H-8 was not only indicated an acylation at this position but also attachment of the side chain at C-8. The location of the 2-hydroxymethyl-but-2-enoyl moiety was directly confirmed by the $^3J_{\text{C-H}}$ correlation between C-1' (δ_{C} 167.5) and H-8 (δ_{H} 5.53) in the HMBC spectrum.

The relative configurations of the stereocenters in **3** were identical with those of **1**. Analysis of the coupling constants between H-5/H-6 ($J_{5,6} = 10.8$ Hz), H-6/H-7 ($J_{6,7} = 10.4$ Hz) and H-7/H-8 ($J_{7,8} = 11.2$ Hz) suggested that **3** had H-5(α)/H-6(β)/H-7(α)/H-8(β) configurations. Based on these findings, the structure of **3** was established as 8 α -O-[2'-(hydroxymethyl)-but-2'-enoyl]-6 α ,15-dihydroxyelema-1,3,11(13)-trien-12-oic acid.

QTOF-MS analysis of **4** suggested a molecular formula of $\text{C}_{16}\text{H}_{24}\text{O}_6$ (m/z 335.1466 $[\text{M}+\text{Na}]^+$). 1D and 2D-NMR spectra of **4** revealed one carbonyl (δ_{C} 168.5), two quaternary carbons (including one olefinic at δ_{C} 139.8), one aldehyde (δ_{H} 9.31; δ_{C} 204.9), one exocyclic methylene as a pair of broad singlet signals (δ_{H} 6.33 and 5.70; δ_{C} 130.0), three oxymethine groups (δ_{H} 4.07, 3.87 and 3.28; δ_{C} 68.1, 71.4 and 78.3), a singlet proton signal attributed to a methoxy group (δ_{H} 3.74, 3H; δ_{C} 52.0), three methines (δ_{H} 2.23, 2.19 and 2.19), three methylene groups (resonating between δ_{H} 1.09 and 2.27; δ_{C} 46.5, 28.8 and 25.4) and a methyl (δ_{H} 0.95, 3H; δ_{C} 12.6).

After subtraction of the methoxy group, the presence of 15 carbon resonances together with overall spectral profile suggested another sesquiterpene structure. The proton and carbon data secured by COSY, HSQC and HMBC spectra revealed a different core structure consistent with an eudesmane framework (Saroglou et al., 2005) (Fig. 2). Comparison of the spectroscopic data with those reported in the literature proved the presence of an aldehyde group at C-15 (δ_{H} 9.31, d; δ_{C} 204.9) rather than a primary alcohol.

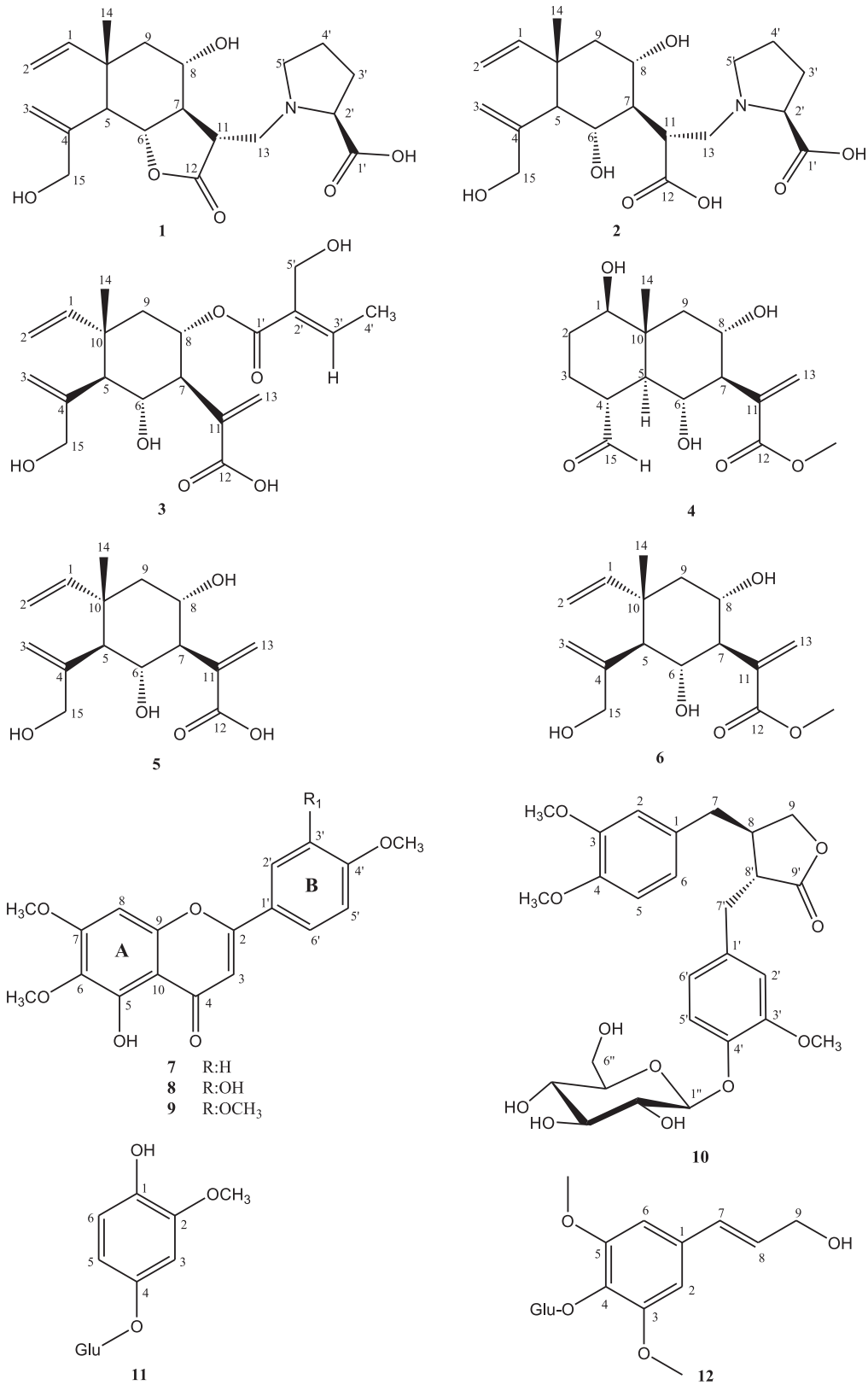


Fig. 1. Structures of compounds 1–12.

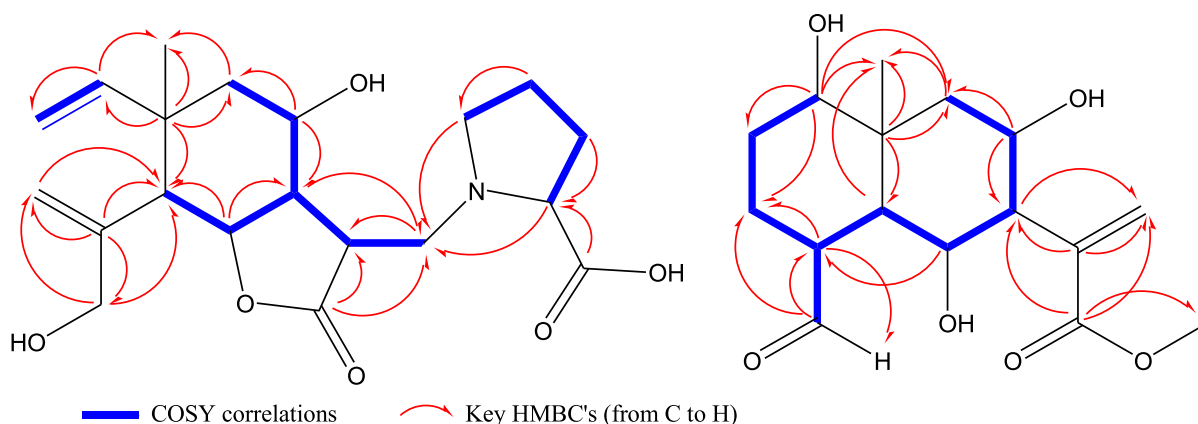


Fig. 2. COSY and key HMBC correlations of compounds **1** and **4**.

The α configuration of C-15 was determined based on the up-field shift of the H-15 and its coupling constant with H-4 ($J_{4,15} = 5.2$ Hz). In the case of 15 β -CHO, the aldehyde proton was reported to be a broad singlet resonating at about δ_{H} 9.91–9.94 (Bruno et al., 2013). Presence of a free β -oriented hydroxyl group at C-1 was also ensured by comparing the spectral data of **4** with the literature (Karioti et al., 2002; Saroglou et al., 2005; Skaltsa et al., 2000).

As in **3**, compound **4** provided characteristic signals for an acrylic acid moiety extending from C-7 and a free hydroxyl group at C-6 instead of a lactone ring. A weak allylic coupling between exocyclic methylene protons of C-13 and H-7 in the COSY spectrum, and the long-range correlations from C-12 to H-7 and H₂-13, C-11 to H-7 and H₂-13, and C-13 to H-7 in the HMBC spectrum were evident for the acrylic acid residue's position. Additionally, $^3J_{\text{C-H}}$ correlation between C-12 (δ_{C} 168.5) and the methyl signal at δ_{H} 3.74 revealed the ester nature of **4** (Skaltsa et al., 2000).

The relative stereochemistry of **4** was resolved on the basis of coupling constant analysis: [H-5/H-6 ($J_{5,6} = 10.8$ Hz), H-6/H-7 ($J_{6,7} = 10.4$ Hz) and H-7/H-8 ($J_{7,8} = 10.4$ Hz)] and comparison of the spectroscopic data with relevant structures establishing the configurations as H-5(α)/H-6(β)/H-7(α)/H-8(β) (Karioti et al., 2002). Based on these findings, the structure of **4** was determined as methyl 1 β ,6 α ,8 α -trihydroxy-15-oxo-eudesm-11(13)-en-12-oate, the decyl derivative of 4-*epi*-carmanin previously reported from *Centaurea achaia* (Skaltsa et al., 2000).

Secondary metabolites of *Centaurea* species are mainly sesquiterpene lactones with guaiane, germacrane, eudesmane and elemene skeletons (Bruno et al., 2013; Cardona et al., 1997; Karamenderes et al., 2007b; Karioti et al., 2002; Skaltsa et al., 2000). As a member of the genus *Centaurea*, *C. polyclada* afforded distinctive sesquiterpene structures. Elemanolides obtained from *Centaurea* species are generally melitensin, dehydromelitensin and their derivatives with different ester chains (Bruno et al., 2013).

In regards to Asteraceae family, a few addition products of aminoacids to the conjugated double bond of sesquiterpene lactones have been reported and the 13-*N*-substitued sesquiterpene derivatives appeared to be the characteristic compounds for *Saussurea* species. Saussureamines (A, B, E) from *S. lappa* (Matsuda et al., 2000) and pulchellamine C from *S. pulchella* (Yang et al., 2008) are the significant representatives of sesquiterpene-amino acid conjugates having guaiane, germacrane and eudesmane skeletons and L-proline as amino acid residue. Several derivatives were also reported from different Asteraceae members as *Inula helenium* (Zaima et al., 2014), *Ixeris dentata* (Cha et al., 2012) and *Scorzonera divaricata* (Yang et al., 2016). Only a germacrane derivative, namely onopordopicrin-valine dimeric adduct from *Centaurea aspera*

(Marco et al., 2005) was reported in the genus *Centaurea*.

To the best of our knowledge, the previously undescribed compounds, 13-*N*-proline melitensin (**1**) and 13-*N*-proline-6 α ,8 α ,15-trihydroxyelema-1,3-dien-12-oic acid (**2**), are very unusual representatives of a group of sesquiterpenes containing amino acid residues, namely L-proline. Elemene-amino acid conjugates were encountered for the first time in nature. 8 α -O-[2'-(hydroxymethyl)-but-2'-enyl]-6 α ,15-dihydroxyelema-1,3,11(13)-trien-12-oic acid (**3**) and methyl 1 β ,6 α ,8 α -trihydroxy-15-oxo-eudesm-11(13)-en-12-oate (**4**) are also previously undescribed natural products with elemene and eudesmane-type sesquiterpene frameworks. On the other hand, 6 α ,8 α ,15-trihydroxyelema-1,3,11(13)-trien-12-oic acid (**5**) is a commercially available elemene derivative with no report from natural sources; therefore, it should be regarded as a new natural product.

3. Experimental

3.1. General experimental procedures

IR spectra were obtained on a Perkin Elmer Spectrum 100 FT-IR spectrometer. Mass spectra analysis was carried out on ThermoScientific TSQ Quantum Access Max LC-MS/MS equipped with an ESI source. High Resolution Mass spectra were obtained on Agilent 6200 Series TOF and 6500 Series QTOF-MS system equipped with an ESI source. Optical rotation measurements were done on a Perkin Elmer Model-351 polarimeter in MeOH at 20 °C. 1D and 2D (COSY, HMBC, HSQC and NOESY) NMR spectra were recorded on Varian Oxford AS400 spectrometer with TMS as internal standard at room temperature. 2D NMR spectra were run using standard Varian pulse programs. Column chromatography was carried out on silica gel 60 (40–63 μm -Merck), Sephadex LH-20 (GE Healthcare) and Lichroprep RP-C18 (25–40 μm , Merck) using analytical grade purity solvents (Merck and Sigma). TLC analyses were carried out on silica gel 60 F₂₅₄ and RP-C18 F_{254s} (Merck) pre-coated aluminum plates. Compounds were detected by UV (244–366 nm) and vanillin/H₂SO₄ reagent followed by heating. Spraying with ninhydrin reagent followed by heating was used to detect the amino acid residue.

3.2. Plant material

Centaurea polyclada DC. (Asteraceae) was collected from the roadside of Kayadibi village district, Izmir, Turkey in June 2010 (500 m, 38°30'21.0" N, 27°14'35.6" E). The plant was confirmed by Assoc. Prof. Serdar Gokhan Senol from Section of Botany,

Department of Biology, Faculty of Science, Ege University and a voucher specimen (IZEF-5914) was deposited in the Herbarium of Ege University, Faculty of Pharmacy, Izmir, Turkey.

3.3. Extraction and isolation

Air-dried and grounded aerial parts of the plant (2.2 kg) were extracted with *n*-hexane, chloroform (CHCl₃) and methanol (MeOH) (3 × 5 L, each) by sonification for 24 h and then filtered. The extracts were separately evaporated under reduced pressure to dryness and yielded 44.3 g *n*-hexane, 35.9 g CHCl₃ and 121.3 g MeOH extracts. Dried MeOH extract was suspended in water and partitioned with *n*-butanol (*n*-BuOH) (H₂O: *n*-BuOH; 1:7) and yielded 34.5 g *n*-BuOH extract.

CHCl₃ extract (30 g) was dry loaded and chromatographed over RP-C18 column (200 g) (H₂O: MeOH, 80:20 to 0:100; 10% decreasing polarity, each 1 L) and 30 main fractions (Frs 1–30) were collected. Based on TLC profiles, 6 main fractions; C1 (Frs. 3–5), C2 (Frs. 8–9), C3 (Frs. 10–12), C4 (Frs. 14–18), C5 (Frs. 19–23) and C6 (Frs. 24–27) were selected for further purification.

Fraction C1 (827 mg) was subjected to Sephadex LH-20 column (80 g) using 100% MeOH and afforded 60 subfractions. Frs. C2/16–27 (335 mg) chromatographed over silica gel column (si-gel) (15 g) using CHCl₃:MeOH:H₂O (90:10:1, 200 ml; 85:15:0.5 and 80:20:2, 100 ml each) and subfractions 17–28 (143 mg) were re-chromatographed over si-gel with EtOAc:MeOH:H₂O (100:5:0.5, isocratic, 300 ml). Subfractions 61–75 (76 mg) were further purified using CHCl₃:MeOH:H₂O (90:10:1, isocratic, 100 ml) to afford **4** (31 mg).

Fraction C2 (970 mg) was chromatographed over si-gel (25 g) using CHCl₃:MeOH:H₂O (90:10:1, 80:20:1 and 70:30:3, 200 ml each) and 95 sub-fractions were obtained. Frs. 3–11 (285) were subjected to si-gel (10 g) with *n*-hexane:EtOAc (60:40 to 50:50, with 2% increasing polarity, 100 ml each). Subfractions 94–156 (137 mg) were re-chromatographed over si-gel (CHCl₃:MeOH:H₂O, 90:10:0.5, isocratic, 100 ml) and **5** (38 mg) was obtained.

Fraction C3 (1979 mg) was subjected to si-gel (35 g) with CHCl₃:MeOH:H₂O (90:10:1, 400 ml and 80:20:1, 200 ml) and subfraction 39–66 (772 mg) were purified with si-gel (EtOAc:MeOH:H₂O, 100:5:1, isocratic, 200 ml) to afford **10** (640 mg).

Fraction C4 (2685 mg) was chromatographed over si-gel column (40 g) using CHCl₃:MeOH:H₂O (90:10:1, 400 ml and 80:20:1, 200 ml) and finally treated with MeOH. MeOH fraction (212 mg) was further purified with CHCl₃:MeOH:H₂O (90:10:1, 200 ml and 85:15:0.5, 300 ml) over si-gel (15 g) and **3** (59 mg) was obtained.

A small amount of residue was observed in the fraction C5. Insoluble part (788 mg) was centrifuged and chromatographed over si-gel column (15 g) using CHCl₃ (300 ml) to afford **8** (247 mg). The soluble portion (1300 mg) was chromatographed over si-gel (25 g) with CHCl₃:MeOH:H₂O (90:10:1, 300 ml and 70:30:3, 100 ml) and subfractions 5–28 (730 mg) were subjected to Sephadex LH-20 column (50 g) using MeOH (500 ml). Subfractions 21–31 (51 mg) were further purified over si-gel (10 g) using CHCl₃ (100 ml) to yield **9** (9 mg).

Fraction C6 (1.4 g) was subjected to Sephadex LH-20 column (80 g) with MeOH (600 ml) and 120 subfractions were obtained. Subfractions 84–120 (78 mg) were chromatographed over si-gel column (CHCl₃, 200 ml) and yielded **7** (35 mg).

n-BuOH extract (30 g) was dry loaded and chromatographed over RP-C18 column (200 g) using H₂O:MeOH (100:0 to 0:100; 10% decreasing polarity, each 1 L). 30 main fractions (Frs 1–30) were collected and based on TLC profiles, 2 main fractions; B1 (Frs. 2–6) and B2 (Frs. 7–8) were selected for further purification.

Fraction B1 (6 g) was chromatographed over RP-C18 (100 g) using H₂O (500 ml) and 100 fractions were collected.

Fractions B1/30–39 (585 mg) were chromatographed over si-gel (45 g) with CHCl₃:MeOH:H₂O (70:30:3, 61:32:7, 64:50:10, 500 ml each) and subfractions 165–224 were re-chromatographed over si-gel (10 g) using CHCl₃:MeOH:H₂O (70:30:3, isocratic, 200 ml) to afford **1** (13 mg). Subfractions 225–261 (102 mg) were further purified with CHCl₃:MeOH:H₂O (61:32:7, isocratic, 300 ml) and yielded **2** (23 mg).

Fractions B1/40–57 (436 mg) were subjected to Sephadex LH-20 column (40 g) using MeOH (500 ml). Subfractions 28–40 (153 mg) were dry-loaded and chromatographed over si-gel (10 g) with CHCl₃:MeOH:H₂O (80:20:1, 100 ml; 80:20:2, 400 ml and 70:30:3, 100 ml) and 80 fractions were collected. Subfractions 20–28 (10 mg) and 45–80 (34 mg) were separately purified with CHCl₃:MeOH:H₂O (80:20:1, isocratic, 100 ml) to afford **11** (7 mg) and **6** (19 mg) respectively.

Fraction B2 (1375 mg) was chromatographed over Sephadex LH-20 column (80 g) with MeOH (500 ml) and afforded 150 subfractions. Fractions 19–39 (830 mg) were subjected to RP-C18 column (10 g) using H₂O:MeOH (100:0 to 80:20, 2% decreasing polarity, 100 ml each). The insoluble portion of subfractions 107–145 was centrifuged and **12** (169 mg) was obtained.

3.4. Acid treatment of 13-*N*-proline-6 α ,8 α ,15-trihydroxyelema-1,3-dien-12-oic acid (**2**)

Acid treatment of **2** (15 mg) with 1% aqueous hydrochloric acid (HCl, 10 ml) at room temperature affording a mixture (Matsuda et al., 2000). An aliquot of the reaction mixture was subjected to normal phase silica TLC (*n*-BuOH:Acetic acid:H₂O, 3:1:1) to detect the amino acid by Ninhydrin reagent followed by heating. After verifying the presence of proline, the mixture was dried and subjected to silica gel column (10 g) with CHCl₃:MeOH:H₂O (70:30:3 and 61:32:7/100 ml each). Subfractions 39–52 were re-chromatographed over Sephadex LH-20 (10 g) using MeOH (100 ml) to afford proline (4 mg). Optical rotation measurement verified the amino acid residue as L-proline ($[\alpha]_D^{20}$ -85, c. 0.001, MeOH) (Convention, 2005).

3.5. Compound characterization

13-*N*-proline melitensin (**1**): Yellowish gum; $[\alpha]_D^{20}$ -17.5 (c. 0.005, MeOH); ¹H NMR (CD₃OD, 400 MHz) and ¹³C NMR (CD₃OD, 100 MHz) data: see Tables 1 and 2; QTOF-MS *m/z* = 380.2028 [M+H]⁺ (calcd. for C₂₀H₃₀NO₆: 380.2073, positive mode) and 378.1921 [M-H]⁻ (calcd. for C₂₀H₂₈NO₆: 378.1917, negative mode).

13-*N*-proline-6 α ,8 α ,15-trihydroxyelema-1,3-dien-12-oic acid (**2**): Colorless gum; $[\alpha]_D^{20}$ -11.3 (c. 0.008, MeOH); IR (KBr) 3389, 2929, 1590, 1411, 1339, 1208, 1135, 1049, 1008 cm⁻¹; ¹H NMR (CD₃OD, 400 MHz) and ¹³C NMR (CD₃OD, 100 MHz) data: see Tables 1 and 2; QTOF-MS *m/z* = 398.1655 [M+H]⁺ (calcd. for C₂₀H₃₂NO₇: 398.2179, positive mode) and 396.2018 [M-H]⁻ (calcd. for C₂₀H₃₀NO₇: 396.2022, negative mode).

8 α -O-[2'-(hydroxymethyl)-but-2'-enoyl]-6 α ,15-dihydroxyelema-1,3,11(13)-trien-12-oic acid (**3**): Yellowish gum; $[\alpha]_D^{20}$ +27 (c. 0.003, MeOH); IR (KBr) 3405, 2931, 1702, 1396, 1238, 1165, 1052, 1002, 915 cm⁻¹; ¹H NMR (CD₃OD, 400 MHz) and ¹³C NMR (CD₃OD, 100 MHz) data: see Tables 1 and 2; QTOF-MS *m/z* = 403.1726 [M+Na]⁺ (calcd. for C₂₀H₂₈O₇Na: 403.1733, positive mode) and 379.1590 [M-H]⁻ (calcd. for C₂₀H₂₇O₇: 379.1757, negative mode).

Methyl 1 β ,6 α ,8 α -trihydroxy-15-oxo-eudesm-11(13)-en-12-oate (**4**): Yellowish gum; $[\alpha]_D^{20}$ -15 (c. 0.003, MeOH); IR (KBr) 3405, 2949, 2353, 1712, 1626, 1442, 1334, 1262, 1198, 1167, 1033 cm⁻¹; ¹H NMR (CD₃OD, 400 MHz) and ¹³C NMR (CD₃OD, 100 MHz) data: see Tables 1 and 2; QTOF-MS *m/z* = 335.1466 [M+Na]⁺ (calcd. for

C₁₆H₂₄O₆Na: 335.1471, positive mode).

6 α ,8 α ,15-trihydroxyelema-1,3,11(13)-trien-12-oiic acid (**5**): Yellowish gum; ¹H NMR (CD₃OD, 400 MHz) and ¹³C NMR (CD₃OD, 100 MHz) data: see Tables 1 and 2; QTOF-MS *m/z* = 587.2832 [2M+Na]⁺ (calcd. for C₃₀H₄₄O₁₀Na: 587.2832, positive mode) and 281.1371 [M-H]⁻ (calcd. for C₁₅H₂₁O₅: 281.1389, negative mode).

Methyl 6 α ,8 α ,15-trihydroxyelema-1,3,11(13)-trien-12-oate (**6**): Yellowish gum; ¹H NMR (CD₃OD, 400 MHz) and ¹³C NMR (CD₃OD, 100 MHz) data: see Tables 1 and 2; QTOF-MS *m/z* = 319.1518 [M+Na]⁺ (calcd. for C₁₆H₂₄O₅Na: 319.1521, positive mode).

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Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.phytochem.2017.07.002>.

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