Bioactivity of the extracts and isolation of lignans and a sesquiterpene from the aerial parts of Centaurea pamphylica (Asteraceae).

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ABSTRACT

Centaurea pamphylica Boiss. & Heldr. (Family: Asteraceae), commonly known as 'pamphylia daisy', is a Turkish endemic species of the genus *Centaurea* that comprises *ca.* 500 species, many of which have been used as traditional medicines.

The *n*-hexane, dichloromethane (DCM) and methanol (MeOH) extracts of the aerial parts of *C. pamphylica* were assessed for antioxidant activity and general toxicity using, respectively, the 2,2-diphenyl-1-picrylhydrazyl (DPPH), and the brine shrimp lethality assays. The reversed-phase preparative HPLC and PTLC were used to isolate compounds from the extracts. The structures of these compounds [**1**-**4**] were elucidated by spectroscopic means, and also by direct comparison with the respective published data.

Both the DCM and the MeOH extract showed significant levels of antioxidant activities with the RC_{50} values of 72.6 x 10^{-2} and 47.3 × 10^{-2} mg/mL, respectively. The MeOH extract exhibited low levels of toxicity towards brine shrimps (LD_{50} = 125.0 × 10^{-2} mg/mL). Three major bioactive components of the MeOH extract were matairesinoside [**1**], arctiin [**2**] and matairesinol [**3**]. An eudesmane-type sesquiterpene, pterodontriol [**4**], was also isolated from the DCM extract.

Since reactive oxygen species are important contributors to various ailments, the antioxidant properties of the extracts as well as the isolated compounds may be of medicinal significance. This is the first report on the occurrence of **1**-**4** in *C. pamphylica*, and **4** in the genus *Centaurea*.

Keywords: *Centaurea pamphylica*, Asteraceae, lignan, DPPH, brine shrimp lethality assay

INTRODUCTION

Centaurea pamphylica Boiss. & Heldr. (Section: *Calcitrapa*; Family: Asteraceae *alt.* Compositae), commonly known as 'pamphylia daisy', is a Turkish endemic species that is distributed in the Mediterranean and Anatolian regions of Turkey (1). It is a biennial herb (~80 cm) with pale pink flowers, and grows abundantly on the roadsides, fields and wastelands. While the genus *Centaurea* is known to produce a variety of secondary metabolites (2), there have been no phytochemical or pharmacological studies carried out on this species to date. As a part of our on-going studies on the genus *Centaurea* (3-9), in this study the assessment of the extracts of *C. pamphylica* for antioxidant activity and general toxicity using, respectively, the DPPH and the brine shrimp lethality assays, and the isolation and identification of three major bioactive lignans from the MeOH extract, and an eudesmane-type sesquiterpene from the DCM extract of aerial parts of this plant is reported.

MATERIALS AND METHODS

General procedures

UV spectra were obtained in MeOH using a Hewlett-Packard 8453 UV-Vis spectrometer (Agilent, Germany). CIMS (Chemical Ionisation Mass Spectrometry) analyses were performed in EPSRC Central Mass Spectroscopy Facility in Swansea, UK, on a Micromass Quattro II triple quadrupole instrument (Waters, UK) in chemical desorption mode using ammonia as CI gas. Mass accuracy was within 0.4 Da. CI source temperature was 170 °C and electron energy was 59 eV. NMR spectra were recorded in CD_3OD on a Varian Unity INOVA 400 MHz NMR Spectro-

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meter 400 (400 MHz for ^{1}H and 100 MHz for ^{13}C) using the residual solvent peaks as internal standard. Chemical shifts were in ppm. The HMBC (Heteronuclear Multiple Bond Coherence) experiment used J = 9Hz and had a 55 ms long-range coupling delay. A NOESY experiment was carried out with a mixing time of 0.8 s. Spectra were recorded with a probe temperature of 25 °C. Preparative reversed-phase HPLC was carried out in a Dionex 580 HPLC system coupled with a UVD340S photo-diode-array detector and Gina50 autosampler (Gynkotek). A Luna C_{18} preparative column (21.2 x 250 mm, 10 μm) from Phenomenex (UK) was used. Sep-Pak Vac (Waters, USA) 10 g cartridge was used for pre-HPLC fractionation of the MeOH extract. 2,2-Diphenyl-1-picrylhydra-zyl (molecular formula $C_{18}H_{12}N_5O_6$, DPPH) and quercetin (quercetin dihydrate >99%) were purchased from Fluka (UK) and were used without further purification. Precoated aluminium sheets silica gel 60 F_{254} (0.25 mm thickness) TLC plates (Merck, Germany) were used for the analyses. TLC mobile phases were *n*-hexane-ethylacetate (EtOAc) mixtures of various proportions, e.g. 5% EtOAc in *n*-hexane, 10% EtOAc in *n*-hexane, etc. Silica 60G was used for vacuum liquid chromatography (VLC).

Plant material

The aerial parts of *C. pamphylica* Boiss. & Heldr. were collected from West and East Anatolia, Turkey, during September-October 2002. A voucher specimen (PHSH0011) has been deposited in the herbarium of Plant and Soil Science Department, University of Aberdeen, Scotland, UK, and Canakkale Onsekiz Mart University, Turkey (COMU).

Extraction, isolation and structure elucidation

Dried ground aerial parts of *C. pamphylica* (100 g) were Soxhlet-extracted successively with *n*-hexane, DCM and MeOH (1.1 L each). All three extracts were concentrated using a rotary evaporator at a temperature not exceeding 45 °C. The MeOH extract was fractionated by solid phase extraction method using a Sep-Pak C_{18} (10 g) cartridge eluting with a step gradient: 40, 60, 80 and 100% MeOH in water (200 mL each). Preparative-HPLC (Luna C_{18} column 10 μm, 250 mm × 21.2 mm, eluted with a linear gradient-water:MeOH= 75:25 to 30:70 over 50 min followed by 70% MeOH for 10 min, 15 mL min^{-1}) of the Sep-Pak fraction, which was eluted with 40% MeOH, yielded lignans **1** (19.7 mg, t_R = 22.1 min), **2** (44.2 mg, t_R = 26.5 min) and **3** (12.6 mg, t_R = 30.5 min). The *n*-hexane extract was fractionated by VLC eluting with *n*-hexane-EtOAc mixture of increasing polarity resulting in eight fractions. The ^{1}H and ^{13}C NMR spectra of the VLC fractions revealed that the fractions contained mainly long chain fatty alcohols and acids, and were not processed further. However, the DCM extract was subjected to normal phase column chromatography on Silica gel eluting with *n*-hexane-DCM-MeOH mixture of increasing polarity. After monitoring by TLC, similar fractions were pooled together and **4** (4.7 mg) was obtained from the column fractions eluted with DCM-MeOH (19:1) by preparative TLC.

Matairesinoside [**1**]. Gum, $[\alpha]^{23}_{D}$ -48.8° (*c* 0.0022, MeOH); UV λ_{max} (MeOH) nm: 279, 222; CIMS *m/z* 538 $[NH_4]^+$; ^{1}H NMR (400 MHz, CD_3OD) and ^{13}C NMR (100 MHz, CD_3OD): as published data (5).

Arctiin [**2**]. Gum, $[\alpha]^{23}_{D}$ -55.3° (*c* 0.0033, MeOH); UV λ_{max} (MeOH) nm: 279, 225; CIMS *m/z* 552 $[M+NH_4]^+$; ^{1}H NMR (400 MHz, CD_3OD) and ^{13}C NMR (100 MHz, CD_3OD): as published data (3, 5).

Matairesinol [**3**]. Gum., $[\alpha]^{23}_{D}$ -47.2° (*c* 0.0022, MeOH); UV λ_{max} (MeOH) nm: 282, 228; CIMS *m/z* 376 $[M+ NH_4]^+$; ^{1}H NMR (400 MHz, CD_3OD) and ^{13}C NMR (100 MHz, CD_3OD): as published data (5).

Pterodontriol [**4**]. Amorphous solid, m.p. 180° C; CIMS *m/z* 274 $[M+NH_4]^+$; ^{1}H NMR (400 MHz, CD_3OD): (ppm) 4.22 m (H-6), 3.67 t (H-1, J=6.2 Hz), 2.25 m (H-7), 2.14 d (H-5, J=11.0 Hz), 1.04-1.98 (H_2-2, H_2-3, H_2-8, H_2-9) m, 1.96 m (H-11), 1.60 s (Me-15), 1.57 m, 1.34 d (Me-12, J=6.6 Hz), 1.22 s (Me-14), 0.96 d (Me-13, J=6.6 Hz) and ^{13}C NMR (100 MHz, CD_3OD): (ppm) 76.6 (C-1), 29.5 (C-2), 32.0 (C-3), 69.0 (C-4), 47.9 (C-5), 73.1 (C-6), 51.2 (C-7), 24.7 (C-8), 34.0 (C-9), 34.0 (C-10), 28.8 (C-11), 24.8 (C-12), 29.1 (C-13), 14.1 (C-14) and 22.7 (C-15).

Antioxidant assay

DPPH solution in MeOH (80 μg/mL) was used in this assay (10) to assess the free radical scavenging (antioxidant) property of the extracts as well as the isolated compounds (**1**-**4**). Quercetin, a well known natural antioxidant was used as a positive control.

Qualitative assay: Test extracts (10 mg/mL) and compounds **1**-**4** (1 mg/mL) were used. While the DCM and MeOH extracts, the test compounds and the positive control quercetin were dissolved in MeOH, the *n*-hexane extract and trolox were in chloroform. Test materials were applied on a

TLC plate and sprayed with DPPH solution using an atomiser and was allowed to develop for 30 min. The white spots against a pink background indicated the antioxidant activity. The same procedure was followed for the positive controls, quercetin and trolox.

Quantitative assay

Stock solutions of the test materials were diluted to obtain concentrations of $10x10^{-1}$, $5x10^{-1}$, $5x10^{-2}$ 5×10^{-3}, 5×10^{-4}, 5×10^{-5}, 5×10^{-6} 5×10^{-7}, 5×10^{-8}, 5×10^{-9}, 5×10^{-10} mg/mL. Diluted solutions (1.00 mL each) were mixed with DPPH (1.00 mL) and allowed to stand for 30 min for any reaction to occurs. The UV absorbance of these solutions was recorded at 517 nm. The experiment was performed in triplicate and the average absorption was noted for each concentration. The same procedure was followed for the positive controls, quercetin and trolox.

Brine Shrimp Lethality assay:

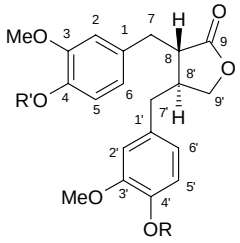
Brine shrimp eggs were purchased from Water Life, Middlesex, UK. The bioassay was conducted following the procedure published previously (11). The LD_{50} values were determined from the 24 h counts using the Probit analysis method (12). Percentage of mortalities were adjusted relative to the natural mortality rate of the control, following Abbots formula P = (Pi – C)/(1– C), where P denotes the observed non-zero mortality rate and C represents the mortality rate of the control.

RESULTS AND DISCUSSION

Reversed-phase preparative HPLC analysis of the MeOH extract of the aerial parts of *C. pamphylica* afforded three dibenzylbutyrolactone-type lignans, matairesinoside [**1**], arctiin [**2**] and matairesinol [**3**], and a combination of CC and PTLC of the DCM extract produced an eudesmane-type sesquiterpene, pterodontriol [**4**] (Fig 1). The structures of these compounds were elucidated by comprehensive spectroscopic analyses, and also by direct comparison with the respective published data.

Compounds **1-3** displayed characteristic UV absorption maxima of dibenzylbutyrolactone-type lignans (5). The ^{1}H and ^{13}C NMR spectra of these compounds also supported this fact. A CIMS spectrum of **2** revealed the $[M+NH_4]^+$ ion peak at *m/z* 552, suggesting *M*r=534, and the molecular formula $C_{27}H_{34}O_{11}$. The ^{1}H and ^{13}C NMR spectral data of **2** were identical to those published for arctiin [**2**] (3, 5). A combination of HSQC, HMBC, COSY and NOESY 2D NMR spectral analyses led to the unambiguous assignment of all ^{1}H and ^{13}C NMR signals of **2** and confirmed unequivocally its identity as arctiin [**2**].

The ^{1}H and ^{13}C NMR spectra of **1** and **3** displayed similar signals with the exception that the signals due to the β-D-glucopyranosyl moiety were absent in the spectra of **3**. The ^{1}H and ^{13}C NMR data of **1** were similar to those of arctiin [**2**] with the exception that one methoxyl signal was missing. The CIMS spectrum of **1** revealed $[M+NH_4]^+$ ion at *m/z* 538, suggesting *Mr*=520, and the molecular formula $C_{26}H_{32}O_{11}$ which also confirmed the findings from the NMR data that it contained 14 mass units less than arctiin [**2**], i.e. instead of a methoxyl group, it had a hydroxyl group present. The ^{1}H and ^{13}C NMR data of **1** were in good agreement with the published data of matairesinoside (5). The CIMS spectrum of **3** revealed $[M+NH_4]^+$ ion at *m/z* 376, suggesting Mr=358, and the molecular formula $C_{20}H_{22}O_6$ which confirmed the findings from the NMR data that, unlike **1**, it did not have a glucosyl moiety.



Compound	R	R'
1	H	Glucosyl
2	Me	Glucosyl
3	H	H

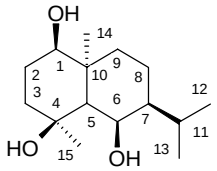


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Figure 1. Structure of compounds [**1-4**] isolated from *C. pamphylica* (Asteraceae)

The spectroscopic data of **3** were in good agreement with the published data of matairesinol (5). Detailed 2D NMR spectral analyses helped to assign unambiguously all the ^{1}H and ^{13}C NMR signals of **1** and **3**, and confirmed unequivocally their identities respectively, as matairesinoside [**1**] and matairesinol [**3**]. The lignans **1-3** were previously reported from a few other species of the genus *Centaurea*. Matairesinoside [**1**] was isolated from *C. americana*, *C. bornmuelleri*, *C. dealbata*, *C. huber-morathii*, *C. macrocephala*, *C. nigra*, *C. scabiosa*, *C. sclerolepis* and *C. schischkinii* (13). In addition to all above species, arctiin [**2**] was also found in *C. alexandria*, *C. isaurica*, *C. melitensis* and *C. sphaerocephala*. Matairesinol [**3**] was reported from *C. affinis*, *C.*

Table 1. Antioxidant activity and general toxicity of the extracts of *C. pamphylica* and isolated compounds (**1-4**)

Compounds/Extracts	DPPH assay (RC_{50} in mg/mL)	Brine Shrimp Lethality assay (LD_{50} in mg/mL)
n-Hexane extract	-	ND
DCM extracts	72.6×10^{-2}	-
MeOH extracts	47.3×10^{-2}	125.0×10^{-2}
1	2.2×10^{-3}	16.0×10^{-3}
2	16.0×10^{-2}	98.0×10^{-3}
3	2.0×10^{-3}	5.5×10^{-3}
4	-	-
Podophyllotoxin	NA	2.79×10^{-3}
Quercetin (in MeOH)	2.88×10^{-5}	NA
Trolox (in DCM)	2.58×10^{-3}	NA

- = No activity detected at test concentrations; NA = Not applicable, ND = Not determined

*americana, C. bornmuelleri, C. calcitrapa, C. huber-morathii, C. macrocephala, C. ptosimopappa, C. scoparia, C. solstitialis, C. schischkin*ii, *C. tweedei C. nicoli, C. nigra, C. persica, C. raphanina, C. scabiosa* and *C. sphaerocephala* (14).

The CIMS spectrum of **4** showed the *pseudo* molecular ion peak $[M+NH_4]^+$ at *m/z* 274 and the molecular formula was calculated as $C_{15}H_{28}O_3$. The ^{13}C NMR spectrum of **4** exhibited signals for fifteen carbons. The ^1H NMR spectrum showed the presence of four methyl groups, of which, two appeared as doublets at δ_H 1.34 and 0.96 (J=6.6 Hz), and the other two as singlets at δ_H 1.22 and 1.60. A signal at δ_C 28.8 in the ^{13}C NMR spectrum was attributable to the methine C-11 which implied the presence of an isopropyl group. Thus, the ^1H and ^{13}C NMR data of **4** suggested the presence of an eudesmane skeleton (15). The ^1H and ^{13}C NMR data were also identical to those published for pterodontriol **4**, previously isolated from *Laggera pterodonta* (16). However, this is the first report on the occurrence of **4** in the genus *Centaurea*.

In the DPPH assay (10), apart from the *n*-hexane extract, the other two extracts, DCM and MeOH, showed considerable antioxidant activity with RC_{50} values of 72.6×10^{-2} and 47.3×10^{-2} mg/mL, respectively (Table 1). All lignans [**1-3**], isolated from the MeOH extract, showed significant antioxidant activity and the RC_{50} values were identical to those published in the literature (3, 5). The antioxidant activity of **1-3**, like any other natural phenolic antioxidants is a consequence of the presence of the phenolic moieties in the structures. The antioxidant activity of phenolic natural products is predominantly due to their redox properties, i.e. the ability to act as reducing agents, hydrogen donors and singlet oxygen quenchers, and to some extent, could also be due to their metal chelation potential. The sesquiterpene **4** did not show any antioxidant activity at test concentrations.

The brine shrimp lethality assay (11), which has been proven to be an effective and rapid assay method to screen compounds for potential general toxicity and cytotoxic activity was used to determine the general toxicity of the extracts as well as compounds **1-4**. However, owing to high degree of lipophilicity, *n*-hexane extract could not be tested in this assay. While only the MeOH extract showed low levels of toxicity towards brine shrimps ($LD_{50} = 125.0 \times 10^{-2}$ mg/mL), the isolated lignans [**1-3**] were toxic towards brine shrimps. The LD_{50} values of **1-3** were identical to those published previously (3,5). The sesquiterpene **4** exhibited no toxicity towards brine shrimp at test concentrations.

CONCLUSION

It has been established that the presence of lignans **1-3** in high amounts in the MeOH extract contributed to the antioxidant property as well as brine shrimp toxicity of the MeOH extract of *C. pamphylica.* Since reactive oxygen species are important contributors to tissue injury, inflammation, cancer and many other ailments, the antioxidant properties of the extracts as well as the compounds **1-3** may be of medicinal significance.

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