Phenolic Compounds with Antioxidant Activity from *Anthemis tinctoria* L. (Asteraceae)

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From the aerial parts of *Anthemis tinctoria* L. subsp. *tinctoria* var. *pallida* DC. (Asteraceae), one new cyclitol glucoside, conduritol F-1-*O*-(6′-*O*-*E*-*p*-caffeoyl)-*β*-D-glucopyranoside (**1**), has been isolated together with four flavonoids, nicotiflorin (**2**), isoquercitrin (**3**), rutin (**4**) and patulitrin (**5**). The structures of the isolated compounds were established by means of NMR, MS, and UV spectral analyses. Methanolic extract and pure isolated compounds were examined for their free radical, scavenging activity, using the 1,1-diphenyl-2-picrylhydrazyl (DPPH) free stable radical, and for their inhibitory activity toward soybean lipoxygenase, using linoleic acid as substrate. Compounds **1** and **5** showed a strong scavenging effect in the DPPH radical assay. In addition **5** also exhibited high inhibitory activity on soybean lipoxygenase.

Key words: Anthemis tinctoria subsp. *tinctoria pallida*, Asteraceae, Flavonoids, Conduritol F

Introduction

The genus *Anthemis* comprises about 130 species predominately distributed around the Mediterranean (Heywood and Humphries, 1978). The species of the *Anthemis* genus are widely used in pharmaceutics, cosmetics and food industry. The flowers of the genus have well-documented use as antiseptic and healing herbs, the main components being natural flavonoids and essential oils. In Europe extracts, tinctures, tisanes (teas), and salves are widely used as anti-inflammatory, antibacterial, antispasmodic, and sedative agents. Extracts are used to allay pain and irritation, clean wounds and ulcers, and aid prevention as well as therapy of irradiated skin injuries, treatment of cystitis and dental afflictions (Mann and Staba, 1986). Continuing our chemotaxonomic examinations of the Greek flora belonging to Asteraceae and our search for new compounds of pharmacological interest, we now report the investigation of the aerial parts of *Anthemis tinctoria* L. subsp. *tinctoria* var. *pallida* DC.

Materials and Methods

General experimental procedures

The $[\alpha]_D$ values were obtained in MeOH at 20 °C on a Perkin-Elmer 341 polarimeter. IR spectra were obtained on a Perkin-Elmer Paragon 500 instrument. UV spectra were recorded on Shimadzu UV-160A and Hitachi U-2000 spectrophotometers according to Mabry *et al.* (1970). The ^{1}H NMR spectra (400 MHz) and ^{13}C NMR spectra (50 and 100 MHz) were recorded in CD_3OD using Bruker DRX 400 and Bruker AC 200 spectrometers. Chemical shifts are reported in δ (ppm) values relative to TMS. COSY, HMQC, HSQC, HMBC and NOESY (mixing time 950 ms) were performed using standard Bruker microprograms. High resolution ESI mass spectral data were recorded on a TSQ 7000 mass spectrometer. Vacuum-liquid chromatography (VLC): silica gel 60H (Merck Art. 7736). Column chromatography: silica gel 60 (Merck Art. 9385), gradient elution with the solvent mixtures indicated in each case; Sephadex

LH-20 (Pharmacia), elution with MeOH. Absorbents for TLC: Merck RP 18 F_{254s}, Art. 5685; Merck silica gel 60 F_{254s}, Art. 5554; Merck cellulose, Art. 5716. Detection on TLC plates (silica gel): UV light, vanillin-H_2SO_4 spray reagent; Neu spray reagent on cellulose (Neu, 1957).

Plant material

Aerial parts of *Anthemis tinctoria* L. subsp. *tinctoria* var. *pallida* DC. were collected on Mount Pelion in June 2002 and authenticated by Dr. Th. Constantinidis (Institute of Systematic Botany, Agricultural University of Athens). A voucher specimen is deposited in the Herbarium of Laboratory of Pharmacognosy, University of Athens under the number Skaltsa & Lazari 135.

Extraction and isolation

Air-dried powdered aerial parts of the plant (0.74 kg) were extracted at room temperature with cyclohexane/Et_2O/MeOH (1:1:1 v/v/v). The extract was washed with brine, the aqueous layer re-extracted with EtOAc, and the organic layer dried with Na_2SO_4 and concentrated under reduced pressure. In continuation, the plant was exhaustively extracted with methanol at room temperature. The residue of EtOAc (5.4 g) was prefractionated by VLC on silica gel (8.5 × 6.0 cm), using hexane/EtOAc/Me_2CO mixtures of increasing polarity as eluents to give eight fractions of 500 mL each: A (hexane/EtOAc, 75:25 v/v), B (hexane/EtOAc, 50:50 v/v), C (hexane/EtOAc, 25:75 v/v), D (EtOAc, 100%), E (EtOAc/Me_2CO, 90:10 v/v), F (EtOAc/Me_2CO, 75:25 v/v), G (Me_2CO, 100%) and H (MeOH, 100%). Fraction H (3.9 g) was subjected to further chromatographic separations as described below. VLC of fraction H (CH_2Cl_2/MeOH, 10:0 to 0:10 v/v) followed by several CC on silica gel and Sephadex LH-20, allowed the isolation of **2** (34.5 mg). The methanol extract was concentrated and 22.0 g of the residue (36.9 g) were subjected to VLC on silica gel using CH_2Cl_2/MeOH mixtures of increasing polarity as eluents to give several fractions. VLC of fraction N (4.1 g) (CH_2Cl_2/MeOH, 70:30 – CH_2Cl_2/MeOH, 60:40), followed by several CC on silica gel and Sephadex LH-20, allowed the isolation of **1** (7.9 mg), **2** (9.4 mg), **3** (2.4 mg), **4** (17.5 mg) and **5** (4.5 mg).

Conduritol F-1-O-(6′-O-E-p-caffeoyl)-β-D-glucopyranoside (**1**): Amorphous yellow powder (7.9 mg). – UV (MeOH): λ_{max} = 290.5 nm (log ε = 3.80), 325 nm (log ε = 3.78). – $[\alpha]_D^{20}$ −11.73° (MeOH, *c* 0.09). – ESI-HRMS (pos.): *m/z* = 471.1498 $[M+H]^+$ (required for $C_{21}H_{26}O_{12}$ 471.1503). – ^1H and ^{13}C NMR spectral data: see Table I.

Table I. ^1H (CD_3OD, 400 MHz, *J* in Hz) and ^{13}C (50 MHz) NMR data of compound **1** (at 295 K).

Position	δ_H	δ_C
Conduritol F		
1	4.18 *dd* (*J* = 4.6, 4.1)	76.2
2	3.50 *dd* (*J* = 9.9, 3.7)	72.3
3	3.67 *dd* (*J* = 9.9, 7.5)	74.8
4	3.94 br *d* (*J* = 7.4)	74.0
5	5.79 *dd* (*J* = 10.0, 2.5)	135.8
6	5.89 *ddd* (*J* = 9.9, 4.6, 1.6)	126.0
Glucose		
1′	4.46 *d* (*J* = 7.9)	103.8
2′	3.28 *dd* (*J* = 7.8, 9.1)	74.7
3′	3.39 *t* (*J* = 7.8)	78.0
4′	3.39 *t* (*J* = 7.8)	71.9
5′	3.58 *ddd* (*J* = 9.9, 6.2, 2.1)	75.9
6′a	4.56 *dd* (*J* = 12.0, 2.1)	64.7
6′b	4.27 *dd* (*J* = 12.0, 6.2)	
Caffeoyl group		
1″	–	128.1
2″	7.05 *d* (*J* = 2.1)	115.9
3″	–	147.6
4″	–	149.8
5″	6.78 *d* (*J* = 8.3)	117.1
6″	6.96 *dd* (*J* = 1.6, 8.3)	123.7
7″	7.60 *d* (*J* = 15.8)	147.4
8″	6.30 *d* (*J* = 16.2)	115.2
9″	–	169.8

Scavenging activity on DPPH radical

The free radical scavenging activity of the extracts and isolates was performed using the DPPH method, as previously described (Kontogiorgis and Hadjipavlou-Litina, 2003). Briefly, 1 mL (0.1 mM) solution of DPPH in ethanol was added to an equal volume of the tested extract (20 μL or 200 μL in DMSO) and compounds (final concentration 0.1 mM and 0.2 mM) and left at room temperature for 20 and 60 min. After incubation the absorbance was recorded at 517 nm

Soybean lipoxygenase inhibition

The bioassay was performed according to a previously described procedure (Kontogiorgis and Hadjipavlou-Litina, 2003). All samples (extract and isolates) were initially dissolved in DMSO (approx. 50 mg in 2 mL DMSO for plant extract).

The incubation mixture consisted of several aliquots of the test sample, 100 μL of sodium linoleate (0.1 mM) and 0.2 mL of the enzyme solution ($1/3 \times 10^{-4}$, w/v in saline). After incubation at room temperature for 3 min the conversion of the sodium linoleate to 13-hydroperoxylinoleic acid was recorded at 234 nm and compared with an appropriate standard inhibitor (quercetin). The same procedure was followed for the pure compounds in order to determine the IC_{50} value of each compound.

Results and Discussion

From the methanolic extract of the aerial parts of *A. tinctoria* subsp. *tinctoria* var. *pallida* one new cyclitol glucoside, conduritol F-1-*O*-(6′-*O*-*E*-*p*-caffeoyl)-β-D-glucopyranoside (**1**) (Fig. 1) has been isolated together with four flavonoids, nicotiflorin (**2**) (Vermes *et al.*, 1976), isoquercitrin (**3**) (Pakudina *et al.*, 1970; Lee *et al.*, 2004), rutin (**4**) (Harborne, 1967) and patulitrin (**5**) (Ulubelen *et al.*, 1980) by repeated chromatographic separation on silica gel 60 (Merck) and Sephadex LH-20.

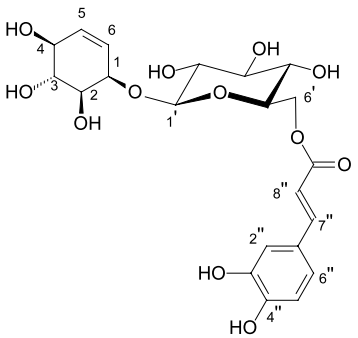

Fig. 1. Structure of **1**.

Compound **1** was obtained as an amorphous yellowish powder and was identified as conduritol F-1-*O*-(6′-*O*-*E*-*p*-caffeoyl)-β-D-glucopyranoside by 1D and 2D NMR spectroscopic analyses and by MS spectrometry. Its ESI-HRMS spectrum exhibited a pseudomolecular ion $[M+H]^+$ at *m/z* 471.1498, compatible with the molecular formula $C_{21}H_{26}O_{12}$. In agreement, 21 carbon signals were observed in the ^{13}C NMR spectrum. Besides the 6 carbon signals of a sugar moiety, the ^{13}C NMR spectrum of **1** exhibited 9 carbon signals indicating the presence of an acyl moiety and 6 carbon signals belonging to a cyclitol group. The IR spectrum showed absorption bands typical of a hydroxy group (3364 cm^{-1}), α,β-unsaturated ester (1691 cm^{-1}) and aromatic ring (1599 and 1518 cm^{-1}). Accordingly, the ^1H NMR spectrum of **1** exhibited proton signals characteristic of an *E*-caffeoyl group (three aromatic protons resonating at δ_H 7.05–6.78 as an ABX system and two *trans* olefinic protons as an AB system at δ_H 7.60, 6.30, J = ~16.0 Hz). A doublet at δ_H 4.46 (J = 7.9 Hz, H-1′), corresponding to C-1′ at δ_C 103.8, pointed to the presence of a β-glucose. This was confirmed by a signal of H-2′ resonating as a *dd* (J = 7.8, 9.1 Hz) at δ_H 3.28. Protons H-3′ and H-4′ appeared together as a triplet (J = 7.8 Hz) at δ_H 3.39, H-5′ resonated as a *ddd* (J = 9.9, 6.2, 2.1 Hz) at δ_H 3.58, and finally, protons H-6′a and H-6′b were shifted downfield to δ_H 4.56 and 4.27, respectively.

The remaining six signals revealed the presence of a cyclitol moiety. From COSY as well as HSQC and DEPT experiments the following sequence was assigned: $-CH_{(5)}=CH_{(6)}-CH_{(1)}(OH)-CH_{(2)}(OH)-CH_{(3)}(OH)-CH_{(4)}(OH)-CH_{(5)}=$. Chemical shifts of protons and carbon atoms, as well as coupling constants revealed the presence of the cyclitol conduritol F (or leucanthe-

Table II. Radical scavenging activities of the methanolic extract and compounds **1**–**5** determined by the reduction of DPPH stable free radical.

Compound	0.1 mM		0.2 mM	
	% interaction with DPPH in 20 min	% interaction with DPPH in 60 min	% interaction with DPPH in 20 min	% interaction with DPPH in 60 min
Caffeic acid	5.2	7.1	11.9	12.0
1	85.5	85.5	92	93
2	52.5	61.2	76.8	76.8
3	65.6	64.5	66.6	65.2
4	3.7	7.1	29.4	19.1
5	81.4	78.1	76.9	66.8
Methanolic extract	98.6	100	nt	nt

nt, not tested.
The results are presented as means of 4–6 measurements (±SD < 10%).
Reference compound was caffeic acid.

mitol) previously isolated as a natural product from several plant sources (Abe *et al.*, 1998; 2000; El-Hassan *et al.*, 2003; Kindl and Hoffmann-Ostenhof, 1966; Kindl *et al.*, 1967; Li *et al.*, 1992).

The linkage of the *trans* caffeoyl group to the sugar at position C-6′ was deduced from the downfield shifted signals of H-6′a and H-6′b. This was confirmed by HMBC crosspeaks between C-9″/H_2-6′a. In the same spectrum a crosspeak between H-1′/C-1 proved the linkage between conduritol F and glucose.

The radical scavenging activities of the methanolic extract and isolates have been evaluated in duplicate in a DPPH assay (in comparison to caffeic acid) and are summarized in Table II. Methanolic extract strongly reacts with the stable free radical DPPH. This reaction was found to be independent by time (20–60 min). The isolated compounds were tested in concentrations of 0.1 mM and 0.2 mM and showed, with the exception of compound **4**, also high radical scavenging activity (**1** > **5** > **3** > **2**). Compound **1** was found to be the most active showing a 85.5% inhibition within one minute (data not shown). Compounds **2** and **3** showed weaker activity, whereas patulitrin (**5**) is nearly as active as the new cyclitol derivative. Interesting enough, compounds showing a very similar caffeoyl substructure showed very different reactivity towards the DPPH radical.

The samples were further evaluated for inhibition of soybean lipoxygenase (LOX). Results are summarized in Table III. Again the examined methanol extract (Table IV) showed a remarkable and concentration-dependent inhibition of LOX.

Table III. IC_{50} inhibition values of soybean LOX by compounds **1**–**5** in 3 min.

Compound	IC_{50} [μM]
Quercetin	184
1	545
2	280
3	395
4	200
5	35.5

Also, all the tested compounds inhibited significantly LOX (IC_{50} from 35.5 μM to 545 μM). Compound **5** (IC_{50} 35.5 μM) seemed to be the most potent inhibitor in comparison to the used reference compound quercetin (184 μM).

Patulitrin (**5**), one of the constituents of the methanol extract, demonstrated significant inhibitory activity against the DPPH radical and soybean LOX. In the context of the results of the other metabolites tested, it can be assumed that the antioxidant and lipoxygenase activity of *A. tinctoria* subsp. *tinctoria* var. *pallida* could be attributed to its high content of phenolic compounds. Thus, the methanol extract of *A. tinctoria* subsp. *tinctoria* var. *pallida* is a product with antioxidant activity and it is proposed to use it in pharmaceutical preparations and cosmetic formulations, since radical scavenging activity is strongly related to the antiaging process.

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Compound	% inhibition				
	IC_{50} [μM]	0.1 mM	0.2 mM	0.5 mM	1.0 mM
Methanolic extract		39.5	80.9	99.7	70.8
Quercetin	184	23.4	55.2	98.7	98.1

Table IV. % inhibition of soybean LOX by the methanolic extract in 3 min.

The results are presented as means of 4–6 measurements (±SD < 10%). Reference compound was quercetin.

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