

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 pharmaceuticals, 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 ¹H NMR spectra (400 MHz) and ¹³C NMR spectra (50 and 100 MHz) were recorded in CD₃OD 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-H₂SO₄ 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/Et₂O/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 Na₂SO₄ 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 pre-fractionated by VLC on silica gel (8.5 × 6.0 cm), using hexane/EtOAc/Me₂CO 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/Me₂CO, 90:10 v/v), F (EtOAc/Me₂CO, 75:25 v/v), G (Me₂CO, 100%) and H (MeOH, 100%). Fraction H (3.9 g) was subjected to further chromatographic separations as described below. VLC of fraction H (CH₂Cl₂/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 CH₂Cl₂/MeOH mixtures of increasing polarity as eluents to give several fractions. VLC of fraction N (4.1 g) (CH₂Cl₂/MeOH, 70:30 – CH₂Cl₂/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

Table I. ¹H (CD₃OD, 400 MHz, *J* in Hz) and ¹³C (50 MHz) NMR data of compound **1** (at 295 K).

| Position | δ _H | δ _C |
|----------------|---|----------------|
| Conduritol F | | |
| 1 | 4.18 <i>dd</i> (<i>J</i> = 4.6, 4.1) | 76.2 |
| 2 | 3.50 <i>dd</i> (<i>J</i> = 9.9, 3.7) | 72.3 |
| 3 | 3.67 <i>dd</i> (<i>J</i> = 9.9, 7.5) | 74.8 |
| 4 | 3.94 <i>br d</i> (<i>J</i> = 7.4) | 74.0 |
| 5 | 5.79 <i>dd</i> (<i>J</i> = 10.0, 2.5) | 135.8 |
| 6 | 5.89 <i>ddd</i> (<i>J</i> = 9.9, 4.6, 1.6) | 126.0 |
| Glucose | | |
| 1' | 4.46 <i>d</i> (<i>J</i> = 7.9) | 103.8 |
| 2' | 3.28 <i>dd</i> (<i>J</i> = 7.8, 9.1) | 74.7 |
| 3' | 3.39 <i>t</i> (<i>J</i> = 7.8) | 78.0 |
| 4' | 3.39 <i>t</i> (<i>J</i> = 7.8) | 71.9 |
| 5' | 3.58 <i>ddd</i> (<i>J</i> = 9.9, 6.2, 2.1) | 75.9 |
| 6'a | 4.56 <i>dd</i> (<i>J</i> = 12.0, 2.1) | 64.7 |
| 6'b | 4.27 <i>dd</i> (<i>J</i> = 12.0, 6.2) | |
| Caffeoyl group | | |
| 1'' | – | 128.1 |
| 2'' | 7.05 <i>d</i> (<i>J</i> = 2.1) | 115.9 |
| 3'' | – | 147.6 |
| 4'' | – | 149.8 |
| 5'' | 6.78 <i>d</i> (<i>J</i> = 8.3) | 117.1 |
| 6'' | 6.96 <i>dd</i> (<i>J</i> = 1.6, 8.3) | 123.7 |
| 7'' | 7.60 <i>d</i> (<i>J</i> = 15.8) | 147.4 |
| 8'' | 6.30 <i>d</i> (<i>J</i> = 16.2) | 115.2 |
| 9'' | – | 169.8 |

(7.9 mg). – UV (MeOH): λ_{max} = 290.5 nm (log ε = 3.80), 325 nm (log ε = 3.78). – [α]_D²⁰ –11.73° (MeOH, *c* 0.09). – ESI-HRMS (pos.): *m/z* = 471.1498 [M+H]⁺ (required for C₂₁H₂₆O₁₂ 471.1503). – ¹H and ¹³C NMR spectral data: see Table I.

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**) (Vermees *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.

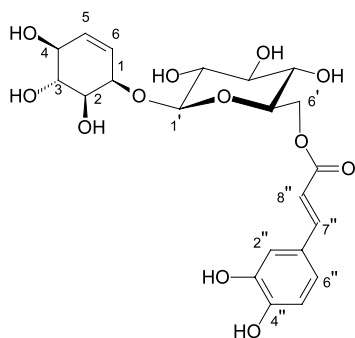


Fig. 1. Structure of **1**.

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 $[\text{M}+\text{H}]^+$ at m/z 471.1498, compatible with the molecular formula $\text{C}_{21}\text{H}_{26}\text{O}_{12}$. 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, $J = \sim 16.0$ 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: $-\text{CH}_{(5)}=\text{CH}_{(6)}-\text{CH}_{(1)}$ (OH)- $\text{CH}_{(2)}$ (OH)- $\text{CH}_{(3)}$ (OH)- $\text{CH}_{(4)}$ (OH)- $\text{CH}_{(5)}=$. Chemical shifts of protons and carbon atoms, as well as coupling constants revealed the presence of the cyclitol conduritol F (or leucanth-

| 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 |

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

nt, not tested.

The results are presented as means of 4–6 measurements (\pm 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''/H₂-6'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.

| 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 III. IC₅₀ 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 (IC₅₀ from 35.5 μM to 545 μM). Compound **5** (IC₅₀ 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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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.

Abe F., Yamauchi T., Honda K., and Hayashi N. (1998), Cyclitols and their glycosides from the leaves of *Marsdenia tomentosa*. *Phytochemistry* **47**, 1297–1301.
 Abe F., Yamauchi T., Honda K., and Hayashi N. (2000), Conduritol F and terpenoid glycosides from *Cynanchum liukiuense* and distribution of conduritol F glu-

cosides in several Asclepiadaceae plants. *Chem. Pharm. Bull.* **48**, 1090–1092.
 El-Hassan A., El-Sayed M., Hamed A. I., Rhee I. K., Ahmed A. A., Zeller K. P., and Verpoorte R. (2003), Bioactive constituents of *Leptadenia arborea*. *Fitoterapia* **74**, 184–187.

- Harborne J. B. (1967), *Comparative Biochemistry of the Flavonoids*. Academic Press, London, New York.
- Heywood V. H. and Humphries C. J. (1978), *Biology and Chemistry of the Compositae* (Heywood V. M., Harborne J. B., and Turner B. L., eds.). Academic Press, London, pp. 852–858.
- Kindl H. and Hoffmann-Ostenhof O. (1966), Untersuchungen über die Biosynthese der Cyclite. XIII. Vorkommen und Biosynthese von Cycliten in Asclepiadaceae. *Phytochemistry* **5**, 1091–1102.
- Kindl H., Scholda R., and Hoffmann-Ostenhof O. (1967), Untersuchungen über die Biosynthese der Cyclite. XV. Zur Bildung von L-(+)-Quercit in *Quercus*-Arten. *Phytochemistry* **6**, 237–244.
- Kontogiorgis C. and Hadjipavlou-Litina D. (2003), Biological evaluation of several coumarin derivatives designed as possible anti-inflammatory/antioxidant agents. *J. Enzyme Inh. Med. Chem.* **18**, 63–69.
- Lee J. H., Ku C. H., Baek N., Kim S.-H., Park H. W., and Kim D. K. (2004), Phytochemical constituents from *Diodia teres*. *Arch. Pharm. Res.* **27**, 40–43.
- Li J., Kadota S., Kawata Y., Hattori M., Xu G.-J., and Namba T. (1992), Constituents of the roots of *Cynanchum bungei* Decne. Isolation and structures of four new glycosides, Bungeiside-A, -B, -C, and -D. *Chem. Pharm. Bull.* **40**, 3133–3137.
- Mabry T. J., Markham K. R., and Thomas M. B. (1970), *The Systematic Identification of Flavonoids*. Springer, New York.
- Mann C. and Staba E. J. (1986), The chemistry, pharmacology, and commercial formulations of chamomile. In: *Herbs, Spices, and Medicinal Plants: Recent Advances in Botany, Horticulture, and Pharmacology*, Vol. 1 (Craker L. E. and Simon J. E., eds.). Oryx Press, Phoenix, AZ, pp. 235–280.
- Neu R. (1957), Chelate von Diarylborsäuren mit aliphatischen Oxyalkylaminen als Reagenzien für den Nachweis von Oxyphenyl-benzo- γ -pyronen. *Naturwissenschaften* **44**, 181–184.
- Pakudina Z. P., Leontev V. B., Kamaev F. G., and Sadykov A. S. (1970), Structure and PMR spectra of isoquercitrin and hirsutrin. *Khim. Prir. Soedin.* **5**, 555.
- Ulubelen A., Kerr K. M., and Mabry T. J. (1980), New 6-hydroxyflavonoids and their methyl ethers and glycosides from *Neurolaena oaxacana*. *Phytochemistry* **19**, 1761–1766.
- Vermes B., Farkas L., Nógrádi M., Wagner H., and Dirscherl R. (1976), The synthesis of afzelin, paeonaside and kaempferol 3-*O*- β -rutinoside. *Phytochemistry* **15**, 1320–1321.