Deoxypodophyllotoxin Content and Antioxidant Activity of Aerial Parts of *Anthriscus sylvestris* Hoffm.

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Deoxypodophyllotoxin content of the aerial parts of *Anthriscus sylvestris* Hoffm. growing at different altitudes was evaluated in comparison to the roots. The lignan accumulation in ground parts was at least double compared to aerial ones.

In addition antioxidant-guided fractionation of the crude methanol extract of aerial parts was performed with the 2,2-diphenyl-1-picrylhydrazyl (DPPH) test. Active fractions contained mainly luteolin-7-*O*-glucoside and chlorogenic acid. Antioxidant properties of both crude extract and isolated compounds were also investigated with the Briggs-Rauscher (BR) oscillating reaction. A satisfactory agreement between the results obtained with the two methods was observed.

Key words: Anthriscus sylvestris, Deoxypodophyllotoxin Content, Antioxidant Activity

Introduction

Anthriscus sylvestris Hoffm. (Apiaceae; wild chervil or cow parsley) is a perennial herb that grows in North America, Europe and Asia. Dried roots are used, mixed with other species, as a haematinic or tonic in China. In Japan the roots and young aerial parts are sometimes used as a food (Kozawa *et al.*, 1982). The roots of *A. sylvestris* produce a different range of lignans, next to the three main lignans deoxypodophyllotoxin, yatein, and anhydropodorhizol (Kozawa *et al.*, 1982; Ikeda *et al.*, 1998a, b). Deoxypodophyllotoxin is an interesting compound correlated to podophyllotoxin, a lignan used as the starting compound for the production of the semi-synthetic anticancer drugs etoposide (VP-16–213), a phosphate analogue, ethopophos, and teniposide (VM-26). However, the isolation of podophyllotoxin from the rhizomes of *Podophyllum* plants is not a very ideal system for large-scale production and the supply becomes increasingly limited due to the intensive collection (Choudhary *et al.*, 1998). For this reason different authors are searching for alternative sources of podophyllotoxin (Van Uden *et al.*, 1997; Koulman *et al.*, 2001). Because of their content of deoxypodophyllotoxin the rhizomes of *A. sylvestris* could become a valuable source for the bio-technological production of podophyllotoxin (Van Uden *et al.*, 1997).

Up to date few data are available about the chemical composition of *A. sylvestris* aerial parts. The aim of this paper is a comparative evaluation of deoxypodophyllotoxin contents in roots and aerial parts of *A. sylvestris* plants growing in northeast Italy. In addition the *in vitro* antioxidant properties of the aerial parts of *A. sylvestris* were evaluated.

Results and Discussion

Phytochemical analysis carried out on the aerial parts of *A. sylvestris* led to the isolation and characterization of seven known compounds, deoxypodophyllotoxin (**1**), nemerosin (**2**), anthriscinol methyl ether (**3**), anthriscusin (**4**), 1-(3′-methoxy-4′,5′-methylenedioxyphenyl)-1-methoxy-2-propene (**5**), luteolin-7-*O*-glucoside (**6**), and chlorogenic acid (**7**) (Fig. 1). The two last compounds were isolated for the first time from this plant.

The amounts of deoxypodophyllotoxin in plants of *A. sylvestris* growing at different altitudes were evaluated by HPLC. Results are given in Table I. Variation in lignan content between plants of dif-
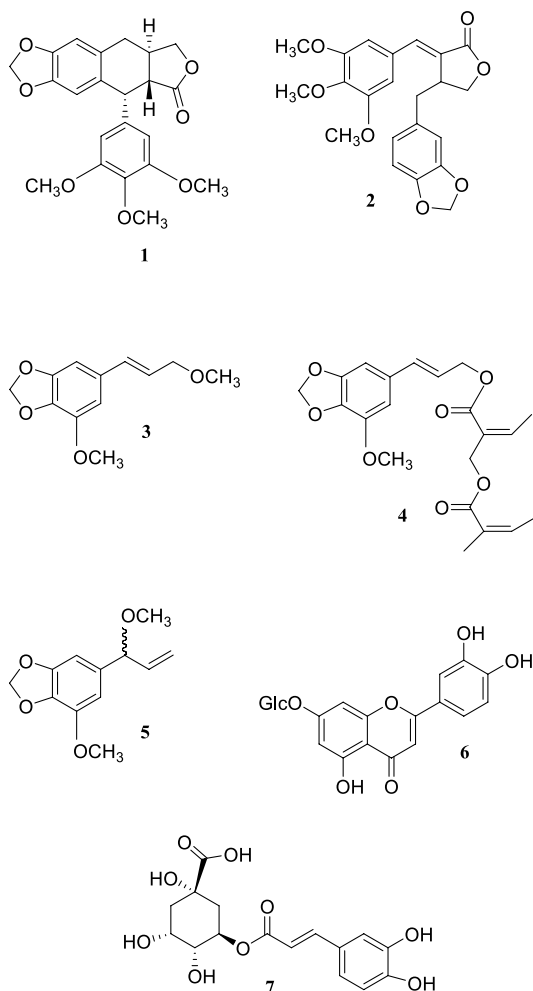
Fig. 1. Compounds isolated from *A. sylvestris* aerial parts: deoxypodophyllotoxin (**1**), nemerosin (**2**), anthriscinol methyl ether (**3**), anthriscusin (**4**), 1-(3′-methoxy-4′,5′-methylenedioxyphenyl)-1-methoxy-2-propene (**5**), luteolin-7-*O*-glucoside (**6**), and chlorogenic acid (**7**).

ferent locations was observed. The deoxypodophyllotoxin content differed at least twofold between the high and low altitude sites in both aerial parts and roots. Many environmental factors like precipitation, mean temperature, soil, duration of snow cover, and intensity of radiation under clear sky condition have been reported to differ between low and high altitude sites in temperate zones (Körner, 1999). These various factors can have an impact on the secondary metabolite profile in higher plants (Harborne, 1982). A large variation in the contents of lignans in *A. sylvestris* plants growing at different locations was also described by Koulman *et al.* (2001).

The deoxypodophyllotoxin content of roots was higher (about 50%) than that found in the aerial parts of two different populations of *A. sylvestris*.

Although, the aerial parts contained lower deoxypodophyllotoxin amounts compared to the roots, they could be regarded as an alternative and renewable source of this lignan.

The antioxidant activity of the crude methanol extract (MTE) of *A. sylvestris* was evaluated by the 2,2-diphenyl-1-picrylhydrazyl (DPPH) test.

Table II. Antioxidant activity of methanol extract (MTE) and fractions (F1–F7) by DPPH test.

Sample	IC$_{50}$ [μg/ml]
MTE	184 ± 10
F1	> 200
F2	> 200
F3	97 ± 10
F4	29 ± 10
F5	25 ± 3
F6	9 ± 2
F7	15 ± 3

Table I. Deoxypodophyllotoxin contents (g/100 g dry weight) in two different populations of *A. sylvestris* specimens.

Sample[a]	Plant part	Content
A	Whole plant sample	0.260 ± 0.004
	Aerial parts	0.130 ± 0.003
	Roots	0.380 ± 0.005
B	Whole plant sample	0.560 ± 0.001
	Aerial parts	0.330 ± 0.001
	Roots	0.780 ± 0.004

[a] A, plants collected at 900 m; B, plants collected at 1200 m.

Table III. Antioxidant activity of the isolated compounds.

Sample	DPPH (IC$_{50}$)[a]	BR (r.a.c.)[b]
Methanol extract (MTE)	184 ± 10	0.046 ± 0.004
Deoxypodophyllotoxin	61.0 ± 2.4	0.22 ± 0.02
Chlorogenic acid	5.6 ± 0.1	3.2 ± 0.2
Luteolin-7-*O*-glucoside	15.5 ± 0.2	0.85 ± 0.04
Rutin[c]	5.2 ± 0.4	0.60 ± 0.10
Resorcinol[c]	n.d.	1

[a] IC$_{50}$ in μg/ml.
[b] r.a.c. in μg/ml for the extract, in μM for pure compounds.
[c] Rutin and resorcinol were used as reference compounds.

The crude extract was bioassay-fractionated by a Sephadex column. The IC$_{50}$ values of the crude extract and the fractions F1 ÷ F7 are reported in Table II. The highest antioxidant activity was mainly related to F6.

From this fraction luteolin-7-*O*-glucoside and chlorogenic acid were isolated and identified. The activity of the crude extract and the two isolated compounds was also investigated with both the DPPH test and the Briggs-Rauscher (BR) method. Results are listed in Table III. As can be seen there is a satisfactory agreement between the results obtained with the two methods.

To better interpret the BR results we report here the relative antioxidant (r.a.c.) value of a methanol extract from *Wulfenia carinthiaca* Jacq., that is (0.15 ± 0.01) μg/ml resorcinol (Re) equivalents: it must be taken into account that the *W. carinthiaca* extract contains the very powerful antioxidants phenylpropanoid glycosides (Cervellati *et al.*, 2004b). Chlorogenic acid is a well-known antioxidant with DNA-protecting properties (Schweiger *et al.*, 2005); its r.a.c. value is indeed high. For comparison the r.a.c. values of rosmarinic acid and cyanidin-3-*O*-β-glucopyranoside are (3.99 ± 0.08) μM Re equivalents and (1.95 ± 0.06) μM Re equivalents, respectively (Cervellati *et al.*, 2002). Luteolin-7-*O*-glucoside shows a relative antioxidant activity similar to that found for epigallocatechin (0.90) and epicatechingallate (0.80) (Bacchelli, 2004).

Experimental

Plant material

The plants of *Anthriscus sylvestris* Hoffm. growing at two different altitude sites, one at Bivio Pian Rosada at 900 m (sample A) and the other at Mt. Calvario-Cansiglio at 1200 m (sample B) (Treviso, Italy), were collected in July 2004. The plants were identified by Prof. E. M. Cappelletti, Botanical Garden of Padova, Padova, Italy, and the voucher specimens were deposited at the Department of Pharmaceutical Sciences, University of Padova, Padova, Italy (n° ASPR0704 and ASCC0704).

Chemicals and apparatus

UV spectra: Perkin-Elmer lambda-25 spectrophotometer; NMR: Bruker AMX-300 spectrometer; HR-MS: Mariner API-TOF mass spectrometer. Podophyllotoxin, DPPH were obtained from Sigma Aldrich; solvents from Carlo Erba were of

analytical or HPLC grade. For BR test: Malonic acid (MA, Merck; reagent grade, > 99%), manganese(II) sulphate monohydrate (Merck; reagent grade, > 99%), NaIO$_3$, anhydrous Na$_2$CO$_3$ (Merck; reagent grade, ≥ 99.9%) and resorcinol (= benzene-1,3-diol; Fluka; reagent grade, ≥ 98%) were used without further purification. HClO$_4$ (Merck; 70–72% v/v), H$_2$O$_2$ (Merck; 35% v/v), and other chemicals were of analytical grade. All stock solutions were prepared from doubly distilled, deionized water. Perchloric acid was analyzed by titration *vs.* standard 0.1 M NaOH (Merck). H$_2$O$_2$ was standardized daily by manganometric analysis. Oscillations in the BR mixtures were followed potentiometrically by recording the potential of the mixture using a coupled bright platinum electrode (Hamilton, model P/N 238 945-S7; Crison, Bologna) – reference electrode (double junction Ag/AgCl; Ingold, model 373-90-WTE-ISE-S7; Urdorf, Switzerland). Electrodes were connected to a pH multimeter (WTW, model pH 540 GLP) controlled by an IBM-compatible PC.

Chemical analysis

Air-dried and powdered aerial parts of *A. sylvestris* (50 g; sample B) were exhaustively extracted in a Soxhlet apparatus with MeOH. The solvent was removed under vacuum. Yield in weight of residue, referring to the weight of dry material extracted, was 12 g (MTE). 6 g of the residue was applied to a Sephadex LH 20 column (250 ml) and eluted with MeOH. Fifty-five fractions were obtained and combined on the basis of their chromatographic behaviour into seven fractions (F1 ÷ F7) and tested for their antioxidant activity by the DPPH method (Hatano *et al.*, 1988). Further chromatographic steps on a silica gel column and silica gel plates led to the isolation of deoxypodophyllotoxin (**1**), nemerosin (**2**), anthriscinol methyl ether (**3**), anthriscusin (**4**), 1-(3′-methoxy-4′,5′-methylenedioxyphenyl)-1-methoxy-2-propene (**5**), luteolin-7-*O*-glucoside (**6**), and chlorogenic acid (**7**). The structures of compounds **1**–**5** were achieved by comparison of their spectral data (UV, HRMS, ^{1}H NMR and ^{13}C NMR) with those reported in the literature for the same compounds (Ikeda *et al.*, 1998a, b). Luteolin-7-*O*-glucoside (**6**) and chlorogenic acid (**7**) were identified on the basis of their UV, NMR and MS spectra by comparison with authentic samples purchased from Roth (Germany) and Sigma-Aldrich Corp., respectively.

Extraction, isolation and evaluation of deoxy-phodophyllotoxin in the plants of A. sylvestris

Extraction and isolation of deoxypodophyllotoxin from roots

35 g of air-dried and powdered roots (sample B) were exhaustively extracted at room temperature using solvents of increasing polarity, namely petroleum ether, chloroform and methanol.

The solvents were removed under vacuum. The petroleum ether extract was chromatographed on a silica gel column (eluted with cyclohexane/ MeOH in increasing ratios). Further chromatographic steps on silica gel plates (toluene/acetone, 2:1 v/v) led to the isolation of deoxypodophyllotoxin. The lignan was identified on the basis of its spectral data (UV, HRMS, 1D and 2D NMR) and by comparison with those reported in the literature for the same compound (Ikeda *et al.*, 1998a, b).

Deoxypodophyllotoxin was further purified by HPLC to 98.5% of purity and used to perform a calibration curve.

Deoxypodophyllotoxin content

The deoxypodophyllotoxin contents in the different samples were performed by HPLC under the following conditions: Spherisorb C18 5 μm (250 × 4.6 mm I.D.) column; AcCN/ammonium acetate 0.02 м (pH 5.00) (60:40; v/v) as mobile phase; 1.0 ml/min flow rate; 10 μl sample was injected; detection at 290 nm. Deoxypodophyllotoxin retention time was 8.86 min.

Quantification of deoxypodophyllotoxin in different samples was carried out using podophyllotoxin (Sigma) as internal standard. A calibration curve was determined using a series of standard solutions containing a constant amount of internal standard with varying amounts of the stock deoxypodophyllotoxin ($R = 0.9998$). Revelation limit was 5.1 μg.

150-mg samples of dried roots or aerial parts with addition of internal standard, were extracted exhaustively with MeOH in ultrasonic cleaning for 5 min (3 × 5 ml). The methanol extracts were filtered through a 0.45 μm filter. The obtained solutions were analyzed by HPLC. Quoted data are the average values of quantitative determinations performed on two different plant samples. Standard deviation was shown to be less than 5%.

Antioxidant activity

The crude methanol extract (sample B) and fractions F1 ÷ F7 were assayed with the DPPH method as previously described (Hatano *et al.*, 1988). Activities of the crude extract and isolated compounds were also evaluated with the BR method, based on the inhibitory effects by antioxidants, free radical scavengers, and the oscillations of the Briggs-Rauscher oscillating reaction, as previously described (Cervellati *et al.*, 2004a). As a value of antioxidant activity the r.a.c. index was used. This index means the relative activity with respect to concentrations, that is, the ratio [std]/ [smp], where [smp] is the concentration of the sample added to the BR mixture giving a certain inhibition time and [std] is the concentration of the standard (resorcinol) that should give the same inhibition time (Cervellati *et al.*, 2004a).

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