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FURANOCOUMARINS FROM *Peucedanum tauricum* Bieb. AND THEIR VARIABILITY IN THE AERIAL PARTS OF THE PLANT DURING DEVELOPMENT ◈

M. Bartnik[*] and *K. Głowniak*

Department of Pharmacognosy with Medicinal Plant Laboratory, Medical University, 1, Chodźki Str., 20-093 Lublin, Poland

SUMMARY

A rapid and sensitive reversed-phase high-performance liquid chromatographic (RP-HPLC) method has been used for determination of furanocoumarins in methanolic extracts of *Peucedanum tauricum* Bieb. HPLC separation was performed on a C_{18} analytical column (250 mm × 4 mm i.d., 5-μm particles) with a methanol–water gradient as mobile phase. A second chromatographic system with an acetonitrile–water gradient was also developed independently. Diode-array detection was performed between 220 and 400 nm and in quantitative analysis compounds were detected at 320 nm. Calibration plots were linear for 0.01–0.2 mg mL^{-1} bergapten and peucedanin in methanol (injection volume 10 μL). Before HPLC, samples were purified by solid-phase extraction (SPE). Oxypeucedanin hydrate (oPh), bergapten (5-MOP), oxypeucedanin (oP), peucedanin (P), 8-methoxypeucedanin (mP), and isoimperatorin (isoIMP) were detected and identified in extracts of *P. tauricum*. Total amounts of furanocoumarins determined by SPE–HPLC were 8.31 mg g^{-1} dry wt (0.83%) in flowers, 20.49 mg g^{-1} dry wt (2.05%) in immature fruits, and 21.30 mg g^{-1} dry wt (2.13%) in mature fruits. The predominant coumarin compound in the reproductive organs was peucedanin – 5.04 mg g^{-1} dry wt (0.50%) in flowers, 13.58 mg g^{-1} dry wt (1.36%) in immature fruits, and 13.51 mg g^{-1} dry wt (1.35%) in completely mature fruits. The predominant furanocoumarin in the leaves, however, was 8-methoxypeucedanin – from 0.55 mg g^{-1} to 1.02 mg g^{-1} dry wt (0.06–0.10%). The furanocoumarin content of the plant extracts correlated with period of development and the part of the plant (generative

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or vegetative). Some of the detected compounds (e.g. peucedanin and 8-methoxypeucedanin, which are both linear furanocoumarins of a rare structural type with restricted occurrence in *P. officinale* and the closely related species *P. coriaceum* and *P. longifolium*) could be a chemotaxonomically characteristic of the species investigated. As we have reported elsewhere, peucedanin is effective at induction of apoptosis and inhibition of heat-shock protein expression in HeLa cells. The fruits of *P. tauricum* are a good source of peucedanin for further biological and pharmacological tests.

INTRODUCTION

Coumarins with an additional ring system (furano or pyranocoumarins) occur in two families only, the *Apiaceae* and *Rutaceae* [1,2]. *Peucedanum tauricum* Bieb. (*Apiaceae*), is an endemic perennial plant, growing in nature on dry hillsides and in pinewoods in the Crimea, the Caucasus, and Romania [3–5]. Qualitative studies of the secondary metabolites present in this species (coumarins [6–9], phenolic acids [8,10], essential oils [8,9,11] and flavonoids [8,12]) have recently been conducted.

In this study the qualitative and quantitative furanocoumarin composition of methanolic extracts of the aerial parts of *P. tauricum* were examined and correlated with the part of the plant and with development time.

EXPERIMENTAL

Plant Material

The aerial parts of *Peucedanum tauricum* Bieb. (*Apiaceae*) were collected in the Botanical Garden of the Maria Curie-Skłodowska University, Lublin, every three weeks during the growing period, from June 9th to September 3rd, 2003. Representative samples of the leaves (before flowering, at the time of flowering, when the fruits were maturing, and when the fruits were completely mature) and reproductive organs (flowers, immature but formed fruits, and mature fruits) were collected in accordance with FP VI rules [13]. The plant material was dried at room temperature, pulverized, and samples (6 × 2 g) were taken from each part of the plant.

Extraction

Samples were extracted with hot (70°C) pure methanol (6 × 50 mL) on a water bath (6 × 30 min). Extracts of the same samples were combined,

partially evaporated under reduced pressure at 50°C, and placed in 25-mL volumetric flasks.

Sample Clean-Up

The procedure used for isolation of furanocoumarins from the methanolic extract was based on a method described elsewhere [8,14]. The compounds of interest were separated from fatty components and chlorophyll by use of SPE microcolumns (LiChrolut RP-18 E; Merck, Germany; 500 mg, 3 mL) attached to a J.T. Baker (USA) SPE-12G manifold vacuum chamber. In the first step, aqueous methanol (50% *v*/*v*) solutions of the samples (2 mL) were passed through the conditioned microcolumns to adsorb furanocoumarins on the adsorbent bed. The microcolumns were washed with 50% methanol (4 mL) and fractions containing phenolic acids were discarded. In the next step the adsorbed furanocoumarins were eluted at a flow-rate 0.5 mL min^{-1} with 80% methanol (6 mL) into vials previously calibrated with a pipette. The samples obtained were analysed by RP-HPLC.

RP-HPLC Analysis

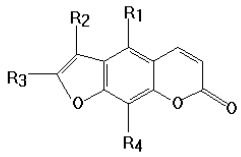
Qualitative and quantitative analysis of the furanocoumarins (Table I) was performed with an Agilent Technologies model 1100 liquid chromatograph equipped with diode-array detector (DAD), degasser, and column thermostat. Compounds were separated on a 250 mm × 4.6 mm i.d. stainless-steel column packed with 5-μm particle Hypersil ODS C_{18} (Shandon, UK). The mobile phase was a methanol–water gradient; the composition of the gradient was 0–5 min, isocratic elution with 60% (*v*/*v*) methanol; 5–20 min, linear gradient from 60 to 80% methanol; 20 to 30 min, linear gradient from 80 to 60% methanol; 30–40 min isocratic elution with 60% methanol [8]. An acetonitrile–water mobile phase gradient was also used (0–8 min, isocratic elution with 50% acetonitrile; 8–25 min, linear gradient from 50 to 70% acetonitrile; 25–28 min, linear gradient from 70 to 50% acetonitrile; 28–40 min isocratic elution with 50% acetonitrile) [7]. The flow rate was 1.0 mL min^{-1}, the volume of sample injected was 10 μL, and for quantitative analysis all compounds were detected at 320 nm.

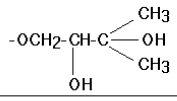
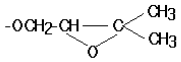
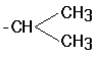
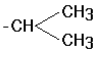
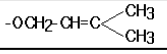
Selectivity

The selectivity of the method was determined by comparison of chromatograms obtained from different parts of the plant with chromato-

Table I

The structures of selected furanocoumarins from *P. tauricum* Bieb.



Compound	R_1	R_2	R_3	R_4
Oxypeucedanin hydrate		H	H	H
Bergapten	$-OCH_3$	H	H	H
Oxypeucedanin		H	H	H
8-Methoxypeucedanin	H	$-OCH_3$	$-OCH_3$	
Peucedanin	H	$-OCH_3$	H	
Isoimperatorin		H	H	H

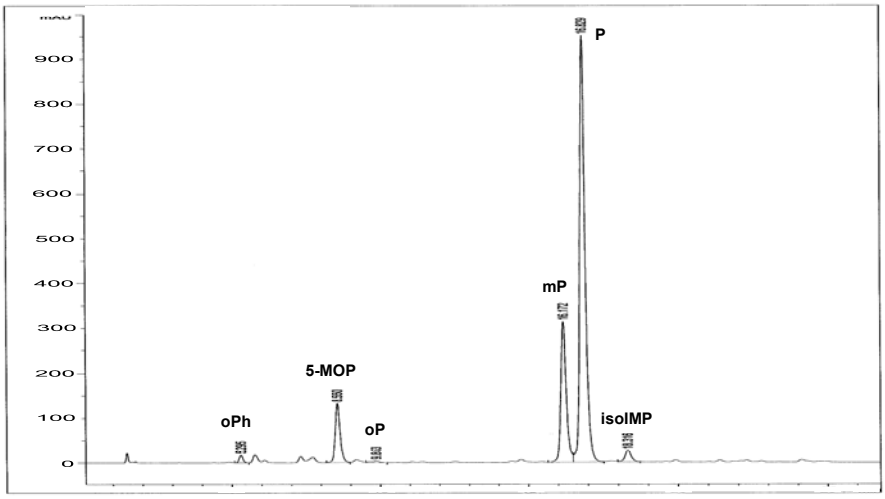


Fig. 1

RP HPLC chromatogram obtained from a methanolic extract of the fruits of *P. tauricum* Bieb. with a methanol–water gradient as mobile phase. oP, oxypeucedanin; 5-MOP, bergapten; mP, 8-methoxypeucedanin; P, peucedanin; isoIMP, isoimperatorin

grams obtained from standards, using the two different mobile phases. Typical chromatograms (of an extract obtained from mature fruits) are shown in Figs 1 and 2. Identification was achieved by chromatography of standard mixtures dissolved in the mobile phase under the same conditions as plant sample extracts and by comparison of retention times and DAD spectra. UV spectra of the compounds and of standard substances, in the range 220–400 nm, were acquired on-line by use of the DAD of the liquid chromatograph (Table II).

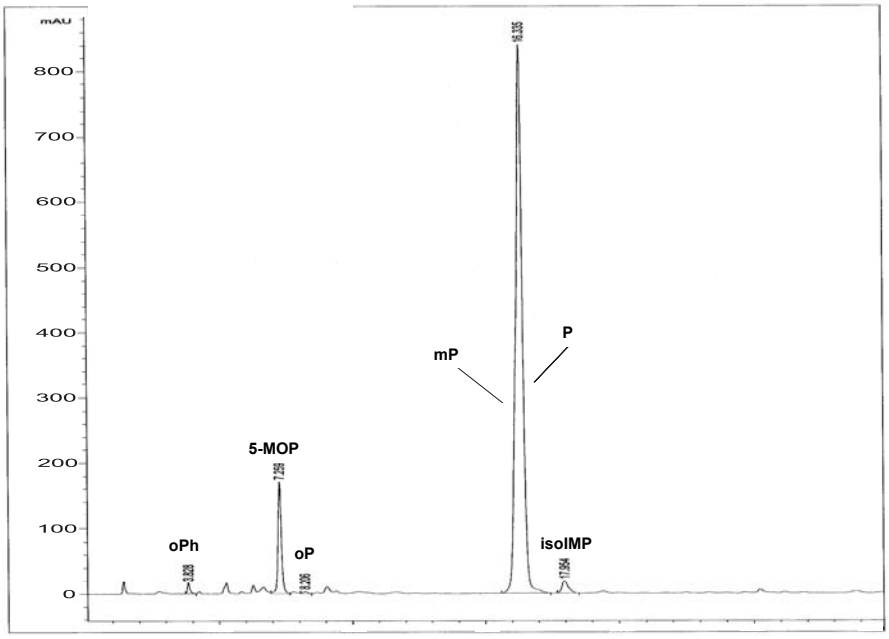


Fig. 2

RP HPLC chromatogram obtained from a methanolic extract of the fruits of *P. tauricum* Bieb. with an acetonitrile–water gradient as mobile phase. oP, oxypeucedanin; 5-MOP, bergapten; mP, 8-methoxypeucedanin; P, peucedanin; isoIMP, isoimperatorin

Linearity and Range

The furanocoumarin content of the plant extracts was estimated by means of calibration plots constructed after chromatography of standards. Identification and quantitative determination of the compounds were accomplished by comparison of retention times and peak areas with those obtained from standard solutions of the furanocoumarins. All compounds were

Table II

Data from the UV spectra of the furanocoumarins analysed, acquired on-line by use of the diode-array detector of the HP 1100 liquid chromatograph

Compound	λ_{max} (nm)	λ_{min} (nm)
Oxypeucedanin hydrate	221, 248, 259, 265, 311	235, 255, 278
Bergapten	222, 249, 259, 267, 310	235, 254, 278
Oxypeucedanin	221, 248, 309	233, 276
8-Methoxypeucedanin	223, 258, 303, 341	237, 285
Peucedanin	216, 256, 296, 341	208, 230, 277
Isoimperatorin	206, 221, 250, 265, 310	208, 230, 277

determined as bergapten or as peucedanin, depending on the similarity of their λ_{max} and λ_{min} values in UV spectra acquired on-line by use of the DAD of the HP 1100 liquid chromatograph [7,10] in the range 220–400 nm (Table II). The peucedanin used as standard was previously isolated from the fruits of *P. tauricum*; the purity of the compound was confirmed by MS, 1D and 2D NMR, COSY, NOESY, HMBC, and HMQC [8,9]. Bergapten standard was purchased from Sigma. The equations of the calibration plots, expressed by the formula $y = ax + b$ for each standard are listed in Table III. Stock solutions (100 μg mL^{-1}) of the furanocoumarin standards were prepared by dissolving the compounds in methanol (Baker).

Table III

Calibration plot data for the standards used

Standard	*a* (mean ± *SD*)	*b* (mean ± *SD*)	*r* (mean ± *SD*)
Bergapten	34573.30 ± 42.13	49.95 ± 4.06	0.9993 ± 0.0053
Peucedanin	46644.17 ± 183.71	46.93 ± 7.77	0.9995 ± 0.0027

Equation of the calibration plot: $y = ax + b$
Concentrations are expressed in mg mL^{-1} methanol.

Precision

The precision of the method was assessed by triplicate analysis of standard solutions at five concentrations (0.01, 0.02, 0.05, 0.1, and 0.2 mg mL^{-1}). The *RSD* values obtained were <0.03.

Recovery

Recovery was determined as described elsewhere [14]. Solutions

of bergapten and peucedanin (0.5 mg) in 50% aqueous methanol (10 mL) were applied to conditioned octadecyl microcolumns and the adsorbed furanocoumarins were eluted with 80% methanol. All the fractions collected were examined for their furanocoumarin content by RP-HPLC. SPE recovery of bergapten from the methanolic extract was 100.3 ± 6.1% for the standard and 99.8 ± 2.0% for fortified samples. Recovery of the peucedanin was 103.0 ± 4.1% for the standard and 100.9 ± 3.7% for fortified samples.

RESULTS AND CONCLUSIONS

Oxypeucedanin hydrate (oPh), bergapten (5-MOP), oxypeucedanin (oP), peucedanin (P), 8-methoxypeucedanin (mP), and isoimperatorin (isoIMP) were identified in extracts of *Peucedanum tauricum* Bieb. (Figs 1 and 2). Total amounts of furanocoumarins determined by SPE–HPLC were 8.31 mg g^{-1} dry wt (0.83%) in the flowers, 20.49 mg g^{-1} dry wt (2.05%) in the immature fruits, and 21.30 mg g^{-1} dry wt (2.13%) in the mature fruits (Table IV, Fig. 3). Only trace amounts of the furanocoumarins were detected in the leaves before flowering, then 1.06 mg g^{-1} dry wt (0.11%) in the leaves at the time of flowering, 0.55 mg g^{-1} dry wt (0.06%) when the fruits were immature, and 0.93 mg g^{-1} dry wt (0.09%) when the fruits were mature (Table IV).

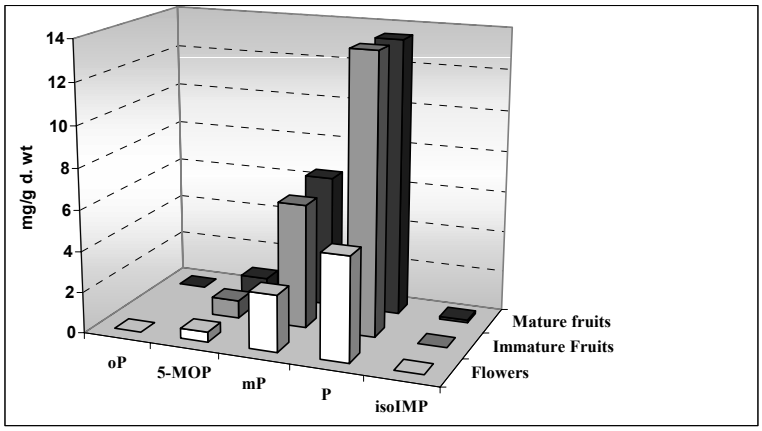


Fig. 3

The furanocoumarin content (mg g^{-1} dry wt) of methanolic extracts of the reproductive organs of *P. tauricum* flowers (white columns), immature fruits (grey columns), and mature fruits (dark grey columns), determined by SPE–RP-HPLC. oP, oxypeucedanin; 5-MOP, bergapten; mP, 8-methoxypeucedanin; P, peucedanin; isoIMP, isoimperatorin

Table IV

Furanocoumarin content (mg g^{-1} dry wt ± *SD*) of the samples of *P. tauricum* Bieb. analysed, determined by SPE–HPLC

Compound	1, Leaves (9 June)		2, Leaves (11 July)		3, Flowers (11 July)	
	Amount	*RSD*	Amount	*RSD*	Amount	*RSD*
oPh[a]	–	–	–	–	–	–
5-MOP[a]	–	–	tr	–	0.49 ± 0.05	0.10
oP[a]	–	–	tr	–	0.02 ± 0.01	0.13
mP[b]	tr	–	1.02 ± 0.06	0.06	2.76 ± 0.10	0.04
P[b]	–	–	0.04 ± 0.03	0.07	5.04 ± 0.12	0.02
isoIMP[a]	–	–	–	–	tr	–
Total	tr	–	1.06 ± 0.06	0.06	8.31 ± 0.23	0.03

Compound	4, Leaves (7 August)		5, Immature fruits (7 August)		6, Leaves (3 September)		7, Mature fruits (3 September)	
	Amount	*RSD*	Amount	*RSD*	Amount	*RSD*	Amount	*RSD*
oPh[a]	–	–	–	–	–	–	tr	–
5-MOP[a]	tr	–	0.87 ± 0.09	0.11	tr	–	0.90 ± 0.06	0.07
oP[a]	tr	–	tr	–	tr	–	0.28 ± 0.01	0.03
mP[b]	0.55 ± 0.02	0.04	6.04 ± 0.33	0.05	0.93 ± 0.06	0.07	6.47 ± 0.42	0.07
P[b]	tr	–	13.58 ± 1.05	0.08	tr	–	13.51 ± 0.89	0.07
isoIMP[a]	–	–	tr	–	–	–	0.14 ± 0.01	0.10
Total	0.55 ± 0.02	0.04	20.49 ± 1.48	0.07	0.93 ± 0.06	0.07	21.30 ± 1.39	0.07

[a] Calculated as bergapten
[b] Calculated as peucedanin
tr, trace; oPh, oxypeucedanin hydrate; 5-MOP, bergapten; oP, oxypeucedanin; mP, 8-methoxypeucedanin; P, peucedanin; isoIMP, isoimperatorin
The dates in parentheses are the harvesting times
t-Student (n=6, α=0.05 p=95%) t=2.571

Amounts of coumarins were greater in the reproductive organs than in the leaves, and the mature fruits were richest in the compounds (Table IV, Fig. 4). The predominant furanocoumarin in the reproductive organs was peucedanin – 5.04 mg g^{-1} dry wt (0.50%) in the flowers, 13.58 mg g^{-1} dry wt (1.36%) in immature fruits, and 13.51 mg g^{-1} dry wt (1.35%) in completely mature fruits. 8-Methoxypeucedanin was the predominant compound in the leaves – from 0.55 to 1.02 mg g^{-1} dry wt (0.06–0.10%) (Table IV).

Our study confirmed that differences between the amounts of the furanocoumarins in the plant extracts depended on the state of development of the plant and on the part extracted (reproductive or vegetative). Some of the compounds detected in the extracts, e.g. peucedanin and 8-methoxypeucedanin, are linear furanocoumarins of a rare structural type

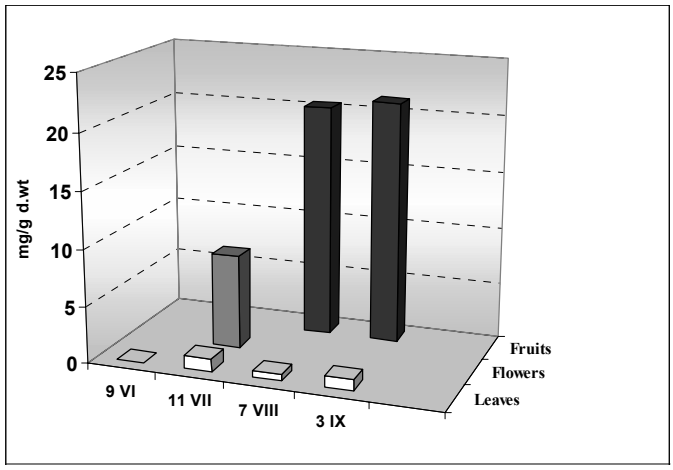


Fig. 4

Total furanocoumarin content (mg g^{-1} dry wt) of methanolic extracts of *P. tauricum* Bieb., determined by SPE–RP-HPLC. 9 VI, 11 VII, 7 VIII, and 3 IX denote the harvesting times of the plant material analysed (9 June, 11 July, 7 August, and 3 September, respectively)

with restricted occurrence in *P. officinale* and closely related species (*P. coriaceum* and *P. longifolium*); these may be chemotaxonomically characteristic of the plant investigated [15,16]. As we have reported elsewhere, peucedanin is effective in inducing apoptosis and in inhibiting expression of heat-shock protein in HeLa cells [8,17,18]. The plant material analysed, particularly the fruits of *P. tauricum*, might be a useful source of the furanocoumarins bergapten and peucedanin for testing the biological and pharmacological activity of these compounds.

The SPE method enabled efficient purification of crude methanolic extracts and separation from ballast substances (chlorophyll and waxes), with good recovery of the analytes of interest. The RP HPLC method is reproducible, precise, and useful for analysis of natural mixtures.

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