Furocoumarins from the Seeds of *Citrus trifoliata* L.

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Citrus trifoliata, Rutaceae, furocoumarins

A detailed study of the furocoumarins present in the seeds of *Citrus trifoliata* L. revealed the presence of isopimpinellin, prangenin and prangenin hydrate in addition to the already reported bergapten and imperatorin. The amount of these compounds, referred to the starting dry material, were: Imperatorin 0.15%, bergapten 0.06%, whereas isopimpinellin, prangenin and prangenin hydrate were found in the order of less than 0.01%.

In relation to the photobiological properties of furocoumarins extensively studied in this institute [1–3] an investigation has been carried out on their presence in the seeds of *Citrus trifoliata* L.

The isolation of two furocoumarins, imperatorin and bergapten, from the seeds of *Citrus trifoliata* L. has already been reported [4, 5], whereas two different coumarinic derivatives, marmesin and seselin have been isolated from the roots [6, 7].

The purpose of this research was the isolation and identification of other furocoumarinic components of the seeds together with their quantitative evaluation.

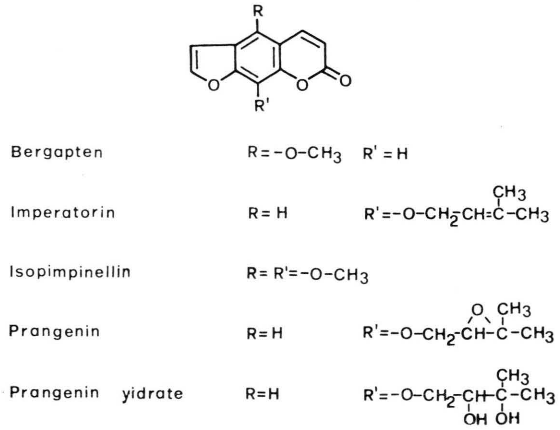
The seeds were obtained from ripe fruit gathered from plants cultivated in Padua or its surroundings either for ornamental purpose or protective hedge.

The seeds dried at 60 °C were extracted with petroleum ether, and, after removal of the solvent, the crystalline residue was chromatographed on a column of deactivated silica gel eluting with solvents of increasing polarity. The elution of furocoumarins was checked by thin layer chromatography.

Bergapten, imperatorin, isopimpinellin, prangenin and prangenin hydrate were isolated from properly pooled fractions in pure crystalline form and identified either by comparison of their properties with those of authentic samples isolated from other natural sources [8, 9] or prepared synthetically.

It is worthwhile to note that the presence of prangenin and prangenin hydrate has not till now been verified in plants of the genus *Citrus*.

An examination by thin layer chromatography revealed in the first fractions of the column other spots with blue and violet-blue fluorescence, probably related to other coumarinic derivatives. Such substances, however, were present in such small quantitaties that they could not be isolated in pure form and identified.



Quantitative determinations revealed that the most abundantly represented furocoumarins are imperatorin, in amount of 0.15% of the dry material, and bergapten 0.06%. On the other hand the other three identified substances isopimpinellin, prangenin and prangenin hydrate are in extremely small amounts ($\leqq 0.01\%$).

Experimental Part

Thin layer chromatography

Merck 5715 silica gel plates were used and, as moving solvents the following systems were used: A. Cyclohexane-ethyl acetate 65 : 35 (v/v); B. chloroform; C. toluene, ethyl formate, formic acid 50 : 40 : 10 (v/v).

The spots were revealed by UV light (Philips HPW 125, 365 nm).

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Table. Separation by chromatography on silica gel column (⌀ 7.8 cm) (1000 g; H_2O 5%) for the extract of seeds of *Citrus trifoliata* (5 g).

Fractions 200 ml	Solvent	Residue [mg]	Thin layer chromatography (System A) Fluorescence	R_F	Substances present	Relative quantity [%]
1–385	benzene-petroleum ether 30–50 °C 70/30 ((v/v)	500	violet-blue blue yellow grey-blue	0.75 0.60 0.52 0.11	unidentified substance unidentified substance bergapten unidentified substance	traces traces ~96 traces
386–390	benzene-petroleum ether 30–50 °C 80/20 (v/v)	130	yellow	0.52	bergapten	100
391–480	benzene-petroleum ether 30–50 °C 80/20 (v/v)	1400	yellow yellow	0.57 0.52	imperatorin bergapten	71.2 28.8
481–595	benzene-petroleum ether 30–50 °C 80/20 (v/v)	1200	yellow yellow	0.57 0.52	imperatorin bergapten	87.5 12.5
596–725	benzene-petroleum ether 30–50 °C 90/10 (v/v)	790	yellow orange-yellow	0.57 0.45	imperatorin isopimpinellin	92.4 7.6
726–800	benzene	200	yellow orange-yellow	0.57 0.45	imperatorin isopimpinellin	50 50
801–810	benzene-ethyl acetate 95/5 (v/v)	40	orange-yellow	0.45	isopimpinellin	100
811–825	benzene-ethyl acetate 90/10 (v/v)	80	violet-blue orange-yellow violet-blue	0.47 0.45 0.41	unidentified substance isopimpinellin unidentified substance	traces ~95 traces
826–845	benzene-ethyl acetate 90/10 (v/v)	220	yellow	0.39	prangenin	100
846–870	benzene-ethyl acetate 90/10 (v/v)	200	yellow yellow	0.39 0.26	prangenin unidentified substance	~95 traces
871–875	ethyl acetate	80	yellow-green	0.06	prangenin hydrate	100

Extraction of seeds and chromatographic fractionation of the extract

The seeds obtained from ripe fruit were dried in a oven at 60 °C to constant weight and finely ground. The powder (3.3 kg) was exaustively extracted with petroleum ether 30–50 °C. The ethereal extract was concentrated to 500 ml and, by storage at room temperature, a solid crystalline material was obtained. The mother waters were concentrated to 400 ml and a further small portion of crystalline substance was obtained. Total yield 8.5 g.

A portion of 5 g of this material was applied on a colum (⌀ 7.8 cm) of 1000 g of deactivated silica gel (5% water p/p) and eluted with a mixture of solvents at gradually increasing polarity: Fractions of 200 ml were collected.

In order to obtain a convenient separation of the different substances, it was necessary a very slow increase of the polarity of the solvent (see Table).

The collected fractions were reduced to a small volume by evaporation at reduced pressure and then examined by thin layer chromatography (system A). The fractions with similar chromatographic pattern were pooled according to the scheme reported in the Table.

Results

Bergapten

From the fractions 386 through 390 a solid white crystalline residue (130 mg) was obtained by evaporation, which on thin layer chromatography moved as a single spot with yellow fluorescence and R_F 0.52, 0.75 and 0.65 respectively in systems A, B, and C.

This residue, crystallized with methanol, yielded a white crystalline substance identified as bergapten from melting point (192 °C, not depressed in a mixture with authentic sample of bergapten), from UV spectrum [95% ethanol: $\lambda_{\max}$ nm ($\log \varepsilon$) 310 (4.18); 268 (4.28); 259 (4.24); 249 (4.26) and $\lambda_{\min}$ 277 (3.67); 263 (4.22); 254 (4.20); 235 (416)] and chromatographic behaviour.

Imperatorin

From the fractions 481 through 595 a slightly yellow crystalline residue (1.2 g) was obtained which, when examined by thin layer chromatography, proved to consist mainly of imperatorin with R_F 0.57, 0.73, and 0.69 respectively in systems A, B, and C, accompanied by a small quantity of bergapten.

After repeated crystallization by cyclohexane, a white substance was obtained, that was identified as imperatorin from m.p. (100 – 101 °C, not depressed in a mixture with authentic sample of imperatorin), UV spectrum [95% ethanol: $\lambda_{\max}$ nm ($\log \varepsilon$) 300 (4.05); 263 (shoulder, 4.12); 249 (4.34) and $\lambda_{\min}$ 277 (3.80); 232 (4.17)] (see Fig. 1) and from chromatographic behaviour.

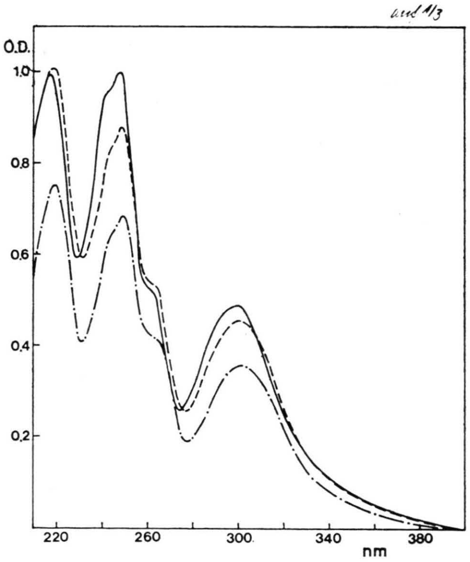

Fig. 1. UV spectra in ethanol 95% of the isolated furocoumarins: ——— Prangenin ($4.04 \cdot 10^{-5}$ M); – – – imperatorin ($4.01 \cdot 10^{-5}$ M); —·— prangenin hydrate ($3.07 \cdot 10^{-5}$ M).

Isopimpinellin

The fractions from 801 to 810, which chromatographically gave one single spot with orangy fluorescence and R_F 0.45, 0.56 and 0.57 respectively in systems A, B, and C, by elimination of the solvent yielded a microcrystalline yellow residue (40 mg). This residue crystallized with 95% ethanol gave straw-yellow crystals of a substance identified as isopimpinellin from m.p. (152 – 153 °C, not depressed in a mixture with authentic sample of isopimpinellin), UV spectrum [95% ethanol: $\lambda_{\max}$ nm ($\log \varepsilon$) 313.5 (4.10); 272 (4.29); 248 (4.18); 241 (4.17) and $\lambda_{\min}$ 287.5 (3.85); 254 (4.09); 245 (4.16); 236.5 (4.15)] and from chromatographic behaviour.

Elemental analysis:

$C_{13}H_{10}O_5$	%	Found:	C 63.53	H 3.93,
	%	Calcd.:	C 63.41	H 4.09.

The residues obtained by concentration of the fractions ranging from 596 to 800 examined by thin layer chromatography showed imperatorin together with smaller quantities of isopimpinellin.

Prangenin

From the fractions 826 to 845 a solid residue (220 mg) was obtained, which by thin layer chromatography showed a single spot with yellow fluorescence and R_F 0.39; 0.39 and 0.47 respectively in systems A, B, and C. Crystallization from 95% ethanol gave a white microcrystalline product identified as prangenin from m.p. (113 °C, not depressed in a mixture with authentic sample of prangenin), UV spectrum [95% ethanol $\lambda_{\max}$ nm ($\log \varepsilon$) 299 (4.09); 262 (4.11); 2.48 (4.39) and $\lambda_{\min}$ 274.5 (3.84); 230 (4.17)] (see Fig. 1) and from the identity of the IR spectrum (recorded by a Beckman IR 9 instrument) with that of a synthetic sample.

Elemental analysis:

$C_{16}H_{14}O_5$	%	Found:	C 67.40	H 5.00,
	%	Calcd.:	C 67.12	H 4.39.

Molecular weight (osmometric method in chloroform; found: 286.5, calcd.: 286.27).

The sample of prangenin used for comparison was obtained by perbenzoic acid oxydation of the imperatorin[10].

As a further evidence 100 mg of the isolated substance was hydrolized in an acetic solution of conc. H_2SO_4 (3 ml, 5 drops). The xantotoxol thus obtained was methylated with diazomethane in ethereal solution to obtain xantotoxin, that was identified by comparison of chromatographic behaviour, UV and IR properties with an authentic specimen [11].

Prangenin hydrate

By elimination of the solvent the fractions 871 to 875 gave a crystalline substance (80 mg), which by thin layer chromatography moved as a single spot with greenish-yellow fluorescence and R_F 0.06; 0.06 and 0.18 respectively in systems A, B, and C. Crystallized by 95% ethanol, a slightly yellow crystalline substance, was obtained that was identified as prangenin hydrate from m.p. [116 – 117 °C, undepressed in mixture with an authentic sample obtained by hydrolysis of the prangein [12]], chromatographic behaviour, UV spectrum [95% ethanol: $\lambda_{\max}$ nm ($\log \varepsilon$) 302 (4.07); 263 (shoulder, 4,14); 249.5 (4.35) and $\lambda_{\min}$ 277.5 (3.79); 231 (4.12) (see Fig. 1)] and the identity of the IR spectrum with that of a synthetic sample.

Quantitative evaluation

The quantitative evaluation of the furocoumarins in the fractions, which by thin layer chromatography were present as a single component, were carried out by eluting the residue in a proper volume of 95% ethanol and determining the absorbance at their relative maxima (Optica CF 4 spectrophotometer)

Bergapten $\lambda = 310$ $\varepsilon = 15240$;
isopimpineliln $\lambda = 313.5$ $\varepsilon = 12650$;
prangenin $\lambda = 299$ $\varepsilon = 12180$;
prangenin hydrate $\lambda = 302$ $\varepsilon = 11640$.

In the fractions in which bergapten and imperatorin were present together the calculation of the respective quantities was carried out determining the optical density at the two wavelenghts: 250 nm (ε imperatorin = 22580; ε bergaten = 18000) and 304 nm (ε imperatorin = 11780, ε bergapten = 14280). These wavelenghts were chosen because by comparison of the spectra of the two furocoumarins present in different ratio it was found the possibility to obtain a differential quantitative evaluation of both.

In the fractions in which imperatorin and isopimpinellin were contemporary present, no pair of wavelenghts was proved satisfactory, on the other hand the separation of the two substances by chromatography appeared difficult. Consequently their evaluation was carried out by hydrolysis of the imperatorin to xantotoxol and spectrophotometric evaluation of the quantity of isopimpinellin after chromatographic separation over alumina according the following procedure: The residues were dissolved in acetic acid (15 ml) and the solution treated with conc. H_2SO_4 (15 drops) to cause hydrolysis of the imperatorin. The reaction mixture was left for three hours at room temperature then neutralized with a $NaHCO_3$ solution and extracted with chloroform. The solvent was removed at reduced pressure and the residue dissolved again with benzene. The dried benzene solution (Na_2SO_4) was applied on a column of deactivated alumina (activity III, Aluminiumoxid Aktiv Neutral, Merck).

The first fractions eluted by benzene were composed exclusively of isopimpinellin, that could now be quantitatively evaluated by its UV absorbance. The imperatorin in the mixture obtained from silica gel column could be evaluated by difference.

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[1] L. Musajo, G. Rodighiero, and G. Caporale, Bull. Soc. Chim. biol. **36,** 1213 [1954].

[2] L. Musajo and G. Rodighiero, Experientia [Basel] **18,** 153 [1962].

[3] L. Musajo and G. Rodighiero, Photochem. Photobiol. **11,** 27 [1970].

[4] G. K. Nikonov and M. M. Molodozhnikov, Med. Prom. SSSR **18,** 24 [1964]; C. A. **61,** 14466 g.

[5] D. L. Dreyer, J. org. Chem. **30,** 749 [1965].

[6] T. Tomimatsu, J. pharmac. Soc. Japan [Yakugakuzasshi] **88,** 643 [1968].

[7] T. Tomimatsu and M. Hashimoto, J. pharmac. Soc. Japan [Yakugakuzasshi] **88,** 1375 [1968].

[8] E. Späth and P. Kainroth, Chem. Ber. **70,** 2272 [1937].

[9] E. Späth and F. Vierhapper, Chem. Ber. **70,** 248 [1937].

[10] E. Späth and H. Holzen, Chem. Ber. **68,** 1123 [1935].

[11] G. Rodighiero, G. Caporale, and G. Albiero, Gazz. chim. ital. **84,** 874 [1954].

[12] Y. N. Sharma, A. Zaman, and A. R. Kidwai, Tetrahedron [London] **20,** 87 [1964].