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Note

High-performance liquid chromatographic study of *Casimiroa edulis*

II. Determination of furocoumarins*

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In the experimental revision of both pharmacological and chemical aspects of *Casimiroa edulis* Llave et Lex carried out by Lozoya and co-workers[1,2] and Romero *et al.*[3], all biodynamic effects were studied in relation to histaminic compounds and more specifically to N^{α},N^{α}-dimethylhistamine, which is present in polar organic or aqueous extracts of seeds and leaves. On the other hand, during the search for active compounds, the hexane extract of seeds was of particular interest as it contains furocoumarins, some of which are known to elicit a series of biodynamic effects, including hypotension[4].

Furocoumarins were first recognized in *C. edulis* in 1911 by Power and Callan[5] as a "yellow phenolic substance"; Kincl *et al.*[6] also noticed it, but its ambiguous physical and chemical data did not allow for a complete structural assignment. Iriarte *et al.*[7] reported the isolation of the furocoumarins bergapten (I) and isopimpinellin (II) from bark. Later, Dreyer[8] reported the isolation of a furocoumarinic substance from the seeds, which was found to be phellopterin (III) and 8-geranyloxypsoralen (IV) and proved to be difficult to separate. Associations of this type have also been noted by Sharma *et al.*[9], who reported a 1:3 complex of IV and imperatorin (V) whose chromatographic behavior differed from that of the pure components. It was attractive in relation to this phenomenon to carry out a study of the problems observed in the separation of furocoumarins in *C. edulis* by high-performance liquid chromatography (HPLC).

In our examination of the hexane extract of the seeds, column chromatography afforded an apparently homogeneous material on silica gel thin-layer chromato-

* Taken in part from the M.Sc. thesis of M. L. Romero.

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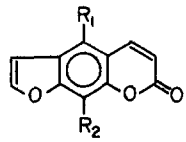
graphy (TLC) but its ^1H NMR spectrum revealed two different furocoumarin moieties. Additionally, III and 5-geranyloxypsoralen (VI) were isolated from other chromatographic fractions.

In this paper we describe the application of an HPLC method to the separation of these furocoumarins, including IV and 5-methoxy-8-geranyloxypsoralen (VII), which were found to occur as a furocoumarinic "complex" in a ratio of approximately 1:1.



I $R_1 = OCH_3$; $R_2 = H$

II $R_1 = OCH_3$; $R_2 = OCH_3$

III $R_1 = OCH_3$; $R_2 = OCH_2CHC(CH_3)_2$

IV $R_1 = H$; $R_2 = O$-geranyl

V $R_1 = H$; $R_2 = OCH_2CHC(CH_3)_2$

VI $R_1 = O$-geranyl; $R_2 = H$

VII $R_1 = OCH_3$; $R_2 = O$-geranyl

VIII $R_1 = OCH_3$; $R_2 = OH$

IX $R_1 = H$; $R_2 = OH$

Although the separation of furocoumarins has been reported using both TLC[10] and HPLC methods[11–13], overlapping of components is often observed, even with the great resolving power of reversed-phase HPLC[13]. Therefore, this work was aimed at providing a useful alternative for the separation of furocoumarin mixtures with very similar chromatographic behaviour as found with IV and VII. Characterization of the compounds was made by means of UV and ^1H NMR spectroscopy and comparison with published data.

EXPERIMENTAL

Equipment

A Varian Model 5000 chromatograph equipped with a Rheodyne Model 7125 injector (20-μl loop) and a variable-wavelength UV detector operated at 270 nm was used. The temperature was maintained constant at 22°C, the flow-rate was 1 ml/min and the recorder chart speed was 1 cm/min. Instrument parameters were controlled by a Vista 401 CDS. The NMR spectrometer was a Varian XL-100 A. UV measurements were carried out with a Perkin-Elmer Model 202 UV–visible spectrophotometer. An analytical MicroPak Si-10 chromatographic column of 30 × 0.4 cm was used.

Conditions

Elution was carried out with a gradient of chloroform and methanol. Reservoir A contained chloroform and reservoir B chloroform–methanol (99:1). The gradient was applied as follows: 0 min, 90% A–10% B; 8 min, 50% A–50% B; and 12 min, 10% A–90% B.

An equilibration time of 10 min was allowed between runs. HPLC solvents and preparative silica gel plates (20 × 20 cm; layer thickness 0.2 cm) were obtained from Merck. NMR determinations were performed in $CDCl_3$ at room temperature;

chemical shifts (δ) are given in ppm from tetramethylsilane (TMS), which was used as an internal standard. Coupling constants (J) are given in hertz (Hz); s = singlet, d = doublet, dd = doublet of doublets and m = multiplet. UV determinations were carried out in spectroscopic-grade ethanol from Merck.

Preparation of extracts

A 7.62-g amount of dry, ground seeds was macerated three times in hexane at room temperature for periods of 24 h. After removal of the solvent *in vacuo* a total yield of 102.2 mg was obtained, and this was percolated through a short chromatographic column packed with silica gel to remove the most polar components, eluting with chloroform–methanol (95:5). After evaporation of the solvent the sample was made up to 10 ml with chloroform in a volumetric flask. Similar steps were followed for preparative purposes.

Separation and characterization of standards

The crude extract obtained from the extraction of 600 g of seeds was chromatographed in a column of deactivated silica gel (5% water), eluting with a gradient varying from pure hexane to hexane–ethyl acetate (85:15, v/v). The chromatographic fractions were monitored for furocoumarins by inspection of their ^{1}H NMR spectra and those giving rise to characteristic furan/lactone unsaturations were separated for further purification. Two furocoumarins were isolated in a pure form directly (III and VI), while the other two (IV and VII) were obtained as an oily substance that showed distinctive furocoumarin signals in the ^{1}H NMR spectrum. These compounds were separated by preparative silica gel TLC in pure dichloromethane. Thus, 20 mg of the oily "complex" were applied per plate and run three times with freshly distilled dichloromethane. Recovery from the plates was effected by elution with chloroform–methanol (90:10).

8-Geranyloxypsoralen (IV). Pale yellowish crystals, m.p. 57–58°C. ^{1}H NMR (δ ppm): lactone AB system δ_A 7.78, δ_B 6.38 (1H each, d, J = 10 Hz); 7.38 (1H, s), furan AB system δ_A 7.71, δ_B 6.83 (1H each, d), 5.04 (2H, d, J = 7 Hz), 2.01 (4H, ~d), 1.68, 1.63 and 1.56 (3H each, s). UV: λ_{max} 302, 265, 250, 243 and 223 nm.

5-Geranyloxyloxypsoralen (VI). Slightly yellowish crystals, m.p. 54–55°C. ^{1}H NMR (δ ppm): lactone AB system δ_A 8.16 (1H, dd, J^3 = 10 Hz, J^5 } 1 Hz), δ_B 6.28 (1H, d), furan AB system δ_A 7.61, δ_B 6.96 (1H, dd, J^3 = 2 Hz, J^5 } 1 Hz), 7.15 (1H, dd, $2J^5 \approx$ 1 Hz), 5.56 (1H, t, J = 7 Hz), 5.09 (1H, m), 4.97 (2H, d, J = 7 Hz), 2.10 (4H, d), 1.68 (6H, s) and 1.60 (3H, s). UV: λ_{max} 310, 259, 250, 244, 220 and 210 nm.

5-Methoxy-8-geranyloxypsoralen (VII). Pale yellow crystals, m.p. 56–58°C. ^{1}H NMR (δ ppm): lactone AB system δ_A 7.01, δ_B 7.63 (1H each, d, J = 10 Hz), furan AB system δ_A 7.64, δ_B 7.01 (1H each, J = 2 Hz), 5.61 (1H, t, J = 7 Hz), 5.03 (1H, m), 4.88 (1H, d, J = 7 Hz), 4.17 (3H, s), 2.00 (4H, ~ d), 1.64 (6H, s), 1.56 (3H, s). UV: λ_{max} 312, 267, 250 and 238 nm.

5-Methoxy-8-dimethylallylpsoralen (phellopterin) (III). Yellow crystals, m.p. 100–101°C. ^{1}H NMR (δ ppm): lactone AB system δ_A 7.01, δ_B 7.63 (1H each, d, J = 10 Hz), 5.62 (1H, t, J = 7 Hz), 4.86 (2H, d, = 7 Hz), 4.18 (3H, s), 1.70 and 1.74 (2H, ~d). UV: λ_{max} 311, 269, 247 and 240 nm.

The reference substances were dissolved in chloroform to a concentration of 0.5 mg/ml each. Calibration graphs were constructed by injecting different volumes of the standard solutions.

TABLE I

CONTENTS OF FUROCOUMARINS IN THE SEEDS OF *CASIMIROA EDULIS*

Compound	*Concentration (wt.-%)**	*Detection limit (ng)*	*k'***
III	0.0529 ± 0.0020	60	6.07
IV	0.0716 ± 0.0029	90	3.41
VI	0.021 ± 0.0012	70	2.57
VII	0.071 ± 0.0031	90	4.53

* Determined from $\bar{u} = \bar{x} \pm ts$ with a confidence level of 95% and $n = 3$, where $\bar{u}$ = population mean, $\bar{x}$ = sample mean, t = student's t-test and s = standard deviation.

** k' calculated as $(t_R - t_0)/t_0$, where t_R = retention time and t_0 = retention time of an unretained.

RESULTS AND DISCUSSION

The yields of four furocoumarins, their detection limits and capacity factors (k') are given in Table I. The calibration graphs (Fig. 1) were linear for sample amounts from 0 to 10 μg. The separation of these compounds is shown in Fig. 2.

In relation to the associated furocoumarins in *C. edulis* reported by Dreyer[8], differences were found regarding both the ratios and the components. Thus, whereas a mixture of phellopterin (III) and 8-geranyloxypsoralen (IV) was previously reported[8], a mixture of 5-methoxy-8-geranyloxypsoralen (VII) and 8-geranyloxypsor-

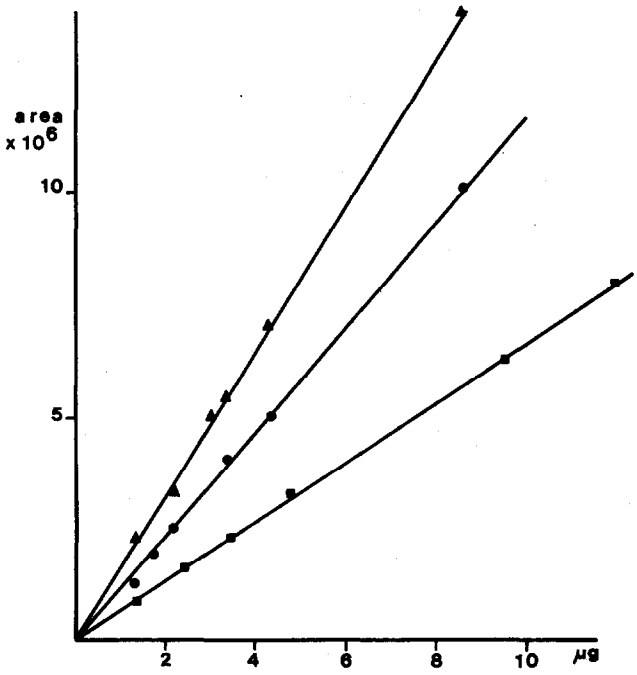


Fig. 1. Calibration graphs for compounds III (△), IV and VI (■) and VII (●).

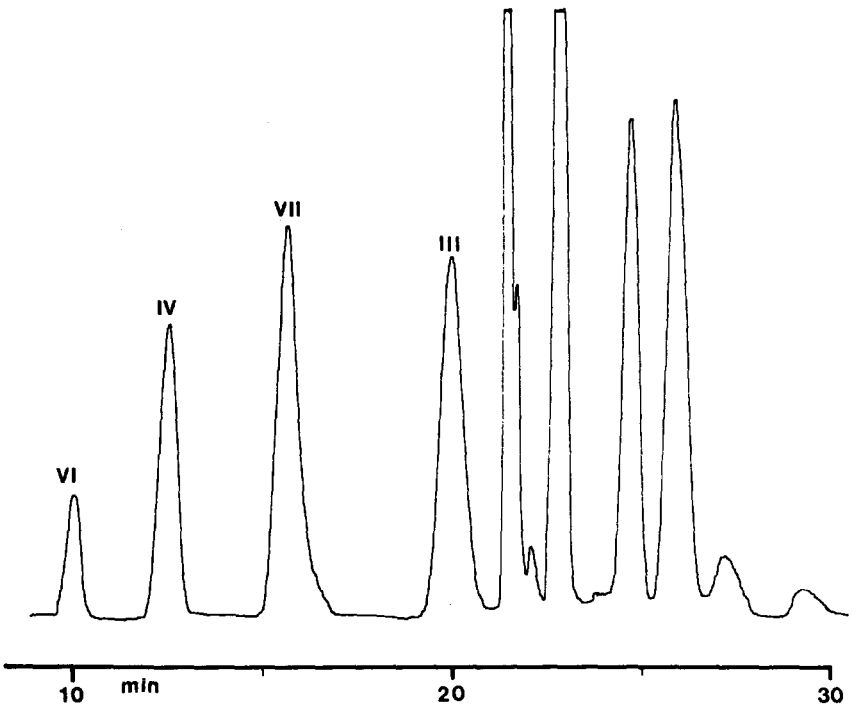


Fig. 2. Separation of furocoumarins in the hexane extract of the seeds of *Casimiroa edulis*.

alen (IV) in a ratio of approximately 1:1 was found in this work. Compound VI, on the other hand, had not previously been reported in *C. edulis*. The distinction between the isomeric pair IV and VI was made on the basis of their ^{1}H NMR spectra. 5-Geranyloxypsoralen shows additional long-range J^5 coupling constants between H-8–H-6 and h-8–H-4 of about 1Hz, while the corresponding ones are absent in IV.

Examination of the data reported by Kincl *et al.*[6] for a furocoumarinic substance allows one to postulate that it is actually a mixture of VIII and IX. These compounds arise from acidic cleavage of either III–IV or VI–VII, respectively.

It was found that in the preparative TLC of IV and VII, the best separations were achieved using pure methylene chloride. However, in the HPLC mode the best results were obtained with gradients of methylene chloride with methanol in the concentration range 0–1%.

Pharmacological assays in relation to hypotension are being carried out with furocoumarins III, IV and VII, and also phytochemical studies of these compounds in the leaves of *C. edulis*.

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