Influence of the Parasite *Viscum cruciatum* Sieber on the Chemical Constituents of *Crataegus monogyna* Jacq.

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Crataegus monogyna, *Viscum cruciatum*, Triterpenes

A phytochemical study of two plant species, *Viscum cruciatum* Sieber and *Crataegus monogyna* Jacq., was completed to investigate the influence of the parasite *Viscum cruciatum* on the host *Crataegus monogyna*. The study was carried out with two samples and consisted of hexane extracts of the *Viscum cruciatum* parasitizing on *Crataegus monogyna* and *C. monogyna*. In these samples ursolic acid, β-sitosterol and a triterpene fraction were found that contained mainly butyrospermol (3β-lanost 8, 24-dien, 3-ol), 24-methylene-24-dihydrolanosterol (24-methylene-5α-lanost-8-en-3β-ol), cycloartenol (9β, 19-cyclo-5α, 9β-lanost-24-en-3β-ol), β-amyrin (olean-12-en-3β-ol) and several aliphatic alcohols identified as the C_{18} to C_{30} members of the 1-alkanol homologous series.

β-Amyrin acetate was only isolated from *Viscum cruciatum* and was not found in *Crataegus monogyna*.

Introduction

Viscum cruciatum Sieber (Viscaceae) parasitizes a variety of hosts in the circummediterranean area (Ahumada *et al.,* 1995; Ayuso *et al*., 1985; Ayuso *et al*., l987; Ayuso *et al*., 1987, 1988). The plant has been evaluated for cytostatic activity, *Crataegus monogyna* Jacq. (Rosaceae) is a small thorny tree distribued in the Iberian Peninsula, the Balearics and Northwest Africa (Font Quer, 1990). This species has been used in folk medicine for its sedative action (Pietta *et al*., 1986), protective effects against arrythmias (Costa *et al*., 1986) and increase of coronary vessel flow (Bezanger-Beauquesne *et al.,* 1990). In our laboratory, the composition of the hexane extracts of *Crataegus monogyna* Jacq. has been previously investigated (Garcia *et al*., 1997). In this paper we studied the composition of hexane extracts of *C. monogyna* Jacq. parasitized with *Viscum cruciatum* Sieber and compared these components to those the hexane extract of the parasite, resulted in the isolation of three triterpenic alcohols, butyrospermol, 24-methylene-24-dihydrolanosterol and cycloartenol, and the C_{18} to C_{30} members of the 1-alkanol homologous series. The triterpene, β-amyrin acetate was only isolated from *Viscum cruciatum* Sieber.

Material and Methods

General experimental procedure

The MS were recorded at 70 eV on a Kratos MS 80 mass spectrometer. The GC operated with a Chrompack-CP 9000 using helium as the carrier gas. GC-MS was performed on a Carlo Erba gas chromatograph linked to a Kratos MS 80 mass spectrometer equipped with a NBSLIB2 data system, using cross-linked 5% phenyl methyl silicone (OV-5, 25 m $\times$ 0.25 mm $\times$ 0.23 μm). Samples were run under at programmed temperatures 230 °C (6 min) to 300 °C at 4 °C min^{-1}. The trimetylsilyl (TMS) ether derivatives of alcohols were obtained by reaction with a mixture pyridine/hexamethyldisilazane/trimethylchlorosilane (9:3:1 v/v/v) at room temperature for 30 min. For analytical TLC silica gel plates (60 F_{254} 0.2 mm, Merck) were used, and detection was made by spraying with oleum reagent: $H_2SO_4/CH_3COOH/H_2O$ (2:40:80 v/v/v) and subsequent heating for 5 min (120 °C).

Plant material

Aerial parts of the parasite *Viscum cruciatum* Sieber and the host *Crataegus monogyna* Jacq including twigs, stems and leaves, were collected in

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Puerto de los Vientos (Serranía de Ronda, Málaga) on February after a cold weather period. A voucher specimen of each species was deposited at the herbarium of the Department of Plant Biology (University of Sevilla) (SEV-F and SEV 137261, respectively), and were authenticated by Prof. S. Silvestre.

Extraction and isolation of triterpenes

Plant material (500 g) of each sample was extracted with hexane in a soxhlet apparatus. From the hexane extracts of *Viscum cruciatum* **V** and *Crataegus monogyna* **C** we obtained a white amorphous powder (0.29% **V1** and 0.05% **C1,** respectively) by precipitation in the cold (5–10 °C), (TLC silicagel developed with n-hexane/diethyl ether (70:30) gave a purple spot with oleum reagent Rf 0.09). The hexane extracts were concentrated under reduced pressure using a rotary evaporator to eliminate organic solvents. Residues of each sample (2 g) were then chromatographed on a silica gel column (60 g, 0.063–0.200 mm and 0.2–0.5 mm, Merck) and successively eluted with n-hexane/CHCl3 (90:10, 80:20, 70:30, 50:50, 30:70 and 10:90 v/v) yielding 169 fractions and 436 fractions respectively (5 ml each).

Fractions 44–67, corresponding to the n-hexane/CHCl3 80:20 v/v eluate, from hexane extracts of *Viscum cruciatum*, yielded a compound **V2** (TLC silica gel developed with n-hexane /diethyl ether (70:30 v/v) gave a orange-purple spot with oleum reagent, Rf (0.92) by preparative chromatography on silica gel.

Fraction 76–106 of the column from the hexane extracts of *Viscum cruciatum* and 151–214 of the column from extract of parasitised *Crataegus monogyna* were again chromatographed on a new column of silica gel using an n-hexane/diethyl ether gradient (n-hexane, n-hexane/diethyl ether 90:10, 80:20, 70:30, 60:40, and 50:50 v/v), yielding 139 and 105 fractions, respectively.

From fractions 11 to 34 and 35 to 83, from the second column of *Viscum cruciatum* the fraction **V3** and compound **V4** were isolated by preparative chromatography on silicagel.

From fractions 8 to 12, from the second column of *Crataegus monogyna*, and from the n-hexane/diethyl ether (70:30) eluate, a crystalline fraction (0.09%) **C2** was obtained, and fractions 24–27 from the n-hexane/diethyl ether (60:40) yielded compound **C3** by preparative chromatography on silicagel.

V3 and **C2** silica gel TLC developed with n-hexane/diethyl ether (70:30), gave a blue-purple spot with oleum reagent (Rf = 0.40). Before injection into the gas chromatograph, the fractions were converted to TMS ether derivatives by reaction with hexamethyldisilazane and trimethylchlorosilane in pyridine, and the TMS derivatives were separated on a OV-5 capillary column at programmed temperature and the GC-MS analysed.

Results and Discussion

Ursolic acid **(V1, C1):** mp 271–272 °C; UV(Cl3CH) λmax 235, 285 nm; EIMS *m/z* (rel. int.%) (M+) 456(6), 411(2), 300(6), 248(100), 219(8), 203(44), 133(33), 119(19).

β-amyrin acetate **(V2):** mp 235–237 °C; UV(Cl3CH) λmax 236 nm; EIMS *m/z* (rel.int.%) (M+) 468(3), 408(3), 218(100), 203(70), 189(40).

β-sitosterol **(V4, C3):** mp 140–141 °C; UV(Cl3CH)λmax 236, 262 nm; EIMS *m/z* (rel.int.%) (M+) 414 (25), 381(11), 145(36), 95(50), 81(68), 69(52), 55(86), 43(100).

Fraction **V3:** The trimethyl silyl derivative of fraction V3, was analyzed by gas chromatography on a capillary column and GC-MS analyses of the fraction showed a predominance of several aliphatic alcohols (74.41%) and four triterpenoids: β-amyrin, butyrospermol, 24-methylene-24-dihydrolanosterol and cycloartenol. Additionally, retention indices (Ip) at programmed temperature were calculated for each compound, in relation with those n-alkanes of Cn and Cn+1 (Dabrio, 1971), and the values obtained are given in Table I.

Peaks 1 to 13 were TMS ethers of a number of aliphatic alcohols (from C18 to C30 members of the 1-alkanol homologous series) that eluted with Ip less than 3328.

Identification of these peaks was carried out by comparison of mass spectra with spectral data in the NBSLIB2 library. The TMS ether derivative of the compound corresponding to peak No. 11 with Ip 3197 was not identified. The MS of the TMS ether of the triterpene alcohols corresponding to peaks No. 14, 15, 16 and 17 (Ip 3340, 3372, 3460 and 3570 respectively) showed the following predominant ions:

Table I. Gas chromatographic retention times and retention indices of TMS ether derivatives of the crystalline fraction isolated fron *Viscum cruciatum* Sieber.
- Retention time (tR), defined as time of solute peak maximum minus time of solvent front.
- Retention index (Ip) , estimated according to Van den Dool and Kratz (1963).

Peak	Retention time (tR)	Retention index (Ip)	Relative area (%)	Compound
1	3.1	2182	0.3	octadecanol-1
2	3.8	2284	0.3	nonadecanol-1
3	4.5	2356	1.1	icosanol-1
4	6.8	2552	0.2	docosanol-1
5	8.1	2632	6.0	tricosanol-1
6	10.1	2754	1.1	tetracosanol-1
7	11.6	2840	16.7	pentacosanol-1
8	14.1	2954	1.8	hexacosanol-1
9	15.2	3028	29.1	heptacosanol-1
10	18.6	3149	1.7	octacosanol-1
11	19.4	3197	13.9	unidentified
12	20.4	3248	0.3	nonacosanol-1
13	21.3	3328	2.1	triacontanol-1
14	22.0	3340	3.7	β-amyrin
15	22.6	3372	13.9	butyrospermol
16	23.7	3460	0.2	cycloartenol
17	25.0	3570	7.6	24-methylene-24-di-hydrolanosterol

Peak 14: *m/z* (rel.int%) (M+) 498(3), 483(11), 218(100), 135(6), 69(16).

Peak 15: *m/z* (rel.int%) (M+) 498(23), 483(37), 393(72), 203(16), 189(22), 145(35), 109(60), 95(42), 69(100).

Peak 16: *m/z* (rel.int.%) (M+) 498(2), 483(31), 408(96), 393(99), 286(26), 189(26) 175(40), 135(51), 109(53), 95(76), 69(100), 55(78). The fragment at *m/z* 286 involves loss of ring A of 9β,19-cyclopropane sterols (Goad,1991). In this mechanism, the cyclopropyl C-19 is retained with loss of C-6. However, an alternative fragmentation involves retention of C-6 with loss of C-19 to give an ion with the same *m/z* value.

Peak 17: *m/z* (rel.int%) (M+) 512(3), 497(14), 483(19), 407(3), 393(47), 339(7), 271(7), 189(17), 69(100).

The M+ at 498 (C30H49OSi-(CH3)3 and 512 (C31H51OSi-(CH3)3) indicated that these compounds were TMS ethers of C30 triterpene alcohols. Spectral data corresponding to those of butyrospermol, cycloartenol and 24-methylene-24-dihydrolanosterol have been previousey reported (Goad, 1991; Itoh, *et al.*, 1981; Lercker *et al.*, 1981; Kornfeldt *et al.*, 1981).

The major constituents of this mixture were the aliphatic alcohols (74.4%): however, the triterpene alcohols were present in minor quantities. Butyrospermol (13.8%), cycloartenol (0.2%) and 24-methylene-24-dihydrolanosterol (7.6%) also were present.

The fraction **C2:** The silyl derivative was analyzed by gas chromatography on a capillary column and the GC-MS analyses of the fraction showed the presence of several aliphatic alcohols (9.2%) and fourth triterpenoids: β-amyrin, butyrospermol, 24-methylene-24-dihydrolanosterol and cycloartenol. Retention index (Ip), retention time (tR) and relative area (%) are given in Table II.

The major constituent of this mixture was cycloartenol. Cycloartenol was the compound corresponding to peak no.16 and accounted for 79.5% of the fraction (equivalent to 67.0 mg of cycloartenol for 100 g of plant material). This compound was accompanied by other triterpene alcohols: butyrospermol (7.8%), 24-methylene-24-dihydrolanosterol (2.7%) and β-amyrin (0.5%), (equivalent to 7.0, 2.4 and 0.4 mg/100 g of plant, respectively). Aliphatic alcohols accounted for 9.2% of the mixture, equivalent to 8.3 mg/100 g plant material.

Table II. Gas chromatographic retention times and retention indices of TMS ether derivatives of the fraction isolated from *Crataegus monogyna* Jacq.
- Retention time (tR), defined as time of solute peak maximum minus time of solvent front.
- Retention index (Ip) , estimated according to Van den Dool and Kratz (1963).

Peak	Retention time (tR)	Retention index (Ip)	Relative area (%)	Compound
1	3.1	2180	0.1	octadecanol-1
2	3.8	2290	0.02	nonadecanol-1
3	4.6	2360	0.4	icosanol-1
4	6.9	2554	0.05	docosanol-1
5	8.1	2640	1.5	tricosanol-1
6	10.1	2760	0.2	tetracosanol-1
7	11.9	2830	2.9	pentacosanol-1
8	14.1	2954	0.2	hexacosanol-1
9	15.6	3030	1.6	heptacosanol-1
10	18.7	3150	0.1	octacosanol-1
11	19.3	3198	0.6	unidentified
12	20.4	3250	0.2	nonacosanol-1
13	22.1	3330	1.3	triacontanol-1
14	22.4	3342	0.5	β-amyrin
15	22.8	3375	7.8	butyrospermol
16	23.6	3450	79.5	cycloartenol
17	25.0	3558	2.7	24-methylene-24-di-hydrolanosterol

Cycloartenol, butyrospermol, 24-methylene-24-dihydrolanosterol and the aliphatic alcohols have been described in *Crataegus monogyna* Jacq. (Garcia *et al.*, 1996), but these compounds are identified from *Viscum cruciatum* Sieber for the first time.

β-Amyrin has not been previously detected in *Crataegus monogyna* Jacq. For this reason, we conclude that this compound, when *Crataegus* is parasitized by *Viscum cruciatum* Sieber, can be synthesized by the parasite.

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