

Epicuticular Leaf Wax of *Euphorbia helioscopia* L. (Euphorbiaceae)

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Dedicated to Prof. Dr. Karimullah on the occasion of his 90th birthday

Euphorbia helioscopia, Epicuticular Wax Composition, Fatty Acids, Hydrocarbons, Wax Esters

The epicuticular wax of *Euphorbia helioscopia* was fractionated into fatty acids, hydrocarbons, wax esters, aldehydes, methyl esters, triterpenol acetates, alcohols, sterols, and polar components. The composition of the fractions was determined by GC, GC-MS, HPLC. Main components within these lipid classes are hentriacontane, wax esters C₄₆ and C₄₈, octacosanal, hexacosanol and octacosanol, hexadecanoic acid, and β -sitosterol. Lupeol and its acetate were also confirmed.

Introduction

Euphorbia helioscopia, a common weed in winter crops, possesses certain medicinal uses [1].

Previously, an alcohol, sterol and an unidentified compound were described from this plant [2] whereas thirteen 12-deoxyphorbol esters were isolated from the fresh latex of this species [3]. Later on, neutral lipids from the leaves and the plant were described [4, 5]. Some minor but highly oxygenated compounds like euphoscopins A and B [6] and some crystalline lactones like helioscopinolides A and B [7] were isolated from the fresh plant. Very recently thirty one new diterpenes including euphoscopins, epieuphoscopins, euphorins, euphohelioscopin and euphohelionone were isolated from the fresh plant [8]. The epicuticular wax of the plant leaves was studied and is being described here.

Materials and Methods

IR: Neat or a dispersion in KBr on a IR spectrophotometer (Hitachi 270-30).

NMR: Solutions in deuterated chloroform with TMS as the internal standard on 60 MHz NMR spectrometer (Hitachi 24B).

GC-MS: Instrument JMS-AX 505 W mass spectrometer, column DB-1, 0.25 μ m film thickness, 0.32 mm \times 30 m, oven temp. programming 50 to 300 °C with a rise of 15 °C/min and initial hold for 1 min, accelerating voltage 3.0 Kv, ionizing voltage 70 V, scan range m/z 20–600, scan speed 0.5 sec cyclic.

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Extraction of wax

Different batches of mature leaves (620 g) collected between Feb. 9 and March 13, 1991 were dipped in hexane for 3 min. The process was repeated twice. The hexane extract was filtered to remove dirt and then freed of the solvent. A greenish semi-solid material (2.07 g, 0.33% of fresh leaves) was obtained.

Isolation of acidic components and their esterification

The above extract (2.07 g) was dissolved in hexane (1 L) and stirred with 0.1 M KOH (300 ml) for 30 min. The layers were separated and the process repeated. The combined alkaline layers were shaken with ether, after separation the water layers were acidified with 2 M hydrochloric acid. The liberated acids were extracted with ether and (after usual work-up) semisolid acids (209 mg, 1.01%) were obtained. These acids (200 mg) were dissolved in benzene (5 ml), boron trifluoride-methanol complex (0.3 ml) was added and the test tube kept in a heated water bath for 15 min. The reaction mixture was diluted with water, the esters were extracted with hexane, freed of solvent, and purified on a PLC plate.

Fractionation into class compounds

The neutral portion of extract (1.00 g) was dissolved in warm hexane and coated on silica gel (10 g) (by warming and swirling the flask till all the solvent got evaporated). This gel was placed on the top of a silica gel 60 (Merck) column (40 g, 42 \times 1.5 cm). The elution was carried out with hexane, hexane-ether, ether and finally with methanol. The fractions were monitored by TLC on Sili-

ca gel 60 G (Merck) and the mixtures were separated by preparative layer chromatography (PLC). The R_f values, quantities present and possible inferences are given in Table I.

Saponification of wax esters

The esters (R_f 0.62, 160 mg) were refluxed in benzene (10 ml) and 2 M ethanolic potassium hydroxide (20 ml) for 2 h. Alcohols, soluble in organic solvents, were obtained (68.7%, 110 mg). The soap solution was processed as in the previous paragraph. After purification by PLC, colourless, semi-solid methyl esters (40 mg, 25%) were obtained.

Gas liquid chromatography of fatty acid methyl esters

Gas liquid chromatography of methyl esters was carried out on a Pye Unicam 104 Instrument equipped with an FID, a carbowax 20 M column (SGE) maintained at 220 °C, and a Spectra-Physics Recorder. The identification was done by retention time measurement and co-injection of standard esters (Merck).

Separation of alkyl acetates and triterpenol acetates

Freshly prepared plates (silica gel-silver nitrate, (9:1, 20 × 20 × 0.1), dried in dark and activated at 100 °C for 30 min were used. The acetates (80 mg) were charged to each plate and the plates developed thrice in a solvent system carbon tetrachloride:methylene chloride (5:1). The zones were visualized by spraying with 2,7-dichlorofluorescein, marked under ultra violet light, scratched and then extracted with distilled chloroform.

High performance liquid chromatography of alcohol acetates

The acetates were analysed using a reversed phase C_{18} column (LichroCART 250–4 mm, Merck), an LC controller Hitachi 638-30, a refractive index detector ERC-7510 (Erma Optical Works Ltd. Japan), Hitachi 833 Data processor and methanol and tetrahydrofuran (80:20 v/v) as the mobile phase. The identification was achieved by comparing the retention volumes and co-injecting the standard acetates (produced by reduction of the corresponding standard fatty acids to alcohols and acetylation of these alcohols).

Gas liquid chromatography (GLC) of the hydrocarbons

GLC of the hydrocarbon fraction was carried out on a Shimadzu GC-7AG chromatograph equipped with an FID, an SE-30, WCOT capillary column with CLH-702 holder, bore 0.2 mm, film thickness 0.25 µm, length 25 m (Shimadzu). The oven temperature was programmed as initial hold at 180 °C for 2 min, 8 °C/min rise, and final hold at 290 °C till no more peaks appeared. The data was processed by a Chromatopac C-R6A (Shimadzu). The identification and quantification was done by comparing the retention times and co-injecting the standard samples (Supelco).

Results and Discussion

The leaves of *Euphorbia helioscopia* contain an epicuticular wax which comprises of hydrocarbons, wax esters, aldehydes, methyl esters of fatty acids, acetates of triterpenols, triterpenols, alcohols, sterols, free fatty acids and resinous material. These class compounds were separated by CC and purified by PLC, whenever necessary. The R_f values and their distribution is recorded in Table I.

Free acids

The free acids isolated by alkali treatment of the extract formed only a small fraction (1%). The

Table I. Distribution of class compounds in Epicuticular wax from *Euphorbia helioscopia*.

S. No.	R_f^*	% contribution	Inference
1.	0.70	9.5	hydrocarbons
2.	0.62	14.1	wax esters
3.	0.45	8.9	aldehydes
4.	0.41	4.6	methyl esters
5.		2.0	acetates of triterpenols
6.	0.31	Traces	
7.	0.11	40.0	primary and triterpenoidal alcohols
8.	0.06	1.4	sterols
9.		12.0	polar oxygenated compounds
10.		1.0	fatty acids
Loss on column		6.5	

* R_f values determined on a precoated silica gel 60 F254, layer thickness 0.2 mm (Merck) using toluene as eluting solvent.

composition of the corresponding fatty acid methyl esters was determined by GC-MS and is reported in Table II. The acids present are similar to those reported in literature [9, 10].

Hydrocarbons

The white waxy fraction (R_f 0.70) was obtained in 9.5% yield. It proved to be saturated hydrocarbons in IR, NMR and argentation TLC. GLC analysis showed the alkanes ranging from octadecane (C_{18}) to nonatriacontane (C_{39}) (Table II). Hentriacontane (C_{31}) was the dominating hydrocarbon in *E. helioscopia* and in many other *Euphorbia* species *e.g.* *E. dendroides* [9] and *E. aphylla* [10], and further *Euphorbia* species [11]. However the distribution pattern was different.

Aldehydes

The fraction eluted by hexane-ether 85:15 comprised of two closely placed spots on TLC plate.

The IR and NMR spectra of the fraction (R_f 0.45) isolated by PLC in toluene in 8.9% yield confirmed the aliphatic and the aldehydic function of the components. On the basis of GC-MS data, hexacosanal, octacosanal and triacontanal were confirmed (Table III).

Methyl esters

The fraction (R_f 0.41) was taken in minimum of boiling hexane and allowed to cool to remove the acetates. The GC-MS data confirmed the presence of methyl behenate, methyl lignocerate, methyl cerotate and methyl 2-methyl hexadecanoate, methyl 2-methyl tetracosanoate and methyl 2-methyl hexadecanoate (Table III). The latter group was identified as branched at C-2 on the basis of their fragments of 88 and 101 instead of 74 and 87 due to McLafferty Rearrangement. To our knowledge the presence of methyl esters in epicuticular waxes is not very common.

Table II. Distribution of fatty acids, hydrocarbons, aldehydes, methyl esters and alcohols in the epicuticular wax of *E. helioscopia* (relative peak areas).

Carbon chain length	Free acid	Combined acid	Hydrocarbons	Aldehydes	Methyl esters	Free alc.	Comined alc.
12	—	0.4	—	—	—	—	0.1
14	3.6	1.8	—	—	—	—	0.2
15	—	0.3	—	—	—	—	—
16	34.7	23.7	—	—	—	—	0.1
17	—	0.2	—	—	2.2	—	—
18	15.9	13.9	0.4	—	—	—	1.0
18:1	6.4	traces	traces	—	—	—	—
19	—	0.1	—	—	—	—	—
20	6.3	31.9	0.2	—	—	0.6	1.9
21	—	0.3	—	—	—	0.1	0.1
22	12.4	21.4	0.3	—	26.3	2.2	2.6
23	—	0.1	0.1	—	—	0.5	0.6
24	4.8	2.7	0.3	—	43.9	2.8	1.8
25	—	0.1	1.9	—	11.0	0.7	0.5
26	4.8	1.0	0.6	7.9	15.3	41.3	52.4
27	—	—	16.7	—	1.3	4.5	0.1
28	9.1	—	0.8	89.9	—	38.4	26.7
29	—	—	20.9	—	—	0.1	—
30	1.7	—	0.5	traces	—	3.5	0.1
31	—	—	29.1	—	—	0.1	—
32	0.5	—	0.3	—	—	0.1	—
33	—	—	15.2	—	—	—	—
34	—	—	0.1	—	—	—	—
35	—	—	6.7	—	—	—	—
36	—	—	0.1	—	—	—	—
37	—	—	3.6	—	—	—	—
38	—	—	—	—	—	—	—
39	—	—	1.0	—	—	—	—

Table III. Characteristic mass fragments of aldehydes and methyl esters.

Component	Scan No.	Characteristic m/z (relative intensity)
Hexacosanal	785	380(3); 362(15); 336(2); 334(5); 292(4); 264(4); 152(5); 137(11); 124(17); 109(23); 96(70); 82(87); 69(49); 57(97); 44(6); 43(100); 41(40)
Octacosanal	903	408(2); 390(18); 360(2); 362(4); 250(2); 208(3); 152(5); 138(10); 123(16); 110(25); 96(74); 82(100); 69(45); 57(90); 44(6); 43(77); 41(38)
Triacontanal	994	436(2); 418(20); 390(4); 320(20); 208(5); 180(3); 152(5); 137(9); 123(15); 110(17); 96(65); 82(84); 69(40); 57(100); 44(5); 43(73); 41(32)
Methyl 2-methyl hexadecanoate	357	284(14); 253(1); 241(7); 213(3); 185(3); 157(8); 143(5); 115(5); 101(48); 88(100); 55(21); 44(2); 43(25); 42(3); 41(18)
Methyl behenate	624	354(32); 323(4); 311(11); 295(1); 255(5); 199(6); 143(22); 129(7); 97(7); 87(67); 74(100); 59(5); 55(22); 43(33); 42(3); 41(16)
Methyl lignocerate	742	382(41); 531(4); 339(11); 283(5); 241(3); 199(6); 143(23); 129(9); 97(9); 87(74); 74(100); 59(4); 55(21); 43(37); 42(4); 41(15)
Methyl 2-methyl tetracosanoate	770	396(37); 365(2); 353(8); 297(3); 213(4); 157(21); 143(7); 101(63); 88(100); 69(8); 57(30); 43(39); 42(3); 41(15)
Methyl cerotate	843	410(43); 379(3); 367(10); 311(4); 255(3); 199(7); 143(25); 129(9); 97(11); 87(75); 74(100); 59(5); 57(35); 55(31); 43(48); 42(4); 41(18)
Methyl 2-methyl hexadecanoate	875	424(42); 381(7); 325(4); 213(4); 157(24); 143(5); 115(8); 101(68); 88(100); 69(17); 57(32); 55(30); 43(40); 42(5); 41(8)

Triterpenyl acetates

The fraction obtained after removal of methyl esters was compared with the acetates from alcoholic fraction (following paragraphs) on argentation TLC, HPLC and IR. Lupeol acetate (65.93%) and the other unidentified triterpenol (34.06%) present in free alcohol were confirmed. The structure of the unidentified triterpenol is in progress.

Free alcohols

The free alcoholic fraction (R_f 0.11) constituted the major portion (40%) of the epicuticular wax. It was an amorphous white solid which softened at 65 °C and melted clearly up to 75 °C. The fraction was homogenous in TLC, and showed a light orange colour on spraying with carbazole-sulphuric acid reagent which indicated the presence of triterpenols [12]. The fraction was acetylated and crystallised from boiling ethanol when the major portion of saturated alkyl acetates appeared as fluffy solid. The mother liquor was removed and the fluffy solid rinsed with hexane. The ethanol mother liquor and the washing were combined, freed of solvent and separated on silver nitrate impregnated plates. Four fractions characterised by IR and NMR-spectroscopy were saturated alkyl acetates

(R_f 0.58, 92.6%), two cyclic alcohol acetates (R_f 0.36, 1.2% and R_f 0.23, 3.7%) and polar material (1.2%). Alkaline hydrolysis of acetate R_f 0.23 and crystallisation from methanol gave needles of lupeol m.p. 210–212 °C (literature [13] m.p. 213–216 °C). Further confirmation was done by comparison of the ^1H NMR spectrum and ^{13}C NMR spectrum [13].

The saturated alcohol acetates (R_f 0.58, above in high performance liquid chromatography analysis ranged from docosanol (C_{22}) to dotriacontanol (C_{32}) (Table II) with the highest percentage of ceryl alcohol (C_{26} , 43.4%).

Sterols

The sterol fraction (R_f 0.06) was obtained in 1.4% yield. On crystallisation from methanol, needles of β -sitosterol m.p. 136–137 °C were obtained. Further confirmation was done by HPLC analysis and by co-injecting the standard β -sitosterol (Aldrich).

Wax esters

The amorphous solid wax esters (R_f 0.62) constituted 14.1% of the epicuticular wax and proved to

be in IR and NMR as straight chain compounds. On saponification with alkali 68.7% of the alcoholic and 25.0% of the acidic fractions were obtained.

The acids were analysed as above. Only traces of unsaturated members were observed. They ranged from lauric acid (C_{12}) to lignoceric acid (C_{26}). Their composition (Table II) resembles those from stems [3, 4]. The alcoholic fraction was acetylated and analysed by HPLC (Table II). The area (87.8%) under this chromatogram was covered by straight chain alcohols ranging from dodecanol-1 (C_{12}) to triacontanol-1 (C_{30}). In agreement to the free alcoholic fraction, hexacosanol-1 formed the highest portion (52.3%).

The epicuticular wax of *E. helioscopia* contains in addition to usual components occurring in *Euphorbia* waxes methyl esters of normal and branched chain fatty acids. The hydrocarbons, alcohols, aldehydes, wax esters identified in the present investigation were found to contain saturated and long chain components with alcohols as the major class (40.0%). The aldehydes have one dominating component *i.e.* octacosanal (89.9%). Although a latex bearing plant, the wax contains only a small amount of triterpenols.

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