Pak. j. sci. ind. res., vol. 35, no. 11, November 1992

EPICUTICULAR WAX OF *EUPHORBIA CADUCIFOLIA* HAINES

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(Received July 28, 1992; revised November 11, 1992)

The stems of *Euphorbia caducifolia* contain epicuticular wax (0.66%) which comprises free fatty acids (2.4%), hydrocarbons (6.7%), wax esters (17.8%), aldehydes (31.1%), fatty alcohols (18.9%), friedelan-3 α-ol (4.4%), unidentified alcohols (6.2%) and resinous material (3.3%). The free and combined fatty acids range from lauric acid (C_{12}) to behenic acid (C_{22}). The hydrocarbons range from pentacosane (C_{25}) to pentatriacontane (C_{35}) with the highest percentage of nonacosane (C_{29}). The aldehydes range from tetracosanal (C_{24}) to dotriacontanal (C_{32}) with the highest concentration of the last member of the series. The free fatty alcohols range from octacosanol (C_{28}) to tetratriacontanol (C_{34}) whereas the alcohols obtained after ester hydrolysis contained two more lower homologues of the series. Triacontanol (C_{30}) and dotriacontanol (C_{32}) were the major fatty alcohols. The esters range from hexacosyl laurate (C_{38}) to tetracontyl behenate (C_{56}).

Key words: *Euphorbia caducifolia*, Epicuticular wax composition, Friedelan-3α-ol.

Introduction

All aerial organs of higher plants are covered with a thin continuous wax layer which represents the interface between the land plants and their aerial environment. The function and physiological role of this wax coating is essential for the healthy growth of plants. The wax layer is responsible for the controlled transpiration and the gas exchange through the stomata. Although containing mainly the same set of known molecules the distribution in the waxes is species specific [1]. The biosynthesis of wax lipids regulates the phytogenetic development of plants.

Euphorbia caducifolia is a tall, armed dense erect and stout bush with thick cylindrical leafless green branches. It is found abundantly in the sandy terrains of Sindh and some areas of Baluchistan province [2,3]. The latex and the root extracts of some of the species are used in the indigenous system of medicine [4]. Previous work on the latex of the plant reveals the presence of cyclolaudenol, 3-epi-cyclolaudenol, cycloartenol, [5] euphol, tirucallol, cycloartenol and cyclocaducinol [6]. Caducifolin and jolkinolide A [7], 3-ketomethyl ursolate [5], were isolated from the total plant. The present work deals with the epicuticular wax of the plant.

Materials and Methods

Melting points are uncorrected. IR-spectra were recorded on a Hitachi 270-30 infrared spectrometer as neat or in KBr. Hitachi R24B NMR spectrometer (60MHz) was used for recording the proton spectra. Deuterated chloroform and tetramethylsilane were used as the solvent and the reference respectively.

Collection of the plant material and extraction of epicuticular wax. The authentic plant material was collected from Karachi area and saplings planted in PCSIR Laboratories Complex Campus, Lahore. The plants were allowed to grow for a year and the samples taken. The stems (80 g) were held vertically in distilled chloroform for 3 min. in such a way that no latex came in contanct with the solvent. The chloroform extract was filtered to remove dirt and then evaporated on a rotary evaporator. A slightly greenish solid material (0.51 g, 0.66%) was obtained.

Isolation of the acidic components and their esterification. The greenish solid material (0.5 g) was dissolved in hexane (500 ml) and stirred with 0.2 M aqueous potassium hydroxide (150 ml) for 20 min. and the layers separated. The process was repeated thrice and finally the hexane layer was washed with water. The alkaline extracts and washings were combined and acidified with 1 M sulphuric acid. Extraction with ether and usual processing gave acids (12 mg, 2.4%) which were dissolved in benzene (1 m) and treated with boron triflouride- methanol (1:10,2ml) in a sealed tube immersed in a boilling water bath for 20 min. The reaction mixture was diluted with water and extracted with hexane. The hexane layer, after washing with saturated sodium bicarbonate solution and brine, was dried and evaporated. The residue was passed over silica gel (2 g) to give colourless methyl esters (12 mg).

Gas liquid chromatography (GLC) of methyl esters. GLC of methyl esters of fatty acids was carried out on a Pye-Unicam gas chromatograph, equipped with the FID detector. Two different columns were used for the resolution of the ester components. A steel column (152 x 0.95 cm) packed with 10% diethylene glycol succinate coated on diatomite C (80–100 mesh) was maintained isothermally at 200°. Nitrogen at a flow rate of 40 ml/min. was used as the carrier gas. A 25 m long WCOT, FFAP column (Supelco) was programmed from 100–200° with a rise of 4 min. Hydrogen at a flow

velocity of 26 cm/sec was used as the carrier gas. The injector and the detector temperatures were maintained at 300°. The signals were recorded and integrated with Spectra Physics 4100 data processor. The peaks were identified by comparing the retention time as well as by coinjecting the respective standards.

Separation of neutral components. The hexane solution after removal of acids was dried and evaporated. The neutral residue (0.45 g) was charged on to eight glass plates (20 x 20cm), coated with a 1 mm layer of silica gel (Kieselgel G 60 F 254 Merck). The plates were developed in toluene, allowed to dry and then sprayed with a 1% solution of 2, 7-dichlorofluorescein in methanol. The zones were marked under UV light, scratched and extracted with distilled chloroform. The R_f values of TLC plate, percentage contribuition and chemical nature of each component was recorded (Table 1).

Gas liquid chromatography (GLC) of hydraocarbon fraction. The equipment and the procedure for the GLC of the hydrocarbon fraction was the same as for the methyl esters except that a 25 m long WCOT BP column (SGE), maintained isothermally at 230° was used for the separations.

TABLE 1. YIELD AND DISTRIBUTION OF CLASS COMPOUNDS IN *E. CADUCIFOLIA* EPICUTICULAR WAX.

Yeld of epicuticular wax Feb, 1990 0.66%
" " " " May, 1990 0.44%.

R_f values*	Contribution by weight %	Nature of fraction	Techniques used for inference
–	2.4	Acids	TLC, IR and neutralization
0.70	6.7	Hydrocarbons	TLC, IR
0.62	17.8	Wax esters	TLC, IR, saponification
0.42	31.1	Aldehydes	TLC, IR, NMR
0.36	2.2	Esters with other functional groups	TLC, IR, NMR
0.21	2.0	—do—	—do—
0.15	0.7	—do—	—do—
0.14	1.5	Alcohols	IR
0.08	27.7	Mix. of aliphatic and cyclic alcohols	TLC, IR, NMR and carbazole test.
0.04	2.0	Esters with other functional groups	TLC, IR, NMR
0.02	2.3	—do—	—do—
0.00	3.3	Resinous material	

*Solvent: Toluene.

Resolution of aliphatic and cyclic alcohols. The alcoholic fraction R_f 0.08 (125 mg) was treated with acetic anhydride- pyridine (1:1, 2 ml) overnight. After work up the crude acetates were charged to 10% $AgNO_3$-silica gel plates (20 x 20 x .1 cm, 3 Nos). The plates were developed thrice in a solvent system (hexane-dichloromethane, 4:1), sprayed with 1% 2,7- dichlorofluorescein, zones marked under UV light, scratched, and extracted. Three bands R_f.77 (80 mg, 18.9%), R_f.55 (30 mg, 6.6%) and R_f.32 (10 mg, 2.2%) were isolated.

Isolation of friedelan-3 α-ol. The sub-fraction R_f .55 in argentation TLC was saponified with alkali and the resulting alcohol (27 mg) was warmed with hexane. The hot hexane layer was removed leaving behind crude friedelan-3 α-ol (18 mg, 4.4%). This was further crystallized out of chloroform-methanol to give needles m.p.304, $[\alpha]_{28}^{D}$ 15.8° (C = 0.0014, $CHCl_3$), Lit. $[\alpha]_{28}^{D} = 16.1°$.

Oxidation of friedelan-3 α-ol to friedelin. The alcohol (10 mg) was dissolved in dry acetone (40 ml) and oxidized with Jones reagent [10] (10 ml) at room temperature. The reaction mixture was diluted with water and friedelin extracted with ether in quantitative yield.

High performance liquid chromatography (HPLC) of fatty alcohol acetates. HPLC of fatty alcohol acetates was carried out on a Hitachi LC Controller 638-30 operated in an isocratic mode. A reversed phase C column (LiChroCART 250-4, 7 µm, Merck) was used for the separation. Methanol: tetrahydrofuran (90:10 v/v) was used as the eluent. A refractive index detector (ERC-7510, Erma Optical Works Ltd. Japan) was used as a monitor. The peaks were registered and processed with a Hitachi M 833 data processor. The components were identified by comparison of the elution times and co-injecting the standards.

Reduction of aldehydic fraction to alcohols. The aldehydic fraction (60 mg) was dissolved in dry ether (40 ml) and lithium aluminium hydride (25 mg) was added portionwise. The mixture was stirred for 4 hrs at room temperature. The excess reagent and the complex were decomposed by the dropwise addition of sat. ammonium chloride solution. The reduced alcohols were extd. with ether, dried and recovered after solvent evaporation (60 mg).

Saponification of wax esters R_f 62. The esters (80 mg) were dissolved in benzene (10 ml) and refluxed with 2M ethanolic potassium hydroxide (15 ml) for 4 hrs. The reaction mixture was diluted with distilled water and extracted with ether (3 x). The combined extracts were washed with water, dried and evaporated to give the alcohol fraction (50 mg, 60.5%).

Liberation of acids and their esterification. The alkaline solution after the saponification of esters was treated in the same way as the free acids. The yield was 25 mg (31.5%).

Results and Discussion

The stems of *Euphorbia caducifolia* contained varying amounts of an epicuticular wax which was 0.66% in Feb. 1990 and 0.44% in May the same year. Thin layer chromatographic analysis revealed the presence of acids, hydrocarbons, wax esters, aldehydes, fatty alcohols, triterpenols as the major constituents and several other minor constituents. These constituents were separated by chemical reaction and preparative layer chromatography on silica gel. The distribution of these components, their R_f values, and the inferred classes of compounds are given in Table 1.

Free acids. Free acids were isolated by the alkali treatment of the epicuticular wax dissolved in hexane and they constituted 2.4% of this wax. These acids were esterified by boron trifluoride-methanol and analysed by gas liquid chromatography. For the purposes of identification two columns a packed diethylene glycol succinate column and a WCOT FFAP column were used. Six saturated acids ranging from lauric to behenic and three unsaturated acids oleic, linoleic, and linolenic were identified. The quantitative results (Table 2) were obtained on a WCOT FFAP column. Palmitic acid and stearic acid were the major components.

TABLE 2. THE AMOUNT OF INDIVIDUAL FATTY ACIDS, HYDROCARBONS, ALDEHYDES AND ALCOHOLS IN VARIOUS FRACTIONS OF EPICUTICULAR WAX FROM *E. CADUCIFOLIA*.

	Acids				Alcohols	
No. of C. atoms	Free	Esterified	Hydrocarbons	Aldehydes	Free	in esters
12	t	0.77	–	–	–	–
14	0.39	0.46	–	–	–	
16	38.62	24.51	–	–	–	–
18(0)	36.27	22.96	–	–	–	–
18(1)	14.72	31.08	–	–	–	–
18(2)	6.96	14.25	–	–	–	–
18(3)	0.91	2.91	–	–	–	–
20	1.32	2.45	–	–	–	–
22	t	0.61	–	–	–	–
24	–	–	–	0.84	–	–
25	–	–	13.21	–	–	–
26	–	–	0.49	2.20	–	0.72
27	–	–	13.93	2.10		0.24
28	–	–	2.98	4.78	1.51	4.61
29	–	–	49.05	3.13	0.18	0.71
30	–	–	0.83	29.63	42.17	54.81
31	–	–	13.33	5.73	–	–
32	–	–	t	46.18	55.90	35.36
33	–	–	2.93	–	–	–
34	–	–	–	–	0.24	0.32
35	–	–	0.94	–	–	–

Hydrocarbons. The least polar fraction (R_f.70) constituted 6.7% of the epicuticular wax. It was a white waxy solid, m.p. 43- 50° and was homogeneous in TLC analysis on silica gel as well as silver nitrate impregnated silica gel plates showing only the presence of saturated compounds. In the IR spectrum strong peaks at 2964, 2928, 2860 medium sized peak at 1464 and small peaks at 1380, 730, 722 cm^{-1} indicated straight long chain hydrocarbon nature of the fraction.

The composition of the fraction was determined by GLC. The *n*-alkanes ranged from pentacosane (C_{25}) to pentatri acontane (C_{35}). The odd-numbered *n*-alkanes predominated over the even numbered ones, a common characteristic of plant hydrocarbons of higher plants. The noticeable concentration of even number hydrocarbons especially octacosane (C_{28}) can be easily explained on basis of previous findings of Herbin and Robins [11] in hydrocarbons from angiosperms. Nonacosane (C_{29}) was found to be the major component (49.05%). This composition was similar to the one from the latex and total plant reported previously [12,13]. However, the n-alkanes forming the maxima in the distribution pattern differed from several other species e.g. *E. dendroides*,[14] *E. aphylla* [15], *E. helioscopia* [16], *E. copiapina, E. lactiflua* [17].

Aldehydes. The fraction (R_f .42) constituted 31.1% of the epicuticular wax. It was an amorphous white solid melting range 45-62°. The IR spectrum showed strong peaks at 2964, 2924, 2856, medium sized peaks at 1718, 1464, and small peaks at 1380, 730, 722 cm^{-1}. All these peaks correspond to straight chain carbonyl compounds. The proton NMR spectral data δ9.77 (t, j = 2Hz, aldehydic proton), 2.25 (m, protons α to carbonyl), δ1.22 (s, - CH_2-), δ 0.84 (t, j = 6Hz, terminal methyl) confirmed the fraction as aliphatic aldehydes. The composition of this fraction was determined by HPLC analysis of the reduced and acetylated material.

The aldehydes ranged from tetracosanal (C_{24}) to dotriacontanal (C_{32}). Both the even and odd numbered aldehydes were present but the content of even numbered aldehydes predominated. The highest concentration was that of dotriacontanal (46.18%). Aldehydes of similar composition have been isolated from other euphorbia species *E. dendroides* [14], *E. niccaensis, E. cyparissias, E. characias, E. lathyris, E. peplus* [18].

*Free alcohols.*Free alcohol constituted the second largest class compounds (29.2%) but were of different chemical structures. The major share were those with an R_f. 08. This fraction which was found to be a mixture of aliphatic and cyclic alcohols (by IR and NMR studies) was acetylated and separated by argentation TLC.

The least polar sub-fraction (R_f. 77, 18.9%) showed IR peaks at 2960, 2924, 2856, 1722, 1465, 1385. 1240, 1050, 720

and 710 cm^{-1}, all corresponding to acetates of n-alkanols. The signal in ^1H NMR, δ3.97 (t, j = 6Hz - CH_2 - 0 -), δ1.98 (s, CH_3 $\overset{O}{\overset{\|}{C}}$), δ1.25 (s, - CH_2 -), δ 0.85 (t, j = 6Hz terminal CH_3) showed the fraction comprising exclusively of acetates of *n*-alkanols. The composition was determined by HPLC where the straight chain primary alcohols ranged from octacosanol to dotri acontanol with the highest percentage of the last member in the homologous series. Similar compositions have been reported in the literature [18,19].

The middle spot in the argentation TLC (R_f .55, 6.6%) was saponified and the resulting alcohols were analysed by HPLC where two major peaks were observed. For resolution of the individual components, the mixture was taken up in hot hexane and decanted to remove the more soluble component. The process was repeated four times until the residue appeared as a single peak of lower retention time in HPLC analysis. This material was crystallised from chloroform-methanol to give friedelan-3α-ol, (4.4%), m.p. 304°, acetate m. p. 315° and 3-keto derivative friedelin m. p. 256°. Friedelan 3α-ol has also been isolated from several other species e.g. *E. antiquorum* [20], *E. neriifolia* [21].

The hexane soluble component with higher retention time in HPLC analysis was freed of solvent, taken up in boiling methanol, filtered to remove traces of friedelan-3-α-ol and allowed to cool. Thread-like long needles (2.5%), m. p. 202°, acetate m. p. 239-240° were obtained.

The most polar acetates in argentation TLC (R_f. 32, 2.2%) were semi-solid. The IR spectrum showed in addition to the ester carbonly and methylenes in a straight chain, unsaturation at 3090 and 1642 cm^{-1}. The inhomogeneity of these acetates was observed in HPLC where at least eight peaks were observed. All these facts showed that it was a mixture of unsaturated and branched aliphatic alcohol acetates.

The free alcohol fraction (R_f. 14) constituted 1.5% of the epicuticular wax. It was oily in nature and co-chromatographed with previously isolated triterpenoidal alcohol glutinol from this species. IR-spectrum (neat) showed a broad peak 3500-3400 cm^{-1} (OH), strong peaks at 2936, 2876, 1462, 1386 cm^{-1} (CH), and small peaks at 1736,1718, 1226, 1168 (carbonyl) and 730 cm^{-1} (CH). The H ^{1}NMR showed signals at δ 1.25 (s - CH_2 -), δ0.92 and δ0.84 (s, angular methyls). These data showed that there is no similarity between glutinol and this alcohol.

Esters. The esters constituted 27.0% of the epicuticular wax. As evidenced from the R_f values (Table 1), they may also contain other functional groups. Among these, the least polar fraction formed the major portion (17.8%). It was a white waxy solid melting range 40-65°. The IR spectrum showed peaks at 2970, 2930, 2850, 1735, 1465, 1375, 1175, 730 and 720 cm^{-1} which all originated from esters containing of long chain primary alcohols and fatty acids. On saponification, 60.5% of an alcohol fraction and 31.3% of a carboxylic acid fraction were obtained.

The IR spectrum of the alcohol fraction was devoid of the ester carbonyl peaks 1735 and 1175 cm^{-1} but contained a peak at 1065 cm^{-1} (C-O). The ^{1}H NMR spectrum confirmed the fractions as n-alkanols. The semi-solid acetates obtained by acetylation of a portion of this fraction was homogeneous in argentation TLC. The HPLC analysis of the acetates showed the n-alkanols to range from hexacosanol to tetratriacontanol. Contrary to the free alcohol fraction triacontanol was the major component (54.81%), however, the difference between two major components was well-marked.

The carboxylic acid fraction was processed as the free acid fraction. The combined acids ranged from lauric acid (C_{12}) to behenic acid (C_{22}). The unsaturated acids oleic, linoleic and linolenic formed 48.24% of this fraction which is a higher figure than for the corresponding acids in the free acid fraction.

Based on the composition of the fatty acid and fatty alcohols one can postulate that the smallest possible ester may be hexacosyl laurate (C_{38}) and the largest possible ester is tetratriacontyl behenate (C_{56}). It is further noted that the esters of chain length C_{46} and C_{48} are predominating.

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