

Epicuticular Leaf Wax of *Euphorbia dendroides* L., Euphorbiaceae

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Quantity and composition of epicuticular leaf wax of *Euphorbia dendroides* L. was examined. The wax contained *n*-alkanes, wax esters, aldehydes, fatty acids and primary alcohols. In addition to these common epicuticular wax constituents several triterpenoids and benzoic acid esters were found. The triterpenols β -amyrin and lupeol occurred free as well as esterified with long chain fatty acids. The ketones Δ 12-oleanen-3-one and lupen-3-one have also been identified.

Introduction

Euphorbia dendroides L. is native to the mediterranean region and prefers rocky places near the sea. It is a perennial shrub and forms aspherical bushes which are flowering in February–March. The leaves are small, oblong-lanceolate, and fall in early summer as an adaptation to the semiarid mediterranean climate. *E. dendroides* contains latex which is characteristic for the Euphorbiaceae [1]. The latex of several *Euphorbia* species has been studied extensively for its possible industrial use and some species represent a renewable resource for hydrocarbons [2]. In addition the triterpenols are studied for application as a taxonomic marker [3–7]. Thus *E. dendroides* is reported to belong to a chemical grouping, which contains β -amyrin and lupeol in comparable amounts in its latex [4]. Triterpenols are also mentioned as constituents of the epicuticular leaf waxes from other *Euphorbia* species, but detailed investigations are rare [8, 9]. The results presented in this report are the first steps to analyze triterpenoid patterns in the leaf waxes of several *Euphorbia* species, and to complement previously reported results on their epicuticular waxes [9].

Abbreviations: CC, column chromatography; GC, gas chromatography; GC-MS, gas chromatography-mass spectrometry; TLC, thin-layer chromatography; SEM, scanning electron microscopy.

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Material and Methods

Plant material

Euphorbia dendroides was cultivated from seeds in the gardens of the Botanical Institute, University of Cologne. They were grown in the greenhouse during winter and in the field from spring to autumn. Leaves were harvested in March. To extract the epicuticular wax the leaves were dipped consecutively into beakers with 600 ml CHCl_3 for 3 min. Care was taken that no latex ran into the CHCl_3 . The extract was filtered and evaporated to dryness. 249 g of fresh leaf material yielded 979 mg of raw wax (0.4%), (1.6% dry wt.).

Silica gel column

The raw leaf wax was redissolved in 50 ml of hot hexane. Only the soluble parts were chromatographed on a silica gel (Merck 60, Darmstadt) column with the following solvents of increasing polarity [10, 11].

- I. Pentane (600 ml) to elute hydrocarbons,
- II. 2-chloropropane (750 ml) to elute esters, aldehydes, and ketones,
- III. methanol (800 ml) to elute alcohols and acids.

The insoluble part was added to the methanol fraction. Yield and composition of the individual fractions are reported in Table I. A flow diagram with the isolated components is given in Fig. 1.

TLC: Silica gel precoated plates (Merck 60, Darmstadt), solvents:
1) C_6H_6 ($R_{\text{f},1}$); 2) CH_2Cl_2 : EtOA_c (24:1)



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(R_{f_2}); 3) plates were impregnated with AgNO_3 in acetonitrile, solvent system CH_2Cl_2 : EtOA_c (24:1) (R_{f_3}); spray reagents used: a) bromothymolblue or b) carbazole for triterpenoids [12].

GC: Hewlett Packard model 5710 A with FID and integrator 3380 S, 25 m glas capillary column DUHT-OV 101, Temp 160 to 340 °C, rate 4 °C/min.

GC-MS: Finnigan - MAT 4510; 70 eV; EI.

MS-Data:

a) Triterpenoids:

Δ 12-Oleanen-3-one: m/z (rel. int.); 55 (100); 203 (45); 218 (90); 257 (0.6); 271 (0.2); 409 (M-15, 0.4); 424 (M $^+$, 0.8).

Lupen-3-one: 55 (100); 205 (30); 218 (10); 232 (5.0); 245 (4.0); 313 (2.5); 381 (M-43, 0.1); 409 (M-15, 1.0); 424 (M $^+$, 2.0).

β -Amyrin: 43 (100); 55 (100); 203 (45); 218 (100); 243 (0.4); 257 (0.9); 411 (M-15, 0.2); 426 (M $^+$, 0.4).

Lupeol: 43 (100); 55 (95); 189 (20); 207 (15); 218 (10); 247 (0.8); 257 (1.0); 315 (0.7'); 383 (M-43, 0.1); 408 (M-18, 0.1); 411 (M-15, 0.2); 426 (M $^+$, 0.9).

MS-data of the isolated lupen-3-one, β -amyrin and lupeol are identical to those from authentic samples (Roth, Karlsruhe).

b) Benzoic acid esters:

Benzoic acid docosanyl ester: m/z (rel. int.); 77 (20); 105 (40); 123 (100); 139 (0.5); 153 (0.2); 430 (M $^+$).

Benzoic acid tetracosanyl ester: 77 (15); 105 (30); 123 (100); 139 (0.5); 153 (0.3); 458 (M $^+$, 0.1).

Benzoic acid hexacosanyl ester: 77 (20); 105 (45); 123 (100); 139 (0.6); 153 (0.8); 486 (M $^+$, 0.1).

MS-data of the isolated benzoic acid esters are identical to those from the synthesized esters.

Methanolysis

The substance was refluxed with 2N HCl/MeOH for 2 h. After evaporation the residue was dissolved in CH_2Cl_2 and evaporated to dryness once more to remove the HCl completely. Finally the product was dissolved in hexane and analyzed.

Ethanolysis

Wax esters were refluxed with 2N HCl/EtOH for 14 h. The reaction products were extracted with

ether/petrolether (1:1) three times, washed for neutrality, dried over MgSO_4 , filtered and evaporated.

Reduction

A solution of the triterpene ketones in benzene was stirred with NaBH_4 deposited on aluminum oxide for 2 h [13], filtered and analyzed.

Hydrogenation

The substance was dissolved in EtOAc with Pd-catalysator and stirred under a H_2 -atmosphere for 2 h, filtered and evaporated.

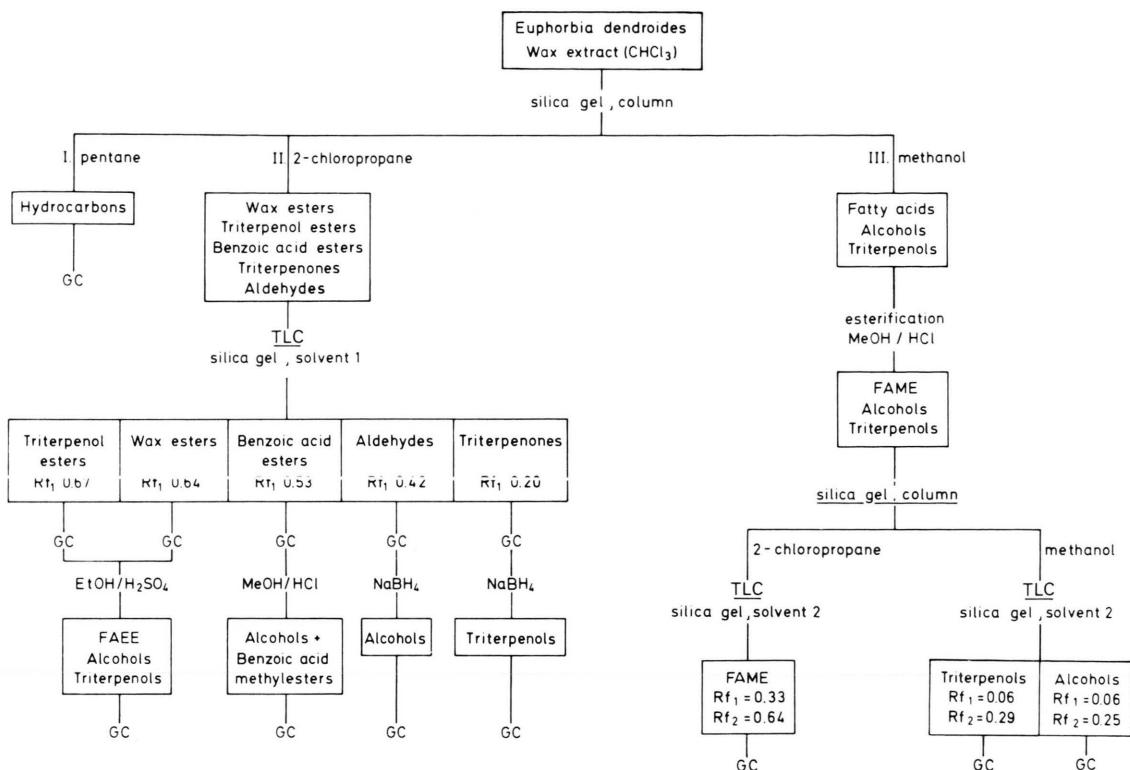
Synthesis of benzoic acid esters.

A mixture of hexacosanol (100 mg) with an excess of benzoic acid and a few drops of H_2SO_4 was heated to 80 °C for 1 h. The reaction products were extracted with ether/petrolether (1:1), washed for neutrality, dried over MgSO_4 , filtered and evaporated. The esterification was checked by TLC, GC and GC-MS.

Results and Discussion

The epicuticular wax (1.63% dry wt) of *E. dendroides* leaves extracted with CHCl_3 was found to contain a complex mixture of different components. Previous analysis of epicuticular waxes from *Euphorbia* species have shown the presence of common lipid components such as alkanes, wax esters, aldehydes, alcohols and fatty acids [9]. Such components resulting from the lipid metabolism are also constituents of the *E. dendroides* epicuticular wax. In addition to the components mentioned above, a number of triterpenoids were found which originate from the isoprene metabolism. Benzoic acid esters were also discovered, a rather uncommon group and therefore rarely described as epicuticular wax constituents.

Fractionation of the raw wax was carried out on a silica gel column with solvents of increasing polarity into three fractions [10, 11]. Fraction I, the hydrocarbons, could be detected directly by GC. Fraction II (2-chloropropane) and III (methanol) had to be separated again by TLC. A flow diagram for fractionation, separation and identification of the individual components is shown in Fig. 1. Yield and composition of *E. dendroides* leaf wax is listed in Table I.

Fig. 1. Flow diagram for fractionation, separation and identification of epicuticular leaf wax from *E. dendroides*.Table I. Yield and composition of *E. dendroides* epicuticular leaf wax.

Fraction	mg	% wax	% dry wt	mg	% fract.	R_{f_1}
I. pentane	68.5	7.0	0.11	68.5	100	0.7
<i>n</i> -Alkanes						
II. 2-chloropropane	249.7					
Triterpenol esters		4.6	0.08	45.0	18	0.67
Wax esters		3.9	0.06	38.2	15	0.64
Benzoic acid ester		2.1	0.04	20.6	8	0.53
Aldehydes		1.9	0.03	18.6	7	0.42
Triterpenones		13.0	0.21	127.3	51	0.20
III. methanol	502.8					
Triterpenols		8.6	0.14	84.2	17	0.06
pr. Alcohols		27.7	0.45	271.0	54	0.06
Fatty acids		15.1	0.25	147.6	29	0.02
unidentified	83.6	8.5				
lost on column	74.4	7.6				
	979.0	100.0				

Alkanes

Fraction I contained only saturated *n*-alkanes in a series of 21 homologues ranging from C_{19} to C_{39} . Main components in almost comparable amounts were heptacosane ($C_{27}H_{56}$) with 32.0% and hexatriacontane ($C_{31}H_{64}$) with 33.0%. Odd numbered *n*-alkanes are predominant (97%) (see Table II).

Wax esters

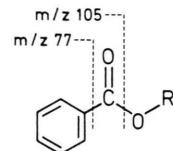
Wax esters occurred together with triterpenol esters in fraction II (2-chloropropane). They had to be separated from aldehydes by TLC (benzene). Triterpenol esters could be separated in most cases and are discussed later. Wax esters (R_f , 0.64) were present in homologous series ranging from C_{36} to C_{52} . The esters C_{42} (32.7%) C_{44} (26.4%) and C_{46} (23.4%) were dominant (see Table II). As reported previously the individual ester peaks represent indeed wax esters of the same total carbon number but mostly they contain mixtures of isomeric esters which differ in the chain length of the alcohol and acid components [14].

Ethanolysis of the wax esters yielded alcohols and fatty acids in form of fatty acid ethyl esters (FAEE). Alcohols ranged from C_{18} to C_{30} with hexacosanol

(C₂₆H₅₄O) as the dominating main component (83.7%). FAEE were always saturated and ranged from C₁₄ to C₃₀. Palmitic acid (C₁₆H₃₂O) with 38.2% and stearic acid (C₁₈H₃₆O) with 30.3% were the dominant fatty acids (see Table II).

Benzoic acid esters

The 2-chloropropane fraction contained esters with R_{f_1} 0.53 which could be separated by TLC (see Fig. 1). Analysis with GC resulted in three peaks. These esters were saponified with HCl/MeOH and heated for 2 h. This procedure yielded even numbered primary alcohols ranging from C_{22} to C_{26} with hexacosanol ($C_{26}H_{54}O$) as the main component (92.2%). The structure of the resulting combined acid was elucidated by GC-MS. The mass spectra of



$$R = C_{22}, C_{24}, C_{26}$$

Table II. Quantitative composition of epicuticular wax components of *E. dendroides* (peak area %).

Table III. Composition of benzoic acid esters in *E. dendroides* leaf wax.

No. of C-atoms	TLC R_{f_1}	GC r.e.t.*	GC-MS M^+	ester. No. of C-atoms	Alcohols %
29	0.53	1.000	430	22	2.1
31	0.53	1.078	458	24	5.7
33	0.53	1.157	486	26	92.2

* Relative emergence temps.

all three esters showed significant fragment ions, namely m/z 77 and m/z 105 which are characteristic for benzoic acid esters [15]. In addition to this the molecular ion M^+ was visible in all mass spectra. The coordination of the benzoic acid to the corresponding alcohols is listed in Table III.

The structure of these esters was verified by synthesis. These synthetic benzoic acid tetracosanyl and hexacosanyl esters show the same chromatographical behaviour and MS-data as the isolates from *E. dendroides* leaf wax. Benzoic acid esters in the form of analogue esters were just found in *Citrus halimii* leaf wax, too, [16] and benzyl-benzoate has been reported earlier in the essential oil of *Cistus* leaves [17].

Aldehydes

Aldehydes (R_{f_1} 0.42) could be detected in fraction II. They were separated by TLC (benzene) and analyzed by GC. Aldehydes are present with chain lengths from C_{22} to C_{32} , with octacosanol ($C_{26}H_{54}O$) dominating (74.3%). Reduction with $NaBH_4$ yielded primary alcohols. Aldehydes ($C_{28}H_{56}O$) and free alcohols ($C_{26}H_{54}O$) showed defined main components but of different chain length. In the same fraction one additional band with R_{f_1} 0.20 could be separated by TLC. These substances were found to be triterpenones.

Fatty acids

Fraction III contained pr. alcohols, triterpenols and fatty acids. The similarity of their R_f values (alcohols R_{f_1} 0.06) (fatty acids R_{f_1} 0.02), and the non feasibility to gas chromatograph of fatty acids in their free form, necessitated their esterification into methyl esters (FAME). These FAME could be separated completely by C.C. on silica gel with 2-chloropropane. The GC analysis showed chain lengths ranging from C_{14} to C_{30} . Dominating was eicosanoic acid ($C_{20}H_{40}O_2$) with 34.2% followed by

stearic acid ($C_{18}H_{36}O_2$) (24.3%) and palmitic acid ($C_{16}H_{32}O_2$) (14.9%). Only saturated fatty acids could be detected (see Table II).

Alcohols

Primary alcohols (R_{f_2} 0.25) were separated by TLC with the solvent $CH_2Cl_2/EtOAc$ (24:1) and analyzed by GC. They ranged in chain lengths from C_{22} to C_{30} . The dominating main component was hexacosanol ($C_{26}H_{54}O$) with 95.4%, see Table II.

Triterpenols and derivatives

Free triterpenols were also present in the methanol fraction. Their separation succeeded by TLC with the solvents $CH_2Cl_2/EtOAc$ 24:1 (R_{f_2} 0.29). The coloration with carbazole was positive [12]. Two individual peaks were obtained in the subsequent GC analysis and identified with authentic samples as β -amyrin and lupeol. These triterpenols showed different R_f values on silica gel plates, impregnated with $AgNO_3$ (β -amyrin R_{f_3} 0.30, lupeol R_{f_3} 0.20).

In addition to this their identity was confirmed by GC-MS which gave data identical to those from authentic samples. The results were in agreement with data reported by Tulloch [18, 19]. Both triterpenols were also found to be esterified with long chain fatty acids. Separation of the triterpenol esters was carried out by TLC (benzene) with R_{f_1} 0.67. Ethanolysis of these esters resulted in the triterpenols β -amyrin and lupeol, and fatty acids in form of ethyl esters (FAEE) ranging in chain lengths from C_{16} to C_{30} . Triterpene derivatives were also found as ketones and were present in fraction II (R_{f_1} 0.20). These ketones were separated by TLC (benzene) and identified as oleanen-3-one and lupen-3-one. Both ketones showed a positive colour reaction with carbazole and were reduced with $NaBH_4$ to the corresponding alcohols β -amyrin and lupeol. MS data of the isolated oleanen-3-one and lupen-3-one were

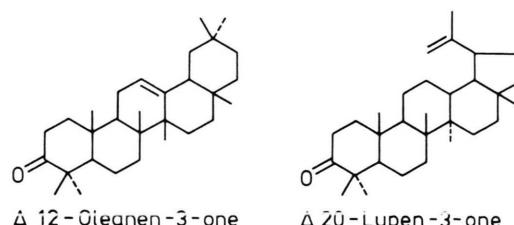


Fig. 3. Structures of triterpene ketones.

Table IV. Characterisation of triterpenoids in *E. dendroides* leaf wax.

	R_{f_1}	R_{f_3}	TLC	GC r.e.t.*	GC-MS M^+
			Carbazole		
I. Triterpenol esters					
a) Triterpenol esters					
C ₄₈ -C ₆₀	0.67		+		
b) ester. β -Amyrin	0.06	0.30	+	1.000	426
ester. Lupeol	0.06	0.20	+	1.033	426
II. Triterpene-3-ones					
a) Δ 12-Oleanen-3-one	0.20		+	0.981	424
NaBH ₄ \rightarrow β -Amyrin	0.06	0.30	+	1.000	426
b) Lupen-3-one	0.20		+	1.018	424
NaBH ₄ \rightarrow Lupeol	0.06	0.20	+	1.033	426
III. Triterpenols					
a) β -Amyrin	0.06	0.30	+	1.000	426
b) Lupeol	0.06	0.20	+	1.033	426

* Relative emergence temps.; β -Amyrin = 1.000.

identical to those from authentic samples (lupen-3-one) and were in agreement with MS data reported by Budzikiewicz *et al.* [20] (see Table IV).

Yield and composition of the epicuticular wax of *E. dendroides* leaves were in agreement with data of other *Euphorbia* species in respect to alkanes, wax esters, aldehydes, free alcohols and free fatty acids [9]. Triterpenoids are known to be present in *Euphorbia* leaf waxes but detailed analysis exist only sparsely. Free triterpenols, namely β -amyrin and lupeol in *E. dendroides* epicuticular leaf wax were found to be the same as reported from the latex [4]. The predominance of primarily saturated and long

chain components as well as the high amounts of primary alcohols, free fatty acids, triterpenones and triterpenols in the epicuticular leaf wax of *E. dendroides* result in crystalline wax layers. Platelets with fringed edges arising from a liquid layer were observed on the SEM pictures (not documented). They are similar to the surface structures of other *Euphorbia* species recently described by Hemmers *et al.* [21].

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