Epicuticular Wax of *Euphorbia aphylla* Brouss. ex. Willd., Euphorbiaceae

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Dedicated to Professor Hans Reznik on the occasion of his 65th birthday

Euphorbia aphylla, Epicuticular Wax Composition, Triterpenols, Triterpenones, Triterpenol Esters

Quantity and composition of epicuticular wax of *Euphorbia aphylla* were examined. The wax contained the common lipid components *n*-alkanes, wax esters, pr. alcohols, fatty acids and in traces aldehydes and acetates all occurring in homologous series. Additionally, several triterpenols such as β-amyrin, α-amyrin, lupeol and simiarenol were found. With the exception of simiarenol these triterpenols occurred free as well as esterified with acetic acid and fatty acids. The ketones, oleanen-3-one, ursen-3-one and lupen-3-one have also been identified.

Introduction

Euphorbia aphylla Brouss. ex. Willd. is a small, compact, succulent and extensively ramified shrub growing to 50 cm in height. Stems are about 5–8 cm long, grey green, richly limbed, slender, pencil like in diameter and leafless. The flowers are more or less sessile and found in small clusters at the tips of stems. Fruits are very small, light brown or reddish. Seeds are also small, brown, caruncle sessile and plate like. This shrub is native to the central- and western islands of the Canary Island group and found frequently near the coastal rocks [1–3].

Materials and Methods

E. aphylla was cultivated in the greenhouse of the Botanical Gardens of the City of Cologne. The stems were dipped consecutively into two beakers with 600 ml $CHCl_3$ each for a total of 6 min. Care was taken that no latex from the cuttings ran into the $CHCl_3$. The extract was filtered, evaporated to dryness and yielded 4015 mg crude wax. The wax was redissolved in hexane and chromatographed on a silica gel column into three fractions by eluting with pentane, 2-chloropropane and methanol as described previously [4–6]. These fractions were separated further by preparative TLC on silica gel precoated plates. For preparative and analytical TLC the following solvents were used: 1) Toluene (R_{f1}); 2) CH_2Cl_2:EtOAc (24:1) (R_{f2}) and 3) $AgNO_3$ impregnated plates, CH_2Cl_2:EtOAc (24:1) (R_{f3}). The isolated compounds were identified with chemical reactions such as methanolysis, ethanolysis, acetylation and reduction with $NaBH_4$ as well as TLC and GC analysis and comparison with authentic samples as described recently [5–6].

GC was carried out with a Hewlett Packard 5710, equipped with FJD and an integrator 3380 S. The column used was 20 m glas capillary DUHT OV 101; the temperature was programmed from 160 °C to 340 °C at 4 °C/min. The triterpenols were identified by GC-MS: Finigan-MAT 4510; 70 eV; EI; with a 15 m fused silica column DB-1.

Yield and composition of the isolated wax compounds are shown in Table I.

The quantitative composition of the lipid wax compounds are listed in Table II.

Composition and characterization of the triterpenoids are given in Table III.

Results and Discussion

The epicuticular wax extracted from *E. aphylla* stems was found to contain a complex mixture of

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different components. The common long chain and saturated lipid components were found in homologous series of alkanes, wax esters, primary alcohols, fatty acids and aldehydes and acetates in trace amounts. These substances are resulting from the lipid metabolism and were present with 62.7% of the crude wax.

On the other hand, triterpenoids, which originate from the isoprene metabolism were found in amounts of 31.4% of the wax. These substances were identified free as well as in form of their ketones and esterified with acetic acid and fatty acids (see Table I).

Table I. Yield and composition of *E. aphylla* epicuticular wax.

	mg	% wax	R_{f1}	R_{f2}
n-Alkanes	61	1.5	0.70	
Wax esters	547	13.6	0.62	
Aldehydes	35	0.9	0.43	0.77
Acetates	30	0.8	0.35	0.68
pr. Alcohols	1654	41.2	0.06	0.30
Fatty acids	189	4.7	0.02	
Triterpenol esters	60	1.5	0.62	
Triterpenol acetates	291	7.2	0.35	0.68
Triterpenones	85	2.1	0.19	0.53
Triterpenols	827	20.6	0.06	0.30
Unidentified	80	2.0		
Lost on column	156	3.9		
	4015	100		

Common lipid components

Hydrocarbons (1.5% wax) were fractionated with pentane from the silica gel column. GC analysis showed a homologous series of prevailing odd numbered *n*-alkanes ranging from C_{23} to C_{35} in chain lengths with hentriacontane as the main component (60.9%).

Wax esters (13.6% wax) were present in the fraction eluted with 2-chloropropane and were separated from the other compounds by TLC (R_{f1}: 0.62). Esters of previously even numbered chain lengths ranged from C_{36} to C_{52} with C_{42} (35.0%) and C_{44} (30.4%) as the main components. The last two esters derived mainly from the fatty acid C_{16} and the alcohols C_{26} and C_{28} as shown by their saponification products. However individual ester peaks are not documenting individual wax esters. Such peaks contain mixtures of isomeric esters with the same total carbon number [7, 8]. Ethanolysis of the wax esters yielded primary alcohols ranging from C_{24} to C_{30} in chain lengths and fatty acids as FAEE ranging from C_{14} to C_{30} (see Table II).

Table II. Quantitative composition of epicuticular lipid wax components of *E. aphylla*.

No. of C-atoms	Alkanes	Alcohols Free	Alcohols Esterified	Fatty acids Free	Fatty acids Esterified	No. of C-atoms	Wax esters
12				3.5	+	36	+
14				10.8	11.6	38	7.7
16				31.1	61.5	39	+
18:1					5.0	40	6.1
18				7.8	5.6	41	+
19						42	35.0
20				1.4	4.6	43	+
21						44	30.4
22				+	+	45	+
23	+					46	10.0
24	+	+	+	1.5	6.9	47	
25	2.9					48	+
26	+	81.7	63.8	29.8	5.0	49	
27	8.5					50	+
28	+	18.3	36.2	12.0	+	51	
29	15.5					52	+
30	0.8	+	+	+	+		
31	60.9						
32	0.7						
33	5.9						
34	+						
35	+						

Aldehydes (0.9% wax) were found in very small amounts presumably with chain lengths of C_{24} to C_{30}. Of these, the aldehyde C_{26} was identified by GC-MS. Acetates (0.8% wax) were found in similar small amounts containing C_{26} and C_{28} alcohols.

Alcohols and fatty acids were eluted from the silica gel column with methanol. This fraction was esterified with HCl/MeOH to obtain the corresponding fatty acid methyl ester (FAME). These FAME (4.7%) were separated from the alcohols by silica gel column chromatography as described before [5]. GC analysis of the FAME (R_{f1} 0.33) showed numerous acids ranging from C_{12} to C_{30} with two maxima in their distribution pattern, at C_{16} and C_{26}.

Primary alcohols (41.2% wax) were the dominating compounds in this surface wax and of these hexacosanol was present in the highest amounts of 81.7%. These alcohols had the same chain lengths as those found in the wax esters (see Table II). Alcohols usually show the highest concentrations in epicuticular waxes of Euphorbia species [5, 9]. They have an unique and steep distribution pattern with hexacosanol always as the dominating component. This alcohol was found also to be dominating in the wax esters of this genus. A very high chain length specificity in the biosynthesis of the alcohols may be postulated from these results in contrast to the fatty acids biosynthesis.

Triterpenoids

In the epicuticular waxes of all *Euphorbia* species studied a high concentration of triterpenoids (26%–61%) was found. Not only the free triterpenols could be identified but also their derivatives in form of ketones and esters.

In the wax of *E. aphylla* several triterpenols (20.6% wax) accompanied the primary alcohols. Thus β-amyrin was identified by TLC (R_{f3}: 0.30) with a positive carbazole colour reaction [10] as well as by GC comparison with authentic samples and also by GC-MS data [5, 6]. The MS spectra show the characteristic ions: *m/z* (rel. int.) 426 (M^+); 218 (100); 203 (40) for β-amyrin. This substance was taken as reference for the r.r.t. = 1.000. In similar concentrations as β-amyrin, α-amyrin and lupeol were present. They also give a positive carbazole colour reaction. On silica gel plates which were impregnated with $AgNO_3$, lupeol showed a quite different R_f value (R_{f3}: 0.20) than those of β-amyrin and α-amyrin (R_{f3}: 0.30), according to another position of the double bond namely in Δ 20 [6]. Depending on the quality of the column α-amyrin and lupeol are not always completely separated by GC. Nevertheless both compounds were identified definitely by their characteristic GC-MS data and comparison with authentic samples (see Table III). The MS spectra showed the characteristic ions for α-amyrin: 426 (M^+); 218 (50); 203 (15) and for lupeol: 426 (M^+); 315 (0.5); 205 (20) [5, 6].

Additionally simiarenol, a triterpenol usually not occurring in waxes, could also be identified. This compound showed a deviation in R_f-values (R_{f1}: 0.10) from the above mentioned triterpenols (R_{f1}: 0.06) and based on this behaviour could thus be isolated by TLC. Simiarenol showed a positive colour reaction with carbazole. The presence of a free alcohol group was proven by a positive acetylation. GC-MS data showed the following characteristic ions: 426 (M^+); 411 (M^+-15); 408 (M^+-18); and 245 (2); 259 (20); 274 (20); 281 (5) [11]. The same triterpenol was isolated and identified in detail from *E. lathyris* by Hemmers *et al.*, showing identical chromatographic and spectroscopic behaviour [12]. Simiarenol was found in a concentration of only 10% of the total of the other triterpenols present.

Wax esters as well as triterpenol esters (R_{f1}: 0.62) and triterpenol acetates (R_{f1}: 0.35) were found in the

Table III. Composition and characterization of triterpenoids in *E. aphylla* epicuticular wax.

	TLC R_{f1}	R_{f3}	Carbazole	GC r.r.t.*	GC-MS M^+
Triterpenol esters					
β-Amyrin esters	0.62		+		
α-Amyrin esters	0.62		+		
Lupeol esters	0.62		+		
Triterpenol acetates					
β-Amyrin acetate	0.35	0.64	+	1.097	468
α-Amyrin acetate	0.35	0.64	+	1.126	468
Lupeol acetate	0.35	0.46	+	1.129	468
Triterpenones					
Oleanen-3-one	0.19	0.53	+	0.983	424
Ursen-3-one	0.19	0.53	+	1.013	424
Lupen-3-one	0.19	0.42	+	1.016	424
Triterpenols					
β-Amyrin	0.06	0.30	+	1.000	426
α-Amyrin	0.06	0.30	+	1.029	426
Lupeol	0.06	0.20	+	1.034	426
Simiarenol	0.10	0.04	+	1.098	426

* Relative retention times.

same fraction eluted with 2-chloropropane from the silica gel column. Triterpenol esters could not be separated from the wax esters in this case. The esterified triterpenols (1.5% wax) were analyzed by ethanolysis and yielded β-amyrin, α-amyrin and lupeol each in similar concentrations. They showed the same TLC and GC behaviour as the free triterpenols, described above (see Table III). These triterpenols were esterified with fatty acids, predominantly with chain lengths of C_{16}, C_{18} and in smaller amounts of C_{20}.

Triterpenol acetates (R_{f1}: 0.35) were present in this fraction in remarkable amounts of 7.2% of the wax and could be isolated very well by TLC (Toluene). The acetates were transesterified with HCl/MeOH to yield the triterpenols β-amyrin, α-amyrin and lupeol again each in similar concentrations. They were identified by TLC and GC analysis as described above and showed the same chromatographic behaviour as the free triterpenols (see Table III).

Triterpenols as well as triterpenol acetates were accompanied by triterpenones (R_{f1}: 0.19). These ketones (2.1% wax) could be isolated by TLC. They were identified by their TLC behaviour, a positive carbazole colour reaction, reduction with $NaBH_4$ to the corresponding alcohols and especially by their GC-MS data. According to their MS spectra the triterpenones oleanen-3-one, ursen-3-one and lupen-3-one could be identified (see Table III). The MS spectra of these triterpenones showed the characteristic ions for oleanen-3-one: 424 (M^+); 409 (M^+-15); 218 (100); 203 (40), ursen-3-one: 424 (M^+); 409 (M^+-15); 218 (50); 203 (20), lupen-3-one: 424 (M^+); 409 (M^+-15); 313 (1); 205 (20). These triterpenones were found also in similar concentrations and were identical to those described recently in *E. dendroides* and *Citrus halimii* [5, 6].

Yield and composition of *E. aphylla* epicuticular wax are in agreement with results of other *Euphorbia* species concerning the common lipid components. Always present are large amounts of free pr. alcohols with hexacosanol dominating and also free triterpenols. The wax esters occur in lower concentrations and are found to be accompanied always with triterpenol esters. The composition of free triterpenols is species specific. These species specific patterns differ from each other in composition and concentration. Most of them however are present also in form of triterpenones. Triterpenol acetates could not be shown in all *Euphorbia* waxes.

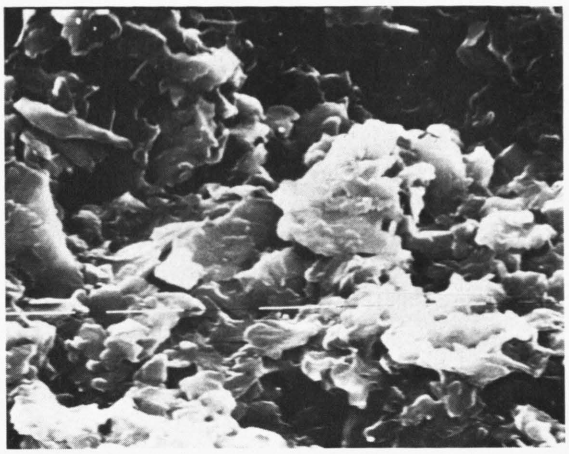

Fig. 1. SEM picture from the surface of a *E. aphylla* stem. (1 Bar = 10 μm).

The triterpenols definitely identified in the epicuticular wax of *E. aphylla*; β-amyrin, α-amyrin, lupeol and simiarenol are not in agreement with those described from the latex of *E. aphylla*. Ponsinet et Ourisson reported the presence of cycloartenol, lanosterol and lanostenol in *E. aphylla* latex [13]. Nielsen *et al.* described tirucallol, lanosterol, lanosterol isomere, cycloartenol and 24-methylencycloartenol as compounds of this latex [14].

In *E. aphylla* surface wax the following three groups of substances are dominating: pr. alcohols (41.2% wax), triterpenols (20.6% wax) and wax esters (13.6% wax). The alcohols and wax esters are very long chained and saturated lipids. The alcohols have one dominating component hexacosanol (81.7%). These wax compounds, especially hexacosanol may primarily determine the surface structure of the *E. aphylla* wax layer.

SEM pictures (Philips PSME 500) (Fig. 1) show a cristalline surface structure of *E. aphylla* stems. This surface consists of a continuous wax layer, which is superimposed by a formation of numerous cristalline wax plates with fringed edges. The dense wax cristals seem disarranged in and against each other and emerge from a waxy ground layer.

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