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Occurrence and characterization of oils rich in γ-linolenic acid Part II: fatty acids and squalene from Macaronesian *Echium* leaves

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Abstract

Leaves from 25 Macaronesian *Echium* (Boraginaceae) species have been surveyed for hydrocarbon compounds. These plants were previously reported as the major source of γ-linolenic acid so far found in nature. In addition, six European *Echium* species and the common *Borago officinalis* have been analysed for comparative purposes. High squalene amounts were found in all *Echium* plants from the Macaronesia, ranging from 3.73% in *E. simplex* to 20.11% in *E. fastuosum*. Squalene was almost absent from all European *Echium* species, and the same is true for *B. officinalis*. The relatively high oil content (2.27%) in leaves of *E. fastuosum* raises the total squalene amount to about 0.46% within this tissue. The main fatty acid component in the leaf was α-linolenic acid (ALA, 18:3ω3), ranging in the Macaronesian *Echium* from 9.32% in *E. acanthocarpum* to 54.45% in *E. simplex*. Possible utilisation of these plants as a commercial source of squalene and hypotheses about its physiological role in the plant are discussed.

Keywords: Squalene; Fatty acids; Macaronesia; *Echium*; Leaf lipids

1. Introduction

Squalene is a triterpene precursor for all steroids of animals and plants. The biosynthesis in animals begins with metabolites while in plants carbon dioxide is the main precursor. In the recent years, it has been demonstrated that squalene confers some beneficial effects. Recently, cellular and systemic radioprotection by this compound have been observed on mice receiving lethal whole-body radiation doses (Storm et al., 1993). It has also been reported that squalene has a preventive and therapeutic efficacy on tumour proliferation (Rao et al., 1998). Based on this hypothesis, cancer-protective properties of olive oil have been attributed to the presence of squalene (Newmark, 1997; Smith et al., 1998). Other beneficial effects on health can be attributed to its hypocholesterolaemic action, in combination with the administration of tocotrienols (Khor and Chieng, 1997). A similar effect has been described in the coadministration of pravastatin and squalene to elderly patients with hypercholesterolaemia, to prevent a higher incidence of side effects when using larger doses of pravastatin alone (Chan et al., 1996). Additionaly, the use of squalene alone has been demonstrated effective in decreasing serum cholesterol levels (Miettinen and Vanhanen, 1994). Squalene is also used as a raw material in the cosmetic field due to its photoprotective role (Gasparoli et al., 1998).

The traditional sources of squalene are fish liver oils. It occurs in certain shark liver oils, as in the sting ray, which has been recently proposed as a good source. However, a great number of related compounds such

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Table 1
Saponifiable oil, squalene and hydrocarbons in the hexane extract of *Echium* plant leaves

Macaronesian species	Leaf oil[a]	Saponifiable lipids[b,c]													Hydrocarbons[b]	
		16:0	18:0	18:1ω9	18:2ω6	18:3ω6	18:3ω3	18:4ω3	20:0	20:1ω9	22:0	22:1ω9	24:0	24:1ω9	Squalene	Others[d]
Sect. *Echium*																
E. plantagineum	1.40	20.26	3.04	5.83	6.97	2.19	29.43	5.34	0.89	0.32	0.43	0.23	0.12	0.34	10.23	8.32
Sect. *Gigantea*																
E. triste	1.12	24.99	3.01	7.86	5.50	1.77	22.80	3.33	1.20	0.89	1.83	0.00	0.42	0.00	6.61	6.98
E. leucophaeum	1.87	25.60	3.49	4.83	6.87	3.92	18.99	5.78	2.48	0.81	0.31	0.00	0.93	0.31	9.83	5.19
E. aculeatum	1.17	21.03	5.90	7.07	7.21	0.98	14.17	1.65	3.92	0.45	0.97	0.00	1.83	0.83	16.76	7.64
E. giganteum	1.46	23.16	4.51	4.88	8.51	2.08	23.29	3.48	0.43	0.23	1.27	0.00	2.38	0.58	13.46	10.65
Sect. *Virescentia*																
E. webbii	1.85	23.34	2.12	3.34	9.38	1.05	26.34	3.04	1.23	0.84	1.42	0.37	0.90	0.00	14.87	7.38
E. x bondspraguei	1.46	17.44	1.32	4.23	11.60	1.43	19.34	3.48	0.73	0.94	1.98	0.29	1.63	0.29	11.45	5.92
E. candicans	1.35	19.72	5.73	4.38	8.73	1.99	17.51	4.21	1.28	0.12	0.64	0.21	2.32	0.72	17.34	8.48
E. nervosum	2.05	22.24	5.73	6.04	6.92	4.30	16.31	3.85	4.57	0.34	3.98	0.39	0.54	0.31	12.25	6.37
E. hierrense	1.74	17.64	4.32	3.89	7.02	3.19	15.47	3.65	0.32	0.00	1.09	0.00	0.78	0.00	13.67	7.56
E. virescens var. *angustissimum*	1.09	25.95	3.78	5.84	7.46	1.30	17.58	4.45	1.24	0.59	0.94	0.28	1.79	0.89	13.79	8.83
E. v. var. *virescens*	1.23	24.24	4.34	3.58	5.34	1.40	17.34	2.32	1.89	0.76	1.38	0.52	1.30	0.75	17.43	11.48
E. onosmifolium	1.25	18.97	4.07	6.49	8.23	1.61	14.04	1.56	2.87	0.32	0.73	0.98	0.84	0.21	14.25	12.39
E. bethencourtianum	1.72	25.30	3.83	7.40	8.15	4.94	18.91	3.89	1.10	0.00	0.89	0.43	0.12	0.00	12.79	8.89
E. acanthocarpum	1.68	12.81	3.23	7.62	14.30	1.88	9.32	1.45	2.21	0.00	1.34	0.00	1.84	0.00	14.95	18.84
E. sventenii	0.76	20.14	5.88	5.73	8.68	1.56	16.59	1.67	2.89	0.21	1.53	0.00	0.97	0.12	10.27	13.32
E. callithyrsum	1.05	17.62	4.33	7.19	9.41	2.64	15.86	3.21	2.19	0.00	2.87	0.00	2.08	0.00	10.65	12.65
E. brevirame	1.98	19.22	3.94	4.82	10.23	2.49	19.87	3.29	1.74	0.15	2..54	0.57	1.39	0.00	14.45	11.89
E. fastuosum	2.27	23.48	3.45	4.56	3.54	1.55	21.23	3.74	1.29	0.00	2.40	0.00	0.81	0.00	20.11	4.38
Sect. *Simplicia*																
E. simplex	2.12	21.45	3.23	4.37	16.08	1.89	32.70	3.74	2.12	0.13	1.21	0.21	0.27	0.87	3.73	4.87
E. pininana	1.87	16.93	1.34	3.22	8.45	0.94	24.58	2.18	1.29	0.71	1.38	0.76	0.93	0.36	18.77	12.92
E. wildpretti	1.29	25.30	4.14	3.98	8.08	0.86	22.60	1.85	2.37	0.12	0.31	0.41	0.85	0.13	13.15	5.21
Sect. *Stricta*																
E. strictum	1.35	19.41	5.39	8.46	8.62	3.61	19.64	7.98	1.29	0.00	1.84	0.23	1.42	0.00	8.61	12.93
Sect. *Auberia*																
E. auberianum	1.46	22.91	3.40	2.29	8.73	0.89	25.30	0.38	1.38	0.00	0.32	0.87	0.76	0.00	13.23	9.90
Sect. *Decaisnea*																
E. decaisnei	1.28	16.52	2.88	5.91	8.39	1.73	15.93	1.78	0.23	0.32	0.42	0.00	1.23	0.53	7.83	16.83
Other European Boraginaceae species																
Borago officinalis	3.31	12.34	3.09	1.92	7.58	4.73	35.22	17.38	1.36	0.31	1.13	0.23	0.23	0.12	Traces	1.91
Echium species																
Sect. *Echium*																
E. asperrimum	3.25	18.05	2.33	4.09	6.55	1.04	41.34	7.24	1.35	0.38	1.23	0.00	0.00	0.23	Traces	11.92
E. boissieri	2.28	14.20	2.47	3.18	2.14	0.26	54.45	3.05	1.20	0.00	2.38	0.00	0.89	0.58	Traces	10.38
E. creticum	3.34	12.93	2.76	1.73	7.10	1.94	41.23	14.24	1.04	0.39	0.70	0.42	0.90	0.31	Traces	8.89
E. flavum	2.90	18.32	1.99	2.09	11.23	0.98	39.98	9.45	1.32	0.34	1.04	0.14	1.11	0.25	Traces	10.84
E. sabulicola	2.61	12.94	2.07	2.57	8.54	0.94	46.83	11.18	1.13	0.00	1.13	0.00	0.81	0.74	Traces	7.83
E. vulgare	1.98	16.41	2.40	4.28	5.28	0.83	41.74	4.99	2.30	0.00	2.71	0.00	0.33	0.93	Traces	8.94

[a] Oil percentage over the leaf dry weight, and are obtained as the mean value of two replicate experiments. [b] Figures represent the compound percentage over the total leaf oil, and are obtained as in footnote a. [c] Other fatty acids present in low and variable amounts mainly are: 12:0, 14:0 and 16:1ω7. [d] This fraction is mainly composed by variable amounts of eicosane, tetracosane, pentacosane, octacosane, and triacontane.

as cholesterol are also present at high concentration, while squalene is only present at 3.5% of the hydrocarbon fraction (Debasish et al., 1998). Other fishes having squalene in their liver oils are *Centrophorus squamosus*, with an squalene content of 27% in the oil (Peyronel et al., 1984; Bordier et al., 1996), and the dog fish (*Squalus acanthias*), with 40 mg/100 g of the oil (Sunarya and Taylor, 1996).

The presence of similar compounds in the oil, such as cholesterol, can make the squalene extraction process difficult. Purification can be also hampered in fish oils due to the presence of several heavy metals as major contaminants.

At present, plant sources are being widely prospected in a search for squalene. Several species, among them *Amaranthus cruentus*, have been proposed as an alternative source to obtain squalene from their seeds (Lee et al., 1996; Sala et al., 1998). In this case, squalene was present at 0.43% of the total seed weight (Lee et al., 1996; Sala et al., 1998). Other plants containing squalene, although in a lower amount, are: *Solanum elaeagnifolium* and *Solanum nigrum* (Hanna et al., 1996); *Jessenia bataua* oil, with a yield of 204 mg/kg (Rios et al., 1997); green friable calluses induced from *Origanum vulgare* subsp. *virens* (Alves-Pereira and Fernandes-Ferreira, 1998); Malvaceae seeds (Awatif and Mohamed, 1997); and rice bran oil, 320 mg/100 g (Rukmini and Raghuram, 1991). Although the squalene content in the olive oil is very low, a method has been proposed for its recovery from deodorising distillates (Bondioli et al., 1993).

Squalene is present in several plant oils, although their contents are not high enough to be considered as a good source for extraction. In this paper we report the presence of high levels of squalene in the leaf oil of Macaronesian *Echium* plants and the fatty acid composition within this tissue. Seeds from these plants have been recently reported as a major source of γ-linolenic acid (Guil-Guerrero et al., 1999).

2. Results and discussion

Leaf oil content, fatty acid composition and other hydrocarbon compounds from the plant species analysed are given in Table 1.

The saponificable oil content (s.o.) in leaves of the analysed *Echium* plants ranged from 0.76% in *E. sventenii* to 3.25% in *E. asperrimum*. Leaf oil content was higher in European species (2.72% mean) than in Macaronesian *Echium* plants (1.52%). Plants from the Macaronesian area are subjected to a higher solar irradiance, leading to the development by the cell of a smaller chloroplast membrane surface. As chloroplastic fatty acids contribute up to 75% of the total fatty acids (Forde and Steer, 1976), this might explain the lower oil content in the Macaronesian *Echium* leaves.

The main saponifiable component was the α-linolenic acid (ALA, 18:3ω3), ranging within Macaronesian *Echium* plants from 9.32% in *E. acanthocarpum* to 54.45% in *E. simplex*, while for European *Echium* species it ranged from 39.98% in *E. creticum* to 54.45% in *E. boissieri*. Another Boraginaceae species (*Borago officinalis*) had a lower ALA content of 35.22%. The γ-linolenic acid (GLA, 18:3ω6), which was found at high levels in seeds of all the analysed species (Guil-Guerrero et al., 2000) was found in Macaronesian *Echium* leaves as minoritary, ranging from 0.86% in *E. wildpretii* to 4.94% in *E. bethencourtianum*. Low amounts were also found in the European *Echium* plants, ranging from 0.26% in *E. boissieri* to 1.94% in *E. creticum*. *B. officinalis* had the highest GLA content (4.76%). Stearidonic acid (SA, 18:4ω3) was present in relatively high amounts, mainly in the European *Echium*, with values varying from 3.05% in *E. boissieri* to 14.24% in *E. creticum*. Among the Macaronesian *Echium* plants, *E. auberianum* had the lower SA content (0.38%), while *E. strictum* showed the higher value (7.98%). In the top of the range was *B. officinalis*, with a SA content of 17.38%. Linoleic acid (LA, 18:2ω6) was present in Macaronesiam *Echium* ranging from 3.54% in *E. fastuosum* to 16.08% in *E. simplex*. LA content for European *Echium* plants ranged from 2.14% in *E. boissieri* to 11.23% in *E. flavum*. The remaining fatty acids were found in similar percentages to those described in literature for leaf lipids.

Among the hydrocarbon compounds, squalene was found only in the Macaronesian *Echium* surveyed. However, squalene was found in minute amounts in all European *Echium* species and *B. officinalis*. Squalene content ranged from 3.73% in *E. simplex* to 20.11% in *E. fastuosum*. The relatively high oil content (2.27%) in leaves of *E. fastuosum* raises the squalene percentage to 0.46% over the leaf dry weight. Although seeds from *Amaranthus cruentus*, which is considered a good squalene source (Lee et al., 1996; Sala et al., 1998), have a higher oil content than the *Echium* leaves, the lower squalene proportions in the seed oil make them a source with a similar squalene richness.

A possible role for such a high squalene content in the leaves of Macaronesian *Echium* species can be inferred from data found in literature, where a photoprotective function has been attributed to this compound as a part of epicuticular structures (Nordby and McDonald, 1990; Pilon et al., 1999). Thus, we can speculate that the common ancestor of the Echium species that colonised the Macaronesian islands (Böhle et al., 1996) evolved to acquire high squalene levels on the leaf surface, thus providing the plant with a protec-

tive shield against the relatively high irradiances found in the Macaronesian area.

The high squalene percentage in the *Echium* leaf oil can aid the purification process, as the main difficulty is to obtain a high purity in the presence of similar compounds such as cholesterol, which are normally present in fish oils and are not recommended for human consumption. According to these data, *Echium* species from the Macaronesia appear to be a potential new source of squalene. Seeds from these plants have also been established as an important source of GLA, thus increasing the interest in the cultivation and integral exploitation of these species.

3. Experimental

3.1. Materials

Leaves were collected in the natural habitats of the plants. Leaves of *E. nervosum*, *E. plantagineum* and *E. candicans* were collected from Madeira in July 1998. Leaves of *E. decaisnei*, *E. onosmifolium*, *E. callithyrsum* and *E. strictum* were gathered from the island of Gran Canaria in July 1998, while leaves of *E. simplex*, *E. virescens* var. *virescens*, *E. virescens* var. *angustissimum*, *E. triste*, *E. leucophaeum*, *E. aculeatum*, *E. sventenii* and *E. giganteum*, were obtained from the island of Tenerife in May 1998. Leaves of *E. wildpretii* and *E. auberianum* were obtained from the island of Tenerife in July 1998. Leaves of *E. acanthocarpum* were collected from the island of La Gomera in July 1998. Leaves of *E. hierrense* were obtained from the island of Hierro in July 1998. Leaves of *E. fastuosum* were collected from garden plants in Almería (Spain) in May 1998. Leaves of *E. pininana*, *E. webbii*, *E. brevirame*, *E. bethencourtianum* and *E. x bondspraguei* were obtained from the island of La Palma in May 1999. European *Echium* leaves were obtained from Spain in May 1999.

Due to the fact that Macaronesian plants are endemics, a great number of precautions were taken during their harvesting. Only the necessary leaves to accomplish the analyses were collected, usually two leaves per plant.

3.2. Oil extraction and transesterification

Leaves were freeze-dried and ground to powder with a mortar. Rapid simultaneous oil extraction and transesterification was made according to the method of Rodríguez-Ruiz et al., (1998). Around 40 mg of each sample was transferred to test tubes with 1 ml of the methylation mixture (methanol/acetyl chloride, 20:1 v/v) and 0.5 ml hexane and heated at 100°C for 10 min. After cooling to room temperature, 1 ml of distilled water was added and the upper hexane layer was extracted for GC analyses. This method for fatty acid analysis provides routinely a variation less than 5%, and all *Echium* analysis were within this range. Duplicates of every sample were done and mean values are shown in the table.

3.3. Gas–liquid chromatography (GLC)

Mixed fatty acid methyl esters (FAME) were analysed in an Hewlett-Packard HP5890 series II gas chromatograph provided with FID and HP3394 integrator. A capillary column of fused silica of high polarity (Supelco SP2330; length: 30 m; internal diameter: 0.25 mm; thickness of the film: 0.2 μm) was used. The flow of the carrier gas (N_2) was 0.75 l/min. Split ratio in the injector was 100:1. Injector temperature was 240°C, and detector temperature was 260°C. The oven starting temperature was 205°C, and it was increased at a rate of 6°C/min until 240°C (5.83 min). Injection volume was 5 ml, and a blank was run after every ten analyses. Peaks were identified by comparison with known methyl ester standards ('Rapeseed oil mix' and 'PUFAS-1', from Sigma), and oil and fatty acid contents in the leaves were determined by comparison of the peak areas, using a known amount of methyl heptadecanoate (17:0) as an internal standard. The absence of 17:0 in the plant oils was previously checked. Unidentified peaks were taken into account for further calculations.

3.4. GLC–mass spectrometry (GLC/MS)

Verification of double bonds and hydrocarbons was achieved by GLC/MS. In particular, identity of the squalene peaks obtained by GLC was confirmed by GLC coupled to MS, by comparing the pattern obtained to that of pure squalene analysed in the same apparatus. Analyses were performed in a Hewlett-Packard HP5890A GLC provided with a Hewlett-Packard 5988A MS. A capillary column of methyl silicone (HP-1; length: 25 m; internal diameter 0.2 mm; thickness of the film: 0.33) was used. The flow of the carrier gas (He) was 1 ml/min. Injector temperature was 260°C, and the pressure at the head of the column was 15 psi. The oven starting temperature was 100°C, it was increased at a rate of 10°C/min until 280°C, and then kept at 280°C for 25 min. The temperature in the inter-phase was 280°C, and the temperature of the source in the detector was 180°C.

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