# EPICUTICULAR COMPONENTS FROM PSEUDOGNAPHALIUM ROBUSTUM (ASTERACEAE): CHEMOSYSTEMATIC CONSIDERATIONS

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## ABSTRACT

Surface compounds were obtained by a methylene chloride extraction of fresh aerial parts of *Pseudognaphalium robustum*. The methylene chloride extract was fractionated by CC and the fractions were analyzed by GC-MS. Since flavonoids and terpenes are present only in small amounts in the epicuticular exudate of *P. robustum*, 80% of the surface compounds actually correspond to a complex mixture of saturated fatty acid esters, unsaturated fatty acid esters, alcohols, aldehydes, fatty acids, alkenes, triglycerides and monoglycerides. A minor hydrocarbon fraction of n-alkanes from C23 to C37 was also identified. On the contrary, in *P. vira vira*, *P. cheiranthifolium,* and *P. heterotrichium,* the epicuticular extracts contain from 70% to 80% of a mixture of diterpenoids and n-alkanes. These results show a remarkable distance between *P. robustum* and other species of the genus, which share the same ecosystem, when the whole pool of epicuticular compounds is taken into account. Also, these considerable differences in chemical composition are in agreement with authors that consider *Pseudognaphalium* as a heterogeneous taxonomic group.

**Keywords:** Pseudognaphalium robustum; Asteraceae; Methylene chloride extract, Epicuticular components; GC-MS; Saturated fatty acid esters; Unsaturated fatty acid esters; Epicuticular triglycerides; monoglycerides; Chemosystematic.

## INTRODUCTION

The genus *Pseudognaphalium* (Asteraceae) is well represented in Chile by 14 species. All of them show a distinctive combination of non-glandular and glandular trichomes and the morphology of the latter varies according to species [1,2]. When present, the contribution of these specialised secretory structures to the surface chemistry of the plant is significant.

From the epicuticular extract of *Pseudognaphalium robustum* (initially reported as *Gnaphalium*), simple and acylated B-ring deoxyflavonoids had been isolated [3-6]. Monoterpenes and sesquiterpenes had been identified in the trichome secreted exudates [7] and by headspace analysis [8].

Flavonoids, mono- and sesquiterpenes are present only in small amounts in the epicuticular components of *P. robustum*, representing less than 8% of that fraction. In order to clarify the structure of the unidentified metabolites and establish any differences with other Chilean species of *Pseudognaphalium* with known epicuticular chemistry, a new study of *P. robustum* was undertaken.

Results shows that the chemical composition of the epicuticular components of *P. robustum* is considerable different to that of other *Pseudognaphalium* species that share with *P. robustum* the same ecosystem. These results are in agreement with authors that consider *Pseudognaphalium* as a heterogeneous taxonomic group.

## EXPERIMENTAL

### Plant material

Aerial parts of *P. robustum* (Phil.) A. Anderb. were collected during the flowering season, October 2003, between Zapallar and Papudo (V Región, Chile, 32º 30'S, 71º 30'W). Voucher specimens were deposited in the Herbarium of the National Museum of Natural History, Santiago, Chile (SGO 133617-03).

### Plant extraction and column chromatography separation of the extracts

Aerial parts of *Pseudognaphalium robustum* (340 g) were extracted by dipping the fresh plan material in 1.5 L of cold $CH_2Cl_2$ for 15-20 s. The extraction was repeated twice to assure the total and selective extraction of the epicuticular components [9,10]. The $CH_2Cl_2$ extract (1.2 g, 0.35 %) was fractionated by CC (silica gel) using pentane – $CH_2Cl_2$ and $CH_2Cl_2$ – MeOH step gradients. Fractions eluted with pentane (47 mg), pentane-$CH_2Cl_2$ (9:1), (47 mg), pentane- $CH_2Cl_2$ (7:3), (440 mg), and pentane- $CH_2Cl_2$ (6:4), (440 mg) were submitted to extensive GC-MS analysis.

### GC-MS analysis of the $CH_2Cl_2$ extract

The fractions were analysed in triplicate by two GLC/EI/QI-MS analysis in FISONS MD-800 equipment with a HP Ultra-2 capillary column (12 m x 0.20 mm) in one case and a HP Ultra-2 capillary column (25 m x 0.20 mm) in the other. In the former analysis, injector temperature was 270ºC and column temperature was programmed starting at 80ºC, for 2 min, followed by a rise to 320ºC at 20ºC $min^{-1}$. Helium was the carrier gas at 7 lb psi. In the later analysis, injector temperature was 270ºC and column temperature was programmed starting at 40ºC, for 3 min, followed by a rise to 280ºC at 15ºC $min^{-1}$. Helium was the carrier gas at 0.7 $kp/cm^2$.

Fractions were derivatised with 200 µL of N-methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA) (Aldrich). The samples were heated at 70º during 45 min and the excess of reagent was eliminated with a gentle stream of $N_2$ to yield the TMS derivatives[11].

### Yields of fractions and compounds

Extract and compound yields were calculated in relation to fresh plant material. The percentage of different families and individual compounds was calculated from the peak areas of the chromatograms.

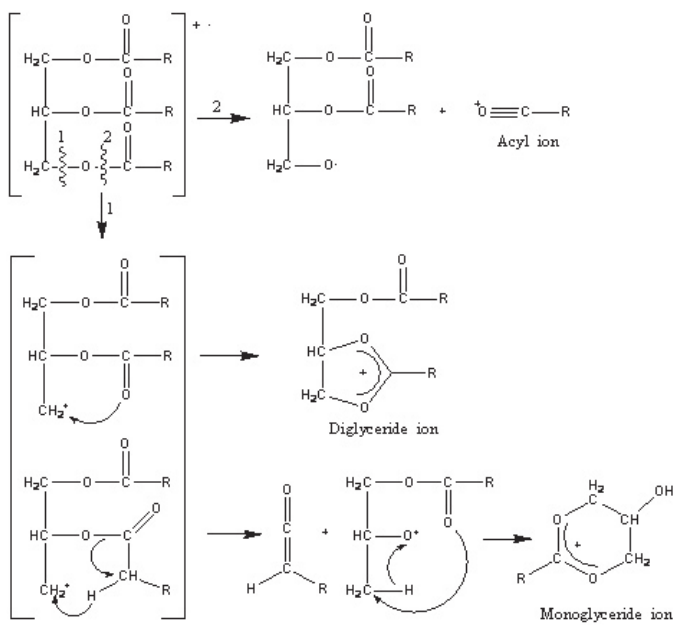


**Figure Nº1.** Detailed mechanisms of ion fragmentation of triglycerides

## RESULTS AND DISCUSSION

**Identification of the epicuticular components**

n-Alkanes, n-alkenes, alcohols, fatty acid esters, aldehydes and fatty acids were identified by comparison of their retention time and MS spectra with standards (Sigma, Aldrich, Supelco) and of the mass spectra with data from the NIST library (1998) linked to the mass detector. Only correlation indexes greater than 98% were accepted.

The structures of triglycerides and monoglycerides (TMS derivatives) were obtained by interpretation of their mass spectra. In the case of triglycerides characteristics acyl, diglyceride and monoglyceride fragment ions were used[12].

The structure of monoglycerides was obtained by interpretation of the mass spectra of their TMS derivatives. In these compounds two ion fragments were considered: the acyl and the M+ -103 (base peak) formed by the heterolytic cleavage of the C1-C2 bond in 1-monoacylglycerols [13]. These were: m/z 163 and m/z 315 for 2, 3-dihydroxypropyl laurate, m/z 187 and m/z 343 for 2, 3-dihydroxypropyl myristate, and m/z 211 and m/z 371 for 2, 3-dihydroxypropyl palmitate.

Finally identification was performed by comparison of their retention time and MS spectra with standards (Sigma).

**Chemical composition**

From the fractions eluted with pentane and pentane-$CH_2Cl_2$ (9:1), the following not previously reported epicuticular compounds for *P. robustum* were identified: n-Alkanes (4%): $C_{23}H_{48}$ ; $C_{24}H_{50}$ ; $C_{25}H_{52}$ ; $C_{26}H_{54}$ ; $C_{27}H_{56}$ ; $C_{28}H_{58}$ ; $C_{29}H_{60}$ ; $C_{30}H_{62}$ ; $C_{31}H_{64}$ ; $C_{32}H_{66}$ $C_{33}H_{68}$; $C_{34}H_{70}$; $C_{35}H_{72}$ ; $C_{36}H_{74}$; $C_{37}H_{76}$. n-Alkenes (0.3%): 1-nonadecene. Alcohols (1%): 1-tetradecanol; 1-hexadecanol. Saturated fatty acid esters (3%): methyl dodecanoate (methyl laurate); methyl tetradecanoate (methyl myristate); methyl hexadecanoate (methyl palmitate); methyl heptadecanoate (methyl margarate); methyl docosonoate; methyl 5,9-dimethyldecanoate; methyl 9-oxononanoate; methyl 12-methyltridecanoate (methyl isomyristate); methyl 14-methylpentadecanoate; methyl 6-methylheptanoate; methyl 16-methyl heptadecanoate (methyl isoestearate); methyl 2-hexylcyclopropaneoctanoate. Unsaturated fatty acids esters (2.5%): methyl (Z)-7-hexadecenoate (methyl palmitoleate); methyl (Z)-9-hexadecenoate; methyl (Z)-9-octadecenoate (methyl oleate); methyl acetylricinoleate; methyl (Z)-12 acetoxy-9-octadecenoate. Aldehydes (0.5%): nonanal; decanal.

From the fraction eluted with pentane-$CH_2Cl_2$ (7:3) the following not previously reported epicuticular compounds for *P. robustum* were identified: Triglycerides (40%): glyceryl tridodecanoate (trilaurin); glyceryl trimyristate (trimyristin); glyceryl tripalmitate (tripalmitin); and several other triacylglycerol isomers, with combinations of lauric, myristic, palmitic and stearic acids, that were not identified.

From the fraction eluted with pentane-$CH_2Cl_2$ (6:4) the following not previously reported epicuticular compounds for *P. robustum* were identified: Fatty acids (5%): myristic acid; lauric acid. Monoglycerids (35%): 2, 3-dihydroxypropyl laurate; 2, 3-dihydroxypropyl myristate; 2, 3-dihydroxypropyl palmitate.

**Chemotaxonomic significance**

The composition profile of the hydrocarbon fraction of *P. robustum* is very much like that found in three other species of the genus: *P. vira vira* (Mol.) A. Anderb., *P. cheiranthifolium* (Lam.) Hilliard and Burtt. and *P. heterotrichium* (Phil.) A. Anderb. [2]. In contrast, the yields of these fractions remarkably differ among the four species. While *P. robustum* hydrocarbon yield corresponds to around 4% of the epicuticular components, in *P. vira vira* this percentage increases up to 50%, and in *P. cheiranthifolium* and *P. heterotrichium* up to 35%. These differences would not be ascribed to environmental conditions because the four species share the same eco-system. Another common composition profile among these four species is that of phenolic compounds, since their resinous exudates contain minute amounts of simple and acylated flavonoids lacking ring-B substitution [3-6].

Considerable differences were found between *P. robustum* and the other *Pseudognaphalium* species concerning epicuticular terpenes. In relation to diterpenoids, *ent*-16-kauren-19-oic acid and *ent*-3-β-hydroxy-16- kauren-19-oic acid have been isolated from *P. heterotrichium*, *P. cheiranthifolium* and *P.vira vira*. In addition, *ent*- 9(11)-16-kauradien-19-oic acid and 13-*epi*-sclareol have also been isolated from *P. heterotrichium,* and *ent*-3-α- hydroxy-9(11),16-kauradien-19-oic acid and 13-*epi*-sclareol from *P. cheiranthifolium*. In these three species, diterpenoid fractions account for around 30% to 40% of the epicuticular compounds [3,5]. In contrast, only trace amounts of *ent*-16-kauren-19-oic acid were found in *P. robustum* in a previous work [3] and none in the samples analysed here.

Since flavonoids and terpenes are present only in small amounts in the epicuticular exudate of *P. robustum*, 91% of the surface compounds actually correspond to a complex mixture of hydrocarbons, alcohols, fatty acid esters, aldehydes, triglycerides and monoglycerides. On the contrary, in *P. vira vira*, *P. cheiranthifolium* and *P. heterotrichium,* the epicuticular extracts contain from 75% to 85% of a mixture of diterpenoids and n-alkanes [2,3]. These results show a remarkable distance between *P. robustum* and other species of the genus in the same eco-system, when the whole pool of epicuticular compounds is taken into account. In contrast, a correlation was not found between these chemical results and the glandular trichome morphology, frequency and distribution in each species [14]. In the four cases, glandular trichomes consist of bi-cellular glandular heads on top of multi-cellular columns that are extensions of the leaf epidermis. Those of *P. robustum* and *P. heterotrichium* are very similar, abundant and regularly distributed. In comparison to these, glandular trichomes in *P. vira vira* are scarce and distributed around and along leaf enervations. Finally, *P. cheiranthifolium* shows two types of glandular trichomes evenly distributed on both sides of leafs [2].

*Pseudognaphalium* consisted originally of 10 species[15] and Anderberg remodelled it to comprise about 80 [2,16]. Dillon and Sagástegui are reluctant to accept the present definition of this genus but, at the same time, they recognise that a potential reorganisation of *Pseudognaphalium species* cannot be proposed without the support of further studies on other related genus *Gnaphalium, Achyrocline*, and *Stenocline* [17].

The considerable differences between the chemical composition of the cuticle of *P. robustum* found in this work and that of other species of the genus, which share the same ecosystem, are in agreement with Dillon and Sagástegui, who consider *Pseudognaphalium* as a heterogeneous taxonomic group and propose an extensive revision of the same[17]. In this context, the chemical data here reported may be supportive as part of relevant information in the genus revision.

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