

IN-MIXTURE ANALYSIS OF TRITERPENES FROM *Raphiodon echinus*

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Abstract

The acetone crude extract of *Raphiodon echinus* aerial parts (Lamiaceae) yielded pure betulinic acid and three mixtures of pentacyclic triterpenes. The mixtures constituents were identified by ¹³C NMR spectroscopy (decoupled, APT) and confirmed by GC-MS and comparison with previously published data. Betulinic, oleanolic, ursolic, micromeric and siarsinolic acids have been identified in different combinations and proportions.

Key Words: *Raphiodon echinus*, Lamiaceae, In-Mixture Analysis, Triterpenes

Resumen

El extracto crudo en acetona de las partes aéreas de *Raphiodon echinus* (Lamiaceae) forneció el ácido betulínico solo y tres mezclas distintas de triterpenos pentacíclicos ácidos. Los constituyentes de las mezclas fueron identificados por la utilización de espectroscopia de RMN ¹³C (desacoplado, APT) y confirmado por CG-EM y también por la comparación con datos publicados previamente. Desde así, los ácidos betulínico, oleanólico, ursólico, micromérico y siarsinólico fueron identificados en diferentes mezclas con distintas combinaciones.

Palabras Clave: *Raphiodon echinus*, Lamiaceae, Análisis de mezclas, Triterpenos

INTRODUCTION

Raphiodon echinus (Nees et Mart.) Schauer belongs to the family Lamiaceae, subfamily Ocimoideae, subtribe Hyptidineae (Bentham and Hooker, 1862-1883). This plant is abundant in the Northeast region of Brazil, mainly in the States of Ceará and Paraíba, where it is known as “betônica” and used for the treatment of cough. By the chemical

point of view, *R. echinus* had not still been studied. As a base to start the chemical study of this species, a literature survey was made to ascertain the chemical composition of the genus *Hyptis*, the most studied of the subtribe Hyptidineae. The literature survey showed the richness of species belonging to this genus in the production of flavonoids, triterpenoids, α -pyrones and lignans (Pere-da-Miranda, 1995).

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RESULTS AND DISCUSSION

From chromatography of the acetone crude extract of *R. echinus* aerial parts, four fractions (**I**, **II**, **III** and **IV**) apparently pure on TLC, showed after analysis by ^1H NMR and ^{13}C NMR to be actually mixtures, except for **I**. The structure of **I** was characterized as betulinic acid and confirmed by comparison of its spectrometric data with those published for an authentic sample of betulinic acid. Since **II**, **III** and **IV** were characterized as triterpene acid mixtures, a reaction with diazomethane in ether was performed in order to obtain their derived methyl esters. The mixtures of methyl esters were then analysed by GC-MS in the same conditions used for methyl ester of **I**.

By this way, it was possible to visualize in the chromatogram of **II** the presence of four compounds with the following retention times: 72.52 min., 73.37 min., 76.01 min. and 78.00 min. Mass spectra obtained for those compounds evidenced the following molecular ions: 470 (RT=72.52), 470 (RT=73.37), 470 (RT=76.01) and 468 (RT=78.00). Mass spectrum for compound with retention time 73.37 min. was very similar to that observed for betulinic acid methyl ester, suggesting this compound as one of the representatives of this mixture. Analysis of the ^{13}C NMR spectrum of **II** confirmed the presence of betulinic acid in this mixture due to the presence of characteristic signals for that substance: δ (ppm) 177.1 (C_{28}); 150.2 (C_{20}); 109.5 (C_{29}) and 76.8 (C_3). In the same spectrum it was possible to see three other characteristic group of signals belonging to three other triterpene skeletons: oleanolic acid δ (ppm) 178.5 (C_{28}); 143.7 (C_{13}), 121.5 (C_{12}) and 76.8 (C_3); ursolic acid δ (ppm) 178.2 (C_{28}); 138.1 (C_{13}); 124.5 (C_{12}) and 76.8 (C_3); and micromeric acid δ (ppm) 177.6 (C_{28}); 152.8 (C_{20}); 137.8 (C_{13}); 125.1 (C_{12}); 105.0 (C_{30}) and 76.8 (C_3). Comparison of these data with those of the literature (Siddiqui *et al.*, 1988; Maillard *et al.*, 1992; Seo *et al.*, 1975 and Kojima *et al.*, 1987) confirmed this

proposal. The multiplicity of carbon atoms for each compound was obtained by the use of APT technique. The ^1H NMR spectrum of **II** presented very characteristic signals: δ (ppm) 5.25 (*broad singlet*) regarding to the H_{12} of ursolic, oleanolic and micromeric acids, 4.55 and 4.68 regarding to the hydrogens 29 of betulinic acid, 3.00 (*dd*) regarding to the carbinol hydrogens belonging to the four triterpenes in the mixture, 2.75 (*dd*) regarding to the H_{18} of oleanolic acid, 2.15 (*dd*) regarding to the H_{18} of betulinic acid, 2.10 (*d*) regarding to the H_{18} of ursolic and micromeric acids, 1.62 (*s*) regarding to the hydrogens belonging to the methyl group of the isopropylene moiety of betulinic acid, besides the other signals belonging to the methyl hydrogens of the four triterpenes in the mixture. All the spectroscopic data made possible to characterize the compound with RT=72.52 as oleanolic acid methyl ester, the compound with RT=76.01 as ursolic acid methyl ester and the compound with RT=78.00 as micromeric acid methyl ester.

Gas chromatogram of **III** showed the presence of three compounds with the following retention times: 71.98 min., 75.67 min. and 77.90 min. Mass spectra obtained for those compounds evidenced the following molecular ions: 470 (RT=71.98), 470 (RT=75.67) and 468 (RT=77.90). Mass spectrum for these compounds are in agreement with those observed for the methyl esters of oleanolic, ursolic and micromeric acids in **II**. In fact, analysis of the ^{13}C NMR spectrum of **III** clearly showed the presence of oleanolic, ursolic and micromeric acids. Comparison of these data with those of the literature (Maillard *et al.*, 1992; Seo *et al.*, 1975 and Kojima *et al.*, 1987) confirmed this proposal. The multiplicity of carbon atoms for each compound was also obtained by the use of APT technique. The ^1H NMR spectrum of **III** presented very characteristic signals: δ (ppm) 5.25 (*broad singlet*) regarding to the H_{12} of ursolic, oleanolic and micromeric acids, 4.55 and 4.68 regarding to the hy-

drogens 30 of micromeric acid, 3.00 (*broad signal*) regarding to the carbinol hydrogens belonging to the three triterpenes in the mixture, 2.75 (*dd*) regarding to the H₁₈ of oleanolic acid, 2.10 (*dd*) regarding to the H₁₈ of ursolic and micromeric acids, besides the other signals belonging to the methyl hydrogens of the three triterpenes in the mixture. All the spectroscopic data made possible to characterize the compound with RT=71.98 as oleanolic acid methyl ester, the compound with RT=75.67 as ursolic acid methyl ester and the compound with RT=77.90 as micromeric acid methyl ester.

Gas chromatogram of **IV** showed the presence of two compounds with the following retention times: 71.98 min., 75.67 min. and 77.90 min. Mass spectra obtained for those compounds evidenced the following molecular ions: 470 (RT=71.98), 470 (RT=75.67) and 468 (RT=77.90). Mass spectrum for these compounds are in agreement with those observed for the methyl esters of oleanolic, ursolic and micromeric acids in **II**. In fact, analysis of the ¹³C NMR spectrum of **III** clearly showed the presence of oleanolic, ursolic and micromeric acids. Comparison of these data with those of the literature (Maillard *et al.*, 1992; Seo *et al.*, 1975 and Kojima *et al.*, 1987) confirmed this proposal. The multiplicity of carbon atoms for each compound was also obtained by the use of APT technique. The ¹H NMR spectrum of **III** presented very characteristic signals: δ (ppm) 5.25 (*broad singlet*) regarding to the H₁₂ of ursolic, oleanolic and micromeric acids, 4.55 and 4.68 regarding to the hydrogens 30 of micromeric acid, 3.00 (*broad signal*) regarding to the carbinol hydrogens belonging to the three triterpenes in the mixture, 2.75 (*dd*) regarding to the H₁₈ of oleanolic acid, 2.10 (*dd*) regarding to the H₁₈ of ursolic and micromeric acids, besides the other signals belonging to the methyl hydrogens of the three triterpenes in the mixture. All the spectroscopic data made possible to characterize the compound with RT=71.98 as oleanolic acid methyl ester, the compound

with RT=75.67 as ursolic acid methyl ester and the compound with RT=77.90 as micromeric acid methyl ester.

Gas chromatogram of **IV** showed the presence of two compounds with the following retention times: 75.10 min. and 78.36 min. Mass spectra obtained for those compounds evidenced the following molecular ions: 470 (RT=75.67) and 488 (RT=78.36). Mass spectrum for the compound with retention time 75.10 min. was very similar to that observed for the ursolic acid methyl ester, suggesting this compound as one of the representatives of this mixture. In fact, analysis of the ¹³C NMR spectrum of **IV** clearly showed the presence of ursolic acid due to the presence of characteristic signals of that substance: δ (ppm) 178.2 (C₂₈); 138.1 (C₁₃); 124.5 (C₁₂) and 76.8 (C₃). In the same spectrum it was possible to see other characteristic groups of signals belonging to the other triterpene skeleton derived from oleanolic acid. Comparison of these data with those of the literature (Seo *et al.*, 1975 and Inada *et al.*, 1987) confirmed the presence of ursolic acid and suggest the presence of siaresinolic acid in this mixture. The multiplicity of carbon atoms for each compound was also obtained by the use of APT technique. The ¹H NMR spectrum of **IV** presented very characteristic signals: δ (ppm) 5.20 (*m*) regarding to the H₁₂ of ursolic and siaresinolic acids, 3.45 regarding to the H₂₀ of siaresinolic acid, 3.05 (*tl*) regarding to the carbinol hydrogens belonging to the two triterpenes in the mixture, 2.75 (*d*) regarding to the H₁₈ of siaresinolic acid, 2.15 (*d*) regarding to the H₁₈ of ursolic acid, besides the other signals (0.7-1.1) belonging to the methyl hydrogens of the two triterpenes in the mixture.

In-Mixture analysis is an important methodology for natural products evaluation leading not only to economy of operation time but very often it can preserve the scientist to use modern (2D) techniques to solve very known structures. In the present work, the richness in triterpene production by *R. echinus* proved to be of very important appliance

the use of in-mixture analyses dispensing even more expensive techniques and lack of time to ascertain known structures, mainly triterpenes, which are very easy to identify the skeletons by the assignment of double bond carbon atoms avoiding the use of 2D techniques like HMBC, HMQC and HETCOR for these molecules.

METHODOLOGY

Plant Material- Aerial parts of *R. echinus* were collected by Prof. Dra. Maria de Fátima Agra, Laboratório de Tecnologia Farmacêutica (UFPB), on October 17, 1994, in the district of Santa Rita, João Pessoa, PB, Brazil. A representative sample was deposited in the New York Botanical Garden Herbarium (voucher number = 1590). The plant material was dried at 50°C and further ground.

Extract Preparation- Dried and ground aerial parts (630g) of *R. echinus* were extracted by cold percolation with acetone. The acetone extract was concentrated under reduced pressure yielding a total of 11g.

Chemical Study of Acetone Extract- The acetone extract of *R. echinus* aerial parts was chromatographed over a silica gel column (internal diameter of 70mm, length of 80cm, and total silica gel 250g) eluted with hexane, gradients of hexane and ethyl acetate, ethyl acetate, gradients of ethyl acetate and methanol, and methanol, yielding 25 fractions. Fractions 8-9, eluted with hexane/ethyl acetate (8:2) was filtered in the presence of active charcoal, giving after evaporation of the solvent 100.9mg of **I**, identified as betulinic acid.

I- Betulinic acid- m.p.=250-255°C. ¹H NMR (200 MHz, DMSO-D₆, TMS): δ 0.66; 0.77; 0.90 and 0.96 (s, 5 X 3H; H₂₃, H₂₄, H₂₅, H₂₆ and H₂₇), 1.62 (s, 3H, H₃₀), 2.97 (m, 1H, H₃), 4.55 (sl, 1H, H_{29a}), 4.68 (sl, 1H, H_{29b}), 12.0 (sl, 1H, acid H). ¹³C NMR (50 MHz, DMSO-D₆, TMS): δ 14.3 (C₂₇), 15.7 (C₂₄), 15.8 (C₂₅), 15.9 (C₂₆), 18.0 (C₆), 19.0 (C₃₀), 20.5 (C₁₁), 25.1 (C₁₂), 27.2 (C₂), 28.0 (C₂₃), 29.1 (C₂₁), 30.1 (C₁₅), 31.7 (C₁₆), 34.0 (C₇), 36.7

(C₂₂), 37.6 (C₁₀), 38.3 (C₁₃), 38.5 (C₁), 38.6 (C₄), 40.3 (C₈), 41.9 (C₁₄), 46.6 (C₁₈), 48.5 (C₁₉), 49.9 (C₉), 54.9 (C₅), 55.4 (C₁₇), 76.8 (C₃), 109.5 (C₂₉), 150.2 (C₂₀), 177.2 (C₂₈). GC [betulinic acid methyl ester prepared with diazomethane (DB-1 30m X 0.20mm, helium as carrier gas and temperature programming from 50 to 270° C - 4°C min⁻¹, isotherm for 25 min. at 270°C)] RT=73.77 min. MS-EI *m/z* (%): 470 [M⁺.] (3.3), 452 (1.1), 411 (5.5), 262 (33.3), 233 (7.7), 207 (35.5), 189 (66.7), 175 (26.6), 119 (41.1), 107 (44.4), 95 (50.0), 69 (65.5), 55 (93.3), 43 (100).

Fractions 10, 11-13 and 16-20, eluted respectively with hexane/ethyl acetate (7:3), hexane/ethyl acetate (6:4) and ethyl acetate/methanol (9:1), were filtered in the presence of active charcoal, to give respectively **II** (72.0 mg), **III** (76.4 mg) and **IV** (150.1 mg). ¹H NMR and ¹³C NMR analysis showed that **II**, **III** and **IV** were actually triterpene mixtures containing: **II** – oleanolic acid (41%), ursolic acid (24%), betulinic acid (29%) and micromeric acid (6%); **III** – oleanolic acid (29%), ursolic acid (57%) and micromeric acid (14%); and **IV** – ursolic acid (67%) and siaresinolic acid (33%). The percentage of each triterpene in the mixtures were estimated by the amplitude of double bond carbon signals in the ¹³C NMR triterpene mixture spectra.

GC-MS- Mixtures of triterpene acids obtained from the acetone extract of *R. echinus* (**II**, **III** and **IV**) as well as pure betulinic acid (**I**) were transformed in the corresponding methyl esters by reaction with diazomethane in ether. The methyl ester mixtures were analysed by gas chromatography coupled to mass spectrometry using DB-1 column (30m X 0.20mm) helium as carrier gas and temperature programming from 50 to 270° C - 4°C min⁻¹, isotherm for 25 min. at 270°C.

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