Antinociceptive activity of triterpenes isolated from *Clusia ellipticifolia*

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**Summary**

On the base of the traditional use, from the methanolic extract of the stem bark of *Clusia ellipticifolia* of the family Guttiferae, three pure triterpenoid compounds were isolated and identified as betulinic acid, friedelanol and friedelene, by means of chemical and spectrometric methods. The pharmacological study by the abdominal constriction model induced by acetic acid showed significant antinociceptive activities to the studied substances and the highest effect was attributed to the betulinic acid.

**Keywords:** *Clusia ellipticifolia* – Guttiferae – Betulinic acid – Friedelanol – Friedelene – Antinociceptive activity.

**Resumen**

**Actividad antinociceptiva de triterpenos aislados de Clusia ellipticifolia**

Con base en los usos tradicionales de varias especies de la familia Guttiferae, tres compuestos fueron aislados del extracto metanólico de la corteza de *Clusia ellipticifolia* y identificados por medio de métodos químicos y espectroscópicos como ácido betulínico, friedelanol y friedelena. El estudio farmacológico por el modelo de contracciones abdominales inducidas por ácido cético mostró actividad antinociceptiva muy significante para los compuestos aislados siendo la más alta atribuida al ácido betulínico.

**Palabras clave:** *Clusia ellipticifolia* – Guttiferae – Ácido betulínico – Friedelanol – Friedelena – Actividad antinociceptiva.

**Introduction**

They are numerous medicinal uses of the species of the family Guttiferae in the popular medicine among them the use of the latex of the bark and of the seeds of *Galiphophilum fluviatile* (Tree of Oil), *Galiphophilum lucidum* (Stick of Oil) and *Galiphylum martinense* (Oil of Marta) in dermatology and to heal the navel of those recently born ones when it does not heal. The resin of *Clusia olana* is used in the treatment of the cephalalgia and the leaves are detestive. The decocation of the root and of the bark of the shaft of *Clusia amazonica* is used in bathroom form in the treatment of the leprosy. The fruits of *Manuana americana* known popularly as “Mammy” are used as nutritious fruit for their very pleasant flavor. The emulsion prepared in water from the nilled seeds is used to eliminate the parasites, fleas and lice of the animals and man and in the treatment of the scabies in the dogs (1). The vegetable species of the Guttiferae are rich in xanthones (2-7), triterpenes and sterols (8-10).

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The flavonoids (11, 12) and antimicrobial α- and β-Glutathion (13) were reported in some species. It was also reported that isopentenitrin (1, 3-dihydroxy-7-methoxystyphon) and their 3-O-glucoside, which are characteristic constituents of plants of the Guttiagraceae and Gentianaceae, inhibit the rat brain mitocon- drial monoamine oxidase in vivo (14).

Previously friedeline and friedelinal were isolated from the petroleum ether extract of the stem bark of *Cusus ellipsoidea* (13). Benzophenone- chrysophenone A, B, C, and D, viniaphenone B and isoviniaphenone B were also isolated from the fruits of the same plant (16, 17).

Betulinic acid has been isolated of various species of different family (8, 18 - 25) and showed a wide range of pharmacological activities among them anti-inflammatory activity on the carageenan induced rat paw edema (19) and on the edema in the rat's ear showing a mecha- nism of similar action to that of the glucocorticoids (22), inhibitory effect in the synthesis of the prostaglandins in vivo (23), antitumor activity against the human melanoma (26), antiin- fectious activity with an inhibitory minimum concentration superior to 10 μg/mL against *E. coli* ssp. (27). Meanwhile friedeline was also isolated of several plants among them *Cusus ellipsoidea* (15), *Stema lancea* (33), *Dicksonia for- mosa* (28) and *Lepidophyllum ssp. eugeniifforme* (34).

The above-mentioned motivates us to study the alcoholic extract of the stem bark of *Cusus ellipsoidea*, which allowed the isolation of the betulinic acid, friedeline and friedelinal and the de- termination of their antimicrobial properties.

Methodology

**Plant material**

*Cusus ellipsoidea* was collected in the region between Fusagasugá and Bogotá, Department of Cundinamarca, Colombia; a specimen was classified by Doctor Hernando García Barriga, of the Institute of Natural Sciences of the Na- tional University of Colombia. A voucher speci- men was deposited with the number Col 229095 in the Colombian National Herbarium.

**Extraction and isolation of compounds**

The dried powdered stem bark of *Cusus ellipsoidea* (1035 g) was exhaustively extracted with petro- leum ether 37-40°C, in Soxhlet equipment. The defatted bark again was exhaustively extracted with methanol. The dried residue of the metha- nolic extract (250 g) was heated in 200 mL of methanol, it was left during 24 hours, after which it was filtered, and a precipitate was obtained (residue A, 12.5 g). The concentration of the filtrate allowed the obtaining of a residue which weighed 238 g (residual B), which revealed the presence of a mixture of flavonoids, saponins, phenols and terpenic compounds by chemical tests. 5 g of the residue A insoluble in methanol was deposited on a column of silica gel G, Merck, U06-0.200 mm (80g). Elution with benzene and increasing concentration of ethyl acetate in benzene → preparative TLC [cyclohexane: ethyl acetate (90:10)] permitted the isolation of friedeline (I) (54 mg), friedelinal (II) (5 mg) and belutinic acid (III) (62.5 mg).

**Identification of the isolated compounds**

Friedeline (I). White needles, solubles in CHCl3, ethyl acetate, slightly soluble in benzene, petroleum ether and methanol, insoluble in
water, gave positive test of Liebermann-Burchard for triterpenes. MP 268-270°C (ethyl acetate) with decomposition; IR (KBr): 1720 (C=O), 1397, 1390 cm⁻¹ (s). 1-HNMR (200 MHz, CDCl₃): δ 0.72 (3 H, s, Me-C-17 β), 0.86 (3 H, s, Me-C-5 β), 0.87 (3 H, δ) = 6 H, Me, C-2 (β), 0.93 (3 H, s, Me-C-9 (β), 1.00 (6 H, s, 2 Me-C-20α and 2β), 1.04 (3 H, s, Me-C-14 (β), 1.17 (3H, s, Me-C-13 (α), 1.95 (1 H, m, 10 (β), 2.25 (1 H, q, J = 7.0, Hz, H-4 (α), 2.35 (2 H, m, H-30 and H-2β); MS, m/z: 426 [M⁺] (0.18), 411 (0.10), 341 (0.05), 302 (0.18), 287 (0.08), 273 (0.36), 218 (28), 203 (40), 191 (21), 179 (28), 125 (73), 93 (94), 69 (100). Accurate mass measurement: found 426.3863 ± calculated 426.3861 for C₂₅H₃₉O₅.

Friedelolin (II). White prisms, soluble in CHCl₃, mp 286-289°C (ethyl acetate), it was isolated from the comparison of its melting point and Rₚ with a patron sample (15).

Betulinic acid (III). White prisms, slightly soluble in CHCl₃ and ethyl acetate, mp 325-328°C with decomposition (ethyl acetate–isopropanol), gave positive test of Liebermann-Burchard for triterpenes; IR (KBr): 3460 (OH) 3090 (C=CH), 1690 (C=O), 1645 (C=CH₂) cm⁻¹; UV (MeOH): 205 nm; 1-HNMR (200 MHz, CDCl₃): δ 4.74 (1 H, d, J = 2 Hz, H-29), 4.73 (1 H, d, J = 2 Hz, H-29), 4.60 (1H, m, H-1), 1.65 (3H, s, Me-C-30), 1.22 (9H, s, 3 Me), 1.20 (6H, s, 2 Me); MS m/z: 456 [M⁺], 438, 423, 411, 395, 382, 287, 248, 107, 189, 175, 135, 81, 69. Accurate mass measurement: found 456.3595 and calculated 456.3593 for C₂₅H₃₉O₅.

Betulinic acid acetate: The residue A obtained from the methanolic extract was heated at 90°C during 4 hours in pyridine and acetic anhydride. After the usual work, it was purified by chromatographic columns and sequential preparative TLC [cyclohexane–ethyl acetate, (90:10)] x 4. Crystallization in benzene gave white plate crystals, soluble in CHCl₃, ethyl acetate and methanol, mp 275-276°C; UV, (MeOH): 206 nm; IR, (Nujol): 3100 (C=CH₂), 1738 (CH₂COO), 1695 (C=O), 1645 (C=CH₂), 1380, 1370 (gem dimethyl group), 1250 (CH₂COO) cm⁻¹; 1-HNMR (200 MHz, CDCl₃): δ 0.81 (3H, s, Me-C-25), 0.83 (3H, s, Me-C-27), 0.92 (3H, s, Me-C-26), 0.96 (3H, s, Me-C-24), 1.03 (3H, s, Me-C-23), 1.68 (3H, s, Me-C-30), 2.03 (3H, s, Me-OAc), 4.45 (1H, t, J=6H), 4.60 (1H, d, δ=CH₂-C-29), 4.72 (1H, d, δ=CH₂-C-29).

1H-NMR (50.3 MHz, CDCl₃): δ 14.0 (C=COO), 15.99 (C-26), 16.12 (C-25), 15.42 (C-24), 18.09 (C-2), 19.30 (C-23), 20.79 (C-6), 21.27 (CH₂COO), 23.64 (C-11), 25.37 (C-12), 27.89 (C-23), 29.64 (C-21), 30.52 (C-15), 32.10 (C-16), 34.16 (C-7), 37.02 (C-10, 22), 37.74 (C-4), 38.37 (C-1, 13), 40.62 (C-8), 42.34 (C-14), 46.89 (C-19), 49.20 (C-18), 50.32 (C-9), 55.34 (C-5), 56.35 (C-17), 80.93 (C-3), 109.69 (C-29), 150.35 (C-20), 171.12 (C=O of the OAc), 182.55 (C-28); MS, m/z: 498 [M⁺], 483 [M⁺-CH₂], 466 [M⁺-CH₂-OH], 438 [M⁺-CH₂-OOC], 423 [M⁺-CH₂-OOH], 395, 248, 219, 215, 203, 189, 175, 135, 107 and 81.

Anticoagulative activity

Following the method described by Rahola (35), the antithrombotic activity was investigated employing ICR fasted female mice weighing 25 to 30 g. The triterpenoid compounds were evaluated by oral administration in doses of 30, 60 and 100 mg kg⁻¹. Groups each of six mice were employed for each dose, the vehicle [Tween 80: water (20:80)], blank (water) and the standard patent acetonylthiacylic acid (ASA) in dose of 200
mg/kg. One hour after the substances were administered, the mice were injected intraperitoneally with 0.25 ml/kg of 1% acetic acid. The mice were observed for the next 20 minutes and the number of writhings shown in each mouse was recorded. The data on antinociceptive activity was analyzed using analysis of variance (ANOVA) and the group means were considered statistically significant if p < 0.05.

Results and discussion

Isolated Compounds

Friedelane (I): C₆H₆O₂, molecular weight 426, mp 268-270°C, gave a positive test of Liebermann-Burchard for triterpenes. In the IR spectrum shows bands at 1720 for (C=O), 1397 and 1390 cm⁻¹ for phenyl dimethyl. The ¹H-NMR spectrum showed 6 singlet signals between 6.70 and 7.17 for seven tertiary methyl groups and a double signal at 6.87 for a secondary methyl group. Also signals at 6.195 (1H, m, H8 [8]), 2.25 (1 H, q, J=7.0 Hz, for H-4 α and 2.35 (2 H, m, H2α and H2β). The above-mentioned and the mass fragmentation pattern confirmed that the compound is a triterpene of the friedelane group. More evidence for the structure of the compound is the agreement of the spectroscopic data and its melting point with those reported in the literature (15).

Friedelinal (II): C₅H₉O₂, molecular weight 428, mp 286-289°C, gave a positive test of Liebermann-Burchard for triterpenes and it was identified by comparison of its Rf and the melting point with those of a patron sample (15).

Betaulnic acid (III): C₃H₄O₅, molecular weight 456, mp 323-328°C (ethyl acetate - iso-propanol), gave a positive test of Liebermann-Burchard for triterpenes. The compound shows in the IR spectrum a wide band at 3460 cm⁻¹ for a hydroxyl group that was confirmed by obtaining the acetate derivative with mp 275-276°C, which shows in its IR spectrum bands at 1738 and 1550 cm⁻¹ for the carbonyl group of the acetate ester, and a single signal at 6 2.03 for the methyl group of OAc in the ¹H-NMR spectrum. The acid also shows bands at 3090, 1645 cm⁻¹ for C=CH₂ and 1690 cm⁻¹ for the carbonyl group of the COOH function that were confirmed by the appearance of bands at 3100, 1645 (C=CH₂) and 1695 cm⁻¹ (C=O) in the IR spectrum of the acetate derivative.

The above was confirmed by the presence of a double signal at 6.74 and other at 6.73, each one for one proton of a methylene (C=CH₂) group, and a third triplet signal at 6 4.60 for the H30 in the NMR-¹H spectrum of the triterpenic acid, which appear as double signals at 6.72 and 4.60 (C=CH₂) and other multiple one at 6 4.45 for a proton (H30) in
the spectrum of RMN-1H of the acetate derivative. The 13C-NMR spectrum of the triterpenic acid and its acetate derivative show 5 singlet signals for 6 methyl groups, one of which appears at δ 1.69 (C-CH3). The 1H-NMR spectrum of the triterpenic acid, 13C-NMR, 11C NMR of the acetate derivative data and the mass fragmentation pattern confirm that the isolated compound is a triterpenic acid of the lupane series and was identified as 3β-OH-biap-20(29)-en-28-oic, betulinic acid (III). More evidence for the identification of the compound is the agreement of the 11C NMR spectrum data of the acetate derivative with those reported in the literature for patron sample (36).

The present work presents the isolation for the first time of the betulonic acid from the stem bark of Clausia elliptica, family Gutiferae, which suggests that it can be a biotaxonomic indicator. Betulonic acid previously was isolated from the wood of Calypsothamnus lanceolatus Thw. (8) and the bark of Kayapo stylosa Thw. (18) (Guttiferae) native in Sri Lanka.

Antinociceptive activity

Study results showed a significant decrease in the number of the writhings induced by 1% acetic acid in mice for all the evaluated compounds and the highest activity was attributed to the betulonic acid. Friedelindol showed an antinociceptive activity of 54.5 ± 4.10% in the dose of 30 mg/kg that did not increase in the doses of 60 and 100 mg/kg (51.3 ± 4.10% and 50.0 ± 4.10% respectively) which means there is no direct relationship between dose and activity, while friedelindol showed antinociceptive effect of 46.2 ± 4.44%, 57.1 ± 4.44% and 60.9 ± 4.44% in doses of 30, 60 and 100 mg/kg respectively and betulinic acid showed the effect of 65.5 ± 3.79%, 59.8 ± 3.79% and 75.0 ± 3.79% in the same doses of 30, 60 and 100 mg/kg respectively, manifesting a direct relationship dose activity at the range of the evaluated doses for these two compounds (Figure 1).

The antinociceptive activity manifested by the studied compounds is very important since it is caused by small doses in comparison with the patron acetyl salicylic acid which showed antinociceptive activity of 65.6 ± 4.11% in dose of 200 mg/kg.

The antinociceptive activity of betulonic acid can be attributed to an inhibitory effect in the synthesis of the prostaglandins (23). Possibly its high activity is due to the higher polarity and the presence of a double bond near to a carboxylic group in its structure as compared with friedelindol and friedelindol. Those characters of betulonic acid can also be the responsible for its multiple reported pharmacological activities (19, 22, 23, 25-27). The results mean that all the studied substances have higher antinociceptive activities than that of the patron acetyl salicylic acid if we consider that the employed doses of these compounds (30, 60 and 100 mg/kg) correspond to 15, 30 and 50% of the employed dose (200mg/kg) of the patron.

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