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, friedelinol and friedeline, by means of chemical and spectroscopic methods. The pharmacological study by the abdominal contortions model induced by acetic acid showed significant antinociceptive activities to the studied substances and the highest effect was attributed to the betulinic acid.

Key words: *Clusia ellipticifolia* – Guttiferae - Betulinic acid – Friedelinol – Friedeline - Antinociceptive activity.

Resumen

Actividad antinociceptiva de triterpenos aislados de *Clusia ellipticifolia*

Con base en los usos tradicional de varias especies de la familia Guttiferae, tres compuestos fueron aislados del extracto metabólico de la corteza de *Clusia ellipticifolia* y identificados por medio de métodos químicos y espectroscópicos como ácido betulínico, friedelinol y friedelina. El estudio farmacológico por el modelo de contorsiones abdominales inducidas por ácido acético mostró actividad antinociceptiva muy significante para los compuestos aislados siendo la más alta atribuida al ácido betulinico.

Palabras clave: *Clusia ellipticifolia* – Guttiferae – Ácido betulínico – Friedelinol – Friedelina – 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 *Callophyllum brasiliense* (Tree of Oil), *Callophyllum lucidum* (Stick of Oil) and *Callophyllum mariae* (Oil of María) in dermatology and to heal the navel of those recently born ones when it does not heal. The resin of *Clusia alata* is used in the treatment of the cephalalgy and the leaves are detersive. The decoction 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 *Mammea americana* known popularly as "Mamey" are used as nutritious fruit for their very pleasant flavor. The emulsion prepared in water from the milled 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-Guttiferin (13) were reported in some species. It was also reported that isopentisin (1, 3-dihydroxy-7-methoxyxanthone) and their 3-*O*- glycoside, which are characteristic constituents of plants of the Guttiferae and Gentianaceae, inhibit the rat brain mitocondrial monoamineoxidases *in vitro* (14).

Previously friedeline and friedelinol were isolated of the petroleum ether extract of the stem bark of *Clusia ellipticifolia* (15). Benzophenones clusiaphenone A, B, C and D; vismiaphenone B and isovisiaphenone 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 carrageenan induced rat paw edema (19) and on the edema in the rat's ear showing a mechanism of similar action to that of the glucocorticoids (22), inhibitory effect in the synthesis of the prostaglandins *in vitro* (23), antitumor activity against the human melanoma (26), antimicrobial activity with an inhibitory minimum concentration superior to 10 μg/mL against *Bacillus subtilis*, *Escherichia coli* and *Micrococcus luteus* (25) and an antiviral effect against the virus of human immunodeficiency (27).

Friedeline was isolated of several plants (15, 28-31) and pharmacological study has shown an anti-inflammatory activity suggesting a mechanism action of inhibition of the receptor H1 (32). Meanwhile friedelinol was also isolated of several plants among them *Clusia ellipticifolia* (15), *Stevia lucida* (33), *Dischidia formosana* (28) and *Lepidophyllum cupressiforme* (34).

The above-mentioned motivates us to study the alcoholic extract of the stem bark of *Clusia ellipticifolia*, which allowed the isolation of the betulinic acid, friedeline and friedelinol and the determination of their antinociceptive properties.

Methodology

Plant material

Clusia ellipticifolia 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 National University of Colombia. A voucher specimen was deposited with the number Col 229095 in the Colombian National Herbarium.

Extraction and isolation of compounds

The dried powdered stem bark of *Clusia ellipticifolia* (1035 g) was exhaustively extracted with petroleum ether 37-40°C, in Soxhlet equipment. The defatted bark again was exhaustively extracted with methanol. The dried residue of the methanolic 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, xanthones, 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, 0.063-0.200 mm (80g). Elution with benzene and increasing concentration of ethyl acetate in benzene and preparative TLC [cyclohexane: ethyl acetate (90:10)] permitted the isolation of friedeline (I) (54 mg), friedelinol (II) (3 mg) and belutinic acid (III) (625 mg).

Identification of the isolated compounds

Friedeline (I). White needles, soluble in $CHCl_3$, ethyl acetate, slightly soluble in benzene, petroleum ether and methanol, insoluble in

water, gave positive test of Liebermann Burchardt for triterpenes. Mp 268-270°C (ethyl acetate) with decomposition; IR ((KBr): 1720 (C=0), 1397, 1390 cm^{-1} (Gemdimethyl); ^{1}H-NMR (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, d, J = 6 Hz, Me, C-4 β), 0.95 (3 H, s, Me, C-9 β), 1.00 (6 H, s, 2 Me, C-20α and 20β), 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-2α and H-2β]; MS, *m/z*: 426 [M^+] (0.18), 411 (0.10), 341 (0.05), 302 (0.18), 287 (0.08), 273 (36), 218 (28), 205 (40), 191 (21), 179 (28), 125 (73), 95 (94), 69 (100). Accurate mass measurement: found 426.3863 and calculated 426.3861 for $C_{30}H_{50}O$.

Friedelinol (II). White prisms, soluble in $CHCl_3$, mp 286-289°C (ethyl acetate), it was identified as 3α-OH-friedelane (friedelinol) by the comparison of its melting point and R_f with a patron sample (15).

Betulinic acid (III). White prisms, slightly soluble in $CHCl_3$ and ethyl acetate, mp 325-328°C with decomposition (ethyl acetate - isopropanol), gave positive test of Liebermann - Burchardt for triterpenes; IR, (KBr): 3460 (OH) 3090 (C=CH_2), 1690 (C=0), 1645 (C=CH_2) cm^{-1}; UV, ((Methanol): 205 nm; ^{1}H-NMR (200 MHz, $CDCl_3$):δ 4.74 (1 H, d, J = 2 Hz, H-29), 4.73(1H, d, J = 2Hz, H-29), 4.60 (1H, m, H-3), 1.69 (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, 369, 302, 287, 248, 207, 189, 175, 135, 95, 81, 69. Accurate mass measurement: found 456.3593 and calculated 456.3603 for $C_{30}H_{48}O_3$.

Betulinic acid acetate: The residue A obtained of 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 column and sequential preparative TLC [cyclohexane-ethyl acetate, (90:10)] x 4). Crystallization in benzene gave white plate crystals , soluble in $CHCl_3$, ethyl acetate and methanol, mp 275-276°C; UV, (Methanol): 206 nm; IR, (Nujol): 3100 (C=CH_2), 1738 (CH_3COO), 1695 (-COOH), 1645 (C=CH_2), 1380, 1370 (gem dimethyl group), 1250 (CH_3COO) cm^{-1}; ^{1}H-NMR (200 MHz, $CDCl_3$): 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, 3α-H), 4.60 (1H, d, =CH_2, C-29), 4.72 (1H, d, =CH_2, C-29); ^{13}C-NMR (50.3 MHz, $CDCl_3$: δ 14.60 (C-27), 15.99 (C-26), 16.12 (C-25), 15.42 (C-24), 18.09 (C-2), 19.30 (C-30), 20.79 (C-6), 21.27 (CH_3COO), 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^+ - CH_3], 466 (M^+ - CH_3 - OH), 438 (M^+ - CH_3 - COOH), 423 (M^+ - CH_3 - COOH - CH_3), 395, 248, 219, 215, 203, 189, 175, 135, 107 and 81.

Antinociceptive activity

Following the method described by Rahola (35), the antiwrithing 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. Groups each of six mice were employed for each dose, the vehicle [tween 80: water (20:80)], blank (water) and the standard patron acetylsalicylic acid (ASA) in dose of 200

mg/kg. One hour after the substances were administrated, 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

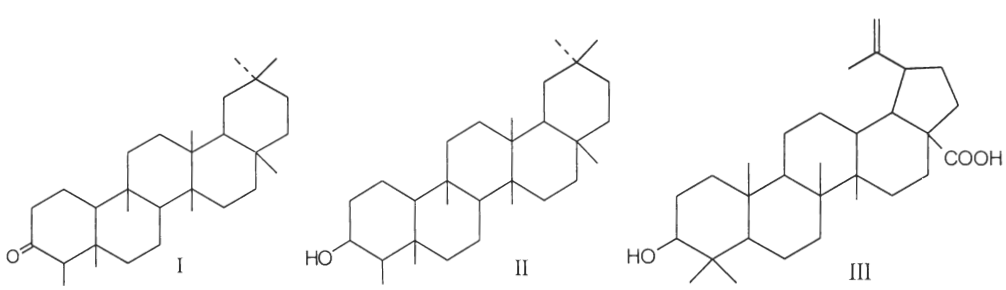
Isolated Compounds

Friedeline (I): $C_{30}H_{50}O$, molecular weight 426, mp 268-270°C, gave a positive test of Liebermann Burchardt for triterpenes. In the IR spectrum shows bands at 1720 for (C=O), 1397 and 1390 cm^{-1} for gem dimethyl. The ^{1}H-NMR spectrum showed 6 singlet signal between δ 0.72 and 1.17 for seven tertiary methyl groups and a double signal at δ 0.87 for a secondary methyl group. Also signals at δ1.95 (1H, m, H 10 β), 2.25 (1 H, q, J=7.0 Hz, for the H-4 α and 2.35 (2 H, m, for 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).

Friedelinol (II): $C_{30}H_{52}O$, molecular weight 428, mp 286-289°C, gave a positive test of Liebermann Burchardt for triterpenes and it was identified by comparison of its R_f and the melting point with those of a patron sample (15).

Betulinic acid (III): $C_{30}H_{48}O_3$, molecular weight 456, mp 325-328°C (ethyl acetate - isopropanol), gave a positive test of Liebermann - Burchardt 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 1250 cm^{-1} for the carbonyl group of the acetate ester, and a singlet signal at δ 2.03 for the methyl group of OAc in the ^{1}H-NMR spectrum. The acid also shows bands at 3090, 1645 cm^{-1} for $C=CH_2$ and 1690 cm^{-1} for the carbonyl group of the COOH function that were confirmed by the appearance of bands at 3100, 1645 ($C=CH_2$) 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 δ 4.74 and other at δ 4.73, each one for one proton of a methylene ($C=CH_2$) group, and a third triplet signal at δ 4.60 for the H3α in the NMR-^{1}H spectrum of the triterpenic acid, which appear as double signals at δ 4.72 and 4.60 (C=CH2) and other multiple one at δ 4.45 for a proton (H3α) in



the spectrum of RMN-^{1}H of the acetate derivative. The NMR-^{1}H spectrum of the triterpenic acid and its acetate derivative show 6 singlet signals for 6 methyl groups, one of which appears at δ 1.69 (=C-CH3). The ^{1}H-NMR spectrum of the triterpenic acid, ^{1}H-NMR, ^{13}C 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-lup-20(29)-en-28-oic, betulinic acid (III). More evidence for the identification of the compound is the agreement of the ^{13}C NMR spectrum data of the acetate derivative with those reported in the literature for patron sample (36).

The present work presents the isolation for first time of the betulinic acid from the stem bark of *Clusia ellipticifolia*, family Guttiferae, which suggests that it can be a biotaxonomic indicator. Betulinic acid previously was isolated from the wood of *Calophyllum bracteatum* Thw. (8) and the bark of *Kayea 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 betulinic acid. Friedelinol 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 (51.5 ± 4.10% and 50.0 ± 4.10% respectively) which means there is no direct relationship between dose and activity, while friedeline 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 100mg/kg respectively, manifesting a direct relationship dose activity in the range of the evaluated doses for these two compounds (Figure 1).

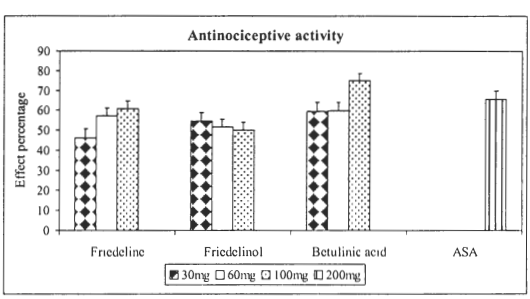


Figure 1. Antinociceptive activity of friedeline, friedelinol and betulinic acid against the patron acetylsalicilic acid.

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 betulinic 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 friedeline and friedelinol. Those characters of betulinic 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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