PRELIMINARY PHYTOCHEMICAL STUDY OF THE ECUADORIAN PLANT *CROTON ELEGANS* KUNTH (EUPHORBIACEAE)

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ABSTRACT

The species *Croton elegans* Kunth, belonging to the family Euphorbiaceae and known with the common name "mosquera", is a native medicinal plant of Ecuador. Due to its medical applications, we are interested in secondary metabolite composition. After extraction with hexane and preparation of the alkaloid extract, preparative chromatographic fractionation in both normal and direct phase was performed. ^{1}H NMR, ^{13}C NMR, HSQC and HMBC experiments, together with polarimetric measurements, were conducted in order to characterize the purified compunds. Two triterpene metabolites (friedelin and cycloeucalenol) and two morphinan alkaloids (*(+)*-pallidine and *(+)*-*O*-methylpallidine) were obtained in a pure form. This work represents the first report on the phytochemistry of *C. elegans* and probably the second publication on the natural *(+)*-pallidine alkaloid.

Keywords: *Croton elegans*; alkaloid; secondary metabolite; Ecuador; NMR; mosquera; friedelin; cycloeucalenol; *(+)*-pallidine; *(+)*-*O*-methylpallidine.

1. INTRODUCTION

Ecuador is one of the 17 recognized *megadiverse* countries [1], i.e. one of the countries with the highest concentration of different botanical and animal living species on the planet. For this reason, Ecuador is a privileged place for chemical research on plants that were never studied before. Furthermore, in Ecuador many indigenous nations live with their ancestral knowledge, which includes traditional application of biological active plants to medicine and supernatural rites [2].

Croton elegans Kunth is an endemic species of Ecuador, belonging to the family Euphorbiaceae and known with the common name "mosquera". According to the Catalogue of the Vascular Plants of Ecuador [3], this species corresponds also to *Croton ferrugineus* var. *elegans* (Kunth) Moell. Arg.

Phytochemistry of *C. elegans* has not been reported previously in literature, for this reason we consider this work as the first chemical report on this plant. According to botanical literature, this plant is a shrub of the dry interandean vegetation, growing between 1500 and 3500 m a.s.l. and spread in the canton of Loja, south of Ecuador, where it is described as "locally very abundant" [4]. *C. elegans* was selected as an interesting item for two reasons. First of all because it presents many applications in the traditional medicine of Ecuador, in fact *C. elegans* is used as an anti-inflammatory, in the treatment of toothache, wounds, tonsillitis an warts. It is also a powerful purgative, that should be used very carefully as it can produce vomit and strong abdominal pain. It has also been used in the treatment of rheumatism, gout, neuralgia and bronchitis. On the other hand, it was selected also due to the phytochemical richness and phylogenetic variety of the genus [2]. *Croton* spp. are well known for producing terpene compounds, mainly diterpenes of the cembranoid, clerodane, neoclerodane, halimane, isopimarane, kaurane, secokaurane, labdane, phorbol and trachylobane families. Other important terpenic compounds present in *Croton* spp. are triterpenes and volatiles isoprenoids, that are constituents of essential oils, together with volatile shikimic acid derivatives [5]. The other great category of metabolites commonly found in this genus is constituted by alkaloids, whose presence is of the greatest interest due to the biological activities they often show [5].

From the pharmacological point of view, *Croton* spp. are known to possess a lot of activities, among them: anti-lipidemic, wound healing, anti-diarrheic, immunomodulatory, antibacterial, antifungal, antiviral, antimalarial, anticancer, anti-mutagenic , mutagenic, antioxidant, myorelaxant, antispasmodic, anti-hypertensive, anti-inflammatory, anti-nociceptive, etc [5].

2. EXPERIMENTAL

2.1 General information

The structural identification of friedelin, cycloeucalenol, *(+)*-pallidine and *(+)*-*O*-methylpallidine was performed through NMR, with a Varian Agilent NMR instrument (Walnut Creek, CA, USA, 400 MHz for ^{1}H and 100 MHz for ^{13}C experiments). Deuterated solvents were purchased from Sigma-Aldrich®. Chemical shifts were reported in units (ppm) relative to the signal of tetramethylsilane (TMS) and coupling constants (J) in Hz. Rf values are referred to silica normal phase TLC, friedelin and cycloeucalenol were revealed with vanillin/sulphuric acid reagent [6], *(+)*-pallidine and *(+)*-*O*-methylpallidine were revealed with Dragendorff's reagent [6]. Yields are expressed as % w/w referred to extracts.

2.2 Plant material

A few specimens of *C. elegans* were collected in February 2013 at the parish of Sagrario, canton Cotacachi, province of Imbabura, Ecuador. Plants were located at 2676 m a.s.l. After collection, they were reproduced in greenhouse at the conservation garden of Tumbaco (Pichincha). Leaves were collected and selected by a technician of INIAP and a botanical sample was prepared with voucher number MT-JA116, preserved by INIAP with the Germoplasm Bank number ECU-20046. The collection was performed under scientific investigation permission N. 004-12-IC-FLO-DNB/MA. After selection, the plant material was dried at 35°C and stored in a dark, dry and fresh place until processing.

2.3 Extraction

500 g of dry leaves were milled and exhaustively extracted with hexane, the solvent was then removed by vacuum distillation obtaining 20.6 g of dry extract. It was directly submitted to chromatographic fractionation, while the residual plant was submitted to acidic extraction in order to obtain the alkaloid fraction. In this case the plant was suspended in diluted aqueous sulphuric acid (2% v/v), the solid was filtered and the process repeated until negative reaction to Dragendorff's reagent. The reunited aqueous phases were alkalinized until pH 11 by addition of concentrated ammonia, then extracted with chloroform until negative reaction to Dragendorff's reagent. After drying with anhydrous sodium sulphate, the reunited organic phases were distilled at reduced pressure, affording 1.9 g of alkaloid fraction. All organic solvents were technical grade, bought from Brenntag (Guayaquil, Ecuador) and carefully distilled before using.

2.4 Isolation and purification

2.0 g of the hexane extract were fractionated by preparative liquid chromatography, applying a ratio of 100:1 w/w between the stationary phase and the mixture. The process was performed on normal phase silica gel 60 Å (40-63 μm) purchased from Sigma-Aldrich, eluting through an increasing polarity gradient. The elution was performed with a mixture of hexane/EtOAc, from 95% of hexane to EtOAc 100%. The fractions were analysed on silica TLC plates with fluorescent indicator at 254 nm, purchased from Sigma-Aldrich, and reunited in relation to their composition. After exposure to UV

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light (254 and 366 nm), TLC plates were revealed with sulphuric acid/vanillin reagent. Subsequent direct phase chromatographic fractionation of the impure fractions, performed in line with the same principles, afforded two major pure compounds, identified as friedelin (**1**) and cycloeucalenol (**2**).

For what concerns the alkaloid extract, the whole amount was submitted to both direct and C-18 reversed phase chromatography. The reversed phase fractionation was performed on the whole alkaloid extract, with LiChroprep® RP-18 (40-63 μm) from Merck. The process was performed following an increasing gradient of eluotropic strength, eluting with a MeOH/H_2O from 80% until 100% MeOH. The column was packed with a ratio 100:1 w/w between stationary phase and alkaloid mixture. The fractions obtained were analysed on Merck C-18 reversed phase TLC, with fluorescence indicator at 254 nm. After exposure to UV light (254 and 366 nm), TLC plates were revealed with Dragendorff's reagent [6]. Two major alkaloid fractions were then purified on silica, with the same stationary phase and ratio of the hexane extract. However, in this case the elution was performed with a mixture of hexane/CH_2Cl_2/MeOH, in an increasing polarity gradient from 50:40:10 to CH_2Cl_2/MeOH 90:10. Two pure alkaloid compounds were obtained and identified as *(+)*-pallidine (**3**) and *(+)-O*-methylpallidine (**4**). To purify both alkaloid and non-alkaloid compounds, the same solvents of the extraction process were applied.

2.5 Physical and Spectral Data of Isolated Compounds

Friedelin (**1**): $C_{30}H_{50}O$; white crystals; yield 0.14%; soluble in CH_2Cl_2 and $CHCl_3$; Rf = 0.50 (hexane/EtOAc 90:10); ^{1}H NMR ($CDCl_3$): δ 2.39 (1H, dd, J=2.5, 5.0, H-2β), 2.31 (1H, dd, J=7.0, 13.0, H-2α), 2.25 (1H, q, J=6.8, H-4α), 1.97 (1H, m, H-1α), 1.76 (1H, dt, J=5.0, 2.5, H-6β), 1.68 (1H, dd, J=5.0, 13.0, H-1β), 1.18 (3H, s, H-28), 1.05 (3H, s, H-27), 1.01 (3H, s, H-30), 1.00 (3H, s, H-26), 0.95 (3H, s, H-29), 0.88 (3H, d, J=6.8, H-23), 0.87 (3H, s, H -25), 0.73 (3H, s, H-24); ^{13}C NMR ($CDCl_3$): δ 213.4 (C-3), 59.6 (C-10), 58.3 (C-4), 53.2 (C-8), 42.9 (C-18), 42.2 (C-5), 41.6 (C-2), 41.4 (C-6), 39.8 (C-13), 39.4 (C-22), 38.4 (C-14), 37.5 (C-9), 36.1 (C-16), 35.7 (C-11), 35.4 (C-19), 35.1 (C-29), 32.9 (C-15), 32.5 (C-21), 32.2 (C- 28), 31.9 (C-30), 30.6 (C-12), 30.1 (C-17), 28.3 (C-20), 22.4 (C-1), 20.4 (C-26), 18.8 (C-27), 18.3(C-7), 18.1 (C-25), 14.8 (C-24), 6.9 (C-23).

Cycloeucalenol (**2**): $C_{30}H_{50}O$; white crystals; yield 0.08%; soluble in CH_2Cl_2, $CHCl_3$, MeOH; Rf = 0.30 (hexane/EtOAc 90:10); ^{1}H NMR ($CDCl_3$): δ 4.70 (1H, brs, H-28), 4.64 (1H, d, J=1.2, H-28), 3.22-3.16 (1H, m, H-3), 2.22 (1H, septet, J=6.8, H-25), 1,01 (3H, d, J=7.2, H-26), 1.00 (3H, d, J=6.8, H-27), 0.96 (3H, d, J=7.2, H-30), 0.95 (3H, s, H-18), 0.88 (3H, d, J=6.4, H-21), 0.87 (3H, s, H- 32), 0.56 (1H, dq, J=2.8, 12.8, H-6β), 0.37 (1H, d, J=4.0, H-19β), 0.18 (1H, d, J=4.0, H-19α); ^{13}C NMR ($CDCl_3$): δ 157.1 (C-24), 106.1 (C-28), 76.7 (C-3), 52.3 (C-17), 49.0 (C-14), 47.0 (C-8), 45.5 (C-13), 44.7 (C-4), 43.5 (C-5), 36.3 (C-20), 35.5 (C-15), 35.1 (C-22), 35.0 (C-2), 33.9 (C-25), 33.0 (C-12), 31.5 (C-23), 30.9 (C-1), 29.7 (C-10), 28.2 (C-16), 27.4 (C-19), 27.1 (C-11), 25.3 (C-7), 24.8 (C- 6), 23.7 (C-9), 22.1 (C-26), 22.0 (C-27), 19.3 (C-32), 18.5 (C- 21), 17.9 (C-18), 14.5 (C-30).

(+)-pallidine (**3**): $C_{19}H_{21}NO_4$; yellow solid; yield 0.73%; soluble in CH_2Cl_2, $CHCl_3$, MeOH; Rf = 0.25 (hexane/ $CHCl_3$/MeOH 40:55:5); $[\alpha]_D^{20}$= +20.3 (c = 0.10, $CHCl_3$); ^{1}H NMR ($CDCl_3$): δ 6.78 (1H, s, H-4), 6.70 (1H, s, H-1), 6.34 (1H, s, H-5), 6.33 (1H, s, H-8), 3.90 (3H, s, 3-MeO), 3.80 (3H, s, 6-MeO), 3.73 (1H, d, J=6.0, H-9), 3.36 (1H, d, J=18.0, H-10a), 3.04 (1H, dd, J=18.0, 6.4, H-10b), 2.67-2.62 (2H, m, H-16), 2.49 (3H, s, N-Me), 1.96 (1H, dt, J=6.0, 12.8, H-15a), 1.83 (1H, dt, J=12.8, 2.0, H-15b); ^{13}C NMR ($CDCl_3$): δ 180.8 (C-7), 160.1 (C-14), 151.6 (C-6), 146.0 (C-3), 145.1 (C-2), 129.5 (C-12), 129.0 (C-11), 122.9 (C-8), 118.8 (C-5), 113.7 (C-1), 107.6 (C-4), 61.0 (C-9), 56.3 (3-MeO), 55.3 (6-MeO), 45.8 (C-16), 42.3 (C-13), 41.6 (N-Me), 40.8 (C-15), 32.6 (C-10).

(+)-O-methylpallidine (**4**): $C_{20}H_{23}NO_4$, yellow solid; yield 2.78%; soluble in CH_2Cl_2, $CHCl_3$, MeOH; Rf = 0.35 (hexane/ $CHCl_3$/MeOH 40:55:5); $[\alpha]_D^{20}$= +14.4 (c = 0.08, $CHCl_3$); ^{1}H NMR ($CDCl_3$): δ 6.81 (1H, s, H-4), 6.63 (1H, s, H-1), 6.35 (1H, s, H-5), 6.33 (1H, s, H-8), 3.88 (3H, s, 2-MeO), 3.86 (3H, s, 3-MeO), 3.80 (3H, s, 6-MeO), 3.69 (1H, d, J=6.0, H-9), 3.34 (1H, d, J=18.0, H-10a), 3.04 (1H, dd, J=18.0, 6.0, H-10b), 2.60-2.57 (2H, m, H-16), 2.47 (3H, s, N-Me), 1.99-1.91 (1H, m, H-15a), 1.86-1.82 (1H, m, H15b); ^{13}C NMR ($CDCl_3$): δ 181.0 (C-7), 161.8 (C-14), 151.6 (C-6), 148.5 (C-3), 148.2 (C-2), 130.1 (C-12), 128.9 (C-11), 122.4 (C-8), 118.9 (C-5), 110.6 (C-1), 108.8 (C-4), 61.0 (C-9), 56.4 (6-MeO), 56.0 (3-MeO), 55.2 (2-MeO), 45.8 (C-16), 42.4 (C-13), 41.9 (N-Me), 41.3 (C-15), 32.8 (C-10).

3. RESULTS AND DISCUSSION

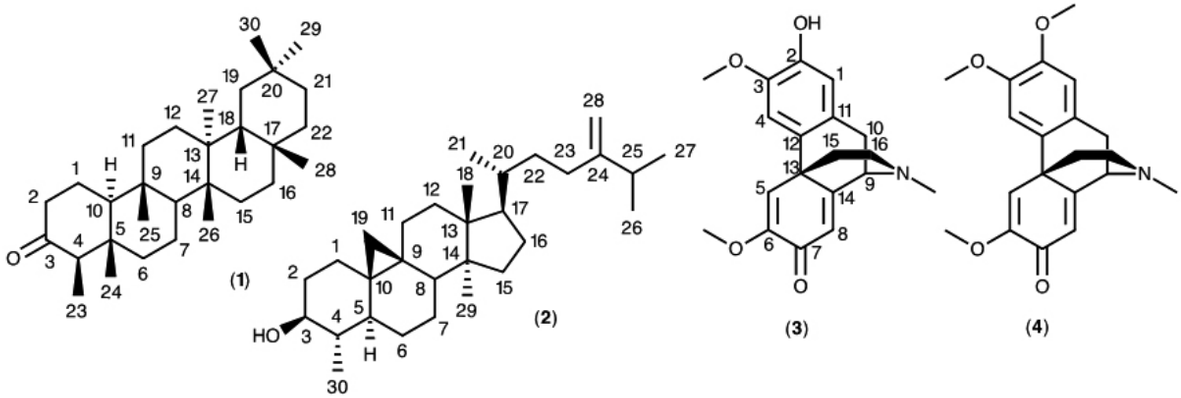

Figure 1. Secondary metabolites isolated from *Croton elegans* Kunth.

Friedelin (**1**) is a triterpenic carbonyl compound, quite common and well known since a long time as it was obtained in XIX century, in mixture with the α-hydroxy derivative cerin. The structure was not completely elucidated until 1956, due to the study of Corey and Ursprung [7]. Since then, compound (**1**) has been found in many species, such as *Kalanchoe daigremontiana* [8], *Acer mandshuricum* [9], *Maytenus rigida* [10], *Azima tetracantha* [11] and *Pterocarpus erinaceus* [12].

Cycloeucalenol (**2**) is also a triterpenic metabolite, belonging to the family of cycloartanes. This class of molecules is also quite common and it is characterized by the presence of a cyclopropane condensed to ring B. It has been isolated from *Boophone disticha* [13], *Fritillaria hupehensis* [14], *Brassica rapa* [15], *Erythrina stricta* [16], *Erythrina subumbrans* [16], among others. According to bibliographic information, no one of these metabolites has been previously found in *Croton* sp.

Compounds (**1**) and (**2**) have been identified through ^{1}H and ^{13}C NMR experiments, by comparison of the respective spectra with literature [17,18].

(+)-pallidine (**3**) and *(+)-O*-methylpallidine (**4**) are typical alkaloids of genus *Croton*, belonging to the family of morphinandienones [5,19]. *O*-methylpallidine (also known as *O*-methylflavinantine and sebiferine) is a quite common alkaloid in many vegetal species, both in *(+)* and *(-)* enantiomeric forms (*Meconopsis horridula* [20], *Alseodaphne perakensis* [21], *Stephania bancroftii* [22], *Guatteria multivenia* [23], *Ocotea acutangula* [24], *Croton flavens* [25,26]). On the other hand, pallidine is almost exclusively known as a natural compound in the levorotatory form. In fact, while *(-)*-pallidine has been found in many botanical species such as *Ocotea acutangula* [24], *Corydalis pallida* [27], *Corydalis saxicola* [28], *Meconopsis cambrica* [29] and *Fumaria vaillantii* [30], *(+)*-pallidine is mainly known as a synthetic compound [31]. Apparently, to date, the only known natural source of the dextrorotatory

pallidine is *Croton chilensis* [32], the only *Croton* sp. growing in Chile [33].

Compounds (**3**) and (**4**) were characterized through ^{1}H and ^{13}C NMR experiments and optical activity. The spectra were compared with literature, resulting almost identical. Furthermore, HSQC and HMBC experiments were performed in order to confirm the position of MeO groups and respective signals (Table 1).

Table 1. HSQC and HMBC coupling for MeO groups of compounds (**3**) and (**4**).

^{1}H NMR (ppm)	HSQC ^{13}C signal (ppm)	HMBC ^{13}C signal (ppm)
3.90 (3H, s)[a]	56.3	146.0
3.80 (3H, s)[a]	55.3	151.6
3.88 (3H, s)[b]	55.2	148.2
3.86 (3H, s)[b]	56.0	148.5
3.80 (3H, s)[b]	56.4	151.6

[a]*(+)*-pallidine, [b]*(+)-O*-methylpallidine

4. CONCLUSIONS

The first preliminary phytochemical study on *Croton elegans* Kunth has been performed and described in this article. The most important result was the isolation of *(+)*-pallidine, an unusual enantiomer of the well-known pallidine alkaloid. In fact this enantiomer, whose stereochemistry has been proved by measuring its optical activity, is not usually the common stereoisomer found in nature. Apparently only in *Croton chilensis*, the only chilean plant of the genus, the same enantiomer has been described.

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