Magnoflorine and Phenolic Derivatives from the Leaves of *Croton xalapensis* L. (Euphorbiaceae)

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The alkaloid magnoflorine **1**, has been isolated for the first time from *Croton xalapensis* (Euphorbiaceae), in addition two phenylpropenols derivates, 3,4-dimethoxy-(*E*)-cinnamyl alcohol **2** and 3,4-dimethoxy-5-hydroxy-(*E*)-cinnamyl alcohol **3**, 3,4,5-trimethoxycinnamic acid **4**, gallic acid **5**, methyl gallate **6** and 3,4-dihydroxybenzoic acid **7** have been also found; these compounds were identified by spectroscopic analysis particularly, 2D NMR and ESI-MS/MS techniques. The high concentration of magnoflorine, calculated with quantitative HPLC, of the aqueous extract, probably contributes to the remarkable medicinal properties of this plant. In addition this is the first phytochemical study on *Croton xalapensis* to be reported.

Keywords: Euphorbiaceae, *Croton xalapensis*, magnoflorine, phenylpropenol derivatives.

Croton (Euphorbiaceae) is one of the largest genera of flowering plants, with nearly 1300 species of herbs, shrubs, and trees that are ecologically prominent and often important elements of secondary vegetation in the tropics and subtropics worldwide [1]. The *Croton* species of plants, are used in South America as folk medicines for the treatment of wounds, inflammation and cancer [2]. The genus *Croton* is one of the richest sources of alkaloids with aporphine, proaporphine and morphinandienone skeletons [3a-3c], in addition, flavonoids [4], lignans [5], phenols [6] and diterpenes with the clerodane skeleton [7a-7d] are also commonly found in this genus. The major constituents of the essential oils from leaves, inflorescences and stalks were monoterpenes [8,9]. *C. xalapensis,* commonly named "china native", is a small tree that grows in the forests of southern Honduras. Literature phytochemical studies on *C. xalapensis* have not been reported; this is the first chemical investigation of *C. xalapensis* leading to the isolation of 7 compounds including the alkaloid magnoflorine and

representative phenolic compounds. The leaves of *C. xalapensis* are used as a decoction to treat wounds, infections, malaria, fever, gastrointestinal disease and diabetes in the Honduran folk medicine. Mature trees of genus *Croton* produce a blood-red to yellowish orange colored sap, or latex, which is highly regarded for its ability to speed the healing of wounds. Due to this blood-like appearance, the latex is termed "sangre de drago" in Spanish and "dragon's blood" in English [10]. Over the last two decades a chemical examination of this latex has led to the isolation of the major wound healing constituent of *C. lechleri*, which has been identified as the benzylisoquinoline-derived alkaloid taspine, which results from the conversion of magnoflorine [11a-11c]. The leaves of *C. xalapensis* were extracted in order to investigate its chemical constituents; a part of the methanol extract was partitioned between *n*-butanol and water. The water soluble portion was chromatographed on a RP-18 column and a fraction obtained was further purified by RP-HPLC to yield the aporphine, magnoflorine **1**. The *n*-butanol soluble portion was

fractionated by Sephadex LH-20 to yield pure compounds 3,4-(*E*) dimethoxycinnamyl alcohol **2**, 3,4-dimethoxy-5-hydroxy-(*E*)-cinnamyl alcohol **3**, 3,4,5-trimethoxycinnamic acid **4**, gallic acid **5**, methyl gallate **6** and 3,4-dihydroxybenzoic acid **7**. The structure of all compounds were unambiguously established by comparison of physical and spectroscopic properties including mass spectrometry, 1D (^{1}H, ^{13}C) and 2D (HSQC, HMBC and COSY) NMR spectroscopy with those reported in literature [12a-12d].

Compound **1** was identified as magnoflorine with the physical and the MS and NMR data [13,14]. The quantitative analysis of magnoflorine from *C. xalapensis* leaves was performed by HPLC. The concentrations of compound in the extract, calculated from the experimental peak areas by interpolation to standard calibration curve was 139.58 mg/g dry weight. This compound was found to inhibit the copper-mediated (Cu^{2+}) oxidation of LDL, as well as of glycated and glycoxidated LDL by increasing the lag time of conjugated diene formation and preventing the generation of thiobarbituric acid reactive substances (TBARS) [15]. These results suggest that magnoflorine may be useful for preventing the oxidation of various LDL forms. Magnoflorine is reported to have multiple pharmacologies as a neuromuscular blocking agent (AChR blocking agent), lipoxygenase inhibitor [16], as well as cytotoxic [17], immunosuppressive [18] and antimicrobial activities [12d].

Compound **3**, was isolated as a yellow amorphous solid and its molecular formula was determined by HRESIMS to be $C_{11}H_{14}O_4$. The ^{1}H and ^{13}C NMR spectra suggested a similar skeleton to that of **2**. The concerted interpretation of the ^{1}H-NMR and COSY spectra allowed us to evidence the observation of H-2/H-6 *meta*-coupling (*J*=1.5 ppm); the position of the substituents were deduced as occurring at C-3 and C-4 and C-5 (two methoxyl groups and one hydroxyl group) using HMBC connectivities between H-2 and H-6 with C-7 (131.6 ppm). The coupling constant (J_{AB}=15.9 Hz) of the olefinic protons suggested a *trans* double bond. These data suggest that compound **3** is identical with 3,4-dimethoxy-5-hydroxy-(*E*)-cinnamyl alcohol reported from *Ferula sinica* [14] without ^{13}C NMR data. Thus we report the NMR data here for compound **3** in CD$_3$OD.

Alkaloids have been proposed as chemotaxonomic markers in the infraspecific classification of the genus *Croton* [3a]. Aporphine alkaloids are widely

distributed among members of *Croton* genus but this is the first report of the occurrence of magnoflorine in *Croton xalapensis*.

Experimental

General Experimental Procedure: A Bruker DRX-600 NMR spectrometer, operating at 599.19 MHz for ^{1}H and at 150.86 MHz for ^{13}C, was used for NMR experiments; chemical shifts are expressed in δ (parts per million) referring to the solvent peaks δ H 3.34 and δ C 49.0 for CD$_3$OD; coupling constants, *J*, are in Hertz. DEPT, ^{13}C, DQF-COSY, HSQC, and HMBC NMR experiments were carried out using the conventional pulse sequences as described in the literature. Electrospray ionization mass spectrometry (ESIMS) was performed using a Finnigan LCQ Deca instrument from Thermo Electron (San Jose, CA) equipped with Xcalibur software. Instrumental parameters were tuned for each investigated compound: capillary voltage was set at 3 V, the spray voltage at 5.10 kV and a capillary temperature of 220°C and the tube lens offset at - 60 V was employed; specific collision energies were chosen at each fragmentation step for all the investigated compounds, and the value ranged from 15-33% of the instrument maximum. Data were acquired in the MS1 scanning mode (*m/z* 150-700). All compounds were dissolved in MeOH : H$_2$O (1:1) and infused in the ESI source by using a syringe pump; the flow rate was 5 µL/min. Chromatography was performed over Sephadex LH-20 (Pharmacia, Uppsala, Sweden) employing MeOH as solvent. Column chromatography was carried out employing Silica gel RP18 (0.040–0.063 mm; Carlo Erba) and MeOH:H$_2$O gradients. HPLC separations were performed on a Waters 590 series pumping system equipped with a Waters R401 refractive index detector and a Kromasil C18 column (250 x 10 mm i.d., 10 µm, Phenomenex). HPLC-grade methanol was purchased from Sigma Aldrich (Milano, Italy). HPLC-grade water (18 mΩ) was prepared by a Milli-Q50 purification system (Millipore Corp., Bedford, MA). TLC analysis was performed with Macherey-Nagel precoated silica gel 60 F$_{254}$ plates. Quantitative HPLC analysis was performed on Agilent 1100 series system consisting of a G-1312 binary pump, a G-1328A Rheodyne injector (20 µL loop), a G-1322A degasser, and a G-1315A photodiode array detector, equipped with a 250 x 4.6 mm C-18 Thermo column.

Plant Material: The leaves of *C. xalapensis* were collected in Lange, Valle, Honduras, in January 2004. The plant was identified by Dr. Cirilo Nelson. A

voucher specimen was deposited in the herbarium of the Botanical Department of the Universidad Nacional Autonoma de Honduras, Tegucigalpa, Honduras (Voucher No. 214)

Extraction and Isolation Procedure of Compounds 1-7: Dried and powdered leaves (500 g) of *C. xalapensis* were extracted for a week, three times, at room temperature using solvents of increasing polarity; namely, petroleum ether, chloroform, and methanol. Part (8 g) of the MeOH extract (220 g) was partitioned between *n*-butanol and water. The H_2O soluble portion was chromatographed on a RP-18 column using MeOH-H_2O (from 60% to 20% of MeOH). The fraction obtained (3 g) was successively purified by RP-HPLC on a 250 mm x 10 mm, Kromasil C18, Phenomenex column at a flow rate of 3.0 mL/min with MeOH-H_2O (20:80) to yield magnoflorine **1** (2.3 g). The *n*-butanol soluble portion (5 g) was chromatographed on a Sephadex LH-20 column (100 cm X 5.0 cm) using CH_3OH as mobile phase with a flow rate of 1 mL/min; 60 fractions collected of 8 mL each and monitored by TLC [Si-gel plates, using solvent system *n*-BuOH-AcOH-H_2O (60:15:25)]. TLC plates were developed using UV 254 nm, 366 nm, Ce(SO_4)/H_2SO_4. Using the above criteria the 60 fractions were combined into 4 major fractions (1-4). Fraction 1 and 2 (2.0 g) were purified by RP-HPLC on a 250 mm x 10 mm, Kromasil C18, Phenomenex column at a flow rate of 3.0 mL/min with MeOH-H_2O (20:80) to yield pure compound 3,4-(*E*)-dimethoxycinnamyl alcohol **2** (18.5 mg) and 3,4,5-trimethoxycinnamic acid **4** (2.4 mg). Fractions 3 and 4 (2.4 g) were purified by RP-HPLC at the same chromatographic conditions described for earlier fractions to yield pure compounds 3,4-dimethoxy-5-hydroxy-(*E*)-cinnamyl alcohol **3** (12.1 mg), gallic acid **5** (23.8 mg), methyl gallate **6** (11.1 mg) and 3,4-dihydroxybenzoic acid **7** (6.9 mg).

Magnoflorine (1)
Yellow amorphous solid.
UV λ_{max} (MeOH): 230.2, 278.6, 310.4 nm.
^{1}H and ^{13}C NMR were similar to literature [13].
ESI-MS *m/z* 342 [M-H][-]; MS/MS *m/z* 297 [(M - H)-45][-].

3,4-dimethoxy-(*E*)-cinnamyl alcohol (2)
Yellow amorphous solid.
^{1}H and ^{13}C NMR were similar to literature [12c].
ESI-MS *m/z* 193 [M-H][-].

3,4-dimethoxy-5-hydroxy-(*E*)-cinnamyl alcohol (3)
Yellow amorphous solid.

UV λ_{max} (MeOH): 280.0, 254.2 nm.
^{1}H NMR (CD$_3$OD): δ 6.66 (1H, d, *J* = 1.5, H-2), 6.51 (1H, d, *J* = 1.5, H-6), 6.50 (1H, d, *J* = 15.6, H-7), 6.27 (1H, dt, *J* = 15.6 and 6.8, H-8), 4.31 (1H, d, *J* = 6.8, H-9), 3.89 (3H, s, 3-OMe), 3.87 (3H, s, 4-OMe).
^{13}C NMR (CD$_3$OD): 130.1 (C-1), 110.9 (C-2), 148.1 (C-3), 141.3 (C-4), 147.8 (C-5), 102.8 (C-6), 131.7 (C-7), 127.5 (C-8), 62.4 (C-9), 56.2 (3-OMe), 56.1 (4-OMe). ESI-MS *m/z* 209 [M-H][-].

Trimethoxycinnamic acid (4)
Yellow amorphous solid.
^{1}H and ^{13}C NMR were similar to literature [12a].
ESI-MS *m/z* 237 [M- H][-].

Gallic Acid (5)
White amorphous solid.
^{1}H and ^{13}C NMR were consistent with the literature [12a].
ES-MS, *m*/ 169 [M-H][-].

Methyl Gallate (6)
White amorphous solid.
^{1}H and ^{13}C NMR were consistent with the literature [12a].
ES-MS, *m/z* 183 [M-H][-]

3,4-Dihydroxy Benzoic Acid (7)
White amorphous solid.
^{1}H and ^{13}C NMR were consistent with the literature [12a].
ES-MS, *m/z* 153 [M-H][-]

C. xalapensis **Infusion preparation:** The aqueous extract of *C. xalapensis* was obtained by pouring 50 mL of boiling distilled water on 1.5 g of dried leaves and steeping it for 10 min; then the infusion was filtered through filter paper and freeze-dried. The yield of the lyophilized aqueous extract was 447 mg (29.8% of dried leaves). The extractions were performed in triplicate.

HPLC Quantitative Analysis: Quantitative HPLC of the *C. xalapensis* infusion was carried out using a isocratic solvent, MeOH-H_2O (20:80). Detection wavelength was 280 and 320 nm, the flow rate of 1.0 mL/min and the injection volume was 20 μL. Magnoflorine was identified by comparing the retention time of the peak in the aqueous extract with that of the standard compound.

Quantification: The lyophilized infusion was diluted a volume of 10 mL in a volumetric flask. Quantification was performed by reporting the

measured integration area in the calibration equation of the corresponding standard of magnoflorine. The linearity of responses for magnoflorine (**1**) was determined on six level of concentration with three injections for each level. The concentration of magnoflorine calculated from the experimental peak areas by interpolation to standard calibration curves was 139.58 mg/g in dry.

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