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Chemical composition and antioxidant activity of chemical constituents from *Croton zehntneri* (Euphorbiaceae)

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Abstract
Croton zehntineri is an aromatic plant native to the northeast of Brazil which is popularly used in folk medicine as a sedative and for the relief of gastrointestinal disturbances. The present work deals with the chromatographic analysis of the ethanolic extract of roots and stem of *C. zehntineri* allowed the isolation and characterization for the first time for this species of seven compounds named *trans*-docosanyl ferulato, acetyl aleuritolic acid, 3-*O*-methylquercetin, *E*-anethole, 2-hydroxy-4,6-dimethoxyacetophenone and a mixture of β-sitosterol and stigmasterol. Structural elucidation was done on the basis of spectral data, mainly by high field NMR and EIMS. The antioxidant activity (DPPH radical scavenging activity) was determined, only compound named 3-*O*-methylquercetin showed antioxidant activity (IC_{50} $2.76.10^{-3} \pm 9.6.10^{-5}$ mg/mL).

Keywords: *Croton zehntineri*, ferulic acid, antioxidant activity, 3-*O*-methylquercetin

Introduction
Croton is an extensive genus comprising around 1,300 species from Euphorbiaceae family. This genus with wide range of bioactive compounds have been found to exert vasorelaxant activity (Baccelli *et al*. (2007) [1]. Popular uses include treatment of cancer, constipation, diabetes, digestive problems, dysentery, external wounds, fever, hypercholesterolemia, hypertension, inflammation, intestinal worms, malaria, pain, ulcers, and weight-loss (Salatino *et al*. (2007) [15].
Previous phytochemical investigations show that this genus possesses alkaloids (Murillo *et al*. (2001) [12], flavonoids (Peres *et al*. (1997) [14]; Maciel *et al*. (2000) [9]; triterpenoids and steroids (Guadarrama *et al*. (2004) [6], and a large number of diterpenoids Giang *et al*. (2004) [5]; Santos *et al*. (2008) [18]; Santos *et al*. (2009) [17].
C. zehntneri (Euphorbiaceae) is an aromatic plant native in northeastern Brazil; and popularly known as "canela de cunhã". The specie is used in traditional medicine as sedative, appetite stimulating, antianorexigen and for the relief of gastrointestinal disturbances and antinociceptive (Oliveira *et al*. (2001) [13]. The essential oil also acts as intestinal muscle relaxant (Coelho-de Souza *et al*. (1998) [3], depressor central effect (Lazarini *et al*. (2000) [8], cholinesterase inhibition (Santos *et al*. 2010) [16], antispasmodic (Magalhães *et al*., 1998) [10] and larvicidal activity (Morais *et al*. (2006) [11]; Santos *et al*. (2007) [19]. The literature reports the isolation of a single compound named crototropone (Bracher *et al*. (2008) [2]. This work we report an evaluation of the antioxidant activity, as well the chemical composition of the ethanolic extract of stem and roots of *C. zehntneri*.

Material e methods
General Experimental Procedures
Optical rotations were measured on a Perkin Elmer 341 polarimeter. IR spectra were recorded using a Perkin Elmer 1000 spectrophotometer. ^{1}H and ^{13}C NMR where recorded on a Bruker Avance DRX-500 (500 MHz for ^{1}H and 125 MHz for ^{13}C); chemical shifts were given in ppm (d_C and d_H), relative to residual $CHCl_3$ (7.24 and 77.0 ppm). Mass spectra were registered using Shimadzu LCMS-IT-TOF and Shimadzu QP5050A mass spectrometers. Silica gel 60 (230-400 mesh, Merck) was used for analytical TLC. Silica gel 60 (70-230 mesh, Merck) was used for column chromatography. All compounds were visualized by TLC using vanillin-perchloric acid-EtOH followed by heating.

Plant Material

The leaves, stem and roots of *C. zehntineri* was collected in March 2006, in Tianguá, Ceará State, Brazil. The plant material was identified by Dr. Edson Paula Nunes at the Herbário Prisco Bezerra (EAC), Departamento de Biologia, Universidade Federal do Ceará, Fortaleza, CE, Brazil, where a voucher specimen (No. 42389).

Extraction and Isolation

The stem (1.5 kg) of *C. zehntineri* was powdered and extracted with ethanol (10 L x 3, at room temperature, for four weeks). The solvent was removed under reduced pressure to give an EtOH extract. The EtOH extract (208.0 g), was fractionated coarsely on a silica gel column by elution with hexane (fractions 1-10), $CHCl_3$ (fractions 11-18), EtOAc (fractions 19-27), and EtOH (fractions 28-40), affording a total of 40 fractions of 100 mL each. The hexane fractions (42.39 g) were pooled and fractionated on a silica gel column using hexane (F'1-2), hexane/EtOAc (9:1 F' 3-4; 8:2 F' 5-9; 6:4 F' 10-14; 4:6 F' 15-17), EtOAc (F' 18-20), and EtOH (F' 21), affording a total of 20 fractions of 50 mL each. Fractions (F' 8-9), obtained with hexane/EtOAc (8:2), yielded compound 1 (90 mg). The fractions (F' 2-4) 3.20 g was fractionated coarsely on a silica gel column by elution with petroleum ether (F" 1-30), petroleum ether/hexane (8:2 F" 31-44; 4:6 F" 45-56), hexane (F" 57-72), hexane/EtOAc (1:1 F" 73-83) and EtOH (F" 84-100) affording a total of 100 fractions of 50 mL each. Fractions (F" 80-83), obtained with hexane/EtOAc (1:1), yielded compound 2 (100 mg). The fractions (F'1-2) 11.6 g was fractionated coarsely on a silica gel column by elution with hexane (F"'1-15), hexane/EtOAc (9:1 F"' 16-25; 8:2 F"' 26-35; 6:4 F"' 36-45; 3:7 (F"' 46-65), EtOAc (F"' 66-75), and EtOH (F"' 76-77), affording a total of 77 fractions of 50 mL each. Fractions (F"' 1-15) obtained with hexane, yielded compounds 3 e 4 (70 mg). The fraction $CHCl_3$ (4.8 g) were pooled and fractionated on a silica gel column using hexane (F'1-60), hexane/EtOAc (9:1 F' 61-95; 8:2 F' 96-102; 7:3 (F' 103-105; 6:4 F' 106-120; 1:1 F' 121-125; 4:6 F' 126-130; 3:7 F' 131-135; 2:8 F' 136-140; 1:9 F' 141-145), EtOAc (F' 146-150), and EtOH (F' 151), affording a total of 151 fractions of 50 mL each. Fractions (F' 82-83), obtained with hexane/EtOAc (9:1), yielded compound 5 (30 mg). The root (3 Kg) was powdered and extracted with ethanol (10 L x 3, at room temperature, for four weeks). The solvent was removed under reduced pressure to give an EtOH extract. The EtOH extract (212. 2g), was fractionated coarsely on a silica gel column by elution with hexane (F 1-10), $CHCl_3$ (F 11-20), EtOAc (F 21-30) e EtOH (F 31-40), affording a total of 40 fractions of 500 mL each. The $CHCl_3$ fractions (161.60 g) were pooled and fractionated on a silica gel column using hexane (F' 1-7), hexane/EtOAc (9:1 F' 8-50; 7:3 F' 51-60; 1:1 F' 61-70; 3:7 F' 71-80), EtOAc (F' 81-20), and EtOH (F' 89-90), affording a total of 90 fractions of 100 mL each. Fractions (F' 1-5), obtained with hexane, yielded compound 6 (40 mg). The EtOAc fractions (21.4 g) were pooled and fractionated on a silica gel column using hexane (F" 1-6), hexane/EtOAc (9:1 F" 7-18; 8:2 F" 19-31; 7:3 F" 32-45; 6:4 F" 46-56; 1:1 F" 57-67; 4:6 F" 68-77; 3:7 F" 78-87; 2:8 F" 88-97; 1:9 F" 98-108), EtOAc (F" 109), and EtOH (F" 110), affording a total of 110 fractions of 50 mL each. Fractions (F" 59-78), obtained with hexane/EtOAc 1:1 and 4:6, yielded compound 7 (40 mg).

Antioxidant activity

The antioxidant activity of compounds was measured in terms of hydrogen-donating or radical scavenging ability, using the stable radical DPPH (Hegazi (2002) [7]. The compounds at various concentrations (0.001, 0.005, 0.01, 0.05, 0.1 and 1.0 mg/mL) were added to a solution of DPPH in methanol. After incubation, the absorbance of each solution was determinate at 520 nm. Percentage inhibition of the DPPH radical was obtained from following equation:

$$\text{DPPH radical inhibition (\%)} = \frac{\text{Control absorbance} - \text{sample absorbance}}{\text{Control absorbance}} \times 100$$

The antioxidant activity of the samples was also expressed as IC_{50} (inhibitory concentration), which was defined as the concentration (in mg/mL) of sample required to inhibit the formation of DPPH radicals by 50%. Trolox (IC_{50} $2.6.10^{-3} \pm 2.3.10^{-4}$ mg/mL) and vitamin C (IC_{50} $4.3.10^{-3} \pm 1.9.10^{-2}$ mg/mL) were used as positive control.

trans-docosanyl ferulato (**1**)**:** white, amorphous powder; melting point 56,9-58,2 °C; IR (KBr) n_{max} 3490, 2918, 1714, 1634, 1599, 1517 e 1471, 1276 e 1175 cm^{-1}; ^1H NMR ($CDCl_3$, 500 MHz), see Table 1; ^{13}C NMR ($CDCl_3$, 125 MHz), see Table 1; EMIE m/z (%) 502 ($[M]^{\bullet+}$), 194 (80), 150 (52), 177 (53).

Results and Discussion

The ferulic acid **1** was isolated as a white solid with the molecular formula, $C_{32}H_{54}O_4$, as deduced by ESIMS of $[M^+]$ at *m/z* (rel. int) 502, in combination with the ^1H and ^{13}C NMR spectra. Its IR spectrum showed bands indicating the presence of ester (n_{max} 1714 and 1276 cm^{-1}), and olefin (n_{max} 1634 cm^{-1}) functionalities. The ^1H NMR spectrum (Table 1) of **1** displayed signals assignable to a feruloyl moiety [d_H 7.62 d (*J* = 15.8 Hz, 1H, H-7), d_H 7.08 dd (*J* = 8.2, 2.0 Hz, 1H, H-6), d_H 7.04 d (*J* = 2.0 Hz, 1H, H-2), d_H 6.92 d (*J* = 8.2 Hz, 1H, H-5), d_H 6.30 d (*J* = 15.8 Hz, 1H, H-7), and 3.94 s (3H, OCH_3-3)]. The coupling constant between H-2 and H-3 (*J* = 15.8 Hz) clearly suggested its *trans*-geometry. The signals for aliphatic methylene groups (d_H 1.40-1.15, 13-30 H), a terminal methyl [d_H 0.89 t (*J* = 6.4 Hz, 3H), carbinyl methylene [d_H 4.20 t (*J* = 6.7 Hz, 2H)] and the neighbouring methylene (d_H 1.71 m) were assignable of a long-chain alcoholic residue. An analysis of the $\{^1H\}$-^{13}C NMR (Table 1) spectrum of **1** with the aid of DEPT 135 and HMQC experiments revealed the signals of 32 carbons: one carbonyl [δ_C 167.8 (ester), two olefinic [δ_C 145.0 (CH), 116.8 (CH)], two methyl [δ_C 56.5 (H_3CO), 14.5 (H_3C), one oxygenated methylene δ_C 65.1 (H_2CO)], as well as of twenty simple methylenes, three hydrogenated aromatic carbons, two oxygenated aromatic carbons, and one non-hydrogenated aromatic carbon.

In the HMBC spectrum of 1, the proton signal at d_H 7.62 (d, 15.8) and 6.30 (d, 15.8) showed long-range ^1H-^{13}C correlations with the carbonyl group at δ_C 167.8 (C-9) and non-hydrogenated carbon at δ_C 127.5 (C-1). Additionally, the resonance at d_H 3.94, as well as, at d_H 7.04 and 7.08 exhibited long-range ^1H-^{13}C correlation with the carbons at δ_C 147.1 (C-3) and 148.3 (C-4), respectively. These observations permitted the methoxyl and hydroxyl groups to be placed at C-3 and C-4. Thus, the chemical structure of compund 1 was assigned as *trans*-docosanyl ferulato, which is being reported for the first time in the genus *Croton*. Structural elucidation of the compounds *E*-anethole (2) (Santos *et al*. (2007) [30], β-sitosterol (3) and stigmasterol (4) (Santos *et al*. (2008) [29], acetyl aleuritolic acid (5) (Salatino *et al*. (2007) [26], 2-hydroxy-4,6-dimethoxyacetophenone (6) (Santos *et al*. (2008) [29], and 3-*O*-methylquercetin (7) (Silva *et al*. (2003) [34] was done on the basis of spectral data, mainly by high field NMR

and EIMS, and by comparison with their published data. All these compounds are reported for the first time in this species. The antioxidant activity (DPPH radical scavenging activity) was determined and only compound named 3-*O*-methylquercetin showed antioxidant activity (IC_{50} $2.76.10^{-3}$ ± $9.6.10^{-5}$ mg/mL).

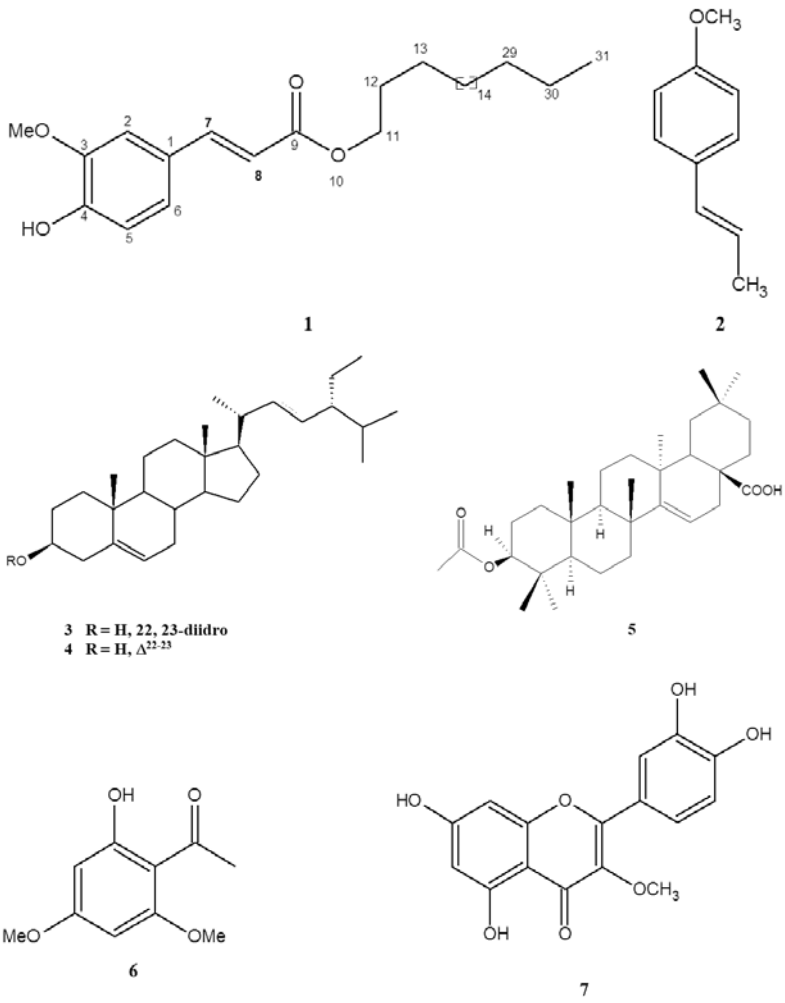

Fig 1: Compounds isolated from *C. zehntneri*.

Table 1: NMR Spectroscopic Data for 1 (^{1}H: 500 MHz; ^{13}C: 125 MHz; in $CDCl_3$) [a]

	1			
Positions.	**δ_C**	**δ_H (*J*)**	**$^2J_{CH}$**	**$^3J_{CH}$**
1	127.48		H-7	H-8
2	109.69	7.04		H-6; H-7
3	147.15		H-2	H-5; MeO
4	148.29			H-2; H-6
5	115.10	6.92 (d, 8.2)		
6	123.45	7.08 (dd, 8.2)		H-2; H-7
7	145.03	7.62 (d, 15.8)		H-2; H-6
8	116.10	6.30 (d, 15.8)	H-7	
9	167.81		H-8	H-7; 2H-11
11	65.10	4.20 (t)		
12	29.12	1.71 (m)		
13	26.41	1.40-1.15 (m)	2H-12	2H-11
14-28	30.11-29.72	1.40-1.15 (m)		
29	32.33	1.40-1.15 (m)		3H-11
30	23.10	1.40-1.15 (m)	3H-11	
31	14.52	0.89 (t, 6.4)		
MeO	56.52	3.94 (s)		

[a] Number of hydrogens bound to each carbon atom was deduced by comparative analysis of {^{1}H}- and DEPT-^{13}C NMR spectra. Chemical shifts (d values) and coupling constants [*J* (Hz), in parentheses)] was obtained from 1D ^{1}H NMR spectrum.

Conclusions

The analysis of the ethanolic extract of stem and roots of *C. zehntineri* allowed the isolation and characterization of seven compounds, of which *trans*-docosanyl ferulato and 3-*O*-methylquercetin are being reported for the first time in the genus *Croton*, while the other compounds are being reported for the first time in this species. Regarding the potential antioxidant only compound 3-*O*-methylquercetin showed activity. These results are very important since it confirms the chemotaxonomic profile of this genus.

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