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Isolation and antioxidant activity of chemical constituents from *Vitex gardneriana* Schauer

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Abstract
Vitex gardneriana Schauer (Verbenaceae) popularly known as "jaramataia", is a shrub commonly found in caatinga biome located in Northeast Brazil. In folk medicine, its leaves have been used as analgesic and anti-inflammatory agents. Chromatographic analysis of leaf extracts, stem bark, wood and roots of V. gardneriana allowed isolation of the mixture ursolic and oleanolic acids (1 and 2), 4-hydroxy-3-methoxybenzoic (3) and 4-hydroxybenzoic acid (4) and four more compounds: 4-carboxyphenyl 4-hydroxy-3-methoxybenzoate (5), acid 2α, 3α, 19α-trihydroxyursan-12-en-oic (6), 3,4-dihydroxybenzoic acid (7) and 5-Hydroxy-3,7,4'-trimethoxyflavone (8) all compounds are being reported for the first time in the genus *Vitex*. Structural elucidation was done on the basis of spectral data, mainly by high field NMR. For the antioxidant assays, 4-carboxyphenyl 4-hydroxy-3-methoxybenzoate was able to reduce DPPH free radicals, as well as inhibit ß-carotene oxidation on a level of 95 and 50%, respectively, demonstrating high and moderate antioxidant ability when compared with controls quercetinand BHA.

Keywords: *Vitex gardneriana*, secondary metabolites, antioxidant activity

Introduction
The use of plants for therapeutic purposes is a millenary tradition present in the cultures of several nations and is still an alternative resource of great acceptance, not only in urban centers, but especially in small rural communities. This behavior has been drawing the attention of the scientific community to prove its efficacy and promote the safe use of these natural resources (Silva *et al*., 2016) [1].

The genus *Vitex* is the largest in the family *Verbenaceae* which comprises 250 species distributed all over the world. It consists of shrubs and trees that are distributed in the tropics and subtropics. The civilization has used *Vitex* plants for treating many health problems such as malaria, herpes, itching, dermatitis, and controlling menstruation (Sharma and Rani, 2013) [2]. A phytochemical study of the genus *Vitex* revealed the presence of diterpenoids (Kiuchi *et al*., 2004; Ono *et al*., 2002, 2000) [3, 4, 5], alkaloids (Suksamrarn *et al*., 2000) [6], triterpenoids (Sridhar *et al*., 2005) [7], flavonoids (Sathiamoorthy *et al*., 2007; Azhar-UL *et al*., 2004) [8, 9] and many others. Some of them exhibited pharmacological activities such as anticancer, inhibitory α-glucosidase, and hepatoprotective (Ban *et al*., 2017) [10].

Vitex gardneriana (Verbenaceae) popularly known as "jaramataia", is a shrub commonly found in caatinga biome located in Northeast Brazil. In folk medicine, its leaves have been used as analgesic and anti-inflammatory agents (Sá-Barreto *et al*., 2008, 2005) [11, 12]. This work is a contribution in the description of the chemical composition of leaves, bark of trunk, wood and roots of *V. gardneriana* (Figure 1).

Fig 1: *Vitex gardneriana*

Material e methods

General Experimental Procedures

^{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 (^{13}C and ^{1}H). Silica gel 60 (230-400 mesh, Merck) was used for analytical TLC. Silica gel 60 (70-230 mesh, Merck) was used for column chromatography. TLC using vanillin-perchloric acid-EtOH followed by heating visualized all compounds.

Plant material

Vitex gardneriana were collected in April 2014 at the experimental farm of the Acaraú Valley State University (03º 36' 44" S 40º 18' 37" W), located 11 km from the city of Sobral, Ceará, Brazil. The plant authentication was performed by Professor Elnatan Bezerra de Souza (a plant taxonomist) and a voucher specimen (N° 17, 703) has been deposited at the herbarium Francisco José de Abreu Matos (Sobral, Brazil).

Extraction and Isolation

Stem bark (900 g) of *V. gardneriana* was powdered and extracted with ethanol (10 L x 3, at room temperature). The solvent was removed under reduced pressure to give an EtOH extract. The EtOH extract (79.4 g), was fractionated coarsely on a silica gel column by elution with hexane, $CHCl_3$, EtOAc and EtOH. The EtOAc fractions (18.2 g) were pooled and fractionated on a silica gel column using hexane, hexane/$CHCl_3$, $CHCl_3$, $CHCl_3$/EtOAc, EtOAc, EtOAc/MeOH and MeOH. Fractions (F' 150-171; 1.5 g) obtained with $CHCl_3$/EtOAc (1:1) was fractionated coarsely on a silica gel column by elution with $CHCl_3$, $CHCl_3$/EtOAc, EtOAc, EtOAc/MeOH and MeOH. Fractions (F" 60-110; 5.7 g) obtained with $CHCl_3$/EtOAc (1:1) was fractionated coarsely on a silica gel column by elution with $CHCl_3$, $CHCl_3$/EtOAc, EtOAc, EtOAc/MeOH and MeOH, affording a total of 384 fractions. Fractions (F"' 88-90) obtained with EtOAc/MeOH (2:8) yielded a mixture of ursolic (1) and oleanolic (2) acids (56, 3 mg). Wood (2.3 kg) of *V. gardneriana* was powdered and extracted with ethanol (10 L x 3, at room temperature). The solvent was removed under reduced pressure to give an EtOH extract. The EtOH extract (73.5 g), was fractionated coarsely on a silica gel column by elution with hexane, $CHCl_3$, EtOAc and EtOH. $CHCl_3$ fractions (3.7 g) were pooled and fractionated on a silica gel column using hexane, hexane/$CHCl_3$, $CHCl_3$, $CHCl_3$/EtOAc, EtOAc, EtOAc/MeOH and MeOH, affording a total of 131 fractions. Fractions (F' 116-118) obtained with $CHCl_3$/EtOAc (9:1) yielded the compound 4-hydroxy-3-methoxybenzoic acid (3) (116.4 mg). EtOAc fractions (20.2 g) were pooled and fractionated on a silica gel column using $CHCl_3$, $CHCl_3$/EtOAc, EtOAc, EtOAc/MeOH and MeOH, affording a total of 315 fractions. Fractions (F" 131-141), (F" 142-155) and (F" 156-166) obtained with EtOAc/MeOH (3:7 and 2:8) yielded the compounds 4-hydroxybenzoic acid (4) (29.4 mg), 4-carboxyphenyl 4-hydroxy-3-methoxybenzoate (5**)** (75.8 mg) and acid 2α, 3α, 19α-trihydroxyursan-12-en-oic (6) (38.7 mg), respectively. Roots (1.27 kg) of *V. gardneriana* were powdered and extracted with ethanol (10 L x 3, at room temperature). The solvent was removed under reduced pressure to give an EtOH extract. The EtOH extract (56.4 g), was fractionated coarsely on a silica gel column by elution with hexane, $CHCl_3$, EtOAc and EtOH. The EtOAc fractions (18.2 g) were pooled and fractionated on a silica gel column using hexane, hexane/$CHCl_3$, $CHCl_3$, $CHCl_3$/EtOAc, EtOAc, EtOAc/MeOH and MeOH. EtOAc fractions (5.0 g) were pooled and fractionated on a silica gel column using hexane, hexane/ $CHCl_3$, $CHCl_3$, $CHCl_3$/EtOAc, EtOAc, EtOAc/MeOH and MeOH, affording a total of 305 fractions. Fractions (F' 57-77) and (F' 131-149) obtained with $CHCl_3$/EtOAc (7:3 and 4:6) yielded the compounds 3, 4-dihydroxybenzoic acid (7) (30.1 mg). Leaves (5.6 kg) of *V. gardneriana* were powdered and extracted with methanol (10 L x 3, at room temperature). The solvent was removed under reduced pressure to give an MeOH extract. The MeOH extract (100 g), was fractionated coarsely on a silica gel column by elution with hexane, $CHCl_3$, EtOAc and MeOH. The EtOAc fractions (14.8 g) were pooled and fractionated on a silica gel column using hexane and hexane/EtOAc affording a total of 295 fractions. Fractions (F' 80-87) obtained with hexane/EtOAc (8:2) yielded the compound 5-hydroxy-3, 7, 4'-trimethoxyflavone (8) (48.8 mg).

Antioxidant Activity

Sequestration of the 2, 2-diphenyl-1-picrylhydrazyl (DPPH)

The ability of compounds tested in sequestering DPPH radicals were determined according to the method described by Duan *et al*. (2006) [13]. A methanolic solution containing 0.16 mM DPPH was added to compounds tested at concentrations ranging from 500 to 7.8 µg/mL. The data obtained were compared to blank solution (a mixture of compounds and methanol) and control of the assay (0.16 mM DPPH solution). The samples were incubated in the absence of light at 25 °C for 30 minutes and the optical density was measured at 517 nm by a microplate reader (Biochrom Asys UVM 340). Quercetin was used as a positive control. The percentage of DPPH radical sequestration was determined by the following equation:

$$\text{Scavenging effect (\%)} = \left[1 - \frac{\left(\text{Abs}_{\text{sample}} - \text{Abs}_{\text{sample blank}}\right)}{\text{Abs}_{\text{control}}}\right] \times 100\%$$

Ferrous ion chelating assay (FIC)

The FIC assay was performed according to Wang *et al*. (2009) [14]. Compounds tested were suspended in deionized water to obtain concentrations ranging from 500 to 7.8 µg/mL. In addition, 2 mM ferrous chloride ($FeCl_2$) and 5 mM ferrozine were also added to the suspension. Both, blank and control were performed with ferrozine solution plus compounds tested and ferrous chloride solution, respectively. Samples were incubated at 25 °C for 10 minutes and the optical density was measured at 562 nm using a microplate reader (Biochrom Asys UVM 340). EDTA (ethylenediaminetetraacetic acid) was used as positive control. The FIC activity was calculated by the following equation:

$$\textit{Ferrous ion chelating activity (\%)} = \frac{\left[Abs_{\,control} - \left(Abs_{\,sample} - Abs_{\,sample\ blank}\right)\right]}{Abs_{\,control}} \times 100\%$$

Iron Reduction Method

The FRAP assay was performed according to methodology described by Ganesan *et al*. (2008) [15]. Initially, compounds tested were diluted in 0.2 mol/L sodium phosphate buffer (pH

6.6) with 1% potassium ferricyanide at concentrations ranging from 500 to 7.8 µg/mL. Samples were incubated at 50 °C for 20 minutes. After cooling, 10% trichloroacetic acid was added. An aliquot was mixed with 0.1% ferric chloride in distilled water. After 10 minutes, the optical density was measured at 700 nm in the microplate reader (Biochrom Asys UVM 340). Butylhydroxyanisole (BHA) was used as a positive control.

Method of inhibition of β-carotene oxidation

The ability of compounds tested in inhibiting the oxidation of β-carotene and linoleic acid was determined by the method described by Dhongade and Chandewar (2013) [16] with some modification. The solution for this assay had 3 mL of 0.1 mg/mL of β-carotene dissolved in chloroform, 40 mg of linoleic acid and 400 mg of Tween 40. The chloroform was evaporated using a rotary evaporator and then ultra-pure water was added to the solution. Compounds tested was tested in concentrations ranging from 500 to 7.8 µg/mL. At the first moment, the optical density of the solutions measured at 470 nm (Biochrom Asys UVM 340 microplate reader) and again after 3 hours of incubation at 50 °C. BHA was used as a positive control for this assay. The antioxidant activity was calculated by the following equation:

$$\text{Antioxidant activity } (\%) = \left(\frac{\text{Abs}_{3h}}{\text{Abs}_{initial}} \right) \times 100\%$$

Statistical analysis

All tests were performed in triplicates and with significance level $p<0.05$. For the antimicrobial assays the difference between the means of the triplicates was verified by one-way ANOVA test with Bonferroni post-test, executed by GraphPad Prism version 5.0 for Windows (San Diego, California, USA). For antioxidant assays, percentiles obtained in all concentrations tested were converted to absolute values, submitted to angular transformation and compared through Student's t test for independent values.

Results and Discussion

4-Carboxyphenyl4-hydroxy-3-methoxybenzoate (5) was isolated as a white solid. ^{1}H-NMR data showed signs δ_H 7.87 and 6.82 (2H, d, J = 8.8 Hz, H-2'/H-6', H-3'/H-5'), confirms the presence of *p*-substituted aromatic ring. Signals were recorded in δ_H 6.3 (2H, d, J = 8.7 Hz) and 3.88 (3H, s, OCH_3) attributed to respective aromatic end methoxyl hydrogen. An analysis of the {^{1}H}-^{13}CNMR with the aid of DEPT 135 and HMQC experiments revealed the signals of 15 carbons, one carbonyl δ_C 178.2 (ester), one carbonyl δ_C 170.2 (acid), seven carbons aromatics δ_C 113.9, 115.9, 125.4 (CH), 116.2, (2CH), 133,1 (2CH), five carbons aromatics δ_C 122.8, 123.2 (C) not oxygenated, 148.8, 152.8 163.4 (C) oxygenated, oxygenated methoxyl carbon δ_C 56.5 (OCH_3). These data allowed to identify the presence of two aromatic rings in the formation of the molecule. In the HMBC spectrum the signal protons δ_H 7.87 showed long-range ^{1}H-^{13}C correlations with the carbonyl group at δ_C 170.2 (C-7') and carbon aromatic oxygenated at δ_C 163. 4 (C-4'). Additionally, the resonance at δ_H 3.88, as well as, δ_H 6.83 exhibited long-range 1H-^{13}C correlation with the carbons at δ_C 148.8 (C-3), respectively. These observations permitted the methoxyl and hydroxyl groups to be placed at C-3 and C-4 (Table 1).

Table 1: NMR Spectroscopic Data for 5 (^{1}H: 500 MHz; ^{13}C: 125 MHz; in $CDCl_3$) [a]

	HSQC		HMBC	
	δ_C	δ_H	$^2J_{CH}$	$^3J_{CH}$
C				
1	123.21			H-5
3	148.79		H-2	H-5; MeO-3
4	152.79		H-5	H-2
7	170.18			H-2; H-6
1'	122.86			H-3'/H-5'
4'	163.47		H-3'/H-5'	H-2'/H-6'
7'	170.22			H-2'/H-6'
CH				
2	113.99	7.55 (d)		H-6
5	115.98	6.83 (d *J*=8.7 Hz)		
6	125.43	7.57 (dd)		H-2
2'/6'	116.18	7.87 (d, *J*=8.8Hz)		
3'/5'	133.14	6.82 (d, *J*=8.8Hz)		
CH_3				
MeO-3	56.55	3.88 (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 (δ values) and coupling constants [*J* (Hz), in parentheses)] was obtained from 1D ^{1}H NMR spectrum.

Acid 2α, 3α, 19α-trihydroxyursan-12-en-oic (6) was isolated as a white solid. ^{1}H-NMR data showed a group of signs in δ_H 0.76 - 2.50 ppm of methyl and methylene characteristic of triterpenes. The signal in δ_H 2.50 (1H, s) described to H-18 justifies substituent presence in C-19. The value δ_C 73.7 ppm suggests the presence of oxygen attached to C-19, confirming the triterpene of the ursan class. Signals were reported for six methyls, with a doublet in δ_H 0.99 ppm (3H-30) and an olefin hydrogen in δ_H 5.25 ppm. Signals in δ_H 3.83 (m, H-2) and 3.30 (H-3) suggest the presence of two hydroxyl groups on ring A. An analysis of the {^{1}H}-^{13}CNMR reveled the signals of 30 carbons consistent with a triterpenoid structure. Signals were recorded for seven methyl groups and signals referring to olefinic carbons, two signals for methinic carbons and one signal for oxygenated quaternary carbon. The position of the signal for the quaternary C-17 in δ_C 47.23 that is superimposed on the signal of the solvent methanol was elucidated through the spectrum of the acetylated compound in chloroform. The presence of a signal relative to carboxylic carbon in δ_C 182.4 (C-28) (Table 2).

Table 2: NMR Spectroscopic Data for 6 (^{1}H: 500 MHz; ^{13}C: 125 MHz; in CD_3OD) [a]

	HMQC		HMBC	
	δ_C	δ_H	$^{2}J_{CH}$	$^{3}J_{CH}$
C				
4	39.54		3H-23; 3H-24	
8	41.43		3H-26	3H-27
10	39.62		3H-25	
13	140.23			3H-27
14	42.91		3H-27	3H-26
17	47.23			
19	73.76		3H-29	3H-30
28	182.43			
CH				
2	67.34	3.93 (m)		
3	80.28	3.30		3H-23; 3H-24
5	49.49	1.20		3H-23; 3H-24; 3H-25
9	48.38	1.70		3H-25; 3H-26
12	129.53	5.25 (br, s)		
18	55.25	2.50 (s)		3H-29
19				
20	43.25	1.35	3H-30	3H-29
CH_2				
1	42.66	1.58, 1.30		
2				
6	19.44			
7	34.23	1.50, 1.30		3H-26
11	24.87	1.98		
15	29.74			3H-27
16	26.77			
19				
21	27.46			3H-30
22	39.17			
CH_3				
23	29.39	0.99 (s)		3H-24
24	22.61	0.87 (s)		3H-23
25	17.04	0.99 (s)		
26	17.70	0.76 (s)		
27	27.23	1.20 (s)		
29	25.31	1.35 (s)		
30	16.76	0.93 (*J*, 6.7 Hz)		

[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 (δ values) and coupling constants [*J* (Hz), in parentheses)] was obtained from 1D ^{1}H NMR spectrum.

Structural elucidation of the compounds ursolic and oleanolic acids (1**)** and (2) (Mahato and Kundu, 1994) [17], 4-hydroxy-3-methoxybenzoic acid (3) (Canuto *et al*., 2010) [18], 4-hydroxybenzoic acid (4) (Pouchert and Behnke, 1993) [19], 4-carboxyphenyl 4-hydroxy-3-methoxybenzoate (5), acid 2α, 3α, 19α-trihydroxyursan-12-en-oic (6), 3, 4-dihydroxybenzoic acid (7) (Pouchert and Behnke, 1993) [19] and 5-hydroxy-3, 7, 4'-trimethoxyflavone (8) (Maria *et al*., 1997) [20] and was done on the basis of spectral data, mainly by high field NMR and by comparison with their published data. All compounds are being reported for the first time in the genus *Vitex* (Figure 2).

The literature has been describing the antioxidant properties of extracts, fractions and secondary metabolites of plant origin (Santos *et al*., 2017; Aderogba *et al*., 2012; Gouveia *et al*., 2011) [21, 22, 23]. For the antioxidant assays, 4-carboxyphenyl 4-hydroxy-3-methoxybenzoate (5) was able to reduce DPPH free radicals, as well as inhibit ß-carotene oxidation on a level of 95 and 50%, respectively, demonstrating high and moderate antioxidant ability when compared with controls quercetinand BHA (Figure 3).

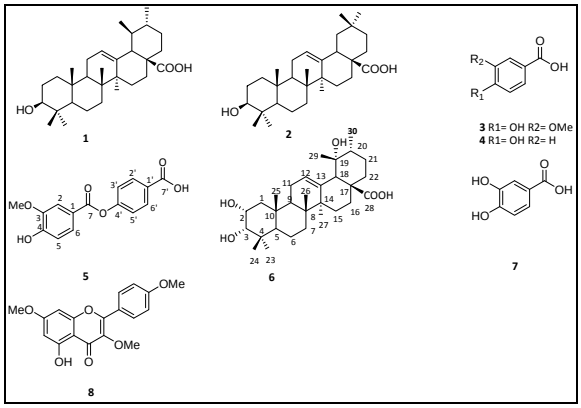
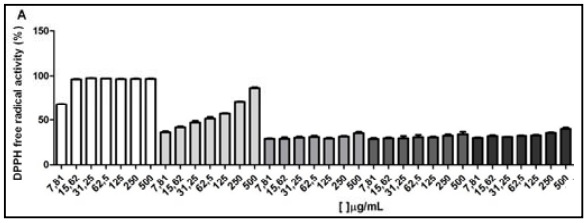


Fig 2: Compounds isolated from *V. gardneriana*.



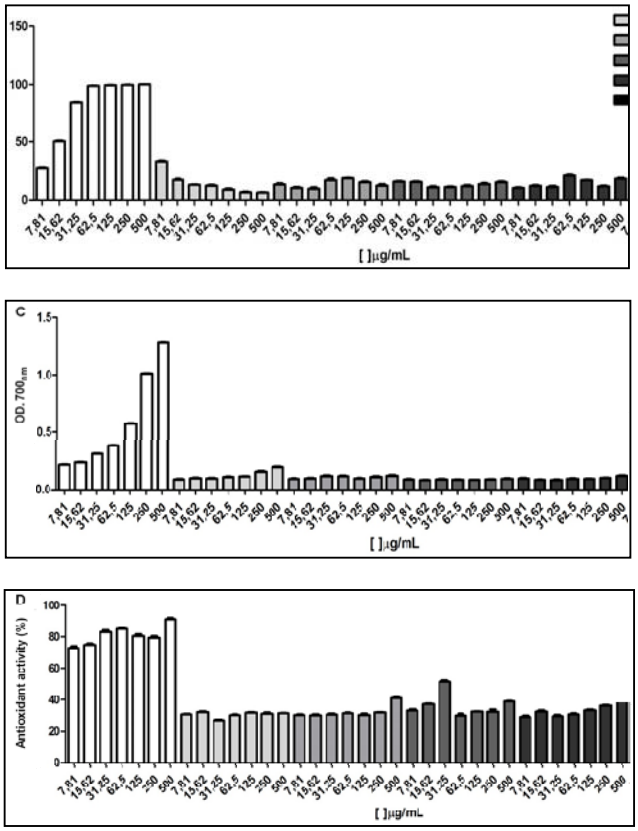


Fig 3: Antioxidant activity of compounds by DPPH a; FIC b, FRAP c and inhibition of ß-carotene oxidation assay d. Compouds 5(▭), 6 (▭), 1 and 2 (▭), 3 (▭); positive control (▭). Quercetin was used as positive control for DPPH assay; EDTA was used as positive control for FIC assay and BHA was used as positive control for FRAP and BCB assays.

Conclusions

The analysis of the extracts of leaves, stem bark, wood and roots of *V. gardneriana* allowed the isolation of the mixtures ursolic and oleanolic acids, 4-hydroxy-3-methoxybenzoic and 4-hydroxybenzoic acid and four more compounds: 4-carboxyphenyl 4-hydroxy-3-methoxybenzoate, acid 2α, 3α, 19α-trihydroxyursan-12-en-oic, 3,4-dihydroxybenzoic acid and 5-hydroxy-3,7,4'-trimethoxyflavone all compounds are being reported for the first time in the genus *Vitex*. These results are very important since it confirms the chemotaxonomic profile of this genus. For the antioxidant assays, 4-carboxyphenyl 4-hydroxy-3-methoxybenzoate was able to reduce DPPH free radicals, as well as inhibit ß-carotene oxidation on a level of 95 and 50%, respectively, demonstrating high and moderate antioxidant ability when compared with controls quercetinand BHA

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