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Chemical constituents isolated from extracts of *Annona vepretorum* Mart. (Annonaceae) leaves

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Annona vepretorum **Mart. is a medicinal plant endemic to Brazil, popularly known as "araticum", "bruteira" and "pinha da Caatinga". In this study, the chemical composition of different leaf extracts obtained from this species was evaluated. Chemical compounds were isolated by silica gel column chromatography, resulting in a mixture of steroids (β-sitosterol and stigmasterol) and a triterpene (lupeol acetate). Ethanol extract presented a precipitate insoluble in chloroform, which after washing was identified as the flavonoid rutin (quercetin-3-*O*-α-*L*-rhamnopyranosyl-(1'''→6'')-β-glucopyranoside). These compounds are being reported for the first time in *A. vepretorum*.**

Key words: *Annona vepretorum*, leaf extracts, phytochemical investigation, ^{1}H and ^{13}C NMR.

INTRODUCTION

The Anonnaceae family is composed of 135 genera and about 2500 species (Chatrou et al., 2004), distributed mainly in tropical regions. Among these genera, 34 can be found in South America. *Annona* L., *Duguetia* St. Hil., *Guatteria* Ruiz et Pavon, and *Xylopia* L. are the predominant genera of Annonaceae (Fechine et al., 2002).

Annona L. comprises 114 species, 110 Neotropical and 4 African species (Costa et al., 2011). In Brazil, there are 82 species, where 24 of them are endemic and distributed mainly in the Amazon, Caatinga, Cerrado, Atlantic Forest and Pantanal biomes (Maas et al., 2017). Some phytochemical studies with *Annona* species reported the isolation of alkaloids, acetogenins, flavonoids, terpenoids, steroids and lignoids. These compounds presented important biological activities, such as cytotoxic, antitumor, pesticide, vermicide, antimicrobial, immunosuppressive, anti-emetic and

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antimalarial (Dutra et al., 2012; Costa et al., 2011; Cruz et al., 2011; Santos et al., 2007; Di Stasi and Hiruma-Lima, 2002).

Annona vepretorum Mart. is a tree popularly known as "araticum", “bruteira”, and “pinha da Caatinga”, predominantly tropical, endemic to Brazil and distributed in the Caatinga biome (Maas et al., 2017; Dutra et al., 2014; Santos et al., 2012; Costa et al., 2011). *A. vepretorum* is widely used in human nutrition (Costa et al., 2012). Phytochemical studies have shown spathulenol as the major component of its essential oil (Araújo et al., 2015). Seven diterpenes, two sesquiterpenes and three steroids were reported in the stem bark of *A. vepretorum* (Dutra et al., 2014). Other studies described the isolation and identification of six alkaloids from the leaves of *A. vepretorum* (Teles et al., 2015).

Previous reports showed that the crude ethanol extract and fractions (hexane and chloroform) from the leaves of *A. vepretorum* exhibited cytotoxic activity against human ovarian, human colorectal and human glioblastoma tumor cell lines. These samples also exhibited strong antibacterial activity against *Escherichia coli* (Almeida et al., 2014). Studies performed by our research group have shown that the crude ethanol extract from the leaves of *A. vepretorum* had sedative activity without affecting the motor coordination in mice. In addition, significant antinociceptive and anti-inflammatory properties were demonstrated and other results suggested that the extract may be useful in the orofacial pain treatment (Diniz et al., 2013; Silva et al., 2015; Silva et al., 2016).

In this paper, we described for the first time the isolation and characterization of four chemical constituents obtained from the ethanol extract of *A. vepretorum* leaves.

MATERIALS AND METHODS

General experimental procedures

^{1}H and ^{13}C NMR spectra were obtained on a Bruker NMR spectrometer (DRX 500), operating at a frequency of 200 MHz for ^{1}H and 50 MHz for ^{13}C. The samples were prepared in deuterated solvents CIL (Cambridge Isotopes Laboratories) ($CDCl_3$ and CD_3OD). Chemical shifts (δ) were referenced to the ^{1}H-NMR peaks characteristic of protons belonging to the non-deuterated solvents in relation to TMS: Chloroform (δ_H = 7.24, δ_C = 77.0) and CD_3OD (δ_H = 4.84 and 3.30, δ_C = 49.0). For adsorption column chromatography (CC), it was used silica gel 60 (70-230 mesh, ASTM), with 0.063 to 0.200 mm particles (Merck®). For thin layer chromatography (TLC), silica gel 60 PF_{254} was used (Merck®). Fractions were monitored by analytical thin layer chromatography (TLC - Aluminum F_{254}), determining the purity of the sample when a single spot was observed after staining under UV irradiation chamber (254 and 365 nm), eluted with at least three solvent systems.

Plant material

Leaves of *A. vepretorum* were collected in December 2010 and January 2012, in Jaguarari-BA and Petrolina-PE, respectively. The plant material was identified by the botanist José Alves de Siqueira Filho. The material of the first collection was compared to the voucher specimen #946 and the voucher specimen of the species of the second collection was deposited in the Herbarium of San Francisco Valley (HVASF) at the Federal University of San Francisco Valley (UNIVASF) under number #18350.

Extraction and isolation

The plant material was dried in an oven with circulating air at an average temperature of 40°C for 72 h, obtaining the dried and pulverized plant material for the first (1400 g) and the second sample (431 g). The dried and powdered plant material was submitted to exhaustive maceration with 95% ethanol. The extractive obtained solution was concentrated on a rotatory evaporator (50°C) to give the crude ethanol extract of the first sample (Av-EEB1, 600 g) and ethanol extract of the second collection (Av-EEB2, 135 g). The material was partitioned to isolate the chemical constituents.

Av-EEB1 was solubilized in a mixture of MeOH:H_2O (3:7) and then fractionated with hexane and chloroform solvents in ascending order of polarity (Oliveira et al., 2010), yielding two fractions: Hexane (Av-Hex1) and chloroform (Av-$CHCl_3$1).

Av-EEB2 had a precipitate which was washed with chloroform to give a yellow amorphous powder, soluble in methanol, identified as compound **1** (36.0 mg).

Av-$CHCl_3$1 fraction (4.0 g) was subjected to column chromatographic using silica gel 60 as stationary phase and hexane, chloroform and methanol as eluents, alone or in binary solvent mixtures, in an ascending concentration gradient. Fraction 68 was washed with methanol to achieve a soluble phase (supernatant) and an insoluble phase (precipitate). The precipitate, white in color, was subsequently identified as a mixture of two substances, **2** and **3** (12.3 mg).

Fraction 110 to 133 was purified by preparative thin layer chromatography (TLC preparative), using a mixture of hexane/chloroform (50:50) as eluent, and the procedure was performed twice, in succession, resulting in the isolation of compound **4** (61.3 mg).

RESULTS AND DISCUSSION

Av-EEB2 presented a yellow precipitate, which was washed with chloroform and identified as the flavonoid rutin (**1**). The chloroform fraction (Av-$CHCl_3$1) was subjected to classic chromatography, obtaining three substances, β-sitosterol (**2**), stigmasterol (**3**) and lupeol acetate (**4**). The chemical structures of the compounds are shown in Figure 1.

Rutin (quercetin-3-*O*-α-*L*-rhamnopyranosil-(1'''→6'')-β-glucopyranoside) **(1)** was obtained as a yellow amorphous powder, which presented a melting point between 177 and 179°C as well as the R_f value of 0.76 (acetone/acetic acid 10% - 5:1). The compound also revealed a yellow color when analyzed with polyethylene glycol diphenylborinate amino-2-ethyl reagent (NEU-PEG) in TLC plates. Such result suggests a positive reaction to flavonoids. The ^{13}C-NMR spectrum showed the presence of 27 signals, of which 15 belong to the aglycon unit. Among them, the signal at δ_C 179.5 that corresponds to the carbonyl carbon C-4 was highlighted.

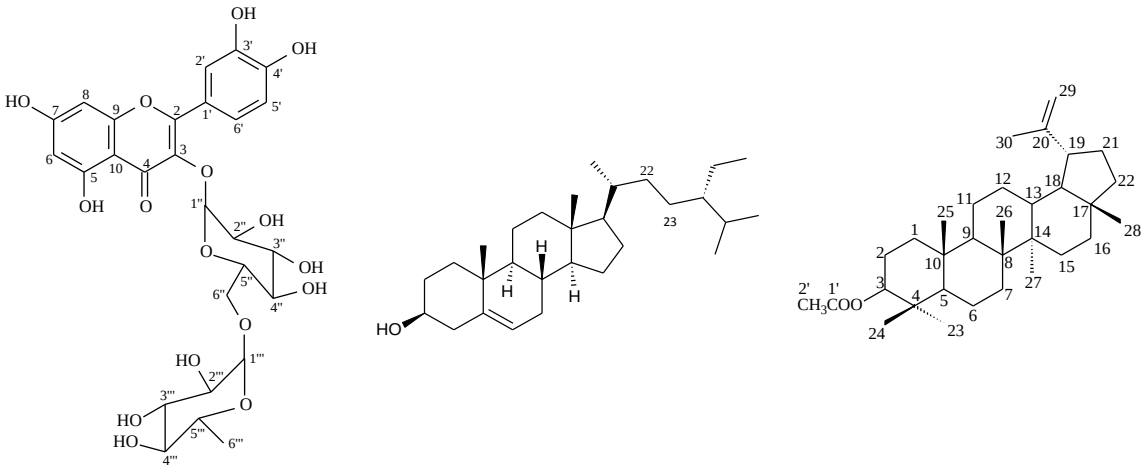

Figure 1. Chemical constituents isolated from the leaves of *A. vepretorum*.

DEPT 135 technique revealed 17 signals, including fifteen signals corresponding to CH carbons. In addition, characteristic signals of anomeric carbons for two glycosidic units at δ_C 105.8 and 104.9 and a methyl carbon signal at δ_C 18.0 (C-6''') was observed which corroborates the presence of a rhamnose glycoside unit. The signal at δ_C 68.7, a methylene carbon, indicated the presence of a glucose unit. The other signals, in the range of δ_C 69.7 to 78.1, were attributed to glycoside units. The ^{1}H-NMR spectrum showed signals of aromatic hydrogens at δ_H 7.61 (d, *J* = 2.1 Hz, H-2'), 7.66 (dd, *J* = 2.1 and 8.4 Hz, H-6'), 6.87 (d, *J* = 8.4 Hz, H-5 '), 6.38 (d, *J* = 2.0 Hz, H-8) and 6.19 (d, *J* = 2.0 Hz, H-6). In addition, the hydrogen spectrum revealed the presence of signals related to two glycosidic units with multiple signals that were verified in the range of δ_H 3.29 to 3.81. Signals for two anomeric protons in δ_H 5.10 (d, *J* = 7.4 Hz, H-1'') and 4.52 (d, *J* = 1.3 Hz, H-1''') also were observed. According to these results, the first unit has been identified as glucose and the second one as rhamnose. The observed hydrogen correlations provided by the HOMO COSY spectrum confirmed the couplings between the signals at δ_H 6.87 (H-5') and δ_H 7.66 (H-6'). In the HMQC spectrum, it was observed a correlation between δ_H 5.10 (H-1'') and δ_C 135.8 (C-3), δ_H 4.52 (H-1''') and δ_C 68.7 (C-6''), confirming the position of the glycosidic groups in the structure (Table 1). From the obtained data and subsequent comparison with literature's information (Vandresen et al., 2010), the compound **1** was identified as rutin.

In studies conducted by Afanas'ev et al (1989), it was observed the effect of rutin on the smooth muscle without promoting potential toxicity. Several other routine activities have been also elucidated, such as the effectiveness of rutin in treating arthritis and anti-candida activity (Han, 2009), antihyperlipidemic activity (Santos et al., 1999), anticonvulsive (Nassiri-Asl et al., 2008), and anti-inflammatory effects (Guardia et al., 2001).

The mixture of β-sitosterol (**2**) and stigmasterol (**3**) was obtained as white crystals soluble in chloroform. The ^{13}C-DEPTQ NMR spectrum showed the presence of three non-hydrogenated carbons (δ_C 140.7, 36.5, 42.2), fourteen methine carbons, twelve methylene carbons and nine methyl carbons. Among them, the presence of the oximethinic carbon at δ_C 71.8 related to C-3 and four olefinic carbons were highlight, of which two are common for both compounds: δ_C 140.7 and δ_C 121.7 (corresponding to C-5 and C-6, respectively). Besides, two olefinic signals at δ_C 138.2 (C-23) and δ_C 129.2 (C-22) were attributed to the double bond of the side chain present in stigmasterol structure (Table 2). The ^{1}H-NMR spectrum revealed signals in the region of δ_H 0.8 to 2.0 related to methine, methylene and methyl groups. A multiplet at δ_H 3.51 and signals between δ_H 5.00 and 5.40 indicated the presence of the olefinic protons. In comparison with literature data (Chaturvedula and Prakash, 2012), the sample was identified as a mixture of β-sitosterol (**2**) and stigmasterol (**3**).

Lupeol acetate (**4**) was obtained as white crystals with R_f value of 0.95 (hexane:chloroform 1:1). The ^{13}C-NMR spectrum using the APT technique ($CDCl_3$) revealed characteristic signals of terpenes: seven non-hydrogenated carbons, seven methine carbons, ten methylene carbons, and eight methyl carbons. In addition, it was observed the signal at δ_C 168.4 corresponding to a carbonyl carbon (C-1') as well as signals at δ_C 148.4 and 106.8 that are characteristic of double bonds between a carbon non-hydrogenated (C-20) and other methylene (C-29). NMR data led to the characterization of Av-4 as a pentacyclic triterpene lupane type (Table 2). The ^{1}H-NMR spectrum showed signals in the region of δ_H 0.77 to 1.67 ppm related to

Table 1. ^{1}H NMR (200 MHz; MeOD) and ^{13}C NMR (50 MHz; MeOD) spectral data for compound **1** including results obtained by heteronuclear 2D shift-correlated HMQC and HMBC spectra.

Position		^{1}H × ^{13}C - HMQC	^{1}H × ^{13}C - HMBC		^{1}H × ^{1}H - COSY
C	δ_C	δ_H	$^{2}J_{CH}$	$^{3}J_{CH}$	
2	158.6	-	-	H-2', H-6'	-
3	135.8	-	-	H-1''	-
4	179.5	-	-	-	-
5	163.1	-	H-6	-	-
7	166.1	-	H-8, H-6	-	-
9	159.5	-	H8	-	-
10	105.8	-	-	H-6, H-8	-
1'	123.3	-	H-2', H-6'	H-5'	-
3'	145.9	-	H-2'	H-5'	-
4'	149.9	-	H-5'	H-2', H-6'	-
CH					
6	100.1	6.19 (d, *J* = 2.0)	-	H-8	-
8	95.0	6.38 (d, *J* = 2.0)	-	H-6	-
2'	117.9	7.61 (d, *J* = 2.1)	-	H-6'	-
5'	116.2	6.87 (d, *J* = 8,4)	H-6'	-	H-6'
6'	123.7	7.66 (dd, *J* = 2.1; 8.4)	H-5'	H-2'	H-5'
1''	104.9	5.10 (d, *J* = 7.4)	-	-	H-2''
2''	77.34	3.25-3.51 (m)	H-3''	H-4''	H-1'', H-3''
3''	75.9	3.25-3.51 (m)	H-2'', H-4''	H-5''	H-2'', H-4''
4''	71.5	3.25-3.51 (m)	H-3'', H-5''	H-6'''	H-3'', H-5''
5''	78.3	3.25-3.51 (m)	-	-	H-4'', H-6''
1'''	102.5	4.52 (d, *J* = 1.3)	-	-	H-2'''
2'''	72.4	3.64 (dd, *J* = 1.3; 3.3)	H-3'''	-	H-1''', H-3'''
3'''	72.2	3.54 (dd, *J* = 3.3; 9.5)	H-2'''	-	H-2''', H-4'''
4'''	74.1	3.29 (m)	H-3''', H-5'''	H-2''', H-6'''	H-3''', H-5'''
5'''	69.8	3.45 (m)	H-4''', H-6''', H-3'''	H-1'''	H-4''', H-6'''
CH_2					
6''	68.7	α: 3.39 (m); β: 3.81 (d, *J* = 10.0)	H-5''	H-1''', H-4'''	H-5''
CH_3					
6'''	18.0	1.12 (d, *J* = 6.2)	H-5'''	H-4'''	H-5'''

Chemical shifts in δ (ppm) and coupling constants (*J*, in parenthesis) in Hz.

Table 2. ^{13}C NMR (50 MHz; $CDCl_3$) spectral data for compounds **2/3** and **4**.

Position	δ_C	
C	**Compounds 2/3**	**Compound 4**
1	37.3	38.4
2	31.7	23.7
3	71.8	81.0
4	42.3	37.7
5	140.7	55.4
6	121.7	18.2
7	31.9	34.2
8	31.9	40.8
9	50.1	50.3
10	36.5	37.1
11	21.1	20.9

Table 2. Cont'd.

12	39.8/39.7	25.1
13	42.2	38.1
14	56.8	43.0
15	24.3	27.4
16	29.1	35.6
17	56.0	42.8
18	11.9/12.0	48.3
19	19.4	48.0
20	36.2/40.5	150.9
21	18.8/21.2	28.7
22	33.9/138.2	40.0
23	26.0/129.2	27.9
24	45.8/51.2	16.1
25	28.9/29.7	16.0
26	19.8	16.5
27	19.0	14.5
28	23.1/25.4	18.0
29	12.0/12.3	109.3
30	-	19.3
1'	-	170.9
2'	-	19.7

methine, methylene and methyl groups. In addition, a singlet at δ_H 4.56, a doublet at δ_H 4.67 compatible with H-2' and olefinic hydrogen (H-29), and the signal at δ_H 2.02 compatible with H-30 were observed. After NMR analyses and subsequent comparison with the literature data (Silva et al., 1998), it was possible to identify the substance as lupeol acetate (**4**).

Conclusion

Phytochemical investigation of *Annona vepretorum* extracts led to the isolation and identification of four compounds, a triterpene (lupeol acetate), two steroids (β-sitosterol and stigmasterol) and a glycosylated flavonoid (rutin). These compounds have been reported for the first time in this species.

CONFLICTS OF INTERESTS

The authors have not declared any conflict of interests.

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