Tropical Journal of Pharmaceutical Research March 2017; 16 (3): 597-604
ISSN: 1596-5996 (print); 1596-9827 (electronic)

Available online at http://www.tjpr.org
http://dx.doi.org/10.4314/tjpr.v16i3.14

Original Research Article

Phytochemical screening, cytotoxicity and acute toxicity of Annona vepretorum Mart (Annonaceae) leaf extracts

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Received: 22 January 2016 *Revised accepted: 9 February 2017*

Abstract

Purpose: *To investigate the phytochemistry, cytotoxicity and acute toxicity of leaf extracts from Annona vepretorum.*
Methods: *The crude extracts were obtained by maceration with hexane and methanol. The crude methanol extract was suspended in a 3:7 (v/v) mixture of methanol (MeOH) and water (H_2O) and partitioned with hexane, chloroform ($CHCl_3$) and ethyl acetate (AcOEt) in ascending order of polarity to obtain the respective extracts. In the investigation of phytochemical profile, the extracts were evaluated on thin layer chromatography (TLC) plates of silica gel. Cytotoxicity was tested using 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) and (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) (MTS) assays against tumor cell lines, viz, HCT-116 (colon), SF-295 (brain), HL-60 (leukemic) and Sarcoma-180. Acute toxicity study was performed by administration of a single oral dose of 2 g/kg body weight of the extracts to mice and the animals were observed for 14 days.*
Results: *Phytochemical screening results showed that A. vepretorum extracts contain alkaloids, flavonoids and terpenes. Methanol and chloroform extracts exhibited high cytotoxic activity against HCT-116, HL-60 and Sarcoma-180. Moreover, the extracts displayed low toxicity in mice, as no deaths and pronounced toxic effects were observed.*
Conclusion: *A. vepretorum contains a variety of secondary metabolites which may confer on this species high cytotoxic activity. In addition, the oral administration of the extracts produced low toxicity in mice.*

Keywords: *Annonaceae, Annona vepretorum, Phytochemistry, Tumor cell lines, Acute toxicity*

Tropical Journal of Pharmaceutical Research is indexed by Science Citation Index (SciSearch), Scopus, International Pharmaceutical Abstract, Chemical Abstracts, Embase, Index Copernicus, EBSCO, African Index Medicus, JournalSeek, Journal Citation Reports/Science Edition, Directory of Open Access Journals (DOAJ), African Journal Online, Bioline International, Open-J-Gate and Pharmacy Abstracts

INTRODUCTION

The Annonaceae family comprises about 2300-2500 species and over 130 genera distributed mainly in the tropical and subtropical regions [1,2]. In Brazil there are about 29 genera and about 260 species, the majority occurring in forests, with few representatives in open areas [3]. This family is characterized by the presence of terpenoids (mainly diterpenes), alkaloids (particularly isoquinoline derivatives) and essential oils with predominant composition of monoterpenes and sesquiterpenes [4,5]. Another class of substances found in this family is the acetogenins of Annonaceae, which have many

biological activities, and one of the most important is the antitumor activity [6].

The genus *Annona* L. comprises approximately 175 species of trees and shrubs [7]. It has geographical distribution in Brazil where there are about 81 species of which 24 are endemic and occur in regions like the Amazon, Caatinga, Cerrado, Atlantic Forest and Pantanal [8]. This genus has great economic importance because of their edible fruits and it has been heavily researched due to the isolation and characterization of several classes of substances with important pharmacological properties such as cytotoxic, antitumor, pesticide, vermicide, antimicrobial, immunosuppressive, anti-emetic, inhibiting appetite and antimalarial [4,9]. *Annona vepretorum* Mart. is an endemic species of Brazil (Caatinga biome) and is popularly known as "pinha da Caatinga" [8]. Its fruits are an important source of nutrition, being consumed raw or in the form of juices. Its roots have popular medicinal indication to bite of bees and snakes, inflammation and pain, while the leaves are used in bath to allergies, skin diseases, yeast and bacterial infections [10].

Previous studies on this species demonstrated that the essential oil from the leaves of *A. vepretorum* showed trypanocidal and antifungal activity and that bicyclogermacrene, spathulenol and α-phellandrene are the major constituents of the essential oil [11]. Another study showed the presence of spathulenol, limonene, caryophyllene oxide and α-pinene in essential oil and a weak antioxidant activity [12]. It was reported that the ethanol extract of the leaves of *A. vepretorum* has sedative effect in mice without affecting motor coordination of the animals [13]. In addition, research has shown that the ethanol extract has anti-inflammatory and anti-nociceptive activity, which is related probably with the activation of opioid receptors and inhibition of release of mediators of the inflammatory process [14]. Antioxidant, cytotoxic and antimicrobial activities have also been reported for this plant [15].

In this paper, we carried out the phytochemical investigation and evaluation of cytotoxicity and acute toxicity of leaf extracts from *Annona vepretorum*.

EXPERIMENTAL

Plant material

The leaves of *Annona vepretorum* Mart. were collected in the city of Petrolina, State of Pernambuco, Brazil, in April 2015. The samples were identified by Prof José Alves de Siqueira Filho, from Centro de Recuperação de Áreas Degradadas da Caatinga (CRAD). A voucher specimen (#18350) was deposited at the Herbarium Vale do São Francisco (HVASF), of the Federal University of São Francisco Valley (UNIVASF).

Preparation of extracts

A. vepretorum leaves (1.100 g) were dried in an air circulation oven at 40 °C during 72 h and then were sprayed in a knife mill. The dried and pulverized leaves of *A. vepretorum* (412 g) were subjected to exhaustive maceration with hexane (Hex) and then with methanol (MeOH) with renewal of the extractor fluid every 72 h. The crude extracts were concentrated under vacuum, yielding 10 g of crude hexane extract (Av-HexC) and 83 g of crude methanol extract (Av-MeOH). A portion of the methanol extract (80 g) was suspended in a 3:7 (v/v) mixture of methanol (MeOH) and water (H_2O) and partitioned with hexane, chloroform ($CHCl_3$) and ethyl acetate (AcOEt) in ascending order of polarity to obtain the respective extracts (Av-Hex 9.15 g; Av-$CHCl_3$ 4.35 g; Av-AcOEt 5.45 g and Av-H_2O 40.0 g).

Qualitative analysis of phytochemicals

The extracts were evaluated on thin-layer chromatography plates of silica gel 60 F_{254} aluminum supports, applied with a micropipette and eluted in different solvent systems as described by Wagner and Bladt [16], seeking to highlight the main groups of secondary metabolites (Table 1).

Cell lines and culture

Human tumor cell lines used, HCT-116 (colon), SF-295 (glioblastoma) and HL-60 (leukemic) were supplied by the National Cancer Institute (USA) and were cultured in RPMI 1640 medium supplemented with 10 % fetal bovine serum and 1 % of antibiotics, kept in oven at 37 °C and atmosphere containing 5 % CO_2.

Murine tumor cell line, Sarcoma-180, were maintained in the peritoneal cavity of Swiss mice. The adult animals (90 days age, 30 - 40 g) were randomly housed in appropriate cages at 22 ± 2 °C on a 12 h light/dark cycle (lights on at 6:00 am) with access to food and water *ad libitum*. For the *in vitro* cytotoxicity assay, the cell line were cultured in RPMI-1640 supplemented with 10 % fetal bovine serum, sodium bicarbonate, 25 mM HEPES, *L*-glutamine 300 mg/L, gentamicin sulfate 50 mg/L and amphotericin B 2 mg/L, kept in oven at 37 °C.

Table 1: Elution systems and reagents used to characterize the main secondary metabolites in the extracts of *Annona vepretorum* by thin-layer chromatography

Phytochemical	Elution system	Standard	Reagent
Alkaloids	Toluene:ethyl acetate: diethylamine (70:20:10, v/v)	Yohimbine	Dragendorff
Anthocyanins	Ethyl acetate:formic acid: glacial acetic acid:water (100:11:11:26, v/v)	Methylene blue	Anisaldehyde sulfuric
Anthraquinones aglycons	Petroleum ether:ethyl acetate:formic acid (75:25:1, v/v)	Anthraquinone	Phosphomolybdic acid/ ethanolic H_2SO_4 10%
Flavonoids	Ethyl acetate:formic acid: glacial acetic acid:water (100:11:11:26, v/v)	Quercetin	NEU
Coumarins	Toluene:ethyl ether: (1:1 saturated with acetic acid 10 %, v/v)	Scopoletin	10 % ethanolic KOH
Anthracene derivatives	Ethyl acetate:methanol: water (100:13.5:10, v/v)	Aloin	10 % ethanolic KOH
Lignans	Chloroform:methanol:water (70:30:4, v/v)	Flaxseed extract	Vanillin sulfuric
Monoterpenes, sesquiterpenes and diterpenes	Toluene:ethyl acetate (93:7, v/v)	Thymol and carvacrol	Vanillin sulfuric
Naphthoquinones	Toluene:formic acid (99:1, v/v)	Lapachol	10 % ethanolic KOH
Saponins	Chloroform:acetic acid: methanol:water (64:32:12:8, v/v)	Saponin	Sulfuric anisaldehyde
Condensed tannins	Ethyl acetate:glacial acetic acid: acid formic:water (100:11:11:26, v/v)	Catechin Epicatechin	Vanillin hydrochloric
Hydrolysable tannins	n-Butanol:acetone: Phosphate buffer (40:50:10, v/v)	Gallic acid Tannic acid	Ferric ammonium sulfate (1%)
Triterpenes and steroids	Toluene:chloroform: ethanol (40:40:10, v/v)	Lupeol Sitosterol	Liebermann-Burchard
Xanthine	Ethyl acetate:Methanol: Water (100: 13.5: 10, v/v)	Caffeine	Iodine-KI-HCl

Determination of cytotoxicity

Human tumor cell lines were plated in 96-well plates: HCT-116 (0.7×10^5 cells/mL), SF-295 (0.1×10^6 cells/mL) and HL-60 (0.3×10^6 cells/mL). The plates were incubated with the extracts (50 µg/mL) dissolved in 1% DMSO for 72 h in an incubator at 5% CO_2 at 37 °C. At the end of this, they were centrifuged and the supernatant removed. Thereafter, a 150 µL solution of 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) was added, and the plates incubated for 3 h. After incubation, the plates were centrifuged again to remove the MTT solution. The absorbance was read after solubilization of the formazan precipitate with 150 µL of pure DMSO at 595 nm spectrophotometer plate.

Sarcoma-180 cells maintained *in vivo* were added with phosphate buffer (PBS) and subsequently centrifuged (1200 rpm for 3 min). The supernatant was discarded and Cells were re-suspended in RPMI-1640 medium. The cells were then seeded (1×10^5 cells/well suspended in 50 µL of medium) in 96-well plates and incubated with the extracts (50 µg/mL) dissolved in 1% DMSO for 24 h at 37 °C. After 24 h, 0.02 mL of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxy methoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) in a concentration of 5 mg/mL was added to each well. After incubation for 3 h, the absorbance (Abs) was read at 492 nm on spectrophotometer plate and the cell proliferation inhibition (P) was calculated using Eq 1.

$$P\ (\%) = 100 - \{(At/Au)\}100 \quad \ldots\ldots\ldots\ldots\ldots\ldots \quad (1)$$

where At and Au are the absorbance of treated and untreated cells, respectively.

The concentration that caused 50 % cell growth inhibition (IC_{50}) was determined from the concentration-response curves by non-linear regression with a confidence interval of 95 %. An intensity scale was used to assess the cytotoxic

potential of the tested samples: samples without activity (SA), with weak activity (cell growth inhibition ranging from 1 to 50 %), with moderate activity (cell growth inhibition ranging from 50 to 75 %) and strong activity (cell growth inhibition ranging from 75 to 100 %).

Acute toxicity test

The acute toxicity study was performed according to OECD 423 Guidelines for testing of chemicals [17]. The mice were randomly divided into 7 groups of 3 female mice. Group I: normal control group, and the mice received vehicle (saline/0.2 % Tween 80); Group II: 2 g/kg body weight Av-MeOH; Group III: 2 g/kg body weight Av-HexC; Group IV: 2 g/kg body weight Av-Hex; Group V: 2 g/kg body weight Av-$CHCl_3$; Group VI: 2 g/kg body weight Av-AcEtOH; Group VII: 2 g/kg body weight Av-H_2O. After administration of the extract, the animals were observed every 30 min for 4 h on the first day and once daily for 14 days. During this period the emergence of general toxic signs was assessed [18]. The LD_{50} of the extracts was investigated based on the eventual observation of mortality. The mice were assessed daily throughout the study to monitor their body weight variation, consumption of food and water. Experimental protocols and procedures were approved by the Federal University of San Francisco Valley Animal Care and Use Committee (protocol no. 0018/140415).

Statistical analysis

Statistical analysis was performed using GraphPad Prism® 5.0 software. The experimental results were analyzed employing the Student's *t* test. Data are presented as mean ± standard deviation (SD) and differences were considered significant when $p < 0.05$.

RESULTS

Phytochemical profile

The phytochemical analysis of *A. vepretorum* showed that almost all extracts, except the crude hexane extract (Av-HexC), were positive for the presence of flavonoids. The methanol, hexane, chloroform and ethyl acetate extracts also showed positive reaction for the presence of alkaloids. Only the aqueous extract was not positive for the presence of monoterpenes, sesquiterpenes, diterpenes, triterpenes and steroids. The methanol and ethyl acetate extracts were positive for anthocyanins. The chloroform and ethyl acetate extracts were positive for naphthoquinones and hydrolysable tannins. Only the ethyl acetate extract was positive for saponins. All extracts of *A. vepretorum* showed negative results for the presence of anthraquinones, anthracene derivatives, lignans, naphtoquinones, condensed tannins and xanthine. These results are presented in Table 2.

Table 2: Phytochemical profile of extracts of *Annona vepretorum*

Phytochemical	Av-MeOH	Av-HexC	Av-Hex	Av-$CHCl_3$	Av-AcOEt	Av-H_2O
Alkaloids	+	-	+	++	+	-
Anthocyanins	+	-	-	-	++	-
Anthraquinones aglycons	-	-	-	-	-	-
Flavonoids	++	-	+	+++	++	++
Coumarins	-	-	-	+	-	-
Anthracene derivatives	-	-	-	-	-	-
Lignans	-	-	-	-	-	-
Monoterpenes, sesquiterpenes and diterpenes	+	+++	+++	+++	+	-
Naphthoquinones	-	-	-	+	+	-
Saponins	-	-	-	-	+	-
Condensed tannins	-	-	-	-	-	-
Hydrolysable tannins	-	-	-	+	+	-
Triterpenes and steroids	+	+++	+	+	+	-
Xanthine	-	-	-	-	-	-

(-) Not detected; (+) low presence; (++) moderate presence; (+++) strong presence. Av-MeOH (crude methanol extract), Av-HexC (crude hexane extract), Av-Hex (hexane extract), Av-$CHCl_3$ (chloroform extract), Av-AcOEt (ethyl acetate extract), Av-H_2O (aqueous extract)

Cytotoxicity

The methanol and chloroform extracts showed a high cytotoxic potential in three of the tested lines (HCT-116, HL-60 and Sarcoma-180). The crude hexane extract had a high cytotoxic potential in glioblastoma line (SF-295) and Sarcoma-180. The hexane partition and aqueous extracts

showed high cytotoxic activity only in sarcoma-180 cell line. The ethyl acetate extract had an activity ranging from low to moderate in cell lines (Table 3).

Acute toxicity

In the evaluation of the acute toxicity of extracts obtained from the leaves of *A. vepretorum* there was no death or signs of toxicity in any of the groups that received a single dose of extract and were assessed for 14 days of the experiment. The animal treated with the extracts showed some significant changes in body weight when compared with the control group (Table 4). The results for evaluation of acute toxicity are presented in Table 4, Table 5 and Table 6, and represent the mean values obtained in the first and in the last 7 days as well as the mean values after 14 days.

Table 3: Cell proliferation inhibition (%) of extracts of *Annona vepretorum* (50 µg/mL)

Extract	Cell proliferation inhibition (%)				IC_{50} (µg/mL)
	HCT-116	***SF-295***	***HL-60***	***Sarcoma-180***	
Av-MeOH	98.16 ± 0.92	63.98 ± 3.40	82.23 ± 4.84	82.34 ± 1.36	2.81 ± 0.41
Av-HexC	29.96 ± 1.60	86.54 ± 3.31	17.11 ± 7.34	79.43 ± 4.39	4.87 ± 0.83
Av-Hex	56.04 ± 21.00	65.43 ± 6.52	55.85 ± 3.56	86.24 ± 1.09	45.82 ± 9.07
Av-$CHCl_3$	74.28 ± 0.25	82.05 ± 24.67	29.79 ± 1.82	81.32 ± 6.79	2.88 ± 1.39
Av-AcOEt	17.87 ± 6.45	27.98 ± 5.16	68.72 ± 38.28	63.15 ± 6.57	22.82 ± 3.76
Av-H_2O	9.52 ± 11.68	6,72 ± 1.25	- 8.20 ± 3.33	78.57 ± 5.91	71.18 ± 1,69

HCT-116 (colon), SF-295 (brain), HL-60 (leukemia). Values are mean ± SD (n= 3); 95% confidence intervals

Table 4: Mice weight after administration of a single dose of different extracts of *A. vepretorum* (2 g/kg)

Group	Animal weight (g)		
	1 - 7 days	***8 - 14 days***	***14 days***
Control	32.19 ± 0.37	32.14 ± 0.32	32.17 ± 0.33
Av-MeOH	32.95 ± 0.44*	32.33 ± 0.38	32.64 ± 0.51*
Av-HexC	29.71 ± 0.48*	29.00 ± 0.43*	29.36 ± 0.57*
Av-Hex	31.10 ± 0.41*	30.67 ± 0.67*	30.88 ± 0.48*
Av-$CHCl_3$	30.33 ± 0.33*	30.29 ± 0.35*	30.31 ± 0.33*
Av-AcOEt	30.95 ± 0.23*	31.24 ± 0.49*	31.10 ± 0.40*
Av-H_2O	28.76 ± 0.49*	28.86 ± 0.46*	28.81 ± 0.46*

Values are mean ± SD; *$p < 0.05$

Table 5: Water consumption after administration of a single dose of different extracts of *A. vepretorum* (2 g/kg)

Group	Water consumption (mL)		
	1 - 7 days	***8 - 14 days***	***14 days***
Control	22.57 ± 2.14	20.71 ± 2.49	21.64 ± 2.43
Av-MeOH	25.43 ± 1.90*	26.86 ± 3.80*	26.14 ± 2.98*
Av-HexC	26.57 ± 5.12	27.57 ± 1.13*	27.07 ± 3.60*
Av-Hex	29.29 ± 10.01	37.14 ± 3.43*	33.21 ± 8.26*
Av-$CHCl_3$	33.71 ± 8.90*	30.86 ± 6.09*	32.29 ± 7.47*
Av-AcOEt	26.57 ± 8.14	24.29 ± 1.79*	25.43 ± 5.78*
Av-H_2O	30.29 ± 13.68	32.14 ± 5.61*	31.21 ± 10.09*

Values are mean ± SD; *$p < 0.05$

Table 6: Feed consumption after administration of a single dose of different extracts of *A. vepretorum* (2 g/kg body weight)

Group	Food consumption (mL)		
	1 - 7 days	***8 - 14 days***	***14 days***
Control	15.00 ± 1.63	15.43 ± 1.13	15.21 ± 1.36
Av-MeOH	16.57 ± 1.39	16.43 ± 1.71	16.50 ± 1.50*
Av-HexC	13.71 ± 1.38	14.71 ± 0.95	14.21 ± 1.25
Av-Hex	14.71 ± 2.62	16.86 ± 2.19	15.79 ± 2.57
Av-$CHCl_3$	14.00 ± 1.29	15.57 ± 1.13	14.79 ± 1.42
Av-AcOEt	15.00 ± 1.41	15.57 ± 2.14	15.29 ± 1.77
Av-H_2O	13.86 ± 2.03	16.14 ± 2.26	15.00 ± 2.38

Values are mean ± SD; *$p < 0.05$

Water consumption was increased in the treated groups compared to the control (Table 5). As well as for the feed intake, there was only increased consumption in the group treated with the methanol extract when we calculate the average consumption for 14 days of evaluation (Table 6).

DISCUSSION

Investigations about the chemical composition of plants of Caatinga that are traditionally used to treat various diseases had identified secondary metabolites with promising pharmacological activities [13]. Phytochemical analysis showed the presence of flavonoids, except in crude hexane extract (Av-HexC), confirming previous studies with *A. vepretorum* [15]. A large number of biological activities have been attributed to these compounds, including anticancer activity [19]. It was also demonstrated the presence of alkaloids and terpenes in the extracts [4].

MTT assay is a well-known colorimetric assay that is based on the enzymatic reduction of the tetrazolium salt MTT to a formazan product in viable cells. It has been largely used to determine cytostatic/cytotoxic potential of medicinal agents in screening programs [20]. The MTS assay has the same general principle of MTT test. Those extracts that caused more than 75% cell growth inhibition in any cell line were considered active. Thus, only the methanol extract and chloroform extract showed strong activity against human tumor cell lines. On Sarcoma-180, the extracts showed strong activity, but the ethyl acetate extract showed moderate activity.

According to the American National Cancer Institute, the IC_{50} threshold for considering a promising extract for cancer treatment should be less than 50 µg/mL [21]. Thus, the methanol extract, crude hexane extract, partitioned hexane extract, chloroform and ethyl acetate presented promising results based on the IC_{50} values, which could be attributed to the presence of alkaloids, flavonoids and terpenes, since these classes of secondary metabolites have cytotoxic activity [22-24].

The majority (60 %) of anticancer drugs introduced into therapy has its origin in natural products, however, most chemotherapeutic drugs used in clinical practice are not selective for tumor cells, causing severe side effects. Thus, it is critical to characterize the toxicity profile of potential candidates for antitumor agents [25]. It was observed that a single oral administration of 2 g/kg body weight extracts of *A. vepretorum* leaves did not cause any mortality or alteration in behavioural or physiological state of animals indicating that the plant extracts are not toxic at the tested dose and that its LD_{50} is likely to be greater than 2 g/kg.

Analysis of the body weight (Table 4) of treated animals with the extracts showed some differences when compared with the control. However, there was no marked weight loss of animals over the 14 days of evaluation. Generally, changes in these parameters reflect its toxic effects, especially if such loss is greater than 10% of the initial weight [26].

The analysis of the ingestion of food and water is an important parameter to investigate the safety of the substances studied for therapeutic purpose, particularly for substances which exhibit indicative of gastrointestinal toxicity, which may cause decreased intake of feed and water, and hence weight loss [27]. In contrast, there was an increase of water consumption in the treated groups compared with the control. As well as for the feed intake, there was only a statistically significant increase in consumption in the group treated with methanol extract compared to the control, demonstrating the low toxicity of the extracts.

CONCLUSION

A. vepretorum contains a variety of secondary metabolites which may confer on this species the capacity to inhibit the growth of human tumor cell lines and sarcoma-180 *in vitro*. Moreover, the administration of a single dose of the extracts shows low toxicity in mice. Thus, *A. vepretorum* is a potential source of bioactive compounds with cytotoxic activity and low oral toxicity, which should be further investigated in the search for new molecules with potential anticancer activity.

DECLARATIONS

Acknowledgement

The authors thank Brazilian National Agencies CAPES, CNPq, FACEPE and FUNCAPE for financial support for the work.

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

The authors declare that this work was done by

the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them.

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