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Original Article

Chemical characterization and toxicological assessment of hydroethanolic extract of *Mandevilla velame* xylopodium

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ABSTRACT

The present study was aimed to characterize the chemical profile and evaluate the cytotoxicity and sub-chronic toxicity of the hydroethanolic extract of the xylopodium *Mandevilla velame* (A.St.-Hil.) Pichon, Apocynaceae. Chemical profile was analyzed by high performance liquid chromatography. Cytotoxic potential of hydroethanolic extract of the xylopodium *M. velame* was evaluated using Chinese hamster ovary cells. The sub-chronic assessment was done on rats with hydroethanolic extract of the xylopodium *M. velame* (50, 200 and 800 mg/kg) was orally administered daily for 30 consecutive days. High performance liquid chromatography analysis confirmed the presence of gallic acid, ellagic acid, catechin, epigallocatechin gallate, naringin, myricetin, quercetin and naringenin. hydroethanolic extract of the xylopodium *M. velame* tested concentrations did not alter the viability of Chinese hamster ovary cells. In the sub-chronic test, 50 and 200 mg/kg were safe, but there were significant changes in relation to weight gain and water consumption by animals that received 800 mg/kg of hydroethanolic extract of the xylopodium *M. velame*. Among the haematological and biochemical parameters evaluated, only the number of neutrophils, lymphocytes, and creatinine concentration were changed at 800 mg/kg. Phytochemical profile of hydroethanolic extract of the xylopodium *M. velame* revealed the presence of phenolics and flavonoid compounds. The *in vitro* cytotoxicity assay result demonstrated that hydroethanolic extract of the xylopodium *M. velame* had no cytotoxic effects in Chinese hamster ovary cells. In the *in vivo* models, hydroethanolic extract of the xylopodium *M. velame* was shown to be relatively safe after sub-acute administration in rats which is relation to that the population daily takes a total dose of the plant xylopodium decoction or infusion about 23.29 times lower than the no-observed-adverse effect level dose in rats.

Introduction

The medicinal uses of plants and any medicine derived from them have increased in recent decades, but there is a mistaken consensus that the natural product only produces beneficial effects without any health risks (Ekor, 2014).

In spite of the rising popularity and presumed safety of herbal medicines, adverse effects have become a major safety issue for plant products/extracts (Han et al., 2016). Consequently, in response to public health concerns, it is essential to validate the safety of traditional herbal medicines before their utilization. Experimental data on the toxicity profile of medicinal plants and their extracts should be obtained to increase confidence in their safety for human use and in the development of pharmaceuticals (Yuet Ping et al., 2013). Therefore, scientific validation (systemic) of medicinal plants for potential toxicity is a necessary footstep for the corroboration of their standard therapeutic use (El Kabbaoui et al., 2017).

Mandevilla velame (A.St.-Hil.) Pichon, Apocynaceae, has as synonym *Macrosiphonia velame* (A. St.-Hil.) Müll. Arg. and *Echites velame* A.St.-Hil., is a sub-shrub up to 82 cm height when with flowers, grows in rocky soil or on the stone, native but not endemic, that

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can be found in Central West, Southeast and South regions of Brazil in the phytogeographic domains of *Cerrado* and *Pampa* (Flora do Brasil, 2020).

It is popularly known as "velame-branco", "velame-do-cerrado", "losna-do-campo" and "velame". Traditional populations of the Brazilian *Cerrado* use medicinal infusion or decoction prepared respectively by putting about 10 g of *M. velame* xylopodium in boiled water (1 l) and then drown for 15 min or to boiled with water (1 l) for 5 min for treating inflammation, syphilis, diuretic, kidneys and to promote blood depuration (Rodrigues and Carvalho, 2001; Ribeiro et al., 2017).

In the treatment of diseases, a cup (̃200 ml) of the resulting prepared is taken four to five times for day until the symptoms disappear (Velame branco, 2018). Since it is a small sub-shrub, there is the risk of extinction of the species due to farming, construction of dams or roads and predatory extractive for medicinal use (Rodrigues and Carvalho, 2001).

There is only one report published in the literature was made by our research group (Ribeiro et al., 2010) and involved the anti-inflammatory, antinociceptive and antipyretic properties of the hydroethanolic extract of *M. velame* xylopodium (HEMv), as well its acute toxicity in rodents. In this same work, the author's described about preliminary phytochemical analysis and acute toxicity in mice.

Despite the widespread medicinal use, there is no report demonstrating its safety and phytochemical profile. Thus, this study aims to evaluate pre-clinical toxicological studies and carry out the phytochemical analysis of the HEMv.

Materials and methods

Animals

Male Wistar rats (*Rattus norvegicus*, 180–200 g) obtained from the Central Animal House of the Federal University of Mato Grosso (UFMT), Brazil were used. The animals were kept in polypropylene cages at 24 ± 1 °C with controlled cycles of light/dark of 12 h, and maintained on standard laboratory feeds (Purina®, Labina, Goiás, Brazil) and water *ad libitum*. The experimental protocols for this study was designed in accordance with the ethical principles of animal experimentation (adopted by the Brazilian College of Animal Experimentation) and approved by the Ethics Committee on Animal Research of UFMT (No. 23108.005276/08-5).

Drugs and reagents

Gallic acid, ellagic acid, catechin, epigallocatechin gallate, naringin, myricetin, quercetin and naringenin, dimethylsulfoxide (DMSO), Dulbecco's Modified Eagle Medium (DMEM), doxorubicin, Alamar blue® and ethylenediamine tetra-acetic acid (EDTA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Ultrapure water was produced using a Milli-Q system (Millipore, MO, USA), ketamine and xylazine (Syntec, SP, Brazil), haematoxylin and eosin (HE) (Easypath, SP, Brazil). All drugs and other reagents were used analytical grade.

Plant collection and extraction

The xylopodium (1 kg) of *Mandevilla velame* (A.St.-Hil.) Pichon, Apocynaceae, was collected in the municipality of Acorizal, Mato Grosso, Brazil (co-ordinates: S15° 04′17 0.3 W56° 20′55 0.5) on July 2013 after authorization to access traditional knowledge associated with Genetic Heritage issued by the *Conselho de Gestão do Patrimonio Genético do Ministério do Meio Ambiente* (CGen/MMA n° 135/2013). Witness samples containing plant material with flowers were deposited at the UFMT-Herbarium and identified by Dr. Rosilene Silva Rodrigues under voucher number 38289.

For the extract preparation, the xylopodium was clean, dried at room temperature and ground into powder in an electric mill (5 mm sieve), (Tecnal TE-625, Brazil). The powdered material was extracted with 75% water/ethanol at a 1:3 (w/v) ratio, macerated for 7 days, and concentrated using rotary evaporator (model 801, Fisatom, Brazil) under reduced pressure (600 mmHg) at a temperature of 40 °C. The residual solvent was removed in an oven (Marconi MA037, Brazil) at 40 °C, and frozen at −86 °C in a bio freezer (Indrel® Ultra Freezer IULT 335D, Brazil), lyophilised (Scientific LJJ02, Brazil), yielding 60.1 g (6.0%) HEMv. It was then packaged in an amber bottle and kept in bio freezer (Indrel, Model CPS 10D, Brazil) at −30 °C.

High-performance liquid chromatography (HPLC) analysis of HEMv

The chemical profiling of HEMv was analyzed by HPLC (Shimadzu, Japan). It consisted of a chromatograph (LC-10 Avp series) equipped with a degasser (DGU-14A), a pump (LC-10 AT) to pump the mobile phase, manual Rheodyne injector (20 μl loop) and class integrator (LC-10AT), UV-VIS (SPD - 10A) detector and a column oven (CTO 10A). The extract solutions and standards were prepared with methanol and filtered through Millipore® (0.20 μm pore size) membrane. The separation was carried out by the gradient system, using a reverse-phase C18 Phenomenex Luna 5 μm (2) (250 × 4.6 mm^2) column with direct-connect C18 Phenomenex Security Guard Cartridges (4 × 3.0 mm^2) filled with a material similar to the main column's. Mobile Phase A was 0.1% phosphoric acid in Milli-Q water and mobile phase B was 0.1% phosphoric acid in Milli-Q water/acetonitrile/methanol (54:35:11). Program gradient: 0 to 0.01 min, 0% B; 0.01–5 min, 0% B, 5–10 min, 30% B, 10–20 min 40% B, 20–29 min, 40% B, 29–30 min 50% B, 30–50 min 100% B, 50–80 min, 100% B. Flow rate: 1 ml/min, temperature: 22 °C. UV detection was done at 280 nm.

The compounds were identified by comparing the retention times of the samples with authentic standards, such as gallic acid, catechin, epigallocatechin gallate, ellagic acid, naringin, myricetin, quercetin and naringenin (Sigma–Aldrich, USA). Concentrations of the compounds were expressed in micrograms per milligram of extract (μg/mg) by correlating the area of the analyte with the calibration curve of built in concentrations of 4.5–18 μg/ml standards.

Cytotoxicity assay

Epithelial cells of Chinese hamster ovary line (CHO-K1, code 0067) acquired from the Rio de Janeiro Cell Bank (BCRJ) were used. Cells were plated at density 2 × 10^4 cell/ml in 96-well plates containing 200 μl culture medium DMEM + HAM F10 supplemented with 10% fetal bovine serum (FBS, Sigma–Aldrich, USA), penicillin (100 U/ml, Sigma–Aldrich, USA) and streptomycin (100 μg/ml, Sigma–Aldrich, USA) at 37 °C in a 5% CO_2 and 90% humidity atmosphere after that treated with HEMv concentrations from 3.125 to 200 μg/ml obtained by serial dilution. Culture medium was used as negative control. After incubation for 72 h at 37 °C, 5% CO_2, the treatments were removed and added to 200 μl of 10% solution of Alamar blue in each well. After 5 h, absorbance reading at 540 nm (oxidized state) and 620 nm (reduced state) were taken using a microplate-reader spectrophotometer (Multiskan EX, Thermo Scientific, USA). The results were expressed as inhibitory concentration (IC_{50}), and the values considered to be non-cytotoxic were IC_{50} > 30 μg/ml for the mixture and IC_{50} > 4 μg/ml for pure substances (Suffiness and Pezzuto, 1990).

Sub-chronic toxicity (repeated doses)

Subchronic toxicity was evaluated as previously proposed by Chan et al. (1982). Animals (n = 6/group), kept in metabolic cages

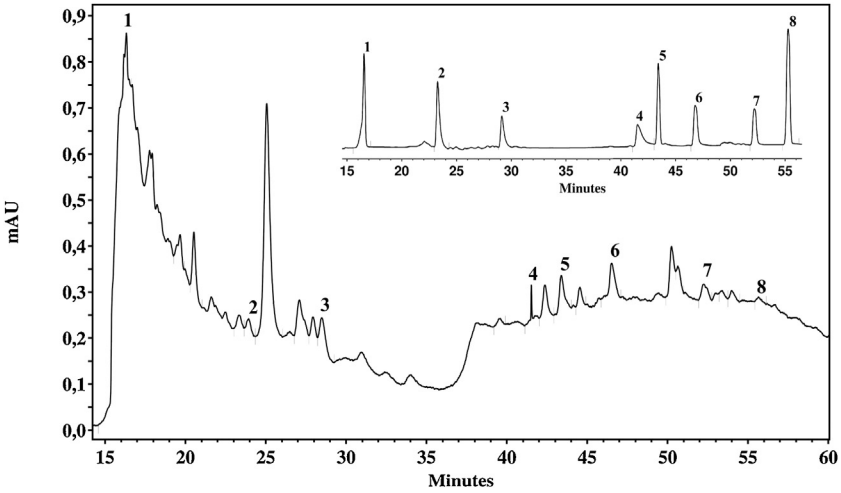

Fig. 1. High performance liquid chromatography (HPLC) fingerprint of the hydroethanolic extract of *Mandevilla velame* xylopodium (HEMv) detected at 280 nm. (1) gallic acid; (2) Catechin; (3) epigallocatechin gallate; (4) ellagic acid; (5) Naringin; (6) myricetin; (7) quercetin; (8) naringenin. Inside HPLC chromatogram showed the authentic standards of phenolic compounds.

(Ugo Basile, model 41800, Italy), were treated daily by oral gavage with the vehicle (distilled water, 0.01 ml/g) and HEMv (50, 200 and 800 mg/kg b.w.) for 30 days. Body weight gain and intake of food and water were determined every 3 days and grouped every 6 days (D1, D2, D3, D4 and D5) over the 30-day period. Signs and symptoms of behavioral alterations were recorded, as skin, eyes, gastrointestinal, respiratory, central nervous system and peripheral alterations, including any other general changes. At the end of the treatments, animals were anesthetized with intraperitoneal (*i.p.*) injection of xylazine/ketamine (10/100 mg/kg) on the 30th day after a 12 h overnight fasting. Blood was collected *via* vena cava for biochemical and hematological analysis. Animals were then sacrificed by cervical dislocation and the organs (liver, heart, lung, kidneys, stomach and spleen) were removed. The relative weights were calculated (weight of the organ/body weight × 100) and subjected to a complete necropsy, including external body macroscopic examinations. The organs were fixed in 10% formalin solution for histopathological analysis.

Hematological and biochemical analysis

At the end of 30 days, the animals were anesthetized by *i.p.* injection of xylazine/ketamine (10/100 mg/kg) in a final volume of 1 ml/100 g and their blood was collected in Vacutainer® tubes containing EDTA (BD, Brazil), was used for the haematological examinations [hematocrit (Ht), hemoglobin (Hb), total erythrocyte count, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), total leukocytes, rods, neutrophils, lymphocytes, eosinophils, monocytes and platelets] on an automatic cell counter (Cell Dyn 3700, Abbott Laboratories, USA) and serum biochemical analysis [glucose, urea, creatinine, uric acid, aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase, cholesterol, triacylglycerides, total protein and albumin) was quantified by colorimetric assays using Labtest® kits (Labtest diagnostics, Brazil).

Histopathological analysis

The organs removed at the end of the sub-chronic toxicity study were stored in 10% formalin, embedded in paraffin, cut and processed for preparation of slides and stained with hematoxylin and eosin (H & E) for examination under an optical microscope at 40 x magnifications.

Statistical analysis

Results were expressed as mean ± S.E.M. (standard error of mean). Comparisons between means were performed by one-way analysis of variance (ANOVA) followed by post-test of Student-Newman-Keuls for multiple comparisons, using GraphPad Prism Version 6.07 software (USA). *P*- values less than 0.05 were considered statistically significant. The IC_{50} was determined by the linear regression curve, relating the percentage of inhibition versus the logarithm of the tested concentrations and assuming a confidence interval of 99% ($p < 0.01$) for the resulting curve.

Results

Qualitative analysis of phytochemicals using high performance liquid chromatography (HPLC)

The HEMv chemical profile was identified by HPLC (Fig. 1), and revealed the presence of the phenolic acids gallic acid (8.18 μg/mg, retention time 16.5 min) and ellagic acid (2.05 μg/mg, retention time 41.5 min) respectively, the polyphenol epigallocatechin gallate 4.44 μg/mg (Rt 28.9 min) and the flavonoids catechin 4.31 μg/mg (Rt 23.7 min), naringin 3.31 μg/mg (Rt 43.4 min), myricetin 5.27 μg/mg (Rt 46.8 min), quercetin 3.13 μg/mg (Rt 52.2 min) and naringenin 1.11 μg/mg (Rt 55.6 min).

Cytotoxicity assay

The cytotoxicity result demonstrates the viability of CHO-K1 cells treated for 72 h with various concentrations of HEMv. The extract showed $IC_{50} = 173.36 \pm 3.30$ μg/ml, and the doxorubicin used as a standard drug showed an $IC_{50} = 0.30 \pm 0.01$ μg/ml (Fig. 2).

Sub-chronic toxicity study

Table 1 shows the effect of a 30-day daily treatment with vehicle or HEMv (50, 200 or 800 mg/kg, *p.o.*) for 30 days, relative organs weight of animals were observed, there was no gross effect on the heart, lung, liver, stomach, spleen and kidneys, and no significant difference of the indexes of relative weight (%) of these organs of the animals when compared the control group (data not shown).

The results of daily treatment with control or HEMv on the accumulated weight gain, feed and water intake and excretion of

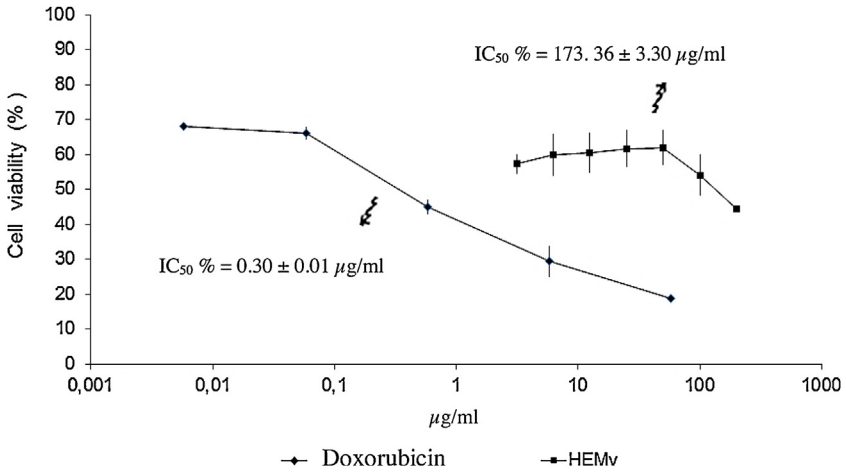

Fig. 2. Cytotoxicity of CHO-K1 cells exposed to varying concentrations of hydroethanolic extract of *Mandevilla velame* xylopodium (HEMv) and doxorubicin for 72 h, expressed as minimum inhibitory concentration (IC_{50}) of three independent assays.

Table 1
Effect of the hydroethanolic extract of *Mandevilla velame* (HEMv, mg/kg *p.o.*) on the body weight, accumulated body weight gain, water consumption, fecal and urine excretion in male rats.

Parameters analyzed	Period of treatment				
	D_0	D_1	D_2	D_3	D_4
Control (vehicle)					
Body weight (g)	156.80 ± 7.23	191.80 ± 7.69	226.50 ± 8.38	267.80 ± 7.17	299.70 ± 4.69
Commulative weight gain (g)	–	35.00 ± 5.02	71.00 ± 5.34	107.80 ± 6.44	129.60 ± 7.32
Water consumption (ml)	–	54.30 ± 2.88	54.80 ± 1.54	53.40 ± 1.43	49.90 ± 1.36
Food intake (g)	–	36.60 ± 1.90	37.70 ± 1.42	40.60 ± 1.43	39.90 ± 1.64
Feces (g)	–	17.00 ± 1.05	15.90 ± 1.13	18.50 ± 1.02	18.50 ± 0.98
Urine (ml)	–	18.50 ± 1.67	17.70 ± 1.17	18.30 ± 0.83	19.40 ± 0.51
HEMv 50 mg/kg					
Body weight (g)	162.60 ± 5.02	209.00 ± 4.75	248.50 ± 4.06	289.50 ± 4.54	319.50 ± 5.39
Commulative weight gain (g)	–	46.40 ± 6.12	83.10 ± 2.19	119.00 ± 3.40	141.00 ± 4.07
Water consumption (ml)	–	53.10 ± 2.66	55.50 ± 3.13	58.70 ± 2.70	55.10 ± 3.10
Food intake (g)	–	41.10 ± 1,30	42.30 ± 0.99	43.80 ± 0.94	43.20 ± 1.28
Feces (g)	–	18.50 ± 0.78	17.80 ± 0.95	19.70 ± 0.81	18.60 ± 0.99
Urine (ml)	–	22.50 ± 1.89	22.00 ± 0.93*	23.70 ± 1.39*	25.10 ± 1.23[a]
HEMv 200 mg/kg					
Body weight (g)	157.40 ± 6.83	198.70 ± 6.05	236.10 ± 5.00	278.70 ± 7.20	315.40 ± 11.3
Commulative weight gain (g)	–	41.30 ± 3.40	81.70 ± 3.82	120.50 ± 4.80	133.80 ± 5.94
Water consumption (ml)	–	52.90 ± 2.10	56.40 ± 2.10	57.10 ± 2.27	51.90 ± 2.27
Food intake (g)	–	40.10 ± 2.17	40.60 ± 0.98	42.20 ± 1.28	37.90 ± 1.23
Feces (g)	–	18.30 ± 0.77	18.30 ± 0.55	19.40 ± 0.75	18.20 ± 0.82
Urine (ml)	–	21.40 ± 1.56	24.70 ± 1.88[a]	24.90 ± 1.66[b]	25.20 ± 1.33[b]
HEMv 800 mg/kg					
Body weight (g)	159.50 ± 3.12	202.30 ± 2.20	232.80 ± 2.60	231.80 ± 2.60	278.30 ± 4.71
Commulative weight gain (g)	–	42.80 ± 4.18	73.40 ± 4.70	99.60 ± 6.40	111.50 ± 5.24[a]
Water consumption (ml)	–	58.90 ± 3.38	61.20 ± 3.35	63.80 ± 1.99[a]	59.80 ± 2.35[a]
Food intake (g)	–	38.10 ± 1.08	38.80 ± 0.83	37.80 ± 1.26	37.50 ± 1.16
Feces (g)	–	17.10 ± 0.62	18.60 ± 0.67	17.90 ± 0.50	18.40 ± 0.97
Urine (ml)	–	23.30 ± 1.95	26.80 ± 1.86[b]	31.10 ± 1.22[c]	29.30 ± 1.52[c]

Values represent mean ± S.E.M. for $n = 6$ animals/group. One way ANOVA followed by Student–Newman–Keuls test.
[a] $p < 0.05$ compared to the vehicle control group.
[b] $p < 0.01$ compared to the vehicle control group.
[c] $p < 0.001$ compared to the vehicle control group.

feces and urine are shown in Table 1. On these parameters, there was no statistically significant difference between the body weight of the animals treated with HEMv at doses of 50 and 200 mg/kg, compared to the negative control group (vehicle). However, at the highest dose (800 mg/kg), it significantly reduced the increase in body weight in the D_4 by (13.96%, $p < 0.05$) period compared with the vehicle group (129.60 ± 7.32 g). Similarly, treatment of animals with HEMv in smaller doses (50 and 200 mg/kg) did not change significantly water intake at any time, only equally at dose of 800 mg/kg at periods D_3 and D_4 (19.47% and 11.98%, $p < 0.05$) compared to the vehicle group. As for the excretion of feces no significant change was observed except with urine excretion where there was significant increase during periods D_2, D_3 and D_4.

Biochemical profile of HEMv

After 30 days of oral treatment with HEMv (50, 200 and 800 mg/kg, *p.o.*), biochemical analysis showed the absence of significant changes in serum concentrations of uric acid, albumin, alkaline phosphatase, total cholesterol, glucose, AST, ALT, triacylglyceride and urea. As can be seen in Table 2, only the serum creatinine levels were increased by 11.93% ($p < 0.05$) at higher dose of 800 mg/kg, compared to vehicle group (62.00 ± 2.62 mg/dl).

Table 2
Effect of sub-chronic orally administration of hydroethanolic extract of *Mandevilla velame* xylopodium (HEMv) for 30 days on the biochemical parameters in male rats.

Parameters	Vehicle	HEMv (mg/kg, p.o.)		
		50	200	800
Uric acid (mg/dl)	1.45 ± 0.23	1.24 ± 0.17	1.25 ± 0.16	1.45 ± 0.30
Albumin (g/l)	2.57 ± 0.05	2.47 ± 0.11	2.47 ± 0.10	2.46 ± 0.09
Alkaline phosphate (U/l)	130.00 ± 3.46	121.00 ± 5.42	118.00 ± 7.71	115.00 ± 4.51
Total cholesterol (mg/dl)	80.80 ± 6.10	78.20 ± 7.74	80.20 ± 3.68	86.50 ± 5.90
Creatinine (mg/dl)	62.00 ± 2.62	58.80 ± 3.30	58.70 ± 1.20	69.40 ± 2.90[a]
Glocuse (μg/dl)	101.70 ± 8.74	110.80 ± 7.03	98.40 ± 3.41	113.30 ± 8.50
Protein (g/dl)	6.67 ± 0.10	6.62 ± 0.12	6.88 ± 0.09	6.98 ± 0.180
Oxaloacetic transaminase (U/l)	127.30 ± 5.52	124.10 ± 8.94	120.10 ± 9.67	121.00 ± 3.88
Pyruvic transaminase (U/l)	50.80 ± 6.61	56.50 ± 9.48	47.30 ± 0.94	57.00 ± 2.76
Triacylglycerides (mg/dl)	66.10 ± 7.50	72.50 ± 12.90	74.30 ± 8.23	79.30 ± 14.50
Urea (mg/dl)	30.10 ± 1.05	34.50 ± 1.26	33.50 ± 6.00	33.70 ± 2.13

Values represent mean ± S.E.M. for n = 6 animals/group. One way ANOVA followed by Student–Newman–Keuls test.
[a] $p < 0.05$ compared to the vehicle control group.

Table 3
Effect of sub-chronic orally administration of hydroethanolic extract of *Mandevilla velame* xylopodium (HEMv) for 30 days on hematological parameters in male rats.

Parameters	Vehicle	HEMv (mg/kg)		
		50	200	800
Erythrocytes ($\times 10^6/\mu l$)	6.95 ± 0.24	7.12 ± 0.19	7.05 ± 0.11	7.13 ± 0.18
Hemoglobin (g/dl)	14.40 ± 0.42	14.40 ± 0.20	14.40 ± 0.15	14.90 ± 0.29
Hematocyte (%)	41.80 ± 1.36	42.20 ± 0.92	41.90 ± 0.40	42.40 ± 0.74
Total platelets ($\times 10^3/\mu l$)	1056.00 ± 40.60	1118.00 ± 58.10	1020.00 ± 44.5	1053.00 ± 64.0
Total leukocytes ($\times 10^3/\mu l$)	7.69 ± 0.45	8.11 ± 1.02	8.22 ± 0.61	8.78 ± 1.14
Neutrophils (%)	25.90 ± 1.32	27.70 ± 2.00	29.90 ± 2.35	36.60 ± 2.32[a]
Lmyphocytes (%)	69.50 ± 1.39	68.60 ± 2.10	66.20 ± 1.63	59.60 ± 1.58[a]
Monocytess (%)	0.38 ± 0.10	0.37 ± 0.10	0.33 ± 0.08	0.34 ± 0.12
Eosinophils (%)	3.10 ± 0.31	2.70 ± 0.23	2.90 ± 0.32	2.94 ± 0.18
Basophils (%)	1.01 ± 0.19	0.93 ± 0.16	0.90 ± 0.17	0.96 ± 0.19

Values represent mean ± S.E.M. for $n = 6$ animals/group. One way ANOVA followed by Student–Newman–Keuls test.
[a] $p < 0.01$ compared to the vehicle control group.

Haematological analysis

Table 3 shows the effect of treatment with HEMv at doses of 50, 200 and 800 mg/kg, compared to vehicle group on haematological parameters. On these parameters, only the percentage of neutrophils (41.30% increased) and lymphocytes (14.20% decreased) on HEMv treated group at a dose of 800 mg/kg differed significantly from vehicle group ($p < 0.01$). The other haematological parameters showed no statistically significant change.

Anatomical and histopathological analysis

Subchronic treatment of rats with HEMv (50, 200 or 800 mg/kg) did not cause macroscopic changes in the major organs like kidneys, stomach, liver, lungs, heart and brain. In histopathological examination two of the six animals that received the vehicle and two of the six animals in each group receiving the HEMv (50, 200 and 800 mg/kg, *p.o.*) showed multifocal pulmonary edema with peribronchial lymphocytic infiltrate. In other organs (heart, liver, stomach, spleen and kidneys) no significant histological changes in the animals receiving HEMv were observed (data not shown).

Discussion

One of the most relevant aspects of pre-clinical validation of an herbal remedy widely used by the population is the safety of these products. In order to evaluate the toxicity of HEMv, two tests were conducted: cell cytotoxicity test in CHO-K1 cells and sub-chronic toxicity in rats.

The cytotoxic profile of therapeutic agents is important in view of the fact that many drugs can cause irreversible damage on the cellular level. This is one of the tests recommended to be carried out on plant extracts or its derivatives by the agency that regulates registration of drugs in Anvisa (2013). Results from this study showed that HEMv at different tested concentrations (3.125–200 μg/ml) did not alter the cell viability of CHO-K1 lineage's ability to reduce Alamar blue®, indicating that the HEMv does not have cytotoxic potential. Even though HEMv has been shown to be non-cytotoxic *in vitro*, a single *in vitro* toxicity study cannot be used as conclusive evidence that an agent is not toxic.

Acute toxicity studies in animals are usually necessary for any pharmaceutical intended for human use (Balogun et al., 2014). The information obtained from these studies is useful in choosing doses for repeat-dose studies, providing preliminary identification of target organs of toxicity, and, occasionally, revealing delayed toxicity (OECD, 2001).

Our research group has been published preliminary acute toxicity study of HEMv (Ribeiro et al., 2010). It was demonstrated that single oral administration of high-dose in mice (greater than 2000 mg/kg doses) caused significant adverse reactions like signs of central depression through decreased motility and respiratory rate, increased passivity, moderate analgesia and loss of seizure of the legs. The higher dose at 5000 mg/kg of HEMv culminated in the death of 2/3 animals. In the same study was also determined the LD_{50} of HEMv in mice which has been presented relatively high value (4176.0 ± 218.5 mg/kg, p.o.).

Brazilian law classifies natural products in five classes: highly toxic products (I), toxic products (II), the product of average toxicity (III), low-toxicity products (IV) and virtually non-toxic products (V). Therefore, from the data obtained, it can be assumed that HEMv falls in "Plant Protection Products Class IV," according to Ordinance No. 04 - DISAD/MS April 30, 1980, which ranks as products of low toxicity formulations oral LD_{50} present that between 4000 and 6000 mg/kg *p.o.* (Garcia et al., 2005).

Pre-clinical evaluation of drug safety and plant-derived products is performed in animals, particularly in rats due to good correlation between rat and human being toxicological effects, as a lower correlation is observed between human and mice (Clark and Steger-Hartmann, 2018). Numerous studies relayed on investigating the acute effects of high doses in mice and chronic effects of lower doses in rats, including potentially useful doses in humans (Mu et al., 2011).

Considering that *M. velame* has been widely used by communities that inhabit the Midwest of Brazil for various medical purposes, primarily in the therapy of acute and chronic inflammatory processes (Rodrigues and Carvalho, 2001; Ribeiro et al., 2010) and that its use generally lasts for a long period and that there are still no studies that demonstrate safety in long-term treatment, the study of the sub-chronic toxicity of HEMv in rats, which is backed of by National Health Surveillance Agency (ANVISA), which provides a guide for conducting toxicity studies pre-clinical herbal was conducted, which is oriented so that the sub-chronic administration of the trial substance to be tested is administered by the same route used by the population and the treatment period is not less than 30 days (Anvisa, 2013).

In order to assess the effect of repeated and non-lethal doses of HEMv (50, 200 and 800 mg/kg) sub-chronic toxicity test was performed in rats. Based on the acute toxicity and lethal dose experimental results these doses were selected for sub-chronic studies which assess the undesirable effects of continuous or repeated exposure of plant extracts or compounds over a portion of the average life span of experimental animals, such as rodents. Specifically, they provide information on target organ toxicity and are designed to identify no observable adverse effect level (NOAEL). Sub-chronic evaluation can also help to determine appropriate dose regimens for longer-term studies (NRC, 2006). Consequently, in this study the three geometric doses of HEMv were administered for 30 days, assessing the behavioural, physiological (weight gain, water and feed intake, and feces and urine excretions), haematological, biochemical, anatomical and histopathological changes in target organs. In this study, no deaths or clinical signs and symptoms of toxicity were observed in the animals at any of the doses tested, except for relative heart weight and creatinine levels.

Changes in body weight have been used as an indicator of side effects of drugs and chemicals (Santos, 2000). In the sub-chronic study between clinical parameters evaluated, there were significant changes ($p < 0.05$) on body weight gain and water consumption by rats treated orally with HEMv, but was present only at higher dose (800 mg/kg), which was neither persistent, nor dose-dependent manner. These signs were not accompanied by observable changes in biochemistry, macroscopic analysis of organs of animals and neither on the histopathological evaluation, indicating that these two changes have little or no toxicological relevance (Mu et al., 2011).

Administration of HEMv causes an increase in urination similar to a green tea effect reported by Oz (2017). Interestingly, HPLC analysis revealed that HEMv is rich in phenolic constituents such as flavonoids and phenolic acids, including catechin and epigallocatechin gallate, the most prevalent and bioactive polyphenol in green tea.

With regard to the excretion of urine, it was found that doses of the HEMv promoted a diuretic effect in a dose-dependent from the 2nd week. To elucidate this result makes it necessary to conduct additional studies for evaluate the mechanism involved in increasing urination.

In biochemical evaluation the only change observed was an increase in creatinine levels of the animals treated with the highest dose of HEMv. However, although statistical significance compared to the vehicle group, it is apparent that in agreement with previous studies by Baracho et al. (2015) creatinine levels were observed in the physiological patterns for rats (0.30 ± 0.10 mg/dl). Moreover, in our histopathological analysis no renal abnormalities were observed.

In haematological analysis the only significant changes were related to the number of neutrophils and lymphocytes. It was found that rat receiving HEMv at a dose of 800 mg/kg showed increased numbers of neutrophils numbers than in the vehicle control group, indicating a possible inflammatory process. On the other hand, the treatment of HEMv at a dose of 800 mg/kg decreased the numbers of lymphocytes compared to the control group. It is unclear whether the splenic changes reflect a decreased production of cells or a reduced cellular life span (De Porto et al., 2010).

The analysis of rats of relative weight and macroscopic anatomical aspects of vital organs showed no significant change. Therefore, this result was judged to be of absent of toxicological significance, since no remarkable treatment related histological alterations in these two organs were observed (Tasaki et al., 2008).

Histopathological analysis revealed the presence of nonspecific chronic inflammation, found in the lungs of some animals of both vehicle and treated HEMv groups. However, these alterations seem not related with drug treatment, since similar observations were noticed in the vehicle group. This type of observation is not usual in rats subjected to this type of experiment. This anomaly may be explained as follows: (i) probably have been due to inhalation of inspired air by these animals, (ii) this type of injury has been reported to occur spontaneously in rats (Tasaki et al., 2008; Beserra et al., 2011), and (iii) there were no alterations of clinical relevance in the biochemical parameters related to kidney function. This observation is therefore of no toxicological effect relevance. Generally, the few clinical signs and symptoms observed after administration and the few changes observed in some biochemical and haematological parameters in the sub-chronic toxicity test do not seem to indicate toxicity of HEMv. Therefore, the use of appropriate levels of the HEMv as a traditional medicine remedies should have a wide margin of safety for its therapeutic use.

The NOAEL dose, which consists of the highest level of exposure to a substance where there is no statistically or biologically increased frequency or severity of adverse effects (Park and Cho, 2011) for HEMv was 800 mg/kg/day in rats.

Traditional populations of the Brazilian *Cerrado* use medicinal decoction or infusion made with approximately 10 g of dried *M. velame* xylopodium in 1 l of boiled water, corresponding to 2.6 mg/ml in extractive terms (Ribeiro et al., 2017). Considering the dose of 1 shallow cup of tea ($\tilde{2}$00 ml) 4–5 times a day, it can be inferred that an adult weighing 60 kg consumes 2080–2600 mg/kg of HEMv, equivalent to a daily dose of 34.34–43.33 mg/kg/day. Thus, the maximum dose in rats (800 mg/kg) was about 23.29 times higher than ingested by humans, suggesting that the traditional population consumes safety dose of tea.

The results of cytotoxicity test and sub-chronic demonstrated that oral administration of HEMv low dose ($\leq$200 mg/kg), acutely or over an extended period is safe and has low toxicity. HPLC fingerprint of the HEMv demonstrated pharmacologically important phytochemicals, namely, the phenolic acids (gallic acid and ellagic acid), flavonoids (catechin, epigallocatechin gallate, naringin, myricetin, quercetin and naringenin). In the previous study reports, ellagic acid and gallic acid have antigenotoxic activity in comet and micronucleus models and no sub-chronic toxicity (Niho et al., 2001; Tasaki et al., 2008; Rehman et al., 2012; Silva et al., 2017). Naringin is widely distributed in plant foods and has not previously been evaluated for safety through standard *in vivo* toxicological studies. It is practically non-toxic for rats in oral acute toxicity study and the NOAEL of naringin in rats is greater than 1250 mg/kg/day when administered orally for 13 consecutive weeks (Li et al., 2013).

The extensive investigations into health impacts of flavonoid/phenolic, their mechanisms of action have garnered considerable interest. Because flavonoids are polyphenolic and are able to quench free radicals, they have commonly been termed antioxidants (Corcoran et al., 2012). It is well known that such flavonoid/phenolic compounds have many potential activities, *e.g.* antimicrobial, anti-inflammatory and anti-allergic activities (Mierziak et al., 2014). Several studies have addressed the potential toxicity of flavonoid/phenolics, and have been shown to be non-toxic even at high doses for treatment of hepatobiliary dysfunction and digestive complaints, such as sensation of fullness, loss of appetite, nausea, and abdominal pain (Galati and O'Brien, 2004); Spencer et al., 2009; Kim et al., 2011). To the best of our knowledge, there is no available information in the literature citing in details, the presence of these phytochemical substances from the xylopodium of HEMv.

Conclusion

The HEMv presented phenolic and flavonoid compounds as main secondary metabolites. Based on the toxicological tests evaluated in this present study and literature data, HEMv has no toxicological potential in rodents. In *in vivo* model, sub-chronic results indicate that HEMv is safe in rats at a NOAEL of oral dose of 800 mg/kg b.w./day for 30 days. However, the doses which are popularly used in infusion can be consumed safely because they did not show any relevant cytotoxic, acute or sub-chronic effects in rats. The results presented in this study suggest that promising alternatives phytotheraputic drug for treatment of common ailments, especially those were indicated for *Mandevilla velame* by traditional community.

Author's contributions

RVR participated in the design of the experiments, conducted the experimental work and the data analysis, and contributed to manuscript writing. DBM contributed in the phytochemical analysis. KA participated in the experimental work and the data analysis, and contributed to manuscript writing. IMS contributed in the phytochemical studies and data analysis. RWSA contributed in the phytochemical experimental work and data analysis. SDA contributed in the phytochemical experimental work, data analysis and manuscript writing. MR contributed the histopathological study; EMC contributed to the histopathological study and data analysis. DTOM designed the study, supervised the work, discussed the results, data analysis and contributed to the manuscript writing.

Ethical disclosures

Protection of human and animal subjects. The authors declare that the procedures followed were in accordance with the regulations of the relevant clinical research ethics committee and with those of the Code of Ethics of the World Medical Association (Declaration of Helsinki).

Confidentiality of data. The authors declare that no patient data appear in this article.

Right to privacy and informed consent. The authors declare that no patient data appear in this article.

Conflicts of interest

All authors declare no conflicts of interest.

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