Gastroprotective and anti-inflammatory activities integrated to chemical composition of *Myracrodruon urundeuva* Allemão - A conservationist proposal for the species

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A B S T R A C T

Ethnopharmacological relevance: Myracrodruon urundeuva Allemão (Aroeira-do-Sertão), Anacardiaceae, is one of the most used plants in folk medicine in Northeastern Brazil as an anti-inflammatory, healing and antiulcer. This species is threatened with extinction due to anthropogenic exploitation. The importance of this study is to demonstrate the feasibility of a conservationist model of replacement of the *M. urundeuva* adult tree (inner bark) for its under developing plants (shoots) in order to ensure the preservation of this species, but also to ensure sufficient raw material for pharmaceutical purposes.
Aim of the study: To characterize chemically and assess the gastroprotective and anti-inflammatory activities of the fluid extracts from *M. urundeuva* innebark (adult plant) as well as stem and leaves of shoots (young plant).
Materials and methods: The fluid extracts were prepared by maceration-percolation with hydroalcoholic solution according to the methodology described in the Brazilian Pharmacopoeia. These extracts were cleaned-up through solid phase extraction (SPE) and chemically characterized by ultra-performance liquid chromatography coupled to mass spectrometry (UPLC-ESI-QTOF MS/MS). Gastroprotective and anti-inflammatory activities of the extracts (700 or 1000 mg/kg) were assessed on ethanol-induced gastric lesions and Croton oil-induced ear edema in rats, respectively. The extracts were evaluated for cytotoxicity in vitro.
Results: The UPLC-ESI-QTOF-MS/MS analysis evidenced the presence of chalcones, flavonoids and tannins. Gastroprotective and anti-inflammatory activities achieved with fluid extracts from the stems and leaves was similar to inner bark. The fluid extracts were not toxic.
Conclusion: It is possible to replace the inner bark of the adult tree for the stems and leaves from the shoots as raw material to be used in the preparation of its the phytotherapeutics. Therefore, this finding may help in the implementation of public policies that ensure the conservation of the species along with its sustainable use for pharmaceutical purposes.

1. Introduction

Ethnopharmacological studies have shown that in Northeast Brazil, *Myracrodruon urundeuva* Allemão (aroeira-do-sertão), Anacardiaceae, is one of the most used plants in folk medicine because of its anti-inflammatory, healing, and antiulcer properties (Bandeira et al., 2013; Carlini et al., 2010; Souza et al., 2007; Matos, 2007; Rodrigues et al., 2002; Braga, 1976). Furthermore, its innerbark has been used to prepare two phytotherapeutics - the elixir and vaginal ointment of aroeira - that are widely produced and used in Farmácias Vivas (Mourad et al., 2015), a Brazilian phytotherapy program.

However, *M. urundeuva* is threatened with extinction (National

Center for Plant Conservation, 2012), because of the predatory extractivism of its innerbark for medicinal purposes and the use of its wood in carpentry. Reports on the oldest community of the District of Guassussê, Orós, Ceará, Brazil, have recorded that in the 1960s, many of these trees were exterminated to use their wood as railway sleepers (Honório, 2000). It is noteworthy that in this part of the caatinga, is the Orós dam, which was under construction in 1960, when a partial collapse of the dam caused a great flood - Orós deluge. The flood left several villages completely flooded and without communication. The most famous village among them in the District of Guassussê (an indigenous term for what is born of the waters) is Sítio Aroeiras (Honório, 2006), an allusion to the species *M. urundeuva*. The plant material used in the present study was collected from Sítio Aroeiras, as a way of preserving the living memory of this place.

The species *M. urundeuva* exhibits secondary growth, based on which Bandeira (2002) proposed the following premise: "the tissues of innerbark and of the shoots are in intense metabolic activity; therefore, they must produce the same chemical constituents and show similar pharmacological actions". To prove this premise and to make a proposition to conserve this species, based on the aforementioned ethnopharmacological information, agronomic studies have been carried out integrating the pharmacological and chemical studies. These studies demonstrated that the cultivated species (shoots of height 40 cm) maintains its genetic characteristics regarding the pharmacological activity and produces qualitatively the same pharmacologically active constituents of the inner bark, the dimeric chalcones urundeuvines A, B, and C (Fig. 1) (Bandeira et al., 2003) and tannins (Souza et al., 2007).

Further, the flavonoids quercetin, aromadendrinole and agathisflavone (Fig. 2) (Bandeira, 2002) were identified in the leaves of 40-cm-tall shoot. It is known that the flavonoids and chalcones have a common biosynthetic precursor and both exhibit anti-inflammatory activity (Cecilio et al., 2016), which demonstrates the perspective of medicinal use of the shoot leaves.

In this context, the aim of this study is to evaluate and compare the gastroprotective and anti-inflammatory activities of the fluid extracts, prepared from the innerbark, stems and leaves of the shoots, providing a scientific basis for substituting the inner bark with shoot of *M. urundeuva* in the preparation of phytotherapeutics.

2. Materials and methods

2.1. Plant Material

The inner bark and shoots (including stems and leaves) of *M. urundeuva* were evaluated in the present study. The inner bark was collected from the District of Guassussê, Orós County, Ceará State, Brazil (6°20′28.0′S 38°59′57.2′W –6.34109779, –38.99921983). The shoots were propagated through seeds collected from selected adult tree matrixes found in the abovementioned locality. They were cultivated in flowerbeds with a spacing of 0.5 m × 0.8 m, in February 2016, at the Núcleo de Fitoterápicos (3°49′23.1′S, 38°29′26.0′W –3.823096,

–38.490564), Fortaleza, Ceará. The shoots were harvested in June 2016. The selection of matrixes was necessary, because the species exhibits a dioecious reproduction, which is common in plants growing in tropical environments. The plants are characterized by the presence of individuals of separate sexes in the population of a species. Dioecy, as well as other types of reproduction in plants that exhibit sexual dimorphism, has been interpreted as a mechanism to increase genetic variability and reduce endogamic depression in the population (Rolim et al., 2016; Fuzato et al., 2001). The voucher specimen of the adult (wild) and cultivated plant material were identified and deposited at Prisco Bezerra Herbarium (Federal University of Ceará) with the numbers 14.199 and 14.810, respectively.

The average height of the shoot sampled was 40–50 cm (Calou et al., 2014; Bandeira, 2002). Before drying, the stems was manually separated from the leaves. The inner bark, stems, and leaves were dried separately in an oven with air circulation at 40 °C (Calou et al., 2014; Bandeira et al., 2013), manually reduced to smaller pieces, and ground in a mill to obtain a powder of moderate particle size (Brazilian Pharmacopeia, 2010).

2.2. Extracts preparation

The preparation of fluid extract of innerbark (FEI), stems (FES), and leaves (FEL) was performed according to the method recommended by the Brazilian Pharmacopeia (Brazil, 2010). The dried material (180 g) was grinded and subjected to two sequential extractions. Initially, a mixture of ethanol/water (H_2O)/glycerol (6:4:0.5, v/v) was used for maceration of the dried material for 6 h in a percolator, to obtain 144 mL of extractive solution. The residue was then subjected to a second extraction with a mixture of ethanol/H_2O (6:4, v/v), up to exhaustion. The resulting liquid of this last extraction was evaporated in a water bath at 60 °C to a syrupy consistency. This syrup was then added to hydroalcoholic solution of the first extraction and the volume was made up to 180 mL with hydroalcoholic solution (6:4, v/v).

Additionally, a compound extract was prepared by mixing FES and FEL in the ratio of 1:1 (FESL), for evaluation of gastroprotective activity.

2.3. Chemical characterization by ultra- performance liquid chromatography coupled to mass spectrometry (UPLC-ESI-QTOF MS/MS)

All fluid extracts (1 mL) were cleaned up by solid phase extraction (SPE) using polymeric resin-based cartridges (Strata-X® 500 mg/3 mL; Phenomenex) and eluted with 75% methanolic solution acidified with glacial acetic acid (pH = 3.0). The samples were then filtered through 0.22 μm PTFE syringe filters (Allcrom®).

The chemical characterization was performed using an Acquity ultra-performance liquid chromatography (UPLC) chromatograph coupled to a Xevo quadrupole and time-of-flight mass spectrometers (UPLC-Q-TOF, Waters®). The chromatographic separation was achieved on a Waters Acquity UPLC BEH column (ID 150 mm × 2.1 mm, 1.7 μm
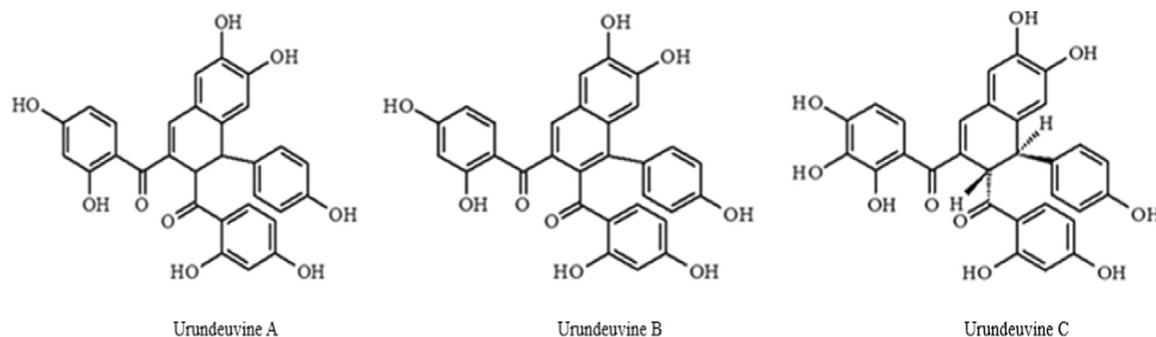

Fig. 1. Chemical structures of dimeric chalcones isolated from *M. urundeuva* Allemão – Urundeuvines A, B and C. Source: Bandeira et al., 2003.
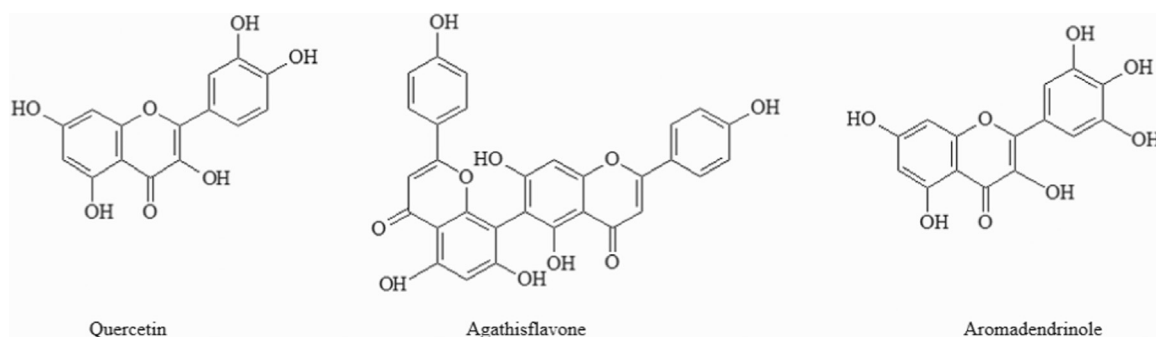
Fig. 2. Chemical structures of flavonoids isolated from the leaves of a 40-cm-tall shoot of *Myracrodruon urundeuva* Allemão – Quercetin, Agathisflavone e Aromadendrinole. Source: Bandeira, 2002.

diameter) at 40 °C. The sample (5.0 μL) was eluted with a mobile phase composed of water (A) and acetonitrile (B), both acidified with 0.1% of formic acid, with the following elution gradient: 2–95% of B (0–15 min), 100% of B (15.1–17 min), and equilibrating with 2% of B (17.1–19.1 min) at a flow rate of $0.4 \, mL \, min^{-1}$. The ionization was performed using an electrospray ionization source in a negative mode at 120 °C with a desolvation gas flow rate of $500 \, L \, h^{-1}$ at 350 °C, extraction cone of 0.5 V, and capillary voltage of 2.6 kV. Leucine enkephalin was used as the lock mass. The mass spectra (MS) were acquired in the range of 110–1180 Da in MS^E mode. The UPLC-Q-TOF system was managed by MassLynx 4.1 software (Waters®). The extract constituents were tentatively characterized using the molecular formula provided by MassLynx 4.1 software based on their accurate masses (error < 5 ppm) and isotopic patterns (i-fit). Subsequently the compounds were annotated based on the MS fragmentation pattern and previous reports of their occurrence in the same species or botanical family. Furthermore, the compounds were assigned by comparison with authentic standards (when available) and public MS database (ChemSpider, PubChem, and MassBank and KNapsack). Urundeuvine standards were previously isolated by Bandeira et al. (2003).

2.4. Assessment of the gastroprotective and anti-inflammatory effects of fluid extracts from Innerbark (FEI), Stem (FES) and Leaves (FEL) of shoots

2.4.1. Animals
Swiss albino mice (20–25 g) from the experimental animal facility at the University of Fortaleza (UNIFOR) were kept in a controlled environment (circadian cycle, 22 °C) with free access to water and standard pellet diet (Purina, São Paulo, Brazil). Prior to the induction of gastric damage, the animals were fasted for 6 h to ensure an empty stomach (water was allowed). The experimental protocols followed the ethical guidelines of CONCEA (Brazilian Council for the Control of Animal Experimentation) and were approved by the UNIFOR Animal Research Ethics Committee and filed under entry number 012/2015.

2.4.2. Gastroprotective activity – gastric injury induced by Ethanol
The mice were divided into six groups (n = 6/each) and pretreated with 0.3 mL (700 or 1000 mg/kg) of the following fluid extracts: inner bark (FEI), stems (FES), leaves (FEL), as well as stem and leaves at a ratio of 1:1 (FESL), besides vehicle (VC) (hydroalcoholic solution (6:4, v/v)), which was used as control group. The inner bark extract, commonly used in traditional formulations, at the doses of 700 or 1000 mg/kg, was considered the positive control (PC). After 30 min of the drug administration, each animal group received 0.2 mL of ethanol (96%) orally. Thirty minutes later on, the animals were euthanized and their stomachs were excised, opened along the greater curvature to observe the injury level macroscopically (Robert et al., 1979). The area of glandular gastric injury was determined using and the software ImageJ® (National Institutes of Health – USA) and the percentage of gastric damage was calculated.

2.4.3. Anti-inflammatory activity – croton oil multiple application-induced mouse ear edema
The mice were divided into six groups (n = 6/each). The chronic inflammatory process was induced by the application of 0.2 mL of Croton oil in acetone (5% v/v) in the right ear. In the left ear only acetone was applied. Both Croton oil and vehicle were applied on alternate days for 9 days (Stanley et al., 1991). From the 5° day, the right ear of animals was treated with 0.2 mL (700 or 1000 mg/kg), twice a day, of the following fluid extracts: inner bark (FEI), stems (FES) and leaves (FEL), besides vehicle (VC) (hydroalcoholic solution (6:4, v/v)), which was used as control group. The inner bark extract, commonly used in traditional formulations, at the doses of 700 or 1000 mg/kg, was considered the positive control (PC). The edema was evaluated at the times 24, 48, 72, 96, 120, 144, 168 and 192 h after the first application of Croton oil, by measuring the thickness of the ears with digital caliper. On day 9, 192 h after the first application of Croton oil, the animals were euthanized and their ears were excised for light microscopy.

2.4.4. Light microscopy
Tissue samples were fixed in 10% neutral-buffered formalin solution, sectioned and embedded in paraffin. Sections of thickness 4 μm were deparaffinized, stained with hematoxylin and eosin and examined under a light microscope (Silva et al., 2010). Subsequently, the sections were evaluated by an experienced pathologist, in a blind evaluation.

2.4.5. Statistical analysis
The results are presented as means ± standard error (S.E.M.) for each group. The data were subjected to one-way or two-way analysis of variance (ANOVA), followed by the Tukey *post-hoc* test. The level of statistical significance was set at 5% ($p < 0.05$).

2.5. Cytotoxicity to HEK-293

The fluid extracts of inner bark (FEI), stems (FES) and leaves (FEL) were subjected to the test of specific cytotoxicity to HEK-293 (human embryonic kidney cells).

2.5.1. Cell line
HEK 293 cell line (normal human embryonic kidney cell line ATCC® CRL-1573) was used for the analysis of cytotoxicity of fluid extracts. Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM - Gibco®-Thermo Fisher Scientific) supplemented with 10% heat-inactivated fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin (Sigma®). Subsequently, the cell line was grown at 37 °C in a humidified atmosphere containing 5% CO_2 (Butler and Dawson, 1992).

2.5.2. Alamar blue cytotoxicity assay
This assay was used to evaluate the cell viability of HEK-293 cells

Table 1
Phytochemical compounds detected and characterized in *M. urundeuva* Inner bark (I), Stem (S) and Leaves (L) by using HPLC-DAD/QTOF-MS in negative ionization modes.

Peak no.	Rt Min	[M-H]⁻ Observed	[M-H]⁻ Calculated	Product Ions (MS/MS)	Empirical Formula	Ppm (error)	i-fit	Putative compound	Plant Part	References
1	1.78	169.0142	169.0142	125.0232 (100)[a]	$C_7H_6O_5$	3.0	0.0	Gallic Acid	I, L	Silva et al. (2011); Sandhu and Gu (2010)
2	2.13	243.0511	243.0505	169.0111 (18)[a]; 124.0139 (30)[a]	$C_{10}H_{11}O_7$	2.5	0.0	GalloylGlycerol	L	Nonaka and Nishioka (1983)
3	2.62	635.0869	635.0884	483.0746 (48)[a]; 465.0702 (16)[a], 169.0151 (80)[a]	$C_{27}H_{23}O_{18}$	−2.4	0.1	Tri-galloyl-hexoside	L	Abu-Reidah et al. (2015), Silva et al. (2011)
4	2.80	483.0769	483.0775	331.0819 (30)[a], 169.0123 (40)[a]	$C_{20}H_{19}O_{14}$	−1.2	1.2	Gallic acid-galloyl hexoside	I	Abu-Reidah et al. (2015)
5	2.86	577.1357	577.1346	425.0832 (50)[a], 407.0836 (80)[a], 289.0668 (100)[a]	$C_{30}H_{25}O_{12}$	0.5	0.6	B-type procyanidin dimer	S	Silva et al. (2017)
6	3.13	183.0297	183.0293	168.0040 (12)[a]; 124.0164 (92)[a]	$C_8H_7O_5$	2.2	0.0	Methyl gallate	L	Abu-Reidah et al. (2015); Silva et al. (2011)
7	3.15	635.0907	635.0884	483.0962 (10)[a], 465.0715 (15)[a], 169.0128 (40)[a]	$C_{27}H_{23}O_{18}$	3.6	1.1	Tri-galloyl-hexoside isomer	I	Silva et al. (2011)
8	3.88	787.1031	787.0994	635.0836 (18)[a], 169.0105 (12)[a]	$C_{34}H_{27}O_{22}$	4.7	3.4	Tetra-O-galloylhexoside	S	Silva et al. (2011)
9	4.17	463.0875	463.0877	301.0323 (42)[a], 300.0237 (54)[a]	$C_{21}H_{19}O_{12}$	0.4	0.0	Quercetin 3-O-glucoside[b]	S	Silva et al. (2011)
10	4.21	939.1085	939.1104	787.0920 (2)[a], 769.0876 (13)[a], 617.0815(3)[a]	$C_{41}H_{31}O_{26}$	2.0	0.4	Pentagalloyl-hexoside Isomer	L	Abu-Reidah et al. (2015); Silva et al. (2011)
11	4.26	441.0818	441.0822	289.0741 (40)[a], 169.0081 (100)[a], 125.0267 (41)[a]	$C_{22}H_{17}O_{10}$	0.9	0.2	Epicatechin gallate[b]	S	Abu-Reidah et al. (2015); Sandhu Gu (2010)
12	4.26	467.0927	467.0919	357.0578 (23)[a], 300.0578 (15)[a], 217.0123 (22)[a], 169.0125 (11)[a]	$C_{31}H_{15}O_5$	1.7	3.4	Gallic Acid derivative I	I	Sandhu and Gu (2010)
13	4.37	939.1142	939.1104	787.1089 (8)[a], 769.0854 (16)[a], 617.0786 (5)[a]	$C_{41}H_{31}O_{26}$	4.0	2.5	Pentagalloyl-hexoside isomer	L	Abu-Reidah et al. (2015); Silva et al. (2011)
14	4.45	939.1161	939.1104	787.1029 (12)[a], 169.0125 (12)[a]	$C_{41}H_{32}O_{26}$	1.0	0.0	Pentagalloyl-hexoside isomer	L	Abu-Reidah et al. (2015); Silva et al. (2011)
15	4.57	1091.1228	1091.1213	939.1082 (100)[a], 787.1124 (05)[a], 769.0859 (13)[a], 617.0868 (3)[a], 599.0796 (02)[a]	$C_{48}H_{35}O_{30}$	1.4	2.0	Hexagalloyl-hexoside	L	Silva et al. (2011)
16	4.62	1091.1240	1091.1213	939.1080 (100)[a], 787.1099 (6)[a], 769.0908 (16)[a], 617.0868 (4)[a], 599.0799 (2)[a]	$C_{48}H_{35}O_{30}$	2.5	1.5	Hexagalloyl-hexoside	L	Silva et al. (2011)
17	4.68	447.0928	447.0927	301.0338 (79)[a]; 300.0222 (97)[a]	$C_{21}H_{19}O_{11}$	0.2	0.0	Quercetin rhamnoside[b]	S	Santos et al. (2012)
18	4.74	467.0988	467.0978	357.0578 (30)[a], 341.0605 (16)[a], 217.0113 (26)[a], 169.0124 (10)[a]	$C_{24}H_{19}O_{10}$	2.1	0.3	Gallic Acid derivative II	I	Sandhu and Gu (2010)
19	4.78	461.0723	461.0720	315.0131 (78)[a]	$C_{21}H_{17}O_{12}$	0.9	0.4	Isorhamnetin O-rhamnoside	S	Abu-Reidah et al. (2015); Iwashina et al. (2012)
20	4.94	939.1126	939.1104	617.0991 (25)[a], 301.0332 (32)[a], 169.0110 (42)[a]	$C_{41}H_{31}O_{26}$	2.3	0.9	Pentagalloyl-hexoside Isomer	L	Abu-Reidah et al. (2015); Silva et al. (2011)
21	5.09	619.1070	619.0994	449.0854 (16)[a], 357.0658 (12)[a], 169.0123 (13)[a]	$C_{20}H_{27}O_{22}$	3.7	3.8	Gallic Acid derivative III	I	Sandhu and Gu (2010)
22	5.45	599.1081	599.1037	447.0975 (2)[a]; 301.0264 (68)[a]	$C_{28}H_{23}O_{15}$	0.3	0.0	Quercetin galloyl-O-deoxy-hexoside	L	Silva et al. (2011)
23	5.84	541.1126	541.1135	519.0967 (10)[a], 387.0849 (22)[a], 169.0125 (14)[a]	$C_{30}H_{21}O_{10}$	−1.7	0.2	Urundeuvine C	I	Bandeira (2002)
24	5.85	583.1100	583.1088	297.0617 (4)[a], 285.0354 (93)[a], 169.0099 (4)[a]	$C_{28}H_{23}O_{14}$	2.1	0.1	Afzelin O-gallate	L	Abu-Reidah et al. (2015); Kirby et al. (2013)
25	5.86	285.0392	285.0399	–	$C_{15}H_9O_6$	2.5	0.1	Luteolin[b]	S	–
26	6.12	525.1183	525.1186	415.0803 (27)[a], 389.1042 (58)[a], 135.0094 (48)[a]	$C_{30}H_{21}O_9$	−0.6	0.0	Urundeuvine A	I, S	Bandeira (2002)
27	6.24	755.2556	755.2551	584.2369 (8)[a], 525.1169 (100)[a], 415.0863 (14)[a], 389.0993 (20)[a], 135.0092 (18)[a]	$C_{38}H_{43}O_{16}$	0.7	0.2	Urundeuvine A derivative	S	Bandeira (2002)
28	6.29	525.1164	525.1186	415.0873 (17)[a], 389.0999 (42)[a], 135.0088 (27)[a]	$C_{30}H_{21}O_9$	−4.2	0.0	Urundeuvine A isomer I	I	Bandeira (2002)
29	6.61	809.3039	809.3021	755.2727 (13)[a], 525.1288 (50)[a], 389.1154 (25)[a], 135.0065 (17)[a]	$C_{42}H_{49}O_{16}$	2.2	1.9	Urundeuvine A derivative	S	Bandeira (2002)
30	6.62	525.1157	525.1186	415.0806 (10)[a], 389.1004 (24)[a], 135.0074 (22)[a]	$C_{30}H_{21}O_9$	−3.8	0.1	Urundeuvine A isomer II	I	Bandeira (2002)
31	6.74	523.1028	523.1029	387.0817 (30)[a], 371.0907 (18)[a], 251.0657 (24)[a], 135.0089 (24)[a]	$C_{30}H_{19}O_9$	−0.2	1.2	Urundeuvine B	I	Bandeira (2002)
32	6.81	327.2110	327.2113	229.1417 (10)[a], 211.1326 (14)[a], 171.1001 (8)[a]	$C_{18}H_{31}O_5$	−0.9	0.0	9,12,13- Trihydroxyoctadecadienoic acid	S, L	Rodríguez-Pérez et al. (2013)

against FEI, FES, and FEL samples. The log-growing cells were seeded in 96-well plates at the density of 20.000 cells per well and incubated for 24 h at 37 °C, with 5% CO_2 and 95% humidity before application of the samples. Samples (FEI, FES, FEL) and the vehicle (hydroalcoholic solution (6:4, v/v)) were pre-filtered on 0.22 μm membranes and an aliquot of stock solution from each sample was diluted in DMEM culture medium. The concentrations tested ranged from 1500 μg/mL to 0.73 μg/mL. For cell growth control, only the complete medium was used. After application of the samples, the plates were incubated for 72 h at 37 °C, with 5% CO_2 atmosphere and 95% humidity. Four hours before the end of the incubation period, 10 μL/well of Alamar blue solution (0.312 mg/mL Sigma®) was added. At the end of the 72 h, fluorescence was measured by ELISA reader (BioTek Synergy HT) using excitation wavelength at 530–560 nm and emission at 590 nm. The following formula, considering relative fluorescence units (RFU), was used to calculate the cell viability (%): Viability (%) = (RFUsamples/ RFUcontrol) × 100.

Non-linear regression was used to obtained the inhibition concentration required to produce a 50% reduction in cell viability (IC_{50}). The cytotoxicity potential (CTP) of the sample was classified according to Fadeyi et al. (2013) into (a) Toxic ($IC_{50} \leq 30$ μg/mL), b) Nontoxic ($IC_{50} > 30$ μg/mL).

3. Results

3.1. Chemical characterization by ultra- performance liquid chromatography coupled to mass spectrometry (UPLC-ESI-QTOF MS/MS)

Table 1 presents a list of 34 compounds tentatively identified by UPLC-ESI-QTOF MS/MS (negative mode) associated with their respective retention times (RT), accurate masses, (mass error in ppm), molecular formula and isotopic pattern (i-fit) as well as the MS/MS fragment ions and the bibliographic references surveyed for the chemical characterization of the fluid extracts from the innerbark, shoot stems and shoot leaves of *M. urundeuva*.

All extracts were basically constituted by polyphenols (29 compounds), including gallic acid derivatives, flavonoids, chalcones, and two hydroxy-fatty acids. The inner bark extract contained 12 components, while extracts from shoot stem and leaves comprised 11 and 15 compounds, respectively. Although the chemical profile of the three extracts was almost similar in terms of chemical classes, only three compounds were detected in at least two of them simultaneously: gallic acid (**1** - innerbark and shoot leaves extracts), urundeuvine A (**26** - innerbark and shoot stem extracts), and trihydroxy-octadecenoic acid (**33** and **34** - all the extracts).

Gallic acid derivatives were the main components, which were found in both free and esterified forms with sugar units (hydrolyzable tannins or gallotannins) and flavonoids. Gallic acid (**1**) showed a typical precursor ion at *m/z* 169.0142 and was found in the inner bark and shoot leaves. However, its methyl ester (**6**) and glyceryl (**2**) derivatives were observed only in the shoot leaves. Compound **1** was confirmed by comparing with an analytical standard of gallic acid. The galloylation degree of the hydrolyzable tannins ranged from 2 to 6 galloyl moieties depending on the part of the plant These compounds were characterized by neutral loss of gallic acid and galloyl moiety (170/152 Da) and also sugar residues (162 Da for glucose and 146 for rhamnose). The inner bark presented di- and trigalloylated gallotannins assigned to ions at *m/z* 483.0769 (**4**) and 635.0907 (**7**). The [M-H]- ions of the shoot leaves were observed at *m/z* 635.0869, 787.1031, 939.1142, 939.1161, 1091.1228 and 1091.1240, corresponding to tri-galloyl-hexoside (**3**), tetra-galloyl-hexoside (**8**), penta-galloylhexoside (**13** and **14**) and hexagalloyl-hexoside (**15** and **16**), respectively.

In addition to hydrolyzable tannins, a condensed tannin was also detected in the young stems as a B-type procyanidin dimer with the precursor ion at *m/z* 577.1357 (**5**) along with fragment ions at *m/z* 425 and 407. These fragments are generated from the breakage of

Table 1 (*continued*)

Peak no.	Rt Min	[M-H]⁻ Observed	[M-H]⁻ Calculated	Product Ions (MS/MS)	Empirical Formula	Ppm (error)	i-fit	Putative compound	Plant Part	References
33	7.02	537.0837	537.0822	479.2250 (15)[a], 375.0480 (22)[a], 327.2154 (35)[a], 301.0358 (20)[a], 211.1073 (10)[a], 183.0395 (12)[a], 169.0136 (52)[a], 124.0086 (21)[a]	$C_{30}H_{17}O_{10}$	2.8	1.3	Agathisflavone	L	Bandeira (2002)
34	7.23	329.2300	329.2328	229.1426 (13)[a], 211.1311 (21)[a], 171.0981 (18)[a]	$C_{18}H_{33}O_{5}$	− 2.4	0.0	9,12,13-trihydroxyoctadecaenoic acid	I, S	Farag et al. (2015)

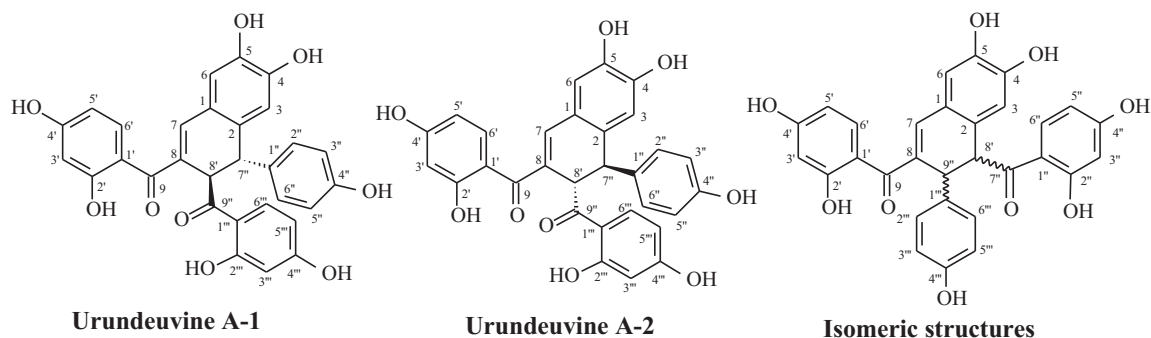[a] Relative intensity (%).
[b] Sta (standard).

Fig. 3. Isomeric structures of urundeuvine A.

interflavan bond following a retro Diels-Alder rearrangement, yielding a fragment at *m/z* 289, which is typical of (*epi*)catechin (Silva et al., 2017).

The dimeric chalcones urundeuvines A (**26**), B (**31**) and C (**23**) were observed at RT 6.12, 6.74 and 5.84 min in the UPLC-MS chromatogram of the inner bark extract, respectively. These substances were isolated earlier from the *M. urundeuva* inner bark by Bandeira et al. (2003) and employed as analytical standards to confirm their presence. Furthermore, two novel urudeuvine A isomers were identified in the inner bark, with their peaks were observed at different RTs (6.29 and 6.62 min). However, their high-resolution electrospray ionization mass spectra (HRESIMS) were identical, differing marginally only in the intensity of the fragment ions. Fig. 4 represents fragmentation mechanisms that reinforces the assumption of stereoisomerism of urundeuvine A: urundeuvine A-1 [(*S*)-2,4-dihydroxybenzoyl and (*S*)-*para*-hydroxyphenyl] and A-2 [(*R*)-2,4-dihydroxybenzoyl and (*R*)-*para*-

hydroxyphenyl] as *trans* isomers (Fig. 3), along with the isomers exchanging the substituents at the carbons 7′ and 8″. Also, Figs. 5 and 6 represents fragmentation mechanisms of urundeuvines B and C, respectively.

In the present study, urundeuvine A (**26**, $C_{30}H_{21}O_9$) was also found in the shoot (stem). Furthermore, to the best of our knowledge, the present study is the first to report two urundeuvine A derivatives: a peak at 6.24 min with *m/z* 755.2556 (**27**) and other one at 6.61 min with *m/z* 809.3018 (**29**). The fragmentation proposal has been depicted in the Fig. 7.

The flavonoids were found exclusively in the shoots (Table 1). They were present in many forms: galloylated, glycosylated, dimerized, and free. The UPLC-MS chromatogram of the leaves extract exhibited precursor ions [M-H]⁻ at *m/z* 599.1081, 583.1100 and 537.0837 corresponding to quercetin galloyl-*O*-deoxy-hexoside (**22**), afzelin-*O*-gallate (**24**) and agathisflavone (**33**), respectively. In the stem, quercetin-3-*O*-
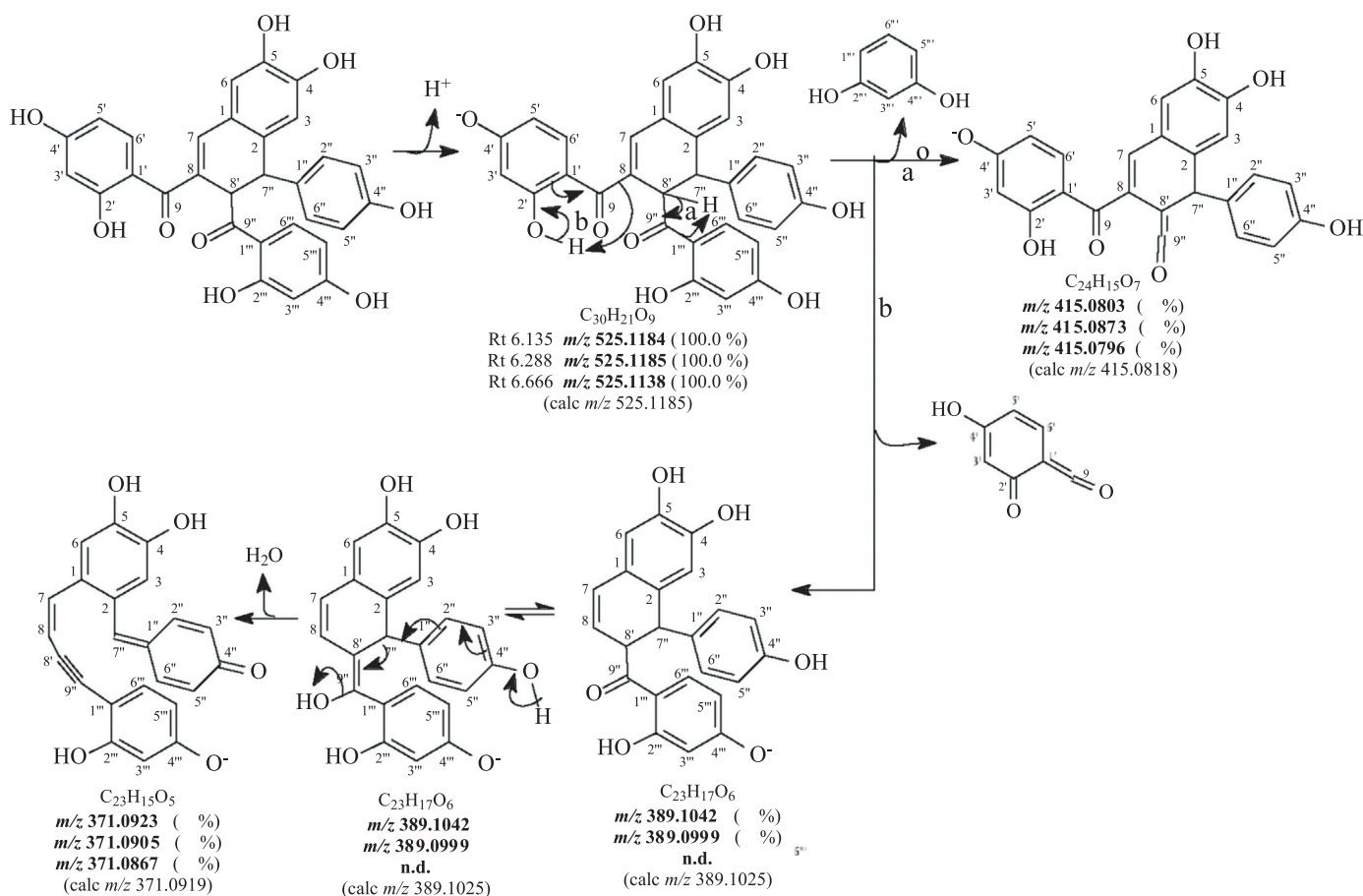

Fig. 4. Proposed fragmentation mechanisms of urundeuvines A and its isomers urundeuvines A-1 and A-2.
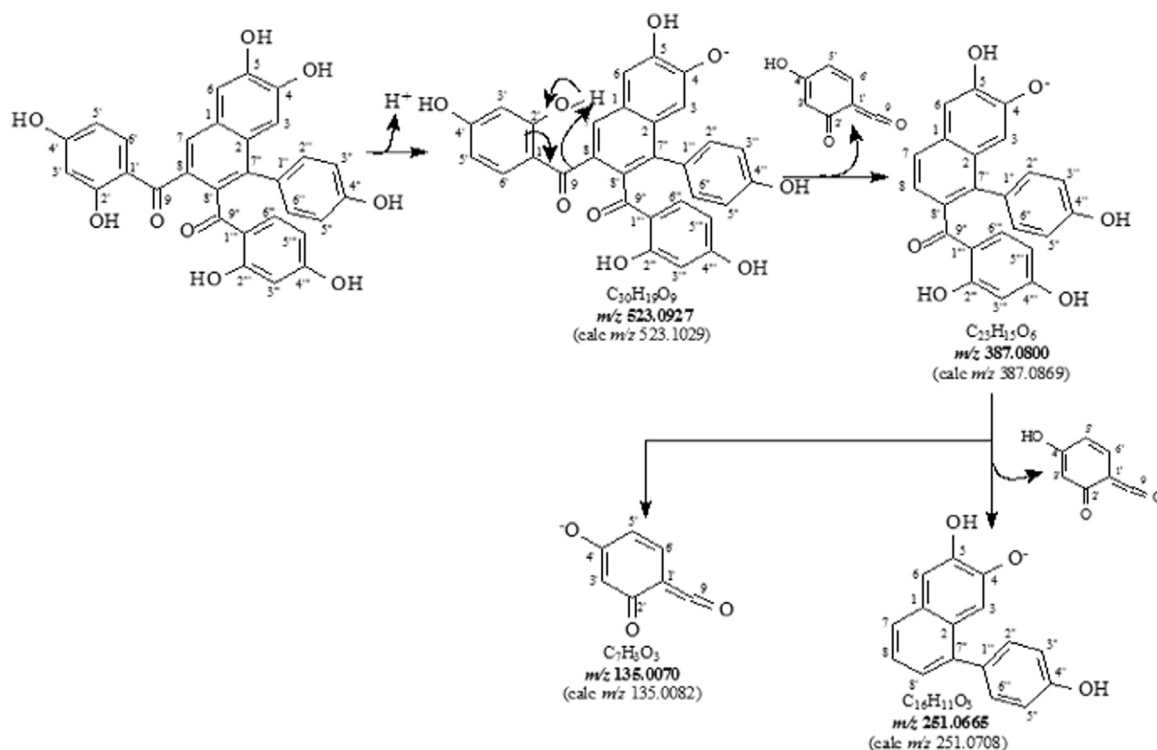
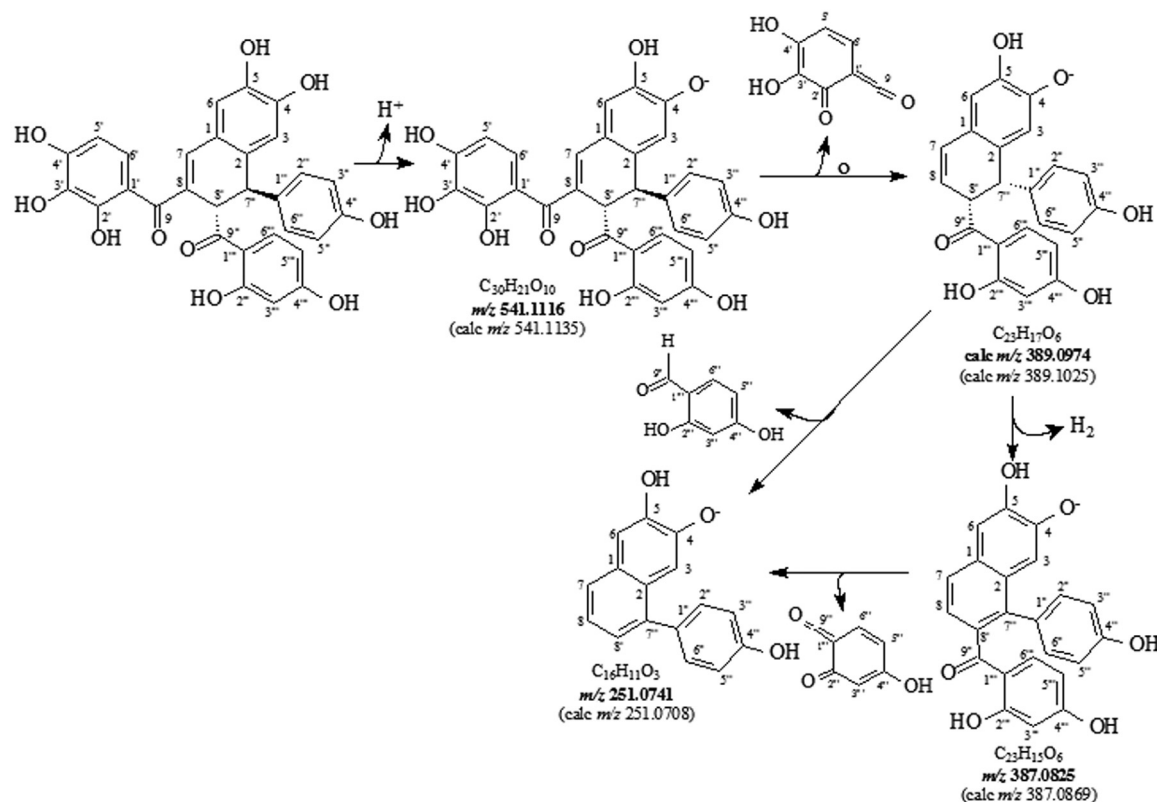Fig. 5. Proposed fragmentation mechanisms of urundeuvine B.

Fig. 6. Proposed fragmentation mechanisms of urundeuvine C.

glucoside (**9**), epicatechin gallate (**11**), quercetin-rhamnoside (**17**), isorhamnetin *O*-rhamnoside (**19**), luteolin (**25**) were characterized through their precursor ions [M-H]⁻ at *m/z* 463.0875, 441.0818, 447.0928, 461.0723 and 285.0392, respectively. Furthermore, their aglycones kaempferol, quercetin, isorhamnetin and epicatechin showed

fragment ions at *m/z* 285, 301, 315 and 289, respectively, while the galloyl moiety showed fragment ions at *m/z* 169. The compounds **9**, **11**, **17** and **25** were confirmed by comparison with authentic samples.

Rt 6.24 min
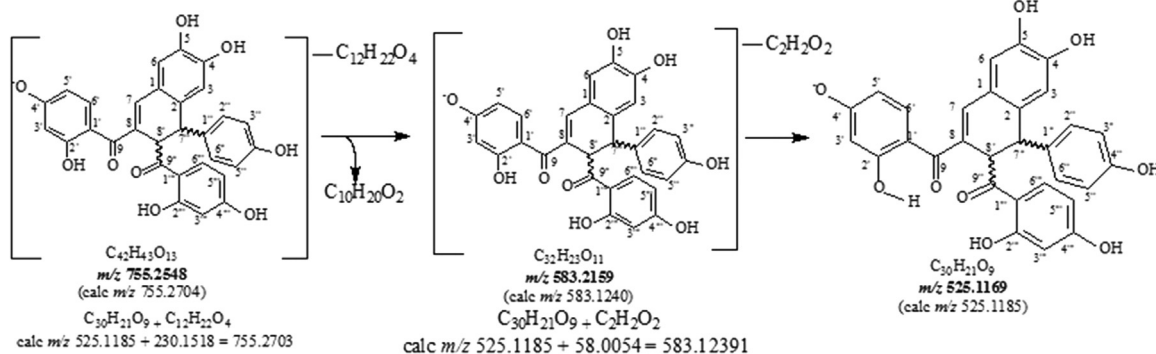
Rt 6.61 min

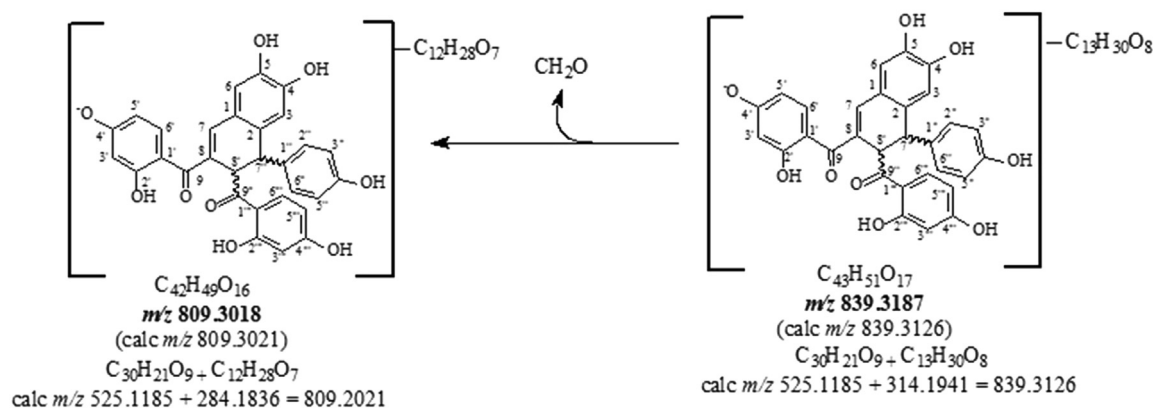
Fig. 7. MS fragmentation proposal for urundeuvine A derivatives.

3.2. Gastroprotective activity – gastric injury induced by ethanol

As show in Fig. 8, the oral pre-treatment with all extracts reduced ($p < 0,0001$) the ethanol-induced acute gastric ulcer when compared the vehicle control group.

Fig. 9 shows light micrographs of the mucosa 30 min after administration of 0.2 mL absolute ethanol.

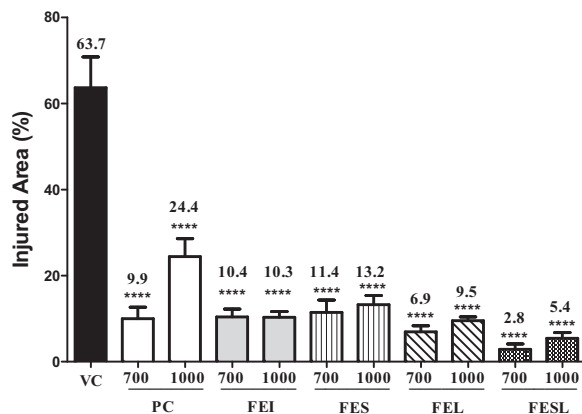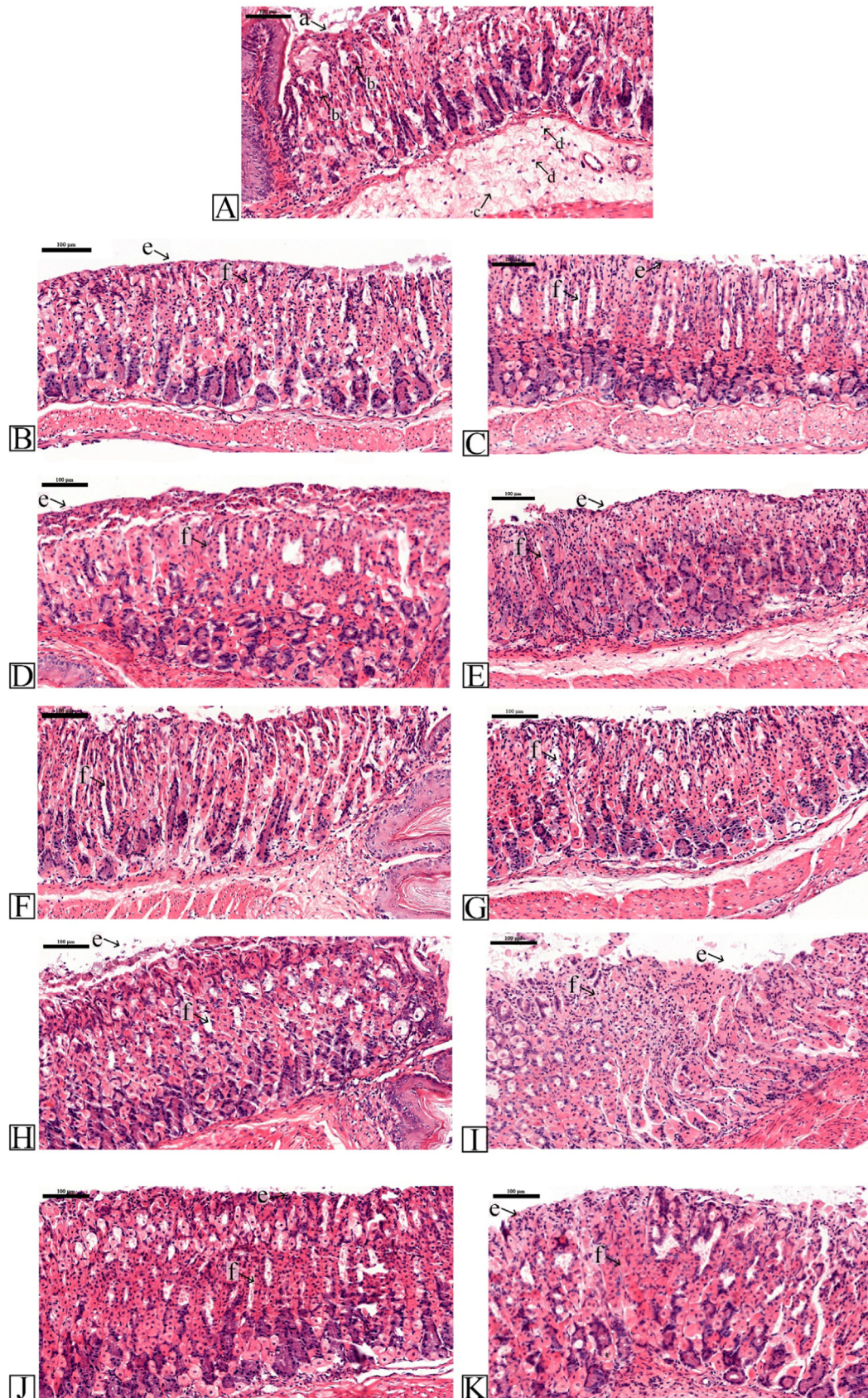The animals in vehicle group (VC) exhibited partial loss of the glandular portion of the stomach in the apical portion of the stomach villi. This was evidenced by the loss of the architecture of the crypts of the glandular epithelium. The basal portion is partially preserved. Furthermore, these animals, in the submucosal portion, exhibited intense edema with the presence of discrete inflammatory infiltrate of diffuse nature with polymorphonuclear predominance. In the positive controls, there was no significant difference in the gastroprotective activity between the different doses. It was observed the preservation of the glandular epithelium with the absence from edema, inflammatory infiltrate or vascular congestion. Some fragments revealed the presence of interstitial hemorrhagic foci in the apical portion of the glandular epithelium (not observed throughout the glandular epithelium), a preserved basal portion, a discrete hydropic degeneration of the glandular epithelium villi and edema in a discreet manner.

The fluid extracts evaluated showed similar activity since tissues presented similar morphostructural or histopathological characteristic. The results revealed that stomachs from animals treated with FEI, FES, FEL, and FESL demonstrated absence of edema, congestion, inflammatory infiltrate and necrosis, with gastroprotective action possible to be observed by the preservation of the epithelium glandular, with preserved histological architecture. This results show that the fluid extracts obtained from stems and leaves of the shoots have pharmacological activity similar to the inner bark of the adult plant, since there was no significant difference between the treated groups.

Fig. 8. Effect of fluid extracts from *Myracrodruon urundeuva* on ethanol-induced gastric damage. ****$p < 0.0001$ *vs* Control (Vehicle). ANOVA followed by Tukey. VC: vehicle control, PC: positive control, FEI: innerbark, FES: stem, FEL: leaves, FESL: stem and leaves (1:1).

Fig. 9. Histological analysis of the effects of the fluid extracts of the innerbark (FEI), stem (FES), leaves (FEL) of the shoots, and the compound fluid extract stem and leaves 1:1 (FESL) in gastric injury induced by ethanol. 15× magnification. A: vehicle control, B: positive control 700 mg/kg, C: positive control 1000 mg/kg, D: FEI 700 mg/kg, E: FEI 1000 mg/kg, F: FES 700 mg/kg, G: FES 1000 mg/kg, H: FEL700 mg/kg, I: FEL1000 mg/kg, J: FESL700 mg/kg, K: FESL1000 mg/kg. a: partial loss of the apical glandular epithelium portion, b: loss of the architecture of the crypts of the glandular epithelium, c: submucosal portion, evidencing intense edema, d: polymorphonuclear leukocyte, e: apical glandular epithelium portion preserved, f: crypts of the glandular epithelium preserved.
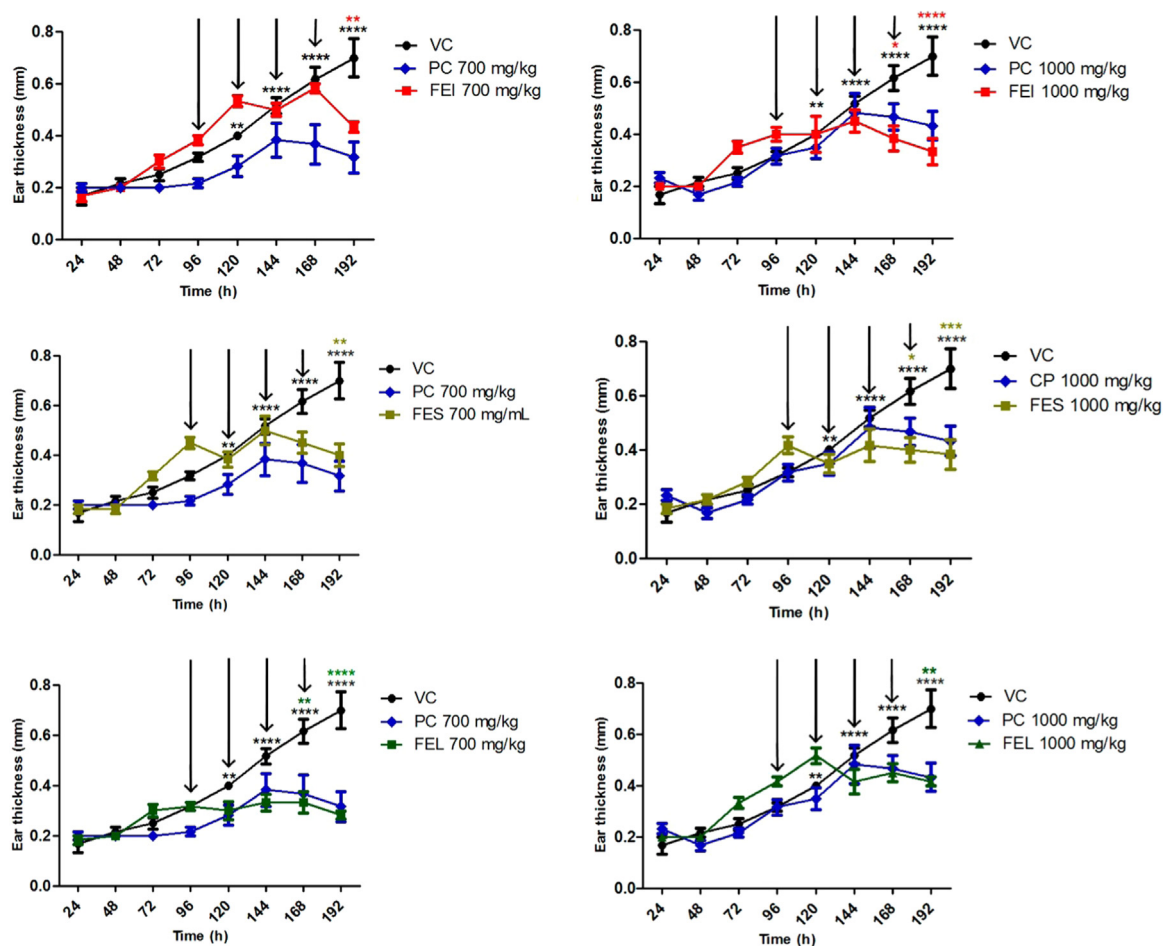
Fig. 10. Effect of fluid extracts from *Myracrodruon urundeuva* on Croton oil multiple application-induced ear edema. Arrows indicate the days of treatment. The points represent the mean of 6 animals and vertical bars S.E.M. (two-way ANOVA followed by Tukey). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$ vs Control (Vehicle), VC: vehicle control, PC: positive control, FEI: innerbark, FES: stem, FEL: leaves.

3.3. Anti-inflammatory activity – croton oil multiple application-induced mouse ear edema

Topical application of Croton oil caused a significant inflammatory response in mice, as determined by the increase of ear thickness in vehicle control group (Fig. 10). The application of FEI (1000 mg/kg), FES (1000 mg/kg) and FEL (700 mg/kg) caused a significant reduction in ear thickness, when compared to the vehicle group, with three days after the first treatment with the extracts. On day 9, which correspond to 192 h to first application of Croton oil, was observed a significant reduction of the thickness of the ears for all groups treated with FEI, FES and FEL (700 mg/kg or 1000 mg/kg), as evidenced in Fig. 10.

Fig. 11 shows light micrographs of the ears 192 h after the application of Croton oil.

The ears of animals from vehicle control group (VC) revealed intense inflammatory infiltrates, diffuse with polymorphonuclear predominance and vascular congestion, when compared to the non-inflamed ear (acetone vehicle only). However, there were no areas of edema or necrosis in the tissue samples analyzed. In the positive control (700 mg/kg), moderate to severe vascular congestion can be observed, with the presence of a discrete lymphocytic infiltrate, but without any change in the tissue architecture worthy of note. At the dose of 1000 mg/kg, no relevant histopathological alteration was observed, being evidenced in the tissue fragments analyzed, only the discrete presence of lymphocytes, cellular structures normally found in the dermis region.

For the FEI, (700 mg/kg), a significant inflammatory process of

diffuse nature, with a predominance of lymphocytes was observed, whereas at the dose of 1000 mg/kg it was possible to detect evident anti-inflammatory activity, when compared to the inflamed ear, with preserved morpho-functional architecture.

For the histological section of the ears treated with the FES and FEL, in both doses, it is possible to observe anti-inflammatory activity, when compared to the inflamed ear, other than a mild to moderate vascular congestion, possibly pertinent to the sampling method. No relevant morpho-functional changes were seen in the groups treated with both doses.

3.4. Cytotoxicity to HEK-293

FEI, FES and FEL were not toxic to HEK-293 cells (IC$_{50}$ = 210.7, 1486 and 185.5 µg/mL, respectively).

4. Discussion

The UPLC-MS analysis revealed that the fluid extracts from inner bark and shoots (stem and leaves) are basically constituted by polyphenols, mainly gallotannins, flavonoids and chalcones. The results corroborate with the findings of Bandeira (2002), who isolated urundeuvines from the ethyl acetate extract of the inner bark. The results of present study also demonstrates that the species is rich in tannins and evidencing the phytotherapeutic complex present in *M. urundeuva*. Based on the works developed by Bandeira (2002), urundeuvine A was detected in *Astronium graveolens*, other species of Anacardiaceae
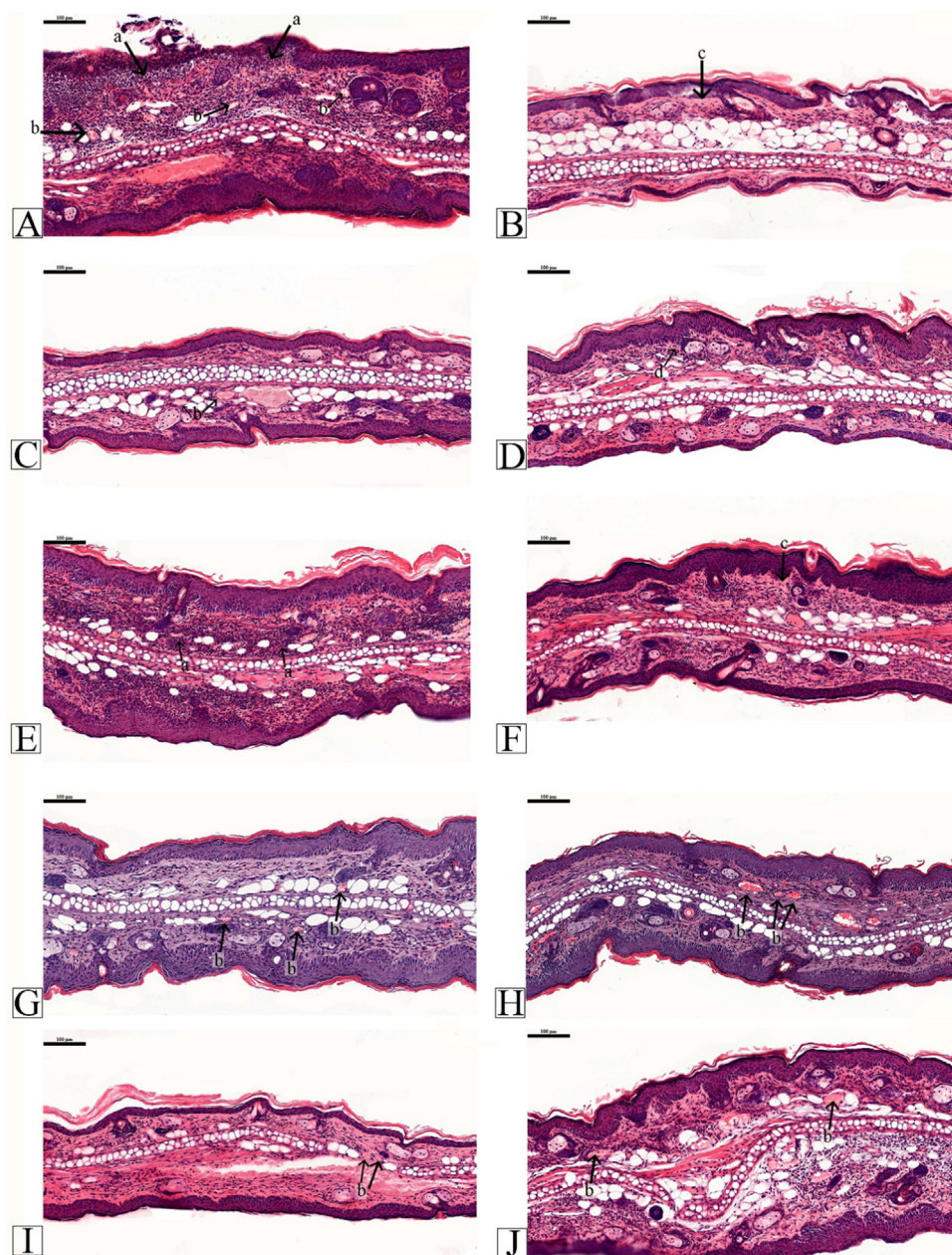
Fig. 11. Histological analysis of the effects of the fluid extracts of the innerbark (FEI), stem (FES), leaves (FEL) of the shoots, on Croton oil multiple application-induced ear edema. 15× magnification. A: vehicle control (Croton oil 5% (v/v) in acetone), B: vehicle acetone only, C: positive control 700 mg/kg, D: positive control 1000 mg/kg, E: FEI 700 mg/kg, F: FEI 1000 mg/kg, G: FES 700 mg/kg, H: FES 1000 mg/kg, I: FEL 700 mg/kg, J: FEL1000 mg/kg. a: inflammatory infiltrates, b: vascular congestion, c: non-inflamed tissue, d: discrete lymphocytic infiltrate.

(Villari, 2011). Previously, Silva et al. (2011) reported that the ethanol extracts from adult *M. urundeuva* leaves and stems consisted of gallic acid (**1**), methyl gallate (**6**), flavonoid glycosides and hydrolysable tannins such as the compounds: **3**, **7–10, 13–16** and **20**. The highest degree of galloylation was observed in the leaves. However, the gallo-tannins were present with up to 10 galloyl moieties.

It has been reported that the presence of flavonoids in Anacardiaceae is common. Agathisflavone is present, for example, in *Anacardium occidentale* (Taiwo et al., 2017), *Schinus terebithinfolius* (Feuereisen et al., 2017) and *Rhus coriolaria* L (Abu-Reidah et al., 2015). Quercetin has been identified in other species of Anacardiaceae, such as *Mangifera indica* (Oliveira et al., 2016) and as quercetin-3-O-galactoside in *Anacardium occidentale* (Brito et al., 2007) and *Rhus coriolaria* L (Abu-Reidah et al., 2015).

In the shoot stems, the presence of urundeuvine A is observed, as described by Bandeira (2002), as well as a derivative of urundeuvine A. Chalcones represent a class of compounds with pronounced gastro-protective activity (Mota et al., 2009). Urundeuvines isolated from *M. urundeuva* have a pronounced antiulcerogenic effect, in addition to the anti-inflammatory, analgesic (Viana et al., 2003; Albuquerque et al., 2011; Rodrigues et al., 2002) and also neuroprotective effects (Calou et al., 2014; Nobre-Júnior et al., 2009).

In the shoot leaves, the presence of gallotannins, such as tri-galloyl hexoside, tetragalloylglucose, penta galloyl-hexoside and hexagalloyl-hexoside was observed in the present study. The tannins represent a class of bioactive compounds that is characteristic of *M. urundeuva* (Carvalho et al., 2017; Bandeira, 2002). The tannin rich fraction exhibited antiulcer activity (Souza et al., 2007). It is believed that the galloylation of polyphenols modifies their biological properties, thus affecting their ability to donate electrons and chelate ions.

Furthermore, gallotannins exhibit anti-cancer, anti-angiogenic, anti-oxidant, anti-inflammatory and anti-ulcerogenic activities (Karas et al., 2017; Adzu et al., 2015), partially justifying the effects found for the shoot leaves of *M. urundeuva*.

The presence of the flavonoid derivatives quercitrin 2″-O-gallate, afzelin O-gallate and agathisflavone, has been reported by Bandeira (2002). A similar observation was made in the present study. These compounds have been reported to exhibit gastroprotective activity, improve prostaglandin content in tissues, scavenge free radical and decrease gastric acid secretion (Zakaria et al., 2014; Mota et al., 2009).

The assessment of gastroprotective and anti-inflammatory activities reveal that the fluid extracts obtained from shoots stems and leaves have pharmacological activity similar to the inner bark of the adult plant, as there was no significant difference between the treated groups. These results are consistent with findings of Bandeira (2002), validating the substitution the innerbark of the adult plant for its shoots of height 40 cm. In fact, the traditional use validated by Bandeira (2002) can be extended to the shoots of this species. To the best of our knowledge, the present data for the shoots are being presented for the first time. It is noteworthy that the importance of the data demonstrated for the compound fluid extract, which presented similar results in gastro-protective activity to other extracts. Also, the absence of toxicity points to the potential use of FEI, FES and FEL in phytotherapeutic preparations, since the samples showed non toxic to HEK-293 cells will not be toxic to humans (Magalhães et al., 2018; Fadeyi et al., 2013). These results corroborate with findings of Viana et al. (1995), which demonstrated that hydroalcoholic extract of inner bark is destitute of toxicity.

It has been reported that the pharmacological activity of the extracts occurs as a consequence of the phytotherapeutic complex formed by the synergistic action of the polyphenols found in *Myracrodruon urundeuva* (Bandeira, 2003). It was observed, in the present study, the occurrence of flavonoids and tannins, as described in Table 1. The relevant biological activity of tannins is due to the interaction of these compounds with a variety of molecules, especially proteins (Barrett et al., 2017; Sekowski et al., 2017). This, added to the antioxidant properties of tannins (Karas et al., 2017; Deng et al., 2016) and anti-inflammatory (Spinaci et al., 2018; Souza et al., 2007) explain, at least in part, the pharmacological actions of these compounds.

Flavonoids, especially those with catechol-like groups, such as quercetin and its derivatives, might be related to gastroprotective, anti-inflammatory and antioxidant activities. These compounds stimulate the mucosal defense system by stimulating gastric mucus secretion, sequestering of reactive oxygen species produced by ethanol and reactive intermediates that are potentially implicated in ulcerogenicity (Minozzo et al., 2016; Zakaria et al., 2014; Santos et al., 2012; Mota et al., 2009). Furthermore, quercetin and its derivatives demonstrated anti-inflammatory activities by inhibition of metabolism and productions of inflammatory mediators (Lesjak et al., 2018). In this way, the pharmacological activities evidenced for the extracts of *M. urundeuva* can be attributed to the synergistic activity of its multiple constituents.

The results revealed that the fluid extracts obtained from shoot stems and leaves have pharmacological activities similar to that of the inner bark of the adult plant, as there was no significant difference between the treated groups. The insertion of shoot leaves, especially in the mixture with shoot stem (1:1) will be important not only from the pharmacochemical context, because flavonoids and tannins present in the extracts have relevant pharmacological actions (Hoensch and Oertel, 2015), but also from the point of view of the yield of the raw material. When deciding on the use of the leaves, it is possible to generate a significant amount of green matter.

The ethnopharmacological data are useful, not only to direct the pharmacological studies with the inner bark, but also as an instrument indicative of extinction risk of *Myracrodruon urundeuva*. Thus, necessitating an ecological proposal for the conserve the species. The present study proposes a substitution of the innerbark of the adult tree with the stems and leaves of the shoots.

Authors' contributions

Aguiar-Galvão, Bandeira, Fonseca, Braz-Filho, Canuto, Campos and Moreira conceived and designed the experiments. Aguiar-Galvão and Mesquita-Filho did the extracts preparations. Aguiar-Galvão, Mesquita-Filho, Canuto and Ribeiro did the UPLC-ESI-QTOF MS/MS analysis, as well as data interpretation. Aguiar-Galvão and Gonçalves performed cytotoxicity assay. Aguiar-Galvão, Campos, Mesquita-Filho, Melo-Junior and Santos performed the evaluation of gastroprotective and anti-inflammatory activities. Silva did the light microscopy and the histological analysis. Aguiar-Galvão and Bandeira wrote the article. Everyone read the article and suggested some changes.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.jep.2018.04.024.

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