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Original Article

The role of tannins as antiulcer agents: a fluorescence-imaging based study

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ABSTRACT

Condensed tannins have been used for many years in folk medicine to treat gastric problems. The mechanism of action that explains why tannins improve gastritis symptoms is based on their ability to chelate metals, antioxidant activity, and their complexation power with other molecules. Even though these uses are well-known, the requirements to become an herbal medicine are much more complex. Herein, we analyzed *Stryphnodendron rotundifolium* Mart., Fabaceae, extract using MALDI for tannin characterization and carried out a fluorescence-imaging study to prove the gastroprotective effects of tannins as coating agents. Through these methods we show that condensed tannins form a gastroprotective layer. Moreover, we revise and discuss other possible mechanisms of action for phenolic-rich plant extracts and their potential in the development of herbal medicines to treat ulcers and gastritis.

Introduction

The term “tannins” arose in 1796, when it was used by Seguin to designate substances found in plant extracts that combine with proteins from animal skins, converting this complex to leather and preventing putrefaction of the skin. Currently, they are defined as a class of phenols with high molecular mass, usually 500–3000 Da, originating from the polymerization of simple polyphenols (Li et al., 2006). The high molecular weight is directly related to a peculiar characteristic of this molecular group: the astringent activity. Ecologically, the astringency of these molecules acts as a defense mechanism, which protects the plant from its natural predators (Remis, 2006).

Tannins are chemically classified into two groups that differ in their phenolic core: condensed tannins (proanthocyanidins) and hydrolysable tannins. Hydrolysable tannins are products of esterification of sugar with gallic acid units, while condensed tannins are two or more units of catechin derivatives, which polymerize and form complex structures. Plants with condensed tannins are used in folk medicine to treat diarrhea, gastritis, and ulcers, and are commonly reported in ethnopharmacology with these purposes (Elseweidy et al., 2008; Balluff et al., 2012; Prado et al., 2014).

The mechanism of action that explains why tannins improve treatment and prevention of diarrhea and gastritis symptoms is based on their ability to chelate metals, antioxidant activity, antibacterial action and complexation power with other molecules (Haslam, 1989, 1996; Haslam et al., 1989; Ruggiero et al., 2006). Due to such capabilities, tannins are thought to comprise a protective layer, which improves gastric problems. However, this mechanism of action is only based on these theoretical principles (Prado et al., 2014).

In Brazil, *Stryphnodendron rotundifolium* Mart., Fabaceae, known as barbatimão, is a tannin-rich species whose bark is extensively used in folk medicine to treat gynecological and gastrointestinal problems, including diarrhea and gastritis (de Oliveira et al., 2014; Luiz et al., 2015). Considering the wide use of tannins for gastric problems, and the knowledge about their activity associated with their chemical properties, herein, we present the action of tannins as coating agents through a fluorescence-imaging based study and tannin characterization using matrix-assisted laser desorption/ionization laser ionization (MALDI-MS). Moreover, we revise and discuss the other possible mechanisms of action for

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phenolic-rich plant extracts and their potential for the development of herbal medicines that treat ulcers and gastritis.

Materials and methods

Extracts and fractions

Stryphnodendron rotundifolium Mart., Fabaceae, bark was collected in Campo Grande, Mato Grosso do Sul state, in April/2015 and identified by Professor Flávio Macedo Alves. A voucher specimen was deposited under registration number 64224 into the CGMS herbarium (at the Federal University of Mato Grosso do Sul), Campo Grande, MS, Brazil (Brazilian Council for the Administration and Management of Genetic Patrimony – CGEN 010808/2014-0). The bark was dried in circulating air stoves (40 °C) and ground in a knife mill (20 mesh). The extraction was done by maceration: 1 kg was immersed in acetone:water (7:3) and rested for 48 h. The solvent was removed and the process was repeated four times. The crude extract obtained (CrEx; yield 20%) was dried and lyophilized. The CrEx (20 g) was submitted to partition with ethyl acetate, dried, and resuspended in methanol. An aliquot was eluted in Sephadex LH-20 column with methanol. After thin layer chromatography analysis (eluent ethyl acetate/acetone 7:3), the fractions were gathered in five new fractions (Fr1–4, 6%; Fr5–7, 3%; Fr8–10, 11%; Fr11–15, 18%; and Fr16–31, 34%).

MALDI-TOF analysis

Analyses of fractions and extracts were performed using the MALDI-TOF UltrafleXtreme (Bruker) in positive mode. To evaluate the best relation of sample and matrix, we prepared extracts at the following concentrations: 10, 5, and 2.5 mg/ml (2.5 mg/ml was chosen). DHB with sodium chloride solution (0.1 M) was used as the matrix (20 mg/ml) and a mixture of peptides was used for external calibration (peptide calibration standard of Bruker: angiotensin I and II, substance P, bombesin, ACTH clip 1–17, ACTH clip 18–39 and somatostatin 28). An equal amount of 5 μl of matrix and extracts were mixed and applied to MALDI plate (1 μmol). After spectra acquirement, the fragmentation pattern of tannins was analyzed (Guaratini et al., 2014).

Flavan-3-ols and gallic acid quantification

Quantification of procyanidins in the CrEx administered to the animals was done using commercially available compounds gallocatechin, catechin, and gallic acid (Sigma–Aldrich). For this, we used an HPLC Shimadzu (LC-20AD) coupled with an ESI triple quadrupole mass spectrometer API 3200 (ABSciex). 10-hydroxycascaroside A (10OHCasc-A) was used as internal standard, isolated in a previous study (Demarque et al., 2017). For chromatographic separation we used a column Supelco Ascentis Express C18 (5 cm × 2.1 mm, 2 μm of particle size), coupled with pre-column of the same material. The mobile phases were optimized to obtain the best signal/noise ratio and water (solvent A) and methanol (solvent B), both with 0.1% formic acid, were adopted. The elution method started with 15% of B, kept isocratic for 0.7 min, and then increased to 35% until 1 min and to 45% until 2.5 min. After this period, a new gradient to 100% was adopted until 3 min. For wash and stabilization 5 min were added to the method. The injection volume was 10 μl, the column temperature was 45 °C and the autosampler temperature was 5 °C.

The negative ionization mode was employed, and the ionization source parameters were CUR 12 (Curtain Gas), source temperature 450 °C, ionization voltage (IS) −4000 V, CAD gas (Collisionally-activate dissociation gas) 8, Gas1 (nebulization gas) 50 and Gas2 (turbo heaters gas) 50. The parameters DP (declustering potential), EP (entrance potential), CE (collision energy), CXP (collision cell exit potential), and Dwell time (monitoring time for each transition about 18 scans/peak) were optimized for each monitored transition and are shown in Table 1.

The quantification methodology was validated for precision (curve with nine points and six replicates each), inter-run accuracy (six replicates in nine points), selectivity, and linearity. The assumed concentrations were 20, 50, 100, 125, 250, 375, 500, 1000, and 2000 ng/ml. The CrEx extract was weighed in triplicate and quantified using validated methodology conditions.

Total tannins content

Total tannins content of the crude extract was performed using a Brazilian Pharmacopeia method (Farmacopeia Brasileira, 2010). An aliquot (0.25 g) of the crude extract was solubilized in distilled water and transferred to a 250 ml volumetric flask ("initial solution"). The *sample solution for quantification of total phenolics (TP)* was prepared by diluting 5 ml of the initial solution in a volumetric flask with distilled water to 25 ml. From this solution, 2 ml was added to a volumetric flask (25 ml) with 1 ml of phosphomolybdotungstic reagent and 10 ml of distilled water. The volume was completed with 29% sodium carbonate solution (p/V). After 30 min the absorbance was determined in spectrophotometer using wavelength 760 nm and water as blank reference.

The *sample solution for polyphenols not absorbed by skin powder (PNASP)* was prepared using 10 ml of the initial solution with 0.1 g and stirred for 60 min. Afterwards, the solution was filtered and transferred to a volumetric flask (25 ml) with distilled water. From this solution, 2 ml was transferred to another 25 ml volumetric flask with 1 ml of phosphomolybdotungstic reagent and 10 ml of distilled water. The volume was completed with 29% sodium carbonate solution (p/V). After 30 min we determined the absorbance in the spectrophotometer using wavelength 760 nm and water as blank reference.

The *standard solution (SS)* was prepared by diluting 50 mg of pyrogallol in a volumetric flask (100 ml) with distillated water. From this solution, 5 ml were transferred to another volumetric flask (100 ml) and filled with distilled water. Afterwards, 2 ml was transferred to a volumetric flask (25 ml), with 1 ml of phosphomolybdotungstic reagent and 10 ml of distilled water. The volume was completed with 29% sodium carbonate solution (p/V). After 30 min the absorbance in spectrophotometer was determined using wavelength 760 nm and water as blank reference. The total tannin content (TT) was calculated according to the formula:

$$TT = \frac{62.5 \times (TP - PNASP) \times m_2}{SS \times m_1}$$

where m_1 is the sample weight in grams and m_2 is pyrogallol weight in grams.

Biological test

The biological test was done using male Wistar rats that weighed between 200 g and 250 g. The experimental procedures were approved by the Ethics Committee of Ribeirão Preto campus, University of São Paulo (Protocol 14.1.722.53.2, December 18th, 2014). The animals were kept in collective cages (41 length × 32 width × 18 height), maintained under controlled temperature (22 ± 2 °C) and lighting (12/12 light/dark cycle), with water and food *ad libitum*. The animals fasted for a maximum of 12 h before the treatment. Animals were euthanized through anesthesia thiopental (Cristalia) overdose (150 mg/kg – intravenous). The CrEx extract was dissolved in water and administered by oral gavage (400 mg/kg of animal) (Melo et al., 2007). Experimental groups were divided randomly: six animals in the control group and six animals treated

Table 1
Mass spectrometer conditions for quantification of catechin (CAT), gallocatechin (GCT), gallic acid (GC) using 10-hydroxycascaroside A (10OHCasc-A) as internal standard.

		Q1	Q3	DT	DP	EP	CE	CXP
CAT	QT	289.0	109.0	20	−40	−4	−30	0
	Conf	289.0	125.1	10	−40	−4	−28	−2
GCT	QT	305.0	167.1	110	−40	−4	−26	0
	Conf	305.0	125.0	10	−40	−4	−26	0
GC	QT	169.0	125.0	110	−30	−10	−24	−2
	Conf	169.0	106.8	10	−30	−10	−18	0
10OHCasc-A	QT	595.0	269.0	90	−60	−10	−64	−6
	Conf	595.0	432.0	10	−60	−10	−34	−15

DP: declustering potential, EP: entrance potential, CE: collision energy, CXP: collision cell exit potential, DT: Dwell time, Q1 and Q3: quadrupoles, QT: monitored transition for quantification, Conf: monitored transition for confirmation.

30 min before euthanasia (for three of these animals the stomachs were washed three times with 5 ml of PBS and for the other three the stomachs were immersed in PBS to remove the excess blood). The organs were frozen in a nitrogen chamber and kept in the freezer at −80 °C until analysis procedures.

Biochemical parameters evaluation

To evaluate the acute toxicology, we collected blood from a third group of three animals. They were treated with CrEx (400 mg/kg of animal, dissolved in water, administered by oral gavage) 1.5 h before euthanasia and anesthetized with ketamine (Syntec, 100 mg/kg) and xylazine (Syntec, 10 mg/kg, intraperitoneally) (Adinortey et al., 2013). Time of blood collection was chosen according to the T_{max} reported for the compounds in the literature (Huo et al., 2016). The blood was collected from the femoral artery (1 ml from each animal). After coagulation, the blood was centrifuged for 20 min (4 °C, Boeco M-240R, 400 × *g*). The serum was stored at −70 °C and sent to Setor de Análises Clínicas (SAC) of Faculdade de Ciências Farmacêuticas de Ribeirão Preto, Universidade de São Paulo to evaluate creatinine, uric acid, urea, HDL (High Density Lipoprotein), LDL (Low Density Lipoprotein), VLDL (Very Low Density Lipoprotein), GOT/ASAT (glutamate oxaloacetate transaminase/aspartate aminotransferase), SGPT/ALT (glutamate-pyruvate transaminase/alanine transaminase), LDH (lactate dehydrogenase), alkaline phosphatase, glucose, cholesterol, and triacylglycerides. Evaluation was carried out by comparing specimens with non-treated animals. The results were evaluated according the difference between averages and the statistical significance was evaluated using the *t*-test in the software Statistica 8.0.

Fluorescent microscopy

Organ sections were made using a Leica CM1860 cryostat. Optimal cutting temperature compound was used to fix the stomach to the plate using Tissue Tek and to position it for transverse cuts. The temperature used in the cryostat was −15 °C and the thickness for confocal microscopy was 30 μm. We used the Leica TCS SP8 confocal laser scanning microscope, and performed excitation using 488 nm laser with 28.9% intensity. Absorption was adjusted to the 575–634 nm range.

Results and discussion

Stryphnodendron rotundifolium is a phenolic-rich plant with diverse structures (De Mello et al., 1996a, 1996b, 1999; Antonelli Ushirobira et al., 2007; Luiz et al., 2015). To analyze the tannin sizes present in the extract, we used MALDI ionization method, which has been shown to be a good resource for tannin analysis (Guaratini et al., 2014). For this, we made a tannin-enrichment from crude extract (CrEx) using a Sephadex LH-20 column, which lead to a better resolution in MALDI analysis for tannin identification. In fact, compounds that are extensively present in tannin fractions (*i.e.* sugars) affect the MALDI analysis. They can suppress the ionization process due to charge competition, which reduces the analyte desolvation/declustering, while an increase in laser energy can induce source dissociation (Silva and Lopes, 2015). This influence is commonly found with electrospray ionization (ESI) and, although MALDI is more susceptible to contaminants, sugar reduction favors the ionization process (Rue et al., 2017). Fig. 1 shows a typical tannin spectrum in MALDI analysis, obtained from Fr16-31.

The high polymerization degree of tannins makes structural elucidation difficult; however, MALDI provides reliable identification of units and the degree of polymerization, considering the fragmentation pattern followed by units: Retro–Diels–Alder (RDA; −136 Da) fragmentation succeeded by the loss of the remaining unit through remote hydrogen rearrangement (RHR; −152 Da). Following this pattern is possible to identify the monomers that which constitute each oligomer. Fig. 2 shows the fragmentation pattern and identification of the units according to this resource. It was identified four different series according the 3-first monomer unit (Fig. 2A). The sequential loss of monomer units (loss of 152 Da by RDA and 136 Da by remote hydrogen rearrangement; Fig. 2B) allows the units to be identified even in mixtures. For instance, the first series *m*/*z* 2041.4491 ($C_{105}H_{86}O_{42}$ + Na) has a correspondent fragment *m*/*z* 1905.4330 ($C_{98}H_{82}O_{39}$ + Na) due to RDA fragmentation (−132 Da). The loss of a procyanidin unit occurs after a RHR fragmentation (−152 Da). Thus, following this fragmentation pattern we could differentiate the units proposed in Fig. 1: four different series differing in the 3-first units. The first series consists of seven PCY units, while in the second, third, and fourth series the PCY units is changed by one, two and three PDE units, always with a total of seven units in their oligomers (Table 2) (Pasch et al., 2001; Navarrete et al., 2010; Drovou et al., 2015).

Tannins content was 291 ± 0.27 mg/g of CrEx. The compounds catechin, gallocatechin, and gallic acid represented 2, 10 and 8 mg/g of CrEx, respectively. Thus, the 400 mg/kg dose administered to animals for the image study represented ~116 mg of tannins and 8 mg of the other quantified phenol compounds. According to Audi et al. (1999), a 400 mg/kg dose of the CrEx inhibited about 70% of acid-ethanol induced ulceration, while cimetidine inhibited only 40%. However, the authors did not quantify the total tannin content.

The extractive process determines the efficiency of extraction. Brazilian Pharmacopeia specifies that a vegetal drug should contain a minimum of 8% total tannins (Farmacopeia Brasileira, 2010), 0.02% gallic acid and 0.03% gallocatechin, when decoction extraction is adopted. In addition, the present extraction yielded a suitable content of other phenols compounds, with an estimated tannin content of 6% relative to the vegetal drug. Considering the extractive process adopted herein, our results agree with the contents obtained for this species using the extractive process of maceration (Fiori et al., 2013).

After chemical analysis and extract standardization, we performed the biological test. The comparison between biochemical parameters for treated and non-treated animals did not present

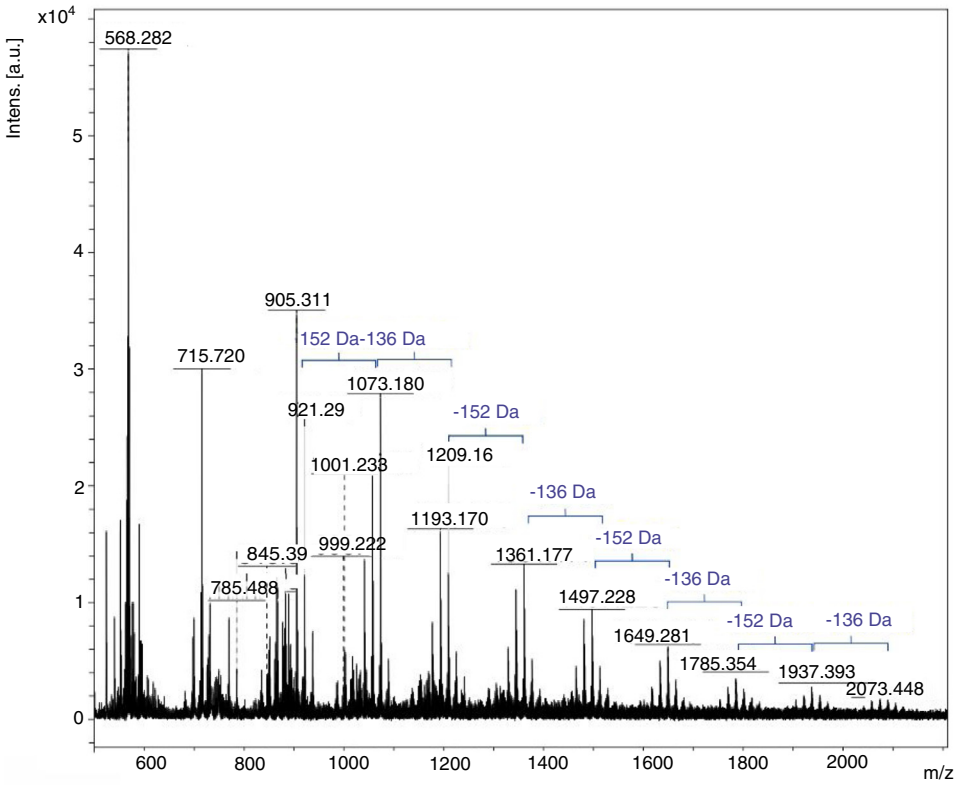

Fig. 1. MALDI spectrum of tannin-enriched fraction obtained after chromatography column.

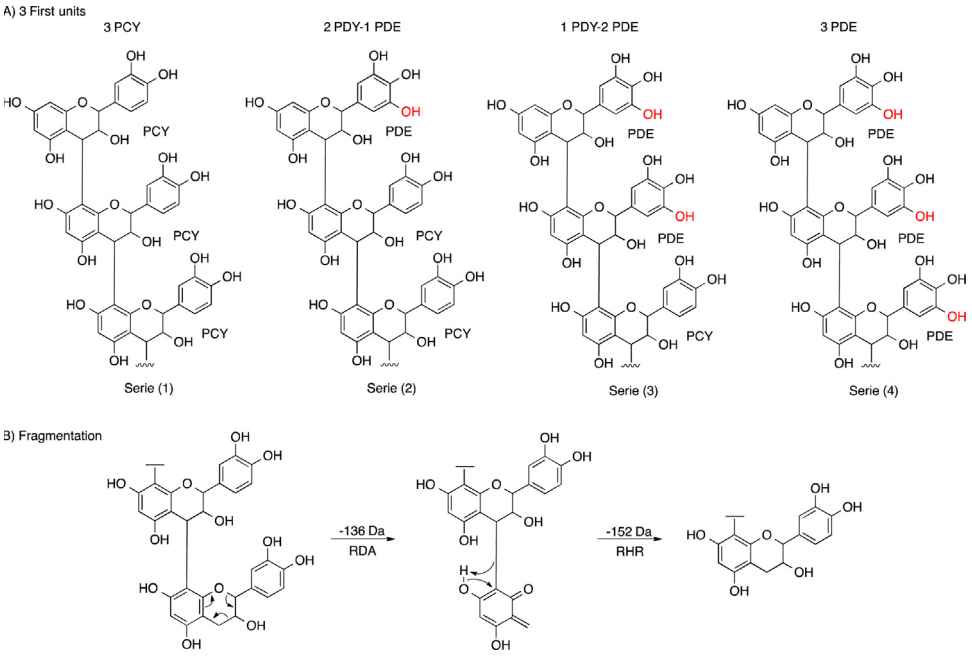

Fig. 2. Fragmentation pathway used to identify tannins.

toxicity, since they only differed in glucose dosage (Table 1S). Animals treated with CrEx had reduced glucose levels (108 ± 16 mg/dl) compared to the non-treated animals (172 ± 18 mg/dl, $p < 0.05$). A polyphenol-rich diet is known to reduce glucose levels, considering that these substances affect the absorption of sugar and other dietary components, micronutrients, as well as drugs (Johnston et al., 2005; Ma et al., 2011; Alzaid et al., 2013). Such findings should be considered in dosage schedules to avoid interference with nutrition and the absorption of other drugs.

The action of antiulcer drugs involves blocking H2 receptors, inhibiting the cholinergic pathway (muscarinic antagonists), the proton pump, local antacids and antibacterial and gastric surfactant agents. The proposed mechanism of action for tannins is based on the complexation power of these molecules on the gastric

Table 2
Identification of tannins (series 1–4) according molecular formula and fragmentation type.

Series	*m/z* $[M+Na]^+$ (error - ppm)	Calculated	MF	Fragmentation type	Fragmentation and identification of tannin's monomers units
1	889.1955±0.1	889.1955	$C_{45}H_{38}O_{18}$	RHR (-152 Da)	3PCY, -PCY
	1041.2266±15.7	1041.2429	$C_{53}H_{46}O_{21}$	RDA (-136 Da)	
	1177.2743±13.0	1177.2589	$C_{60}H_{50}O_{24}$	RHR (-152 Da)	4PCY, -PCY
	1329.2951±8.4	1329.3063	$C_{68}H_{58}O_{27}$	RDA (-136 Da)	
	1465.3425±13.8	1465.3223	$C_{75}H_{62}O_{30}$	RHR (-152 Da)	5PCY, -PCY
	1617.3544±9.5	1617.3696	$C_{83}H_{70}O_{33}$	RDA (-136 Da)	
	1753.4000±8.1	1753.3857	$C_{90}H_{74}O_{36}$	RHR (-152 Da)	6PCY, -PCY
	1905.4227±5.4	1905.4330	$C_{98}H_{82}O_{39}$	RDA (-136 Da)	
	2041.4539±2.3	2041.4491	$C_{105}H_{86}O_{42}$		Tannin (1) 7PCY
2	905.1923±2	905.1904	$C_{45}H_{38}O_{19}$	RHR (-152 Da)	2PCY-1-PDE, -PCY
	1057.2231±13.9	1057.2378	$C_{53}H_{46}O_{22}$	RDA (-136 Da)	
	1193.2683±12.1	1193.2538	$C_{60}H_{50}O_{25}$	RHR (-152 Da)	3PCY-1-PDE, -PCY
	1345.2900±8.3	1345.3012	$C_{68}H_{58}O_{28}$	RDA (-136 Da)	
	1481.3326±10.4	1481.3172	$C_{75}H_{62}O_{31}$	RHR (-152 Da)	4PCY-1-PDE, -PCY
	1633.3557±5.5	1633.3646	$C_{83}H_{70}O_{34}$	RDA (-136 Da)	
	1769.3939±7.5	1769.3806	$C_{90}H_{74}O_{37}$	RHR (-152 Da)	5PCY-1-PDE, -PCY
	1921.4120±8.3	1921.4279	$C_{98}H_{82}O_{40}$	RDA (-136 Da)	
	2057.4610±8.2	2057.4440	$C_{105}H_{86}O_{43}$		Tannin (2) 6PCY-1-PDE
3	921.1982±13.9	921.1854	$C_{45}H_{38}O_{20}$	RHR (-152 Da)	1PCY-2-PDE, -PCY
	1073.2270±5.4	1073.2327	$C_{53}H_{46}O_{23}$	RDA (-136 Da)	
	1209.2601±9.4	1209.2487	$C_{60}H_{50}O_{26}$	RHR (-152 Da)	2PCY-2-PDE, -PCY
	1361.2855±7.8	1361.2961	$C_{68}H_{58}O_{29}$	RDA (-136 Da)	
	1497.3281±10.6	1497.3121	$C_{75}H_{62}O_{32}$	RHR (-152 Da)	3PCY-2-PDE, -PCY
	1649.3518±4.7	1649.3595	$C_{83}H_{70}O_{35}$	RDA (-136 Da)	
	1785.3945±10.6	1785.3755	$C_{90}H_{74}O_{38}$	RHR (-152 Da)	4PCY-2-PDE, -PCY
	1937.4072±8.1	1937.4229	$C_{98}H_{82}O_{41}$	RDA (-136 Da)	
	2073.4553±6.2	2073.4389	$C_{105}H_{86}O_{44}$		Tannin (3) 5PCY-2-PDE
4	937.1779±2.6	937.1803	$C_{45}H_{38}O_{21}$	RHR (-152 Da)	3-PDE, -PCY
	1089.2195±7.5	1089.2276	$C_{53}H_{46}O_{24}$	RDA (-136 Da)	
	1225.2540±8.4	1225.2437	$C_{60}H_{50}O_{27}$	RHR (-152 Da)	1PCY-3-PDE, -PCY
	1377.2896±1.1	1377.2910	$C_{68}H_{58}O_{30}$	RDA (-136 Da)	
	1513.3150±5.2	1513.3070	$C_{75}H_{62}O_{33}$	RHR (-152 Da)	2PCY-3-PDE, -PCY
	1665.3383±9.7	1665.3544	$C_{83}H_{70}O_{36}$	RDA (-136 Da)	
	1801.3780±4.2	1801.3704	$C_{90}H_{74}O_{39}$	RHR (-152 Da)	3PCY-3-PDE, -PCY
	1953.4151±1.4	1953.4178	$C_{98}H_{82}O_{42}$	RDA (-136 Da)	
	2089.4303±2.4	2089.4338	$C_{105}H_{86}O_{45}$		Tannin (4) 4PCY-3-PDE

wall tissue, since condensed tannins do not undergo absorption. However, other derivative constituents can be absorbed by the degradation of monomers performed by the microflora (Deprez et al., 2000), which occurs with proanthocyanidins that are absorbed as dimers and trimers, whereas polymeric molecules are limited to the intestinal lumen (Deprez et al., 2001; Scalbert et al., 2002; Koleckar et al., 2008).

In order to prove the binding ability of polyphenols in gastric tissues, animals were treated and their stomachs were removed and analyzed using fluorescence microscopy. Fig. 3 shows the images obtained in the biological test. The stomachs of animals treated with CrEx (Fig. 3C and D) were distinct from non-treated animals (Fig. 3A) when observed in fluorescence microscopy. Fig. 3A shows that there are no elements in the organ that emit fluorescence. On the other hand, organs impregnated with CrEx extract (Fig. 3B) showed fluorescence throughout the tissue. This demonstrates a clear permeation and fixation of phenolic compounds throughout the tissue. Organs of animals euthanized after 0.5 h of treatment, which were not washed with PBS, showed the fluorescent substance distributed along the parietal cells (Fig. 3C). Such fluorescence remained, even when the organ was washed with PBS before freezing (Fig. 3D), revealing a longstanding effect.

Considering the sections obtained from several organ parts, we noticed a wide distribution of fluorescence related to the presence of phenolic compounds on the tissue surface. Fluorescence microscopy is an excellent tool to determine tissue distribution of molecules in cellular structures, especially for natural product-derived compounds. Molecules from natural sources have an intrinsic fluorescence and have been attached to molecules that do not display this property as fluorescent probes. This practice shows the efficiency of natural products as probes, as well as the possibility of using this technique to determine natural compound distributions in tissues (Alexander et al., 2006). Other techniques have been useful to generate images of metabolites from tissues, like MALDI-imaging (Silva et al., 2014). When compared to fluorescence imaging, MALDI-imaging's main advantage is the direct identification of compounds attached to the tissue. However, sample preparation and optimal conditions for high quality image acquisition are much more complex and demanding. In the present study, fluorescence was an effective tool to present tannin complexation on the surface of gastric tissue.

Complexation is a surface phenomenon and conformation and flexibility are important factors that contribute to the level of interaction. In this context, small proteins are better binders than large proteins, which have complex secondary and tertiary structures. Considering the size of polyphenols, the binding efficacy increases exponentially from the trimmers to larger tannin series (Haslam et al., 1989; Saito et al., 1998). However, some authors state that large tannins (>3000 Da) make the binding process more difficult, based on molecular diffusion in collagen fibers (Haslam, 1989). According to the series found in the present work, the tannin content in CrEx extract was of suitable size for binding activity.

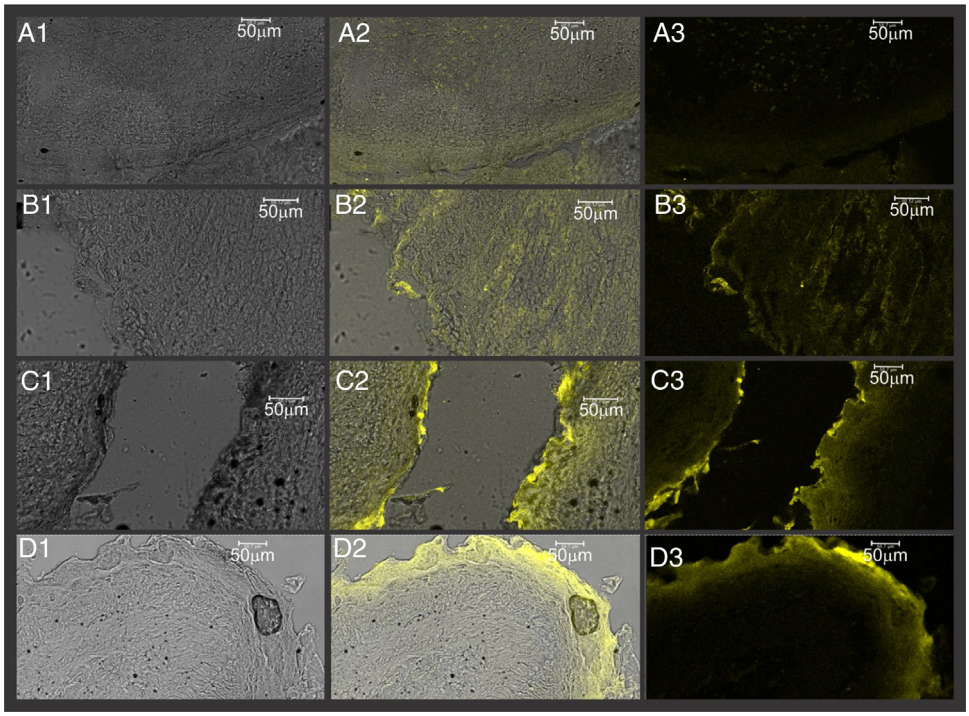

Fig. 3. Fluorescence confocal microscopy images of stomach cross sections. First column (A1-D1): visible light image; second column (A2-D2): overlap of visible and fluorescence light images; third column (A3-D3): fluorescence light image. Line A: Stomach tissues from untreated animals. Line B: Stomach tissues from untreated animals impregnated with *S. rotundifolium* crude extract. Line C: Stomach tissues from treated animals with *S. rotundifolium* crude extract (400 mg/ml); non-washed tissues (euthanized after 0.5 h). Line D: Stomach tissues from treated animals with *S. rotundifolium* crude extract (400 mg/ml); PBS-washed tissues (euthanized after 0.5 h).

The efficacy of tannins that create a layer on the surface of gastric tissue explains why CrEx improves gastric problems. The present results corroborate with the effectiveness of *Stryphnodendron* spp. to treat gastric lesions reported in previous studies (Audi et al., 1999). Prado et al. (2014) state that the absence of tannins in *Eugenia dysenterica* extract suppressed antiulcerogenic activity, supporting the fact that tannins are related to this activity.

Other components of vegetable extracts, along with the cytoprotective action of condensed tannins, can also improve gastric problems. Hydrolysable tannins are important substances for treating gastric problems and are related to the antibacterial activity against *H. pylori*, an agent that also causes gastritis (Funatogawa et al., 2004). In fact, around 10% of individuals who have *H. pylori* in their flora develop peptic ulcers and its presence is the target of pharmacological therapy (Harsha et al., 2017). *Stryphnodendron* spp. are also a source of hydrolysable tannins and can be used when *H. pylori* is in the gastric tissue.

Herbal medicines present considerable advantages when compared to conventional synthetic drugs: synergy and additive effects. Additive effects are the sum of an individual compounds' efficacy to improve symptoms. On the other hand, synergy is the concept that the chemical complexity of a vegetable extract is better than the arithmetical sum of each component separately. Considering the chemical diversity of the vegetable extract of *Stryphnodendron* spp., different compounds can act on multiple mechanisms to improve gastric problems.

The complexation process is due to the main binding forces derived from hydrophobic effects and hydrogen bonding, specifically the phenol groups with the hydrogen acceptor bond in the protein (Haslam, 1996). Although tannin properties are well establish and have been used to tan leather for many years, the requirements for use as herbal medicine are much more complex. In fact, tannins present in a tissue may contribute to more than just as a physical barrier. Sucralfate, which has the same mechanism of action proposed for tannins, has been shown to play an important role in angiogenesis (Szabo, 2014). Therefore, supporting re-epithelization is a crucial step in restoring gastroduodenal mucosal integrity (Szabo et al., 1991). Phenolic compounds have been studied in vascular protection and studies have shown the importance of maintaining gastric mucosal blood flow. Thus, considering histopathological studies that show epithelization, fibroblast proliferation, and angiogenesis by tannins in healed wound tissues, these molecules act on more than just physical barriers (Lai et al., 2016).

Using gastroprotective agents for ulcer and gastritis treatment instead of other pharmacological agents, as proton pump inhibitor, does not modify the chemical environment necessary for digestion and protection. In fact, the gastric juice acts as a barrier to prevent the proliferation of microorganisms and clinical studies report increased infections related to proton pump inhibitors (Giuliano et al., 2012).

In the present work we observed the gastroprotective barrier formed by tannins. We noticed that even when we washed the organ with PBS the layer of tannins on the stomach wall was not removed (Fig. 3D), showing the formation of a long-term layer. Although tannins are water-soluble, their bind forces are strong enough to remain bonded to the parietal cells. These results, combined with the non-acute and chronic toxicity found for *Stryphnodendron* spp. (Costa et al., 2013) extract, support the development of an antiulcer herbal remedy from this plant species. However, clinical studies are needed to develop the herbal medicine.

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 they have followed the protocols of their work center on the publication of patient data.

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

Authors' contributions

DPD contributed in running the laboratory work, analysis of the data and drafted the paper. DRC contributed in running the experiments with animals. GGO contributed to biological studies, histological cuts and with fluorescence microscopy. DBS contributed with MALDI analysis, supervised the laboratory work and contributed to critical reading of the manuscript. CAC contributed collecting plant sample and identification, confection of herbarium and designing the study. NPL supervised the laboratory work, analysis of the data and drafted the paper. All the authors have read the final manuscript and approved the submission.

Conflicts of interest

The authors declare no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bjp.2018.03.011.

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