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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

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