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Possible mechanisms of action of *Caesalpinia pyramidalis* against ethanol-induced gastric damage

Polyana B.F. Diniz [a], Ana Roseli S. Ribeiro [a], Charles S. Estevam [a], Cristiane C. Bani [b], Sara M. Thomazzi [a,*]

[a] Departamento de Fisiologia, Universidade Federal de Sergipe, Cidade Universitária, Av. Marechal Rondon, CEP 49100-000, São Cristóvão, Sergipe, Brazil
[b] Departamento de Morfologia, Universidade Federal de Sergipe, Cidade Universitária, Av. Marechal Rondon, CEP 49100-000, São Cristóvão, Sergipe, Brazil

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ABSTRACT

Ethnopharmacological relevance: *Caesalpinia pyramidalis* Tul. (Fabaceae), known as "catingueira", is an endemic tree of the Northeast region of Brazil. This plant, mainly inner bark and flowers, has been used in traditional medicine to treat gastritis, heartburn, indigestion, stomachache, dysenteries, and diarrheas.
Materials and methods: The ethanol extract of *C. pyramidalis* inner bark was used in rats *via* oral route, at the doses of 30, 100, and 300 mg/kg, in the ethanol-induced ulcer model and some of the mechanisms underlying to the gastroprotective effect of this plant investigated.
Results: The ethanol extract of *C. pyramidalis* inner bark (100 mg/kg) produced reduction ($P < 0.001$) on the total lesion area in the ethanol-induced gastric damage. The gastroprotective response caused by the ethanol extract (100 mg/kg) was significantly attenuated ($P < 0.05$) by intraperitoneal treatment of rats with DL-Propargylglycine (PAG, a cystathionine-γ-lyase inhibitor; 25 mg/kg), but not by N_w-nitro-L-arginine methyl ester hydrochloride (L-NAME, an inhibitor of nitric oxide synthase; 70 mg/kg), and confirmed by microscopic evidence. The ethanol extract significantly decreased the number of mucosal mast cells compared to vehicle-treated group. The inflammatory cells of the ethanol extract (100 mg/kg)-treated ulcerated rats exhibited an upregulation of interleukin (IL)-4 protein expression and down-regulation of inducible nitric oxide synthase (iNOS) expression, observed by immunohistochemistry and flow cytometer.
Conclusions: The present results suggest that the ethanol extract of *C. pyramidalis* produced dose-related gastroprotective response on ethanol-induce ulcer in rats through mechanisms that involved an interaction with endogenous hydrogen sulfide and reduction of inflammatory process with imbalance between pro-inflammatory and anti-inflammatory mediators, supporting the popular usage of this plant.

1. Introduction

Peptic ulcer (gastric and duodenal ulcers) has long been and is a continuing serious medical problem, with higher mortality for gastric ulcer than for duodenal ulcer (Overmier and Murison, 2013). It is a worldwide disease, with 10% of the global population experiencing ulcers (Zapata-Colindres et al., 2006), despite variations in incidence among countries.

Several factors play an important role in the pathogenesis of gastric ulcer, such as alcohol abuse, smoking, inadequate dietary habits, stress, trauma, sepsis, hemorrhagic shock, burns, *Helicobacter pylori*, and steroidal and non-steroidal anti-inflammatory drugs (Saxena and Singh, 2011) as well as inflammatory mechanisms, the generation of free radical (Polat et al., 2011), and endogenous factors related to the pathophysiology of gastroprotection (prostaglandin, ATP-sensitive K^+ channels, and nitric oxide) (Saxena and Singh, 2011).

The standard drugs to treat peptic ulcers have decreased the morbidity rates, but produce many side effects including relapse of the disease, and are often expensive for the poor population (Maity and Chattopadhyay, 2008) leading the search for alternative treatments, such as medicinal plants. Approximately 65–80% of the population in developing countries depends solely on medicinal plants for primary health care (WHO, 1999).

In Brazil, *Caesalpinia pyramidalis* Tul. (Fabaceae) is an endemic tree of the Northeast, mainly in the Caatinga region, which is known popularly as "catingueira" (Agra et al., 2007, 2008; Albuquerque et al., 2007). Inner bark or flowers are traditionally used to treat colic, stomachache, gastritis, diarrhea, collision, cough, bronchitis, asthma,

* Correspondence to: Department of Physiology Center of Biological and Health Sciences Federal Universityof Sergipe 49100-000, São Cristóvão (SE), Brazil. Tel.: +55 79 21056640; fax: +55 79 21056474.
E-mail address: sthomazzi@gmail.com (S.M. Thomazzi).

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respiratory infection, influenza, diabetes, dysenteries, and fever (Agra et al., 2007, 2008; Albuquerque et al., 2007).

We recently showed that the ethanol extract of *Caesalpinia pyramidalis* inner bark displays pronounced gastroprotective activity in rats, as evidenced by significant inhibition of the formation of ulcers induced by different agents (absolute ethanol, indomethacin, and pylorus ligature) and by increasing the amount of mucus, supporting the folkloric usage of the plant to treat various gastrointestinal disorders (Ribeiro et al., 2013). In the same study, we also demonstrated that the EE of *Caesalpinia pyramidalis* presents weak antimicrobial activity against *Helicobacter pylori* using the agar-well diffusion and broth microdilution methods (Ribeiro et al., 2013). In addition, our previous studies have demonstrated that the ethanol extract of *Caesalpinia pyramidalis* presents anti-inflammatory and antinociceptive activities in rodents (Santana et al., 2012; Santos et al., 2011, 2013).

Therefore, the present study was conducted to further investigate possible mechanisms of action that participate in the ethanol extract of *C. pyramidalis* inner bark-induced gastroprotection.

2. Materials and methods

2.1. Plant collection and extraction of the ethanol extract

The inner bark of *C. pyramidalis* was collected in the municipality of Canindé de São Francisco-SE, Brazil (09°66'00''S, 37°78'98''W), in September 2008. The plant was authenticated by Professor Ana Paula Prata, Department of Biology, Federal University of Sergipe. A voucher specimen has been deposited at the Herbarium of the Federal University of Sergipe (number ASE 13,164). The inner bark was dried at 40 °C in a forced air oven for two days and subsequently powdered (2,840 g) and extracted by maceration at room temperature with 90% ethanol for five days. The extract was filtered in vacuum, and the solvent was removed using a rotary evaporator (45 °C). The percentage of yield of the ethanol extract (EE) was 2.6% (73.8 g). Phytochemical screening showed that the EE of *C. pyramidalis* inner bark contains flavonoids, phenols, saponins, steroids, tannins, and triterpenes (Santos et al., 2011).

The EE of *C. pyramidalis* was analyzed using high performance liquid chromatography (Shimadzu, Prominence model, Kyoto, Japan) consisting of a vacuum degasser DGU-20A3 model, SIL-10A auto sampler, two high pressure pumps LC-6A, and an SPDM20Avp photodiode array detector system coupled with a CBM 20A interface. Analysis was performed in an analytical Phenomenex LUNA® C18 column ($250 \times 4.6\ mm^2$ i.d., 5 μm of particle diameter, Torrance, CA, USA). Separation of compounds was done by reverse mode gradient elution and demonstrated the presence of rutin as previously described (Santana et al., 2012).

2.2. Animals

Young adults Wistar rats (180–300 g) of both genders were obtained from the Central Biotery of the Federal University of Sergipe (São Cristóvão, Brazil). Animals were maintained at controlled room temperature (21 ± 2 °C) with free access to food (Purina®) and water, under a 12 h light/dark cycle. Twenty-four hours before the experiments, they were transferred to the laboratory and given only water, *ad libitum*. The experiments were performed after approval of the protocol by the Institutional Ethics Committee (CEPA/UFS 63/2011) and were carried out in accordance with the current guidelines for the care of laboratory animals.

2.3. Analysis of possible involvement of gaseous mediators (nitric oxide and hydrogen sulfide) in the protective effect of C. pyramidalis on absolute ethanol-induced ulcer

After 24 h of fasting, to investigate the role of nitric oxide (NO) and hydrogen sulfide (H_2S) in the protective effects of the EE from *C. pyramidalis*, the animals ($n=8$/group) were pre-treated with an inhibitor of NO synthase (NOS), N_w-nitro-L-arginine methyl ester hydrochloride (L-NAME, 70 mg/kg, i.p.), or with an inhibitor of cystathionine-γ-lyase (CSE), DL-Propargylglycine (PAG, 25 mg/kg, i.p.). After 30 min, the rats received the EE (100 mg/kg, p.o.) or vehicle (0.2% Tween 80 in 0.9% NaCl solution, 10 mL/kg, p.o.). The choice of the dose of the EE was based on the preliminary findings (Ribeiro et al., 2013). Thirty minutes later, gastric damage was induced by intragastric instillation of absolute ethanol (4 mL/kg, p.o.; Robert et al., 1979). The control groups ($n=8$/group) received only vehicles, vehicle plus the EE, or vehicle plus carbenoxolone (200 mg/kg; Sigma-Aldrich, St. Louis, MO, USA). One hour later, the animals were anesthetized and euthanized by cervical dislocation, and the stomachs were removed and opened along the greater curvature. The stomachs were gently rinsed with water to remove the gastric contents and blood clots, for subsequent scanning. The images obtained were analyzed using specific "EARP" software (developed by Dr. Eros Comunello, Universidade do Vale do Itajaí, São José, SC, Brazil) measure each lesion point. The results were expressed as total lesion area (mm^2).

2.4. Histopathologic analysis

For the histological analysis, the absolute ethanol-induced ulcerative response in the stomachs was carried out after 1 h. The stomach samples were embedded in Tissue-Tek (OCT compound; Sakura Finetek, Torrance, CA, USA) and frozen in liquid nitrogen. The samples were cut in serial 10 μm thick sections (Leica CM1850; Leica Biosystems, Wetzlar, Germany) and stained in hematoxylin–eosin. A microscopic score was determined for the following parameters: (i) disruption of the superficial region of the gastric gland with epithelial cell loss and (ii) interstitial edema, using a scale ranging from 0 to 3 (0: none; 1: mild; 2: moderate; and 3: severe) for each criterion. The sections were assessed by an experienced pathologist without the knowledge of the treatments (three histological sections/animal, $n=4$/group).

2.5. Mast cells staining

The identification of mast cells was performed as previously described by Meuser-Batista et al. (2008). For the mast cells staining, the stomach samples were embedded in Tissue-Tek (OCT compound; Sakura Finetek, Torrance, CA, USA) and frozen in liquid nitrogen. The samples were cut in serial 16 μm thick sections (Leica CM1850; Leica Biosystems, Wetzlar, Germany). Stomach mast cells in tissue slices (triplicate, $n=3$/group) were stained with a mixed solution composed by 0.36% toluidine blue (TB), 0.02% alcian blue (AB), and 0.01% safranin (S) in acetate buffer, pH 1.42. This protocol identifies mucosal mast cells (MMC), connective tissue mast cells (CTMC), and hybrid mast cells. According to this procedure, mast cells can be classified as AB-positive cells (MMC identified in blue and rich in chondroitin sulfate) or as S- and/or TB-positive cells (CTMC identified in brown with S and purple with TB, and rich in heparin). Combinations of these colors are referred as hybrid mast cells. The images were observed in an optical microscope (LEICA DM500; Leica Microsystems, Wetzlar, Germany) and photographed (NIKON, XM-100) in order to quantify the number of mast cells (in mm^2). The quantification of the different phenotypes of mast cells was made using an ImageJ/Fiji 1.46s software (Bethesda, MD, USA).