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In vitro and *in vivo* anti-*Helicobacter pylori* activity of *Calophyllum brasiliense* Camb.

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ABSTRACT

Aims of the study: Calophyllum brasiliense (Camb.) is a medicinal tree that grows particularly in the hilly and forested regions of Brazil. Preparations from its stem bark are popular remedies for the treatment of chronic ulcers. Since earlier investigations on bark extracts evidenced gastroprotective and gastric acid inhibitory properties, this study evaluated the effects of hydroethanolic extract (HEE*Cb*) and the dichloromethanic fraction (DCMF), from *Calophyllum brasiliense* stem bark, against *Helicobacter pylori*, *in vitro* and *in vivo*.

Materials and methods: The *in vitro* assays were performed using the disk diffusion and broth microdilution methods to determine the minimum inhibitory concentration (MIC) values. The test substances were evaluated *in vivo* taking into account the delay in the gastric ulcer healing in Wistar rats, infected with *Helicobacter pylori*.

Results: DCMF appeared the most active and potent *in vitro* against *Helicobacter pylori* growth with an MIC of 31 μg/mL. In the *in vivo* assays, rats ulcerated by acetic acid, and inoculated with *Helicobacter pylori* showed a marked delay in ulcer healing. Treatment with HEE*Cb* (50, 100 and 200 mg/kg) and DCMF (100 and 200 mg/kg) reduced the ulcerated area in a dose-dependent manner. While DCMF, at 200 mg/kg, increased the prostaglandin E_2 (PGE_2) level, both HEE*Cb* and DCMF decreased the number of urease-positive animals, as confirmed by the reduction of *Helicobacter pylori* presence in histopathological analysis.

Conclusion: The results suggest that the antiulcer activity of *Calophyllum brasiliense* is due, in part, to its anti-*Helicobacter pylori* action, validating the popular use of this species.

1. Introduction

Peptic ulcer is generally a chronically evolving disease, which results from the circumscriptive loss of tissue in regions of the digestive tract that may come into contact with the stomach's chloride peptic secretion (Coelho, 2003). In general, it is caused by an imbalance between aggressive and defensive factors of the gastric mucosa (Rao et al., 2000). The identification and isolation of *Helicobacter pylori* allowed for a considerable development of knowledge about peptic ulcer-related pathology (Marshall and Warren, 1984). This pathogen is considered the main etiological agent of human peptic ulcer, with a worldwide prevalence rate of about 40% in developed countries and over 80% in developing countries (Shi et al., 2008).

Traditional medicine has made use of plants since ancient times to treat varied gastrointestinal diseases including peptic ulcers (Rodríguez et al., 2006). Numerous studies have been published involving tests with medicinal plants showing antiulcer activity (Falcão et al., 2008); however, few of these studies have targeted *Helicobacter pylori*. The bark of *Calophyllum brasiliense* Camb. (Clusiaceae), also known as guanandi, has been popularly used for treatment of gastric and hepatic disturbances (Corrêa, 1984; Guarim Neto, 1987). Sartori et al. (1999) demonstrated that the dichloromethanic fraction from the stem bark of *Calophyllum brasiliense* has gastroprotective activity in experimental models for gastric ulcer. Reyes-Chilpa et al. (2006), showed inhibition

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of the gastric H^+, K^+-ATPase pump by prenylated xanthones isolated from the methanolic extract of *Calophyllum brasiliense* heartwood. Caneppele et al. (2008) have isolated, from the same dichloromethanic fraction, three chromanones—brasiliensic, isobrasiliensic and inophylloidic acids.

The present work was aimed to evaluate the *in vitro* and *in vivo* anti-*Helicobacter pylori* activity of the hydroethanolic extract and the dichloromethanic fraction from *Calophyllum brasiliense*, as well as to investigate the possible mechanisms involved.

2. Material and methods

2.1. Animals

Male Wistar albino rats (160–210 g) and male Swiss-Webster mice (25–30 g), from the Central Animal House of the Universidade Federal de Mato Grosso (UFMT), were used. The animals were maintained in propylene cages at 26 ± 2 °C under 12 h light–dark cycle, with free access to water and restricted access to food, 2 h/day (9–10 a.m. and 6–7 p.m.). Groups of 4–10 animals were used for the experiments. The experimental protocols were approved by the Committee for Ethics in Animal Experimentation (CEPA/UFMT), according to the Federal Government legislation on animal care.

2.2. Microorganism

Helicobacter pylori strain ATCC 43504 (VacA and cagA positives) was obtained from the Fundação Oswaldo Cruz – Fiocruz/RJ, Brazil. Stock cultures were maintained in Mueller–Hinton broth (Himedia, Mumbai, India) at −20 °C.

2.3. Botanical material

Calophyllum brasiliense stem bark was collected at the headwaters of the Coxipó River, adjacent to the road leading to Santo Antônio do Leverger, in the city of Cuiabá-MT, GPS coordinates: (S15°38′40.8″) (W056°03′05.6″) on July 09, 2007 at 09:30 a.m. The collection was authorized by the Brazilian Institute of Environment and Renewable Natural Resources. The flowered voucher for *Calophyllum brasiliense* is deposited and registered under the number 37.993 at the UFMT Herbarium, having been taxonomically validated by MSc. Harri Lorenzi, from Instituto *Plantarum* de Estudos da Flora, in Nova Odessa, SP.

2.4. Extract and dichloromethanic fraction preparation and phytochemical analysis

Calophyllum brasiliense stem bark was cleaned, dried at room temperature and shredded in an electric mill with a sieve with a mesh size of 40, until powder was obtained. The dried powder was successively macerated (1:5, w/v), with hexane, dichloromethane, ethyl acetate, methanol and water–ethanol 75%, for 7 days each (yield 10.4, 4.2, 5.2, 16.7 and 10.3%, respectively). Every extract was separated by filtration and concentrated under reduced pressure at, approximately, 40 °C, with the residual solvent being eliminated in an incubator at 40 °C. To prepare the dichloromethanic fraction, the crude hexanic extract was submitted to silica filtration using dichloromethane (yield, 0.67%). The preliminary phytochemical analyses of the hydroethanolic extract and the dichloromethanic fraction followed the methodology described by Matos (1988).

2.5. In-vitro assays

2.5.1. Disk diffusion

For the disk diffusion assay, serial dilutions of the hexanic (HE*Cb*), dichloromethanic (DE*Cb*), ethyl acetate (EAE*Cb*), methanolic (ME*Cb*) and hydroethanolic (HEE*Cb*) extracts and of the dichloromethanic fraction (DCMF), from *Calophyllum brasiliense* stem bark, were prepared, hexanic (HE*Cb*), dichloromethanic (DE*Cb*) and ethyl acetate (EAE*Cb*), which are the most apolar ones, were dissolved in Tween 2% (v/v), whereas methanolic (ME*Cb*) and hydroethanolic (HEE*Cb*) were dissolved in sterilized distilled water, in order to obtain the following doses: 62.5; 125; 250; 500 and 1000 μg/disk. The sterile disks utilized (6 mm – CECON®, São Paulo – SP, Brazil) were imbibed in 25 μL of each dose of extract and fraction. The extract- or fraction-imbibed disks were deposited on the surface of the plate inoculated with *Helicobacter pylori*, in a suspension of 6×10^8 CFU/mL (McFarland turbidity standard 2), using clarithromycin (15 μg – CECON®, São Paulo – SP, Brazil) as the standard drug, incubated at 37 °C under microaerophilic conditions in an atmosphere of 5–15% O_2 and 5–10% CO_2 for 3–5 days. After this period, the growth inhibition halos were quantified with a digital pachymeter. The diameters of inhibitory zones were measured in duplicate and mean values ≥10 mm were considered active.

2.5.2. Broth microdilution

The broth microdilution assay allows the determination of the Minimum Inhibitory Concentration (MIC). To each well in the microplate was added 100 μL of Mueller–Hinton broth (Himedia, Mumbai, India), supplemented with 10% fetal calf serum inoculated with 6×10^8 *Helicobacter pylori* (McFarland turbidity standard 2), 100 μL of the HE*Cb*, EAE*Cb*, DE*Cb* ME*Cb*, HEE*Cb* and DCMF, all from *Calophyllum brasiliense*, prepared as described in Section 2.5.1, were also added to reach the final concentrations of 15.6; 31.2; 62.5; 125; 250; 500 and 1000 μg/mL. Clarithromycin 5 μg/mL (Medley, São Paulo – SP, Brazil) was used as the standard drug for growth inhibition. Next, the microplate was incubated at 37 °C under microaerophilia in an atmosphere of 5–15% O_2 and 5–10% CO_2, for 3–5 days. After incubation, the plates were visually examined and each well was replicated in blood agar (Mueller–Hinton agar with 5% sheep blood, Newprov, Pinhais – PR, Brazil), to determine whether growth had occurred, with the MIC defined as the lowest concentration to cause complete bacterial growth inhibition (bactericidal activity).

2.6. In-vivo assays

2.6.1. Acute toxicity evaluation

The acute toxicity evaluation of the HEE*Cb* and DCMF from *Calophyllum brasiliense* stem bark was performed in mice ($n = 4$). The animals were treated orally (p.o.) with HEE*Cb* at 250, 500, 1000, 3000 and 5000 mg/kg doses and with DCMF at 250, 500, 1000 and 3000 mg/kg doses. A control animal was used for each dose, having received the vehicle (distilled water, 10 mL/kg p.o.). After the administration of the HEE*Cb*, DCMF or vehicle, the animals were observed individually in appropriate cages (open field) at 0, 15 and 30 min; 1, 2, 4 and 8 h and, once every day, for 14 days. The results for the general behavioural observations were recorded in a table adapted from Malone (1977).

2.6.2. Ulcer induction and colonization by Helicobacter pylori

Rats were ulcerated by using acetic acid according to the method described by Takagi et al. (1969), with modifications. Briefly, under anesthesia, laparotomy was performed in rats through a midline epigastric incision, the stomach was exposed, and 20% acetic acid (0.03 mL) was injected into the subserosal layer of the glandular portion, using a microsyringe (0.05 mL). After closing the abdominal incision, the animals were maintained in individual cages, with daily access to commercial food restricted to the time periods of 9–10 a.m. and 5–6 p.m., thus allowing adequate fasting for administrations of *Helicobacter pylori*, and HEE*Cb* (50, 100 and 200 mg/kg) and DCMF (100 and 200 mg/kg) of *Calophyllum brasiliense* as well