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ANTI- *Helicobacter pylori* ACTIVITY OF PLANT EXTRACTS TRADITIONALLY USED FOR THE TREATMENT OF GASTROINTESTINAL DISORDERS

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ABSTRACT

The antibacterial activity of plant extracts obtained from *Bixa orellana* L., *Chamomilla recutita* L., *Ilex paraguariensis* A. St.-Hil., *Malva sylvestris* L., *Plantago major* L. and *Rheum rhaponticum* L. has been evaluated against two reference strains and eleven clinical isolates of *Helicobacter pylori*. All the plant species chosen are used in popular Brazilian cuisine and folk medicine in the treatment of gastrointestinal disorders. Initial screening was made by the disk diffusion test and then minimum inhibitory concentration was determined by the agar dilution method. The results presented in this work demonstrated that among the plant preparations analyzed, *B. orellana* L., *C. recutita* L., *I. paraguariensis* A. St.-Hil. and *M. sylvestris* L. were capable of inhibiting the *in vitro* growth of *H. pylori*.

Key words: *Helicobacter pylori*, antibacterial activity, plant extracts.

INTRODUCTION

Helicobacter pylori is a Gram-negative spiral-shaped bacterium that was first isolated by Barry Marshall and J. Robin Warren. Since its discovery in 1983, the microrganism has been associated with the etiopathogenesis of several diseases of the digestive system, such as gastritis, peptic ulcer disease and gastric cancer (11). Conventional treatment for eradication therapy of these infections is mainly based on the use of multiple drugs, such as clarithromycin, amoxicillin, furazolidone, tetracycline and metronidazole with bismuth or a proton pump inhibitor (15).

Although the conventional treatment for eradication therapy of *H. pylori* allows obtaining high cure rates, eradication failure rate remains of 5-20 %. This fact may be partially explained by non-compliance in some patients who do not follow the treatment properly and by the development of resistance to antibiotics used (10). Therefore, there is a growing need to search new therapeutic agents that can hopefully eradicate this significant human pathogen and medicinal plants are a useful source of novel drugs. Several natural products have demonstrated antibacterial activity against *H. pylori* (18) and for centuries a wide variety of plants and substances derived from plants have been used to treat gastrointestinal disorders (2).

Many plants used in Brazil to treat these infections do not

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present any scientific evidence of efficacy. It is interesting to determine whether their traditional uses are supported by pharmacological effects or merely based on folklore. Within this context, extracts obtained from *Bixa orellana* L. (annatto), *Chamomilla recutita* L. (chamomile), *Ilex paraguariensis* A. St.-Hil.(roasted and green yerba maté), *Malva sylvestris* L. (mallow), *Plantago major* L. (plantain) and *Rheum rhaponticum* L. (rhubarb) - all of which are used in popular Brazilian cuisine and folk medicine in the treatment of gastrointestinal disorders - were investigated for their anti-*H. pylori* activity.

MATERIALS AND METHODS

General

Roots, rhizomes or aerial parts (leaves, stems, seeds, inflorescence) of the plants *Bixa orellana* L., *Chamomila recutita* L., *Ilex paraguariensis* A. St.-Hil., and *Plantago major* L. were collected in Paraná state, Southern region of Brazil (cities of Morretes, Lapa, Piraquara, and Curitiba respectively) and identified by Dr. Gerdt Hatschbach from Museu Botânico Municipal da Prefeitura de Curitiba, Paraná (MBM), where the vouchers have been deposited. The plants *Malva sylvestris L.* and *Rheum rhaponticum L.* were obtained commercially (Flores & Ervas, Piracicaba, SP, Brazil); the voucher specimens, including identification and classification of plant materials, had been preserved by the company.

The parts of each plant examined and voucher numbers are shown in Table 1.

Extraction of materials

A total of 50g of each plant species was exhaustively extracted with aqueous 96% ethanol (v/v) by maceration at room temperature. The extracts were obtained after filtration and concentration of the material under reduced pressure until the final volume of 50 ml.

Stock solutions of the extracts were made with sterile distilled water at concentration of 100 mg/ml which were used in the disk diffusion test. Another was made at the same concentration, now with dimethylsulphoxide (DMSO), to perform the determination of the minimum inhibitory concentration. Final concentration of DMSO in the culture medium did not exceed 1% (12).

Bacterial strains

A total of eleven clinical isolates of *H. pylori* obtained from the gastric mucosa of patients submitted to upper endoscopy and subsequently diagnosed with gastritis, peptic ulcer disease or gastric cancer were used in the present study. Clinical isolates were coded with the numbers of access BP-84, BP-667, BP-660, BH-27, BP-446, BP-650, BP-118, BP-713, BP-132, BP-652 and F-39 in order to preserve the identity of the patients from whom they were obtained and were previously approved by the Ethics Committee with the issuing of protocol number 982.021/2005-01.

Reference strains *H. pylori* 26695 (23) and J99 (1), that had their genomes completely sequenced, were tested as control. All the strains were previously evaluated against clarithromycin, amoxicillin, furazolidone, tetracycline and metronidazole, which are antibiotics commonly used in conventional therapy.

Preparation of bacterial suspensions

An inoculum of each strain used in susceptibility tests was prepared by transferring fresh colonies of the microrganisms in tubes containing sterile physiological saline solution and adjusting the turbidity to the 2.0 McFarland standard (7). This turbidity produces a suspension that corresponds to approximately 6.0 x 10^8 CFU/mL of *H. pylori*.

Disk diffusion test

In the initial phase, the disk diffusion test was used as screening to analyze the susceptibility of reference strains *H. pylori* 26695 and J99 against to different plant extracts. The bacterial suspensions were spread-plated onto Columbia Agar plates (Oxoid, Basingstoke, UK) supplemented with 10% defibrinated sheep blood (Newprov, Curitiba, Brazil). Filter paper disks of 6mm diameter impregnated with 5mg of each

extract (50µl of stock solutions) were placed onto the surface of the inoculated agar. The plates were incubated at 37ºC under microaerophilic conditions and observed after 3 to 5 days. The tests were performed in triplicate and the antimicrobial activity was expressed in terms of the mean diameter of the inhibition zone around the disks impregnated with the plant extracts tested, as presented in Table 1.

Determination of the minimum inhibitory concentration

All the extracts that had produced an inhibition zone greater than 6 mm in the disk diffusion test were separated to determinate the MIC by the agar dilution method. In addition to reference strains, 11 clinical *H. pylori* isolates were subjected to this test.

The stock solutions made with DMSO were further serially diluted in distilled sterile water and 1 mL of each dilution was incorporated into 19 mL of molten Columbia agar (Oxoid, Basingstoke, UK) containing 10% defibrinated sheep blood (Newprov, Curitiba, Brazil) to be then transferred separately into Petri dishes. The final concentrations of the extracts in the culture medium ranged from 5.0 to 0.625 mg/mL.

Bacterial suspensions were prepared as described above, and 1 µL of each suspension was spotted with a multipoint inoculator onto the surface of the agar plates containing consecutive dilutions of plant extracts. After that, plates were incubated at 37ºC in a microaerophilic atmosphere for 72 hours and MIC, which is defined as the lowest concentration of an extract that inhibits the visible growth of a microrganism, was determined. For clinical isolates, MIC_{50} and MIC_{90} were determined and defined as the concentrations that inhibited, respectively, 50 and 90% of the strains evaluated. All tests were conducted in triplicate, in addition to growth controls with and without DMSO.

RESULTS AND DISCUSSION

According to the data reported in Table 1, of all the plant extracts submitted to the screening test, *B. orellana* L., *C. recutita* L., *I. paraguariensis* A. St.-Hil. (green and roasted Yerba Maté varieties) and *M. sylvestris* L. produced inhibition zone diameters by the disk diffusion test. However, there is a disadvantage to this method in that it yields only qualitative results. The absence of objective quantification inherent in the method makes it impossible to compare the degree of antimicrobial activity of the extracts against the *H. pylori* strains investigated (3). For that reason, in the next stage of the study, MIC values were determined by the agar dilution method. The results obtained are shown in Table 2.

The agar dilution test confirmed an anti-*H. pylori* activity of all the plant extracts evaluated, with *C. recutita* L. and *I. paraguariensis* A. St.-Hil. (green Yerba Maté variety) showing to be more potent (MIC_{50}: <0.625 mg/ml) than *B. orellana* L. (MIC_{50}: 1.25 mg/ml), *I. paraguariensis* A. St.-Hil. (roasted Yerba Maté variety) (MIC_{50}: 1.25 mg/ml) and *M. sylvestris* L (MIC_{50}: >5.0 mg/ml). The MIC_{90} values demonstrated that *I. paraguariensis* A. St.-Hil. was able to inhibit a higher number of clinical isolates when compared with other extracts, although the green Yerba Maté variety (MIC_{90}: 5.0 mg/ml) was slightly less active than the roasted variety (MIC_{90}: 2.5 mg/ml).

Previous investigations have demonstrated that *I. paraguariensis* A. St.-Hil., widely consumed as part of the usual diet in Brazil in the form of *tea* (roasted yerba maté) and *chimarrão* (green yerba maté), presents several secondary metabolic products that have antimicrobial activity, including phenolic compounds, triterpenes and flavonoids (21). As for *C. recutita* L., this plant has anti-inflammatory and calming properties and is also used to treat gastric colic, and several forms of gastritis, stomatitis, laryngitis and pharyngitis (17). Flavonoids - particularly apeginine - and essential oils are among the main constituents of the plant extract (13).

Research conducted by Stamatis *et al*. (22) confirmed the anti-*H. pylori* activity of *C. recutita* L. extract. Although, the plant part used to produce the extract in their work was not specified, which may directly influence the development of results (5).

B. orellana L. and *M. sylvestris* L. were other plant extracts evaluated by the agar dilution method. The first plant -

Table 1. Analysis of anti-*Helicobacter pylori* activity of plant extracts by disk diffusion test.

Species (voucher numbers)	Family	Plant part used	Mean of inhibition zone * (mm)	
			***H. pylori* J99**	***H. pylori* 26695**
Bixa orellana L. (MBM 212752)	Bixaceae	Seed	7	10
Chamomilla recutita L. (MBM 189637)	Asteraceae	Inflorescence	10	11
Ilex paraguariensis A. St.-Hil. (MBM 113738)	Aquifoliaceae	green leaves	9	10
Ilex paraguariensis A. St.-Hil. (MBM 113738)	Aquifoliaceae	roasted leaves	9	9
Malva sylvestris L. (Flores & Ervas)	Malvaceae	inflorescence and leaves	10	8
Plantago major L. (MBM 243458)	Plantaginaceae	above-ground parts	< 6	< 6
Rheum rhaponticum L. (Flores & Ervas)	Polygonaceae	Root	< 6	< 6

*Final concentration of each extract = 5 mg/disk

Table 2. MIC (mg/mL) values of plant extracts against clinical isolates and reference strains of *Helicobacter pylori*.

***H. pylori* strains**	**Plant extracts**				
	B. orellana	***C. recutita***	***I. paraguariensis* (green yerba mate)**	***I. paraguariensis* (roasted yerba mate)**	***M. sylvestris***
H. pylori 26695	< 0.625	< 0.625	< 0.625	< 0.625	< 0.625
H. pylori J99	< 0.625	< 0.625	< 0.625	2.5	1.25
BP-84	>5.0	>5.0	5.0	1.25	>5.0
BP-667	>5.0	>5.0	5.0	5.0	>5.0
BP-660	>5.0	>5.0	5.0	1.25	>5.0
BH-27	1.25	< 0.625	< 0.625	< 0.625	>5.0
BP-446	1.25	< 0.625	< 0.625	2.5	>5.0
BP-650	>5.0	>5.0	5.0	< 0.625	>5.0
BP-118	< 0.625	< 0.625	< 0.625	< 0.625	0.625
BP-713	>5.0	< 0.625	2.5	5.0	2.5
BP-132	>5.0	< 0.625	< 0.625	2.5	5.0
BP-652	< 0.625	< 0.625	< 0.625	2.5	2.5
F-39	1.25	< 0.625	< 0.625	< 0.625	>5.0
MIC_{50}	1.25	<0.625	<0.625	1.25	>5.0
MIC_{90}	>5.0	>5.0	5.0	2.5	>5.0

widely used in Brazilian home cooking - is known to contain an essential oil rich in *all*-E-geranylgeraniol, oxygenated monoterpenes and sesquiterpenes (8). The second one is composed of mucilage, tannins, essential oils and flavonoids (4) reasons why it is used as anti-inflammatory and support in the treatment of different types of infections (14).

Moreover, it is important to note that the most active substances found in the plants screened in these experiments have recognized properties in gastrointestinal digestive diseases and presented stable activity at acid pH (9).

Increasing antimicrobial resistance is a serious global problem that is present in this important human pathogen (6). Mendonça *et al*. reported the susceptibility profile involving Brazilian *H. pylori* strains. Resistance rates were observed as to metronidazole, amoxicillin and clarithromycin of 42%, 29% and 7% respectively; values of furazolidone (4%) and tetracycline (7%) were also presented (16).

In this study, for each *H. pylori* strain evaluated for the antimicrobial activity of plant extracts, susceptibility to antibiotics used in conventional therapy, was also characterized as shown in Table 3. These strains presented different susceptibility profiles and, in some cases, resistance to one or more antibiotics. Interestingly, the resistant strains evaluated against the different extracts, demonstrated a similar profile when compared to sensitive ones (Table 2).

Table 3. Susceptibility test of *Helicobacter pylori* reference strains and clinical isolates.

	Antibiotics				
Strains	**Cla**	**Am**	**Fu**	**Tet**	**Met**
26695*	S**	S	S	S	S
J99*	S	S	S	S	S
BP-84	S	R***	S	S	S
BP-667	S	S	R	S	S
BP-660	S	S	S	S	S
BH-27	S	R	S	S	S
BP-446	R	S	S	S	R
BP-650	S	S	S	S	S
BP-118	S	R	S	R	S
BP-713	S	S	S	S	S
BP-132	S	R	S	S	S
BP-652	S	S	S	S	S
F-39	S	S	S	S	S

Cla - Clarithromycin, Am - Amoxicillin, Fu - Furazolidone, Tet - Tetracycline, Met – Metronidazole
* Reference strains, ** Susceptibility, ***Resistance.

In summary, a variety of plant species is capable of synthesizing many substances which show antibacterial activity. These properties have been described to extracts of many plants found in Brazilian flora (19,20). However, as regards the plant extracts included in this work, there are no previous studies that evaluate the proposed feature, except for *C. recutita* L. (22). Results demonstrate that the extracts obtained from plants *B. orellana* L., *C. recutita* L., *I. paraguariensis* A. St.-Hil. and *M. sylvestris* L. were capable of inhibiting the *in vitro* growth of *H. pylori* and could form a promising basis for further investigation in the discovery of new natural anti-*H. pylori* compounds.

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