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In Vitro and *In Vivo* Anti-*Helicobacter pylori* Activity of Natural Products

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1. Introduction

Since old times plants have been a resource used by human beings an important sources of biologically active products. Recently, efforts have been made in order to identify new antiulcerogenic drugs from natural sources, having as the main target the *Helicobacter pylori*, a bacterium considered as the most important etiological agent of the human peptic ulcer. It has been shown to be a rich source of bioactive substances, having antifungal, gastroprotective, analgesic, anti-HIV, antibacterial, antitumoral properties and inhibitor of gastric H+,K+-ATPase and angeotensin-converting enzyme. This chapter aims to demonstrate methods anti-*Helicobacter pylori in vitro* and *in vivo* for screening of plant extracts.

Helicobacter pylori was identified in 1982 by Marshall and Warren (1984) and quickly became the subject of countless microbiological, histological, epidemiological, immunological, ecological and clinical studies (Vaz, 2005). This organism has its nomenclature revised, starting with Campylobacter pyloridis, and a correction of the name was originally Greek to Latin, Campylobacter pylori (Marshall and Goodwin, 1987) and organisms like Campylobacter. Taxonomic studies have led to reclassification, resulting in the name Helicobacter pylori (Goodwin and Armstrong, 1990).

H. pylori is a bacillus, Gram negative, microaerobic, spiral and curved (Dunn et al. 1997). It has two to six flagella that provide motility to it to resist the rhythmic contractions of stomach and penetrate the gastric mucosa. It has 2.4 to 4.0 mm in length and 0.5 to 1.0 mm in width (Brown, 2000).

The identification and isolation of *Helicobacter pylori* allowed for a considerable development of knowledge about peptic ulcer (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).

H. pylori produces a number of virulence factors that may have different associations with the disease. The establishment of chronic infection may be influenced by host genetic factors as well as the blood group ABO and Lewis-blood group antigen and differences in susceptibility to particular strains of *H. pylori* (Brown, 2000).

To establish and maintain the infection, H. pylori expresses a variety of different types of maintenance factors that allow bacteria to colonize and remain within the host and virulence factors, which contributes to the pathogenic effects of bacteria, with emphasis on gastric inflammation, mucosal barrier disruption gastric and changes in gastric physiology (Dunn

et al. , 1997). *H. pylori* is a genetically highly diverse bacterium, featuring several genotypes which have been associated with virulence factors and risk of gastric disease and other outcomes of infection. Among these, the vacA gene, which encodes a cytotoxin of vacuolization, is present in all types of *H. pylori*. This gene is also strongly associated with high levels of inflammation and epithelial damage in the gastric mucosa, caused by cagA gene is a marker for the presence of PAI pathogenicity (cag pathogenicity island) (Ladeira et al., 2003).

In previous studies, infection with different types of Helicobacter, as H. mustela, H. felis, H. heilmanii, and H. pylori in mice, cats, pigs, monkeys and gerbils have been described suggesting its relevance to human infection, but these animal models do not mimic the infection of H. pylori in humans because of the lack of virulence factors of infecting organisms, such as vacA and cagA encoded cytotoxins, required for the mucosal damage, inflammation and ulcer formation (Konturek et al., 2000). Moreover, some of these animals are large and unwieldy, there is a need to test commonly used in animal models such as mice, which could be used to study various aspects of infection by H. pylori, ulcer healing and therapy of infection (Ross et al., 1992).

Results in experimental studies in animal models using rodents, concluded that H. pylori alone causes little or no effect on the gastric mucosa of intact rats. However, this organism can cause persistence of pre-existing ulcers and chronic active inflammation. Presence of predisposing factors leading to disruption of the integrity of the gastric mucosa may be necessary for the H. pylori enhancement inflammation and tissue damage to the stomach of these animals (Konturek et al 2000). Whereas peptic ulcer is generally a 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). It seems that the Helicobacter pylori takes advantage of this situation to colonize and settle in the gastric mucosa.

2. Experimental protocols

2.1 Animals

Male Wistar albino rats (160-210 g) and male Swiss-Webster mice (25-30 g), can be used. The animals should be kept in propylene cages at 26±2ºC under 12h light-dark cycle, with free access to water and restricted access to food, 2 hours/day (9-10a.m. and 6-7p.m.).

2.2 Microorganism

The strain of *Helicobacter pylori* ATCC 43504 (vacA and cagA positives) can be used to express the factors that determine their virulence. Stock cultures can be maintained in Mueller-Hinton broth at -20ºC.

2.3 Botanical material

Plants should be carefully collected and treated to prevent fungal contamination. The plants should be deposited, registered and taxonomically verified.

2.4 Extract preparation and phytochemical analysis

Plants should be cleaned, dried at room temperature and shredded in an electric mill with a sieve with a mesh size of 40μm, until powder be obtained. The dried powder should be

successively macerated (1:5, w/v), with hexane, dichloromethane, ethyl acetate, methanol and water-ethanol 75%, for 7 days each. Every extract should be separated by filtration and concentrated under reduced pressure at, approximately, 40ºC, with the residual solvent being eliminated in an incubator at 40ºC. To prepare the dichloromethanic fraction (Fig. 1), the crude dichloromethanic extract should be submitted to silica filtration using dichloromethane. The preliminary phytochemical analyses of the hydroethanolic extract and the dichloromethanic fraction may follow the methodology described by Matos (1998).

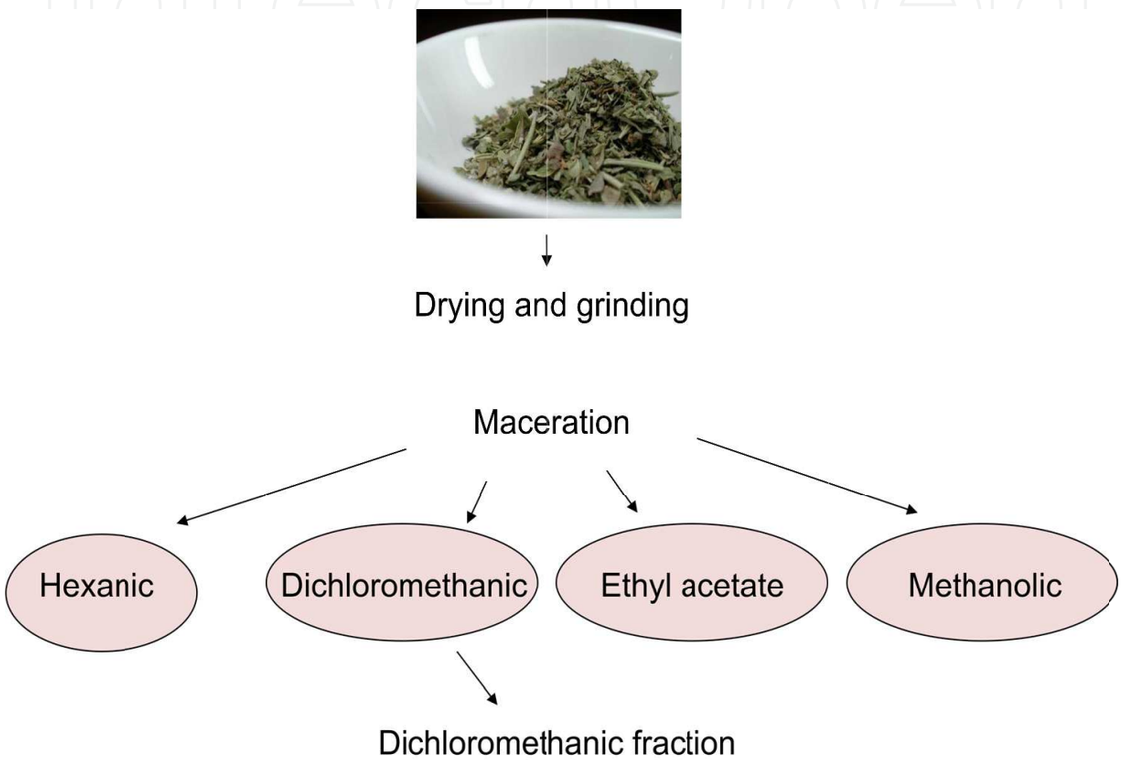

Fig. 1. Scheme of preparation of extracts and fractions

2.5 In-vitro assays

2.5.1 Disk diffusion

For the disk diffusion assay, serial dilutions of the hexanic, ethyl acetate, dichloromethanic, methanolic and hydroethanolic extracts from plants, should be prepared, in order to obtain the following doses: 0.0625; 0.125; 0.25; 0.5 and 1 mg/disk. The sterile disks utilized (6 mm - CECON®) should be imbibed in 25µL of each dose of extract and fraction. The extract- or fraction-imbibed disks should be deposited on the surface of the plate inoculated with *H. pylori*, in a suspension of $6x10^8$ CFU/mL (McFarland turbidity standard 2), using clarithromycin (15µg - CECON®) as the standard drug, incubated at 37°C under microaerophilic conditions in an atmosphere of 5 to 15% O_2 and 5 to 10% CO_2 for 3-5 days. After this period, the growth inhibition halos should be quantified with a digital pachymeter. The diameters of inhibitory zones should be measured in duplicate and mean values ≥10 mm are considered active (Fig. 2).

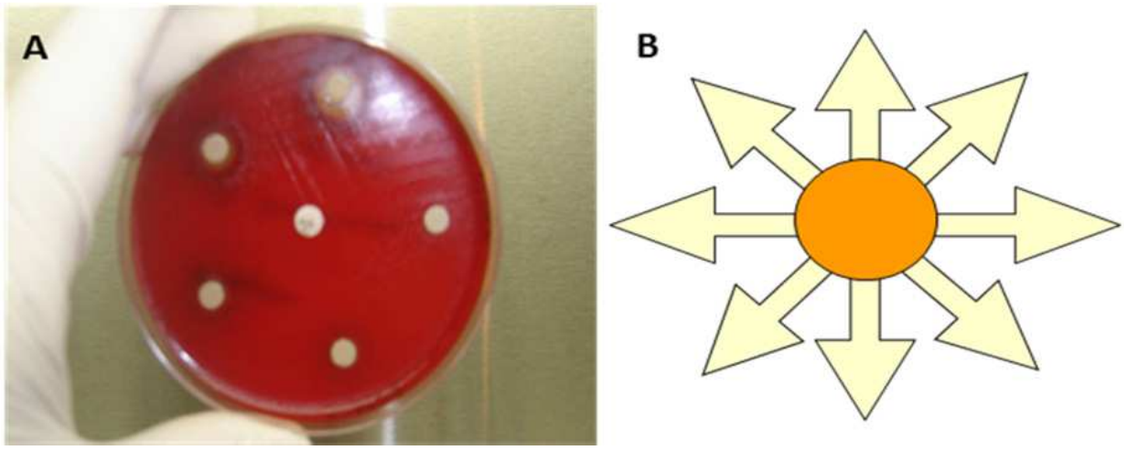

Fig. 2. Photograph (A) and scheme (B) of a disk diffusion

2.5.2 Broth microdilution

The broth microdilution assay allows the determination of the Minimum Inhibitory Concentration (MIC). To each well in the microplate should be added 100µL of Mueller-Hinton broth, supplemented with 10% foetal calf serum inoculated with $6x10^8$ *H. pylori* (McFarland turbidity standard 2), 100µL of the hexanic, ethyl acetate, dichloromethanic, methanolic and hydroethanolic extracts from plants, should be also added to reach the final concentrations of 0.0625; 0.125; 0.25; 0.5 and 1 mg/mL. Clarithromycin (5 mg/mL) is used as the standard drug for growth inhibition. Next, the microplate (Fig. 3), should be incubated at 37°C under microaerophilia in an atmosphere of 5 to 15% O_2 and 5 to 10% CO_2, for 3-5 days. After incubation, the plates should be visually examined and each well should be replicated in blood agar (Mueller-Hinton agar with 5% sheep blood), to determine whether growth had occurred, with the MIC defined as the lowest concentration to cause complete bacterial growth inhibition (bactericidal activity).

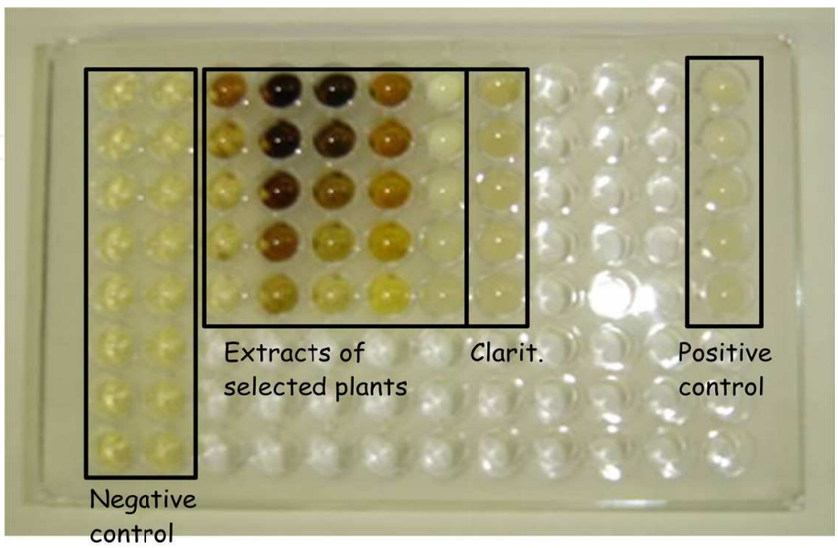

Fig. 3. Photograph of a microdilution plate

2.6 In-vivo assays

2.6.1 Acute toxicity evaluation

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

2.6.2 Ulcer induction and colonization by *H. pylori*

Rats should be ulcerated by acetic acid according to method described by Takagi et al. (1969), with modifications. After ulcer induction, the animals should be kept in propylene cages, with daily access to commercial food restricted to the time periods of 9-10 a.m. and 5-6 p.m., allowing for adequate fasting for administration of *H. pylori*, and of extract at 50, 100 and 200mg/kg doses of preparations, as well as of the standard drugs (amoxicillin 50mg/kg + clarithromycin 25mg/kg + omeprazole 20mg/kg).

According to the method described by Konturek et al. (1999), with modifications, 24h after ulcer induction by acetic acid, the animals should be inoculated intragastrically with 1 mL of *H. pylori* ATCC 43504 ($9x10^8$) suspended in Mueller-Hinton broth, by using a cannula appropriate for orogastric gavage. For the animals in the control, Sham and acetic acid-induced ulcer groups without *H. pylori* infection, only Mueller-Hinton broth should be orally administered. The orogastric inoculation of *H. pylori* should be maintained twice a day for 7 days, whereas the administration of extracts from plants and of the standard drugs, twice a day, for 14 consecutive days, starting from the third day after ulcer induction by acetic acid. After treatment, the animals should be sacrificed by cervical dislocation; blood can be collected from the inferior vena cava. The stomachs should be removed for evaluation of gastric lesions, the ulcerated area (mm^2) should be measured and the healing rate (%) and then determined according to method described by Takagi et al. (1969). Prostaglandin E2 (PGE2) levels should be measured from gastric mucosal scrapings and a fragment from each stomach can be used for the histopathological exam and for the urease determination.

2.6.3 Determination of PGE_2 concentration

The concentration of PGE_2 in scrapings of gastric mucosa should be quantified by ELISA using a commercial kit (Parameter®, R&D Systems). The mucosal scrapings (100 mg) should be homogenized with 1 mL phosphate buffer and centrifuged at 3,000 RPM at 4^oC for 10 min. The PGE_2 levels should be determined according to the manufacturer's instructions.

It has been demonstrated that PGE_2, derived from COX-1 and COX-2, is involved in the regulation of gastric mucosa inflammation and also contributes to maintaining its integrity during infection by *H. pylori* through several mechanisms, including augmentation of the gastric mucosal flow, synthesis of mucus and bicarbonate, inhibition of gastric motility, and the release of enzymes, free radicals from neutrophils and gastric secretion (Chao et al., 2004).

2.6.4 Urease production determination

With the aid of tweezers, a fragment of gastric tissue should be inserted in the centre of a minitube containing urease gel (NEWPROV®). Inoculation times should be recorded, the minitubes should be kept at room temperature and the change in colour should be evaluated after 1 hour, and whenever it is negative, a final reading should be taken after 24 hours. A urease test should be considered positive if an alkaline reaction has developed (red or dark pink colour), and negative when there are no changes in the medium's colour (yellow or light orange).

Urease, an enzyme produced by *H. pylori*, acts by promoting the hydrolysis of urea, a substrate that is present in gastric juice under physiological conditions, leading to the production of ammonium, that behaves as a receptor for H^+ ions and generates a neutral pH inside the bacteria, thus contributing to the survival of these organisms in the highly acidic environment of the stomach (Ladeira et al., 2003). The rapid urease test is considered one of the most useful and cheapest tests among the invasive assays, with a 100% sensitivity and 89.5% specificity (Ogata et al., 2002), although false positive results may occur given the other bacterial species that might be isolated from the oral and/or gastric cavities (*Proteus mirabilis*, *Citrobacter freundii*, *Klebsiella pneumoniae*, *Enterobacter cloacae* and *Staphylococcus aureus*) and that also produce urease (Osaki et al., 2008).

2.6.5 Determination of cytokines IL-1β, TNF-α, IL-10 and VEGF

Total blood should be collected from the inferior vena cava, in tubes containing 5% EDTA, centrifuged at 3000 RPM for 10 min., and the plasma should be separated and frozen at -20ºC until the assay. For measuring plasma levels of IL-1β, TNF-α, IL-10 and VEGF, a plex kit for rat cytokines and chemokines (RCYTO-80K) should be used according to the manufacturer's instructions, and the fluorescence should be determined through a Luminex ® device.

The literature refers to IL-1β as being the most potent among the known gastroproctective agents (Kondo et al., 1994; El-Omar, 2004). TNF-α also inhibits gastric secretion, although to a lesser extent than IL-1β and is found, together with IL-10, elevated in patients with chronic gastritis associated with *H. pylori* infection (El-Omar, 2004). VEGF has been mentioned as a cicatrisation-promoting factor in gastric ulcer (Okabe and Amagase, 2005).

2.6.6 Histopathological analysis

After their removal, half of each stomach should be fixated in 10% buffered formalin and embedded in paraffin. From each block, two 5µm sections should be made, one being stained by hematoxilin and eosin (HE) and the other by a modified Giemsa stain for H. pylori detection.

All tissues should be examined by a pathologist, according to criteria established by Dixon et al. (1996), and the following parameters should be analyzed:

- Inflammation - presence of lymphocytes and plasmocytes in the lamina propria;
- Activity – characterized by the presence of neutrophils inside the superficial and glandular epithelial layers;
- Regeneration - characterized by a proliferative response to epithelial lesion, in which epithelial cells presenting larger hyper-stained / excessively stained nuclei, with an increase in the nucleus-cytoplasm ratio, and observation of occasional mitotic figures;
- Atrophy – reduction of glandular structures;

- Metaplasia - presence of caliciform cells with an intestinal morphology.

2.6.7 Statistical analyses

Results for the parametric tests should be expressed as mean ± standard error of the mean (S.E.M.). Statistical significance should be determined by one-way analysis of variance (ANOVA), followed by Tukey-Kramer or Dunnett's post-test. For frequency comparisons, Fisher's exact test should be used, and p values <0.05 should be considered significant.

3. Some considerations

Gastrointestinal diseases are one of the most important causes of the previously high morbidity rates in non-industrialized countries, which have been lowered, in part, by the many drugs employed for treatment of peptic ulcers. However, such drugs may have several side effects, on top of their high financial cost for underprivileged populations (Borrelli and Izzo, 2000). Therefore, the use of medicinal plants and the development of phytotherapies, at a low cost, would represent an alternative for treatment of gastrointestinal problems for a large population segment that does not have access to medication (Sartori 1997). In Brazil, numerous plant extracts are used in conventional medicine to treat many digestive disorders (Falcão et al., 2008).

With respect to the *in vitro* assays, it is important to emphasize that the disk diffusion method is recommended for studying polar substances, given that it allows the evaluation of different compounds against a microorganism and, therefore, establishes its antibacterial spectrum. For non-polar extracts the employment of diffusion techniques seems inadequate, since they do not readily diffuse in agar. In the broth microdilution method, the compound to be tested is mixed into the proper liquid medium that had been previously inoculated with the microorganism, allowing determination as to whether the compound is bacteriostatic (minimum bacteriostatic concentration) or bactericidal (minimum bactericidal concentration – MBC). It presents a higher sensitivity to drugs than the disk diffusion method because it permits direct contact between the drug and the microorganism and is, therefore, appropriate for assays assessing either polar or nonpolar substances (Rios and Recio, 2005).

With respect to the *in vivo* assays, the establishment of a persistent infection by *H. pylori* in laboratory animals that completely reproduces the basic characteristics of human infections (an intense active chronic gastritis, either antral or diffuse), their complications (mucosa atrophy and intestinal metaplasia) and their associated pathologies (peptic ulcer, gastric adenocarcinoma and lymphoma) is not easy to accomplish. Chronic ulcer by acetic acid injection into the gastric subserosa area, differently from acute ulcers, penetrates the muscle layer of the glandular area and, occasionally, relapses after wound healing, and is highly similar to the human gastric ulcer in light of its pathological characteristics and healing process (Okabe and Pfeiffer, 1972).

Previous results indicate that Wistar rats with pre-existing gastric ulcers, experimentally produced by acetic acid injection, developed active ulcers when exposed to *H. pylori*, similar to the results of Konturek et al. (1999), which were obtained with a different species (Souza *et al*, 2009).

In order to evaluate the *in vivo* anti-*H. pylori* activity and to verify the presence of this bacteria in the gastric mucosa of ulcerated infected rats, the urease test and histopathological

analysis should be carried out and monitored by the degree of cicatrisation of the gastric lesion.

The histopathological exam is considered one of the most specific tests for diagnosing *H. pylori* infections, presenting 98% sensitivity and 97% specificity (Lin et al., 1996; Ogata et al., 2002). The histopathological findings confirm the results found in the urease test, in which treatment of animals, ulcerated and inoculated with *H. pylori*. Moreover, parameters such as inflammation, ulcer persistency and neutrophilic activity, which are characteristics of *H. pylori* infection.

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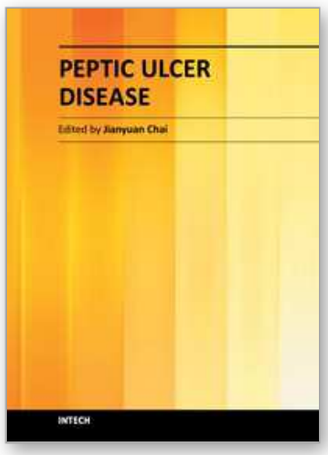

Peptic Ulcer Disease

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Peptic ulcer disease is one of the most common chronic infections in human population. Despite centuries of study, it still troubles a lot of people, especially in the third world countries, and it can lead to other more serious complications such as cancers or even to death sometimes. This book is a snapshot of the current view of peptic ulcer disease. It includes 5 sections and 25 chapters contributed by researchers from 15 countries spread out in Africa, Asia, Europe, North America and South America. It covers the causes of the disease, epidemiology, pathophysiology, molecular-cellular mechanisms, clinical care, and alternative medicine. Each chapter provides a unique view. The book is not only for professionals, but also suitable for regular readers at all levels.

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