Full Length Research Paper

In vitro inhibition of *Helicobacter pylori* by methanolic extract of *Stenocereus marginatus* and *Castela texana*

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Research on plants has as its purpose the development of new drugs to treat diseases that affect humans. The increasing prevalence of multidrug resistant strains of bacteria and the recent appearance of strains with reduced susceptibility to antibiotics raises the specter of untreatable bacterial infections and adds urgency to the search for brand new infection-fighting strategies. Current advancements in drug discovery technology and search for new drugs from plants have always been of great interest for the scientists working in this field. The biological activity of methanolic extracts of *Castela texana* and *Stenocereus marginatus* was evaluated *in vitro* in the growth of *Helicobacter pylori,* both extracts inhibited the growth, but the methanolic extract of *S. marginatus* completely inhibited biofilm formation; this structure is important for the protection of *H. pylori* and increased resistance to antimicrobial agents. The toxicity was determined in *Artemia salina*; the methanolic extract of *S. marginatus* is less toxic than the extract of *C. texana*. The phytochemical analysis of the methanolic extract of *S. marginatus* revealed that potential secondary metabolites with anti- *H. pylori* activity are flavones, sesquiterpene lactones and alkaloids. As a result, future research should be conducted to obtain pure substances from *S. marginatus* for possible treatment of *H. pylori*.

Key words: *Helicobacter pylori, Stenocereus marginatus, Castela texana.*

INTRODUCTION

Helicobacter pylori is a microaerophilic, Gram-negative spiral-shaped bacterium that was first isolated by Barry Marshall and J. Robin Warren. Since its discovery in 1984, the microorganism has been associated with the etiopathogenesis of several diseases of the digestive system, such as gastritis, peptic ulcer disease and gastric cancer (Marshall and Warren, 1984; Kusters, 2006; Cogo et al., 2010). World Health Organization has categorized *H. pylori* as a class-1 carcinogen (Sugiyama, 2004). *H. pylori* lives exclusively in the human stomach, the only known organism that can survive in an extremely acidic environment (Perez and Blaser, 1996). It is one of the most common chronic bacterial pathogens in human. Approximately 50% of people in the world are infected with it, and its prevalence is significantly higher in developing countries than in developed countries (Goodwin et al., 1997).

Current therapy is based on a combination of multiple drugs, such as clarithromycin, amoxicillin, furazolidone, tetracycline and metronidazole with bismuth or a proton pump inhibitor (Megraud and Lehours, 2007). Conventional treatment for eradication therapy of *H. pylori* allows obtaining high cure rates, the eradication

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failure rate remains 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 (Gadhi et al., 2001), only 80% of patients show absence of pathogen and the infection sometimes returns after treatment completion (Graham and Shiotani, 2008). The main reason for eradication failure can be *H. pylori*'s resistance to one of the antibiotics used for treatment (Megraud, 2004). Furthermore, chemical drugs have side effects such as diarrhea and colitis (Ogudo et al., 2014). Therefore, it is necessary to introduce alternative remedial regimens (Moghaddam, 2011).

Accordingly, there is a growing need to search new therapeutic agents who can hopefully eradicate this significant human pathogen and medicinal plants are a useful source of novel drugs (Yousuf et al., 2012). Plants represent an important source of diverse biomolecules with unique properties, some of which make them attractive candidates for the development of novel antimicrobials. Plant extracts have been evaluated *in vitro* for their activity against human-infecting gastrointestinal microorganisms (Haslam et al., 1989), and nontoxic effects (Beg and Ahgmad, 2000). Secondary metabolites of the plants, poses several pharmacology properties: antimutagenic, antiviral, antimycotic, antiprotozoal (Dunsmore et al., 2001; Chen et al., 2003) and antibacterial activities (Kalemba and Kunicka, 2003). One of these resources is medicinal plants that some of their therapeutic properties have been recognized in folk medicine. Most people have positive attitude toward natural products due to their natural origin and lesser toxicity (Naik et al., 2003).

Several natural products have demonstrated antibacterial activity against *H. pylori* (Nostro et al., 2005) and for centuries a wide variety of plants and substances derived from plants have been used to treat gastrointestinal disorders (Borrelli and Izzo, 2000). Many plants used to treat this infection do not present any scientific evidence of efficacy for anti- *H. pylori*. 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 *Castela texana* and *Stenocereus marginatus* were investigated for their anti-*H. pylori* activity.

Castela texana (T. and G.) Rose (syn. *C. tortuosa* Liebm, *C. nicholsonii* Hook) is a shrub commonly known in México as "chaparro amargo", is a plant of northeast of México that has been used in amoebic human dysentery and other parasitosis treatment since the beginning of this century (Nixon, 1916; Sellards and McIver, 1918); *C. texana* belongs to the Simaroubaceae family. Several quassinoids have been isolated of the methanolic extract obtained from the root or the aerial parts (Chaudhuri and Kubo, 1992; Dou et al., 1996), and the amebicidal effect is mainly attributed to one of them named chaparrin (Calzado et al., 1998).

S. marginatus belongs to the Cactaceae family, genus *Stenocereus spp.* (also named as *Pachicereus spp.*) and is represented by about 19 species; this species is semi-domesticated as it is used to create living fences. As a result, the species has been introduced to many parts of México and it has subsequently become naturalized and these populations are likely increasing and spreading (Yetman, 2007). The pulp of this fruit is a fresh and sweet food, the nutritional importance of which is derived from its high-sugar content plus considerable amounts of vitamins B, C, E and antioxidant activity (Beltran et al., 2009). The species is used as fodder, living fences, fuel wood and for medicinal purposes (Pimienta and Nobel, 1994).

The present study was carried out to evaluate the *in vitro* anti-*Helicobacter pylori* activity of methanolic extract of *C. texana* and *S. marginatus*.

MATERIALS AND METHODS

Plant materials

Samples of the plants *C. texana* and *S. marginatus* were collected from San Isidro, Villa Hidalgo, San Luis Potosí México in the following coordinates: 100°41' west longitude and 22°27' north latitude, with a height of 1,670 m above sea level; specimens were identified by the Herbarium of the Department of Botany, Facultad de Ciencias Biológicas, Universidad Autónoma de Nuevo Léon (México). A voucher specimen was maintained for reference in the Herbarium (#026948).

Preparation of extracts

C. texana

C. texana were dried at room temperature in the shade and ground separately before extraction, after which leaf, stem and root were obtained.

S. marginatus

The stem was washed and subsequently cut into thin slices and immediately placed in methanol. Each material was extracted using maceration with methanol. The powder of materials was macerated in methanol for 7 days and the mixture was subsequently filtered and the solvent evaporated using the rotary evaporator (Büchi Rotavapor R-114) at 40°C. Dried extracts were stored at 4°C.

Phytochemical screening

Chemical tests for the screening and identification of bioactive chemical constituents in the methanolic extract of *C. texana* (leaf, stem and root) and *S. marginatus* (stem) under study were carried out in extracts as well as

powder specimens using standard procedures. Tests were carried out for unsaturations, carbonyl group, phenolic hydroxyls, triterpenes and sterols, carbohydrates, lactones, sesquiterpenes lactones, flavonoids, alkaloids, saponin and aromaticity according to standard methods (Quiñones et al., 2013).

Preparation of plant extracts

The test solutions were prepared by weighing 5 g each of the different methanolic extracts, which were diluted in 15 mL Brucella broth (BBL™), filtered (Millipore disc 0.22 µm diameter) and collected in a borosilicate tube (Pyrex); when the sterile test was positive, the solution was employed.

Brine shrimp lethality test

Brine shrimp (*Artemia salina*) eggs (0.1 g) were hatched in artificial sea water prepared from commercial sea salt 38 g/L (Sasidharan et al., 2008). A lamp was placed above the open side of the tank to attract the hatched shrimps close to the tank wall. After 24 h, the shrimps matured as nauplii (*A. salina*) and were ready for the assay. The brine shrimp lethality bioassay was carried out on the *C. texana* (leaf, stem and root) and *S. marginatus* (stem) methanol extract using the standard procedure (Meyer et al., 1982; McLaughlin and Rogers, 1998). In a microplate (96 wells), 100 µL of the suspension of nauplii/well (approximately 10 nauplii) were added plus 100 µL of dilutions of the extracts to be tested (0.333 µg/µL) and incubated for 24 h. The microplate assays were then examined, and the number of dead larvae in each well was counted after 24 h. The total number of shrimps in each bottle was counted and recorded. Each concentration was tested in triplicate. A microplate test was then performed in which potassium dichromate (Sigma-Aldrich) to 400 ppm was used as positive control, and sea water as negative control. The death percentage (Equation 1) of the lethal doses 50% (LD_{50}) was determined using statistical analysis:

Percentage of death (%): (Total nauplii – Alive nauplii) × 100 / Total nauplii (Equation 1)

Microbiological studies

Biological activity was evaluated by turbidimetric method which consisted of placing 3 mL of culture medium of *H. pylori* (ATCC43504) in 13×100 mm tubes, to which were added each of the extracts in concentrations of 0.1, 1, 5, 7 and 10 mg/mL; each tube was inoculated with 75 µL of *H. pylori*, incubated for 24 h, and the absorbance was read at 635 nm in a Spectronic® GenesysTM5 (previously a test solution of 5 g/15 mL of distilled water was the prepared solution). As a positive control for inhibition, commercial drug ceftriaxone (10 mg/mL) was used, each bioassay was evaluated in three separate events in triplicate.

Statistical analysis

Data used for the analyses were the means of inhibition of antibacterial activity of plant extracts experiments, which have been repeated three times. The results were analyzed initially using analysis of variance (ANOVA) and then by Duncan's test. A value of p<0.05 was considered statistically significant. To determine the lethal doses 50% (LD_{50}) toxicity testing, the extracts were analyzed in triplicate by PROBIT analysis.

RESULTS

Partial chemical screening of the methanolic extracts of *C. texana* and   *S. marginatus*

The results of phytochemical screening are summarized in Table 1. The results of the tests used to identify the phytochemical functional groups and secondary metabolites present in the methanolic extracts of leaf, stem and root of *C. texana* and stem of *S. marginatus* are presented in Table 1. *S. marginatus* were identified in 9 functional groups (unsaturations, carbonyl group, phenolic hydroxyls, triterpenes and sterols, carbohydrates, lactones, sesquiterpenes lactones, flavonoids and alkaloids), whereas *C. texana* 12, 10 and 10 were identified in the functional groups in stem, leaf and root respectively, with the presence of saponins and aromaticity being evident.

Toxicity of methanol extracts of *C. texana* and *S. marginatus* on *A. salina*

The values corresponding to the median lethal dose (LD_{50}) of the methanol extracts of *C. texana* and *S. marginatus* on *A. salina* were: The methanol extract of *C. texana*-leaf (Ct-l) presents its LD_{50} for *A. salina* to 22.46 µg/mL, being the most toxic dose, followed by 35.06 µg/mL corresponding to *C. texana*-stem (Ct-s), and the dose of 56.52 µg/mL of methanol extract of *C. texana*-root (Ct-r); three doses are highly toxic in comparison to the LD_{50} of *S. marginatus* which was 164.118   µg/mL for *A. salina*. The methanol extracts of *C. texana* on *A. salina* showed marked toxic activity with lower doses of 100 µg/mL. These results demonstrated that *C. texana* methanol extracts were highly toxic, while *S. marginatus* proved nontoxic, representing the opportunity to search for an active substance for the treatment of *H. pylori*.

Biological activity

The cell yield of *H. pylori* did not show significant inhibition in the presence of methanolic leaf extract of *C. texana* (Ct-l) in concentrations of 0.1 and 1 mg/mL, contrary to what was observed with extracts of stem (Ct-s) and root (Ct-r) at the same concentrations. At doses of 5, 7 and 10 mg/mL, significant inhibition was observed with treatments Ct-l and no significant inhibition by Ct-s.

Table 1. Phytochemical screening data of crude methanolic extract of *C. texana* (leaf, stem and root) and *S. marginatus* (stem).

| Functional group | Test | C. texana | | | S. marginatus |
		Stem	Leaf	Root	Stem
Unsaturations	KMnO$_4$	-	+	+	+
Carbonyl group	2,4-Dinitrofenilhidracine	+	+	+	+
Phenolic hydroxyls	FeCl$_3$	+	+	-	+
Triterpenes and sterols	Salkowski	+	+	+	+
Carbohydrates	Molish	+	+	+	+
	Cumarines	+	-	+	-
Lactones	Lactones	+	+	+	+
Sesquiterpenes lactones	Baljet	+	-	+	+
Flavonoids	H$_2$SO$_4$	F	F	F	F
Alkaloids	Dragendorff	+	-	-	+
Saponins	Sodium bicarbonate	+	+	+	-
	Salkowski	+	+	+	-
Aromaticity	Sulfuric acidformaldehyde	+	+	-	-

- indicates absence; + indicates presence; F indicates flavonas.

The root extract (Ct-r) in 5 doses showed significant cell growth compared to the control (Figure 2).

The methanolic extract of the stem of *S. marginatus* in concentration of 0.1 mg/mL promoted a non-significant increase in cell performance slightly. Concentrations of 1, 5, 7 and 10 mg/mL significantly inhibited cell growth, with respect to control, however with 7 mg/mL, this inhibition was significantly higher. The growth of *H. pylori* to the drug of choice: ceftriaxone at a dose of 7 mg/mL was significantly inhibited. Moreover, the methanol extract of *C. texana* (leaf) and *S. marginatus* (stem) at a dose of 7 mg/mL marked significant inhibition with respect to *H. pylori* growth as observed in Figure 2.

The culture of *H. pylori* is characterized by the formation of a thick film or biofilm on the surface of the culture broth, whereas turbidity and precipitate are observed in the background. As regards *H. pylori* growth in the presence of the methanolic extract of *S. marginatus* (7 mg/mL) and drug ceftriaxone, no biofilm formation or precipitate was observed, indicating growth inhibition in the presence of these treatments (Figure 3).

DISCUSSION

Extracts of *S. marginatus* and *C. texana* show a clear inhibition of *H. pylori* as antibiotic ceftriaxone (Figure 1). The capacity of the methanolic extract of *S. marginatus* inhibits production of biofilm characteristic of *H. pylori*. Figure 3 gives a good overview and an option to conduct research aimed at finding new metabolites as alternative treatment of anti-*H. pylori*; this is supported by the observations made by Cole et al. (2004), who reported that drug resistance may be due the ability of this bacterium to produce or synthesize biofilm. In this regard,

Gilbert et al. (1990) mentioned that the production of biofilm represents a defense mechanism and adaptation of the cell to the environment as well as it possibly represents a means of resistance to the environment and a dispersal mechanism. In this sense, the methanol extract of *S. marginatus* and *C. texana* inhibited the growth of *H. pylori* and also the biofilm. As such, it is suggested that research should be conducted for identification of the metabolites that are involved in the inhibition of *H. pylori* biofilm.

Besides the fact that resistance increases biofilm defense mechanisms both specific and non-specific, the exopolysaccharides (EPS) that form the biofilm can modulate cytokine responses, changing the type of local immune response to infection (Trinchieri, 1995). *H. pylori* capacity to form biofilms in patients also has implications for the treatment of infection because bacteria in biofilms may show greater resistance to antibiotics (Gilbert et al., 1990; Evans et al., 1991; Costerton et al., 1995), and is one of the possible causes of frequently occurring processes of reinfection (Andersen and Rasmussen, 2009). *H. pylori* has an intrinsic resistance either as a barrier or the biofilm presents the abundant proliferation of it. Also it has acquired resistance via gene transfer, for which it is suggested that future therapies against such infections should act on the biofilm for quick and prompt eradication.

The importance of biofilm in the pathogenesis of *H. pylori* infection is not yet fully understood. Many researchers suggest that it may be responsible for the failure of eradication of *H. pylori* and contribute to the survival of microorganisms in the focal infection, leading to reinfection. So it is necessary to design strategies to inhibit biofilm formation by *H. pylori* which we believe will
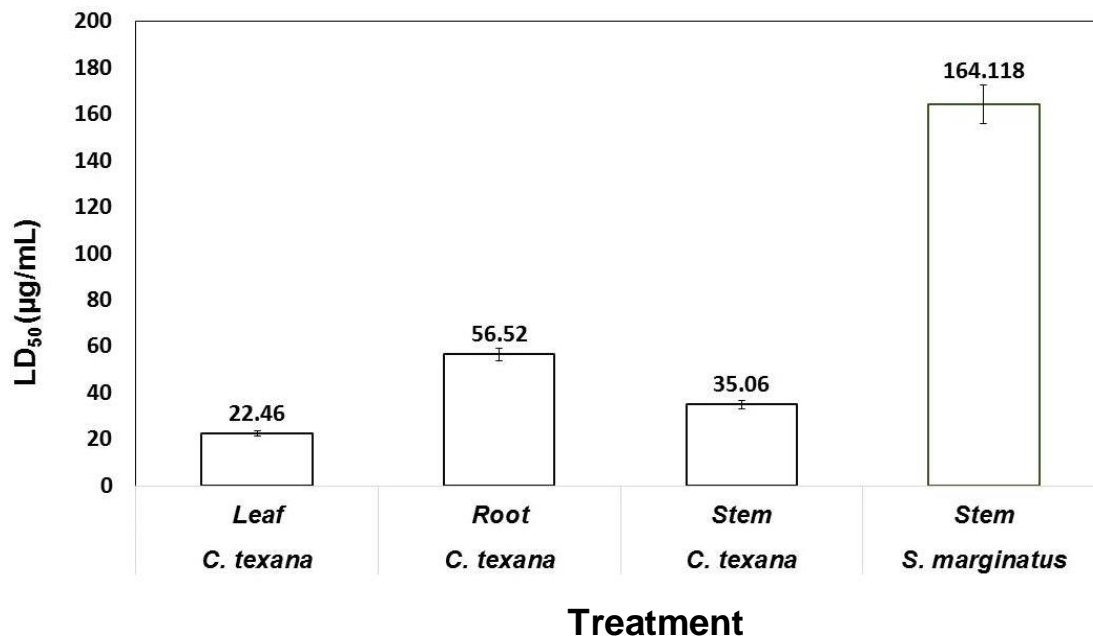
Figure 1. Comparison of the LD_{50} value (µg/mL) of methanol extract of *S. marginatus* and *C. texana* on *A. salina*.
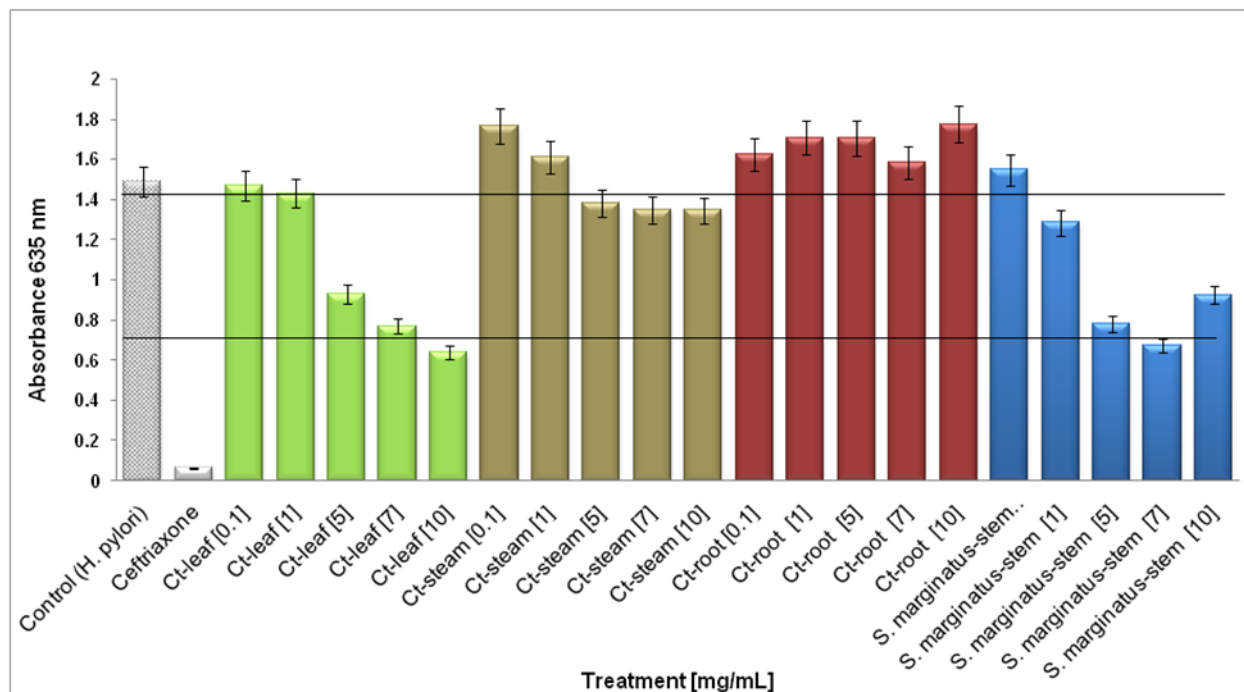

Figure 2. Comparison of the biological activity of methanol extract of *C. texana* (leaf, stem and root), *S. marginatus* and ceftriaxone on the culture of *H. pylori*.

help in the future weakening and eradication of infection or re-infection by *H. pylori*.

These results agree with those reported by different researchers who have evaluated a number of plant species against *H. pylori*, regardless of the plant species and the solvent used for extraction. There is variability in the MIC reported to inhibit the growth of *H. pylori*. In this regard, Cellini et al. (1996) reported an MIC of 5 mg/mL
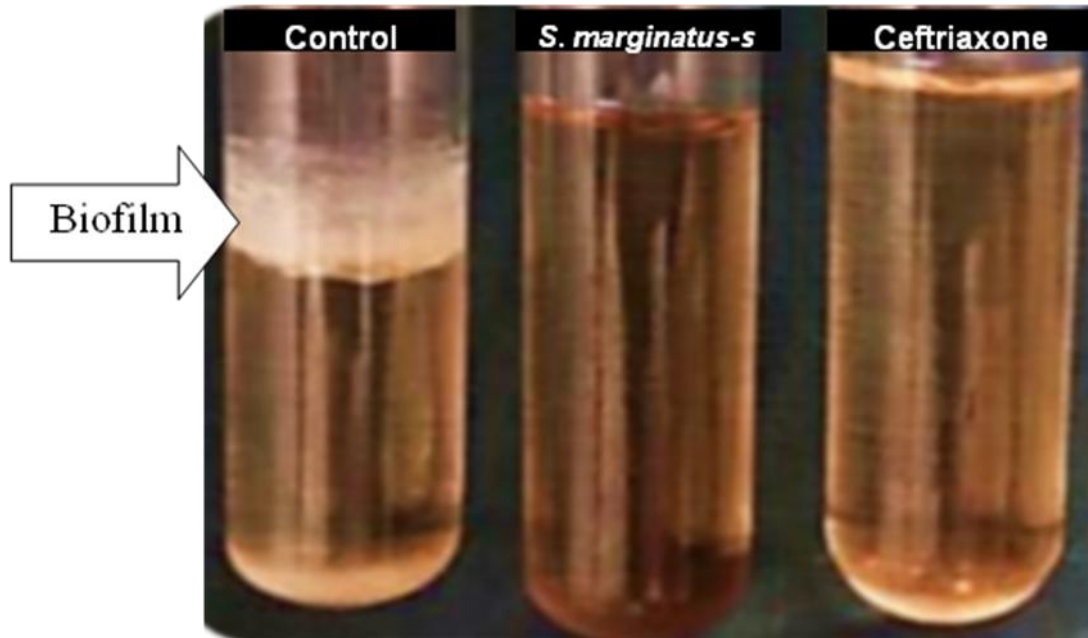
Figure 3. Comparison of macroscopic appearance of the culture of *H. pylori* (control) in the presence of methanol extract of *S. marginatus* and ceftriaxone.

of *Allium sativum* against 19 strains of *H. pylori*. Tabak et al. (1996) reported that 3 to 5 mg/mL of the aqueous extract of thyme was completely inhibited. Malekzadeh et al. (2001) reported that 125 mg/L of *Terminalia chebula* were required. Moghaddam (2011) evaluated the antibacterial activity of methanol extracts of some species commonly used as medicinal, finding that the crude extracts of peel granada (*Punica granatum*) showed the highest levels of inhibition of *H. pylori*, of which the MIC was 312.5 mg/mL.

Gadhi et al. (2001) found that hexane and methanol extract of the rhizome and leaves of *Paucinervis aristolochia* exhibited inhibitory activity at a concentration of 128 g/mL against *H. pylori*, rhizome showing higher inhibitory activity (4 mg/mL) and leaves (16 mg/mL). The dichloromethanic fraction of *Calophyllum brasiliense* showed an MIC of 31 mg/mL against *H. pylori* (Stamatis et al., 2003).

In this study, the methanolic extracts of *S. marginatus* (stem) and *C. texana* (leaf) showed significant inhibition on the growth of *H. pylori*, particularly in concentrations of 5, 7 and 10 mg/mL, although lower than the positive control ceftriaxone. Consistent with the results obtained for secondary metabolites, this effect is mainly attributed to the presence of the components of the functional groups of phenolic, hydroxyls, sesquiterpene lactones and alkaloids which are supported by the results obtained by different researchers which highlighted the relevant inhibitory activity of plant species on different bacterial strains. Yan et al. (2009) reported minimum inhibitory concentrations (MIC) of 0.01, 6.25 and 3.13 mg/mL

against *Staphylococcus aureus*, *Escherichia coli* and *Staphylococcus typhimurium*, to make use of the essential oil of *Lindera strychnifolia*. Moreover, it has been reported that the alkaloids 2-quinolone of *Toddalia aculeata* (Rutaceae) inhibit 70% of the growth of *E. coli* (Jain et al., 2006). According to Cowan (1999) and Scalbert (1991), the antimicrobial effects of the peel of granada are due to the presence of phenolic compounds and tannins.

Toxicity of the extracts of *C. texana* can be attributed to the presence of saponins, which are not present in the extracts of *S. marginatus* that showed low toxicity. Ekabo et al. (1996) indicate that the antifungal and molluscicidal properties are due to the saponins, which is consistent with high concentrations of this phytochemical functional group found in the ethanol extract. Recently, Thakur (2011) pointed out that saponins have a clinical importance due to its utilization for cancer treatment as chemotherapeutic agents, though this property has generated a lot of potential. For this reason, the search for active compounds for the control of *H. pylori* using low saponin extracts is necessary because they can have detrimental effects, and act as anti-nutrients or toxic. This is due to the fact that its high capacity surfactants disrupt cell membranes and are not absorbed in the digestive tract; as such its effect would produce membrane alterations, and possibly increase its permeability (Prince et al., 1987 and Baumann et al., 2000).

H. pylori strain ATCC43504 is susceptible to the methanolic extracts of *C. texana* and *S. marginatus*, though the latter proved to be less toxic to the methanolic

extracts of *C. texana* on *A. salina*.

According to the results, it is concluded that the methanolic extract of *S. marginatus* shows inhibition in the growth of *H. pylori*, as well as biofilm formation, and it is nontoxic, thus it is of great interest to identify the functional group(s) that confers the ability to inhibit *H. pylori*.

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