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Abstract

Background: Prolonged use of antibiotics may lead to the selection of drug-resistant bacteria; as a result, efforts are being made to identify new and effective antimicrobial agents, particularly, from medicinal plants, against bacterial infections. Antimicrobial activity of *Gymnosperma glutinosum* against *Helicobacter pylori* has not yet been reported.

Materials and methods: The antibacterial *in vitro* effect of *Gymnosperma glutinosum* methanol leaf extracts against *Helicobacter pylori* (ATCC 43504) was evaluated in liquid medium by the colorimetric 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) reduction assay and in solid medium by the colony forming units (CFU) method.

Results: Methanol extracts significantly ($p < 0.05$) inhibited *in vitro* *H. pylori* growth in liquid medium from 24% to 82% at concentrations ranging from 31.25 mg/ml to 500 mg/ml, respectively, and in solid medium the extracts caused significant ($p < 0.05$) 52% and 100% bacterial growth inhibition at concentrations of 250 µg/mL and 500 µg/mL, respectively, as compared with untreated control. Methanol vehicle did not affect *H. pylori* growth.

Conclusion: The observed antibacterial effect of *G. glutinosum* extracts may be of benefit as an adjuvant treatment of diseases caused by *H. pylori*.

Key words: *Gymnosperma glutinosum*, *Helicobacter pylori*, methanol extract, minimal inhibitory concentration (MIC).

Introduction

Helicobacter pylori (*H. pylori*) is a Gram-negative spiral-shaped bacteria, which is the main factor for the development of duodenal ulcer disease and has been involved in the development of gastric ulcer, which increases the risk of stomach cancer, the most common digestive tract neoplasia worldwide (Arana y Corona, 2009). In developed countries, 70 to 90% of the population becomes infected before 10 years of age and the modes of transmission include oral-oral or fecal-oral, and iatrogenic when performing endoscopy with a contaminated tube from person to person (Dunn et al., 1997). In addition to surgery, including partial gastrectomy, a variety of antibiotics approved by the United States Food and Drug Administration to treat gastric ulcer have been suggested, among which it is common the use of bismuth subsalicylate, metronidazole, and tetracycline, in addition to an antacid agent; however, this can cause systemic damage such as pseudo-membranous colitis (11%) and vaginal candidiasis (above 10%) in women under treatment (Dunn et al., 1997).

H. pylori infections are currently treated with antibiotics, plant extracts, and various types of immunization with the aim of preventing the colonization of the bacterium, as well as eradicate it when it is established in gastric epithelial cells. Different antibiotics have been suggested to treat *H. pylori*; among these, the use of 10 to 14 days of 20 mg omeprazole twice daily or using plus amoxicillin 1g, 2 times per day, can remove 80 to 90% of these bacteria (Ramakrishnan et al., 2007). An antibiotic scheme 7 days in patients infected with *H. pylori* on the basis of 400 mg of ranitidine, metronidazole 500 mg, and 500 mg of clarithromycin was used before meals in another study, in which they found elimination of the bacteria in 91% of 47 patients (Hoffman et al., 1999).

There is a general warning about the potential benefits of medicinal plants for health, as part of complementary medicine in the world (Gomez-Flores et al., 2010, 2009). Plants play a vital role in the medicinal practices of many Native Americans who use them not only for diagnosis and treatment, such as Ginseng (*Panax quinquefolius*, *Panax ginseng*, *Eleutherococcus senticosus*), *Echinacea* (*Echinacea purpurea*, *Echinacea angustifolia*, *Echinacea pallida*), and Goldenseal (*Hydrastis canadensis*), but also to enhance immune responses against many diseases (Borchers et al., 2000). Interest in botanical medicine has increased over the years, not only by physicians, but also by public in general who seem to prefer natural products than synthetic ones (Borchers et al., 2000; Torrado-Truiti et al., 2003). Although there are a great number of plants in Mexico, the percent of species studied for their antimicrobial activity is low, and their effectiveness must be scientifically validated to increase the credibility of their use (García-Alvarado et al., 2001). Some plant

Gomez et al., Afr J Tradit Complement Altern Med. (2016) 13(4):55-59

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families have been demonstrated to produce antimicrobial substances against a wide variety of bacteria (Akihisa et al., 2005) and fungus (Stein et al., 2006).

Gymnosperma glutinosum is a gummy shrub, 1-1.5 m high, distributed from Guatemala and Mexico to the southeastern United States. In traditional medicine in Mexico, this plant (known as tatalencho, ladybug, motita, jarilla, fox tail, xonequitl, sticky grass, among other names) is used for the treatment of diarrhea, ulcers, pain, and rheumatism (Martinez, 1989). The few phytochemical studies to date have resulted in the discovery of more than 20 flavonoids (Dominguez and Torre, 1974) from the alcoholic extracts and 4 diterpenes (Maldonado et al., 1994; Calderon et al., 2001) of the hexane extracts. Serrano et al. (2009) reported antifungal activity of an ent-labdane type diterpene from *G. glutinosum*, whereas Quintanilla-Licea et al. (2012) found antitumor activity of compounds hentriacontane (1) and (+)-13S,14R,15-trihydroxy-ent-labd-7-ene, as well as the ent-labdane diterpene (-)-13S,14R,15-trihydroxy-7-oxo-ent-labd-8(9)-ene from this plant. Furthermore, *G. glutinosum* has been reported to possess antibiotic activity against *Sarcina lutea*, *Aspergillus niger*, and *Trichophyton mentagrophytes* (Canales et al., 2007), *Mycobacterium tuberculosis* (Gomez-Flores et al., 2008). Canales et al. (2004) reported that *G. glutinosum* extracts possessed antibacterial activity against *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Vibrio cholera* and *Shigella boydii*.

The present study was designed to evaluate the effects of the *Gymnosperma glutinosum* leaf extracts on *H. pylori* growth. The plant belongs to the family Asteraceae, but has not been previously reported to have antimicrobial activity against this bacterium. We found significant *in vitro* growth inhibition activity of *G. glutinosum* extracts against *H. pylori*.

Material and Methods

Reagents, culture medium, and bacterial strain

Brucella broth was obtained from Difco (Detroit MI), 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) was purchased from Sigma-Aldrich (St. Louis, MO), and dimethyl sulfoxide (DMSO) was obtained from Amresco (Solon, OH). *H. pylori* ATCC 43504 was donated by the Laboratorio de Odontología from Centro de Investigación y Desarrollo en Ciencias de la Salud at Universidad Autónoma de Nuevo León, México, and maintained in Brucella broth (Difco, Detroit, MI).

Plant extract preparation

Gymnosperma glutinosum (Spreng.) Less. was collected in Escobedo, NL, México and identified by Biol. Ma. del Consuelo González from Facultad de Ciencias Biológicas Herbarium at Universidad Autónoma de Nuevo León, México, with the specimen voucher number 024784. Leaves were washed, dried, and macerated. To prepare aqueous extracts, 5 grams of leaves powder were allowed to stand in boiling water (80ml) for 10 minutes, freeze dried (Freeze Dry Systems, Labconco Corporation, KC) and stored at -20°C, until use. To produce methanol extracts, 5 grams of leaves powder were allowed to stand in 100% methanol (80ml) for 24 hours at room temperature, solution was then dried in a Speed Vac (Milford, MA) and stored at 6°C, until use. Aqueous and methanol extracts were then diluted to 1mg/ml in sterile media. Under aseptic conditions, the products were then filtrated through 0.22µ-pore size diameter filters (Whatman filters, Whatman International Ltd., Maidstone, England) and one milliliter aliquots were stored in 1.5ml Eppendorf tubes at -20°.

Plant extracts susceptibility testing

In brief, 50 µl of *H. pylori* suspensions at 2.5×10^5 bacteria/mL were plated in Brucella broth (Difco), in flat-bottomed 96-well plates (Corning Incorporated, Corning, NY), in the presence or absence of serial dilutions (1:2) of the *G. glutinosum* aqueous or methanol extract (50 µl) at 1mg/ml, and 50µl antibiotic control (tetracycline), methanol vehicle control or culture medium. The methanol vehicle control was similarly processed as with plant methanol extractions, but without plant material. Plates were then incubated for 6 h at 37°C, after which the tetrazolium salt MTT was added to all wells at a final concentration of 0.5 mg/ml, and plates were incubated for 15 additional minutes. At the end of the incubation period, 80 µl of DMSO were added to all wells and plates were incubated for 15 minutes. Optical densities resulting from dissolved formazan crystals were then read in a microplate reader (DTX 880 Multimode detector, Becton Dickinson, Austria) at 570 nm (Gomez-Flores et al., 2009, 2010; Caballero-Hernandez et al., 2009). In regard to CFUs determination, 50 µl of the microbial suspensions were plated in Brucella broth, in flat-bottomed 96-well plates (Corning Incorporated), in the presence or absence of serial dilutions (1:2) of the *G. glutinosum* aqueous or methanol extract (50 µl), antibiotic control (5 µg/ml tetracycline), and vehicle controls (methanol and culture medium). Then, 1:10,000 dilutions were prepared from the wells and 100 µl were plated on Brucella agar plates (Difco). Agar plates were then incubated at 37°C for 24h and colonies were counted in a colony counter (ULB-100, Lightbox 37864-2000, Scienceware BEL-ART products, Pequannock, NJ). Percent growth inhibition was calculated as follows:

$$\% \text{ Growth inhibition} = 100 - \frac{A_{570} \text{ or CFU/ml of cells treated with extract}}{A_{570} \text{ or CFU/ml untreated cells}} \times 100$$

Statistical analysis

Data represent means \pm SEM of triplicate determinations from three independent experiments. Level of significance was assessed by the Student *t* test and one-way ANOVA.

Results

G. glutinosum aqueous extract did not alter *H. pylori* growth (data not shown), however, methanol extract showed minimal inhibitory concentration (MIC) of 31.25 μ g/ml and 250 μ g/ml and induced a maximum of 82% and 100% growth inhibition against *H. pylori*, as measured by the MTT reduction assay and CFU method, respectively (Fig. 1). Methanol extract caused from 24% to 82% significant ($p < 0.05$) *H. pylori* growth inhibition at concentrations from 31.25 μ g/ml to 500 μ g/ml, respectively, as measured by the MTT reduction assay, and significant ($p < 0.05$) 52% and 100% growth inhibition at concentrations of 250 μ g/ml and 500 μ g/ml, respectively, as measured by the CFU method (Fig. 1). Methanol vehicle control or medium alone did not alter bacterial growth (data not shown).

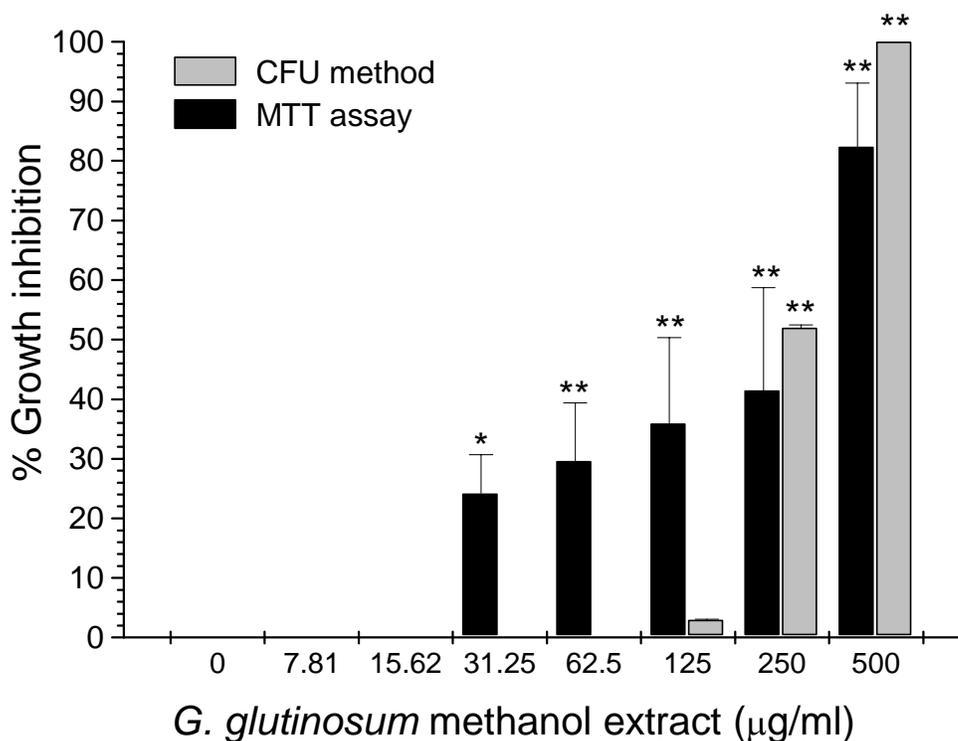


Figure 1: Antimicrobial effect of *G. glutinosum* methanol extract on *H. pylori* growth. *H. pylori* culture suspensions were incubated in the presence or absence of various concentrations of *G. glutinosum* methanol extract, after which growth was measured by the MTT reduction assay and CFU method, as explained in the text. Data represent means \pm SEM of triplicate determinations from three independent experiments. * $p < 0.05$, ** $p < 0.01$, compared with *G. glutinosum* extract-untreated control. Optical density at 570 nm for untreated cells was 0.85 ± 0.03 for the MTT reduction technique, whereas CFU control value for untreated cells was $3.5 \times 10^8 \pm 8 \times 10^6$ CFU/ml.

Discussion

Medicinal plants have become popular worldwide because of their potential health benefits; these plants can be consumed or directly applied to treat infections (Rojas et al., 2006). Compounds synthesized by plants have great therapeutic potential due to their chemical constituents, for which the evaluation of their antimicrobial activity is important to develop alternative pharmacological products (Rodriguez-Fragoso et al., 2008).

There are studies with *Asteraceae* plant extracts as antibacterial agents, but none is related to their effects against *H. pylori*; the selection of *G. glutinosum* for the present study was based on its medicinal traditional use in Mexico. The use of aqueous and methanol plant extracts is common for this type of studies (Akihisa et al., 2005; Jiménez-Arellanes et al., 2003; Rai and Acharya, 1999).

Various biological activities of plants of the *Asteraceae* family including *Baccharis gaudichaudiana* (Guo et al., 2006), *Anthemis aciphylla* (Baser et al., 2006), *Echinops ritro* (Fokialakis et al., 2006), and *Pterocaulon spp* (Stein et al., 2006) have been reported. *G. glutinosum* hexane extracts were shown to possess antibacterial and antifungal activities (Canales et al., 2007). In the present study, *G. glutinosum* extract MICs against *H. pylori* ranged from 31.25 µg/ml to 250 µg/ml. It is accepted that antimicrobial agents from plants have clinical potential if their MICs are in the range from 100 to 1000 µg/ml (Drusano, 2004); thus, the results of the present study may be an indication of an important antibiotic activity of *G. glutinosum* extracts against *H. pylori*.

Medicinal plants have proven to be important sources of antimicrobial agents, many of which have been the basis for the development of new pharmaceuticals drugs, which may overcome the increasing resistance of many pathogens to common antibiotics (Chinedum, 2005; Moreillon, 2000; Rusell, 2000).

Conclusion

To our knowledge, this is the first report showing that *G. glutinosum* methanol extracts inhibit *H. pylori* growth in-vitro. There are still a number of plant compounds that remain to be evaluated at the molecular, cellular, and physiological levels for their potential to treat human diseases.

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Gomez et al., Afr J Tradit Complement Altern Med. (2016) 13(4):55-59

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