Antibacterial activity of aqueous extracts of *Lippia alba* (Mill.) N.E. Brown against *Helicobacter pylori*

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Abstract

Lippia alba (Mill) N.E. Brown is a medicinal plant known in Colombia as "prontoalivio" (= fast relief). It is used for the treatment of multiple gastro-intestinal ailments. The essential oil, the aqueous and alcoholic extracts obtained from the plant have demonstrated antibacterial, antiviral, anti-parasitic and antimycotic power.

In the current *in vitro* study of the aqueous extracts from *Lippia alba* leaves against three isolated *Helicobacter pylori* cultures, it was determined that there was an antibacterial effect.

There are needed additional studies that allow to establish the potential clinical use of the aqueous extracts in combination with the antibiotic treatment.

Key words

Helicobacter pylori, Lippia alba, inhibition, antibacterial activity.

INTRODUCTION

Helicobacter pylori colonize and remain in the human gastric mucosa where the bacteria trigger significant inflammatory responses. The infection is acquired primarily in childhood and persists for years or for life. The presence of bacteria in the gastric mucosa is associated with different types of chronic gastritis, peptic ulcers and cancers including MALT (Mucosa-Associated Lymphoid Tissue) and adenocarcinomas (1). Various studies have associated *H. pylori* with extra-gastro-intestinal diseases including coronary disease, Raynaud's phenomenon and immune diseases (2). Since 1994 the International Agency for Research on Cancer (IARC) of the World Health Organization (WHO) has included it among type 1 carcinogens. It is estimated that over 60% of the world's population is infected with *H pylori,* making it the most common chronic bacterial infection in human beings (3, 4).

Colombia has a reported prevalence of infection in children and adults undergoing upper endoscopy of 61% and 70% respectively (5, 6).

To eradicate these bacteria, the administration of proton pump inhibitors such as omeprazole and lansoprazole combined with two antibiotics including amoxicillin and clarithromycin is currently recommended for 10 days or more. However about 20 % of these treatments fail to eradicate the bacteria, primarily due to bacterial resistance to antibiotics and lack of patient adherence to treatment regimes (7, 8).

In Colombia, more than 80% of all *H. pylori* strains studied have been determined to have metronidazole resistance. Consequently, use of this drug is not recommended for medical purposes in our country (9). Also an increasing primary resistance to clarithromycin has been reported around the world. International reports show rates of resistance to this drug ranging from 1% to 58% for isolates of *H. pylori*. In Colombia resistance has been determined in 15% of *H. pylori* bacteria isolates (8, 10-12).

The use of plants or extracts to cure various infections is an ancient practice, known in many cultures and widely used nowadays (13-17).

The high frequency of gastrointestinal symptoms (dyspepsia, abdominal pain, altered intestinal habits, etc) and of recognized diseases (gastroesophageal reflux, gastritis, peptic ulcers, acute diarrhea, etc.) in the human population makes the use of plants the first therapeutic measure that is chosen due to easy availability, low economic costs and effectiveness (18-21).

In recent years, the high prevalence of diseases associated with *H. pylori* infection, the increasing resistance of microorganisms to antibiotics, subsequent treatment failure, and the increased economic costs of treatments have led to tests of treatment with traditional plants from various regions of the world (22-33).

Lippia alba (Mill.) Nebr. ex Britt. & Wils. *(Verbenaceae),* known in Colombia as "fast-relief", is a shrub which is native to American tropical and subtropical dry forests. It grows in environments with very intense sunlight. The natural distribution of this aromatic plant extends from the southern United States south through Central America and all the way through South America to northern Argentina. Traditional infusions of leaves and flowers are used as sedatives, digestives, febrifuges, carminatives, antispasmodics, emmenagogues, antidiarrheal medicines, and for relief of pain in general and in various stomach ailments (34-37).

Studies of the essential oil of *Lippia alba* leaves grown in different regions of Colombia have shown the existence of three different chemotypes in the departments of Cundinamarca, Tolima, Boyacá, Valle del Cauca, Santander, Antioquia, Quindío and Cesar. Chemotype A, also called chemotype III, is characterized by its high content of carvone (41%) and limonene (36%) (38).

The aim of this study was to evaluate the antibacterial activity of aqueous extracts of *Lippia alba* plant against *H. pylori.* This plant has been traditionally used in Latin America for management of dyspepsia, abdominal pain and diarrhea.

MATERIALS AND METHODS

Lippia alba (Mill.) NE Brown. ex Britt. & Wils (Verbenaceae) plants were collected in September 2009 on a farm called Las Alicias located in the village of Peñitas in the municipality of Puente Nacional in Santander Department, at 05º 52' 41" N 73º 40´ 33¨ W. Average annual temperatures there are 20 ° C, relative humidity is 86% and relative effective annual precipitation is 338 μmol/m^2 sec (National Herbarium COL record 511 011, JL Fernández-2005).

Preparation of extracts

To obtain the lyophilized extract of *Lippia alba,* 50 g of dried ground leaves were used. Later a part of the plant in ten volumes of water was taken. For initial maceration 1 part of the plant and 3 parts hot water (90 º C) were mixed. The mixture obtained was macerated with a mortar and pestle and allowed to rest for fifteen (15) minutes. Then the solution was placed in a water bath at a temperature of less than 90º C. The water was constantly agitated for 5 minutes. Then it was allowed to cool to room temperature. Subsequently, it was filtered with the Buchner funnel method. Finally, the extract was lyophilized. It yielded 2.419 g of *Lippia alba* (39).

Two *H pylori* isolates from the collection of strains at the Laboratory of Bacteriology in the Department of Microbiology at National University of Colombia were tested. One of these isolates came from a patient who had been diagnosed with gastric ulcers and the other came from a patient with erosive chronic gastritis. Reference strain NCTC 11637 was also tested. These samples were preserved at -70 ° C in a mixture of BHI broth (Merck) and 20% glycerol. These isolates were recuperated from Chalgren Wilkins agar supplemented with horse serum (DIFCO), 7% Vitox (Oxoid SR0091) and antibiotic supplement for *H. pylori* (Oxoid SR0147). Cultures were placed in an incubator of CO_2 under microaerophilic conditions of humidity and temperature sufficient for the growth of the cultures. Bacteria were identified by Gram staining and urease, catalase and oxidase reactions.

Evaluation of Aqueous Extracts

Preparation of Bacteria. One inoculate was prepared for each bacterial strain in sterile distilled water. They were homogenized in a Vortex mixer for 30 seconds, and then the suspension was calibrated nephelometrically using the McFarland scale (10^8 CFU).

Preparation of aqueous extract. The freeze-dried *Lippia alba* powder was diluted in sterile distilled water at final concentrations of 100, 50, 25, 5, 0.5 and 0.25 mg of plant extract per ml of water.

Determination of antibacterial activity of extract. The bactericidal activity of aqueous extracts was determined using the technique described by O'Mahony et al. (27). Each concentration of *Lippia alba* extract (100, 50, 25, 5, 0.5 and 0.25 mg / ml) was put into contact with the three bacterial inoculums: strain NCTC 11637, M8 isolates from a patient with a gastric ulcer, and M10 isolates from a patient with erosive gastritis. Contact was maintained for a period of 20 minutes. From each of the trials 150 ml were sown in Petri dishes containing agar-Chalgren Wilkins (Becton-Dickinson) supplemented with horse serum and 7% Vitox (Oxoid). They were then incubated under microaerophilic conditions. All tests were conducted in duplicate. The bactericidal effects of the aqueous extracts against the isolates

of *H. pylori* were evaluated by observing the inhibition of bacterial growth and comparing each rate to the control of viability for each sample. Results are expressed as percentages of inhibition of bacterial growth (27, 40, 41).

RESULTS

Different concentrations of the aqueous extracts of *Lippia alba* were evaluated to determine the degree to which they inhibited *H. pylori* growth and to determine the minimum concentration of the extract bactericidal needed to produce a bactericidal effect in a 20 minute long exposure period. The results are summarized in Table 1 which shows the concentrations of the aqueous extracts tested, the times tested for, and the percentage of growth inhibition of isolates from patients with gastric diseases and from reference strain NCTC 11637.

Table 1 shows that the aqueous extract of *Lippia alba* totally inhibits growth of all three strains tested after 20 minutes. We found that the minimum bactericidal concentration (MBC) was 0.5 mg/ml, and that the longer the time the bacterial inoculum was exposed to the aqueous extract, the greater was the bactericidal activity. This extract has a highly inhibitory effect on growth of *H. pylori.*

Table 1. Bactericidal effects of aqueous extracts of *Lippia alba* against *Helicobacter pylori.*

	Helicobacter pylori isolate		
Lippia alba aqueous extract Concentration	NCTC 11637	M 8	M 10
100 mg/ml	100% inhibition	100% inhibition	100% inhibition
50 mg/ml	100% inhibition	100% inhibition	100% inhibition
25 mg/ml	100% inhibition	100% inhibition	100% inhibition
5 mg/ml	100% inhibition	100% inhibition	100% inhibition
0,5 mg/ml	100% inhibition	100% inhibition	100% inhibition
0,25 mg/ml	0% inhibition	0% inhibition	0% inhibition

DISCUSSION

Factors such as the high prevalence of benign and malignant gastroduodenal diseases associated with *Helicobacter pylori* infections, lack of patient adherence to treatment regimens, side effects involved in combination therapy, increasing resistance of microorganisms to antibiotics, the subsequent failure of treatment, and the resulting cost increases of treatment, make it necessary to conduct additional research to find safe new therapeutic options which can improve eradication rates for this bacteria. In developing countries, especially in rural areas and marginal urban centers, the use of infusions, decoctions, and other preparations made from plants are the first therapeutic option. Because of their availability, low cost and effectiveness, they are also emerging as therapeutic alternatives.

In Colombia the traditional use of medicinal plants for the treatment of various gastrointestinal diseases is widely recognized (35). Background information about other medicinal use of plants includes a study of aqueous extracts of different plant species with wide culinary and medicinal applications in Asia *(Curcuma longa, Borago officinalis, Capsicum Annum, Zingiber officinale, Origanum vulgare, Nigella sativa and Cuminum cyminum)*. That study reports inhibitory effects on the growth of seven strains of *H. pylori* (27). In addition, Castillo-Juarez and colleagues studied the *in vitro* antibacterial activity of 53 medicinal plants used traditionally in Mexico. They used the agar dilution technique on several strains of *H. pylori* and found that the greatest inhibitory effects occurred with the aqueous extract of *Artemisia ludoviciana subsp. mexicana, Cuphea aequipetala, Ludwigia repens* and *Mentha* × *piperita* (42).

The aim of our study was to evaluate whether aqueous extract of *Lippia alba,* a plant with wide distribution in Colombia which is inexpensive and widely available, has properties which inhibit the growth of *H. pylori* isolates. The main traditional uses reported for this plant are related to gastrointestinal problems, respiratory problems, cardiovascular disorders, sleep disorders and anxiety. In addition, various publications have evaluated the antiviral, antifungal, antiprotozoal, neurosedative, analgesic, anti-inflammatory, antioxidant and cardiovascular properties of extracts obtained from *Lippia alba* (37, 48-50).

The essential oil of *Lippia alba* have demonstrated antibacterial activity especially against Gram-positive bacteria. Values of minimum inhibitory concentrations range from 0.3 to 0.63 mg/MLW. The ethanol and methanol extracts from the roots of *Lippia alba* have shown bactericidal activity against strains of *Staphylococcus aureus* (ATCC 6538P, ATCC 6538) and *Klebsiella pneumonia* (ATCC 10031) (44). The essential oils of *Lippia alba* have demonstrated growth inhibitory activity on strains of *C. albicans* (ATCC 14053), *S. aureus* (ATCC 6538) and *E. coli* (ATCC 25992) (45). The chloroform, acetone and ethanol extracts of root of *Lippia alba* have shown antibacterial activity in vitro (using the paper disc technique) against *Staphycoccus aureus* strains*, Micrococus luteus, Bacillus subtilis, Mycobacterium smegmatis, Candida albicans,* and *Monilia sitophila.* The ethanol extract of *Lippia alba* serves as an antibacterial (inhibits growth of *Vibrio parahaemolyticus)* and antifungal *(Aspergillus niger)* (43-47).

Essential oils of *Lippia alba* including citral and carvone have demonstrated antimycotic activity against *Aspergillus fumigatus and Candida krusei* (48). In addition, it has recently been shown to have inhibitory

effects on human pathogenic viruses with high prevalence in Latin America including the yellow fever virus and dengue virus (49,50).

At the time of preparation of this manuscript, no references related to the use of aqueous extracts of this plant for bactericidal action against *H. pylori* could be found. Perhaps, the study which came closest to indicating this possibility was the research performed by Pascual and colleagues. They demonstrated that the aqueous extracts of *Lippia alba* have antiulcerogenic effects, but did not demonstrate effects against *H. pylori*. They used doses of 12.5 g of dried *Lippia alba* plant/kg. They also demonstrated a protective effect against aggression by indomethacin at doses of 50 mg/kg for five days with no detrimental effects on the gastric mucosa of Wistar rats (51). Their findings, coupled with the results obtained in this study which show that growth of three isolates of *H. pylori* (a reference strain and two isolates from patients) is inhibited by aqueous extracts of *Lippia alba* at a minimum bactericidal concentration of 0.5 mg/ml with contact time of 20 minutes or less, raise the possibility that the gastroprotective and inhibitory effects of this plant on *H. pylori* may have therapeutic usefulness for treating people with different forms of gastritis and peptic ulcers.

It is important to note that the use of *Lippia alba* infusion is a logistical advantage over the processes used to obtain essential oils and alcoholic extracts of *Lippia alba* commonly reported to be antibacterial. Their extraction requires specific equipment and qualified personnel, two conditions which are not always available to the economically most vulnerable populations.

In conclusion, we report for the first time *in vitro* inhibitory effect of aqueous extract of *Lippia alba* on the growth of *H pylori.* Further studies are needed to establish the potential clinical use of these extracts either alone or in combination with currently used triple therapy. Something similar to what we hope can be done in the future with *Lippia alba* has recently been published for other species. The research was a clinical study that showed that *Nigella sativa* plant used at a dose of 2 g/day in combination with 40 mg/day of omeprazole has a 67% rate of eradication for *H. Pylori* whereas triple therapy for 10 days with omeprazole, amoxicillin, and clarithromycin has an eradication rate of 82% (52).

Increasing prevalence of *H. pylori* resistance to antibiotics raises the need to find safe new therapeutic modalities for eradication of the infection and for treatment of various gastroduodenal diseases. The use of plant extracts on humans, alone or in combination with antibiotics, is one such potential alternative to be explored.

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Conflicts of interest

The authors declare that they have no conflicts of interest.

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