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Original Article

Effect of Liquorice-Extract-Containing Antimicrobial Mouthwash on *Helicobacter Pylori*: An *In Vitro* Study

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Abstract

Background: The oral route is one of the main portals for *Helicobacter pylori* transmission. The elimination of this bacterial species from the oral cavity might be useful in oral health and decreasing infections due to *H. pylori*. This study aimed to evaluate the effect of a liquorice-extract-containing mouthwash at different concentrations on the proliferation of *H. pylori in vitro*.

Methods: *H. pylori* bacterial species was cultured, and the isolated strains from the specific culture medium were prepared for the welling procedures. The liquorice (*Glycyrrhiza glabra*) mouthwash at 12.5% and 25% concentrations was added to the case group wells at 1,1/2,1/4,1/8, and 1/16 dilutions. In the control group, regular daily mouthwash containing cetylpyridinium chloride and sodium fluoride components was used. The growth inhibition zones were analyzed in the study groups. The data were analyzed by SPSS and reported using descriptive statistics (means ± standard deviation).

Results: In both the mouthwashes containing 25% and 12.5% concentration, the means of growth inhibition zones at 1, 1/2, 1/4, 1/8, and 1/16 dilutions were larger than those in the control group. Further, the largest growth inhibition zone was seen with the undiluted 25% mouthwash. There were no significant differences in the *H. pylori* growth inhibition zones between 25% and 12.5% mouthwashes ($P = 0.14$).

Conclusions: Mouthwashes containing liquorice extracts inhibited the growth of *H. pylori* more significantly than mouthwash with no liquorice extract. Therefore, it is suggested that liquorice extract-containing mouthwashes be used to prevent *H. pylori* infections in the oral cavity in clinical studies.

Keywords: *Helicobacter pylori,* Plant extract, Mouthwash

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Introduction

Helicobacter pylori is a bacterial species from the *Helicobacteraceae* family and the most common microorganism to affect humans all over the world. More than half of the world's population is contaminated with this microorganism, and more than 81% of the infected individuals are asymptomatic. It might also have a role in the normal ecology of the stomach (1-4). *H. pylori* was identified by two Australian scientists, Barry Marshal and Rabin Warren, in 1982, who discovered this bacterial species in patients with chronic gastritis and peptic ulcers. They received the Nobel Prize in 2005 in the field of pharmaceutics for discovering *H. pylori* and its role in peptic ulcers (5).

Helicobacter pylori is a microaerophilic gram-negative microorganism, generally found in the stomach. Its role has been reported in peptic ulcers, chronic gastritis, and malignancies such as adenocarcinoma and lymphoma of mucosa-associated lymphoid tissue cells as an important etiologic agent (6-9). Some studies have investigated *H. pylori* in coronary artery disorders, atherosclerosis, gallstones, malabsorption, metabolic syndrome, type I diabetes, and insulin resistance (10-12).

The co-occurrence of *H. pylori* has been reported in oral cancer lesions, dental plaque, recurrent aphthous stomatitis, and dental caries (13-15). A relationship has been also reported between *H. pylori* counts and the severity of periodontitis in patients with periodontitis. Moreover, there is a relationship between the periodontal health status of the elderly with periodontitis and *H. pylori* contamination of the stomach (16). In addition, *H. pylori* DNA was extracted from some pathologic lesions of the oral cavity, including leukoplakia and oral lichen planus. However, this microorganism has not been detected in

normal and healthy mucosa (17).

Helicobacter pylori contamination of the stomach was treated with systemic antibiotics such as metronidazole, tetracycline, bismuth, and omeprazole (18). Such treatment not only led to resistance to antibiotics (19) but also was ineffective in some patients, and resistant bacterial infection and recurrence were observed in some patients. Some researchers have suggested that transmission through the oral cavity might be the primary portal of entry for *H. pylori*, with the dental plaque and saliva serving as a reservoir for *H. pylori*, inoculating the infectious agent into the stomach after its elimination from the stomach (20). Since indiscriminate use of antibiotics results in resistance to antibiotics, such treatments for *H. pylori* complicate the treatment process for this microorganism (21).

In recent years, attention has been shifted to the production of new drugs, especially those with a natural origin with antimicrobial effects on this microorganism with fewer side effects. On the other hand, there has been an increase in the tendency of patients to use herbal-derived antibiotics instead of chemical agents in recent decades (22).

Liquorice (*Glycyrrhiza glabra*) is an herbal medicine with some components that have therapeutic properties. It consists of valuable ingredients that have turned it into a significant agent for the synthesis of useful medications. *In vitro* studies have shown that the extract of liquorice root inhibits the growth and proliferation of *H. pylori* and prevents its adhesion to the human gastric mucosa (23,24). In clinical studies on patients with peptic ulcers due to *H. pylori*, the use of liquorice extract tablets has resulted in the elimination of *H. pylori* contamination, with a healing effect of this extract on ulcers (25).

Since the oral cavity is one of the principal routes for the transmission of *H. pylori*, and the dental plaque and saliva can serve as a reservoir and a factor for the proliferation of this infectious agent, its inhibition in the oral cavity can be useful for the oral health and more effective treatment of medical conditions due to this infectious agent. The present *in vitro* study aimed to evaluate the antibacterial effect of a mouthwash containing liquorice extract on *H. pylori*'s growth and proliferation. Similar studies were conducted on the effect of liquorice on these bacteria, but this is the first time that this material is evaluated in the mouthwash form.

Materials and Methods

The liquorice extract-containing mouthwash used in the present study was prepared by the Rojin Cosmetic Co. The standard strain of *H. pylori* (ATCC 43 504) was procured from the Pasteur Institute, Tehran, Iran, to culture on Columbia solid agar plates. To prepare the agar culture plates, 4 grams of the powder of Columbia base agar was dissolved in 100 mL of deionized sterile distilled water and boiled to achieve a transparent solution from the culture medium, which was autoclaved for 15 minutes at 121°C at 15 pounds pressure. After sterilization, it was cooled up to 45–50°C. Then, 5 mL of sheep-defibrinated blood (at a 5% ratio) was added to the culture medium, and the culture medium was then placed on plates and stored at 4°C.

Microbial Culture

After preparing and activating standard lyophilized bacteria, they were suspended in the culture medium, and 6 mL of distilled water was added to it for activation, followed by homogeneous culturing on the culture medium on all the plate surfaces. The inoculated plates were incubated under a microaerophilic condition, an anaerobic jar system, and a special gas pack for 72 hours at 37°C under an anaerobic condition so that the bacteria can grow, proliferate, and form colonies. Then, urease, catalase, and gram staining tests were used to confirm bacterial growth.

Sampling

McFarland 0.5 Standard

The bacterial counts used for a test should be compared with a standard because if an adequate number of bacteria is not used, false negative results (in terms of sensitivity) may be achieved. Therefore, the 0.5 McFarland standard was used in the present study. First, concentrated sulfuric acid was used to prepare a 1% concentration of sulfuric acid. To this end, 99 mL of distilled water was mixed with 1 mL of concentrated sulfuric acid. Great care was exercised during the procedure due to the exothermic nature of the reaction because the acid should be added to water. Then, 1.175 grams of barium chloride powder was added to 100 mL of distilled water to achieve a 1.175% concentration of barium chloride. Then, 99 mL of 1% sulfuric acid was mixed with 1 mL of barium chloride. Turbidity was seen in the tube due to the precipitation of barium sulfate, which is the 0.5 McFarland standard. Finally, the resulting microbial suspension contained 1.5×10^8 CFU/mL.

Determination of Minimum Inhibitory Concentration

First, some wells were produced in the blood agar culture plates. A colony of freshly cultured bacteria was homogeneously spread, and different dilutions of pure extract of liquorice were prepared at 1/2, 1/4, 1/8, and 1/16 concentrations by adding distilled water. Then, 40 µL of the different concentrations was placed in each well and incubated for 72 hours. The same procedure was repeated for the pure and undiluted liquorice extract (100%) at different volumes of 40, 60, and 90 µL, and the diameter of the growth inhibition zone for each well was measured. Based on the results, 1/4 and 1/8 dilutions were the minimum concentrations of the extract that exhibited a favorable growth inhibition zone. Accordingly, a request was sent to the Rojin Company to prepare 25% and 12.5% concentrations of liquorice extract.

Evaluation of Antimicrobial Effects Using the Plate Well Technique

In the present study, the plate well technique was used

to evaluate the antimicrobial effect of two types of mouthwashs (1 and 2) containing liquorice extract at two concentrations of 25% and 12.5%. The plates containing the culture medium were concentrated with the standard samples of the tested bacterial species. Then, a Pasteur pipette was used to create four wells with 5 mm in diameter in each plate, and 40 µL of the pure liquorice-containing mouthwash at 1/2,1/4,1/8, and 1/16 dilutions (6.25,12.5, 25, 50, and 100 vol%) were separately added to each well. All procedures were repeated for the control mouthwash (with a brand name for daily use), which contained cetylpyridinium chloride and sodium fluoride. These procedures were repeated for different volumes of undiluted mouthwashes 1 and 2 at volumes of 90, 60, and 40 µL. Therefore, the bacteria were exposed to all five concentrations of liquorice-containing mouthwashes 1 and 2 and the mouthwash without liquorice extract. The plates were incubated for 72 hours at 37°C, along with an anaerobic gas pack. Then, the diameters of growth inhibition zones were measured around the wells in the three groups (control and mouthwashes 1 and 2). This procedure was repeated three times in each group for greater validity of the test.

Statistical Analysis

The study data were reported using descriptive statistics (mean ± standard deviation). The means of three measurements of the growth inhibition zones created by different dilutions of mouthwashes 1 and 2 in each well were compared with those created by the control mouthwash. In addition, the means of the growth inhibition zones created by the different volumes of mouthwashes 1 and 2 were compared.

Results

In the present study, first, the MIC of the liquorice extract was determined. Table 1 presents the effects of different volumes of pure liquorice extract on the growth inhibition zone diameters of *H. pylori*. In the next stage, different dilutions of liquorice extract were prepared with distilled water to determine MIC. Table 2 presents the effects of different liquorice extract concentrations with a 0.4-mL volume and the bacterial growth inhibition zone diameters.

Table 1. The Effects of Different Volumes of Pure Liquorice Extract and the Diameters of Bacterial Growth Inhibition Zones

The First Measurement				
The volume of pure liquorice extract (µL)	40	60	90	Control
The diameter of inhibition zone (mm)	10	11.5	14	0
The Second Measurement				
The volume of pure liquorice extract (µL)	40	60	90	Control
The diameter of inhibition zone (mm)	10	11.5	14.5	0
The Third Measurement				
The volume of pure liquorice extract (µL)	40	60	90	Control
The diameter of inhibition zone (mm)	11	12	14	0

Since the control mouthwash (without the extract) had a minor effect on the bacterial growth inhibition zone, these effects were considered baseline or zone effects. Further, since the 1/16 dilution did not have a favorable effect, and the effects of 1/4 and 1/8 dilutions were almost similar, both dilutions were used for comparisons.

Table 3 presents the inhibition zone diameters under the effect of 25% and 12.5% liquorice extract at different dilutions. Table 4 presents the growth inhibition zones of *H. pylori* induced by 25% and 12.5% liquorice extract-containing mouthwashes at different dilutions with a volume of 40 µL. A comparison of the two types of mouthwash samples containing 25% and 12.5% concentrations of liquorice extract did not indicate any significant difference ($P = 0.41$). The results showed that both mouthwash samples containing liquorice extract had larger growth inhibition zones compared to the control group, and the difference was significant ($P < 0.05$).

Discussion

The present study evaluated the effects of two daily types of mouthwashes containing liquorice extract at 25% and 12.5% concentrations and the effect of a daily mouthwash containing cetylpyridinium chloride and sodium fluoride without liquorice extract on the growth inhibition of *H. pylori in vitro*. The results of the present study showed

Table 2. The Effects of Different Dilutions of Liquorice Extract and the Diameters of Bacterial Growth Inhibition Zones

The First Measurement					
The dilution of liquorice extract	1/2	1/4	1/8	1/16	Control
The diameter of inhibition zone (mm)	10	9	8	7	0
The Second Measurement					
The dilution of liquorice extract	1/2	1/4	1/8	1/16	Control
The diameter of inhibition zone (mm)	9	9.5	8.5	7	0
The Third Measurement					
The dilution of liquorice extract	1/2	1/4	1/8	1/16	Control
The diameter of inhibition zone (mm)	10.5	9	8	7	0

Table 3. The Effects of Different Volumes of the Two Mouthwashes Containing 25% and 12.5% Concentrates of Liquorice Extract and the Diameter of Growth Inhibition Zones (in mm)

The First Measurement				
Volume (µL)	40	60	90	Control
The diameter of inhibition zone (25%)	13	14	15	0
The diameter of inhibition zone (12.5%)	12	13	14	0
The Second Measurement				
Volume (µL)	40	60	90	Control
The diameter of inhibition zone (25%)	12.5	14.5	14	0
The diameter of inhibition zone (12.5%)	10.5	12	14	0
The Third Measurement				
Volume (µL)	40	60	90	Control
The diameter of inhibition zone (25%)	12.5	14.5	14.5	0
The diameter of inhibition zone (12.5%)	12	13	13.5	0

Table 4. The Effect of Different Dilutions of the Two Mouthwashes Containing 25% and 12.5% Concentrations of Liquorice Extract With a Volume of 40 µL and the Diameters of Growth Inhibition Zones (in mm)

The First Measurement						
Dilution	1/16	1/8	1/4	1/2	Undiluted	Control
The diameter of inhibition zone (25%)	7	8	10	13	14	0
The diameter of inhibition zone (12.5%)	7	7	8.5	10	12.5	0
The Second Measurement						
Dilution	1/16	1/8	1/4	1/2	Undiluted	Control
The diameter of inhibition zone (25%)	7	7	10	11	14	0
The diameter of inhibition zone (12.5%)	6.5	7	8	11	13	0
The Third Measurement						
Dilution	1/16	1/8	1/4	1/2	Undiluted	Control
The diameter of inhibition zone (25%)	7	8	10.5	12.5	13	0
The diameter of inhibition zone (12.5%)	6.5	9	8	10.5	13	0

that, compared with the control group mouthwash, both types of mouthwashes containing liquorice extract at the two concentrations mentioned above were more effective in inhibiting *H. pylori*'s growth and proliferation. The maximum diameter of the growth inhibition zone in the brucella agar culture medium in this study was 15 mm with 90 µL of the 25% liquorice extract-containing mouthwash. Considering the results of a study by Chaves et al, the diameter of this growth inhibition zone is in the range of sensitivity to antibiotics (26). A study by Taghipour et al showed that the diameter of the growth inhibition zone produced by liquorice at a concentration of 33 g/dL was almost similar to that produced by metronidazole against *H. pylori*, confirming that the components in liquorice can have a role as an effective antibiotic against *H. pylori* (27).

Several mechanisms have been reported on the mechanism of the effect of liquorice ingredients on the activity of *H. pylori*. The anti-*H. pylori* activity of liquorice is attributed to the ingredients found in the extract of its root, referred to as GutGard, which inhibits DNA gyrase, protein synthesis, and dihydrofolate reductase. DNA gyrase is an essential bacterial enzyme that catalyzes the ATP-dependent negative supercoil in the double-stranded circular DNA. It is also necessary for the transcription of bacteria (23,28-30).

A study by Wittschier et al revealed that the glycyrrhizin ingredients in liquorice extract significantly affect the adhesion of *H. pylori* to the human gastric tissue (24). A comparison of the effects of mouthwashes containing liquorice extract with chlorhexidine mouthwash on *Streptococcus mutans* and *Lactobacilli* indicated a higher bacterial inhibitory effect of the liquorice extract-containing mouthwash (31). Moreover, a clinical study by Hajiaghamohammadi et al on patients with dyspnea and peptic ulcer evaluated the effect of liquorice extract on the elimination of *H. pylori* using the urease test in six weeks. The results indicated a higher rate of *H. pylori* elimination in peptic ulcer patients receiving liquorice extract (32). Further, in a clinical trial by Momeni et al, patients with *H. pylori* were assigned to two groups. Liquorice extract was used in one group in addition to their conventional treatment modality for *H. pylori*. After four weeks, in the group treated with liquorice, *H. pylori* counts in the urease test and stool analysis were significantly lower than those in the control group (33). Likewise, a study by Park et al showed that special licorice compounds derived from liquorice decreased the expression of genes related to vascular endothelial growth factor, inducible nitric oxide synthase, cyclooxygenase-2, and interleukin 8 in rats contaminated with *H. pylori* due to their anti-oxidative and anti-inflammatory properties, decreasing the incidence of gastritis and gastric masses in the rats (34).

The different ingredients in liquorice extract might interfere with the different stages of the growth and proliferation of *H. pylori*. The use of the active ingredient of this plant might eliminate the oral reservoir of *H. pylori* and prevent the recurrence of gastrointestinal contamination due to the transmission of this bacterial species from the oral cavity to the stomach.

Conclusions

Mouthwashes containing 25% and 12.5% concentrations of liquorice extract led to a higher mean of bacterial growth inhibition zone for *H. pylori* compared to the mouthwash without this extract.

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Author Contributions

The study was planned by SB and ZA. The statistical analyses and interpretation of data were carried out by BS and RN. ME and HSK contributed to the literature review. All the authors have read and approved the final manuscript.

Conflict of Interest Disclosures

The authors declare that they have no conflict of interests.

Ethical Statement

The study protocol was approved by the Ethics Committee of the Research Council of Tabriz Dental School, Tabriz, Iran (approval

number: IR.TBZMED.VCR.REC.1397.408). All the procedures of the study were performed based on the Declaration of Helsinki.

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