

Full Length Research Paper

Comparison of *in vitro* antimicrobial effect of ethanol extracts of *Satureja khuzestanica*, *Rhus coriaria*, and *Ocimum basilicum* L. on *Helicobacter pylori*

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Helicobacter pylori is the main reason of gastritis, gastric ulcer and cancer, which cause many therapeutic problems for man, throughout the world. Therefore, the aim of the current study is to compare the antimicrobial effect of the extracts of *Satureja khuzestanica*, *Rhus coriaria*, and *Ocimum basilicum* L. on *H. pylori*. To isolate *H. pylori*, sampling was performed in 30 patients of the endoscopy ward of Be'sat Hospital, Sanandaj. Ethanol extracts of *S. khuzestanica*, *R. coriaria* and *O. basilicum* L. were prepared. Using disc diffusion and agar dilution methods, the antimicrobial effects of the plant extracts against *H. pylori* were evaluated. Seven strains of *H. pylori* were isolated from the biopsy samples. The results of disc diffusion demonstrated that the mean diameter of no-growth halo for the discs containing 20 mg of *S. khuzestanica*, *R. coriaria* and *O. basilicum* L. were ≥ 12.28 , ≥ 19.42 , and ≥ 11.42 mm, respectively. The values for the discs containing 40 mg of the extracts were ≥ 20.14 , ≥ 28.57 , and ≥ 13.57 mm, respectively. The mean diameter of no-growth halo for gentamicin (10 $\mu\text{g/ml}$ con.) as the positive control was obtained to be ≥ 37.85 mm. The results of agar dilution method showed that the minimum inhibitory concentration (MIC) of the extracts of *S. khuzestanica*, *R. coriaria*, and *O. basilicum* L. were 307.14, 214.28, and 392.8 $\mu\text{g/ml}$, respectively. The study showed that the ethanol extract of *R. coriaria* and *S. khuzestanica* had antibacterial effect against *H. pylori*.

Key words: *Satureja khuzestanica*, *Rhus coriaria*, *Ocimum basilicum* L., *H. pylori*, antibacterial effect.

INTRODUCTION

Helicobacter pylori is a spiral-shaped Gram-negative micro-aerobic bacterium, which is found in the stomach of most people. The bacterium has infected more than half of the world population, and is the agent of gastrointestinal diseases such as peptic ulcer, chronic gastritis, and dyspepsia in 10% of infected individuals (Wen and Moss, 2009). In the past 20 years, some evidence indicating the role of the bacterium in development of

gastric cancer has been obtained. Therefore, the World Health Organization (WHO) in 1994 introduced *H. pylori* as the first carcinogen factor, and reported that the bacterium is responsible for 63% of the gastric cancer cases (Wen and Moss, 2009). In developing countries such as Iran, the infection rate is high, such that in some areas, 90% of the adults are infected (Saber-Firoozi and Nejabat, 2006). Gastric cancer is a known fatal disease around the world. It is estimated that in 2010 more than 1.1 million individuals were affected by the disease. Gastric cancer is the second cancer in men and fourth cancer in women that leads to death. Epidemiological studies have shown that the developing countries,

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particularly in the Middle East, have the highest rate of *H. pylori* infection and gastric cancer (Lochhead and El-Omar, 2007). Gastric and duodenal ulcers are treated with multi-drug regimens, which are usually a combination of metronidazole, furazolidone, bismuth, tetracycline, amoxicillin, and recently clarithromycin, accompanied with omeprazole, famotidine, or pantoprazole (Saber-Firoozi and Nejabat, 2006; Lochhead and El-Omar, 2007; Kuipers et al., 2003). It should be noted that the cost of treatment with clarithromycin is several times higher than with other antibiotics, and many patients cannot afford it. Furthermore, considering the studies carried out in different countries, the rate of resistance of *H. pylori* to clarithromycin and other antibiotics is increasing (Cowan., 1999; Malekzadeh et al., 2001).

Medicinal plants have less adverse effects and lower costs and are better tolerated by the patients. Considering these and also the known adverse effects of synthetic drugs, the use of medicinal plants has increased in recent years (Malekzadeh et al., 2001). The studies on the antimicrobial effect of *Rhus coriaria* (Sumac) have shown that the fruit extract of the plant contains tannin as the major constituent. This compound is easily dissolved in alcohol and is effective against both Gram-negative and Gram-positive bacteria. This effect is more prominent on intestinal bacteria (Rayne and Mazza, 2007). *Satureja khuzestanica* is a plant, native to Iran, which has been employed as a medicinal plant since ancient times. The plant has antibacterial, anti-inflammatory, antifungal, and antioxidant properties. One of the major constituents of the plant is carvacrol, which can be easily dissolved in ethanol and brings about the antibacterial and antioxidant properties of the plant (Amanlou et al., 2007). In previous studies, it was demonstrated that *Ocimum basilicum* L., which is known as a fragrant edible plant, has antibacterial effects against *Salmonella* and *Bacillus cereus* (Budka and Ahmed, 2010; Rattanachaiakunsopon and Phumkhachorn, 2010).

As earlier mentioned, *H. pylori* infection has high prevalence in our society, and a wide range of disease with high costs of treatment is caused by the organism, in human. Considering these items and since some medicinal plants with confirmed anti-inflammatory and antioxidant effects such as *S. khuzestanica*, *R. coriaria* and *O. basilicum* L., are native to Iran, the current study compared the antimicrobial effect of ethanol extract of *S. khuzestanica*, *R. coriaria*, and *O. basilicum* L. with the antibacterial activity of gentamicin used as a standard antibacterial agent against *H. pylori* under *in vitro* conditions.

MATERIALS AND METHODS

Sampling and culture of *H. pylori* strains

Sampling of the gastric antrum was performed in 30 patients

presented with gastritis and peptic ulcer to the endoscopy ward of Be'sat Hospital, Sanandaj, after obtaining patients' information. The biopsy samples were placed in tubes containing fluid thioglycollate medium and immediately transferred to the laboratory. After homogenizing, the samples under sterile condition, the cells were cultured on brucella agar culture medium (Merck) containing 5% fresh defibrinated sheep blood, 7% inactivated fetal bovine serum (FBS) and 0.25 mg/L of polymyxin B (Sigma), 10 mg/L of vancomycin (Sigma), and 2 mg/L of amphotericin B (Sigma). Then, the cultures were incubated in the anaerobic jar with Gas pack C (Merck) at 37°C for 5 to 7 days. When the bacteria grow, catalase, oxidase, and urease tests and Gram staining were used for detection of the strains. After confirmation of the bacterium for *H. pylori*, culture was repeated on the media without antibiotics (Esmaeilli et al., 2009; Ndpi et al., 2003).

Preparation of the plant extracts

In the study, we used ethanol extracts of the *R. coriaria* fruits, and *S. khuzestanica* and *O. basilicum* L. leaves. To this end, for each plant, 100 g of the plant was macerated in ethanol 80% (Merck) and then mixed well. Then, the mixture of ethanol and the plant was placed in shaker incubator at 35°C. As the alcohol was evaporated, the extracts were collected and kept at 4°C until the time of use (Malekzadeh et al., 2001; Fazeli et al., 2007).

Disc diffusion method

In this method, concentrations of 20 and 40 mg/ml were prepared from the extracts of *S. khuzestanica*, *R. coriaria*, and *O. basilicum* L. and sterilized using membrane filters of 0.4 µm. Then, to each sterile blank disc (Padtan Teb, Iran), 50 µl of each concentration of the extracts were added. In the following, from the fresh culture of *H. pylori*, 1 McFarland solution in brucella broth (Himedia) was prepared and 100 µl of the solution was taken by sterile swab and cultured on brucella agar medium containing 5% defibrinated sheep blood 7% inactivated fetal bovine serum (FBS). The dried discs of the extracts were placed on the culture an appropriate distances. In this method, the gentamicin disc was used as the standard control. After placing the discs, the media were kept in CO₂ incubator (with 10% CO₂) at 37°C for 72 h. The results were obtained by measurement of the diameter of no-growth halo (Malekzadeh et al., 2001; Fazeli et al., 2007; Yang et al., 2010).

Agar dilution method

In this method, *S. khuzestanica*, *R. coriaria*, and *O. basilicum* L. extracts with concentrations of 50, 100, 150, 200, 250, 300, 350, and 400 µg/ml were prepared in sterile distilled water. Each concentration was added to a plate of brucella agar medium (Merck) containing 5% defibrinated sheep blood 7% inactivated fetal bovine serum (FBS). Moreover, from the fresh culture of *H. pylori*, concentration of 3×10^8 was prepared in brucella broth (Himedia) and with a sterile swab, 10 µl of it was cultured on media containing different concentrations of the extracts. The culture media were then placed in CO₂ incubator (with 10% CO₂) at 37°C for three days. The lowest concentration of the extracts that inhibit the observable growth after adequate (72 h) incubation time was considered as the minimum inhibitory concentration (MIC) (Malekzadeh et al., 2001).

Statistical analysis

The data obtained were analyzed using SPSS software, version 12,

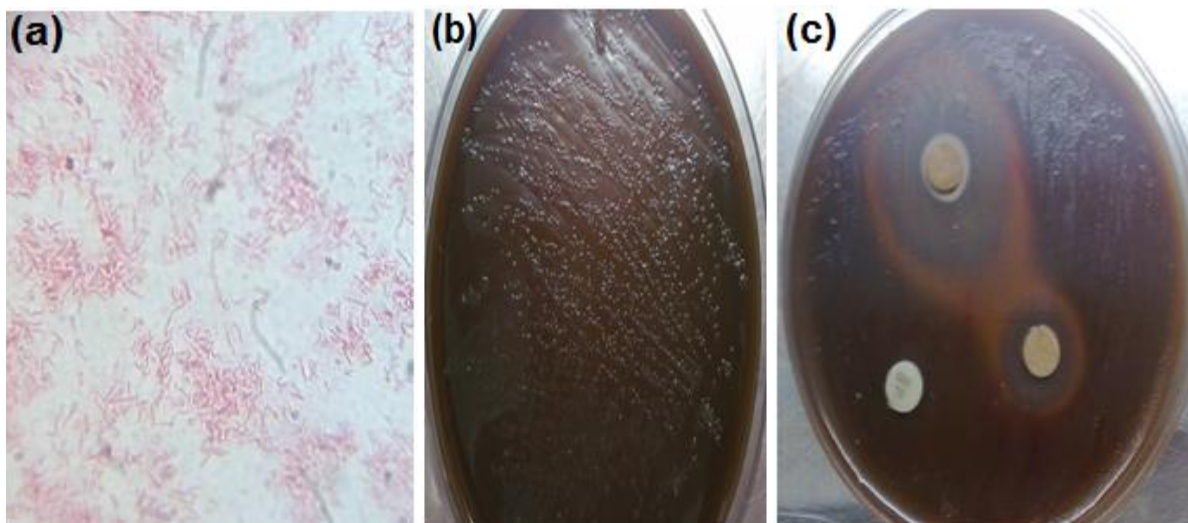


Figure 1. (a) Gram staining of isolated bacteria. (b) Colony growth of *H. pylori*. (c) An example of disc diffusion method. The upper disc and the right disc contain 40 and 20 mg/ml of *R. coriaria* respectively, and the left disc contain 10 µg/ml of gentamicin.

Table 1. Results of statistical analysis of the data obtained.

Extracts	Result of statistical analysis
Comparison of <i>S. khuzestanica</i> (20 mg) with <i>R. coriaria</i> (20 mg)	$p = 0.001$
Comparison of <i>S. khuzestanica</i> (20 mg) with <i>O. basilicum</i> L. (20 mg)	$p = 0.28$
Comparison of <i>S. khuzestanica</i> (40 mg) with <i>R. coriaria</i> (40 mg)	$p = 0.001$
Comparison of <i>S. khuzestanica</i> (40 mg) with <i>O. basilicum</i> L. (40 mg)	$p = 0.001$
Comparison of <i>S. khuzestanica</i> (40 mg) with gentamicin	$p = 0.001$
Comparison of <i>R. coriaria</i> (20 mg) with <i>O. basilicum</i> L. (20 mg)	$p = 0.001$
Comparison of <i>R. coriaria</i> (40 mg) with <i>O. basilicum</i> L. (40 mg)	$p = 0.001$
Comparison of <i>R. coriaria</i> (40 mg) with gentamicin	$p = 0.001$
Comparison of <i>O. basilicum</i> L. (40 mg) with gentamicin	$p = 0.001$

by non-parametric Mann-Whitney test.

RESULTS

From the 30 biopsy samples, we isolated seven strains of *H. pylori* using culture and the confirmatory biochemical tests (Figure 1a and b). The disc diffusion tests demonstrated that the mean diameters of the no-growth halos for discs containing 20 mg of *S. khuzestanica*, *R. coriaria* and *Ocimum basilicum* L. were ≥ 12.28 , ≥ 19.42 , and ≥ 11.42 mm, respectively. The values for the discs containing 40 mg of the extracts were ≥ 20.14 , ≥ 28.57 , and ≥ 13.57 mm, respectively. Moreover, the mean diameter of no-growth halo for gentamicin as the positive control was ≥ 37.85 mm (Figure 1c). The results obtained from agar dilution test showed that the mean MIC of *S. khuzestanica*, *R. coriaria* and *O. basilicum* L. extracts were 307.14, 214.28, and 392.8 µg/ml,

respectively. Also, no-growth halo was not observed for the blank disc, which was used as the negative control. According to the standard table of National Committee for Clinical Laboratory Standards (NCCLS) for slow-growing bacteria and comparison of the diameters of no-growth halos of *R. coriaria* and *S. khuzestanica* extracts with the no-growth halo of gentamicin, it was observed that *R. coriaria* and *S. khuzestanica* extracts had significant bactericidal effect against the isolated strains of *H. pylori*. The results of statistical analysis are provided in Table 1.

DISCUSSION

H. pylori is the most common bacterium that has affected human communities, and man can be carrier of the bacterium from neonatal period to old ages. The antibiotics that are used for prophylaxis and treatment of the infection caused by *H. pylori*, have different adverse

effects. Thus, evaluation of medicinal plants for this purpose is worthy. Many studies have been carried out or are underdeveloped in this field (Shirazi et al., 2003). In this study, we evaluated the antibacterial effect of the extracts of *S. khuzestanica*, *R. coriaria* and *O. basilicum* L. against clinically isolated *H. pylori* strains. The results of disc diffusion test showed that the mean diameters of no-growth halos for the discs containing 20 and 40 mg of *S. khuzestanica*, *R. coriaria* and *O. basilicum* L. were ≥ 12.28 and ≥ 20.14 mm, ≥ 19.42 and ≥ 28.57 mm, and ≥ 11.42 and ≥ 13.57 mm, respectively. Moreover, the mean diameter of no-growth halo for gentamicin as the positive control was observed to be ≥ 37.85 mm. According to the results of agar dilution test, the mean MIC of *S. khuzestanica*, *R. coriaria*, and *O. basilicum* L. extracts were determined to be 307.14, 214.28, and 392.8 $\mu\text{g/ml}$, respectively. The *O. basilicum* L. extract at the concentrations used (400 $\mu\text{g/ml}$) did not show any bactericidal effect.

In a study reported in 1999 by Jonkers et al. (2009), antibacterial effects of garlic and omeprazole against five clinical strains of *H. pylori* were studied. The results show that the MIC of garlic extract against *H. pylori* was 10000 to 17500 mg/ml. It was also shown that garlic extract in combination with omeprazole has a synergistic effect against *H. pylori*. Compared with this study, the anti-*H. pylori* effect of *S. khuzestanica*, *R. coriaria* and *O. basilicum* L. extracts with MIC equal to 307.14, 214.28, and 392.8 $\mu\text{g/ml}$, respectively is much higher than the garlic extract. In 2009, Bonacorsi et al. (2009) reported the antimicrobial and immunostimulatory effects of chloroform and methanol extracts of *Byrsonima crassa* Nied against *H. pylori*. In this study, they used a standard strain of *H. pylori* that was resistant to metronidazole. The MIC was obtained based on the broth micro dilution method. The results show that the MIC level of these extracts against *H. pylori* was 1024 $\mu\text{g/ml}$. This study has also shown that these extracts stimulate immune cells and to produce immune factors such as NO and H_2O_2 . Previous studies have shown that the production of NO and H_2O_2 in the stomach tissue, leads to inflammation and further damage of epithelial cells, and even malignant stomach tissue such as gastric cancer (Koh et al., 1999; Smith et al., 2006). Compared with the study of Bonacorsi et al. (2009) in the present study, seven clinical strains of *H. pylori* that were isolated from patients with gastritis and peptic ulcer were used. *S. khuzestanica*, *R. coriaria*, and *Ocimum basilicum* L. were selected because they have anti-inflammatory properties (Panico et al., 2009; Marawan et al., 2011; Vosough-Ghanbari et al., 2010) and also these plants have antimicrobial activity that can help to reduce gastric colonization of *H. pylori* in the stomach, but there is need to further studies *in vivo* conditions.

Shirazi et al. (2003) reported the antimicrobial effect of ten plant extracts on *H. pylori* and compared with selected antibiotics. Their results showed that the

diameters of no-growth halos for extracts of *Artemisia absinthium* L., *Melia ozedarach* L., *Glycyrrhiza glabra* L., *Salvia officinalis* L., *Myrtus communis* L., *Cichorium intybus* L., *Peganum harmala* L., *Heraclim persicum* Desf. ex Fischer, *Achillea millefolium* L., and *Citrus bigaradia* L. were 15, 14, 14, 13, 11, 9, 9 and 9 mm, respectively, and other plants, have no anti-bacterial activity. In our study, the effect of 20 mg of *R. coriaria* extract against *H. pylori* using the disc diffusion method was 19.42 mm. Thus, showing that the effect of ethanol extract of *R. coriaria* against *H. pylori* was higher than all the ten plant extracts evaluated by Shirazi et al. (2003); Cogo et al. (2010) reported that *Bixa orellana* L. (annatto), *Chamomilla recutita* L. [chamomile/llex paraguariensis A. St-Hil. (roasted and green yerba maté)], *Malva sylvestris* L. (mallow) have significant effects against *H. pylori*. Compared with this study, the extracts of *S. khuzestanica*, *R. coriaria*, and *O. basilicum* L. are less effective against *H. pylori*.

Conclusion

In previous studies, the antioxidant and anti-inflammatory effects of *R. coriaria* and *S. khuzestanica* were confirmed. *H. pylori* is the known cause of gastric cancer, and antioxidants are robust anticancer agents. Moreover, most patients infected with *H. pylori* have gastritis. So the current study was planned. Anti-*H. pylori* activity of *R. coriaria* and *S. khuzestanica* are significantly lower than the effect of gentamycin, but these plants can be used as medicine in patients infected by *H. pylori* and those with gastritis.

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