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An experimental *in-vitro* study to evaluate the anti-helicobacter activity of Glycyrrhetinic acid

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Abstract

***Aim**: The aim of this study was to investigate the in-vitro efficacy of Glycyrrhetinic acid against Helicobacter pylori (H. pylori) strains, as compared with conventional antibacterial agents.*

***Methods**: A total of 41 H. pylori isolates were used, 6 of which were of standard strains (NCTC 1637), 8 of which were drug-sensitive, and 27 were resistant to drugs isolates. Clarithromycin and metronidazole resistance in all strains of H. pylori were determined by the Epsilometer test (E-test) method. MIC study was performed by using microdilution broth method.*

***Results**: Glycyrrhetinic acid was found to be effective against H. pylori NCTC 1637 in doses of 12.0±4.38 µg/mL, while the MIC value of clinical H. pylori isolates susceptible to antimicrobials was 20.8±10.11 µg/ml. It was found that the MIC values for antimicrobial-sensitive clinical H. pylori isolates was higher when compared with H. pylori NCTC 1637 strains. The MIC values of the standard antimicrobial agents against drug-resistant H. pylori strains were higher than H. pylori NCTC 1637 strains and drug-sensitive H. pylori strains. The MIC value was found to be 14.22±7.77 µg/ml for metronidazole, 3.89±1.90 µg/ml for clarithromycin, 2.33±1.0 µg/ml for amoxicillin, 2.44±0.88 µg/ml for levofloxacin and 4.89±2.47 µg/ml for tetracycline, whereas the MIC value of Glycyrrhetinic acid was 26.67±8.0 µg/ml in metronidazole-resistant H. pylori isolates. Besides, MIC values of the antimicrobials and 18ß-Glycyrrhetinic acid among the strains resistant to clarithromycin were as follows: 3.25±2.12 µg/ml for metronidazole, 9.71±4.54 µg/ml for clarithromycin, 2.06±1.32 µg/ml for amoxicillin, 3.88±4.22 µg/ml for levo-floaxacin and 3.25±1.04 µg/mL for tetracycline and 22.0±11.11 µg/ml for Glycyrrhetinic acid.*

***Conclusion**: Glycyrrhetinic acid had significant antimicrobial activity against H. pylori strains. Although further in-vivo studies are needed on antimicrobial activity of Glycyrrhetinic acid, increased resistance to drugs currently used in treatment suggests that Glycyrrhetinic acid may be a potential agent for the treatment of H. pylori.*

***Keywords**: Glycyrrhetinic acid, Helicobacter pylori, drug, resistance, metronidazole, clarithromycin*

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Introduction

Helicobacter pylori (*H. pylori*), a Gram negative curved and spiral rod, is one of the most common bacterial pathogens in humans colonized in more than half of the world's population (1,2). It has been reported that the bacterium is present in almost all patients with active chronic gastritis, duodenal ulcer and gastric ulcer; thus, it may be a significant factor in the etiology of these diseases. Therefore, eradication of *H. pylori* in infected patients is very important for the treatment of diseases associated with this agent (3). For the treatment and eradication of this infectious agent, the conventional approach relies on using multiple antibiotics such as clarithromycin, amoxicillin, tetracycline, metronidazole in combination with bismuth sulphate or a proton-pump inhibitor (4). In clinical practice, *H. pylori* eradication rates vary from 60% to 80% (6). However, the resistance to antibiotics used in therapy especially to metranidazole and clarithromycin causes failure in *H. pylori* eradication (5). Because of treatment failures, antibiotics such as levofloxacin, rifabutin, and furazolidone have been used as alternative agents in *H. pylori* eradication. Nevertheless, both the rapid development of quinolone resistance (6) and adverse side effects related to the use of rifabutin and furazolidone have limited the effectiveness and use of these agents in the treatment of *H. pylori* infections (7,8). These adversities in treatment have increased the need for the development of news agents. In addition, this also has encouraged studies investigating herbal medicinal products with antimicrobial activity as an alternative in the *H. pylori* treatment (9). It has been shown that some natural products have antibacterial activity in the treatment of *H. pylori* (10).

Licorice is the root of the *Glycyrrhiza glabra*. The active ingredient in licorice is glycyrrhizin (*Glycyrrhizic acid*, glycyrrhizinate). *Glycyrrhetinic acid*, a hydrolytic product of *glycyrrhizic acid*, is a component of licorice. *Glycyrrhiza glabra*, also called Licorice, is a plant belonging to Fabaceae family that is found in South Europe and some parts of Asia and has antiviral activity (11,12). Animal studies on rats have shown that Licorice has significant hepatoprotective activity (13,14). A study conducted by Japanese scientists found that licorice reduced transaminase levels in patients with chronic viral hepatitis (15).

Glycyrrhetinic acid major bioactive triterpene glycoside in Licorice root extracts has a wide range of pharmacological activities including anti-inflammatory, anti-ulcer, anti-allergic, antidote, anti-oxidant, anti-tumor, and antiviral effects (16). In the present study, we aimed to investigate the *in-vitro* efficacy of *Glycyrrhetinic acid* against the standard and clinical *H.pylori* strains as compared with conventional antibacterial agents.

Material and Method

This study used 41 *H. pylori* isolates, 6 of which were of standard strains (NCTC 1637), 8 of which were drug-sensitive and 27 were resistant to drugs isolates. Ethics approval for the present study was obtained from the local Ethics Committee. The experiments were carried out using the following strains: Eight susceptible strains to all antimicrobials; only metronidazole resistant (nine isolates); only clarithromycin resistant (nine isolates); both clarithromycin and metronidazole resistant strains (nine isolates); and standard *H. pylori* NCTC 1637 strains (six strains).

Antimicrobial susceptibilities of the isolates were investigated by E-test (AB Biodisk, Sweden) method (17). The clinical samples were inoculated to Mueller-Hinton agar with 5% sheep blood and *H. pylori* agar. Plates were incubated at 37°C in a microaerophilic environment (Camy-Gen, Oxoid). *H. pylori* identification was performed using conventional techniques (18.19). The standard *H. pylori* strain used in the study was supplied by the Microbiology Depart-

ment of Hacettepe University, Medicine School in Ankara.

Preparation of Bacterial Suspension

Glycyrrhetinic acid used in the study was purchased from Sigma (18 β-*Glycyrrhetinic acid*, Sigma, USA). DMSO was used as solvent for *Glycyrrhetinic acid*. MIC values were calculated by microdilution broth method. Standard antibiotics and *Glycyrrhetinic acid* were diluted from 256 to 0.5 µg/ml by 2-fold serial dilution.

The clinical isolates and *H. pylori* NCTC 1637 strains were suspended in Brucella Broth (BBL 4311086) containing 5% fetal calf serum (FCS), and incubated for 48 hours at 37°C in microaerophilic conditions. *H. pylori* cell density was adjusted to $1x10^8$ cells/ml. MIC values were assessed after incubation at 37°C under microaerophilic conditions over 5 days (20).

Determination of Minimum Bactericidal Concentration (MBC)

Antimicrobial activity of *Glycyrrhetinic acid* against *H. pylori* NCTC 1637 and the clinical isolates were evaluated at the concentration of 512 µg/ml. It was determined that *Glycyrrhetinic acid* inhibited the proliferation of *H. pylori* strains by 100% at this concentration. Serial dilution was performed from 512 to 0.5 µg/mL. Bactericidal activity was studied according to the method described by O'Mahony et al. (21). Briefly, 900 µl of solution containing different concentrations of *Glycyrrhetinic acid* were added to 100 µl of bacterial suspension. At the end of the incubation, 100 µl of each dilution were inoculated to *H. pylori* agar for evaluation of bacterial growth. Metronidazole (100 µg/ml), clarithromycin (10 µg/ml), amoxicillin (10 µg/ml), levofloxacin (20 µg/ml), and tetracycline (40 µg/ml) were selected as the standard drugs. DMSO was used as negative control. All experiments were performed in triplicate. Bactericidal concentration was defined as the lowest concentration in which no growth turbidity and bacterial growth on *H. pylori* agar (Sigma, USA) was observed.

Effect of DMSO

In order to test the effects of DMSO against *H. pylori* strains and clinical *H. pylori* isolates, $1x10^8$ bacterial cells were inoculated in each well of 12-well plates containing Brucella Broth medium with 5% fetal calf serum. Bacterial isolates were allowed to grow for an additional 48 h in the presence of decreasing amounts of DMSO (8%, 4%, 2%, 1%, 0.5%). The non-toxic concentration was determined up to 2%. A concentration of 0.5% and 1% DMSO did not influence the growth of the *H. pylori* isolates as determined microscopically. Therefore, bacterial isolates were dissolved in 1% DMSO.

Cytotoxicity Testing

Cell Culture

HEp-2 (Human laryngeal carcinoma cell line) was used to perform cytotoxicity tests for *Glycyrrhetinic acid*. RPMI-1640 containing 10% FCS, 100 IU/ml penicillin and 0.1 mg/ml streptomycin was used as the cell culture medium. DMSO (1% v/v) was selected as solvent to dissolve *Glycyrrhetinic acid*.

For cytotoxicity testing, HEp-2 cells were inoculated to microplates at a concentration of $1x10^6$ cells/ml. Culture plates were incubated at 30°C for 8 hours for cell adhesion. At the end of the 8-hour incubation, various concentrations (3.200, 1.600, 400, 200, 100, 75, 50, 25 µg/ml) of *Glycyrrhetinic acid* were added to the plates where cell adhesion was completed. Subsequently, all plates were incubated at 37°C with 5% CO_2 over 72 hours. All experiments were performed in triplicate.

Antimicrobial Sensitivity Tests

The presence of clarithromycin and metronidazole resistance in the clinical isolates was as-

sessed using E-test as described by Fukazawa et al. (17). Bacteria colonies obtained from *H. pylori* cultures isolated were incubated in Mueller-Hinton agar with 7% equine blood. The bacterial suspension was prepared in Brucella Broth according to McFarland 3 turbidity. Plates were incubated at 37°C over 3 days under microaerophilic conditions (CampyGen). After 3 days of incubation, antibiotic concentration corresponding to the point where the ellipsoid inhibition zone intersects the E-Test stripe was accepted as MIC value. In the present study, the following values were accepted as resistance threshold: ≥8 µg/ml for metronidazole and ≥1 µg/ml for clarithromycin (17).

Results

DMSO was used as solvent to prepare stock solution of *Glycyrrhetinic acid*. In the studies on HEp-2 cell cultures, it was found that 1% DMSO did not inhibit cell proliferation when compared to a control group (Figure 1). There was no statistically significant difference in cell viability at 24, 48, 72 and 96 hours of incubation in cultures between the cultures containing DMSO (1% v/w; DMSO control) and the cultures with neither antimicrobial agent nor DMSO (negative control).

In experiments, it was determined that *Glycyrrhetinic acid* was not toxic for the cells up to a concentration of 512 mg/ml. There was no significant difference between the DMSO-containing group and the control group in terms of cell viability.

Moreover, in morphological evaluation by inverted microscope, it was determined that there were no pathological changes morphologically in the cell cultures containing DMSO and *Glycyrrhetinic acid* at concentrations up to 256 µg/ml (including 256 µg/ml) and these cells were found to be intact, like the control cells. When the *Glycyrrhetinic acid* concentration in the medium was used at 512 µg/ml and higher, cytopathological changes such as nuclear growth, rounding and granulation were observed in the cells and the cells were found to be atypical in form.

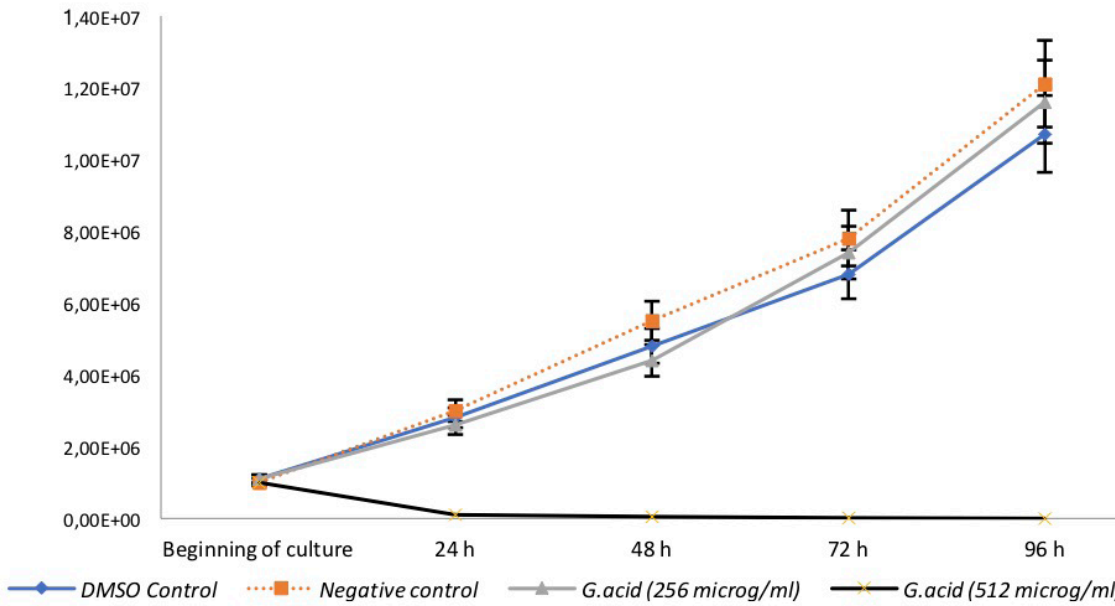

Fig. 1. The cytotoxic effect of *Glycyrrhetinic acid* on HEp-2 Cell line.

The antimicrobial activity results of *Glycyrrhetinic acid* against *H. pylori* NCTC 1637 strain and the drug-susceptible clinical *H. pylori* isolates are shown in Figure 2. It was found that *Glycyrrhetinic acid* showed bactericidal activity at the 12.0±4.38 µg/ml for *H. pylori* NCTC 1637, while MIC value was 20.8±10.11 µg/ml for the antimicrobial-sensitive clinical *H. pylori* isolates (Table 1). The MIC values of *Glycyrrhetinic acid* against the drug-sensitive *H. pylori* clinical isolates were found to be higher than *H. pylori* NCTC 1637 ($p<0.01$). When the efficacy of *Glycyrrhetinic acid* was compared with the commonly used antibiotics (metronidazole, clarithromycin, amoxicillin, levofloxacin, and tetracycline) for the treatment of *H. pylori* infections, the MIC value of the *Glycyrrhetinic acid* was found to be higher than those.

MIC value of *Glycyrrhetinic acid* against the metronidazole, clarithromycin-resistant and both metronidazole and clarithromycin resistant isolates were compared with the standard antimicrobial agents. Our study found that MIC values of the standard antimicrobial agents against drug-resistant *H. pylori* strains were higher when compared with those against *H. pylori* NCTC 1637 and the drug-sensitive strains ($p<0.05$). The MIC values of the antibacterials and *Glycyrrhetinic acid* against metronidazole-resistant *H. pylori* isolates were as follows: 14.22±7.77 µg/ml for metronidazole; 3.89±1.90 µg/ml for clarithromycin; 2.33±1.0 µg/ml for amoxicillin and levofloxacin; 2.44±0.88 µg/ml for tetracycline, and 4.89±2.47 µg/ml for *Glycyrrhetinic acid* (Table 1).

MIC value was found to be 3.25±2.12 µg/ml for metronidazole, 9.71±4.54 µg/ml for clarithromycin, 2.06±1.32 µg/ml for amoxicillin, 3.88±4.22 µg/mL for levofloxacin and 3.25±1.04 µg/ml for tetracycline, whereas 22.0±11.11 µg/ml for *Glycyrrhetinic acid* against the clarithromycin-resistant *H. pylori* isolates (Table 1).

In the *H. pylori* isolates resistant to both metronidazole and clarithromycin, the MIC values were found to be 26.88±17.30 µg/ml for metronidazole, 6.25±4.46 µg/ml for clarithromycin, 3.38±2.20 µg/ml for amoxicillin, 2.38±1.06 µg/ml for levofloxacin and 4.75±2.12 µg/ml for tetracycline, and 37.00±18.60 µg/ml for *Glycyrrhetinic acid*.

Discussion

HP is one of the most common causes of bacterial infections in human (22). *H. pylori* infection is generally transmitted in childhood by

Table 1. MIC values of *Glycyrrhetinic acid* and antimicrobials against dug-sensitive and resistant isolates.

	Metronidazole	Claritromycin	Amoxicillin	Levofloxacin	Tetracycline	Glycyrrhetinic acid
6-H.pylori NCTC 11637	2.67±0.83	0.75±0.27	0.83±0.26	2.67±1.03	2.16.0±0.98	12.0±4.38
8-Drug sensitive clinical isolates	1.05±0.55	0.80±0.48	0.80±0.26	0.9±0.21	1.8±0.92	20.8±10.11
9-Metronidazole-resistant clinical isolates	14.22±7.77	3.89±1.90	2.33±1.0	2.44±0.88	4.89±2.47	26.67±8.0
8-Clarithromycin-resistant clinical isolates	3.25±2.12	9.71±4.54	2.06±1.32	3.88±4.22	3.25±1.04	22.0±11.11
8-Both metronidazole and clarithromycin resistant isolates	26.88±17.30	6.25±4.46	3.38±2.20	2.38±1.06	4.75±2.12	37.00±18.60

"maternal-to-infant transmission". As a result of this type of transmission, multiple strains can be colonized in the stomach during childhood. The majority of these strains are eradicated spontaneously; however, a genotype adapting to the gastric mucosa and host immune system may lead to permanent colonization (23). The microorganism causes changes in gastric epithelium through direct bacterial toxicity and indirect inflammation-mediated injury (24). Several studies have found that *H. pylori* infection may be associated with chronic gastritis, peptic ulcer, gastric adenocarcinoma and pathogenesis of mucosa-related tissue lymphoma (25-29).

Although several antimicrobial agents are used effectively for the treatment of *H. pylori*, research on natural drugs has become important due to increasing antibiotic resistance in recent years. There has been an increasing popularity of medicinal plants worldwide. Studies have shown that many plants or herbal extracts can be used for the treatment of gastrointestinal system diseases. Among these plants, it is known that the roots and rhizomes of *G.glabra* from the family Leguminosae have been used in folk medicine for centuries. In various studies, *G.glabra* has been shown to have some pharmacological activities such as diuretic, laxative, sedative, antipyretic, antimicrobial, hepatoprotective, and antioxidant properties (30,31).

Previous studies have shown that *G.glabra* has anti-allergic and anti-inflammatory activities, and that it is used for the treatment of various diseases such as allergy, asthma, and eczema. In addition, *G.glabra* is known to be useful for the treatment of chronic hepatitis and viral infections caused by human viruses such as human immunodeficiency virus, cytomegalovirus, and *Herpes simplex* virus in Japan (32-38).

In a study conducted by Kalaigandhi et al. in 2011, the activity of *G.glabra* against peptic ulcer caused by *H. pylori* was investigated. Ethanol and acetone extracts of *G.glabra* leaves were used in their study. The study reported that *G.glabra* had antimicrobial activity against *H. pylori* which was thought to be related to tannin, alcoholic and triterpenoid components of *G.glabra* (39).

Our study also found that *Glycyrrhetinic acid*, one of the most important components of glycyrrhiza, had a strong antimicrobial activity against *H. pylori*. The MIC value of *Glycyrrhetinic acid* against the *H. pylori* standard stain was found to be 12.0±4.38 µg/ml while it was found to be 20.8±10.11 µg/ml against the drug-sensitive *H. pylori* clinical isolates. Given the MIC value of metronidazole was 2.67±0.83 µg/ml against the *H. pylori* standard stain and 1.05±0.55 µg/ml for the drug sensitive clinical isolates, it was apparent that *Glycyrrhetinic acid* activity was significant (Figure 2). It was found that the activity of *Glycyrrhetinic acid* was rather high against the drug-resistant *H. pylori* strains (Figure 3). The MIC value of metronidazole was found to be 14.22±7.77 µg/ml against the resistant *H. pylori* strain while the MIC value of *Glycyrrhetinic acid* was 26.67±8.0 µg/ml for the same strains. It was found that the MIC value of clarithromycin was 9.71±4.54 µg/ml against the clarithromycin-resistant strains while the MIC value of *Glycyrrhetinic acid* was 22.0±11.11 µg/ml for the same strain. The MIC value of *Glycyrrhetinic acid* was found to be 37.00±18.60 µg/ml for strain resistant to both clarithromycin and metronidazole, while the MIC values of metronidazole and clarithromycin for the same strains were found to be 26.88±17.30 µg/ml and 6.25±4.46 µg/ml, respectively. In our study, we found that the MIC value of *Glycyrrhetinic acid* was lower against the *H.pylori* NCTC 1637 strain and the drug-sensitive clinical isolates, while it was increased against the drug-resistant strains.

In a randomized, placebo-controlled trial, the efficacy of *Glycyrrhiza glabra* extracts in patients with functional dyspepsia was investigated and 75 mg plant extracts were given (twice

daily for 1 month) to these patients and evaluated as comparable with the control group. When symptom severity was assessed by Likert scale, it was found that there was significant decrease in symptom severity in the patient group (40). In addition, it was shown that *Glycyrrhiza glabra* extracts increases gastric mucus release and has anti-ulcerative activity (41).

In an *in-vitro* study conducted by Asha et al. in 2013, flavonoids of *Glycyrrhiza glabra* were shown to be antimicrobially effective against *H. pylori* (42). In an *in-vivo* study, Wang et al. showed that it has beneficial effects on gastric mucosal damage (43).

In conclusion, it was found that *Glycyrrhetinic acid* had significant antimicrobial activities against the *H. pylori* strains in our study. There is a need for advanced *in-vivo* studies of antimicrobial activity of *Glycyrrhetinic acid*. Especially in recent years, resistance to drugs used in the treatment of *H. pylori* infections is a serious health problem. We think that *Glycyrrhetinic acid* may be a potential new agent in the treatment of diseases caused by *H. pylori*.

Author statement and acknowledgements

The second author named is lead and corresponding author. All other authors are listed in alphabetical order.

We describe contributions to the paper using the taxonomy provided above. Writing-Original Draft: M.M.C. and N.D.; Writing-Review & Editing: M.M.C. and N.D.; Conceptualization: M.M.C. and N.D.; Investigation: M.M.C. and N.D. Methodology: M.M.C. and N.D.; Formal Analysis: M.M.C. and N.D.; Project Administration: M.M.C. and N.D.

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