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# *In vitro* anti-*Helicobacter pylori* activity of a flavonoid rich extract of *Glycyrrhiza glabra* and its probable mechanisms of action

Mannanthendil Kumaran Asha, Debnath Debraj, D'souza Prashanth, Jothie Richard Edwin, H.S. Srikanth, Nithyanantham Muruganantham*, Shekhar Michael Dethe, Bhaskar Anirban, Balachandran Jaya, Mundkinajeddu Deepak, Amit Agarwal

*R&D centre, Natural Remedies Pvt. Ltd., Bangalore, Karnataka, India*

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ABSTRACT

*Ethnopharmacological relevance:* *Glycyrrhiza glabra* Linn. is regarded useful for peptic ulcer in traditional systems of medicine in India and *Helicobacter pylori* has been considered as one of the causative factors for peptic ulcer. Aim of the present study is to evaluate the anti-*Helicobacter pylori* action of GutGard®, a flavonoid rich extract of *Glycyrrhiza glabra* and further to elucidate the possible mechanisms of its anti-*Helicobacter pylori* action.

*Materials and methods:* Agar dilution and microbroth dilution methods were used to determine the minimum inhibitory concentration of GutGard® against *Helicobacter pylori*. Protein synthesis, DNA gyrase, dihydrofolate reductase assays and anti-adhesion assay in human gastric mucosal cell line were performed to understand the mechanisms of anti-*Helicobacter pylori* activity of GutGard®.

*Results:* GutGard® exhibited anti-*Helicobacter pylori* activity in both agar dilution and microbroth dilution methods. Glabridin, the major flavonoid present in GutGard® exhibited superior activity against *Helicobacter pylori* while glycyrrhizin did not show activity even at 250 μg/ml concentration. In protein synthesis assay, GutGard® showed a significant time dependent inhibition as witnessed by the reduction in $^{35}S$ methionine incorporation into *Helicobacter pylori* ATCC 700392 strain. Additionally, GutGard® showed a potent inhibitory effect on DNA gyrase and dihydrofolate reductase with $IC_{50}$ value of 4.40 μg/ml and 3.33 μg/ml respectively. However, the extract did not show significant inhibition on the adhesion of *Helicobacter pylori* to human gastric mucosal cell line at the tested concentrations.

*Conclusion:* The present study shows that, GutGard® acts against *Helicobacter pylori* possibly by inhibiting protein synthesis, DNA gyrase and dihydrofolate reductase.

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## 1. Introduction

*Helicobacter pylori* has been considered as the leading cause of peptic ulcer disease. About 50% of the world's population and 90% population in developing countries have been reported to be infected with this gram-negative bacterium (Bardhan, 1997; Dunn et al., 1997; Farthing, 1998). Treatment for eradication of *Helicobacter pylori* infection is continuously evolving from the standard triple therapy using a combination regime with proton pump inhibitor (PPI), amoxicillin, and clarithromycin or PPI, amoxicillin, and metronidazole to sequential therapy with PPI plus amoxicillin for five days followed by PPI plus clarithromycin and tinidazole for five days. Various other treatment regimens used as second line therapy for *Helicobacter pylori* eradication include quadruple therapy, levofloxacin, and rifabutin based therapy. In addition to these options, adjuvant therapy with probiotics, bovine lactoferrin, and curcumin are being used to reduce the side effects (associated with the standard triple therapy), increase the patient compliance and thereby the treatment efficacy (Egan et al., 2007).

*Glycyrrhiza glabra* Linn. (Leguminosae), commonly called as licorice has a long history of consumption for its many medicinal properties. It has been traditionally used for the treatment of peptic ulcer (McKenna et al., 2008). Roots and stolons of this plant were considered as a primary medicine for peptic ulcer until the advent of cimetidine (Davis and Morris, 1991). Various preclinical studies showed the effectiveness of *Glycyrrhiza glabra* in the treatment of peptic ulcer (De et al., 1997; Khayyal et al., 2001, Aly et al., 2005). Glycyrrhetinic acid, the major metabolite of glycyrrhizin was reported to inhibit *Helicobacter pylori* (Kim et al.,

*Abbreviations:* PPI, Proton pump inhibitor; DGL, Deglycyrrhizinated licorice; MAG, Monoammonium glycyrrhizinate; DMSO, Dimethyl sulfoxide; FBS, Fetal bovine serum; MIC, Minimum inhibitory concentration; DHFR, Dihydrofolate reductase; PBS, Phosphate buffered saline; FITC, Fluorescein isothiocyanate

* Correspondence to: Plot No. 5-B, Veerasandra Industrial Area, 19th K.M. Stone, Hosur Road, Bangalore - 560100. Tel.: +91 80 4020 9999; fax: +91 80 4020 9817.
*E-mail address:* muruganantham@naturalremedy.com (N. Muruganantham).

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2000; Krausse et al., 2004). Side effects of licorice, viz., edema, hypertension and hypokalemia were attributed to glycyrrhizin and its derivatives (Conn et al., 1968; Monder et al., 1989; Souness and Morris, 1989; Morris et al., 1990). Flavonoids, present in licorice were identified to be active against *Helicobacter pylori* (Fukai et al., 2002). A flavonoid rich extract of *Glycyrrhiza glabra* (GutGard®) has been reported to possess antiulcer property in rats (Mukherjee et al., 2010) and found to be clinically safe and efficacious in patients with functional dyspepsia at the dose of 150 mg/day when administered for 30 days (Raveendra et al., 2012). In addition, the extract was found to be non-genotoxic in a battery of *in vitro* genotoxicity tests viz., bacterial reverse mutation test, chromosome aberration and micronucleus tests (Chandrasekaran et al., 2011b). Recently, a randomized double blind clinical trial on GutGard® showed it to be effective against *Helicobacter pylori* (Communicated). The present study is carried out to evaluate the anti-*Helicobacter pylori* action of GutGard® and its possible mechanisms of action.

## 2. Materials and methods

### 2.1. Test substances

GutGard® is a flavonoid rich standardized extract of *Glycyrrhiza glabra* as described in previous publication (Chandrasekaran et al., 2011a). The extract is standardized to contain glabridin ( $\geq$ 3.5% w/w), glabrol ( $\geq$ 0.5% w/w), eicosanyl caffeate ( $\geq$ 0.1% w/w), docosyl caffeate ( $\geq$ 0.1% w/w) and total flavonoids ( $\geq$ 10% w/w).

Dimethyl sulfoxide (DMSO, Sigma, USA) was used as the solvent for dissolving GutGard®, glabridin (Natural Remedies, Bangalore), deglycyrrhizinated licorice (DGL, Natural Remedies, Bangalore), and monoammonium glycyrrhizinate (MAG, Sigma, USA). For anti-adhesion assay, working solution of GutGard® was made using Dulbecco's phosphate buffered saline. Sterile MilliQ water was used as the solvent for tetracycline hydrochloride (Sigma, USA), ciprofloxacin (Sigma, USA) and rebamipide hydrochloride (Sigma, USA). Amoxicillin (Sigma, USA) was dissolved in phosphate buffer; clarithromycin (Sigma, USA) in acetone and omeprazole (Sigma, USA) in methanol. Methotrexate (Sigma, USA) was dissolved in assay buffer provided with the dihydrofolate reductase assay kit (Sigma, USA).

### 2.2. Bacterial strains

*Helicobacter pylori* strain ATCC 700392 was obtained from American Type Culture Collection (Rockville, MD, USA); the strain NCTC 11916 was obtained from National Collection of Type Cultures (Salisbury, UK). Clinical strains AB 976 and AB 977 were obtained from a hospital in India. The clinical strains were confirmed by usual microbiology and molecular biology methods (Tiwari et al., 2007, 2008). All the *Helicobacter pylori* strains were propagated in Brucella agar medium (Becton Dickinson, Le Pont de Claix, France) supplemented with 7% horse serum (Gibco, New Zealand). The colonies were preserved in 20% sterile glycerol saline medium and stored at −86 °C in an ultra freezer until used for the microbroth dilution method and inhibition of protein synthesis. *Helicobacter pylori* strain ATCC 700392 cultivated using Brain heart infusion agar (Difco, Detroit, MI, USA) supplemented with 10% fetal bovine serum (Gibco, USA), which was used for the agar dilution method.

### 2.3. Minimum inhibitory concentration (MIC)

Agar dilution and microbroth dilution methods were used to determine the minimum inhibitory concentration (MIC).

#### 2.3.1. Agar dilution method

Agar dilution method was performed as per Mitscher et al. (1972) with slight modifications. GutGard®, glabridin, DGL and MAG were tested in this method against *Helicobacter pylori* strain ATCC 700392. Amoxicillin, clarithromycin and omeprazole, were used as positive controls. Test samples were mixed with sterile Brain heart infusion agar containing 10% fetal bovine serum (FBS, heat inactivated) and poured on to sterile petri plates such that the final volume was kept at 5 ml (pH, 7.2 $\pm$ 0.1). Solvents used for solubulising the samples were used as respective controls. Test organisms were streaked on to the surface of agar using a calibrated loop that delivers 10 µl of the inoculum. The plates were then incubated at 37 °C for 5 days in an incubator under microaerophilic conditions. The growth was examined after 5 days. MIC, the lowest concentration at which the test sample completely inhibited the visible growth of microorganism was determined.

#### 2.3.2. Microbroth dilution method

Microbroth dilution method was performed as per the recommendations of Clinical and Laboratory Standards Institute (CLSI). GutGard® and positive controls viz., amoxicillin and tetracycline, were tested by this method using four *Helicobacter pylori* strains viz., ATCC 700392, NCTC 11916, AB 976 and AB 977. The bacterial colony suspension equivalent to 2.3–2.5 McFarland's standard was prepared and diluted 100 times with the Brucella broth media (pH, 7.3). One hundred microlitre of diluted culture was added to a microtitre plate containing 100 µl of diluted sample or media, such that the final inoculum contained 0.7–1 $\times$ $10^6$ CFU/ml. The assay plates were maintained at 35 °C in a 5% $CO_2$ incubator for 72 h under microaerophilic condition. After 72 h, the assay plates were read visually for growth inhibition and also at 600 nm using a multi-detection microplate reader.

### 2.4. Inhibition of protein synthesis

Inhibition of protein synthesis was performed in ATCC 700392*Helicobacter pylori* strain by $^{35}$S methionine incorporation assay by following the method of Patrzykat et al. (2002). GutGard® was tested at concentrations of 4 and 8 µg/ml while the positive control, tetracycline hydrochloride, was tested at 0.03 µg/ml. ATCC 700392*Helicobacter pylori* strain was inoculated in Brucella broth supplemented with 7% horse serum and incubated for 72 h. Then the cells were harvested by centrifugation and re-suspended in warm synthetic M9 medium plus Brucella broth and incubated with $^{35}$S methionine for 10 min. After incubation, the cells were pelleted and washed twice with M9 medium. Thereafter, it was re-suspended in M9 medium plus Brucella broth and incubated with and without test samples for 48 h at 35 °C in an incubator under microaerophilic condition. Controls (Organism+$^{35}$S Methionine) without sample were included in the study.

Evaluation of protein synthesis was performed by removing 100 µl of sample at different time points of 0 (immediately before the addition of test sample), 1, 2, 3, 5, 24 and 48 h (after the test sample addition) and adding them individually to 1 ml of ice-cold 10% trichloroacetic acid (with excess unlabeled precursors in order to precipitate the macromolecules). The resultant precipitates were kept for 40 min on ice and 15 min at 35 °C. After incubation, the samples were filtered by Whatman glass fiber filter and $^{35}$S activity was measured using a microplate liquid scintillation counter for 1 min. The results were represented as percentage inhibition of protein synthesis calculated by considering the counts per minute with untreated cells as 100%. Viability evaluation was also performed at different time points of

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