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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 ± 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 $0.7\text{–}1 \times 10^6$ 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

0 (immediately before the addition of test sample), 1, 2 3 5, 24 and 48 h (after the test sample addition) by using Brucella agar supplemented with 7% horse serum.

2.5. DNA gyrase inhibition assay

DNA Gyrase inhibition assay was performed as per the protocol of DNA topoisomerase II (gyrase) assay kit (ProFoldin, Catalog No. DSA020K) with slight modifications. In brief, the reaction mixture (10 μl) contained 2 μl of sample/standard at various concentrations, 2 μl of 5× T2 buffer, 2 μl of 5× relaxed DNA (substrate), 2 μl of 5× DNA gyrase (enzyme) and 2 μl of 5 mM ATP. Reaction mixture without ATP was used as the negative control. The reaction mixture was incubated at 25 °C for 60 min. After incubation, 62.5 μl of freshly prepared 1× H19 dye was added to the reaction mixture and again incubated at 25 °C for 5 min. The fluorescence intensity was measured at 535 nm with the excitation wavelength set at 485 nm using Fluostar Optima (BMG Labtech Instruments, Germany). Ciprofloxacin was used as the positive control and IC_{50} (concentration of the inhibitor at which 50% inhibition was seen) values were calculated for each tested sample.

2.6. Dihydrofolate reductase inhibition assay

The assay was performed using the dihydrofolate reductase (DHFR) assay kit as per Sigma protocol with slight modifications. Briefly, assay buffer (1×), dihydrofolate reductase solution (0.0015 U), β-Nicotinamide adenine dinucleotide phosphate reduced tetrasodium salt (NADPH, 0.06 mM) and different concentrations of test samples were mixed together. The reaction was initiated by adding dihydrofolic acid (0.05 mM). The total volume of the reaction mixture was 500 μl. The reaction was read at 340 nm in a kinetic mode for 5 min. Methotrexate was used as the positive control and IC_{50} (concentration of the inhibitor at which 50% inhibition was seen) values were calculated for each tested sample.

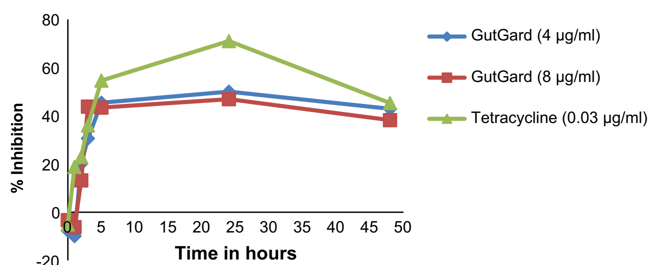

Fig. 1. Inhibition of protein synthesis by GutGard®. Inhibition of protein synthesis was performed by ^{35}S methionine incorporation assay and the results of samples are represented as percentage inhibition calculated by considering the activity of untreated cells (control) as 100%.

2.7. Helicobacter pylori anti-adhesion assay in AGS cells

2.7.1. Cell Viability assay with GutGard® in human gastric adenocarcinoma (AGS) cells

Cultured AGS cells were seeded in the complete medium for each treatment condition at approximately 5×10^4 cells per well (200 μl/well) in a 96 well plate and incubated for 20 h at 37 °C in a humidified atmosphere of 5% CO_2. After 20 h, the medium was decanted and washed once with phosphate buffered saline (PBS, pH 7.4). Complete medium (175 μl) and 25 μl of GutGard® at various concentrations were added to each well and incubated for 90 min. Then the cells were washed twice with PBS, treated with 5 mg/ml of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, 10 μl) and again incubated for 1 h at 37 °C. The medium was discarded, 200 μl of DMSO was added and absorbance was read at 570 nm (Mosmann, 1983).

2.7.2. Helicobacter pylori culture

Helicobacter pylori strain was cultured in brain–heart infusion broth supplemented with 10% FBS (heat inactivated) under microaerophilic condition. After 5 days of incubation, the culture was centrifuged, and the pellets obtained were suspended in a fresh, complete medium (Ham's F12 medium supplemented with 10% FBS). The cells were washed thrice, and cell counts were adjusted to 2×10^{10} cells/ml by reading in a spectrophotometer at 450 nm.

GutGard® was then evaluated for its anti-adhesion effect on *Helicobacter pylori* in AGS cells by three different techniques viz., fluorescence, urease detection technique and immuno-fluorescence. Rebamipide hydrochloride was used as the positive control. Results of the anti-adhesion assay are represented as percentage change from control, which was set as 100% (Fig. 2.).

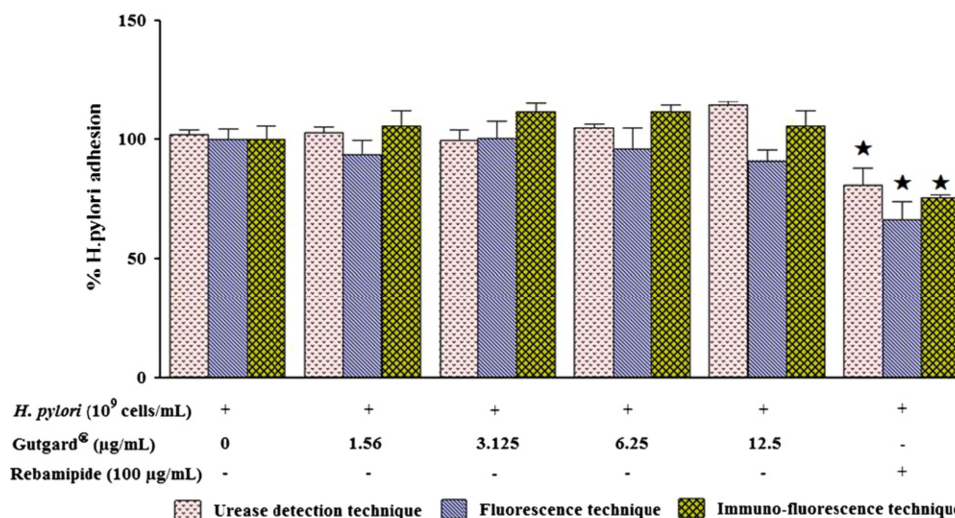

Fig. 2. Effect of GutGard® on *Helicobacter pylori* treated AGS (human gastric carcinoma) cells. GutGard® was tested for its anti-adhesion activity at concentrations ranging from 1.56 μg/ml to 12.5 μg/ml in *Helicobacter pylori* treated AGS cells. Rebamipide at 100 μg/ml was used as positive control, which showed significant effect ($p<0.05$) in comparison to concurrent control. *$p<0.05$ compared to control.

2.7.3. Fluorescence technique

Fluorescein isothiocyanate (FITC, Isomer I, Sigma) solution (10 µl, 5 mg/ml) in DMSO (Sigma), was added to bacterial suspension (1×10^9 cells/ml) and incubated for 45 min with continuous shaking. Suspensions were washed thrice with PBS and resuspended in PBS. Incubation and washing steps were carried out in the dark at room temperature.

In brief, 25 µl of GutGard® at various concentrations and 175 µl of complete medium were added to each well containing AGS cells (prepared as described previously under Section 2.7.1). The mixture was incubated for 90 min and washed twice with PBS. Then, 100 µl of FITC tagged *Helicobacter pylori* (10^9 cells/ml) was added to each well and incubated for further 90 min at 37 °C in a humidified atmosphere of 8% CO_2. After 90 min, unattached bacterial cells were washed; 100 µl PBS added and the fluorescence intensity measured at 530 nm with excitation wavelength set at 485 nm (Wadstrom et al., 1997).

2.7.4. Urease detection technique

In brief, 100 µl of *Helicobacter pylori* (10^9 cells/ml) was added to each well containing test samples and AGS cells (prepared as described previously under Section 2.7.1) in 200 µl complete medium. The mixture was incubated for 90 min at 37 °C in a humidified atmosphere of 8% CO_2. After 90 min, unattached bacterial cells washed; 100 µl of urease detection medium added and incubated at room temperature for 15 min. Absorbance was read at 550 nm after incubation (Wadstrom et al., 1997).

2.7.5. Immuno-fluorescence technique

The assay was performed similarly as described under Section 2.7.4. Instead of the urease detection medium, 100 µl of anti-*Helicobacter pylori* antibody (FITC, Abcam, Cambridge, UK) was added to each well. The plate was then incubated at room temperature for 60 min; washed thrice with PBS, 100 µl of PBS added and fluorescence intensity measured at 530 nm with excitation wavelength set at 485 nm (Hayashi et al., 1998; Eftang et al., 2012).

2.8. Statistical analysis

Raw data obtained in the study were statistically evaluated by one-way analysis of variance (ANOVA) followed by Dunnett's test for multiple comparisons (GraphPad Prism) in order to find the significance of the results.

3. Results

GutGard® exhibited anti-*Helicobacter pylori* activity in both agar dilution and microbroth dilution methods. Moreover, glabridin, a flavonoid present in GutGard® showed better activity compared to omeprazole and other substances of *Glycyrrhiza glabra*. DGL and MAG did not show activity up to 250 µg/ml concentration. MIC of the tested sample by agar dilution and microbroth dilution methods are shown in Tables 1 and 2 respectively. In both these methods, positive controls gave expected MICs validating the MIC results of the tested samples.

Table 1
MIC by agar dilution method against ATCC 700392 *Helicobacter pylori* strain.

Test samples	MIC[a] (µg/ml)
GutGard®	100
Glabridin	12.5
DGL	>250
MAG	>250
Positive controls	
Amoxycillin	0.1
Clarithromycin	0.12
Omeprazole	25–50

[a] MIC—the lowest concentration at which the test sample did not show any visible growth of *Helicobacter pylori.*

Table 2
MIC by microbroth dilution method.

Test Samples	MIC[a] (µg/ml) against different *Helicobacter pylori* strains			
	ATCC 700392	NCTC 11916	AB 976	AB 977
GutGard®	32	64	64	64
Positive controls				
Amoxycillin	0.03	0.03	0.25	0.25
Tetracycline HCl	0.25	0.125	0.125	0.125

[a] MIC—the lowest concentration at which the test sample did not show any visible growth of *Helicobacter pylori.*

In protein synthesis assay, GutGard® showed time-dependent inhibition as witnessed by a reduction in ^{35}S methionine incorporation into *Helicobacter pylori* ATCC 700392 strain. GutGard® inhibited *Helicobacter pylori* (6×10^7 CFU/ml) protein synthesis by 45.4% and 43.4% at 4 and 8 µg/ml (sub MIC) concentrations respectively at 5-hour post treatment. A 50% and 46.9% inhibition was observed at 24-hour post treatment by GutGard® at 4 and 8 µg/ml concentrations respectively. Tetracycline hydrochloride (0.03 µg/ml), which was used as a positive control displayed 54.6% and 71% inhibition at 5 and 24-hour post treatment respectively. A decline in the inhibition of protein synthesis at 48-hour post treatment for GutGard® and tetracycline hydrochloride (Fig. 1) was observed. No inhibition in bacterial cell growth was observed at all tested concentrations and time points (data not shown).

GutGard® strongly inhibited DNA gyrase with IC_{50} value of 4.40 µg/ml while the positive control (ciprofloxacin) showed an IC_{50} value of 12.31 ng/ml. In DHFR assay, GutGard® was found to have an IC_{50} value of 3.33 µg/ml while the positive control (methotrexate) showed an IC_{50} value of 1.71 ng/ml.

GutGard® was found to be cytotoxic to AGS cells at 25 µg/ml concentration. Hence, in the anti-adhesion assay, GutGard® was tested at concentrations ranging from 1.56 to 12.5 µg/ml. GutGard® was not found to inhibit the adhesion of *Helicobacter pylori* to AGS cells, which was confirmed by three different techniques viz., fluorescence, urease detection and immunofluorescence. However, the reference standard rebamipide significantly inhibited the adhesion of *Helicobacter pylori* to AGS cells at 100 µg/ml. The three detection techniques were checked for assay performance measures like Z' factor and signal to noise ratio, and the assays were found to qualify the criteria for a good assay (Data not shown).

4. Discussion

Susceptibility testing by agar dilution method and cultivation under microaerophilic condition is most often recommended for *Helicobacter pylori* (McDermott et al., 2001; Luber et al., 2003). Initially, MIC of different test samples viz., GutGard®, glabridin, DGL and MAG against *Helicobacter pylori* strain ATCC 700392 was evaluated by agar dilution method. Glabridin, the major flavonoid present in GutGard® exhibited good anti-*Helicobacter pylori* activity, which is in agreement with the earlier report (Fukai et al., 2002). Both DGL and glycyrrhizin were not active against *Helicobacter pylori* up to 250 µg/ml. This is in agreement with the

past report on licorice extract and glycyrrhizin by Krausse et al. (2004). The fact that both GutGard® and glabridin were active against *Helicobacter pylori* while DGL and MAG were inactive gave an indication that flavonoids could be the active constituents.

GutGard® was additionally tested for its anti-*Helicobacter pylori* action by microbroth dilution method using four *Helicobacter pylori* strains, including the ATCC 700392. Results obtained in this method were utilized for selecting sample concentration used in the protein synthesis assay, which was also a microtitre based method. Timely determination of the susceptibility of the pathogen isolated from the gastric mucosa of patients during or after treatment is critical in ensuring successful therapy. While agar dilution method, is inconvenient to perform in most clinical microbiology laboratories, and more time consuming; broth microdilution method is easier to handle, less time-consuming and can be routinely used (Kobayashi et al., 2004). Varied MIC values for GutGard® (100 and 32 μg/ml in agar dilution and microbroth dilution methods respectively) against the *Helicobacter pylori* strain ATCC 700392 is understandable from the nature of different methods. Similar differences in MIC between various methods have been reported previously (Waites et al., 1991; Reynolds et al., 2003).

In this study, anti-*Helicobacter pylori* activity of GutGard® was performed at the pH 7.2 ± 0.1. According to Cheng et al. (in press) reduction in pH from neutral to acidic leads to decrease in antimicrobial activity of two antibiotics out of eight antibiotics tested. It is worthy to mention that GutGard® was found to be clinically effective against *Helicobacter pylori* in a recently conducted randomized double blind clinical trial (communicated) indicating its efficacy even at stomach pH.

In order to understand the mechanisms of anti-*Helicobacter pylori* activity, protein synthesis inhibition, DNA gyrase inhibition, dihydrofolate reductase inhibition and *Helicobacter pylori* anti-adhesion assays were carried out. Protein synthesis inhibitors viz., clarithromycin and tetracycline, have been successfully used in the eradication of *Helicobacter pylori* in human (Berardi, 2002). In the present study GutGard® was found to inhibit *Helicobacter pylori* protein synthesis *in vitro* and this mechanism is being reported for the first time for a standardized licorice extract. Fluroroquinolones viz., ciprofloxacin and levofloxacin, are being considered for *Helicobacter pylori* eradication in infected patients. These drugs are inhibitors of DNA gyrase, the enzyme needed to overcome topological problems encountered during DNA replication, transcription, recombination and maintenance of the genomic stability of *Helicobacter pylori* (Rimbara et al., 2011). The present study shows that GutGard® might inhibit DNA gyrase in *Helicobacter pylori*. DHFR is a ubiquitous enzyme present in all eukaryotic and prokaryotic cells, playing a key role in thymidine synthesis. It catalyzes the reduction of 7,8-dihydrofolate to 5,6,7,8-tetrahydrofolate, utilizing NADPH as a cofactor. This reaction is an essential step in the biosynthesis of nucleotidic bases of DNA (Gready, 1980; Blakley, 1995 and Costi and Ferrari, 2001). Blockage of DHFR enzyme causes cell death as a result of the inhibition of DNA synthesis. Reduced folate derivatives participate in numerous reactions of bacterial intermediary metabolism (Myllykallio et al., 2003) and hence play an essential role in the survival of bacteria like *Helicobacter pylori* in the human body. Inhibition of DHFR has been shown to be a suitable target for the inhibition of *Helicobacter pylori* growth and survival (Mendz et al., 1997). GutGard® was found to inhibit DHFR moderately when compared to the positive control methotrexate, a well-known dihydrofolate reductase inhibitor. GutGard® did not inhibit the adhesion of *Helicobacter pylori* to AGS cells when tested up to a concentration of 12.5 μg/ml. Higher concentrations were cytotoxic to AGS cells and therefore could not be tested. It may however be noted that aqueous extract and polysaccharides of licorice have been reported to inhibit the adhesion of *Helicobacter pylori* to human gastric mucosa, when tested at 50–300 times higher concentrations than adopted in this study (Wittschier et al., 2009).

It is interesting to note that apart from anti-*Helicobacter pylori* activity, GutGard® has also been reported to possess anti-inflammatory (Chandrasekaran et al., 2011a) and antioxidant (Mukherjee et al., 2010) properties. Flavonoids in general are known to possess diverse pharmacological actions including anti-inflammatory and antioxidant activities (Xiao et al., 2011). It is therefore possible to speculate that multiple actions of GutGard® would have contributed to the beneficial effects observed in the clinical study (Raveendra et al., 2012).

5. Conclusions

Results of the present study reveal that GutGard®, a flavonoid rich extract of *Glycyrrhiza glabra* exerts anti-*Helicobacter pylori* activity possibly by inhibiting protein synthesis, DNA gyrase and dihydrofolate reductase. These mechanisms might have played a role in the observed beneficial effects of GutGard® against functional dyspepsia in humans (Raveendra et al., 2012). The present study lends a scientific support for the traditional use of *Glycyrrhiza glabra* in peptic ulcer disease. Future studies may be aimed at understanding other possible mechanisms involved in anti-*Helicobacter pylori* action of *Glycyrrhiza glabra*.

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References

Aly, A.M., Al-Alousi, L., Salem, H.A., 2005. Licorice: a possible anti-inflammatory and anti-ulcer drug. AAPS PharmSciTech 6, E74–82.

Bardhan, P.K., 1997. Epidemiological features of *Helicobacter pylori* infection in developing countries. Clinical Infectious Diseases 25, 973–978.

Berardi, R.R., 2002. Peptic ulcer disease. In: Dipiro, J.T., Talbert, R.L., Yee, G.C., Matzke, G.R., Wells, B.G., Posey, L.M. (Eds.), Pharmacotherapy: A Pathophysiology Approach, fifth ed. McGraw-Hill, New York, pp. 612.

Blakley, R.L., 1995. Eukaryotic dihydrofolate reductase. In: Nord, F.F., Meister, A. (Eds.), Advances in Enzymology and Related Areas of Molecular Biology, vol. 70. John Wiley & Sons Inc., Hoboken, NJ, pp. 23–102.

Chandrasekaran, C.V., Deepak, H.B., Thiyagarajan, P., Kathiresan, S., Sangli, G.K., Deepak, M., Agarwal, A., 2011a. Dual inhibitory effect of *Glycyrrhiza glabra* (GutGard™) on COX and LOX products. Phytomedicine 18, 278–284.

Chandrasekaran, C.V., Sundarajan, K., Gupta, A., Srikanth, H.S., Edwin, J., Agarwal, A., 2011b. Evaluation of the genotoxic potential of standardized extract of *Glycyrrhiza glabra* (GutGard™). Regulatory Toxicology and Pharmacology 61, 373–380.

Cheng, A., Sheng, W.H., Liou, J.M., Wang, H.P., Wu, M.S., Lin, J.T., Chang, S.C. Comparative *in vitro* antimicrobial susceptibility and synergistic activity of antimicrobial combinations against *Helicobacter pylori* isolates in Taiwan. Journal of Microbiology, Immunology and Infection, http://dx.doi.org/10.1016/j.jmii.2012.08.021, in press.

Conn, J.W., Rovner, D.R., Cohen, E.L., 1968. Licorice-induced pseudoaldosteronism. Hypertension, hypokalemia, aldosteronopenia, and suppressed plasma renin activity. Journal of the American Medical Association 205, 492–496.

Costi, M.P., Ferrari, S., 2001. Update on antifolate drugs targets. Current Drug Targets 2, 135–166.

Davis, E.A., Morris, D.J., 1991. Medicinal uses of licorice through the millennia: the good and plenty of it. Molecular and Cellular Endocrinology 78, 1–6.

De, B., Maiti, R.N., Joshi, V.K., Agrawal, V.K., Goel, R.K., 1997. Effect of some Sitavirya drugs on gastric secretion and ulceration. Indian Journal of Experimental Biology 35, 1084–1087.

Dunn, B.E., Cohen, H., Blaser, M.J., 1997. Helicobacter pylori. Clinical Microbiology Reviews 10, 720–741.

Eftang, L.L., Esbensen, Y., Tannæs, T.M., Bukholm, I.R., Bukholm, G., 2012. Interleukin-8 is the single most up-regulated gene in whole genome profiling of *Helicobacter pylori* exposed gastric epithelial cells. BMC Microbiology 12, 9.

Egan, B.J., Katicic, M., O'Connor, H.J., O'Morain, C.A., 2007. Treatment of *Helicobacter pylori*. Helicobacter 12, 31–37.

Farthing, M.J., 1998. *Helicobacter pylori* infection: an overview. British Medical Bulletin 54, 1–6.

Fukai, T., Marumo, A., Kaitou, K., Kanda, T., Terada, S., Nomura, T., 2002. Anti-*Helicobacter pylori* flavonoids from licorice extract. Life Sciences 71, 1449–1463.

Gready, J.E., 1980. Dihydrofolate reductase: binding of substrates and inhibitors and catalytic mechanism. Advances in Pharmacology and Chemotherapy 17, 37–102.

Hayashi, S., Sugiyama, T., Amano, K., Isogai, H., Isogai, E., Aihara, M., Kikuchi, M., Asaka, M., Yokota, K., Oguma, K., Fujii, N., Hirai, Y., 1998. Effect of rebamipide, a novel antiulcer agent, on *Helicobacter pylori* adhesion to gastric epithelial cells. Antimicrobial Agents and Chemotherapy 42, 1895–1899.

Khayyal, M.T., el-Ghazaly, M.A., Kenawy, S.A., Seif-el-Nasr, M., Mahran, L.G., Kafafi, Y.A., Okpanyi, S.N., 2001. Antiulcerogenic effect of some gastrointestinally acting plant extracts and their combination. Arzneimittel-Forschung 51, 545–553.

Kim, D.H., Hong, S.W., Kim, B.T., Bae, E.A., Park, H.Y., Han, M.J., 2000. Biotransformation of glycyrrhizin by human intestinal bacteria and its relation to biological activities. Archives of Pharmacal Research 23, 172–177.

Kobayashi, I., Muraoka, H., Saika, T., Nishida, M., Fujioka, T., Nasu, M., 2004. Microbroth dilution method with air-dried microplate for determining MICs of clarithromycin and amoxycillin for *Helicobacter pylori* isolates. Journal of Medical Microbiology 53, 403–406.

Krausse, R., Bielenberg, J., Blaschek, W., Ullmann, U., 2004. *In vitro* anti-*Helicobacter pylori* activity of Extractum liquiritiae, glycyrrhizin and its metabolites. Journal of Antimicrobial Chemotherapy 54, 243–246.

Luber, P., Bartelt, E., Genschow, E., Wagner, J., Hahn, H., 2003. Comparison of broth microdilution, E test, and agar dilution methods for antibiotic susceptibility testing of *Campylobacter jejuni* and *Campylobacter coli*. Journal of Clinical Microbiology 41, 1062–1068.

McDermott, P.F., Bodeis, S.M., Walker, R.D., 2001. Abstracts. 11th international workshop on *Campylobacter*, *Helicobacter* and related organisms. International Journal of Medical Microbiology 291, 109–110.

McKenna, D.J., Jones, K., Hughes, K., 2008. Botanical Medicines: The Desk Reference for Major Herbal Supplements, second ed. Viva Books, New Delhi.

Mendz, G.L., Shepley, A.J., Hazell, S.L., Smith, M.A., 1997. Purine metabolism and the microaerophily of *Helicobacter pylori*. Archives of Microbiology 168, 448–456.

Mitscher, L.A., Leu, R.P., Bathala, M.S., Wu, W.N., Beal, J.L., 1972. Antimicrobial agents from higher plants-I: introduction, rationale, and methodology. Lloydia 35, 157–166.

Monder, C., Stewart, P.M., Lakshmi, V., Valentino, R., Burt, D., Edwards, C.R., 1989. Licorice inhibits corticosteroid 11 beta-dehydrogenase of rat kidney and liver: *in vivo* and *in vitro* studies. Endocrinology 125, 1046–1053.

Morris, D.J., Davis, E., Latif, S.A., 1990. Licorice, tobacco chewing, and hypertension. New England Journal of Medicine 322, 849–850.

Mosmann, T., 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. Journal of Immunological Methods 65, 55–63.

Mukherjee, M., Bhaskaran, N., Srinath, R., Shivaprasad, H.N., Joshua Allan, J., Shekhar, D., Agarwal, Amit, 2010. Anti-ulcer and antioxidant activity of GutGard^{TM}. Indian Journal of Experimental Biology 48, 269–274.

Myllykallio, H., Leduc, D., Filee, J., Liebl, U., 2003. Life without dihydrofolate reductase FolA. Trends in Microbiology 11, 220–223.

Patrzykat, A., Friedrich, C.L., Zhang, L., Mendoza, V., Hancock, R.E., 2002. Sublethal concentrations of pleurocidin-derived antimicrobial peptides inhibit macromolecular synthesis in *Escherichia coli*. Antimicrobial Agents and Chemotherapy 46, 605–614.

Raveendra, K.R., Srinivasa, V., Sushma, K.R., Joshua Allan, J., Shankargouda Goudar, K., Shivaprasad, H.N., Venkateshwarlu, K., Geetharani, P., Sushma, G., Agarwal, Amit, 2012. An extract of *Glycyrrhiza glabra* (GutGard) alleviates symptoms of functional dyspepsia: a randomized, double-blind , placebo-controlled study. Evidence-Based Complementary and Alternative Medicine 2012, 216970. http://dx.doi.org/10.1155/2012/216970, Article ID: 216970.

Reynolds, R., Shackcloth, J., Felmingham, D., MacGowan, A., 2003. Comparison of BSAC agar dilution and NCCLS broth microdilution MIC methods for *in vitro* susceptibility testing of *Streptococcus pneumoniae*, *Haemophilus influenzae* and *Moraxella catarrhalis*: the BSAC respiratory resistance surveillance programme. Journal of Antimicrobial Chemotherapy 52, 925–930.

Rimbara, E., Noguchi, N., Kawai, T., Sasatsu, M., 2011. Fluoroquinolone resistance in *Helicobacter pylori*: role of mutations at position 87 and 91 of GyrA on the level of resistance and identification of a resistance conferring mutation in GyrB. Helicobacter 17, 36–42.

Souness, G.W., Morris, D.J., 1989. The antinatriuretic and kaliuretic effects of the glucocorticoids corticosterone and cortisol following pretreatment with carbenoxolone sodium (a liquorice derivative) in the adrenalectomized rat. Endocrinology 124, 1588–1590.

Tiwari, S.K., Khan, A.A., Manoj, G., Ahmed, S., Abid, Z., Habeeb, A., Habibullah, C.M., 2007. A simple multiplex PCR assay for diagnosing virulent *Helicobacter pylori* infection in human gastric biopsy specimens from subjects with gastric carcinoma and other gastro-duodenal diseases. Journal of Applied Microbiology 103, 2353–2360.

Tiwari, S.K., Manoj, G., Kumar, G.V., Sivaram, G., Hassan, S.I., Prabhakar, B., Devi, U., Jalaluddin, S., Kumar, K., Ahmed, S., Abid, Z., Habeeb, M.A., Khan, A.A., Habibullah, C.M., 2008. Prognostic significance of genotyping *Helicobacter pylori* infection in patients in younger age groups with gastric cancer. Postgraduate Medical Journal 84, 193–197.

Wadstrom, T., Hirmo, S., Novak, H., Guzman, A., Ringnér-Pantzar, M., Utt, M., Aleljung, P., 1997. Sulfatides inhibit binding of *Helicobacter pylori* to the gastric cancer Kato III cell line. Current Microbiology 34, 267–272.

Waites, K.B., Figarola, T.A., Schmid, T., Crabb, D.M., Duffy, L.B., Simecka, J.W., 1991. Comparison of agar versus broth dilution techniques for determining antibiotic susceptibilities of *Ureaplasma urealyticum*. Diagnostic Microbiology and Infectious Diseases 14, 265–271.

Wittschier, N., Faller, G., Hensel, A., 2009. Aqueous extracts and polysaccharides from liquorice roots (*Glycyrrhiza glabra* L.) inhibit adhesion of *Helicobacter pylori* to human gastric mucosa. Journal of Ethnopharmacology 125, 218–223.

Xiao, Z.P., Peng, Z.Y., Peng, M.J., Yan, W.B., Ouyang, Y.Z., Zhu, H.L., 2011. Flavonoids health benefits and their molecular mechanism. Mini Reviews in Medicinal Chemistry 11, 169–177.