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# Anti-ulcer and antioxidant activity of GutGard™

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The present study was undertaken to determine the anti-ulcer and antioxidant potential of GutGard™, a standardized extract of *Glycyrrhiza glabra* commonly known as licorice. Effect of various doses (12.5, 25, and 50 mg/kg, po) of GutGard™ was studied on gastric ulcers in pylorus ligation-, cold-restraint stress- and indomethacin induced gastric mucosal injury in rats. Anti-ulcer activity was evaluated by measuring the ulcer index, gastric content, total acidity, and *p*H of gastric fluid. GutGard™ dose dependently decreased gastric content, total acidity, ulcer index and increased *p*H of gastric fluid in pylorus ligation ulcer model. In cold-restraint stress- and indomethacin induced ulcer models all the doses of GutGard™ decreased the ulcer index and increased the *p*H of gastric fluid. The antioxidant activity was evaluated by the oxygen radical absorbance capacity (ORAC) assay. GutGard™ exhibited potent antioxidant activity with high hydrophilic and lipophilic ORAC value. GutGard™ possessed anti-ulcerogenic properties that might be afforded via cytoprotective mechanism by virtue of its antioxidant properties. These results supported the ethnomedical uses of licorice in the treatment of gastric ulcer.

**Keywords:** Anti-ulcer, Cold-restraint, *Glycyrrhiza glabra,* GutGard™, Indomethacin, ORAC assay, Pylorus ligation

Gastric hyperacidity and ulceration of the stomach mucosa due to various factors are serious health problems of global concern. Peptic ulcer disease (encompassing gastric ulcer and duodenal ulcer) affect a large portion of the world population and are induced by several factors, including stress, smoking, nutritional deficiencies, and ingestion of non-steroidal anti-inflammatory drugs[1]. The pathophysiology of these ulcers involves an imbalance between offensive (acid, pepsin, and *Helicobacter pylori*) and defensive factors (mucin, prostaglandin, bicarbonate, nitric oxide and growth factors). There is evidence concerning the participation of reactive oxygen species in the etiology and pathophysiology of human diseases, such as neurodegenerative disorders, inflammation, viral infections, autoimmune pathologies and digestive system disorders such as gastrointestinal inflammation and gastric ulcer[2]. Drugs with multiple mechanisms of protective action, including antioxidant properties, may be one way forward in minimizing tissue injury in human disease[3]. Oxygen radical absorbance capacity assay (ORAC), is one of the most popular and best standardized chemical *in vitro* antioxidant assay[4-7]. It is widely used for evaluation and comparison of the antioxidant capacity in natural products[8].

Today, there are two main approaches for treating peptic ulcer. The first deals with reducing the production of gastric acid and the second with re-enforcing gastric mucosal protection[9,10]. Although a number of anti-ulcer drugs such as $H_2$ receptor antagonists, proton pump inhibitors and cytoprotectants are available, all these drugs have side effects and limitations[11]. There is, thus, a need to search for natural alternatives having anti-ulcer properties. This has been the basis for the development of new anti-ulcer agents, which include herbal substances.

In traditional medicine, several plants and herbs have been used to treat gastrointestinal disorders, including gastric ulcers[12]. The first drug effective against gastric ulcer was carbenoxolone, discovered as a result of research on a commonly used indigenous plant, *Glycyrrhiza glabra*[13]. Studies on cabbage previously employed as an anti-ulcer agent in folk medicine, has led to the development of Gefarnate[14]. Banana fruit has also been found to inhibit peptic ulceration[15].

Licorice, the roots and rhizomes of *Glycyrrhiza glabra* Linn. (family: Leguminosae) is one of the

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most extensively researched medicinal plants and has a history of consumption for the past 6000 years. The roots have expectorant, diuretic, laxative, sedative[16], antipyretic[17], antimicrobial and anxiolytic activities[18]. The main constituent of licorice is glycyrrhizin which has antiviral[19], anti-inflammatory[20] and antioxidant activities[21]. Licorice has historically been regarded as an excellent medicine for peptic ulcer. It has been reported clinically and pre-clinically for anti-ulcer activity[22-24]. However, due to side effects of the licorice compound glycyrrhetinic acid (causes hypertension, water retention and hypokalemia), a procedure was developed to remove this compound from licorice and form deglycyrrhizinated licorice (DGL). The anti-ulcer activity of DGL was demonstrated using a rat model of aspirin-induced gastric mucosal damage[25]. Many clinical trials have confirmed the use of DGL in the treatment of gastric ulcer[26-29]. Glabridin and glabrene, flavonoids present in licorice roots have shown to possess anti-*Helicobacter pylori* activity *in vitro*[30]. Licorice extract appears to be a promising agent for the treatment of all forms of *H. pylori* infection[31].

The present study was aimed to investigate the anti-ulcer potential of GutGard™ using three experimental gastric ulcer methods (*viz* pylorus ligation, cold-restraint stress and indomethacin induced gastric lesions) and its antioxidant activity by ORAC assay.

## Materials and Methods

*Test substance*—GutGard™ is a flavonoid rich, standardized extract of *Glycyrrhiza glabra* developed by Natural Remedies Pvt. Ltd, Bangalore. GutGard™ was standardized to the content of the following bioactive constituents' *viz.,* glabridin (≥3.5% w/w), glabrol (≥0.5% w/w), eicosanyl caffeate (≥0.1% w/w), docosyl caffeate (≥0.1% w/w) and total flavonoids (≥10.5% w/w). It was further standardized using the following *in vitro* bioassays *viz.,* 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging assay ($IC_{50}$<100 µg/ml) and 2,2'-azino-di-[3-ethylbenzthiazoline sulphonic acid] (ABTS) radical scavenging assay ($IC_{50}$<75 µg/ml).

*Chemicals used*—AAPH [2,2'-azobis (2-methyl-propionamidine) dihydrochloride] was purchased from Aldrich (Milwaukee, WI). Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) was obtained from Fluka A.G (Buchs SG, Switzerland). Indomethacin and fluorescein sodium salt were purchased from Sigma-Aldrich (St Louis, MO). Sodium phosphate monobasic anhydrous and di-sodium hydrogen phosphate dehydrate were obtained from Himedia (Bombay, India). Methylated β –cyclodextrin was purchased from Cyclolab R&D Ltd. (Budapest, Hungary), and Omeprazole from Zydus Cadila (Mumbai, India) were used for the study.

### *In vitro* antioxidant assay (oxygen radical absorbance capacity; ORAC)

*ORAC-hydrophilic assay*—The ORAC-hydrophilic assay was performed as per Davlos *et al.*[32] with slight modifications. The reaction mixture contained 75 m*M* of sodium phosphate buffer (*p*H 7.4), 67 n*M* of sodium fluorescein and various concentrations of Trolox or test solution (GutGard™–concentration ranging from 0.1-10 µg/ml). The mixture was pre-incubated for 10 min at 37°C. AAPH [2,2'-azobis (2-methylpropionamidine) dihydrochloride] solution (12 µ*M*; final concentration) was added to all the wells except the negative control well and mixed for 15 sec at 37°C. The fluorescence was measured at 485 nm excitation and 520 nm emission wavelengths using a fluorescence plate reader (FLUOstar BMG, Labtech, Germany). The fluorescence was recorded every minute for 90 min. The microplate was automatically shaken prior to each reading. A blank using phosphate buffer instead of the antioxidant solution was carried out.

*ORAC-lipophilic assay*—The lipophilic ORAC assay was performed as per Huang *et al*[33]. An aliquot of GutGard™ sample solution was appropriately diluted with 7% randomly methylated β-cyclodextrin (RMCD) solvent (w/v) made in a 50% acetone-water mixture (v/v) and shaken for 1 h at room temperature on an orbital shaker at 265 rpm. The final assay mixture contained 75 n*M* of sodium fluorescein, 30 m*M* of AAPH, and different concentrations of standard/sample/negative control in a total volume of 200 µl. RMCD (7%) solution was used as the blank, Trolox was used as the control standard and GutGard™ (0.1-8 µg/ml) was used as test sample. The fluorescence measurements were made every minute for 90 min immediately after addition of AAPH using a fluorescence plate reader (FLUOstar BMG, Labtech, Germany) at 485 nm excitation and 520 nm emission wavelength.

Raw data from both hydrophilic and lipophilic assays were exported from the FLUOstar optima software to an Excel sheet for further calculations. The standard curve was obtained by plotting Trolox

(standard) concentrations against the average net AUC of the two measurements for each concentration. ORAC values were expressed as Trolox equivalents per gram of the test sample.

*Animals*—Experiments were carried out on 48 h starved Wistar rats of both sex, weighing 180–200 g obtained from Central Animal Facility, Research and Development Centre, Natural Remedies Pvt. Ltd., Bangalore. The animals were acclimatized for a week and maintained in a 12 h light/dark cycle at 25°±2°C with free access to feed ('Amrut' M/s Pranav Agro Industries Ltd., Sangali) and UV purified water, *ad libitum*. All experiments were carried out according to the guidelines for care and use of experimental animals and approved by Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA).

*Animal treatment*—For each experiment, the animals were divided into six groups of 6 animals each. Group I served as the vehicle control (de-mineralized water; 10 ml/kg). Group II served as the negative control [pylorus ligation control - 4 h; cold stress control-kept in refrigerator for 4 h at 4°±1°C; Indomethacin (40 mg/kg body wt), respectively in three different models]. Group III served as positive control (Omeprazole; 10 mg/kg). Group IV, V and VI were treated with GutGard™ at the dose levels of 12.5, 25 and 50 mg/kg body wt, respectively. The doses were administered orally by gavage using feeding needle.

## Anti-ulcer activity

*Pylorus ligated ulcer technique*—Pylorus ligation was done by ligating the pyloric end of the stomach of rats 1 h after test substance administration[34]. Animals were allowed to recover and stabilized in individual cage and were deprived of water during post-operative period. After 4 h of surgery, rats were sacrificed and gastric juice was collected, centrifuged and the supernatant was measured. Gastric contents were analysed for total acidity by titrating against 0.01 N NaOH using phenolphthalein as indicator. The *p*H of gastric juice was measured using *p*H meter. The ulcers were examined on the dissected stomachs as described below and ulcer index was measured.

*Cold-restraint stress-induced ulcers*—One hour after test substance treatment, the experimental rats were immobilized by strapping all the four limbs on a wooden plank and kept for 4 h, at 4°±1°C[35]. After 4 h, the animals were sacrificed, gastric juice was collected, ulcers were examined on the dissected stomachs and ulcer index was measured. The *p*H of gastric juice was measured using *p*H meter.

*Indomethacin-induced ulcers*—One hour after test substance administration, each animal received orally 40 mg/kg of indomethacin. All animals were sacrificed 6 h later and gastric juice was collected. The stomach was incised, examined for lesions and ulcer index was measured. The *p*H of gastric juice was measured using *p*H meter.

*Measurement of ulcer index*—Immediately after the animals were sacrificed, their stomach was dissected out, incised along the greater curvature and the mucosa were rinsed with cold normal saline to remove blood contaminant, if any. Tissues were kept overnight in 10% formalin solution. Next day, the ulcers were examined under a magnifying lens. The ulcers were measured with the help of Vernier caliper using the following arbitrary scale [Score 0 = No ulcers; Score 1 = Petechial hemorrhages; Score 2 = Ulcers < 2 mm; Score 3 = Ulcers > 2 < 4 mm; Score 4 = Ulcers > 4 mm].

*Statistical analysis*—The data was analyzed using one way ANOVA. In case of heterogeneous data after transformation, Dunnett T3 method was used as post-hoc test. All values were reported as mean ± SEM. Statistical significance was set at $P \leq 0.05$.

## Results

*ORAC assay*—The ORAC value has been expressed as relative Trolox equivalents. The hydrophilic ORAC value based on the net area under the curve (AUC) of fluorescence decay curve for various concentrations of GutGard™ was found to be 1290 ± 0.71 µmole TE/g, whereas the lipophilic ORAC value was 5279 ± 35 µmole TE/g.

*Pylorus ligated ulcer technique*—GutGard™ was tested at the dose levels of 12.5, 25 and 50 mg/kg on pylorus ligation-induced gastric lesions. The results obtained when ulceration of the gastric mucosa was induced by pylorus ligation were shown in Table 1. In gastric secretion studies, it was found that pylorus ligation control rats having the gastric *p*H of 2.15 ± 0.06 was increased when treated with GutGard™ in a dose-dependent manner. Omeprazole, the positive control showed highest gastric *p*H (6.89 ± 0.14). The pylorus ligation control animals showed total acidity of 120.67 ± 5.56 mEq/L/100 g, whereas GutGard™ decreased the total acidity in a dose dependent manner. Total acidity was significantly decreased at

Table 1—Effect of GutGard™ in pylorus ligation induced ulcer model

[Values are expressed as mean ± SEM; n=6]

| Group | Treatment | Dose (mg/kg) | Gastric content (ml) | *p*H | Total acidity (mEq/L/100 g) | Ulcer index |
|---|---|---|---|---|---|---|
| I | Vehicle control | — | 0.00 ± 0.00 | 2.00 ± 0.00 | 0.00 ± 0.00 | 3.17 ± 0.87 |
| II | Pylorus ligation (control) | — | 6.33 ± 0.31* | 2.15 ± 0.06 | 120.67 ± 5.56* | 30.00 ± 4.33* |
| III | Omeprazole | 10 | 2.42 ± 0.24# | 6.89 ± 0.14# | 15.33 ± 0.92# | 3.33 ± 1.38# |
| IV | GutGard™ | 12.5 | 3.83 ± 0.28# | 2.45 ± 0.14 | 92.33 ± 5.40 | 6.00 ± 1.37# |
| V | GutGard™ | 25 | 3.58 ± 0.35# | 2.55 ± 0.17 | 84.83 ± 8.34 | 5.50 ± 1.54# |
| VI | GutGard™ | 50 | 3.33 ± 0.33# | 2.72 ± 0.22 | 57.50 ± 4.89# | 3.00 ± 1.37# |

*$P \leq 0.05$ pylorus ligation control Vs vehicle control; # $P \leq 0.05$ treated groups Vs pylorus ligation control.

Table 2—Effect of GutGard™ in cold-restraint stress-induced ulcer model

[Values are expressed as mean ± SEM; n=6]

| Group | Treatment | Dose (mg/kg) | *p*H | Ulcer index |
|---|---|---|---|---|
| I | Vehicle control | — | 2.00 ± 0.00 | 0.33 ± 0.21 |
| II | Cold stress (control) | — | 2.00 ± 0.00 | 29.67 ± 3.62* |
| III | Omeprazole | 10 | 6.50 ± 0.22# | 0.67 ± 0.49# |
| IV | GutGard™ | 12.5 | 3.67 ± 0.33 | 5.83 ± 1.87# |
| V | GutGard™ | 25 | 3.33 ± 0.42 | 2.83 ± 0.95# |
| VI | GutGard™ | 50 | 3.00 ± 0.45 | 0.50 ± 0.34# |

*$P \leq 0.05$ cold stress control Vs vehicle control; # $P \leq 0.05$ treated groups Vs cold stress control.

Table 3—Effect of GutGard™ in indomethacin induced ulcer model

[Values are expressed as mean ± SEM; n=6]

| Group | Treatment | Dose (mg/kg) | *p*H | Ulcer index |
|---|---|---|---|---|
| I | Vehicle control | — | 2.00 ± 0.00 | 0.83 ± 0.40 |
| II | Indomethacin (control) | 40 | 2.00 ± 0.00 | 71.50 ± 3.87* |
| III | Omeprazole | 10 | 6.50 ± 0.22# | 2.83 ± 1.45# |
| IV | GutGard™ | 12.5 | 3.00 ± 0.45 | 27.17 ± 6.86# |
| V | GutGard™ | 25 | 3.33 ± 0.42 | 15.00 ± 2.71# |
| VI | GutGard™ | 50 | 3.67 ± 0.33 | 28.67± 2.54# |

*$P \leq 0.05$ indomethacin control Vs vehicle control; # $P \leq 0.05$ Treated groups Vs indomethacin control.

50 mg/kg dose of GutGard™. The gastric content of pylorus ligation control animals were 6.33 ± 0.31 ml, whereas GutGard™ significantly decreased the gastric content in a dose dependent manner. In measurement of ulcer index, the pylorus ligation control animals showed ulcer index of 30.00 ± 4.33 whereas GutGard™ decreased the ulcer index significantly in a dose-dependent manner.

*Cold-restraint stress-induced ulcers*—In gastric secretion study, the cold stress control rats having the gastric *p*H of 2.00 ± 0.00 was increased when treated with different doses of GutGard™ (12.5, 25 and 50 mg/kg). Omeprazole, the positive control showed highest gastric *p*H (6.50 ± 0.22). In measurement of ulcer index, the cold stress control animals were showing the ulcer index of 29.67 ± 3.62, whereas GutGard™ decreased the ulcer index significantly in a dose-dependent manner (Table 2).

*Indomethacin-induced ulcers*—As shown in Table 3, animals treated with GutGard™ exhibited significant reduction in the ulcer index. It was found that indomethacin control rats having the gastric *p*H of 2.00 ± 0.00 which was increased when treated with different doses of GutGard™ (12.5, 25 and 50 mg/kg) in a dose-dependent manner. Omeprazole, the positive control showed highest gastric *p*H (6.50 ± 0.22).

## Discussion

Although in most of the cases the etiology of ulcer is unknown, it is generally accepted that ulcer results from an imbalance between aggressive factors and the maintenance of the mucosal integrity through the endogenous defense mechanism[36]. To regain the balance, different therapeutic agents including herbal preparations are used to inhibit the gastric acid secretion or to boost the mucosal defense mechanism by increasing mucus production.

Antioxidants play a major role in repairing the gastric damage.[37] The Oxygen Radical Absorbance Capacity assay (ORAC) is a classic tool for measuring the antioxidant capacity of natural products[8]. It is a simple, sensitive, and reliable way to measure the peroxyl radical absorbing capacity of antioxidants. The ORAC method is unique in its analysis, it takes into account the inhibition time and

degree of inhibition into a single quantity by measuring the area under the curve. The ORAC value is expressed as relative Trolox equivalent. In the present antioxidant study, GutGard™ exhibited potent antioxidant activity with high hydrophilic and lipophilic ORAC value.

The results of the anti-ulcer study demonstrated that the GutGard™ exerted protective effects against pylorus ligation, cold-restraint stress and indomethacin induced ulcer. Pylorus ligation and cold-restrained stress induced ulcers are results of auto digestion of the gastric mucosal barrier probably due to excess production and accumulation of hydrochloric acid in the stomach[38]. GutGard™ at the dose levels of 12.5, 25 and 50 mg/kg significantly reduced the volume of gastric juice, total acidity, ulcer index and increased the gastric juice *p*H. These effects of GutGard™ treatment on the parameters that influence the initiation and induction of ulceration may be considered as highly desirable property of anti-ulcerogenic agent. In order to further probe the effectiveness of GutGard™ in preventing gastric ulcer, it was tested against indomethacin induced ulcer.

Indomethacin is a cyclooxygenase inhibitor which suppresses gastroduodenal bicarbonate secretion, reduces endogenous prostaglandin biosynthesis and disrupts the mucosal barrier as well as mucosal blood flow in animals[39]. Roles of toxic oxygen radicals has been determined in etiopathogenesis of indomethacin-induced gastric damage[40]. Antioxidant parameters have been shown to be reduced in stomach tissue damaged by indomethacin[41]. It is also well known that prostaglandins synthesized in large quantities by gastrointestinal mucosa can prevent experimentally induced ulcers by ulcerogens. Thus, when the gastric lesions are induced by indomethacin, the cytoprotective effect of the anti-ulcer agent can be mediated through endogenous prostaglandins[42]. The results obtained in our study showed that the ulcer surface area and the mean ulcer index were significantly reduced. Therefore, it can be concluded that GutGard™ might have stimulated the secretion of prostaglandins or possess prostaglandins like-substances.

The present study revealed that the GutGard™ was a significant inhibitor of gastric mucosal lesions caused by pylorus ligation, cold-restraint stress and indomethacin in rats thereby confirming its anti-ulcerogenic activity. The cytoprotective effect could be partially due to flavonoid content of GutGard™ and its reactive oxygen species scavenging property. The antioxidative mechanism of GutGard™ against gastric mucosal lesions was supported by its *in vitro* antioxidant potency as evidenced by its high ORAC value. These results support the ethnomedical uses of licorice in the treatment of ulcer.

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