Original research article (Experimental)

Protective effect of hydro-alcoholic extract of *Ruta graveolens* Linn. leaves on indomethacin and pylorus ligation-induced gastric ulcer in rats

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1. Introduction

Peptic ulcer is a sore on the lining of the stomach or duodenum. It may also develop in the esophagus just above the stomach. A person can have both the duodenal and gastric ulcers at the same time [1]. The duodenal ulcer is many times more common than gastric ulcer and is mainly a disease of men [2]. The lifetime risk for developing a peptic ulcer is approximately 10%. It can develop more than 1 time in the lifetime of a man. It is quite common. It is developing a peptic ulcer is approximately 10%. It can develop more than 1 time in the lifetime of a man. It is

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to the severity of the ulcer that often precipitates serious complications of ulcer like perforation, stenosis or bleeding [10].

Most of the commonly used drugs such as proton pump inhibitors (rabeprazole, pantoprazole, and omeprazole), M1 blockers (telenzepine, pirenzepine) and H2 blockers (famotidine, ranitidine) decrease the secretion of acid while drugs like cromogenoxolone and sucralfate promote mucosal defenses. These drugs balance the defensive factors (cell turnover, mucosal blood flow, mucin secretion, bicarbonate secretion, and cellular mucus) and aggressive factors (bile salts, pepsin, acid, and Helicobacter pylori) [11]. However, there are incidences of danger of drug interactions and relapses during the therapy of ulcer by synthetic drugs. Further, herbal drugs mostly augment the defensive factors such as bicarbonate secretion, mucosal blood flow, cell turnover, cellular mucus, and mucin secretion [10]. Hence, the search for an ideal and new antulcer drug continues, and it has been extended to herbs in search for novel and new molecules which decrease the incidence of relapse and afford better protection.

Ruta graveolens Linn. belonging to family Rutaceae is commonly known as garden rue. It contains quinolone alkaloids, glycosides, flavonoids (rutin and querceitin) and furanocoumarins (psoralens and methoxy psoralens) [12]. Above-ground parts of the plant have the highest rutin content at the beginning of blooming that decreases after blooming [13]. Leaves of the plant are collected in early summer just prior to the beginning of blooming [14]. Phytoconstituents alcohol, aliphatic ketones, and acids were also isolated from its volatile oil [15]. Volatile oil obtained from R. graveolens is being used as flavoring agent and also being used for therapeutic purposes. In Unani system of medicine, it is reported as abortifacient, anti-vitiligo and on local application, it increases blood supply and has anti-inflammatory property, relieve joint and gut pain. It is also an ingredient of Unani formulations such as jawarish kamuni, safoof muhazzil, and majoon halteet [16]. The jawarish kamuni is carminative, digestive, stomachic and relieves stomach pain, and colitis whereas safoof muhazzil is used for weight loss [17]. According to homeopathy, fresh leaves of R. graveolens are useful in rheumatism, arthritis, neuropathic pain, and varicose vein [12]. It is used as antispasmodics, digestive and for intestinal gases in Ayurvedic system of medicine [18]. Hence, keeping in view the effects of R. graveolens on gastrointestinal tract in traditional system of medicines such as Ayurveda and Unani, an attempt has been made in this study to evaluate antulcer activity of hydro-alcoholic extract of R. graveolens (RGE).

2. Materials and methods

2.1. Collection and authentication of crude drug

The leaves of R. graveolens Linn. were obtained from Sami Labs Limited, Bengaluru and authenticated by Dr. Shekhar Chaturvedi, botanist and manager at Sami Labs Limited. The voucher specimen was deposited for future reference (Ref. no.: slab/cif/0359/02).

2.2. Special reagents or instruments

5,5-dithiobis-2-nitro benzoic acid (DTNB, Sigma-Aldrich), albumin (Sd fine), Alcian blue (Sd fine), ethylene diamine tetra acetate (EDTA, Sd fine), indomethacin (IND, Jagnonpal), omeprazole (Sd fine), phosphate buffer pH-6 (prepared as per IP, 2010), thiobarbituric acid (TBA, Spectrochem), Topfer’s reagent (Sd fine), tris buffer (Sd fine), double beam UV spectrophotometer (UV-1700, Shimadzu), centrifuge (Almicro).

2.3. Evaluation of hydro-alcoholic extractive value of R. graveolens

Air-dried leaves of R. graveolens Linn. were powdered. About 100 g of dry coarse powder was taken in a closed flask and defatted with petroleum ether. The marc was dried under shade and extracted with hydro-alcoholic azetric mixture (ethanol:water — 70:30) by Soxhlet extractor. The extract was filtered and concentrated to a semisolid mass in a rotavapor. Finally obtained hydro-alcoholic RGE Linn. leaves were weighed and extractive value was calculated. RGE was stored in a cool place for its use in research [19].

2.4. Preliminary phytochemical screening of the extract

Phytochemical screening of RGE in favor of carbohydrates (Benedict’s test), protein (Biuret test), alkaloid (Maeyer’s test), steroid (Lieberman–Burchard’s test), saponins (Foam test), phenolics (ferric chloride test) and flavonoids (Shinoda test) was carried out according to standard methods [20].

2.5. Experimental animal

Wistar rats of either sex weighing between 200 and 250 g were procured from the animal house facility, Faculty of Pharmacy, Integral University. Lucknow and kept in polypropylene cages as six rats in each cage under standard laboratory environment of 12/12 h light and dark cycle with free access to standard pellet diet with drinking water ad libitum. They were randomized into experimental and control groups. The animal house was maintained at 22 ± 2 °C temperature and 50 ± 15% relative humidity. Ethical clearance was obtained from Institutional Animal Ethics Committe, Faculty of Pharmacy, Integral University (IU/Pharm/M.Pham/ CPCSEA/12/07).

2.6. Acute toxicity study

The procedure was followed as per the Organization for Economic Cooperation and Development-423 guidelines (acute toxic class method). Wistar rats of either sex selected by random sampling were used [21]. They were deprived of food (but not water) for overnight, after which the extract was administered orally at 5 mg/kg body weight (bw) and changes in the behavior of rats were observed for 24 h after RGE administration. For any signs of toxicity and mortality, rats were observed for 14 days. If, mortality was observed in two out of three rats, then the dose administered was assigned as toxic dose. If, mortality was observed in one rat then the same dose was repeated again to confirm the toxic dose. If, mortality was not observed, the procedure was repeated for higher doses (200, 500 and 2000 mg/kg bw).

2.7. Experimental protocol

The antiulcerogenic activity of hydro-alcoholic RGE leaves was evaluated using six groups of Wistar rats with each group consisting of six rats [22]. Rats were deprived of food (but not water) for 24 h prior to being subjected to ulceroens. The first group (negative control) received 1 ml/kg/day p.o. of 1% carboxymethyl-cellulose calcium (CMC), second group (positive control) 1 ml/kg/ day p.o. of 1% CMC and third group 20 mg/kg/day p.o. of IND. Fourth and fifth groups received 200 and 400 mg/kg/day p.o. of RGE, respectively while the sixth group 10 mg/kg/day p.o. of standard omeprazole. After 30 min, last three groups received 20 mg/kg/day p.o. of IND also. All these treatments after food deprivation were repeated each day for 5 consecutive days. Pylorus ligation was performed on the 6th day in last five groups under ether anesthesia.
and then, drinking water was withheld. Gastric juice was allowed to accumulate for a period of next 4 h. The rats were sacrificed and stomach was removed after clamping the esophagus. Gastric juice and gastric tissues were collected and then assessed for the ulcer index (UI), gastric wall mucus, biochemical estimations (estimation of TBA-reactive substances and estimation of tissue GSH), free acidity and total acidity, gastric pH, and gastric pepsin activity.

2.8. Estimation of ulcer index

The stomach was opened along the greater curvature and pinned on a cork plate [22]. The mucosa was examined with a magnifying glass of $\times 10$. The number of ulcers was noted and recorded for the severity (0 for no ulcer, 1 for superficial ulcer, 2 for deep ulcer, and 3 for perforation). The UI and percent protection were calculated by the equations ($UI = U_{ct} - U_s + U_s/10$) and (% protection $= [U_{ct} - U_{ct}/U_{ct}] \times 100$); where $U_{ct}, U_s$, $U_n$, and $U_t$ are average of number of ulcers per animal, average of severity score, percentage of animals with ulcer, UI of control group and UI of treated group respectively.

2.9. Estimation of gastric wall mucus

The glandular segment from stomach that had been opened along the greater curvature was removed and weighed [23]. It was immediately immersed to 10 ml of 0.1% w/v Alcian blue dye solution (prepared in 0.16 M sucrose solution buffered with 0.05 M sodium acetate and adjusted to pH 5.8 with hydrochloric acid [HCl]) for 2 h. The excess dye was removed by two successive rinses for 15 min and then for 45 min with 10 ml of 0.25 M sucrose. Dye complexed with gastric wall mucus was extracted with 10 ml of 0.5 M magnesium chloride (MgCl2) by shaking intermittently for 1 min after every 30 min intervals for 2 h. The resulting blue solution was shaken vigorously with an equal volume of diethyl ether and the resulting emulsion was centrifuged at 3000 rpm for 10 min. The absorbance of the aqueous layer was read at 580 nm against blank MgCl2 solution. The quantity of gastric wall mucus was calculated by standard curves of Alcian blue, and the result was articulated in μg of Alcian blue per gram of glandular tissue.

2.10. Estimation of thiobarbituric acid reactive substances

A volume of 0.5 ml of 30% aqueous trichloroacetic acid (TCA) was added to 1 ml of suspension medium taken from the 10% tissue homogenate, followed by 0.5 ml of 0.8% aqueous TBA reagent in a test tube [24]. The tube was then covered with aluminum foil and kept shaking on water bath at 80 °C for 30 min. The tube was taken out, kept in ice-cold water for 30 min and centrifuged at 3000 rpm for 15 min. The absorbance of the supernatant was read at 540 nm against reagent blank.

2.11. Estimation of tissue glutathione

Five hundred milligrams of gastric tissue were homogenized in 10 ml of 0.02 M aqueous EDTA and then 4.0 ml of cold distilled water was added to it [25]. One milliliter of 50% aqueous TCA was added after proper mixing and shaken intermittently for 10 min using a vortex mixer. The mixture was centrifuged at 6000 rpm for 15 min and then, 2 ml of the obtained supernatant was mixed with 4.0 ml of 0.4 M Tris buffer of pH 8.9, 0.1 ml of 0.01 M methanolic DTNB was added to it after proper mixing. The absorbance of resulting yellow colored solution was read at 412 nm against reagent blank within 5 min. The tissue GSH (mmol/mg of protein) was calculated from the equation ($GSH = [\text{absorbance} \times 50 \times 3.5 \times 2.25 \times 1]/[0.337 \times 2 \times \text{mg of protein}]$).

2.12. Estimation of free acidity and total acidity

One milliliter of gastric juice was taken into a conical flask and diluted to 10 ml with distilled water [26]. Two to three drops of Topfer’s reagent was added and finally titrated with 0.01 N sodium hydroxide until all traces of red color disappeared, and the solution turned to yellowish orange. The volume of the alkali corresponding to free acidity was noted. Then, 2 to 3 drops of the indicator phenolphthalein solution was added, and titration was continued until a definite red tinge reappeared. Again, the total volume of alkali corresponding to total acidity was noted. Acidity (meq/l/100 g) was calculated by the equation ($\text{Acidity} = \frac{\text{Volume}_{\text{NaOH}} \times \text{Normality}_{\text{NaOH}}}{100}$).

2.13. Estimation of gastric pH

The gastric content earlier transferred into centrifuge tube was used for the estimation of gastric pH. The supernatant was collected and estimated for the pH using a digital pH meter [27].

2.14. Estimation of gastric pepsin activity

A volume of 0.2 ml of centrifuged gastric juice plus 3 ml of 3% albumin for each rat test and blank were used [28]. Then, 10 ml of 6% TCA was added to blank to stop enzyme activity. Both blank and test were incubated in water bath at temperature 37 °C for 30 min. Then, 10 ml of TCA was added to test tubes, shaken well and filtered. Proteolytic activity was estimated spectrophotometrically by reading absorbance at 280 nm. Gastric pepsin content was estimated by extrapolation of constructed standard plot.

2.15. Histopathological examination

Small pieces of stomach from each group were cut, washed with ice-cold saline, fixed in 10% buffered neutral formalin solution and embedded in paraffin wax. Sections of 5 μm thick were cut in a microtome and mounted on glass slides using standard techniques. After staining the tissues with hematoxylin-eosin stain, the slides were observed under a light microscope equipped for photography and photomicrographs were captured.

2.16. Statistical analysis

The results were expressed as mean ± standard error of mean. The data were analyzed by Student’s t-test with multiple comparisons with the groups using GraphPad Prism software (GraphPad Software, Inc., San Diego, California, USA). The $P < 0.05$ was considered statistically significant.

3. Results

The hydro-alcoholic extractive value of *R. graveolens* leaves was found to be 13.73% w/w. The preliminary phytochemical screening showed the presence of the phytoconstituents carbohydrates, proteins, alkaloids, steroids, flavonoids, and phenolic compounds in hydro-alcoholic RGE Linn, leaves. RGE was found to be devoid of mortality of all the rats up to the dose of 2000 mg/kg bw in the acute toxicity study. Hence, the optimum doses selected for the study of “protective effect of RGE on IND and pylorus ligation-induced gastric ulcer in rats” were 200 and 400 mg/kg/day p.o. of RGE.

The effects of RGE on UI, percent protection, pepsin activity, gastric wall mucus, TBA-reactive substance (TBARS), GSH, pH, free acidity and total acidity levels are shown in Table 1.
Group II rats showed increase in UI (P < 0.001) and TBARS level (P < 0.001) while decrease in gastric mucus (P < 0.001) and GSH level (P < 0.001) as compared to Group I rats. Group III rats showed increase in UI (P < 0.001 and P < 0.05) and TBARS level (P < 0.001 and P > 0.05) while decrease in gastric mucus (P < 0.001 and P > 0.05) and GSH level (P < 0.001 and P > 0.05) as compared to Group I and Group II rats, respectively. Group III rats showed decrease in UI (P > 0.05) while increase in total acidity (P > 0.05), free acidity (P > 0.05) and pepsin activity (P > 0.01) as compared to Group II rats.

Group IV rats showed decrease in UI (P < 0.05 and P < 0.001), TBARS level (P > 0.05 and P < 0.05), total acidity (P > 0.05 and P > 0.05), free acidity (P > 0.05 and P < 0.05) and pepsin activity (P < 0.01 and P < 0.001) while increase in gastric mucus (P < 0.05 and P < 0.01), GSH level (P > 0.05 and P < 0.001) and pH (P < 0.05 and P < 0.01) as compared to Group II and Group III rats, respectively.

Group V rats showed decrease in UI (P < 0.001 and P < 0.001), TBARS level (P > 0.05 and P < 0.01), total acidity (P > 0.05 and P > 0.01), free acidity (P > 0.05 and P < 0.01) and pepsin activity (P < 0.01 and P < 0.001) while increase in gastric mucus (P < 0.01 and P < 0.001), GSH level (P < 0.01 and P < 0.001) and pH (P < 0.01 and P < 0.01) as compared to Group II and Group III rats, respectively.

Group VI rats showed decrease in UI (P < 0.001 and P < 0.001), TBARS level (P < 0.001 and P < 0.001), total acidity (P < 0.01 and P < 0.01), free acidity (P < 0.01 and P < 0.01) and pepsin activity (P < 0.001 and P < 0.001) while increase in gastric mucus (P < 0.001 and P < 0.01), GSH level (P < 0.001 and P < 0.01) and pH (P < 0.001 and P < 0.01) as compared to Group II and Group III rats, respectively. Group VI rats showed increase in UI (P < 0.001 and P < 0.001), TBARS level (P < 0.001 and P < 0.001), total acidity (P < 0.01 and P < 0.01), free acidity (P < 0.01 and P < 0.01) and pepsin activity (P < 0.001 and P < 0.001) while increase in gastric mucus (P < 0.001 and P < 0.01), GSH level (P < 0.001 and P < 0.01) and pH (P < 0.001 and P < 0.01) as compared to Group II and Group III rats, respectively.

The Group V rats showed highly significant decrease in UI (10.33 ± 0.67), TBARS (0.33 ± 0.03 mmol/mg of protein), free acidity (48.78 ± 5.12 meq/l/100 gm bw), total acidity (99.33 ± 9.31 meq/l/100 g bw) and pepsin activity (8.47 ± 0.41 μg/ml) while highly significant increase in gastric mucus (412.4 ± 21.6 μg/g tissue), GSH (57.9 ± 4.8 mmol/mg of protein) and pH (3.32 ± 0.27) levels as compared to Group III rats. Percent protections in Group IV, Group V and Group VI rats as compared to Group II and Group III rats were found to be (15.19% and 27.20%), (57.27% and 63.32%) and (70.38% and 74.57%), respectively.

The gross structure of stomach is shown in Fig. 1 and histopathological examination of Group I showed normal appearance of the gastric tissues. Group II and Group III showed gastric ulcer due to pylorus ligation alone or pylorus ligation plus IND respectively indicated by denudation of lining epithelium, degenerative changes in the cells, intracellular and interstitial cell edema along with degenerative changes in glandular epithelial cells, areas of hemorrhage and pigment laden macrophages. The Group IV, Group V and Group VI showed protection from the gastric ulcer due to pretreatment with RGE 200 mg/kg/day, RGE 400 mg/kg/day or omeprazole 10 mg/kg/day respectively indicated by intact mucosal lining with flattened epithelial cells, glands separated by thin strands of fibro connective tissue, thick and intact basement membrane, few bundles of fibrous tissue, occasional blood vessels and compactly arranged mucosal glands.

Hence, the protective effect of hydro-alcoholic RGE leaves, RGE (400 mg/kg/day) was comparable to that of standard drug omeprazole (10 mg/kg/day) on IND and pylorus ligation-induced gastric ulcer in rats. Results of the protective effects of RGE are well supported by the histopathological examination of gastric tissues.

4. Discussion

IND causes the depletion of the mucosal layer [29]. The digestive effect of accumulated gastric juice and interference of gastric blood circulation due to pylorus ligation are responsible for the induction of ulceration [29]. Hence, IND plus pylorus ligation was used in the study to induce severe ulceration in rats. Results of the study clearly demonstrated that Group II rats showed increase in UI and TBARS level while decrease in gastric mucus and GSH level as compared to normal control Group I rats. Group III rats showed increase in UI and TBARS level while decrease in gastric mucus and GSH level as compared to Group I and Group II rats. Group III rats also showed increase in UI and TBARS level while decrease in gastric mucus and GSH level as compared to Group II rats. Extent of UI may be the indicator of intensity of stress or ulcerogenic potential of pharmacologic agents [30]. Results of the study clearly demonstrated that pretreatment with hydro-alcoholic RGE leaves in different doses significantly decreased the UI elevated due to IND and pylorus ligation suggesting that RGE has prevented the ulcerogenic potential of IND plus pylorus ligation.

If some oxygen radicals are generated in the surface epithelium containing mucus, the intracellular mucus scavenge them and act as an antioxidant agent reducing mucosal damage mediated by oxygen free radicals [31]. Results of the study clearly demonstrated that pretreatment with RGE in different doses significantly increased the mucus production, which was reduced due to IND and pylorus ligation, suggesting the likely mechanism of gastroprotection is related to the factors that lead to increased production of mucus.

| Treatment groups and ulcer specific variables | Control (vehicle control) | Negative (control) | IND | IND + IND (200) | IND + IND (400) | IND + IND (OM) |
|---------------------------------------------|--------------------|-----------------|----|----------------|----------------|----------------|
| UI                                          | 24.17 ± 1.43$     | --              | -- | 28.16 ± 0.48*** | 20.50 ± 0.99**** | 10.33 ± 0.67***** |
| Percentage of protection compared to Group II| --                | --              | -- | 15.19          | 57.27          | 70.38          |
| Pepsin activity (mg/ml)                      | --                | --              | -- | 27.20          | 63.32          | 74.57          |
| Mucosal barrier (μg/g of tissue)             | 14.23 ± 0.71      | 19.31 ± 0.71**  | 10.11 ± 0.62**** | 8.47 ± 0.41**** | 7.74 ± 0.53***** |
| TBARS (mmol/mg of protein)                  | 0.27 ± 0.02       | 0.48 ± 0.04*    | 0.41 ± 0.04***  | 0.33 ± 0.03**** | 0.25 ± 0.03***** |
| GSH (mmol/mg of protein)                     | 69.80 ± 5.3       | 34.3 ± 3.3**    | 51.1 ± 5.2**    | 57.9 ± 4.8***** | 68.1 ± 4.2****** |
| Free acidity (meq/l/100 g bw)                | 71.46 ± 6.11      | 86.21 ± 7.93    | 60.88 ± 5.89**  | 48.78 ± 5.12**** | 42.38 ± 4.22**** |
| Total acidity (meq/l/100 g bw)               | 139.34 ± 11.32    | 154.11 ± 13.61* | 121.72 ± 11.34* | 99.33 ± 9.31*** | 88.21 ± 7.63**** |
| pH                                          | 1.38 ± 0.11       | 1.27 ± 0.09*    | 2.12 ± 0.21**** | 3.32 ± 0.27**** | 4.33 ± 0.39**** |

Values were expressed as mean ± SEM (n = 6). *P < 0.01 as compared to control group I; **P < 0.05; ***P < 0.01 and ****P < 0.001 as compared to respective Group II; *P < 0.05; **P < 0.01; ***P < 0.001 and ****P < 0.0001 as compared to respective Group III. SEM: Standard error of mean, RGE: Extract of Ruta graveolens, TBARS: Thiobarbituric acid reactive substances, GSH: Glutathione, IND: Indomethacin.
Increased formation of reactive oxygen metabolite like TBARS has been implicated in the pathogenesis of many inflammatory conditions including gastrointestinal tract disorders and peptic ulcer [32]. The excessive generation of oxygen radicals in the extracellular space and depletion of GSH are responsible for oxidative damage of the gastric mucosa causing peptic ulcer [33]. Results of the study clearly demonstrated that pretreatment with RGE significantly decreased the level of TBARS elevated and significantly increased the level of GSH reduced due to IND and pylorus ligation suggesting that the RGE prevents the depletion of nonprotein sulfhydryl groups caused by IND and pylorus ligation treatment [34].

The causes of gastric ulcer in pylorus ligation are believed to be due to increase in gastric HCl secretion leading to autodigestion of the gastric mucosa and breakdown of mucosal barrier [35]. Results of the study clearly demonstrated that pretreatment with RGE...
significantly decreased the levels of total acidity and free acidity elevated; and also significantly increased the level of pH reduced due to IND and pylorus ligation suggesting that RGE treatment has prevented the autodigestion of the gastric mucosa and breakdown of mucosal barrier by HCl.

Ulcers induced due to pylorus ligation are thought to be caused due to increased presence of gastric acid and pepsin [11]. IND also increases pepsin secretion [36]. Results of the study clearly disclosed.

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Source of support

due to increased presence of gastric acid and pepsin [11]. IND also prevented the autodigestion of the gastric mucosa and breakdown due to IND and pylorus ligation suggesting that RGE treatment has

were significantly decreased the levels of total acidity and free acidity elevated; and also significantly increased the level of pH reduced due to IND and pylorus ligation, to normal level and the effect was comparable to that of omeprazole suggesting that RGE has prevented the excess pepsin secretion.

Antioxidant activities of flavonoids have been well documented in the literature. Furthermore, flavonoids have been already reported for their gastric protection and antiulcerogenic activity [37,38]. Results of the study clearly demonstrated that RGE contains flavonoids and phenolic compounds suggesting that the protective effect of RGE on ulcer is probably due to the antioxidant activity of RGE.

Hence, it was found that the RGE has antisecretory activity as observed by decrease in total and free acidity. Further, the RGE offers cytoprotection by antioxidant activity and increasing thickness of gastric wall mucus. The protective effects of RGE may be predominantly due to its activity on defensive mucosal factors. The inherent antioxidant activity of RGE may be one of the important factors contributing toward its activity. All the results of the study were significantly supported by the results of histopathological examination which showed protection of mucosal layer from ulceration.

5. Conclusion

The present findings conclude that the hydro-alcoholic RGE Linn. leaves have antiulcerogenic activity as it exhibited protective effect on gastric ulcer in rats.

Source of support

Nil.

Conflicts of interest

The authors declare that they have no conflicts of interest to disclose.

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