Potential Protective Effect of Achillea fragrantissima against Indomethacin Induced- Gastric Ulcer in Male Rats

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Abstract: Gastric ulcer is one of the major gastrointestinal disorders with increasing incidence and prevalence globally. The present study was carried out to investigate the potential protective effect of Achillea fragrantissima extract (AFE) alone or combined with ranitidine (RAN) on gastric ulcer induced by indomethacin (IND) in male rats. Male albino rats (n=42) were divided into six equal groups: I. control negative; II. AFE (800 mg/kg /day, p.o); III. IND; IV. RAN+IND (20 mg/kg /day, p.o); V. AFE+IND; VI. AFE + RAN + RAN. After 2 weeks, groups IV, V and VI received a single oral dose of IND (25 mg/kg b.wt) to induce peptic ulcer. Oxidative stress markers and biomarkers of gastric ulcer (gross evaluation of gastric lesions, ulcer index, pH and total gastric juice acidity) were determined, as well as histopathological and histochemical examinations of stomach were also performed. The results showed that pre-treatment with AFE combined with RAN for 2 weeks significantly decreased the gastric acidity and ulcer index, as well as oxidative stress markers were significantly improved as compared to the IND group. Histopathological examination of the stomach showed alleviation of histological degeneration changes as well as histochemical changes caused by IND. Therefore, the study recommends that, intake of AFE with RAN treatment may be beneficial for patients suffering from acute gastric ulcer.

Key words: Peptic ulcer • Indomethacin • Achillea fragrantissima • Ranitidine • Rats

INTRODUCTION

Gastric ulcer disease, with increasing incidence and prevalence globally, is a gastrointestinal disorder induced by mucosal damage and free oxygen radicals associated with gastritis [1]. It is characterized by lesions in the gastrointestinal mucosa that may penetrate the muscularis layer [2-4]. Gastric ulcer is caused by an imbalance between aggressive factors and defensive factors, which can in turn affecting on the inflammatory processes involving the roles of neutrophils, eosinophils and mast cells [5]. The defensive factors as gastric mucus, antioxidant enzymes, nitric oxide (NO) and prostaglandins protect from aggressive agents. Exogenous damaging etiologic factors as poor diets, stress, smoking, prolonged ingestion of non-steroidal anti-inflammatory drugs (NSAIDs) and Helicobacter pylori (H. pylori). All these factors effect on acid secretion in the gastric mucosa and all relevant to the formation of gastric ulcer [6, 7].

Non-steroidal anti-inflammatory drugs (NSAIDs) are among the most widely used drugs in the world. They are extensively used for their analgesic, anti-inflammatory and antipyretic properties [8]. Indomethacin (IND) is considered to be the most common NSAIDs. It is known to induce experimental gastric ulcer and has been documented to have a higher potential to cause gastric injury than other commonly-used NSAIDs [9]. Anti-ulcer medication as ranitidine (RAN) and Omeprazole have been extensively used as model drug in prophylaxis and treatment of gastric ulcer, duodenal ulcer, gastritis,
gastroesophageal reflux, stomach hypersecretion in endocrine multiple adenoma and inhibiting proton pumps [10, 11]. Although these therapy could potentially achieve complete gastric acid suppression. However, the risks which may be associated with this level of suppression including enteric infections and malabsorption of nutrients as vitamin B12, iron and calcium [12]. Hence, the search for a natural ulcer agent that decreases the incidence of relapse and affords better protection is still needed. Recently, there is a growing attention on the use of traditional medicine as treatment options for different ailments [5]. Plants have been proven to be safe and effective with better patient tolerance, as well as being cheaper, making traditional medicine globally competitive [13].

*Achillea fragrantissima* is a medicinal shrub plant that belongs to the *Asteraceae* family. It is a desert plant that has been used for many years in traditional medicine in the Arabia region for the treatment of respiratory diseases and gastrointestinal disturbances [14]. In Saudi Arabia it is known locally as Gaisoom. It is used as carminative, anthelmintic, antiseptic to various infections for the urinary tract and also has antiviral and antioxidant properties [15]. Because, the main mechanism beneath IND-induced gastric ulcer is pro-oxidants and AFE has strong antioxidant properties. Therefore, the present study was conducted to investigate the protective efficacy of AFE against IND-induced gastric ulcer in rats, which have not been studied to date.

**MATERIALS AND METHODS**

**Materials**

**Plant Material:** Dried aerial parts of *Achillea fragrantissima* were collected from Al-Jawf in Northern Saudi Arabia. Taxonomic identification of the plant material was established by Prof. Dr. Alaa Eldin M.S. Khedr, Department of Pharmaceutical and Phytochemistry, Faculty of Pharmacy, KAU, Jeddah, Saudi Arabia.

**Acute Toxicity Test:** Acute toxicity study was carried out using the limit test dose of 2000 mg/kg as described by OECD 425 guideline. Three female albino rats were fasted for 24 h but allowed free access to water. A limit dose of 2000 mg/kg of AFE was administered sequentially and animals were observed individually for behavioral profile (alertness, restlessness, irritability and fearfulness), autonomic profiles (defecation and urination), neurologic profile (spontaneous activity, reactivity, touch response, pain response and gait), physical states such as lacrimation, loss of appetite, tremors, hair erection, salivation, diarrhea and for morbidity or mortality, after dosing continuously for 2 h, periodically during the first 24 h (with special attention given during the first 4 h) and daily thereafter, for a total of 14 days.

**Experimental Animals:** Male albino Wistar rats (*n*=42, 180-200 g) were purchased from the animal house of King Fahd medical research center (KFMRC) under the rules of Canadian ethical approval from the Local Biomedical ethical committee of KAU. They were kept under standard laboratory conditions and were given free access of food and water. They were left for acclimatization for one week before starting the experiment.

**Drugs, Kits and Chemicals:** Indomethacin (Rothacin capsule, 25 mg) and Ranitidine (Zydac tablet, 150 mg) were purchased from Nahdi Pharmacy, Jeddah, Saudi Arabia. Thiobarbituric acid reactive substances (TBARS) and reduce glutathione ELISA kits were purchased from BioAssay systems, CA, USA. Other chemicals were obtained from Sigma-Aldrich (St Louis, MO, USA).

**Methods**

**Preparation of Plant Extract:** *Achillea fragrantissima* plant was grounded to powder, then 100 g was extracted using 80% hydroalcoholic solvent. The final extract was filtrated using filter paper no. 1 and then the solvent was evaporated under reduced pressure vacuum system by a rotary evaporator (Büchi labortechnik AG, R-215, Switzerland) to maintain semisolid material. Finally the semisolid extract was transferred to a Gamma 2-20 freeze dryer (Christ, Germany), then stored at 4°C until further use [16]. (100 g yield 5 mg).

**Experimental Design:** Rats were fasted for 24 h with free access to water except for the last 4 h before the experiment. Then, they were randomly divided into six groups (7 rats each):

- **Group I:** in which rats were given distilled water orally and served as control group.
- **Group II:** rats were given AFE (800 mg/kg/day orally) [17].
- **Group III:** in which gastric ulceration was induced by a single oral dose of IND (25 mg/kg) dissolved in distilled water [18].
- **Group IV:** rats were pretreated with RAN (20 mg/kg, orally) for 14 days before IND administration and served as a standard drug [19].
Group V; rats were pretreated with AFE for 14 days before IND administration

Group VI; rats were concurrently pretreated with RAN+AFE in the same previous doses for 14 days before IND administration.

Four h after ulcer induction, rats were sacrificed. Stomach was removed immediately and opened along the greater curvature, then it was rinsed with saline to remove blood clots and gastric juice and then photographed for macroscopic assessment of gastric mucosal injury. Part of the gastric tissue after being photographed was fixed in 10% buffered formaldehyde solution for histopathological examination. The rest was stored at - 80°C until biochemical analysis.

Biochemical Analysis

Determination of pH and Total Gastric Acidity: An aliquot of 1 ml of gastric juice was diluted with 1 ml of distilled water and pH of the solution was measured using pH meter [20]. The gastric contents were collected and centrifuged at 3000 rpm for 15 min, the total acidity were determined [21].

Tissue Homogenate and Determination of Oxidative Stress Biomarkers: The mucosal surface of the stomach were collected by scraping and kept in ice-cold phosphate buffer saline (PBS) after estimation of ulcer index. 500 mg of stomach tissues were homogenized with 5 ml of PBS using a Teflon pestle (Ultra- Turrax, IKA: T25 digital, Germany) and centrifuged at 3000 rpm for 20 min at 4°C (Centurion, K280 R, UK). The supernatant were used for the estimation of malondialdehyde (MDA) and reduced glutathione (GSH). The procedures were performed according to the manufacturer’s protocols.

Gross Evaluation of Gastric Lesions: The data were obtained using Image Pro Express analyzer computer system. The image analyzer consisted of a coloured video camera; coloured monitor, hard disc of personal computer connected to the microscope and controlled by image Pro software. Using the measuring field menu the gastric ulcer area in each stomach was assessed. The sums of the areas of all lesions for each stomach were used in the calculation of the ulcer area (UA, mm²). The total mucosal area and total ulcerated area were measured. The ulcer index and % inhibition of ulceration were then calculated using the following equations [22]:

\[
\text{Ulcer index} = \frac{10}{x} \quad \text{Where} \quad x = \frac{\text{Total mucosal area}}{\text{Total area of mucosal lesions}}
\]

\[
\% \text{Inhibition of ulceration} = \frac{\text{UI (IND group)} - \text{UI (test group)}}{\text{UI (IND group})} \times 100
\]

Histopathological and Histochemical Examination: Stomach tissue samples were taken after washing by cold saline followed by fixation in 10% buffered formalin solution, processed through graded alcohols and xylene and embedded in paraffin blocks in automatic processor. Serial sections were made on longitudinally oriented specimens. Sections were stained with hematoxylin and eosin (H&E) to study the changes in the structure and periodic acid Schiff (PAS) to observe changes in mucus secretion [23]. The photographs were taken by a camera (Olympus DP72- USA) in the microscope unit at KFMRC.

Statistical Analysis: The results were expressed as mean ± standard deviation (SD), the difference between groups were determined by analysis of variance (ANOVA), using Statistical Package for the Social Sciences version 22 (SPSS Inc., Chicago, IL, USA), the significant difference was considered at \(p \leq 0.05\).

RESULTS

Acute Toxicity Test: With the acute toxicity test at the limit test dose of 2000 mg/kg, neither mortality nor changes related to behavioral, autonomic, neurologic and physical profiles were observed within the first 24 h and during the 14-days follow-up.

Gross Evaluation of Gastric Lesions: Gastric of control and AFE groups revealed regular stomach mucosa. The glandular part showed up pink with normal rugae. However, stomach of rats from IND group showed multiple ulcerations with hemorrhage and the glandular part revealed macroscopic mucosal patches of different sizes, form and shade that range from hyperemia to darkish patches. Rats pretreated with RAN as well as AFE showed minute lesions and apparently few linear dark brown lesions, while nearly normal appearance in group pretreated with both AFE+ RNA was showed Fig. (1).

Assessment of Gastric Oxidative Stress Biomarkers

Non-Enzymatic Antioxidant Malondialdehyde (MDA): In IND ulcer group, MDA showed a significant increase \((p<0.001)\) in the gastric mucosa compared with control rats. A significant decrease in the gastric MDA level was
GI: Control, GII: AFE, GIII: IND, GIV: RAN+IND, GV: AFE+IND, GVI: AFE+RAN+IND

Fig. 1: Gross appearance of gastric mucosa. (GI) showing the pink glandular part (normal rugae); (GII) showing noticeable likeness to the control; (GIII) mucosa seems to be hyperaemic with obviously few darkish patches showing macroscopic mucosal areas of different sizing and color; (GIV and GV) show nearly normal mucosae with tiny ulcers; (GVI) has nearly normal mucosa in rats pretreated with both RAN + AFE.

Data are represented as mean ± SD (n = 7). *Significant versus control, † Significant versus IND group, ‡ Significant versus RAN+ IND, § Significant versus AFE + IND, p < 0.05.

Fig. 2: Effect of AFE and/or RAN on gastric MDA level against IND-induced ulcer in rats.

Data are represented as mean ± SD (n = 7). *Significant versus control, † Significant versus IND group, ‡ Significant versus RAN+ IND, § Significant versus AFE + IND, p < 0.05.

Fig. 3: Effect of AFE and/or RAN on gastric GSH activity against IND-induced ulcer in rats.

Data are represented as mean ± SD (n = 7). *Significant versus control, † Significant versus IND group, ‡ Significant versus RAN+ IND, § Significant versus AFE + IND, p < 0.05.
Table 1: Effect of AFE and/or RAN pretreatment on gastric ulcer biomarkers against IND-induced ulcer in rats

<table>
<thead>
<tr>
<th>Experimental groups</th>
<th>Gastric pH</th>
<th>Total gastric acidity</th>
<th>Ulcer index</th>
<th>% Ulcer inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>3.34 ± 0.07</td>
<td>52.22 ± 4.49</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>AFE</td>
<td>3.23 ± 0.05</td>
<td>49.74 ± 4.83</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>IND</td>
<td>2.29 ± 0.06*</td>
<td>112.62 ± 4.33*</td>
<td>23.3 ± 1.82*</td>
<td>-</td>
</tr>
<tr>
<td>RAN+ IND</td>
<td>3.11 ± 0.40*</td>
<td>64.32 ± 2.60*</td>
<td>6.0 ± 0.53*</td>
<td>74.25</td>
</tr>
<tr>
<td>AFE+IND</td>
<td>3.05 ± 0.28*</td>
<td>67.04 ± 4.46*</td>
<td>11.67 ± 0.88*</td>
<td>49.91</td>
</tr>
<tr>
<td>AFE+RAN+IND</td>
<td>3.37 ± 0.39*</td>
<td>59.44 ± 3.68*</td>
<td>2.33 ± 0.21*</td>
<td>90.00</td>
</tr>
</tbody>
</table>

Data are represented as mean ± SD (n = 7). * Significant versus control, † Significant versus IND group, ‡ Significant versus RAN+ IND, § Significant versus AFE + IND, p ≤ 0.05

Fig. 4: Photomicrography sections of gastric mucosa. Control showing fundic glands in the lamina propria and muscularis mucosa (Mm); AFE showing maintained glandular architecture with predominance of mucous secreting cells (arrows); IND showing dead cells with acidophilic cytoplasm and pyknotic nuclei in the damaged glandular area (arrows), with vacuolization of the cytoplasm of parietal cells (V); RAN+IND showing apparently near-normal glands, with some areas of deep eosinophilic surface epithelial cells (arrows). The lumen of the gland is dilated (D); AFE+IND showing apparent improvement in the gastric mucosa, except slight vacuolization (V) of some cells with dilated and congested capillaries; AFE+RAN+IND showing minimal areas of congested blood capillaries (BV) in the lamina propria between glands. (H&E stain)

Enzymatic Antioxidant Reduced Glutathione (GSH): A significant decrease in the gastric GSH activity was observed in IND group compared with control (p<0.001). Pretreatment with RAN and/or AFE restored significantly gastric GSH activity (p<0.001) compared with IND group. While in the group pretreated both AFE+RAN, there was a significant increase in the gastric GSH level compared with RAN pretreated group alone (p<0.05). At the same time there was a significant increase in gastric GSH level observed in RAN and/or AFE pretreatment groups as compared with IND group (p<0.001). The most effective pretreatment was seen in the group co-pretreated with AFE+RAN. There was a significant difference in the gastric MDA level between pretreated group with AFE+RAN and the rats pretreated with RAN alone (p<0.05), while no significant different between pretreated group with AFE+RAN and pretreated with AFE alone (p >0.05) Fig. (2).
between AFE pretreated and RAN pretreated group (p<0.05), thus indicated high antioxidant effect of AFE Fig. (3).

**Gastric Ulcer Biomarkers:** The IND caused significant decrease in pH value associated with significant increase in total gastric acidity and ulcer index (p<0.001) as compared with control group. Pretreatment with RAN or AFE produced significant increase in pH associated with significant decrease in total gastric acidity and ulcer index (p<0.001) compared with IND group. Co-administration of AFE+RAN showed more potent efficacy in elevation gastric pH, there was a significant increase (p= 0.006) in gastric pH value, decrease in ulcer index (p<0.001) compared with IND group. At the same time the potent ulcer inhibition was seen in the group pretreated with AFE+RAN compared with group pretreated with either RAN or AFE alone Table (1).

**Histopathological Results:** The luminal epithelium of the fundic mucosa in control group was columnar mucous-secreting cells with basal oval nuclei. The fundic glands were perpendicular to the surface epithelium extending in the lamina propria. Gastric sections of AFE group were nearly similar to that of the control group. Variable grades of mucosal injuries in between areas were demonstrated in IND group, focal areas of cellular vacuolization, necrotic cells in different parts of the glands, edema and mononuclear cellular infiltration was detected in the submucosa. Pretreated with RAN decreased the mucosal ulcerogenic effect of IND, gastric glands appeared mostly near normal, except few areas showed cellular vacuolation. Some of the surface epithelial cells had deep eosinophilic cytoplasm, whereas other areas showed mucous-secreting cells. Rats pretreated with AFE showed apparent improvement in the gastric mucosa, except the basal parts of the glands showed vacuolization of some cells with dilated and congested capillaries. Pretreated with AFE+RAN was apparently normal, with minimal areas of congested blood capillaries in the lamina propria between glands Fig. (4).

**Histochemical Results:** Control and AFE groups showing positive mucous secreting cells that fill mainly the gastric pits. In IND group there was an interrupted faint PAS positive mucous film and scattered glandular PAS
positive cells surrounding the ulcer area. In RAN+IND and AFE+IND groups there was a PAS positive stained mucous film over the epithelial surface. Deeply stained PAS positive mucous-secreting cells were also seen in the whole parts of the gland. Highly PAS positive mucous-secreting cells were observed in the elongated gastric pits and along the whole length of the fundic gland in AFE+RAN+IND group (Fig.5).

**DISCUSSION**

Peptic ulcer (PU) is a benign lesion on the gastrointestinal mucosa [24]. Indomethacin, as a NSAIDs, widely used to treat arthritic diseases [25]. Several synthetic antiulcer drugs are used in the treatment of PU and manage NSAIDs side effects. Unfortunately, no one of antiulcer drugs is without side effects or gives a complete cure or curative rate. In addition to the high rate of recurrence, thus prompted a search for non-toxic, easily accessible and affordable antiulcer medication [26]. Many plants prove to have beneficial effects on gastrointestinal disorders and can be used as alternatives in stomach diseases. *Achillea* species have been reported to have medicinal properties [27]. Therefore, the present study aimed to assess the antiulcer activity of AFE against IND-induced PU in rats comparable to RAN as a reference drugs.

In the present study, the results revealed that IND induced a significant increase in gastric MDA level with a decrease in gastric GSH activity in ulcerated rats compared with control rats. Reactive oxygen species (ROS) have been implicated in the etiology of IND-induced gastric mucosal damage. The obtained results agreed with Parvan et al. [28] who reported that IND induces an increase in lipid peroxidation and produces free radicals in gastric mucosa. This IND effect could be explained by IND-induced gastric damage by inhibiting the release of protective factors like cyclooxygenase-1 (COX-1), prostaglandin E2 (PGE2), bicarbonate and mucus [25].

In the present study pretreatment with AFE showed an antioxidant effect; there was a marked improvement in oxidative stress biomarkers, where a significant decrease in gastric MDA with a significant increase in gastric GSH levels were found in compared with IND rats. The obtained results agreed with Giorg et al. [29]. This could be attributed to the phytochemical contents in AFE as flavonoids, saponins and tannins, which have been reported to exert antiulcer activity [30]. Moreover, Zayachkivska et al. [31] reported that plant-originated flavonoid substances are highly gastroprotective probably due to enhancement of the expression of constitutive NOs. Several pharmacological properties in the gastro-protective area of flavonol act as an anti-secretory. This flavonol has antihistamine properties, thus decreasing histamine levels, as well as preventing the release of histamine from gastric mast cells and inhibiting the gastric H+/K+ proton pump, diminishing acidic gastric secretion; cytoprotective and antioxidant agents which increase the mucosal blood flow, stimulate the synthesis of mucosubstances in the gastric mucosa and increase PGs levels [32].

In the present study, IND induced significant increase in total gastric acidity and ulcer index with a significant decrease in pH value compared with the control group. Pretreatment with AFE as well as RAN showed marked improvement on gastric lesion biomarkers, there was a significant increase in the pH value with a significant decrease in total gastric acidity and ulcer index compared with IND group. The most effective, protective pretreatment was seen in the rats co-pretreated with both AFE + RAD compared with other pretreated groups.

The obtained results agreed with Taha et al. [33] who revealed that in the ulcer group there was a significant decrease in pH of the gastric juice and mucus content of gastric mucosa compared with the control group, while the groups pretreated with antiulcer drugs showed a significant increase in the mucus production and the pH of the gastric juice. The observed effect of IND could be explained by NSAIDs inhibiting cyclooxygenase and consequently the synthesis of prostaglandins E2, which have cytoprotective effects in gastric mucosa, these effects on prostaglandins have been thought to be a major cause of NSAIDs-induced ulceration [34].

Ranitidine is an antagonist of the histamine-2 receptor. It is employed to treat peptic ulcer and other conditions in which gastric acidity must be reduced [35]. It is a proton pump inhibitor, which suppresses gastric acid secretion by specific inhibition of the gastric H+/K+/ATPase enzyme at the secretary surface of the gastric parietal cells [36]. The anti-ulcer effect of RAN could be explained by it interfering in the gastric acid secretion, such as H2 antagonists and proton pump inhibitors [37].
The marked antiulcer activity of AFE could be attributed to the presence of glycosides, sterols, flavonoids and triterpenes. These active compounds would be able to stimulate mucus, bicarbonate and the prostaglandin secretion and counteract with the deteriorating effects of reactive oxidants in gastrointestinal lumen [38, 39].

In the present study the macroscopic examination of gross appearance of gastric in the IND rats showed multiple ulcerations and hemorrhage. Damaged glandular areas, focal areas of cellular vacuolization, edema and mononuclear cellular infiltration was detected in the histopathological examination, also there was an interrupted faint PAS positive mucous film in the histochemical examination. The obtained results agreed with the previous studies [40, 41]. In addition, Moram et al. [42] revealed that in the control group there were no gross mucosal lesions, while IND-administerated rats showed marked gross mucosal lesions including long hemorrhagic bands of different sizes and petechial lesions. Rats pretreated with RAN showed a few minute mucosal lesions and there was a significant reduction in PAS positive reaction in epithelial cells and basal lamina [43]. Thus could be explained by NSAID-induced peptic ulcers by interfering with the action of growth factors, decreasing epithelial cell proliferation in the ulcer margin, decreasing angiogenesis in the ulcer bed and slowing maturation of the granulation tissues [44].

Oral pretreatment with AFE as well as RAN showed nearly normal appearance with a few linear dark brown lesions in gross gastric macroscopic examination decreased the mucosal ulcerogenic effect of IND and there was apparent improvement in the gastric mucosa, while deeply stained PAS positive mucous-secreting cells were observed in the gastric microscopic examination. The antiulcer effects may account for a variety of actions including facilitation of gap functional intercellular communication, inhibition of the reduced gastric mucosal blood flow response and suppression of reactive oxygen generation [45]. This effect could be attributed to a variety of pharmacological properties of the Achillea, where it contains sesquiterpenes, diterpenes, flavonoids, lignans, which have been reported for their anti-ulcerogenic and gastric protection activity [46, 47].

The present study concluded that AFE had beneficial effects in the prevention of peptic ulcer induced by IND, these effects could be attributed to the antioxidant properties of this plant. Therefore, the intake of AFE with ulcer therapy may be beneficial for patients suffering from acute gastric ulcer.

REFERENCES