Abstract

Purpose: To assess the gastroprotective potential of the stem bark ethanol extract of Delonix regia (EDR) on ethanol and cold restrain stress-induced ulcer in experimental rats. Methods: EDR (100, 200 and 400 mg/kg doses, orally) was evaluated on ethanol and cold restrain stress-induced ulcer in experimental rats. In ethanol induced ulcer model, ulcer index, percent protection, reduced glutathione (GSH), malondialdehyde (MDA), superoxide dismutase (SOD), catalase (CAT), myeloperoxidase (MPO), cytokines and nitric oxide (NO) levels in stomach tissue were evaluated. In the cold restrain stress model, ulcer index, percent protection, and GSH levels were evaluated. 2,2-Diphenyl-1-picryl hydrazyl (DPPH) radical scavenging assay of EDR was also performed. Results: EDR caused a significant (p < 0.05-0.001) decreased ulcer index in ethanol (61.33-76.00%) and cold restrain stress (47.34-84.28 %) models. The EDR caused a significant (p < 0.05 - 0.001) increase in SOD (0.20 - 0.27 U/mg protein, CAT (200 - 270 µmole H₂O₂/mg of protein/minute), GSH (1.63 - 1.17 µg/mg protein) and reduction in nitric oxide (NO) level, pro-inflammatory cytokine (TNF-α and IL-6) levels and inhibition in neutrophil accumulation (p < 0.001) in ethanol-induced model. EDR exhibited significant antioxidant activity with IC₅₀ value of 45.23 ± 3.23 µg/ml. Conclusion: The results suggest that EDR has gastroprotective effect in the two ulcer models and this may be due to its antioxidant effect. Keywords: Delonix regia, Fabaceae, Ethanol-induced ulcer, Cold restrain stress-induced ulcer, Gastroprotective, Pro-inflammatory cytokines

INTRODUCTION

Gastric epithelium is often attacked by physical, chemical or microbiological agents acting from the gastric lumen. Among the numerous injurious luminal agents and irritants of both exogenous and endogenous origin, the stomach is a site of massive production and concentration of reactive oxygen species (ROS), far higher than other tissues or biological fluids [1-2]. ROS provoke severe changes at the cellular level leading to cell death because of their extreme reactivity. They attack essential cell constituents, leading to the formation of toxic compounds [3]. Both superoxide anion radical O₂•⁻ and hydroxyl radical (•OH) are involved in tissue damage through initiation of lipid peroxidation and disruption of the interstitial matrix [4]. Since prehistoric civilizations, people have relied on flora for their prophylactic or curative properties, to uphold and renovate health, and
flora are well-known as a central source of numerous biologically active compounds. In recent times, we have observed an upward interest in plants as an important source of new pharmaceuticals [5]. Delonix regia (Boj. Ex Hook.) Raf. (Fabaceae, sub family: Caesalpiniaceae) is commonly called as Gul mohor in Hindi [6]. Delonix regia is an umbrella-shaped, medium-sized tree reaching up to 40 feet high and with large red-orange flowers (in April-May) grown in most tropical countries [7]. Stem bark of Delonix regia contains lupeol, epilupeol, β-sitosterol, stigmasterol and p-methoxybenzaldehyde [8]. It has folkloric reputation as diuretic, anthelmintic, leucorrhoea and astringent [8]. Also, Delonix regia is used to treat bruises, wounds, piles, gastric problems, body pain, and rheumatic pain of joints [9,10].

On the basis of literature survey, the aim of the present study was to assess gastroprotective potential of ethanol extract from stem bark of Delonix regia (EDR) on ethanol and cold restrain stress-induced ulcer in experimental rats.

EXPERIMENTAL

Collection of plant material

The stem bark of Delonix regia was collected from the garden of the Bazikhera of Unnao district of Uttar Pradesh in the month of May to June 2011. Plant materials were taxonomically identified and authenticated by Dr Anamika Tripathi, Associate Professor, Hindu College, Moradabad as Delonix regia with registration no. HC/Bot/PERL-26. The plant materials to be used were dried under the shade and were later ground to a fine powder. The powdered drug was extracted successively using soxhlet apparatus (Borosil, India) with various solvents in an increasing order of polarity viz., Petroleum ether, chloroform, ethyl acetate and ethanol. Yields for petroleum ether (40 – 60 °C), chloroform, ethyl acetate and ethanol extracts were 0.76, 0.77, 0.80 and 1.63 % w/w, respectively. In initial pilot studies of stem bark, ethanol extract showed antiulcer activity.

Preliminary phytochemical studies

EDR obtained was subjected to preliminary qualitative tests for various plant constituents by chemical tests [11,12].

Experimental animals

Wistar albino rats of either sex weighing 180 - 220 g were used for all experiments. All animals were housed in polypropylene cages in an air-conditioned area at 25 ± 2°C with 12:12-h light and dark cycle, respectively. They were given feed and water ad libitum. The optimum conditions for experiments were decided on the basis of initial pilot experiments. The Institutional Animal Ethics Committee affiliated to CPCSEA (837/ac/04/CPCSEA), India, approved the protocols used for animal experiments.

Acute oral toxicity

According to Organization for Economic Cooperation Development (OECD) guideline 423 [13], EDR at a dose level of 5, 50, 300 and 2000 mg/kg [orally (p.o.)] was used for acute oral toxicity study. Three female rats, each sequentially dosed at intervals of 48 h, were used for the test. Once-daily cage-side observations for changes in skin, fur, eyes, mucous membrane (nasal), autonomic nervous system (salivation, lacrimation, perspiration, piloerection, urinary incontinence and defaecation) and central nervous system (drowsiness, gait, tremors, and convulsions) changes were recorded. Mortality, if any, was determined over a period of 2 weeks.

Selection of doses

For the assessment of antiulcer activity by animal models, three dose levels of EDR were chosen in such a way that middle dose was approximately one-tenth of the maximum dose during acute toxicity studies, and a low dose, which was 50 % of the one tenth dose, and a high dose, which was twice that of one-tenth dose (100, 200 and 400 mg/kg).

Evaluation of ethanol-induced ulcers

The antiulcerogenic activity of EDR was performed in ethanol induced ulcer model as described by Hollander et al [14]. In this study, 24 h fasted rats were randomly distributed into 5 groups as follows:

Group 1: Animals received normal saline and served as control.
Groups 2, 3 and 4: Animals received 100, 200 and 400 mg/kg body weight. of EDR, respectively.
Group 5: Animals received Lansoprazole (20 mg/kg) orally and served as the reference drug for comparison.

Gastric ulcers were induced by oral administration of 1 ml of absolute ethanol per rat. Test substances were given 30 min before the ulcerative agent and after 1 h animals were
sacrificed by cervical dislocation and stomach was incised along the greater curvature and examined for ulcers in the glandular region. Stomach of all treated and control rats were subjected to visual macroscopic examination and ulcer score was calculated as follows: 0 = normal mucosa, 0.5 = blushing, 1 = spot ulcers, 1.5 = hemorrhage streaks, 2 = 3 mm < ulcers < 5 mm and 2.5 = ulcers >5 mm. Finally, GSH, superoxide dismutase (SOD), catalase (CAT), myeloperoxidase (MPO), cytokines and nitric oxide (NO) levels in stomach tissue were measured and compared with that of control rat group.

Evaluation of reduced glutathione (GSH) content

All groups of rats treated were utilised to estimate the reduced glutathione (GSH) content in stomach tissues as non-protein sulfhydryls according to the method described by Sedlak and Lindsay [15]. Glandular segment from each stomach was homogenised in 5 ml ice-cold 0.02 M EDTA solution (0.02 M). Aliquots (4 ml) of tissue homogenate were mixed with 3.2 ml of distilled water and 0.8 ml of 50 % (w/v) trichloroacetic acid (50 %) in glass tubes and centrifuged at 1000 × g for 15 min, 2 ml supernatant were mixed with 4 ml Tris buffer (0.4 M, pH 8.9) and 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB; 0.01 M) was added. After shaking the reaction mixture, its absorbance was measured at 412 nm within 5 min of the addition of DTNB against blank with no homogenate.

Superoxide dismutase activity (SOD) assay

Superoxide dismutase activity was assayed spectrophotometrically as previously described by Misra and Fridovich [16]. This method is based on the capacity of SOD to inhibit the autoxidation of epinephrine to adrenochrome. The colour reaction was measured at 480 nm. One unit of enzyme was defined as the amount of enzyme required to inhibit the rate of epinephrine autoxidation by 50 % at 36 °C.

Catalase activity assay (CAT)

The stomach was homogenized in 50 mM Tris–HCl, pH 7.5 (1/10, w/v) and centrifuged at 2400 g for 15 min. The supernatant was assayed spectrophotometrically [17], which involve monitoring the degradation of H2O2 in the presence of cell homogenate at 240 nm.

Assay of myeloperoxidase (MPO) in gastric tissue

Myeloperoxidase, an enzyme found primarily in neutrophil azurophilic granules, has been used extensively as a biochemical marker for granulocyte infiltration into various tissues, including the gastrointestinal tract [18,19]. MPO activity was determined using an MPO activity measurement kit by adding 0.2 ml of o-dianisidine hydrochloride and 0.0005 % hydrogen peroxide to 4 ml buffer containing 0.2 ml homogenates. MPO activity was assayed at room temperature by measuring the increase in absorbance at 460 nm due to the fluorescent product oxidized by the H2O2 generated redox intermediate. MPO activities were expressed as units per gram of tissue.

Evaluation of cytokines in gastric tissue

The cytokine levels of IL-6 and TNF-α in gastric tissue were evaluated using ELISA kits according to the manufacturer's instructions (Minneapolis, USA). Supernatant of homogenates or cytokine standards (100 µl) were respectively loaded into each well and then followed with biotin conjugated secondary antibodies. To obtain colour reaction, streptavidin–HRP and substrate solution were added. The absorbance was measured at 450 nm. A standard curve was run on each assay plate using recombinant IL-6 and TNF-α in serial dilution. The results were expressed pg/mg tissue.

Determination of NO level in gastric tissue

The level of nitric oxide in the gastric tissue was evaluated as total nitrate/nitrite using Griess reagent [20] and the operational processes were measured in accordance with the NO kit instructions. Briefly, 50 µl of tissue supernatant was added to 50 µl Griess reagent [0.1 % N-(1-naphthyl) ethylenediamine dihydrochloride, 1% sulphanilamide and 2.5 % H3PO4] and mixed. After incubation at room temperature for 10 min, the absorbance was measured at 540 nm. The results were expressed as µmol/g protein.

Assessment of cold restrain stress-induced ulcer

Cold restrain stress in the 36 h fasted experimental rats, with free access to water, were administered normal saline (1 ml/100g, p.o.), EDR (100, 200 and 400 mg/kg, p.o.) or PGE2 (20 mg/kg s.c.). One hour later after vehicle or EDR and 15 min after PGE2, rats were immobilized by strapping the fore and hind limbs on a wooden plank and were kept at 4-6 °C for 4
h [21]. Later, the animals were sacrificed by cervical dislocation. Stomachs were dissected out, opened along greater curvature and examined for ulcers. Ulcer index were calculated as per method described in previous ulcer model. Also, reduced glutathione (GSH) content in stomach tissues as non-protein sulfhydryls were estimated according to the method described by Sedlik and Lindsay [15].

DPPH radical scavenging assay

The radical scavenging activity of the EDR against 2,2-diphenyl-1-picrylhydrazyl (DPPH•) radical was determined [22]. Aliquots of the EDR of various concentrations (10-100 µg/ml) were prepared in methanol. One milliliter of these EDR concentrations was placed in test tubes and methanol (3 ml) was added followed by 1 mM methanol solution of DPPH• (0.5 ml). A blank solution containing the same amount of methanol and DPPH• was also prepared. After 30 min incubation at room temperature, the absorbance was read against blank at 517 nm. Inhibition (%) of free radical by DPPH• in percent (%) was calculated using following formula:

\[
\text{Inhibition of DPPH•} \ (%) = (1 - \frac{A_{a}}{A_{b}})100 \quad \ldots\ldots (1)
\]

where \(A_b\) is the absorption of the blank sample and \(A_a\) is the absorption of the extract. IC\(_{50}\) values, which represented the EDR concentration providing 50% inhibition of DPPH• radicals, were calculated from the plot of inhibition percentage against EDR concentration.

Statistical analysis

The results were expressed as mean ± SEM and were analyzed using one-way analysis of variance followed by Dunnett’s test using GraphPad Prism 5.0 (Graph-Pad Software Inc, San Diego, California, USA). \(P < 0.05\) was considered statistically significant.

RESULTS

Phytochemical profile

On the basis of preliminary phytochemical analysis of ethanol extract of stem bark of Delonix regia the results revealed the presence of glycosides, flavonoids, phenols and tannins.

Acute oral toxicity

In the present study, EDR had no effect on mortality, clinical signs, body weight change or gross observation in rats. Therefore, no acute toxicity was found in rats treated with EDR and LD\(_{50}\) might be higher than 2000 mg/kg.

Ethanol induced ulcers

The antiulcerogenic effects of EDR on ethanol induced ulcer model in rats are shown in Table 1. Oral administration of ethanol produced severe ulcer index in the untreated animals. However, pretreatment with EDR decreased the ulceration in a dose dependent manner. At a dose of 400 mg/kg, the extract significantly \((p < 0.001)\) reduced the intensity of gastric mucosal damage and the gastroprotective activity (76.00 %) was comparable to that of lansoprazole (80.67%).

**Table 1:** Effect of EDR on ethanol induced ulcers in rats

| Treatment  | Ulcer Index  | Protection (%) |
|------------|--------------|----------------|
| Control    | 16.17 ± 1.40 | -              |
| EDR 100    | 4.83 ± 0.67*** | 61.33          |
| EDR 200    | 3.50 ± 0.82*** | 72.00          |
| EDR 400    | 3.00 ± 0.39*** | 76.00          |
| Lansoprazole | 2.42 ± 0.24*** | 80.67          |

Values are expressed as mean ± SEM (n = 6). ANOVA followed by Dunnett’s test with control group. Significance represented as *** \((p <0.001)\)

Effect on GSH, SOD and CAT level

EDR replenished the GSH level in all doses and it was significant \((p < 0.001)\) in doses of 200 and 400 mg/kg. In the control group the SOD activity was 0.12 ± 0.11 (U/mg protein). SOD activities in treatment with different doses of EDR and reference were comparable and statistically significant. The dose dependent antioxidant activity of EDR has been suggested to play a role in the relief of long-term complications and the oxidative stress. CAT were 90 ± 12 (µmoleH\(_2\)O\(_2\)/mg of protein/minute) in control group rats while it was increased significantly \((p < 0.001)\) in rats pre-treated with EDR (200 and 400 mg/kg) (Table 2).

Effect on MPO, IL-6, TNF-α and NO level

The levels of MPO in gastric tissue were decreased and it was statistically significant in all doses of EDR \((p < 0.001)\). The level of TNF-α were decreased significantly \((p < 0.001)\) in group of rats pre-treated with EDR comparable to standard drug Lansoprazole (Fig 1).
Table 2: Effect of EDR on SOD, CAT and GSH in ethanol induced ulcer

| Treatment | SOD (U/mg protein) | CAT activity (µmole H₂O₂/mg of protein/minute) | GSH (µg/mg protein) |
|-----------|--------------------|-----------------------------------------------|--------------------|
| Control   | 0.12 ± 0.01        | 90 ± 12                                       | 0.89 ± 0.05        |
| EDR 100   | 0.21 ± 0.03        | 200 ± 30**                                   | 1.17 ± 0.07*       |
| EDR 200   | 0.24 ± 0.02*       | 250 ± 26***                                 | 1.52 ± 0.6**       |
| EDR 400   | 0.27 ± 0.02*       | 270 ± 32***                                 | 1.63 ± 0.09***     |
| Lansoprazole | 0.28 ± 0.05**     | 300 ± 40***                                 | 1.70 ± 0.08***     |

Values are expressed as mean ± SEM (n=6). ANOVA followed by Dunnett’s test with control group. *p < 0.01, **p < 0.01 and ***p < 0.001.

The EDR decreased the level of IL-6 in all groups of rats pre-treated with EDR and it were statistically significant (p < 0.01) at 100 mg/kg and (p < 0.001) at remaining dose level. NO level in gastric tissue was also significantly decreased (p < 0.001) in doses of EDR (200 and 400 mg/kg) and comparable to Lansoprazole (Fig 2).

Fig. 1: Effect of EDR on the level of MPO and TNF-α in gastric tissue of ethanol induced ulcer in rats. Bars are expressed as means ± SEM (n = 6). Analysis of variance followed by Dunnett’s test with control group; ***p < 0.001 were considered significant

Fig. 2: Effect of EDR on the level of IL-6 and NO in gastric tissue of ethanol induced ulcer in rats. Bars are expressed as means ± SEM (n = 6). Analysis of variance followed by Dunnett’s test with control group; **p < 0.01 and ***p < 0.001 were considered significant
Table 3: Effect of EDR on ulcer index and GSH level Cold restrain stress-induced ulcers in rats

| Treatment | Ulcer Index | Protection (%) | GSH (µg/g tissue) |
|-----------|-------------|----------------|-------------------|
| Control   | 11.83 ± 1.99 | -              | 130.5 ± 17.4      |
| EDR 100   | 6.23 ± 0.97* | 47.34          | 157 ± 12.4        |
| EDR 200   | 4.65 ± 1.12*** | 60.69       | 191 ± 10.56*      |
| EDR 400   | 1.86 ± 1.02*** | 84.28        | 213 ± 11.8***     |
| PGE₂      | 1.48 ± 1.36*** | 87.49        | 205.4 ± 12.3**    |

Values are expressed as mean ± SEM (n=6). ANOVA followed by Dunnett’s test with control group. Significance represented as *(p < 0.05), **(p < 0.01) and ****(p < 0.001)

Cold restrain stress-induced ulcer

As observed in Table 3, ulcerated regions in the gastric body after 4 h of cold restrain-stress was observed in normal saline treated animals with ulcer index of 11.83 ± 1.99. EDR at doses of 100, 200 and 400 mg/kg significantly reduced ulcer index by 47.34 % (p < 0.05), 60.69 % (p < 0.001) and 84.28 % (p < 0.001) respectively. PGE₂ (20 mg/kg; s.c.) administration also prevented the ulcer injury (p < 0.001). The GSH level were replenished by both EDR (200 and 400 mg/kg) and PGE₂ but EDR at dose 100mg/kg was unable to do so.

Effect of EDR on anti-oxidant assay

EDR exhibited strong antioxidant activity in the DPPH inhibition assay as evidenced by the low IC₅₀ value, 45.23 ± 3.23 µg/ml, were comparable to ascorbic acid’s IC₅₀ value, 41.54 ± 2.92 µg/ml.

DISCUSSION

Ethanol induced gastric ulcers are believed to arise as a result of stasis in gastric blood flow, which contributes to the development of the hemorrhagic and necrotic aspects of tissue injury [23]. Occurrence of these ulcers, which is predominant in the glandular part of the stomach, was reported to stimulate the formation of reactive oxygen species [24], resulting in damage to rat gastric mucosa [25]. The obtained results suggest that the EDR, exhibiting strong antioxidant and radical scavenging potential, has remarkably restricted ethanol-induced ulceration.

Neutrophil infiltration plays a very important role in the progression of injury and inflammation by aggregation and release of tissue-disrupting substance in various tissues, plus gastric mucosal lesions [26,27]. Prior study has established that neutrophil infiltration into gastric mucosal tissues is involved in the progress of acute gastric mucosal lesions [28]. The accumulation of neutrophil infiltration into the gastric mucosal tissues is estimated by MPO [29]. Furthermore, a reduction in the activity of MPO can be interpreted as a manifestation of the anti-inflammatory activity. In this study, neutrophil infiltration in the gastric mucosa was evidenced from the increased MPO activity in the ulcer control group, and pre-treatment of EDR showed a notable inhibition on the accumulation of MPO, revealing that EDR may possess gastroprotective properties through alleviating gastric mucosal inflammation.

Ethanol ingestion may activate the innate immune system leading to the release of the pro-inflammatory cytokines, such as TNF-α and IL-6 [30]. Previous findings implicate that the levels of IL-6 and TNF-α remarkably increased in the gastric tissue of ethanol-induced ulcer [31,32]. TNF-α, a representative inflammatory cytokine with pleiotropic functions, plays a key role in the process of inflammation. It is well known that TNF-α has disadvantageous effects, such as inducing tissue injury, inflammations as well as bacterial and viral infections. Thus, we believe that the inflammatory process, which happens as a part of the body’s natural defense against tissue damage, is generally associated with an increase in oxidative stress. This stress generates the reactive oxygen species and produces the lipid peroxidation which increases the MPO activity. The results of this study represent direct evidence that the EDR has a marked anti-inflammatory effects. These properties are achieved by a decrease in pro-inflammatory mediators, including myeloperoxidase, IL-6 and TNF-α, and a reduction in leukocyte activation [33,34].

Stress is associated with inactivation of mucosal prostaglandin syntheses by accumulating hydrogen peroxide, an endogenous pathway of prostaglandin synthesis inhibition, which also causes reactive oxygen species (ROS) generation [35]. It is well known that ROS deplete NP-SH levels in the gastric mucosa [3,36]. It is also interesting to note that EDR was able to significantly prevent depletion of NP-SH levels in the gastric mucosa compared to control rats, thus confirming the gastroprotective mechanism of EDR to be partly modulated by NP-SH replenishment.
The preliminary phytochemical screening of EDR revealed the presence of glycosides flavonoids, phenols and tannins. Previous studies have shown these compounds may be related to their free-radical scavenging and antioxidant properties and play a major role in the mechanism of gastroprotection [37]. It is, therefore, possible that the gastroprotective effects observed with the EDR may be attributable to its phytochemical constituents (secondary metabolites) and strong antioxidant activity.

**CONCLUSION**

The present results revealed that ethanol extract of *Delonix regia* protects gastric mucosa from acute gastric mucosal injury probably by its potent antioxidant action. *Delonix regia* warrants additional attention because it could represent a new interesting pharmacological tool for the treatment of acute erosive gastropathy.

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