ORIGINAL ARTICLE



Gastroprotective Potential of *Dalbergia sissoo* Roxb. Stem Bark against Diclofenac-Induced Gastric Damage in Rats

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Received: August 20, 2013 Revised: September 4, 2013 Accepted: September 5, 2013

KEYWORDS:

antioxidant, antiulcer, Dalbergia sissoo, gastric mucosa, lipid peroxidation

Abstract

Objectives: Dalbergia sissoo Roxb. stem bark possesses anti-inflammatory, antipyretic, and antioxidant properties. This plant is used traditionally in the Indian system of medicine to treat emesis, ulcers, leucoderma, dysentery, stomach complaints, and skin disorders. This study was conducted to evaluate the antiulcer effects of *D. sissoo* stem bark methanol extract (DSME) against the diclofenac sodium-induced ulceration in rat.

Methods: The DSME (200 mg/kg and 400 mg/kg body weight) was orally administered to rats once a day for 10 days in diclofenac-treated rats. The gastroprotective effects of DSME were determined by assessing gastric-secretory parameters such as volume of gastric juice, pH, free acidity, and total acidity. Biochemical studies of gastric mucosa were conducted to estimate the levels of nonprotein sulfhydryls (NP-SHs), lipid peroxidation [thiobarbituric acid reactive substances (TBARSs)], reduced glutathione (GSH), hydrogen peroxide (H₂O₂), levels of scavenging antioxidants, catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), glutathione-S-transferase (GST), and myeloperoxidase (MPO). Moreover, adherent mucus content and histological studies were performed on stomach tissues.

Results: Administration of DSME significantly decreased the ulcer index, TBARSs, H_2O_2 , and MPO activity in gastric mucosa of the ulcerated rats. Activities of enzymic antioxidants, CAT, SOD, GSH-Px, GST and GSH, and NP-SH contents were significantly increased with DSME administration in the gastric mucosa of diclofenac-treated rats. Volume of gastric juice, total and free acidity were decreased, whereas pH of the gastric juice was increased with the administration of DSME + diclofenac. Our results show that DSME administration is involved in the prevention of ulcer through scavenging of free radicals. Results of histopathological studies supported the gastroprotective activities of DSME.

Conclusion: The results of this study showed that DSME exhibit potential gastroprotective activity probably due to its antioxidant and cytoprotection ability.

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1. Introduction

Nonsteroidal anti-inflammatory drugs (NSAIDs) such as diclofenac sodium [Sodium {2-[(2,6-dichlorophenyl)amino]phenyl}acetate] is used to induce ulcer in animal models. In humans, chronic administration of diclofenac sodium for the treatment of various diseases such as rheumatoid and osteoarthritis induces gastric ulcer in 35–60% of patients [1]. In general, diclofenac sodium is prescribed for its analgesic, antipyretic, and anti-inflammatory properties; its action is mediated by inhibition of the biosynthesis of prostaglandins, cyclooxygenase, and leukotriene [2].

Diclofenac sodium induces gastric mucosal lesions because of its acidic properties. A highly acidic gastric environment favors the migration of nonionized lipophilic diclofenac sodium into the epithelial cells, and at the surface these are dissociated into ions, trapping hydrogen ions and inducing mucosal injury. This action is further enhanced by the decrease of the following: mucosal blood flow, secretion of mucous and bicarbonates, and the defensive factors of the gastric layer [3]. Diclofenac sodium is also suggested to be involved in oxidative stress in mucosal cells, another etiopathogenic factor inducing gastric ulcer. All these factors cause an imbalance between the acid—pepsin secretion and defensive factors including secretion of mucin and shedding of cells [4].

The effect of diclofenac can be minimized by the proper use of antioxidants that ameliorate the free radicals. Plants possess valuable phytochemicals in the form of secondary metabolites of which flavonoid and phenolics are of great concern for antioxidant properties. In recent years, studies on antioxidants have received much attention as these chemicals can help defend the biological systems from diseases and injuries. The traditional drugs used in the treatment of a gastric ulcer are histamine (H₂) receptor antagonists, proton-pump inhibitors, antacids, and anticholinergics. Most of these drugs have severe unwanted side effects and drug interactions [5]. However, alternative and complementary systems in medicament can provide additional therapeutics for gastric damages.

Dalbergia sissoo is native to Pakistan, India, Bangladesh, Nepal, and Afghanistan. Chemical characterization of *D. sissoo* bark revealed the presence of flavonoids, furans, benzophenone, styrenes, and terpenoids [6]. Its bark exhibits anti-inflammatory, antipyretic, and antioxidant properties [7]. This plant is traditionally used to treat emesis, ulcers, leucoderma, dysentery, stomach complaints, and skin disorders [8].

To the best of our knowledge no experimental evidence is available to prove the gastroprotective effect of *D. sissoo* stem bark extract. This study was undertaken to evaluate the antiulcer effects of crude methanol extract of *D. sissoo* (DSME) stem bark on a diclofenac sodium-induced gastric ulcer in rats.

2. Materials and methods

2.1. Plant collection and extract preparation

Shade-dried bark (2 kg) of *D. sissoo* collected in September 2010 from the Sargodha district (Pakistan) was mechanically grinded into a powdered form and extracted twice in 4 L of 95% methanol for 1 week. The filtrates obtained were combined and evaporated through rotary vacuum evaporator to get 7.36% (147.25 g) of DSME and were stored at 4 °C.

2.2. Animal treatment

Twenty-five Sprague-Dawley rats of either sex with weight ranging from 150 g to 200 g were acclimatized for 2 weeks in ordinary cages at a room temperature of 25 ± 3 °C with a 12-hour dark/light cycle. Use of animals for all experimental procedures was conducted in accordance with the guidelines of the National Institutes of Health (Islamabad, Pakistan). The study protocol was approved by the Ethical Committee of Quaid-i-Azam University (Islamabad, Pakistan).

Animals were divided into five groups with five rats in each group. All animal groups were fasted for 12 hours prior to each administration. Rats in Group I were untreated (control) and had free access to food materials. Diclofenac sodium [50 mg/kg body weight (bw)] was intragastrically administered to animals of Groups II, III, and IV once a day for 10 days. However, rats of Groups III and IV were also administered with 200 mg/kg and 400 mg/kg bw of DSME once a day for 10 days. Animals of Group V were treated with 400 mg/kg bw of DSME alone [9].

2.3. Pyloric ligation

Twenty four hours after the last treatment, pyloric ligation was done for 4 hours to collect the gastric juice. The animals were anesthetized, the abdomen was opened by making a small midline incision, and the pyloric stomach was ligated with a thread by avoiding damage to its blood supply. The abdominal wall was closed by interrupted sutures.

2.4. Determination of acid-secretory parameters

The animals did not have access to both food and water during the postoperative period, and were killed after 4 hours of pyloric ligation. The stomach was dissected out along the greater curvature, the gastric juice was drained off and centrifuged at 4000 rpm for 10 minutes. The volume of gastric juice (mL/100 g/4 hours) and pH were estimated. Free acidity and total acidity were estimated according to Card and Marks [10].

2.5. Ulcer index studies

For ulcer index studies, any damage to gastric mucosa, bulging, and/or inflammation were recorded (in millimeter) for each lesion in the stomach [11].

2.6. Histopathological studies

Gastro-mucosal tissues from animals of all the groups were isolated and stored in fixative sera for histological analysis. Thin sections of $4-5 \mu m$ were stained in hematoxylin–eosin stain and examined under a microscope.

2.7. Gastro-mucosal studies

The stomach was washed in ice-cold saline, dried with blotting paper, and weighted. One portion of the stomach was used to collect the mucosa, which was immediately frozen in liquid nitrogen and stored at -70 °C for the determination of different parameters. Mucosa (100 mg) was homogenized in Tris—HCl buffer (0.1 M, pH 7.4) at 4 °C, and centrifuged at 12,000 g for 30 minutes. The supernatant obtained was used for the analysis of biochemical parameters. The total soluble-protein estimation of the mucosa was determined according to the procedure suggested by Lowry et al [12]. The second portion of the stomach was used for the estimation of barrier mucus [13].

2.8. Estimation of nonprotein sulfhydryl groups and myeloperoxidase

Nonprotein sulfhydryl (NP-SH) groups were determined according to a previously described method [14], and Krawisz et al's [15] method was applied to measure the myeloperoxidase (MPO) activity in the gastric mucosa of the rats.

2.9. Assessment of tissue biochemical studies

Catalase (CAT) activity was determined by following the method suggested by Chance and Maehly [16], whereas superoxide dismutase (SOD) activity was determined as suggested by Kakkar et al [17]. The activities of gammaglutamyl transpeptidase (γ -GT), glutathione-S-transferase (GST), and glutathione peroxidase (GSH-Px) were estimated as suggested previously [18–20]. The level of lipid peroxidation in gastric tissues was carried out following the protocol of Iqbal et al [21]. The amount of reduced glutathione (GSH) in each sample was assessed by following the protocol of Jollow et al [22].

2.10. Statistical analysis

The values obtained were analyzed for mean and standard error and were subjected to *post hoc* comparison by least significance difference (LSD) at 0.05% level of probability.

3. Results

3.1. Effect of DSME on gastric ulcer and histopathology in rats

Treatment with diclofenac sodium causes extensive gastric erosions on the glandular mucosa with an ulcer index of 14.9 \pm 0.7. By contrast, post-treatment with

DSME (200 mg/kg and 400 mg/kg bw) caused a dose-related reduction in ulceration (7.4 \pm 0.6 and 2.6 \pm 0.3, respectively; Table 1). All animals treated with DSME alone did not show gastric damage. Treatment with diclofenac sodium induced severe damage to the gastric mucosa as revealed by deep alteration of glandular epithelium (Figure 1). The lesion exhibited infiltration of leucocytes, abundant granulation, and intense inflammation. Administration of DSME along with diclofenac prevented the intense damage and inflammation.

3.2. Effect of DSME on mucus weight, NP-SH, and MPO activity in gastric mucosa

The quantity of bound mucus on the glandular mucosa and NP-SH contents in gastric mucosa were depleted significantly (p < 0.05), whereas the MPO activity was enhanced by diclofenac treatment when compared with the control samples (Table 1). Posttreatment with DSME at both doses augmented (p < 0.05) these parameters in a dose-dependent manner.

3.3. Effect of DSME on antioxidant enzymes

The activities of CAT and SOD in the gastric tissues were significantly lowered in the diclofenac group as compared with the control group (Table 2). Treatment of rats with diclofenac sodium along with DSME increased (p > 0.05) the activity of CAT and SOD in the gastric tissues in a dose-dependent way as compared with the diclofenac sodium-treated group. Levels of GST and GSH-Px in the gastric tissues were decreased, whereas γ -GT level was increased significantly (p < 0.05) after diclofenac sodium treatment when compared with that of the control group. However, DSME treatment in combination with diclofenac sodium dose dependently restores the level of GST, GSH-Px, and γ -GT in the gastric tissues. Treatment of DSME alone did not change the activity level of these antioxidant enzymes as compared with the control group.

3.4. Effect of DSME on GSH, lipid peroxidation, and H_2O_2

Diclofenac sodium treatment to rats significantly (p < 0.05) decreased the level of GSH, an antioxidant substance, whereas it increased the level of thiobarbituric acid reactive substances (TBARSs), an indicator of lipid peroxidation, and H₂O₂ in the gastric tissues as compared with the control group (Table 3). The levels of TBARSs and H₂O₂ were significantly (p < 0.05) lowered in the gastric tissues with the combined treatment of diclofenac sodium and DSME compared to the group administered with only diclofenac sodium. Equally, co-treatment of DSME and diclofenac sodium increased the level of GSH content, dose dependently, in the gastric tissues in the group

Treatments	Lesion index	Bound mucus $(\mu M/g \text{ tissue})$	NP-SH (µM/g tissue)	Myeloperoxidase (U/g tissue)
Control	0.0*	48.56 ± 3.76*	$1.64 \pm 0.23^{*}$	9.24 ± 1.34*
Diclofenac (50 mg)	$14.9\pm0.7^{\dagger}$	$24.63\pm2.65^\dagger$	$0.87\pm0.17^{\dagger}$	$16.78\pm1.65^\dagger$
Diclofenac + DSME (200 mg)	$7.4\pm0.6^{*,\dagger}$	$36.86 \pm 2.43^{*,\dagger}$	$1.25\pm0.18^{\dagger}$	$14.54 \pm 1.25^\dagger$
Diclofenac + DSME (400 mg)	$2.6\pm0.3^{*,\dagger}$	$45.76 \pm 2.68*$	$1.48 \pm 0.21*$	$10.21 \pm 0.86*$
DSME (400 mg)	0.0*	$50.32 \pm 2.76*$	$1.68 \pm 0.28*$	$8.76 \pm 0.46*$

Table 1. Effect of methanol extract of Dalbergia sissoo stem bark on gastric ulcer in rats.

Data are presented as mean \pm standard deviation (n = 6). * Indicates significance at p < 0.05 from the diclofenac-treated group. [†] Indicates significance at p < 0.05 from the control group. DSME = D. sissoo stem bark methanol extract; NP-SH = nonprotein sulfhydryl.



Figure 1. Hematoxylin and eosin stain. (A) Control rats showing normal histology with no necrosis of the surface epithelium as well as the absence of edema and leukocyte infiltration. (B) Section showing severe epithelial surface disruption and edema of the submucosal layer with leukocyte infiltration in diclofenac sodium (50 mg/kg bw)-treated rats. (C) Rats treated with diclofenac + DSME (50 mg/kg and 200 mg/kg bw) show normal histology with repaired serosa and subserosa layers. (D) Rats treated with diclofenac + DSME (50 mg/kg and 400 mg/kg bw) showing almost normal histopathology. (E) Rats treated with DSME (400 mg/kg bw) showing normal histology. bw = body weight; DSME = *Dalbergia sissoo* stem bark methanol extract.

Treatments	CAT (U/min)	SOD (U/mg protein)	GSH-Px (nM/mg protein)	GST (nM/mg protein)
Control	$2.5\pm0.6*$	$16.8 \pm 2.1*$	$39.9\pm8.3^{*}$	$157.8 \pm 7.9*$
Diclofenac (50 mg)	$0.9\pm0.7^{\dagger}$	$10.0\pm1.5^{\dagger}$	$32.4\pm3.2^{\dagger}$	$135.0 \pm 11.6^{\dagger}$
Diclofenac + DSME (200 mg)	$1.1\pm0.4^{\dagger}$	$13.4 \pm 1.0^{\dagger}$	$38.5 \pm 7.4*$	$143.8\pm7.7^{\dagger}$
Diclofenac + DSME (400 mg)	$1.5\pm0.5^{\dagger}$	$14.4\pm0.7^{*,\dagger}$	$40.7 \pm 8.7*$	$148.1 \pm 4.4^{*,\dagger}$
DSME (400 mg)	$2.2\pm0.4*$	$16.3 \pm 1.4*$	$37.5 \pm 12.0*$	$156.9 \pm 7.3*$

Table 2. Effect of methanol extract of Dalbergia sissoo bark on CAT, SOD, GSH-Px, and GST in gastric tissues.

Data are presented as mean \pm standard deviation (n = 6). * Indicates significance at p < 0.05 from the diclofenac-treated group. [†] Indicates significance at p < 0.05 from the control group. CAT = catalase; DSME = *D. sissoo* stem bark methanol extract; GSH-Px = glutathione peroxidase; GST = glutathione-*S*-transferase; SOD = superoxide dismutase.

treated with diclofenac sodium alone. The DSME treatment alone did not statistically change the level of GSH, TBARSs, and H_2O_2 in the gastric tissues when compared with the controls.

3.5. Effect of DSME on acid-secretory parameters

Table 4 depicts the level of acid-secretory parameters in the gastric juice of control and experimental groups of rats. Diclofenac sodium treatment showed a significant increase in gastric volume, free acidity, and total acidity with a significant decrease in pH compared with control animals. However, the co-treatment of diclofenac sodium + DSME significantly decreased the gastric volume, free acidity, and total acidity, and increased the pH when compared with diclofenac sodium-ulcerated rats. There were no significant alterations in DSMEonly-administered rats in gastric volume, free acidity, total acidity, and pH level.

4. Discussion

Gastroprotective activity of DSME has been evident at various levels in this experiment. Ulcer induction with diclofenac sodium treatment was significantly (p < 0.05) decreased by the simultaneous administration of DSME. Similar results have been reported in previous studies as well [23]. Presence of various secondary metabolites, such as flavonoids, terpenoids, tannins, and phenolic compounds exhibit diversified biochemical and pharmacological activities including antioxidant and antitumor properties [24]. Moreover, these phytochemicals possess the ability to interact with other molecules, such as proteins and polysaccharides to form an impervious microlayer on the ulcer site by precipitating the microproteins, thereby protecting the underlying tissues from toxins and other irritants [25].

Exposure of rats to diclofenac sodium may induce an overwhelming generation of free radicals resulting in a significant (p < 0.05) decrease in gastric NP-SH contents and depletion (p < 0.05) of CAT, SOD, GSH-Px, and GST in mucosal tissues. The results presented in this study corroborate with earlier reports where NSAID was reported to induce a significant depletion of SHs in gastric lesions [26]. These enzymes are endogenous defenses, which are primarily involved in maintaining the integrity and physiology of tissues. The SODs are very crucial in eliminating the superoxide radicals by converting them into H2O2 and are catalytically converted by CAT into ground-state oxygen and hydroxyl radicals, whose accumulation can play a critical role in the pathophysiology of ulceration. The protective potential of DSME to augment antioxidant enzymes against the diclofenac-induced toxicity indicates its possible preventive ability in the amelioration of gastric lesions involving free-radical reactions probably by the mediation of SH contents [26].

The GSH is a remarkable endogenous antioxidant, whose activity remarkably decreased in this investigation. It is used as a cofactor in the removal of hydrogen peroxide and lipoperoxides by the GSH-Px family

Table 3. Effect of methanol extract of *Dalbergia sissoo* bark on GSH, TBARSs, H_2O_2 , and γ -GT in gastric tissues.

	GSH	TBARSs	H_2O_2	γ-GT
Treatments	(nM/mg tissue)	(nM/mg protein)	(nM/min/mg tissue)	(U/mg protein)
Control	$76.8\pm6.7*$	$55.1\pm4.5^{*}$	$0.90 \pm 0.25^{*}$	$0.20\pm0.07*$
Diclofenac (50 mg)	$59.7 \pm 6.2^{\dagger}$	$71.0\pm5.3^{\dagger}$	$1.76\pm0.50^{\dagger}$	$0.44\pm0.07^{\dagger}$
Diclofenac + DSME (200 mg)	$65.7\pm6.4^\dagger$	64.9 ± 4.2	$1.34 \pm 0.61^{*,\dagger}$	$0.38\pm0.09^{\dagger}$
Diclofenac + DSME (400 mg)	$73.3 \pm 5.9*$	$60.9 \pm 3.4*$	$1.21 \pm 0.12*$	$0.31\pm0.09^{*,\dagger}$
DSME (400 mg)	$75.0 \pm 5.2*$	$54.9 \pm 5.2^{*}$	$0.99 \pm 0.32^{*}$	$0.25 \pm 0.06*$

Data are presented as mean \pm standard deviation (n = 6). *Indicates significance at p < 0.05 from the diclofenac-treated group. [†]Indicates significance at p < 0.05 from the control group. DSME = D. sissoo stem bark methanol extract; GSH = glutathione; H₂O₂ = hydrogen peroxide; TBARSs = thiobarbituric acid reactive substances; γ -GT = gamma-glutamyl transpeptidase.

Treatments	Gastric volume (mL/100 g/4 h)	рН	Free acidity (mEq/1/100 g)	Total acidity (mEq/1/100 g)
Control	$2.32\pm0.37*$	$4.5 \pm 0.12^{*}$	$40.30 \pm 2.12^*$	$69.40 \pm 7.92*$
Diclofenac (50 mg)	$3.67\pm0.61^{\dagger}$	$2.1 \pm 0.17^{\dagger}$	$60.13 \pm 2.45^{\dagger}$	$86.68 \pm 4.96^\dagger$
Diclofenac + DSME (200 mg)	$3.21 \pm 0.43^{*,\dagger}$	$3.6 \pm 0.51*$	$47.66 \pm 3.64*$	$67.60 \pm 6.85^*$
Diclofenac + DSME (400 mg)	$2.74\pm0.42^{*,\dagger}$	$4.0 \pm 0.38*$	$39.41 \pm 1.37*$	$56.67 \pm 6.32^{*,\dagger}$
DSME (400 mg)	$2.27\pm0.32*$	$4.5\pm0.24*$	$36.65 \pm 2.35*$	$52.71 \pm 7.56^{*,\dagger}$

Table 4. Effect of methanol extract of *Dalbergia sissoo* bark on gastric volume, pH, free acidity, and total acidity in gastric juice of rats.

Data are presented as mean \pm standard deviation (n = 6). *Indicates significance at p < 0.05 from the diclofenac-treated group. [†]Indicates significance at p < 0.05 from the control group. DSME = D. sissoo stem bark methanol extract.

during which it is converted into the oxidized form of glutathione (GSSG). Availability of GSH is crucial for the integrity of mucosa whereas its depletion causes severe ulceration. The protective effects of DSME in maintaining the GSH levels toward control have rendered the restoration of steady state of GSH and/or its synthesis, which increases the endogenous efficacy for oxidative stress induced by diclofenac sodium in the gastric mucosa of rats [27].

Quantification of MPO activity in the gastric mucosa provides another approach for the detection of diclofenac-induced tissue damage. We obtained significant (p < 0.05) increase in MPO activity with diclofenac sodium in gastric mucosa samples against the respective control samples. As a response to NSAID-induced inflammation, neutrophils are stimulated, which results in the release of MPO and other tissue-damaging substances in the extracellular space [28]. The results from this study indicate that dimethyl sulfoxide exhibits a dose-dependent decrease of MPO in gastric tissues.

Secretion of acid from gastric mucosa, its pH, and acidity are critically involved in the development of an ulcer. Diclofenac sodium induced the higher secretion of gastric acid, decrease in pH, and increase in acidity in ulcerated rats. Developing drugs that accelerate and improve the quality of ulcer healing is very important. The DSME provoked a marked increase in pH, whereas it decreased the volume of acid and acidity, thus restoring a balance highly desirable for antiulcer effects in ulcerated rats [27].

In conclusion, the results of this study suggest that diverse phytochemicals present in DSME might synergistically offer gastrointestinal protection at different levels such as scavenging of free radicals, restoration of enzymic antioxidants, cytoprotection, as well as provide barriers against NSAIDs.

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