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Gastroprotective Effects of Methanolic Leaves and Stem Extracts of *Sphagneticola trilobata* on Indomethacin-Induced Gastric Ulcer in Rats

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The medicinal plant *Sphagneticola trilobata* native to South America is used in local folk medicine to treat inflammation and analgesics. In present study, gastroprotective effects of methanolic extracts of *S. trilobata* leaves and stem were investigated in indomethacin-induced gastric ulcers in rats at doses of 200 mg/kg for leave extract and 350 mg/kg for stem extract. Indomethacin produced stomach ulcers and increased neutrophil percentage and MDA levels compared to the control group ($p < 0.001$). Co-administration of indomethacin and omeprazole, methanolic extracts of leaves (200 mg/kg) ($p < 0.001$) and methanolic extracts (350 mg/kg) ($p < 0.05$) of stem compared to indomethacin group to ulcers was low ($p < 0.001$). Methanolic extracts (200 mg/kg) of leaves and methanolic extracts (350 mg/kg) of stem reduced MDA levels ($p < 0.001$). Methanolic extracts (200 mg/kg) of leaves and methanolic extracts (350 mg/kg) of the stem significantly decreased neutrophil percentage compared to indomethacin group ($p < 0.001$). The results suggest that the methanolic extracts of *Sphagneticola trilobata* leaves and stem have a protective effect on indomethacin-induced gastric ulcers.

Keywords: *Sphagneticola trilobata*, Indomethacin Induced ulcer, Peptic ulcer, Gastro protection, Histopathology.

INTRODUCTION

NSAIDs are non-steroidal anti-inflammatory drugs. All drugs in this class have analgesic, antipyretic and anti-inflammatory actions in various measures. They are more commonly employed and are on-the-counter drugs. These show their activity due to prostaglandins (PG) synthesis inhibition [1]. However, these drugs have some side effects, such as gastric mucosal damage, bleeding, renal blood flow limitation, delayed/prolonged labour, asthma and anaphylactic reaction in suspected individuals [2]. Peptic ulcer also caused due to NSAIDs and it occurs in the part of the gastrointestinal tract that comes in contact with gastric acid and pepsin, *i.e.* stomach and duodenum [3].

The primary mechanism for NSAID damage to gastroduodenal mucosa involves systemic prevention of expressed cyclooxygenase 1 (COX-1)-derived prostaglandins [4]. COX inhibition by NSAIDs increases the synthesis of leukotrienes by pushing arachidonic acid metabolism towards the 5-lipoxygenase (5-LOX) pathway. Cysteinyl leukotrienes (CysLTs), such as LTC4, LTD4 and LTE4 are the primary inflammatory lipid mediators and metabolites of the 5-LOX pathway [5]. LTB4 is a principal factor in several neutrophile activities like adherence and chemotaxis. Additionally, leukotrienes are involved in gastric mucosal complications by triggering tissue ischemia and inflammation [6]. The other mechanisms include the generation of reactive oxygen species (ROS) and nitric oxide (NO), the beginning of lipid peroxidation and penetration of neutrophils secondary to the generation of inflammatory factors [7]. As a strong beneficial NSAID, indomethacin has been considered a drug of choice to induce gastric ulcers [8]. Indomethacin changes the arachidonic acid metabolism in neutrophils by inhibiting COX pathways, which directs this metabolism to the 5-LOX pathway, leading to an increase in leukotriene levels [9]. This drug through a neutrophil-dependent process, accumulation and neutrophil adhesion and inducible nitric oxide synthase (iNOS) as a producer of NO, initiates and advances gastric damages [10].

In recent years, the gastroprotective effects of various plants and their secondary metabolites were reported. In this context, *Sphagneticola trilobata* (L.), Pruski (*S. trilobata*) Asteraceae

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is a creeping evergreen perennial herb [11,12]. In South America, it is used to treat colds, flu, fever and inflammation [13]. In folk medicine, *S. trilobata* is used to treat back pain, muscle cramps, gout, stubborn wounds, sores and swelling and painful joints of arthritis [14]. The methanolic extracts of the aerial part of *Sphagneticola trilobata* possess a wide range of activity like anti-inflammatory activity [15], antidiabetic activity [16], antileishmanial activity [17], antioxidant activity [18], hepatoprotective activity [19], antimicrobial activity [20], antineoplastic activity [21], neuropharmacological activity [22] and anticarcinogenic activity [23], *etc*.

Literature survey on this plant also suggests that *Sphagneticola trilobata* shows antioxidant activity [18], which is essential for gastric protection by eliminating the irritant species causing gastric mucosal damage. The literature also shows the anti-inflammatory and wound healing activity of *Sphagneticola trilobata,* which is also important in treating gastric peptic ulcers, which cause inflammation and bleeding in the lining of the gastric mucosa. However, no reports were found on the antiulcerogenic activity of methanolic leaves and stem extract of *S. trilobata*. Thus, the main aim of the present study was to evaluate the gastroprotective effect of methanolic lextracts of *S. trilobata* of eaves and stem on indomethacin-induced gastric ulcer lesions in rats.

EXPERIMENTAL

Plant collection: *Sphagneticola trilobata,* which grows as a weed, were collected from Noida Institute of Engineering and Technology (Pharmacy Institute), Greater Noida, India, in October 2020. The plant parts were washed with water and shade dried at ambient temperature for 4-5 days. Dried pieces of the plant were powdered for disintegration using a grinder and stored in an air-dried container in a cool and dry place to prevent the sample from any potential contamination [24]. A voucher plant specimen (NIET/Pharmacy Institute/R&D/07) was preserved and authenticated by the Botanical Garden of the Indian Republic, Noida, India.

Extraction: The dried powder of leaves and stem of plant *Sphagneticola trilobata* was extracted by methanol using the Soxhlet apparatus. The extraction was conducted in 800 mL of methanol for 15 h/solvent. Each crude extract was filtered and dried using a rotatory evaporator at 60 ºC and stored in a cooled and dry place until further use in the experiments [25].

Animals: A total of 30, 150-200 g weighted albino-Wistar rats and 15-20 g Swiss albino mice have been used for the experiment. The animals were provided from the Central Animal Facility of Noida Institute of Engineering and Technology, Greater Noida, India. The Ethics Committee approved the experiment of the Institutional Animal Ethics Committee having protocol no. IAEC/NIET/2020/01/20. The animals were grouped before experiments and kept under standard conditions.

Acute toxicity studies: To determine the acute toxicity of methanolic extract of *S. trilobata* of leaves and stem, according to OECD guidelines 423 applying toxic doses, mortality doses and safe non-toxic doses. The test drug will be administered orally at an initial dose of 300 mg/kg. If mortality is observed, the procedure will be repeated with a low dose of 50 and 5 mg/kg. If no mortality is observed, the procedure will be repeated with a high dose of 2000 mg/kg. The dose at which mortality is observed in two out of three mice would be considered a toxic dose. Toxic manifestations include changes in water or food intake, respiration, observed for 6 h and mortality for 24 h [26].

Indomethacin-induced gastric damage: *S. trilobata* on indomethacin-induced gastric damage in this series of experiments. The protective effects of methanolic extracts were compared with the proton pump inhibitor, omeprazole. The animals were divided into five groups, each consisting of six rats. Leaves extract 200 mg/kg (dose calculated after acute toxicity studies), stem extract 350 mg/kg (dose calculated after acute toxicity studies) and omeprazole 20 mg/kg body weight, doses prepared by suspending in normal saline solution. One group was administered with only normal saline solution 10 mL/kg to determine whether this solution has gastroprotective effects. One group is a negative control group on which only indomethacin 25 mg/kg is administered. All the doses are administered orally [27].

After 14 days, the pretreatment through leaves and stem extract, normal saline and omeprazole administrations orally, rats are kept at fasting for 24 h then indomethacin 25/kg body weight was administered to animals orally. After 6 h of administration of indomethacin to all 5 groups, animals were sacrificed using ketamine 80-100 mg/kg. The abdomen was removed and opened with greater curvature and then washed with serum physiological solution. The width of the ulcer areas was determined using a magnifier and millimeter paper. The protective effect of aryl extract was compared with the results obtained from the indomethacin and omeprazole groups.

Percentage of neutrophils in blood: After the six hours administration of indomethacin, blood samples were collected from cardiac puncture and transferred to heparin vials and sent to the laboratory to determine neutrophil counts [28].

Determination of macroscopic gastric ulcer score: To determine the antiulcer score leaves extract, stem extract, omeprazole and normal saline was administered orally to rats for 14 days before the oral administration of indomethacin. For the experiment day, the rats did not feed food for 24 h and water for 4 h. Then indomethacin was administered orally. Rats were sacrificed after 6 h of indomethacin administration and the stomach was removed. The stomach was removed and opened along with greater curvature and then washed with serum physiological solution.

Finally, each stomach was placed in a separate tube and kept at a temperature of -80 ºC for other experiments [29].

Histopathological evaluation: The tissues were fixed in 10% buffer formalin solution for 24 h for histological evaluation. After fixation, tissue samples were immersed in paraffin. Sections that were 5 µm thick were cut from paraffin-submerged tissue samples and placed on positively charged slides. The samples underwent decentralization and rehydration and were visualized with Meyer's hematoxylin and eosin. Sections were examined under the microscope for histopathological changes using a light photomicroscope [30].

Measurement of lipid peroxidation indices (malondialdehyde (MDA) level): First, 10% homogenate of the stomach tissue was mixed with 1.15% KCl in a container of ice. Then 0.5 mL of it was mixed with 3 mL of phosphoric acid, 1% and 1 mL of thiobarbituric acid (TBA), which was 0.6 % in some tubes. The tubes were placed in boiling water for 45 min and after cooling, 4 mL of *n*-butanol was added to each tube to remove the coloured complex and after that, they were vortexed for 1 min. Finally, the tubes were placed inside the centrifuge at 4000 rpm for 20 min at 4 ºC. After completion of centrifugation, the supernatant was separated and the absorbance was read with a spectrophotometer at 532 nm. The standard curve was drawn up in a concentration range of 0-100 nmol/mL for MDA and the concentrations were calculated and reported as nmol/g tissue value [31].

Statistical analyses: Statistical calculations were performed using Graph Pad Prism 9.0.0.121 software. To determine the statistical significance of the results, one-way variance analysis (ANOVA) was applied. Differences between groups were considered significant ($p < 0.05$).

RESULTS AND DISCUSSION

Acute toxicity determination: Observations included changes in the skin, fur, eyes and mucous membranes. The presence of toxicity related to the central nervous system, the cardiovascular system and the autonomic nervous system such as tremors, convulsions, sedation, stereotypic behaviour, saliva, diarrhea, posture, gait, limb paralysis, lethargy, sleep, coma and mortality (Table-1). The results revealed no treatment-related death or signs of toxicity in the treated animals in all the doses throughout the study. Bodyweight gain of both male and female mice was also observed (Table-2) compared with before and after treatment of the experimental groups. Further, there were no gross pathological abnormalities, which prove the doses for the methanolic extracts of leaves and stem of *S. trilobata* were found to be 200 and 350 mg/kg, respectively.

Gastric ulcer index: The gastric ulcer index was determined by evaluating the ulcer score on the stomach mucosal wall. Indomethacin administration increases the ulcer index and pathological score values in the stomach tissues compared with the control group. The methanolic extracts of leaves and stem of *S. trilobata* significantly reduce the ulcer index compared with the indomethacin group (Table-3). The macroscopic observation and scores of ulcers are shown in Fig. 1.

Percentage of neutrophils in blood: Exposure to indomethacin remarkably increases the percentage of blood neutrophils compared with the control group. Pretreatment with methanolic extracts of leaves and stem of *S. trilobata* at the dose of 200 and 350 mg/kg, respectively, significantly decrease the blood neutrophils compared with the indomethacin group. The results of methanolic leaves and stem of *S. trilobata* extract on the percentage of blood neutrophils after gastric ulcers induced by indomethacin are shown in Table-4.

TABLE-1
CLINICAL OBSERVATION OF MICE OF METHANOLIC EXTRACTS OF *Sphagneticola trilobata*

Sign and symptoms	Methanolic extract of leaves (200 mg/kg)			Methanolic extract of stem (350 mg/kg)		
	Day 1	Day 7	Day 14	Day 1	Day 7	Day 14
Behaviour	Normal	Normal	Normal	Normal	Normal	Normal
Somatomotor activity	Normal	Normal	Normal	Normal	Normal	Normal
Skin and fur	Normal	Norma	Norma	Norma	Normal	Normal
Eye and muscle membrane	Normal	Norma	Norma	Norma	Normal	Normal
Salivation	Absent	Absent	Absent	Absent	Absent	Absent
Diarrhea	Absent	Absent	Absent	Absent	Absent	Absent
Tremors/convulsions	Absent	Absent	Absent	Absent	Absent	Absent
Death	Nil	Nil	Nil	Nil	Nil	Nil
Other symptoms	Nil	Nil	Nil	Nil	Nil	Nil

TABLE-2
EFFECT OF METHANOLIC EXTRACT ON BODY WEIGHT, FOOD CONSUMPTION AND NECROPSY OF MICE

Animals	Body weight (g)			Food consumption (g)			Observed lessons during study
	Day 1	Day 7	Day 14	Day 1	Day 7	Day 14	Day 14
Leaves extract (200 mg/kg)	25.2 ± 1.23	27.6 ± 1.12	30.5 ± 1.20	4.56 ± 0.82	4.78 ± 1.12	5.12 ± 0.56	Nil
Stem extract (350 mg/kg)	24.5 ± 0.96	28.6 ± 1.08	29.6 ± 0.05	4.52 ± 0.48	4.96 ± 1.03	5.23 ± 0.85	Nil

TABLE-3
GASTROPROTECTIVE EFFECT OF DIFFERENT GROUPS ON INDOMETHACIN-INDUCED GASTRIC ULCERS IN RATS

Treatment	Dose (mg/kg)	Animal of number	Ulcer index (mm^2)[a]	Inhibition (%)[b]	*p*
Normal saline	–	6	0.00 ± 0.00	100	≥ 0.05
Indomethacin	25	6	30.74 ± 6.98	-	< 0.05
Omeprazole	20	6	3.63 ± 3.62	88.4	< 0.05
Methanolic leaves extract	200	6	6.23 ± 3.44	80.3	< 0.05
Methanolic stem extract	350	6	8.18 ± 2.14	72.6	< 0.05

[a]Average values of indomethacin-induced gastric damage; [b]% Inhibition was based on the indomethacin group.

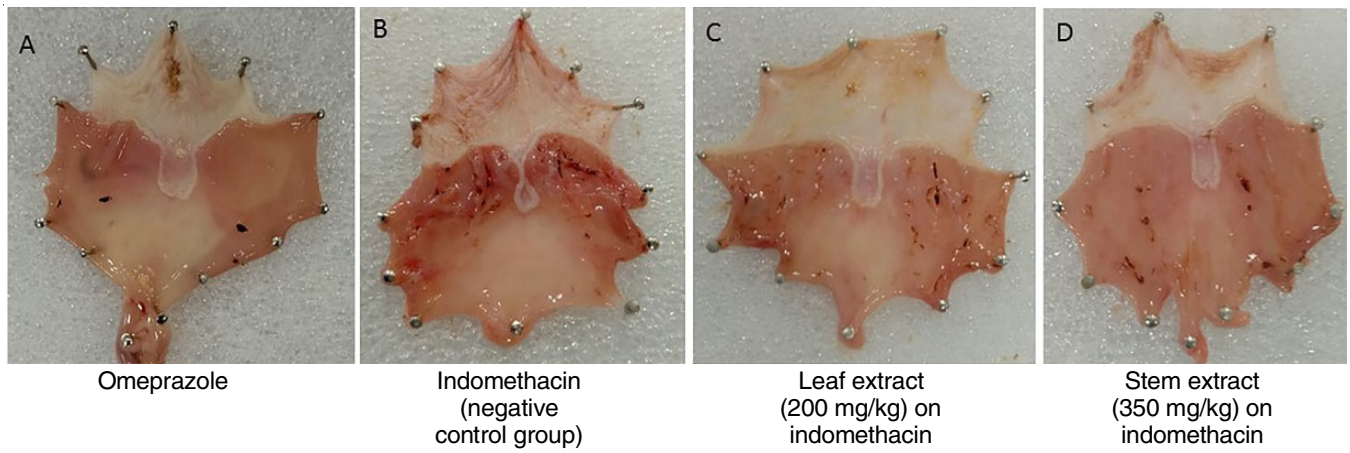

Fig. 1. Macroscopic observation of the gastric ulcers

TABLE-4
PERCENTAGE OF NEUTROPHILS IN BLOOD IN DIFFERENT GROUPS

Groups	Mean neutrophils count (%)
Group 1: Control group	14.3
Group 2: Negative control group	55.1
Group 3: Standard	18.5
Group 4: Plant extract (leaves)	25.1
Group 5: Plant extract (stem)	30.6

Effect of indomethacin and extracts of *S. trilobata* induced by indomethacin in gastric tissue: In the gastric tissue, the MDA level in the indomethacin treatment group was remarkably higher when compared with the control group ($p < 0.001$). Meanwhile, pretreatment with the methanolic extract of leaves of *S. trilobata* (250 mg/kg) and methanolic extract of stem of *S. trilobata* (350 mg/kg) has shown a marked decrease in MDA amounts of the gastric tissue in comparison with the indomethacin alone group ($p < 0.001$). Fig. 2 presents the summary of the effects of both extracts on MDA levels in the stomach tissue after indomethacin-induced gastric ulcer.

Histopathological evaluation: The section of tissue was evaluated for histopathological changes under a microscope. In the healthy group, it was observed that the gastric pits were normal and the parietal and surface mucous cells had a healthy appearance. In indomethacin group, the epithelial losses and irregular gastric pits were observed in the mucosa; the necrotic appearance of the surface mucous cells and the increase of lymphatic cells in the lamina propria were remarkable. There was also an increase in eosinophilic staining properties of some parietal cells. The methanolic extract of leaves had a similar appearance to a healthy group. Nevertheless, some epithelial cells were cast. Histopathological ulcerated area scoring results are shown in Table-5. Microscopic observation of the gastric ulcers in different groups are shown in Fig. 3.

The present study demonstrates the gastroprotective effects of doses of methanolic extracts of leaves and stem of *S. trilobata* on indomethacin-induced gastric ulcers in rats. Indomethacin produced obvious macroscopic stomach ulcers compared with the control group. It also increased the percentage of neutrophils in the blood and increased MDA levels compared with the control group. The methanolic extracts of

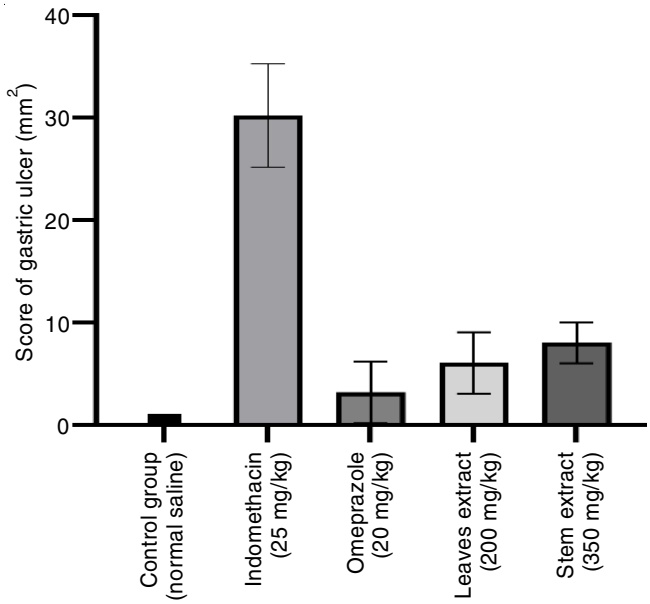

Fig. 2. Effect of oral dose of methanolic extract of leaves and stem, omeprazole on the gastric ulcers induced by indomethacin

TABLE-5
HISTOPATHOLOGICAL ULCERATED AREA SCORING RESULTS

Treatment	Lymphatic cell increase	Hemorrhage	Epithelial cell loss
Normal saline	–	–	–
Indomethacin	+++	+	+++
Omeprazole	–	–	–
Methanolic extract of leaves	+	–	+
Methanolic extract of stem	++	++	++

Histopathological damage: – (none), + (little damage), ++ (moderate damage), +++ (severe damage).

S. trilobata (stem extract 350 mg/kg and leaves extract 200 mg/kg), like omeprazole, significantly decrease nucleophiles' percentage in the blood indomethacin had elevated. Up indomethacin, omeprazole and methanolic extract of *S. trilobata* remarkably reduce stomach ulcer compared with the indomethacin 25 mg/kg ($p < 0.001$) alone group, it was also found

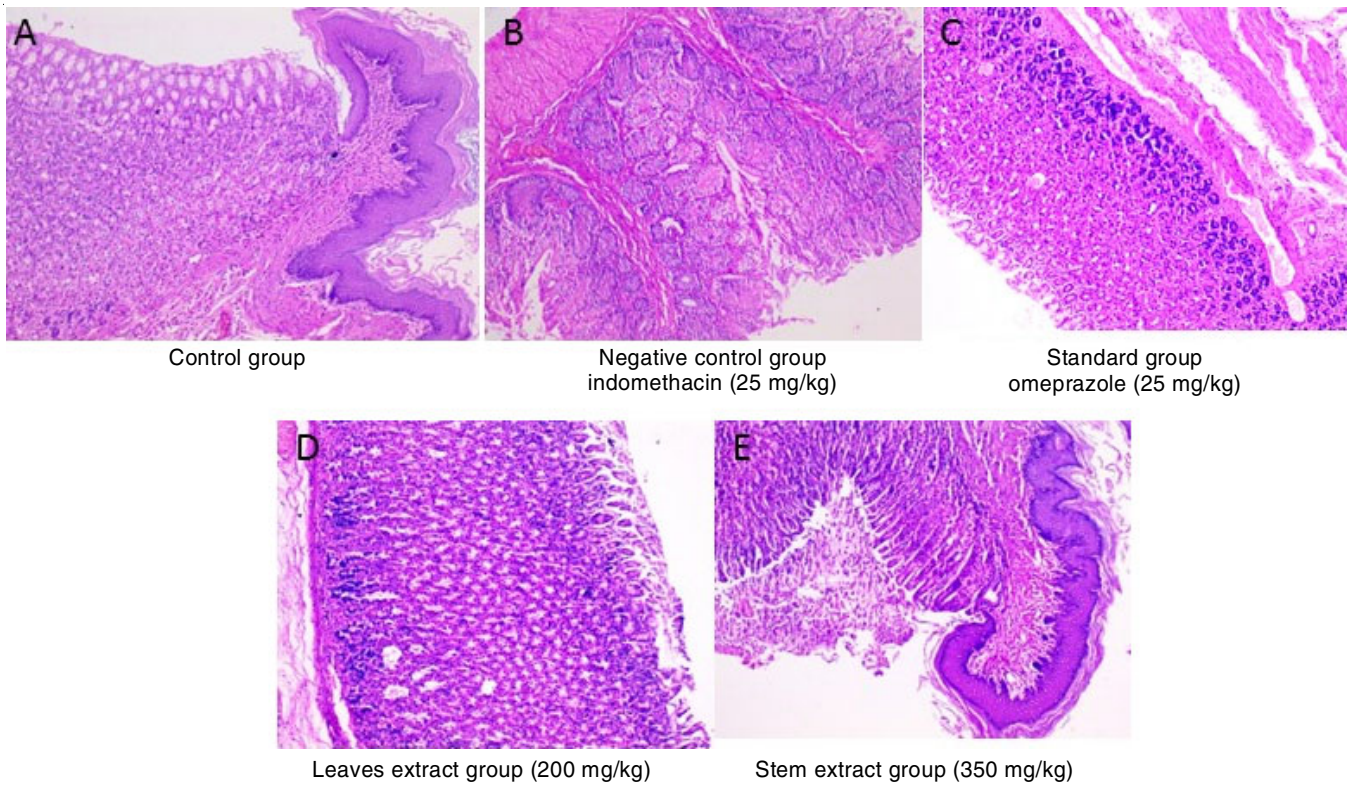

Fig. 3. Microscopic observation of the gastric ulcers with different groups

that 350 mg/kg of methanolic stem extract ($p < 0.05$) is less effective than 200 mg/kg of methanolic extract of leaves. Both the methanolic extracts of *S. trilobata* could also decrease the MDA level in gastric tissue. Furthermore, omeprazole also diminished the MDA contents in the stomach tissue.

Administration of NSAIDs, especially indomethacin leads to microscopic and macroscopic wounds in animals that resemble gastric ulcers in humans. In present study, oral administration of indomethacin at a dose of 25 mg/kg induced an obvious macroscopic gastric ulcer in rats. Inhibiting the COX enzyme by NSAIDs leading to a decrease in mucosal synthesis and bicarbonate secretion and an increase in leukocyte accumulation is the main cause of gastric ulcers [32]. Several studies revealed indomethacin, through the prevention of prostaglandins (PGs) synthesis in the arachidonic acid metabolism pathway, stimulates the 5-LOX enzyme leading to elevated leukotriene's amounts [33]. On the other hand, LTB 12-HD/PGR is a crucial enzyme for eicosanoid inactivation and acts an important part in the metabolism of PGs E and F and LTB4. This enzyme is a potent chemoattractant agent for inflammatory leukocytes and 15-oxo-lipoxin A (15-oxo-LXA) [34]. Some NSAIDs like indomethacin apart from inhibition of COX can act as LTB 12-HD/PGR inhibitor and thus cause an increase in leukotriene's levels. Some studies have found that LTB4, LTC4 or LTD4 are the main factors in the development of vascular damage and mucosal lesions in the gastric tissues [35]. One attempt has been made to investigate the protective impact of 5-LOX inhibitors and leukotriene antagonists on the development of gastric ulcers after indomethacin intake. The results of the study highlighted that the overproduction of metabolites of the 5-LOX pathway plays a significant role in the development of gastric injury and 5-LOX inhibitors and leukotrienes antagonists' agents reverse these gastric damages induced by indomethacin [36]. The most important sources of leukotrienes are neutrophils, which are more effective in the pathogenesis of gastrointestinal tract ulcers induced by NSAIDs, especially indomethacin. For this reason, we experimented to investigate the effect of indomethacin on neutrophil counts [37]. The main functions of leukotrienes are the invitation of neutrophils and chemokines and causing neutrophil adhesion to epithelial cells [38]. Moreover, leukotrienes activate neutrophils to release mediators, leading to degranulation. Activated neutrophils exhibited microvascular disturbance and then resulted in gastric erosions following NSAIDs intake [39]. Elevated LTB4 concentrations trigger chemotaxis, adhesion and degranulation of neutrophils. These processes stimulate gastric mucosal injuries [40]. Previous studies proved that gastrointestinal ulceration resulting from indomethacin intake is due to increased leukotrienes production followed by an increment in neutrophil infiltration and gastric MPO activity [41].

Sphagneticola trilobata owing to having terpenoids, flavonoids and polyacetylenes as well as steroids and exhibit strong antioxidant, anti-inflammatory and lipoxygenase activity [42]. Luteolin, kaurenoic acid, wedelolactone, wedeolide A, oleanolic acid, norwedelic acid are the main secondary metabolites present in the *S. trilobata* [43]. A study after various extraction procedures reported that the methanolic extracts of leaves of *Sphagneticola trilobata* exhibit a higher capacity of recovery of organic compounds in comparison with the other extract of *Sphagneticola trilobata*.

It appears that the present study contributes the evidence which suggests the methanolic extracts of leaves and stem of *S. trilobata* show significant effects against the indomethacin-induced gastric ulcer in rats. Daily administration of *S. trilobata* extract leads to a significant decrease in the level of MDA in neutrophils and protected these cells against oxidative stress lesions compared with the control group.

In brief, this experiment substantiates strong gastro-protection of leaves of *S. trilobata* against indomethacin-induced gastric ulcers with comparable efficacy to CysLT inhibitors such as omeprazole. The prospective usage of *S. trilobata* as a protective compound *versus* gastric ulcer remains an open area for future research.

Conclusion

In brief, this is the first study to evaluate the protective effects of methanolic extract of leaves and stems of *S. trilobata* on the indomethacin-induced gastric ulcers in rats. The results showed that the oral administration of methanolic extracts of leaves (200 mg/kg) and stem (350 mg/kg) has a protective effect on the development of gastric ulcers induced by indomethacin inhibiting LOX enzyme or inhibiting leukotrienes receptor and lipid peroxidation. The evidence of the protective effects of the methanolic extracts of leaves and stems of *S. rilobata* is supported by histopathological studies, lipid peroxide count and neutrophil count, indicating the antiulcerogenic effects. Concerning the evidence on the protective effect of the extracts of *S. trilobata* in reducing the gastrointestinal damage induced by indomethacin, the future experiment is needed to investigate the protective effect of the active ingredients of *S. trilobata* on healing the gastric ulcer and to explore the involved mechanisms regarding protective effects of *S. trilobata* against gastrointestinal damage caused by indomethacin.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this article.

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