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Gastroprotective Effects of Hexanic Extract of the Leaves of *Spondias purpurea* and Its Underlying Mechanism of Action

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ABSTRACT

Spondias purpurea is used in ethnopharmacology as an antidiarrheal, antispasmodic, diuretic, antianemic, antipyretic, and analgesic. Studies carried out by our research group attested to the preliminary antioxidant and gastroprotective activities of this species; however, its mechanism of action remains unknown. This study aimed to evaluate the mechanisms of action underlying the healing activity of hexanic extract of *S. purpurea* leaves (SpHE). In this study, 144 Wistar rats were used, 72 males and 72 females. For this, antisecretory capacity by pylorus ligation, effects of nitric oxide and sulfhydryl (—SH) groups, and the ability to stimulate mucus production were evaluated. Healing activity was evaluated in vitro using a scratch test and in vivo using the acetic acid-induced rat ulcer model. Values were expressed as mean ± standard deviation (SD). Statistical significance between groups was determined using one-way analysis of variance (ANOVA) followed by Tukey's test, with $p < 0.05$ indicating significance. SpHE (25 mg/kg) decreased acid secretion in the gastric contents and increased mucus production in rats; this effect was —SH-dependent but nitric oxide-independent. Cell migration assays demonstrated an increase in the rate of endothelial cell confluence. Ulcer healing and marked cell proliferative activities were observed in the rat ulcer model using histopathological and immunohistochemical analyses with Ki-67 and BrdU. These data indicate that *S. purpurea* has antiulcerogenic properties, possibly related to antisecretory, cytoprotective, and healing pathways.

1 | Introduction

A peptic ulcer is considered a public health problem with a high prevalence and morbidity rate within the world population and is the focal point of experiments and clinical investigations (Zhang et al. 2020). This disease affects approximately 10% of the world's population, equivalent to approximately four million people diagnosed each year (Ercan et al. 2020).

Ulcers arise from an imbalance between aggressive (endogenous and exogenous) and protective factors in the esophagus, stomach, or duodenum. Endogenous destructive factors in the stomach include HCl, pepsin, blood flow reduction, lipid peroxidation, and free radical formation. Exogenous destructive factors include excessive use of alcohol, indiscriminate use of non-steroidal anti-inflammatory drugs (NSAIDs), stress, smoking, and *Helicobacter pylori* infection. Defensive mechanisms are

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mediated by factors such as mucus and bicarbonate barrier, surface phospholipids, prostaglandins (PGs), nitric oxide (NO), mucosal blood flow, cell renewal, growth factors, and antioxidant enzymes (Brito, de Almeida, et al. 2018; Mendonça et al. 2000).

Drugs that inhibit gastric acid secretion, such as histamine H_2 receptor antagonists and proton pump inhibitors, are used to treat gastric ulcers. However, when used for a long time, these drugs may lose their effectiveness and present various side effects such as hypersensitivity reactions, gynecomastia, arrhythmias, impotence, increased risk of gastric cancer, and hematopoietic changes (Kavitt et al. 2019). Thus, encouraging investigations and development of alternative gastroprotective and healing therapies with fewer side effects is extremely important.

From this perspective, natural products, used since ancient times, are a source of potential metabolites for the development of new drugs (David et al. 2015). Developed countries also have a history of the traditional use of natural products similar to many parts of the world; approximately 30% of the drugs used are derived from natural products, mainly from medicinal plants (World Health Organization 2011).

Spondias purpurea L. (Anacardiaceae), popularly known as "serigueleira," has antidiarrheal, antispasmodic, diuretic, antianemic, antipyretic, and analgesic properties (Agra et al. 2007). Studies carried out by our research group attested to the preliminary antioxidant and gastroprotective activities of the hexanic extract obtained from the leaves of *S. purpurea*. These activities were probably due to the presence of flavonoids, cinnamic derivatives, terpenes, and steroids in the hexane extract; however, the underlying mechanisms have not been elucidated (de Almeida et al. 2017). Therefore, this study investigated the mechanisms underlying the antiulcerogenic and ulcer-healing potential of hexanic extract of *S. purpurea* leaves (SpHE).

2 | Materials and Methods

2.1 | Reagents and Chemicals

Ethanol, sodium chloride, Tween 80, lansoprazole, Alcian blue, N-ethylmaleimide, N_ω-nitro-L-arginine methyl ester, ranitidine, and carbenoxolone were obtained from Sigma-Aldrich (St. Louis, MO, USA). Acetic acid and glucose were obtained from Vetec (Duque de Caxias, Rio de Janeiro, Brazil). Ethyl ether, formaldehyde, and phenolphthalein were obtained from Maia (Cotia, São Paulo, Brazil), and xylazine and ketamine were obtained from Vetbrands (Paulínia, São Paulo, Brazil). Ki-67 (code: sc-23900) and BrdU (code: sc-32323) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

2.2 | Plant Material and Preparation of the Extract

S. purpurea leaves were collected from Alhandra, Paraíba, Brazil (7°19′54.8″ S, 34°57′39.5″ W), and the exsiccate (registration number 89986) was deposited in the Herbarium Dárdano de Andrade Lima (Agronomic Institute of Pernambuco, Recife, Brazil). Leaves were oven-dried and milled in a knife mill. The sample (500 g) was macerated with hexane (0.5 L) for 3 days. The solvent was then removed using a rotary evaporator, and the hexanic extract of *S. purpurea* leaves (SpHE) was obtained (10% w/v).

2.3 | Animals

Male and female Wistar rats (*Rattus norvegicus*), weighing between 180 and 250 g, 90 days old (12 weeks), were obtained from the Department of Physiology and Pharmacology (Federal University of Pernambuco, UFPE, Pernambuco, Brazil). One hundred and forty-four animals were used, 72 males and 72 females. The animals had access to water and food (Presence, Purina, Brazil) *ad libitum* and were exposed to a 12-h dark/light cycle at 22°C ± 2°C. All protocols were approved by the UFPE Animal Experimentation Ethics Committee under registration 23076.013615, in 2015. The experimental protocols were carried out in accordance with the standards established by the Brazilian Society for Laboratory Animal Science (SBCAL) and the National Institute of Health's Guide for the Care and Use of Laboratory Animals.

2.4 | Determination of Gastric Acid Secretion

SpHE at doses of 12.5, 25, and 50 mg/kg was tested in an ethanol-induced gastric ulcer rat model, according to de Almeida et al. (2017). A dose of 25 mg/kg was considered the best based on statistical comparison and was used to test the mechanisms of action. Briefly, the animals ($n = 6$) were fasted for 18 h with free access to a 5% glucose solution. The animals were then anesthetized with xylazine (6 mg/kg) and ketamine (60 mg/kg) via intraperitoneal, and pyrolus ligation was induced by a surgical incision. Next, aqueous solution containing 1% Tween 80 (injured control—IC, 0.1 mL/100 g) (vehicle), ranitidine (60 mg/kg), or SpHE (25 mg/kg) was administered intraduodenally (i.d.). The incisions were sutured; 4 h after ligation, the rats were euthanized, their esophagus was clamped to prevent loss of secreted material, and their stomach was removed. The gastric contents were collected and weighed to determine the amount of gastric juice expressed in g/4-h. The stomach contents were centrifuged (790 × g, 10 min), and pH was determined using a digital pH meter. The total acidity (meq[H^+]/l/4 h) was determined in the gastric juice supernatant by titration (pH 7.0) with 0.1 N NaOH solution and phenolphthalein as an indicator, modifying the procedure of Shay (1945).

2.5 | Involvement of Nitric Oxide (NO) in Gastroprotection

After fasting for 18 h, Wistar rats were divided into six groups ($n = 6$) according to pre-treatment: three groups received the vehicle, and three groups received L-NAME (N_ω-nitro-L-arginine methyl ester, 70 mg/kg, intraperitoneally), a nitric oxide synthase (NOS) enzyme blocking agent. After 30 min of administration, each group was orally administered the vehicle, carbenoxolone (100 mg/kg; positive control), or SpHE (25 mg/kg). After 1 h of treatment, the rats received 4 mL/kg ethanol (v.o.). After another hour, the animals were euthanized, and their stomachs were removed, opened, and

photographed. The lesions were quantified by computerized planimetry using ImageJ software (Bethesda, MD, USA). Results are expressed as the ulcerative lesion area (ULA, mm^2) relative to the total area of the gastric mucosa (Arrieta et al. 2003).

2.6 | Involvement of Sulfhydryl Compounds (—SH Groups) in Gastroprotection

Wistar rats were fasted for 18 h and divided into six groups. As a pre-treatment by intraperitoneal injection, three groups received the vehicle, and the other three groups received N-ethylmaleimide (NEM) 10 mg/kg, a blocker of sulfhydryl groups (Matsuda et al. 1999). Thirty minutes after administration, each group was orally administered vehicle (aqueous solution containing 1% Tween 80), carbenoxolone (100 mg/kg), or SpHE (25 mg/kg). After 1 h of treatment, the rats received 4 mL/kg ethanol (v.o.). Sixty minutes later, the animals were euthanized, and their stomachs were removed, opened, and photographed. The lesions were quantified by computerized planimetry using ImageJ software (Bethesda, MD, USA). Results are expressed as ULA (mm^2) relative to the total area of the gastric mucosa.

2.7 | Determination of Mucus Adhering to Gastric Mucus

The animals were fasted for 18 h, divided into groups ($n = 6$), and treated (v.o.) with an aqueous solution containing 1% Tween 80 (injured control, IC), carbenoxolone (200 mg/kg), or SpHE (25 mg/kg). After 1 h, the animals were anesthetized, and pylorus ligation was induced. The incisions were sutured; 4 h after ligation, the rats were euthanized, and the stomach was removed and opened. The stomach samples were placed in contact with an Alcian blue solution (0.1%, 10 mL) for 2 h. Excess Alcian blue was removed by gastric lavage twice with sucrose solution (0.25 mol/L, 7 mL). Alcian blue attached to the glandular region of the stomach was extracted using magnesium chloride solution (0.5 mol/L, 10 mL). An aliquot of 4 mL was added to 4 mL of ethyl ether and stirred for 2 min. The emulsion obtained was centrifuged for 10 min at 1134 × g, and the supernatant was discarded. The absorbance was recorded using a spectrophotometer (Epoch model; BioTek, Curitiba, Brazil) at a wavelength of 595 nm. The results were expressed as μg Alcian blue/g tissue, according to Rafatullah et al. (1990), with modifications.

2.8 | Evaluation of Healing Properties of SpHE

2.8.1 | Scratch Test Assay

Human umbilical vein endothelial cells (HUVEC) from the Cell Bank of Rio de Janeiro were used. Cells were cultured in six-well plates at a concentration of 5×10^5 cells/mL and maintained in DMEM supplemented with 10% fetal bovine serum. Upon reaching 100% confluence, the medium was removed from the wells and 3 mL of PBS was added. Then, using a 200-μL tip, a scratch was made at the bottom of the plate in a previously delimited location. The PBS solution was removed; SpHE (50 μg/mL) and phytohemagglutinin, a mitogenic agent used in cell permeability studies, were added to the wells along with 10% PBS solution. Then, the lesion field was photographed at 0, 2, 4, 6, 8, 10, 12, 24, 48, and 72 h. The results were expressed as the percentage of confluence (Buachan et al. 2014).

2.8.2 | Acetic Acid-Induced Gastric Ulcer

Wistar rats were fasted for 18 h ($n = 6$) and randomly assigned to the following groups: sham, injured control (IC, aqueous solution containing 1% Tween 80), ranitidine (60 mg/kg), or SpHE (25 mg/kg). The animals were anesthetized with 5% ketamine (60 mg/kg) and 2% xylazine (6 mg/kg); their abdominal wall was opened by laparotomy. Their stomach was exposed, and 0.05 mL of acetic acid (30%) was injected into the outer surface of the stomach serous layer. The stomachs were washed with distilled water, and the abdominal wall was sutured. After 48 h, the animals were orally administered an aqueous solution containing 1% Tween 80 (vehicle), ranitidine, and SpHE (25 mg/kg) once a day for 14 days, according to Takagi et al. (1969), with modifications. After 14 days, the animals were euthanized; their stomachs were removed and photographed. The ULA was determined by computerized planimetry (ImageJ Software), and the data were expressed in mm^2.

2.8.3 | Histological Analyses

After macroscopic evaluation, stomach samples were collected and fixed in 10% buffered formalin for 24 h at room temperature. The tissues were then washed in running water, dehydrated through increasing ethanol concentrations, cleared in xylene, and embedded in paraffin. Paraffin blocks were sectioned using a microtome to obtain tissue sections of approximately 5 μm thickness. The sections were mounted on glass slides, deparaffinized in xylene, rehydrated through decreasing ethanol concentrations, and stained with hematoxylin and eosin (H&E) for general histopathological evaluation. Additional sections were stained with periodic acid-Schiff (PAS) to evaluate gastric mucus/glycoprotein content.

The stained slides were analyzed under a light microscope by an observer blinded to the experimental groups. Histopathological evaluation included epithelial integrity, mucosal edema, hemorrhage, inflammatory cell infiltration, necrosis, and preservation of the gastric mucosal architecture. Representative photomicrographs were obtained for each experimental group. The histological procedures were performed according to a previously described method, with minor modifications (Almeida et al. 2014).

2.8.4 | Immunohistochemical Analysis

Immunohistochemical staining was carried out on 4 μm-thick sections of samples from regions ulcerated by acetic acid and used to obtain histological slides. The samples were

deparaffinized with xylene and hydrated in ethanol (Liboska et al. 2012). The methodology of free biotin conjugated with horseradish peroxidase was used to identify Ki-67 and BrdU proteins. Antigens were recovered in a steam pressure cooker for 120 s. The slides were cooled; endogenous peroxidase was blocked with BSA for 60 min and then incubated overnight with the primary anti-mouse monoclonal antibody against Ki-67 protein (Santa Cruz Biotechnology; code: sc- 23900, 1:200 dilution) and BrdU protein (Santa Cruz Biotechnology; code: sc-32323, 1:200 dilution). The slides were washed, immersed in a chromogen solution containing diaminobenzidine (Dako Corporation, Carpinteria, CA, USA), stained with hematoxylin, dehydrated, and mounted. The HRP visualization system was used, and Ki-67 and BrdU immunoreactive cells were detected by the presence of a reddish-brown chromogen in the nucleus and/or cytoplasm of the epithelial cells in the injured area. Reactivity was observed in the analyzed cells: negative (less than 10% of cells), mild (10%–15%), moderate (25%–50%), or strong (> 50%).

2.9 | Statistical Analysis

Values were expressed as mean ± standard deviation (SD). Statistical significance between groups was determined using one-way analysis of variance (ANOVA) followed by Tukey's test, with $p < 0.05$ indicating significance. All statistical analyses were performed using GraphPad Prism 7.0 (GraphPad Software Inc., La Jolla, CA, USA).

3 | Results

3.1 | Determination of Gastric Acid Secretion

Administration of SpHE (25 mg/kg) and ranitidine (60 mg/kg), an H_2-receptor antagonist for histamine, significantly reduced gastric secretory content in rats compared to that in the injured control (IC). However, there was no change in the concentration of H^+ or the pH of the group treated with the extract (Table 1).

3.2 | Evaluation of the Effect of NO

In animals pre-treated with the vehicle, treatment with SpHE (25 mg/kg) had a gastroprotective effect, as expected, since the extract inhibited the formation of ethanol-induced gastric lesions. In rats pre-treated with L-NAME, SpHE also maintained a gastroprotective effect. Comparing the groups blocked with L-NAME with those not blocked, the results showed that there was a significant exacerbation of ULA only in those treated with the control and carbenoxolone. The group treated with SpHE maintained the ability to protect the gastric mucosa from ethanol-promoted lesions, even when pre-treated with the NOS

TABLE 1 | Effect of intraduodenal administration of SpHE and ranitidine on the biochemical parameters of gastric juice after pylorus ligation in rats.

Treatment	pH (units)	$[H^+]$ (meq/ml/4-h)	Gastric content (g)
IC	2.67 ± 0.24	15.9 ± 1.6	0.88 ± 0.09
SpHE (25 mg/kg)	3.25 ± 0.46	16.9 ± 1.6	0.76 ± 0.06*
Ranitidine (60 mg/kg)	4.57 ± 0.53***	11.6 ± 1.5***	0.19 ± 0.05***
Sham	3.93 ± 0.35***	4.43 ± 0.89***	0.15 ± 0.02***

Note: *$p < 0.05$, ***$p < 0.001$ compared to injured control (1% Tween 80 aqueous solution) ($n = 6$).
Abbreviation: IC, injured control.

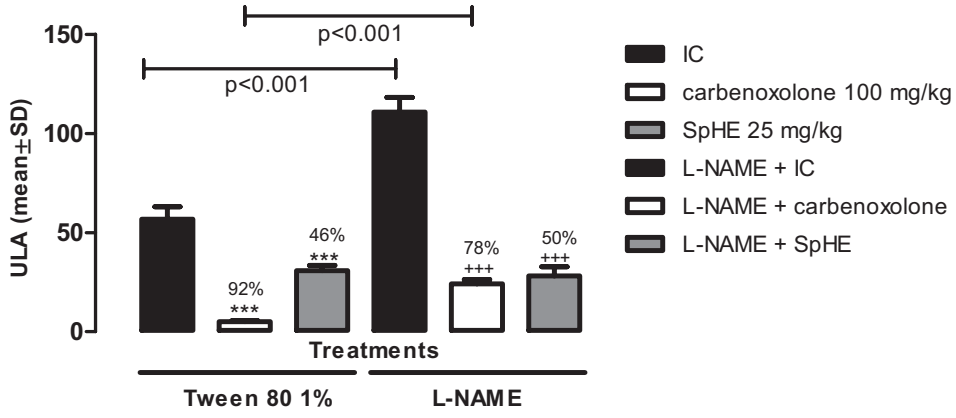


FIGURE 1 | Effect of oral administration of SpHE and carbenoxolone after pretreatment with L-NAME (i.p.) on gastric ulcer induced by absolute ethanol in rats. ***$p < 0.001$ when compared with IC; $^{+++}p < 0.001$ when compared with L-NAME + IC. The comparison between non-blocked and blocked groups was performed using Student's "*t*" test. The percentages indicate the percentage of protection in relation to the control groups. ULA, ulcerative lesion area.

inhibitor (Figures 1 and 2). This suggests that NO is not involved in the gastroprotective effects of *S. purpurea*.

3.3 | Evaluation of the Effect of Sulfhydryl Groups

In rats pre-treated with the vehicle, treatment with SpHE (25 mg/kg) showed a gastroprotective effect, as expected, since the extract inhibited the formation of ethanol-induced gastric lesions. In animals pre-treated with NEM, the blockade of -SH compounds significantly reduced the gastroprotective effect of SpHE. Comparing the groups blocked with NEM with those not blocked, the results showed that there was a significant exacerbation of ULA in all groups (Figures 3 and 4). These results indicated that sulfhydryl groups were involved in the gastroprotective mechanism of SpHE.

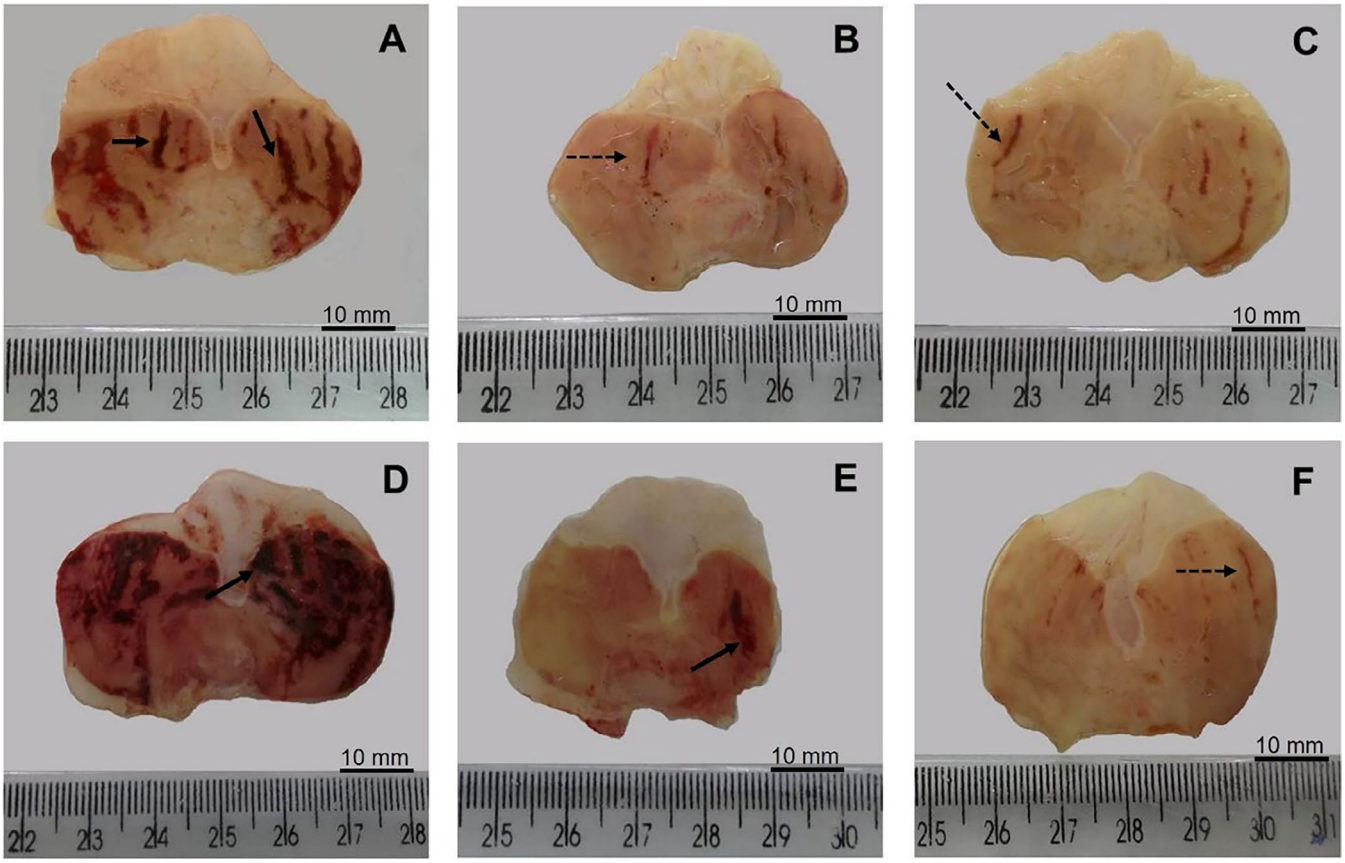


FIGURE 2 | Stomachs of rats pretreated with vehicle and treated with vehicle (A), carbenoxolone 100 mg/kg (B), and SpHE (25 mg/kg) (C); or pre-treated with L-NAME and treated with vehicle (D); carbenoxolone 100 mg/kg (E); and SpHE (25 mg/kg) (F), in the evaluation model of the participation of nitric oxide in gastroprotection. In A, D, and E, observe more evident intense hemorrhagic lesions (arrows); in B and C (dashed arrows), observe regions with discreet lesions.

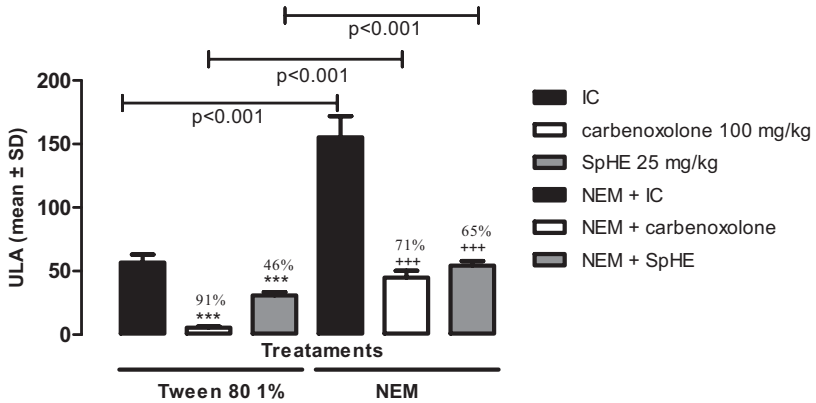


FIGURE 3 | Effect of oral administration of SpHE and carbenoxolone after pretreatment with NEM (i.p.) on gastric ulcer induced by absolute ethanol in rats. ***$p < 0.001$ when compared with IC; $^{+++}p < 0.001$ when compared with NEM + IC. The comparison between non-blocked and blocked groups was performed using Student's "*t*" test. The percentages indicate the percentage of protection in relation to the control groups. ULA, ulcerative lesion area.

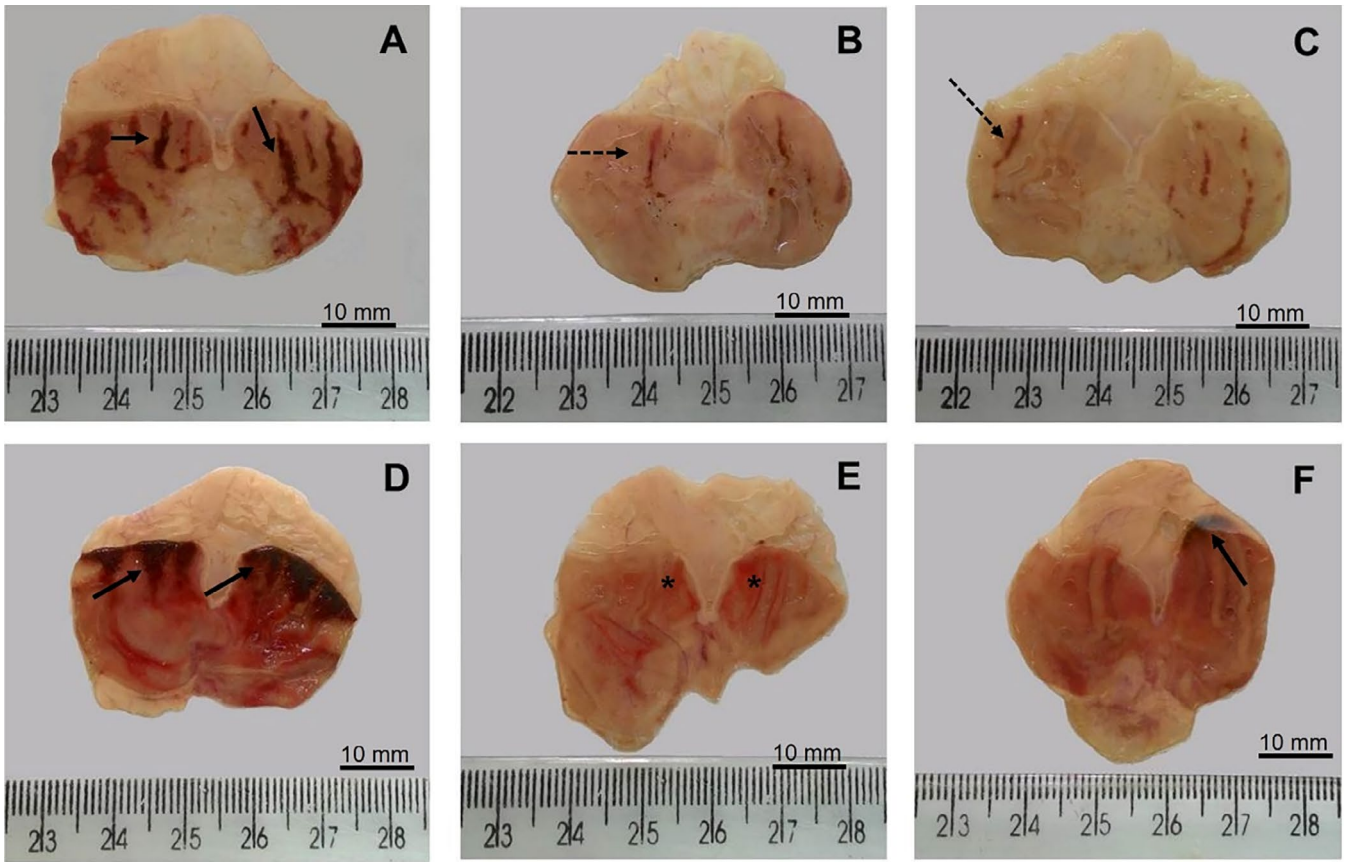


FIGURE 4 | Stomachs of rats pretreated with vehicle and treated with vehicle (A), carbenoxolone 100 mg/kg (B), and SpHE (25 mg/kg) (C); or pre-treated with NEM and treated with 1% Tween 80 aqueous solution (D); carbenoxolone 100 mg/kg (E); and SpHE (25 mg/kg) (F) in the evaluation model of the participation of sulfhydryl groups in gastroprotection. In A, D, and F, observe necrotic areas (arrows); in B and C (dashed arrows), observe regions with discreet lesions; in E, observe for diffuse hemorrhagic areas (asterisk).

3.4 | Determination of the Concentration of Mucus Adhered to the Gastric Wall

In this model, it was possible to evaluate the effect of mucus in gastroprotection mediated by SpHE (25 mg/kg) through the concentration of Alcian blue in contact with the stomach mucus. The results presented in Figure 5 show a significant increase in the concentrations of mucus adhered to the gastric wall induced by the extract when compared to the control (IC).

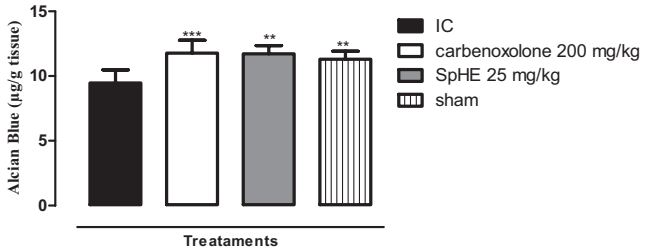


FIGURE 5 | Effect of oral administration of SpHE on gastric mucus content after pylorus ligation in rats. $^{**}p < 0.01$ and $^{***}p < 0.001$ when compared with IC.

3.5 | Evaluation of Healing Properties of SpHE

3.5.1 | Scratch Test Assay

In the scratch test, the percentage of confluent cells indicated the endothelial closure rate of the lesion at different time intervals. It was observed that 8 h after the addition of SpHE to the medium, significant migration of HUVEC was detected when compared to the control group (Figure 6). The microscopic images of HUVEC migration showed that the total closure of the injury induced during the test was observed after 12 h (Figure 7). This suggests that SpHE exerts an important proliferative effect, which can aid in the healing process of damaged tissues.

3.5.2 | Acetic Acid-Induced Gastric Ulcer

After 14 days of treatment with SpHE (25 mg/kg) and ranitidine (60 mg/kg), a reduction in ULA of 74% and 69%, respectively, was observed in rats. As expected, the sham group, which consisted of animals in which the ulcer was not induced, did not present with lesions. According to the results illustrated in Figure 8, it was possible to affirm that SpHE showed healing activity of gastric ulcers induced by acetic acid.

3.5.3 | Histological Analysis

In histological studies of gastric mucosa injured with acetic acid and stained with HE and PAS, it was observed that the sham group (stomachs not exposed to the injurious agent) presented preserved mucosa and submucosal layers (black asterisk), as well as the presence of barrier protection of intact basal mucus (black arrow) (Figure 9A,B). In the injured control group—IC, the absence of the epithelial layer (red arrow), evident destruction of the mucus barrier and mucosal and submucosal layers with intense epithelial exfoliation (red dashed arrow), and vasodilation and edema (red asterisk) were observed (Figure 9C). There was also a loss of surface lining cells and mucous cells of the colon, which produced mucus, with a reduction in the amount of mucus and impairment of the protective barrier (red dashed arrow) (Figure 9D). However, analyses of stomachs treated with SpHE and ranitidine demonstrated regeneration of

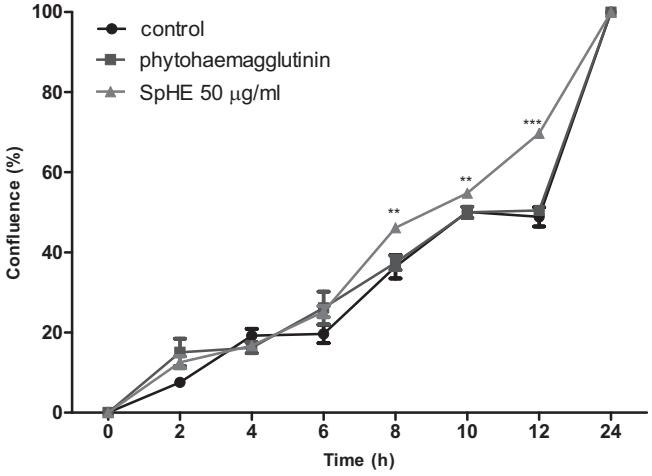


FIGURE 6 | Effect of SpHE on endothelial injury closure and cell migration. The percentage of confluence indicates the rate of endothelial closure of the lesion at different time intervals. $^{**}p < 0.001$ and $^{***}p < 0.001$ compared to control.

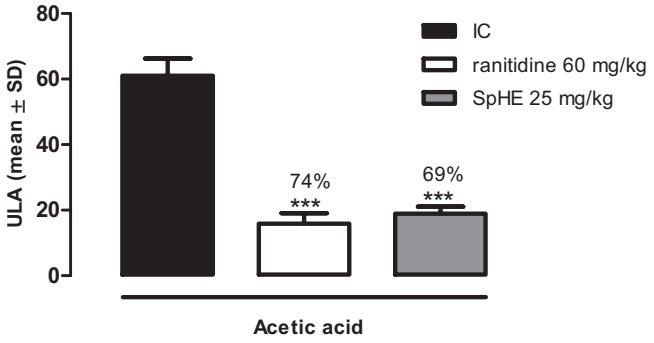


FIGURE 8 | Effect of oral administration of SpHE and ranitidine on acetic acid-induced ulcers in rats. Values represent the mean ± SD ($n = 6$–7). $^{***}p < 0.001$ when compared with IC.

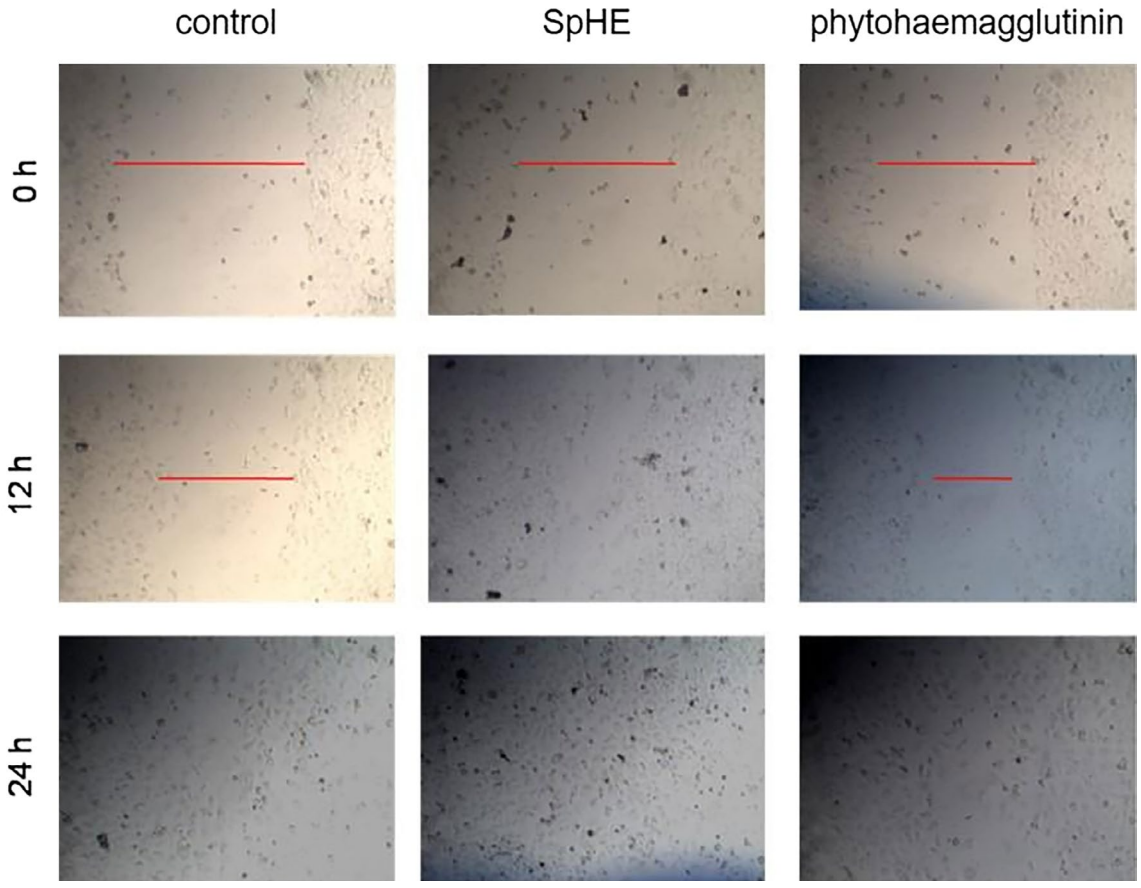


FIGURE 7 | Microscopic images of endothelial cell migration (HUVEC) after injury at different time intervals (0, 12, and 24 h). The red lines indicate the cell-free scratch area at each time point. Scale bar: 200 μm.

the gastric mucosa, with the reappearance of the epithelial and stromal layers (black asterisk), compared to IC (Figure 9E,G). PAS staining highlighted the presence or absence of mucus in gastric mucosal glands. In this case, the pits and gastric glands appeared well preserved (black circular area), and it was possible to verify the presence of the protective mucus barrier (black arrow), represented by areas of intense purple color in the superficial epithelial layer of the mucosa (Figure 9F,H).

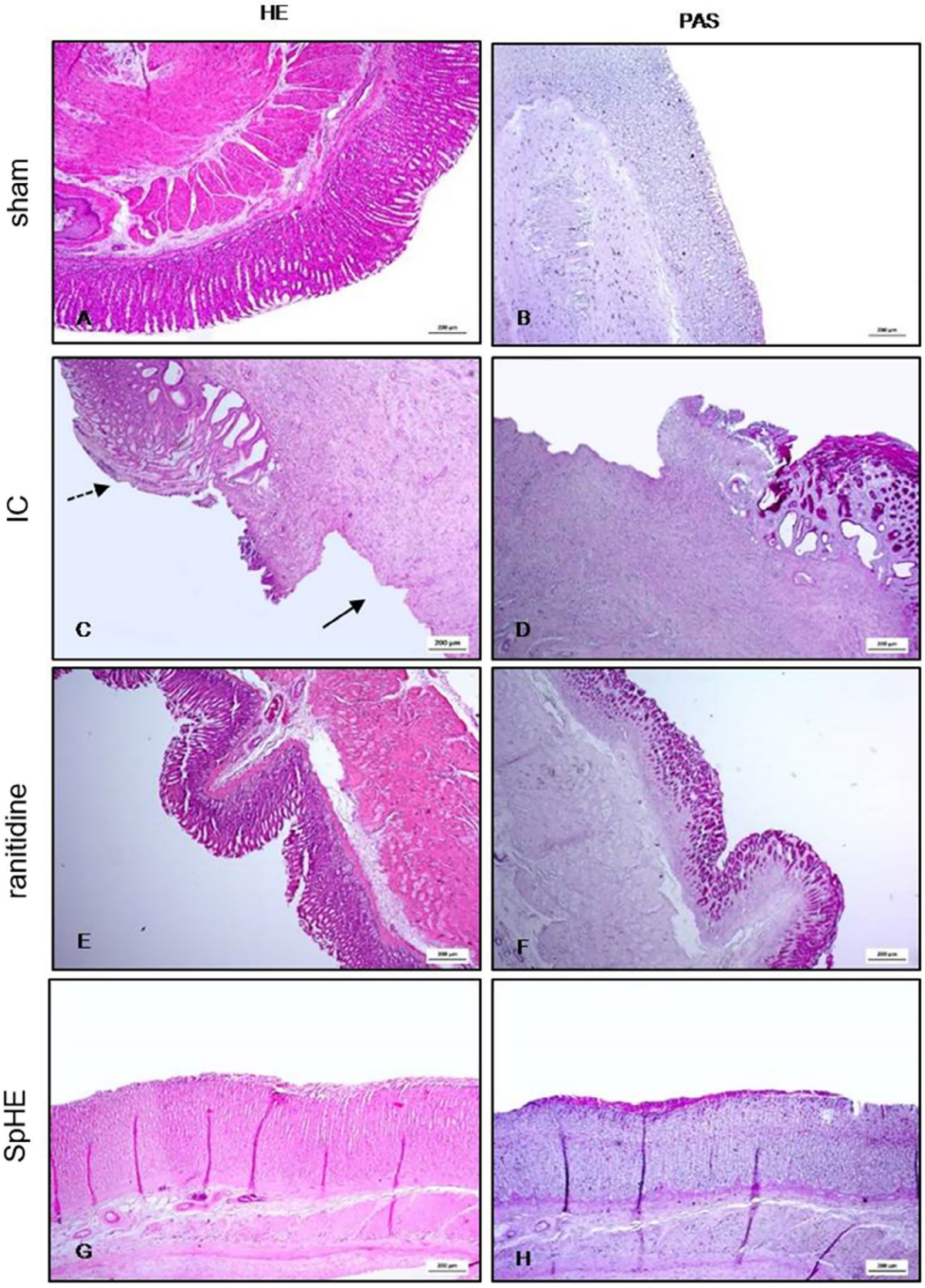


FIGURE 9 | Legend on next page.

FIGURE 9 | Histopathological analysis of gastric mucosa stained with HE and PAS of rats submitted to induction of ulcers with acetic acid. The animals were treated orally with vehicle (injured control), ranitidine and SpHE (25 mg/kg) for 14 days. In A and B, observe preserved mucosa and submucosal layers (black asterisk) and presence of the barrier protection of intact basal mucus (black arrow); in C and D, note the absence of the epithelial layer (red arrow), evident destruction of the mucus barrier and mucosal and submucosal layers with intense epithelial exfoliation (red dashed arrow), and edema (red asterisk); from E to H, observe regeneration of the gastric mucosa, reappearance of the epithelial layer (black asterisk), pits and gastric glands appear well preserved (black circular area), and production of evident mucus (black arrow). Hematoxylin/eosin (HE) and periodic acid Schiff (PAS) staining, 40× magnification. Scale bar: 200 μm.

3.5.4 | Immunohistochemical Analysis

In this analysis, it was possible to verify that in the injured control group (IC), there was no reactivity for the markers due to the destruction of the epithelial layer. In the treatment with ranitidine, there was strong reactivity for BrdU labeling but moderate reactivity for Ki-67. In the groups treated with SpHE, antibodies against Ki-67 and BrdU showed strong reactivity (intense brown staining) in the gastric mucosa, which indicated a marked proliferative activity of cells in the reconstruction of the region injured by acetic acid (Figure 10).

4 | Discussion

Research on the biological activities of *S. purpurea* is limited, but this species is widely used as a therapeutic agent in ethnopharmacology. Studies with other species of *Spondias* have demonstrated gastroprotective, antioxidant, and ulcer-healing potential. Aqueous, ethanolic, and nebulized extracts from *S. mombin* leaves, as well as the juice extracted from the pulp and fruit peels, were tested in different animal models. The methanolic extract of *S. tuberosa* branches also showed in vitro antioxidant properties and gastroprotective effects in an ethanol-induced gastric ulcer model of mice (Brito, Barbosa, et al. 2018; Sabiu et al. 2015; Araruna et al. 2021; de Araujo et al. 2020; Saheed et al. 2015; Oluwatoyin and Deborah 2019; Alves et al. 2022; Cartaxo et al. 2022).

Studies carried out by our research group showed relevant in vitro antioxidant activity and in vivo gastroprotective potential in models of acute induction of HCl/ethanol, ethanol, and NSAID, which must be related to the presence of caffeic acid and epigallocatechin, the major compounds identified in SpHE (de Almeida et al. 2017). Caffeic acid and epigallocatechin are recognized as antioxidants and have gastroprotective effects on injury induced by different agents in rat models (Pellizzon et al. 2017; Kolgazi et al. 2021; Xu et al. 2021).

Despite the relevant results achieved in previous studies, no reports in the literature describe the mechanisms of action involved in the antiulcerogenic effect of *S. purpurea*; therefore, our study brings novelty to this field.

In antisecretory activity, the mechanism of gastric ulcer formation by pylorus binding is related to stress induced by increased acid secretion, which is mediated by vagovagal reflexes independent of G cells and enterochromaffin cells. The contention of acid in the stomach lumen favors self-digestion of the mucosa and disruption of its protective barrier, decreases the levels of prostaglandins, promotes lipid peroxidation, reduces the concentrations of endogenous glutathione, and increases the concentration of intracellular calcium, which acts as a generator of free radicals (Schubert 2014; Adinortey et al. 2013; Muthuraman and Sood 2010).

Thus, pylorus ligation is the most efficient model to investigate the probable antisecretory potential of an intraduodenally administered sample, based on the analysis of changes in gastric content, in addition to indicating the possibility of a systemic effect of the extract (Lakshmi et al. 2010). SpHE administration significantly reduced gastric secretory content, suggesting that gastroprotection is related to antisecretory mechanisms and that its activity is systemic under the conditions tested.

Nitric oxide is an endogenous mediator involved in gastric mucosal integrity mechanisms and is important in the regulation of acid and alkaline secretion and the regulation of blood flow. In addition, it participates in ulcer healing and stimulates growth factors that promote angiogenesis. Although this mediator plays an important role in the maintenance of mucosal integrity, studies have demonstrated that its biphasic action is related to ulcerogenic effects. Thus, it can promote the protection or ulcerogenic action, depending on the type of NOS isoenzyme activated (Wallace 2008; Aragão et al. 2018; Rovere-Querini et al. 2014). In the experimental protocol of NO involvement in gastroprotection, an acute model of the ethanol-induced ulcer was established in rats in the absence and presence of L-NAME, a non-specific inhibitor of the NOS enzyme. The results of this study showed a significant reduction in ulcerative lesions induced by absolute ethanol exerted by SpHE both in the absence and presence of L-NAME, with no significant difference between the groups tested. This suggests that NO is probably not involved in the gastroprotective effects of *S. purpurea*.

Sulfhydryl compounds are responsible for increasing mucus production and maintaining mucus stability through disulfide bonds and are involved in maintaining gastric integrity by combating reactive oxygen species generated during tissue damage. Reduced glutathione (GSH) participates in many aspects of oxidative metabolism, including neutralizing hydroperoxides and maintaining the physiological conditions of sulfhydryl group proteins. A high GSH concentration indicates its possible role in the treatment of peptic ulcers (Rocha Caldas et al. 2015; Rozza et al. 2014; Sánchez-Mendoza et al. 2020; Almasaudi et al. 2016). In this study, an exacerbation of the ULA was observed in the animal groups pre-treated with NEM blocker when compared to the groups pre-treated with the vehicle, indicating that the -SH groups are involved in the gastroprotective mechanism of action of SpHE. These results corroborate the relevant levels of GSH present in the gastric mucosa of rats subjected to gastric ulcers induced by HCl/ethanol, ethanol, and indomethacin and pre-treated with SpHE (de Almeida et al. 2017).

Mucus present in the gastrointestinal tract is an important tissue defense factor that acts as an antioxidant agent, and its production is stimulated by prostaglandins. Different ulcerogenic substances compromise the mucus gel and phospholipid layers, leading to gastric mucosal injury. The presence of mucus and membrane phospholipids forms a hydrophobic physical layer

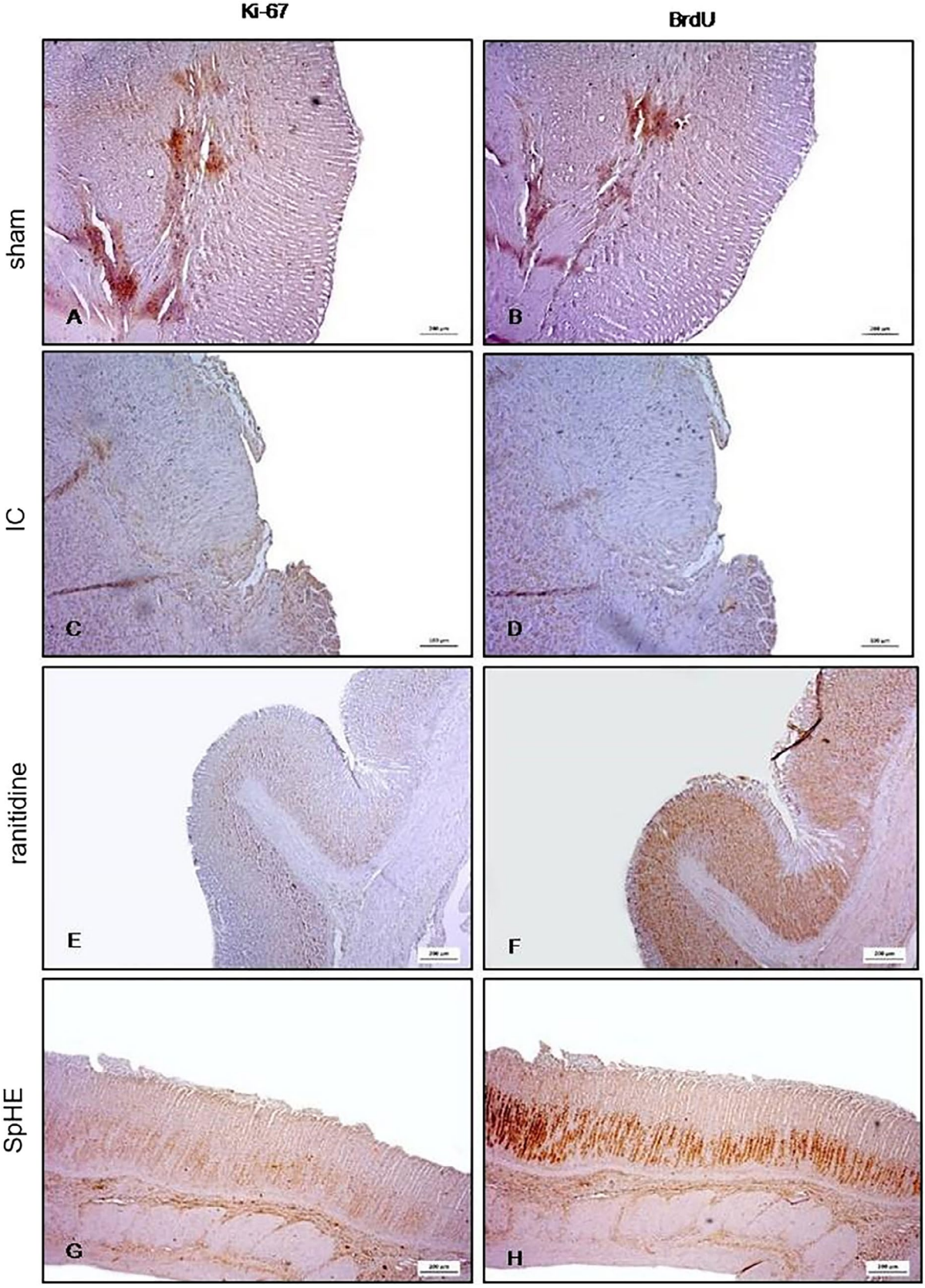


FIGURE 10 | Legend on next page.

FIGURE 10 | Immunohistochemical analysis by Ki-67 and BrdU of gastric mucosa from rats submitted to ulcer induction with acetic acid. The animals were treated orally with vehicle (injured control), ranitidine and SpHE (25 mg/kg) for 14 days. In A and B, note that *sham* group shows relevant reactivity for Ki-67 and BrdU, evidenced by the brown coloration (black arrow); in C and D, note that there was no reactivity for the markers and intense destruction of the epithelial layer (red arrow) in the IC group; in ranitidine group, there was moderate reactivity for Ki-67 labeling (E) and strong reactivity for BrdU (F) (black arrow); in the group treated with SpHE, antibodies against Ki-67 and BrdU showed strong reactivity—intense brown staining (G and H) (black arrow). Microphotographs depict the immunoreactivity for Ki-67 and BrdU in the groups, magnification 200×. Scale bar: 200 μm.

that prevents the diffusion of H^+ ions, and together with the bicarbonate ions secreted by the epithelium, they act to contain the action of pepsin and other gastric secretions with acidic pH, and thus provide a microenvironment close to neutrality. This barrier also prevents the ability of bacteria to penetrate the epithelium (Wallace 2008; Laine et al. 2008; Martins et al. 2014). SpHE increased mucus production, which is an important defense mechanism involved in gastroprotection.

To evaluate the possible healing activity of SpHE, an in vitro cell migration test and an in vivo evaluation were performed. Healing is a continuous and orderly process involving essential tissue changes such as inflammation, chemotaxis, and wound repair (Tarnawski and Ahluwalia 2021). Endothelial cells are elongated squamous epithelial cells that line the walls of blood vessels and establish a close relationship between the blood and tissues. In response to tissue damage, they release fibroblast growth factors, which are responsible for stimulating the synthesis of proteoglycans and extracellular matrix components, promoting cell migration and proliferation, and allowing tissue remodeling. In addition, they play an important modulatory role in vasomotricity; hemostasis; angiogenesis; and the expression of adhesion molecules, von Willebrand factor, and vascular endothelial growth factors (Tarnawski et al. 2021; Yeh et al. 2017). Assays with primary human endothelial cells are important for evaluating healing ability, and HUVECs are the most used, as they are easily available (Losi et al. 2020).

The scratch test mimics the wound in vitro and assesses the rate of cell migration and cell proliferation, which are important parameters in healing. In this case, the organization of the cells in the monolayer is interrupted. Owing to the low interaction between the cells, there is an increase in the concentration of growth factors and cytokines (Lu et al. 2021). It was possible to observe that SpHE exerted an important proliferative activity, which can aid in the healing process of damaged tissues.

The acetic acid-induced rat ulcer model resembles gastric ulcers in humans, both in its pathological aspects and in relation to healing mechanisms (Ercan et al. 2020). Acetic acid stimulates the release of histamine, allows the back-diffusion of H^+ ions, and increases the capillary permeability in the injured region. Furthermore, the ulcer is composed of two regions: the base, formed by granulation tissue rich in macrophages and fibroblasts, and the margin region, formed by a non-necrotic mucosa with signs of healing (Yang et al. 2018; da Silva et al. 2019; Okabe and Amagase 2005). SpHE showed healing activity in gastric ulcers induced by acetic acid, corroborating the results found in a study of a tea rich in epigallocatechin, one of the major compounds of this extract, for this model (Hamaishi et al. 2006).

Naturally, gastric mucosa ulcerated by acetic acid initiates a healing process that encompasses inflammatory reaction, re-epithelialization, angiogenesis, and tissue remodeling. However, the presence of therapeutic agents can accelerate this repair process (Liu et al. 2015). Histopathological analysis of the stomachs stained with HE and PAS revealed that SpHE accelerated mucosal healing. Tissue regeneration promoted by SpHE was also verified using immunohistochemical analyses with monoclonal antibodies against Ki-67 and BrdU, which are markers used to evaluate cell proliferation and DNA repair. The Ki-67 protein is exclusively present in the cell nucleus in the active phases of the cell cycle (G1, S, and G2). During mitosis, part of this protein is directed to chromosomes and is absent in cells in a resting state (G0 phase). Thus, only actively proliferating cells are positive for Ki-67 staining (Shahrokhi et al. 2015; Cuylen et al. 2016).

BrdU is a synthetic nucleoside and a thymidine analog that is incorporated into the cell nucleus in the S phase of the cell cycle. BrdU-positive cells were detected due to the incorporation of BrdU instead of thymidine during DNA replication (Webster et al. 2014).

According to the results obtained in our study, it was possible to conclude that the hexane extract of *S. purpurea* leaves (SpHE) acts via antisecretory mechanisms by reducing gastric content, acting as a cytoprotective factor, stimulating sulfhydryl groups, and increasing mucus production, in addition to the relevant healing activity on endothelial cells in vitro and in the acetic acid-induced injury model in vivo.

5 | Conclusion

The results of our study indicate that SpHE effectively inhibits gastric injury through different mechanisms of action, such as the involvement of sulfhydryl groups, increased mucus production, cell proliferation, and healing effects. Thus, *S. purpurea* may be an alternative treatment for gastric injury caused by various injurious agents.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

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