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Acacia Saligna alleviates Stress Induced Gastric Ulcer in Rats by TLR4/NF-κB Downregulation

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ABSTRACT

Background: One of the most prevalent types of peptic ulcers is stress-induced stomach ulceration. Various research have reported the biological and pharmacological potentialities of various Acacia saligna (AS) extracts, including their anti-inflammatory and antioxidant properties.

Objective: This study aimed to illustrate Acacia saligna's underlying processes and gastroprotective effects in rats suffering from stress-induced stomach ulcers.

Material and methods: Thirty male albino rats were split into: Control, ulcer, and ulcer + AS. Stomach ulcer index values, stomach PH, serum corticosterone, body weight change, gastric prostaglandin E2 (PGE2), gastric malondialdehyde (MDA), gastric superoxide dismutase (SOD), gastric TNF-α, gastric IL-6, and the expression of TLR4 and NF-κB genes were taken into consideration after two weeks. Additionally, stomach tissue was evaluated histopathologically and using NF κB immunohistochemistry.

Results: While, the ulcer group had significantly higher serum corticosterone, gastric ulcer index values, gastric MDA, gastric TNF-α, gastric IL-6, and gastric gene expression of TLR4 and NF-kB, along with upregulation of NF-kB immunoreaction. They also showed significantly lower change in BW, gastric PH values, gastric SOD, and gastric PGE2 when compared to the control group. Stress-induced alterations in stomach tissue were significantly ameliorated by Acacia saligna.

Conclusion: Through the downregulation of the TLR4/NF-kB signaling pathway and the presence of anti-inflammatory and antioxidant properties, Acacia saligna provides gastroprotective advantages to rats with stress-induced stomach ulcers.

Keywords: Gastric ulcer, Acacia saligna, Stress, NF-κB, TLR4.

INTRODUCTION

Globally, gastric ulcers (GU) are the most prevalent disease and cause 5–10% of deaths. The risk of stomach cancer is highest for long-term GU incidence. Numerous factors, including stress, ethanol, pepsin, Helicobacter pylori, acid, and NSAIDs, can cause GU by interfering with defensive mechanisms like microvascular blood circulation, bicarbonate secretion, prostaglandin availability, and nitric oxide [1].

One of the most prevalent types of peptic ulcers is stress-induced gastric ulceration. Due to its significant prevalence across various socioeconomic strata, peptic ulcers are considered a dangerous medical condition that can lead to costly medical care and incapacity. Clinical reports have shown a positive association between stress and ulcers because stress can change parameters related to mucosal integrity, resulting in worn-out mucosal defensive systems [2].

One important component of the neuroendocrine system that detects stress and regulates responses is the hypothalamic-pituitary-adrenal (HPA) axis. The HPA axis's stress response includes the release of several hormones with various effects [3].

Mucosal ischemia and increased back diffusion of hydrogen ions have been suggested as key pathophysiological phenomena in these stress-induced injuries, despite the fact that the pathophysiology of stomach lesions is not fully understood. To understand the mechanisms of acute gastric lesion formation linked to stress, researchers have looked at the production of oxygen free radicals, which are triggered by the reactive oxygen species (ROS) that are generated. In both people and experimental animals, lipid peroxidation brought on by oxygen-free radicals may be a key factor in the pathophysiology of acute gastric mucosal damage [4]. Furthermore, the pathophysiology of stress-induced stomach ulcers is influenced by inflammation and a reduction in the generation of gastric prostaglandins [5].

A transmembrane protein called the Toll-like receptor (TLR) is essential for recognizing the pathogen-associated molecular pattern in bacterial and viral products. It has been demonstrated that TLR4 in

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these TLRs is crucial for triggering an inflammatory response, which in turn causes inflammatory damage. Inflammatory reactions are mediated by the TLR4/nuclear factor-κB (NF-κB) signaling pathway. Nearly every type of cell contains NF-κB, which is involved in several biological functions such as apoptosis, differentiation, inflammation, and immunology. By activating NF-κB, TLR4 promotes the large synthesis of pro-inflammatory cytokines [6].

Proton pump inhibitors and other traditional antiulcer drugs have a number of adverse effects that restrict their use in preventing stress-induced stomach ulcers. Therefore, it is necessary to look for safe anti-ulcer medications. Numerous medicinal herbs have been found to have anti-peptic ulcer properties in the scientific literature [7].

The utilization of herbal medications as therapeutic agents has recently drawn a lot of interest from all over the world for the treatment of various illnesses. Flavonoids and other phenolic chemicals are the primary constituents of nearly all herbal plants. Strong bioactive substances with antiulcer, antioxidant, anti-inflammatory, cardioprotective, anticancer, hepatoprotective, antibacterial, and anti-diabetic properties are phenolic compounds, which include flavonoids and phenolic acids [8].

Numerous research have reported various biological and pharmacological properties of Acacia saligna extracts, including antioxidant, antibacterial, anti-hyperglycemic, and anti-ulcerative colitis. The extracts of A. saligna were used to describe several types of chemicals, such as phenolic acids and flavonoids [9]. Numerous articles have characterized these plants' biological activity as anti-inflammatory [8].

In order to determine the potential protective effect of Acacia saligna against stress-induced stomach ulcers and the potential underlying processes including referral to the TLR4/NF-kB signaling pathway, we conducted this study.

MATERIAL AND METHOD

Experimental animals: Male albino rats, weighing 155–205 grams at eight weeks of age, were kept in plastic cages in a hygienic animal room at Menoufia University's Faculty of Medicine.

Experimental conditions: Temperature of 20°C, a typical light-dark cycle and food and water that are always available. A week prior to the commencement of the trial, the animals were acclimated. Every procedure was carried out at Menoufia University's Faculty of Medicine, Physiology Department.

Ethical statement: Every animal experiment utilized in this study complied with the guidelines set forth by the Faculty of Medicine's Research Ethics Committee at Menoufia University in Egypt with IRB NO:2/2025ANAT15 . Every rat (three per cage) was kept in a polypropylene cage in regular laboratory settings, with unlimited access to food and water. Prior to sample collection, the animals were handled gently, housed in appropriate environmental and nutritional conditions, evaluated for overall health and body weight, and had their cervical dislocation.

Study groups: Thirty adult male rats were allocated into 3 equal groups (10 rats each):

Group 1 (control group): In this group, rats were not subjected to stress, were free to roam around.

Group 2 (stress induced gastric ulcer group) (Ulcer): As previously mentioned, rats were placed in individual plastic restrainers for two hours each day for 14 days in order to expose them to chronic immobilization stress [10].

Group 3 (stress-induced gastric ulcer + Acacia Saligna-treated group) (Ulcer+AS): Rats in this group received Acacia Saligna extract dissolved in 0.5 ml distilled water orally by gavage at the dose of 30 mg/kg /day for 14 days. It was given I hour before exposure to chronic immobilization stress [9].

Every rat was weighed at the start and finish of the study, and the body weight (BW) change was computed. The rats were starved overnight at the conclusion of the trial. The serum was then separated for the determination of serum corticosterone after retro-orbital blood samples were obtained at 10 a.m. Finally, once the rats were killed, the stomach was dissected along its larger curvature and examined for gastric ulcers and acidity. Each stomach was separated into two sections. A portion was ready for immunohistochemical and histopathological examinations. For the purpose of biochemical analysis, the other one was homogenized.

Determination of gastric acidity: Following scarification, a 1000 µl micropipette was used to wash each stomach with 1 ml of phosphate-buffered saline before collecting the gastric juice. A pH meter was used to determine the collected gastric juice's pH [11].

Macroscopic assessment of stress-induced gastric lesions: After the stomachs were cleaned with cold saline, two researchers scored the gross mucosal lesions and presented the results in terms of the ulcer index (U.I.), as previously explained: A lesion is 0 if it is <1 mm long, 2 if it is 2-4 mm long, and 3 if it is > 4 mm long. The number of lesions in each rat was then multiplied by the corresponding factor to determine the score for each rat [12].

Gastric homogenate preparation: Using a tissue homogenizer gastric tissues were weighed and homogenized independently. Gastric tissues were homogenized in order to measure the amounts of prostaglandin E2 (PGE2), interleukin 6 (IL-6), TNF-α, gastric MDA and SOD. After centrifuging the crude tissue homogenate for 15 minutes at 11,000 rpm in an ice-cold centrifuge, the supernatant was gathered and kept at -20°C for further tests.

Biochemical analysis: The rat ELISA kit (catalog No. CSB-E07014r, CUSABIO Life Science Inc., Washington, DC, USA) was used to measure serum corticosterone level in accordance with the manufacturer's instructions. Using the appropriate rat ELISA kits (TNF-α: ERT2010 1, Assaypro LLC, Saint Charles, Missouri, USA, IL-6: ab100772, Abcam, Cambridge, UK, PGE2: MBS262150, MyBioSource, San Diego, CA, USA), the levels of TNF-α, IL-6, and PGE2 in stomach homogenate were measured in accordance with the manufacturer's instructions. Malondialdehyde (MDA) and SOD in stomach homogenate were determined using colorimetric kits (Biodiagnostic Company, Giza, Egypt).

Histopathological evaluation: Prior to being dried in ethyl alcohol, washed with xylol, and then placed in paraffin, the stomach tissue was fixed at 10% neutral buffered formalin (pH=7.0). Ordinary Hematoxylin & Eosin (H&E) stain and Periodic Acid Schiff's (PAS) stain were used to stain the sections. NF-kB: (monoclonal, dilution 1:200, Abcam) was used for immunohistochemical evaluation.

Quantitative RT-PCR (qRT-PCR): Rat stomach tissue was removed, weighed, and utilized for quantitative RT-PCR or stored at -80. Gene-specific primers were created using Primers Express Software Version 3.0.1 (Applid Biosystems, USA). The NF-kB forward primer was (TCGACCTCCACCGGATCTTTC). The reverse primer was (GAGCAGTCATGTCCTTGGGT).
The forward primer for TLR4 was (TCAGCTTTGGTCAGTTGGCT). The Reverse was (GTCCTTGACCCACTGCAAGA). β-actin was the housekeeping control gene. All primers were obtained from Sigma-Aldrich (Chemie GmbH, Germany). RT-PCR assays were conducted in duplicate using an Applied Biosystems 7500 FAST 96-well PCR apparatus (USA). Following the homogenization of fresh or frozen rat gastric tissue samples using TRI reagent (Sigma-Aldrich, UK), total RNA was extracted. Using a high-capacity RNA-to-cDNA kit (Applied Biosystems, CA, USA), the RNA from rat stomach tissue was reverse-transcribed. The mRNA expression of the target gene was then measured using the generated cDNA. The comparative Ct ($2^{-\Delta Ct}$) method was employed to ascertain the relative level of mRNA expression of the target gene, with β-actin serving as a reference.

Statistical analysis

The mean ± standard deviation (SD) was used to represent all data. Version 16 of the SPSS software (SPSS Inc., Chicago, IL, USA) was used to analyze the data. To find significance between the groups, a post hoc Tukey test was performed after ANOVA. Statistical significance was defined as a $P \leq 0.05$.

RESULTS

The ulcer group exhibited significantly higher serum corticosterone, gastric ulcer index values, gastric MDA, gastric TNF-α, gastric IL-6, and gastric gene expression of TLR4 and NF-kB, but substantially smaller change in BW, gastric PH values, gastric SOD, and gastric PGE2 than the control group. While, the Ulcer + AS group had substantially lower levels of serum corticosterone, gastric ulcer index values, gastric MDA, gastric TNF-α, gastric IL-6, and gastric gene expression of TLR4 and NF-kB, they had a substantially higher changes in BW, gastric PH values, gastric SOD, and gastric PGE2 compared to the ulcer group (Table 1).

Table (1): Change in BW, serum corticosterone, gastric ulcer index, PH, MDA, SOD, PGE2, TNF-α, IL-6, gastric TLR4 and NF-kB genes expression evaluation in the research groups (Total 30 rats, 10 for each group)

	Control group	**Ulcer group**	**Ulcer +AS group**
Change in body weight (gm)	125.9±5.44	61.18±3.99*	90.5±4.9 *#
Serum Corticosterone (ng/mL)	50.5±2.3	130.2±2.12*	96.8±3.1 *#
Macroscopic ulcer Index	0±0	35.9±2.12*	21.5±2.1 *#
Gastric PH	3.5±0.12	2.22±0.09*	2.99±0.14 *#
Gastric MDA (nmol/ gm. Tissue)	5.18 ±0.26	21.6±1.125*	12.9± 1.22*#
Gastric SOD (U/gm. Tissue)	8.9 ±0.98	4.2±0.44*	6.65±0.12*#
Gastric PGE2(pg/mg.tissue)	35.9±1.1	20.9±2.02*	27.8±0.72*#
Gastric TNF-α (ng/ml)	25.9±1.12	50.9±1.98*	36.5±1.1*#
Gastric IL-6 (pg/mL)	110.2±3.99	220.9±3.14*	170.3±4.1*#
Gastric TLR4 gene expression	1	4.18±0.11*	2.71±0.14*#
Gastric NF-kB gene expression	1	3.29±0.09*	2.11±0.11*#

* Significant compared with control, # Significant compared with Ulcer.

Histopathological results: The stomach of the control group showed normal gastric tissue with intact mucosa. Ulcer group showing erosion of the surface epithelial cells with noticed ulcer and congested submucosa. Ulcer group treated with acacia saligna showing improvement in the gastric architecture but the submucosa showing some inflammatory cells (Figure 1).

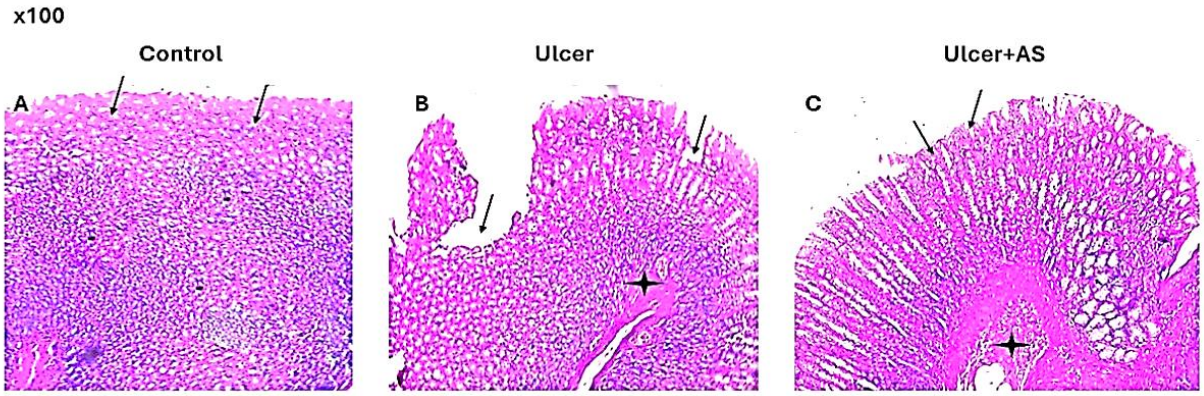

Figure (1): H & E-stained gastric sections of the studied groups: (A) Photomicrograph of the control group showing normal gastric tissue with intact mucosa (Arrows). (B) Photomicrograph of ulcer group showing the erosion of the surface epithelial cells with noticed ulcer (Arrows) and congested submucosa (Star). (C) Photomicrograph of the ulcer + AS group showing improvement in the gastric architecture (arrows) but the submucosa showing some inflammatory cells (Star).

The mean value of the PAS intensity in the ulcer was substantially decreased than that of the control (2.05 ± 0.2 vs. 25.**3** ± o.22, respectively, p<0.05). PAS intensity in the Ulcer + AS group was dramatically elevated than that of the ulcer (15.25±4.5, P<0.05), however, it was still substantially lower than that in the control. (Fig. 2 A-D)
The mean values of NF-kB intensity in the Ulcer was substantially higher than that of the control (73.5 ± 0.2 vs. 15.4 ± 0.33, P<0.05). NF-kB intensity in the Ulcer + AS group was substantially decreased than that of the ulcer group (33.8 ± 0.14 vs. 73.5 ± 0.2, respectively, P<0.05) however, it was still substantially higher than that in the control (p<0.05) (Fig. 2 E-H).

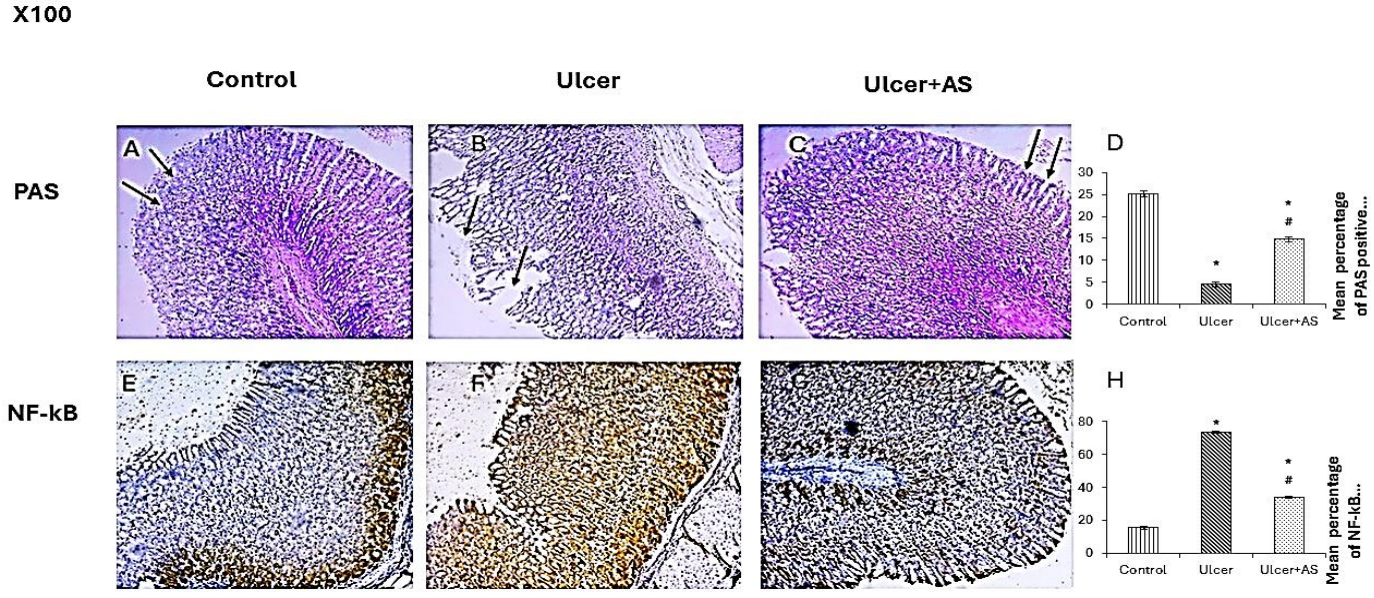

Figure (2): Representative Photomicrographs showing strong PAS reaction (Arrows) indicated by a thick layer of mucous (A). Ulcer group showing weak PAS reaction (Arrows) (B). Ulcer+ AS group showing moderate PAS reaction (Arrows) (C). Also, there is a significant increase in the NF-kB (E-H) immunoreaction in the ulcer group but a significant decrease in Ulcer + AS group (x100).

DISCUSSION

In today's world, stress-related diseases have grown in severity and importance. Stress ulcers is gaining attention [3]. Stress and long-term NSAID usage are two of the many factors linked to the pathophysiology of peptic ulcers in humans. Reactive oxygen species (ROS) and cytokines both directly and indirectly promote damage to the stomach mucosa. Several treatments are available to treat peptic ulcers, but regrettably, the ones currently in use have a number of negative side effects, slow down the healing process, and increase the risk of recurrence. This promotes curiosity about alternative natural items as a form of treatment [1]. The goal of the current study was to look into Acacia saligna's possible therapeutic effects on rats' immobilization stress-induced stomach ulcers. It has long been known that stress and stomach injury are related.

In the present investigation, immobilization stress for two hours per day for fourteen days caused a considerable drop in body weight and a large rise in blood corticosterone in the ulcer when compared to the control, suggesting the production of a chronic stress state. Additionally, it created gastric stress lesions, as demonstrated by both microscopic and macroscopic analysis of the stomach. This is consistent with earlier research [7].

The hypothalamic-pituitary-adrenal axis is stimulated by immobilization stress, resulting in the secretion of cortisol. Free radicals are also produced, and the hormones in the stomach are disturbed. The expression of the inflammatory mediator is enhanced by elevated catecholamine levels. The biological tissues are disrupted and injured as a result of oxidative stress. Peroxidation of the lipids in the cell membrane, which is linked to the release of intracellular components, is one aspect of gastric cell injury. Consequently, the gastric mucosa's inflammatory process begins [13].

Based on the available information, it can be said that stress-induced gastric ulceration causes an increase in gastric acid output, as seen by a lower PH in the ulcer group. This could be because stress-induced ulceration is linked to increased histamine secretion by the parietal cells. It might also have something to do with the vagal stimulation of gastrin, which promotes the secretion of acid [14]. It is acknowledged that stressful situations cause changes in GIT motility and acid secretion, which might result in many ulcerations [15].

Since Acacia plants, and particularly Acacia saligna, have been found to have a large number of polyphenolic chemicals. As evidenced by a much lower gastric ulcer index and higher PH values as compared to the ulcer group, the current treatment with Acacia saligna herb provided protection against stress-induced gastric ulcers. Additionally, it lowered serum corticosterone levels in comparison with the ulcer group. Previously, Acacia saligna provided protection against aberrant ulcer indices and unpleasant morphological alterations brought on by acetic acid [8]. Therefore, the herb is recommended as a safe, natural treatment for ulcerative lesions. All of the substances found have the potential to cure ulcers since they are enriched extracts of flavonoids and phenolic compounds that act as anti-inflammatory and antioxidant agents [16].

A further defense against stress-induced stomach ulcers was provided by Acacia salgina, which significantly reduced the gastric PH values in comparison with the ulcer group. The pathophysiology of acute stomach lesions brought on by experimental stress is known to be significantly influenced by oxygen-free radicals [17]. Lipid peroxidation is the mechanism by which these radicals damage tissue. ROS can cause phospholipid fatty acid peroxidation, which damages cellular membranes. Consistent with earlier research, the current investigation demonstrated that rats subjected to immobilization stress had markedly elevated MDA and decreased SOD in their stomach tissues as compared to the control [7].

In contrast to the ulcer group, the Acacia saligna-treated group exhibited a substantial decrease in MDA levels and elevation in SOD, a recognized antioxidative marker. Acacia saligna's antioxidant qualities were demonstrated in a prior study [9]. The polyphenolic composition and flavonoid content of Acacia saligna, which have the capacity to boost the antioxidant enzyme and scavenge free radicals, may be responsible for the antioxidant qualities of its bioactive substances. When analyzed separately, these substances demonstrated potent antioxidant properties [17].

PGE2 and other PGs are important lipid mediators in the stomach mucosa. By decreasing permeability, preventing the release of gastric acid, and improving blood flow to the mucosal lining, these mediators are essential for preserving the integrity of the epithelial layer. They also efficiently control gastric motility and inhibit the release of inflammatory mediators [18]. PGE2 in the stomach mucosa was much lower in ulcer rats than in control rats, which is consistent with other research [7].

The development of stomach ulcers is significantly influenced by proinflammatory cytokines. Due to elevated gastric expression of NF-kB, which regulates the production of pro inflammatory cytokines. Stress has been shown to raise the level of several inflammatory markers in the gastric mucosa [19].

Our findings, which are consistent with those of other study [1] showed significantly higher levels of inflammatory cytokines and an elevation of NF-kB immunoreaction in the stomach mucosa as compared to the control. In comparison with the ulcer group, Acacia saligna significantly reduced proinflammatory cytokines, upregulated PGE2, and downregulated NF-kB immunoreaction. Characterization of Acacia saligna

showed that it was rich in a variety of bioactive components, such as flavonoids and phenols, which have potent anti-inflammatory effects [9].

Yousof *et al.* [9] ascribed the anti-inflammatory properties of Acacia saligna to the extract's abundant phenolics and flavonoids, which have the capacity to downregulate inflammatory pathways (PI3K, AKT ERK, NF-κB) and cytokines, resulting in improved histological outcomes, decreased oxidative stress, and increased antioxidant status.

TLR4/NF-kB pathway play a vital role in gastric ulcer healing [20]. One important receptor that mediates innate immune responses is toll-like receptor 4 (TLR4), which recognizes chemicals linked to pathogens. It has the ability to trigger signaling pathways involved in the intracellular inflammatory response. Numerous genes involved in immunological and inflammatory responses, cell differentiation, cell proliferation, and cell survival are regulated by NF-kB, the TLR4 downstream [21]. Numerous inflammatory genes are modulated as a result of ROS activation, which also sets off the NF-kB pathway. As a result, this triggers the production of proinflammatory mediators, which in turn causes damage to the stomach mucosa [22].

In line with a prior study, which found that ethanol increased TLR4 and NF-kB in rats, our data showed up-regulation of TLR4/NF-kB gastric gene expression and up-regulation of NF-kB immunoreaction in Ulcer group when compared to control [23]. However, compared to the ulcer group, Acacia saligna significantly reduced the expression of TLR4/NF-kB gastric genes and NF-kB immunoreaction, which is consistent with earlier research [9].

Additionally, the results of PAS staining, which identified mucopolysaccharides in the stomach mucosa and indicated its integrity [7] supported the normal H & E stain's weak PAS reactivity in the ulcer group, which is in line with earlier research [7]. Strong PAS-reactive mucosal cells were seen in the ulcer + AS group, which is consistent with earlier research showing that Acacia saligna restored the normal PAS-stained reactivity of the basement membrane with minor reaction loss in the testis's QET-100 [9].

CONCLUSION

Based on the available information, it can be said that Acacia saligna is useful in treating stress-induced stomach ulcers and has encouraging ulcer-healing properties. It restored the architecture of the mucosal layer in rats, improving gastric pH and stomach function. Its antioxidant action resulted in suppression of the inflammatory cascade, encouragement of gastric barrier repair, and inhibition of the TLR4/NF-kB pathway. All may contribute to these benefits. Overall, the findings made it abundantly evident that Acacia saligna's effects might be matched to those of medications that are frequently prescribed to treat stomach ulcers.

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