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Allicin Mitigates Immobilization Stress Induced Gastric Ulcer in Rats by Upregulation of Nrf2/HO-1 Pathway

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ABSTRACT

Background: Gastric ulcers brought on by stress offer an intriguing area of study. The persistent challenges in properly controlling and treating these ulcers have attracted a lot of research interest. The primary biologically active ingredient in freshly crushed garlic extract is thought to be allicin. Research has demonstrated that allicin possesses antibacterial, antioxidant, and anti-inflammatory qualities.

Objective: To determine any potential underlying processes and assesses the gastroprotective impact of allicin on immobilization stress-induced stomach ulcers.

Material and Methods: Three groups of thirty male rats were created: control, ulcer, and ulcer+allicin (10/group). The following factors were assessed: gastric ulcer index values, stomach pH, serum corticosterone, body weight change, stomach PGE2, stomach MDA, stomach GSH, stomach TNF-α, stomach IL-10, and the expression of Nrf2 and HO-1 genes in the stomach. Additionally, stomach tissue was evaluated by histopathology and COX2 immunohistochemistry.

Results: Compared to control the ulcer group exhibited dramatically lower change in BW, gastric pH values, gastric GSH, gastric PGE2, gastric IL-10 and gastric gene expression of Nrf2 and HO-1, while also exhibiting significantly higher serum corticosterone, gastric ulcer index values, gastric MDA and gastric TNF-α together with upregulation of COX2 immunoreaction compared to control. Allicin dramatically improved stress-induced changes in gastric tissue.

Conclusion: Through the upregulation of the Nrf2/HO-1 signaling pathway, as well as its anti-inflammatory and antioxidant properties, allicin provides gastroprotective effects to rats with stress-induced stomach ulcers.

Keywords: Allicin, COX2, Gastric Ulcer, HO-1, Nrf2.

INTRODUCTION

Gastric ulcers (GU) have a rate of around 6–11% over the course of a person's lifetime and represent a significant public health concern. Numerous studies have demonstrated that the critical imbalance between invasive and protective factors of the gastric mucosa causes GU, despite the fact that its etiology and pathogenesis are still up for debate [1]. Long-term stress, smoking, alcohol, and NSAIDs are the most frequent harmful variables causing this condition [2].

Although there are many causes of ulcers, stress-induced stomach ulcers offer an intriguing area of study. Because it is still difficult to manage and treat these ulcers successfully, they have attracted a lot of scientific interest [3]. Ulcers can develop as a result of altered stomach physiology brought on by stress, including increased production of gastric acid, decreased blood supply to the mucosa, and compromised defensive systems. The intricacy of treating stress-induced stomach ulcers is highlighted by this complicated interaction of variables [4].

Stress results in increased mast cell degranulation and reduced prostaglandin production. There is strong evidence that both psychological and physical stresses are major contributors to the development of ulcers because they cause the release of histamine, which is triggered by increased acid secretion [5].

Stress-induced stomach ulcers resulted from oxidative stress, and lipid peroxidation results from a disturbance in the equilibrium between the antioxidant system and ROS. Damage to the stomach mucosa consequently worsens. Furthermore, stress activates mast cells, which raises the number of inflammatory cells linked to long-term stress. Stress triggers the HPA axis, which has detrimental effects on the stomach by causing the mast cells to produce chemical mediators including histamine and proteases. By inducing the formation of stomach acid, these mediators raise the risk of ulcers [6].

Overproduction of ROS damages negatively impacts the stomach cells. Additionally, the NF-kB pathway is triggered by ROS activation, which modifies a large number of inflammatory genes. Consequently, this leaks proinflammatory mediators, which causes damage to the stomach mucosa [7]. The involvement of

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mucosal ischemia and reperfusion damage in the pathophysiology of stress ulcers sets them apart from regular peptic ulcers [8].

While fewer side effects are often unavoidable and restrict therapeutic value, conventional therapies are effective. Therefore, it is necessary to look for safe anti-ulcer medications. Medicinal herbs have been shown to have positive benefits on stomach ulcers with less negative effects in both experimental and clinical investigations [5]. Cells, particularly those in the gastrointestinal tract, are crucially protected against oxidative damage by the nuclear factor erythroid 2-related factor 2 (Nrf2) signaling pathway. Numerous mechanisms can be ascribed to the impact of Nrf2. Free radicals are neutralized by Nrf2 through the activation of natural antioxidant enzymes including heme oxygenase 1 (HO1). Furthermore, Nrf2 lowers inflammation by suppressing NF-κB and the proinflammatory signaling that follows [9].

For the treatment of ulcers, a number of drugs are easily accessible. However, these medications' poor effectiveness against gastric ulcer and serious adverse effects are causing serious issues. In order to prevent and cure GU, novel medication candidates with great effectiveness and minimal toxicity are thus needed [1].

The primary biologically active ingredient in freshly crushed garlic extract is allicin. Antimicrobial, antioxidant, anti-inflammatory, cardioprotective, anti-cancer and anti-genotoxic properties have all been demonstrated for allicin [10].

The utilization of plant-derived natural chemicals, which are proved to contain non-toxic characteristics, efficacy, and cost-effectiveness, seems to be a promising option for treating stomach ulcers. Their predicted decreased incidence of side effects when compared to synthetic alternatives is the main reason for this conclusion. The aim of this study was to investigate the potential preventive impact of allicin against stress-induced stomach ulcers as well as the potential underlying processes.

MATERIAL AND METHODS

Ethical considerations:

This study was conducted in accordance with ethical procedures and policies approved by Animal Care and Use Committee of faculty of Medicine, Menoufia University Ethics Committee gave its approval for the use and care of the animals with IRB NO:2/2025BIO13-2.

Animals

For the current investigation, thirty male Wistar rats were procured from a nearby animal care facility. Before the studies begin, the rats were given ten days to acclimate. Rats had free access to water and a standard chow diet. Rats were scarified by cervical dislocation at the conclusion of the trial.

Experimental design

Rats were divided into (10 rats per group):

1) Control group, rats were free to walk about and weren't subjected to stress. For 14 days, they were given an oral gavage once daily of an equal amount of DMSO diluted in saline. Less than 1 percent of DMSO was utilized in the end.

2) Ulcer group, rats were given an equivalent dose of DMSO diluted in saline by oral gavage once daily for 14 days after being subjected to chronic immobilization stress for two hours each day for 14 days in an individual plastic restrainer, as previously reported [11]. Less than 1% was the final DMSO concentration that was utilized.

3) Ulcer-Allicin treated group (Ulcer+Allicin), for 14 days, rats were given oral gavage of 45 mg/kg bw/day of allicin (purity, ≥95%; Shanghai Macklin Biochemical Co., Ltd., Shanghai, China) one hour prior to their exposure to chronic immobilization stress [10,12]. Allicin was dissolved in DMSO and diluted in saline. The used concentration of DMSO was the same as in the other 2 groups.

Every rat was weighed at the start and finish of the research, 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 retroorbital 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 halves. 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 [13].

Macroscopic assessment of gastric lesions

After washing the mucosa with regular saline, it was examined visually for signs of ulceration and bleeding. The ulcer index, as defined by **Peskar *et al.*** [14], was used to quantify the degree of damage to the stomach mucosa. Each rat's ulcer index was determined by multiplying the total number of lesions by the severity factor associated with each lesion. Based on the lesion's length, a 0–3 scoring system was used to estimate the severity factor. The severity factor would be zero if there were no lesions. Severity factor = 1 for lesions less than 1 mm, 2 for lesions between 1 and 4 mm, and 3 for lesions more than 4 mm.

Gastric homogenate preparation

Using a tissue homogenizer, gastric tissues were weighed and homogenized independently. Gastric tissues were homogenized in order to measure the levels of prostaglandin E2 (PGE2), IL-10, TNF-α, MDA and GSH. After centrifuging the crude tissue homogenate

for 14 minutes at 11,000 rpm in an ice-cold centrifuge, the supernatant was collected and kept at -80°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 the blood corticosterone level in accordance with the manufacturer's instructions. Following the manufacturer's instructions, the levels of TNF-α, IL-10, and PGE2 in stomach homogenate were measured using the appropriate rat ELISA kits (TNF-α: ab100785, Abcam, Cambridge, UK), IL-10: ab100765, Abcam, Cambridge, UK, and PGE2: MBS262150, MyBioSource, San Diego, CA, USA). Using colorimetric assays, the levels of GSH and MDA in stomach homogenate were measured (Biodiagnostic Company, Giza, Egypt).

Gene expression quantification using RT-PCR

Using RT-PCR test, we investigated the levels of stomach Nrf2 and HO-1 mRNA expression. After stomach tissues were homogenized using the TRI reagent (Sigma-Aldrich, UK), total RNA was extracted. The high-capacity RNA-to-cDNA kit (Applied Biosystems, CA, USA) was then used to reverse-transcribe the stomach RNA. The produced cDNA was then utilized to quantify the chosen genes' mRNA expression. Gene specific primers were designed using Primer Express Software version 2.0 (Applied Biosystems, USA). As the housekeeping control gene, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was employed. All target and housekeeping genes underwent repeated RT-PCR experiments using an Applied Biosystems 7500 FAST 96-well PCR equipment (USA). The comparative Ct ($2-\Delta Ct$) approach was used to determine the relative mRNA expression of the gene of interest, with GAPDH acting as the endogenous control.

The following primers were used for the Nrf2 gene:

(1) Forward primer: 5-GGTTGCCCACATTCCCAAATC-3.

(2) Reverse primer: 5-CAAGTGACTGAAACGTAGCCG-3.

The following primers were used for the HO-1 gene:

(1) Forward primer: 5-AGGTGCACATCCGTGCAGAG-3.

(2) Reverse primer: 5-CTTCCAGGGCCGTATAGATATGGTA-3.

Histopathological methods:

Prior to being dried in ethyl alcohol, washed with xylol, and then placed in paraffin, the stomach tissue was fixed at 12% formalin. Ordinary Hematoxylin & Eosin (H&E) stain and periodic acid Schiff's (PAS) stain were used to stain the sections. Cyclooxygenase-2 (COX-2) immunohistochemical (IHC) was carried out using rabbit polyclonal anti-COX-2 antibody (Catalog No. A1253; Abclonal, Woburn, United States).

Statistical analysis

Version 16 of the SPSS software (SPSS Inc., Chicago, IL, USA) was used to analyze the data. The mean ± standard deviation (SD) was used to represent all data. To find significance between the groups, a post hoc Tukey test was used after a one-way analysis of variance (ANOVA) test. Statistical significance was defined as a P value of less than 0.05.

RESULTS

Compared to control, the ulcer group exhibited dramatically lower change in BW, gastric pH values, gastric GSH, gastric PGE2, gastric IL-10 and gastric gene expression of Nrf2 and HO-1, while also exhibiting significantly higher serum corticosterone, gastric ulcer index values, gastric MDA and gastric TNF-α. In contrast to the Ulcer group, the Ulcer+Allicin group had significantly higher change in BW, gastric pH values, gastric GSH, gastric PGE2, gastric IL-10 and gastric gene expression of Nrf2 and H0-1, while the Ulcer+Allicin group had significantly lower levels of serum corticosterone, gastric ulcer index values, gastric MDA and gastric TNF-α (Table 1).

Table (1): Change in BW, serum corticosterone, gastric ulcer index, pH, MDA, GSH, PGE2, TNF-α, IL-10, gastric Nrf2 and HO-1 genes expression evaluation in the research groups (total 30 rats, 10 for each group)

	Control group	Ulcer group	Ulcer+Allicin group
Change in body weight (gm)	109.86±7.36	46.3±4.98*	92±3.18 *#
Serum corticosterone (ng/mL)	45.89±3.1	110.9±4.13*	89.9±4.12 *#
Macroscopic ulcer Index	0.1±0.32	30.8±2.9*	18.6±0.3*#
Gastric pH	4.6±0.22	3.02±0.24*	3.98±0.17 *#
Gastric MDA (nmol/ gm Tissue)	3.17 ±0.11	27.89±2.35*	11.99± 1.08*#
Gastric GSH (mmol/mg protein)	1.2 ±0.09	0.33±0.06*	0.85±0.02*#
Gastric PGE2 (pg/mg tissue)	30.12±2.1	16.3±1.99*	25.1±0.99*#
Gastric TNF-α (pg/mg protein)	350.9±7.1	648±9.18*	450.89±11.69*#
Gastric IL-10 (pg/g tissue)	304.8±8.97	102.9±7.99*	248.9±9.62*#
Gastric Nrf2 gene expression	1±0	0.48±0.06*	0.78±0.02*#
Gastric HO-1 gene expression	1±0	0.39±0.01*	0.62±0.02*#

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

Histopathological results:

The stomach of the control group showed normal gastric structure. Ulcer group showed the erosion of the surface epithelial cells with areas of hemorrhage. Ulcer group treated with allicin showed amelioration in gastric architecture, with the absence of erosion (Fig. 1).

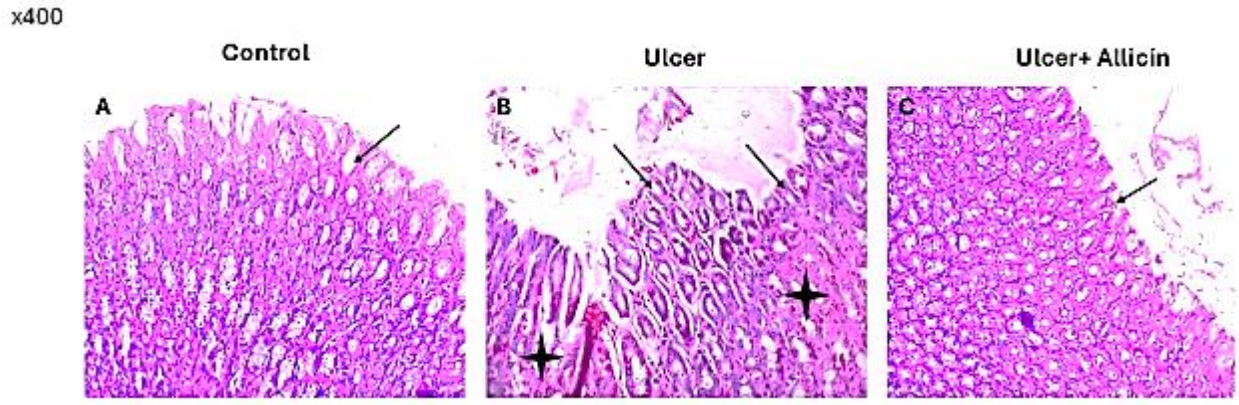

Fig. (1): H& E-stained gastric sections of the studied groups: (A) is a photomicrograph of the control group showing normal gastric mucosa (arrow). (B) is a photomicrograph of ulcer group showing the erosion of the surface epithelial cells (arrow) with areas of hemorrhage (stars). (C) is a photomicrograph of the Ulcer+Allicin group showing marked amelioration in gastric architecture, with the absence of epithelial erosion (x 400).

Compared to the control group, the ulcer group's mean PAS intensity value was substantially lower (4.5±0.2 vs. 25.2±0.02, respectively, $p<0.05$). Though it was remained considerably lower than that of the control group, the PAS intensity in the Ulcer+Allicin group was significantly greater than that of the ulcer group (14.8±0.5 vs. 4.5±0.2, respectively, $p<0.05$) (Fig. 2 A-D).

Compared to control, the ulcer group's mean COX2 intensity levels were substantially greater (72.5±1.2 vs. 15.2±0.03, respectively, $p<0.05$). The Ulcer+Allicin group's COX2 intensity was substantially greater than the control group's ($p<0.05$), but it was significantly lower than the ulcer group's (34.3±0.34 vs. 72.5±1.2, respectively, $p<0.05$) (Fig. 2 E-H).

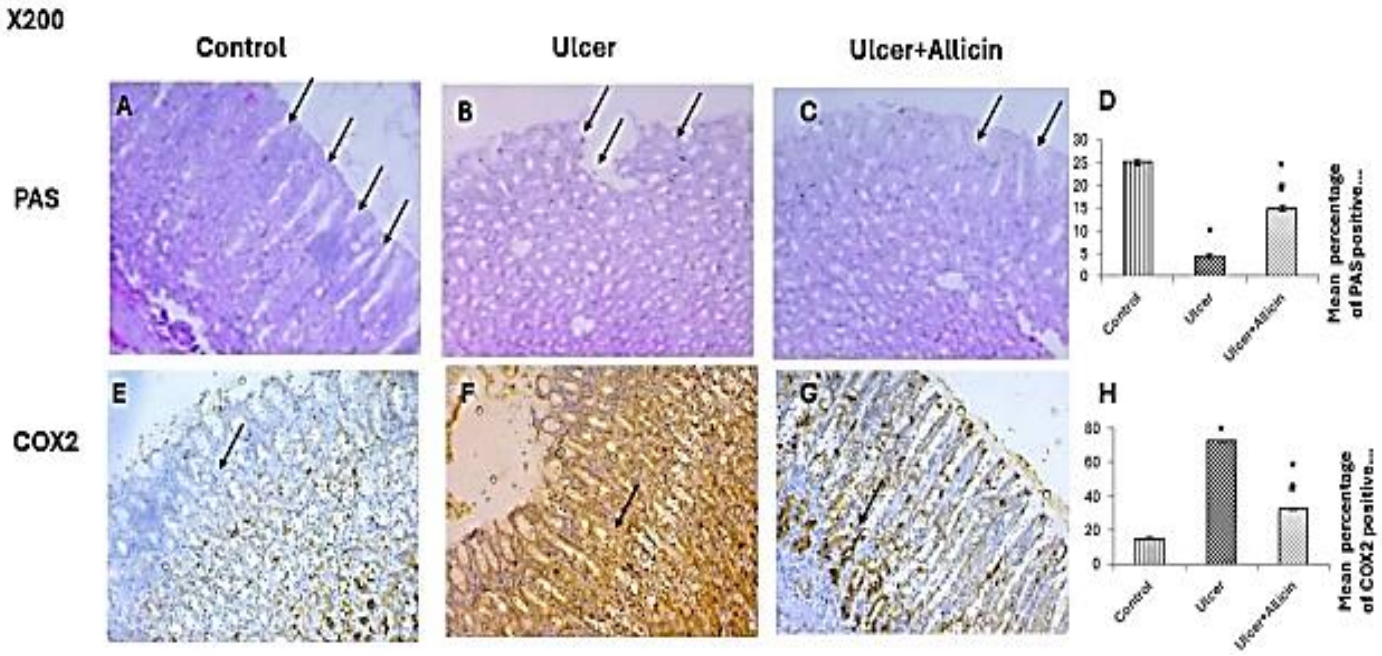

Fig. (2): Representative photomicrographs of the control group showing strong PAS reaction (arrows) indicated by a thick layer of mucous (A). Ulcer group showing faint PAS reaction (arrows) (B). Ulcer+Allicin group showing moderate PAS reaction (arrows) (C). Also, there is a significant increase in the COX2 (E-H) immunoreaction in the ulcer group (arrows) with dramatically downregulation in Ulcer+Allicin groups (arrows) (x200).

DISCUSSION

The pathogenesis of several gastrointestinal illnesses, including stomach ulcers, has been linked to stress [5]. Allicin contains anti-inflammatory and antioxidant qualities. [10] This study's primary goal was to determine whether giving rats supplements of allicin might shield them against stomach lesions brought on by immobilization stress. Therefore, serum corticosterone, oxidative stress indicators, inflammatory markers, and PGE2 in the stomach mucosa were evaluated in order to investigate the protective impact of allicin and the underlying likely processes. COX-2 immunostaining was also done in the stomach, along with the expression of the Nrf2 and HO-1 gastric genes. Allicin showed antioxidant, anti-inflammatory, and anti-stress qualities.

According to **Tian *et al.*** [11]**,** the current investigation confirmed the production of a chronic stress state by showing that immobilization stress for two hours per day for 14 days caused a large rise in blood corticosterone and a substantial drop in body weight in the ulcer group compared to control. Additionally, it created gastric stress lesions, as demonstrated by both microscopic and macroscopic analysis of the stomach. This is consistent with earlier research [15].

Because of its anti-inflammatory and antioxidant qualities, allicin significantly reduced changes in body weight and corticosterone levels as compared to the ulcer [10].

The macroscopic ulcer index values were significantly improved by allicin. It was verified by histopathological studies. Prior research demonstrated the gastroprotective effects of garlic extract [16]. Additionally, the treatment of black garlic extract demonstrated partial gastroprotective benefits [17].

Both the volume of stomach output and the degree of acidity are indicated by the pH value. A drop in pH indicates an increase in the amount of hydrogen ions in gastric juice, which causes stomach damage [18]. In the current investigation, ulcer rats showed a much higher level of stomach acidity than control rats. This is consistent with the findings of **Fatemeh *et al.*** [18] who found that stress-rats had significantly higher levels of both stimulated and baseline stomach acid than control rats. Acid causes direct damage to the mucosa, increases leucocyte infiltration, and stimulates the generation of TNF-α [19]. When compared to control, allicin considerably raised the stomach pH in the Ulcer+Allicin group. Garlic extract significantly raised stomach pH values in ethanol-induced gastric ulcers in prior research [16].

PGE2 are important lipid mediator in the stomach mucosa. By decreasing permeability, preventing the release of stomach 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 production of inflammatory mediators [20].

PGE2 in the stomach mucosa was much lower in ulcer rats than in control rats, which is consistent with earlier research [21]. Due to its anti-inflammatory properties, allicin raised PGE2 levels in the Ulcer+Allicin group relative to the Ulcer group, which explains its gastroprotective effects.

The reaction to injury in different tissues seems to be the cause of the production of the inducible isoform COX-2. COX-2 activation in inflammatory cells has been shown in a number of prior investigations in inflammatory regions [22]. It has also been observed that gastric ulceration induces COX-2 expression [23].

In line with earlier research, the current investigation found that the ulcer group had higher levels of COX-2 expression than the control group [8]. Supplementing with allicin significantly reduced the gastric COX2 immunoreaction in comparison to ulcer. This may be because allicin has anti-inflammatory properties, as **Park *et al.*** [24] demonstrated that the elevated TNF-α level may have caused the increased COX-2 expression.

By encouraging lipid peroxidation, inflammatory indicators such as TNF-α and COX-2 were hypothesized to trigger the chain reaction of stress-induced mucosal injury and tissue destruction [25].

Furthermore, the results of PAS staining (which identified mucopolysaccharides in the stomach mucosa and indicated its integrity [8]) supported the normal H&E stain's weak PAS reactivity in the ulcer group, which was in line with earlier research [8]. In agreement with other researchers, the Ulcer+Allicin group showed robust PAS-reactive mucosal cells with the addition of gastroprotective drug treatments [26].

The pathophysiology of acute stomach lesions brought on by experimental stress is known to be significantly influenced by oxygen-free radicals. Lipid peroxidation is the mechanism by which these radicals damage tissue. ROS can cause phospholipid fatty acid peroxidation, which damages cellular membranes. This is consistent with the study's findings. Our findings, are consistent with earlier research [27].

In keeping with earlier research, allicin significantly reduced oxidative stress as compared to the ulcer group [28]. Allicin's capacity to scavenge free radicals and its successful restoration of the antioxidative defense system are responsible for its antioxidant activity [29], in addition to its ability to upregulate Nrf2/HO-1 pathway [28].

According to our findings, stress causes alterations in the levels of cytokines such TNF-α and IL-10, which may be connected to the etiology of the relevant organ disorders [30]. The current study found that the stomach homogenate of ulcer rats had significantly higher levels of the inflammatory marker TNF-α and lower levels of the anti-inflammatory marker IL-10 when compared to the control. As a potent NF-κB activator and one of the primary pro-inflammatory cytokines that promote inflammation, TNF-α promotes the breakdown of stomach tissue.

Moreover, TNF-α is linked to the formation and synthesis of superoxide molecules as a result of neutrophil activation and accumulation in the stomach tissue, which disrupts the microcirculation and produces free radicals [31].

In comparison to the ulcer group, allicin decreased proinflammatory cytokines and increased anti-inflammatory IL-10. This is consistent with a prior study that linked allicin's anti-inflammatory effects to its capacity to reduce proinflammatory cytokine and downregulate NF-kB [28].

It is commonly known that Nrf2 plays a critical impact in controlling antioxidant processes in a variety of cellular settings. Hemoxygenase-1 (HO-1) and other antioxidant enzymes are therefore encouraged to manifest themselves. By controlling HO-1 expression, the oxidant-responsive transcription factor Nrf2 promotes cytoprotection against oxidative stress. Similarly, the Nrf2/HO-1 cascade's activation promotes the buildup of antioxidant enzymes in the stomach mucosa, which significantly reduces stomach damage and the development of stomach ulcers. By downregulating NF-kB, Nrf2 has been shown to suppress proinflammatory signaling and lessen inflammation [32].

According to our findings, Nrf2/HO-1 was downregulated in ulcer compared to control, which is consistent with earlier research [33]. Allicin had a gastroprotective effect in our study by upregulating Nrf2/HO-1 compared to ulcer, which is consistent with other researches [28, 34].

CONCLUSION

Through the upregulation of the Nrf2/HO-1 signaling pathway and its anti-inflammatory and antioxidant qualities, allicin provides gastroprotective effects to rats with stress-induced ulcers. Therefore, allicin could have a therapeutic impact on stomach stress ulcers.

Conflict of interest: None.
Financial disclosures: None.

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