

# Macroscopic and Microscopic Evaluation of Burn Wound Healing Infected with Methicillin-Resistant *Staphylococcus aureus* in Immunocompromised Rats Using Clove Extract

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## Abstract

**Background:** Severe or moderate burns can be life-threatening, and being infected by multidrug resistant bacteria (MRSA strain) makes treating burns a significant issue. **Objectives:** To evaluate the healing effect of clove extract in improving burn wound healing infected with MRSA in experimentally immunocompromised rats. **Materials and Methods:** Thirty rats (200-250 g) were experimentally immunocompromised with prednisone and azathioprine, and the burn (deep II-degree) was induced using heating bar end and directly infected with MRSA inoculum. All 30 rats were divided randomly into three groups ( $n = 10$ ); named as control negative (burn not infected), burns infected with MRSA and treated with clove extract 5%, and burns infected with MRSA and treated with silver sulfadiazine (SSDI%). The treatment was daily and locally for 14 days. Burn wound healing diameter, histopathology, and immunohistopathology examination were carried out at the end of days 7 and 14. **Results:** The topical delivery of clove extract gel into the burn skin showed significantly reducing burn diameter when compared with silver sulfadiazine-treated group and control group at  $P < 0.05$ . In addition, the microscopic examination of histological sections of burn skin in clove extract-treated group showed low infiltration of inflammatory cells in comparison with other groups. Also the immunohistopathological examination of local expression of tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) in clove extract and silver sulfadiazine-treated group showed a relative decrease in the expression of TNF- $\alpha$ . **Conclusion:** Clove extract can provide effective therapy for infected burn wound because it improves/immunosuppresses wound healing by exerting immunomodulatory/anti-inflammatory effects.

**Keywords:** Burn skin, clove extract, histopathology, MRSA, wound healing

## INTRODUCTION

Burn injuries are a significant public health issue that may have a high morbidity and fatality rate.<sup>[1]</sup> Burns are considered as one of the most serious injuries in the world.<sup>[2]</sup> Considering the most recent data from the World Health Organization, burns are responsible for 180,000 fatalities per year. In 2004, over 11 million individuals required medical care due to serious burns.<sup>[3]</sup> According to the Iraq injury surveillance system, burns are the second most common reason for emergency department visits; however, there are few local research on the epidemiology of burns.<sup>[4,5]</sup> Mortality following such an injury is most often due to infection; in recent years, infections in burn

patients have been shown to be associated with reduced host defenses such as diabetes mellitus, nutritional status, and medications with corticosteroids that one of its major side effects is immunosuppression.<sup>[6,7]</sup> In fact, it has been noted that corticosteroids may interfere

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with the healing process at multiple stages through the healing cascade.<sup>[8]</sup> Many diseases, including those caused by allergies, inflammation, immune-mediated illnesses, spinal injuries and hypoadrenocorticism, are treated with corticosteroids.<sup>[9]</sup> Immunosuppression is one of the most significant negative effects of long-term use of corticosteroids.<sup>[10]</sup> The topical antimicrobials are always given prophylactically to prevent infectious complications because burn wound patients are more susceptible to infection with microorganism, specifically multidrug resistant infections (MDR),<sup>[11]</sup> which usually are resulting in more prolonged hospital stays, slower wound healing, more expensive, and higher mortality rate.<sup>[12,13]</sup> Antibiotics failure to treat infections is caused by multidrug resistant bacteria (MDR) bacteria, particularly methicillin-resistant *Staphylococcus aureus* (MRSA) which has recently grown to be a significant global public health issue. The prevalence of this strain had shown continuous increase and as colonization rates rise, so are infection rates in the community and in hospitals.<sup>[14]</sup> MRSA is considered the most common bacterium which may infect the burns, and has triggered more studying effort to identify new antimicrobial strategies that are more effective and encourage the development of less bacterial resistance.<sup>[15]</sup> Pharmacological investigations have shown that many herbal medications that comprise organic elements and chemicals have an enhanced biological activity and can expedite the healing of wounds caused by injuries and specific illnesses.<sup>[16-18]</sup> The advantages of clove oil are related to its antimicrobial properties, antifungal effect, antiviral properties, and disinfectant effect.<sup>[19]</sup> In addition to antibacterial, antifungal, antiviral and, antioxidant activities, in that clove oil extract has cytotoxic and anti-inflammatory properties.<sup>[20]</sup> This study's objective was to assess biological activity of clove oil extract on the healing of burn wounds infected with MRSA in immunocompromised rats.

## MATERIALS AND METHODS

### Preparation of clove extract

The clove (*Syzygium aromaticum*) was obtained from a local market of Iraq. In order to obtain powder form of the plant, the plant was dried by using a mechanical mortar, and was later ground into powder. The hydro-distillation extraction was performed by cleveger equipment. The amount of *S. aromaticum* L. used for hydro-distillation was 200 g, and the operation was let to continue for 3 h. The flask was firstly joined to the machine and electrically heated. The temperature raised progressively to 100°C from a starting point of roughly 80°C. The oil was collected after isolating the water, and to increase the water disposal a little of anhydrous sodium sulfate was used. The extract oil was kept in the refrigerator at a temperature of 4°C.<sup>[21,22]</sup>

### Preparation of gel formula of clove extract

Gel formulation has been prepared by the use of the physiologically and pharmaceutically inert and non-sensitizing carbopol polymer which is used as stabilizer and suspender for many pharmaceutical products and prepared as per the followings method<sup>[23]</sup>: Carbopol 1% was prepared by dispersion of 1.0 g of carbopol powder in 80 mL of deionized water with continuous stirring using a magnetic stirrer at 700 rpm and 30°C for about 30-60 min. Then the volume was completed to 100 mL by deionized water. To neutralize the pH to  $\approx 7$  added few drops of triethanolamine (TEA). In order to prepare a carbopol gel containing clove extract at 0.05%, 50 mg of clove extract added to 100 g of carbopol gel as they prepared separately with constant stirring for 0.5–1 h. The resulting mixtures were homogenized by ultrasound to obtain a homogeneous transparent colored gel solution.

### Study design and animals

This study was approved by Baghdad University's Ethics Committee and carried out in accordance with the Faculty of Veterinary Medicine's Animal Care Review Board's regulations for the handling and use of laboratory animals. A total of 30 male Wistar rats which aged around 9-10 weeks and weighted 200-250 g received from the University of Baghdad's College of Veterinary Medicine's Animal House were utilized as model to perform the experiment of the current study. Commercial pellets were given to the animals, and tap water was also supplied. Each group of rats were kept in a plastic cage with bedding made of hard wood chips, which was changed every three days to maintain a clean environment.

### The bacterial isolates used in induction of infection

The challenge bacteria MRSA was obtained from the Physiology, Biochemistry and Pharmacology Department in College of Veterinary Medicine, University of Baghdad. In Muller-Hinton broth, the bacteria were cultured, and the suspension part was centrifuged for 15 min at 1000 rpm. The supernatant had been discarded and sterile phosphate-buffered saline was used to dilute the bacteria to  $10^8$  colony forming unit (CFU) mL<sup>-1</sup>.

### Surgical procedures and grouping

Firstly, the immunosuppression was attained by the oral administration of azathioprine (50 mg/tablet) at a dose of 2 mg/kg once a day for 14 days, together with prednisone (5 mg/tablet) at a dose of 2 mg/kg. Rats in the control group did not receive any medication during this time.<sup>[24]</sup> Under general anesthesia by intra muscular injection of xylazine 2% 4 mg/kg body weight (BW) and ketamine 10% 50 mg/kg BW,<sup>[7]</sup> 30 rats were subjected to skin burn by hot bar brass round end of 2.0 cm in diameter, placed on their backs for 15 s without applying any pressure after being submerged in boiling water (100°C) for 10 min.<sup>[25]</sup> The infection with

MRSA was induced by applying topically 100 µL of the MRSA inoculum (containing 10<sup>8</sup> CFU of total bacteria) that was previously prepared at the burn site directly after induction of burn. The rats were monitored daily for the symptoms. After that, the rats were divided into three equal groups, as follows: (*n* = 10) randomly named as control negative (burn not infected), burns infected with MRSA were treated with clove extract (5%), and burns infected with MRSA were treated with silver sulfadiazine (1%). The wounds were covered with burn dressings, which were changed every 24h. At the end of days 7, 14 the relevant tests, such as measurement of burn wound diameter, histopathology, and immunohistochemistry study were done by resecting the tissue biopsy from the burned skin, and then the rats were euthanized.

### Measurement of burn wound diameter

A vernier caliper was used to measure the diameter of the induced burn. The procedure for the measurement of burn area had been measured four times, and the average of those values was determined. Immediately following burn induction, as well as at 7 and 14 days later, the burns' diameter was measured.

### Histopathological examination of the burn wounds healing

Samples of burned skin were taken from each animal under general anesthesia on the day zero, 7, and 14 after burn treatment. Samples were excised from the edge of the burn along with about 5 mm surrounding normal skin and fixed in neutral-buffered formalin 10%. Slides were prepared according to reference.<sup>[26]</sup>

### Immunohistochemistry study of burned skin section

Immunohistochemistry was carried out using Dako EnViision detection immunohistochemistry kit (Enviision FLEX, Dako, K8000, Denmark) and as per the manufacturer's instructions. The anti-tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) primary Antibody (Polyclonal Rabbit Antibody: E-AB-40015, Elabscience, China) was employed in the current investigation to identify and assess the expression of TNF- $\alpha$  in skin tissue.

### Statistical analysis

The Statistical Analysis System (SAS) program was utilized to determine how various factors affected the research parameters. Least significant difference (LSD) test (Analysis of Variance—ANOVA) was employed in this study to compare the means in a significant way.

### Ethical consideration

The study was conducted in accordance with the ethical principles that have their origin in the Declaration of Helsinki. It was carried out with patients verbal and analytical approval before sample was taken. Ethical

approval was granted through the local committee of animal care and use at the college of Veterinary Medicine/ University of Baghdad before starting this study. A local ethics committee evaluated and approved the study protocol according to the document number 1604 on July 26, 2023 by Veterinary Medicine/ University of Baghdad.

## RESULTS

### Macroscopic evaluation of burn wounds healing

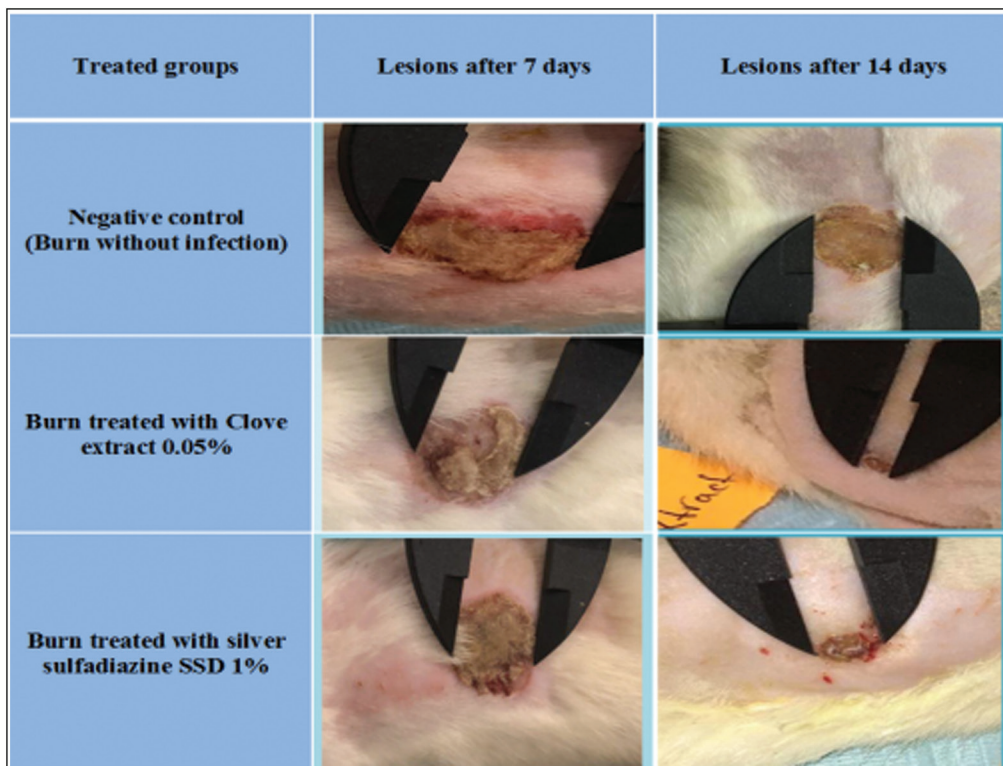
On the physical appearance and the reduction in burn diameter, the therapeutic effect was evaluated. Instantly after the onset of the burn, the burned skin displayed paleness in all experimentally induced immunocompromised rats as shown in Figure 1. In a rat model, the efficacy of clove extract in the healing of burnt wounds was investigated, in which administrated as gel formula for topical application of clove extract placed on a polymer or polysaccharide to produce a hydrogel formulation which makes administration of these agents convenient. At day 7 post burn, the results of treated groups showed enhancement in burned skin by development thin superficial layer beneath the scab in groups treated with clove extract and SSD as compared with control group which showed no signs of healing only reduction in inflammation sings with persistence of the scab. At day 14 post burn, clove extract group demonstrated better healing indications, where the superficial layer completely formed with hair growing and to a lesser extent in the SSD group compared to the control group, which possessed only a thin superficial layer

The diameter of the burns on the first day was 20mm and burns diameter on subsequent days (7 and 14 days) were compared with diameter of the burns on the first day. In the experimental induced immunocompromised rats and after burn induction, the comparison was made between periods (at 7 and 14 day) to assess the reduction in burn diameter of the same group which showed significant decrease ( $P < 0.05$ ) in burn diameter, as well as significant reduction in burn diameter was observed between all treated groups at the same periods [Table 1]. The results of the comparison that made between burns diameters extents at day 7 and 14 days after burn induction revealed that clove extract showed a significant difference when compared with control and SSD groups [Table 1 and Figure 1].

### Microscopic evaluation of burn wounds healing

#### *Histological study of burned skin section*

Microscopic appearance of skin samples taken from negative control group in experimentally induced immunocompromised rats at day 7 post burn revealed thick epidermis supported by dermal immature granulation tissue which infiltrated with inflammatory cells, hemorrhage and little of immature collagen fibrils [Figure 2A]. At day 14 post burn there was very thick



**Figure 1:** The burn wound healing activity of clove extract compared with silver sulfadiazine and negative control treatment at 7 and 14 days of post burn

**Table 1: Means of burn diameter (mm) in immunocompromised rats infected with MRSA after 7, 14, days of treated with clove oil and hamazin or kept without treatment**

Groups	Periods		
	Zero day	7days	14 days
Negative control	20.00 ± 0.00	18.30 ± 0.32	14.90 ± 0.50
Burn without infection	A a	A a	A b
Burn treated with Clove extract 0.05%	20.00 ± 0.00	15.54 ± 0.24	6.02 ± 0.32
	A a	B b	C c
Burn treated with silver sulfadiazine SSD 1% (Hamazin)	20.00 ± 0.00	18.48 ± 0.51	9.10 ± 0.37
	A a	A a	B b
LSD value		2.23	

Values represent mean ± SE

Means with different capital letters in the same column and small letters in the same row are significantly different ( $P \leq 0.05$ ) among the groups

epidermis layer that supported by dermal granulation tissue which infiltrated with little inflammatory cells and blood vessels in addition for numerous sebaceous glands and hair follicles [Figure 2B].

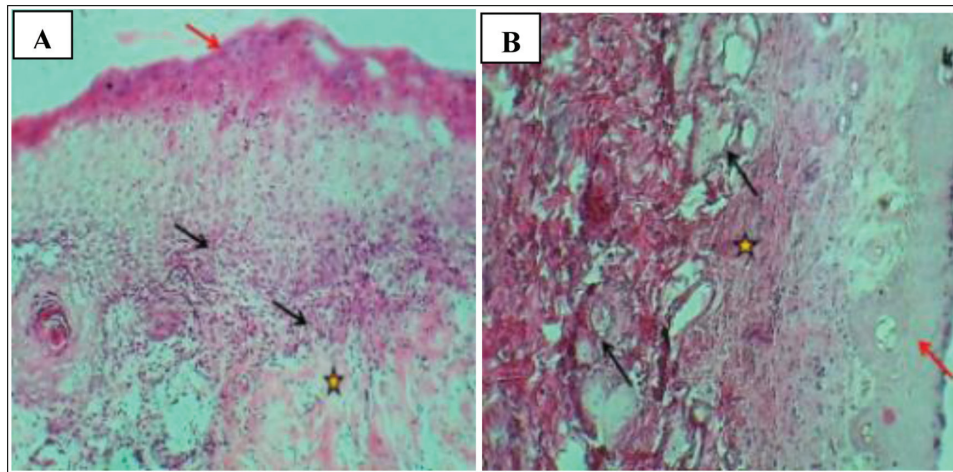
On the other hand, the histomorphological analysis of clove extract-treated group, at day 7 post burn infected with MRSA in immunocompromised rats, showed dermis composed of mature fibrous tissue with numerous hair follicles development of epidermal pads (early epidermis repair) [Figure 3A]. At day 14 post burn showed very

thick epidermis and revealed mature collagen bundles of dermis with well angiogenesis (dilated blood vessels) and hair follicles [Figure 3B].

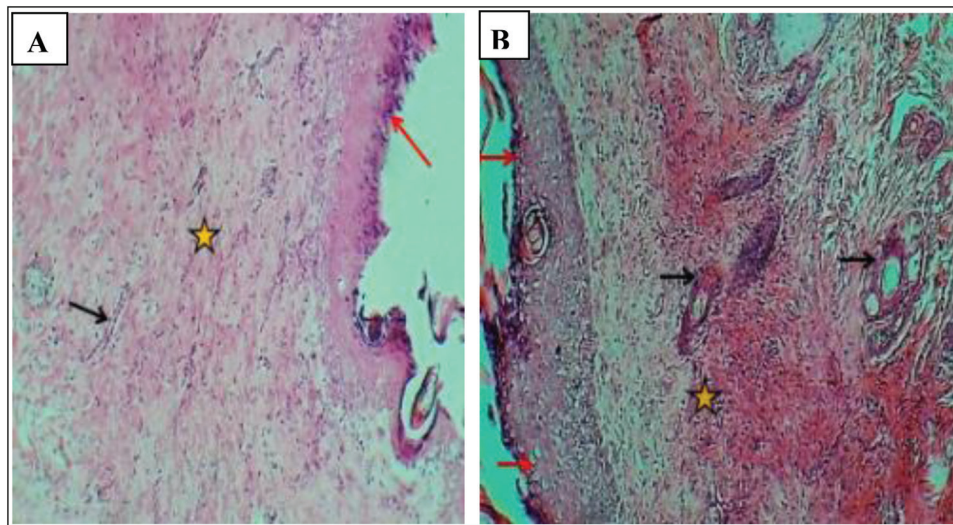
Microscopic examination of burn skin section at day 7 post burn of group infected with MRSA and treated with SSD in immunocompromised rats revealed epidermis and sub epithelial dermis show proliferating in epithelial cells, little hemorrhage, within immature granulation collagen fibrils and little mononuclear leukocytes [Figure 4A]. At day 14 after burn induction, histopathological figure of the skin revealed that the epidermis was very thick layer that completely formed with marked parakeratosis. The dermis comprised of fibrous tissue that invaded with meshwork of severely dilated blood vessels and numerous hair follicles [Figure 4B]

**Immunohistochemistry study of burned skin section**

The microscopic examination of the burn skin section infected with MRSA in immunocompromised rats in the control group (negative control) showed overexpression of TNF- $\alpha$  in squamous epithelial cells of all layer of epidermis at wound area margin, that involved 50% of affected area of burn skin section as shown in Figure 5 also overexpression of TNF- $\alpha$  was seen in the SSD treated group as was clearly observed in Figure 6, whereas, weak expression of TNF- $\alpha$  was observed in stratum basale of epidermis at margin of wound area in clove extract-treated group as noted in Figure 7.



**Figure 2:** (A) Histopathological section of burn skin in in negative control group at 7 days post burn shows: thick epidermis (red arrow), immature granulation tissue infiltrated with inflammatory cells (black arrows), immature collagen fibrils (Asterisk). H&E stain 100×. (B) Histopathological section of burn skin in in negative control group at 14 days post burn shows: thick epidermis (red arrow), granulation tissue infiltrated with inflammatory cells (asterisk) and sebaceous glands (black arrows). H&E stain 100×



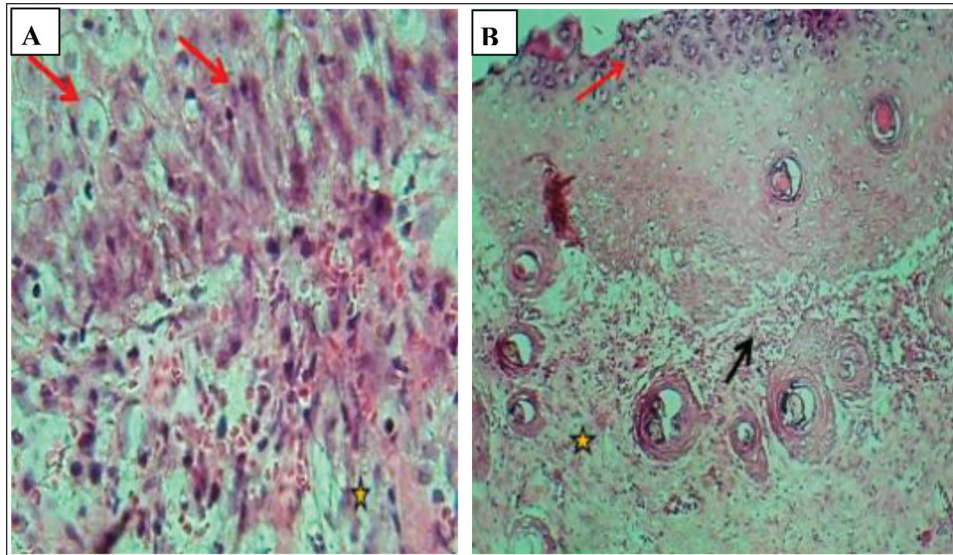
**Figure 3:** (A) Histopathological section of burn skin infected with *MRSA* in group treated with clove extract at 7 days post burn shows: early epidermis repair (red arrow), maturing fibrous tissue with little inflammation (asterisk) and numerous blood vessel (black arrows). H&E stain 40×. (B) Histopathological section of burn skin infected with *MRSA* in group treated with clove extract at 14 days post burn shows: thick epidermis (red arrows), mature dermis fibrous tissue (asterisk) and numerous hair follicles (black arrows). H&E stain 40×

## DISCUSSION

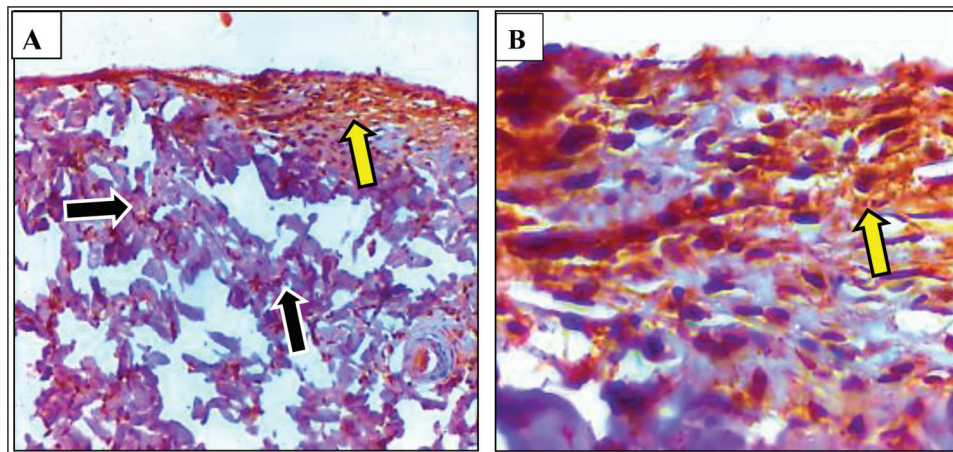
There are several local and systemic factors that may delay wound healing. Many of these factors treated with many medications may delay wound healing such as steroids, particularly glucocorticoids, are known for having negative effects on the healing of wounds.<sup>[27]</sup> On other hand, *S. aureus* attacks eschar, enters subcutaneous and the unburned tissues and producing abscesses of various sizes. These abscesses protect *S. aureus* from the host immune system and antibiotics and also offer suitable conditions for penetrating this bacteria into the circulation because of their virulence's factors.<sup>[28]</sup> At the site of the wound infection, bacterial invasion prompts the release

of cytokines by the inflammatory cascade, which causes inflammation.<sup>[29]</sup> For this reason, a significant number of inflammatory cells can be observed in the negative control group's high power resolution picture, indicating that the inflammatory stage is still ongoing and the tissue has not yet reached the stage of reepithelization.<sup>[30]</sup>

This study investigates the impact of topical application of hydrogel containing clove extract on the histopathological alterations of *MRSA*-infected wounds in immunosuppression rats during a period of 14 days. The results indicate that the application of clove oil resulted in an elevation of epidermal height and capillary density. In addition to a decrease in the presence of the



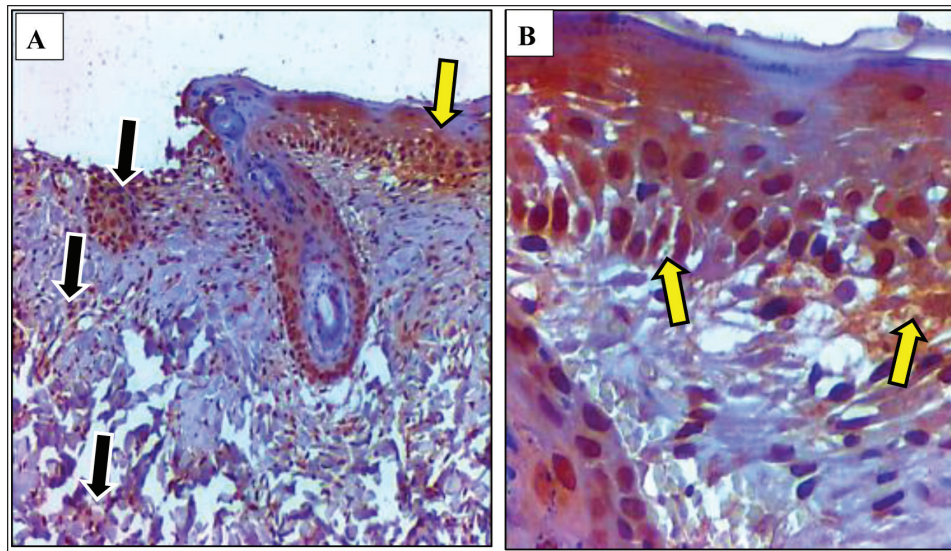
**Figure 4:** (A) Histopathological section of burn skin infected with *MRSA* in group treated with SSD at 7 days post burn shows: proliferating epithelial cells (red arrow) sub epithelial hemorrhage within, immature granulation tissue (asterisk). H&E stain 400 $\times$ . (B) Histopathological section of burn skin infected with *MRSA* in group treated with SSD at 14 days post burn shows: epidermis with marked parakeratosis (red arrow), dermis fibrous tissue (asterisk) with numerous hair follicles and meshwork of dilated blood vessels (black arrow). H&E stain 100 $\times$



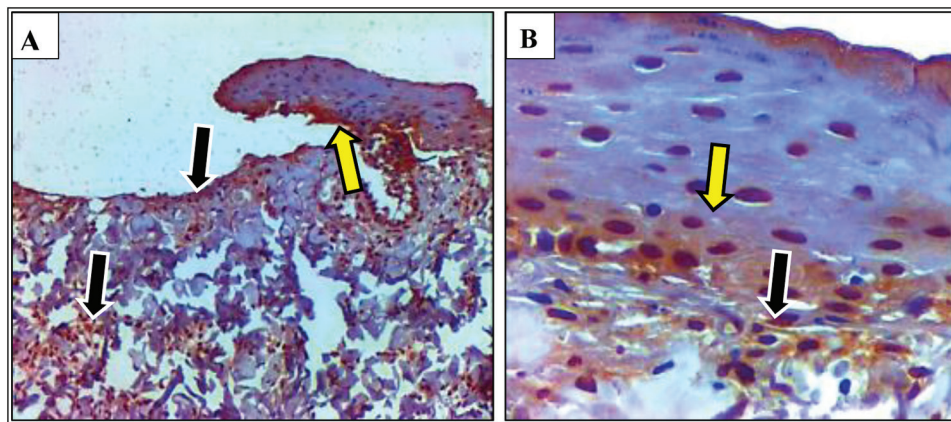
**Figure 5:** Immunohistological section of burn skin in negative control group. (A and B) Overexpression of TNF- $\alpha$  (yellow arrow) was observed in squamous epithelial cells of all layer of epidermis at wound area margin. Also, TNF- $\alpha$  expression (black arrow) was observed in dermis in wound area. DAB & hematoxylin. (A) 100 $\times$  and (B) 400 $\times$

inflammatory cells, which collectively indicate a potential improvement in wound healing, and this is a comply.<sup>[31]</sup> Clove oil's potential ability to heal wounds rapidly may be linked to the antioxidant and anti-inflammatory effects of its phytochemical components, as well as its antibacterial efficacy, particularly in relation to phenolic compounds.<sup>[32]</sup> Clove oil is considered an effective healing substance for burns and wounds in conventional medicine. Burns typically have high oxidative stress, which requires antioxidant therapy such as clove oil which have antioxidant properties can be therefore promising agents, and it has been demonstrated to be effective in reducing inflammation and promoting tissue healing by combating off these free radicals and without suitable antioxidant therapies, burn wound healing may

be delayed or serious tissue damage may happen.<sup>[33]</sup> Also a study<sup>[34]</sup> who reported the anti-MRSA potential of clove oil and have demonstrated the major constituent of clove oil that is responsible for antibacterial effect which is important in wound-healing process. On other hand, the use of silver sulfadiazine, a synthetic ointment commonly employed in the treatment of burns, has demonstrated efficacy as an antibacterial and antifungal agent. However, its use is associated with a notable drawback, namely the inhibition of fibroblast and keratinocyte proliferation.<sup>[35]</sup> In accordance with our findings, the group that received silver sulfadiazine ointment did not exhibit any significant progress in managing MRSA-infected burns in comparison to the other treated groups.



**Figure 6:** Immunohistological of burn skin infected with MRSA in group treated with SSD ointment. (A and B) Over expression of TNF- $\alpha$  (yellow arrow) was observed in squamous epithelial cells of all layer of epidermis at wound area margin. However, moderate expression of TNF- $\alpha$  (black arrow) was observed in dermis layer in wound area. DAB & hematoxylin. (A) 100 $\times$  and (B) 400 $\times$



**Figure 7:** Immunohistological of burn skin infected with MRSA in group treated with clove extract. (A and B) Weak expression of TNF- $\alpha$  (yellow arrow) was observed in stratum basale of epidermis at margin of wound area. However, moderate expression of TNF- $\alpha$  (black arrow) was observed in dermis layer in wound area. DAB & hematoxylin. (A) 100 $\times$  and (B) 400 $\times$

With regarded to the immunohistochemistry examination that conducted in this study to evaluate anti-inflammatory and immunomodulation effect of clove extract in burn wound healing, the level of TNF- $\alpha$  had been examined locally in burned skin section since TNF- $\alpha$  exhibits a significant upregulation in burn injuries.<sup>[36]</sup> Several of the most efficacious pharmaceuticals presently available depend on the inhibition of TNF- $\alpha$  and are utilized to manage a variety of inflammatory medical conditions.<sup>[37]</sup>

The topical administration of clove extract can generate an anti-inflammatory microenvironment that is related to the bioactive substance that was present in the extract. This mechanism enhances the wound microenvironment and minimizes additional tissue damage.<sup>[38]</sup> Numerous investigations have highlighted the anti-inflammatory properties of clove and eugenol. These studies have

reported that these substances possess the ability to regulate various inflammatory markers, including to nitric oxide, COX-2, iNOS, leukotriene C4, prostaglandin E2, mast cell degranulation, nuclear factor kappa B (NF- $\kappa$ B) and the transcription factors nuclear factor of activated T cells (NF-AT).<sup>[39]</sup> Altering the inflammatory mechanism through cytokines modulation, or the resultant activated immune cells, is a potential therapeutic strategy for protecting viable tissue from burn-induced pathogenesis. The topical administration of clove extract in a immunocompromised rat model had demonstrated significant decreased local TNF- $\alpha$  levels on burn injuries. The findings regarding the effect of clove extract on the decrease the formation and release of TNF- $\alpha$  presented in this study are consistent with previous research conducted by,<sup>[40]</sup> in their study they demonstrated that the administration of eugenol significantly decreased

the levels of transforming growth factor, interferon, and TNF- $\alpha$  in vivo.

In general, the histological and immunohistochemical observations provided support for the macroscopic examination results and confirmed that the clove extract group was more effective in accelerating the healing process of burn wounds in immunosuppression rats by exerting anti-inflammatory, antibacterial effect when compared it to the untreated group (control group) and the SSD groups.

## CONCLUSION

Burn wound healing studies in immunocompromised rats have showed that the topical delivery of clove extract gel into the burn skin had shown significantly reducing burn diameter in the clove extract-treated group when compared with the untreated group (negative control group) and silver sulfadiazine-treated group. In addition, the microscopic examination of histological and immunohistological sections of burn skin in clove extract-treated group showed low infiltration with inflammatory cells in comparison with other groups of study with better histological regeneration by accelerating epithelium layer formation; therefore, this indicates clove extract had positive effects when used locally to improve burn healing.

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Nil.

## Conflicts of interest

There is no conflict of interest.

## Authors' contribution

All the authors have contributed equally in terms of giving their technical knowledge to frame the article.

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