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ORIGINAL ARTICLE

The Antibacterial and Wound-Healing Effects of Clove (*Syzygium aromaticum*) Essential Oil on Burn Wounds Contaminated with *Pseudomonas aeruginosa* in Rats

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Clove Oil for Burn Wound Healing and Antibacterial Action in Rats

Abstract

One of the major causes of death in burn patients is bacterial infections. An effective approach to speeding up the healing process and preventing antibiotic resistance in infected burn wounds is to use natural antibiotics such as clove (*Syzygium aromaticum*) extract. This study assesses the medicinal and anti-bacterial effects of clove essential oil on burn wounds infected with Pseudomonas aeruginosa compared to Silver Sulfadiazine (SSD). Forty male Wistar rats were randomly divided into five groups. After anesthesia, bilateral paralumbar burns were inflicted, resulting in a total of 80 wounds. The wounds were artificially infected with *Pseudomonas aeruginosa* 24 hours post-burn. Wound areas were measured daily for 14 days (days 0, 1, 2, 3, 5, 7, 9, 11, 13, and 14). Pathological indices and bacterial colony counts were determined on days 7 and 14. Additionally, the in vitro inhibitory effect of clove extract concentration on the growth of Pseudomonas aeruginosa was investigated. Macroscopic assessment showed a significant reduction in wound size in both the 10% concentration group and the positive control group. While histopathological examination on day 7 indicated a slight advantage for the 30% clove concentration group in some pathological indices, but no significant differences were observed between groups ($p < 0.05$). By day 14, the positive control and 10% clove groups demonstrated superior healing, while the 30% clove group exhibited the worst outcomes, suggesting that prolonged exposure to high concentrations of clove may hinder wound repair.

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Introduction

Every year, numerous patients suffer from burns, one of the most common and devastating forms of trauma due to various accidents. This issue has been recognized as a major problem worldwide (1). Up to 75% of fatalities in individuals who have suffered thermal injuries are caused by nosocomial burn wound infections (2). With disappearing skin as the primary defense and a crucial barrier against microbial invasion in burn wounds, a suitable condition is provided for implantation and invasion of opportunistic pathogens to cause infection (3, 4). Although burned patients are prone to a range of bacterial and fungal colonization, the most common micro-organism that has a prominent role in the infection of burn wounds is *Pseudomonas aeruginosa* (*P. aeruginosa*) (5, 6). *P. aeruginosa* is considered one of the most resistant gram-negative bacteria (7). Septicemia, endocarditis, keratitis, and skin infections are just a few acute illnesses that can be caused by this bacterium colonizing in patients (8). These infections are sometimes very difficult to cure due to their intrinsic medication resistance. The issue of growing drug resistance as well as the paucity of new antimicrobial medication discoveries in recent years has further exacerbated this issue (5).

A variety of ointments, and antibiotics are used to treat burn wound infections, and in local burn treatment, silver sulfadiazine (SSD) has been introduced as the gold standard with antibacterial properties (9). SSD has an enviable safety record, but due to its side effects, it is not applicable to premature babies or newborns during the first 2 months of life and pregnancy as it is considered a pregnancy category B drug (10). Considering the increasing resistance of *P. aeruginosa* to chemicals and drugs, the fatal risks of its infection, and the side effects of SSD treatment, treatment based on medicinal plants with different antibacterial and healing effects can be a sufficient alternative (11).

Antibacterial and anti-quorum sensing phytoconstituents from medicinal plants may be helpful in lowering the dosage of antibiotics, hence reducing the risk of toxicity and the emergence of resistance (12). Herbal medicines are gaining popularity due to perceived lower side effects, with around 80% of the global population relying on them for healthcare (13). Clove (*Syzygium aromaticum*), *S. aromaticum*, a dried flower bud from the Myrtaceae family, is a valuable spice that has been used for centuries as a food preservative and medicinal plant (13). Studies have reported antibacterial, antioxidant, anti-cancer, anti-inflammatory, antiseptic, antispasmodic properties for clove extracts (13, 14). Approximately 89% of clove essential oil is eugenol, and 5% to 15% is eugenol acetate and β-caryophyllene. Another important compound found in clove essential oil in concentrations up to 2.1% is α-humulene. Other volatile compounds present in lower concentrations in clove essential oil include β-pinene, 2-heptanone, benzaldehyde, farnesol, and ethyl hexanoate (15, 16).

Animal models of burn wounds are essential for analyzing and development of burn wound treatments. Although the natural progression of infection in full-thickness burn lesions is not replicated by them, nor do they imitate what happens in burn centers. Pigs, mice, and rats are commonly used animal models for studying burn wound infections. However, the use of porcine and mice models is limited despite their advantages for simulating this complication. Pigs incur numerous costs and moral consequences, while mice are often limited to an initial burn wound infection (less than 48 hours after inoculation) before sepsis and death rapidly ensue. Since the initial Walker-Mason model of burns was reported in the 1960s, rats have been crucial in the development and introduction of antimicrobial burn wound therapies (17, 20).

Therefore, this study aimed to investigate the antibacterial and healing effects of different concentrations of *S. aromaticum* essential oil on *P. aeruginosa* burn wound infection in comparison to SSD in experimental rats.

Materials and Methods

Plant Material and Preparation of *S. aromaticum* Essential Oil

Fresh clove spices were powdered using an electric mixer after being acquired from Kerman's old bazaar in July 2022. Three and a half hours were spent combining 200 grams of the powder with 700 mL of distilled water in a Clevenger apparatus fitted with an electric mantel heater. The essential oil was extracted by hydrodistillation, and gas employed to evaluate its chemical components (21).

Formulation of *S. aromaticum* Essential Oil Ointment

The fusion method was used to create *S. aromaticum* essential oil ointments (10%, 20%, and 30% w/w) using emulsifying wax, liquid paraffin, and soft paraffin (24). The stability, diffusion, and physicochemical characteristics of the prepared extract ointment were evaluated (24, 25). A base composed of emulsifying wax, liquid paraffin, and soft paraffin was employed for control animals.

Pathogenic Bacteria and *In Vitro* Antimicrobial Activity Assay

The standard strain of the bacterial pathogen *P. aeruginosa*

(PAO-1) was acquired from Kerman University of Medical Sciences (Kerman province, Iran) and inoculated onto Nutrient-Agar (N.A) plates using the streaking method. After 24 hours of incubation at 37 °C, a single colony was transferred from the plates to injectable normal saline (NS) using an inoculation needle. The culture solution was diluted with sterile saline to a standard dilution of 0.5 McFarland (1.5×10^8 CFU/ml), and then sterile cotton swabs dipped in the solution were spread on N.A plates according to the method described by Henry (22).

The Kirby and Bauer disk diffusion method was used to measure the antibacterial activity and obtain a sufficient and efficient concentration of clove oil (23). Commercial SSD and different concentrations of clove essential oil (from 10% to 100%) were prepared in 1 ml microtubes by mixing clove extract and dimethyl sulfoxide (DMSO) as a diluent. The blank disks were autoclaved for 20 minutes at 120°C for sterilization, then dipped in each concentration individually and pressed into the bacteria-seeded agar plates (5 per plate). To prevent the crossing of inhibition zones, a 20 mm gap was maintained between the plates' borders. A filter paper disc loaded with 20 µL DMSO (10%) was used as a negative control, and SSD was employed as a positive control. The plates were incubated for 24 hours at 37°C, and the inhibition zone was monitored.

Animals

Forty sexually mature male Wistar albino rats weighing 250–300 g (2–3 months old) were purchased from the animal laboratory of Kerman University of Medical Sciences, Kerman province, Iran. They were housed in the Animal Care Center laboratory at the Veterinary Medicine Faculty of Shahid Bahonar University of Kerman for one week before treatment. The rats were randomly divided into five groups of eight animals. They were housed in standard polypropylene cages with wire mesh tops at 22 ± 2 ˚C and 55 ± 5% humidity in a 12 hour/12 hour dark-light cycle. During the study, the animals had free access to water and pellet food (Javaneh Khorasan Co., Mashhad, Iran).

The in vivo experiments were designed according to the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes of ETS N123 (26). All ethical concerns in the usage of animals were considered and the experimental protocol was conducted following the guidelines of the Ethics Committee of Shahid Bahonar University of Kerman. All tests were conducted in an aseptic environment, and the procedure for anesthesia, surgery, and postoperative care was the same for all animals. The animals were kept in individual cages throughout the investigations.

Experimental Design and Creation of Infected Burn Wounds

All rats were anesthetized using ketamine (100 mg/kg) and xylazine (20 mg/kg). Hypothermia was controlled with the help of a warm plate during surgery. Their right and left paralumbar areas were shaved, prepared surgically and exposed. Burn wounds were induced by a hot cylindrical metal rod (1.5 cm in diameter). The metal rod was placed in hot boiling water for 1 minute and then applied to the prepared areas on the rat's body and held for 20 seconds to reach the standard of second-degree burns.

In this study, tramadol, an opioid analgesic, was administered to induce analgesia in rat burn models. A dose of tramadol ranging from 10 to 40 mg/kg was administered intraperitoneally immediately following the induction of the burn injury.

After 24 hours, dead tissues were excised using a sterile surgical blade and wounds were contaminated with *P. aeruginosa* (1 McFarland). Contamination was done again after 24 hours for assurance that infection was successful. Twenty-four hours later (on day 3 after burn injury) the treatments began with ointments, which were topically applied 1–1.5 mm once a day with the following drugs: Group-N (negative control) received no treatment at all. Groups A, B, and C were treated with ointments containing 10%, 20%, and 30% clove oil, respectively. Group P (positive control) received the standard drug (1% SSD).

On days 0 (burn injury day), 1, 2, 3, 5, 7, 9, 11, 13, and 14, all rats were anesthetized, clinical assessments including observations concerning the appearance and wound size measurement were performed, and digital photography was taken for image processing and analysis. On the 7th and 14th days of the experiment, half of the rats in each group were sacrificed and the wounds were removed along with the surrounding skin for histological evaluation. All animals were euthanized by CO2 according to AVMA Guidelines for the Euthanasia of Animals (27).

Macroscopic Wound Contraction Evaluation

Wounds were monitored daily with digital photographs taken using an Olympus digital camera. An L-shaped ruler was included in all photos for calibration of measurements. The images were analyzed using Image J software (Imaging Processing and Analysis in Java, National Institutes of Health). Wound areas were measured daily for 14 days (days 0, 1, 2, 3, 5, 7, 9, 11, 13, and 14).

Microbial Infection Assessment of the Induced Burns

On the 7^{th} and 14^{th} days of the test, a swab was taken from

the burn site (rotation for 30 seconds clockwise) and immediately sent to the microbiology laboratory of the Faculty of Veterinary Medicine at Shahid Bahonar University of Kerman for evaluation.

In the quantitative count study, 2 ml of normal saline was added to each of the samples. The sample was thoroughly vortexed, and a 10-fold serial dilution was performed. Eight hundred microliters of each sample dilution was spread onto Tryptic Soy Agar (TSA). Two replicates were carried out for each dilution, and the agar plates were incubated at 37°C for 24 hours. The colonies were counted, and the results were tabulated.

Histopathological Assays

Skin samples were taken from 3 rats in each group for histopathology evaluation on the 7th and 14th days of the experiment. The samples were fixed in a 10% formalin solution for 24 hours and then transferred to the pathology department of the Faculty of Veterinary Medicine at Shahid Bahonar University of Kerman for preparation of histopathological sections. Five micron-thick serial sections were cut and stained with hematoxylin and eosin (H&E). The histopathological study evaluated factors such as the percentage of vein formation, amount of covering tissue formation, fleshy bud tissue, collagen deposition, and tissue order.

Pathological indices were assessed using non-parametric statistical tests. Specifically, the Mann-Whitney U test was employed for comparisons between two groups, while the Kruskal-Wallis test was used for comparisons among multiple groups.

Statistical Analysis

The collected data was analyzed using software called IBM SPSS Statistics 27. In order to summarize the distribution of the studied variables, descriptive statistics including mean and standard deviation were calculated. First, a test for normality was conducted using the Kolmogorov-Smirnov test, which was then followed by a variance homogeneity assessment using the Levene's test. Considering the fact that wound area data follows a normal distribution and the percentage of wound contraction in the study groups, one-way ANOVA was used to determine significant differences between these parameters on the 3rd, 5th, 7th, 9th, 13th, 14th, and 15th days after the burn injury. Tukey's post hoc test was used for multiple comparisons between groups. The level of significance was set at 5% throughout the tests.

Results

Physicochemical Properties of the Essential Oil

The created ointment was stable, yellowish cream with a sharp and somewhat bitter taste. It has satisfactory spread ability, homogeneity, wash ability, and diffusion.

Chemical Constituents of Clove Essential Oil Analyzed by GC-MS

A GC-MS analysis was conducted to determine the compositional profile of clove essential oil. The results indicated that eugenol was the predominant constituent, accounting for 85.04% of the total oil. Other significant components identified included acetoeugenol (12.02%), trans-caryophyllene (1.86%), and various minor compounds such as methyl salicylate, chavicol, beta-selinene, caryophyllene oxide, adamantane, and dehydrodieugenol. These findings highlight the substantial presence of eugenol and its derivatives within clove essential oil (21).

Bacteriology

The results of in vitro microbial evaluation are presented in Table 1 and in vivo in Figure 1.

For the in vivo experiment, groups A, B, and C were treated with 10%, 20%, and 30% concentrations of clove oil, respectively. These concentrations were chosen based on their effectiveness in the in vitro experiments.

To assess the relationship between bacterial load and wound healing, bacterial CFU was measured in scabs, wounds, and healed skin. All groups were infected with *P. aeruginosa* colonies at the end of the first and second weeks. It appears that the use of clove extract ointments at concentrations of 10%, 20%, and 30% on day 7, as well as at 20% concentration on day 14, demonstrated superior efficacy compared to the positive control group, but none of the groups significantly differed from the control groups in terms of did not suppress the bacterial load.

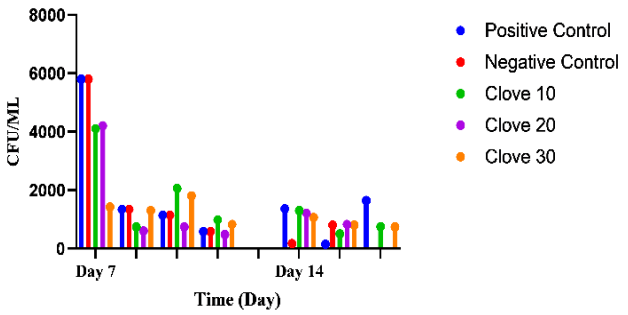

Figure 1. Effect of clove extract (10%, 20%, and 30%) versus SSD on *P. aeruginosa* density in burn wound infection.

Concentration-Dependent Effects of Clove on the Growth of *Pseudomonas aeruginosa In Vitro*

The results indicate that clove essential oil exhibited antimicrobial activity against *P. aeruginosa*, as evidenced by the formation of inhibition zones around the discs impregnated with the oil.

As the concentration of clove essential oil increased from 10% to 100%, the diameter of the inhibition zone generally decreased. This trend suggests that while clove essential oil possesses antimicrobial properties against *P. aeruginosa*, its efficacy is concentration-dependent, with higher concentrations yielding larger inhibition zones. However, it is important to note that beyond a certain concentration (in this case, 20%), further increases in concentration did not result in a proportional increase in the inhibition zone.

The positive control, SSD, demonstrated a significant inhibition zone, confirming its antimicrobial activity. In contrast, the negative control, DMSO (10%), did not exhibit any inhibitory effect, indicating that the observed inhibition zones were due to the antimicrobial properties of clove essential oil and not the solvent.

Table 1. Inhibitory effects of various concentrations of clove essential oil on the growth of *Pseudomonas aeruginosa* in vitro

Concentration (%)	Inhibition zone (mm)
10	15
20	14
30	12
40	10
50	9.5
60	9
70	9
80	8
90	7
100	6.5
SSD	14

Comparison of Mean Wound Area in Rats

Macroscopic assessment revealed that wounds treated with 10% *Arnebia euchroma* and the positive control group exhibited accelerated healing compared to the other groups. This visual observation was further supported by quantitative analysis using Image J software and qualitative histological examination (Figure 2).

Histopathological Evaluation

The histopathological results on the 7th and 14th days have been presented in Figures 3 and 4 which show the histopathological changes of the groups on the 7th day after exposure to different treatments.

The results of the statistical analysis of microscopic data on day seven demonstrated a consistent response of different tissues to the experimental interventions, indicating no significant differences ($p>0.05$) in histopathological parameters among the groups.

A significant difference ($p<0.05$) was observed in epithelialization rates between the 30% clove extract group and both the 20% group and the positive control. Neutrophil infiltration was significantly lower in the 30% extract group compared to the positive control and the 10% group. Furthermore, the 30% extract group demonstrated a significant reduction in coagulative necrosis when compared to both the negative and positive control groups.

Descriptive Histopathological Evaluation

By day 7 following burn wound induction, a layer of purulent-fibrinopurulent crust was observed covering the wound surfaces. Centripetal epidermal regeneration was observed, with epithelial tongues extending from the wound margins toward the center. Granulation tissue with high angiogenesis, proliferated fibroblasts and collagen deposition was formed in the wound area instead of heat-induced necrotic tissues. Treatment with 20% clove oil extract enhanced the rate of re-epithelialization. In addition, this group showed high angiogenesis in the wound area compared to with other groups. New blood vessels placed in a perpendicular orientation.

The 30% clove oil group and positive control demonstrated higher granulation tissue density than the other groups. Inflammatory cells, especially lymphocytes were dispersed in all wounds but the density of cells was higher in all burned wounds. There was not any significant difference between various histopathologic parameters in all experimental groups (Figure 5).

On the 14th day after the burn wound, re-epithelialization was mostly completed in most wounds, covering the surface except for those treated with 30% clove. The new epithelium structure consisted of stratum basalis, stratum spinosum, stratum granulosum, and stratum corneum, respectively. The maturity of the new epithelium was better in the positive group, followed by the clove 20 and 10 groups. The weakest re-epithelialization was observed in wounds treated with clove 30. The stage of remodeling, which includes the maturation and organization of granulation tissue, was in progress. The number of fibrocytes and the amount of deposited collagen increased. A delay in wound healing was observed in the negative control and clove 30 groups. The scores for maturation and organization of granulation tissue were lower in these groups compared to the other groups. A regress in the number of neovessels was observed after 14 days in all

groups, with higher angiogenesis in wounds treated with clove 30. Inflammatory cells especially lymphocytes, were dispersed in the wound area in the negative control, clove 20, and 30 groups. Cutaneous adnexa including sebaceous glands and hair follicles were not regenerated in any of the control or treated wounds (Figure 6).

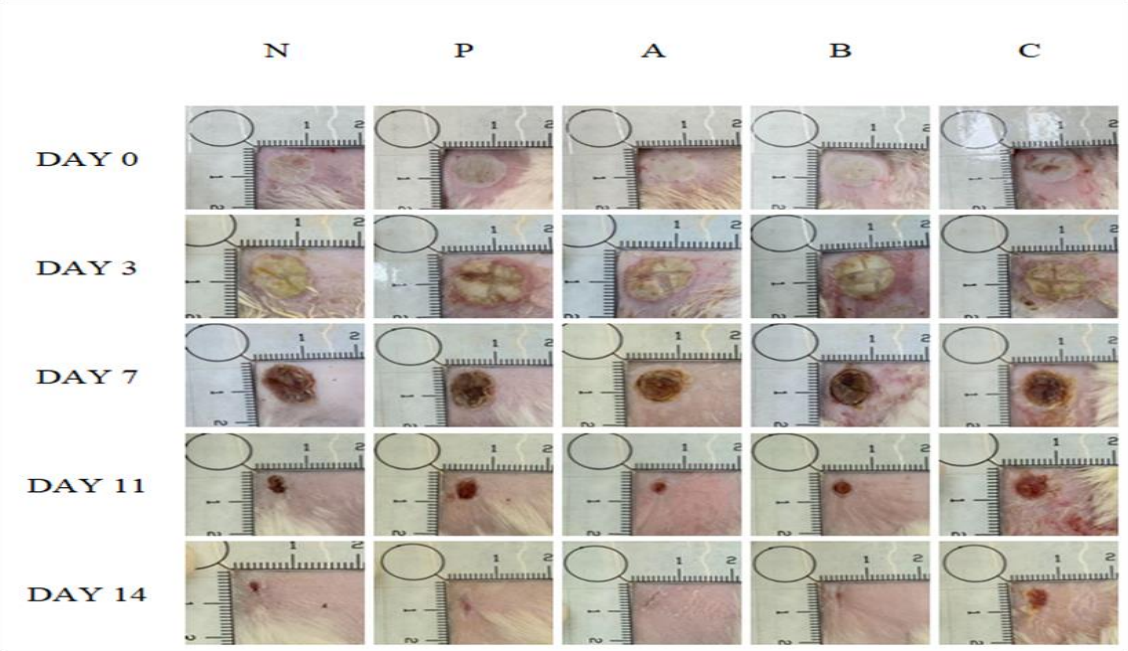

Figure 2. Serial measurements of wound area were taken at days 0, 3, 7, 11, and 14 to monitor healing progression. Treatment groups included: positive control (P), negative control (N), 10% clove extract (A), 20% clove extract (B), and 30% clove extract (C).

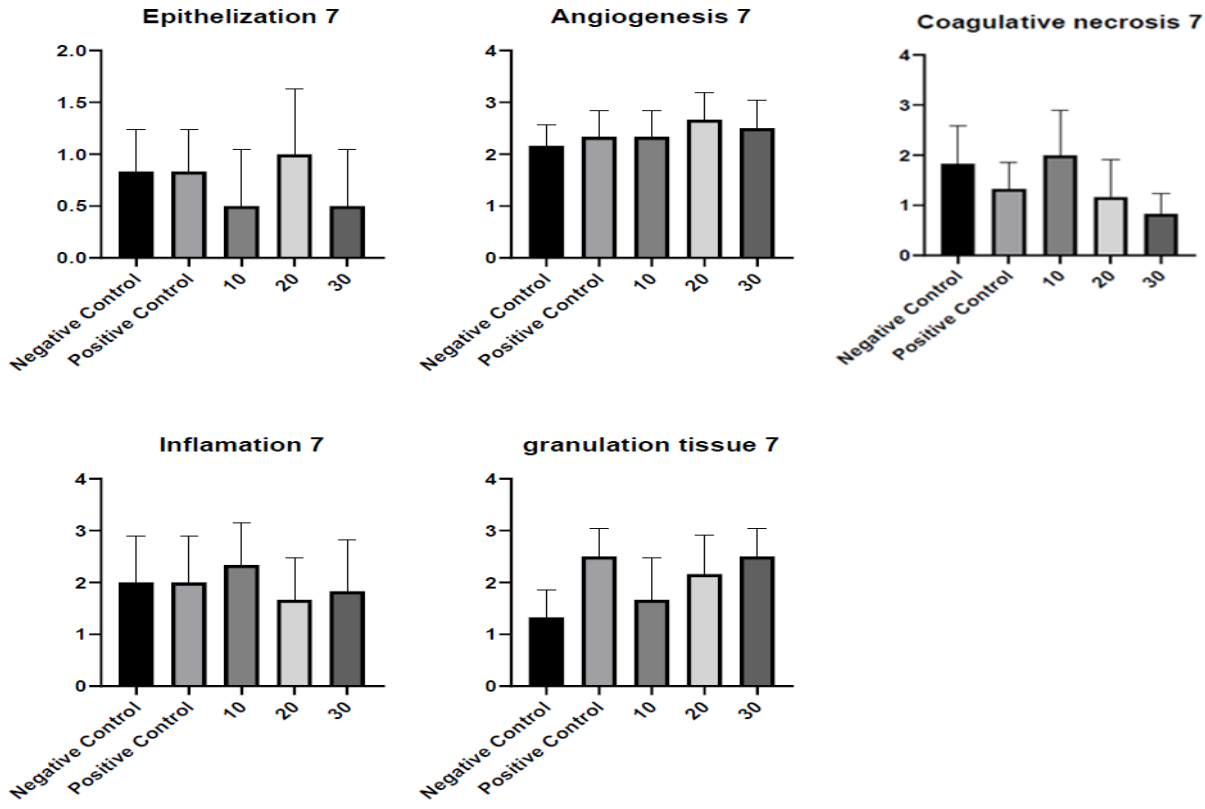

Figure 3. Comparison of histopathological indices as mean ± SEM among different groups on day 7 ($p < 0.05$).

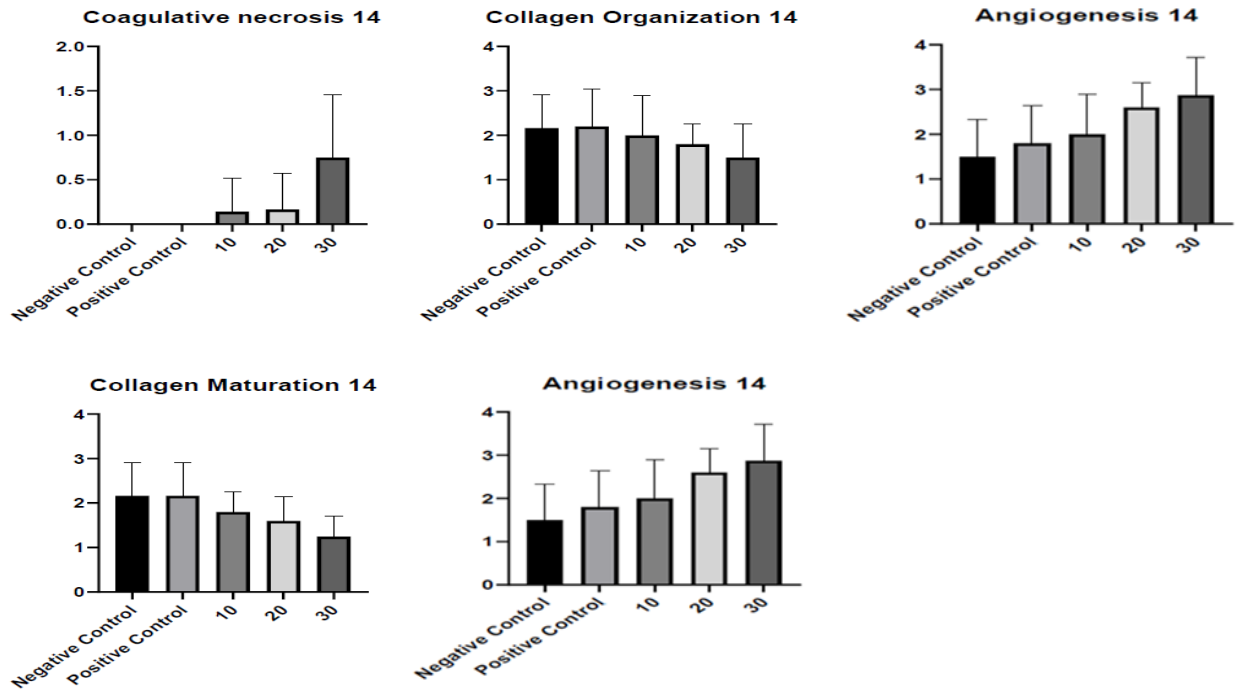

Figure 4. Comparison of histopathological indices as mean ± SEM among different groups on day 14 ($p < 0.05$).

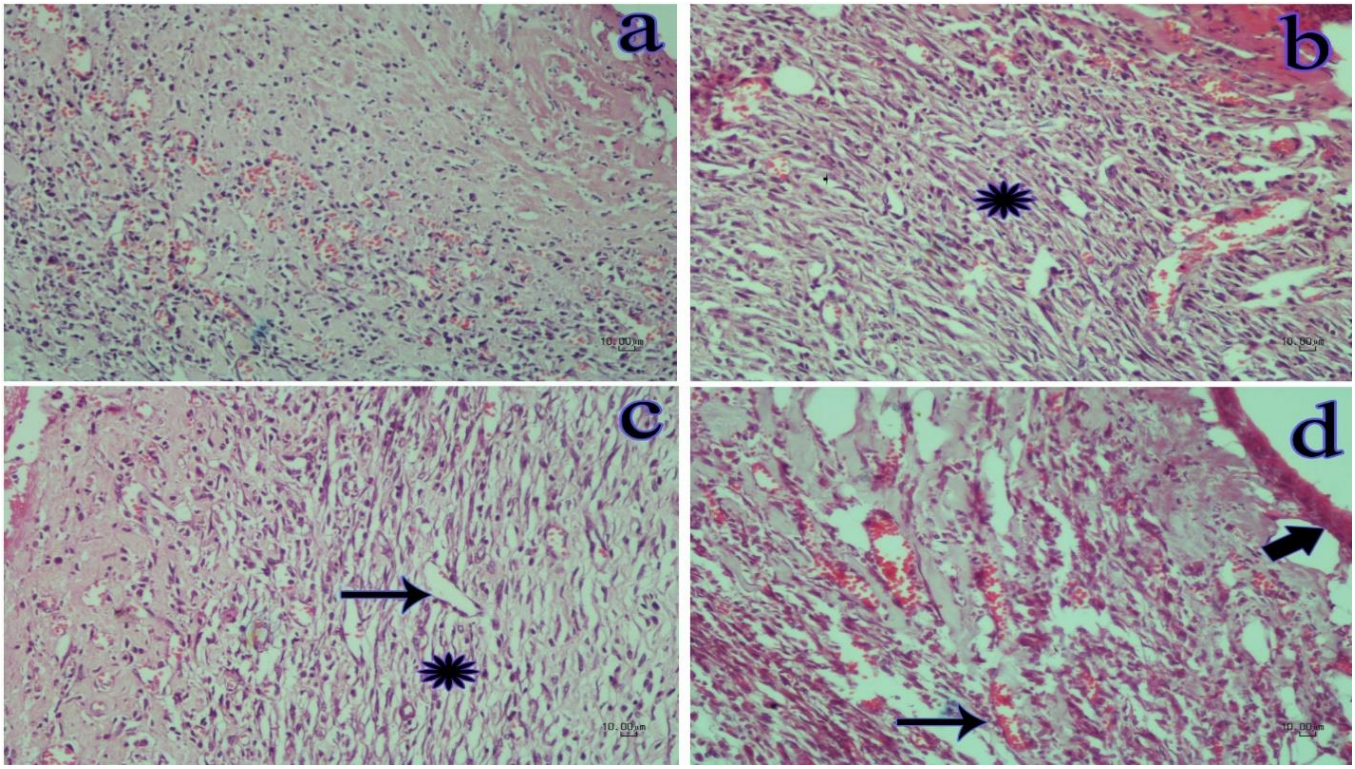

Figure 5. Histopathological evaluation of burns on day 7 in different groups.7 days after the burn wound: the treated group with the clove 30 (b) and then, the positive group (c) show a higher amount of granulation tissue (asterisk) in comparison with the negative control (a) and the clove 20 (d) groups. The group treated with clove 20 (d) shows more angiogenesis (thin arrow) and faster re-epithelialization (thick arrow) rather than the other groups (Hematoxylin-eosin, Bar = 10 µm).

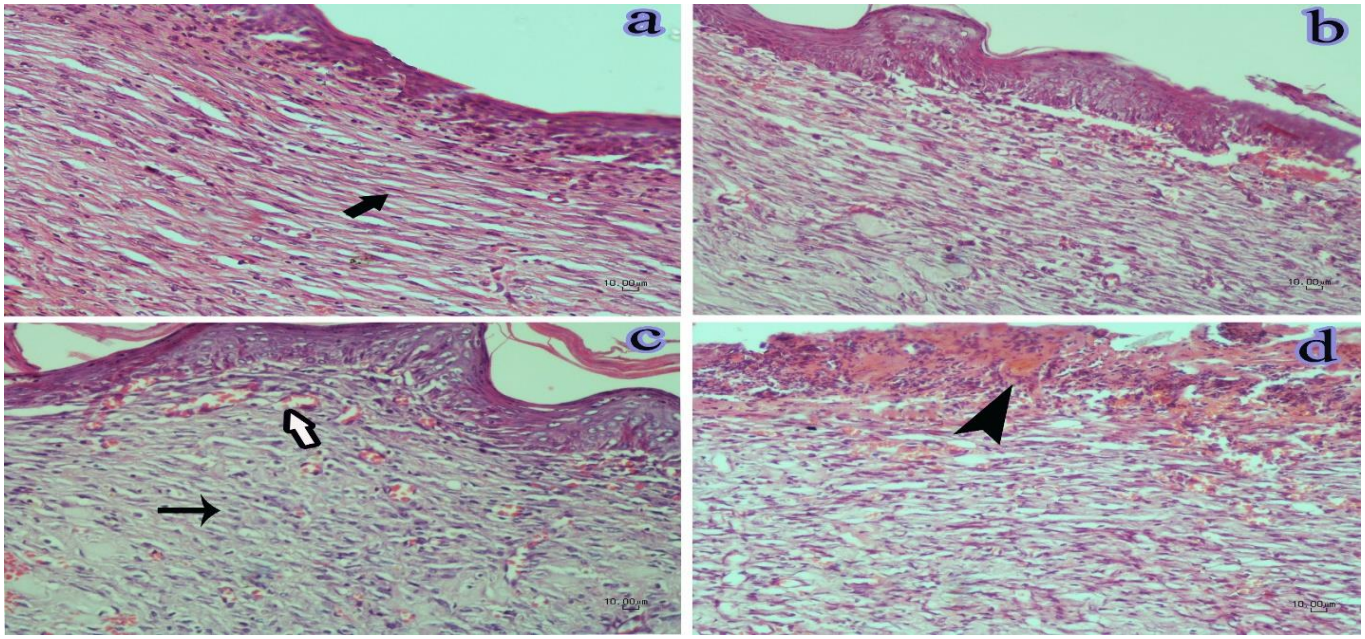

Figure 6. 14 days after the burn wound: better maturation and organization of granulation tissue is observed in the positive control (a) compared to the clove 10 (b) and the clove 20 (c) groups, respectively. The number of fibrocytes (thick arrow) is more than fibroblasts (thin arrow) in the figure (a). The group treated with clove 30 (d) shows the lowest level of maturation and organization. In addition, re-epithelialization (arrowhead) is not completed. Angiogenesis (white arrow) is high in the groups (c) and (d) (Hematoxylin-eosin, Bar = 10 µm).

Discussion

Wound healing is a multi-stage process characterized by the coordinated interplay of various growth factors, cells, and extracellular matrix components. These phases, which often overlap, include hemostasis, inflammation, proliferation, and remodeling (28).

Consistent with the findings of Kumar and colleagues (2021), clove extract has a long history of traditional use in India for wound healing due to its anti-toxic and anti-inflammatory properties (29). Our study further supports these claims by demonstrating that low-dose clove extract significantly accelerates wound healing. This acceleration was attributed to enhanced re-epithelialization, maturation of granulation tissue, increased fibroblast proliferation, and elevated collagen deposition (29).

The in vitro results from this study demonstrate that clove extract exhibits a potent inhibitory effect on the growth of *P. aeruginosa*, comparable to that of SSD cream. Additionally, the study suggests that the maximum efficacy of clove extract is achieved at concentrations below 20%. These results support the findings of Ameen et al. (2024), which indicated that clove extract possesses significant antibacterial properties and is effective against important pathogens like *Staphylococcus aureus* and *Streptococcus pyogenes* (30).

This study examined the antibacterial effects of clove extract against *P. aeruginosa* in both *vitro* and in vivo. Results demonstrated the potent ability of clove extract to inhibit the growth and proliferation of this bacterium. These findings are consistent with previous studies by Alanazi et al. (2022), which confirmed the antibacterial effects of clove oil against methicillin-resistant *Staphylococcus aureus* (MRSA) in both in vitro and in vivo models (31). The results of these studies highlight the potential of natural compounds such as clove extract in controlling bacterial infections and accelerating wound healing.

Our study has shown that clove extract exhibits a marked stimulatory effect on cell proliferation and wound healing when applied at lower concentrations (e.g., 10% compared to 30%) over a 14-day period. These findings are consistent with the in vitro research conducted by Mumtaz and colleagues (2023), which demonstrated that clove extract, especially at a concentration of 0.05 mg/mL, can significantly stimulate cell proliferation (32). The results suggest that lower concentrations and longer application durations create an optimal environment for promoting cellular repair mechanisms.

Despite extensive in vitro and in vivo studies investigating the optimal concentration of clove extract, results have been inconsistent and sometimes contradictory. Nonetheless, there is a general agreement that clove extract is effective in reducing inflammation and infection in wounds.

The findings of this study are inconsistent with those reported by Prashar et al (2006) (33). While Prashar et al.

(2006) observed high cytotoxicity in clove oil at certain concentrations, mainly due to eugenol, our study demonstrates that increasing doses of clove extract led to a decrease in antimicrobial, anti-inflammatory, and wound healing effects (33). This contradiction suggests that the relationship between clove extract dosage and its biological effects may be more complex than previously thought.

While the cytotoxicity of clove oil is not necessarily detrimental, the research conducted by Kumar et al. (2014) revealed its substantial potential in inhibiting the growth of cancer cells (34). Specifically, clove essential oil demonstrated the highest cytotoxicity against MCF-7 breast cancer cells, with IC50 values of 36.43 µg/mL and 17.6 µg/mL at 24 and 48 hours, respectively (34). These results underscore the promising anticancer properties of clove oil and emphasize the need for further research to determine the optimal therapeutic dosage and cytotoxic threshold for various applications.

The current study investigated the therapeutic efficacy of clove (*S. aromaticum*) essential oil in treating *P. aeruginosa*-infected burn wounds in a rat model. Findings revealed that the essential oil demonstrated potent antibacterial and anti-inflammatory activities, comparable to the effects of SSD. Surprisingly, lower concentrations, particularly below 20%, were found to be the most effective in enhancing wound healing.

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Authors' Contributions

Navid Kor: Conceptualization, investigation, **Mohammad Mahdi Alinaghizadeh:** writing original draft, **Fatemeh Heydari:** methodology**,** validation **Reza Molaei:** methodology**,** investigation, **Pouneh Hajipour:** writing – original draft, writing – review & editing**, Shahrzad Azizi:** histophatological evaluation**, Ehsanollah Sakhaee:** Conceptualization, formal analysis, investigation, methodology, supervision, validation, visualization, review & editing**.**

Data Availability

All data generated or analyzed during this study are included in this published article.

Ethical Approval

All experimental procedures involving animals in this study were conducted in accordance with the ethical standards and guidelines for the care and use of laboratory animals established by the Ethical Committee of Shahid Bahonar University of Kerman, Kerman, Iran. Efforts were made to minimize animal suffering and to use the minimum number of animals necessary to achieve the scientific objectives of the research. All protocols were reviewed and approved by the relevant ethical review board prior to the commencement of the study.

Conflict of Interest

The authors declared no potential conflicts of interest.

Consent for Publication

Not applicable.

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