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Original Article

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Topical application of *Dianthus* essential oil improved the infected healing process of wounds infected with *Staphylococcus aureus* in an experimental model

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Abstract

***Background & Aims*:** In recent years, wound infections caused by *Staphylococcus aureus* have substantially grown. Lack of easy access, medications expensive, side effects, and in particular the development of drug resistance, the use of biological materials are proposed as an alternative solution. The recent study was aimed to evaluate the topical effect of *Dianthus* essential oil on cutaneous excisional wound healing in mice infected with *S. aureus*.

Materials & Maethods: This study was performed on 36 mice (weight 25±3 g). After general anesthesia, 0.5 mm circle wound was created with biopsy punch between the shoulder, and immediately 50 γ of the suspension containing 10^7 CFU/ml *S. Aureus* was applied to the wound. Then tested animals, grouping in three groups of 12 mice each (control, treated with ointment 2% and 4%). During the project, image was obtained on days 3, 6, 9 and 12 images for assessment of wound area, and in 3, 7 and 14 from wounds in order to histopathology assessment.

Results: The results of the wound size showed that the wound area decreased significantly in the treated groups ($p< 0.05$) compared with the control group. On histological examination, a significant ($p< 0.05$) reduction was observed in the migration of immune cells, the migration of fibroblasts and fibrocytes into the wound, the collagen synthesis and secretion. In addition, the thickness of the epithelium thickest increased in the treatment groups compared with the control group ($p< 0.05$).

Conclusion: Based on the results of this study, topical application of *Dianthus* essential oil, especially at higher therapeutic doses, can be considered a viable option for treatment of infected wounds by the bacteria *S. aureus*.

***Keywords*:** Dianthus essential oil, Infected wound healing, Staphylococcus aureus, Ointment, Mice

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Introduction

During last decades, the number of surgical operations performed on humans has increased dramatically due to the development of advanced techniques, and wound infection is still a critical and life-threatening complications of these operations (1). *Staphylococcus aureus* is considered the most common cause of a broad range of human infections, especially

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skin and soft tissue infections. These types of infections include microbial invasions of the primary defense barriers epidermis and soft tissues (2).

Although wound healing is an innate sophisticated multistep process, it is generally mandatory to manage wound healing, which is essential for infection prevention, reduction in healing time, and prevention of unsightly scars (3). Routinely, to prevent wound skin infection, suturing, antiseptic therapies such as using antimicrobial agents, and tissue aseptic handling are applied, however, these management strategies are not sufficient in most cases and there is an urgent need to develop and design novel and effective therapeutic practice (4). Particularly, development of drug resistance against multiple antibiotics used as an antimicrobial remedy for *S. aureus* infections, is a burden against effective treatment of wound infection (5). Among various introduced therapeutic strategies to combat wound infection, an increasing amount of attentions have been paid to application of natural products from traditional medicines and natural resources (6). In this regard, a cumulative number of recent studies have demonstrated the efficacy of plant derived essential oils, as novel antimicrobial candidates, which have been proven to have antibacterial, antiviral, antifungal and antioxidant properties (7, 8). In addition, some researchers suggest that plant essential oils prevent resistance in some infectious strains, especially methicillin-resistant *S. aureus* (9). The use of essential oils and herbal infusions has been practiced in traditional medicine since ancient times (10). Following the advancement of science and more attention to health, the use of drugs has increased (10). Due to the side effects, high prices and complex stages of production of chemical drugs, the use of medicinal plants has been considered. The present study was performed to evaluate the antibacterial effect of *Dianthus* flowers in the laboratory. *Dianthus* is a plant of the Caryophyllaceae family, used in traditional medicine to treat some gastrointestinal diseases as well as toothache (11). To our knowledge, there are no recorded reports on the antibacterial effects of *Dianthus* essential oil on infectious wounds, therefore, this study was designed and performed to investigate the clinical, pathological and laboratory effects of this essential oil.

Material & Methods

Animals:

In this study, 36 adult mice, of both sexes, with an age range of 10-12 weeks and a weight range of 25±3 g were used. These animals were transported to the intended location for testing, and kept in standard rat shelves under controlled exposure conditions for 12 hours of light and 12 hours of darkness at a constant temperature of 25±3 °C. During the whole period, the mice were fed with pellets and had free access to water and food (pellets). All methods were followed in accordance with the relevant guidelines and regulations of the Animal Care.

Dermal wound healing model:

Induction of anesthesia was performed intraperitoneally with a combination of xylazine hydrochloride 2% (5 mg/kg) and ketamine hydrochloride 5% (50 mg/kg). After anesthesia, and the dorsal surface of the mice was prepared, and a 5-mm-diameter circular wound was made between the two shoulders by a biopsy punch. By creating this type of wound by excisional wounding, the layers of epidermis, dermis, hypodermis and paniculus were completely removed. After trauma, mice were randomly divided into 3 groups (n=12/group) including control group, 2% *Dianthus* ointments and 4% *Dianthus* ointments. Then, each of the main groups was divided into two subgroups (n=6) to measure the wound surface on days 3, 6, 9 and 12, and for pathological examination of the samples taken on days 3, 7 and 12. Generally, on the same day of surgery and after wounding on all tested mice, 50 microliters of prepared bacterial inoculation by the sampler were poured on each wound to infect the wounds.

Preparation of bacterial inoculation and wound infection:

In order to inoculation, as well as evaluating the antibacterial activity of *Dianthus* ointments, *S. aureus*

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was used as positive standard for inoculation and assessment of antibacterial activity of *Dianthus* ointments, 1-2 × 10^8 inoculum was applied to infecting wound in mice as described previously (12, 13).

Experiment to determine the minimum inhibitory concentration and the minimum bactericidal concentration of *Dianthus* ointments:

Broth Micro dilution MIC testing was used to determine the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC). In this method, sterile polystyrene panel containing 96 wells was used. To adjust the bacterial inoculation rate, first using the McFarland 0.5 standard method, 1.5×10^7 F CFU/ml was obtained from the studied bacteria, then to achieve 10^7 CFU / ml bacterial inoculation, it was diluted. To determine MIC and MBC, first 160 µl of Müller Hinton broth, then 20 µl of prepared *Dianthus* ointments concentrations and finally 20 µl of bacterial inoculation were added to microplate wells. For positive control (sterilization), 200 µl of Müller-Hinton broth and also 180 µl of Müller-Hinton broth and 20 µl of 10% DMSO solvent and for negative control (bacterial growth), 180 µl of Müller-Hinton broth with 20 µl of bacterial inoculum were added. After capping, the microplates were shaken at 300 rpm in a plate thermocirculator and then incubated at 37 ° C for 24 hours. After 24 hours, the microplates were removed and the wells were visually inspected for turbidity and the first clear MIC well and the second clear MBC well were considered. Then, for confirmation, 10 µl of the first clear well was removed along with a clear well and adjacent turbidity and cultured superficially on nutrient agar. The MIC by definition is the lowest concentration of an antimicrobial agent that reduces by 90% or more the initial inoculation. Also, the MBC is the lowest concentration of antimicrobial agent that reduces 99.9% or more of the initial inoculation.

Ointment preparation from *Dianthus caryophyllus* flower:

Dianthus caryophyllus flower essential oil was prepared from Aria Company and by Clevenger machine. The essential oil obtained after dehydration with anhydrous sodium sulfate was stored at 4 ° C and then used for making ointments and experiments with MIC and MBC. In addition, in order to prepare therapeutic ointment, 2 grams and 4 grams of *Dianthus* essential oil were added to 100 grams of commercial ointment base (Pars Daroo Company - Tehran) in two separate cans, respectively.

After 24 hours, the infected wounds in each group were treated daily with the appropriate ointment until complete healing. It is noteworthy that the wounds were kept open. The animals were then kept in the designated cages of each treatment according to their treatment and were cared for days until the end of the sampling day. It should be noted, however, that the ointment was applied to the wounds without anesthesia at a set time (approximately 7 to 8 pm).

Tissue sampling method for histopathological examination:

Tissue samples were taken from the wounds of treatment and control groups on days 3, 7 and 14. Thus, 2 animals are randomly selected from each group on each of these days, and after anesthesia by the method mentioned by the skin punch, a full-thickness tissue sample with a diameter of 5 mm of healing tissue with 2 mm of healthy tissue was removed from the wound margin. The samples were placed in 10% neutral formalin for histopathological examination and stored.

Preparation and examination of histopathological sections:

The tissue sample was placed in 10% neutral medium with formalin for stabilization and transferred to the pathology laboratory for further stabilization, blocking and cross-sectional preparation. Then, after fixing and molding tissue samples in paraffin (blocking), by microtome, sections with a thickness of 5 µm were prepared and stained by trichromason method and quantitatively examined for immune cell count, fibroblasts and fibrocytes, as well as the thickness of the formed epithelial tissue and qualitative examination for edema rate and collagen synthesis.

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Statistical analysis:

Statistical analysis was performed using the ---. One-way analysis of variance (ANOVA), followed by Tukey's and Dunnett's test, was used to evaluate the statistical significance of differences in each parameter among the different groups, and a P-value of < 0.05 was considered statistically significant.

Results

Wound contraction:

The effect of *Dianthus* ointment containing on *S. aureus* -infected wounds are shown in Table 1. The wound surface size in the treated animals (2% and 4% *Dianthus* essential oil) significantly decreased compared to the control group on the 9th and 12th days after surgery (P <0.05).

Table 1. Mean area of infected wounds in the experimental groups

Groups	3rd day	6th day	9th day	12th day
Controls	28.06 ± 0.0[a]	25.13 ± 4.88[a]	21.02 ± 3.98[a]	8.15 ± 3.09[a]
2% *Dianthus* ointments	23.10 ± 4.14 [a]	22.10 ± 1.12[a]	15.96 ± 2.87[b]	2.99 ± 0.78[b]
4% *Dianthus* ointments	21.01 ± 4.02 [a]	20.54 ± 0.97[a]	12.06 ± 2.99[b]	0.81 ± 0.42[b]

All data are presented as mean±SD (n=6/group)
a P < 0.05 vs control. c P < 0.05 vs -------.

Pathological analysis:

Fibroblast cell proliferation at the wound site:

The results of fibroblast cell count per square millimeter of connective tissue were shown in Table 2. In this study, administration of ointments containing 2 and 4% *Dianthus* essential oil in treated animals resulted in the significant increase in fibroblast cell distribution only on the seventh day of sampling, increased as compared to the control group (P<0.05). Also on the same day, there was no significant difference in fibroblast cell distribution between the 2% and 4% treatment group of (P> 0.05).

Table 2. Mean distribution of fibroblasts per square millimeter of tissue in wound tissue

Groups	3rd day	7th day	14th day
Controls	0.83±0.28[a]	6.33±1.52[a]	8.00±2.00[a]
2% *Dianthus* ointments	5.60±2.08[b]	15.33±3.05[b]	9.00±1.00[a]
4% *Dianthus* ointments	8.00±2.00[b]	19.00±1.00[b]	11.33±1.52[a]

All data are presented as mean±SD (n=6/group)
a P < 0.05 vs control. c P < 0.05 vs -------.

The distribution of fibrocyte cells to the wound site:

The results of fibrocyte cell count per square millimeter of connective tissue were represented in Table 3. Administration of ointments containing *Dianthus* essential oil significantly increased the distribution of fibrocytes compared to the control group on all sampling days (3, 7, 14) after wounding (P<0.05). Also, a significant difference was observed for the distribution of fibrocytes between the treatment group of 2% and 4% on the seventh day (P <0.05).

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Table 3. Mean distribution of fibrocyte cells per square millimeter of tissue in wound tissue

Groups	3rd day	7th day	14th day
Controls	0.16±0.02[a]	1.66±0.57[a]	4.00±1.00[a]
2% *Dianthus* ointments	5.00±1.00[b]	9.66±1.52[b]	11.66±1.52[b]
4% *Dianthus* ointments	6.00±1.00[b]	12.00±1.52[c]	12.66±1.54[b]

All data are presented as mean±SD (n=6/group)

a P < 0.05 vs control. c P < 0.05 vs -------.

Examining and counting the release of immune cells to the wound site:

Table 4 shows the results of counting immune cells per square millimeter of connective tissue. In this study, cell diffusion significantly reduced in groups treated with ointment containing 2 and 4% *Dianthus* essential oil, on all sampling days after wounding compared to the control group (P <0.05). Also, no significant difference (P> 0.05) was found for the distribution of these cells between the treatment group of 2$ and 4% on any of the sampling days.

Table 4. Mean distribution of immune cells per square millimeter of tissue in wound tissue

Groups	3rd day	7th day	14th day
Controls	53.28±3.22a	32.00±4.58a	4.00±1.00a
2% Dianthus ointments	38.17±4.02b	20.33±3.66b	16.35±4.33b
4% Dianthus ointments	25.25±3.55a	8.66±2.33b	6.55±1.66b

All data are presented as mean±SD (n=6/group)

a P < 0.05 vs control. c P < 0.05 vs -------.

Qualitative evaluation of edema:

In the groups treated with 2% and 4% ointments containing *Dianthus* essential oil, compared to the control group, the rate of edema significantly decreased on all sampling days after wounding (3, 7, 14) (P<0.05). But no significant difference was observed between the treatment groups.

Quantitative study of epithelial tissue regrowth:

The results of a quantitative study of the regrowth of the epithelial tissue of the repairing tissue in the different groups tested are shown in Table 5. Regrowth of epithelial tissue was seen in all animals from the seventh day after wounding; this growth was significantly increased in the treatment groups (P <0.05). Comparing the treatment groups with each other, no significant difference was observed on any of the sampling days (P> 0.05).

Table 5. Results of quantitative analysis of epithelial tissue thickness in the experimental groups.

Groups	3rd day	7th day	14th day
Controls	-	-	-
2% Dianthus ointments	24.34±4.60a	39.67±5.30b	60.75±3.56b
4% Dianthus ointments	34.26±5.64a	95.22±6.31b	103.37±7.31c

All data are presented as mean±SD (n=6/group)

a P < 0.05 vs control.

Qualitative study of collagen synthesis and secretion:

The results of qualitative study of the amount of collagen synthesis and secretion of repairing tissue in

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different groups tested are shown in Figure 1. A significant amount of collagen synthesis and secretion was observed in both treatment groups, unlike the control group, on the third day. Over time, in animals treated with *Dianthus* essential oil ointment, especially higher doses of *Dianthus* essential oil ointment (4%) showed more synthesis and secretion.

Figure 1. Microscopic view of the wound surface in sampling on day 7 after wound induction to show the amount of collagen deposition and collagen clusters; A: control, B: group therapy with 2% *Dianthus* essential oil ointment and C: group treated with ointment 4% *Dianthus* essential oil. Unlike the control group, the germ tissue in both treatment groups, especially the higher dose, is well formed. Mason trichrome staining, magnification A, B, C: 400 ×.

Determining MIC and MBC:

Our results showed that the MIC and MBC of *Dianthus* essential oil on *S. aureus* strain 29312 were 0.8 and 0.4 mg/ml, respectively. In addition, the MIC and MBC values for *S. aureus* strain 1442, *Pseudomonas* strains 27853 and *Escherichia coli* strain 15922 were 6.4 mg/ml for all bacteria.

Discussion

Wound healing is a set of cellular and molecular events that require the recruitment of cells into the wound site, cell proliferation, and the synthesis and accumulation of new connective tissue (14). Although this process begins and continues naturally in wounds, both in terms of speed and quality of healing tissue, the result of this natural process under the influence of pathogens, especially *S. aureus*, is not always desirable (15, 16). Many studies have been performed to positively affect or prevent the impact of negative factors on this process in terms of both the speed of formation and the proper quality of healing tissue (17).

There are reports that only 1 to 3 percent of the drugs listed in Western Pharmacopeia are intended for use on the skin and in wounds, in comparison, at least one third of herbal remedies are for such applications (18). Therefore, some herbal products contain potential agents for wound healing, and due to their high dispersion and easy access to them, are seen as alternative solutions to wound healing problem in developing countries (19).

In this regard, the essential oil of *Dianthus caryophyllus* flower due to its high content of antibacterial compounds, including eugenol, with the chemical formula C10H12O2, which is one of the phenolic compounds with strong antibacterial as well as antioxidant, astringent (spasmodic) and antiseptic properties, has gained increasing attention in wound healing research (20-22).

From the perspective of cellular and pathological changes, wound healing is divided into three stages: inflammation, proliferation and maturity (23). The first stage (inflammatory phase) of the wound healing process under normal conditions (absence of infectious agents) lasts three to four days (3). During this stage, immune cells are recruited to the wound site, in order to destroy carrying infectious agents and dead tissues. An increase or decrease in the number of these cells indicates the extent of wound infection (24). In our study, the results of immune cell migration in the pathological study and comparison of different groups with each other showed that in both treatment groups *Dianthus caryophyllus* flower ointment significantly fewer immune cells migrated to the wound site. At the same time, the amount of tissue edema in the treatment groups showed a significant reduction in a dose-dependent manner. In general, these changes indicate local and antibacterial effects of *Dianthus caryophyllus* flower essential oil, which ultimately reduces the rate of induction of infection with *S. aureus*, leading to a reduction of local infection and subsequently duration of the inflammatory phase, during the first period of wound healing (25, 26).

In this regard, experiments to determine the MIC and MBC of *Dianthus caryophyllus* flower essential oil in different dilutions, showed that this essential oil inhibited the growth of *S. aureus* and *Pseudomonas aeruginosa*. The results confirm other reports that due to the structural differences in the walls of gram-positive

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and gram-negative bacteria, this essential oil had the ability to have a greater effect on gram-positive bacteria.

The second stage (proliferative phase) of the wound healing process under normal conditions (absence of infectious agents) takes three to four weeks (27). This stage continues with vascular regeneration, formation of granular tissue (budding), regeneration of epithelial tissue (epithelialization) and finally simultaneously with wound contraction (28). The results of pathological studies in this study demonstrated that *Dianthus caryophyllus* flower ointment significantly increased the regrowth of epithelial tissue and migration of fibroblast cells at the wound site in animals, compared with the control group. At the same time, with increasing collagen synthesis and secretion, the amount of wound surface size decreases, which is consistent with the results obtained in this study. According to the results obtained in this stage, it can be said that topical application of *Dianthus caryophyllus* flower essential oil has increased the rate of reproduction and regeneration in the process of healing of infected wounds compared to the control group.

The stage three (Maturation) of the wound healing process under normal conditions begins in the second week and lasts for several weeks (29). This phase consists of tissue remodeling and vascular regression, as well as formation of new extracellular matrix components. During this phase, type III collagen is replaced with newly synthesized type I collagen (30). Pathological examination on the last day of sampling in the present study (fourteenth day) showed an increase in the presence of fibrocyte cells as well as an increase in collagen bundles at the wound site followed by a maximum increase in wound contraction. Dose-dependent topical application of *Dianthus caryophyllus* flower essential oil in the treatment of infectious wounds has accelerated wound healing in treated mice.

Conclusion

By pathologically examining the different stages of wound healing process, the results of wound surface size as well as the results of the MIC and MBC of *Dianthus caryophyllus* flower essential oil, it can be said that topical application of *Dianthus caryophyllus* flower essential oil on mice skin wound infections with standard strains of *S. aureus* increased the healing process by reducing tissue inflammation, increasing cell proliferation, as well as collagen synthesis and secretion.

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