

Wound healing activity of *Origanum vulgare* against surgical wounds infected by *Staphylococcus aureus* in a rat model

Moslemi, H.R.^{1*}, Tehrani-sharif, M.¹, Mohammadpour, S.¹, Makhmalbaf, A.¹, Kafshdouzan, Kh.², Ahadi, T.³, Mazaheri Nezhad Fard, R.⁴

¹Department of Clinical Sciences, Faculty of Veterinary Medicine, Garmsar Branch, Islamic Azad University, Garmsar, Iran

²Department of Microbiology, Faculty of Veterinary Medicine, University of Semnan, Semnan, Iran

³Department of Physical Medicine and Rehabilitation, Iran University of Medical Sciences, Tehran, Iran

⁴Rastegar Central Laboratory, Faculty of Veterinary Medicine, University of Tehran, Tehran, Iran

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Correspondence

Moslemi, H.R.
Department of Clinical Sciences,
Faculty of Veterinary Medicine,
Garmsar Branch, Islamic Azad
University, Garmsar, Iran
Tel: +98(23) 34552121
Fax: +98(23) 34552020
Email: hmoslemi@iau-garmsar.
ac.ir

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Abstract:

BACKGROUND: Wound infection has become a major medical problem in recent years. This is usually caused by Gram-positive bacteria, especially *Staphylococcus aureus*. Since antimicrobial resistance to current drugs has critically been developed in these causative microorganisms, substitution medicine has become one of the main interests within researchers. **OBJECTIVES:** The aim of this study was to evaluate the healing activity of *Origanum vulgare* against surgical wounds infected by *S. aureus*. **METHODS:** Twenty male Sprague-Dawley rats were randomly divided into two groups. Excisions were created surgically on the animals' skin and then infected with *S. aureus*. Group 1 was treated with an extract of *O. vulgare* while Group 2 was untreated. Wound biopsy specimens were collected on Days 5, 10 and 16 and analyzed. **RESULTS:** Results showed that the hydroxyproline content in the treatment group was significantly higher in various post wounding days. The mean of hexosamine in the treated group was higher than in the control group. Protein content increased gradually in Day 10. Results of histopathological studies showed moderate to intense granulation tissue formation and neovascularization in the treated group on Day 10. Furthermore, the histopathological studies showed that intense matrix formation and collagen fiber deposition occurred in treatment group on Day 16 post wound, while intense granulation tissue formation was the prominent feature in control group. **CONCLUSIONS:** The present study has demonstrated that the ethanol extract of *O. vulgare* contains properties that accelerate wound healing activities compared to control group.

Introduction

Wound is defined simply as the disruption of the cellular and anatomic continuity of a tissue. Wound may be produced by physical,

chemical, thermal, microbial or immunological insult to the tissue. Wound healing is a programmed biological process that restores tissue continuity after injury and is a combination of physical, chemical and cellular events

that restore the wounded tissue or replace it with collagen. Wound healing can be divided into three stages, inflammation, proliferation and remodeling and maturation phases which involve the interaction of various cells, cytokines and growth factors (Teoh et al., 2009). The normal healing response begins immediately after an injury. When blood spills into the site of an injury, the blood platelets contact with collagen and other elements of the extracellular matrix. This triggers the release of clotting factors as well as essential growth factors and cytokines such as platelet-derived growth factor (PDGF) and transforming growth factor beta (TGF- β). The inflammatory phase begins after the migration of neutrophils to the wound site to clean the tissue. Then, fibroblasts migrate into the tissue to begin the proliferative phase and deposit new extracellular matrix. This new collagen matrix becomes cross linked and organized (Diegelmann and Evans, 2004).

Due to poor hygienic conditions in developed and developing countries, wound infection has become a common disease in recent years (Kumar et al., 2007). Gram-positive bacteria, especially *Staphylococcus aureus* are early colonizers of burn wounds which makes the occurrence of multidrug resistant *S. aureus* a troublesome event. *S. aureus* is a major cause of hospital acquired (nosocomial) infection of surgical wounds and infections associated with indwelling medical devices (Cook, 1998). A public tendency towards the use of herbal wound healers is increasing, possibly due to lower side effects and prices of herbals compared to chemical drugs. One of these natural herbs, oregano, was scientifically named *Origanum vulgare* by Swedish botanist Carl Linnaeus. This genus is a member of the mint family (Lamiaceae). *Origanum* spp. is native to Western and Southwestern Eurasia and the Mediterranean region. The *Origanum* oil possesses carminative and diuretic properties. Furthermore, it is used in chronic rheumatism and

tooth and ear aches. The oil is also used in veterinary ointments and stimulates hair growth. In homoeopathy, it has been used for hysteric conditions. Moreover, the *Origanum* oil is topically used with other herbs for wound healing. Previously, the antibacterial activities of *O. vulgare* have been reported (Derwich et al., 2010; Saeed and Tariq, 2009; Sivropoulou et al., 1998). The aim of this study is to assess the healing activity of *O. vulgare* against surgical wounds infected by *S. aureus* in a rat model for which no data has been published.

Materials and Methods

The plants and extract preparation: Five hundred grams of *O. vulgare* were purchased from an official herbal drug center. The aerial parts of the plant were dried and grounded into fine powder using an electric blender. The extract was prepared by cold maceration with distilled water for 24 h. 50 gr of powder were suspended at 100 ml ethanol for 24 h at room temperature. The mixture was then filtered using a fine muslin cloth followed by Whatman's No. 1 filter paper. The extract was concentrated using vacuum distillation.

Bacterial dilution: *S. aureus* (ATCC 29213) was used as standard for inoculation and characterization of antibacterial activity of *O. vulgare*. A total of 1×10^4 CFU/ml of the bacteria was used as described in previous studies (Barker et al., 1982; Stratford et al., 2002). *S. aureus* was inoculated onto blood agar plates. Colonies were isolated and suspended in sterile distilled water. 0.5 McFarland dilutions were prepared and using spectrophotometry to achieve a wave length of 630 nm. The bacterial concentration was calculated as 1×10^4 CFU/ml in the dilution.

Minimum inhibitory concentration (MIC): To assess the minimum inhibitory concentration (MIC) of the extract, a stock solution of *O. vulgare* (30,000 ppm) was made. Four drops of solvent were added to 0.3 g of hydroalcohol

extract of *O. vulgare* in a sterile manner after which 10 ml of sterile phosphate buffer saline (PBS) were added to the solution and homogenized. A two-fold dilution of the extract in nutrient broth was made in sterile tubes and then adjusted inoculum of *S. aureus* (1×10^4 CFU/ml) was added to each tube. Tubes were mixed gently and incubated at 35°C for 24 h. Then bacterial turbidity was assessed by visual examination. The last dilution at which the growth of the bacteria was inhibited was selected as the MIC of the extract.

Animals: Twenty male Sprague-Dawley rats weighing 254 ± 14 g were selected for the study. The animals were housed in a standard animal house (Faculty of veterinary medicine, Islamic Azad University, Garmsar Branch) and left for seven days at room conditions for acclimatization. They were supplied with water and food ad libitum and maintained at $25 \pm 2^\circ\text{C}$, 45–57% RH and 10:14 hr Light: Day Light: Day cycle during the study. The animals were periodically weighed before and after the experiments. All experiments on the animals were carried out according to the “Guide for the Care and Use of Laboratory Animals” published by the National Institute of Health (NIH).

Wound excision: Animals were anaesthetized with a combination of 10% ketamine hydrochloride (50 mg/kg) and 2% xylazine hydrochloride (5 mg/kg) intraperitoneally. Dorsal fur of the animals was shaved with an electric clipper and the anticipated area of the wound was marked. The site of excision was sterilized with povidine iodine followed by 70% ethanol. A full thickness of the excision wound of area 225 mm² was created and left open. One milliliter of bacterial dilution (1×10^4 CFU/ml) was inoculated topically at the wound site. Animals were divided in two equal groups. Group 1 was topically treated with hydro alcohol extract of *O. vulgare* while Group 2 was not treated with any solution. *O. vulgare* extract was used topically 4 h after bacterial in-

oculation. Treatment was continued daily for seven consecutive days (every 24 h).

Biochemical analysis: Wound biopsy specimens were collected from each group on Days 5, 10 and 16 post wound excision. At the end of the experiment, animals were scarified, serum harvested and kept at -80°C until analysis. Hydroxyproline concentration was assessed as described by Woessner (1961). Hexosamine was assessed using an original method by Elson and Morgon (1933). Total protein was measured using Lowry (1951) method.

Histopathological studies: Wound biopsy specimens from each group were collected on Days 5, 10 and 16 post excision. Tissues were fixed in 10% buffered formalin and routinely processed by standard procedures and then stained with hematoxylin and eosin (H&E). Stained specimens were microscopically evaluated to assess histopathological changes during wound healing.

Statistical analysis: Statistical analyses were carried out using SPSS, v16.0 (SPSS, USA) and the data were expressed as mean \pm standard deviation (SD). Independent t test and analysis of variance were used to test data significance. Differences were considered significant at $p < 0.05$.

Results

Mean concentrations of hydroxyproline, total protein and hexosamine are shown in Table 1. A common pattern of changes in hydroxyproline was seen in all groups. Collagen content increased gradually with time and reached the maximum level on Day 16 of post wounding. The hydroxyproline content in the treatment group was significantly higher on various post wounding days ($p < 0.05$). Hexosamine content decreased gradually during the study. The mean concentration of hexosamine was higher in the treatment group than in the control group ($p < 0.05$). Protein concentration increased gradually until ten days post wound-

Table 1. Effects of *O. vulgare* extract on wound healing biochemical parameters. Values are expressed as mean \pm SD; (*) $p < 0.05$.

Group	Hydroxyproline (mg/g)			Hexosamine (mg/g)			Total protein (mg/100 ml)		
	Day 4	Day 10	Day 16	Day 4	Day 10	Day 16	Day 4	Day 10	Day 16
<i>O. vulgare</i>	15.9 \pm 1.9	20.4 \pm 1.4*	21.7 \pm 2.6*	58.2 \pm 5.7*	47.8 \pm 3.8*	36.4 \pm 8.2	6417 \pm 486.3*	6488 \pm 573*	5815 \pm 485
Control	13.3 \pm 1.7	16.3 \pm 1.6	16.5 \pm 1.2	47.9 \pm 4.3	41.8 \pm 4.6	32.6 \pm 4.9	5034 \pm 287	5271 \pm 545	5491 \pm 284

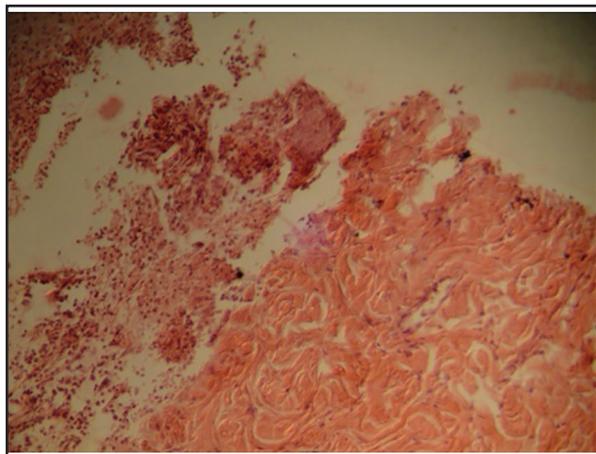


Fig 1. H & E sections of the wounds on Day 5 ($\times 10$). An intense inflammatory response was seen and neutrophils were the predominant cells in both groups.

ing but decreased dramatically on Day 16. Total protein increased significantly in wound tissues of the treatment group ($p < 0.05$). Histopathological studies descriptively showed an intense inflammatory response on Day 4 post wounding and neutrophils were the predominant cells in both groups (Fig. 1). A mild inflammatory response was continued in the control group until Day 10 post wounding while no inflammatory response was seen in the treatment group. Results of histopathological studies showed moderate to intense granulation tissue formation and neovascularization characterized by increased fibroblasts in the treatment group on Day 10 post wounding (Fig. 2). The granulation tissue formation seemed to be delayed in the control group and only newly formed granulation tissues were seen. Histopathological studies revealed severe matrix formation and collagen fiber deposition in the treatment group on Day 16 post wounding while intense granulation tissue formation was the prominent feature in the control group (Fig. 3).

Discussion

Wound healing is a complex and dynamic process of restoring cellular structures and tissue layers in damaged tissues as closely as possible to normal (Nayak and Pereira, 2006). In normal conditions, wound healing is considered as an accurately programmed insult that does not require considerable medical interventions. However, efficient and organized healing processes are absent in pathological conditions such as ulcers and infections. In such conditions, chronic inflammation is the predominant event characterized by abundant neutrophil infiltration, associated reactive oxygen species and destructive enzymes (Diegelmann and Evans, 2004). Infection of wounds is one of the frequent complications in patients who have undergone surgeries. Nowadays, an increased interest is seen amongst researchers to use herbal agents in complicated infectious wounds due to increased antibiotic resistance in microorganisms. Plant products are potential agents for wound healing and are largely preferred because of their widespread availability, non-toxicity, lack of unwanted side effects and their effectiveness as crude preparations. A therapeutic agent selected for wound healing must improve at least one phase of the healing process without producing any side effects (Moslemi et al., 2012). Many plants are used as topical wound healers in Persian traditional medicine. One of these plants, *O. vulgare*, is used as an antiseptic agent in traditional medicine.

Hydroxyproline is the major constituent of collagen and found almost exclusively in collagen. The estimation of hydroxyproline is an accepted method of biochemical evaluation of

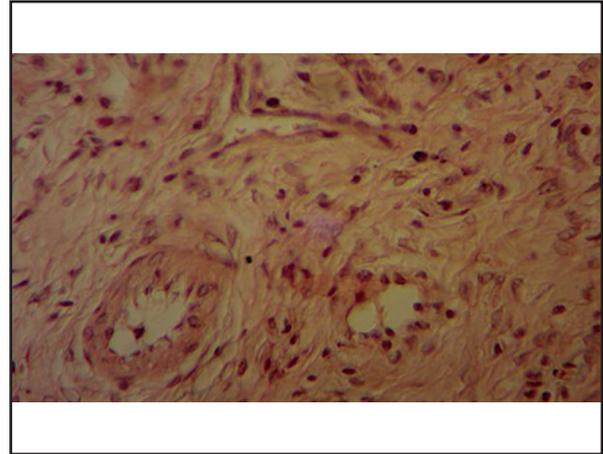
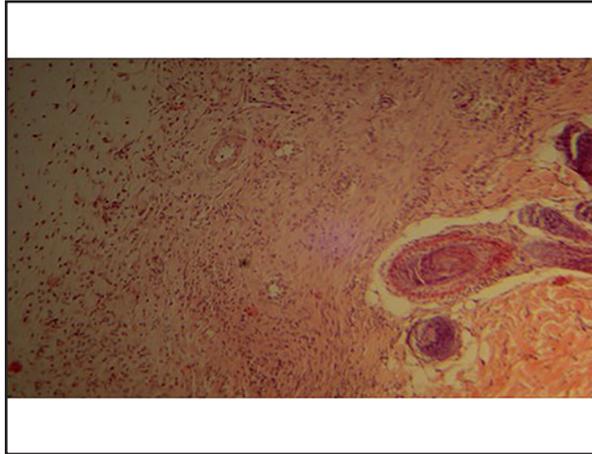


Fig 2. H & E sections of the wounds on Day 10 in treatment group. A) Moderate to intense granulation tissue formation and neovascularization ($\times 10$), B) Increased fibroblasts ($\times 40$).

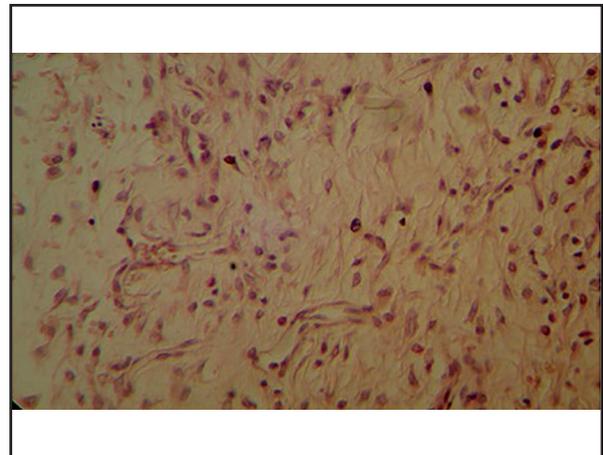
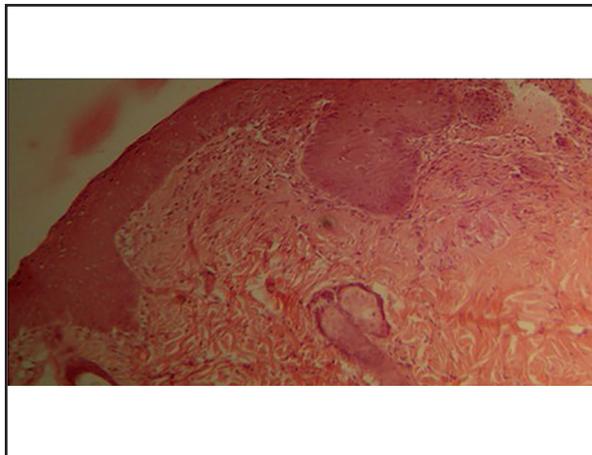


Fig 3. H & E sections of the wounds on Day 16. A) Matrix formation and collagen fiber deposition are seen in treatment group ($\times 40$), B) Granulation tissue formation is the prominent feature in control group ($\times 40$).

total collagen content of a sample (Lin et al., 2003) and is also used as a marker of collagen synthesis (Rasik et al., 1999). An increase in the collagen content of the extracellular matrix is a characteristic change observed in the proliferative phase of wound healing (Lin et al., 2003). Biochemical analysis of the excisional wound tissue of *O. vulgare* treated wounds demonstrated a significant increase in total collagen content compared to that of untreated wounds. The high level of hydroxyproline in the treatment group indicates that more collagen has been produced. This is possibly seen due to increased collagen synthesis following fibroblast proliferation. In the current study, hydroxyproline concentration increased gradually and reached its maximum on Day 16.

Chithra et al. (1998) and Pather et al. (2011) showed that collagen content reached a peak on Day 8 and then a mild continuous decline started until Day 16 (Chithra et al., 1998; Pather et al., 2011).

In 2005, Gupta et al. (2005) demonstrated that topical application of 1% seabuckthorn leaf extract significantly improved the healing process as evidenced by an increase in hydroxyproline and protein contents as well as a reduction in the wound area on Day 8 post wounding. In a study by Nayak et al. (2006), contents of protein, hydroxyproline, and hexosamine increased significantly in groups treated with *Cecropia peltata* (Nayak, 2006) showing that the proliferative stage reached a peak on Day 7 or 8 post wounding (Chithra

et al., 1998; Pather et al., 2011). In the current study, biochemical assessment of collagen content (with maximal collagen content on Day 16) showed different results compared to other studies. This might have occurred due to moiety of the wound induced in the current study. Infection in wounds is considered as a complication and leads to delayed proliferation responses. Overall, higher hydroxyproline concentrations over several days revealed that the healing process proceeded in a usual manner in the treatment group compared to control group. A relationship was seen between hydroxyproline concentration and histopathological findings. An intense inflammation was seen in wound tissues in both groups on Day 4 post wounding thus indicating low hydroxyproline concentration in both groups. Furthermore, an increased concentration of hydroxyproline was seen on Day 10 in the treatment group while the control group had a lower hydroxyproline concentration. This possibly occurred because of moderate tissue granulation formation and neovascularization in treatment group while subsided inflammatory response was the prominent feature in control group.

Glycosaminoglycans and proteoglycans are matrix molecules which act as ground substratum for the synthesis of newly formed extracellular matrix. These molecules are produced by fibroblast in wound tissues as a highly hydrated gel-like ground on which collagen fibers are embedded. These molecules stabilize collagen fibers by enhancing an ionic and electrostatic interaction (Ricard-Blum and Ruggiero, 2005). An increase in the level of these components is seen during the early stages of wound healing after which normal levels are restored (Dunphy and Udupa, 1956). As the collagen content increases, hexosamine level decreases (Chithra et al., 1998; Dunphy and Udupa, 1956). Therefore, hexosamine estimates the amount of ground substance in a wound. In the current study, a similar pattern was observed in *O. vulgare* treated wounds as the level of

hexosamine reached a peak on Day 5 followed by a steady decrease to Day 16.

The results of the current study are similar to those reported by Chitra et al. (1998), Pather et al. (2011) and Sumitra et al. (2005) who showed replacement of granulation tissue by collagen in the wound area. This is verified by increased collagen deposition seen in the histological sections of the treated wounds. As collagen deposition increases, it begins to occupy a greater area at the wound site. In this study, concentration of hexoseamine was higher in the treatment group compared to the control group. It shows that granulation tissue formation began earlier and faster in *O. vulgare* treated rats. Total protein content is an indicator of protein level and cellular proliferation in the wound tissue (Teoh et al., 2009). Results of this study showed that the total protein concentration in wound tissue in the treatment group was higher than that in control group, which might be explained due to cellular infiltration or increased collagen synthesis rate.

The present study has shown that the ethanol extract of *O. vulgare* possesses properties that accelerate wound healing activities. According to literature, this study is the first in vivo study that evaluates the healing activity of *O. vulgare* against wounds infected by *S. aureus*. However, further and complementary studies are recommended to better understand the antimicrobial effects of *O. vulgare*.

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مطالعه فاکتورهای التیامی عصاره گیاهی پونه کوهی در یک مدل زخم عفونی جراحی ناشی از استافیلوکوکوس اورئوس در مدل حیوانی رت

حمیدرضا مسلمی^{۱*}، میثم تهرانی شریف^۱، سعید محمدپور^۱، علیرضا مخملباف^۱، خاطره کفشدوزان^۲، طناز احدی^۳، رامین مظاهری نژاد فرد^۴

(۱) گروه علوم درمانگاهی، دانشکده دامپزشکی دانشگاه آزاد اسلامی واحد گرمسار، گرمسار، ایران

(۲) گروه میکروبیولوژی، دانشکده دامپزشکی دانشگاه سمنان، سمنان، ایران

(۳) گروه طب فیزیکی و توانبخشی، دانشگاه علوم پزشکی ایران، تهران، ایران

(۴) آزمایشگاه مرکزی رستگار، دانشکده دامپزشکی دانشگاه تهران، تهران، ایران

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چکیده

زمینه مطالعه: پونه کوهی یکی از گیاهان دارویی است که اسانس بدست آمده از این گیاه دارای خواص ضدباکتریایی می باشد. استافیلوکوکوس اورئوس عامل درصد بالایی از عفونت‌های زخم جراحی می باشد. **هدف:** مطالعه حاضر به منظور بررسی تغییرات فاکتورهای التیامی در استفاده از عصاره هیدروالکلی گیاه پونه کوهی در درمان زخم عفونی ناشی از استافیلوکوکوس اورئوس در مدل حیوانی موش صحرایی انجام گرفت. **روش کار:** در این تحقیق تعداد ۲۰ سر موش صحرایی نر سفید تهیه و در ناحیه پشت و بین دو کتف، برشی تمام ضخامت به قطر ۱/۵ Cm ایجاد گردید. جهت عفونی نمودن زخم‌ها، پس از گذشت ۲۴ ساعت از ایجاد زخم، ۵-۶ قطره از باکتری با رقت 10^4 cfu/ml در محل زخم تلقیح گردید. در گروه درمانی، سطح ضایعه کاملاً با عصاره پونه کوهی آغشته گردید و سپس تا ۱۶ روز و به فاصله ۲۴ ساعت، سطح ضایعه کاملاً با این عصاره آغشته می شد. اما در گروه کنترل، هیچ گونه تیماری بر روی زخم‌ها در طول دوره آزمایش انجام نشد. سپس در روزهای ۵، ۱۰ و ۱۶ از محل زخم‌ها نمونه برداری شده و فاکتورهای بیوشیمیایی و هستیوپاتولوژی مورد بررسی قرار گرفت. **نتایج:** نتایج فاکتورهای بیوشیمیایی افزایش معنی داری را در میزان هیدروکسی پرولین و پروتئین تام در گروه درمان شده در مقایسه با گروه کنترل نشان داد. همچنین میزان هگزوزآمین در گروه درمانی بالاتر از کنترل بود. نتایج هستیوپاتولوژی نیز افزایش روند التیام در گروه درمانی را نسبت به گروه کنترل نشان داد. **نتیجه گیری نهایی:** در نتیجه می توان بیان داشت که، عصاره حاصل از این گیاه می تواند باعث تسریع در روند التیام در زخم عفونی شده با باکتری استافیلوکوکوس اورئوس در مدل حیوانی موش صحرایی گردد.

واژه‌های کلیدی: زخم عفونی جراحی، پونه کوهی، استافیلوکوکوس اورئوس