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Original Article
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In vitro Antibacterial Activity and Wound Healing Effects of *Achillea millefolium* Essential Oil in Rat

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Objectives: In this study we aimed to evaluate the *in vitro* antibacterial activity and wound healing properties of *Achillea millefolium* essential oil (AMEO) in full-thickness wound model in rat. The antibacterial activity of AMEO was evaluated against *Staphylococcus aureus* and *Pseudomonas aeruginosa* using the broth dilution method.
Methods: The 2 cm × 2 cm full-thickness excisional wounds were created on the back of animals. Topical therapy was applied twice a day using 1%, 2%, and 3% w/w AMEO ointments, and the measurement of the wounds area was carried out every 3 days, after that the wound closure percentage was calculated in these days. Hydroxyproline content and histopathological evaluation of wound tissue samples were carried out on day 7 and 14 post wounding. Eucerin was used for the treatment of vehicle control group and negative control group received no treatment.
Results: Our results revealed the bacteriostatic activity of AMEO against *S. aureus* and *P. aeruginosa*. Wound healing activity evaluation of AMEO showed the significant increase ($p < 0.05$) in the wound closure percentages in rats treated with AMEO 1% and 2% comparing to those of non-treatment group. In addition, hydroxyproline contents of tissue significantly ($p < 0.01$) increased in AMEO 1% and 2% comparing to non-treatment group. Histopathological evaluations of wound tissue samples on day 7 and 14 demonstrated higher accumulation of collagen fibers, reduction of edema and inflammation and also formation of tissue appendages in 1% and 2% AMEO treated groups in comparison with non-treatment group.
Conclusion: The results of this study indicated that AMEO has the potential to be used as a safe and effective wound healing agent.

Keywords: wound healing, *Achillea millefolium*, essential oil, antibacterial

INTRODUCTION

Wound healing is a complex process consisting of four complex steps: hemostasis, inflammation, proliferation, and remodeling. The hemostasis phase of wound healing, comprising vasoconstriction, platelet aggregation, and coagulation, occurs rapidly. The second phase is initiated right after injury and consists of cell signaling, migration of different cells such as macrophages to the wound site, the cell cascade signaling process, led by cytokines, and finally fibroblast activation and proliferation, which leads to the reconstruction and rehabilitation of the collagen structure. The final phase is the restoration of normal characteristics of the wound site [1].

Various factors may interrupt or delay the processes men-

tioned above, and these include opportunistic bacterial infections [2, 3]. The clinical signs of an infected wound are topical pain and erythema, infectious exudate, and malodor. Infection in a wound caused by bacteria is the main reason for chronic wounds, which can lead to mobility disorders, multiple-organ dysfunction, and even more life-threatening situations. *Staphylococcus aureus* and *Pseudomonas aeruginosa* are the two most common pathogens isolated from chronic wounds [4, 5].

There are a variety of therapeutic agents and techniques for treating wounds. However, chronic and non-healing wounds are one of the most common health problems worldwide and can often lead to disability or even mortality [5]. Therefore, researching new techniques and drugs to accelerate wound healing is critical. In recent years, medicinal plants and their derivatives, such as essential oils, have been considered by researchers to be sources for the production of new agents effective in wound healing [6].

Achillea millefolium, also known as yarrow, is a species of the Asteraceae family and has a long history of traditional uses in treating gastrointestinal disorders, hepatic diseases, rheumatic pain, pneumonia, and wound healing in several cultures across Asia and Europe [7, 8]. The anti-inflammatory effects of *A. millefolium* essential oil (AMEO), caused by the inhibition of proteases, have been widely demonstrated in several studies [9, 10]. GC-Mass analysis of AMEO identified several active components such as Eucalyptol, camphor, α-terpineol, β-pinene, and borneol [11].

In vitro studies of the AMEO have demonstrated antioxidant and antimicrobial properties against *Streptococcus pneumoniae*, *Clostridium perfringens*, *Candida albicans*, *Mycobacterium smegmatis*, *Acinetobacter lwoffii* and *Candida krusei* [12].

The main purpose of this study was to evaluate wound-healing potential and the antibacterial activity of AMEO against two common microorganisms (*S. aureus* and *P. aeruginosa*) involved in wound infection.

MATERIALS AND METHODS

1. Evaluation of the antibacterial activity of AMEO

Standard strains of *S. aureus* (*S. aureus*, ATCC6538) and *P. aeruginosa* (*P. aeruginosa*, ATCC 27853) were purchased from the Persian Type Culture Collection at the Iranian Research Organization for Science and Technology (IROST), Tehran, Iran. The bacteria were cultured on nutrient agar (NA). A 0.5 McFarland standard (the final concentration of 10^8 CFU/mL of each organism individually) was prepared by harvesting the organisms and suspending them in a saline solution [13].

Minimum inhibitory concentrations (MICs) of AMEO against *S. aureus* and *P. aeruginosa* were determined using the broth dilution method. Serial dilutions of AMEO in broth (0.0625, 0.125, 0.5, 1, 1.5, and 2 mL/mL) were prepared in test tubes, next, 100 μL of bacteria suspension (10^7 CFU/mL of *S. aureus* and *P. aeruginosa* strains) with 1 mL SCDB (Soya Bean Casein Digest Broth) were inoculated to each test tube, before being incubated at 37℃ for 24 h. The control tubes (media control and organism + media control) were also prepared for each strain [14]. The lowest concentration of the AMEO that produces no turbidity (no visible growth) was considered the MIC.

2. Preparation of AMEO ointments

Achillea millefolium essential oil was purchased from Shafa Kurdistan Inc. (Iran, Kurdistan) and 1, 2, and 3 g of essential oil were mixed in 100 g of Eucerin in order to prepare 1%, 2%, and 3% W/W ointments of AMEO, respectively.

3. Animals

30 Wistar rats (weighing 200 ± 30 g) of both sexes were used in this study. The animals were kept in individual cages under standard conditions of temperature (22 ± 2℃) relative humidity (50-55%), and 12:12 h light-dark cycles. Animals were allowed to adapt to laboratory conditions for seven days before the experiment.

4. Wound procedure

The rats were divided randomly into six groups (n = 5). The negative control group received no treatment, the vehicle group received topical Eucerin, and the test groups received 1%, 2%, and 3% AMEO ointment, respectively. Phenytoin 1% cream was used as a positive control. After anesthetizing the rats with an IP injection of ketamine (60 mg/kg) and xylazine (5 mg/kg), the hairs of the wound site were shaved and the area was disinfected with 70% ethanol, then a full-thickness excisional wound with dimensions of 2 cm × 2 cm was created on the back of each animal [15]. Wound treatments were typically applied twice a day, beginning two hours after wound creation until complete wound closure.

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The wound areas were measured every three days by analyzing photographs of the wounds using ImageJ software [16], while wound closure percentage was calculated using the following equation:

$$100 \times \frac{\text{Initial wound area} - \text{wound area on a specific day}}{\text{Initial wound area}} = \text{Percentage of wound closure}$$

5. Biopsy sampling

Full-thickness cross-section specimens were collected on days 7 and 14 post-wounding. For the histopathological study, samples were fixed in 10% formalin, while for the hydroxyproline assay skin samples were homogenized and centrifuged, and the supernatant was aliquoted and stored at –80℃ until needed.

6. Hydroxyproline assay

Hydroxyproline is one of the structural components of collagen. Determining the amounts of hydroxyproline in tissue indicates the amount of synthesized collagen in the tissue [17]. The hydroxyproline assay kit was purchased from KiaZist life sciences, Iran. The assay of the hydroxyproline content of tissue samples was conducted according to the manufacturer's guidelines seven and 14 days after wound creation.

7. Histopathology

Histopathological studies of the samples were carried out using hematoxylin and eosin (H&E) and Masson's trichrome staining. Inflammation, re-epithelialization, angiogenesis, granulation tissue formation, and extracellular matrix deposition were evaluated by H&E staining, while the degree of collagen deposition was observed using Masson's trichrome staining.

8. Statistical analysis

Data are presented as mean ± SD, and data analysis was performed using GraphPad Prism via one-way ANOVA and Tukey post-hoc tests. The significance level was $p < 0.05$ for all analyses.

RESULTS

1. Antibacterial activity

Determining the MIC by the broth dilution method was used to evaluate the antibacterial activity of AMEO. The results showed that AMEO can inhibit the growth of both *S. aureus* and *P. aeruginosa*. The MICs of AMEO for both microorganisms were at a concentration of 1 m/mL.

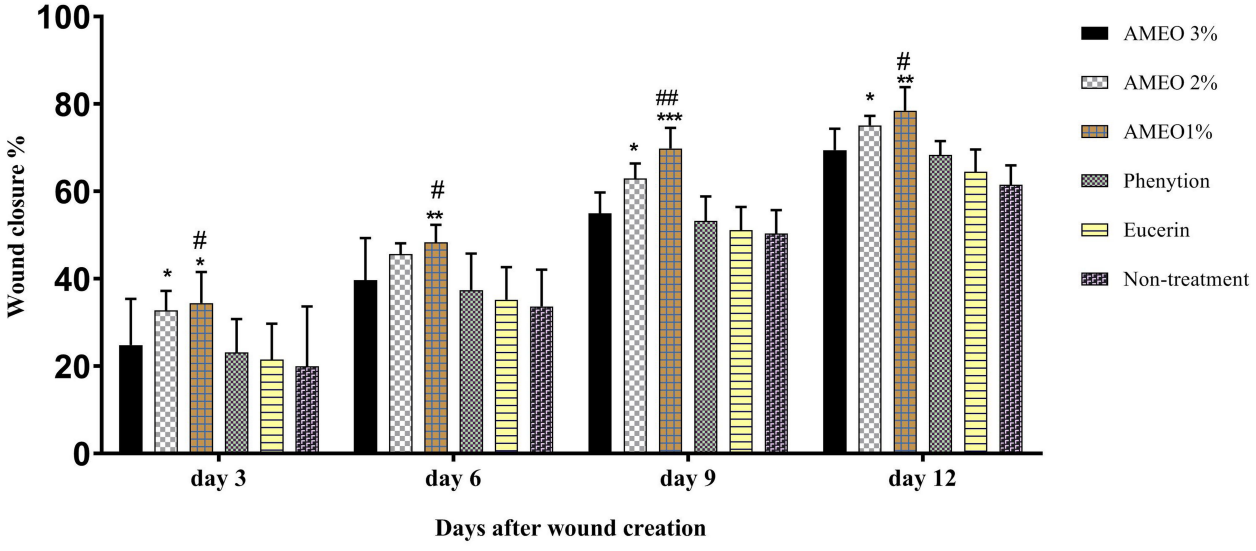

Figure 1. Wound closure percentages in studied groups on days 3, 6, 9, 12 post injury. Data are presented as mean ± SD% of wound closure. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ indicate the significant differences from non-treatment group. $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ indicates significant differences from Eucerin treated group.

2. Wound closure

The average time to complete wound closure for the AMEO 1%, AMEO 2%, AMEO 3%, Phenytoin, Eucerin, and negative control groups were 19.4, 22, 26, 27, 26, and 26 days, respectively. The complete wound closure time of the AMEO 1% treated group was significantly shorter than those of the other groups ($p < 0.01$) except for the AMEO 2% group. The complete wound closure time of AMEO 2% was significantly shorter than those of the AMEO 3%, Phenytoin, Eucerin, and negative control groups ($p < 0.01$). Interestingly, the positive control group, which was the wounds treated with Phenytoin, had the slowest closure rate.

Three days after wound creation, the AMEO 1% and 2% treated groups demonstrated a significantly higher wound closure rate (34.4% ± 7.1 and 32.75 ± 4.43, respectively) in comparison with the negative control group (19.92% ± 137.2) ($p < 0.05$). On day 6 post-wounding, the wound closure rate was significantly higher in rats treated with AMEO 1% ointment (48.3% ± 4.02) compared with the negative control (33.65% ± 8.42) and vehicle groups (35.2% ± 7.44) ($p < 0.01$). On day 9 and 12 post-wounding, wound closure rates in rats treated with AMEO 1% (69.8% ± 4.7 and 78.45% ± 5.9, respectively) and AMEO 2% (62.95% ± 3.39 and 75.1 ± 2.17, respectively) were significantly higher when compared to those of the negative control group (50.31% ± 5.39 and 61.45% ± 4.45, respectively) ($p < 0.05$). There were no significant differences in wound closure rate between Eucerin (vehicle) treated animals and the negative control group on days 3, 6, 9, or 12 (Fig. 1).

3. Hydroxyproline assay

The results of the hydroxyproline assay are shown in Fig. 2. The hydroxyproline contents of tissue samples obtained from animals treated with AMEO 1% (170.4 ± 5.98 and 196 ± 7.6 μg/mg tissue on days 7 and 14, respectively) and AMEO 2% (171.6 ± 6.94 and 193.6 ± 4.636 μg/mg tissue on days 7 and 14, respectively) were significantly ($p < 0.01$) higher than those of negative control group (153.2 ± 8.87 and 164.8 ± 4.76 μg/mg tissue on days 7 and 14, respectively) (Fig. 2).

4. Histopathological study

The histopathological study of the wound tissue in the different experimental groups showed attenuated inflammation response and more collagen deposition in the AMEO 1% treated group compared with the other experimental groups on day 7 post-wounding (Fig. 3, 4).

The formation of the epidermal layer with more cell layers, the formation of epidermal protrusions and dermal papillae, the reappearance of skin appendages, and a reduction of edema and inflammation in granulation tissue were observed in samples obtained from AMEO 1%-treated animals on day 14 post-wounding. The histopathological study of samples obtained from the AMEO 3%, Eucerin-treated, and negative control groups showed no new epidermal layer or coagulum, as

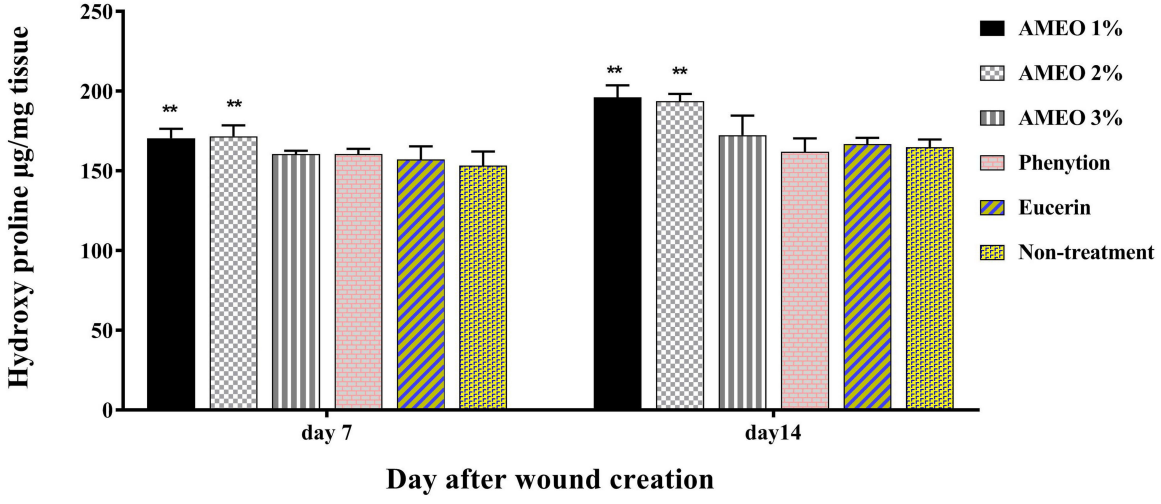

Figure 2. The hydroxyproline content of wound tissue samples in studied groups on days 7 and 14 post injury. Data are presented as mean ± SD μg/mg tissue of hydroxyproline. **$p < 0.01$ show the differences from non-treatment group.

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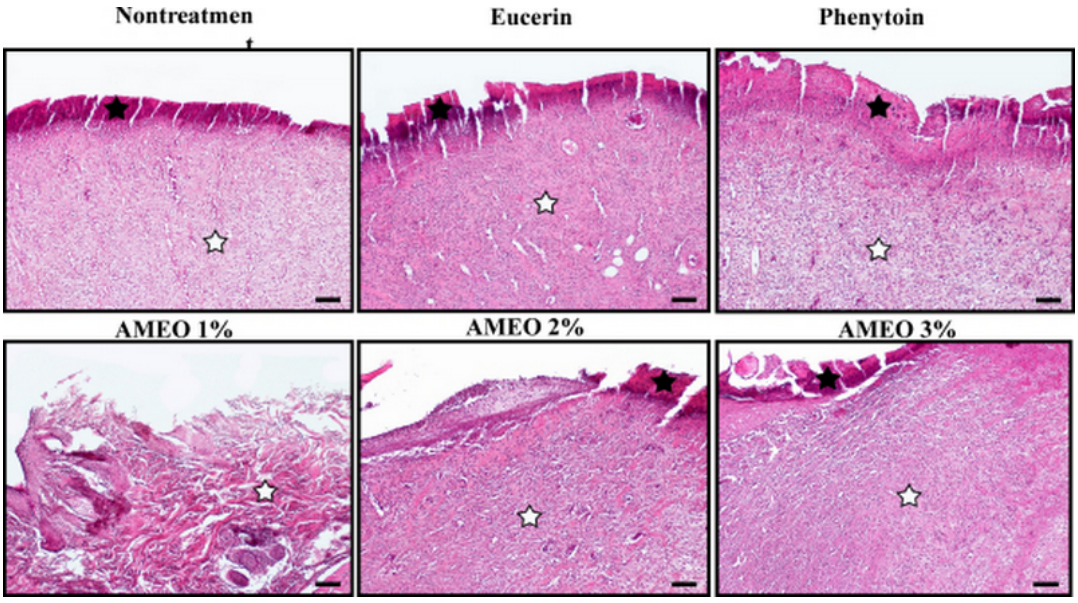

Figure 3. Histopathological micrographs of wound tissue samples obtained from studied groups on day 7 post wounding using H&E staining. Scale bar is 100 μm, AMEO, *Achillea millefolium* essential oil; Black star, coagulum; White star, granulation tissue.

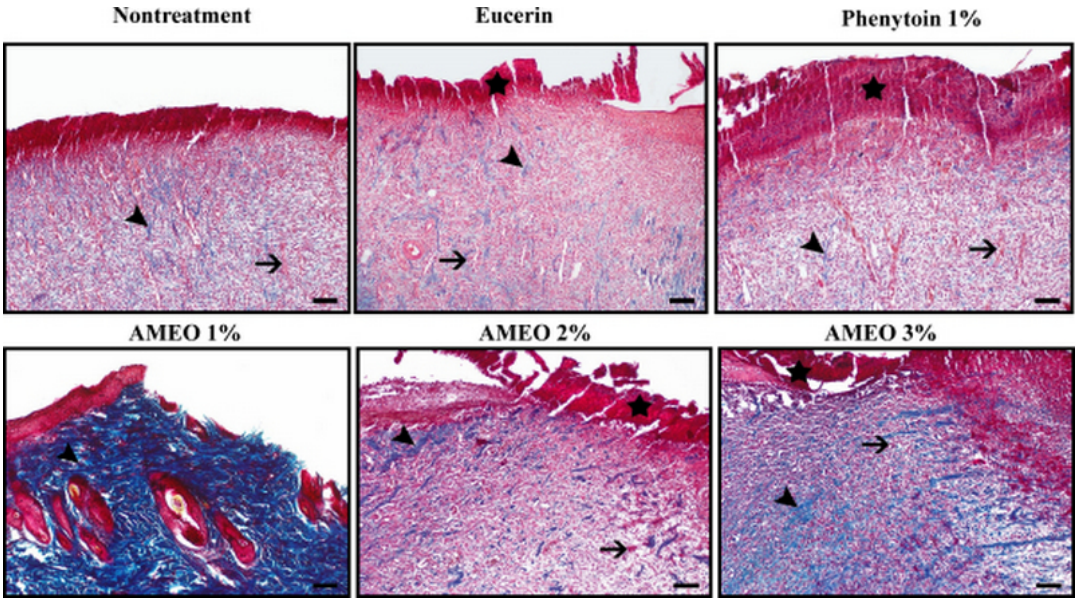

Figure 4. Histopathological micrographs of wound tissue samples from studied groups on day 7 post wounding using Mason's trichrome staining. Scale bar is 100 μm, AMEO, *Achillea millefolium* essential oil; Arrow tip, collagen; Arrow, blood vessels; Black star, coagulum.

well as a reduction in edema and inflammation in granulation tissue. In addition, reduction of vascular hyperemia and accumulation of collagen fibers in granulation tissue was observed in these groups on 14 post-injury. A study of samples obtained from animals treated with AMEO 2% on day 14 revealed the formation of a thin epidermal layer and a reduction in edema, inflammation, and vascular hyperemia as well as a significant accumulation of collagen fibers in granulation tissue (Fig. 5, 6).

DISCUSSION

Skin wounds are the most common health issues people encountered throughout their lives, so developing new methods and drugs for encouraging optimal wound healing is essential.

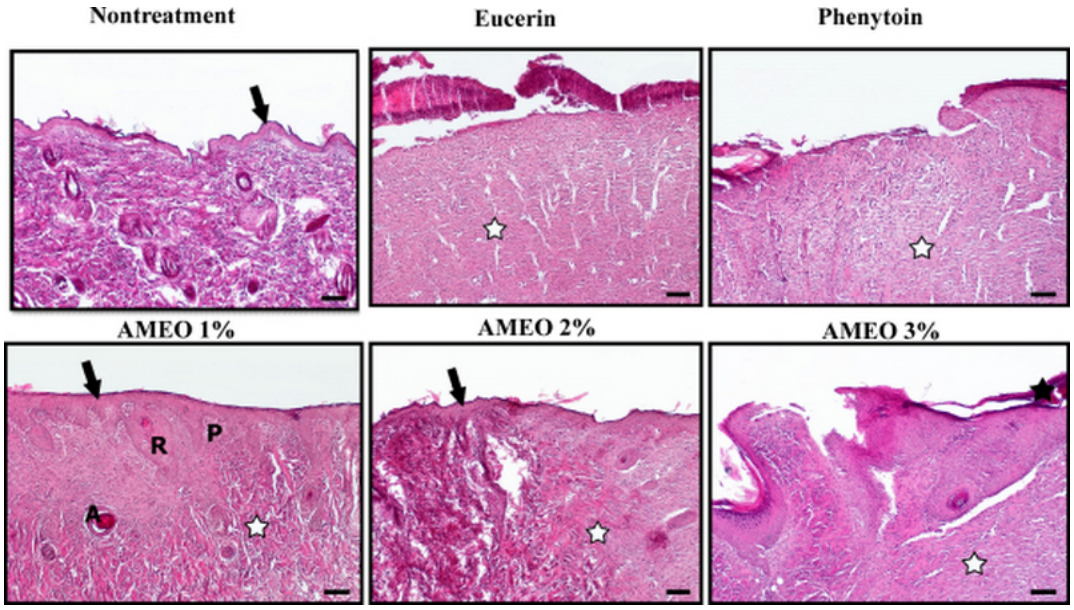

Figure 5. Histopathological micrographs of wound tissue samples obtained from studied groups on day 14 post wounding using H&E staining. Scale bar is 100 μm, AMEO, *Achillea millefolium* essential oil; White star, granulation tissue; A, skin appendages; P, dermal papilla; R, epidermal bulges; Arrow, epidermis.

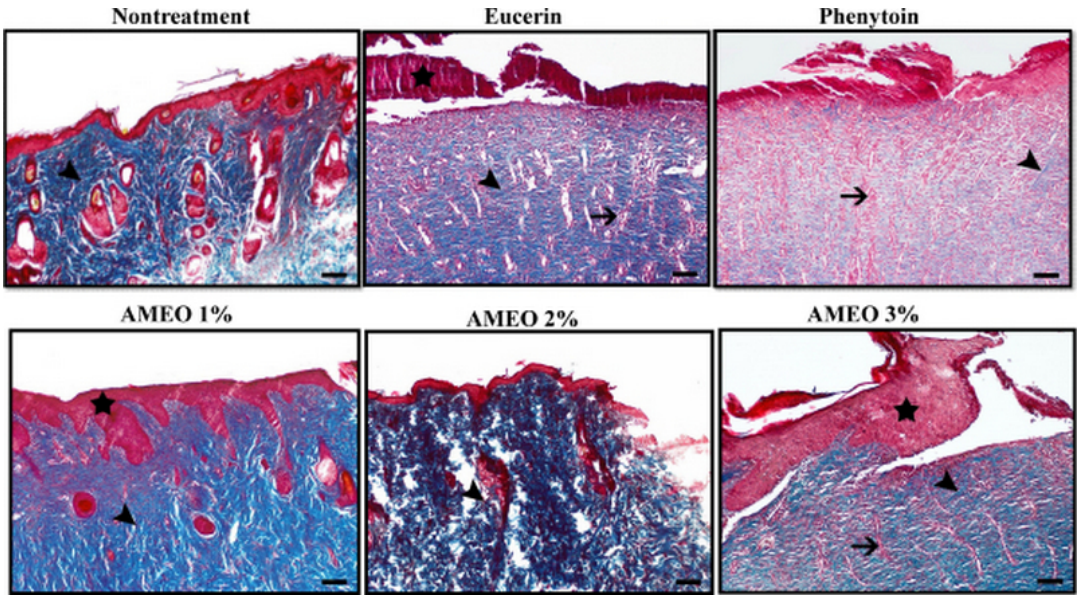

Figure 6. Histopathological micrographs of wound tissue samples from studied groups on day 14 post wounding using Mason's trichrome staining. Scale bar is 100 μm, AMEO, *Achillea millefolium* essential oil; Arrow tip, collagen; Arrow, blood vessels; Black star, coagulum.

Medicinal plants and their derivatives can be used as an important source for developing potential new drugs effective for wound healing.

In this study, we evaluated the wound-healing potential of *Achillea millefolium* essential oil in a full-thickness wound rat model. Considering that one of the main reasons for delayed wound healing is an infection caused by bacteria such as *Pseudomonas aeruginosa* and *Staphylococcus aureus*, the *in vitro* antibacterial activity of *A. millefolium* essential oil against these two microorganisms was also investigated. The results of this study showed that AMEO at a concentration of 2% or less (with AMEO 1% ointment being the most effective concentration)

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has the potential to accelerate wound healing by attenuating the inflammatory response and increasing collagen synthesis and antibacterial activity. *Achillea* has been used in traditional medicine for the treatment of wounds throughout the world; and the name *Achillea* is derived from Achilles, the Greek hero who used yarrow to heal soldiers' wounds during the Trojan War [8]. The wound-healing activity of *A. millefolium* extracts has been evaluated in several studies [18, 19], although, to date, no studies have investigated the effects of *A. millefolium* essential oil on wound healing.

Monoterpenes and sesquiterpenes are the most representative molecules of *Achillea* essential oil and each one of the evaluated monoterpenes demonstrated wound-healing properties [20]. The antioxidant and anti-inflammatory effects of monoterpenes and sesquiterpenes are often associated with wound-healing activity [21].

Collagen is the most abundant protein in the body and the most important component of the extracellular matrix. Considering that hydroxyproline amino acid exists exclusively in collagen protein, we measured the amount of hydroxyproline in the tissue samples obtained from the studied groups to estimate the collagen content in healing wound tissue from the different experimental groups. The hydroxyproline assay showed that AMEO 1% and 2% significantly increased the hydroxyproline content in tissue. These results seem to indicate that increasing collagen synthesis is one of the possible mechanisms by which AMEO can heal wounds. The increased collagen synthesis may be because of the presence of large amounts of terpenes in AMEO [22, 23]. It is likely that AMEO stimulates the migration of fibroblasts to the wound site and activates them to produce collagen. Increased collagen production in the AMEO 1% and 2%-treated groups was also confirmed by histological studies.

Another important finding of this study was the antibacterial activity demonstrated by AMEO against *S. aureus* and *P. aeruginosa*, the most common bacteria isolated from chronic wounds, which often form a biofilm resistant to many antibiotics. Co-infection with these two bacterial strains is a major challenge in the treatment of chronic wounds [24]. Antibacterial wound dressings decrease the risk of bacterial infection during wound healing and prevent the wounds from becoming chronic. Based on the results of this study, AMEO has the potential to be used as an antibacterial wound dressing in combination with synthetic biomaterials.

CONCLUSION

This study has shown that AMEO has the potential to treat wounds. AMEO accelerated the wound-healing process by attenuating the inflammatory response and stimulating both collagen synthesis and angiogenesis. In addition, AMEO's antibacterial activity against *S. aureus* and *P. aeruginosa* can reduce the risk of wound infection, meaning that it has the potential to be used in combination with standard antibiotics in the treatment of infected wounds. Also, it could be used in combination with synthetic biomaterial for the preparation of antibacterial wound dressings.

ETHICAL APPROVAL

All animal experimentation procedures were carried out in accordance with guidelines for care and use of laboratory animals approved by ethics committee in biomedical research of Hamadan University of Medical Sciences (ethical code: IR.UMSHA.REC.1400.097).

CONFLICTS OF INTEREST

The authors declare no potential conflicts of interest with respect to the research, authorship, and publication of this article.

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