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Anti-inflammatory and Wound Healing Activities of *Aloe vera*, Honey and Milk Ointment on Second-Degree Burns in Rats

Parviz Farzadinia, PhD[1], Niloofar Jofreh, MSc[2], Saeed Khatamsaz, PhD[2], Ali Movahed, PhD[3], Samad Akbarzadeh, PhD[3], Mostafa Mohammadi, BSc[4], and Afshar Bargahi, PhD[5]

Abstract

The aim of the present study was morphological and morphometric investigation of burn healing impacts of an honey, milk, and *Aloe vera* (HMA) ointment on experimentally induced second-degree burns, to approve the medicinal basis of its use in Iranian traditional medicine. A total of 21 male Albino rats weighing 200 to 300 g were divided into 3 groups of 7, including (1) control group, (2) positive control group, and (3) the treatment group that were treated with eucerin, silver sulfadiazine 3% and HMA ointment 5% respectively. After anesthetizing, the second-degree burns (1 cm^2 areas) were made on the back of the animals using a digital controlled hot plaque, and each group was treated topically, based on the time scheduled. Then, skin punch biopsies were obtained on the 1st, 14th, and 28th days of post–burn induction; processed; and stained using hematoxylin and eosin and Masson's trichrome methods. The results showed that HMA ointment induces cell proliferation, increasing the wound closure rate, blood vessel counts, and collagen fiber density in treated animals. It also reduced the wound secretions, inflammation, and scar formation. According to the obtained morphological, morphometric results, we concluded that the traditional HMA ointment, which is rich in therapeutic biomaterials and minerals, has multiple healing effects on burn wounds in rats.

Keywords

burn, wound healing, biomaterial, morphometric

Burn injuries are the most devastating accidents and one of the major causes of disability and mortality worldwide.[1] After traffic accidents, burn injuries are the second most common cause of mortality.[2] Skin is a coated barrier against chemicals and microorganisms because it has stratified squamous cornified epithelium tissue.[3,4] The degree of burn depends on the heat temperature, contact time, and anatomical region of the skin. Some health problems arising from burns are necrosis, trauma, damage to fibroblasts, inflammation, blood and lymphatic capillary stasis, changes in the local pH, mass production of collagen, and disorders in gene expressions such as frequent mistakes in RNA polymerases and t-RNA functions.[5] For successful treatment of burns, it is necessary to improve knowledge of the biology, pathology, and use of modern medical technology along with natural therapy. So far, many drugs have been used in the control and treatment of burns, including chemical medications such as nitrofurazone,[6] silver sulfadiazine,[7] alpha ointment,[8] phenytoin,[9] and many others. Usually, chemical drugs have side effects and drug resistance; therefore, researchers have attempted to find alternative natural source drugs. The occurrence of many biologically active compounds in *Aloe vera* gel may be the reason for its use in traditional medicine. *Aloe vera* heals the skin by reinitiation of angiogenesis, increasing blood flow, stimulation of fibroblast proliferation, anti-inflammation and antimicrobial activities, and moisturizing effects.[10,11] Moreover, there are many scientific studies about the burn healing effects of honey.[12] Honey has been

[1]Department of Biology and Anatomical sciences, Faculty of Medicine, Bushehr University of Medical Sciences, Bushehr, Iran
[2]Department of Biology, Kazerun Branch, Islamic Azad University, Kazerun, Iran
[3]Department of Biochemistry, Faculty of Medicine, Bushehr University of Medical Sciences, Bushehr, Iran
[4]Young researchers and elite club, Bushehr branch, Islamic azad University, Bushehr, Iran
[5]The Persian Gulf Marine Biotechnology Research Centre, Bushehr University of Medical Sciences, Bushehr, Iran

Corresponding Author:
Afshar Bargahi, PhD, The Persian Gulf Marine Biotechnology Research Center, Boostan 19th Alley, Imam Khomeini St, P. O. Box-3631, Bushehr, Iran.
Email: abargahi@yahoo.com

used in wound care in traditional medicine, including in ayurvedic, Chinese, and Roman traditions.[13-15] It contains approximately 30% glucose, 40% fructose, 5% sucrose, and 20% water and amino acids, vitamins, minerals, and enzymes.[16] There have also been many traditional scientific studies on the wound healing effects of milk. It is rich in bio-organics and minerals.[17] So the aim of the present study was investigation of the burn healing effects of honey, milk, and *Aloe vera* (HMA), in the ointment form, to approve its use in our traditional medicine.

Material and Methods

Preparation of HMA Ointment

Aloe vera (Liliaceae) is a semitropical plant having fibrous roots and a short stem with spiral greenish leaves. *Aloe vera* leaves were collected from farms in the southern region of Bushehr Province, Iran. The samples were authenticated at the Agriculture and Natural Resources Research Center, Bushehr, Iran. The *Aloe vera* leaves were placed in the refrigerator at 4°C, and the gel was extracted from leaves. To inhibit enzymatic degradation, air oxidation, and loss of active ingredients, the gel was separated and chopped on ice with time intervals of less than 30 minutes.[18] The gel was immediately transported to the freeze-drier apparatus (Beta 2-8 LD plus, Christ, Germany), then dried under cold vacuum conditions. The dried material was ground to a fine powder with a grinder in cold conditions and kept in the dark bottles. Sterilized low-fat dairy milk was purchased from PAK Dairy Co, Tehran, Iran. It was freeze dried immediately and powdered. Also, wild honey from the honeybee (*Apis mellifera*) was harvested from Ziziphus-rich forests in the south-east regions of Bushehr province, Iran, in late summer or early autumn. Our previous histological study at concentrations of 1%, 3%, 5%, 7%, and 9% of HMA ointment showed that the burn healing effect increased with increase in concentration from 1% to 5%, but reached a plateau at 7% to 9%. With this in mind, a dosage of 5% HMA ointment was selected. Then, according to our traditional medicine practices, 5 g of powdered aloe gel was added to 5 g of filtered honey and homogenized. Then, 5 g of dry milk was added to the obtained compound and stirred until homogenized. Finally, the whole compound was added to 85 g of anhydrous eucerin to obtain the 5% HMA ointment.

Animals

A total of 21 male Albino rats (Wistar strain) weighing 200 to 300g were divided into 3 groups of 7 each: (1) the control group, (2) positive controls, and (3) treated with eucerin, silver sulfadiazine 3%, and HMA ointment 5%, respectively. Animals were housed in cages (20°C-25°C, 70%-80% humidity, 12-hour light/dark cycle) in an animal house and allowed free access to water and food. All experiments were performed according to the guidelines of the animal ethics committee of Bushehr University of Medical Sciences (Permission Number Et/Anim/32180).

Burn Inducing and Treatments

Burn inducing was performed according to the method of Bazzi et al.[19] Animals were anesthetized with intramuscular injection of 10% ketamine (50 mg/kg) and 2% xylazine (10 mg/kg) mixture. For inducing the second-degree burn, the back area of animals were shaved and disinfected with 1% polyvinylpyrrolidone iodine. Then, a homemade aluminum plaque (1 cm^2 areas), which was attached to the digitally controlled thermal electric soldering tip was placed on the back region of the animal with constant pressure for 16 s. Immediately, the analgesic with sodium dipyrone (40 mg/kg) was injected intramuscularly and maintained for 2 consecutive days using oral administration of sodium dipyrone (200 mg/kg in drinking water). The second-degree burn was approved by microscopic and macroscopic evaluations after 24 hours of burn inducing. Animals in groups (1), (2), and (3) were treated with eucerin, silver sulfadiazine 3%, and HMA ointment 5%, respectively. The wounds were treated topically according to the study time scheduled: twice a day in the first week with intervals of 12 hours, once a day in the second week, followed by alternate days in the third and fourth weeks.

Histological Evaluations

After anesthetizing animals with intramuscular injection of 10% ketamine and 2% xylazine at doses of 10 and 90 mg/kg of body weight, respectively,[20] the punch biopsies (2-3 mm) were taken from the edges of burn wounds using a biopsy puncher. Biopsy samples were fixed in 10% formalin, dehydrated in alcohol, molded in paraffin, and 3-μm sections were prepared.[21] Samples were stained with hematoxylin and eosin (H&E) for detection of vessels, keratinocytes, hair follicles, sweat glands, and collagen fibers. Also, Masson's trichrome staining was used for the detection of collagen fibers.[22] Microscopic images of sections were taken using a light microscope equipped with a digital camera (Moticam, model A-352, Netherlands & China). The morphometric analysis of photomicrographs, including fibroblast and blood densities (as a ratio of fibroblast or vessel counts per 1 mm^2 area at 40× magnification),[23] percentage wound area, tissue necrosis, and epidermal thickness, were done using Image tool software (version 3).[24]

The wound area was calculated using the following equation:

$$\text{Percentage wound area} = \left(A^0 - A_X / A^0\right) \times 100, \quad (1)$$

where A^0 is the wound area on the first day of burn induction and A^X the wound area on the Xth day of burn

induction. The morphological changes, including epidermis and cornified layer, blood vessel congestion, and collagen fiber arrangement in the dermis, were evaluated.

Experimental Assessment

For morphological evaluations, the clinical criteria for wound healing were scored according to secretion type (*purulent* = 1, *sanguinous* = 2, *serous* = 3, *none* = 4); Secretion amount (*heavy* = 1, *low* = 2, *moderate* = 3, *none* =4), wound color (*dark gray* = 1, *creamy* = 2, *reddish* = 3, *bright red* = 4), and dermal stiffness or scar formation (*none* = 1, *stiff* = 2, *moderate* = 3, *soft* = 4).[25,26]

Statistical Analysis

Statistical evaluation of quantitative data was done using SPSS software (version 11.5, Chicago, IL) and parametric 1-way ANOVA followed by Duncan tests. Also, qualitative data were analyzed by the nonparametric Kruskal-Wallis method followed by the Mann Whitney test. Data were expressed as the mean values ± SD for quantitative and median ± standard error of mean for qualitative data. Differences in quantitative and qualitative data were considered significant at $P < .05$; n = 7.

Results

Experimental Assessments

Our results showed that on the 5th day of the experiment, the amount of secretions in the treated group (HMA ointment) was moderate, but in the control group, it was high. No secretions were observed in the positive control and treated groups. However, on the 10th and 15th days of the study, in the control group, the secretion was moderate (Table 1). The type of secretion was more purulent in the control group compared with the other groups on the 5th day of the study (Table 1). The color of wound secretion was more reddish in the treated group as compared with the controls on the 5th, 10th, and 15th days (Table 1). Also, the wound scar was evaluated on the 10th, 20th, and 30th days of the study. The result showed that the scar tissue was softer (not stiff or coarse) in the treated group when compared with the controls (Table 1 and Figure 1).

Morphological Evaluations

As shown in Figure 2, the epithelial and dermal tissues were relatively healed in positive control and treated groups, but in the controls, the skin did not have clear epithelium on the 14th day. The H&E staining showed that epithelial thickness and consistency of the dermis and granular vascular tissues were increased in the treated group at the end of the study. Photomicrographs of Masson's trichrome–stained sections of dermis showed the existence of tissue damage and inflammation in the control group, whereas in the positive control and treated groups, it was uniform and regular, with normal structure at the end of the study (Figure 3).

Table 1. Experimental Assessment of Anti-inflammatory and Wound Healing Activities of *Aloe vera*, Honey, and Milk Ointment on Second-Degree Burns in Rats.[a]

Experimental Criteria	Day	Group I	Group II	Group III
Wound secretion	5	2 ± 0.184	2 ± 0.202	2 ± 0.142
	10	2 ± 0.142	2 ± 0.184	3 ± 0.000***
	15	2 ± 0.000	3 ± 0.142*	3 ± 0.143*
Secretion type	5	1 ± 0.000	2 ± 0.202	3 ± 0.184***
	10	3 ± 0.000	4 ± 0.421	4 ± 0.143*
	15	3 ± 0.000	4 ± 0.000*	4 ± 0.184*
Wound color	5	1 ± 0.142	2 ± 0.184	3 ± 0.143***
	10	2 ± 0.000	3 ± 0.185*	3 ± 0.000*
	15	2 ± 0.000	2 ± 0.202	4 ± 0.184***
Dermal stiffness	10	1 ± 0.142	1 ± 0.202	1 ± 0.184
	20	2 ± 0.000	2 ± 0.202	3 ± 0.000*
	30	3 ± 0.000	3 ± 0.202	4 ± 0.000*

[a](I) Control group, (II) positive control, and (III) treated with eucerin, silver sulfadiazine 3%, and HMA ointment 5%, respectively. The data were analyzed with nonparametric Kruskal-Wallis followed by the Mann-Whitney test. Data were expressed as the median ± standard error of mean. Significant difference with control group (*) and positive control group (**); $P < .05$; n = 7. Wound secretion: *high* = 1, *low* = 2, *moderate* = 3, *none* = 4. Wound color: *dark gray* = 1, *creamy* = 2, *reddish* = 3, *bright red* = 4. Secretion type: *purulent* = 1, *sanguineous* = 2, *serous* = 3, *none* = 4. Dermal stiffness: *none* = 1, *stiff* = 2, *moderate* = 3, *soft* = 4.

Morphometric Evaluations

The thickness of the epidermis, fibroblast and blood vessel counts, and wound closure were increased in the treated group on the 14th and 28th days of the study (Table 2). Also, the wound area on the 7th, 14th, 21st, and 28th days was decreased (Table 2 and Figures 2 and 3).

Discussion

Global epidemiological studies have shown that burns are one of the main causes of mortality in children and one of the most common problems in health organizations.[27-29] Burns cause damage to the tissues through destabilizing the cell membrane, protein coagulation, depletion of energy resources, and cellular hypoxia, which finally leads to tissue damage and necrosis. Moreover, burns pose severe threats to other parts of the body when exposed to infectious agents, antigen challenges, and trauma.[30] Wound healing is an essential biological response to the regeneration of damaged connective and epithelial tissues. In the present study, the *Aloe vera* gel, as a constituent of HMA ointment, has been

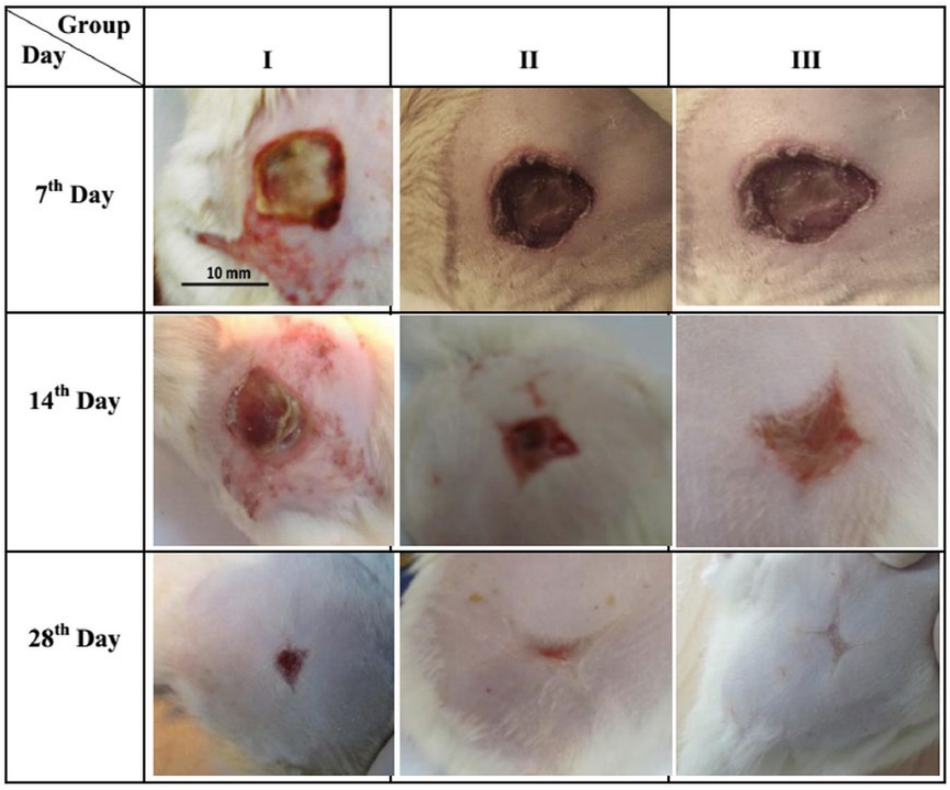

Figure 1. Skin photographs of second-degree burns treated with *Aloe vera*, honey, and milk (HMA) ointment in rats. (I) control group, (II) positive control, and (III) treated with eucerin, silver sulfadiazine 3%, and HMA ointment 5%, respectively.

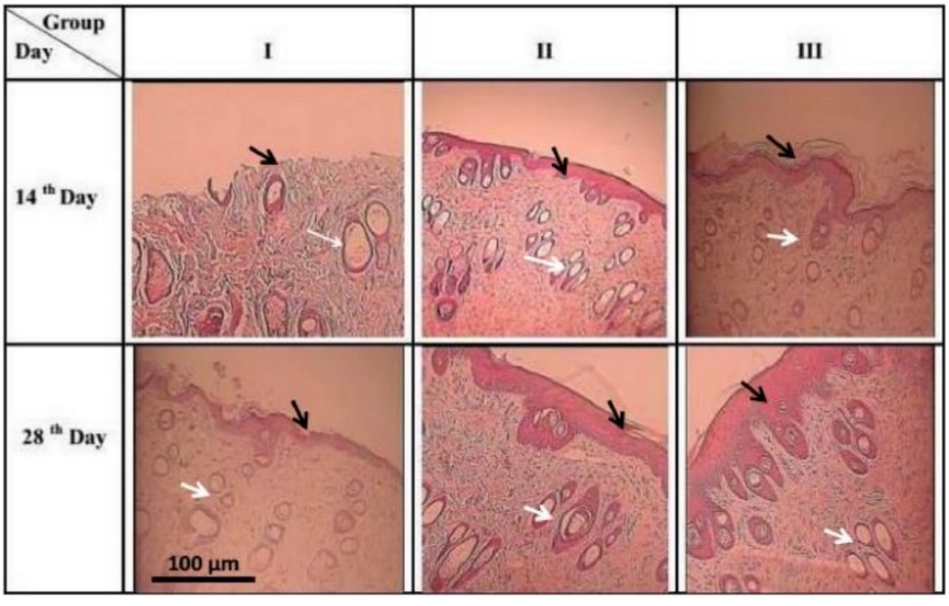

Figure 2. Skin photomicrographs of second-degree burns treated with Aloe vera, honey, and milk (HMA) ointment in rats: (I) control group, (II) positive control, and (III) treated with eucerin, silver sulfadiazine 3%, and HMA ointment 5%, respectively. Epithelium and hair follicles are shown by black and white arrows, respectively. (hematoxylin and eosin staining; ×100).

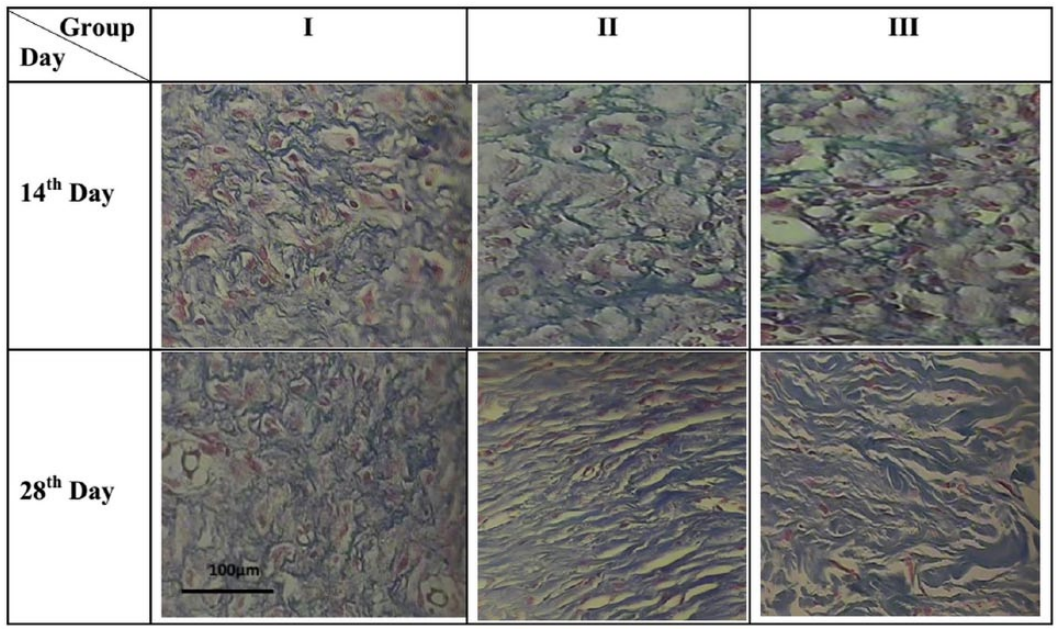

Figure 3. Light micrographs of rat dermal skin treated with *Aloe vera*, honey, and milk (HMA) ointment: (I) control group, (II) positive control, and (III) treated with eucerin, silver sulfadiazine 3%, and HMA ointment 5%, respectively (Masson's trichrome staining, ×100).

Table 2. Morphometric Analysis of Anti-inflammatory and Wound Healing Activities of *Aloe vera*, Honey, and Milk Ointment on Second-Degree Burns in Rats.[a]

		Group		
Morphometric Parameters	Day	I	II	III
Vessel density[b]	1	7.17 ± 1.23	8.17 ± 1.35	7.33 ± 2.43
	14	5.32 ± 3.57	11.32 ± 2.65*	15.12 ± 1.66***
	28	8.45 ± 6.15	9.33 ± 2.48	10.11 ± 3.24
Fibroblast density[b]	1	19.34 ± 2.34	20.15 ± 1.12	21.23 ± 4.15
	14	21.34 ± 2.05	32.12 ± 3.25*	38.10 ± 3.13***
	28	21.23 ± 1.46	19.11 ± 2.12	20.22 ± 1.46
Percentage wound area	7	88.12 ± 5.57	83.23 ± 3.21	81.21 ± 1.18
	14	85.41 ± 1.56	56.13 ± 2.34*	51.67 ± 2.46***
	21	66.34 ± 1.67	33.34 ± 2.11*	31.63 ± 2.24*
Epithelial thickness (µm)	1	145.11 ± 5.25	149.41 ± 1.45	150.56 ± 0.21
	14	No epithelium	29.21 ± 1.28*	33.31 ± 3.11***
	28	155.63 ± 2.49	158.46 ± 2.44	159.32 ± 0.23

[a](I) Control group, (II) positive control, and (III) treated with eucerin, silver sulfadiazine 3%, and HMA ointment 5%, respectively. The data were analyzed with 1-way ANOVA followed by the Duncan test. Values are expressed as the mean ± SD. Significant difference compared with the control group (*) and positive control group (**); $P < .05$; n = 7.
[b]Fibroblast or vessel count per 1 mm^2 area at 40× magnification.

reported to contain amino acids, tannins, lipids, steroids, enzymes, flavonoids, anthraquinones, phlorotannins, carbohydrates, alkaloids, terpenes, and saponins.[31] The presence of these compounds in *Aloe vera* makes it an important plant in traditional and new medicine, especially because of its wound healing properties. On the basis of morphological photographs and histopathological micrographs, our results showed that epithelial thickness and dermis connective tissue were better healed in rats that were treated with HMA ointment. In addition, it was observed that the wound area was significantly reduced and became reddish in color; this was in line with the study by Emsen.[32] According to our

findings, the wound appearance in terms of the type of secretion was serosanguinous, and the inflammation was decreased. Moreover, in agreement with the previous studies, it was found that the wound color in the group treated with HMA ointment was bright red, whereas in the control group, it was dark-creamy,[33-36] indicating that the honey in HMA ointment may induce the anti-inflammatory activity and promote growth and repair of the tissue. It was reported that a mixture of milk and honey has a healing effect on burns, with low scar formation,[32] edema absorption by dehydration, granulation and closing of the wound edges, and increase in catalase activity.[37] These are in agreement with our results, which showed that in the group treated with HMA ointment, the collagen fibers were reduced and had a soft consistency, and no scar tissue was found. Also, HMA ointment caused an increase in the blood vessel counts and blood supply in the treated group. This can be explained by the presence of saponins in honey, which promotes the speed of wound healing by increasing angiogenesis and blood vessel counts.[38] In addition, our finding showed that the epithelial thickness and fibroblast counts in animals treated with HMA ointment were increased significantly, leading to the increase in connective tissue regeneration and acceleration in wound healing. This is in agreement with reports showing the healing mechanism of honey on burns—namely, by differentiation and proliferation of monocytes and fibroblasts.[12,39-41] The wound closure effects of the honey and milk mixture[42]; the presence of glycosides, tannins, and vitamins in *Aloe vera*[33,43]; and the re-epithelializing[35] and tissue debriding effects of honey[42,44-46] all play important roles in the re-epithelialization and proliferation of epidermal cells, which finally increased the wound healing in the group treated with HMA ointment. Thus, the HMA ointment, which contains all the above-mentioned bioactive ingredients, effectively healed the burn wounds, as indicated. The present results indicate that HMA ointment healed the wounds, as measured by the following parameters: proliferation and re-epithelialization of epidermal cells, increase in epithelial thickness, and fibroblast counts.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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