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The efficacy of a traditional medicine preparation on second-degree burn wounds in rats

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ABSTRACT

Ethnopharmacological relevance: Lime Salve (L.S) has been well documented from the 9th to the 19th century AD by traditional Iranian medicine (TIM) as an effective remedy for burn healing.
Aim of the study: The present study was undertaken to evaluate the healing effect and related underlying mechanisms of Lime Salve in a model of deep second-degree thermal burn in male Wistar rats.
Materials and method: L.S was made up of a combination of refined calcium hydroxide powder, beeswax and sesame oil and its quality control was assessed. A deep second-degree burn was created by a hot plate in 48 male Wistar rats. Afterwards, they were randomly divided into four groups including normal saline (C group), L.S (T group), basement of formulation composed of beeswax and sesame oil (B group) and silver sulfadiazine (S group). On days 5, 10, 17 and 24, the wounds were digitally photographed by a camera and after sacrifice of the rats, skin samples were obtained for performing qRT-PCR, immunohistochemistry staining and histological examination.
Results: L.S prominently augmented the wound closure rate, neovascularization on day 10 and collagen formation on days 17 and 24 in comparison with the C group. Furthermore, the Salve-exposed specimens showed a significant higher epithelialization during the experiment with a peak on day 24. qRT-PCR also showed that on day 10, VEGF and TGF-β1 genes were significantly higher in the T group as compared with the C group. Also, MMP-9 and MMP-2 genes had a significant peak of expression on day 17 and rapid reduction of expression on day 24. Expression levels of IL-6 and TNF-α genes peaked on day 10 in the T group, followed by a progressive reduction until the end of the examination.
Conclusion: L.S could effectively accelerate the healing process of deep second-degree burn wounds and therefore, it may be recommended as a promising topical medication for treating burn wounds in the future clinical trials.

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1. Introduction

The skin is the largest organ in the body with a wide range of functions and the ability to heal itself (Balañá et al., 2015). However, this ability fails to be perfect in deep and extensive burns and in people with specific health problems, e.g. diabetic and immune-compromised patients; hence, therapeutic intervention is required (Mehrabani et al., 2015; Pereira and Bartolo, 2016; Tatara et al., 2018). Burn treatments can be either systemic or topical depending on the degree of the burn (Yibrah et al., 2011). Generally, the topical burn treatments can be classified into two categories, namely common and modern. The most widely known local treatment for burn wounds is the use of silver compounds (Aziz et al., 2012). The efficacy and safety of these compounds, especially in severe and extensive burns, are unclear (Aziz et al., 2012; Rosanova et al., 2012). Modern burn treatments include new dressings, bioengineered skin substitutes, tissue scaffolds, stem cells, healing promotive factors, and gene therapy (Augustine et al., 2014; Oryan et al., 2017). These methods are frequently costly and difficult to access, particularly in developing countries (Pereira and Bartolo, 2016) Accordingly, further research is required to identify more appropriate medicines in terms of efficacy, price, and availability for optimal burn treatment.

Medicines traditionally used by different communities are considered one of the most useful sources for research (Mehrabani et al., 2016; Qi, 2013). Traditional Iranian Medicine (TIM) comprises a great extent of inscribed experimental approaches to burn wounds that have been screened for more effective ones over the centuries (Sarhadynejad et al., 2016). In the written sources of TIM, there are multiple topical medications that have been proved in terms of efficacy over consecutive centuries (Choopani et al., 2017; Oloumi et al., 2011; Rezaeizadeh et al., 2009). Topical drugs used in TIM for burn treatment include plant, animal, and mineral products (Aliasl and Khoshzaban, 2013). Lime salve (L.S), which is a mixture of hydrated lime (calcium hydroxide powder), sesame oil (*Sesamum indicum* L.), and beeswax, is one of the topical medications that is highly recommended to treat burn wounds due to its noticeable efficacy (Aghili, 2009; Akhavini, 2007; Chashty, 2004; Jorjani, 2012). This salve has been mentioned in the books of TIM from 4th to 14th century AH (9th to the 19th century AD) and it has been suggested to be applied immediately after the burn and preferably before blistering (Avicenna, 2010; Razi, 2005b). There is also some evidence on the application of the lime compounds in complementary and alternative medicine. In a study that reviewed various methods for burn wound treatments in traditional Chinese medicine, the applied lime component was reported to decrease pain and accelerate lesion healing (Kopp et al., 2003).

Calcium hydroxide (CH), a low water soluble substance, is mostly used in medicine as the gold standard for direct pulp capping (Zhu et al., 2015). There is strong evidence for the antibacterial effect of CH (Garima et al., 2018; Mohammadi et al., 2012). Additionally, CH can release some of the growth factors involved in pulp repair (Chen et al., 2016). The present study sought to investigate the effect of L.S (prepared according to TIM textbooks) and some of its underlying mechanisms on the burn wound healing in an animal model. Even though the rat skin structure is not totally similar to human skin, a broad range of factors such as availability, affordability, and relative anatomical and functional similarities with human skin, have led us to select rat as the animal model for the present experiment (Abdullahi et al., 2014).

2. Materials and methods

2.1. Preparation and quality controls assessment of L.S and basement of formulation

L.S was made according to the principles of TIM Pharmacy (Chashty, 2004; Jorjani, 2012; Razi, 2005a) as follows: calcium oxide (Sigma-Aldrich, USA) was added to a certain amount of water to be converted to CH. After 24 h, the settled powder was filtrated, dried at room temperature and sieved (80 mesh). Twenty grams of beeswax (Sigma-Aldrich, USA) and 60 g of sesame oil (Manilla®, Adonis Gol Darou Co, Iran) were heated up to 60 °C until the wax completely melted. Then, CH (40 g) was added and the materials were mixed until reaching room temperature (Table 1). The basement of formulation was prepared in the same way without CH. The microbial challenge tests were done based on World Health Organization's protocol for herbal topical formulation.

Table 1
The Lime Salve components applied to the wound surface and their calculated ratios.

Material	Lime Salve	
	Amount (gr)	Ratio (%)
$Ca(OH)_2$	40	33
Sesame oil	60	50
Beeswax	20	17
Total	120	100

2.2. FTIR fingerprint

The samples (A; Lime Salve, B; Basement of formulation composed of beeswax and sesame oil, C; Sesame oil and D; Beeswax) were prepared by mixing 2 mg of samples with 200 mg of dried potassium bromide followed by pressing under pressure 15 MPa for 3 min to make a disk pellet. The samples were then subjected to mid-IR measurements, and the spectral range (4000–400 cm − 1) was recorded using a Bruker ALPHA-II instrument (Bruker Optics GmbH, Ettlingen, Germany) with a resolution of 4 cm − 1 and 64 scans per sample. The results were then analyzed using OPUS 7.0 data processing software.

2.3. Experimental animals

The study was accepted by the Ethical Committee of Kerman University of Medical Sciences [IR.KMU.REC.1395.879] and was performed in accordance with the guideline of the National Institutes of Health Principles of Laboratory Animal Care (NIH publication no. 85–23, revised 1985). 48 Male Wistar rats aged 8–11 weeks, weighing 200–250 g were purchased from the animal facility of Neuroscience Research Center, Kerman University of Medical Sciences. They were maintained in standard conditions: individual plastic cages, temperature 20–26 °C and humidity of 40–70%, with regular light cycles of 12/12 h light/dark.

2.4. Wound induction

The rats were anesthetized using ketamine/xylazine (100/10 mg/kg, intraperitoneally) (Cai et al., 2014). The dorsal portion of the body was shaved using an electrical clipper, and 70% alcohol was used to disinfect the dorsal area. A deep second-degree burn was created by an iron metal cube with dimensions of 2.5cm × 2.5cm × 0.3 cm. It was heated over the flame for 30 s and pressed gently (only 200 g weight of metal cube and the handle without any additional pressure) on the dorsal region of animals for 7 s (Fatemi et al., 2014; Pannerselvam et al., 2017). The degree of burn and the uniformity of wounds were confirmed by a pathologist in a pilot study.

2.5. Groups and treatment protocol

Forty-eight burned animals were randomly divided into four groups of twelve rats. The groups were topically treated by normal saline (C group), L.S (T group), basement of formulation composed of beeswax and sesame oil (B group) and silver sulfadiazine cream 1% (Iran Najo

Pharmaceutical Company, Tehran, Iran; S group). After cleaning the burn wounds with normal saline, about 2 g (8 g/kg) of materials were applied to completely cover the wound surface of each animal. Immediately after burn induction, treatment was performed twice daily until the animals were sacrificed.

2.6. Wound area analysis

Digital photographs of the wounds were captured on days 0, 5, 10, 17 and 24. The wound areas were blindly measured using NIH Image J software. Then, wound closure rates were calculated, using the following formula (Mehrabani et al., 2015):

$$\text{Wound closure rate (\%)} = \frac{\text{Wound area on day 0} - \text{Wound area on day x}}{\text{Wound area on day 0}} \times 100$$

X = days 5, 10, 17, 24.

2.7. Assessment of the physical appearance of the wounds

The macroscopic appearance of the wounds was blindly evaluated by an independent researcher based on the described criteria (Table 2).

2.8. Histological and immunohistochemistry examination

The appropriate time for sampling was selected based on a pilot study under the supervision of a pathologist. Three animals of each group were sacrificed on days 5,10,17 and 24, using an overdose of ketamine/xylazine; after that, skin samples, including the cross sectional full thickness specimens, plus 5 mm of the surrounding skin were collected for histological examination. Skin specimens were fixed in 10% neutral buffered formalin and embedded in paraffin wax blocks. Afterwards, 3 μm sections were stained with hematoxylin and eosin (H &E) and Masson's trichrome. Also, sections were immune-stained for cluster of differentiation 34 (CD34) in order to verify neovascularization. All samples were blindly assessed at 5–6 fields per section by a specialist. Each sample was given a score using the Abramov's histological scoring system (Abramov et al., 2007) for angiogenesis and collagen levels are as summarized in Table 3. As well, epithelialization was evaluated according to the percentage of wound re-epithelialization in each sample.

2.9. Real-time PCR

RNA was extracted according to the recommendations in Bio Basic Kit, Cat. No (BS82312) and stored at -80 ˚C. The Thermo Scientific Revert Aid RT Kit (K1621) (Thermo Fisher Company, Germany–Darmstadt) was used for the synthesis of cDNA from RNA. Real QPlus 2x Master Mix Green with high ROX™ (Ampliqon Company, Denmark) and the primers of VEGF (vascular endothelial growth factor), TGF-β1 (tumor growth factor- β1), MMP-2 (matrix metalloproteinase-2), IL-6 (interlukin-6), TNF- α (tumor necrotizing factor- α), MMP-9 (matrix metalloproteinase-9) genes and GAPDH gene (housekeeping gene) were used for performing real-time PCR. The sequences of rat primers were noted in Table 4. A sample without cDNA was subjected to an identical protocol as a negative control. Expression of the target gene was normalized with GAPDH (housekeeping gene). Fold change or a fold-difference of expression levels in T/B/S groups *versus* C group was assessed based on $2^{-\Delta\Delta CT}$ formula.

Table 2
Macroscopic properties of burn wound.

Swelling of surface	without swelling, mild, moderate, severe
Exudate	dry, mild, moderate, large exudate
Scab	without scab, small scabs, large scabs, scab with cracked surface, bloody, creamy, black, thick, thin
Bed color	red, bruise, black, creamy, gray

Table 3
Histological scoring based on Abramov's histological scoring system.

Scores	Collagen level	Angiogenesis (per HPF)
0	None	None
1	Scant	1–5
2	Moderate	6–10
3	Abundant	≥10

Table 4
Rat primers for qRT-PCR.

Gene	Sense strand	Antisense strand
VEGF	GTGTGTGTGTGTATGAAATCTGTG	GCAGAGCTGAGTGTTAGCAA
TGF $-\beta_1$	TGCTAATGGTGGACCGCAA	CACTGCTTCCCGAATGTCTGA
MMP-2	CCCTCCCCTGATGCTGATACT	GTCACTGTCCGCCAAATAAACC
MMP-9	CCACTAAAGGTCGCTCGGAT	GAGTTGCCCCCAGTTACAGT
IL-6	GTCAACTCCATCTGCCCTTC	TGTGGGTGGTATCCTCTGTG
TNF-α	AGTCCGGGCAGGTCTACTTT	TGAGCCACAATTCCCTTTCT
GAPDH	ACA GTC CAT GCC ATC ACT	GCC TGC TTC ACC ACC TTC

2.10. Statistical analysis

Statistical significance of the mean differences between groups was analyzed by one-way ANOVA with Tukey's post-test. Statistically, differences were assumed significant at $P < 0.05$. Results are presented as mean and standard error of the mean (SEM).

3. Results

3.1. Physical properties of L.S

Proper formulation of L.S with adequate consistency and spread ability was formed from a composition of CH, beeswax and sesame oil. In the examination of the microbial challenge test, no contamination was observed in the L.S.

3.2. FTIR fingerprint

The FT-IR spectrums were used to identify the functional groups of the active components present in formulations on the peaks values in the region of IR radiation. When the samples were passed into the FT-IR, the groups of the components were separated based on its peaks ratio. The results of FT-IR analysis confirmed the presence of O–H, C═C, C–H, C–O and C═C functional groups (Fig. 1 and Table 5). FTIR spectroscopy is proved to be a reliable and sensitive method for detection of bio molecular composition.

3.3. The physical appearance of the wounds

The wounds in the T group on 5th and 10th days had a mild to moderate swelling with mild exudate and small cracked gray scabs on a bright bruise bed surface. On day 17, the T group showed a dry wound surface with partially separated scabs. However, the scabs of the B group were oily and soft and the wound surface was more bruised as compared with the T group. During all days of the experiment, the wounds of the S group showed more exudate and red swelled surfaces with thin bloody scabs. Moreover, during the experiment, the wounds in the C group were covered by a thick necrotic scab with a high amount of exudate accumulated below the scab.

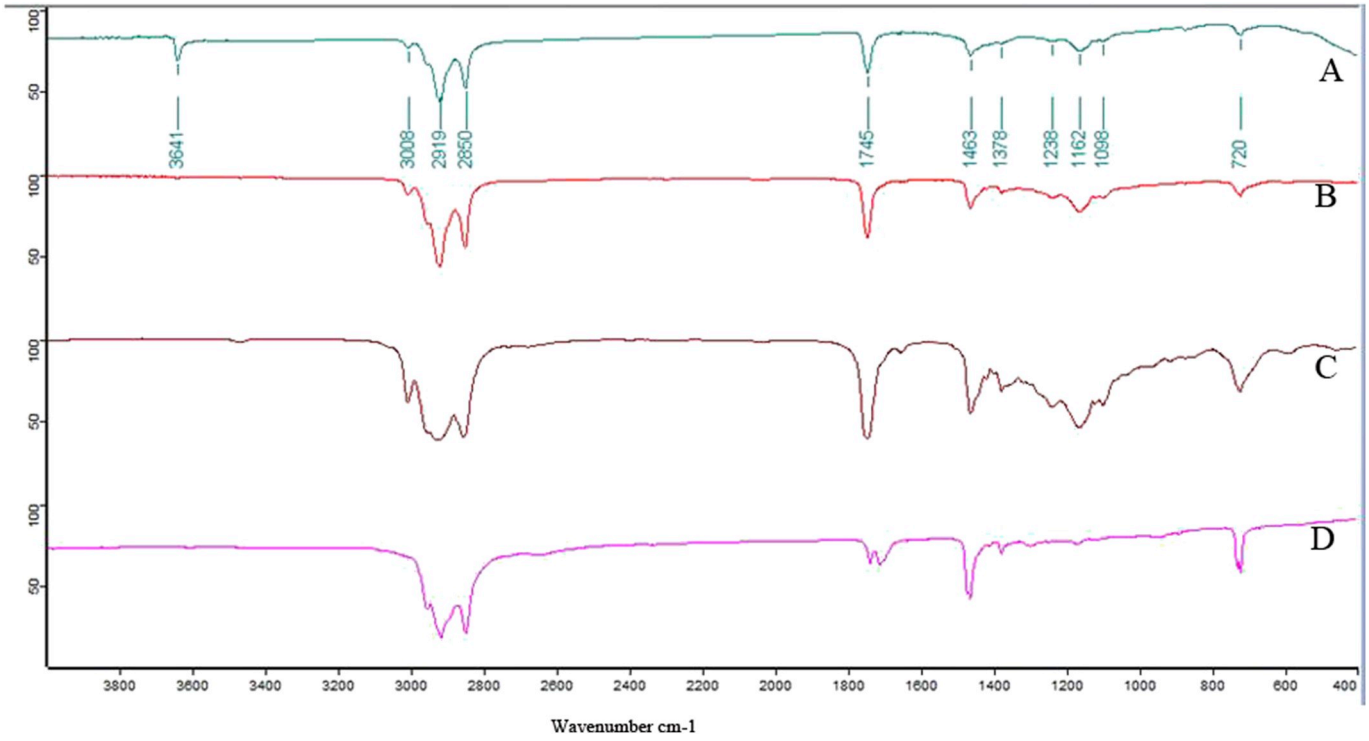

Fig. 1. FTIR analysis of A to D samples (A; Lime Salve, B; Basement of formulation composed of beeswax and sesame oil, C; Sesame oil and D; Beeswax).

Table 5
FTIR peak values of formula.

Peak values (cm $^{-1}$)	Functional groups
3641	O–H (Ca $(OH)_2$)
2850, 1238	C=O (ester, acid)
3008, 2919, 720	C–H
1745, 1162, 1098	C–O (ester)

3.4. The effect of L.S on wound closure

On the first day, the mean wound area was 7.036 ± 0.13 cm^2. Analysis showed that there was no remarkable difference among the four groups regarding the primary wound area. The wound area increased in all groups on day 5 compared to day 0. On other days, a reduction was observed in the wound size of all groups. On days 10, 17, and 24, the T group had higher wound closure rates compared with other groups. Significantly, on day 10, the closure rate of only the T group was higher than the C group ($P < 0.05$). On day 17, the T group showed a significant difference with the C ($P < 0.001$) and S ($P < 0.01$) groups. On day 24, the T group had significantly higher rates of closure compared to the other groups (Fig. 2).

3.5. Histological findings

On day 10, the T group showed a remarkably higher angiogenesis score in comparison with the C group ($P < 0.05$). However, regarding the histological score, there were no obvious differences between the T and B groups. On day 17, the T group showed a maximum angiogenesis score. However, no distinct difference was observed between the groups. On day 24, the T group had a significantly lower angiogenic score as compared with the C/B groups ($P < 0.05$) (Fig. 3). In the histological examination, re-epithelialization was observed on day 5 at the margin of the wound. On days 10 and 17, the T group showed a significant increase in epithelialization as compared with the C group ($P < 0.05$). On day 24, in the T group, re-epithelialization was

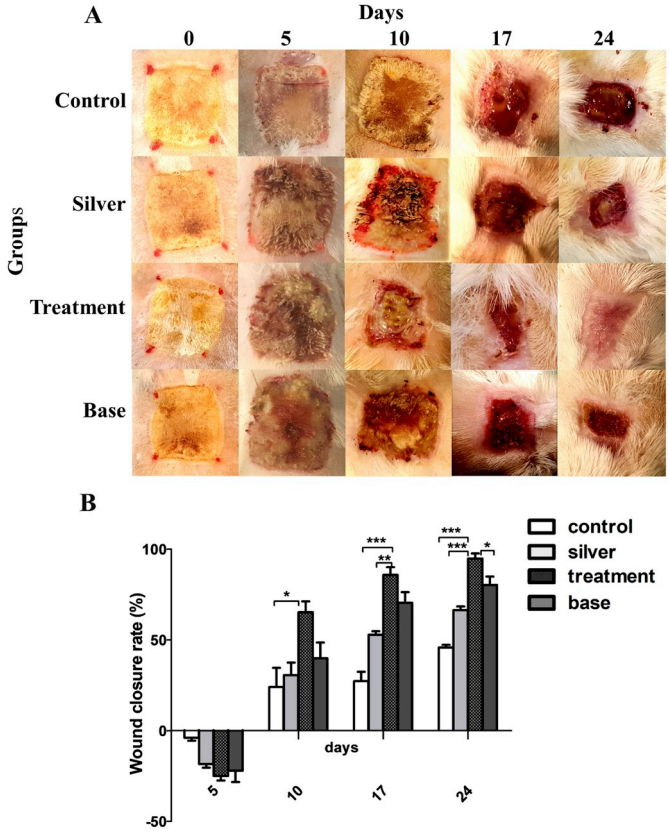

Fig. 2. Gross appearance of burn wounds following treatment with L.S (A): Representative images of wounds in groups on days 0,5,10,17 and 24 revealing faster wound closure in L.S group. (B): Plot of wound closure rate in groups on different days. Data are expressed as means ± standard error of the mean (n = 3). *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$ vs other group(s) on the same day.

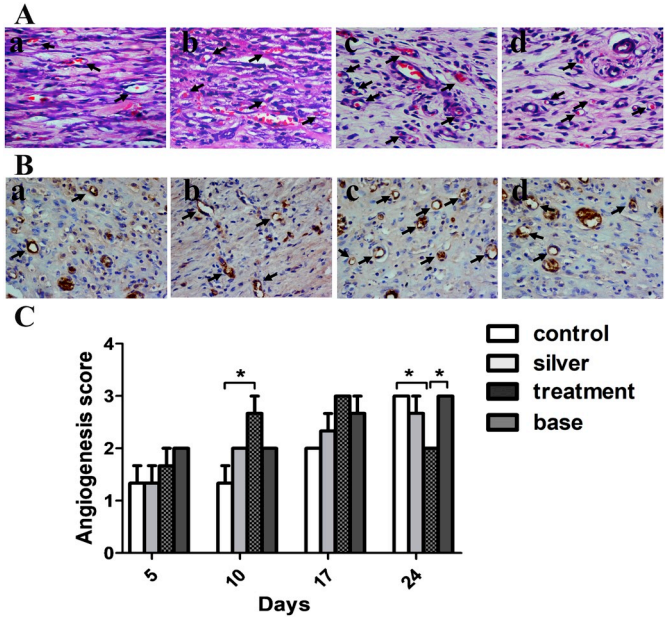

Fig. 3. Effect of L.S on angiogenesis in burn wound healing of rat. (A): Representative images of histological view of hematoxylin and eosin (H&E) stained sections (40 × magnification) of burn wounds following treatment with L.S (c) compared with the base of L.S (d), silver sulfadiazine (b) and control (a) on day 10, focused on angiogenesis. (B): Representative images of immunohistochemical cluster of differentiation 34 (CD34) stained sections (40 × magnification) of different groups on day 10. Arrows denotes the newly forming micro vessels. (C): Plot of angiogenic scores in groups on days 5,10,17 and 24, using Abramov's histological scoring system. Scoring Data are expressed as means ± standard error of the mean (n = 3). *P < 0.05, **P < 0.01 and ***P < 0.001 vs other group(s) on the same day.

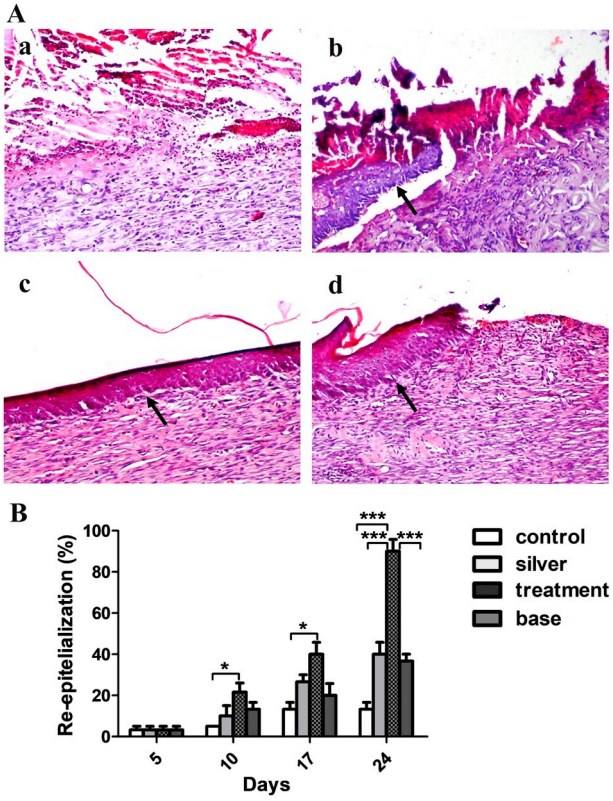

Fig. 4. Effect of L.S on epithelialization in burn wound healing of rat. (A): Representative images of histological view of hematoxylin and eosin (H&E) stained sections of burn wound on day 24 focused on the healing margin (40 × magnification) following treatment with L.S (c): complete re-epithelialization, severe and regular collagen deposition, silver sulfadiazine (b): incomplete and thin re-epithelialization, mild to moderate collagen deposition, the base of L.S (d): incomplete re-epithelialization, moderate collagen deposition, and control (a): incomplete re-epithelialization, mild collagen formation. Markers denote newly formed epithelial layer. (B): Plot of percentage of re-epithelialization in groups on days 5,10,17 and 24. Scoring data are expressed as means ± standard error of the mean (n = 3). *P < 0.05, **P < 0.01 and ***P < 0.001 vs other group(s) on the same day.

significantly increased compared to the C/S (P < 0.001) and B (P < 0.001) groups (Fig. 4). In a histological view, collagen in the T group was more distributed than the other groups. The one-way analysis showed that the T group, on day 17, had higher collagen formation scores compared with the C group (P < 0.05) (Fig. 5).

3.6. Effect of L.S on mRNA expression of VEGF, TGF-β, MMP-2, MMP-9, IL-6 and TNF-α

Quantitative real-time PCR was performed on all groups, where mRNA expression levels of VEGF in the T group (2.405 ± 0.148 fold) were significantly higher as compared with the C (1.304 ± 0.209 fold), S (1.452 ± 0.146 fold) and B (1.535 ± 0.243 fold) groups on day 10 (P < 0.05) (Fig. 6A). On day 10, the relative expression of TGF-β in the T group (23.870 ± 1.538 fold) was significantly higher than the C (3.376 ± 2.268 fold), S (0.361 ± 0.043 fold) (P < 0.001), and B (15.23 ± 0.528 fold) (P < 0.05) groups (Fig. 6B). The relative mRNA expression of MMP-2 was significantly up regulated in the T group (3.205 ± 0.657 fold) in comparison with the C group (1.000 ± 0.035 fold) on day 5 (P < 0.05). The results showed a significantly higher expression of this gene on day 10 in the T group (26.020 ± 1.325 fold) as compared with the C (2.934 ± 0.384 fold) (P < 0.01), S (4.944 ± 0.424 fold) (P < 0.05) and B (10.340 ± 1.067 fold) (P < 0.05) groups. On day 17, mRNA expression of MMP-2 was significantly higher in the T group (51.98 ± 1.801 fold) compared with the C group (3.762 ± 0.415 fold) (P < 0.001). (Fig. 6C). On days 10 and 17, the relative mRNA expression of MMP-9 in the T group (5.203 ± 0.553 fold on day 10, 7.676 ± 1.148 fold on day 17) was significantly higher in comparison with the C (1.967 ± 0.232 fold on day 10; P < 0.01, 0.837 ± 0.056 fold on day 17; P < 0.001), S (2.514 ± 0.388 fold on day 10; P < 0.01, 1.802 ± 0.451 fold on day 17; P < 0.01) and B (2.742 ± 0.170 fold on day 10; P < 0.01, 3.614 ± 0.778 fold on day 17; P < 0.05) groups (Fig. 6D). The relative mRNA expression of IL-6 on day 5 in the T group (0.303 ± 0.036 fold) was significantly lower as compared with the C group (1.000 ± 0.138 fold) (P < 0.05). The results indicated a significant increase in the expression of this gene on day 10 in the T group (1.740 ± 0.340 fold) compared with the C (0.129 ± 0.013 fold) (P < 0.001) and S (0.267 ± 0.017 fold) (P < 0.05) groups; and on day 17 in the T group (1.386 ± 0.015 fold) compared with the C (0.296 ± 0.040 fold) (P < 0.001) and S (0.125 ± 0.055 fold) (P < 0.001) groups (Fig. 6E). On day 10, the results showed that TNF-α gene expression in the T group (2.306 ± 0.176 fold) was significantly higher as compared with the C (1.314 ± 0.269 fold) and B (1.586 ± 0.063 fold) groups (P < 0.05). On day 24, the expression in the T group (0.592 ± 0.052 fold) was significantly lower in comparison with the C group (2.074 ± 0.046 fold) (P < 0.001) (Fig. 6F).

4. Discussion

The purpose of this study was to investigate the burn wound healing effect of L.S. on an animal model. We prepared this combination based on the protocols described in traditional Iranian sources (a combination of processed CH powder, sesame oil and beeswax) (Razi, 2005a). The healing effects of sesame oil (Kiran and Asad, 2008; Shenoy et al., 2011) and beeswax (Fu et al., 2007) on wound have been reported in some

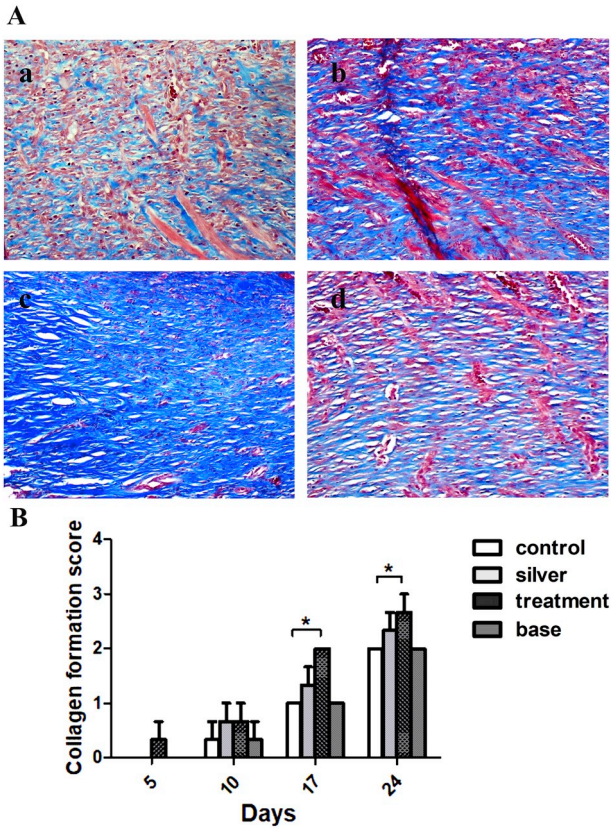

Fig. 5. Assessment of collagen formation in burn wound of rat after treatment with L.S. (A): Representative images of histological view of Masson's trichrome stained sections (40 × magnification) of burn wound following treatment with L.S (c) compared with the base of L.S (d), silver sulfadiazine (b) and control (a) on day 17. (B): Plot of collagen formation scores in groups on days 5,10,17 and 24, using Abramov's histological scoring system. Scoring data are expressed as means ± standard error of the mean (n = 3). *P < 0.05, **P < 0.01 and ***P < 0.001 vs other group(s) on the same day.

previous studies.

The positive effects of L.S on the healing processes reported in the current study are consistent with a large number of studies on CH (as the main component of L.S) in the field of dentistry (Chen et al., 2016; Pannu and Berwal, 2017; Sathorn et al., 2007). Due to its antibacterial and anti-inflammatory properties, CH is used as the first line of treatment for intracanal medicaments (Prabhakar et al., 2013). CH, in contact with dental tissue, creates an alkaline environment containing calcium ions, which, in addition to prolonged and continuous anti-microbial effects, causes calcification and induces mineralization (Fava and Saunders, 1999; Pannu and Berwal, 2017). Combining CH with an oily substance improves its physical properties and, because of its low solubility in oil, reduces the diffusion of hydroxyl ions in the tissues (Fava and Saunders, 1999). It can be assumed that L.S, in contact with a wounded surface, slowly releases ions of calcium. Calcium ion concentration plays a key role in both intracellular and extracellular matrix (ECM) during wound healing processes (Chen et al., 2016; Huang et al., 1999; Lansdown, 2002; Oda et al., 2016). Extracellular calcium modulates proliferation and maturation of keratinocytes and fibroblasts and also epidermal lipid barrier formation through signal transduction and gene expression (Lansdown, 2002). Ca^{2+} environment is a major regulator of the expression of markers related to epidermal differentiation (Yuspa et al., 1989). Wounding triggers a rapid and sustained increase in epidermal calcium ions (Xu and Chisholm, 2011). Calcium demand is high during haemostasis, keratinocyte proliferation, and maturation (Menon et al., 1985). Calcium alginate is the most commonly used calcium-containing compound, especially used in the treatment of deep wounds or those with excessive exudates (Boateng et al., 2008). The calcium ion existing in this compound substitutes the sodium ion in the wound exudate, affecting cell proliferation (Barnett and Varley, 1987; Limová, 2003). However, calcium alginate is more appropriate for chronic wounds such as venous ulcers and wounds with moderate to high exudation (Abdelrahman and Newton, 2011; Kawai et al., 2011). As a conclusion, the calcium ions released from L.S are responsible for some of the effects of this compound on burn wound healing.

In the current study wound closure rate and epithelialization were measured in the all groups. Surprisingly, the wound size increased in all groups on 5th day. In agreement, some previous studies reported the same observation (Lee et al., 2015; Nasiri et al., 2015; Otsuka et al., 2015; Wall et al., 2002). It may be related to the activity of cytokines and tissue degradation processes triggered by hypo-perfusion, microthrombosis and autophagy that increase the ulcer margins (Salibian et al., 2016; Singh et al., 2007; Tan et al., 2013). Moreover, burn wounds may not immediately reflect the extent of the burn after being created. However, the wound surface of C group was significantly lower compared with the other groups on day 5. Probably, the effect of treatment might accelerate the healing process, especially in T group. The other finding was significant positive effect of L.S on wound closure rate and epithelialization when it was compared with control on days 10, 17 and 24. These effects of L.S may be related to an alkaline environment created by CH in the wound bed. Altered pH ranges are necessary for different phases of wound healing (Schneider et al., 2007). During the healing process, both acute and chronic wounds move to a neutral and then acidic state (Gethin, 2007). Proliferation and migration of keratinocytes occur at pH higher than the physiological pH (Sharpe et al., 2009). Alkaline milieu improves graft survival (Sayegh et al., 1988). In opposite to our results, acidic environment may be considered favorable for inducing fibroblast proliferation, promoting epithelialization, controlling bacterial colonization (Power et al., 2017). However, efforts to reduce wound surface pH by using topical agents have had varying degrees of efficacy (Gethin, 2007). Modulating wound pH and alkaline compounds have not been examined as a strategy to improve healing in acute wounds (Sharpe et al., 2009); hence the necessity for further investigation is required.

As showed by the current study, L.S increased the relative gene expression of VEGF factor on day 10, which is consistent with the angiogenic scoring in histological observation. Conclusively, modulation of this factor is one of the possible effects of L.S on wound healing. VEGF is currently recognized as one of the most important angiogenesis mediators, especially in the early stages of wound healing (Johnson and Wilgus, 2014; Mirzamohammadi et al., 2016; Raeiszadeh et al., 2018). Endothelial cell migration and proliferation were promoted by VEGF (Pastar et al., 2014), at least in part, through VEGF-receptor-mediated calcium influx into the endothelial cell (Hamdollah Zadeh et al., 2008).

Furthermore, our results showed the up-regulation of TGF-β expression on day 10 in the presence of L.S. TGF-β plays a role in wound healing processes such as inflammation, angiogenesis, collagen formation and remodeling of extracellular matrix (Kubo et al., 2014; Penn et al., 2012). TGF-β exerts some of its control by the modulation of calcium channels. The rate of Ca^{2+} influx in response to TGF-β is dependent on the concentration of extracellular calcium (Muldoon et al., 1988; Nesti et al., 2007). As a conclusion, L.S can accelerate both TGF-β production and the response of calcium channels to TGF-β by increasing extracellular Ca^{2+}, which is in agreement with more collagen formation detected by current histological assay.

Matrix metalloproteinase enzymes (MMPs) are a group of calcium-dependent zinc-containing enzymes secreted from keratinocytes, fibroblasts and inflammatory cells, playing major roles in the degradation and modulation of ECM (Caley et al., 2015; Martins et al., 2013; Weremijewicz et al., 2018). MMP-2 and MMP-9 act as protease (gelatinase) and signaling factors in the angiogenesis and the migration of keratinocytes and inflammatory cells, particularly in tissue remodeling stages (Martins et al., 2013; Raina et al., 2008; Weremijewicz et al.,

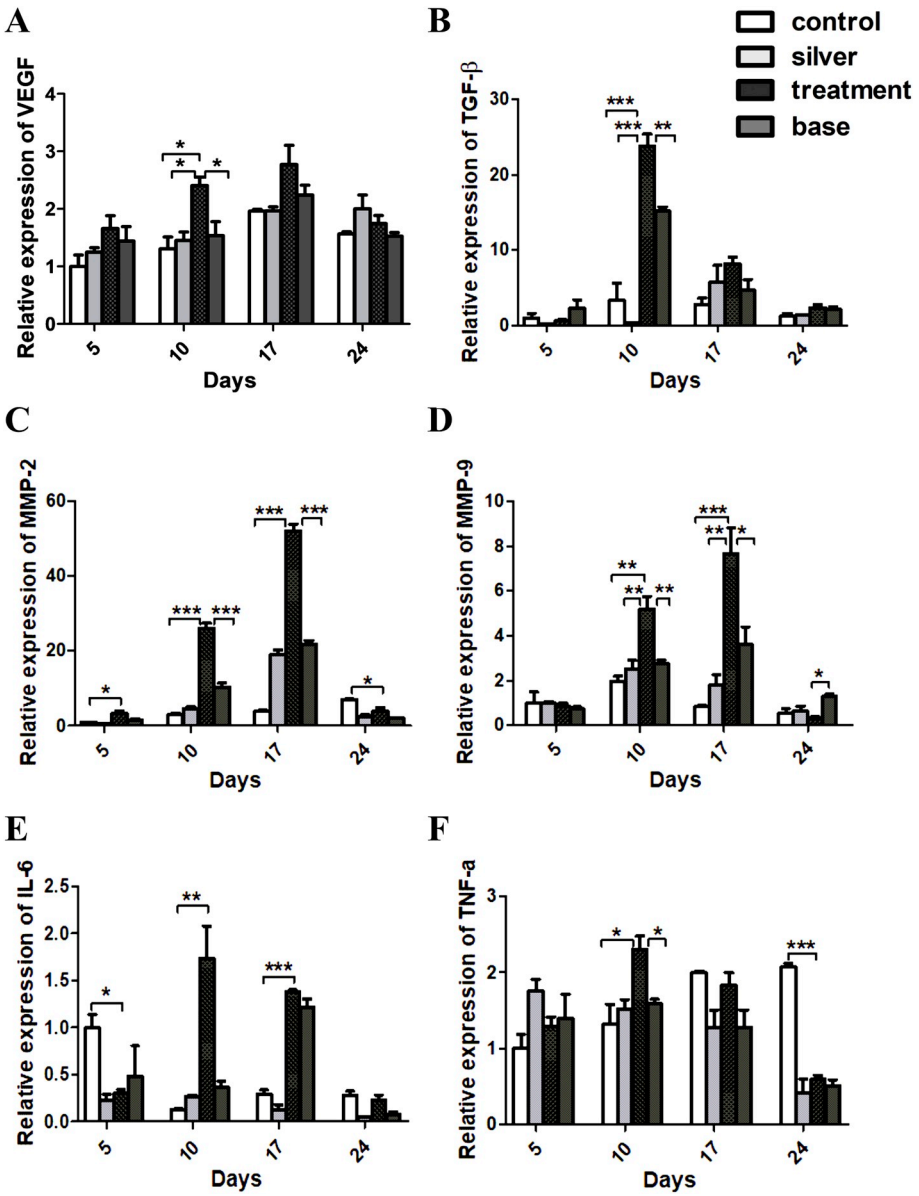

Fig. 6. Effect of L.S on relative gene expression in burn wound healing of rat. Plots of mRNA expression of VEGF (A), TGF-β (B), MMP-2 (C), MMP-9 (D), IL-6 (E) and TNF-α (F) following treatment with L.S compared with the base of L.S, silver sulfadiazine and control on days 5,10,17 and 24. Data are represented as means ± standard error of the mean (n = 3). *P < 0.05, **P < 0.01 and ***P < 0.001 vs other group(s) on the same day.

2018). Based on our findings, in the T group, the activity of MMP-2 and MMP-9 were significantly higher than the other groups on day 10 and 17. Given the role of these enzymes in proliferation and remodeling processes, it can be concluded that L.S influences these two stages of tissue repair by changing the presence of MMP-2 and MMP-9. Elevated levels of MMPs have been reported in chronic ulcers (Caley et al., 2015; Lazaro et al., 2016). In this report, the rapid reduction of MMP-2 and MMP-9 on day 24 in the T group shows the effectiveness of L.S on the prevention of delayed wound healing.

Then, the present study evaluated the expression of two important inflammatory mediators including TNF-α and IL-6. IL-6 is a well-known and important inflammatory mediator generated by a number of cells such as fibroblasts, immune cells, mesenchymal and endothelial cells in response to various stimuli (Akira et al., 1993). IL-6 is a mitogenic factor and has proliferative effects on keratinocytes and acts as a chemoattractant for neutrophils (Pastar et al., 2014). In the T group, a significant up-regulation was seen in IL-6 on 10th and 17th days compared with C group. TNF-α is a pro-inflammatory factor produced by a variety of immune cells (Barrientos et al., 2008; Heo et al., 2011). Impaired wound healing has been reported, which is related to the elevated levels of TNF-α (Ashcroft et al., 2012). L.S induced a significant up-regulation of TNF-α on 10th day and down-regulation on 24th day compared with the control, probably meant that the healing processes in the T group was faster than the C group.

5. Conclusion

We investigated the topical application of L.S, a traditional Iranian preparation, on burn wound healing in an animal model and our results showed that it accelerated some indicators of healing. Although this topical medication for burn wounds has been used for centuries by people, it is recommended that this compound should be evaluated in a clinical trial. Some studies have separately investigated the components of L.S and often reported positive outcomes. It is necessary to determine the synergistic, counteractive or moderating role of the components in wound healing processes.

Author's contributions

Nasser Ebrahimpour: Experimental work and scientific writing, Mehrnaz Mehrabani: Data analysis and manuscript revision, Maryam Iranpour: Pathological assessment, Zeinab Kordestani: Experimental work, Mitra Mehrabani: Study design and preparation of traditional drug, Mohammad Hadi Nematollahi: Experimental work, Ali Asadipour: Study design, Mahboobeh Raeiszadeh: Experimental work, Mehrzad Mehrbani: Study design and manuscript revision.

Declaration of competing interest

The authors confirm that there are no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jep.2020.112570.

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Abbreviations

CD34: cluster of differentiation 34
CH: calcium hydroxide
L.S: Lime Salve
C: control or normal saline treated
S: silver sulfadiazine treated
T: Lime Salve treated
B: base of formulation treated
VEGF: vascular endothelial growth factor
TGF-β1: tumor growth factor- β1
MMP-2: matrix metalloproteinase-2
IL-6: interlukin-6
TNF-α: tumor necrotizing factor- α
MMP-9: matrix metalloproteinase-9
GAPDH: glyceraldehyde-3-phosphate dehydrogenase
H&E: hematoxylin and eosin
qRT-PCR: quantitative reverse transcription - polymerase chain reaction