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Original Article
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Hydroalcoholic Extract of *Scrophularia Striata* Attenuates Hypertrophic Scar, Suppresses Collagen Synthesis, and Stimulates MMP2 and 9 Gene Expression in Rabbit Ear Model

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Objectives: Hypertrophic scars (HSs) are caused by abnormal wound healing. To date, no standard treatment has been made available for HSs. *Scrophularia striata* has been reported to accelerate wound healing and has the potential to prevent HS formation. In this study, we investigated the anti-scarring effects of *S. striata* extract (SSE) in a rabbit ear model of scarring.
Methods: In this study, New Zealand white rabbit (weight: 2.3-2.5 kg) were used. In the prevention phase of the study, three test groups received 5%, 10%, and 15% ointments of SSE in the Eucerin base, the fourth group received Eucerin, and the fifth group received no treatment. The samples were obtained on day 35 after wounding. In the treatment phase of the study, the test groups received an intralesional injection of SSE (5%, 10%, and 15%), the fourth group received an intralesional injection of triamcinolone, the fifth group received a solvent (injection vehicle), and the sixth group received no treatment. To evaluate the anti-scarring effects of SSE, the scar elevation index (SEI), epidermis thickness index (ETI), collagen deposition, and MMP2 and MMP9 gene expression were evaluated.
Results: A significant reduction in SEI, ETI, and collagen deposition was noted in animals treated with SSE compared with the control groups. In addition, topical SSE stimulated MMP2 and MMP9 gene expression.
Conclusion: The findings of this study demonstrate the potential for SSE in the prevention and treatment of HS. SSE could be prepared as an appropriate formulation to treat wounds and prevent abnormal scarring.

Keywords: hypertrophic scar, *scrophularia striata*, scar elevation index, epidermal thickness index, mmp2, mmp9

INTRODUCTION

Hypertrophic scars (HSs) are a common problem during the wound healing process. HSs are caused by abnormal wound healing [1]. They are defined as visible, erythematous, and raised lesions that do not expand into adjacent tissues [2]. HSs are usually limited to the wound site and may improve over time as the underlying cause resolves [3]. These scars are characterized by dermal tissue proliferation and excessive deposition of extracellular matrix (ECM) proteins, especially collagen,

produced by fibroblasts during a long period. HSs have large amounts of fibroblasts that express higher amounts of TGF-β1 than produced by normal skin. HSs are formed by the overproduction and excessive deposition of collagen by TGF-β1 [4].

Matrix metalloproteinases (MMPs) are zinc-dependent proteinases that play a crucial role in the remodeling phase of wound healing. MMPs break down collagen and other ECM proteins [5]. Some MMPs are associated with HS formation; MMP-2 and MMP-9 are two important types of such enzymes, and their roles in HS formation have been studied. The modulation of MMP2 and MMP9 expression levels via the interaction of fibroblasts and keratinocyte may improve the quality of wound healing [6]. Low MMP-2 and MMP-9 expression levels in tissue samples noted in patients with HS [7, 8] suggest that enhancing the expression of these enzymes during wound healing may decrease the probability of HS formation by these enzymes.

HSs are often accompanied by esthetic and psychological problems and social dysfunction and may influence the quality of life of affected individuals [2]. To date, no optimal treatment has been developed for HSs. The development of anti-scarring agents is a challenging problem in HS treatment. Recent studies have suggested the use of medicinal plant extracts and phytochemicals in the treatment and prevention of HS formation because of their demonstrated efficiency in traditional medicine, better safety profile, and cost-effectiveness [9].

Scrophularia. striata (Scrophulariaceae) is a medicinal plant that grows in the western regions of Iran. People living in these regions consider it a magic plant because the plant extract has been used for the treatment of second- and third-degree burn wounds, with excellent treatment outcomes. The plant extract accelerates wound closure rates and prevents abnormal scarring after burn injuries [10, 11]. The phytochemical analysis of *S. striata* extract (SSE) showed the presence of bioactive phytochemicals such as cinnamic acid and quercetin [12]. Antioxidant, antimicrobial, antianxiety, antiproliferative, anti-inflammatory, neuroprotective, analgesic, antidepressant, and healing effects of this plant have been reported [13]. The wound healing effects of *S. striata* have been evaluated in human and animal models of ulcers and burns [10, 14]. Based on the anti-inflammatory and antioxidant properties as well as the modulatory effects of SSE on ECM [15], in this study, we investigated the effects of *S. striata* in the prevention and treatment of HSs in a rabbit ear model of HSs.

MATERIALS AND METHODS

1. Preparation of hydroalcoholic SSE and ointment

S. striata was collected from the growing areas of this plant in the Ilam province of Iran and identified in the Department of Pharmacognosy, School of Pharmacy, Hamadan University of Medical Sciences. The plant was dried under shade at room temperature. Hydroalcoholic SSE was prepared by maceration.

Ointments containing 5%, 10%, and 15% SSE were prepared by adding 5, 10, and 15 g of SSE to 95, 90, and 85 g of Eucerin base. The ointments were prepared by levigation. Briefly, the particle size of the extract powder was decreased by triturating the powder with a mortar and pestle along with a small amount of glycerin. Then, the fine powder was incorporated in Eucerin.

2. Animals

Twenty-three male and female New Zealand rabbits (weight: 2.3-2.5 kg) were used in this study. Before and during the experiment, the animals were kept in individual cages and provided with standard food, fresh vegetables, and water. The animal room temperature was maintained at 20 ± 2℃, and the duration of dark-light period was 12:12 h. All animal procedures were performed in accordance with the animal ethics guidelines of the Hamadan University of Medical Sciences, Hamadan, Iran, and approved by the research ethics committee of Hamadan University of Medical Sciences (ethics approval code: IR.UMSHA.REC.1398.435).

3. Evaluation of skin irritation effects of SSE ointment by Draize test

To perform this test, a male rabbit was selected. The hairs on the back of the rabbit were shaved, and after 24 hours, the back area was divided into four parts. The 15% SSE ointment was applied to the test area, Eucerin was applied to the vehicle area, and two adjacent areas were kept untreated and served as controls for the test. Thereafter, at intervals of 24, 48, and 72 h, the presence of erythema and edema was assessed based on the Draize scoring system (scores 0-4, with 0 indicating the absence of erythema and edema and 4 indicating severe erythema and edema).

4. Rabbit ear model of HS and study design

Wounds were created according to the Morris et al. [16] method. Briefly, the rabbits were anesthetized with ketamine (60 mg/kg) and xylazine (5 mg/kg), and four wounds were created on the ventral surface of each ear using a 6-mm-diameter biopsy punch, and the cartilage cover (perichondrium) was removed with a scalpel and microsurgical technique.

In the prevention phase of the study, five groups of animals were studied. Each group consisted of one male and one female rabbit; 16 wounds were evaluated in each group. Group 1 served as a negative control and received no treatment, group 2 rabbits were treated with 5% SSE ointment, group 3 rabbits with 10% SSE ointment, group 4 rabbits with 15% SSE ointment, and group 5 rabbits with Eucerin as the ointment vehicle. The treatments were applied topically once a day for 35 days.

In the treatment phase of the study, six groups of animals were studied. Each group consisted of one male and one female rabbit; 16 wounds were evaluated in each group. The treatments were started 6 weeks after wounding and confirmation of HS formation as follows: the test groups were intralesionally treated with 50 μL of 5%, 10%, and 15% SSE solutions (SSE was dissolved in ethanol and normal saline [NS] in a ratio of 1:1) once a week for 4 weeks. The negative control group received no treatment, the injection vehicle group received 50 μL of ethanol and NS, and the positive control group received 50 μL of triamcinolone.

5. Sampling

In the prevention phase of the study, the sampling was performed on day 35 after wounding (immediately after treatment completion). In the treatment phase, the samples were obtained 30 days after treatment completion. For sampling, the rabbits were anesthetized with ketamine (60 mg/kg) and xylazine (5 mg/kg), the wound site was cleaned with 70% ethanol, and the surrounding hair was shaved; the scar tissue was then removed with a scalpel, and the wound was bandaged. The samples were divided into two groups: samples from one group were placed in a 10% formalin container for 3 days for histological examination, and those from the other group were placed in a microtube, fixed in nitrogen, and kept at –70℃ for the evaluation of MMP2 and MMP9 gene expression.

6. Scar elevation index (SEI) determination

Hematoxylin-eosin staining was used to determine SEI. SEI evaluation is a reliable method for the quantification of scar formation [17]. SEI is the ratio of the height of the dermal area of wound tissue to the height of the dermal area of normal tissue under the scar. In this study, SEI measurement was performed at 100X magnification using Image-Pro Plus 6 software. SEI > 1 indicates HS formation [17, 18].

7. Epidermal thickness index (ETI) determination

ETI indicates the degree of hypertrophy of the epidermal scar, in which the thickness of the epidermis is measured from the basal septum to the upper limit of the granular layer. Five sections of the epidermal scar samples were viewed under a microscope with 400X magnification. Five non-wounded areas around the scar were evaluated to measure the thickness of the epidermis in normal skin. ETI is the ratio of the mean height of the epidermis at the scar to the mean height of the epidermis in normal unwounded tissue, where ETI > 1 indicates epidermal hypertrophy [19].

8. Determination of collagen deposition

For the determination of collagen deposition, tissue sections were placed in paraffin and stained with Masson's trichrome staining method to identify collagen. The collagen fibers turn blue and the cytoplasm and nucleus of the cell turn light pink and dark brown, respectively. Using light microscopy, the characteristics of collagen fibers, namely, band thickness, density, and placement, and collagen deposition were compared between the treatment and control groups. Four images per slide were taken at 100X magnification for the histological study. Using the Color Deconvolution ImageJ software, the blue color area in the whole area of each image was evaluated [20].

9. Evaluation of MMP2 and MM9 gene expression

To evaluate MMP2 and MMP9 gene expression, at first, total RNA was extracted from the rabbit ear scar tissue samples using an RNA extraction kit (SinaPure RNA, Iran). cDNA was then synthesized using the SinaClon First Strand cDNA Synthesis Kit. MMP2F, MMP2R, MMP9R, MMP9F, beta-actinF, and beta-actinR primers were designed and synthesized by Sinaclone.

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The beta-actin housekeeping gene was selected as the control gene. cDNA samples were prepared by master real-time PCR with the master program (Sina SYBER blue HS-qPCR mix, 2x). Using real-time PCR and the ΔΔCT method, the expression of the desired genes was examined. In the real-time PCR program using the LongGene device, first, preliminary denaturation at 95℃ was performed for 5 min, followed by 40 cycles of denaturation at 95℃ for 15 s, annealing at 60℃ for 30 s, and elongation at 72℃ for 30 s at the end of melting curve. Primers used in real-time PCR are presented in Table 1.

10. Statistical analysis

The samples were analyzed using SPSS 26 software, and the results are reported as mean ± SD one-way analysis of variance.

Table 1. Primers used in real-time PCR analysis

Gene	Primer sequence
MMP2 Forward	5'-GCGACCTCAACCAACTACGA-3'
MMP2 Reverse	5'-TGTAAATGGGCGCCATCAGG-3'
MMP9 Forward	5'-AAGACGCAGACGGTGGATTC-3'
MMP9 Reverse	5'-ACTCACACGCCAGAAGAAGC-3'
Beta-actin Forward	5'-CGTCTTCCCCTCCATCGTG-3'
Beta-actin Reverse	5'-GGATGCCTCTCTTGCTCTGG-3'

A significance level of $p < 0.05$ was set to analyze the data. A post hoc Tukey's test was used to evaluate the significance between different groups.

RESULTS

1. Draize skin irritation test

Results of the skin irritation test for SSE ointments showed that after topical application of SSE, no erythema, edema, or irritation was noted after 24, 48, and 72 h.

2. Macroscopic examination

As shown in Figs. 1, 2, the healed wound tissues in animals who received different SSE concentrations were flat and had a color similar to that of normal skin. However, in the negative control and vehicle-treated groups, the wound areas were raised and appeared red, resembling HSs (Figs. 1, 2).

3. Effect of SSE on SEI

SEI measurement in the different groups showed that 5%, 10%, and 15% SSE ointment caused a significant reduction ($p < 0.001$) in SEI compared with the control and Eucerin groups

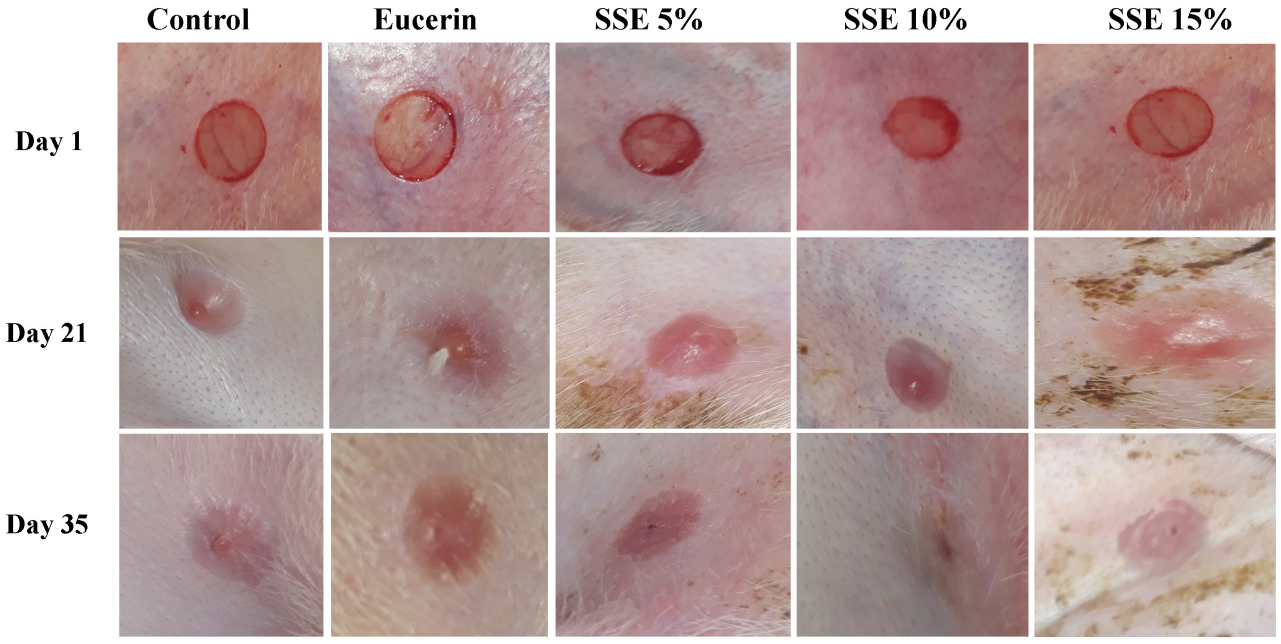

Figure 1. Macroscopic view of scar samples obtained from studied groups (in the prevention phase of the study) on day 1, 21 and 35 post injury. SSE, *Scrophularia striata* extract.

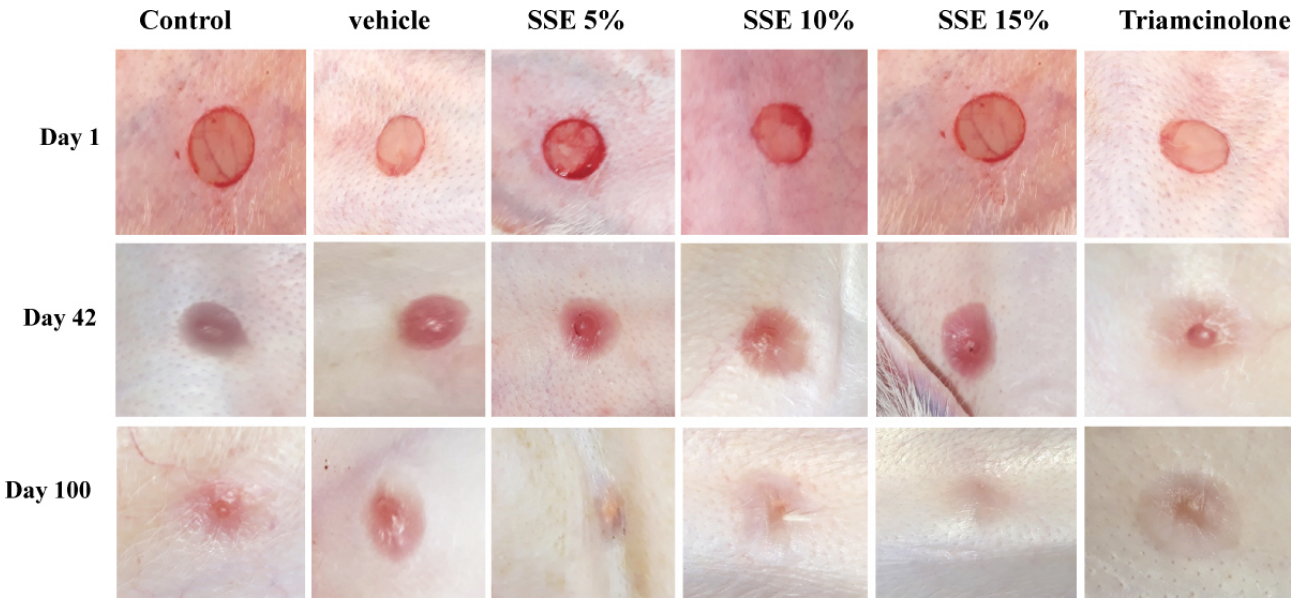

Figure 2. Macroscopic view of scar samples obtained from studied groups (in treatment phase of study) on day 1, 42 (immediately before treatment initiation) and 100 (30 days after treatment completion). SSE, *Scrophularia striata* extract; vehicle, NS + ethanol.

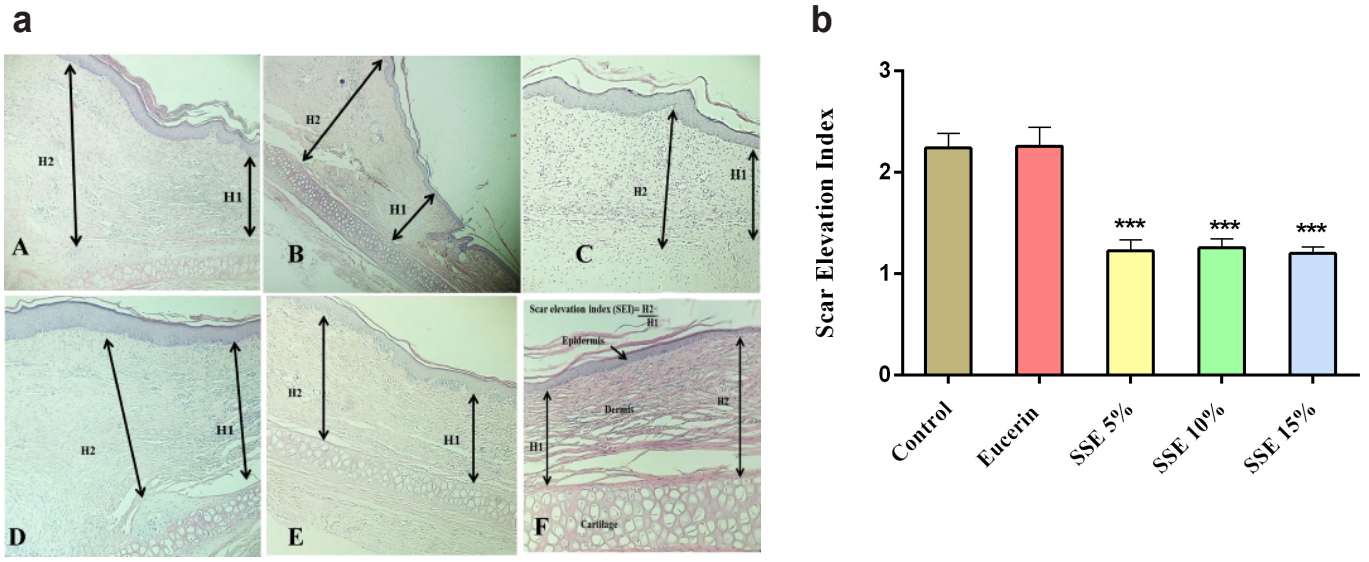

Figure 3. Effect of topical SSE on scar elevation index (a) Micrographs of H&E stained scar tissue sample (100X) obtained from (A) Eucerin (B) control, (C) SSE 5% ointment, (D) SSE 10% ointment, (E) SSE 15% ointment groups, (F) In this image the scar elevation index calculation method is shown. (b) Scar elevation index. Data are shown as mean ± SD, ***($p < 0.001$) indicates significant differences from Eucerin and control groups. p value < 0.05 was consider as statistical significant. n = 4, SSE, *Scrophularia striata* extract.

(Fig. 3b). As shown in Fig. 3a, the thickness of the dermis in animals treated with topical SSE was lesser than that in the untreated and Eucerin-treated groups. Intralesional injection of SEI (all three doses) and triamcinolone also significantly ($p < 0.001$) reduced SEI in tissue samples compared with the untreated and vehicle-treated groups (Fig. 4).

4. Effect of SSE on ETI

The results of ETI determination in the studied groups are shown in Figs. 5, 6. Topical application and intralesional injection of all concentrations of 5%, 10%, and 15% SSE ointment caused a significant reduction ($p < 0.001$) in ETI compared

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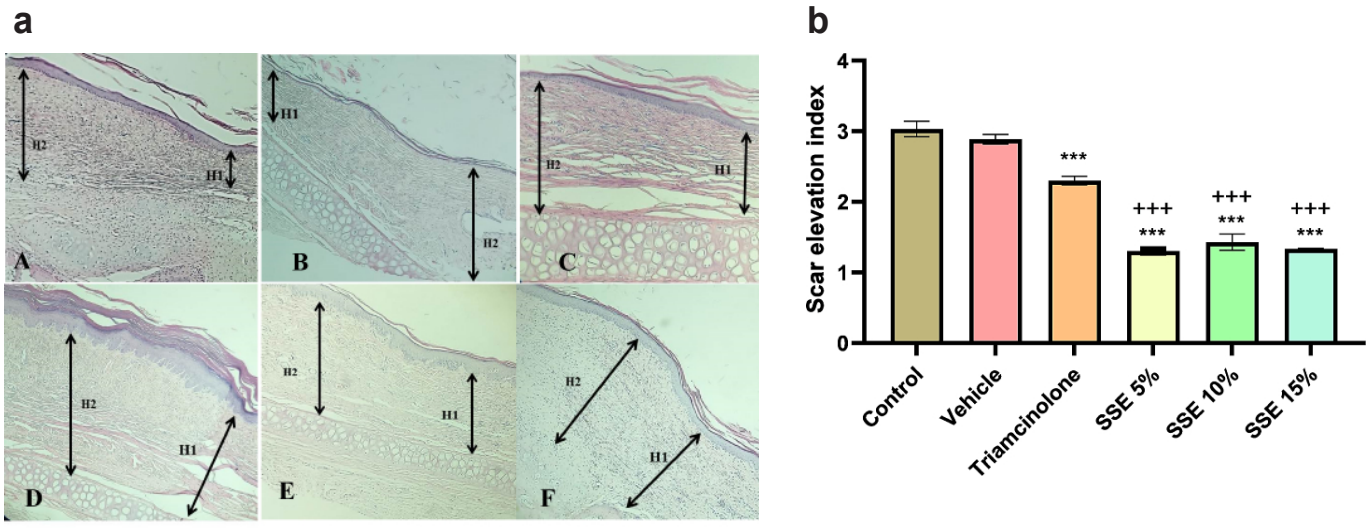

Figure 4. Effect of intra-lesional injection of SSE on SEI. (a) H&E staining of tissue samples was performed to evaluation of microscopic evaluation of hypertrophic scar (100X). (A) Control, (B) vehicle (solvent), (C) Triamcinolone, (D) SSE 5%, (E) SSE 10%, (F) SSE 15%. (b) Quantification and statistical analysis of SEI. ***($p < 0.001$) indicates differences from control and vehicle (solvent) groups, $^{+++}$($p < 0.001$) indicates differences from Triamcinolone group. Data are shown as mean ± SD. n = 4, SSE, *Scrophularia striata* extract.

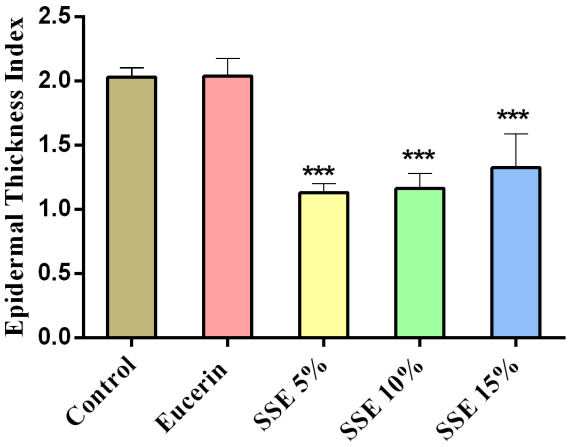

Figure 5. Effect of topical SSE on ETI. Data are expressed as mean ± SD, ***($p < 0.001$) indicates significant differences from control and Eucerin groups, $p < 0.05$ was considered as statistical significant. n = 5, SSE, *Scrophularia striata* extract.

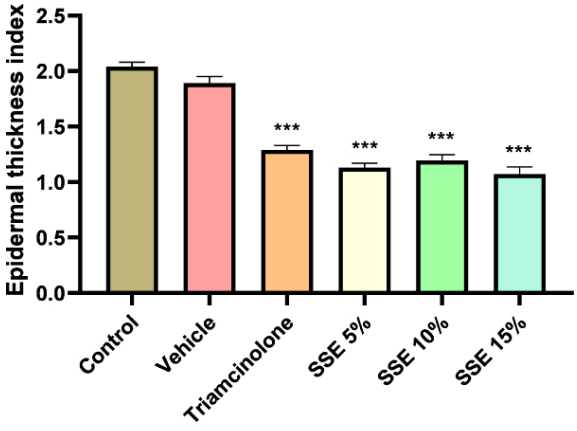

Figure 6. Effect of intra-lesional injection of SSE on ETI. Data are expressed as mean ± SD, ***($p < 0.001$) indicates significant differences from control and vehicle (solvent) groups, $p < 0.05$ was considered as statistical significant. n = 5, SSE, *Scrophularia striata* extract.

with the control and vehicle-treated groups. As shown in Figs. 5, 6, the epidermis in SSE-treated groups was thinner than that in the control and vehicle-treated groups. ETI of tissue samples obtained from triamcinolone-treated groups was also significantly ($p < 0.001$) lower than that of samples from the control and vehicle-treated groups.

5. Investigation of the effect of SSE on collagen deposition

Determination of collagen deposition using Masson's trichrome staining method in tissue samples obtained from different groups showed that 15% SSE ointment caused a significant reduction ($p < 0.05$) in collagen deposition compared with the control and Eucerin groups (Fig. 7b). Dermal cellularity was markedly reduced in wounds treated with topical SSE compared with control and Eucerin-treated wounds (Fig. 7a). In addition,

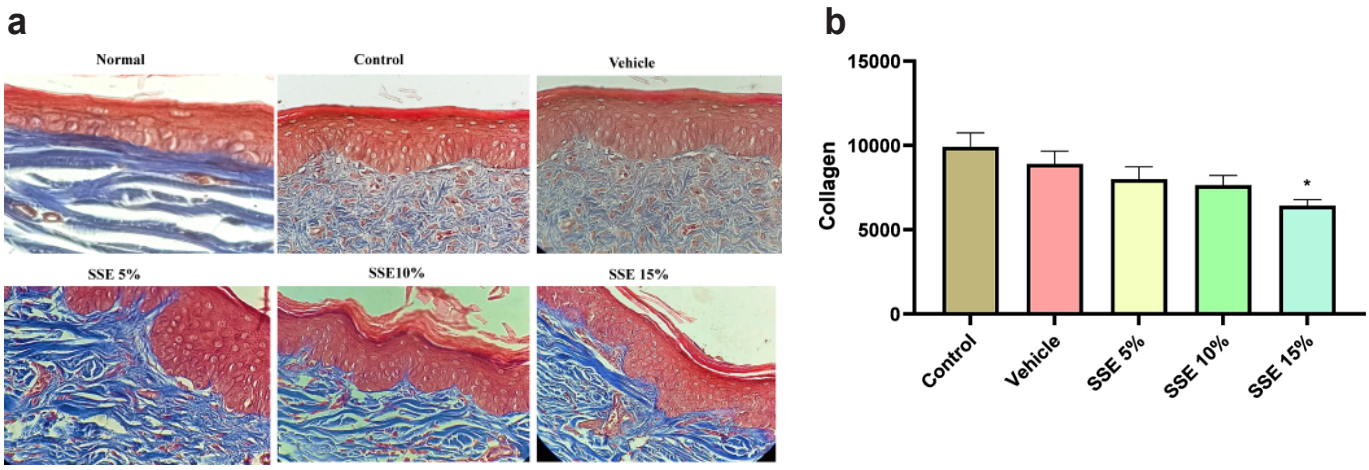

Figure 7. Effect of topical SSE on collagen deposition. (a) Evaluation of collagen deposition using Mason's Trichrom staining on day 35 post injury (100X). The collagen fibers of SSE treated groups are more similar to those of normal skin tissue. (b) Quantification of collagen deposition and statistical analysis. *($p < 0.05$) indicates the significant reduction in collagen deposition comparing to control and Eucerin groups. Data are shown as mean ± SD, n = 4, SSE, *Scrophularia striata* extract.

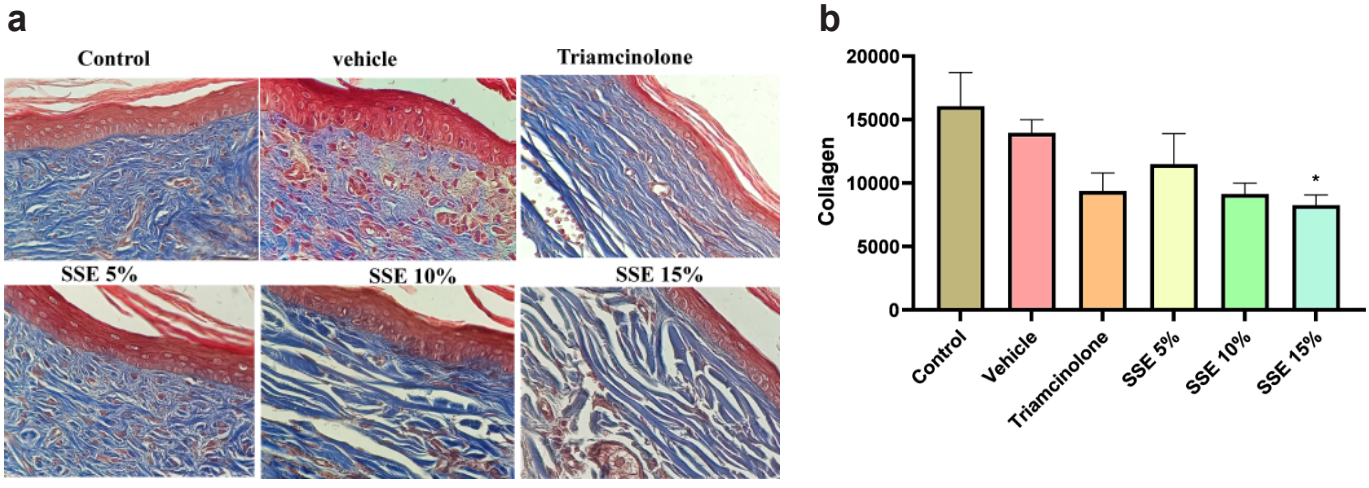

Figure 8. Effect of intra-lesional injection of SSE on collagen deposition. (a) Mason's Trichrome staining was used to evaluation of collagen deposition in scar tissue samples (100X). The collagen fibers are more regular in Triamcinolone, SSE 10 and 15% treated groups comparing to those of control and vehicle (solvent) groups. The more cellularity (brown to red spots) are seen in tissue samples obtained from control and vehicle groups. (b) Quantification of collagen deposition. Data are shown as mean ± SD, *($p < 0.05$) indicates significant differences from vehicle and control groups. p value < 0.05 was consider as statistical significant. n = 4, SSE, *Scrophularia striata* extract.

intralesional injection of 15% SSE ointment caused a significant reduction ($p < 0.05$) in collagen deposition compared with the control and vehicle-treated groups (Fig. 8b). In samples obtained from animals treated with an intralesional injection of 15% SSE, the collagen fibers appeared regular and were more similar to those of normal skin (Fig. 8a).

6. Evaluation of the effect of SSE on MMP2 and MMP-9 gene expression

Topical application of 10% ($p < 0.01$) and 15% ($p < 0.001$) SSE significantly enhanced MMP2 gene expression compared with the control and Eucerin groups. MMP9 gene expression was significantly increased in the wound tissue of animals treated with SSE of all three doses (Fig. 9). Intralesional injection of SSE and triamcinolone showed no significant effect on MMP2 and MMP9 gene expression (Fig. 10).

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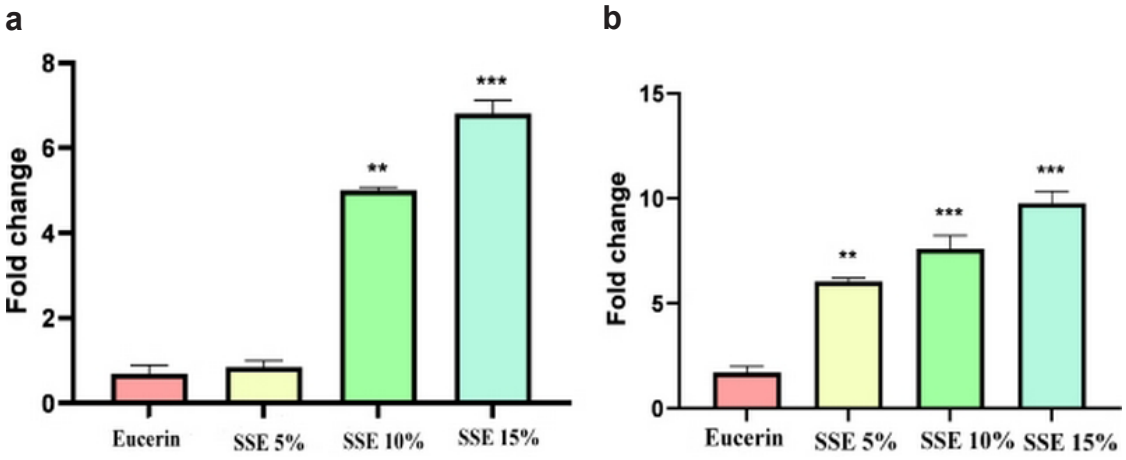

Figure 9. Effect of topical SSE on MMP2 (a) and MMP9 (b) gene expression. Data are shown as mean ± SD. **($p < 0.01$) and ***($p < 0.001$) indicate the significant differences from control and Eucerin groups. n = 6, SSE, *Scrophularia striata* extract.

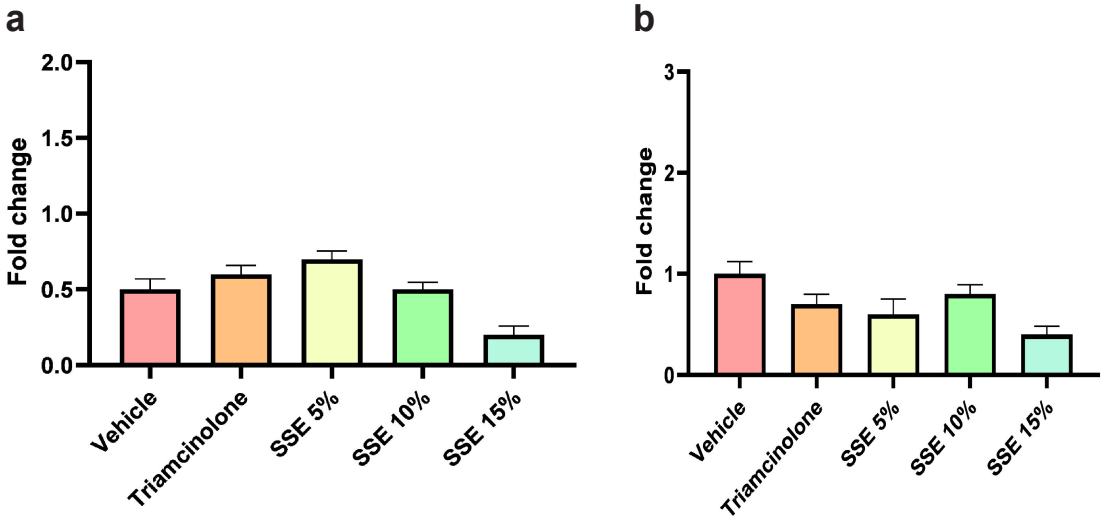

Figure 10. Effect of intra-lesional injection of SSE on MMP2 (a) and MMP9 (b) gene expression. Data are shown as mean ± SD, n = 6, SSE, *Scrophularia striata* extract.

DISCUSSION

HSs occur following trauma and burn injuries affecting the deep dermis. These scars affect the quality of life of affected individuals as they cause functional and esthetic deformities. Medical modalities that are commonly applied for the treatment and prevention of HSs, including intralesional steroid treatment, cryotherapy, silicone gel sheeting, pressure therapy, and laser surgical intervention, may cause side effects such as pain and local irritation and may not be completely satisfactory. Therefore, new and effective treatments are required for the prevention of abnormal scarring. Recently, phytochemicals and medicinal plant extracts have been reported to have the potential to resolve this problem as a safe and effective treatment [9].

Based on previous findings of the effectiveness of hydroalcoholic SSE in preventing abnormal scarring when used in traditional medicine to treat burn injuries as well as its healing effects [11, 21], in this study, we examined the anti-scarring effects of hydroalcoholic SSE in a rabbit ear model of HS. The rabbit ear model of HS is a sensitive, reliable, and practical model to evaluate the potential of agents in the treatment and prevention of HS formation [22]. Although there are some limitations, including significant differences between rabbit and human skin physiology, this model remains an appropriate animal

model for HS evaluation.

Abnormalities during the wound healing process lead to the disruption of balance between ECM degradation and deposition. Either insufficient degradation of ECM proteins due to decreased MMP expression or excessive deposition of ECM proteins caused by increased activity and fibroblast proliferation might lead to abnormal scarring [18].

Chronic inflammation is another mechanism underlying HS formation, which extends the duration of wound healing and causes excessive scarring [4, 22]. It is essential to decrease the duration of complete wound healing to prevent HS formation [23]. Several studies have reported the anti-inflammatory and wound healing activity of *S. striata* [14, 24, 25]. As indicated by the findings of this study, by preventing prolonged inflammation and increasing the wound healing rates, S. striata can prevent excessive scarring.

In this study, we evaluated the effects of topical and intralesional injection of *S. striata* on MMP2 and MMP9 gene expression, and our results revealed that topical, but not intralesional, injection of SSE stimulated MMP2 and MMP9 expression in scar tissue samples. Our previous study also showed that SSE stimulates MMP1 expression at the mRNA and protein levels in cultured fibroblasts [15]. The role of MMP2 and MMP9 in scarless wound healing has been demonstrated in a study by Dang et al. (2003) [26], in which scarless fetal wound healing was associated with an increase in the MMPs to tissue-derived MMP inhibitor ratio. They reported more rapid and greater MMP1 and MMP9 expression in scarless wounds than in scarring wounds. In addition, greater decrease in MMP2 expression was observed in scarring wounds than in scarless wounds [26]. Another study revealed MMP9 upregulation as a major contributor of scarless wound healing [27]. In addition, MMPs control inflammation via the regulation of cytokine and chemokine activity. Therefore, MMPs play a key role in wound healing process [28]. These findings suggest that the mechanism underlying the anti-scarring effect of topical SSE may involve an increase in MMP1, MMP2, and MMP9 expression during the wound healing process.

The results of this study indicated that intralesional injection of SSE has no effect on MMP2 and MMP9 gene expression. This seems like a logical conclusion because MMP2 and MMP9 are primarily involved in inflammation and remodeling of ECM [29], and probably, several weeks after wounding, the levels of these enzymes are usually undetectable.

In summary, our results indicated that topical application of SSE could prevent HS formation in our rabbit ear model. This effect might be achieved through the upregulation of MMP2 and MMP9 expression. In addition, intralesional injection of SSE was effective in the treatment of HS; however, its mechanism of action in HS treatment remains unknown. Further studies are needed to confirm our results and elucidate the mechanism of action of SSE in HS.

CONCLUSION

The results of this study showed that hydroaloholic SSE exhibits anti-scarring properties, and the plant can be used as a source for novel anti-scarring preparations.

CONFLICT OF INTEREST

The authors declare there is no conflict of interest.

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