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# The healing effects of herbal preparations from *Sambucus ebulus* and *Urtica dioica* in full-thickness wound models

Esmaeil Babaei[1], Mohammad Hossein Asghari[2,3], Fatemeh Mehdikhani[4], Milad Moloudizargari[5*], Emad Ghobadi[3], Seyedeh Rokhsane Hosseini Pouya[6]

[1]*Department of Medical Sciences, Sari Branch, Islamic Azad University, P.O. Box 48161-19318, Sari, Iran*

[2]*Department of Pharmacology, Faculty of Medicine, Babol University of Medical Sciences, Babol, Iran*

[3]*Department of Toxicology and Pharmacology, Faculty of Pharmacy, Tehran University of Medical Sciences, Tehran, Iran*

[4]*Faculty of Medicine, Urmia University of Medical Sciences, Urmia, Iran*

[5]*Student Research Committee, Department of Immunology, School of Medicine, Shahid Beheshti University of Medical Sciences, Tehran, Iran*

[6]*Food and Cosmetic Health Research Center, Hormozgan University of Medical Sciences, Bandar Abbas, Iran*

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ABSTRACT

**Objective:** To investigate the healing effects of two herbal preparations.
**Methods:** For this purpose, 106 wistar rats were divided into 9 groups including a control, eucerine, phenytoin, *Urtica dioica* (*U. dioica*) (2%), *U. dioica* (5%), *Sambucus ebulus* (*S. ebulus*) (2%), *S. ebulus* (5%), combination (2%), and combination (5%) groups. The control group remained untreated, the eucerin and phenytoin groups were considered as the negative and positive controls respectively, and the remaining groups received different concentrations of the ointments. Full thickness wounds were made. The healing process of the wounds was investigated on day 7, 14 and 21 of the experiment. Several factors including the number of fibroblasts, new vessel formation (angiogenesis), thickness of the granulomatous tissues (GT), and the overlying epithelium were analyzed.
**Results:** Among the studied groups, all of the treatment groups were significantly different from the control, eucerin, and phenytoin groups in a positive manner with regard to all studied factors ($P \leq 0.05$). However, the best results were observed with the *S. ebulus* (2%) and the combination 2% groups ($P \leq 0.05$).
**Conclusions:** Topical ointments prepared from the extracts of *U. dioica* and *S. ebulus* and their combination possess strong wound healing properties. It is postulated that a synergistic effect may exist between the two extracts since the combination 2% showed better results than the sole extracts.

## 1. Introduction

The skin is among the most important organs in the human body which protects against the invasion of environmental agents and prevents the body from dehydration [1]. Wound healing process is a complicated pathophysiologic scenario of five major phases including: hemostasis, inflammation, cellular migration and proliferation, protein synthesis and wound contraction [2]. Some wounds and ulcers including diabetic foot ulcers, venous ulcers and pressure wounds are among the most common ones with a chronic healing process. Long lasting inflammation and inadequate neovascularization are the most prominent causes of delay in wound healing [3]. An aberrant wound healing process which involves fibrotic tissue formation and the abnormal accumulation of collagen, ends in hypertrophic scar formation [4]. Thus far, different herbal preparations have been used to aid in the treatment of wounds some of which include: the grape seed, lemon, green tea, rosemary and lavender. The presence of

*Corresponding author: Milad Moloudizargari, Student Research Committee, Department of Immunology, School of Medicine, Shahid Beheshti University of Medical Sciences, Tehran, Iran.
Tel: +98 9141482523
E-mails: miladmoludi@gmail.com, miladmoludi@sbmu.ac.ir

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phenolic compounds is the common feature among all the mentioned plant extracts [5].

*Sambucus ebulus* (*S. ebulus*) is a member of Caprifoliaceae family which commonly grows in north of Iran, locally known as Palem [6,7]. The plant has been used in traditional folk medicine of Iran and Turkey for the treatment of inflammatory conditions such as snake bite, rheumatoid pain and sore-throat and for gastric disorders such as peptic ulcer [8,9]. It has been also used as a therapy for open wounds [10]. Previous studies have shown the presence of ebulin, ebulitins, flavonoids, caffeic acid, steroids, cardiac glycosides and tannins in this plant [6,9]. The presence of flavonoids has been attributed to its anti-inflammatory and anti-ulcerogenic activities [9].

*Urtica dioica* (*U. dioica*), known as the stinging nettle is a plant of Urticaceae family [11]. It has a widespread use in Iranian folklore medicine as a remedy for wounds, ulcers, and dermatological disorders such as acne and eczema [12]. Phyto-pharmacological evaluation of the plant has revealed the presence of several constituents of medicinal importance in this plant including: terpenoids, phenylpropanoids, coumarins, steroids and flavonol glycosides [11]. There are reports on the efficacy of *U. dioica* in the treatment of blood pressure and rheumatoid arthritis. The plant has been also shown to possess anti-allergic, anti-microbial, hypoglycemic, immune-stimulatory and anti-carcinogenic properties [13–15].

With respect to the phyto-pharmacological constituents of *S. ebulus* and *U. dioica* and their medicinal properties and based on the centuries-old folklore use of these plants for the treatment of wounds and conditions of this type, the present study was conducted to evaluate the comparative healing efficacy of the hydroalcoholic extract of these two plants in rat models of full-thickness wound.

## 2. Materials and methods

### 2.1. Plant extraction and ointment preparation

The two plants *S. ebulus* and *U. dioica* were prepared from Kia-Kola region, Mazandaran Province, Northern Iran and after pharmacognotic verification of the species in the herbarium of Mazandaran University of Medical Sciences, voucher numbers were deposited (*S. ebulus*: E-28-131 and *U. dioica*: E-14-411). In order for the preparation of the extract, the leaves and the aerial parts of the plant were isolated and 350 g of the root of the plant ground into a mild powder was dissolved into 800 mL of 70% methanol. The mixture remained for three days and was agitated every 24 h to complete the maceration process. Next, the mixture was filtered through a Whatman paper No. 1 and was processed into a gelatinous substance using a rotator machine for evaporation [16]. The extract was finally lyophilized and the powder was incorporated with euserin as the ointment base for the preparation of 2% and 5% topical ointments.

### 2.2. Animal studies

A number of 48 male Wistar rats with an average weight of 200 g were used in the present study. The animals were purchased from the animal house of Urmia University of Medical Sciences and were subjected to two weeks of acclimatization. During this period and the study process, the animals had access to *ad libitum* amounts of feed and water and a standard light cycle and temperature was considered. All experimental procedures involving animals were conducted in accordance to NIH Guide for the Care and Use of Laboratory Animals and approved by the Committee on Animal Research, Urmia University.

### 2.3. Animal grouping

The animals were divided into 9 groups of 12 each in which 12 rats were placed. In each group, full-thickness wounds were surgically made under aseptic condition, each time 4 of which were sacrificed for sampling at the end of the week one, two and three. The groups included: a negative control which remained untreated, a positive control which received topical 1% phenytoin, and six treatment groups which received 2% and 5% extracts of the plants *S. ebulus*, *U. dioica* and a combination of both, respectively.

### 2.4. Wound induction

After reaching a reliable depth of anesthesia, the selected site was initially trimmed using a razor machine and was then carefully shaved and scrubbed. Based on the results of the pilot study, the best site for wound induction was decided to be the dorsal region of the neck, two cervical vertebrae behind the imaginary line connecting the two ears. The mentioned site is normally out of reach of the animal and is the least contaminated. The difference between the healing processes of different wounds depends on the cranial or caudal position of the wound and has no link to the size of the animal [17].

A 1.5 × 1.5 cm stencil was placed in position and the target area of the skin was marked using a marker. After a second assessment of the depth of anesthesia, the skin was grabbed with two forceps so that a skin fold would develop. Due to the high elasticity of rat skin, creating such a fold on the skin seemed necessary in order to increase the accuracy. As the next step, the skin fold was cut out carefully using a surgical blade and the resulting flap of skin was removed. A sterile tampon was placed on the wound site with a gentle pressure in order to stop dermal capillary bleeding. Two simple single sutures were placed on the two corners of the square-shaped wound to prevent the elastic skin of the rats from moving on the hypodermis tissue around the wound [18].

At the end of the surgery and before the recovery of the animals from anesthesia, 10 mg/kg of tramadol was injected intra-peritoneally to each rat for analgesia. The animals received two further injections of tramadol for two successive days post-surgery to achieve the maximum analgesia. The body temperature was continuously observed and kept balanced before the complete gaining of consciousness. The rats were then kept in isolated cages with soft straw beds [18].

### 2.5. Sampling and preparation

On each sampling day (7, 14, and 21) the animals were anesthetized and specimens were obtained from the granulation tissues (GT) at the wound sites. The specimens were kept in 10% form aldehyde solution, microscopic sections were prepared and were stained with H&E for histopathological evaluation.

### 2.6. Histopathological analysis

In order to study the wound healing process in different groups, four major factors were considered while studying the microscopic sections as indicators of the repair process and new tissue formation including: epidermal thickness, the extent of GT formation, fibroblast count, and neovascularization. The epidermal and GT thickness of each specimen was measured in its both sides and middle at a magnification of 400 using a graticule microscope and the data were presented as means of diameters. Angiogenesis (neovascularization) and fibroblast count were measured in 10 microscopic fields in each section and the means were calculated as well.

### 2.7. Statistical analysis

All data were analyzed using One-way and Two-way ANOVA tests and Dunnett and Bonferroni *post hoc* tests were used for the determination of differences between specific groups. $P$ values less than 0.05 were considered statistically significant. The results were expressed as mean ± SD for experimental groups.

## 3. Results

### 3.1. The results obtained at the end of day 7

The average epidermal thickness in the control group was measured as 49 μm. The calculated numbers for the eucerin, phenytoin, *U. dioica* 2%, *U. dioica* 5%, *S. ebulus* 2%, *S. ebulus* 5%, combination 2%, and combination 5% were 76, 72, 78, 80, 99, 74, 110, and 95, respectively (Figure 1). A significant difference was observed in the epidermal thickness between the control group and the all other tested groups ($P < 0.05$). The most effective preparations which increased the epidermal thickness were *S. ebulus* 2% (Figure 1), combination 2%, and combination 5%.

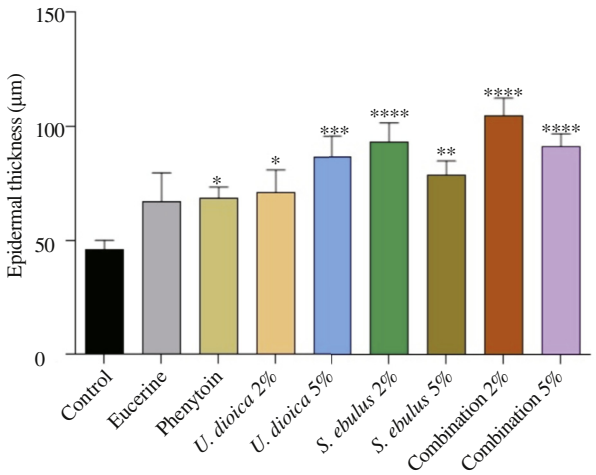


**Figure 1.** The epidermal thickness in each group at the end of week one. There is a significant difference between the control group and all the other tested groups. The significance level is indicated as $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, and $^{****}P < 0.0001$.

The mean diameter of the GT was 835 μm in the control group. This factor was measured as 915 μm in the eucerin and 909 μm in the phenytoin group. After treatment with *U. dioica* 2%, *U. dioica* 5%, *S. ebulus* 2%, *S. ebulus* 5%, combination 2%, and combination extracts, the diameters were 923, 945, 976, 900, 993, and 975, respectively (Figure 2). The extent of GT formation was significant on day 7 of the experiment only in the *S. ebulus* 2% and combination 2% groups, in comparison to the control group ($P < 0.05$).

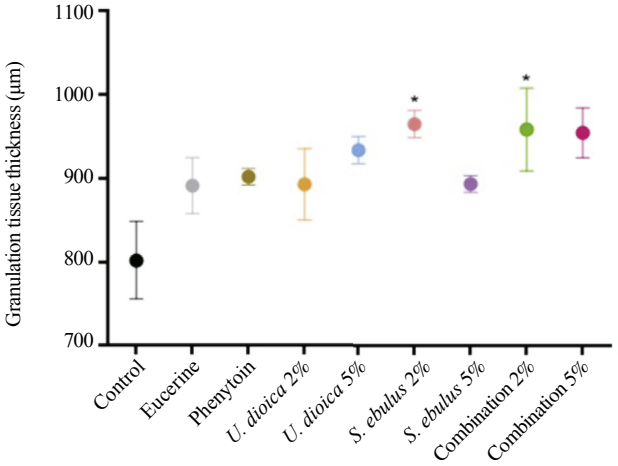


**Figure 2.** The GT diameter in each group at the end of week one. A significant difference can be only seen the *S. ebulus* 2% and combination 2% groups ($P < 0.05$).

An average number of 15 new capillary vessels were formed in the control group at the end of day 7. The number of new capillaries was 21 in the eucerin and 20 in the phenytoin group. In the groups receiving *U. dioica* 2%, *U. dioica* 5%, *S. ebulus* 2%, *S. ebulus* 2%, *S. ebulus* 5%, combination 2%, and combination extracts, the average number of new vessels was 20, 22, 24, 21, 24, and 23, respectively (Figure 3). With the exception of the groups phenytoin and *U. dioica* 2%, new vessel formation in all tested groups was higher than the control group on day 7 of the experiment ($P < 0.05$). The most prominent effect was observed in the group treated with the 2% extract of *S. ebulus*.

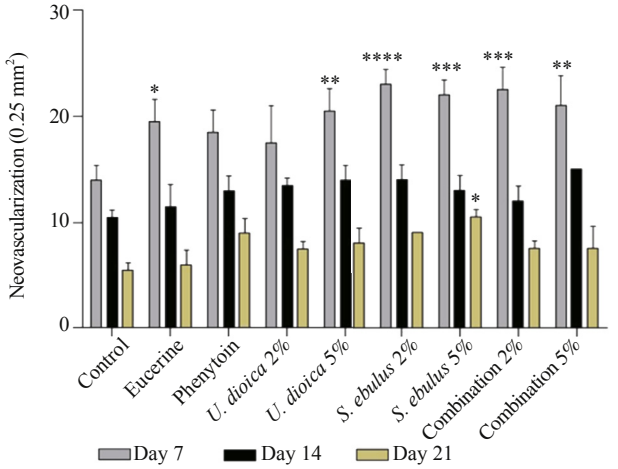


**Figure 3.** The number of new capillary vessels formed at the site of wounds expressed as mean ± SD. On day 7, the extent of neovascularization is significantly higher in all groups except the phenytoin and *U. dioica* 2% group, in comparison to the control group. The significance level is indicated as $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, and $^{****}P < 0.0001$.

The average number of fibroblasts was 165 for the control group, 213 for the eucerine and 220 for the phenytoin group. Fibroblast count in the groups administered with *U. dioica* 2%, *U. dioica* 5%, *S. ebulus* 2%, *S. ebulus* 5%, combination 2%, and

combination extracts was measured to be 211, 243, 295, 232, 303, and 285, respectively. All data obtained are presented in Figure 4. Treatment with all the extract preparations as well as phenytoin and the ointment base (eucerin) itself, significantly increased the number of wound site fibroblasts, compared to the control group ($P < 0.001$). The best results were observed with *S. ebulus* 2% and the combination 2% (Figure 5).

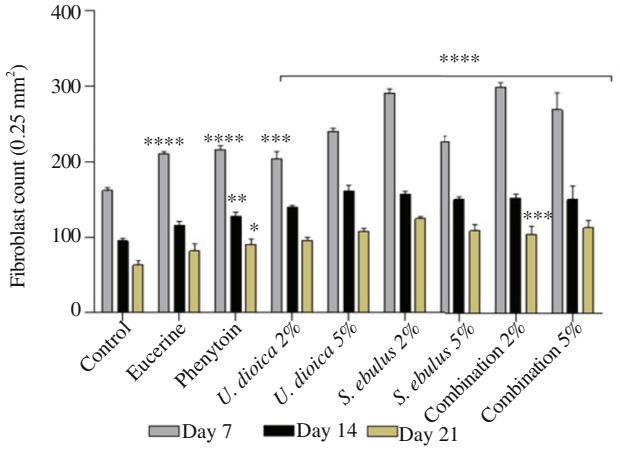


**Figure 4.** Fibroblasts count on days 7, 14, and 21 in different groups (fibroblasts/0.25 mm$^2$).
Treatment with all the extract preparations as well as phenytoin and the ointment base (eucerin) itself, significantly increased the number of wound site fibroblasts, compared to the control group. The significance level is indicated as $^{****}P < 0.0001$, $^{***}P < 0.001$, $^{**}P < 0.01$ and $^{*}P < 0.05$.

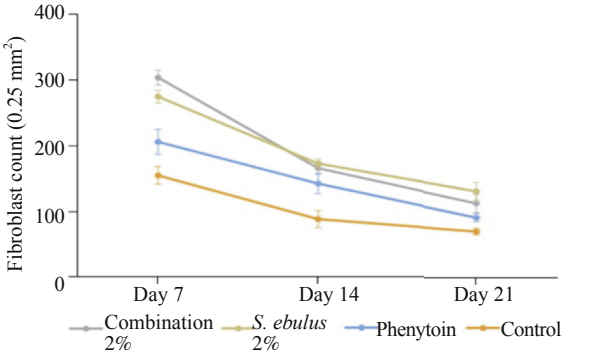


**Figure 5.** Comparison of the fibroblast count between the control, phenytoin, *S. ebulus*, and combination 2% groups on day 7, 14 and 21 on the experiment. The best results were achieved with the combination 2% group.

### 3.2. The results obtained at the end of days 14 and 21

Treatment with neither the extract preparations nor the phenytoin and eucerin, did not exert any significant change in the epidermal thickness, GT diameter, and neovascularization (Figures 1–3) on days 7 and 14 ($P > 0.05$). However, in addition to day 7, the fibroblast count was positively affected by all tested groups at the end of days 14 and 21 (Figure 4). As it is shown in Figures 6–8; *S. ebulus* 2% and combination 2% have been the most successful treatment groups in maintaining the fibroblast number at a higher level than that of the control group and even the phenytoin group as the routine medicine used in the treatment of open wounds, on all tested days of the experiment (Figure 8).

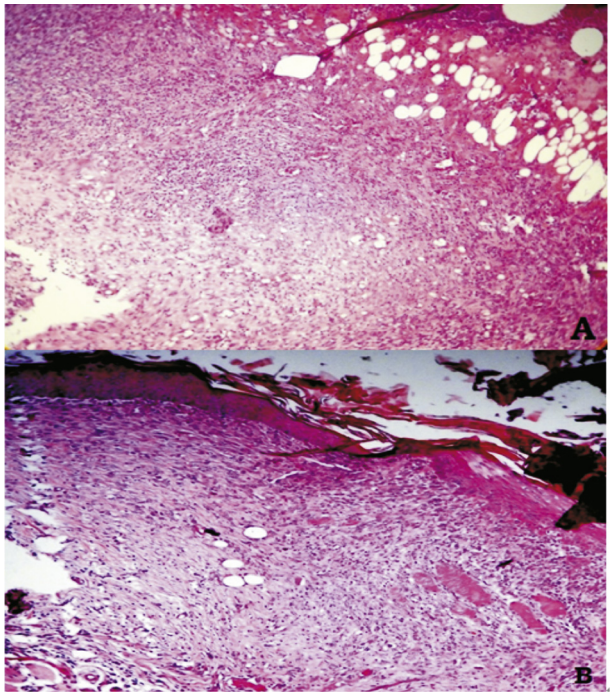


**Figure 6.** Cross section of the wound in the control group on day 7. Increased number of inflammatory cells and accumulation of serotic secretions under the epiderm (A) (H&E, 100×). Cross section of the wound in the *S. ebulus* 2% group on day 7. Increased diameter of the epidermis and GT can be seen. The epidermal edges have reached and the wound is closed (B) (H&E, 100×).

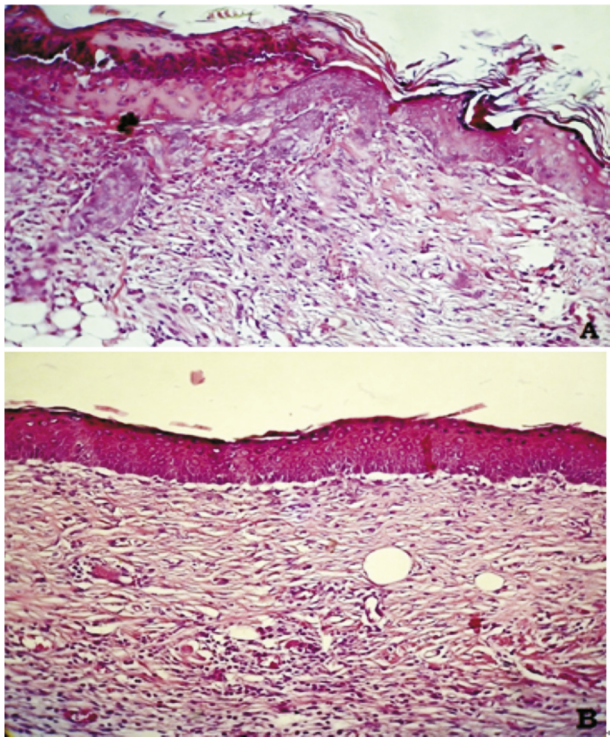


**Figure 7.** Cross section of the wound in the control group on day 14. The wound edges are still open. Unorganized GT and various capillary sections can be seen (A) (H&E, 200×). Cross section of the wound in the *S. ebulus* 2% group on day 14. The wound edges have completely reached and increased collagen deposition and further organization in the granulation tissue is observed (B) (H&E, 200×).

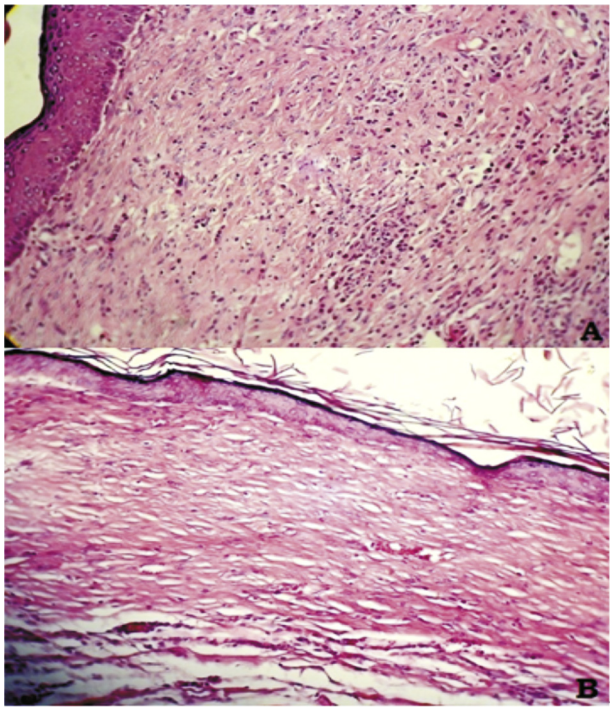


**Figure 8.** Cross section of the wound in the control group on day 21. The wound edges have reached and the epidermal and GT diameter is high (A) (H&E, 100×). Cross section of the wound in the *S. ebulus* 2% group on day 21. Dramatic decrease in GT diameter and epidermal thickness is seen. The number of fibroblasts and newly formed vessels has also decreased. Further organization in collagen is present; the healing process is mature and the granulation tissue is transforming into scar tissue (B) (H&E, 100×).

## 4. Discussion

Among the major goals in basic and clinical medicine in modern societies, is to achieve a desirable state of wound healing in a shorter time with less adverse effects [19]. Based on its importance, widespread research has been conducted in this regard and various therapeutic measures have been taken [20]. The aim of all these methods is the rapid, safe, and at the same time cost-effective treatment of the wound [21]. There are a number of factors which affect wound healing. Among them, fibroblasts, inflammatory factors and keratinocytes, interact and organize a state of cell division, differentiation, and migration which ultimately ends in collagen deposition, connective tissue formation and neovascularization [19]. After any injury, an inflammatory response is initiated and the cells underlying the dermis begin to increase their production of collagen while the epithelial tissue is gradually healing [22]. Any factor which interrupts wound healing could result in excessive fibrosis [23]. *S. ebulus* has a long history as an herbal medicine in Iran [7]. The leaves and the root of the plant have been used in traditional folk medicine of Iran and Turkey for the treatment of inflammatory conditions such as snake bite, rheumatoid pain and sore-throat and for gastric disorders such as peptic ulcer [8,9]. *U. dioica* has been also used as a folk remedy for wounds and ulcers [12]. The anti-inflammatory, anti-microbial, and blood pressure lowering effects of this plant have been previously reported [13,24].

The present study was carried out to investigate the healing effects of topical preparations from *S. ebulus* and *U. dioica* alone and in combination and to compare their efficacy in improving the healing process of the wounds.

Based on the results of this study, the GT thickness at the end of days 7 and 14 in the group treated with *S. ebulus* 2% was 976 and 353 μm, respectively; indicating that during the second week, the GT diameter has decreased by 64%. In other words, the wound in the *S. ebulus* 2% group has had a 64% contraction in this period while the extent of wound contraction in the control group was 45%. In addition to *S. ebulus* 2%, it was the combination 2% ointment which induced a significant GT formation during the first week and also a noticeable reduction in GT mass during the second week, compared to the control group (Figure 3). This shows that, treatment with *S. ebulus* 2% has caused a 19% increase in wound contraction on day 14, compared to the control group. Wound contraction is normally initiated within 2–3 days after the injury and lasts for two weeks [25].

It is obvious that any factor that may accelerate the wound contraction, would aid in a more rapid repair process. The *S. ebulus* and combination 2% ointments caused the highest epidermal thickness and GT mass on day 7 among all tested groups; while, the lowest epidermal thickness on day 14 is seen in the *S. ebulus* 2%; which indicates better contraction. Moreover, despite the rapid grow of GT in the early days of the experiment induced by this ointment, a 74% shrinkage was observed on day 21 which represents a more rapid contraction rate. This plant seems to contain constituents that have caused such a rapid grow in GT and its consequent maturation and contraction at the end of day 21. Since similar effects were exerted by the combination 2% group which contained less amounts of *S. ebulus* extract (compared to the sole *S. ebulus* 2%), it could be concluded that *U. dioica* also contains compounds which contribute to this strong effect of this ointment. This might suggest the existence of a probable synergistic effect between the compounds present in the extracts of these two plants; for a potent effect similar to that of *S. ebulus* 2% was seen when both extracts were used in combination even in smaller amounts. With regard to a previous study conducted in north of Iran, most of the anti-inflammatory property of *S. ebulus* extract is attributable to its hexane fraction which is comparable to the effect of diclofenac [6]. Several *in vitro* studies have reported the anti-inflammatory effects of *S. ebulus* which is due to its flavonoid content. The anti-inflammatory effects of flavonoids and steroids have been previously reported [9]. Among the compounds stated above that play a positive role in the healing process of wounds, is quercetin and mucilage. The latter is shown to have such wound healing effects in rabbit models [26]. Whether or not the mentioned compounds are the main constituents responsible for the positive effects of the ointment prepared from *S. ebulus* extract remains to be pointed out.

Fibroblast count has been long considered as an index of connective tissue healing quality, since Ross *et al*. used it in a study of wound healing. Usumez *et al*. also used the number of fibroblasts around the suture line as an indicator of wound healing process [27]. In the present study, the results obtained from fibroblast count and neovascularization were in harmony with that of wound contraction studies in a way that the maximum number of fibroblasts being 295 cells/microscopic field, was recorded on day 7 in the *S. ebulus* 2% group and the utmost neovascularization was recorded in the same group, as well (24 capillaries/0.25 $mm^2$). These results support the idea that this plant may contain compounds which accelerate fibrogenesis and angiogenesis. It is clear that this can result in

better GT formation. Although, different degrees of significance were recorded in the other groups including the *S. ebulus* 5%, combination 2%, and 5% at the end of day 7; however, none of these ointments were as strong as *S. ebulus* 2% ($P < 0.0001$) in the induction of neovascularization (Figure 4). Moreover, the decrease in the fibroblast count and neovascularization during the days 7–21 is in parallel with the decrease in GT mass and wound contraction in this period which was observed in the *S. ebulus* 2% group. Based on a previous study by Romo *et al*., whenever blood supply is adequate, the migration and proliferation of endothelial cells would decrease and the unnecessary blood vessels would undergo apoptosis [28]. With respect to these findings, such an initial neovascularization and subsequent reduction of blood vessels at the end stages of wound healing was best observed with *S. ebulus* 2% with the most capillaries formed on day 7 and the least remaining vessels at the end of the experiment (a 63% decline). Comparison of this scenario with the control group better indicates the effectiveness of this ointment. As stated previously, the *S. ebulus* 2% ointment has caused a more rapid wound contraction. The fibroblast count was 47% more than the control group on day 7 which was followed by a greater reduction of 55% on day 21, in comparison to the control group. In addition, the neovascularization induced by the administration of this ointment on day 7, was 60% more than the control group. It also caused a greater reduction in the number of excess blood vessels at the end stages. Interestingly, the best results for the epidermal thickness was also observed with the *S. ebulus* 2% among other groups with the greatest thickness on day 7 and the least on days 14 and 21.

These data all together show that the active constituents present in *S. ebulus* extract, not only has increased fibrogenesis and angiogenesis at the end of day 7, but it has also increased the epidermal cells proliferation on the initial days. Subsequently, during the days 7–21, the epidermal thickness has significantly had a better decrease, compared to the control group.

Among the tested groups, the phenytoin and the eucerine groups also significantly aided in a better wound contraction by altering the GT mass and fibroblast count, however their effectiveness was less than the *S. ebulus* group. The antibiotic properties of phenytoin may play a role in its effectiveness. The suggested mechanism through which eucerine might have exerted its effect can be due to its covering of the wound surface and thus preventing the wound from infection and further inflammation by inhibiting the contact of pathogenic bacteria. In conclusion, the *S. ebulus* 2% ointment has exerted the strongest and the most desirable effects among all other tested groups.

The results obtained from *U. dioica* ointments also showed the positive effects of the plant in ameliorating the wound healing process; however it was not as effective as *S. ebulus*. *U. dioica* contains several compounds with anti-inflammatory and anti-bacterial effects [26]. The anti-bacterial effect of extracts prepared from the both plants has been previously shown against several bacterial species including methicillin-resistant *Staphylococcus aureus* [24]. This effect may have a major role in the wound healing properties of these two plants.

The results of the present study all together show that both *S. ebulus* and *U. dioica* possess potent wound healing properties. This effect can be multiplied when both extracts are used together which suggest the probable existence of a synergistic effect between the compounds present in these two plants. The 2% topical ointment of *S. ebulus* showed a stronger effect in comparison to the other sole extract groups. The wound healing efficacy of these herbal preparations might be at least partially due to their anti-inflammatory and anti-microbial effects which have been previously proven. Indeed, more studies are required to precisely determine the major responsible compounds and the underlying mechanisms.

## Conflict of interest statement

We declare that we have no conflict of interest.

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