Licorice cream promotes full-thickness wound healing in Guinea pigs

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ABSTRACT: Impaired wound healing may cause economic and social problems and seriously reduce the quality of life. The uses of herbal medicines as new alternative treatments are under investigation. This study investigated the effect of Glycyrrhiza glabra L. extract on the full-thickness wound healing in Guinea Pig model. Eight male Guinea Pigs (700-800 g) were used to be created a square full-thickness wound with 1.5 × 1.5 dimensions on the lumbar dorsal area. The wounds were randomly divided into four groups: I; control, II; 1% phentoin, III; 5% G. glabra and IV; 10 % G. glabra. On day 3, 5, 7, 9 and 12, wound size was measured for assessment of the percent of wound healing. In order to determine the wound healing activity, excisional biopsies were evaluated histopathologically on the 12th days of treatments. Acute inflammation, granulation tissue fibroblast maturation, collagen deposition, epidermal layer formation, neovascularization, keratin layer formation were evaluated according to the Abramov score method. Hydroalcoholic extract of G. glabra exhibited total phenolic and flavonoid contents of 114.1 ± 5.45 and 82.85 ± 6.38 mg, respectively. G. glabra creams (5% and 10% w/w) were significantly increased the epidermal formation, collagen deposition and neovascularization, and was decreased acute inflammation in comparison to the control group. Wound healing rate were increased in the G. glabra groups. G. glabra creams 10 % was more effective than 5% w/w. Our findings proved that 5% and 10% w/w G. glabra creams were effective in acute dermal wound healing. More studies with different doses of G. glabra extract are recommended.

KEYWORDS: Glycyrrhiza glabra L.; dermal wound healing; flavonoid, phenol; Guinea pigs models.

1. INTRODUCTION

Wound healing, a physiological process, is repair of damaged cells and tissues that occurs with an accurate and proper process. Phases of wound healing includes: hemostasis, inflammation, proliferation, and remodeling, which that overlap with each other [1]. Loss of skin integrity by trauma leads to an imbalance of hemostasis and so, wound healing process become impaired [2]. Dermal wounds are a major concern and seriously reduce the quality of life for patients. As well, dermal wounds, with high cost and an inert treatment is a public health problem [3]. So, trying to quickly close the cutaneous wound with functional and aesthetic results would be ideal goal of treatment [4]. On the other hand, impaired wound healing is the main source of mortality and morbidity, that is associated with high costs [5]. Natural products are safe, less side effect, cultured acceptability and with having excellent physiological properties are the source of new treatments for cutaneous wound healing [6]. Herbal drugs are inexpensive compared with synthetic drugs. So some researchers have focused on the potential healing properties of herbs and there are various reports on using herbal drugs in healing of skin injuries [7, 8].

Glycyrrhiza glabra L. (G. glabra, Licorice), a native of south-east Europe and south-west Asia, is one of the most widely used as a herbal medicine, natural sweetener, as an additive for flavoring and sweetening.
tobacco, candies, and beverages [9] and skin-cosmetics [10]. Pharmaceutical therapeutic properties, such as antifungal [11] and antimicrobial [12] efficacy, antiviral and antitumoral activity [13], anti-bacterial [14], antioxidant [15], antiulcer [16] have been reported in the numerous articles, that are only some of possible therapeutic properties. The main biologically active components of licorice include: triterpene saponins, flavonoids, isoflavonoids, chalcones and glycyrrhizic acid which are responsible for the observed activities [17, 18]. G. glabra is a medicinal plant that abundantly used in ancient times to treat bacterial and fungal infections but so far only one study reported the wound healing activity of this plant on full-thickness dermal wound in rat model [19]. Studies have shown that G. glabra can have a role in wound healing of gastric [20], oral [21] and colitis [16] mucosal ulcers. Also, the extract of this plant was effective on burning wound healing [22]. Since pig skin is similar to human skin physiologically and anatomically [23], and based on above facts about the G. glabra properties, this study was conducted to evaluate the effects of G. glabra on full thickness wound healing in guinea pigs.

2. RESULTS

2.1. Total phenolic and total flavonoid contents

The total phenolic and total flavonoid contents of the extract were 114.1 ± 5.45 mg gallic acid equivalents and 82.85 ± 6.38 mg quercetin equivalents per gram dried extract respectively.

2.2. Macroscopic observation of wound healing

All animals studied survived in the surgical procedures and during study period without any complications. Symptoms of secondary infection were not observed in any wounds. Wound surface in the phenytoin and G. glabra groups were moist. In macroscopic observation, necrotic tissue covers most of the surface of the wound in control group and clearly became visible on the wound areas from the early days in the control group, while necrotic tissue was not seen on wounds in the treated groups. New epithelium was totally obvious at the edges of the wound with a pinkish color in the treatment (phenytoin and G. glabra) groups (Figure 1).

Figure 1: Macroscopic morphology of the wound healing in groups after 12-day treatment by application of G. glabra cream. A) control, B) phenytoin, C) 5% and D) 10% G. glabra cream. The ratios of wound areas to the initial wound area (WHR%) were greater in the treatment group compared with the control group.

2.3. Wound healing rate findings

Figure 2 shows the wound healing rate (WHR) in the groups. The ratios of wound areas to the initial wound area on days 3, 5, 7, 9 and 12 were calculated. In the 5% G. glabra group, the wound area decreased and scab of wounds healed on day 12, being smaller in wound area than other groups. Difference between groups was statistically significant. As shown in Table 1, until the 5th day, the average of WHR in the groups was seen a similar increase. The mean WHR in wound area were presented in figure 2. Consider the following graph:
Figure 2: The ratios of wound areas to initial area on days 3, 5, 7, 9 and 12 were drawn on a line graphs. There were significant differences between the treatment groups with control groups. In all groups, the WHR increased gradually until day 5 during the inflammatory phase and the proliferative phase, increased with more speed until day 12 in the phenytoin and G. glabra groups during the remodeling phase and it was a gradual increase in the control groups (P<0.05).

As was shown in Table 1, there was significant difference in WHR between G. glabra groups with control group regarding the wound healing score determined by microscopic analysis of wounds. The most important changes that can be seen from the image are the formation of epidermis after G. glabra cream treatment compared to control group. G. glabra stimulated epithelialization. New epidermis in 5 and 10% G. glabra covered 62.02 and 64.5% respectively, of the wound surface on day 12, while in the control and phenytoin groups, new epithelium covered 39.86 and 54.89 % of the surface of the wound, and these differences were statistically significant in G. glabra groups compared with control group. G. glabra stimulated epithelialization, and seemed that is not dose-dependent. Analysis of WHR is showed in the table 1.

Table 1. WHR during days 3, 5, 7, 9 and 12 after the wound creation.

<table>
<thead>
<tr>
<th>WHR/Groups</th>
<th>3&lt;sup&gt;rd&lt;/sup&gt; day Mean±SD</th>
<th>5&lt;sup&gt;th&lt;/sup&gt; day Mean±SD</th>
<th>7&lt;sup&gt;th&lt;/sup&gt; day Mean±SD</th>
<th>9&lt;sup&gt;th&lt;/sup&gt; day Mean±SD</th>
<th>12&lt;sup&gt;th&lt;/sup&gt; day Mean±SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>5.7±2.55</td>
<td>13.84±5.61</td>
<td>21.15±8.23</td>
<td>35.44±9.36</td>
<td>39.86±8.27</td>
</tr>
<tr>
<td>Phenytoin</td>
<td>8.84±2.26&lt;sup&gt;a&lt;/sup&gt;</td>
<td>14.15±1.94</td>
<td>30.37±7.75</td>
<td>41.24±10.23</td>
<td>54.89±9.85&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>5% cream</td>
<td>8.41±1.6</td>
<td>12.83±1.51</td>
<td>31.52±11.49</td>
<td>51.94±9.91&lt;sup&gt;a&lt;/sup&gt;</td>
<td>62.02±9.74&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>10% cream</td>
<td>8.54±1.23&lt;sup&gt;a&lt;/sup&gt;</td>
<td>13.68±1.4</td>
<td>30.34±3.73&lt;sup&gt;a&lt;/sup&gt;</td>
<td>45.86±6.23</td>
<td>64.5±11.83&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Control; without any prescription, Phenytoin; wounds treated with 1% phenytoin, 5% cream; wounds treated with 5% G. glabra cream, 10% cream; wounds treated with 10% G. glabra cream. a significant P<0.05 versus control. WHR expressed in cm<sup>2</sup> mean ± S.D. WHR; Wound Healing Rate

2.4. Microscopic Observation

Figure 3 has been shown the photomicrographs of the wound area in different groups that taken 12 days after the wound creation. The histopathological findings showed the granulation tissue formation, keratin, neoepiderm and neovascularization are marked. Each wound was given a score from 0 to 3 and a mean score was calculated in each group. Then the average scores of wound healing criteria were compared for each group.

Wound healing mean scores of all groups are presented in Figure 4. The number of macrophages and neutrophils were counted on day 12 in each group. The inflammatory cell infiltration in the control group was significantly larger than the G. glabra and phenytoin groups (P<0.05 and P<0.01, respectively). The number of macrophages in the phenytoin and 5% and 10% G. glabra groups no difference was seen.

In this study, the G. glabra cream induced fibroblast cell proliferation in wound area. The amount of granulation tissue increased significantly on 12 days in phenytoin and G. glabra groups. Granulation tissue filled the wound space. Maturation of granulation tissue increased in 5% and 10% G. glabra group compared...
with control and phenytoin groups. Besides that, maturation of granulation tissue was increased on 12 day in phenytoin compared with control group.

![Figure 3](image-url)

**Figure 3.** Photomicrographs of the biopsy specimens taken from the Guinea Pig 12 days after the surgery. A more perfect epidermis layer (red arrow) can be seen G. glabra cream treatment in acute wound models. The granulation tissue formation (white arrow) and keratin layer formation is clearly seen in treated groups. G. glabra cream was effective in formation of the granulation tissue, new epidermis and keratin layer compared with the control group, and this difference was significant ($P<0.05$). H & E. Mag: ×40. Scale bar = 100 µm.

Keratin layer formation, as a key role in the wound healing process in the skin surface, was appeared in the G. glabra groups. Furthermore, G. glabra cream caused the formation of epidermal and keratin layer compared with control and phenytoin groups ($P<0.05$). Phenytoin increased keratin layer formation, but not significant compared with control group.

The number of new blood vessels per HPF ×40 in the G. glabra and phenytoin groups increased rapidly. G. glabra and phenytoin stimulated angiogenesis, and this increase was significant compared with control group. In addition, it can be prove that G. glabra cream treatment induced neovascularization greatly in the dermis layer.

The collagen fibers stained with Trichrome Masson colored in blue (Figure 5). The content collagen deposition in the granulation tissue in the wound area increased in all groups, but in the 5% and 10% G. glabra groups were larger than control group ($P \leq 0.03$, $P \leq 0.03$, resp.). There was no significant difference in collagen fiber deposition between the two treatment groups. The amount of collagen fibers in the G. glabra groups were more than in the phenytoin group. Evaluation by Mac Biophotonics Image J 1.41 software showed a 50% increase between the two treatment groups compared phenytoin and control groups.
3. DISCUSSION

Healing process is a natural response and do not need to help. But impaired wound healing have helped to promote this process [24]. In the present study, macroscopic study of the cream treated groups also showed the effectiveness of *G. glabra* in improved wound healing. We showed that topical application of *G. glabra* cream accelerates wound healing in acute wound model. There were statistically significant differences in histological criteria between the groups. The top score fibroblast maturation, granulation tissue formation, proliferation of fibroblasts, dermal and epidermal regeneration and enormous angiogenesis, increased WHR...
and collagen synthesis and decreased inflammation in the wound area in *G. glabra* treated (5% and 10%) groups confirmed with histological evaluation.

In present study, *G. glabra* creams increased significantly the WHR rate compared with the control group. Proliferation of epithelial cells and epidermal formation increased the WHR in wounds treated with *G. glabra*. We evaluated the mean WHR till the 12th. In contrast to our findings, Oloumi et al indicated that *G. glabra* are able to be effective in the wound healing in rats, but not effective on wound healing rate. This researcher showed wounds treated with *G. glabra* are associated with less wound closure. They have created full-thickness wound with 7 mm diameter and were treated for 7 days. Also, they used *G. glabra* in the form of an extract [19]. While in this study, we have created wound with 1.5 × 1.5 cm² dimensions and after 12 days were checked. Also, *G. glabra* was prescribed cream form. Increased of WHR rate in treatment wounds can be related to increased re-epithelialization in treated wounds and the presence of flavonoids compounds. Flavonoids to inhibit or reduce cell necrosis plays a role in increasing the synthesis of DNA [25]. In this study, necrotic tissue clearly evident in control group and *G. glabra* cream treatment improved cell proliferation and regrowth of the epidermis.

In this study, we evaluate the level of collagen deposition with Masson trichrome staining that reveal that collagen deposition of the granulation tissue in treated groups with 5% and 10% cream was significantly increased when compared to the control groups (P<0.05). These findings may be related to the presence of flavonoids. Both concentrations of the creams studied have the same effect on collagen synthesis. Collagen, a key protein in the extracellular matrix of granulation tissue, considerably contributes in wound strength [26]. Flavonoids increase the synthesis and cross-linking of collagen and on the other hand, decrease the degradation of collagen solution [27]. In the present study, quantitative analysis of the extract revealed the high levels of total phenolic and flavonoid contents in the roots of *G. glabra*. Recent studies have shown the role of flavonoids in the promotion of wound healing [28]. As well Glycyrrhizin (Gly) and α- and β-glycyrrhetinic acids isolated from *G. glabra*, cause to increase collagen content and the subsequent wound healing [22]. Topical phenytoin with having antibacterial activity and preventing secondary wound infections, accelerated excisional wound healing [29]. In this study, *G. glabra* cream stimulated the synthesis of collagen in the dermis layer.

Data showed the topical application of *G. glabra* by having anti-inflammatory properties and by control of inflammation improved the wound healing process and prevented the secondary wound infections (P<0.01). Inflammation is a biological response to a stimulus, such as trauma that causes the production reactive oxygen species (ROS) [30]. Inhibit of the inflammatory phase cause restored the damaged tissue. Gly as a triterpenoid saponin glycoside, is an active component of *G. glabra* root that at concentrations of 7-10% in the body is converted to glycyrrhetinic acid (GA). Anti-inflammatory properties of *G. glabra* is related to GA. GA by inhibiting the activity of proinflammatory prostaglandins and leukotrienes (as inflammatory mediators), is as an anti-inflammatory [31]. Evans et al showed Gly with having anti-inflammatory properties was improved subacute and chronic dermatoses [32]. Also Gly, with the production of interleukin-1 (IL-1), IL-2, and IL-12 increase antibody production [33]. Interleukin-2, as a cytokine with effect on the T-cell, has a key role in wound healing [5]. It was shown that *G. glabra*, is effective for the treatment of skin diseases such as dermatitis, eczema and psoriasis. Glycyrrhizin by inhibiting local inflammatory pathogens treat skin injuries [34].

HMGB1, as an early mediator of inflammation, increase in traumatic injury and plays a cytokine-like in burn injury [35]. This mediator affect in angiogenesis and wound healing [36]. Shen et al, in their studies showed that administration of Gly before and after burn via intraperitoneal significantly improved thermal skin injury [35]. Gly, with reduction in serum inflammatory factors (TNF-α, IFN-γ, IL-6, IL-1β, and IL-17) and the increase in HMGB1 protein/mRNA expression from damaged cells is effective in wound healing [36]. So, *G. glabra* significantly mitigated thermal injury. This study is consistent with the our investigation, but in study of Tanideh et al 10% licorice extract were used and there was not effective in healing of third degree burns [22]. We think the reason is due to *Pseudomonas aeruginosa* infection of wounds.

Histological findings showed that *G. glabra* decreased acute inflammation in the treated groups. Gly and GA with downregulate expression of inflammatory mediators such as interleukin could be effective in inflammatory diseases [37]. In the inflammation phase of wound healing process, macrophages and neutrophils in the wound bed release inflammatory mediators, such as tumor necrosis factor alpha (TNF-α) and interleukin-1 (IL-1). On the other hand, TNF-α induce production of Matrix metalloproteinase-9 (MMP-9) in keratinocytes [38]. Expression and activity of MMP9, as a protease responsible for the degradation of matrix, can be inhibited by glabridin [39].

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Histopathological observations confirm increased neovessels in the *G. glabra* and phenytoin treated groups (P<0.01 and P<0.05, respectively). Sufficient blood circulation is one of the important factors in wound healing [40]. Neovascularization increases oxygen levels and promotes cell proliferation and collagen synthesis [40]. In this study, the effects of *G. glabra* are clearly seen in neovascularization. In histopathological examination, sections of treated groups were showed neovascularization at a much higher level in compared to the control group. According to the Aly et al study, *G. glabra* promote wound healing through increasing angiogenesis, reepithelialization and wound contraction [41]. Our study was consistent with other study [42].

4. CONCLUSION

The herbal creams of *G. glabra* significantly increased collagen content and the rate of epithelialization and also reduced the severity of inflammation. The extract of *G. glabra* which contains the various types of bioactive compounds, could be used effectively in dermal wound healing and as a candidate for treatment of acute wounds. More studies with more different doses of the *G. glabra* extract are recommend.

5. MATERIALS AND METHODS

5.1. Plant material and extraction

The roots of *Glycyrrhiza glabra* L. were purchased from Grand Bazzar, Tehran, Iran. A voucher specimen (E1-75-1111) was deposited at the herbarium of the Faculty of Pharmacy, Mazandaran University of Medical Science, Sari, Iran.

The roots were powdered and extracted with %80 ethanol by maceration at room temperature. The extract was concentrated using a rotary evaporator at 40 °C and dried by a freeze dryer.

5.2. Determination of total phenolic and flavonoid contents

Total phenolic content of the hydroalcoholic extract was determined by Folin Ciocalteu method [43]. Calibration curve was plotted using various concentrations of gallic acid (12.5, 25, 50, 100, 200 µg/ml). The total phenolic content was expressed as milligrams of gallic acid equivalents per gram of extract. The total flavonoid content was estimated using aluminium chloride colorimetric assay [44]. Quercetin (10, 12.5, 25, 50, 100, 200 µg/ml) was used to make calibration curve and flavonoid content was expressed as milligrams of quercetin equivalents per gram of extract.

5.3. Herbal cream preparations

Herbal creams (5, 10% w/w) were prepared by mixing accurately weighed dried extract of *G. glabra* (5 and 10 g, respectively) to cream base by levigation method using glycerin (5 and 10 g, respectively) as levigating agent to prepare a smooth paste, gradually incorporating more cream base until to form homogeneous cream, finally transferred in a suitable container.

5.4. Animal studies

Eight male guinea pigs, weighing 700-800 g were used in this study. All experiments were performed after getting permission from Animal Ethics Committee of Mazandaran University (code 2457). Instructions regarding the care and use of laboratory animals were completely followed. A week before the test, the animals were transported to the laboratory for acclimatize. They were kept in a separate cage in a room under standard conditions, 25±1°C room temperature, 12 h light/dark cycles. Animals had free access to food and water.

5.5. Create acute wound model

Excision wound was created according to a previous study [45]. After anesthesia with ketamine (50 mg/kg ) and xylazine (5 mg/kg), all of guinea pigs were shaved on the dorsal surface of lumbar area with electric shaver. The shaved area was disinfected with alcohol 70% and four square full-thickness wounds in lumbar area was created with dimensions of 1.5 × 1.5 by surgical blade in each guinea pig. The wounds were randomly classified into 4 groups, with 7 wounds in each group Figure 6.
5.6. Studied groups

Immediately after surgery, the wounds were randomly divided into four groups.

Group 1: negative control, the full thickness wounds was not treated anything.
Group 2: positive control, the full thickness wound was treated with 1% phenytoin cream.
Group 3: the full thickness wound was treated with 5% w/w Glycyrrhiza glabra cream.
Group 4: the full thickness wound was treated with 10% w/w G. glabra cream.

The phenytoin and formulated G. glabra extract cream were applied once daily from the day of the operation until 12 days after surgery. The wounds were uncovered during the whole the experimental period. The animals were kept separately in steel cages. The animal cages cleaned daily to prevent secondary infection.

5.7. Wound healing rate determination

On days 3, 5, 7, 9 and 12, was measured the rate of wound healing (WHR). For measuring the area, the wound was covered with a transparent sheet and then draw around the wound. Wound healing rate determination was calculated using the following formula:

\[ \text{WHR} (\%) = \left( \frac{\text{Wo} - \text{Wu}}{\text{Wo}} \right) \times 100 \]

Wo: Original wound area; Wu: Unhealed wound area.

5.8. Histological evaluation

In addition to the recording macroscopic properties of the wound, fixed skin tissue samples were examined by light microscopy for histological evaluation. To separate the biopsy specimens, 5 mm margin of healthy skin around the edges of the healed wound were collected from each wound. The samples were fixed in 10% formalin and then the preparation steps tissue is done automatically by the processor tissue system. After embedding in paraffin, 5-microns thick sections were provided. Slides stained by using hematoxylin and eosin using the standard protocol. The histological image were assessed according to the Abramov score method [46]. Histological score of criteria wound healing were scored as: Acute inflammation grading were scored as: 0: None, 1: Scant, 2: Moderate, 3: Abundant, Granulation tissue fibroblast maturation grading were scored as: 0: Immature, 1: Mild maturation, 2: Moderate maturation, 3: Fully matured, Collagen deposition grading were scored as: 0: None, 1: Scant, 2: Moderate, 3: Abundant, Epidermis formation grading were scored as: 0: None, 1: Partial, 2: Complete but immature or thin, 3: Complete and mature, Neovascularization grading were scored as: 0: None, 1: Up to five vessels per high-power field (HPF), 2: 6–10 vessels per HPF, 3: More than 10 vessels per HPF, and Keratin layer formation grading were scored as: 0: None, 1: Partial, 2: Complete but immature or thin, 3: Complete and mature.
5.9. Collagen assessment

12 days after surgery, skin samples stained by using Trichrome Masson staining to determine the content of collagen. For the semi-quantitative analysis, photomicrographs were evaluated by densitometry using MacBiophotonics Image J 1.41a software. The blue color severity of collagen fibers was assessed as the ratio of the stained area to the entire field assessment.

5.10. Data analysis

Quantitative data were assessed in all groups, with software spss, version 15. All data were expressed by test of One-Way ANOVA and Tukey. Significant differences between groups were analyzed and P <0.05 was considered significant.

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