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Healing potential of liquorice root extract on dermal wounds in rats

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Abstract: **Liquorice root extract (LE) has been used from long ago as an effective medication, especially in gastric ulcer. In this study, the healing potential of aqueous LE on dermal wounds was evaluated. The study was carried out on 45 male Spragne-Dawly rats. Two uniform 7mm-diameter skin defects were created on the back of each animal by 7mm skin punch (total of 90 wounds). LE was applied once daily on half of the wounds for 7 days, after which the animals were sacrificed for histopathological, biochemical (hydroxyproline content) and biomechanical studies. The ultimate surface area of the wounds was also measured. LE caused a significant increase in the number of fibroblasts and capillary buds, collagen contents and tensile strength of the wounds. The wound surface area in the treatment group was also significantly less than the control group. It can be concluded that LE is an effective herbal remedy in wound healing.**

Key words: liquorice root extract, skin wound healing.

Introduction

Liquorice root extract (LE), derived from the plant Glycyrrhiza glabra has long been used in medicine. It is used as a flavoring and sweetening agent for American-type tobaccos, chewing gums, candies, etc. (Dehpour *et al*., 1995; Fuhrman *et al*., 2002; Nomura *et al*., 2002; Kitagava, 2002; Paolini *et al*., 1998). Clinical studies have shown that LE has spasmolytic and beneficial influence on healing process of gastric ulcer (Bennet *et al*., 1985; Tylor *et al*., 1988). Also, antiarthritic (Paolini *et al*., 1998), antiallergic (Kumagai *et al*., 1967), antiviral (Pompei *et al*., 1979), antihepatotoxic (Kiso *et al*., 1984), anticholinergic, antiestrogenic, anti-inflammatory (Paolini *et al*., 1998), antileukaemogenic (Logemanna and Lauria, 1960), anticarcinogenic (Mirsalis *et al*., 1993) and antiatherosclerotic (Fuhrman *et al*., 2002) actions are only some of the possible therapeutic properties of LE and one of its constituents, glycyrrhizin (Paolini *et al*., 1998, 1999).

On the other hand, wound healing involves a series of well coordinated biochemical and cellular events leading to the growth and regeneration of wounded tissue in a specific manner. Participation of various inflammatory cells such as macrophages and neutrophils is extremely crucial to the repair process. These cells also promote the migration and proliferation of endothelial cells, leading to neovascularization of connective tissue cells, which synthesize the extracellular matrices including collagen; and of keratinocytes leading to re-epithelialization of the wounded tissue (Clark, 1991; Rasik *et al*., 1999). These intervening events are controlled by the coordinated action of certain specific growth factors and cytokines by acting on target cells at the site of injury (Bennet and Schultz,

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1993). Unfortunately, all these processes involved in tissue repair are altered during pathological conditions such as diabetes, immune disorders and ischemia and in injuries such as burns and gunshot wounds. In such conditions, wound does not heal perfectly. It would be interesting to unravel the possible mechanisms involved in such cases as this would help to identify precisely newer agents, which may improve the delayed healing process (Rasik *et al*., 1999).

In an ancient Iranian medical text published between 1895 and1898 (Dagha'egh Al'alaj; "details of treatment", written by Haj Mohammad Karim Kermani), powdered liquorice root was administered directly on the wounds and believed that it could exaggerate wound healing. Considering this old remedy and the ethnopharmacological profile and reputed medicinal use in traditional practice, the present study was undertaken for the first time to evaluate the possible dermal wound healing potential of aqueous LE.

Materials and Methods

Preparation of LE: Powdered roots of Glycyrrhiza glabra were soaked in 80°C distilled water (1:3 weight:volume) for 40 min. The mixture was then passed through paper filter and the clear liquid put in 50°C water bath until a honey like consistency with 20% moisture was achieved. The sterile solution was kept in refrigerator at 4°C.

Animal: 45 male Spragne-Dawley rats, weighing 180-200 grams were housed under controlled conditions (12 h. light-dark cycle, 22°C, 60% humidity). They were fed rodent chow and had tap water ad lib. All the procedures were conducted in accordance with the European community guidelines for laboratory animals.

Wound Creation: The back of animals in the thoraco-lumbar region was surgically prepared for aseptic surgery. A circular wound was created on each side by a 7-mm biopsy punch (total of 90 wounds). The surgical procedures were carried out under general anesthesia (90 mg/kg ketamine + 10 mg/kg xylazine).

Treatment Schedule: All the wounds (treatment and control) were rinsed daily by 10 ml sterile saline solution. In the treatment group (45 wounds) following rinsing, LE was applied topically by sterile cotton tip soabs once daily for seven days, whereas the control wounds (45 wounds) were remained untreated. Wounds remained uncovered in both groups throughout the experiment.

Wound Harvesting: After seven days, the animals were sacrificed by intracardiac injection of 20 mg/kg thiopental sodium. For biochemical analysis, 15 wounds from each group were excised by the same punch used for wound creation and care was taken to chop off only the newly formed regenerated tissue without any contamination with normal skin (Rasik *et al*., 1999). The samples were kept at -70°C for biochemical analysis. For histopathological studies (15 wounds from each group), regenerated tissues were cut in the form of square pieces along with normal skin on either side of the wound and preserved in 10% buffered formalin. For biomechanical studies (15 wounds from each group), a strip of skin, 7 cm. long, with the same widths of wound diameter, in the manner that the wound was located at the middle of the strip, was removed by a double-blade scalpel. The skin was then wrapped in Ringer's soaked gauze, aluminum foils and plastic bags and kept in -20°C freezer until tensile testing.

Measurement of Wound Area: Wound area was measured before wound excision in order to determine unhealed wound area (raw wound) by drawing wound boundaries around it on transparent paper and the area within the boundary was calculated by using graph paper. The values for each treatment were averaged and presented in mm^2.

Collagen Content: Hydroxyproline, the basic constituent of collagen was taken as a marker of collagen synthesis. Seven days treated tissue was dried in hot air oven at 60-70°C till consistent weight was achieved. These dried samples were hydrolyzed with 6N HCl for 4 h at 130°C. The hydrolyzed samples were adjusted to pH 7 and subjected to chloramines T oxidation and finally the colored adduct formed with Ehrllich reagent at 60°C was read at 557 nm after cooling for 5 min. Standard hydroxyproline was also run concurrently and values

Table 1: Wound area and hydroxyproline content of treatment and control group.

	Wound area * (mm^2)	hydorxyproline content * (mg/g dry wt. Tissue)
Treatment	18.28 ± 3.70	83.14 ± 10.15
Control	28.12 ± 3.25	49.35 ± 8.65

*** significant difference between the two groups ($p \leq 0.05$).**

Table 2: Histopathological parameters, evaluated in treatment and control groups.

	Histopathological parameters *		
	Cappillary buds ($No./mm^2$)	Fibroblast ($No./mm^2$)	Epithelial gap (μ)
Treatment	1158.66 ± 157.30	1119.81 ± 116.21	150.75 ± 21.92
Control	402.13 ± 34.87	674.93 ± 94.29	950.48 ± 65.53

*** significant difference between the two groups ($p \leq 0.05$).**

Table 3: Biomechanical parameters, evaluated in treatment and control groups.

	Biomechanical parameters *			
	Y.P (Kg)	U.S (Kg)	Stif. Kg.cm	M.E.S Kg.cm
Treatment	1.13 ± 0.21	1.18 ± 0.22	0.90 ± 0.20	1.23 ± 0.31
Control	0.65 ± 0.16	0.72 ± 0.16	0.44 ± 0.16	0.64 ± 0.19

Y.P: yield point, U.S: ultimate strength, Stif.: stiffness, M.E.S: maximum stored energy.
*** significant difference between the two groups ($p \leq 0.05$).**

reported as mg/g dry wt. of tissue.

Histopathological Study: Following routine preparation of tissues, serial sections of paraffin embedded tissues of 5 μm thickness were cut with a microtome and stained with hemotoxylin and eosin and studied under light microscope for fibroblastic proliferation, angiogenesis and re-epithelialization. A new method was used to quantify the histopathological data. For this purpose, the depth of the granulation tissue in each slide was measured by objective micrometry lens under 40× magnification. A field at the middle of this length was then considered and the number of fibroblasts and capillary buds were counted under 100× and 400× magnifications, respectively and presented in number per mm^2. To evaluate the re-epithelialization, the epithelial gap was measured under 200× magnification (fig. 1). The values were averaged for each group.

Biomechanical Study: The samples were defrosted by immersing in 20°C Ringer's solution. The samples were then mounted on a Strograph mechanical test frame (Toyoseiky Tensile Testing Unit, model R3, Japan) fitted with appropriate clamps, with the distance between the clamps at the start of the testing being 4 cm. The strips were loaded with 0-50 kg load cell, with a strain rate of 1cm/min. and the load-elongation curves were drawn. The following parameters were measured from the load-elongation curves: yield strength (yield point) (kg), ultimate strength (kg), maximum stored energy (kg.cm) and stiffness (kg.cm).

Statistical Analysis: Student's t-test was used to compare two means. A value of $p<0.05$ was considered as significant.

Results

Wound Area: The area of wound in the treatment group was 35% less than the control group (Table 1).

Hydroxyproline Content: Hydroxyproline content in the treatment group was significantly higher than the control group ($p<0.05$), indicating enhanced synthesis of collagen (table 1).

Histopathological Results: As shown in Table 2, the number of fibroblasts and capillary buds was significantly higher in the treatment group ($p<0.05$). Also the epithelial gap in the treatment group (fig. 2) was significantly less than the control group (fig. 3), showing better re-epithelialization in this group.

Biomechanical Results: All biomechanical parameters measured in this study were significantly higher in the treatment group compared with the control group (table 3), which shows better biomechanical properties of the treated tissues.

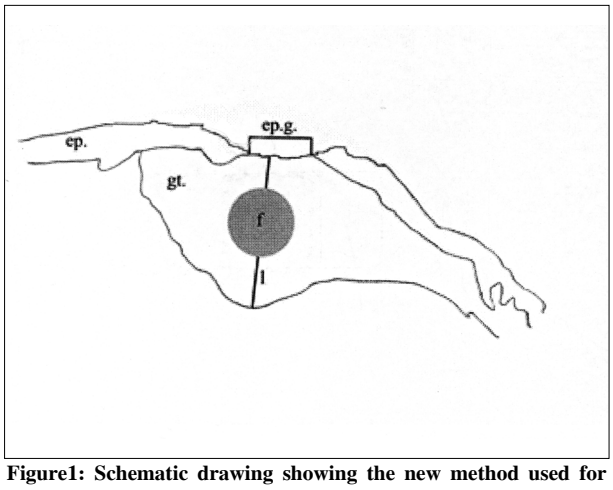

Figure1: Schematic drawing showing the new method used for quantitative tissue characterization. ep: epithelium, ep.g: epithelial gap, f: the field in which the number of fibroblasts and capillary buds were counted. g.t: granulation tissue, l: the depth of granulation tissue.

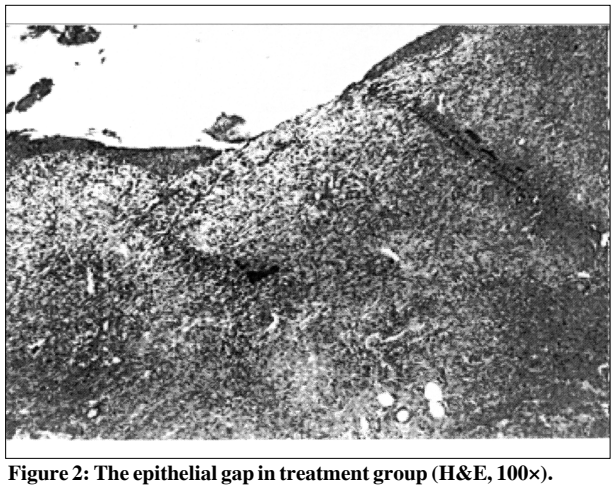

Figure 2: The epithelial gap in treatment group (H&E, 100×).

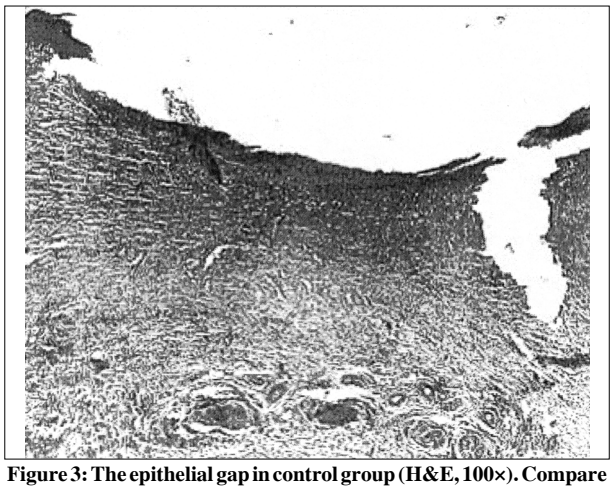

Figure 3: The epithelial gap in control group (H&E, 100×). Compare the gap with figure 2.

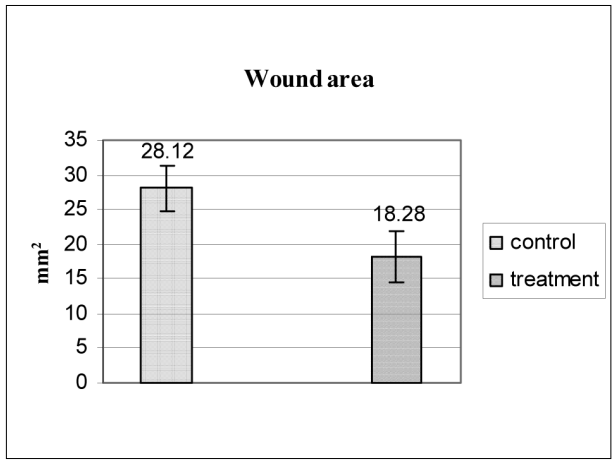

Figure 4. The surface area of the wounds in two groups.

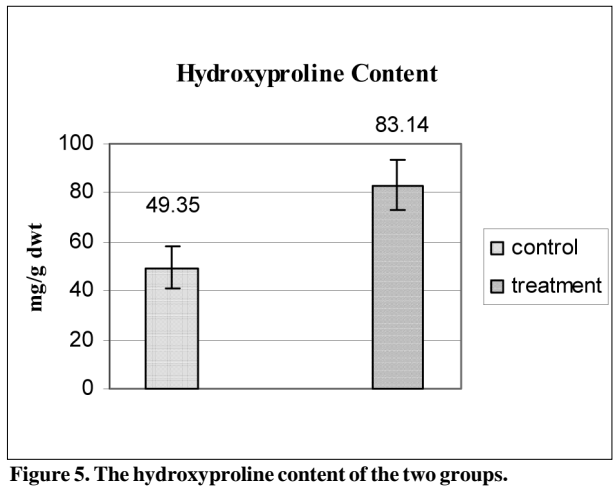

Figure 5. The hydroxyproline content of the two groups.

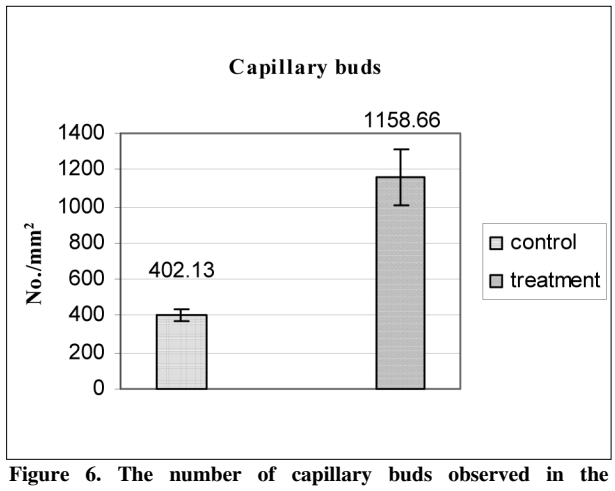

Figure 6. The number of capillary buds observed in the histopathological samples.

Discussion

Wound healing is a complex process characterized by hemostasis, re-epithelialization, granulation tissue formation and remodeling of the extracellular matrix. Though, healing process takes place by itself and does not require much help, but various risk factors such as infection and delay in

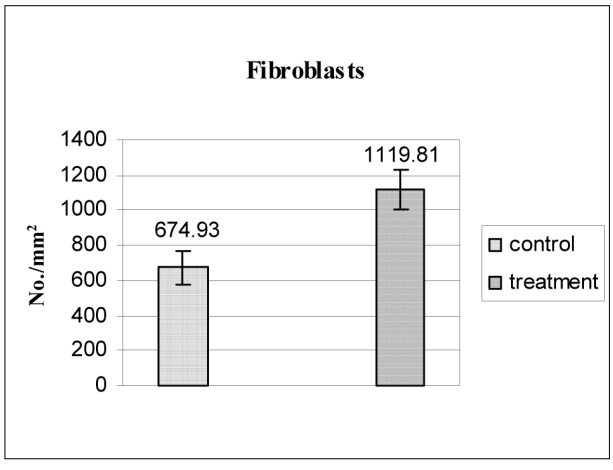

Figure 7. The number of fibroblasts observed in the histopathological samples.

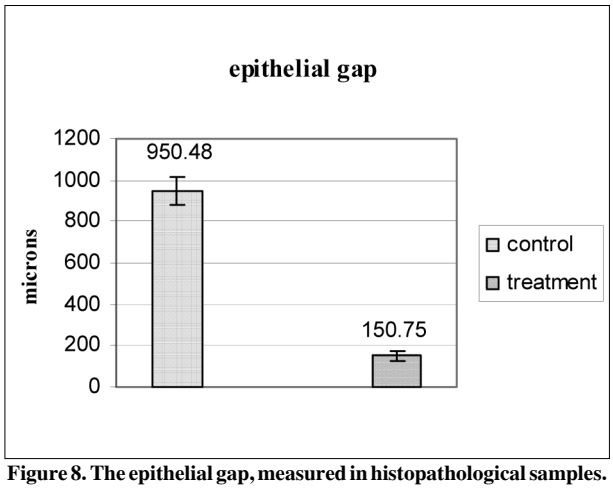

Figure 8. The epithelial gap, measured in histopathological samples.

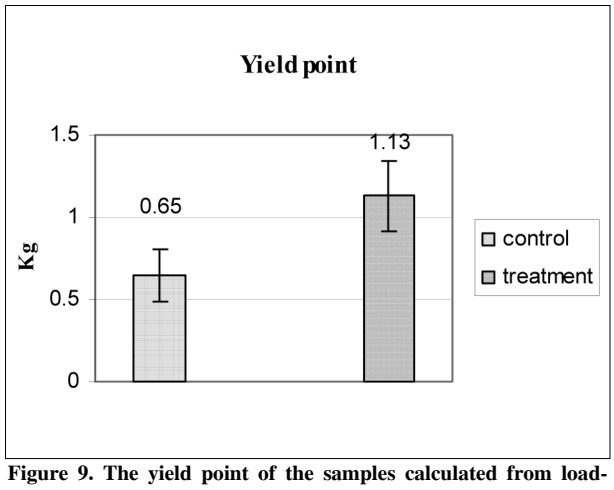

Figure 9. The yield point of the samples calculated from load-elongation curves.

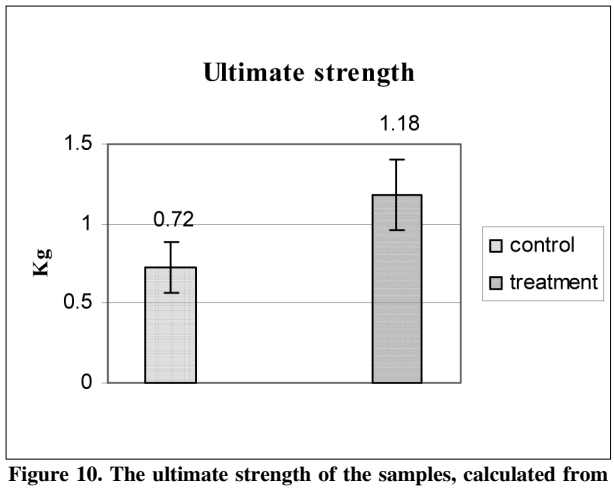

Figure 10. The ultimate strength of the samples, calculated from load-elongation curves.

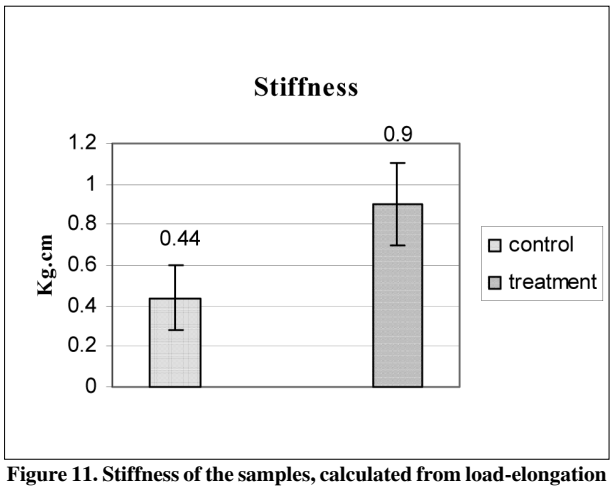

Figure 11. Stiffness of the samples, calculated from load-elongation curves.

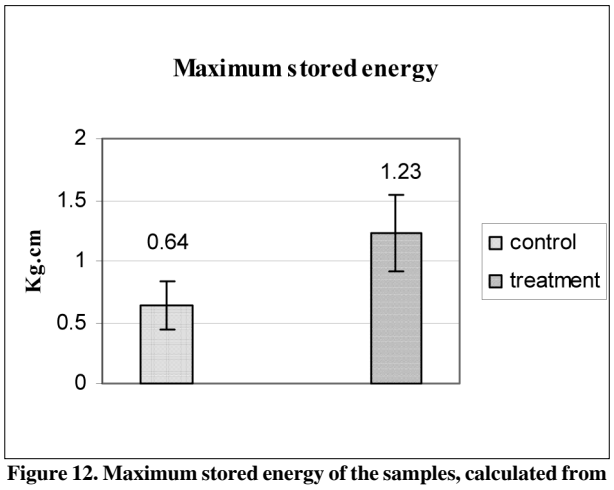

Figure 12. Maximum stored energy of the samples, calculated from load-elongation curves.

healing have been brought to attention to promote this process (Shanmuga Priya *et al*., 2002).

Topical application of LE on the wound caused significant wound healing activity. Increased fibroblastic proliferation may be due to mitogenic activity of the extract, which might have significantly contributed to healing process. Early dermal and epidermal regeneration and massive angiogenesis in treated rats also confirmed that the extract had a

positive effect towards cellular proliferation, granulation tissue formation and re-epithelialization (Shanmuga Priya *et al*., 2002).

Biochemical analysis showed increased hydroxyproline content, which is a reflection of increased fibroblastic proliferation and thereby increased collagen synthesis (Nayak *et al*., 1999). Collagen not only confers strength and integrity to the tissue matrix, but also plays an important role in homeostasis and epithelialization at the later phase of healing (Clark, 1996). Collagen is one of the most dominant extracellular matrix proteins in the granulation tissue, which appears to be significantly high by the fifth day of wounding and after day seven, collagen production is further advanced (Grillo, 1964). Therefore, enhanced synthesis of collagen provides strength to repaired tissue and also healing pattern (Shanmuga Priya *et al*., 2002).

Our biomechanical results well correlate with biochemical and histopathological results. Gain in tensile strength correlates with the rate of collagen synthesis through the first 10 weeks of healing (Capperauld, 1989; Freeman *et al*., 1989; Madden and Peacock, 1971; Miro *et al*., 1995; Paul *et al*., 1997). As shown in Table 1, maximum load and stiffness of the treated tissues were significantly higher than the control one. Maximum load, which is the functionally most important parameter for characterizing healing wounds (Quirinia and Viidik, 1991), reflects the ultimate tensile strength of the specimen, at which complete failure occurs rapidly, and load supporting ability of the tissue is substantially reduced (Carlstedt and Nordin, 1989). This happens as the intermolecular cross links are broken and the collagen fibrils pass each other or as collagen fibrils lose contact with ground substance (Freeman *et al*., 1989). Higher stiffness and maximum load of the treated tissue confirm our biochemical and histopathological observations regarding more fibroblasts, less epithelial gaps and increased collagen synthesis in the treatment group.

Size reduction in treatment wounds was also significantly more than the control ones, which can be attributed to prominent re-epithelialization in treated wounds, which was confirmed microscopically by measuring epithelial gap.

We can conclude that the remedy prescribed by Kermani in 1890s in the book "Dagha'egh Al'alaj" must have been effective on wound healing, although leaving the particles of non-sterile powdered root in an open wound must have had some negative effect on the healing process.

Considering the results of the present study, it can also be concluded that LE posses some pro-healing activity, which can affect the process of wound healing at various phases of tissue repair.

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بررسی خواص التیامی عصاره ریشه گیاه شیرین بیان بر زخم‌های پوستی در موش صحرایی

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عصاره شیرین بیان از زمان های دور به عنوان یک داروی مؤثر بخصوص برای درمان زخم معده مورد استفاده قرار گرفته است. در این مطالعه اثر التیامی عصاره آبی ریشه گیاه شیرین بیان بر زخم های پوستی مورد بررسی قرار گرفت. به این منظور از ۴۵ سر موش صحرایی سفید نر از نژاد اسپراگن-دولی استفاده شد. با استفاده از پانچ پوستی ۷ میلی متری دو زخم متحد الشکل در طرفین ستون مهره ها در پشت هر حیوان ایجاد گردید (مجموعاً ۹۰ زخم). عصاره آبی ریشه شیرین بیان به مدت ۷ روز روزانه بر روی نیمی از زخم ها قرار گرفت. پس از آن حیوانات برای مطالعات هیستوپاتولوژی، بیوشیمیایی (محتوای هیدروکسی پرولین) و بیومکانیک قربانی شدند. سطح نهایی زخم نیز اندازه گیری گردید. عصاره آبی ریشه شیرین بیان سبب افزایش معنی داری در تعداد فیبروبلاست ها و جوانه های مویرگی، محتوای هیدروکسی پرولین و استحکام کششی زخم ها شده بود. همچنین سطح زخم در گروه درمان بطور معنی داری کمتر از گروه شاهد بود. بر اساس این مطالعه می توان چنین نتیجه گرفت که عصاره شیرین بیان داروی گیاهی مؤثری در التیام زخم می باشد.

واژه‌های کلیدی: عصاره شیرین بیان، التیام زخم پوست.

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