



Study of Wound Healing Potential of *Stevia rebaudiana* Ethanol Extract in Male Rats

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Abstract

Background and objectives: *Stevia rebaudiana* has been used in medicine as anti-inflammatory, antioxidant, antipyretic, anti-fungal, and antibacterial agent. The present study was conducted to investigate the healing effects of *S. rebaudiana* ethanol extracts on cutaneous wounds in rats.

Methods: Full-thickness excisional wounds (2×2 cm) were induced on the back of 32 rats. The rats were divided into four groups as follows; untreated (control) and treated with 1 mL basal cream, 1 mL *S. rebaudiana* ethanol extract 10%, and 1 mL tetracycline (3%) for 20 days (short term). Animals of each group were euthanized at 20 day post-injury and wounds were assessed through macroscopic and microscopic analyses. **Results:** During the experiment, *S. rebaudiana* indicated a significant reduction in the wound area compared to other groups. Parameters such as arrangement of the healing tissue, re-epithelization and epithelial formation demonstrated considerable changes when compared to the control. In addition, treatment with *S. rebaudiana* decreased the total number of cells, fibrocytes/fibroblasts ratio, neutrophils, and lymphocytes and enhanced the number of blood vessels and fibroblasts at 20 day. **Conclusion:** The present study demonstrated the wound healing activity of *S. rebaudiana*, lending credence to the folkloric use in the treatment of cutaneous wounds.

Keywords: ethanol extract; *in vivo*; *Stevia rebaudiana*; wound healing

Introduction

Recently, medicinal plants as sources of useful chemical compounds, have received much attention for prevention, control and treatment of many diseases and promotion of human health

[1-3]. Due to recent developments in methodology of plants extraction, medicinal herbs are prepared and examined in different types [4,5]. Types of extraction methods have

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high effects on the medicinal properties of the obtained extracts [6]. Ethanol might be used as the solvent for extraction and the resulting extract is called ethanol extract [7-9]. Some extracts can act as antioxidant and anti-inflammatory agents through inhibiting lipid peroxidation, scavenging free radicals and chelating metal ions [10,11].

Due to the growth in use of medicinal plants, it seems essential to seek plants with healing effects on cutaneous wounds [12] which depends on many factors including types of wound (open wounds and closed wounds), factors affecting wound healing (improper diet, infection at the wound site, diabetes and other diseases), mechanism of wound healing (inflammatory phase, proliferative phase, remodeling phase) and effects of some medicinal plants that have demonstrated wound healing properties [13]. A list of medicinal plants in the worlds that are consumed for their healing properties on cutaneous wounds include *Morinda citrifolia*, *Trigonella foenum-graecum*, *Lycopodium serratum*, *Prosopis Cineraria*, *Catharanthus roseus*, *Lawsonia alba*, *Euphorbia hirta*, *Napoleona Imperialis*, *Pterocarpus santalinus*, *Cecropia peltata*, *Sesamum indicum*, *Alternanthera sessilis*, *Ginkgo biloba*, and *Clerodendrum serratum* [14].

One of the most important herbal medicines which is widely consumed in Iranian traditional medicine for treatment of cutaneous wounds is *Stevia rebaudiana* from order Asterales, Asteraceae family [15]. *Stevia rebaudiana* is an edible plant which has generated a lot of interest throughout human history as a medicinal plant [16]. *S. rebaudiana* could be cultivated for its sweet leaves, which are sources of steviol glycosides (mainly rebaudioside and stevioside). *S. rebaudiana* is 250-300 times sweeter than sugar, so it is used in industry as a sweetener additive. Several extracts of this plant are traditionally used in treating diabetes, gastric ulcer, and parasitic, and viral, fungal, and bacterial diseases [17,18]. As far as we know, there is very little data about wound healing properties of *S. rebaudiana* ethanol extract.

Hence, the aim of the present study was evaluation of effects of the ethanol extract from *S. rebaudiana* on wounds healing in rats. Histological change such as total cells and blood vessels and fibroblasts, fibrocytes, lymphocytes, macrophages, and neutrophils were also estimated.

Material and Methods

Plant material and extraction

Stevia rebaudiana was cultivated for the first time in the Faculty of Agriculture, Razi University, Kermanshah, Iran. In May 2017, the medicinal plant were collected, cleaned and then dried at room temperature (25 °C) without exposure to direct sunshine. About 150 g of the obtained powder of dried aerial part of the plants was extracted with 450 mL ethanol for 2 h at 40 °C with continuous shaking. This process was repeated twice to ensure maximal extraction. Afterwards, the solvent was filtered and then evaporated by Rotavapor®. The obtained extract was then stored at -20 °C until use.

Wound creation and study design

Institutional Ethics Committee of Razi University approval was obtained and all procedures performed in studies were in accordance with the ethical standards of the institution. Thirty two male Sprague-Dawley rats weighing 200-220 g were used. They were housed at 25±3 °C and 12:12 h light-dark cycle and fed with standard pellet diet and water ad libitum (standard environmental and nutritional). At the start of the experiment, the animals were anaesthetized by intraperitoneal injection of 60 mg/kg ketamine HCl (ketamine 5%, Trittau, Germany) for anesthesia and 1 mg/kg xylazine HCl (xylazine 2%, Alfasan, Germany) as premedication ratio of 3 to 1. Under sterile conditions, in the cervical region of back of rats, a square shape full thickness incision of 2×2 cm was made in the skin and the incised piece was removed. The wound was left undressed and no systemic or local anti-microbial factors were used. After wounding, the animals were randomly

divided into four main groups (n=8) as follows: (a) untreated (control): no material was used in the injured area and was left uncovered. (b) Basal cream: the injured area was covered with 1 mL basal cream (eucerin) daily, for 20 days post-injury. (c) *S. rebaudiana* ethanol extract: the injured area was covered with 1 mL *S. rebaudiana* ethanol extract 10% (10 g *S. rebaudiana* ethanol extract dispersed in 90 g eucerin) for 20 day post-injury. (d) Tetracycline: the injured area was covered with 1 mL basal cream and tetracycline cream (3%) daily for 20 day [19].

Sample collection and histological evaluation

At the end of 20th day post-injury, the animals were euthanized by intraperitoneal injection of 60 mg/kg ketamine HCl (Ketamine 5%; Trittau, Germany) and 1 mg/kg xylazine HCl (xylazine 2%, Alfasan, Germany) ratio of 3 to 1, and sampling was done. Full thickness skin samples from the wound site including epidermis, dermis and subcutaneous were carefully dissected and harvested for microscopic (histopathology) studies. The tissue samples were fixed in 10% neutral-buffered formalin, processed routinely, embedded in paraffin, sectioned at 5 µm thickness, stained with Hematoxylin-Eosin and investigated with a routine light microscope. The pictures were taken by a digital camera (Dino capture; version 1.2.7) and transferred to the computer software (Photoshop CS-4; Adobe) for digital analysis. Twenty photomicrographs, equivalent to twenty microscopic fields from all tissue samples in all groups, were applied for histopathologic analysis. The criteria that were studied in histopathological sections consisted of reepithelialization, cornification of the epithelium, fibrin deposition, revascularizations, hemorrhage, mononuclear cell and polymorphonuclear cell infiltration, macrophage content, necrosis, presence of fibroblasts, fibrocytes, maturation and organization of collagen. The number of total cells and blood vessels (magnification×200) and fibroblasts, fibrocytes, neutrophils, lymphocytes, and

macrophages (magnification×800) of the injured area were counted and their mean and standard deviations were computed [20].

Statistical analysis

All data were expressed as mean±standard deviation. Statistical comparison between group means was done through one-way ANOVA followed by Tukey's post-hoc test. $P \leq 0.05$ was considered as significant.

Results and Discussion

Wound healing is a hemostatic process to return physiological equilibrium that includes restoration of the damaged structures by complex interactions between inflammatory cells and reconstruction [21]. There are three main phases for healing cutaneous wounds in the injured area; (a) inflammatory phase, (b) proliferative phase and (c) remodeling phase. The aims of wound caring include decreasing risk agents that prevent wound healing, increasing the healing mechanism and extenuating the incidence of infections of wound. Research on wound healing factors is one of the advanced fields in recent biomedical sciences. The great costs of new medicines indicate that some strategies are necessary for better management of wounds and the related problems [22-24].

The impression of medicinal plants in control, prevention and treatment of diseases such as cutaneous wounds is irrefragable [25-29]. The combination of modern and traditional knowledge especially in the field of traditional medicinal plants can yield better natural drugs for healing cutaneous wounds with fewer side effects [12]. *Stevia rebaudiana* has been indicated to have some optimal treatment properties, due to its antioxidant effects in both *in vitro* and *in vivo* models. It has also presented protective activities against toxicity of some of body organs [17,18]. But, to our knowledge, this is the first time that the ethanol extract of *S. rebaudiana* has been examined on experimentally induced cutaneous wound defects in rats.

At the 20th day post-injury, the wounds presented

the formation of a scar covering a thick granulation tissue in all rats; but, treatment with the ethanol extract of *S. rebaudiana* produced more scar tissue than other groups. In all groups, although the epidermis was thick and disorganized, especially when compared with the adjacent normal skin, its size had decreased and its alignment was ameliorate in the *S. rebaudiana* treated lesions compared to other groups (table 1, figures 1 and 2). Also, the results of the present study demonstrated that topical application of *S. rebaudiana* was able to significantly enhance the wound contraction and re-epithelization rate in rats at the short term (table 1, figures 1 and 2).

Table 1. Wound surface area (cm²) in the different experimental groups on 20th day

Day	Control	Basal cream	<i>Stevia rebaudiana</i>	Tetracycline
20	0.78±0.04 ^a	0.66±0.05 ^a	0.18±0.04 ^c	0.38±0.04 ^b

Values bearing different superscripts differ significantly ($p < 0.05$).

Wound contracture is a mechanism that may occur during healing of cutaneous wounds. Excess of wound contraction leads to physical malformation characterized by skin retraction and functional constraints [30]. Epithelialization is an inseparable and indispensable part of cutaneous wound healing used as a defining factor of a favored wound closure. A cutaneous wound cannot treat completely without re-epithelialization. The epithelialization process is defective in all types of wounds [31]. One of the main results in this study was the notable difference in wound contraction rate and re-epithelization rate between *S. rebaudiana* group and the other groups. The enhanced rate of wound contraction and reducing in healing time in treated lesions with *S. rebaudiana* might be due to the antioxidant properties of this plant together with its effect on maturation and organization of the granulation tissue (table 1, figures 1 and 2) [5].

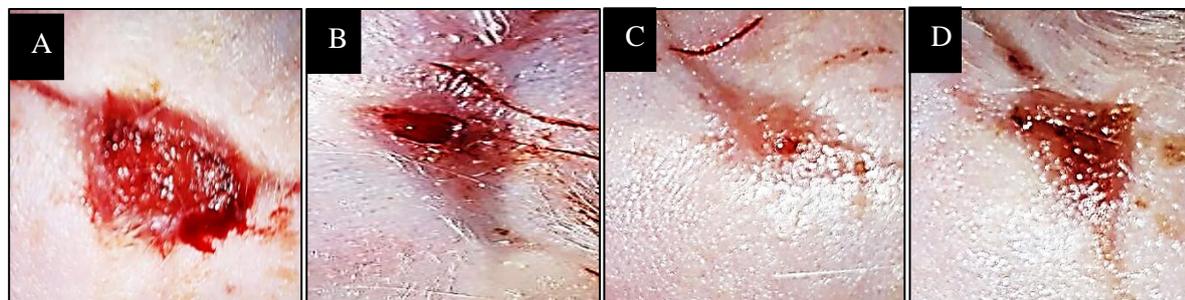


Figure 1. Macroscopic wound images. A: control, B: basal cream, C: *Stevia rebaudiana* and D: tetracycline

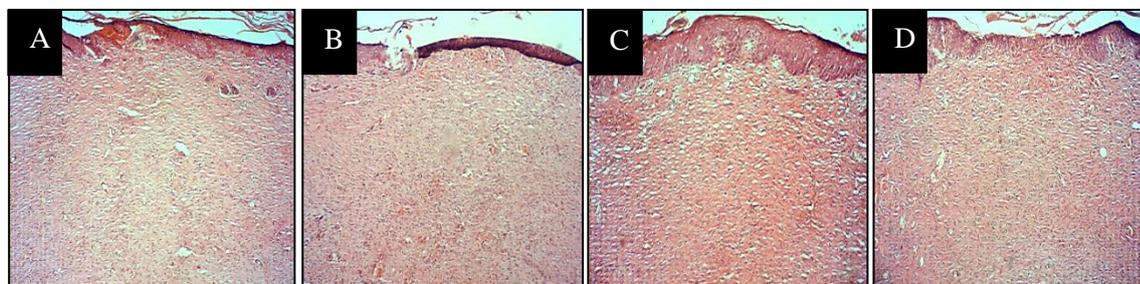


Figure 2. Longitudinal sections of wound images (scale bar for 60 μ m) with Hematoxylin-Eosin staining. A: control, B: basal cream, C: *Stevia rebaudiana* and D: tetracycline

There was no evidence of pus accumulation, or polymorphonuclear cell infiltration, fibrin deposition or edema in the lesions of animals in all groups (table 1, figures 1 and 2). In this experiment, the granulation tissue formation was accelerated by applying the ethanol extract of *S. rebaudiana*. This ability was especially distinct when compared to those of other groups (table 1, figures 1 and 2). Also, the increase in dry granulation tissue weight in the treated animals suggested higher protein content.

The data from the histopathologic analysis have been shown in table 2. Based on histopathological findings, *S. rebaudiana* increased the number of blood vessels in comparison with other groups ($p < 0.05$; table 2, figures 3 and 4). With enhancing the number of blood vessels, amounts of fibroblast and fibrocytes enhanced in surface wound area and the rate of wound healing increased.

Table 2. Histopathologic and histomorphometric analysis of total cells

Day 20	Control	Basal cream	<i>S. rebaudiana</i>	Tetracycline
Total cell	1147.6±214.55 ^a	1147.7±170.88 ^a	540.00±71.49 ^b	1174.8±94.08 ^a
Vascular No.	10.6±4.03 ^b	9.2±3.11 ^b	18.40±5.16 ^a	6.9±3.1 ^b
Fibrocytes	4.0±4.85 ^a	4.1±2.02 ^a	4.5±2.46 ^a	4.7±2.75 ^a
Fibroblasts	22.5±9.02 ^b	19.7±6.78 ^b	46.8±9.27 ^a	13.5±7.66 ^b
Fibrocytes to fibroblasts ratio	0.27±0.37 ^b	0.24±0.15 ^b	0.13±0.1 ^b	0.58±0.57 ^a
Neutrophils	3.0±2.16 ^{ab}	5.1±3.84 ^a	1.5±1.84 ^b	5.6±6.86 ^a
Lymphocytes	11.8±5.49 ^a	8.2±4.39 ^a	0.7±0.67 ^b	10.4±6.85 ^a
Macrophages	0.7±0.94 ^a	0.6±0.84 ^a	0.5±0.9 ^a	0.5±1.08 ^a

Values bearing different superscripts in the same row differ significantly ($p < 0.05$).

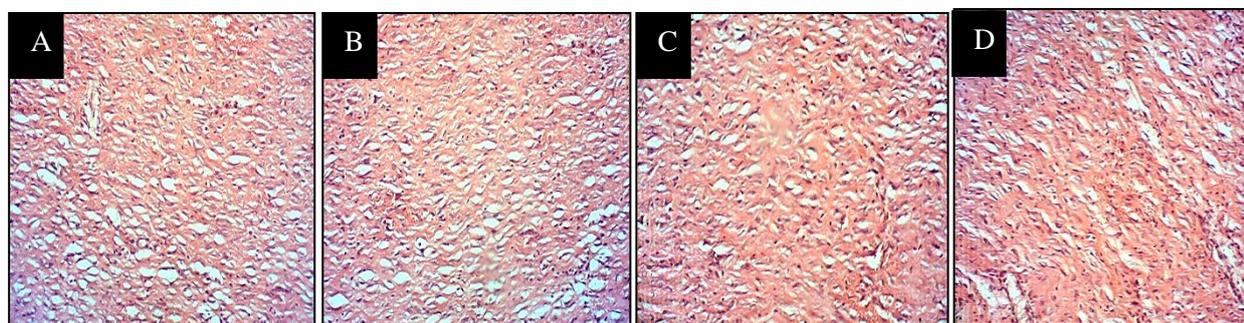


Figure 3. Longitudinal sections of wound images (scale bar for 150 μ m) with Hematoxylin-Eosin staining. A: Control, B: Basal cream, C: *Stevia rebaudiana* and D: Tetracycline.

In granulation tissue formation, fibroblasts and fibrocytes are very active cells. Fibroblasts and fibrocyte are two various states of the same cell, which mainly produce the collagen, extracellular matrix, and the structural framework for animal and human tissues. Fibroblasts and fibrocytes have the potency to differentiate into myofibroblasts in tissues that have been damaged severely; also, as the main components of wound healing they are the most common cells of

connective tissue in injured area. They make collagen and decrease cellularity in wound area [32]. Collagen is the most plentiful structural protein in body and is the component that retains the whole body together. It is found in the skin, tendons, muscles, bones, etc. It makes a lattice to create structure and strength daily activities body. In skin, collagen speeds up wound healing in the injured region by decreasing cellularity. Also, collagen plays a main role in hemostasis [19,33].

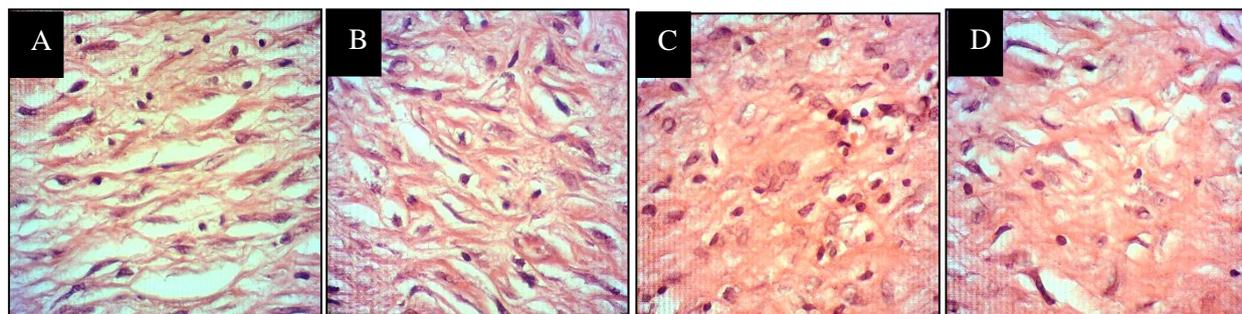


Figure 4. Longitudinal sections of wound images (scale bar for 600 μ m) with Hematoxylin-Eosin staining. A: Control, B: Basal cream, C: *Stevia rebaudiana* and D: Tetracycline

In fact, when collagen is made accessible to the wound bed, closure can happen. In this study, the ethanol extract of *S. rebaudiana* group, significantly increased the number of fibroblasts compared with other groups ($p < 0.05$; table 2). The collagen fibers demonstrated a more organized pattern and the tissue alignment was greater in the *S. rebaudiana* group when compared to the other groups (figures 2-4).

Infection of wound is likely the most usual reason for impaired healing of cutaneous wounds. *Streptococcus pyogenes*, *Staphylococcus aureus*, *Corynebacterium sp.*, *Pseudomonas aeruginosa* and *Escherichia coli* are some main bacteria causing wound infection [34]. In this study, the lower levels of inflammation in *S. rebaudiana* group can be due to the attendance of some phytoconstituents in this plant species which had inhibited the activities of inflammatory cells and production of chemical mediators and consequently decreased inflammation and subsequently increased the organization. Also, *S. rebaudiana* reduced the number of neutrophils (in comparison to basal and tetracycline groups) and lymphocytes (in comparison to the other groups) ($p < 0.05$; table 2, figures 3 and 4).

Based on the obtained results, *S. rebaudiana* the ethanol extract 10% ameliorated angiogenesis and re-epithelization rate, fibroblastic response, collagen content and reduced total cell, inflammation, and wound size significantly; thus, *S. rebaudiana* decreased cutaneous wound area during the experiment compared to other groups. The present research has shown the cutaneous

wound healing activities of the *S. rebaudiana* ethanol extract, offering its possible use as a therapeutics supplement or medication.

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Declaration of interest

The authors declare that there is no conflict of interest. The authors alone are responsible for the content of the paper.

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