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Research Article

Wound healing effect of hydroethanolic extract of *Ribwort plantain* leaves in rabbits

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Abstract

Ribwort plantain is popularly used in the folk medicine as wound healing, anti-inflammatory and for the treatment of diseases of the respiratory tract and muscular pains. In the present study, a hydroethanolic leaf extract of *Ribwort plantain* was evaluated for non-infected wound healing. Forty White New Zealand rabbits were divided into four groups. Full-thickness skin wounds were created on thoracolumabar region on each side of the dorsal midline in each rabbit. Groups A and B received a placebo containing 2 and 4% of hydroethanolic leaf extract of *Ribwort plantain* respectively, group C served as placebo (positive control) and finally group D was kept as control (negative control) receiving no application. The rate of wound size was measured at day 4, 8, 12 and 16 while histological structures were studied on days 3, 7, 14 and 20 after surgery. The wound contraction results show that *Ribwort plantain* hydroethanolic extract reduced wound size especially from day 6 onward. Further, *Ribwort plantain* hydroethanolic extract decreased poly morph nuclear cells, and increased mononuclear cells, fibroblast cell migration and new vascular formation. Thus, this study demonstrated that hydroethanolic extract of *Ribwort plantain* at dose rate of 2% is effective in stimulating the closure of non-infected wounds.

Keywords: Ribwort plantain; wound healing; hydroethanolic extract; ointment; rabbit

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Introduction

The wounds are still a major problem in developing countries, often having severe complications and involving high costs for therapy. An important aspect of the use of traditional medicinal remedies and plants in the treatment of wounds is potential to improve healing and the same time to reduce the financial burden. Several plants and herbs have been used experimentally to treat skin disorders including wound injuries, in traditional medicine.

Ribwort plantain (Plantaginaceae) is a perennial plant species with a worldwide distribution and large ecological amplitude (Reardon et al., 2009). There are lots of reports that Plantago leaves belongs to various species (*P. major, P. lanceolata, P. palmate* and *P. rugelii*) have been good biological activities including treatment of wound (Biringanine et al., 2006, Kumar et al., 2006), pregnancy and lactation troubles (Biringanine et al., 2006), anti-tumour effect (Ozalson et al., 2007), antibacterial and antifungal effect (Kumar et al., 2006; Braga et al., 2007), gastrointestinal

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disorder and diarrhoea (Braga et al., 2007), which have been described in Iranian ancient medical books (Zargari, 1995).

The present study was conducted to evaluate the effect of wound healing activity of hydroethanolic extract of *Ribwort plantain* in rabbits.

Materials and Methods

Plant material and extract preparation

Fresh leaves of *Ribwort plantain* were collected locally during July 2012 in the region of Hamadan city (latitude: 36 46', longitude: 48 34'); and identified by the Department of Botany Sciences, the Hamadan Research Agricultural and Natural Resource Centre. The plant was dried naturally in laboratory at room temperature (23-24ºC) for seven days until crisp, and powdered in an electric blender. Then 200 g of the mentioned plant powder was suspended in 800 ml of hydroethanolic solution for 96 h at room temperature (Saeed et al., 2012). The mixture was filtered using a fine muslin cloth followed by filter paper (Whatman No 1). The filtrate was placed in an oven to dry at 40ºC. The clear residue obtained was used for the study. The extracts were kept at -15°C until it was used in the experiment.

Phytochemical screening

Chemical tests were conducted for identification of coumarins, flavonoids and triterpenes (Harborne 1980).

Experimental animals

Forty New Zealand rabbit (2100 g, 5-week-old and mixed sex) were used in this study. Two weeks prior to the study, animals were housed in individual cages 45×40×50 cm with at standard temperature condition (23 ± 3ºC), stable air humidity, and a natural day/night cycle. In order to acclimatize to experimental procedure, rabbits were handled and mock daily during the 14 days before the experiment. Food and water were given *ad libitum*. Animal handling and experimental procedures were performed according to the guide for the Care and Use of Laboratory Animals by the National Institutes of Health (USA) and the current laws of the Iranian Government. All protocols for animal experiment were approved by the Institutional Animal Ethical Committee in Islamic Azad University, Urmia Branch, West Azarbayjan, Iran.

Preparation of topical test samples for bioassay

The topical applicable ointments were prepared in three different concentrations. All the variants were consisted of base formulation comprising Eucerin (25%) and Vaseline (75%) in 1: 3 proportions.

Wound induction

Animals were anesthetized by intraperitoneal (i.p) administration of ketamine 5% at the rate of 90 mg/kg (Ketaset, Alfasan, Woerden, Netherland) and xylazine hydrochloride 2%, 5mg/kg (Rompun, Bayer, Leverkusen, Germany). The fur was excoriated aseptically and the predetermined area was marked on the back of animals. Each rabbit was fixed on the surgery table in ventral posture. The coordination of experimental wounds (2×2cm dimensions) was outlined through clean transparency sheet template and permanent marker on the dorsolumbar region of the rabbits. A square full thickness skin wound including subcutaneous tissue was made using a #11 BP blade on each side of the midline in rabbits. The wounds were created 2 cm far from the midline at the same location on the trunk (Akela et al., 2013). The haemorrhage was controlled by sterile cotton gauze. The wound area was measured immediately by placing of a transparent paper over the wound and tracing it out; the area of this impression was calculated using the graph sheet. All experimental procedures were performed during 9:00 A.M to 1:00 P.M.

Experimental distribution of rabbits

After making the surgical wounds, all rabbits were randomly coloured with non-toxic colours and divided into four groups of six animals each. In groups A and B (experimental), the rabbits received 2 and 4% of hydroethanolic leaf extract of *Ribwort plantain* mixed with base formulation (RPO) respectively. Group C acted as placebo (positive control) received a base formulation; and finally group D served as control group (Negative control) did not receive any treatment. Each test ointment (0.5g) was applied topically on the wounded site, once a day, immediately from the day of operation until complete epithelialization (Trivellato Grassi, 2013).

Rate of wound size

A graph paper was used in order to measure and record the wound size and percentage of wound contraction. Wound contraction percentage and wound closure times were monitored on 4, 8, 12, 16 and 20 days to assess wound-healing property. The wound healing percentage was calculated by the Walker formula after measuring the wound size (Walker and Mason, 1968).

Percentage of wound size =Wound area on day X / Wound area on day 0 × 100.

Percentage of wound healing = 100 - Percentage of wound size.

Histopathology evaluation

For histological studies, skin's specimens were taken on 3, 7, 14 and 20 day after surgery. Samples were excised 1 to 2 mm in diameter and 3 mm in depth surrounding normal skin, fixed in neutral-buffered formalin 10%. Then tissues were routinely processed, embedded in paraffin wax, sectioned at 5 µm, and stained with hematoxylin and eosin stain. Then stained sections were evaluated by light microscope to assess the predominant stage of wound healing. Three parallel sections were obtained from each specimen. Factors such as cellular infiltration (the number of mononuclear cells, poly morph nuclear cells and fibroblastic aggregation), angiogenesis (the number of blood vessels and capillary buds), were quantitatively evaluated under high power (×400) (Güvenç et al., 2012; Küpeli Akkol et al., 2012). In addition, oedema, haemorrhage, re-epithelialization (epithelium thickness), collagen production and density were evaluated and scored qualitatively and calculated manually (Güvenç et al., 2012; Küpeli Akkol et al., 2012).

Statistical analysis

Data was analyzed by one way analysis of variance (ANOVA) using PASW 18.0 (SPSS, Inc., Chicago, IL, USA), and presented as mean ± SD. Model assumptions were evaluated by examining the residual plot. For treatment showing a main effect by ANOVA, means were compared by Dunnett's test. $P<0.05$ was considered as significant difference between treatments.

Results

Wound closure

The percentage of wound contraction is shown in Table 1. According to the results, after day 8, a significant increase in the rate of wound contraction was observed in the two treatment groups (2% and 4% RPO) compared to the control group. Interestingly, there was no significant effect between two concentrations of RPO groups on all days ($P>0.05$).

Histological evaluation

The qualitative and quantitative features of histological evaluation are presented in Table 2 and 3 respectively. Based on the qualitative results, the amount of oedema and haemorrhage in two treatment groups (2% and 4% RPO) was lower than control group; moreover, the amount of re-epithelialization and collagen deposition in treatment groups (especially group 2%), was higher than control group. As seen in

Table 1: Effects of hydroethanolic leaves extract of *Ribwort plantain* on wound size (mm^2)

Groups	Days				
	4	8	12	16	20
Control	442.1±29.9 (-10.29%)[a]	339.4±39.34(18.1%) [a]	145.1±15.91(63.30%)[a]	90.1±9.71(77.95%)[a]	17.2±3.25(94.3%)[a]
Placebo	436.6±20.68 (-9.91%)[a]	319.8±15.66(25.9%) [a]	119±9.78(69.9%)[a]	80.2±10.58 (80.1%)[a]	13.5±2.19(95.9%)[a]
RPO 2%	426.4±22.45 (-6.19%)[a]	282±22.9(34.8%) [b]	78.9±13.11(80.4%)[b]	27.4±5.02(93.9%)[b]	0.7±0.09(99.80%)[b]
RPO 4%	420.9±16.87 (-4.85%)[a]	296.4±39.03(28.9%)[b]	80.6±11.79(78.90%)[b]	30±9.09(90.5%)[b]	5±1.49(98.5%)[b]

Data are presented as the mean ± SD. There are significant differences between groups with different superscripts in the same column ($P<0.05$)

Table 2: Histological evaluation of *Ribwort plantain* hydroethanolic extract ointment on wound healing process (qualitative study)

Groups	Day	Oed score	Haem score	RE score	CD score
Control	3	++++	++++	–	–
	7	+++	++	–	+
	14	++	+	++	++
	20	–	–	+++	++
Placebo	3	++++	++++	–	–
	7	++	++	+	+
	14	+	+	++	+++
	20	–	–	+++	++
RPO 2%	3	++++	++++	–	+
	7	++	++	+++	+++
	14	+	–	++++	++++
	20	–	–	++++	+++
RPO 4%	3	++++	++++	+	+
	7	+	++	+++	+++
	14	++	-	++++	++
	20	–	–	++++	++

Hematoxylin and eosin stained section were scored as absent (−), present (+), mild (++), moderate (+++) and severe (++++) for Oed: oedema; Haem: Haemorrhage; RE: re-epithelialization; CD: Collagen deposition

Table 3: Mean distribution of vessels, immune cells and fibroblasts/millimetre square of the wound area

Groups	Day	NV	PMN	MNC	Fib
Control	3	2.75±0.71[a]	42.25±0.80[a]	1.75±0.70[a]	27.50±1.20[a]
	7	9.55±0.75[a]	32.00±3.29[a]	3.25±0.70[a]	39.50±1.8[a]
	14	2.75±0.70[a]	16.75±4.24[a]	2.00±0.41[a]	53.50±3.6[a]
	20	1.55±0.57[a]	1.00±0.25[a]	0.75±0.50[a]	15.75±1.75[a]
Placebo	3	5.51±0.19[a]	33.00±1.90[a]	1.00±0.81[a]	30.25±1.9[a]
	7	10.25±3.82[a]	29.75±2.95[a]	2.75±0.95[a]	40.00±5.6[a]
	14	3.25±0.95[a]	11.00±6.75[a]	1.00±0.81[a]	58.75±1.9[a]
	20	2.25±1.25[a]	0.50±0.50[a]	0.25±0.50[a]	24.00±9.4[a]
RPO 2%	3	16.51±0.74[b]	31.75±8.75[a]	9.00±0.16[b]	64.00±5.82[b]
	7	13.55±0.74[a]	18.34±3.25[b]	6.50±0.38[a]	97.50±2.08[b]
	14	6.50±0.65[a]	1.00±0.81[b]	0.57±0.05[a]	80.25±2.03[b]
	20	4.25±0.95[a]	0.50±0.07[b]	0.25±0.00[a]	33.75±1.13[a]
RPO 4%	3	15.90±0.50[b]	25.25±4.11[a]	8.00±0.82[b]	59.50±2.35[b]
	7	14.00±1.63[a]	13.25±1.31[b]	7.50±0.10[a]	89.75±3.79[b]
	14	6.75±0.57[a]	0.55±0.00[b]	0.75±0.05[a]	74.75±2.71[b]
	20	4.25±1.70[a]	0.25±0.00[a]	0.50±0.07[a]	28.75±1.50[a]

NV: number of new vessel formation; PMN: number of poly morph nuclear cells, MNC: number of mononuclear cells, and Fib: number of fibroblast cells. Data are presented as the mean ± SD. There is a significant difference between groups with different superscripts in the same column (P<0.05)

Table 3, based on the quantitative result, on the third day after wound creation, the mean number of the poly morph nuclear cells in RPO-treated groups, significantly decreased compared to the control group (P<0.05). At the same time, RPO caused a significant increase in the mean number of the mononuclear cells and new vascular formation especially at the rate of 2% RPO compared to the control group (P<0.05). According to the result, in RPO-treated animals, the mean number of the fibroblast infiltration into granulation tissue, significantly increased especially in 2% RPO compared to the control group (P<0.05).

Discussion

Wound healing, or wound repair, is the body's natural process of regenerating dermal and epidermal tissue. When an individual is wounded, a set of complex biochemical events take place in a closely orchestrated cascade to repair the damage. These events overlap in time and may be artificially categorized into separate steps: the inflammatory, proliferates, and remodelling phase (Martin, et al., 2009).

Chemical component such as coumarins, flavonoids and terpenes (Nostro et al., 2000), aucubin and catalpol (Reardon et al., 2009) cause therapeutic effects such as wound healing (Nayak et al., 2009; Süntar et al., 2011), antioxidant and anti-inflammatory (Fernandez et al., 2009) and antinociceptive action (Meotti et al., 2006). Flavonoids increase the viability of collagen fibre by inhibition of lipid peroxidation effect and promote the wound healing process (Getie et al. 2002; Shetty et al., 2008). Furthermore, there are other reports which support that the active components of *Ribwort plantain* promote epithelialization and wound contraction (Scortichini and Rossi 1991; Sasidharan et al., 2010). The first cells migrate to the wound site, are PMN, namely neutrophils. This cell, is one of the first-responders of inflammatory cells to migrate towards the site of inflammation. The act of this cell, is preventing of infection (Beldon 2010). Our results show that the topical administration of RPO causes to reduce the number of neutrophils. In this regard, it seems that *Ribwort plantain* hydroethanol leaf extract, due to its phenolic compounds, could reduce the infection. The increase in the mean number of mononuclear cell at wound site indicates reduction of inflammatory phase (Beldon 2010). Our result showed that at early stages (on day 3), decrease in neutrophil cell number was followed by an increase in the macrophages and lymphocytes in RPO-treated animals that illustrates the beginning of the second stage of the healing process.

New vascular bed is needed on the wound site to feed the proliferative phase (Beldon 2010). On the other hand, macrophage is known for secreting endothelial growth factor that stimulates the endothelial cell proliferation, which in turn enhances the new vascular formation in healing wounds. As seen in our result, in RPO-treated animals, the new vascular formation significantly increased compared to the control group. It may be inferred that RPO accelerates the healing process. Fibroblasts have the main role in the wound contraction by secreting collagens (Deodhar and Rana, 1997). Finally, contraction is needed for reducing time for the healing process. Increased wound contraction rate, following 8 days in RPO treated animals may be due to *Ribwort plantain.*

Conclusion

The results obtained in this study demonstrated that topical application of hydro ethanol leaves extract of *Ribwort plantain* at the rate of 2 and 4% reduced the healing time and enhanced wound contraction.

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