

Original article

***Rheum emodi* L. (Rhubarb) promotes wound healing by decreasing inflammatory markers and enhancing accumulation of biomolecules**

Sheikh Bilal Ahmad, Muneeb U. Rehman, Sheikh Parvaiz Ahmad*, Shazada Mudasir and Manzoor Ur Rahman Mir
Molecular Biology Lab., Division of Veterinary Biochemistry, Sher-e-Kashmir University of Agricultural Sciences and Technology,
Shalimar, Srinagar-190006, Jammu and Kashmir, India

*Department of Statistics, University of Kashmir, Hazratbal, Srinagar-190006, Jammu and Kashmir, India

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Abstract

The present study was carried out to investigate the wound healing properties of *Rheum emodi* L. (Rhubarb) aqueous extract in rabbits. Eighteen rabbits of either sex in the age group of 9-15 months were divided in three groups (A, B and C), each group comprising of six animals. The group A which served as control was treated with normal saline, the group B was treated with 0.2% nitrofurazone while group C was treated with aqueous extract of Rhubarb. The tissue collected on 4th, 9th and 14th day after creation of the wound, was estimated for hydroxyproline, glucosamine, protein and DNA. The blood samples were collected on day 1 and 7 and estimated for inflammatory markers; IL-2, IL-6 and TNF- α . Reduction in wound area was recorded on day 1, 4, 8, 11 and 14 after creation of the wound. Group B and Group C showed significant increase in the accumulation of hydroxyproline, glucosamine, protein and DNA as compared to group A animals. There was a significant decrease in IL-2, IL-6, TNF- α and wound area in group B and group C animals when compared to group A animals.

Key words: *Rheum emodi* L., wound healing, inflammatory markers, hydroxyproline, DNA, glucosamine, protein

1. Introduction

Herbal drugs play an important role for the cure of various ailments across the world. In developed countries, 25% of medical drugs are of plant origin (Principe, 2005), and the use of medicinal plants for treatment of various ailments in tribal areas of developing countries is in vogue from ancient times. Though many of the therapeutic properties attributed to plants, need validation through proper research to avoid unwanted health hazard. *R. emodi* locally known as pumba-chalan belongs to family, Polygonaceae and has traditionally been used as diuretic, laxative, tonic and to treat liver disorders, rheumatic pain, wound healing, fever, cough, abscess (impostume) and pimples (Irshad *et al.*, 2012). The plant has been cultivated for more than 5000 thousand years for its medicinal properties (Zargar *et al.*, 2011). The main parts explored as drug are roots and rhizomes. It is distributed in the temperate, sub-alpine and alpine of the Himalayas, especially in Asia and can be cultivated at an altitude of 2000 meters and above. The major phytoconstituents reported from the roots and rhizomes of the plant are anthraquinones (emodin, aloe-emodin, rhein, chrysophenol and physcion) anthrones, flavonoides, lignins, phenols, carbohydrates,

oxalic acid and stilbenes (Parvaiz *et al.*, 2009), tannins both hydrolysable and condensed, saponins, terpenes (Aslam *et al.*, 2012); trace elements K, Ca, Fe, Mn, Na, Zn, Co, Li and Cu (Singh *et al.*, 2010). Compounds isolated from *R. emodi* have been shown to possess antimicrobial (Babu *et al.*, 2003), antidiabetic (Radhika *et al.*, 2010; Arvindkar *et al.*, 2015), nephroprotective (Alam *et al.*, 2005), hepatoprotective (Akhtar *et al.*, 2009), antioxidant (Rajkumar *et al.*, 2010), anticancer (Kuo *et al.*, 2002), anti-inflammatory (Chuhan *et al.*, 1992), immunomodulatory (Kounser *et al.*, 2011), besides wound healing activity (Bilal *et al.*, 2014).

2. Materials and Methods

2.1 Plant material and extract preparation

Plant material was collected from the upper reaches of Sonamarag area of Kashmir, at an altitude of 3000 m and got identified from the centre of plant taxonomy, Department of Botany, University of Kashmir under voucher no. (Kash-bot/KU/Rh-SB-1746). The roots of the plant were washed to remove dust and dried under shade. The dried roots were grinded to fine powder. A weighed amount of powdered root was macerated in double distilled water and agitated occasionally, filtered with filter paper no 1 (Whatman no 1). The extract so obtained was stored at 4°C for further use.

2.2 Experimental animals

18 clinically healthy rabbits of either sex in the age group of 9-15 months were used as experimental animals. These were divided in

Author for correspondence: Dr. Sheik Bilal Ahmad
Associate Professor, Molecular Biology Lab., Division of Veterinary Biochemistry, Sher-e-Kashmir University of Agricultural Sciences and Technology, Shalimar, Srinagar-190006, Jammu and Kashmir, India
E-mail: sbilal@skuastkashmir.ac.in
Tel.: +91-9419400141

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three groups; A, B and C with six animals in each group. In group A which served as control, wounds were treated with normal saline, the wounds of group B animals were treated with 0.2% nitrofurazone while in group C wounds were treated with aqueous extract of *R. emodi*. All the animals were housed in the cages had access to fresh water at a room temperature of $22 \pm 2^\circ\text{C}$. A balanced feed was used throughout the period of study. The experimental protocols involved in this study were approved by the Institutional Animal Ethics Committee (Approval No: Au/FVS/PS-57/8308) and conforms to the guidelines for the Care and Use of Laboratory Animals, published by the US National Institute of Health (NIH Publication No. 85-23, revised, 1996). All the animals were acclimatized for a period of 7 days prior to the commencement of the experiments.

1.	Groups	A	B	C
2.	Number of animals	6	6	6
3.	Ingredient/preparation	Normal saline	0.2% Nitrofurazone	<i>Rheum emodi</i>

2.3 Excision wound model

The animals were sedated on the site with local anaesthetic xylocaine followed by dissociative anaesthetic ketamine, administered @40 mg/kg b.wt by intramuscular route. The anaesthetised animals were shaved on the dorsal aspect of the animal just distal to scapula. The shaved area was swabbed with alcohol and an area of 300 mm² was demarcated with a self-designed stamp on the midline of the shaved area. One wound was created on the midline of the back. The marked skin was excised with the help of a scalpel and scissors to the depth of loose subcutaneous tissue and wound was left undressed. Animals after recovery from anaesthesia were housed individually in properly disinfected cages.

2.4 Measurement of wound area

The wound area of each animal was measured at 2 h interval after the creation of wound. This interval was considered as day 1 measurement and the delay of 2 h after the creation of wound was allowed to accommodate the wound stretching that resulted due to the struggle of animal during recovery from the anaesthesia. The subsequent measurement was recorded on day 4, 8, 11 and 14. A firm but flexible transparent polythene rectangular (4 x 4 cm²) sheet was held just over the wound and its margins were marked with a permanent marker on sheet (Ahanger *et al.*, 2010). The result of wound measurements was expressed as absolute values and relative values or per cent wound contraction was calculated by the formula of Ahanger *et al.* (2010).

$$\text{Percent wound contraction} = \frac{\text{Day one wound area} - \text{unhealed wound area}}{\text{Day one wound area}} \times 100$$

2.5 Measurement of hydroxyproline and glucosamine

Hydroxyproline constitutes major portion of the collagen while glucosamine is an essential moiety of proteoglycans. On day 4, 9

and 14 post wounding a piece of healed tissue was removed and estimated for hydroxyproline and glucosamine by the method of Woessner, (1961) and Rondle and Morgan (1955), respectively.

2.6 Estimation of protein

The tissue was pulverized in ice-cold lysis buffer containing 100 mM Tris-HCl, 0.05 mM EDTA with a proportion of 10 μl of lysis buffer/ mg tissue with the help of chilled pestle and mortar and a pinch of glass wool. The tissues was thoroughly pulverized into a homogenous mixture and transferred to 1.5 ml micro-centrifuge tubes and centrifuged at 10000 rpm for 10 min. The supernatant protein lysate was collected for estimation of protein and estimated by the method of Lowry *et al.* (1951).

2.7 DNA extraction and estimation

A piece of healed tissue collected on day 4, 9 and 14 after creation of the wound was grinded to give a fine powder with the help of chilled pestle and mortar. The DNA from homogenized tissue was estimated by Phenol-Chloroform method (2001).

2.8 Estimation of inflammatory markers

Blood samples were collected on day 1 and 7 from all animals of each group after formation of the wound and estimated for IL-2, IL-6 and TNF- α by commercially available ELISA kits (Abcam Inc. USA) as per the instructions of the manufacturer. The results were expressed as pg/ml by plotting the graph for standard.

2.9 Statistical analysis

The values presented are the mean \pm SEM for each group of the animal. Statistical analysis was performed with one way ANOVA followed with Newman-Keuls Multiple Comparison test. A value of $p < 0.05$ was considered to be statistically significant (Snedecor and Cochran, 1989).

3. Results

3.1 Effect of *R. emodi* on wound contraction

In the experimental protocol, the wound area was strictly adhered to 300 mm² but the actual measurements revealed variations because of the elastic nature of the skin. Figure 1 reflects the trend in the reduction in wound area and allows an insight into the wound contraction. The mean wound closure was recorded on day 1, 4, 8, 11 and 14. *R. emodi* treated wound showed significant increase on day 4 (33.54%) on day 8 (52.84%) on day 11 (74.07%) and (90.18 %) on day 14. Similar effects were observed in rabbits that were treated with 0.2% (w/w) nitrofurazone ointment. In comparison the wounds that received normal saline showed (19.3, 39.87, 62 and 77.84%) reduction in the wound area. The changes in the wound area on day 4, 8, 11 and 14 are shown in Figure 2.

3.2 Effect of *R. emodi* on hydroxyproline and glucosamine

Levels of hydroxyproline and glucosamine are depicted in Figures 3 and 4, respectively. *R. emodi* and nitrofurazone treated wounds showed significant increase in both hydroxyproline and glucosamine content on day 4, 9 and 14 during the healing of wounds as compared to normal saline treatment group.

3.3 Effect of *R. emodi* on protein and DNA content

Protein and DNA contents of *R. emodi* and nitrofurazone treated wounds exhibited significant increase on day 4 and 9 as compared to normal saline treated wounds (Figures 5 and 6). However, on day 14 the DNA content of normal saline treated wounds was higher than *R. emodi* and nitrofurazone treated wounds while there was significant increase in the protein content of normal saline treated wounds on day 14 as against *R. emodi* and nitrofurazone treated wounds.

3.4 Effect of *R. emodi* on inflammatory markers

There was no significant difference in IL-2, IL-6 and TNF- α on day 1 between normal saline and *R. emodi* treated wounds as shown in Table 1. However, on day 7, *R. emodi* treated wounds showed significant decrease in all the inflammatory markers when compared to normal saline treated wounds. The nitrofurazone treated wounds exhibited significant decrease in IL-2, IL-6 and TNF- α on day 1 and 7 as against normal saline treated wounds.

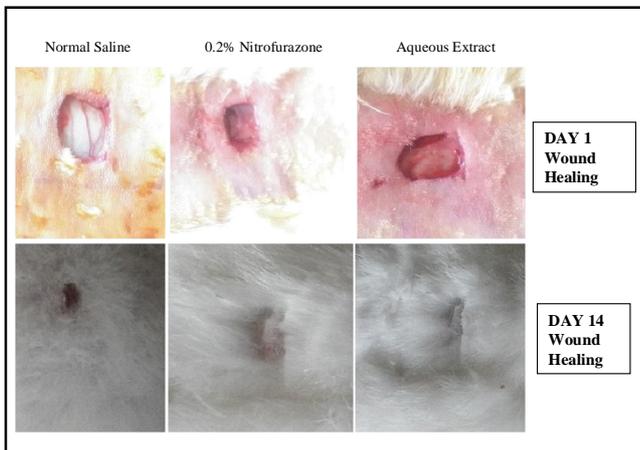
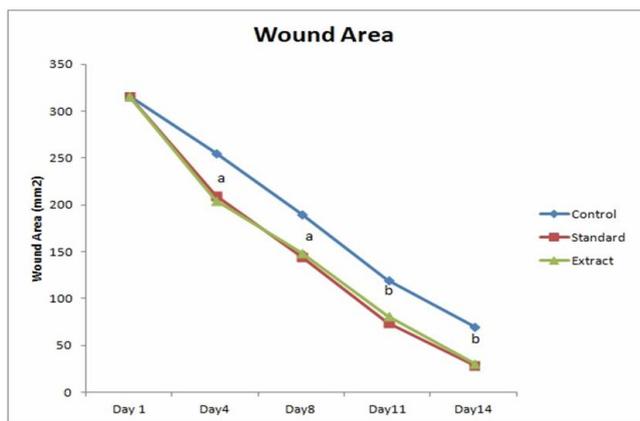
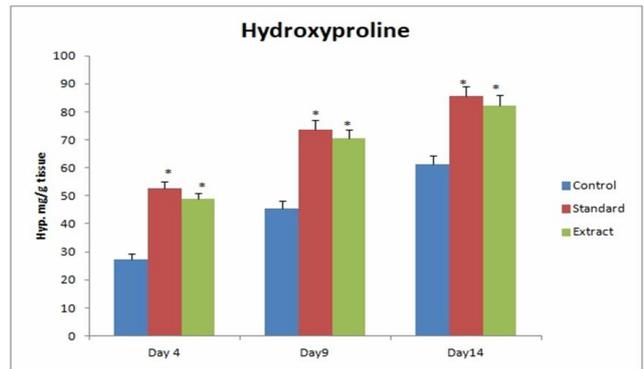


Figure 1: Changes in wound area treated with normal saline, 0.2% nitrofurazone and aqueous extract of *R. emodi* on day 1 and day 14.



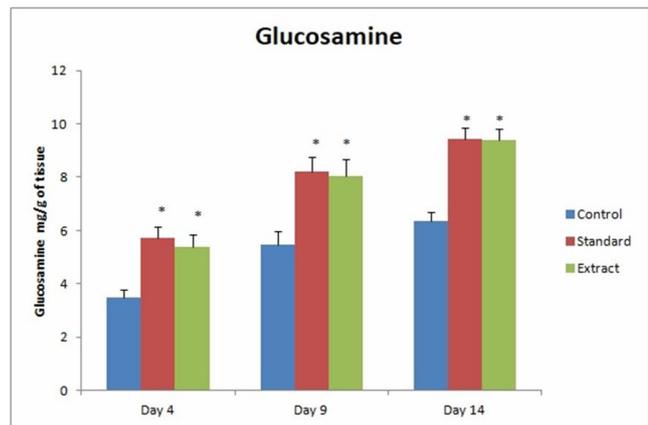
Values are expressed as mean \pm SE; n=6 animals in each group. Analysis done with one-way ANOVA and Newman-Keuls Multiple Comparison test values are significant at a $p < 0.05$ and b $p < 0.01$, respectively.

Figure 2: Effect of aqueous extract of *R. emodi* on wound contraction.



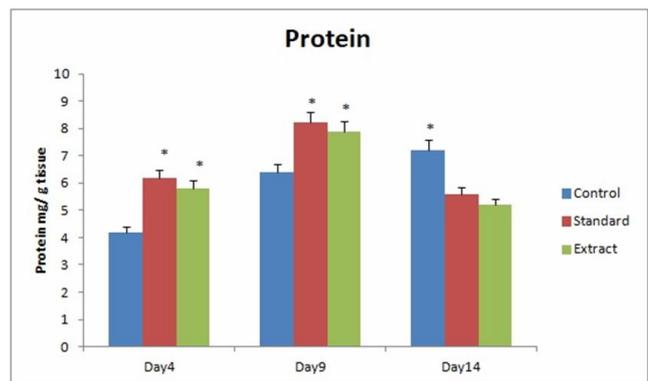
Values are expressed as mean \pm SE; n=6 animals in each group. Analysis done with one-way ANOVA and Newman-Keuls Multiple Comparison test indicates significance at $p < 0.05$.

Figure 3: Effect of aqueous extract of *R. emodi* on hydroxy-proline level in different groups.



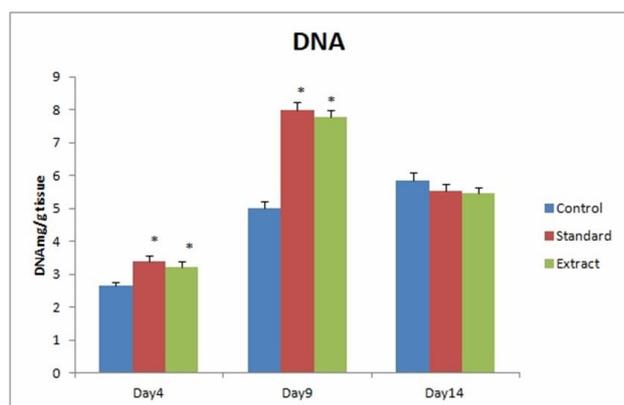
Values are expressed as mean \pm SE; n = 6 animals in each group. Analysis done with one-way ANOVA and Newman-Keuls Multiple Comparison test indicates significance at $p < 0.05$.

Figure 4: Effect of aqueous extract of *R. emodi* on glucosamine level in different groups.



Values are expressed as mean \pm SE; n=6 animals in each group. Analysis done with one-way ANOVA and Newman-Keuls Multiple Comparison test indicates significance at $p < 0.05$.

Figure 5: Effect of aqueous extract of *R. emodi* on protein content in different groups.



Values are expressed as mean \pm SE; n=6 animals in each group. Analysis done with one-way ANOVA and Newman-Keuls Multiple Comparison test indicates significance at $p < 0.05$.

Figure 6: Effect of aqueous extract of *R. emodi* on DNA content in different groups.

Table 1: Effect of aqueous extract of *R. emodi* on inflammatory cytokines

Treatments	Post-wounding day	IL-2 (pg/ml)	IL-6 (pg/ml)	TNF- α (pg/ml)
Normal saline	Day 1	108 \pm 16.2	112 \pm 18.6	256 \pm 19.2
	Day 7	96.7 \pm 12.5	101 \pm 14.9	221 \pm 17.6
Aqueous extract	Day 1	101 \pm 12.9	98.9 \pm 12.7	239.6 \pm 18.9
	Day 7	58.6 \pm 9.73 ^b	73.5 \pm 9.37 ^b	166.2 \pm 13.7 ^b
0.2% Nitro-furazone (w/w)	Day 1	76.2 \pm 11.7 ^a	79.2 \pm 11.2 ^a	203 \pm 11.4 ^a
	Day 7	49.3 \pm 8.43 ^b	67.4 \pm 10.3 ^b	155.4 \pm 20.5 ^b

Values (mean \pm SE) were obtained from each group of 6 animals. a $p < 0.05$ and b $p < 0.01$ compared to the values of normal saline-treated rats on the indicated day in each group, respectively.

4. Discussion

Measurement of wound contraction is an important tool for assessing the progress of wound healing. Wound repair is initiated with the migration and proliferation of keratinocytes forming a new epithelial layer (Falanga, 2005). The healing process depends on the controlled biosynthesis, deposition and maturation of collagen (Nayak *et al.*, 2006). Hydroxyproline is the major component of the collagen while glucosamine is an essential moiety of proteoglycans. Therefore, estimation of hydroxyproline and glucosamine indicates the quality and quantity of the extra cellular matrix. The increased levels of hydroxyproline and glucosamine with *R. emodi* provided the strength to the regenerated tissue. This enhancement in extra cellular matrix content as inferred from higher estimates of hydroxyproline and glucosamine could be attributed to the protective effects offered by phytoconstituents to fibroblast against oxidant insult and apoptosis (Ahanger *et al.*, 2010). The increase in hydroxyproline and glucosamine content with phytochemicals such as flavonoids, tannins and emodin had also been reported by Manjunatha *et al.* (2005).

Protein and DNA synthesis during wound healing shorten the inflammatory phase; promote fibroplasias, collagen and proteoglycan synthesis, wound remodeling and tensile strength of the wound (Chernof, 2004). The *R. emodi* treated wounds revealed a significant increase in the DNA content concomitant with this, there is an increase in the total protein content indicating active synthesis and deposition of matrix proteins in the granulation tissue. The maximum incorporation in DNA and protein occurred on the 9th day after creation of the wound. It is possible that the need for new proteins is high and the wound size has not decreased enough to increase the level of inhibition that may occur with contact inhibition. The results are in concurrence with the Getie *et al.* (2002). The increase in the DNA and protein content may be as a result of increased cell division. This may be attributed to the antioxidant role of *R. emodi*; antioxidants not only prevent or slow down the onset of cell necrosis but also improves vascularity (Nayak *et al.*, 2006; Kounser *et al.*, 2011), increase the viability of collagen fibrils by increasing the strength of collagen fibrils, increasing the circulation, preventing the cell damage and promoting the DNA synthesis (Getie *et al.*, 2002).

Strong induction of IL-2, IL-6 and TNF- α resulted in 24 h after dermal injury; these are the essential players of inflammatory phase of wound healing, enhancing angiogenesis. Treatment with *R. emodi* did not interfere with first phase of wound healing; however on day 7, the levels of these inflammatory markers were reduced. The inflammatory markers are reported to inhibit the collagen formation and hydroxyproline synthesis which are essential for the proliferative phase of wound healing (Siqueira *et al.*, 2010). These findings indicate that *R. emodi* balances inflammatory markers as well thereby enhances wound healing.

5. Conclusion

A large number of studies have demonstrated that various phytoconstituents such as flavonoids, triterpenoids, tannins, emodin and aloe-emodin can enhance wound healing (Havsteen, 2002; Souza *et al.*, 2007). Thus, wound healing property of *R. emodi* may be attributed to the phytoconstituents present in it, which may be either due to individual or additive effect of it that fastens the process of wound healing. Another reason for increased accumulation of DNA and protein at the wound site by *R. emodi* may be due to the presence of Zn. Approximately, 300 enzymes requires Zn for their activities. Zn is an essential trace mineral for DNA synthesis, cell division, protein synthesis, tissue regeneration and repair (Prasad, 1995; Singh *et al.*, 2010). It also reduces inflammatory phase, accelerates granulation tissue formation and epidermal cell proliferation.

Conflict of interest

We declare that we have no conflict of interest.

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