www.roavs.com EISSN: 2223-0343
RESEARCH OPINIONS IN ANIMAL & VETERINARY SCIENCES

Evaluation of the wound healing activity of methanolic extract of *Tragopogon porrifolius* in rat

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Abstract

This study was undertaken to evaluate the wound healing properties of *Tragopogon porrifolius* (TP) on cutaneous wounds in rat. A full-thickness cutaneous defect (2×2cm) was induced on the back of 60 Sprague-Dawley rats. The animals were randomly divided into four equal groups, treated with 1ml basal cream (placebo group), 1ml tetracycline (3%), 1ml TP 10% and untreated (control). Five animals of each group were euthanized at each of 7, 14 and 21 days post-injury (DPI) and wounds were evaluated through histopathological analyses. Treated animals with TP showed a significant reduction in the wound surface area at 14 and 21 DPI compared to other groups. In addition, treatment with TP reduced the number of lymphocytes and enhanced the number of fibroblasts at the earlier stages of wound healing and increased number of fibrocytes at the later stages of wound healing. TP significantly improved alignment of the healing tissue, re-epithelilization and epithelial formation, enhanced maturity of the collagen fibres and fibroblasts and large capillary-sized blood vessels. The present study demonstrated that the methanolic extract of *T. porrifolius* promoted wound healing activity in animal as a preclinical study. These results showed that application of TP extract on wounds induces significant wound contraction and accelerated healing and it may be suggested for treating various types of wounds in animal and human beings.

Keywords: *Tragopogon porrifolius*; methanolic extract; wound healing; rat

To cite this article: Hashemnia M, M Javdani, Z Nikousefat, F Hoseinpour and S Kakaei, 2014. Evaluation of the wound healing activity of methanolic extract of *Tragopogon porrifolius* in rat. Res. Opin. Anim. Vet. Sci., 4(8): 446-452.

Introduction

Wound healing, the result of a complex tissue repairing process, is a continuing challenge in rehabilitation medicine. Despite some recent advances in understanding its basic principles, healing of wound defects has also faced with significant limitations including scar tissue formation and cosmetic concerns (Ruszczak and Schwartz, 2000; Stavrou, 2008).

The quest for better wound healing agents is perhaps one of the oldest challenges for researchers. Because of the high content of tannins, saponins, flavonoids, naphthaquinone, triterpenes and alkaloids in the medicinal plants, they have been used for many years in treatment of cutaneous wounds to increase the quality and rate of healing (Udupa et al., 1994; Ohshima et al., 1998; Baie and Sheikh, 2000; Nayak et al., 2007; Abenavoli et al., 2010; Tong et al., 2011; Oryan et al., 2012).

Traditional forms of medicine practiced for centuries in Africa and Asia are being scientifically investigated for their potential in the treatment of wounds related disorders (Krishnan, 2006). The herbal drugs are prescribed widely because of their effectiveness, fewer side effects and relatively low cost. They also have comprehensive ability to compete with chemical drugs and are more tolerated by patients during treatment period (Ayyanar and Ignacimuthu, 2009).

Tragopogon porrifolius (TP) Known as “sheng” from Compositae (Asteraceae) family is widely consumed as a green vegetable in the west of Iran. It

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has also gained the alternate name “goat’s beard” due to the appearance of its foliage and that of “vegetable oyster” due to the taste of its roots (Tenkerian, 2011).

Tragopogon genus is used for poison elimination and as astringent and wound healer, aseptic property, bleeding inhibitor, healing digestive bleeding and pulmonary and digestive ulcer (Farzaeia et al., 2013).

In different nations, Tragopogon genus is used as anticough, astringent, skin repairing and in the treatment of gastric disorders (Guarrera, 2003; Singh and Lal, 2008). It has also been recently reported that *T. porrifolius* was found to have anticancer, antioxidant, hepatoprotective and moderate radical scavenging activities (Tenkerian, 2011). Active constitutes of *T. porrifolius* are flavonoids which consist of apigenin, luteolin, quercetin, vitexin, isovitexin, vicenin-1and 2, swertisin, orientin, isoorientin, and lucenin; various types of bibenzyl and dihydroisocoumarin derivatives; chlorogenic acid and 3,5-dicaffeoylquinic acid as well as a number of acylated pentacyclic triterpene saponins (Zidorn et al., 2005; Sareedenchai et al., 2009). Vitamin C, K and E have also been isolated from some Tragopogon species (Vardavas et al., 2006).

Based on the present review, Tragopogon genus’s anti-oxidative and anti-inflammatory properties make it a logical adjuvant to improve wound healing. Therefore, the present study was undertaken to evaluate the dermal wound healing potential of *T. porrifolius* after topical application of its methanolic extract on experimentally induced cutaneous wounds in rat models.

Materials and Methods

Plant material and extract preparation

The *Tragopogon porrifolius* plant material was purchased from a retail food store (Kermanshah, Iran) in March 2013. To make the gel thicker, the plant’s leaves were given sufficient time to be relatively dried at room temperature without exposure to direct sunshine. The dried plants were then cut to small pieces and 300 grams were soaked for 72 hours in methanol and then filtered. This process was repeated twice to ensure maximal extraction. After extraction, the solvent was filtered and then evaporated by Rotavapor®. The obtained extract was then stored at -20°C until being used.

Animals

The experiments were performed in adult Sprague-Dawley rats of both sexes, weighing 200 to 220 g. The animals were housed under standard environmental conditions (23±1°C, with 55±5% humidity and a 12 h light/dark cycle) and maintained with free access to water and *ad libitum* standard laboratory diet (70% carbohydrates, 25% proteins, 5% lipids).

Wound creation

The rats were weighed prior to the surgical procedure. The animals were anaesthetized by intramuscular injection of 1mg/kg xylazine HCl (Xylazine 2%; Alfasan) as premedication, and 60 mg/kg ketamine HCl (Ketamine 5%; TRITTAU, Germany) for anesthesia. The backs of the animals, in the cervical region of each animal, were surgically prepared for aseptic surgery. An excision wound was made by cutting out a 2×2 cm piece of skin from the shaved area. The wounds were of full thickness type extending up to the subcutaneous tissue.

Study design

All clinical observations, measurements and analysis of results were undertaken by investigators who were blinded to the experimental design and group allocation. After wound creation, the animals were randomly divided into four main groups, each containing 15 animals, and three subgroups, representing days, 7, 14 and 21 after injury. The groups were numbered as follows: control (1–3), basal cream (4–6), tetracycline (7–9), *T. porrifolius* (10–12). In the control group, no material was used at the injured area, which was left uncovered. In the basal cream and tetracycline groups, the injured area was covered with 1ml basal cream (eucerin) and tetracycline (3%) daily, for 14 days post injury (DPI). In the group 4, the injured area was covered with 1ml TP 10% (10 g *T. porrifolius* powder was suspended in 90 g eucerin) for 14 DPI. Five animals of each group were euthanized at each of 7, 14 and 21 DPI. Samples from all these groups were collected and used for histopathological evaluation.

Measurement of wound area

The progressive changes in wound contraction were monitored planimetrically on days 7, 14 and 21 PI using method as described by Oryan et al. (2008).

Sample collection and histological evaluation

At the end of days 7, 14 and 21 postoperation, the animals were euthanized by IV injection of 50 mg/kg thiopental sodium via tail vein and sampling was done. Tissue samples from both the wound and comparable normal adjacent skin including dermis, epidermis and subcutaneous were carefully dissected and fixed in 10% neutral-buffered formalin, processed routinely, embedded in paraffin, sectioned at 5 μm thickness, stained with hematoxylin and eosin and studied with a routine light microscope. Histological examinations were performed in a double-blind fashion with a procedure reported by Oryan et al. (2012) with some modifications. The pictures were then recorded by a digital camera (Dino capture; version 1.2.7) and transferred to the computer software (Photoshop CS-4;

Adobe) for digital analysis. Five photomicrographs, equivalent to five microscopic fields from each tissue sample, were used for histopathologic analysis. The criteria that were studied in histopathological sections consisted of hemorrhage, fibrin deposition, polymorphonuclear cell and mononuclear cell infiltration, reepithelialization, cornification of the epithelium, fibroblast and macrophage content, revascularizations, necrosis, presence of fibrocytes and maturation and organization of collagen. Total cellularity (magnification ×200) and number of fibroblasts, fibrocytes, neutrophils, lymphocytes, macrophages and blood vessels (magnification×800) of the injured area were counted and their mean and standard deviations were calculated.

Statistical analysis

Descriptive statistics including the mean, standard error, median, minimum and maximum were calculated for all variables. The one-way ANOVA followed by Turkey post hoc test were used for comparison of different parameters. The data were analyzed by SPSS software, version 16.0 (SPSS Inc., Chicago, IL, USA) and P<0.05 was accepted as statistically significant.

Results

Wound surface area was calculated and expressed in cm^2 as shown in Table 1. There was a significant reduction in the wound surface area of the TP group on days 14 and 21 compared to those of the control and cream groups (Fig. 1a-l).

The data from the histopathologic analysis are shown in Table 2. Seven days after injury, treatment with TP and tetracycline significantly reduced total number of cells compared with the cream and control groups (P<0.05; Fig. 1a–d), however, the mean number of cells in the TP group was lower than that in the tetracycline group.

Table 1: Mean ± SD of wound surface area (cm^2) in groups on different days post-injury

Days	Control	Basal cream	Tetracycline[c]	TP[d]
Day 7	2.46±0.12	2.53±0.15	2.25±0.09	2.22±0.16
Day 14	1.5±0.18[a]	1.34±0.14[b]	1.09±0.17[c]	0.93±0.12[d]
Day 21	0.49±0.09[a]	0.47±0.13[a]	0.28±0.06[b]	0.16±0.05[b]

Characters (a=control, b= basal cream, c= Tetracycline, d=TP); Values bearing different superscripts in a row differ significantly (P<0.05).

Table 2: Histopathologic and histomorphometric analysis

Day 7	Control	Basal cream	Tetracycline[c]	TP[d]
Total cell	419±14.3[a]	391±10.8[b]	350±14.6[c]	321±6.4[d]
Vascular no.	13.8±2.8[d]	11±1.5[c]	16±2.5[b]	18±2.2[a]
Fibroblast and fibrocytes	18.4±2.5[b]	20.4±3[a]	11.8±0.8[d]	17.6±2.4[c]
Fibrocytes	3.4±1.1	5±1.5	2.4±1.1	4.2±1.4
Fibroblasts	15±1.5[a]	15.4±1.5[a]	9.4±1.9[B]	13.4±1.8[a]
Ratio	0.22±0.06	0.31±0.07	0.28±0.1	0.31±0.1
Lymphocyte	20.4±2.07	19.4±1.1	21.8±2.3	20.2±1.9
Macrophage	19.8±2.2	17.6±2.4	21.6±2	20.8±1.9
Neutrophil	0.8±0.8	1±0.2	0.4±0.5	0
Day 14				
Total cell	270±9.2[a]	223±12[b]	208±10[c]	201±10[c]
Vascular no.	9.2±1.4[b]	7±1.5[c]	6.2±1.9[c]	11.6±3.2[a]
Fibroblast and fibrocytes	20.4±2[c]	21.2±3.3[c]	27±3.3[b]	31.8±2.6[a]
Fibrocytes	6.8±1[c]	9.2±1.4[c]	16±2.6[b]	20.6±1.1[a]
Fibroblasts	13.6±1.5	12±1.8	11±1.8	11.2±2.5
Ratio	0.50±0.08	0.76±0.01	1.48±0.3	1.92±0.4
Lymphocyte	15.8±1.6	14±1.2	14±3.08	9.6±2.5
Macrophage	25.8±2.2[a]	21.8±2[b]	14.8±1.3[c]	10.8±2.2[c]
Neutrophil	0.8±0.2	0	0	0
Day 21				
Total cell	194±9.8[a]	169±8.4[b]	106±9.6[c]	92±5.7[d]
Vascular no.	5.2±1.9	5±1.5	4.6±1.1	4±1.5
Fibroblast and fibrocytes	23.6±0.54[c]	20±1[c]	29±4.2[b]	44±5.7[a]
Fibrocytes	16±1.2[cd]	12±0.8[cd]	22.2±2.7[d]	32.8±3.5
Fibroblasts	7.6±1.1	7.2±0.8	6.8±1.4[d]	11.2±3.3
Ratio	2.16±0.5	1.80±0.2	3.31±0.3	3.13±0.8
Lymphocyte	17.4±1.8[a]	13.8±1.6[b]	11.2±1.3[c]	9.2±1.4[c]
Macrophage	18.8±2.2[a]	16±1.2[b]	12.4±1.1[c]	10±1.2[c]
Neutrophil	0	0	0	0

Five fields in each of five histopathologic sections were analysed for each group; Characters (a=control, b= basql cream, c= Tetracycline, d=TP) Values bearing different superscripts in a row differ significantly (P<0.05).

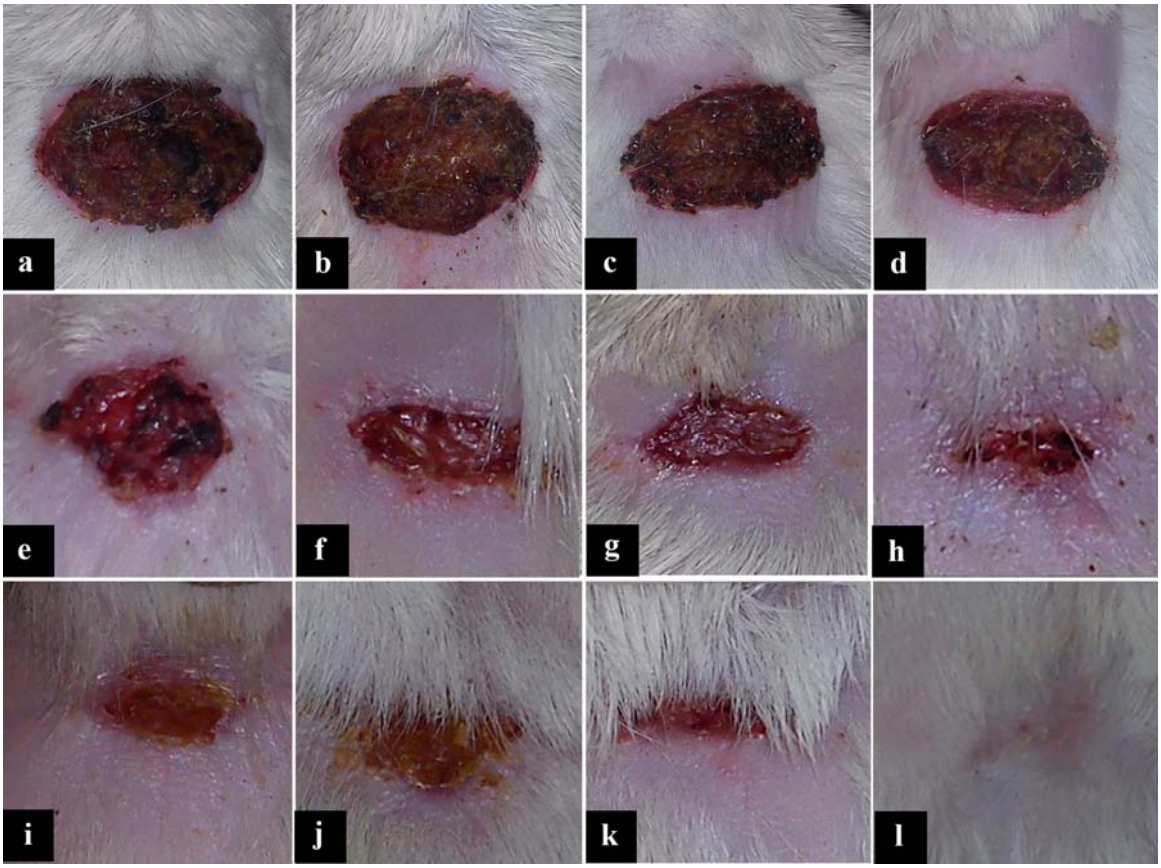

Fig 1: Macroscopic wound images of the control (a,e,i,), basal cream (b,f,j), tetracycline (c,g,k) and TP (d,h,l), on days 7, 14 and 21 post-injury

Fourteen days after injury, animals in TP and tetracycline groups showed significantly lower total number of cells than the control group (P<0.05; Fig 1e-h). Further, it was not statistically significant from the cream group at this stage (P=0.84 and P=0.579; Fig. 1f).

Twenty-one days after injury, TP significantly reduced the total number of cells compared with control but the difference was not significant for the tetracycline group (P=0.620; Fig. 1i-l).

Although TP significantly increased the vascular number in the lesions on day 7 and 14 PI compared with the cream and tetracycline (P<0.05). The number of fibrocytes was significantly higher in the TP and tetracycline groups compared with the cream and control groups on day 14 and 21 PI (P<0.05). In addition, at 14 and 21 DPI, the number of fibrocytes was significantly higher in the TP group compared with the tetracycline group (P=0.014 and P= 0.001).

The mean number of fibroblasts in the TP group was higher than that in the other groups on day 21 PI, but these differences were not significant.

The number of lymphocytes decreased significantly in the TP group compared with the control, cream and tetracycline groups on day 14 PI. At 21 DPI, TP significantly reduced the number of lymphocytes compared with the control and cream groups.

Treatment with TP and tetracycline significantly decreased the number of macrophages compared with the control and cream groups at 14 and 21 DPI (P<0.05); however, the animals in TP group showed a greater reduction in the number of macrophages compared to the tetracycline group at these stage, but these differences were not significant (at 14 DPI: P= 0.075; at 21 DPI: P=0.714).

At 7 DPI, the newly formed collagens were still unorganized and showed a haphazardly distributed pattern in all animals at this stage. The blood vessels within the granulation tissue area were prominent and dilated in all groups. In control and cream groups failure of re-epithelization with persistence of the wound area was seen, while animals in TP group showed minimal re-epithelization and many mitotic figures. In control, cream and tetracycline groups neutrophils, were still detected within the granulation tissue in the absence of signs of infection around the wound area.

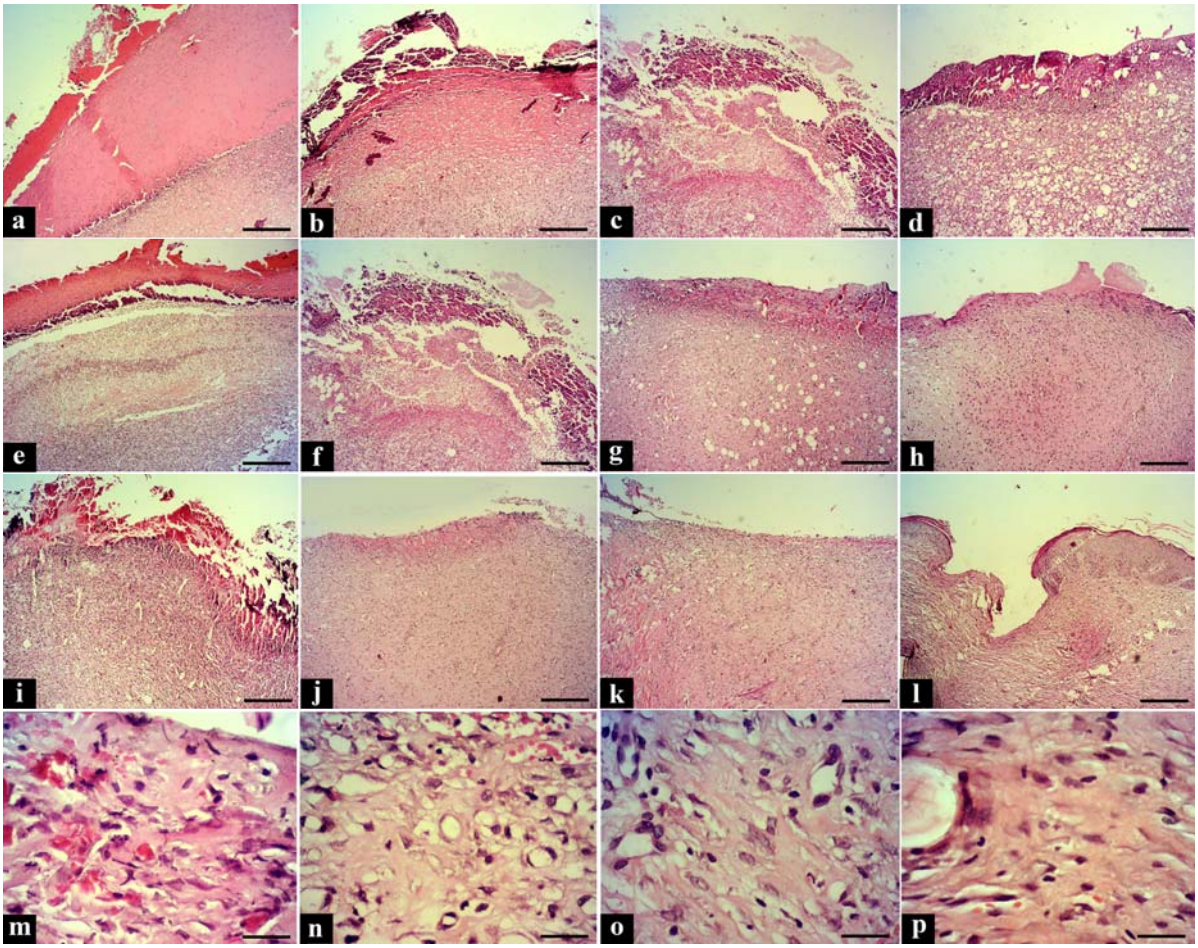

Fig. 2: Longitudinal sections of the control (a,e,i,m), basal cream (b,f,j,n), tetracycline (c,g,k,o) and TP (d,h,l,p), on days 7, 14 and 21 post-injury (scale bar for a–l=600µm, and for m–p=150µm)

At 14 DPI, the collagen fibers showed a more organized pattern and the tissue alignment was greater in the TP group as compared to the other groups. There was full thickness epidermal regeneration which covered completely the wound area. The epidermis was thick and disorganized, especially when compared with the adjacent normal skin. In the control group, the dermis was cellular, with proliferation of fibroblasts, laying down disorganized and poorly oriented collagen fibers. There was no evidence of pus accumulation, or polymorphonuclear cell infiltration, fibrin deposition or edema in the lesions of animals in all groups in this stage.

At 21 DPI, the histopathological sections of the treated animals with TP showed a proper re-epithelilization and epithelial formation. In addition, a lower number of lymphocytes and macrophages, significantly greater maturation, better tissue alignment and large capillary-sized blood vessels were prominent in TP group compared to other groups (Fig.1m-p).

Discussion

Wounds are referred to as disruption of normal anatomic structure and function. Skin wounds could happen through several causes like physical injuries resulting in opening and breaking of the skin (Gerald et al; 1994). Wound healing is an orderly progression of events that establish the integrity of the tissues. The aim in these processes is to regenerate and reconstruct the disrupted anatomical continuity and functional status of the skin. Healing process, a natural body reaction to injury, initiates immediately after wounding and occurs in four stages including coagulation, inflammation, re-epitheliasation and remodeling (Phillips et al., 1991). Many studies have shown that plant products are preferred in wound healing since they are devoid of side effects and are more effective (Jagetia et al., 2013).

T. porrifolius has been shown to have some promising positive effects, due to its antioxidant activity in both *in vitro* and *in vivo*, as well as an anticancer effect against MDA and Caco-2 cell lines. It also exhibited a hepatoprotective potential against liver

toxicity in rats (Tenkerian, 2011). However, to our knowledge, this is the first time *T. porrifolius* has been used on experimentally induced cutaneous wound defects in rats and, except for the anti-inflammatory potential of this plant; there is no information about other beneficial effects on wound healing.

Topical application of *T. porrifolius* at the wound site produced significant wound healing activity, which may be due to its angiogenic and mitogenic potential. Its prohealing activity was conspicuous as all the observed healing parameters were significantly affected. The inflammatory processes of the treated samples displayed enhanced fibroplasia and remodeling stages of wound healing. Decreased total cellularity and improved fibroblast maturation and differentiation in the wound area were also observed.

In this study, a significant increase in wound contraction was observed in TP group compared to control. Wound contraction is the process of mobilizing healthy skin surrounding the wound to cover the denuded area and involves complex and superbly orchesterated interactions of cells, extracellular matrix and cytokines. This centripetal movement of wound margin is believed to be due to the activity of myofibroblast (Kumari et al., 2010). Since *T. porrifolius* improved wound contraction, it would have either enhanced contractile property of myofibroblasts or increased the number of myofibroblasts recruited into the wound area.

The results on day 7 PI showed that healing was well underway in TP and tetracycline groups compared to other groups considering wound contraction and cell proliferation, which are shown in Tables 1 and 2. Although the results showed the beneficial effects of the *T. porrifolius* on the morphology of dermal wound healing in rats on day 7 PI, but the newly formed collagens were still unorganized and showed a haphazardly distributed pattern in all animals at this stage.

It seems at this stage the newly formed collagen fibers are immature; their cross-links were not properly developed and are mostly of type III collagen with small sized collagen fibrils that have a haphazard pattern of distribution (Chithra et al., 1998). Collagen is the predominant extracellular protein in the granulation tissue of wounds. The synthesis of collagen is increased in the wound area immediately following injury. Collagen plays a role in haemostasis and in providing strength and integrity to the wound matrix. It is also essential for re-epithelialisation and cell–cell and cell–matrix interactions (Raghow, 1994; Chithra et al., 1998).

On day 14 PI, higher rate of wound contraction together with significant improvement of histopathological features and full epidermal regeneration were observed in TP group compared to control and cream groups.

The enhanced rate of wound contraction and reduction in cellularity and healing time in treated rats might be due to the anti-inflammatory effects of *T. porrifolius* together with its effect on maturation and organization of the granulation tissue.

A greater degree of organization of the collagen orientation in the treated lesions and a more normal alignment of new collagen in this stage may be due to a modification of the inflammatory reaction or organization of the fibrin network in the tissue spaces at early stages of inflammatory phase of healing by the *T. porrifolius* extract (Oryan et al., 2008).

On day 21 PI, a proper re-epithelilization and epithelial formation were seen in the histopathological sections of the treated animals with TP. In addition, a lower number of lymphocytes and macrophages and higher number of fibroblasts and fibrocytes, significantly greater maturation, better tissue alignment and large capillary-sized blood vessels were prominent in extract group compared to other groups.

Since the fibroblast and fibrocyte count of the treated lesions increased significantly in the present experiment, it seems that the most possible wound healing activity of TP extract is proliferation and stimulating of fibroblasts directly.

The present study demonstrated that the methanolic extract of *T. porrifolius* promote wound healing activity in animal as a preclinical study. These results showed that application of TP extract on wounds induces significant wound contraction and accelerates healing and it may be suggested for treating various types of wounds in animal and human beings. Further study on the fractionation of active components of this plant may provide a better understanding its mechanism in the wound healing process.

Acknowledgment

We thank the authorities of Veterinary School, Razi University for their cooperation. This work was supported, in part, by grants from Vice Chancellor for Research at Razi University (Grant No. 56828).

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