Iranian Veterinary Surgery Association

Iranian Journal of Veterinary Surgery

Journal homepage: www.ivsajournals.com

Original Article

Effect of Extract of *Allium stipitatum* on Excisional Wound Healing in Rats

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ARTICLE INFO

Article History:

Received 16 June 2020
Revised 20 September 2020
Accepted 28 September 2020
Online 28 September 2020

Keywords:

Herbal extract
Allium stipitatum
Wound healing
Full-thickness wounds

ABSTRACT

The aim of the present study was to assess the wound-healing activity of extract of *Allium stipitatum*. Thirty-six male Wistar rats were used in this study. The rats weighing approximately 160-180 g and seven weeks of age were randomized into three groups of 12 rats each: Control surgery group (Control) including the creation of wounds and no treatment, base formulation groups positive (POS) with the creation of wounds and application of base formulation ointment, treatment group 1 (T1) with 2 g of powder extract of the plant material in the ointment. A wound was induced by an excisional based wound model in male rats. The mature green leaves of *Allium stipitatum* were collected and authenticated. Extractions of dried leaves were carried out. For wound-healing activity, the extracts were applied topically in the form of ointment and compared to control groups. The healing of the wound was assessed based on the wound area, histomorphometry, and hydroxyproline estimation studies. Reduction in the wound area and hydroxyproline contents indicated that there was a significant difference (p = 0.001) between group T1 and other groups. Quantitative histological studies and mean rank of the qualitative studies demonstrated that there was a significant difference between group T1 and other groups (p = 0.001). The extract of *Allium stipitatum* leaves enhanced wound-healing activity significantly in both the wound models studied. Enhanced wound contraction, decreased epithelialization time, increased hydroxyproline content, improved histological characteristics studies suggested *Allium stipitatum* leaves extract might have therapeutic benefits in wound healing.

Introduction

Traditional medicine is often described by practitioners of modern (western) medicine using skeptical terms such as alternative, nonconventional, indigenous, and complementary. In fact many of the techniques and practices of modern medicine are little different from traditional practices when it comes to wounds. Traditional approaches depend almost entirely upon natural resources such as water, plants, animals, and minerals, and continue to be valued and widely practiced by a majority of the world's population.[1] Maintaining homeostasis is critical for the survival of the organism; hence, skin needs and possesses a robust and effective repair mechanism. Cutaneous wound healing is the process by which skin

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www.ivsajournals.com© Iranian Journal of Veterinary Surgery, 2021
https://doi.org/10.30500/IVSA.2020.235492.1217

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repairs itself following injury caused by surgery, trauma, and burns.[2]

There is an increasing interest in the potential of traditional and complementary medicines to be used in in the area of wound that has led to investigations of a range of plant extracts and other products as traditionally wound healing agents.[3] These agents usually influence one or more phases of the healing process, and are also involved in disinfection and provide a moist environment to encourage the establishment of a suitable environment for the natural healing process.[3] The plant extracts with wound healing properties have the potential for anti-inflammatory, antioxidant, chelation and antimicrobial activities and may act by one or more of these mechanisms.[4] *Allium stipitatum* Regel (syn. *A. hirtifolium* Boiss., family Alliaceae) is one of the most frequently consumed alliaceous species in Iran, Turkey, and central Asia.[5] Members of the genus *Allium* are known to contain various sulfur compounds that exhibit prominent physiological properties, including anti-inflammatory activity.[6] Persian shallot (*Allium stipitatum*) is a bulbous plant is frequently used in folk medicine for the treatment of a variety of disorders, including inflammation and stress. Anti-inflammatory activity of pyrithione and four related sulfur-containing pyridine N-oxides which are prominent constituents of *Allium stipitatum* were tested. The anti-inflammatory activity was tested by the ability of the compounds to inhibit cyclooxygenase (COX-1 and COX-2). The compounds' affinity for the serotonin transport protein (SERT) and the GABAA–benzodiazepine receptor were also investigated.[7] [(Methylthio) methyldithio] pyridine N-oxide showed very high anti-inflammatory effects which are comparable with those of common pharmaceuticals (IC50 of 7.8 and 15.4 μM for COX-1 and COX-2, respectively).[7]

A literature survey reveals that no systematic approach has been made to study the wound-healing activity of the extract of *Allium stipitatum*. In the present work, the wound-healing activity of the extract of *Allium stipitatum* was investigated in an ointment form in a 2% concentration. Assessment of the healing process was based on wound area, histomorphometery, and hydroxyproline estimation studies.

Materials and Methods

Our study protocol was reviewed and approved by ethical committee of Lorestan University of Medical Sciences under ethical code IR.LUMS.REC.1398.208. All animals received humane care in accordance with the Guide for the Care and Use of Laboratory Animals prepared by the National Academy of Sciences and published by the National Institutes of Health (NIH publication No. 85–23, revised 1985).

Plant Material and Extract Preparation

The plant samples were collected from the Borojerd, Lorestan province, Iran. The values for moisture and ash were 78.4 ± 2.3 and 20.8 ± 0.7, respectively. Specimens from the plant material were deposited and authenticated at the Department of Botany Sciences, the Hamadan Research Agricultural and Natural Rescores Center, Hamadan, Iran. The leaves of the plant were used in the study. The plant material crisp was powdered in an electric blender. For the methanolic extraction, 150 g of the fine powder was extracted with 600 mL of 80 % methanol at 37° C for 3 hours. The sample was then centrifuged at 4500 rpm for 15 minutes and the supernatants were used. The filtrate was placed in an oven to dry at 40° C. The obtained clear residue was used for the study. Moisture and ash contents were determined using standard methods.[8]

Formulation of the Ointment

The base formulation consisting of Eucerin (30%) and Vaseline (70%) in about 1:2 proportions were prepared. The topical application form was prepared comprising 2 g of powder extract of the plant material in ointment.

Excision Wound Model and Planimetric Studies

Thirty-six healthy male Wistar rats weighing approximately 160-180 g and seven weeks of age were randomized into three groups of 12 rats each: Control surgery group (Control) including creation of wounds and no treatment, base formulation groups positive (POS) with creation of wounds and application of base formulation ointment, treatment group 1 (T1) with 2g of powder extract of the plant material in ointment. A wound was induced by an excisional based wound model in male rats. After induction of anesthesia with xylazine HCL 2% (5 mg/kg, IP, Alfasan International, Woerden, Netherland) and ketamine HCL 10% (60 mg/kg, IP, Alfasan International, Woerden, Netherland), rats were fixed in a ventral posture on a surgery table. Following shaving and aseptic preparation, a circular excision wound was made by cutting away approximately 115 mm^2 full thickness of

predetermined area on the anterior-dorsal side of each rat. All the test formulations were applied for 10 days starting from the day of wounding. Wound-healing property was evaluated by wound contraction percentage and wound closure time. Photographs were taken immediately after wounding and on days 0, 7, 14, and 21 post-operation by a digital camera while a ruler was placed near the wounds. The wound areas were analyzed by Measuring Tool of Adobe Acrobat 9 Pro Extended software (Adobe Systems Inc, San Jose, CA, USA) and wound contraction percentage was calculated using the following formula:

Percentage of wound contraction = $(A_0 - A_t) / A_0 \times 100$

Where A_0 is the original wound area and A_t is the wound area at the time of imaging.[9] The animals were left in separate cages for four days at room conditions for acclimatization. Animal houses were in standard environmental conditions of temperature (22 ± 3° C), humidity (60 ± 5%), and a 12h light/dark cycle. The animals were maintained on standard pellet diet and tap water. All rats were closely observed for any infection and if they showed signs of infection were separated, excluded from the study and replaced.

Determination of Hydroxyproline Levels

On the day 21 after surgery, a piece of skin from the healed wound area was collected and analyzed for hydroxyproline content. As a major part of collagen, hydroxyproline has an essential role in collagen stability. The collagen is the major component of extracellular tissue, which gives support and strength. The hydroxyproline contents were estimated using a method described by others.[10] Briefly, tissues were dried in a hot air oven at 60–70° C to constant weight and were hydrolyzed in 6N HCl at 130° C for 4 hours in sealed tubes. The hydrolysate was neutralized to pH 7.0 and was subjected to chloramine-T oxidation for 20 minutes. The reaction was terminated by addition of 0.4 M perchloric acid and color was developed with the help of Ehrlich reagent at 60° C and measured at 557 nm using UV-visible spectrophotometer (CamSpec M330, Cambridge CB2 4BG, UK).

Histological Preparation and Quantitative Morphometric Studies

The tissue samples were taken on 7, 14, and 21 days after surgery from periphery of the wound along with normal skin and fixed in 10% buffered formalin, dehydrated and embedded in paraffin wax, sectioned at 5 µm and stained with hematoxylin and eosin stains. Photomicrographs were obtained under light microscope to assess the predominant stage of wound healing. Three parallel sections were obtained from each specimen. Cellular infiltration including the number of mononuclear cells, polymorphonuclear cells and fibroblastic aggregation were quantitatively evaluated. Acute hemorrhage, congestion, vascularization, epithelialization, collagen production and density were also evaluated qualitatively. Morphological findings were scored using image analyzing software (Image-Pro Express, version 6.0.0.319, Media Cybernetics, Silver Springs, MD, USA). The histological parameters were classified according to the intensity of occurrence in five levels (- absence; + discrete; ++ moderate; +++ intense; ++++ very intense).[10]

Statistical Analysis

Differences among groups in excisional model, hydroxyproline level test were evaluated by Kruskal–Wallis variance analysis. When the *p*-value from the Kruskal–Wallis test statistics was statistically significant, multiple comparison tests were used to know the differences. Student's t-test was used for evaluation of other test results. Comparison among days was assessed by Mann–Whitney U-test. The Bonferroni correction was applied for all possible multiple comparisons. SPSS 11.5 (SPSS Inc., Chicago, IL, USA) was used for statistical analysis. A *p*-value was set at 0.05.

Results

Reduction in Wound Area

Wound contraction percentage in different groups during the course of study is shown in Table 1. The healing rate of the extract treated groups was significantly different compared to the control group ($p = 0.001$). However, time had significant effect on wound contraction of all wounds ($p = 0.001$).

Hydroxyproline Content of the Wounds

Proline is hydroxylated to form hydroxyproline after protein synthesis. Hydroxyproline contents in the groups Control, POS and T3 were found to be 52.76 ± 2.30, 23.55 ± 2.28, and 74.78 ± 2.61 mg g^{-1}, respectively. Hydroxyproline contents were increased significantly in the extract treated groups which implies more collagen deposition compared to Control and POS groups ($p = 0.001$).

Histological and Morphometric Findings

There were significant differences in comparisons of the extract treated and non-extract treated groups, particularly in terms of cellular infiltration, acute hemorrhage, congestion, edema, collagen production and density, reepithelialisation and neovascularization. During the study period, scores for reepithelialisation and neovascularization were significantly higher in animals of the extract treated groups than Control and PO groups (p = 0.001). Polymorphonuclear (PMN) and mononuclear (MNC) cell count, fibroblast cell proliferation and also Mean Rank of the qualitative study of acute hemorrhage, edema and collagen production score in the extract treated groups were significantly higher than those of Control and PO groups (p = 0.001) (Table 2, Figures 1-4).

Discussion

Wound healing is a dynamic process of three overlapping phases: inflammation, proliferative phase with granulation tissue formation and epithelialization, and tissue remodeling.[11-13] Inflammation lasts several days in the acute healing wound but persists in the chronic wound. Driven by pro-inflammatory cytokines,

Table 1. Effect of extract of *Allium stipitatum* on circular excision wound contraction area (mm²). Values are given as mean ± SEM.

	Wound area in days (mm²)					
Groups	**Day 6**	**Day 9**	**Day 12**	**Day 15**	**Day 18**	**Day 21**
Control	234.15 ± 0.77	110.17 ± 1.15	85.15 ± 1.20	41.3±1.87	23.12 ±2 .11	9.19 ± 1.20
POS	275.12 ± 0.65	240.37 ± 1.10	199.19 ± 3.25	165.75±1.32	117.53 ± 2.42	93.11 ± 1.23
T1	133.57 ± 2.40*	92.25 ± 0.37*	53.45 ± 0.28*	35.43±0.12*	19.22 ± 0.50*	5.16 ± 0.14*

The treated groups are compared by Student t test with other groups. *: The mean difference is significant at the .05 level *vs* POS groups.

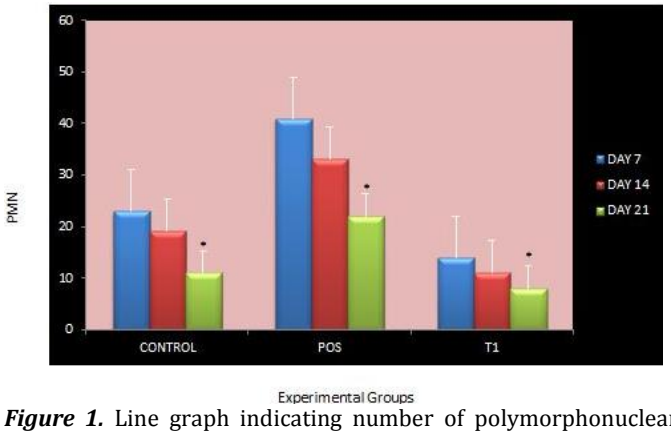

Figure 1. Line graph indicating number of polymorphonuclear cells (PMN) in excisional model of the rat skin in experimental groups. Results were expressed as mean ± SEM. * p = 0.001 *vs* Control and PO groups.

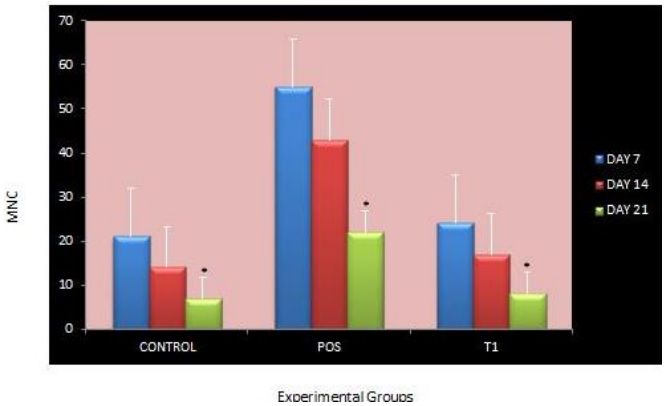

Figure 2. Line graph indicating number of mononuclear cells (MNC) in excisional model of the rat skin in experimental groups. Results were expressed as mean ± SEM. * p = 0.001 *vs* POS group.

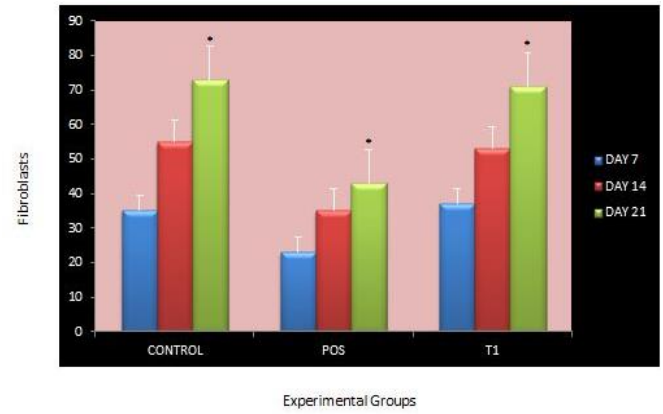

Figure 3. Line graph indicating number of fibroblasts in excisional model of the rat skin in experimental groups. Results were expressed as mean ± SEM. * p = 0.001 *vs* other experimental groups.

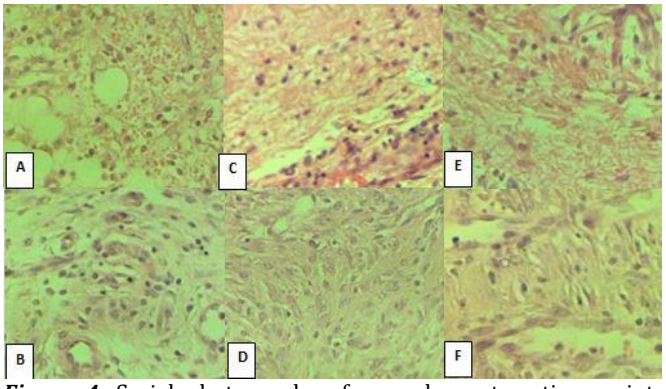

Figure 4. Serial photographs of wounds on two time points (Upper row: Day 7, Lower row: Day 14) in Control (A, B), POS (C, D), T1 (E, F) groups, (H&E, 400×).

Table 2. Intensity of histological parameters assessed in the experimental animals.

		Histological parameters				
Groups	**Days**	**Acute hemorrhage**	**Congestion**	**Vascularization**	**Epithelialization**	**Collagen**
Control	7	++	++	+	-	+
	14	++	++	++	+	+
	21	-	-	+++	++	+++
POS	7	++	++	+	+	+
	14	+	+	+	+	+
	21	-	-	++	++	++
T1	7	+*	+*	++*	+*	+*
	14	-	-	+++*	+++*	++*
	21	-	-	++++*	++++*	++++*

Classification of histological parameters according to the intensity of occurrence: - absence; + discrete; ++ moderate; +++ intense; ++++ very intense. Histopathological damages were assessed as explained under material and methods on days 7, 14, and 21 of lesion. * $p < 0.05$ vs POS group.

the prolonged and overactive neutrophil response leads to increased protease activity, mainly matrix metalloproteinases.[14-18] In some cases, protease activity has been found to be over 100 times higher in chronic compared with acute wounds.[19] Increased metalloproteinases lead to degradation of growth factors, their receptors, and adhesion proteins, such as fibronectin and vitronectin, preventing cell adhesion for normal wound closure.[14,16-21] As a result, topical treatments aimed at inflammation and excess proteases have been developed. Wounding damages the blood supply, leading to hypoxia along with subsequent decreased oxidative bursts and microbicidal activity by polymorphonuclear leukocytes.[22-24] The uncontrolled polymorphonuclear leukocytes respond to low oxygen tension by releasing proteinases and toxic oxygen metabolites, which damages endothelial cells, leading to cellular destruction, deposition of fibrin, and further decreased delivery of nutrients and oxygen, propagating a vicious cycle.[21] Systemic disorders, such as decreased cardiac output, smoking, peripheral vascular disease, past irradiation, and chronic infection, all contribute to hypoxia in the local environment.[24] Reperfusion injury plays a role. During ischemia, substances such as hypoxanthine and xanthine oxidase are made. Reperfusion causes oxygen to react with these substances, producing superoxide bursts that further damage the endothelium.[25]

Collagen, the major component which strengthens and supports extra cellular tissue, is composed of the amino acid hydroxyproline, which has been used as a biochemical marker for tissue collagen.[26] In excisional wound model in the extract treated animals there was a significant decrease in wound area. This indicated improved collagen maturation by increased cross linking. The balance between synthesis and breakdown and so deposition of collagen is important in wound healing and development of wound strength.[27]

Hydroxyproline is a major component of the collagen that permits the sharp twisting of the collagen helix. It helps on providing stability to the triple-helical structure of collagen by forming hydrogen bonds. Hydroxyproline is found in few proteins other than collagen. For this reason, hydroxyproline content has been used as an indicator to determine collagen content.[27] Increase in hydroxyproline content in the extract treated groups indicated increased collagen content, since hydroxyproline is the direct estimate of collagen synthesis.

In the present study, histopathological examination and scoring revealed that there was a significant difference by means of wound healing scores in the extract treated groups compared to other experimental groups. The extract decreased the maturation time of granulation tissue and wound contraction which means that it enhanced reepithelialisation with significant effect on inflammatory infiltration and number of fibroblasts in time-dependent activity.

Although the present study showed the promising effect of the extract of *Allium stipitatum* on wound healing in rats, data regarding the molecular mechanisms leading to its action remain to be investigated in depth. The authors did not provide molecular evidence for the action of the extract which may be considered a limitation of this study.

In conclusion, findings of the present study demonstrated that extract of *Allium stipitatum* had properties that render it capable of promoting accelerated wound-healing activity compared to the controls. On the basis of the results obtained in the

present study, it is possible to conclude that the ointment of the extract of *Allium stipitatum* has significant wound-healing activity.

Acknowledgments

The authors would like to thank the staff of the animal house for their technical expertise and care of animals.

Conflict of Interest

There are no conflicts of interests to declare.

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