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Assessment of antioxidant and cutaneous wound healing effects of *Ornithogalum cuspidatum* hydroalcoholic extract in male Wistar rats

Fatemeh Hoseinpour[1*], Mohammad Hashemnia[2], Hadi Cheraghi[3], Reza Safikhani[3], Tahereh Sheikhi[3], Zahra Hasani[3]

1. Department of Basic Sciences, Faculty of Veterinary Medicine, Razi University, Kermanshah, Iran
2. Department of Pathobiology, Faculty of Veterinary Medicine, Razi University, Kermanshah, Iran
3. Department of Clinical Sciences, Faculty of Veterinary Medicine, Razi University, Kermanshah, Iran

Abstract

Background and Objective: *Ornithogalum cuspidatum* is a medicinal plant in Iranian traditional medicine that has several pharmacological effects. Due to strong antioxidant and anti-inflammatory activities of this plant, the current study was designed to evaluate wound healing activity of *O. cuspidatum* on cutaneous wounds in Wistar rats.

Materials and Methods: A full-thickness excisional wounds was induced on the back of 50 Wistar rats. The animals were randomly divided into five groups, including control, basal cream, phenytoin, *O. cuspidatum* 5%, and *O. cuspidatum* 10%. Five animals of each group were euthanized at 10 and 20 days post-injury (DPI) and wounds were assessed through gross and histopathological analyses. Also, hydroxyproline content and MDA, NO and TOS concentrations were determined.

Results: Treated animals with *O. cuspidatum* showed a significant reduction in the wound surface area at 10 and 20 dpi. Moreover, treatment with this plant reduced the number of lymphocytes and macrophages, increased the number of fibroblasts at the earlier stages and enhanced number of fibrocytes at the later stages of wound healing. *O. cuspidatum* significantly improved re-epithelialization and epithelial formation, enhanced hydroxyproline content and thereby maturity of the collagen fibers. Also, *O. cuspidatum* significantly reduced MDA, NO and TOS concentration as oxidant status in granulation tissue.

Conclusion: The present study demonstrated that application of hydroethanolic extract of *O. cuspidatum* promoted wound healing due to increased re-epithelialization and collagen deposition in wound tissue and also induction of considerable wound contraction, so it can be considered as a therapeutic agent for wound healing.

Keywords: *Ornithogalum cuspidatum*, Antioxidant, Wound healing

1. Introduction

Cutaneous wound healing is a dynamic and intricate process involving three overlapping phases of inflammation, re-epithelialization and remodeling (1), which is synchronized by complex signaling system composed of phagocytosis, chemotaxis, mitogenesis and the synthesis of matrix components (2). The inflammatory phase involves altered activity of neutrophils, macrophages and mast cells (3) leading to the migration of neutrophils primarily and then macrophages return to the injured tissue. The macrophages produce growth factors and cytokines (4, 5). Re-epithelialization and neovascularization are two important events in phase 2. Fibroblasts are dominant cells at stage 3 that produce and organize new extracellular matrix. Collagen is the major protein of the matrix that produced by proteolytic enzymes of fibroblasts (5). The synthesis, secretion and organization of collagen in granulation tissue play a key role in healing process.

Ornithogalum cuspidatum belongs to Liliaceae family. Different species of this family is distributed in

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Corresponding Author: Fatemeh Hoseinpour **E-mail:** fhossainpour@yahoo.com

Europe, Asia and Africa. The species of *Ornithogalum* are used frequently as spice or for treatment of inflammatory and respiratory disease in Iranian traditional medicine (6). Several pharmacological effects of the genus *Ornithogalum* have been reported that include anti-tumor, cytotoxic, anti-bacterial and antioxidant activities (7-10). Phytochemistry and antioxidant activity of the essential oil and extract of *Ornithogalum cuspidatum* have been studied previously. Analysis showed that its essential oil consisted mainly of saturated hydrocarbons, oxygenated hydrocarbons and oxygenated terpenoid compounds (11). Also, methanolic extract of *O. cuspidatum* have shown highest total phenolic content and the strongest antioxidant activity (12). Since oxidative stress has been proven in etiology of many disorders and *Ornithogalum cuspidatum* has relatively strong antioxidant properties, the aim of this study was to evaluate the effect of this medicinal plant on cutaneous wound healing in Wistar rats.

2. Materials and Methods

2.1. Preparation of plant extract

O. cuspidatum was collected from Maragheh, Eastern Azarbaijan province, Iran, in Jan 2020, and was identified by a botanist. One hundred g of dried leaves of this plant was powdered and 500 ml of ethanol 70% was added. Then, it was passed through Whatman filter paper after 24 hours. The excess solvent was evaporated and the extract was dried on separate plates in the laboratory oven gently.

2.2. Animals

Fifty male adult albino Wistar rats weighing 200 to 220 g were kept under standard laboratory conditions [temperature (25 ± 2°C), relative humidity (44–56%), and light and dark cycles (12:12 h) and were allowed free access to water and standard pellet laboratory diet (70% carbohydrates, 25% proteins, and 5% lipids) for 7 days in order to adapt to the new environment. The experimental protocol was approved by the Institutional Animal Ethics Committee, Razi University, Iran (Ethics no. IR.RAZI.REC.1399.052).

2.3. Wound induction

The rats were anesthetized by intramuscular injection of 1 mg/kg of xylazine HCl (xylazine 2%; Alfasan, Woerden, Netherland) as a premedication and 60 mg/kg of ketamine HCl (ketamine 10%; Bremer Pharma Gmbh, Germany) (13, 14). The backs of the animals in the cervical region were clipped and the skin area scrubbed. Under aseptic condition, a square-shaped full thickness incision of 2×2 cm2 was made in skin and the incised piece including epidermis, dermis and subcutaneous tissue was removed (15).

2.4. Study design

After injury induction, the animals were randomly divided into five groups, with 10 animals in each group, including: control, basal cream, phenytoin, O. cuspidatum 5% and *O. cuspidatum* 10%. The groups were treated with daily topical application of normal saline (1 ml), basal cream (1 ml of eucerin), phenytoin 1% as comparative control (standard ointment) (Medipharm Co, Iran), *O. cuspidatum* 5% (5 g of O. cuspidatum powder was suspended in 95 g of eucerin) and *O. cuspidatum* 10% (10 g of *O. cuspidatum* powder was suspended in 90 g of eucerin). Five animals from each group were euthanized at each of 10 and 20 DPI (days post injury) by chloroform inhalation and the granulation tissues were excised. These samples were divided into two parts. Part A was immediately fixed in a 10% buffered formalin solution for histopathological evaluation and the part B was stored at −80◦C for biochemical analysis such as total protein quantification, oxidative stress markers, and hydroxyproline content estimation. Rats were weighted before and after the experiment.

2.5. Macroscopic assessment of wounds healing

To determine the efficiency of wounds healing, wounds were photographed at 10 and 20 DPI using a canon IXY digital camera (Canon Digital IXY 190 3.8X).

2.6. Histopathological examination

Full thickness skin samples were fixed in 10% neutral-buffered formalin, then embedded in paraffin, and sections of 5 µm in thickness were stained using Hematoxylin and Eosin (H&E) and examined by a light microscope. Histological examinations were performed in a double-blind fashion with a procedure reported by Oryan et al. (2012) with some modifications (15). The pictures were then captured 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 of each tissue sample, were used for histopathologic analysis. The parameters that were evaluated in histopathological sections, consisted of hemorrhage, fibrin deposition, polymorphonuclear cell and mononuclear cell infiltration, re-epithelialization, 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.

2.7. Determination of hydroxyproline content

This assay was performed using a hydroxyproline assay kit (Kiazist, Hamedan, Iran). Briefly, 40 mg of skin tissue was homogenized in 100 µl of deionized water and hydrolyzed with 12 M HCl at 120 °C for 4 h. 20 µl of the supernatant was transferred to a 96-well plate, after adding oxidation and chromogen solution, an orange-purple color was formed. The absorbance was read at 550 nm with automatic ELISA microplate reader (ELX-800, BioTek, USA). Skin total protein was determined by Bradford method and hydroxyproline content was presented as µg/mg of total protein.

2.8. Determination of oxidant status

Tissue homogenate of skin was prepared in 100 mM PBS (pH 7.4) (100 mg/ml) and after centrifugation, total oxidant status (TOS), malondialdehyde (MDA) and nitric oxide (NO) levels were assayed in supernatant by colorimetric method at 530, 550 and 570 nm, respectively, using Navand Salamat kits (Navand Salamat, Urmia, Iran). Also, the oxidant parameters were presented as unit/mg of total protein.

2.9. Statistical analysis

Data were expressed as mean±SD and subjected to one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test and $p<0.05$ was considered as the significant level (SPSS software, 22).

3. Results

The wound surface area was calculated and expressed in cm2 as summarized in Table 1. At 10 DPI, the rats in control, basal cream and phenytoin groups showed a significant delay in wound healing as compared with O. cuspidatum groups ($P<0.05$). At 20 DPI, the closure of the wounds with O. cuspidatum 5 and 10% was significantly faster than those of the control and basal cream groups ($P<0.05$). However, the highest rate of wound closure was observed in O. cuspidatum 10% group, but the differences were not significant as compared to the basal cream and O. cuspidatum 5% groups ($P>0.05$).

Table 1. Mean ± SD of wound closure (%) in groups on different days post-injury

Days	Control	Basal cream	Phenytoin	*O. cuspidatum* (5%)	*O. cuspidatum* (10%)
Day 10	32.03±3.40^{a}	34.14±6.25^{a}	32.73±4.84^{a}	44.07±8.57^{b}	57.10±5.15^{c}
Day 20	71.91±3.04^{a}	76.81±5.68^{ab}	79.61±2.28^{abc}	84.65±2.24^{b}	88.15±1.63^{c}

Means within a row with different superscript letters (a, b, c) denote significant differences. $P<0.05$ was accepted as statistically significant.

3.1. Quantitative analysis

The data obtained from the histopathologic evaluations are summarized in Table 2.

At 10 DPI, treatment with low and high dose of *O. cuspidatum* as well as phenytoin significantly decreased total cellularity as compared to the basal cream and control groups ($P<0.05$). Although the mean number of cells in the wounds treated with *O. cuspidatum* 10% group was lower than those in the *O. cuspidatum* 5% and phenytoin, but this difference was not statistically significant ($P>0.05$). The highest number of blood vessels was observed in the *O. cuspidatum* 5% group, which had a significant difference when compared to the control group ($P<0.05$). High-dose of *O. cuspidatum* increased the mean number of fibrocytes as compared to the other groups, but its difference was only significant with the control group ($P<0.05$). The number of fibroblasts in the low and high-dose *O. cuspidatum* groups was significantly higher than those in the basal cream and control groups ($P<0.05$). The control and *O. cuspidatum* 10% groups had the highest and lowest number of lymphocyte, respectively, but this difference was not statistically significant as compared to other groups ($P>0.05$). The treated groups *with O. cuspidatum* had a higher number of macrophages, but this difference was only statistically significant as compared to the basal cream and control groups ($P<0.05$).

At 20 DPI, treatment with low- and high-dose of *O. cuspidatum* significantly reduced the total cellularity compared with the phenytoin, basal cream and control groups ($P<0.05$). Although the number of total cells in the *O. cuspidatum* 10% group was less than the *O. cuspidatum* 5% group, but this difference was not statistically significant ($P>0.05$). At this stage, the treated lesions with *O. cuspidatum* showed significantly lower number of blood vessels in comparison with the basal cream and control groups lesions ($P<0.05$), but this reduction was not significant when compared to the phenytoin group ($P>0.05$). The number of fibrocytes was significantly higher in high-

dose *O. cuspidatum* group as compared with other groups (P<0.05). At this stage, the lowest number of fibroblasts was observed in the *O. cuspidatum* 10%, followed by *O. cuspidatum* 5%, control, basal cream and phenytoin groups. The difference between O. cuspidatum and other groups was statistically significant (P<0.05). Treatment with *O. cuspidatum* 5 and 10% significantly reduced the number of lymphocytes and macrophages compared to the basal cream and control groups (P<0.05). However, the number of lymphocytes and macrophages in high-dose *O. cuspidatum* group was less than low-dose *O. cuspidatum*, but these differences were not significant (P>0.05).

Table 2. Histopathologic and histomorphometric analysis

Day 10	**Control**	**Basal cream**	**Phenytoin**	***O. cuspidatum* (5%)**	***O. cuspidatum* (10%)**
Total cell	461.50±28.12a	443.30±47.76a	375.50±45.81b	344.50±31.24b	325.80±45.63b
Vascular no.	10.90±0.99a	11.20±1.13a	11.20±1.03a	13.10±1.85a	12.70±1.41a
Fibroblast and fibrocytes	16.90±1.44	18.40±1.57	19.30±2.45	24.40±2.67	23.20±2.97
Fibrocytes	4.80±1.03a	5.20±0.78ab	6.00±1.63ab	6.30±1.15ab	7.10±1.10b
Fibroblasts	12.10±2.07a	13.20±1.47a	13.30±2.31ac	18.10±2.42b	16.10±2.76bc
Ratio	0.41±0.14	0.39±0.08	0.46±0.16	0.35±0.07	0.45±0.10
Lymphocyte	16.30±3.23a	16.90±2.28a	16.10±2.37a	15.60±2.59a	15.10±2.13a
Macrophage	12.10±2.13a	12.70±2.35a	15.20±2.34ab	14.80±3.58b	16.40±2.31b
Neutrophil	3.10±2.07	1.50±1.26	1.90±2.55	1.90±1.79	1.30±0.94
Day 20					
Total cell	332.30±40.93a	317.50±61.65a	294.70±36.52a	230.00±47.93b	207.50±30.66b
Vascular no.	9.60±1.17a	8.30±1.33a	6.20±1.47b	5.70±1.63b	6.10±0.87b
Fibroblast and fibrocytes	18.40±2.11	20.00±2.62	24.40±2.79	23.10±2.92	23.60±3.02
Fibrocytes	7.80±1.47a	8.70±1.25a	12.30±1.70ab	14.30±2.49b	16.80±2.25b
Fibroblasts	10.60±1.42a	11.30±1.76a	12.10±2.13a	8.80±1.54ab	6.80±1.54b
Ratio	0.74±0.17	0.77±0.11	1.04±0.22	1.67±0.46	2.57±0.58
Lymphocyte	12.40±1.57a	11.40±2.06a	9.20±1.98ab	8.10±1.72b	6.80±1.22b
Macrophage	11.10±3.72a	10.60±1.89a	9.10±1.28ab	7.70±1.82ab	6.90±1.79b
Neutrophil	2.00±1.33	1.10±1.37	0.90±1.44	0.60±1.07	0.70±1.05

Five fields in each of five histopathologic sections were analyzed for each group.

Means within a row with different superscript letters (a, b, c) denote significant differences. P<0.05 was accepted as statistically significant.

3.2. Qualitative analysis

At 10 DPI, a thick granulation tissue with dilated blood vessels that covered the wounds area was observed in all rats. There was no regenerating epithelium in the basal cream, phenytoin and control groups, while the lesions treated with *O. cuspidatum* revealed minimal re-epithelialization. At this stage, the newly synthesized collagens were still unorganized and had a randomly distributed pattern in all groups. Interestingly, less cellularity, perivascular oedema and fibrin deposition and more collagen fibers were observed in treated lesion with *O. cuspidatum* as compared to other groups. Although, there were no signs of infection around the wound area in *O. cuspidatum* groups, the presence of neutrophils was prominent within the granulation tissue in the control group (Fig. 1a-j).

At 20 DPI, in the control group, the dermis was cellular, and the presence of fibroblasts, lymphocytes and macrophages as well as disorganized and poorly oriented collagen fibers were prominent. The epidermis was thick and disorganized, particularly as compared with the adjacent normal skin. In contrast, in the treated lesions with *O. cuspidatum*, the size of scar tissue was smaller and full thickness epidermal regeneration with thin keratin layer, greater tissue maturation, more organized pattern in the collagen fibers and large capillary-sized blood were observed. Moreover, the number of lymphocytes and macrophages was reduced in the treated lesions. In this stage, there were no evidence of fibrin deposition, polymorphonuclear cells infiltration and edema in the lesions of animals in all groups. Comparing treated groups, high dose of *O. cuspidatum* had a better therapeutic effect (Fig. 1k-t).

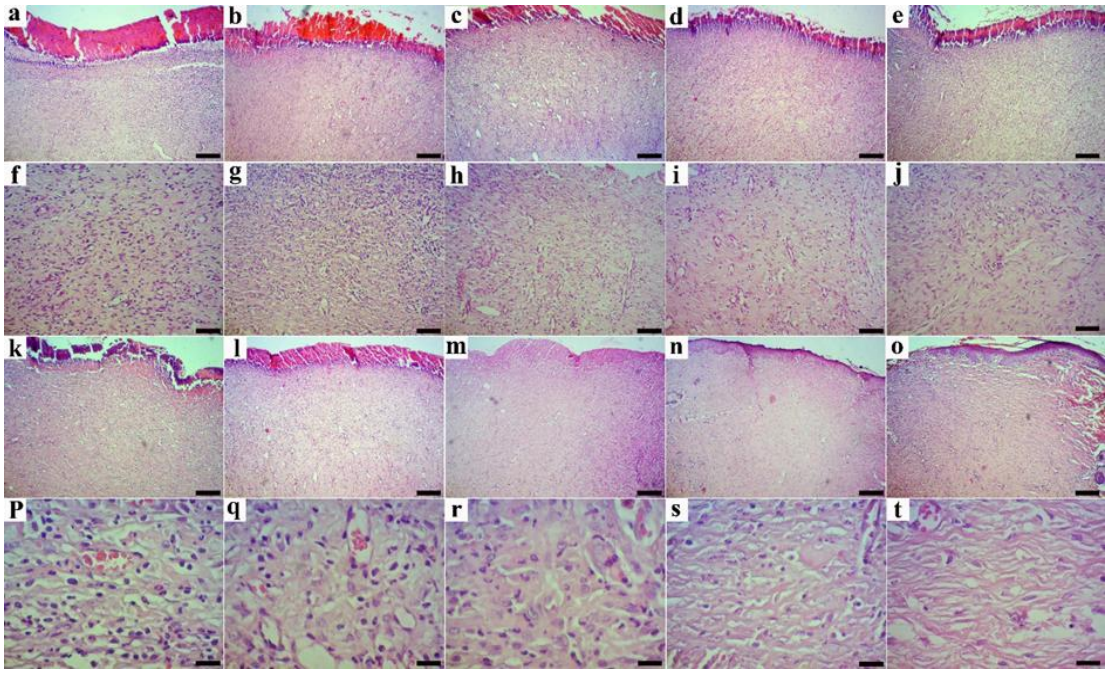

Fig 1. Longitudinal sections of the control (a,f,k,p), basal cream (b,g,l,q), phenytoin (c,h,m,r), *O. cuspidatum* 5% (d,i,n,s), *O. cuspidatum* 10% (e,j,o,t) groups on days 10 and 20 post-injury (Hematoxylin and Eosin staining; Bar = 100 µm for a-e and k-o, Bar = 50 µm for f-j, and Bar = 25 µm for p-t).

3.3. Hydroxyproline content

Animals topically applied with *O. cuspidatum* ($p < 0.05$) showed the highest hydroxyproline content, especially at high dose (10%), 20 DPI. At 10 DPI, the difference between low and high doses was not significant. Moderate effect on hydroxyproline content of granulation tissue was observed in phenytoin group when compared with the control group (Fig. 2).

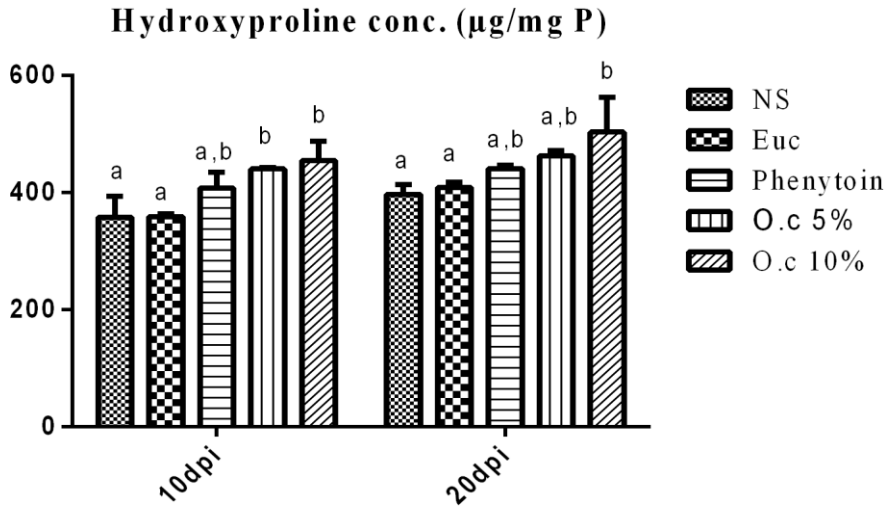

Fig 2. Hydroxyproline concentration in wound excision (mean ± SD) of different groups at 10 and 20 dpi. Different letters are used to demonstrate significant difference among groups ($p<0.05$). NS: normal saline (control), Euc: Eucerin (basal cream), O.c 5%: *Ornithogalum cuspidatum* 5%, O.c 10%: *Ornithogalum cuspidatum* 10%, dpi: days post injury.

3.4. Oxidant Status

It was observed that at 10 DPI, pretreatment with *O. cuspidatum* could significantly lower MDA but reduction of NO and TOS in treatment groups in this stage was not significant. Also, *O. cuspidatum* 10% significantly reduced MDA, NO and TOS at 20 DPI (Figures 3-5). Although application of low dose of *O. cuspidatum* had similar effect, but the difference was not significant when compared to control.

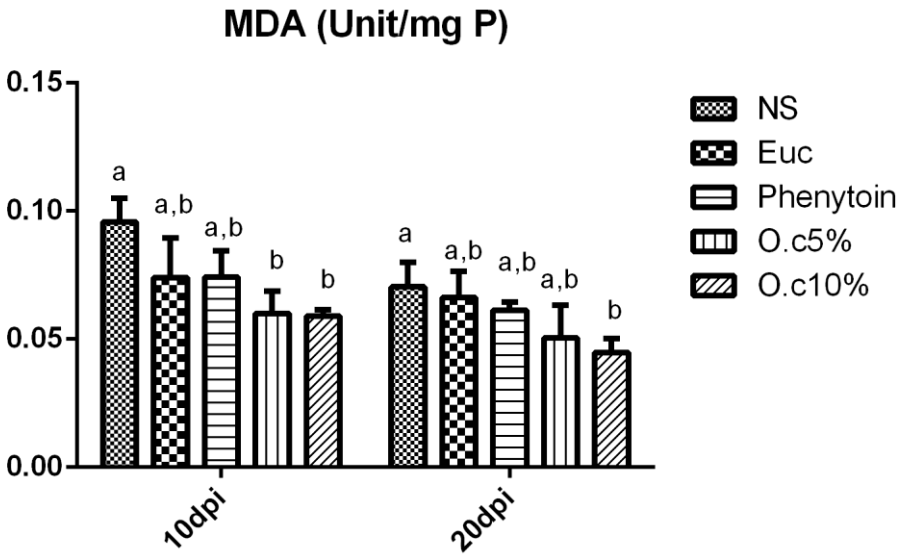

Fig. 3. MDA concentration in wound excision (mean ± SD) of different groups at 10 and 20 dpi. Different letters are used to demonstrate significant difference among groups ($p<0.05$).
NS: normal saline (control), Euc: Eucerin (basal cream), O.c 5%: *Ornithogalum cuspidatum* 5%, O.c 10%: *Ornithogalum cuspidatum* 10%, dpi: days post injury, MDA: malondialdehyde.

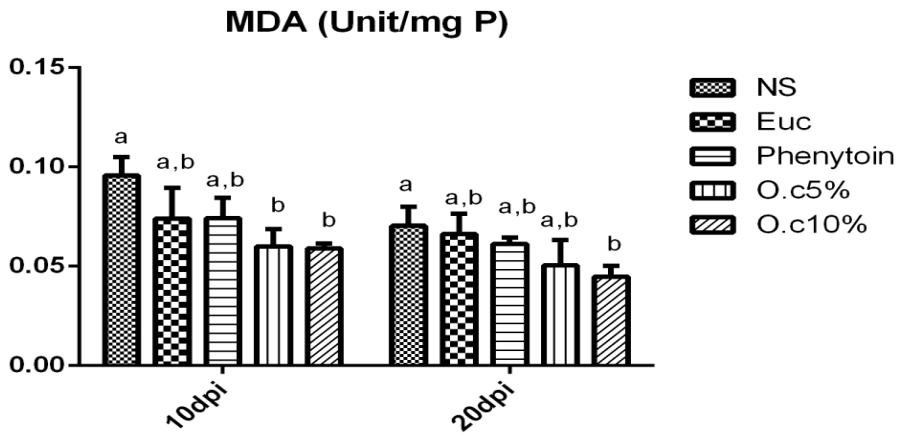

Fig 4. NO concentration in wound excision (mean ± SD) of different groups at 10 and 20 dpi. Different letters are used to demonstrate significant difference among groups ($p<0.05$). NS: normal saline (control), Euc: Eucerin (basal cream), O.c 5%: *Ornithogalum cuspidatum* 5%, O.c 10%: *Ornithogalum cuspidatum* 10%, dpi: days post injury, NO: nitric oxide.

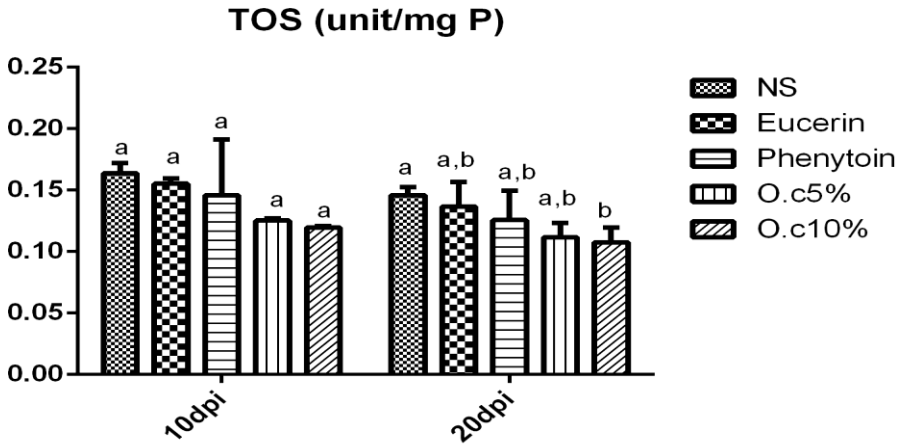

Fig 5. TOS concentration in wound excision (mean ± SD) of different groups at 10 and 20 dpi. Different letters are used to demonstrate significant difference among groups ($p<0.05$). NS: normal saline (control), Euc: Eucerin (basal cream), O.c 5%: *Ornithogalum cuspidatum* 5%, O.c 10%: *Ornithogalum cuspidatum* 10%, dpi: days post injury, TOS: total oxidant status.

4. Discussion

Wounds are defined as damage to skin tissue from cuts, stab wounds or rupture by physical, biological and thermal stimulation (16). Wound healing is an intricate and regulated process in which organized collagen deposition, in response to tissue injury, results in scar formation. This process include inflammation, fibroplasia, and scar maturation. (17). In recent years, the use of botanical medicine as herbal drugs has been increased. *O. cuspidatum* is used traditionally as a medicinal herb for treatment of inflammatory and respiratory disease. Recently novel pharmacological effects of the genus *Ornithogalum* have been discovered, such as anti-tumor, cytotoxic, anti-bacterial and antioxidant activities (6-10). However, no study has been performed yet to investigate the wound healing activity of *O. cuspidatum.*

The major findings of the current study are as follow: *O. cuspidatum* extract accelerates wound healing in rats as assessed by the degree of wound closure, oxidant status, hydroxyproline content, and histopathological score. In this study, a significant increase in wound contraction was observed in *O. cuspidatum* groups as compared to control. Several studies have shown that rapid wound contraction is associated with activation of fibroblasts interceded by specialized myofibroblasts of granulated tissues (18-20). So, the significant effect of *O. cuspidatum* on wound closure in rats may be attributed to the number of myofibroblasts or enhanced contractile property of myofibroblasts crucial for normal wound contraction.

The most significant morphological changes occurred during the second 10 days of wound healing. The presence of the collagen fibers with a greater degree of organization and a more normal alignment in the treated lesions in this stage (20 DPI) may be due to more hydroxyproline content. Hydroxyproline is an important component among three constituents of collagen fibers (21). The increased hydroxyproline obviously explains the increased rate of wound closure, due to rapid collagen organization and accumulation. Also, increase in hydroxyproline content is directly correlated with alleviation in cellular proliferation, and collagen synthesis (22). Increasing the fibrocytes in treated group by *O. cuspidatum* followed by increase in hydroxyproline content and collagen deposition can explain properly morphological changes.

Histopathological analysis showed that collagen deposition, epidermis regeneration, angiogenesis, and proliferation (fibroblasts and epithelial cells) of the treated group were higher than control at 10 DPI. The treated wound areas with *O. cuspidatum* extract were more vascular at this stage and it seems that it is one of the mechanisms that is responsible for providing more blood and oxygen supply and therefore an improved wound healing outcome in the treated animals.

On 20 DPI, the presence of full thickness epidermal layer which covered completely the wound area, reduction in cellularity and vascularity, also the organized collagen were important histopathological features of the treated lesions of *O. cuspidatum* groups when compared to the other groups. Also, the number of lymphocytes and macrophages was reduced and total fibroblasts and fibrocytes were increased. These results can be due to the presence of phenolic compounds of O. cuspidatum that play a key role in acceleration of various wound healing stages, including collagenation, wound closure and re-epithelialization, also the antimicrobial, anti-inflammatory and free radical scavenging (antioxidant effects) properties which is in well agreement with the previous studies (18,23,24). Also, the presence of terpenoids in this medicinal plant can be useful in the management of inflammatory processes. Several studies have shown that terpenoids, especially oxygenated terpenoids, possess strong anti-inflammatory properties and the results of our study are in agreement with them (25-27).

Neutralization of oxidative stress has a key role in wound healing process. Oxidative stress is caused by imbalance in production of reactive oxygen species and endogenous antioxidant defense mechanism that further affects the biomolecules functions (28). In the present study, oxidative stress levels in lesion groups were investigated in 10 & 20 DPI (Figures 4-6). The results showed that oxidative stress during prolonged duration, i.e., 20 DPI, due to over-production of reactive oxygen and nitrogen species, induced significant cytotoxicity within the wound area impairing healing in control group and *O. cuspidatum* had a potent antioxidant activity by reduction of MDA, NO, and TOS, especially at a high dose.

MDA is the major metabolite of arachidonic acid, NO is produced from the amino acid L-arginine by NO synthase (NOS) and both serve as reliable biomarkers for oxidative stress (29). Generally, antioxidant effects of natural products lead to inflammation suppression as well as granulation tissue formation, re-epithelization and epidermis differentiation progression (30).

Conclusion

The present study established that *O. cuspidatum* improves wound healing activity. The results showed that application of *O. cuspidatum* hydroethanolic extract on wounds induces considerable wound contraction and accelerates healing. Significant antioxidant and anti-inflammatory activity of *O. cuspidatum* due to the presence of oxygenated terpenoids, along with phenolic compounds, might be responsible for the healing of wound by rapid wound contraction and increasing hydroxyproline content. However, the precise mechanism of wound repairing action of this medicinal plant is unclear and further researches are needed to clearly understand the exact mechanism of action of such herbal extract.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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