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Research Article

In Vivo Evaluation of Wound Healing Properties of *Platanus orientalis* L.

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Abstract

Background: According to the Iranian Traditional Medicine (ITM) references, *Platanus orientalis* L. possesses wound healing properties. Herein, we developed different topical formulations based on the ethanolic extract of *P. orientalis* leaves and evaluated its wound healing effects through an *in vivo* model.

Methods: Hydroalcoholic extract of the leaves was obtained from ethanol 80% and it was evaluated for DPPH radical scavenging activity, total phenolic and flavonoid contents as well as the presence of tannins. Different topical formulations including ointment (D-O) and polymer film (D-F), were prepared and an *in vivo* test was run for 14 days in an excision wound model consisting of 5 groups of 6 rats.

Results: The results indicated the higher efficacy of D-O compared with D-F, as wound surface area remarkably reduced within 14 days post-injury. Also, histological features including epitheliogenesis score, neovascularization, and collagen density indicated the potential wound healing effect of D-O.

Conclusion: Wound healing properties of the ethanolic extract of *P. orientalis* leaves depended on the type of formulation and D-O was found to be much more potent than D-F, from reducing wound surface area, maximum epitheliogenesis score, proper neovascularization pattern, and early type I collagenization points of view.

Introduction

Wound is described as a damaged living tissue in a cellular, structural, and functional level. It can be caused *via* different routes including physical, thermal, and chemical injuries, divided into two types; open and closed wounds. An open wound is created when the skin is cut or torn, whereas a closed wound is defined as a trauma caused by blunt force, creating bruises and swelling.[1,2] Wound healing process consists of several steps: hemostasis or coagulation, inflammation, proliferation, and maturation or remodeling.[3] Wound care provides desired conditions for the healing process to happen quicker. However, in spite of recent developments, improvement of wound deformities including scar tissue formation and cosmetic concerns, has turned out to be considerably limiting.[4,5] Bacterial infection is another major obstacle in the clinical injuries, as it can delay or stop the wound healing process, resulting in further damages.[6] One of the progressing fields in modern medicine research is development of wound healing approaches since it is one of the most concerning issues worldwide.

Plants and their medicinal properties have always been in the center of attention of traditional and folk medicine. In this respect, phytotherapy is highly preferred by the general population globally and using medicinal plants and preparations thereof, have been a prehistoric tradition for wound healing.[7,8] Healing properties of the botanical extracts have been fully investigated in modern medicine and their anti-inflammatory, anti-microbial and anesthetics effects as well as protective modalities have been proven. It has been demonstrated that herbal extracts can affect different phases of the healing cascade through their various phytochemicals.[9]

Platanus orientalis L. (Chinar or Oriental plane) from the family Plantanaceae is a deciduous, woody, and perennial tree, native to south-western Asia.[10] The Platanus genus consists of nine species that can be found in Asia, Europe, and America and *P. orientalis* is mainly distributed in Turkey.[11] Chinar (leaves, fruit, seeds, and bark) has been traditionally used in various countries for medical purposes and non-medical applications such as the removal of heavy metals and reduction of ozone

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levels.[12-14] Biological activities of Chinar extracts or isolated compounds have been widely described in the literature. They have shown anti-HIV and anti-cancer,[15] anti-septic and anti-inflammatory properties.[12] Also, they have been reported to treat respiratory difficulties and asthma, ophthalmic disease, dysentery, toothache, dermatological and rheumatic problems.[10,16,17] Furthermore, various anti-inflammatory and analgesic properties of its leaves have been studied on wound healing.[18,19] The hydroalcoholic and polyphenolic extracts of *P. orientalis* have also depicted moderate analgesic effects.[12,20] It is worth mentioning that the Iranian Traditional Medicine (ITM) references recommended *P. orientalis* for the treatment of wounds.

Phytochemical analysis of the plant has revealed the presence of various second metabolites including kaempferol 3-*O*-α-lrhamnopyranoside, kaempferol 3-*O*-β-d-glucopyranoside, caffeic acid,[21] platanoside, tiliroside,[22] flavonol glycosides,[23,24] proanthocyanidin glycosides,[25] fatty acids,[26] and phytol derivatives.[27] It has been demonstrated that polyphenolic flavonoids and tannins facilitate wound healing process due to their potent anti-oxidant and anti-bacterial effects. They act synergistically to induce angiogenesis, collagen deposition, epithelization, and wound contraction in the proliferative stage.[28,29]

Focusing on various biological activities of *P. orientalis* involved in wound healing as well as the use of the plant's leaves in ITM, we developed topical formulations prepared from the ethanolic extract of *P. orientalis* leaves and evaluated their efficacy in an *in vivo* excision wound model.

Materials and Methods

Chemicals

Ethanol (Merck), double distilled water, deionized water, gallic acid (standard, Sigma), Folin-Ciocalteu reagent (Merck), sodium carbonate (Merck), HPMC (Merck), chitosan (Sigma), glacial acetic acid (Merck), sodium hydroxide (Sigma), glycerol (Sigma), formaldehyde (Sigma), xylazine, ketamine, Comfeel plus (Coloplast UK), eucerin 200 were used in the experimental section.

Plant material, extraction, and preparation of ethanolic extract

Platanus orientalis leaves were collected from Tehran, Iran. They were identified and voucher specimen of PMP-456 was deposited in the Herbarium of the Faculty of Pharmacy, Tehran University of Medical Sciences, Tehran, Iran.

The plant products were extracted by maceration of plant powder (200 g) with ethanol 80% for three 72 h rounds at room temperature, separately. The extract was concentrated under vacuum (Heidolph, Heizbad Hei-VAP, Germany) at 25 °C and then lyophilized by a laboratory freeze dryer (LTE science LTD, England) to obtain 63 g of a dry and brittle powder.

Phytochemical analysis

The phytochemicals present in the ethanolic extract of *P. orientalis* leaves were investigated through the following assays: measurement of total phenols, total flavonoids, and DPPH radical scavenging activity as well as qualitative tannin test.

Measurement of total phenolic content

Total phenolic content assay was performed through a colorimetric analysis using the modified Folin-Ciocalteu method as described in our previous work.[30]

Measurement of total flavonoid content

The flavonoid content was measured with the aluminum chloride procedure using catechin as a reference compound according to our previous work.[30]

DPPH radical scavenging activity (DPPH)

Antioxidant activity of the extract was evaluated using DPPH (1,1-diphenyl-2-picrylhydrazyl) based on the literature.[31]

Qualitative tannin test

The presence of tannins was evaluated through two types of qualitative tests as described in the literature.[32,33]

Evaluation of wound healing

Experimental animals

Thirty Wistar male rats (200–220 g) of approximately two months of age were studied and randomly divided into five groups of six rats. The animals were housed in the standard environmental conditions (temperature: 22 ± 3°C, humidity: 60 ± 5% and a 12 h light/dark cycle). During the experiment, they were fed a standard pellet diet (Pastor Institute, Iran) and water *ad libitum*. All procedures were carried out according to the institutional guidelines for animal care and use.

Preparation of the topical formulations

To make the extract applicable, two types of topical formulations were prepared for the extract delivery: ointment and polymer film.

Preparation of ointment formulation

The topical formulation (10%) was accordingly prepared: the ethanolic extract (20 g) was dissolved in distilled water (20 mL), resulting solution was gradually added to eucerin 200% to give the final weight of 200 g, and well mixed to afford a homogenous product. Also, an ointment base with no extract was used as placebo (negative control).

Preparation of polymer film formulation

Hydroxypropyl methylcellulose (HPMC) and chitosan were added to an acetic acid solution in deionized water (1%) to obtain the final concentrations of 1% and 3%, respectively. The ethanolic extract and glycerol (to prevent excessive moisture loss) were then added to the mentioned mixture with final concentrations of 10% and 3%, respectively. The mixture was slowly stirred at room temperature to afford a homogenous and viscous solution.

It should be noted that a film base with no extract was used as placebo (negative control). All solutions were kept in the refrigerator to degas and remove any air bubbles. Then, they were poured in petri dishes and dried for 48 h to form a soft film. The films pH was then adjusted at 6.5-7 using a 1N NaOH solution.

Excision wound model

The excision wound model was used to evaluate the wound healing property of the ethanolic extract of *P. orientalis* leaves. After inducing anesthesia *via* the intraperitoneal injection of 2% xylazine (5 mg/kg) and 10% ketamine (90 mg/kg), the rats were fixed in a ventral posture on a surgery table. Then, the dorsal area from the scapula to the ilium was shaved and prepared for surgery. One square (2 cm×2 cm), full-thickness wounds (about 2 mm deep), 1 cm away from both sides of the backbone, was made using a Bistoury surgical knife. The epidermal, dermal, hypodermal, and panniculus carnosus layers were removed.[34]

Grouping animals

The animals were numbered, weighed, and randomly divided into five groups of six rats: O group: Rats treated with ointment base with no extract as placebo (negative control). D-O group: Rats treated with a topical ointment containing 10% (w/w) ethanolic extract of *P. orientalis* leaves. F group: Rats treated with polymer film base with no extract as placebo (negative control). D-F group: Rats treated with a polymer film containing 10% (w/w) ethanolic extract of *P. orientalis* leaves. C group: Rats treated with 'Comfeel® plus' as positive control.

By creating a wound area in the dorsum of animals in this method, all wounds were dressed by only sterile gauze on day zero for 24 h to allow completion of expansion phase. From day 1, the topical formulations (ointments and polymer films) were applied to the surface of the wound. The ointments, both containing the extract and placebo were applied to the wound using supportive sterile gauze. And the polymer films were applied directly to the wound surface and were dressed using supportive sterile gauze. For the positive control group, 'Comfeel® plus' sheets were placed on the wounds. The wound dressing was changed every 24 h. All rats were monitored daily for 14 days and wound healing process was followed. The wound area was calculated using Adobe Photoshop CC 2017 software (Adobe Systems Inc.).

Histopathology studies

Animals from each group were euthanized on 7 and 14 days post-injury and the skin tissues were harvested and immediately fixed in the 10% neutral buffered formalin (pH 7.26) for 48 h. Then, the fixed tissue samples processed, embedded in paraffin, and sectioned to 5 mm thickness. Finally, the sections were stained with haematoxylin and eosin (H&E). The histological slides were evaluated by the independent reviewer, using light microscopy (Olympus BX51; Olympus, Tokyo, Japan). Epithelialization, inflammatory cell infiltration, fibroplasia, and granulation tissue formation have assessed in different groups, comparatively.[35]

Epithelialization on day 14 was assessed semi quantitatively on 5 point scale: 0 (without new epithelialization), 1 (25%), 2 (50%), 3 (75%), and 4 (100%). For these parameters, results were validated by a comparative analysis of one independent observer blinded to the treatment groups. Also, neovascularization and collagen density were calculated and analyzed using computer software Image-Pro Plus® V.6 (Media Cybernetics, Inc., Silver Spring, USA). Masson's Trichrome (MT) staining was used to recognize the progress of collagen synthesis during the formation of granulation tissue (GT) and matrix remodeling. Collagen fibers were stained blue in the MT staining method and the intensity of the color corresponds to the relative amount of deposited total collagen reflecting the advancement of collagen synthesis and remodeling. Magnification ×200 was employed for counting the cells and the calculation was repeated for five fields. The average number of collagen content or collagen density (%) for these fields was then recorded. The number of blood vessels was also counted in five randomly selected high-power field (blood vessels/5HPF).[36]

Statistical analysis

For parametric data, the difference between groups were evaluated by one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test for multiple comparisons. P-values less than 0.05 were considered significant.

Results

Extraction yield and phytochemical analysis

The yield of ethanolic extraction of *P. orientalis* leaves was calculated as 31.5%. The total phenolic and flavonoid contents were shown in Table 1 and the results related to the qualitative tannin tests were portrayed in Table 2.

1,1-Diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity

The ethanolic extract of *P. orientalis* leaves was investigated for its DPPH radical scavenging activity in comparison to butylated hydroxyanisole (BHA) as the reference antioxidant agent.[31] The ethanolic extract showed desirable antioxidant activity with IC_{50} value of 22.94 ± 0.03 µg/mL comparing with BHA (IC_{50} = 91.28 ± 0.13 µg/mL).

Wound healing activity

The decrease in the wound surface area was evaluated in

Table 1. Total phenolic and flavonoid contents of ethanolic extract of *P. orientalis* leaves.

Total phenolic content (µg gallic acid equivalent/mg dry extract)	9.23 ± 0.01
Total flavonoid content (µg catechin acid equivalent/mg dry extract)	111.65 ± 0.01

Table 2. Qualitative analysis of tannins in the ethanolic extract of *P. orientalis* leaves.

Assay	Result	Presence/Absence of tannin
Gelatin test	White precipitate	+
Braymer's test (ferric chloride test)	Green color	+

Table 3. Wound healing effects of the ethanolic extract of *P. orientalis* leaves[a].

Wound surface area (cm^2)	O	D-O	F	D-F	C
Day 1	9.83	8.20	9.44	9.09	10.98
Day 4	7.09	5.15	7.37	7.09	6.32
Day 7	4.97	4.15	5.80	6.38	5.09
Day 14	1.18	0.90	1.28	1.44	0.78

[a]Investigations were performed in two different topical formulations in an excision wound model. O= Group treated with ointment base with no extract, D-O = Group treated with topical ointment containing extract, F= Group treated with polymer film base lacking extract, D-F= Group treated with a polymer film containing extract, and C = Positive control.

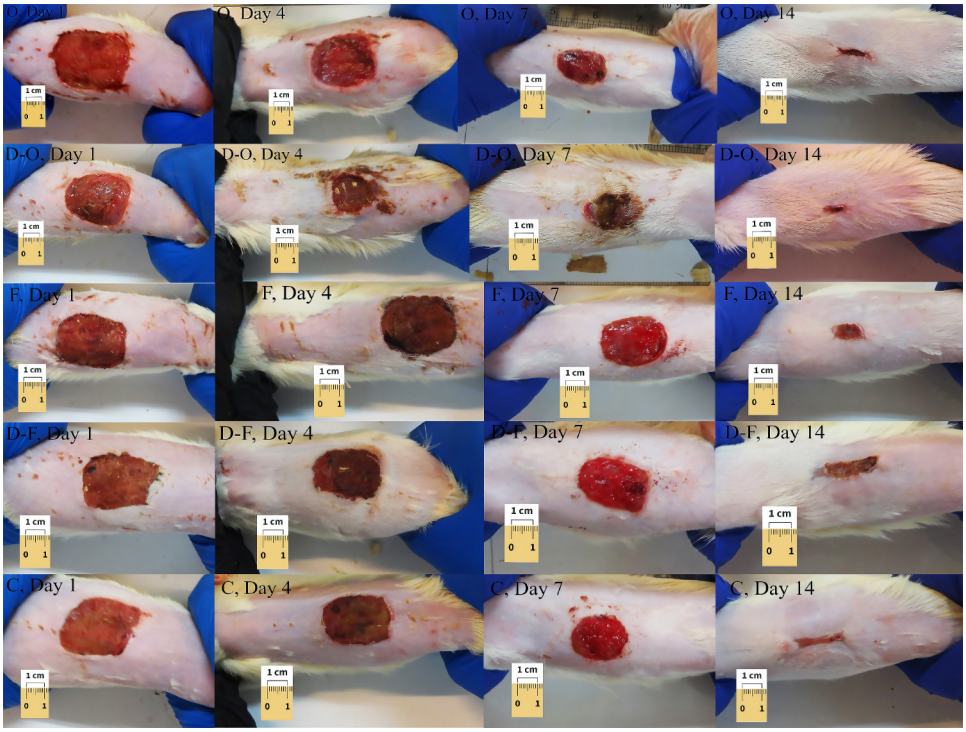

Figure 1. Wound healing process within 14 days

different groups during 14 days. The wound surface area was measured on days 4, 7, and 14 post-injury in all groups (Table 3). The results were demonstrated in Figure 1.

According to the results shown in Table 3, wound surface area of the D-O group were remarkably reduced compared to those of the ointment-negative control group (O) on days 4, 7 and 14 post-injury. On the other hand, the D-F group showed no important reduction in the wound surface area compared to those of the negative control group (F). It could be argued that the anti-inflammatory and astringent effects of polyphenolic compounds and tannins in the ethanolic extract of *P. orientalis* contributed to the obtained results. Poor performance of D-F group despite containing the same amount of extract can be associated with improper release of the extract.

Histopathology study

The study was conducted by the collection of upper layers of treated tissues on days 7 and 14 post-injury, followed by H&E and MT staining as shown in Figures 2 and 3.

Micrographs of the ointment negative control group (O) on day 7 post-injury showed inflammatory cells infiltration and the wound covered by a crusty scab. The epidermal layer has not been formed within 14 days post-injury and the wound area filled by a highly vascularized granulation tissue. Histopathological evaluation of the group D-O on day 7 showed the infiltration of inflammatory cells into the defect area. Finally, on day 14 post-injury, the epithelialization process was started and the inflammatory cells were significantly reduced in comparison to the O treatment group at the same time point.

Histopathology of wounds treated by the film negative control group (F) showed severe inflammatory response

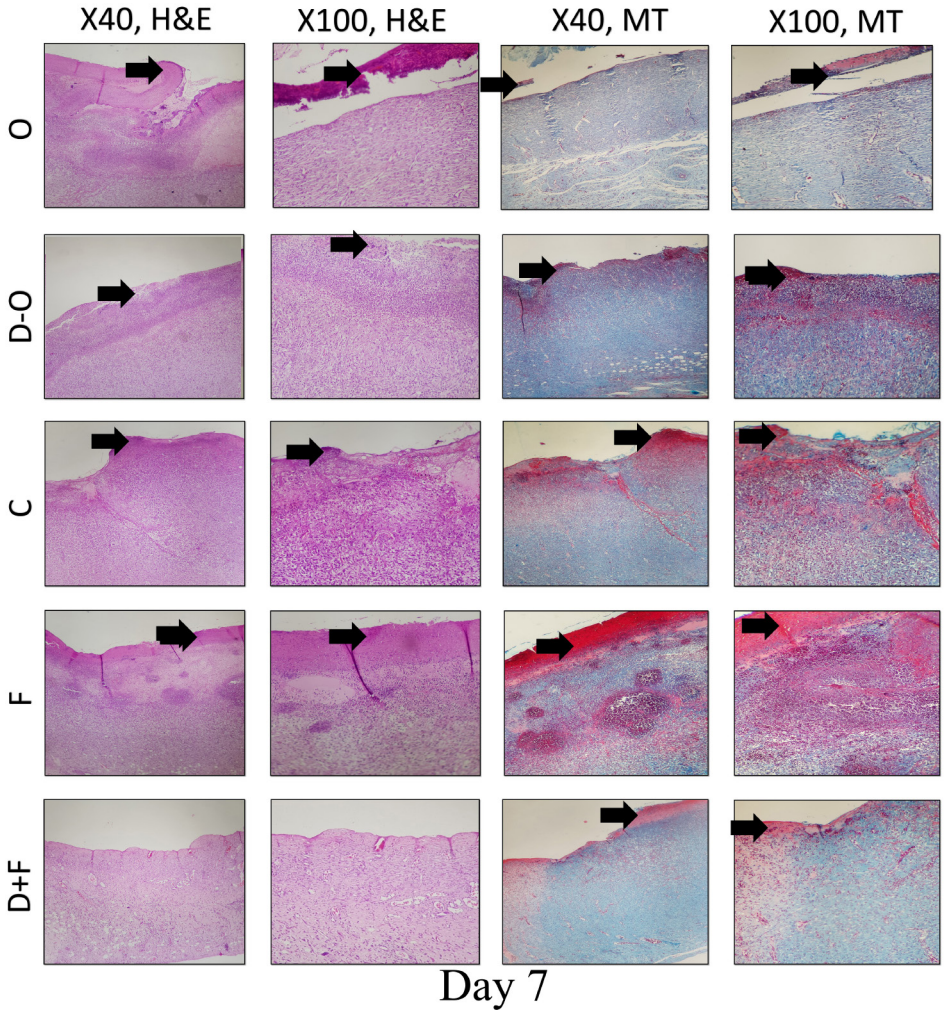

Figure 2. Histopathology of wounds of all groups on day 7 post-injury in X40 and X100 magnifications. Thick arrows reffer to crusty scab. The microscopic sections were stained using hematoxylin and eosin (H&E) and Masson's trichrome (MT).

which significantly decreased within 14 days post-injury. On the other hand, the inflammatory response in the D-F treatment group was considerably lower than the F group on day 7 post-injury. However, in the same manner, the wound area was covered by a crusty scab after 14 days in both D-F and F treatment groups.

Micrographs of the 'Comfeel® plus' (C) treatment group on day 7 post-injury revealed a close similarity to the D-O treatment group, a crusty scab covered the wound area without the epidermal formation and the presence of inflammation in wound area was evident. However, inflammation considerably reduced on day 14 post-injury. Epidermis and dermis also started to regenerate and rejuvenation of skin appendages were also evident on day 14 post-injury.

Overall, the positive group (C) showed more resemblance to normal skin than other groups; with a thin epidermis, the presence of normal rete ridges, and rejuvenation of skin appendages (hair follicles and sebaceous glands).

Analysis of histological features was performed on days 7 and 14 post-injury as reported in Table 4. Among all groups, the best re-epithelialization was observed in the D-O comparing with the positive control group (C). However, the corresponding negative control (O group) displayed poor skin wound re-epithelialization indicating that it was mostly filled with immature granulation tissue. In the case of polymer film formulation (D-F), no remarkable re-epithelialization was observed comparing with related negative (F) as well as positive groups (C).

In the case of neovascularization (blood vessels/5HPF), D-O group depicted the best value (59.75±4.27) on day 7 as compared with positive control (66.50±2.64). In comparison, the O group showed significantly reduced neovascularization (41.25±2.98) ($P<0.0001$). As expected, the desired reduction of neovascularization value was obtained in D-O group (20.75±2.36) on day 14, and the same was observed in the positive control (31.75±2.98). Also, comparing the D-O group with the related negative control (O) (34.75±4.64) demonstrated significant difference ($P = 0.005$).

D-F group showed an increased neovascularization on day 7 (55.50±5.19), significantly higher than its negative control (F) which depicted the lowest values among all groups on day 7 (16.25±2.98, $P<0.0001$). On the other

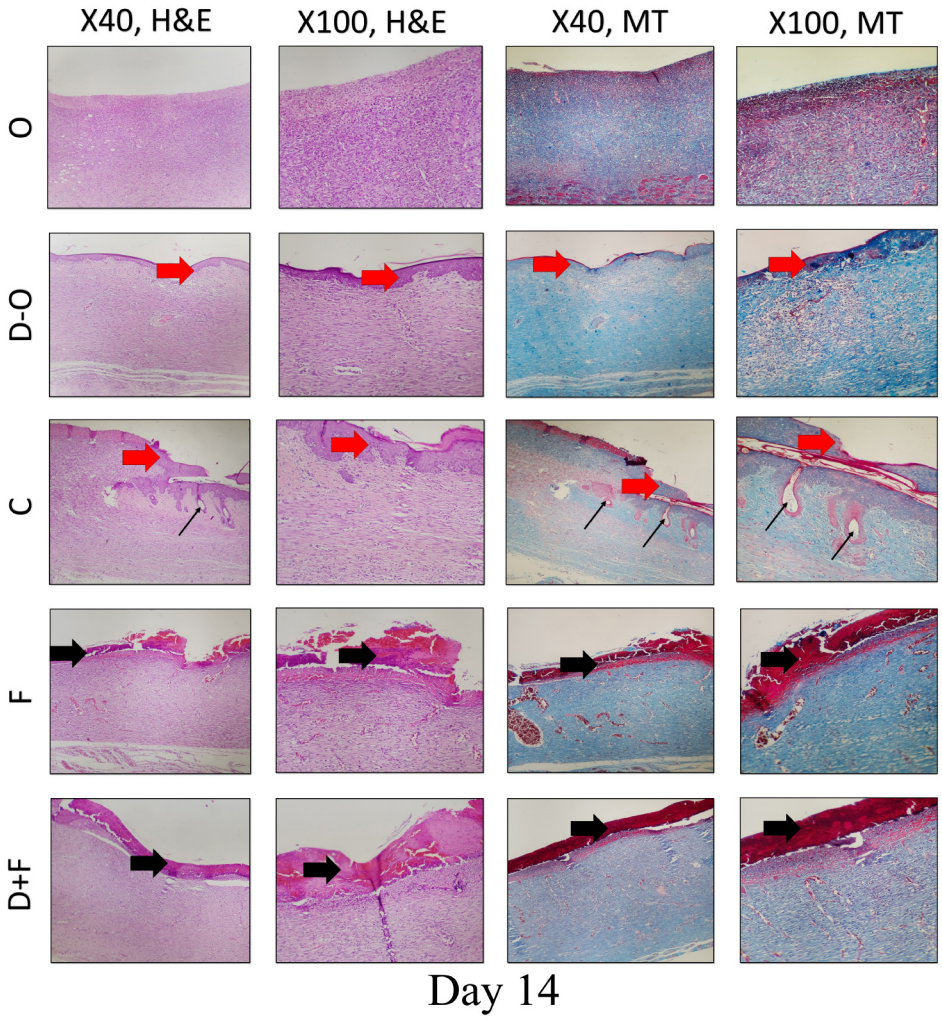

Figure 3. Histopathology of wounds of all groups on day 14 post-injury in X40 and X100 magnifications. Thick black arrows, thick red arrow, and thin arrows refer to crusty scab, re-epithelialization, and rejuvenation of skin appendages, respectively.

hand, both D-F and F groups showed a great increase in the neovascularization on day 14 (74.75±6.70 (P<0.0001) and 71.25±5.05, respectively), comparing with the reduced values of the positive control (C, 31.75±2.98). To the point that there is no statistically significant difference between D-F and its negative control (F) on day 14.

The results related to the collagen density indicated an increased amount of type I collagen in the D-O group on day 7 (55.00±2.58), significantly higher than the positive control (C, 31.25±3.40, P<0.0001) and negative control groups (O, 40.75±3.59, P = 0.0001). The amount of type I collagen on day 14 for both D-O and positive control groups, increased to 65.50±3.41 and 63.50±2.88, respectively. D-O also showed significantly higher collagen content than the negative control (O, 39.50±7.23, P<0.0001). The formation of type I collagen in D-F group (42.75±3.40) significantly increased comparing with both positive (C, 31.25±3.40, P = 0.001) and negative controls (F, 21.25±2.98, P<0.0001) on day 7. On day 14, both D-F and F groups depicted a great increase in the collagen content (63.75±5.43 and 69.50±3.10, respectively), as same as the positive control (C, 63.50±2.88).

Discussion

Wound healing methods based on the natural therapies have been recently in the spotlight,[37] because they are rich in a wide range of phytochemicals possessing potent anti-inflammatory, anti-oxidant, and anti-bacterial properties which synergistically play a crucial role in the wound healing cascade. From the ancient times, herbal remedies were used as a strong tool for the treatment of wounds. Wound healing properties of *P. orientalis* leaves have been recommended in ITM, and herein, we investigated its efficacy in an *in vivo* excision wound model. For this purpose, various formulations including ointment (D-O) and polymer film (D-F) were prepared and evaluated. The ointment formulation was selected as it is the most conventional form of topical treatment of wounds. Also, polymer films have recently emerged as a versatile and efficient type of wound dressing preventing dehydration, providing a barrier to protect the wound from dust and infections, while stimulating epithelialization at the same time.[38] In this respect, it was another candidate for the evaluation of wound healing properties of *P. orientalis*. According to the results reported in Table 3, D-O possessed

Table 4. Histological features of wound healing with different topical formulations of the ethanolic extract of *P. orientalis* leaves.

Day	Treatment groups[a]	Epitheliogenesis Score (n = 4)	Neovascularization[b] (blood vessels/5HPF) (Mean +SD)	Collagen density (%)[b] (Mean + SD)
Day 7	O	0,0,0,0	41.25 ± 2.98	40.75 ± 3.59
	D-O	0,0,1,0	59.75 ± 4.27	55.00 ± 2.58
	F	0,0,0,1	16.25 ± 2.98	21.25 ± 2.98
	D-F	0,0,0,0	55.50 ± 5.19	42.75 ± 3.40
	C	0,0,1,0	66.50 ± 2.64	31.25 ± 3.40
Day 14	O	1,1,1,1	34.75 ± 4.64	39.50 ± 7.23
	D-O	2,3,2,3	20.75 ± 2.36	65.50 ± 3.41
	F	1,2,1,1	71.25 ± 5.05	69.50 ± 3.10
	D-F	1,0, 2,0	74.75 ± 6.70	63.75 ± 5.43
	C	3,2,1,2	31.75 ± 2.98	63.50 ± 2.88

[a]O = group treated with ointment base with no extract, D-O = group treated with topical ointment containing extract, F= group treated with polymer film base lacking extract, D-F= group treated with a polymer film containing extract, and C = positive control.
[b]The differences between groups were evaluated by one-way ANOVA and P-values less than 0.05 were considered significant.

more potent wound healing properties than D-F, based on the surface area of the wounds within 14 days. The D-F group despite having the same amount of *P. orientalis* extract, lacked desired wound healing properties, may be due to improper release of the extract.

The healing property of *P. orientalis* leaves seems to be associated with the presence of phenolic compounds especially flavonoids and tannins. As reported in Table 1, high amounts of phenolic compounds and flavonoids were ubiquitous in the ethanolic extract of leaves. Phytochemical analysis of the leaves has confirmed the presence of flavonoids as the main compounds. In this regard, kaempferol, quercetin, and their derivatives such as kaempferol-3-*O*-α-L-(2′′,3′′-di-*E*-*p*-coumaroylrhamnopyranoside), kaempferol-3-*O*–β-D-(6′′-*E*-*p*-coumaroylglucopyranoside), kaempferol-3-*O*-α-Lrhamnopyranoside, quercetin-3-*O*-α-Lrhamnopyranoside, quercetin-3-*O*-β-D-galactopyranoside, kampferol-3-*O*-β- rutinoside, quercitin-3-*O*rutinoside,[39,40] kaempferol-3-*O*-(2′′′-*p*-*E*-coumaroylrhamnosyl)(1′′′→6′′)-[β-D-glucopyranosyl-(1′′′′→2′′)-β-D-glucopyranoside]-7-*O*-α-L-rhamnopyranoside,[23] kaempferol 3-*O*-α-l-rhamnopyranoside, kaempferol 3-*O*-β-d-glucopyranoside,[21] and quercetin-3-*O*-(2′′′-*p*-*E*-coumaroylrhamnosyl)(1′′′→6′′)-[β-D-glucopyranosyl-(1′′′′→2′′)-β-D-glucopyranoside]-7-*O*-α-L-rhamnopyranoside.[23] Also, acylated flavonol glycosides including 3′,5,7-trihydroxy-4′-methoxyflavonol 3-[*O*-2-*O*-(2,4-dihydroxy)-*E*-cinnamoyl-α-L-rhamnopyranosyl-(1→6)-β-D-glucopyranosyl (1→2)]-β-Dglucopyranoside,[24] and 5,7,4′-Trihydroxy-3,6-dimethoxyflavone-3′-*O*-β-xylopyranoside (axillarin-3′-*O*-β-xylopyranoside),[40] phenolic acids including caffeic acid,[21] *p*-coumaric acid, and 4′-caffeoylquinic acid (cryptochlorogenic acid),[40] tocopherols, and esters of phytol with aliphatic acids have been reported.[27] It has been perceived that the efficacy of medicinal plants in the treatment of wounds is debated to various flavonoids as they are responsible for angiogenesis, collagen deposition, epithelization and wound contraction in the proliferative stage.[29] Furthermore, they have been reported to possess anti-oxidant, anti-viral, anti-microbial, anti-inflammatory, and anti-proliferative activities which can promote wound healing by reducing the complications of the process.[41] Also, qualitative assays as shown in Table 2, revealed the presence of tannins. Tannins have been described to possess effective anti-bacterial,[42,43] and anti-oxidant activities,[44] which can accelerate the wound healing process.[45]

Reactive oxygen species (ROS) play an important role in wound repair and high levels of ROS can complicate the healing process. In this respect, antioxidants can improve all types of wounds.[46] Potent antioxidant activity of the ethanolic extract recommends it as a suitable herbal remedy for the treatment of wounds.

Wounds are usually the source of bacteria leading to cross-contamination and in this respect, fighting against *Staphylococcus* spp. particularly *S. aureus* has been a strong tool in wound healing specially in hospitalized patients.[47] Ucar *et al.*[48] reported significant anti-microbial activity of hydroalcoholic extract of *P. orientalis* leaves against *S. aureus* (MIC = 0.018 mg/mL). This can be considered as a complementary aspect of the plant for inducing wound healing properties.

Using the proper formulation was found to be an important factor playing a significant role in the wound healing process which was demonstrated by results obtained from histopathological studies. As reported in Table 4, D-O group showed much better re-epithelialization than D-F group, even comparable with the positive control group. This may be related to the presence of flavonoids as they have been documented for their re-epithelialization rate enhancement, in the proliferative phase for the improvement of cutaneous impairment.[49]

The neovascularization, formation of new blood vessels, has been found to be a vital process in the four stages of wound healing including hemostasis, inflammation, proliferation and remodeling. In the proliferative phase, extra vessels in the wound bed are essential to provide the metabolic requirements of the cells responsible for

repairing the impaired tissue. In the remodeling phase, the neovascularization value is reduced to that of pre-injury state due to the reduction of metabolic need.[50] Failure of *this process* conflicts with wound healing leading to chronic ulcers.[51]

It has been reported that flavonoid glycosides induce neovascularization.[52] The high amounts of flavonoids in the *P. orientalis* leaves, confirmed the efficacy of D-O group in which the corresponding value on day 14 (Table 4) reduced to approximately one-third of the value on day 7, accelerating the wound healing process. The D-F group which showed no significant difference comparing with F group, showed an increased neovascularization value on day 14 indicating delayed proliferative phase or disrupted remodeling stage.

To sum up, comparison of D-O and D-F groups revealed no significant difference on day 7, however, neovascularization in D-O group was significantly reduced comparing with D-F group on day 14 ($P<0.0001$).

It has been determined that increase of type I collagen in wound healing process can bring about contraction and wound closure. Early increase of type I collagen density in D-O group on day 7 was more significant than the positive control (C). The increase of collagen density was also observed on day 14 in comparison to group C. It may be related to the stimulation of collagen biosynthesis by flavonoids present in the plant leaves extract.[53,54] Although the collagen content of D-F group increased on days 7 and 14, other histological features did not endorse wound healing process.

Comparing the collagen density of D-O and D-F groups on day 7 indicated a significant difference ($P<0.001$), while it was not statistically important on day 14.

The results reported in Table 4, related to epitheliogenesis score, neovascularization, and collagen density, demonstrated more potent wound healing property of the ointment formulation (D-O) than the film polymer type (D-F) which is in good agreement with those results reported in Table 3.

Conclusion

Herein, the wound healing effects of the ethanolic extract of *P. orientalis* leaves were studied in two topical formulations including ointment and polymer film. The results indicated the higher efficacy of ointment (D-O) than polymer film (D-F). Also, anti-bacterial activity of the extract against *S. aureus*, and antioxidant property of the plant were found to be important factors in reducing wound complications. Epitheliogenesis score, neovascularization, and collagen density as significant histological features contributing to wound healing, were also studied. Those results depicted that the early collagenization and induced neovascularization of the ointment group (D-O), have greatly contributed to its efficacy comparing to the negative control (O).

Ethical Issues

The study was approved by the Committee of Ethics of the Faculty of Pharmacy of Tehran University of Medical Sciences with approval number: IR.TUMS.VCR.REC.1397.479.

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Author Contributions

SN participated in the suggestion and preparation of formulations. AR performed experimental part and contributed in the preparation of the manuscript. MB and YV contributed to the selection of the plant and preparation of extracts. MS and TA designed the project and supervised all processes. All authors read and approved the final manuscript.

Conflict of Interest

The authors report no conflicts of interest.

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