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NATURAL DRUGS

WOUND HEALING ACTIVITY OF *MALVA SYLVESTRIS* AND *PUNICA GRANATUM* IN ALLOXAN-INDUCED DIABETIC RATS

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Abstract: The flowers of *Malva sylvestris* Linn. (*Malvaceae*) and *Punica granatum* Linn. (*Punicaceae*) are important medicinal plants in Iranian traditional medicine (*Unani*) whose have been used as remedy against edema, burn, wound and for their carminative, antimicrobial and anti-inflammatory activities. The diethyl ether extract of *M. sylvestris* and *P. granatum* flowers were used to evaluate the wound healing activity at 200 mg/kg/day dose in alloxan-induced diabetic rats. Wounds were induced in Wistar rats divided into six groups as following; Group I, normal rats were treated with simple ointment base. Group II, diabetic rats were treated with simple ointment base (control). Groups III and IV, diabetic rats were treated with simple ointment base containing of extracts (diabetic animals), Groups V, diabetic rats were treated with simple ointment base containing of mixed extracts (1:1), Group VI, diabetic rats received the standard drug (nitrofurazone). The efficacy of treatment was evaluated based on wound area relative and histopathological characteristics. The extract-treated diabetic animals showed significant reduction in the wound area when compared with control. Also, histological studies of the tissue obtained on days 9th and 18th from the extract-treated by extract of *M. sylvestris* showed increased well organized bands of collagen, more fibroblasts and few inflammatory cells. These findings demonstrate that extract of *M. sylvestris* effectively stimulates wound contraction as compared to control group and other groups. *M. sylvestris* accelerated wound healing in rats and thus supports its traditional use.

Keywords*:* wound healing; *Malva sylvestris*; *Punica granatum;* diabetic rats

Healing of wounds, a fundamental response to tissue injury occurs by a process of connective tissue repair. A fibrous scar is the end product of this process, the pre-dominant constituent of which is collagen. Collagen and other components of the ground substance are synthesized by the highly vascular granulation tissue that is formed within the wound space. Collagen provides strength and integrity to the dermis (1). Diabetes mellitus is a condition which is known to be associated with a variety of connective tissue abnormalities. The collagen content of the skin is decreased as a result of reduced biosynthesis and/or accelerated degradation of newly synthesized collagen. These abnormalities contribute to the impaired wound healing observed in diabetes (2).

Wound care can be traced back to early civilizations, and many of these treatments were based on the use of herbal remedies (3). Approximately one-third of all traditional medicines used for the treatment of wounds and skin disorders, such as *Berberis lyceum* Royle. (4), *Cydonia oblonga* Miller. (5), *Inula viscosa* (6), *Lawsonia inermis* Linn. (7) etc., compared to only 1–3% of modern drugs, have been found effective in treating dermal wounds on different wound models. A survey of the ethnobotanical studies, carried out in Iran, indicated the use of several of plant species by the inhabitants of the area, especially by those habiting the rural areas for wound healing purpose (8, 9).

Malva sylvestris Linn. (*Malvaceae*), known locally as "Panirak", is a medicinal plants in Iran whose flowers are used for the treatment of various ailments, including cold, cough and burn and cut wound healing in rural areas of Iran (9, 10). Fluid extracts of *M. sylvestris* flowers and leaves are used as a valuable remedy for cough and inflammatory diseases of mucous membranes (11). The prelimi-

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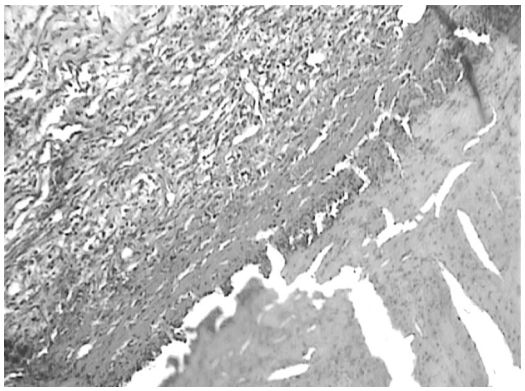
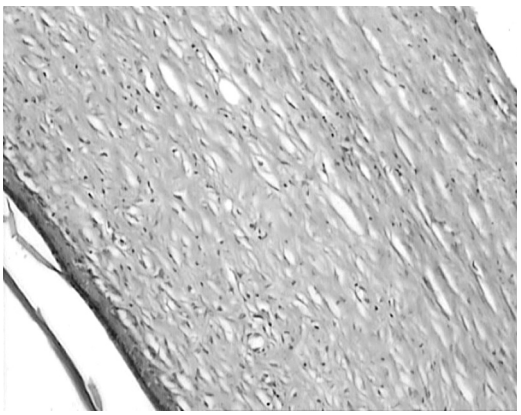
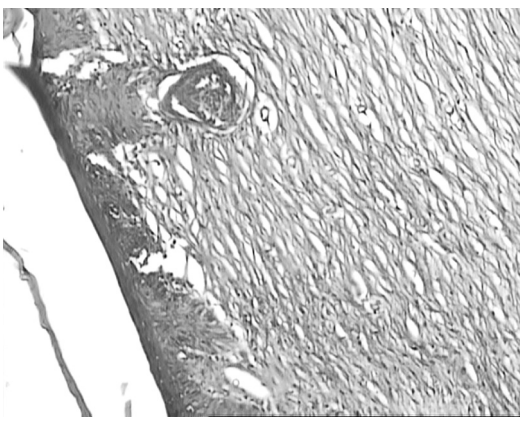


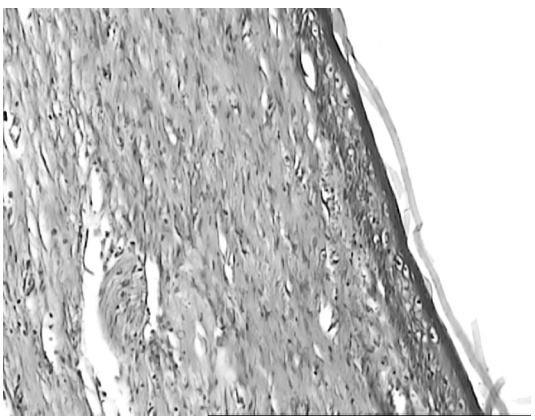
Group I – normal rats treated with simple ointment base



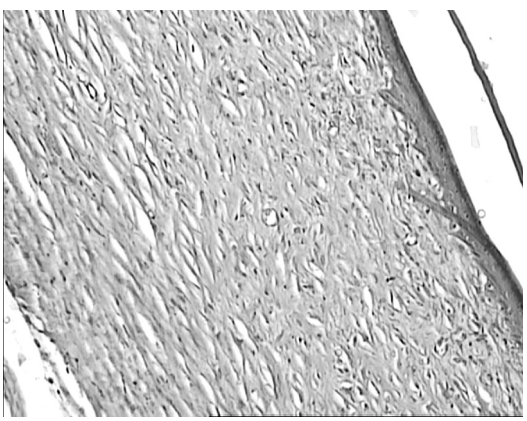
Group IV – diabetic rats treated with simple ointment base containing *Malva sylvestris*



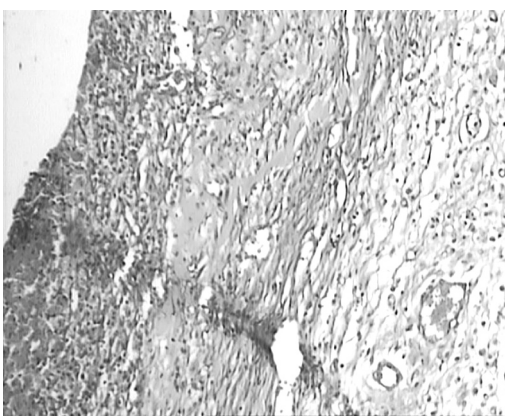
Group II – diabetic rats treated with simple ointment base (control)



Groups V – diabetic rats treated with simple ointment base containing mixed extracts



Group III – diabetic rats treated with simple ointment base containing *Punica granatum*



Group VI – diabetic rats treated with the standard drug (nitrofurazone)

Figure 1. Histological evaluation of normal and diabetic rats of different groups after 18 days of topical application (magnification 400×)

nary phytochemical analysis of the flower extract showed the presence of anthocyanin, malvin, malvidin 3-(6"-malonylglucoside)-5-glucoside, malvaline, niacin and folic acid (12).

Punica granatum Linn. (*Punicaceae*), known locally as "Golnar-e-farsi", is an important medicinal plants in Iran whose flowers are used as astringent, hemostatic, antibacterial, antifungal, antiviral and as a remedy for cut wound, bronchitis, diarrhea, digestive problems, man sex power reconstituent, dermal infected wounds and diabetes in *Unani* medicinal (Iranian Traditional Medicine) literature (9, 10). This flower was also used for the treatment of injuries from falls and grey hair of young man in the traditional Chinese medicine (13). Polyphenol compounds in *P. granatum* are pomegranatate; ellagic acid, 3,3',4'-tri-O-methylellagic acid, ethyl brevifolincarboxylate, urolic and maslinic acids, and daucosterol (14, 15).

No systematic studies have yet been carried out on the experimental evaluation of the wound healing potency of *M. sylvestris* and *P. granatum*, so their effects were investigated using wound and histopathological characteristics in diabetic rats.

MATERIALS AND METHODS

Plant material and extract preparation

The flowers of *M. sylvestris* and *P. granatum* were collected from mountain areas of Zagross, district of Chaharmahal va Bakhtiari, Iran, during May–June, 2008. Their identity was confirmed and voucher specimens were deposited at the Spice, Aromatic and Medicinal Plant Research Center (SAMPRC), Islamic Azad University, Iran. About 100 g of powdered flowers of *M. sylvestris* and *P. granatum* were extracted with absolute diethyl ether (Merck, Germany) using Soxhlet apparatus for 12 h. The concentrated extracts were filtered using Whatman No. 1 filter paper and then lyophilized giving a green residue with yield 5.6% for *M. sylvestris* and a red residue with 10% w/w for *P. granatum*

Experimental animals

Male Wistar rats (150–180 g) two months of age were used. The animals were housed in standard environmental conditions of temperature (22 ± 3°C), humidity (60 ± 5%) and a 12-h light/dark cycle. During experimental time, Wistar rats were given standard pellet diet (Pastor Institute, Iran) and water *ad libitum*. All the procedures were approved by the Medical Ethics Committee of Iran University of Medical Sciences.

Diabetic animals

After 15 h fasting, rats were intraperitoneally treated daily with 125 mg/kg of alloxan monohydrate (Sigma Chemicals, St. Louis, USA) freshly dissolved in distilled water (5%) for two or three consecutive days (16). Blood was drawn from the orbital plexus 24 h after the injection and the glucose level was estimated. Wounds were made on the rats showing elevated blood glucose (> 250 mg/dL). Animals were divided randomly in six groups as following: Group I, normal rats were treated with simple ointment base. Group II, diabetic rats were treated with simple ointment base (control). Groups III and IV, diabetic rats were treated with simple ointment base containing extracts (0.2%), Group V, diabetic rats were treated with simple ointment base containing mixed extracts (1:1), Group VI, diabetic rats received the standard drug (nitrofurazone, Najo® 0.2%, Iran) at 200 mg/kg/day dose for all groups.

Wound healing activity

For wound induction and evaluation of extracts for properties of wound healing, before the beginning of the wound healing experiments, the dorsal skin of the Wistar rats were shaved. Animals were anesthetized with 1.5 mg/kg *i.p.* of ketamine and xylazine. A full thickness of the excision wound (circular area about 150 mm^2 and 2 mm depth) was created along the markings using toothed forceps, a surgical blade and pointed scissors (6). During the wound healing period and at time intervals, the wound area was traced manually and photographed. The wound area was calculated using AutoCAD Version 14 (Autodesk Company) software. At day 3, 6, 9, 12, 15 and 18 the experiment was terminated and the wound area was removed from the surviving animals for histological examination. The excision skin biopsies were fixed in 4% formaldehyde solution after 48 h during the experimentation period.

Analysis of data

The wound relative area was statistically analyzed as the mean ± S.D. and statistical significance between treated and control groups were analyzed. Data are significant at p values ≤ 0.05 compared with control by SAS ver 6.12.

RESULTS AND DISCUSION

Wound area was traced manually and was photographed in each 3 days interval and healed area calculated by subtracting from the original wound area.

On days 3, 6, 9, 12, 15 and 18 the wound area relative of standard and extract ointment treated groups was found to be significant ($p < 0.05$) in comparison to simple ointment base treated group. On days 3, 6, 9, 15 and 18 there was no statistically significant difference in the wound area relative in diabetic animals treated with *M. sylvestris* and *P. granatum* (Table 1 and Fig. 1). But, on the day 12 there was statistically significant difference in the wound area relative in diabetic animals treated with *M. sylvestris* and *P. granatum* (Table 1 and Fig. 1). On the day 18, normal rats were treated with simple ointment base (Group I) and diabetic rats were treated with simple ointment base containing the extracts (Groups III, IV and V), healed 100%, while standard drug showed 57% healing. It was also observed that epithelialization periods of extract groups were shorter in comparison to simple ointment base treated group (Table 1 and Fig. 1).

On the day 9, the study of the histological structure showed the tissue regeneration grater in the skin wound treated with simple ointment base containing extract of *M. sylvestris* and following *P. granatum, M. sylvestris*+ *P. granatum* and nitrofurazone ointment (Table 2 and Fig. 2). On the day 18, incision and dead space type of wounds in groups of plant extracts shown complete healing as in collagenation, fibroblasts cells and angiogenesis (Table 2 and Fig. 2), whereas the skin wound treated with simple ointment base (diabetic rats) presented edema, monocyte cells and area with cellular necrosis that were not observed in the treated with herbal ointments and standard drug (Table 2 and Fig. 2).

Despite the traditional uses of *M. sylvestris* and *P. granatum* in wound healing process in Iran, there are no reported data available in the literature. *M. sylvestris* and *P. granatum* widely distributed plants of Iran are used for the anti-infectious, anti-inflammatory, anti-microbial, skin disease and for wound healing treatment according to several ethnobotanical surveys (8, 9, 17).

Wound healing is a process by which damaged tissue is restored as closely as possible to its normal state and wound contraction is the process of shrinkage of the area of the wound (7). It is mainly dependent upon the type and extent of damage, the general state of health and the ability of the tissue to repair. The aims in these processes are to regenerate and reconstruct the disrupted anatomical continuity and functional status of the skin (18).

Wound contracture is a process that occurs throughout the healing process, commencing in the fibroblastic stage whereby the area of the wound undergoes shrinkage. In the maturational phase, the final phase of wound healing, the wound undergoes contraction resulting in a smaller amount of apparent scar tissue. Granulation tissue formed in the final part of the proliferative phase is primarily composed of fibroblasts, collagen, edema, and new small blood vessels. The increase in dry granulation tissue weight in the test treated animals suggests higher protein content (19).

Table 1. Effect of the treatments on wound healing in rats.

Treatments	Wound area relative (cm^2)					
Day →	3	6	9	12	15	18
Simple ointment (diabetic rats)	1.499 ± 4.590	1.481 ± 4.588	1.469 ± 4.551	1.451 ± 4.369	1.403 ± 4.127	1.347 ± 4.045
Punica granatum + Simple ointment base (diabetic rats)	1.129 ± 3.387	0.968 ± 2.903	0.356 ± 1.069	0.128 ± 0.383	0.086 ± 0.258	0.000 ± 0.000
Punica granatum + *Malva sylvestris* Simple ointment base (diabetic rats)	1.322 ± 3.965	1.084 ± 3.251	0.246 ± 0.737	0.194 ± 581	0.165 ± 0.559	0.112 ± 0.033
Malva sylvestris+ Simple ointment base (diabetic rats)	1.175 ± 3.525	0.998 ± 2.993	0.454 ± 1.362	0.249 ± 0.748	0.108 ± 0.324	0.000 ± 0.000
Nitrofurazone (diabetic rats)	1.450 ± 3.354	1.305 ± 3.768	0.964 ± 2.891	0.814 ± 2.441	0.741 ± 1.224	0.711 ± 1.004
Simple ointment (non diabetic rats)	1.377 ± 4.132	1.184 ± 3.552	1.134 ± 3.402	1.001 ± 3.004	0.231 ± 0.694	0.000 ± 0.000

Each value represents the mean ± S.D. n = 9

Table 2. Effect of the treatments on the evolution of wounds in rats after 9 and 18 days of topical application.

Treatments	Inflammation cells		Collagen ibers		Re-epithelization		Organization of the collagen		Necrosis	
Day→	9	18	9	18	9	18	9	18	9	18
Punica granatum + Simple ointment base (diabetic rats)	+	–	+	++	+	+++	–	++	+	–
Punica granatum + *Malva sylvestris* + Simple ointment base (diabetic rats)	+	–	+	++	+	+++	–	++	+	–
Malva sylvestris+ Simple ointment base (diabetic rats)	+	–	++	+++	+	+++	+	+++	–	–
Nitrofurazone (diabetic rats)	+	+/–	+	++	+	++	–	++	++	+
Simple ointment (non diabetic)	++	+	+	++	–	+	–	+	++	+
Simple ointment (diabetic rats)	+++	+++	–	+	–	–	+	–	+++	+++

+: slight, ++: moderate, +++: extensive, –: absent

In the present study, the wound healing potential in diabetic animals for *M. sylvestris* and *P. granatum* was evident on the day 18 (Table 1 and Fig. 1), this potential was further confirmed in the histological evaluation (Table 2 and Fig. 2). No healing effect was observed with simple ointment in diabetic rats (Table 1 and Fig. 1). On days 3, 6, 9, 15 and 18, animals treated with the *M. sylvestris* showed results similar to animals treated with *P. granatum*, with improving the wound healing process (Table 1 and Fig. 1). The results in this study are in support that the wound healing and repair is accelerated by applying *M. sylvestris* and *P. granatum*, which was highlighted by the full thickness coverage of the wound area by an organized epidermis in the presence of mature scar tissue in the dermis. This ability was especially obvious when these data were compared with those of other groups. The enhanced capacity of wound healing with the *M. sylvestris* and *P. granatum* could be explained on the basis of the anti-inflammatory effects of the plants that are well documented in the literature (8, 9, 17). This outcome was expected according to the wide use of these plants by the inhabitants of Iranian for enhancement of the healing process. The clear difference in the measured wound area between extracts and the control is supported by histological observations. On the day 18, the results of histological evaluation showed that *M. sylvestris* significantly reduced the relative wound area (Table 1 and 2).

Collagen, the major component which strengthens and supports extracellular tissue, is composed of the amino acid, hydroxyproline, which has been used as a biochemical marker for tissue collagen (18). The preliminary phytochemical analysis of the flower extract of *M. sylvestris* showed the presence of anthocyanin, malvin, malvidin, malvaline, niacin and folic acid. Any one of the phytochemical constituents present in *M. sylvestris* may be responsible for antimicrobial activity (12). Also, the results of phytochemical research of *Punica granatum* flower extract showed the absence of polyphenol compound named pomegranatate (14, 15). Polyphenol compound may be responsible for antimicrobial activity. This may be due either to the individual or additive effect of the phyto-constituents that hasten the process of wound healing. The exact component of the extract that is responsible for this effect, however, was not investigated. Further phytochemical studies are needed to isolate the active compound(s) responsible for this pharmacological activity.

CONCLUSION

The results of study showed that the extract ointments of *M. sylvestris* and *Punica granatum* effectively stimulate wound contraction as compared to control group and other groups. These finding could justify the inclusion of this plant in the management of wound healing.

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