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Healing potential of Iranian traditional medicinal plants on burn wounds in alloxan-induced diabetic rats

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Article

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Abstract: *Malva sylvestris*, *Punica granatum, Amygdalus communis, Arnebia euchroma* and *Scrophularia deserti* are important medicinal plants in Iranian traditional medicine (*Unani*) whose have been used as remedy against edema, burn, and wound and for their carminative, antimicrobial and anti-inflammatory activities. The ethanol extracts of *M. sylvestris* and *P. granatum* flowers, *A. communis* leaves, *A. euchroma* roots and *S. deserti* stems were used to evaluate the burn healing activity in alloxan-induced diabetic rats. Burns were induced in Wistar rats divided into nine groups as following; Group-I: normal rats were treated with simple ointment base (control), Group-II: diabetic rats were treated with simple ointment base (control), Groups-III and –VII: diabetic rats were treated with simple ointment base containing of extracts (diabetic animals), Groups VIII: diabetic rats were treated with simple ointment base containing of mixed extracts, Group-IX: diabetic rats received the standard drug (Silver Sulfadiazine). The efficacy of treatments was evaluated based on wound area, epithelialization time and histopathological characteristics. Wound contraction showed that there is high significant difference between the different groups ($p<0.001$). At the 18th day, *A. euchroma, S. deserti, A. communis* and mixed extract ointment treated groups healed 80-90%. At the 9th and 18th days the experiment, the best results were obtained with *A. communis* and standard drug, when compared to the other groups as well as to the controls. It may be concluded that almond leaves (sweet and bitter) formulated in the simple ointment base is effective in the treatment of burns and thus supports its traditional use.

Keywords:
Amygdalus communis
Burn wound healing
diabetic
Iranian traditional medicine

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Introduction

Burn injury is a global public health issue especially for the developing and undeveloped countries, which lack adequate medical facilities. Burn injury may lead to complications such as long-term disability, prolonged hospitalization, loss of body extremities and even death. The skin is maintained by a discrete architecture of cells and extracellular matrix which, serves as the principle barrier to environmental and infectious agents. Tissue injuries resulting from burns, frost-bite, gunshots etc. disrupt this barrier, triggering a healing process (Arturson, 1995). Wound healing is a body's natural process of regenerating dermal and epidermal tissue. The sequence of events that repairs the damage is categorized into three overlapping phases' viz. inflammation, proliferation and tissue remodeling (Singer & Clark, 1999). However, burn is characterized by a hypermetabolic state which compromises the immune system leading to chronic wound healing. Thermal exposure to the body surface causes damage to the skin by membrane destabilization, protein coagulation, associated energy depletion and hypoxia at the cellular level which leads to extensive tissue necrosis. Furthermore, the burn wound is a continuous, severe threat against the rest of the body due to invasion of infectious agents, antigen challenge and repeated additional trauma caused by wound cleaning (Arturson, 1995). 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 (Raghow, 1994). Infection is a major complication of burn injury and is responsible for 50-75% of hospital deaths (Mokaddas et al., 1998).

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 (Goodson & Hunt, 1977). Diabetic patients are a special group of patients, known to have an increased risk of wound complications, such as infection and delayed healing. In burn patients, diabetes may have implications for length of hospitalization, hospital course, number of surgical procedures, and burn outcome. A retrospective study was designed in order to identify burn characteristics in diabetic patients admitted to our burn unit, and the impact of diabetes on their hospital course and outcome (Shalom et al., 2005).

Many of the synthetic drugs pose problems such as allergy, drug resistance, etc., forcing scientists to seek alternative drugs (Shanmuga Priya et al., 2002). More than 80% of the world's population depends upon traditional medicines for various skin diseases (Annan & Houghton, 2008). Recently, the traditional use of plants for wound healing has received attention by the scientific community (Annan & Houghton, 2008; Houghton et al., 2005). Approximately one-third of all traditional medicine in use are for the treatment of wounds and skin disorders, compared to only 1-3% of modern drugs (Mantle et al., 2001).

Several plants used as traditional healing remedies have been reported to treat skin disorders, including burn and cut wounds. In Iran, a survey of the ethnobotanical studies indicated the use of several of plant species by the inhabitants of the area, especially by those habiting the rural areas for burns healing purpose (Ghasemi Pirbalouti, 2009a; Ghasemi Pirbalouti et al., 2009a; Ghorbani, 2003; Zargari, 1989-1992). For example, tribal (Chaharmahal va Bakhtiari) in South West Iran, roots of *Arnebia euchroma* (Ghasemi Pirbalouti et al., 2009a), stems of *Scrophularia deserti* (Ghasemi Pirbalouti et al., 2010a) and leaves of sweet and bitter almond (*Amygdalus communis)* used as remedy burn wound (Ghasemi Pirbalouti, 2009b).

Many traditional remedies are based on systematic observations and methodologies and have been time-tested but for many of them, scientific evidence is lacking. There are only few prospective randomized controlled trials that have proved the clinical efficacy of these traditional burns healing agents. The present study was designed to test the *in vivo* burn wound healing activity of the ethanol extracts of five selected medicinal plants, namely; *Malva sylvestris* L. (Malvaceae), *Punica granatum* L. (Punicaceae), *Amygdalus communis* L. (Rosaceae), *Arnebia euchroma* Rolye. (Johnst) (Boraginaceae) and *Scrophularia deserti* Del. (Scrophulariaceae).

Material and Methods

Plant material

The *Malva sylvestris, Punica granatum, Amygdalus communis* and *Arnebia euchroma* were collected from mountain areas of Zagross, Chaharmahal va Bakhtiari, South-West Iran, and Scrophularia deserti was collected from Ilam, West Iran, during May-June, 2009. Provisional identifications of specimens were made with the help of "Flora of Iran" (Ghahreman, 1987–1989), "Flora of Ilam" (Mozaffarian, 2008), "Encyclopedia of Iranian Plants" (Mozaffarian, 1996), "Flora Iranica" (Rechinger, 1963-1998) etc. In addition Mr Shirmardi and Mr Prirani, Research Centre of Agriculture and Natural Resources, Ministry of Agriculture, Iran, authenticated the plants.

Extract preparation

About 100 g of powdered flowers of *M. sylvestris* and *P. granatum,* leaves of *A. communis* (sweet and bitter), roots of *A. euchroma* and stems of *S. deserti* were extracted with absolute ethanol (Merk, Germany) using Soxhlet apparatus for 12 h. The concentrated extract were filtered using Whatman No. 1 filter paper and then lyophilized gave a green residue with yield 5.8% for *M. sylvestris*, a red residue with 10.5 % w/w for *P. granatum*, a dark red residue with 12 % w/w for *A. euchroma* and green residue with 8.9 % and 6.5 % w/w for *A. communis* and *S. deserti*, respectively. The extract samples were stored in universal bottles and refrigerated at 4 °C prior to use.

Experimental animals

Male Wister rats (150-180 g) of two months were used. The animals were housed in standard environmental conditions of temperature (22±3 ºC), 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*. The rats were used for the experiment after one week of acclimatization period. All the procedures were approved by the Medical Ethics Committee of Shahrekord University of Medical Sciences.

Diabetic animals

After 15 h fasting, rats were intraperitoneally treated daily with 125 mg/kg alloxan monohydrate (Sigma chemicals, St Louis, USA) freshly dissolved in distilled water (5%) for two consecutive days (Diatewa et al., 2004). Blood was drawn from the orbital plexus 24 h after the injection and the glucose level was estimated.

Burns were made on the rats showing elevated blood glucose (>250 mg/dL).

Burn induction

Animals were anesthetized with 1.5 mg/kg *i.p.* of Ketamin and Xylazine and their dorsal surface was shaved with a sterile blade. The shaved area was disinfected with 70% (v/v) ethanol. The burn wounds were created using the method described by Shanmuga Priya et al. (2002) with some modifications. A cylindrical metal rod (15 mm diameter) was heated to 80-90 ºC and pressed to the shaved and disinfected surface for 20 s in rat under Ketamin and Xylazine anesthesia. The animals were randomly divided into nine groups each containing six animals.

Grouping of animals

Burns were induced in Wistar rats divided into nine 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 -VII: diabetic rats were treated with simple ointment base containing of extracts (diabetic animals), Groups VIII: diabetic rats were treated with simple ointment base containing of mixed extracts (1:1), Group-IX: diabetic rats received the standard drug (Silver Sulfadiazine-Najo 1%) at 200 mg/kg/day dose in all groups.

Measurement of wound area

The progressive changes in wound area were measured in cm^2 by tracing the wound boundaries on a transparent paper on every 3-day interval. The burn wound area was calculated using Auto CAD RL 14 software.

Evaluation of histopathology

At the 9th and 18th days the experiment was terminated and the wound area was removed from the surviving animals for histological examination. The excision skin biopsies were fixed in 10% formaldehyde solution 48 h during the experimentation period and were embedded in paraffin wax. A 6 µm thickness sections were stained with hematoxylin–eosin stain and observed for the histopathological changes under light microscope (Olympus BX51). Inflammatory cell (neutrophil), re-epithelisation, angiogenesis, fibroblasts, vascularisation, eExtracellular matrix, vascularization and organization of the collagen were qualitatively evaluated by grading as (-), (+), (++), (+++).

Evaluation of microbiological status

The microbiological status of the wounds was determined by taking sterile swabs of each burn on the 9th and 18th days. These swabs were streaked onto sterile nutrient agar plates and incubated for 24 h at 37 °C. Colony counts were recorded and Gram stains performed on representative colonies.

Analysis of data

Results were expressed as mean±SEM. The differences between experimental groups were compared using one-way Analysis of Variance (ANOVA) and significant means were separated using Duncan's multiple range test (DMRT). Differences were considered significant at $p<0.001$. All data processing was performed with SPSS software Version 11.5.

Results and Discussion

Wound contraction

Wound area was traced manually and was photographed in each three days interval and healed area calculated by subtracting from the original wound area. The percentage wound contraction was determined using the following formula:

Percent wound contraction=Healed area/Total wound area×100

To apply this equation, the wound margins were traced and measured to calculate the non-healed area which was then subtracted from the original wound area to obtain the healed area. Wound contraction on different days is shown in Table 1. Statistically, the percentage of wound contraction showed that there is high significant difference between the different groups ($p<0.001$). The wound healing potential for *A. euchroma, S. deserti, A. communis* and mixed extracts was evident on the 18th day (Table 1). No healing effect was observed with *P. granatum* (Table 1).

Epithelialization time

The Epithelialization time was found be high significantly ($p<0.001$) reduced in groups as depicted in Table 2. A better healing pattern with complete wound closure was observed in treated within 21 days while it was about 34 days in non-diabetic control rats. At the 18th day, *A. euchroma, S. deserti, A. communis* and mixed extract ointment treated groups healed 80-90% and standard drug group showed 51% healing (Table 2).

Table 1. Effect of the treatments on burn wound expressed as percentage of wound contraction in diabetic rats.

Treatments	Percentage wound healing (mean±SEM) on day post burn induction					
	3th	6th	9th	12th	15th	18th
Simple ointment (non diabetic)	0.449±0.203 e*	5.091±0.504 de	11.689±0.975 e	23.111±0.778 d	33.564±0.621 f	52.769±0.570 c
Simple ointment (diabetic)	0.00±0.000 e	0.00±0.000 e	0.00±0.000 g	0.022±0.022 f	0.287±0.022 h	0.507±0.180 e
Silver Sulfadiazine (diabetic rats)	6.620±0.487 d	10.333±0.402 d	17.216±0.394 d	24.618±0.602 d	41.178±0.890 e	51.356±1.908 c
Punica granatum (diabetic rats)	0.601±0.374 e	3.710±0.261 de	3.622±0.408 f	4.862±0.916 e	5.966±0.572 g	38.040±1.885 d
Malva sylvestris (diabetic rats)	9.880±0.013 cd	20.087±5.870 c	35.393±0.140 c	47.250±1.350 c	57.410±0.423 d	77.003±0.797 b
Arnebia euchroma (diabetic rats)	7.742±0.298 d	27.889±0.250 b	43.173±0.522 b	57.056±1.410 b	70.176±0.194 bc	83.549±0.672 a
Scrophularia deserti (diabetic rats)	13.956±1.241 c	35.478±0.503 ab	44.653±0.032 ab	57.229±0.306 b	67.453±0.587 c	88.829±0.332 a
Amygdalus communis (diabetic rats)	28.667±2.467 a	42.664±1.467 a	47.511±0.535 a	64.231±0.452 a	75.920±0.151 a	85.789±0.113 a
Mixed extracts (diabetic rats)	21.84±0.518 b	34.229±0.931 b	44.056±0.650 b	58.356±0.637 b	72.753±1.716 ab	84.844±1.374 a
Significant †	$p<0.001$	$p<0.001$	$p<0.001$	$p<0.001$	$p<0.001$	$p<0.001$

N=6 animals; Each value represents mean+SEM; †One-way Analysis of Variance (ANOVA); *Letters a, b, and etc means in the same column not followed by same letter are significantly different ($p<0.001$).

Table 2. Effect of the treatments on epithelialization time (day).

Groups	epithelialization time (day)
Simple ointment (no diabetic)	34±0.075 b
Silver Sulfadiazine (diabetic rats)	35±0.930 b
Punica granatum (diabetic rats)	-
Malva sylvestris (diabetic rats)	24±0.290 a
Arnebia euchroma (diabetic rats)	21±0.186 a
Scrophularia deserti (diabetic rats)	21±0.109 a
Amygdalus communis (diabetic rats)	22±0.250 a
Mixed extracts (diabetic rats)	22±0.492 a

Each value represents mean+SEM; -no detected.

Histological evaluation

At the 9th and 18th days the experiment, histological evaluation was carried out for the treated and untreated samples. Comparison between controls and some treated animals is shown in Tables 3 and 4. The best results were obtained with *A. communis* and standard drug, when compared to the other groups as well as to the controls. On the 18th day, groups of *A. communis* extract and standard drug showed complete healing as in collagenation, fibroblasts cells and angiogenesis in Table 4. The control groups (diabetic and non-diabetic rats) and some of the groups (*P. granatum*) presented edema, monocyte cells and area with cellular necrosis (Table 4).

Evaluation of microbiological status

At the 9th and 18th days the experiment, microbiological evaluation was carried out for groups. At the 9th and 18th days, lowest colonies per swab of *Bacillus* and *Staphylococcus* species were detected in group of mixed extracts (Table 5), highest colonies were detected in groups of diabetic control and *P. granatum* extract.

Despite the traditional uses *M. sylvestris* and *P. granatum, A. communis* (sweet and bitter), *A. euchroma* and *S. deserti* in burn wound healing process in Iran, there are no reported data available in the literature. These species widely distributed plants of Iran are used for the anti-infectious, anti-inflammatory, anti-microbial, skin disease and for wound and burn healing properties according to several ethnobotanical surveys (Ghasemi Pirbalouti, 2009a; Ghasemi Pirbalouti et al., 2009; Ghasemi Pirbalouti et al., 2010b, Ghorbani, 2003; Zargari, 1989-1992). The present study tested the burn wound-healing properties of the ethanol extracts of *M. sylvestris, P. granatum, A. communis, A. euchroma, S. deserti* and mixed extracts were used to evaluate the burn healing activity in alloxan-induced diabetic rats.

On the different days, the results of morphological evaluation showed that *A. communis, A. euchroma, S. deserti* and mixed extracts high significantly increased the percentage of wound contraction (Table 1). At the 18th day the experiment, *A. communis* extract and standard drug (Silver Sulfadiazine) showed increased collagen turnover (Tables 2-4). 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 (Philips et al., 1991).

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 (Nayak et al., 2007). It is mainly

Table 3. Effect of the treatments on the evolution of wounds in rats after nine days of topical application.

Treatments	Inflammation cells (Neutrophil)	Angiogenesis	Re-epithelization	Fibroblasts	Extracellular matrix
Simple ointment (non diabetic)	++	++	-	++	++
Simple ointment (diabetic)	+++	++	-	+	++
Silver Sulfadiazine (diabetic rats)	+	++	++	++	+++
Punica granatum (diabetic rats)	+++	+	-	+	+
Malva sylvestris (diabetic rats)	++	++	-	++	+++
Arnebia euchroma (diabetic rats)	+	++	+/-	++	++
Scrophularia deserti (diabetic rats)	++	++	-	++	++
Amygdalus communis (diabetic rats)	++	+++	+/-	+++	++
Mixed extracts (diabetic rats)	+	+++	+/-	+++	++

+: slight, ++: moderate, +++: extensive, -: absent.

Table 4. Effect of the treatments on the evolution of wounds in rats after eighteen days of topical application.

Treatments	Inflammation cells (Neutrophil)	Collagen maturation	Re-epithelization	Organization of the collagen	Vascularization
Simple ointment (non diabetic)	++	++	+	++	++
Simple ointment (diabetic)	+++	+	-	+	+++
Silver Sulfadiazine (diabetic rats)	-	++	++++	+++	+
Punica granatum (diabetic rats)	++	++	+	+	+++
Malva sylvestris (diabetic rats)	-	++	+++	++	++
Arnebia euchroma (diabetic rats)	++	++	+	+	+++
Scrophularia deserti (diabetic rats)	-	++	++	+	+++
Amygdalus communis (diabetic rats)	-	+++	++++	+++	+
Mixed extracts (diabetic rats)	-	++	++	++	++

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 (Philips et al., 1991). 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 (Philips et al., 1991).

The results of study showed that the extract ointment of *M. sylvestris, P. granatum, A. communis, S. deserti* and mixed extracts effectively stimulates burn wound contraction as compared to controls group. These finding could justify the inclusion of these plants in the management of wound healing. The result of the present study offers pharmacological evidence on the folkloric uses of *M. sylvestris* flowers, *P. granatum* flowers, *A. communis* leaves and *S. deserti* stems for healing burn wound. Hence, the results support the traditional uses of *M. sylvestris, P. granatum, A. communis* and *S. deserti* to treat skin disorders including burns.

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