

ORIGINAL ARTICLE

Healing Effect of *Perovskia Abrotanoides* Karel and Expression of VEGF and TGF-B Genes in Burn Injury of Rats

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ABSTRACT

Background: Burn is a life-threatening problem which can lead to alteration in the expression of several genes. A folk medicine topical ointment made from *Perovskia abrotanoides* (*P. abrotanoides*) was extensively used in northern region of Iran for burn wound healing properties. Therefore, the current study aimed to examine the healing potency of *P. abrotanoides* ointment and the expression patterns of wound healing-related genes in rat experimental model of burn.

Methods: In an experimental study, scald burn was inflicted in 28 adult male Sprague-Dawley rats by pouring 2.5 cc of boiling water into a firm rubber ring (2 cm diameter) on the dorsal surface of skin for 10 seconds. Rats were randomly divided into four groups and treated for 7 consecutive days: *P. abrotanoides* ointment-treated group; a vehicle-treated group, which received the complete ointment without *P. abrotanoides* part; silver sulfadiazine-treated group; and the control group which received no treatment. Animals were sacrificed and a full thickness skin biopsy was taken from the burn site. Finally, the relative quantification of gene expression was performed using the vascular endothelial growth factor (*VEGF*) and transforming growth factor- β (*TGF- β*) as target genes and β 2-microglobulin (*β 2M*) as a reference gene.

Results: *VEGF* was up-regulated during the first week of healing in *P. abrotanoides* ointment-treated group; however, the local expression of *TGF- β* was similar between the groups.

Conclusion: *P. abrotanoides* ointment utilized in Iranian folk medicine can accelerate wound healing by producing *VEGF*.

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Introduction

Burn is an extensive form of soft tissue injuries potentially leading to serious complications, severe morbidity and mortality (1-3). It can be inflicted by various means; however, the scald burn is the most

common, followed by flame and electrical burns (4, 5). Each type of burn injury seems to represent diverse pathophysiological, cellular, and molecular events; therefore, burn management is associated with a number of critical challenges for clinicians (6).

Wound healing represents a complex biological process in which the tissue arrangement is re-established to recover the integrity, strength and function of the skin. A successful repair depends on the collaborative efforts of various proangiogenic cells, such as the fibroblasts, leukocytes, monocytes, macrophages, endothelial cells, and epidermal cells, as well as a series of cross cascade reactions including hemostasis, inflammation, proliferation, and remodeling (7-9).

Consequently, other cellular and molecular mechanisms are involved in the wound microenvironment to determine the eventual outcome of the healing process. Repair of the wound is initiated by the release of cytokines and various growth factors at the wound site. However, the expression pattern of some wound healing-related genes like vascular endothelial growth factor (*VEGF*) and transforming growth factor- β (*TGF- β*) are believed to have a key role in repairing the skin (9-11).

Herbal medicines have their origins in ancient cultures and have long been used to treat various disorders such as burns. *Perovskia abrotanoides* karel (*P. abrotanoides*) is an important medicinal herb in Iranian folk medicine, which belongs to *Lamiaceae* family and locally known as Hoosh, Visk or Brazambal in the northern region of Iran. The margin of mountainous roads of the arid and cold climate of Northern provinces of Iran is the main sources of *P. abrotanoides* (12-17).

This plant is utilized for the treatment of a broad range of diseases and its pharmacological effects including anti-microbial, antiplasmodial, anti-oxidant, antiseptic, and the anti-inflammatory effects were shown in previous studies. In addition, the sedative, analgesic, and cytotoxic effects of *P. abrotanoides* were also reported (13, 18-20). Therefore, the current study was designed to evaluate the potential healing effect of *P. abrotanoides* ointment on a reliable experimental model of the burn, based on the alterations in the gene expression pattern of *VEGF* and *TGF- β* .

Materials and Methods

The study protocol was approved by the local Ethics Committee of Shiraz University of Medical Sciences, Shiraz, Iran (Code: IR.SUMS.REC.1396.S608; accepted on October 14, 2017). Fresh *P. abrotanoides* whole plant was collected from Shirvan, North Khorasan Province, Iran. The plant was identified and deposited at the herbarium in the Department of Traditional Pharmacy, School of Pharmacy, Shiraz University of Medical Sciences, Shiraz, Iran (Reference voucher no. PM 937).

The folk *P. abrotanoides* ointment was consisted

of the air-dried leaves and flowers of *P. abrotanoides* in combination with black seed, ginger, virgin olive oil, honey wax, and the bee glue. Briefly, the leaves and flowers of *P. abrotanoides* (300 g), black seed (50 g), and ginger (50 g) were powdered separately. Then, they were added to virgin olive oil (500 g) and mixed thoroughly. The mixture was kept at room temperature for 10 days. Afterward, the honey wax (100 g) and bee glue (10 g) were heated in a water bath (100°C) to completely melted and then added to the prepared mixture. Finally, the *P. abrotanoides* ointment was slowly cooled to 5°C until congealed. The vehicle ointment used in this study was prepared in a similar fashion, excluding the leaves and flowers of *P. abrotanoides*.

In an experimental study, 28 adult male Sprague-Dawley rats (weighing 220-230 g) were acclimatized for two weeks at the Center of Comparative and Experimental Medicine, Shiraz University of Medical Sciences, Shiraz, Iran (temperature 22±2°C; humidity 50-60%; light/dark cycle 12:12 h). Maximum of four rats were housed per cage and had access to water and a standard pellet diet ad libitum. Under the general anesthesia using an intramuscular injection of ketamine (90 mg/kg) (Alfasan, Woerden, Holland) and xylazine (10 mg/kg) (Alfasan, Woerden, Netherland), the dorsal surface of the rat's neck was shaved and cleaned with iodine.

A reliable and safe model of scald burn injury was inflicted by pouring 2.5 mL of boiling water (95°C) into a firm rubber ring (2 cm diameter) on the dorsal surface of a rat skin for 10 seconds. Afterward, the hot water was removed by a sterile absorbent cotton wool, and the animals were randomly assigned into four groups as follows: *P. abrotanoides* ointment-treated group; a vehicle-treated group; silver sulfadiazine-treated group (Sobhan Darou, Iran); and the control group which did not receive treatment.

Since the general anesthesia and deep sleep were more effective for absorption of topical ointment in rats, they were anesthetized with a mixture of ketamine and xylazine (40-60 min of anesthesia) for 7 consecutive days after burn injury and different topical ointments were applied in different groups. At the end of the seventh day, the rats were sacrificed with an excessive dose of ether, and a 4×4 cm full thickness skin biopsy was taken from the burn site. Tissues were then cut into small pieces, placed in the cryo-vials, and dropped in liquid nitrogen until further processing.

The skin tissue biopsies were processed by freezing with liquid nitrogen and grinded into a fine powder. Afterward, total RNA was isolated from the specimens using a total RNA purification mini kit (Cat No: YT9065, Yekta Tajhiz Azma, Tehran,

Archive of SID

Iran) according to the manufacturer's protocol. The quantity and quality of the isolated RNAs were checked by a Nanodrop ND-1000 spectrophotometer (Nanodrop, Wilmington, DE, USA) and all extracts had a proper purity to prevent inhibition during downstream steps (relative absorbance ratio 260/280 nm >1.8).

Reverse transcription was performed by a commercially available RevertAid First Strand cDNA Synthesis kit (Thermo Fisher Scientific Inc., Waltham, MA, USA) in accordance with the manufacturer's protocol. Upon completion of this step, cDNAs were diluted 1:5 in nuclease-free water, and subjected to a subsequent SYBR green real-time PCR assay. The reaction mixture containing an equal amount of diluted cDNAs (2 µl), 10 pmol of each primer set (Table 1), and 10 µl of the RealQ Plus 2x Master Mix Green (Ampliqon A/S, Odense, Denmark) was prepared, and the total volume reached 20 µl in nuclease-free water.

Reactions for each sample were run in duplicate and no-template controls (NTC) were also run simultaneously. Reactions were performed using the Step-One instrument (Applied Biosystems, Foster City, CA, USA) under the following conditions: 95°C for 10 min, followed by 40 cycles at 95°C for 15 s, and 60°C for 60 s. After the final cycle, the specificity of the amplicons was verified *via* the conventional melting curve analysis. Finally, the differences in the gene expression between study groups were analyzed by the $2^{-\Delta\Delta CT}$ formula (21), and the expression of the target genes (*VEGF* and *TGF-β*) were normalized by *β2M* housekeeping gene. The

effect of various ointments on the healing of burn wounds was studied by the assessment of the relative expression of *VEGF* and *TGF-β* as target genes and the *β2M* as a reference gene.

Statistical analyses were carried out using the statistical package for the social sciences (SPSS, Chicago, IL, USA). Variables were checked for normality by Kolmogorov-Smirnov test. Comparisons of the two means were made using student *t*-test. One-way analysis of variance (ANOVA) was used to determine significant differences amongst multiple groups followed by Tukey's HSD. A P value < 0.05 was considered to be statistically significant.

Results

The mRNA expression analysis of the skin tissues on the 7th day post-burn-injury showed a significant up-regulation of *VEGF* merely in the *P. abrotanoides* ointment-treated group in comparison to the controls (fold change of 24.833, P=0.021). However, there was no significant difference between the other groups. Furthermore, results of the real-time PCR showed that *TGF-β* gene expression was not significantly altered during the first week in all the study groups in comparison with the control group (Table 2).

Discussion

Wound healing is a complex cascade of events that restores the skin integrity. Significant advancements in burn wound healing have resulted in more effective patient's stabilization and reduced mortality. Although several treatment modalities

Table 1: The list of PCR primer sets used in this experimental study

Primer	Sequence, 5' --- 3'	Primer Length	Product Length (bp)
<i>β2M</i> -F	CGTGCTTGCCATTCAGAAA	19	244
<i>β2M</i> -R	ATATACATCGGTCTCGGTGG	20	
<i>TGF-β</i> -F	AGCAACAATTCCTGGCGTTACCT	23	131
<i>TGF-β</i> -R	CGAAAGCCCTGTATTCCGTCTCC	23	
<i>VEGF</i> -F	ACTTGAGTTGGGAGGAGGATGTC	23	183
<i>VEGF</i> -R	GGATGGGTTTGTCTGTCTTCTGG	23	

β2M: Beta-2-microglobulin, *TGF*: transforming growth factor-β, *VEGF*: vascular endothelial growth factor

Table 2: Results of gene expression of *VEGF* and *TGF-β* in the study groups in comparison to control

Genes	Groups	Fold change	Standard error	95% Confidence interval	P value
<i>VEGF</i>	<i>P. abrotanoides</i> ointment-treated	24.833	6.106-90.979	2.954-239.667	0.021
	Vehicle-treated	5.899	1.189-65.914	0.233-140.671	0.172
	Silver sulfadiazine-treated	4.833	7.532-64.224	2.324-90.957	0.159
<i>TGF-β</i>	<i>P. abrotanoides</i> ointment-treated	1.385	0.781-2.489	0.525-3.601	0.423
	Vehicle-treated	1.945	0.982-3.202	0.839-5.574	0.126
	Silver sulfadiazine-treated	1.670	0.535-6.430	0.360-14.014	0.646

VEGF: vascular endothelial growth factor, *TGF*: transforming growth factor-β

With an acceptable outcome are available, the wound healing is still requires the development of new inexpensive, non-invasive, and innovative treatments to accelerate wound closure with fewer breakdowns (22, 23).

However, novel approaches should be able to accelerate the wound healing response as short as possible. In the last few decades, the world demand on medicinal plant resources has grown exponentially. Due to their benefits, medicinal plants and other plant-derived remedies are widely used and frequently self-prescribed; therefore, the quality, safety, standardization, and efficacy of these products have become a major concern by policy-makers, health professionals as well as the public (24-27).

In Iranian folk medicine, *P. abrotanoides* ointment is extensively used and self-prescribed for wounds and burns management. Hence, we assessed the healing potency of *P. abrotanoides* ointment by evaluating gene expression pattern of *VEGF* and *TGF-β*. Our results showed that *P. abrotanoides* ointment was able to induce the over-expression of *VEGF* in the skin tissues on the 7th day post-burn-injury, but the expression pattern of *TGF-β* was similar to the control group. Therefore, *P. abrotanoides* ointment increased wound healing by the production of *VEGF*, and it was suggested that *VEGF* is up-regulated during the early days of healing and its local release could stimulates collagen deposition, angiogenesis, the migration of fibroblasts, and formation of granulation tissue (28-30).

To the best of our knowledge, this study is the second survey evaluating the healing effect of *P. abrotanoides* in burn injuries. Our previous study confirmed the proliferative effect of *P. abrotanoides* aqueous extract on fibroblast cells after 48 h (31). We also showed that topical application of *P. abrotanoides* ointment could accelerate the wound healing processes by initiating re-epithelialization and formation of granulation tissue during the first week (31). Our molecular and histopathological investigations on experimental model of rat confirmed the potential effects of *P. abrotanoides* ointment on accelerating burn wound healing process.

In addition, our findings suggest that this plant might contain several active compounds to accelerate the healing mechanism. Previous studies reported camphor, cineole, caryophyllene, humulene, myrcene, pinene, careen, camphene, and bisabolol as the main compounds of *P. abrotanoides essential oil* (17, 32, 33). Furthermore, cytotoxic and apoptogenic effects of *P. abrotanoides* extract on MCF-7 and HeLa cell lines (34) as well as its antibacterial activity against both gram positive and gram negative bacteria was also reported (35).

Conclusion

This study confirmed that *P. abrotanoides* ointment utilized in Iranian folk medicine can increase the expression of *VEGF* in the skin tissues within the first week after burn injury; therefore, it can accelerate burn wound healing process. However, to generalize our findings to human being, further comprehensive studies on healing mechanisms of *P. abrotanoides* are warranted.

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Conflict of Interest

None declared.

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