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The healing effect of combined hydroalcoholic extract of *Teocurium polium* and the seed hull of Quercus brantii on burn wounds in rats

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ABSTRACT

Plants and their extracts have considerable potential for wound healing. So, The aim of this study was to evaluate the wound healing effect of combined hydroalcoholic extract of Teocurium Polium (TP) and the seed hull of Quercus brantii (Persian oak) (QB) and comparing with silver sulfadiazine (SSD) ointment (1%) in rats. Animals were divided into six groups consist of normal (untreated), physiologic serum, TP, seed hulls of Quercus brantii (QB), combination of TP and QB and the other group was SSD. Burn area size, epidermal and dermal diameter, number of hair follicle, histopathology of the burn skin and concentration of plasma malondialdehide (MDA) were determined in different groups. Animals treated with QB and TP, SSD and combination of two extracts (QB +TP) showed a significant reduction in the burn area. Serum MDA decreased significantly in animals treated with QB, TP and QB+TP. Histological comparison has shown that TP, QB and combination of them significantly increased re-epithelialization in burn wounds, as compared to normal group. In conclusion, our results demonstrated that topical application of TP and QB has a significant effect on the burn wound healing.

Key words: Burn, Teocurium Polium, Quercus brantii, Wound healing

INTRODUCTION

The skin is the largest organ in the body and performs numerous vital functions such as fluid homeostasis, immunologic, thermoregulation, neurosensory and metabolic functions. The skin also provides primary protection against infection by acting as a physical barrier. When this barrier is damaged, pathogens have a direct route to invade deep tissues. Cutaneous 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 [1]. The normal healing process can be impeded at any step along its path by a variety of factors. Impaired wound healing may be a consequence of pathologic states associated with diabetes, immune disorders, ischemia, venous stasis and injuries such as burn wounds [2]. 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 [3]. However, burn is characterized by a hyper metabolic 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 [3]. Wound is a rupture in the epithelial integrity of the skin which resulted from violence or trauma and may be followed by disruption of the structure and function of underlying normal tissue [4]. It is a

clinical entity and is as old as mankind, often considered as major problem in clinical practice. Wound healing is any process which acts, or induced to act, to restore the integrity of the damaged tissues in order to replace lost tissues. It encompasses the activity of an intricate network of blood cells, cytokines, and growth factors which ultimately leads to the restoration to normal condition of the injured skin or tissue [5]. Research on wound healing agents is one of the developing areas in modern biomedical sciences and many studies have been conducted using different wound healing models. Synthetic chemical moieties are the present treatment regimens for the management of under healing of wound but they possess a wide range of side effects. In alternative and complementary systems of medicines such as oriental and aromatherapy, plants are being used to combat several disease and pathological conditions [6, 7, 8, 9]. Herbal products seem to possess moderate efficacy with little or no toxicity and are less expensive as compared to synthetic drugs [10, 11]. Many plants and plant-derived products have been shown to possess potent wound-healing activity. The presence of bioactive constituents in plants has urged researchers to screen medicinal plants with a view to determine potential wound healing activities [12]. Therefore research has prompted into herbal and natural products which known to increase the healing of different types of wounds and which can eliminate the side effects that were associated with the synthetic chemical drugs [13]. Though some of these drugs have been screened scientifically for evaluation of their wound healing activity in different pharmacological models and patients [14], the potential of many of the traditionally used herbal agents remains unexplored [14]. Hence there is need to identify the various plants or their chemical entities and formulated them for treatment and management of burn wounds.

Persian oak (Quercus brantii) (QB) of fagaceae family, grow in south-western of Iran. The seed hulls of Persian oak contain a high concentration of tannins. Many studies reported the effects of tannins against burns [15, 16, 17]. Tueocurium polium (family lamiaceae) (TP) named calpoureh in Iran and grow in Mediterranean and south-western of Iran [18]. The aerial parts of TP contain polyphenolic substances [12]. A few studies have shown that TP is effective at wound healing [10, 13]. In this regard, combination of these plant extracts may be has synergistic effects on burn wound healing.

The purpose of this study was to determine the healing effects of QB, TP and combination of them on burn wounds in rats and compare the results with silver sulfadiazine (SSD) ointment.

MATERIALS AND METHODS

2.1. Chemicals

All reagents and solvents consist of 1,1,3,3-tetraethoxypropan (TEP), trichloroacetic acid (TCA), and thiobarbituric acid (TBA) were purchased from Sigma–Aldrich (Steinheim, Germany). Hematoxylin and eosine dyes were purchased from Merck Company (Germany). The silver sulfadiazine was purchased from Behvazan Company (Iran).

2.2. Plant materials and preparation of the extract

Fruits of Persian oak (QB, the fagaceae family) and aerial parts of TP were collected in September 2015 from Yasuj mountains and identified at the herbarium of research center of agriculture and natural sources, Yasuj University (Iran) where a voucher specimen was deposited. Seeds of QB were dried under shade in room temperature (30±5 ºC) and after drying, the loosened hulls separated from seeds (fruits) and powdered. Also, the fresh aerial parts of the TP plant were dried under shade and ground in a mortar. The 100 g of powdered plant materials were macerated with ethanol/water (70/30) to obtain the hydroalcoholic crude extract for 48 hours in room temperature. The extract was filtered using a sterile cloth sheet. Solvents were evaporated by drying at 55 ºC in a rotary evaporator prior to storage at 4 ºC. The hydroalcoholic extract solution was freshly prepared and administrated topically. In preliminary experiment the hydroalcoholic extract (70% ethanol) as well as fractions of the plant were screened for the presence of secondary metabolites, including alkaloids, flavonoids, polyphenols and tannins by using standard tests.

2.3. Experimental animals

Male wistar rats (200 - 220 g), from the central animal colony of the Yasuj University were used for this study. The animals were maintained under controlled environment at the Institute's animal house at 25 ± 1$^{\circ C}$ and 12-h light–dark cycle. The experiments were performed in accordance with the regulations specified by the Institute's Animal Ethical Committee and conform to the national guidelines on the care and use of laboratory animals, Iran. Animals were acclimatized for one week before the study, and during the experiment the animals were housed individually in their cages so as to avoid biting and possible wound scratch to each other. The animals were provided with water and food pellets *ad libtum* before and till the end of the experimental period.

2.4. Burn wound model

Sixty male wistar rats (200-220 g) were randomly divided into six groups. The first group was given no treatment. This group did not receive second degree burns. The other groups induced burn. The second group received physiologic serum, the third group as positive control was treated with silver sulfadiazine (%1) ointment. The 4^{th}, 5^{th} and 6^{th} groups were treated with hydroalcoholic extract of QB, TP and combination of QB and TP (50% of each extract) respectively. The animals were kept individually in standardized environmental condition with a temperature of 25±2ºC, under a 12:12-h light-dark cycle. Rats were given *ad libitum* access to food and water. This study was carried out in accordance with ethical principles of laboratory animal care. For creating burns, the animals were anesthetized by intra-peritoneal injection of thiopentone (40 mg/kg, i.p.), the dorsal surface of the rat was shaved, and the underlying skin cleaned with 70% ethanol.

Then the animals were placed in the device with designed puncture on it with dimensions of 2 cm^2. The skin of animals heated to 90–95 ºC with a metal rod heated in boiling water for 10 seconds. The damaged skin was cleaned with normal saline. To prevent hypovolemic shock 2.5 mL of physiologic serum injected to all animals. Single burn wound was created on dorsal part of each rat. After 24 h, dead tissues were excised using sterile surgical blade. Animals were allowed to recover from anesthesia and housed individually in sterile cages. Every day, dead tissues were cleaned using a piece of sterile gauze. During the cleaning of wounds the healing process was disrupted and removes the treatments that were topically applied. By the way, for all groups, the extracts of plants, SSD and physiologic serum were applied topically, once daily for 10 days as described above and total amount of each treatment applied was so as to cover the whole raw area of wound/ulcer. Wounds were covered and wrapped wound with a gauze bandage for protecting from animals licking.

2.5. Histopathological evaluation

The granulation tissues were excised on day 10 post-wounding and fixed in 10% neutral formalin. The animals were scarified and injured tissues were removed carefully, divided into two sections with length of 1cm. Wound tissues were immediately preserved in 10% buffered formalin and passed through different grades of alcohol in order to ensure complete dehydration of tissues and then embedded in paraffin wax. Serial sections of paraffin embedded tissues of 6 µm thickness were cut using a microtome and stained with haematoxylin and eosin stain and observed for the histopathological changes under microscope. The burn surface areas were measured by ruler and were taken from center of burn area. Then a photograph of each section was produced using a microscope (Olympus, Model: BX51, Japan) at the magnification of 400. The captured images were digitized using Olysia software.

2.6. Malondialdehyde (MDA) measurement

Malondialdehyde (MDA) of the treated and untreated wound tissues were analyzed with the UV–Vis spectrophotometer (model V-530, Jusco, Japan) at 10 days after burn tissue harvesting. Recording the absorption spectra and absorbance measurements were carried out with a UV–Vis spectrophotometer using 1.0 cm quartz cells at λ = 532 nm. A part of injured tissues isolated and immediately was transferred to nitrogen tank for measurement of MDA. MDA was assessed based on the reaction of thiobarbituric acid (TBA) with MD^{14}. Three skin samples were randomly chosen in each site for MDA assay and the means were analyzed.

2.7. Statistical analysis

All data were expressed as mean ± S.D. Data were analyzed using one-way ANOVA, followed by a post hoc Tukey's test. Differences were considered to be significantly when $p \leq 0.05$.

RESULTS

In this study the several parameters such as burn area, epidermal thickness, dermis thickness, the number of follicle and the malondialdehyde concentration were investigated and optimized versus to treated animals with QB , TP QB+TP extracts and SSD ointment. According to the obtained results, the animals treated with QB and TP extracts show improved of wound healing, so that after 10 days of treatment, burn areas were reduced to approximately 100% of the original size. Also, SSD and combination of two extracts (QB +TP) showed a significant reduction in the burn area, but the average size of burn was not restore to the normal control in these groups. The figures of merits are summarized in Table 1. The thickness of the epidermis and dermis in the case of TP and QB groups increased with comparison to normal group. Also, serum MDA decreased significantly in animals treated with QB, TP and QB+TP (Table 1). This parameter in SSD group did not show significant difference. The number of hair follicles significantly increased in groups received extract of QB, TP and QB+TP compared to the normal group. According to the obtained results, it is conclude that the combination of the extract from QB and TP have a significant effect on the healing wound burn. In fact, due to synergistic effect of these plants the improvement in wound burn has been seen in comparison to individual extract of TP or QB.

Histhopathalogical survey of the wound healing process

The histological study showed that the tissue formation was more than in treatment groups compare to the normal group. There was no difference between QB+TP and SSD groups. Haematoxylin and eosin stained sections of granulations tissue collected on various days were examined for cellular infiltration, neo-vascularisation, epithelial regeneration and matrix organization. In the 10^{th} Day, sections showed increased cellular infiltration in animals topically treated with the SSD ointment (Fig. 1B than control Fig. 1A) and, epidermal regeneration was not observed. On day 12, a well-advanced organization of granulation tissue and on-going epithelialization was observed in treated groups, either with SSD cream or TP, QB and QB+TP extract (Fig. 1 C, D and E, respectively). The histological examination showed that the original tissue regeneration was much faster in the skin wound treated with extract or SSD without any edema, congestion or inflammatory disorders. There was full thickness epidermal regeneration which covered completely the wound area. The epidermis was thick and disorganized, especially when compared with the adjacent normal skin. The keratin layer was thick with focal parakeratosis. The dermis was cellular with proliferation of fibroblasts, laying down disorganized and poorly oriented collagen fibers. Prominent capillary-sized blood vessels were seen. Scattered collections of inflammatory cells, consisting mainly of macrophages and few neutrophils were also present throughout the whole thickness of the dermis.

DISCUSSION

Medicinal plants have been used extensively in accelerating burn wound healing due to chemical components such as flavonoids and tannin [12]. The existences of the flavonoids materials in the aerial parts of *T. polium* were studied in previous literature [12, 13, 15]. On the other hand it is found that the hydro-alcoholic extract of Q. brantii had active components such as tannins and flavonoids [16]. T. polium has pharmacological effects such and antispasmodic, antibacterial, anti-inflammatory, antioxidant, antiulcerogenic, antinociceptive [17, 18]. Also, it has been reported that Q. Brantii has antioxidant and antimicrobial properties [1]. Therefore, it would be reasonable to assume that the burn wound healing properties of *T. polium and* Q. Brantii *can* be attributed to bioactive components available in these plants. Silver sulfadiazine as a popular drug for treatment of burn has antiseptic, antimicrobial and anti-inflammatory effects [16].

Our results showed that the therapeutic effect of Q. Brantii and T. Polium on burn area was comparable to SSD. A numerous studies have been proposed that T. Polium and Q. Brantii promoted wound healing. Ansari et al. reported that T. Polium extract facilitated the burn wound healing [13]. Similar results have been reported by Karimi and Moradi for Q. brantii possibly due to its antioxidant potential [19]. In addition to favorable action of extracts on Burn area, hydroalcholic extract of these plants significantly reduced plasma MDA levels. Indeed, decrease of MDA was in parallel with reduction of burn area in receiving groups of extracts alone or combination of two extracts. Some studies have proposed that oxidative stress has a pivotal role in the burn wound healing that could be prevented by antioxidants. In this study SSD has not been shown antioxidant effect. It seems that other mechanisms such as anti-inflammatory and antimicrobial effects are important than oxidative stress in wound healing process. We also, postulated that combination of two extracts could potentiate these favorable effects. So, we concluded that, there is a synergistic effect between fractions of these two extracts. The pathological studies revealed a significant change of the epidermal and dermal thickness between treated groups and normal group after ten days. In conclusion, our results demonstrated that the topical extract of T. Polium or Q. Brantii can be used to treat burn injuries. It has also been shown that combination of these two extracts has beneficial healing effect on burn, but it was not more effectively than any of extracts alone.

Table 1: Effect of alcoholic extract of Q.B, T.P and QB+TP Extract on Burn area, epidermal thickness, dermis thickness number of hair follicle and MDA in different groups

Groups / Parameters	Normal	Normal saline	SSD	QB	TP	QB+TP Extract
Burn area (cm^2)	0	0.72±0.25	0.44±0.04*	0**	0**	0.4±0.29*
Epidermal thickness (µm)	75±5.41	41.1±5.87	34.49±9.14	41.37±6.9	52.14±8.25	58.1±0.8
Dermis thickness (µm)	1200±35.2	780±42.4	980±48.8	898±54.5	901±64.4	1051±54.8
Number of hair follicle (mm^2)	8±1.23	2±0.14	1±0.05	5±1.07**	4±.98**	6±0.76*
Malondialdehyde (µmol/L)	1.48±0.25	3.79±0.31	3.43±0.29	3.22±.21**	3.24±.11**	2.24±0.30**

*Data are means ±SE (n = 10 rats/group). * Significant different from treatment groups vs. normal saline group (P < 0.05). ** Significant different from treatment groups vs. normal saline group (P < 0.01).*

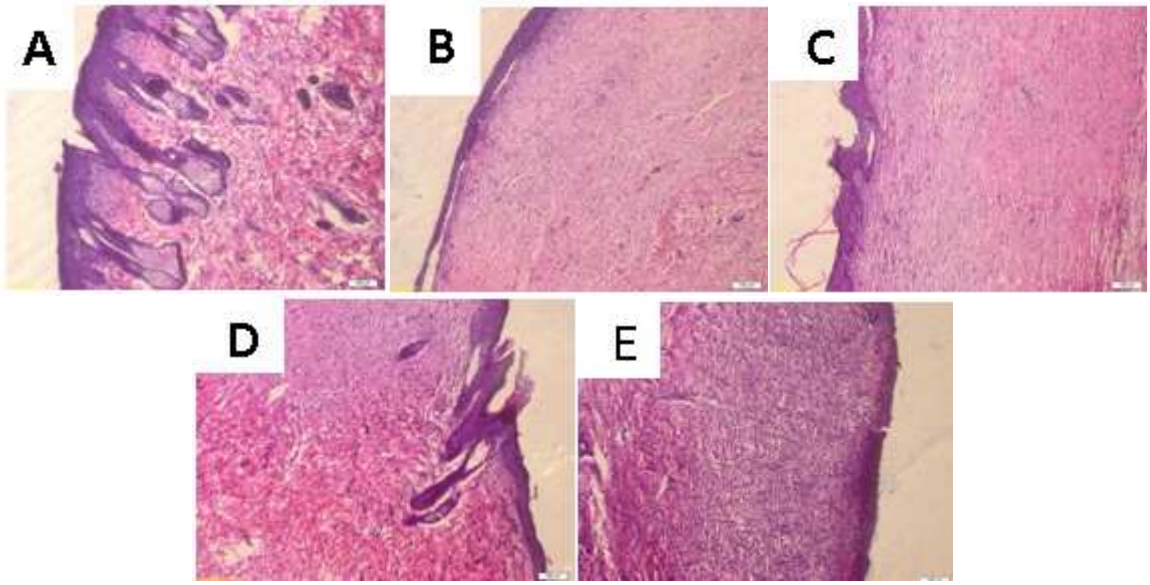

Figure 1. Comparison of the trend of regeneration of skin injured by burn wounds in all groups, EH staining, 40-optical microscope. A- Treated with physicologic serum, B- Treated with silver sulfadiazine, C- Treated with Tueocurium Polium extract, D- Treated with Quersus Brantii extract, E- Treated with Tueocurium Polium - Quersus Brantii extract

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Conflict of Interests
The authors have declared that no conflict of interests exists.

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