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## Enhancement of Fibroblast Proliferation, Vascularization and Collagen Synthesis in the Healing Process of Third-Degree Burn Wounds by Topical *Arnebia euchroma*, a Herbal Medicine

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### Abstract

**Introduction:** The present study was conducted to evaluate the wound healing effect of *Arnebia euchroma* (AE) extract, which is traditionally used in some Indian, Chinese, and Iranian tribes, on histomorphometrical parameters involved in the healing process of third-degree burn wounds by using stereological analyses. **Methods and Materials:** In an experimental study, 48 female Sprague-Dawley rats, each with a standard third-degree burn wound on the posterior surface of the neck, were divided into four groups; AE10 and AE20 groups were treated with carboxymethylcellulose (CMC) gels which contained AE hydroalcoholic extract at the concentration of 10% and 20%, respectively; the untreated burned (UB) group, which received no treatment; and the gel-base treated group. Wound closure rate, fibroblast proliferation, volume density of collagen bundles, length density, and mean diameter of the vessels were measured. Results: Wound closure rate, fibroblast population, volume density of collagen bundles, and length density of vessels were significantly improved by AE10 and AE20 in comparison with the gel-base and UB groups (P value <0.05). **Conclusion:** Although previous investigations on the different aspects of the wound healing effects of AE and the results of this study exhibited the positive effects of topical *Arnebia euchroma* on third-degree burn wound, introducing AE as an alternative wound healing agent requires more investigations on its efficacy on human, safety, and possible adverse effects. [GMJ. 2012;1(2):53-59]

**Keywords:** *Arnebia euchroma*; Stereology; Vascularization; Fibroblast proliferation; Wound closure rate; Collagen synthesis

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## Introduction

**B**urn wounds are common injuries all over the world; however, in developing countries burns constitute a major health problem because of the high incidence of severe complications and limitation of financial resources.<sup>1</sup> Third-degree burns, which are the most severe forms of burns, usually need dressing with appropriate medications in order to prevent infections.<sup>2</sup> The main aim of burn management and therapy is wound healing and epithelization as soon as possible to prevent infections and to reduce functional and aesthetic after effects;<sup>3</sup> nevertheless, although some reports show the efficacy of some biochemical agents<sup>4</sup> as well as procedures such as photodynamic and laser therapy,<sup>5</sup> the healing of third-degree burn wounds seem to be not feasible without skin grafting. Using topical biochemical treatments seems to be essential for improving the survival of patients with major burning injuries and for minimizing the likelihood of the occurrence of burn wound sepsis, a leading cause of morbidity in these patients.<sup>6</sup>

Several groups of topical medications and antibiotics have been used in the treatment of wound infection of burn ulcers, e.g. mafenamide, silver sulfadiazine, neomycine, and polymyxine B.<sup>2</sup> Be that as it may, the risk of the antibiotics resistance, high cost, inability to restore the initial appearance of the skin, and possible side effects have resulted in researches on newer materials for the treatment of burn wounds.<sup>3</sup>

More than 80% of the world's population depend upon traditional medicines for different skin diseases.<sup>7</sup> Recently, the traditional use of plants for wound healing has received attention by scientists, and effectiveness of several plants and herbal medicines in the treatment of skin disorders such as burn and cut wounds has been reported.<sup>7</sup> *Arnebia euchroma* (AE) is an annual herb, distributed in Asia and the drier regions of northern Africa (Figure 1).<sup>8</sup> This plant grows widely in Iran, and it is locally known as "Sorkh Giah" or "Heveh Ghoah".<sup>1</sup> AE is rich in naphthoquinones, alkannins, shikonins, and their derivatives, which are potent pharmaceutical substances with a wide range of biological properties such as wound heal-

ing, antibacterial, antifungal, antiviral (e.g. influenza virus and HIV), antiamebic, anti-inflammatory, antitumor, and anticancer effects.<sup>6,8</sup>

The present study was designed to investigate the effect of topical AE extract on heat-induced third-degree skin wounds in rats by using histomorphometrical and stereological methods.

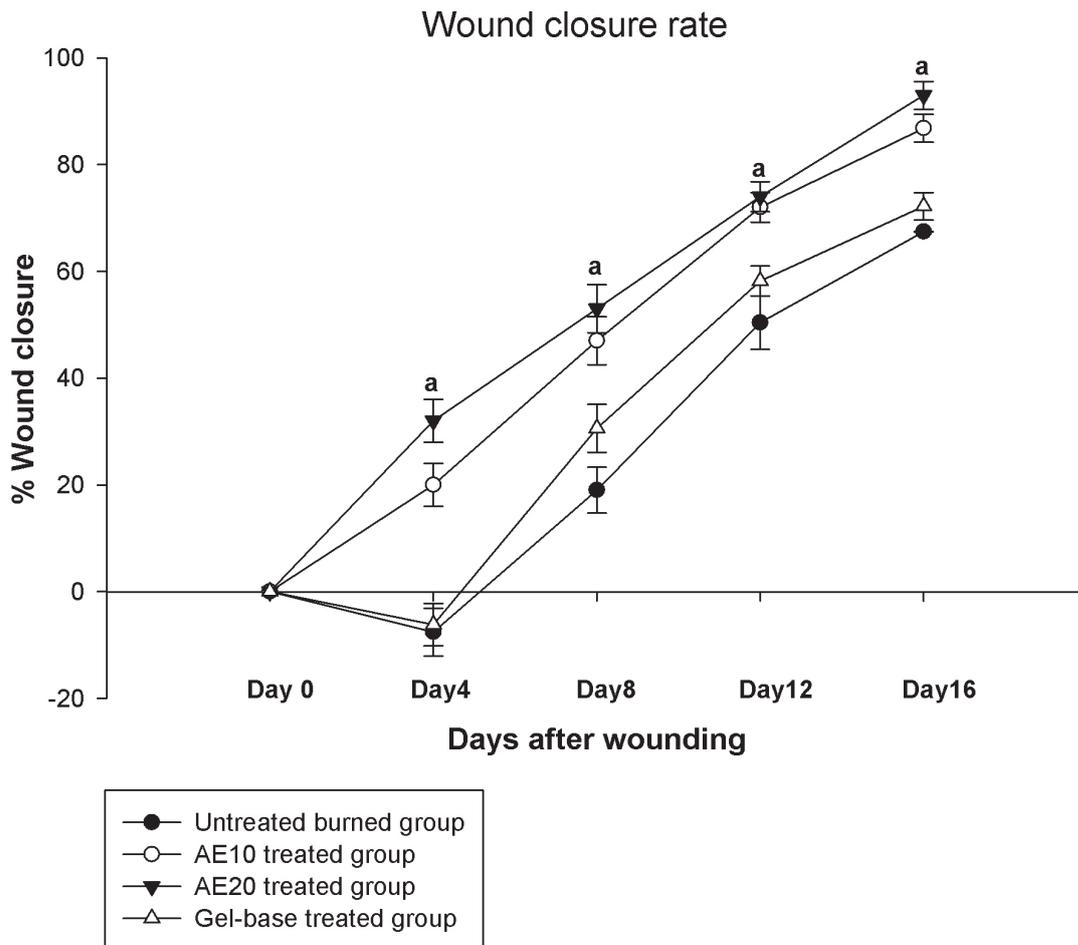
## Materials and Methods

### *Plant Material*

AE plant was collected from the rural areas of Yasuj, the Kohgiluyeh and Boyer-Ahmad province of Iran in June 2009. It was authenticated at the Research Center for Agriculture and Natural sources of Yasuj (Herbal No.151). Preparation of Plant Extract and Vehicle Gel Plant materials, mixture of the all plant organs, were dried at room temperature for 4–7 days. The dried material was ground into powder and subsequently extracted with a mixture of water: ethanol (1:1, v/v) for 72 hours. The obtained material was then filtered and the filtrate was evaporated to obtain a dark hydro-alcoholic extract (yield: 24.25%). In order to facilitate the application of the agent, we provided 10% and 20% AE gel by dissolving 10cc (AE10) and 20cc (AE20) of the extract each in 2cc distilled water and then transferred the solution into 2% carboxymethylcellulose (CMC) (2g CMC dissolved in 98cc distilled water). The gel base was also supplied by creating 2% CMC gel without the AE component.

### *Animals and Excision of Wound Model*

In an experimental study, 48 female Sprague-Dawley rats (200±20 g) between 2 to 3 months of age were randomly divided into four groups (n=12): two groups were treated with the *Arnebia euchroma* gel at concentrations of 10% (AE10) and 20% (AE20); a gel-base group, which received the vehicle gel used as a vehicle; and the untreated burned (UB) group, which received no treatment except cleaning of the wound surface with sterile distilled water every day after making the burn wounds. Gels were provided in the same color and



**Figure 1.** Wound closure rate of the rats with third-degree burn injury treated with 10% (AE10) and 20% (AE20) Arnebia euchroma gel, gel-base, and untreated burned group (UB). Each point represents the mean ( $\pm$ SD) of the wound closure percentage in the related group. "a" shows a p value  $<0.05$ ; AE10 and AE20 vs. UB and gel-base treated groups.

same appearance, and the administration was performed by one person who was unaware of the ingredients of each coded gel. On day 0, under general anesthesia, a circular iron plate ( $1 \times 2 \text{ cm}^2$ , 2gr) heated up to  $100^\circ\text{C}$  was placed on the posterior surface of each rat's neck for 40 seconds, which is equivalent to a third-degree burn in humans, and the burned skin was thereafter removed (down to but not through the panniculus carnosus) to create a full-thickness wound on each rat.<sup>4</sup> The debridement procedure was done in a standard way for all of the animals just after the wounding and repeated every 24 hours, by fully covering the wound site with a layer of the gel, until the end of the study (the day on which at least one wound in any group of rats was completely closed).

After 18 days (the day on which at least one wound in any group was closed), the animals were sacrificed with a high dose of ether, and full thickness skin biopsies were taken from the wound site and fixed in buffered formaldehyde ( $\text{pH}=7.2$ ) for histomorphometrical and stereological evaluations.

The study protocol was approved by the Animal Ethics Committee of Shiraz University of Medical Sciences, and animal care was in accordance with their guidelines.

#### **Stereological Study**

The stereological analyses were performed by an investigator who was unaware of the slide codes. To determine the rate of wound closure, digital photographs were captured

from the wound surfaces every other day with a single-lens digital camera. The wound area (mm<sup>2</sup>) at each visit was estimated by using a software composed of a pointed grid, designed at Histomorphometry and Stereology Research Centre, Shiraz University of Medical Sciences, (9, 10), and the wound closure rate was subsequently calculated as:

Wound closure rate (%) = (area at visit 1 – area at each visit) / area at visit 1) × 100

Nine pieces of the skin samples, each about 1mm<sup>2</sup>, were cut in a systematic random sampling manner and prepared for stereological analysis. The pieces were embedded in a cylindrical block of paraffin. Isotropic uniformly random (IUR) sections<sup>11</sup> of the blocks, 5µm and 15µm in thickness, were provided and stained with both Hedenhain's azan and hematoxylin-eosin. Microscopic analyses of the dermis were done by using a video-microscopy system made up of a microscope (E-200; Nikon™; Japan) linked to a video camera and live imaging on the monitor. The volume densities (V<sub>v</sub>) of the collagen bundles (fraction of the unit volume of the dermis which is engaged by the collagen bundles), the length density (L<sub>v</sub>), and the mean diameters of the vessels were estimated at final magnification of 450× by using 5µm-thick slides and a reported method by Ashkani-Esfahani et al.<sup>11</sup> The numerical density (N<sub>v</sub>; number of the cells per unit volume of the dermis) or the population of the fibroblasts was estimated by employing the 15-µm slides and the "optical dissector" method.<sup>9,11</sup>

### Statistical Analysis

Data were collected, analyzed, and reported as mean and standard deviation (SD). The one-way ANOVA and the Tukey post hoc tests were used for comparing the groups. A P value ≤ 0.05 was considered statistically significant using SPSS 16.0 software.

## Results

### Area of the Wounds

The mean initial area of the wounds before receiving treatment was 207.33 ± 8.66 mm<sup>2</sup> (range = 198.72-214.64 mm<sup>2</sup>) with no signifi-

cant differences between the groups. There were no significant differences in the primary wound surface area between the four groups. The rate of wound closure was similar in AE10 and AE20 (Figure 1) but significantly varied when compared with the UB and gel-base groups (P value < 0.05).

### Fibroblast Population and Volume Density of the Collagen Bundles

Numerical density of the fibroblasts in the dermis of the AE groups was higher than that of the UB and gel-base groups. Numerical densities of fibroblasts in the AE10 group were ~43% (P value = 0.01) and ~27% (P value = 0.03) and in the AE20 group were ~59% (P value = 0.01) and ~40% (P value = 0.02) higher than those in the UB and gel-base groups, respectively (Table-1).

The volume densities of the collagen bundles in AE10 were similar to AE20 but both were significantly higher than those of the UB and gel-base groups (P value < 0.03; Table-1).

### Length Density and Mean Diameter of the Vessels

In comparison to the UB and gel-base groups, length densities of the vessels in AE10 were ~39% (P value = 0.004) and ~72% (P value = 0.002) higher, respectively, and in AE20 were ~31% (P value = 0.004) and ~61% (P value = 0.002) higher, respectively (Table-1). Measurement of the mean diameter of vessels in the AE receiving groups, UB, and gel-base treated group showed inconsequential difference between the groups (Table-1).

**Table 1.** Mean ± SD of the numerical density of the fibroblasts (×103 per mm<sup>3</sup>), volume densities of the collagen bundles (V<sub>v</sub>; %), length density (mm/mm<sup>3</sup>) and mean diameter (µm) of vessels in the dermis of the burned rats treated with 10% and 20% solution of Arnebia euchroma (AE10 and AE20), Gel-base treated group, and untreated burned group (UB).

Groups	Fibroblasts	Collagen	Vessels	
	Numerical density	Volume density	Length density	Mean diameter
UB	184.18±63.92 <sup>a</sup>	0.88±0.03 <sup>a</sup>	49.67±2.73 <sup>a</sup>	10.95±1.84
Gel-base	208.20±16.71 <sup>a</sup>	0.87±0.01 <sup>a</sup>	40.16±2.04 <sup>a</sup>	12.61±2.04
AE10	263.98±29.06 <sup>b</sup>	0.97±0.01 <sup>b</sup>	69.16±9.41 <sup>b</sup>	13.13±2.87
AE20	292.48±38.77 <sup>b</sup>	0.95±0.02 <sup>b</sup>	64.93±7.43 <sup>b</sup>	13.16±2.33

<sup>a,b</sup> Different superscript letters show statistically significant difference in the same column (P value < 0.05).

## Discussion

Burns are severe injuries which are associated with inflammatory and oxidative reactions, tissue damage, infections, disabilities, and even death mostly due to post-traumatic complications.<sup>10</sup> AE belongs to the Boraginaceae family, which is rich in naphthoquinones, Alkannins, and Shikonins. Their derivatives are potent pharmaceutical substances with a wide range of biological properties such as wound healing, antibacterial, antifungal, antiviral (e.g. influenza virus and HIV), anti-amoebic, anti-inflammatory, antitumor, and anticancer effects.<sup>6, 8, 10</sup> Numerous investigations were conducted on wound healing activity of the extracts of some Boraginaceae species, as well as the shikonins, alkannins, and their other derivatives. Pirbalouti et al.<sup>1</sup> reported the anti-inflammatory effect of AE as well as its significant impact on fibroblast proliferation and collagen synthesis in burn wounds (not a specific type of wound) based on pathological analyses. Papageorgiou<sup>12</sup> reported that alkannin esters taken from the plant *Alkanna tinctoria* which are also present in AE possess excellent wound healing effects in a clinical study on 72 patients with indolent ulcers on the lower part of the leg caused by varicose veins. It has been declared that the related naphthoquinone derivative, arnebin-1 ( $\beta$ -dimethylacrylalkannin), significantly enhanced wound healing with or without hydrocortisone treatment in rats.<sup>13</sup> The treatment with arnebin-1 revealed a noticeable reduction rate of the wound area, enhanced cell proliferation, migration, and vessel formation to form a thick granulation tissue and re-epithelialization of the wounds; moreover, an increase in the synthesis of collagen, fibronectin, and transforming growth factor- $\beta$ 1, which promote healing of wounds, was also observed.<sup>13</sup> Oxygen free radicals which are produced during injury play an influential role in the healing of an ischemic injury through impairment of the healing process.<sup>14</sup> Therefore, according to a study done by Sekine et al. (1998),<sup>15</sup> antioxidant activity, which is ascribed to alkannins and shikonins, was assumed to play a consequential role in the

healing enhancement of AE as well as some other Boraginaceae species which were also reported. Previous studies have shown that wound healing preparations based on alkannins and shikonins modulate both the inflammatory and proliferative stages of wound healing.<sup>16,17</sup> Papageorgiou et al. (2008)<sup>18</sup> introduced alkannins and shikonins as the new alternatives for wound treatment through their anti-inflammatory, anti-microbial, antioxidant, and proliferation-inducing effects both in vitro and in vivo. Outcome of the present study showed enhanced re-epithelization and wound closure rate after treatment with AE. AE stimulated fibroblast proliferation, collagen bundle synthesis, and vascularization in the injured site according to stereological analyses. Moreover, our results demonstrated no statistically significant difference between AE10 and AE20 in the measured parameters from which the non-dose-dependent effect of topical AE administration can be assumed. However, higher doses of AE should be investigated for any possible superiority over the doses which were used in this study.

Regarding the outcome of the present study and previous investigations on the different aspects of the wound healing effects of AE and the impacts of this herbal medicine on various wound healing parameters, which are briefly mentioned here, AE can be regarded as an alternative wound healing agent, particularly in burn wounds. Nonetheless, in order to introduce AE as a potent wound healing agent, further investigations are still needed to determine the possible side-effects and effectiveness of this herb in clinical trials.

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