Effect of Aqueous Extract of *Elaeagnus angustifolia* Fruit on Experimental Cutaneous Wound Healing in Rats

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Abstract- The present study was conducted to investigate the histological changes and wound healing effect of aqueous extract of *Elaeagnus angustifolia*. After creating full-thickness skin wounds on the back of 45 male Sprague-Dawley rats they were randomly divided into three groups. Treated group received the extract, positive control group were treated with mupirocin ointment 2% and control group did not receive any treatment. Wound healing rates were calculated on days 3, 5, 8, 10, 12 and 15 post-wounding and the wound tissues were harvested at 5, 10, and 15 days for histological analysis and hydroxyproline content measurement. The results indicated a significant increase in the percentage of wound contraction and hydroxyproline content in the treated group comparing to the control and positive control groups. A significant increase in the assigned histological scores was observed at 10 and 15 days in the treated and positive control groups compared to the control group. The results demonstrate that aqueous extract of *Elaeagnus angustifolia* accelerates cutaneous wound healing, and its effect may be due to the increased re-epithelialization and collagen deposition in wound and so it can be considered as a therapeutic agent for wound healing.

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Keywords: *Elaeagnus Angustifolia*; Histology, Hydroxyproline, Rat; Wound healing

Introduction

Skin is the largest organ of the body and has a critical role in maintaining body homeostasis. Loss of the skin integrity can cause lesions or illness that may lead to a major disability or even death. Wound healing is a dynamic, interactive process initiated in response to injury that restores the function and integrity of damaged tissues (1). Skin wound healing is composed of four precisely and highly programmed phases: hemostasis, inflammation, proliferation, and tissue remodeling or resolution (1). There are many factors, either internal or external, that may negatively affect the process of wound healing in patients, thus causing improper or impaired tissue repair (1). It is estimated that morbidity associated with non healing wounds increases, with the total cost of over $3 billion per year for health services (2). Therefore, a number of laboratory investigations and clinical studies deal with new approaches to improve wound healing using either modern physical and pharmacological methods or phytotherapy (3).

Medicinal plants have been used for the treatment of various diseases for thousands of years. *Elaeagnus angustifolia* (Russian olive, Russian silverberry, Oleander) is one of these herbs applied most in Iran’s traditional medicine (4). Phytochemical studies have shown that aqueous fruit extract of *Elaeagnus angustifolia* contains flavonoids compounds, sitosterols, cardiac glycosides and terpenoids (5). In traditional medicine, oleaster fruit or flower extracts are used for treating nausea, jaundice, vomiting, asthma, and flatulence (6). Extracts of *Elaeagnus angustifolia* are also used as an anti-ulcerogenic agent (7), muscle...
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relaxant (8), antipyretic (4), antinociceptive and anti-inflammatory (4,9,10) in traditional medicine. While the extract of Elaeagnus angustifolia fruit is used for eliminating headaches and rickets, the tea of this fruit is used as an analgesic agent for reducing rheumatoid arthritis pain (4). In folk medicine it has been known as a wound healing accelerator (11). In this research, we try to evaluate the effect of Elaeagnus angustifolia fruit extract on wound healing in a rat animal model.

Materials and Methods

Animals
45 Sprague-Dawley healthy adult male rats (250–280 g) were housed one per cage and maintained under standard housing conditions (room temperature 24-27°C and humidity 60-65% with 12:12 light: dark cycles) for a week prior to the experiment to acclimatize. All procedures were conducted using facilities and protocols approved by Institutional Animal Ethics Committee of Tehran University of Medical Sciences.

Wound model
Rats were anesthetized using an intraperitoneal injection of ketamine (50 mg/kg, Alfasan International, Woerden, Holland) and xylazine (10 mg/kg, Alfasan International, Woerden, Holland). Their back hairs were shaved by electrical clipper and the skins was washed with alcohol 70% and rinsed with sterile water. A full thickness excision wound of circular area 500 mm² and 2 mm in depth was created by excising the skin and panniculus carnosus from the dorsal midline and wounds were left undressed (12). The animals were randomly assigned into three following groups:

Treated Group: Received the extract (n=15).
Positive control group: Standard-treated with mupirocin ointment 2% (Pars Darou Pharmaceutical CO. Iran) (n=15).
Control group: Did not receive any treatment (n=15).

The extract was applied at a dose 500 mg/kg/day once a day for 15 days as a topical solution and mupirocin ointment was applied evenly in sufficient amounts to cover all wound areas(11,13).

Animals were euthanized at day 5, 10, or 15 (n= 5 in each group) and wounds were resected for further analysis.

Extract preparation
The fruits of Elaeagnus angustifolia were collected from mountain area of Ardabil, Iran during October 2010 and was identified and approved by Herbarium Department of Pharmacognosy of Shahid Beheshti University of Medical Science (Tehran, Iran). The voucher specimen (No. 1057) is preserved in the herbarium of this department for reference. The aqueous extract was prepared by adding 2000 ml of distilled water to 100 g of fruit powder (without cores) and the resulting solution was boiled for 10 minute. Then the mixture was filtered and the solution was completely dehydrated for 8 to 10 hours in water bath to provide a crude extract with 20% yield.

Wound analysis
Wound areas were measured manually and calculated in square millimetres. We measured the lengths of the major axis and the minor axis of the wound on days 0, 3, 5, 8, 10, 12, and 15 after wounding with a vernier calliper (14). The measurement of the wound area was calculated by the formula for an ellipse ([0.5 × the length of the major axis][0.5×the length of the minor axis][π]) (15). The wound healing rate was calculated as follows: (Area of original wound/ Area of remaining wound)/Area of original wound×100 (16).

Processing and preparation of skin tissues
Five animals from each group were sacrificed by overdose of ketamine and xylazine on day 5, 10 and 15 post-wounding respectively.

The entire wound as well as a 5 mm margin of surrounding unwounded skin was excised for further analyses. Tissues were cut into two small pieces. One part was placed in formalin (10% formaldehyde in phosphate-buffered saline) for histological examination and the second part was quickly kept frozen (-80°C) for biochemical analysis.

Histopathological analysis
Paraffin-embedded formalin fixed tissue blocks were obtained from the mentioned samples. Representative 5 μm sections from the mid-portion of the wound were stained by hematoxylin-eosin (H&E) and Masson’s trichrome methods. All slides were examined in blind fashion by surgical pathologist. Each slide was given a histological score ranging from 1 to 12, with 1 associated to no healing and 12 associated to a completely re-epithelialized wound (Table 1), according to the method described by Greenhalgh et al. (17).

Hydroxyproline (HP) analysis
Determination of HP was used as an index for assessing collagen content in skin tissue (18). Samples of skin tissue were hydrolyzed in 6 N HCl, at 105°C for
14-16 hours and HP was oxidized by chloramin T (0.14 g chloramine T, 2 ml distilled water, 8 ml citrate/acetate buffer). Then by adding Ehrlich reagent (2.5 g of p-dimethylaminobenzaldehyde, 2.7 ml of 12 N HCl, 16 ml of isopropanol) and incubating at 60°C for 25 min, a chromophore was formed. The absorbance of chromophore was measured at 543 nm, and HP concentration was calculated by comparison to a standard curve. Total protein content of samples was also quantified by Bradford assay (19), using bovine serum albumin as the standard and the results were expressed in μg HP/mg protein (20,21).

**Statistical analysis**

All results were reported as mean ± S.D. and the statistical significance of differences among groups were assessed using one-way ANOVA. A value of \( P < 0.05 \) was considered as significant. All experiments were repeated at least twice.

**Results**

**Wound healing rates**

We evaluated the changes in wound areas in rats as indices of wound closure. The morphology of wound sites was similar in treated, control and positive groups five days after the injury (Figure 1A). The percentage of the wound contraction was significantly increased at days 10 (\( P < 0.05 \)), 12 and 15 (\( P < 0.001 \)) in the treated group compared to the control group. The wound contraction at days 12 (\( P < 0.05 \)) and 15 (\( P < 0.001 \)) was significantly increased comparing to the positive control group (Table 2).

**Histology**

A close correlation between the gross and histological appearances of the wounds was observed. According to the statistical analysis of the mean histological scores, no significant differences were found between groups on day 5 (Figure 1B). Histological scores were significantly (\( P < 0.05 \)) higher at days 10 and 15 in the treated and positive control groups than the control. No significant difference was observed between the positive control and treated group (Figure 1B).

At day 5, wounds in every three groups had thin, immature granulation that were dominated by inflammatory cells but few fibroblasts, capillaries, or collagen deposition and minimal epithelial migration. By 10 days, the wounds in the treated and positive control groups contained granulation tissue rich in fibroblasts, dense collagen, and capillaries (Figure 2A, 2B).

<table>
<thead>
<tr>
<th>Score</th>
<th>Criteria</th>
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<tbody>
<tr>
<td>1-3</td>
<td>None to minimal cell accumulation, no granulation tissue or epithelial travel</td>
</tr>
<tr>
<td>4-6</td>
<td>Thin, immature granulation that is dominated by inflammatory cells but has few fibroblasts, capillaries, or collagen deposition, minimal epithelial migration</td>
</tr>
<tr>
<td>7-9</td>
<td>Moderately thick granulation tissue, can range from being dominated by inflammatory cells to more fibroblasts and collagen deposition, extensive neovascularization, epithelium can range from minimal to moderate migration</td>
</tr>
<tr>
<td>10-12</td>
<td>Thick, vascular granulation tissue dominated by fibroblasts and extensive collagen deposition, epithelium partially to completely covering the wound</td>
</tr>
</tbody>
</table>

**Table 1.** Histologic score of sections, adopted from Greenhalgh et al. (17).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Treated group</th>
<th>Positive control</th>
<th>Control group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 3</td>
<td>22.8 ± 0.4%</td>
<td>20.7 ± 0.05%</td>
<td>18.4 ± 0.3%</td>
</tr>
<tr>
<td>Day 5</td>
<td>33.7 ± 8.3%</td>
<td>28.0 ± 9.4%</td>
<td>31.3 ± 6.9%</td>
</tr>
<tr>
<td>Day 8</td>
<td>61.2 ± 8.0%</td>
<td>52.0 ± 2.5%</td>
<td>49.7 ± 8.0%</td>
</tr>
<tr>
<td>Day 10</td>
<td>87.5 ± 6.8%</td>
<td>73.3 ± 8.0%</td>
<td>65.9 ± 8.0%*</td>
</tr>
<tr>
<td>Day 12</td>
<td>94.1 ± 2.8%</td>
<td>87.0 ± 7.0%*</td>
<td>85.0 ± 3.6%**</td>
</tr>
<tr>
<td>Day 15</td>
<td>95.4 ± 3.1%</td>
<td>91.9 ± 3.3%**</td>
<td>91.4 ± 3.3%**</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± S.D, five animals in each group.

Inter-group comparisons were made using one way ANOVA, * \( P < 0.05 \); ** \( P < 0.001 \)
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Figure 1. Effects of Elaeagnus angustifolia extract on wound closure. A: Macroscopic aspect of wounds under different treatment. I, treated group applied with Elaeagnus angustifolia; II, positive control group treated with mupirocin ointment; III, control group without any treatment B: Histological grading of resected wounds. Treated and positive control group had a significantly higher histological score compared to control. Five animals were used in each group. All values are expressed as mean ± S.D. * P<0.05.

In contrast, focal ulceration of epidermis, minimal cellular infiltrates and granulation tissue developed in wounds of control group 10 days after wounding (Figure 2C). By day 15th, epithelium, partially to completely covered the wound and extracellular matrix dominated by extensive collagen deposition in both treated and positive control groups (Figure 2D, 2E). In control group, 15 days after wounding, focal ulceration of epidermis and moderately thick granulation tissue with lesser collagen fiber were seen (Figure 2F). Histological evaluation revealed increased cellular infiltration, collagen deposition and re-epithelialization in treated and positive control groups.

HP content in wound site

The synthesis and deposition of collagen is another characteristic change observed in the proliferative phase of wound healing process. Since HP concentration is an index of collagen deposition (1,18), we determined HP content in the wound sites. HP content in wound sites increased progressively in treated group. HP contents of treated group increased more rapidly after injury, with significantly (P<0.05) higher levels in 5, 10 and 15 days after injury than positive and control group (Figure 3). On the other hand, treatment with extract significantly increased collagen deposition.
**Figure 2.** Micrographs of wound bed and wound margin in each group at 10 and 15 days after wounding (H&E stain, ×100). At 10 days after wounding, the treated group shows no ulceration with dense collagen deposition (A and B), the control group shows focal ulceration of epidermis (C). At 15 days after wounding, the treated group shows no ulceration with abundant collagen deposition (D and E), the control group shows focal ulceration of epidermis (F).

**Figure 3.** Collagen content in the excision wounds. Hydroxyproline (Hp) measurement as an indicator of collagen deposition was performed. Treated group had a significantly higher hydroxyproline content compared to control and positive control group. Five animals were used in each group. All values are expressed as mean ± S.D. * P<0.05
Discussion

\textit{Elaeagnus angustifolia} fruit extract has been used in traditional medicine as a remedy. This research was designed based on the previous research focused on the effectiveness of \textit{Elaeagnus angustifolia} fruit extract on the wound healing (11). However, the mechanism of its action is unknown. The major findings of this study are as follow: \textit{Elaeagnus angustifolia} fruit extract accelerates wound healing in rat as assessed by the degree of wound closure, hydroxyproline content and histological score. The most significant morphological changes occurred during the second seven days of wound healing. Histopathological analysis showed that collagen deposition, epidermis regeneration and proliferation (fibroblasts and endothelial cells) of the treated group were higher than control. Treatment with fruit extract significantly reduced the process of inflammation. The present study showed that, hydroxyproline content of skin, a specific marker of collagen, was increased in the wounds treated with \textit{Elaeagnus angustifolia}. The increased HP; clearly explains the increased rate of wound closure, due to rapid collagen turnover and accumulation.

Inflammation is the second stage in the wound-healing process, and is important to the removal of contaminating micro-organisms. In this phase of wound healing factors are released that cause the migration and division of cells involved in the proliferative phase (1,22). Successful wound healing after tissue injury requires resolution of the inflammatory response. But, if this phase continues, the wound may enter to chronic phase and fail to heal (1). This prolonged inflammation phase also leads to an increased level of matrix metalloproteinases (MMPs) such as collagenases (MMP-1, MMP-8) and gelatinases (MMP-2, MMP-9) (2,22). Excess MMPs, break down extracellular matrix molecules, growth factors, protease inhibitors and receptors that are essential for healing which results in increased protease activity and reduced protease inhibitors that supports tissue destruction and inhibits normal repair processes (1).

The findings in the present study are consistent with reports from other investigators showing that \textit{Elaeagnus angustifolia} fruit extract reduces pain and inflammation caused by administration of formalin (9,10). Farahbakhsh et al. have shown specifically that extract of \textit{Elaeagnus angustifolia} fruit could inhibit inflammation by inhibiting cyclooxygenase type 1 and 2 enzymes (4). So one of the ways in which \textit{Elaeagnus angustifolia} fruit extract can accelerate the wound healing process is through inhibition of inflammation phase.

Phytochemical studies have shown that aqueous fruit extract of \textit{Elaeagnus angustifolia} contains flavonoids compounds, sitosterols, cardiac glycosides, terpenoids, vitamins B and A and also vitamin K, which are effective in coagulation (23,24). Vitamin A has different roles in wound healing such as: antioxidant activity, increasing fibroblast proliferation, modulating of cellular differentiation and proliferation, increasing collagen deposition and hyaluronate synthesis, and decreasing MMP (1,24).

Also this extract contains a wide array of free radical scavenging molecules and flavonoids which are believed to be responsible for the therapeutic effects of this plant (5).

Previous studies have demonstrate that flavonoids and sitosterols are responsible for anti-inflammatory and analgesic effects of this plant (5). Flavonoids play different roles in biological systems. The flavonoids, due to their phenolic hydroxyl groups, and their capacity to chelate metals, have antioxidant activities, and can reduce levels of free radicals and reduce lipid peroxidation. Antioxidants have been reported to play a considerable role in the wound healing process and significantly promote wound healing and protect tissues from oxidative damage (25). In addition, flavonoids are known to accelerate the wound healing process mainly due to their antimicrobial properties, which appear to be responsible for wound closure and increased rate of epidermis regeneration (26). Hence, if a compound has anti-microbial and antioxidant activity, it can be a good therapeutic agent to increase the viability of collagen fibers by increasing the strength of collagen fibers and their circulation as well as preventing the cell damage by promoting the DNA synthesis which finally accelerate the wound healing process (26,27).

Thus, wound-healing activity of \textit{Elaeagnus angustifolia} may be related to their individual or additive effects of its phytochemicals that promote wound healing process. At this stage, it is difficult to say which component(s) of fruit extracts are responsible for this wound healing activity. However, further phytochemical investigation is required to isolate the active compound(s) responsible for these pharmacological properties and to understand the complete mechanism of wound healing activity of \textit{Elaeagnus angustifolia} fruit extracts.
Acknowledgements

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