WOUND HEALING POTENTIAL OF AQUEOUS EXTRACT OF ELAEAGNUS ANGUSTIFOLIA FRUIT WITH DOWN REGULATION OF TNF-ALPHA IN RAT

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ABSTRACT

Wound healing properties of Elaeagnus angustifolia (EA) fruit has been used for a long time in traditional Iranian medicine. The present study was performed to investigate the biochemical mechanism of wound healing activity of this plant in rats. After creating full-thickness skin wounds on the back of the rats, they were randomly divided into three groups. Two groups as the treated and positive control received the extract of EA and mupirocin ointment, respectively and other group as the control group, was not subjected to any treatments. Isolated tissues from wounds were harvested for 5, 10, and 15 days and were then underwent biochemical analysis. Healing activity was assessed by the rate of wound contraction and collagen content. The in vivo antioxidant and anti-inflammatory activity was investigated to understand the mechanism of wound healing potency. The concentration levels of transforming growth factor beta 1 (TGF-β1) and tumor necrosis factor alpha (TNF-α) were determined in wound tissues by an enzyme-linked immunosorbent assay (ELISA) and a real-time RT-PCR. The results indicated a significant increase in the percentage of wound contraction and collagen content in the treated group, when compared with the control and positive control groups. Both mRNA and protein levels of the TNF-α were significantly reduced in the EA treated group. A significant decrease was also observed in Malondialdehyde and Myeloperoxidase content in the treated group compared to the control group. This study revealed that the EA extract accelerates cutaneous wound healing. This effect may confer its antioxidant and anti inflammatory activities. Therefore, EA extract may be useful in the current management of wound healing.

Keywords: Wound healing, Elaeagnus angustifolia, TNF-α, Antioxidant, Anti-inflammatory.

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Introduction

Cutaneous wound healing is a dynamic and complex biological process, being initiated in response to injury to restore the integrity and function of the damaged tissue[1]. This process is strictly regulated by various growth factors and cytokines, that are released at the wound site[2]. Among these, TNF-α is a potent inflammatory cytokine, being primarily secreted by macrophages and neutrophils, and is expressed during the inflammatory phase of wound healing. Previous studies have shown that high level of TNF-α in non-healing ulcers inhibit synthesis of extracellular matrix (ECM) and activates matrix metalloproteinases[1, 3, 4].

The transforming growth factor-β1 (TGF-β1) is a pluripotent cytokine in normal wound healing and encompasses the broadest spectrum of actions in this process. TGF-β1 stimulates synthesis of ECM; especially collagen I and reduces the expression of matrix metalloproteinases[5].
Today, the demand for herbal medicines is increasing in the developed and developing countries, as they are safe and may be better tolerated in respect to chemical medicines. Numerous medicinal plants have been used for treating wounds. They affect the healing process through modulating the cytokine(s) secretion in different phases of wound healing, which could be achieved by their antioxidant or anti-inflammatory properties. In Iranian traditional medicine, Elaeagnus angustifolia is used as anti-ulcerogenic, anti-inflammatory, and antinociceptive agent. Elaeagnus angustifolia is a member of the Elaeagnus family that is widely distributed in the northern regions of Asia to the Himalayas and Europe. It is commonly known as “senjed” in Iran and “Russian olive” or “Oleaster” in English language countries. Decoction and infusion of the fruits of oleaster is considered to be a good remedy for rheumatoid arthritis. The healing activity of this plant have been reported by the present research group previously, but its mechanism of action remains to be clarified. The present study aimed to determine the underlying mechanisms of wound healing, induced by Elaeagnus angustifolia extract in a rat animal model.

Materials and methods

Plant material

The Elaeagnus angustifolia fruits were collected from mountains of Ardabil, Iran during October 2010. The plant was identified and approved by the Herbarium Department of Pharmacognosy of Shahid Beheshti University of Medical Sciences (Tehran, Iran). The voucher specimen (No. 1057) was conserved in the herbarium of this department for reference. The lethal dose (LD50) of the extract is 20000 mg/Kg body weight in Wistar rats.

Extract preparation

The aqueous extract of the Elaeagnus angustifolia fruits was prepared as described previously. The fruits (without cores) were milled. Two hundred and Fifty gram of milled fruit was added to 1000 ml of distilled water and boiled for 20 minutes, and then the solution was filtered through a whatman filter paper pore size 0.45 micron. The water extract was concentrated on a water bath for 8 to 10 hours and stored in a freezer at -20°C. The moisture of the extract was determined as follows: 5 g of the final extract was placed in an oven in 60°C for 72 hours, then weighed, and the loss weight was used as a moisture indicator. 20% of the final extract contained water. Each 100 g of powdered fruits yielded about 20 g of extract.

Animals

Forty five healthy Sprague-Dawley adult male rats (250-280 g) were obtained from the animal house of the department of pharmacology of Tehran University of Medical Sciences. The animals were housed one per cage and maintained under controlled conditions (room temperature 24-27 °C; humidity 60-65% with 12:12 light: dark cycles) for a week prior to the experiment. All animals received human care in compliance with the Guide for Care and use of Laboratory Animals prepared by the Institute of Laboratory Animal Resources and published by the National Institutes of Health (NIH publication no. 85-23). All of the procedures of this study were approved by the Institutional Animal Ethics Committee of Tehran University of Medical Sciences.

Wound model

The rats were anesthetized with one intraperitoneal injection of ketamine (50 mg/kg, Alfasan International, Woerden, Holland) and xylazine (10 mg/kg, Alfasan International, Woerden, Holland). The hairs on their backs were removed by an electrical clipper and their skins were disinfected with alcohol and rinsed with distilled water. A circular full thickness excision wound (500 mm2: area, and 2 mm in depth) was created on the dorsal midline of the rats and left opened. The animals were randomly divided into three groups of 15. The treated groups received the extract as a topical solution once a day at a dose of 500 mg/kg body weight of the animals for 15 days. The positive control groups were treated with 2% mupirocin ointment (Pars Darou Pharmaceutical CO. Iran), according to (Nayak et al., 2006 and Pandian et al., 2013). This ointment was applied evenly in sufficient amounts to cover all wound area. The control groups were under no treatments.

On days 0, 3, 5, 8, 10, 12, and 15 after wounding the animals, the major and minor axes of each wound were measured manually with a vernier caliper and the wound area was calculated by the formula for an ellipse per square millimeters (Adobe Systems, San Jose, CA). Wound healing rate was expressed as the percentage of the initial wound area.
The Percentage (%) wound contraction = [(initial wound area - unhealed wound area)/ initial wound area] × 100 (15).

To perform biochemical analysis, the wounds and approximately 5 mm margins of the surrounding tissue were harvested on day 5, 10 and 15 post-wounding, before scarifying.

**Myeloperoxidase assay**

Myeloperoxidase (MPO) concentration as a quantitative assessment of neutrophil recruitment in the wound site was measured in the samples of the wound tissue (16). MPO concentration in wound homogenates was determined, using the rat MPO ELISA kit from Hycult Biotechnology (Uden, The Netherlands, intra-assay, CV: 3.66%) according to the manufacturer’s instructions. Total protein concentration of the samples was determined by Bradford assay (17). MPO concentration was further normalized to protein concentration in the samples and expressed as picograms of MPO per milligram of protein.

**Malondialdehyde assay**

Malondialdehyde (MDA) level as an index of lipid peroxidation was measured in the samples of the wound tissue (18). The concentration of MDA protein adducts in the wound tissue was determined, using OxiSelect TM MDA Adduct ELISA Kit according to the manufacturer’s instructions (Cell Biolabs, San Diego, CA, USA, intra- and inter-assay coefficients of the variation 5.91% and 16.39%, respectively). The total protein concentration of the samples was determined by Bradford assay (17). The results were expressed as pmol /mg protein.

**Measurement of hydroxyproline (HP) concentration**

HP concentration was used as an index for the evaluation of collagen content of the samples of wound tissues. The HP content of the samples was measured according to our previous study (11). The concentration of HP was calculated in comparison to the standard curve. Total protein concentration of the samples was also determined by Bradford assay and the results were finally expressed in μg HP/mg protein (17).

**Real-time PCR**

Total RNA from the wound tissue samples was extracted by an RNaseasy Fibrous Tissue Mini Kit (Qiagen, Germany). The purity of RNA was quantified by spectrophotometry (at 260/280 nm) and its quality was examined by electrophoresis. cDNA was synthesized from 1μg sample of total RNA by QuantiTect Reverse Transcription kit (Qiagen, Germany). A quantitative Real-time PCR was performed for cDNA samples, using a QuantiFast SYBR Green PCR kit (Qiagen, Germany). Primers of TGF-β1, TNF-α, and beta-actin, being as the endogenous controls (housekeeping gene) were pre-designed by the QuantiTect Primer Assay (Qiagen, Germany). Thermal cycling conditions consisted of an initial incubation at 95°C for 10 minutes, followed by 40 cycles of denaturation for 10 seconds at 95°C and an annealing and extension for 30 seconds at 60°C. To confirm the specificity of the amplification and the absence of primer dimmers, melting curves were obtained at the end of each PCR run. Efficiencies were determined for the samples and the LinRegPCR software was used to calculate the relative amount of RNA using the ΔΔCt method.

**Enzyme-linked immunosorbent assay (ELISA)**

To extract proteins the samples of wound tissues were frozen in liquid nitrogen and homogenized with Mikro-Dismembrator S (Sartorius, Goettingen, Germany) in 300 μl of lysis buffer (10 mmol/L PBS, 5 mmol/L ethylenediaminetetraacetic acid, 1% Nonidet P-40, 0.1% sodium dodecyl sulfate), containing Complete Protease Inhibitor Cocktail (Roche Diagnostics, Switzerland).

The homogenates were centrifuged in 15,000 rpm for 15 minutes at 4°C to remove the debris. TGF-β1 and TNF-α proteins in the supernatant were measured by the ELISA kits according to the manufacturer’s instructions (TNF-α; RayBiotech Inc., Norcross, GA, USA and TGF-β1; Biosensis, Thebarton, Australia). Then, total protein concentration of the samples were determined (17). The result was expressed as cytokine or growth factor/total protein (pg/mg) for each sample.

**Statistical analysis**

All results were reported as mean ± standard deviation (SD) and the statistical significance of the differences among groups were assessed by one-way ANOVA test followed by a post hoc Tukey’s test. 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 the wound areas in rats as indices of wound closure. Five days after the injury the morphology of the wound sites was similar in treated, control and positive groups (Figure 1A). The percentage of the wound area was significantly decreased in days 10 (P<0.01), 12 and 15 (P<0.001) in the treated group compared to the control group, and in days 12 (P<0.01) and 15 (P<0.001), when compared to the positive control group (Figure 1B).

Myeloperoxidase concentration

In the inflammatory phase of the wound healing different types of leukocytes penetrate into the wound site. It was difficult to determine the accurate number of neutrophils, because many of them were trapped within the clot. Thus, we measured the MPO concentration to evaluate the neutrophil recruitment. The MPO concentration was significantly attenuated 10 days after the injury in the treated (P<0.001) and positive control (P<0.005) groups, when compared with the control group (Figure 2A). These results demonstrated that neutrophil recruitment during the inflammatory phase of wound healing was markedly reduced after the treatment via EA extract. This finding is consistent with the TNF-α expression.

Malondialdehyde concentration

Malondialdehyde content was found to be significantly decreased on days 5, 10 and 15 in the treated group (P<0.05), after comparing to the control group (Figure 2B). The results indicate that EA extract possesses potential antioxidant activity through inhibiting lipid peroxidation.

Hydroxyproline concentration

The proliferative phase of wound healing is characterized by an increased collagen deposition in the extracellular matrix. The HP content in wound sites was progressively increased in all groups. The HP content of the treated group was increased more rapidly after the injury; with significantly higher levels of HP in 5, 10 and 15 days after injury.
injury, when compared to positive and control group (Figure 3). These results suggest that collagen production was enhanced after treatment with EA extract.

**Figure 3**: Collagen content in wound tissue of Elaeagnus angustifolia and mupirocin treated and control rats, 5, 10 and 15 days after injury. Hydroxyproline (HP) as an indicator of collagen content was measured. Data are expressed as mean ±S.D. (n=5). *P<0.05 as compared to control.

**TNF-α and TGF-β1 mRNA and protein expression levels**

The treated group showed a significantly reduced mRNA expression and protein levels of TNF-α, 5, 10 and 15 days after the injury in comparison to the control group (Figure 4A and B). TNF-α mRNA was under-expressed by 4.025 ± 0.68, 28.47 ± 1.9, and 29.72 ± 2.1 in the treated group, 5, 10 and 15 days after injury, respectively. However, treatment by EA could not change the mRNA expression and protein levels of the TGF-β1 (Figure 4 C and D).

**Figure 4**: mRNA and protein expression of chemokines at wound tissue. Relative expression level of TNF-α (A) and TGF-β1 (C) to β-actin in rats treated with EA extract, mupirocin and control were determined by RT-PCR at 5, 10 and 15 days after the injury. Data are expressed as the mean ± SD (n = 5). * P <0.05 as compared to control. Protein levels of TNF-α (B) and TGF-β1 (D) in the wound tissue of rats treated with EA extract, mupirocin and control were analyzed by ELISA. Data are expressed as the mean ±SD, (n = 5). * P < 0.05 as compared to control.

**Discussion**

Various plant and extracts are considered to be potential agents in wound healing. There is an increasing demand for herbal medicines due to its worldwide availability, less or non-toxicity, and lack of side effects(19).

The wound healing efficacy of the aqueous extract of Elaeagnus Angustifolia has been documented previously by our team(11). This finding motivated us to systematically study the underlying mechanisms of efficacy.

The present study demonstrates that the topical application of EA could increase collagen synthesis, wound contraction, decrease lipid peroxidation and neutrophil infiltration, and finally induce TNF-α and MPO secretion. Our study revealed that EA extract could increase HP in the wound site. HP is a specific index for evaluation of the collagen content in a sample of wound tissue. Collagen is a major component of ECM. The production of thicker collagen fibers by fibroblasts results in formation
of condensed granulation tissues, pulling surrounding skin into the lesion, and ultimately yields a wound contraction\cite{20}. The increased HP content of the treated wound by EA extract could clearly explain the increased rate of wound contraction, which may be resulted from rapid synthesis, turnover and accumulation of collagen.

Phytochemical studies have shown that the fruits of EA contain sugar, pectin, flavonoid, terpenoid, phenol acid, saponins and vitamin A\cite{21,22}. Vitamin A plays several roles in wound healing. Vitamin A encompasses antioxidant activity and increases proliferation of fibroblast, collagen deposition, hyaluronate synthesis, and reduces MMP production\cite{22,23}.

Our study could also demonstrate that EA extract reduced production of MDA. MDA is an index of lipid peroxidation, being triggered by reactive oxygen species (ROS). It is well known that ROS produced during the inflammatory phase of wound healing process contributes to tissue damage and healing delay. The presence of a wide range of free radical scavenging molecules and flavonoids in the extract decreases the production of ROS\cite{24}. The antioxidant activity of flavonoids is an outcome of their accompanying phenolic hydroxyl groups\cite{25,26}.

Our study revealed that application of EA could decrease both mRNA expression and protein level of TNF-\(\alpha\). This could be resulted by the flavonoids and terpenoids present in the fruit extract. They may down regulate the production of inflammatory mediators by suppressing NF-\(\kappa\)B pathway at different levels. Another probable responsible mechanism for decreasing TNF-\(\alpha\) expression, is antioxidant activity of the extract\cite{27-29}. TNF-\(\alpha\) decreases the transcription of collagen gene to generate its detrimental function in the process of wound healing\cite{30}. Mori et al. (2002) suggested that in a TNF receptor p55-deficient mice collagen formation, angiogenesis, and wound healing rates were increased\cite{31}. Furthermore, an elevated level of TNF-\(\alpha\) was reported in non-healing wound\cite{32}. Thus, a decrease in the level of TNF-\(\alpha\) is an important contributing factor, being possibly responsible for the wound healing effect of the extract.

This study could show a decrease in MPO activity during the inflammatory phase of the wound healing process. MPO is characterized by powerful pro-oxidative and pro-inflammatory properties\cite{33}. Zhang et al. (2002) reported that MPO serves as a major enzymatic catalyst of lipid peroxidation at the site of inflammation. Free radicals derived from MPO activity could induce the expression of pro-inflammatory genes\cite{33}.

This reduction could contribute to release of flavonoids in fruit, being extremely effective in inhibiting MPO secretion from the stimulated neutrophils\cite{34,35,36}.

### Conclusion

In conclusion, it seems that the underlying mechanism of the wound healing activity by EA is associated with increasing level of collagen synthesis, reduction in neutrophil infiltration, and subsequently, TNF-\(\alpha\) and MPO secretion in the wound site. This could be partly caused by the flavonoids, terpenoids, and vitamins in the extract. Currently, it is difficult to state which component(s) of the fruit extract is responsible for this activity.

Consequently, further phytochemical studies are required to isolate the active compound(s) responsible for manifestation of these pharmacological properties.

### References

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Wound healing potential of aqueous extract of Elaeagnus angustifolia fruit with down regulation of TNF-Alpha in rat


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Declarations of interest
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