Effects of borage extract in rat skin wound healing model, histopathological study

Mohammad Reza Farahpour\textsuperscript{1*} and Amir Hossein Mavaddati\textsuperscript{2}

\textsuperscript{1}Department of Veterinary Surgery, Islamic Azad University, Urmia Branch, Urmia-Iran.
\textsuperscript{2}DVM, Department of Veterinary Surgery, Islamic Azad University, Urmia Branch, Urmia-Iran.

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Nowadays, borage flowers and leaves are used medicinally for wound healing because of plant constituents, gamma-linolenic acid, alpha linolenic acid and delta-6-fatty acid. The aim of the present study was to evaluate the ability of borage extract ointment wound healing process. Under surgical anaesthesia, excisional wounds were made on back of 30 rats by punching. Rats were divided into 3 groups of borage 1.5% ointment, eucerin-vaseline, and control. All the rats were treated with topical ointments daily for 21 days. Histopathological examination was performed on the 3rd, 7th, 14th, and 21st days and the wound healing was assessed. The results showed that wound size of the test groups were reduced early as compared to control group. The significant results of histopathological evaluation were obtained with borage, when compared to the other groups as well as to the control and the placebo. Present study demonstrated that borage extract was capable of promoting wound healing process.

Key words: Borage, herbal extract, histopathological study, rat, wound healing.

INTRODUCTION

Nowadays, the promotion of wound healing in some diseases and chronic disorders with the aid of herbal extracts is more challenging than ever before. That is why new compounds prepared for this purpose have been widely accepted. Borage (Echium amanum) is a large hairy annual herb that is a member of Boraginaceae family. It grows in most of Europe, in the Mediterranean region, and also in northern parts of Iran. The flowers are bright blue and star-shaped and the fruit consists of four brownish-black nutlets. Borage flourishes in ordinary soil and may be propagated by division of rootstocks and by cuttings of shoots in sandy soil in a cold frame in summer and autumn or from seeds sown in good light soil from mid of March to May. The flowers and the leaves of borage are used medicinally in France as an antifebrile, antidepressive, for the treatment of stress and of circulatory heart diseases, for pulmonary complaints, as a poultice for inflammatory swellings (Kapoor and Klimaszewski, 1999), as a diuretic (due to potassium nitrate), as a laxative, emollient and demulcent (due to the mucilage), and as a possible protective factor against cancer (Gonzalez et al., 1993). The plant constituents have been isolated by different investigators; they include gamma-linolenic acid (GLA), alphalinolenic acid (ALA), delta 6 fatty acid denaturase, delta 8 sphingolipid desaturase (Coupland, 2008), pyrrolizidine alkaloids, mucilage, resin, potassium nitrate, and calcium salt combined with mineral acids. Therefore, it can improve wound healing process.

Normal healing response begins immediately after tissue injuring. Platelets, the cells present in the highest numbers shortly after a wound occurs, release a number of things into the blood, including ECM proteins and cytokines, including growth factors. Growth factors stimulate cells to speed their rate of division. Platelets also release other proinflammatory factors like serotonin, bradykinin, prostaglandins, prostacyclins, thromboxane, and histamine, which serve a number of purposes, including to increase cell proliferation and migration to the area and to cause blood vessels to become dilated and porous. Following homeostasis, the neutrophils then
enter the wound site and begin the critical task of phagocytosis to remove foreign materials, bacteria and damaged tissue. As part of this inflammatory phase, the macrophages appear and continue the process of phagocytosis as well as releasing more PDGF and TGF. Once the wound site is cleaned out, fibroblasts migrate in to begin the proliferative phase and deposit new extracellular matrix. The new collagen matrix then becomes cross-linked and organized during the final remodeling phase. In order to this efficient and highly controlled repair process, numerous cell-signaling events are required (Robert and Melissa, 2004). Many biological dressings and indigenous medicines have been reported to possess wound healing properties. However, none of these has been completely effective and free of side effects. Many herbal drugs can be effective in wound healing acceleration.

The wound healing activities of plants have since been explored from ancient times. With the development of scientific research methods, the significant successes reported have led to investigation into medicinal plants with a view to confirming these acclaimed properties. Findings have been shown that different parts of plants contain some active components that are antimicrobial and nutritive in function that could have benefits on wound healing process. Borage is a hairy annual herb commonly known as 'borage'. It has been known for its mood elevating properties. The plant is reputed as antispasmodic, antihypertensive, aphrodisiac, demulcent, diuretic and is also considered useful to treat asthma, bronchitis, cramps, diarrhea, palpitations and kidney ailments (Duke et al., 2002). Decoction of the plant is used as nerve and cardiac tonic and a home remedy for blood purification.

Phytochemical studies reveal the presence of tannins, resins, ascorbic acid, beta-carotene, niacin, riboflavin, thiamine, silicic acid, choline arabinose, unsaturated pyrrolizidines alkaloids including amabiline, lycompsine and supinidine, polyphenolics including phenolic acid, vanillic acid, p-coumaric acid, p-hydroxy benzoic acid, gentisic acid, caffeic acid, rosmarinic acid and chlorogenic acid, scopoletin and flavonoids (Gudej and Tomczyk, 1996).

Borage oil has been reported to lower serum cholesterol, phospholipids and triglyceride levels (Gu et al., 1998) and increases the levels of omega-6 polyunsaturated fatty acids in the plasma, liver, aorta and renal artery tissues. Dietary use of borage oil exhibited immuno-modulatory (Harbig et al., 2000) and blood pressure lowering effects in normal and spontaneously hypertensive rats through unknown mechanism. It exhibited kidney protective potential through angiotensin II receptor blockade (Engler et al., 1998), cytotoxic and free radical scavenging activities (Bandoniene and Murkovic, 2002; Lin et al., 2002). Despite the fact that borage has been used traditionally, it has not been widely studied to justify its use in abdominal colic, diarrhea, asthma and hypertension. In this investigation, we report the presence of Ca antagonist-like constituents, which provide the pharmacological basis for the use of borage in hyperactive gastrointestinal, respiratory and cardiovascular disorders. As a dietary supplement, borage oil (BO) has been reported to exert clinical efficacy in a variety of skin diseases and in the suppression of proliferation and inflammation in the skin (Chung et al., 2002; Mork-Hansen et al., 1983). The major constituent of BO underlying this clinical efficacy is believed to be oxidative metabolites of essential fatty acids (EFA), gamma-linolenic acid (GLA) prostaglandin E1 (PGE1), and 15-hydroxyeicosatetraenoic acid 15HETE), which have been reported to exert antiproliferative and anti-inflammatory effects in vitro (Chung et al., 2002; Miller et al., 1988). Fatty acids belong to a class of compounds formed by a long hydrocarbon chain and a terminal carboxylic group. They have three main functions: They are structural components of biological membranes, they act as precursors of intracellular messengers and they are oxidized to produce adenosine triphosphate (ATP) (Hatanaka and Curi, 2007).

From the beginning of the 1970s, there have been studies on the effects of fatty acids on the immune response. Such compounds interfere with various events of the inflammatory process, such as vascular contraction, chemotaxis, adhesion, diapedesis, activation and cell death, where the majority of these occur via arachidonic acids such as prostaglandins, leukotrienes, thromboxanes and lipoxins (Hatanaka and Curi, 2007). Polyunsaturated fatty acids (PUFAs) should be pointed out among the various fatty acids present in the plasma and in leukocytes. Besides their structural function, they can modulate cell to cell interactions and intracellular signaling. Thus, the alteration of the composition of fatty acids of phospholipids in the cell membrane can modify fluidity and change the binding of cytokines to their receptors (Cardoso et al., 2004). Linoleic acid is an essential fatty acid of 18 carbons. Through a desaturation process, it gives rise to arachidonic acid (20 carbons), a precursor of prostaglandins, leukotrienes, thromboxane and lipoxins, which in turn act as mediators of platelet function and of inflammatory, vascular, motor and sensory processes, among others (Hatanaka and Curi, 2007; Ortonne and Clévy, 1994). It has been observed that linoleic acid is capable of inhibiting the growth of Staphylococcus aureus by inhibiting protein synthesis of cell wall, nucleic acids and cell membrane during division. Linoleic acid has also been shown to participate in cell proliferation and inflammatory process, where in the latter it plays a role as a mediator of leukocyte function having chemotactic and stimulatory effects on neutrophils (Moch et al., 1990).

In present study, number of leukocytes and other parameters that show the repair development such as congestion, neovascularization, leucocytes (neutrophils, lymphocytes and etc), fibroblast level, stratum corneum,
epithelium thickness, collagen mass, collagen maturation, granulation tissue level and fibrin level.

Grading was according following criteria: For example in study region, if lymphocytes are more than 90% of total counted cells, the grade is four, between 60 to 90% the grade is three, between 40 to 60% the grade is two, between 10 to 40% the grade is one and at last less than 10% or none, the grade is zero.

MATERIALS AND METHODS

Plant material and preparation of the extract

The borage found in Iran is *Echium amoenum*, which is different from the borage grown in Europe, *Borago officinalis* (Boraginaceae). Dried borage flowers were collected from Hamedan province, in northwestern Iran in mid August. Cold aqueous extract (pH 5.8) of dried *E. amoenum* flowers (5%, w/v) was used in all the experiments. Dried flowers (15 g) were steeped for 6 h at 4°C in 300 ml distilled water, with constant stirring. The material was centrifuged and the supernatant was filter-sterilized and then freeze-dried.

Experimental animals

Male Wistar rats (150 to 200 g) of 2 to 3 months were used as experimental animals. The animals were housed in standard environmental conditions of temperature (22 ± 3°C), humidity (60 ± 5%), and a 12 h light/dark cycle. During experimental time, rats were given standard pellet diet (Pastor Institute, Iran) and water *ad libitum*.

Surgical procedures

After anaesthesia induction with xylazine 2% and ketamine 10% (I.M. 60 mg/kg) rats were fixed in ventral posture on surgery table. Then the dorsal area from scapula to ilium were scrubbed and prepared to surgery. Two circle shapes, full thickness surgical wounds with 7 mm diameters in both side of the backbone, 1 cm away from backbone and 5 cm away from each other were made with biopsy punch with 7 mm. With this excisional wounding method, epidermis, dermis, hypodermis and Panniculus Carnosus layers were removed completely (Luisa and DiPietro, 2003).

Treatment

After made of surgical wound, all rats randomly were colored with none toxic color and divided to three groups. In group 1, ointment with 1.5% borage extract was administered. Group 2 as control did not receive any administration and group 3 as placebo were administrated with eucerin and Vaseline. All rats were followed 21 days. Daily observation was performed and any wound fluid or any evidence of infection or other abnormalities were noted.

Histopathological study

The healing tissues samples obtained during days 3, 7, 14 and 21, from all four groups of animals and were processed for histological study. The samples fixed in formalin and installed on slides, stained with Hematoxylin and Eosin and were reviewed under light microscope. Recorded factors were eschar, inflammatory cells, kind of inflammatory cells, angiogenesis, fibroplasia, epithelial growth, hyperemia, collagen density fibroblast, fibrin, thickness of corneal layer and Fibroblastic aggregation.

Statistical analysis

The relative wound area was statistically analyzed using one-way ANOVA by the program SPSS 16 and comparison of the means of the wound areas at different days evaluated by Turkey test at $P < 0.05$ level.

RESULTS

Histopathological results

The extract of borage showed significant wound healing activity when topically administered in rats. The wound area measurement showed the wound size of the test groups were reduced early as compared with control group. The best histopathological evaluation were obtained from borage in comparison with other groups as well as compared with control and the placebo (Table 1).

3rd day of 1.5% borage ointments

After evaluation of samples with 1.5% ointment administration, it is observed that eschar formation was low but volume of inflammatory cells in tissue section was high. Neutrophil was the main of these cells. The angiogenesis was mild and the primary stage of fibroplasia was clear. There was no growth in epithelial cells region. Also, there was a low amount of tissue collagen and fibroblastic concentration. But there was a high volume of fibrin in area (Table 1).

7th day of 1.5% borage ointments

In day 7 eschar formation was low but there is medium amount of inflammatory cells in tissue section. The most of these cells were lymphocytes. The angiogenesis and fibroplasia was low. Also, there was a mild epithelial cell growth. Fibrin level was low but collagen level and fibroblastic concentration was medium. The thickness if corneum layer and fibroblastic maturation was not the same as day 3 (Table 1).

14th day of 1.5% borage ointments

In day 14, there was no eschar and levels of inflammatory cells were reduced. The majority of these cells were lymphocytes and angiogenesis is low. But fibroplasia and epithelial growth was increased. Collagen levels were low and fibroblastic concentration was increased. There was little amount of fibrin. The thickness
The true benefit of gamma-3 fatty acids, therefore, may be in their immune modulation of the host rather than in improved wound healing per se. Studies of gamma-3 fatty acids have shown improved survival and reduced infectious complications after the administration of a diet rich in gamma-3 fatty acids (Gottschlich et al., 1990; Alexander et al., 1986).

Up to the 3rd day, subjective observation indicated slow retraction of the wound in the Control group, with hyperemia and edema. Fibrin formation of scab on the edges of the lesion were observed. Hyperemia, edema, and fibrin were seen in the placebo group.

Table 1. Effect of topical administration of borage oil extract on wound healing during days 3, 7, 14 and 21.

<table>
<thead>
<tr>
<th>Studied factors groups</th>
<th>Eschar</th>
<th>Inflammatory cells infiltration</th>
<th>Kind of inflammatory cells</th>
<th>Angiogenesis</th>
<th>Fibroplasia</th>
<th>Epithelial growth</th>
<th>Hyperemia</th>
<th>Collagen density</th>
<th>Fibroblast</th>
<th>Fibrin</th>
<th>Thickness of corneal layer</th>
<th>Fibroblastic aggregation</th>
</tr>
</thead>
<tbody>
<tr>
<td>3rd day 1.5%</td>
<td>1+</td>
<td>3+</td>
<td>Neutrophil</td>
<td>2+</td>
<td>+1</td>
<td>1+</td>
<td>1+</td>
<td>1+</td>
<td>1+</td>
<td>3+</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>7th day 1.5%</td>
<td>1+</td>
<td>2+</td>
<td>Lymphocyte</td>
<td>1+</td>
<td>2+</td>
<td>1+</td>
<td>1+</td>
<td>2+</td>
<td>2+</td>
<td>1+</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>14th day 1.5%</td>
<td>0</td>
<td>1+</td>
<td>Lymphocyte</td>
<td>+1</td>
<td>3+</td>
<td>3+</td>
<td>1+</td>
<td>2+</td>
<td>3+</td>
<td>0</td>
<td>1+</td>
<td>1+</td>
</tr>
<tr>
<td>21st day 1.5%</td>
<td>0</td>
<td>1+</td>
<td>Lymphocyte</td>
<td>0</td>
<td>3+</td>
<td>3+</td>
<td>0</td>
<td>3+</td>
<td>3+</td>
<td>0</td>
<td>2+</td>
<td>2+</td>
</tr>
<tr>
<td>3rd day control group</td>
<td>2+</td>
<td>1+</td>
<td>Neutrophil</td>
<td>+2</td>
<td>1+</td>
<td>0</td>
<td>1+</td>
<td>0</td>
<td>1+</td>
<td>3+</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>7th day control group</td>
<td>2+</td>
<td>3+</td>
<td>Neutrophil</td>
<td>+2</td>
<td>2+</td>
<td>1+</td>
<td>1+</td>
<td>2+</td>
<td>1+</td>
<td>3+</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>14th day control group</td>
<td>0</td>
<td>1+</td>
<td>Lymphocyte</td>
<td>+1</td>
<td>2+</td>
<td>2+</td>
<td>0</td>
<td>3+</td>
<td>1+</td>
<td>0</td>
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<td>1+</td>
</tr>
<tr>
<td>21st day control group</td>
<td>0</td>
<td>1+</td>
<td>Lymphocyte</td>
<td>+1</td>
<td>2+</td>
<td>2+</td>
<td>0</td>
<td>3+</td>
<td>1+</td>
<td>0</td>
<td>2+</td>
<td>2+</td>
</tr>
<tr>
<td>3rd day Placebo group</td>
<td>2+</td>
<td>3+</td>
<td>Neutrophil</td>
<td>+2</td>
<td>1+</td>
<td>0</td>
<td>2+</td>
<td>1+</td>
<td>1+</td>
<td>2+</td>
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<td>0</td>
</tr>
<tr>
<td>7th day Placebo group</td>
<td>3+</td>
<td>3+</td>
<td>Lymphocyte</td>
<td>+3</td>
<td>1+</td>
<td>1+</td>
<td>2+</td>
<td>1+</td>
<td>2+</td>
<td>2+</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>14th day Placebo group</td>
<td>0</td>
<td>2+</td>
<td>Lymphocyte</td>
<td>+2</td>
<td>2+</td>
<td>2+</td>
<td>1+</td>
<td>2+</td>
<td>2+</td>
<td>0</td>
<td>1+</td>
<td>1+</td>
</tr>
<tr>
<td>21st day Placebo group</td>
<td>0</td>
<td>1+</td>
<td>Lymphocyte</td>
<td>+1</td>
<td>2+</td>
<td>3+</td>
<td>0</td>
<td>3+</td>
<td>2+</td>
<td>0</td>
<td>1+</td>
<td>2+</td>
</tr>
</tbody>
</table>

Increased if corneum layer and fibroblastic maturation was increased (Table 1).

21st day of 1.5% borage ointments

In day 21, eschar was same with scar in day 14, but levels of inflammatory cells were reduced. The majority of these cells were lymphocytes. Because of wound constriction, there is no angiogenesis and fibroblastic concentration. Fibrin level was very low. The thickness corneum layer and fibroblastic maturation was medium but overall it was better than control group (Table 1).

DISCUSSION

In this study, the process of tissue repair of experimentally induced cutaneous wounds in rats was evaluated in vivo based on the histopathological over time. Wound healing involves a complex and coordinated number of events which include inflammation, cell proliferation, and contraction of the wound and tissue remodeling. Thus, in this study, the effect of borage oil was analyzed, applied topically, on rat skin wound considering these results comparing to findings from earlier studies that demonstrated the efficacy of these agents in accelerating wound healing.

Borage oil extract contains therapeutics that therapeutic effects are referred to mentioned components. Research has sought to define benefits to wound healing of specific lipid types. The gamma-3 fatty acids, which exhibit anti-inflammatory properties by inhibiting the production of eicosanoids and other mediators, such as platelet-activating factor, interleukin-1, and tumor necrosis factor alpha (Albina et al., 1993; Endres et al., 1989; Simopoulos, 1991; Kremer et al., 1987) are among the most widely investigated.

The true benefit of gamma-3 fatty acids, therefore, may be in their immune modulation of the host rather than in improved wound healing per se. Studies of healing burns in humans and guinea pigs, however, have demonstrated improved immune function, improved survival, and reduced infectious complications after the administration of a diet rich in gamma-3 fatty acids to this specific subset of injured patients (Gottschlich et al., 1990; Alexander et al., 1986). Up to the 3rd day, subjective observation indicated slow retraction of the wound in the Control group, with hyperemia and scab formation. In the Test group, hyperemia and discrete formation of scab on the edges of the lesion were observed. Hyperemia, edema and fibrin were seen in the placebo group.
polyunsaturated fatty acids on the healing of cutaneous wounds were evaluated, a macroscopic difference in lesion repair was demonstrated in the first 48 h after the surgical procedure (Cardoso et al., 2004). The greater infiltration of polymorphonuclear cells and macrophages in the first three days can correspond to the phase of exudation and inflammation in the wounds treated with triglycerides as demonstrated in a study conducted in dogs (De Nardi et al., 2004).

In the same period in the present study, there were no signs of exudates by macroscopic observation. From the 3rd to the 7th day, there was progressive repair of the wound area, which indicated development of granulation tissue in concordance with findings in the literature (Mandelbaum et al., 2003). The macroscopic appearance showed the wound area with the presence of fibrin and scab in the Reference group, complete scab formation in the Control group, and discrete hyperemia in the Test group.

From the 7th to 14th day, a greater rate of repair occurred in the Reference group. The test preparation showed evident granulation tissue and greater tissue contraction around the edges of the wound, which had become irregular. In the Control group, gaps in the scab were seen on the 7th day, along with irregular edges due to contraction of the wound, besides the presence of granulation tissue. The Reference group remained with the largest wound area and with irregular edges, which was also found in a study using linoleic acid (Marques et al., 2004). The treatments in the three groups contributed to the almost complete closing of the lesions, suggesting that growth factors were probably responsible for the hyperplasia of the epithelium as reported in the literature (Mandelbaum et al., 2003).

In this period, there was agreement with a study that considered cytokines as being important mediators of neoangiogenesis, fibroplasia and maturation, which are released by cells such as platelets, neutrophils, macrophages, lymphocytes, mast cells and fibroblasts, making it easy to understand the importance of chemotactic properties of the test preparation in the repair of the lesions (De Nardi et al., 2004).

A study that examined wound healing in sheep showed that linoleic acid constituted a powerful pro-inflammatory mediator, being essential for the regulation of the biochemical events that precede fibroplasia in addition to stimulating growth factors and neovascularization15. It is possible that in this study, the presence of linoleic acid in the test preparation contributed to a similar event. A study carried out with polyunsaturated fatty acids showed a tendency for the wound area to diminish in the first ten days of treatment, and demonstrated overall that PUFAs may play an important role in wound healing (Cardoso et al., 2004).

On the 14th day, clinical observation showed a smaller wound area characterized by the proximity of the edges (contraction) with irregular outlines and better presence of granulation tissue, like that seen in the study with linoleic acid in sheep (Marques et al., 2004). Proliferation (fibroplasia and matrix formation) was demonstrated by scholars in the past as being extremely important in the formation granulation tissue. This depends on fibroblasts which produce elastin, fibronectin, glucosaminoglycans and proteases (Mandelbaum et al., 2003). The repair of wounds by secondary union showed that contraction could have been responsible for the reduction in wound area in the three days of healing in the Control group, in concordance with a study in which contraction was shown to be able to reduce the surface of the cutaneous defect.

In conclusion, present study demonstrated that borage oil extract was capable of promoting wound healing activity. Due to pharmacological evidence and histological observations borage oil extract accelerate wound healing process.

REFERENCES


