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Research Article

COMPARISION EFFECT OF CHAMOMILE (*CHAMOMILLA RECUTITA*) HYDROETHANOLIC EXTRACT AND FLAXSEED OIL (*LINUM USTATISSUM*) ALONE AND SIMULTANEOUS ADMINISTRATION WITH NITROFURAZONE IN WOUND HEALING PROCESS

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ABSTRACT
Present study was conducted in order to compare the provoking impact of *Chamomilla recutita* hydroethanolic extract (CRE) and flaxseed oil (FLO) on wound healing processes with nitrofurazone ointment (NFZ). For this purpose adult Wistar rats were divided into control-sham, 2% CRE-treated, 2% FLO-treated, CRE+FLO-treated, 0.2% NFZ-treated. Following 3, 7, 14 and 21 days from experimentally-induced wound model, the tissue samples were collected. Wound contraction ratio, poly-morphonuclear (PMNI), mono-nuclear immune cells (MNI), fibroblasts and vascular distribution per one mm^2 of the tissue were evaluated. Observations demonstrated that co-administrating of CRE and FLO reduced the time necessary for inflammatory phase. Accordingly, the animals in CRE+FLO-treated group exhibited significantly ($P<0.05$) lower immune cells (PMNI and IMC) versus NFZ and control-sham groups after 7 days. CRE and FLO alone and simultaneous administration resulted in remarkable ($P<0.05$) increase in fibroblasts distribution as well as neovascularization. In conclusion, our data showed that CRE and FLO by shortening the inflammatory stage up-regulated the fibroblasts and vascular proliferation that ultimately in turn accelerated the healing process.

Keywords: *Chamomile, Flaxeed Oil, Nitrofurazone, Wound Healing, Rabbit*

INTRODUCTION
Wound healing is a complex physiological process that requires a series of overlapping phases, each with several factors to come to completion. It is divided into four main continuous stages including; hemostasis, inflammation, cell proliferation and contraction of the collagen lattice formed (Bodeker *et al.*, 1996). Indeed, the tissue injury causes an inflammation leading to homeostasis and clot formation; after that the fibroplasia and neo-vascularization, formation of granulation tissue, re-epithelization and finally the formation of new extracellular matrix and tissue remodeling could occur (Bodeker *et al.*, 1996; Kujath *et al.*, 2008; Houlton and Hom, 2013).
There is an increased interest in administrating plants in medicine for wound healing (Suguna *et al.*, 1993; Oommen *et al.*, 1999; Houghton *et al.*, 2005). Several natural and plant products have shown promoting effects in one or more stages of the wound-healing process, especially contributing to disinfection, debridement, and providing a moist environment to encourage the establishment of an effective healing process. This properties are related to some components like anti-inflammatory, antioxidants and moisturing agents such as; tannins, saponins, flavonoids, napthaquinone (Houghton *et al.*, 2005; Padmaja *et al.*, 1994; Patzelt-Wenczler *et al.*, 2000).
The chamomile (CRE) plant, *Chamomilla recutita* L. Rauschert (synonymous with *Matricaria recutita* L. Rauschert, and *Matricari achamomilla*), belongs to the Asteraceae family. Different groups of effective substances, including; chamazulene, alpha bisabolol, bisabolol oxides, spiroethers, and flavonoids (Mann and Staba, 1986; Domingues *et al.*, 2009) are responsible for CRE-induced therapeutic impacts. Based on these chemicals and agents the CRE is used to treat inflammatory disorders, fever, diarrhoea, menstrual pain, and intestinal and hepatic tumors (Mann and Staba, 1986). Moreover, the CM is also used as an active principle increams administrated for atopic eczema (Patzelt-Wenczler *et al.*, 2000).

Flaxseed (*Linumu sitatissimum*) is known as oldest cultivated plant and is cultivated for its fiber and oil. Flaxseed and its derivatives, flaxseed oil and inseed oil, are rich source of the essential fatty acid and alphalinolenic acid, which are biological precursor to omega-3, fatty acids, such as eicosapentaenoic. Moreover, the flaxseed (FLO) is useful for treating *Vata* disorders including dryness, undernourishment, lack of luster/glow (Patzelt-Wenczler *et al.*, 2000; Joshi *et al.*, 2006).

Topical antibiotics have been used in wound care for many years. A number of reports have indicated the effectiveness of topical antibiotics for prevention of infection in surgical and traumatic wounds (Misra, 1963; Quie *et al.*, 1960). Nitrofurazone (NFZ) as a synthetic chemical is used to treat burns that have become infected. It is also used to treat skin infections due to skin grafts. It acts by eliminating bacteria and/or preventing their growth (Iselin *et al.*, 1990; Saydam *et al.*, 2006). However, like other antibiotics the resistance problems for NFZ resulted in severe risks and effectiveness problems. The rationale for searching an alternative medicine to NFZ was based on the fact that, regardless of the route of administration, significant resistance and some side effects are considered for this drug. In order to reduce the resistance problems as well as side effects, the plant products are potential agents for wound healing of different types because of their widespread availability, non-toxicity, ease of administration, absence of unwanted side effects and their effectiveness as crude preparations. Therefore, the present study was conducted in order to evaluate the promoting impacts of NFZ, CRE and FLO on wound healing process. Moreover, the impacts of these compounds on different stages of wound healing were compared with each other.

MATERIALS AND METHODS

Experimental Animals

Eighty healthy white New Zealand rabbits (2100 g, 5-week-old and mixed sex) were used in this study. Two weeks prior to the study, animals were housed in individual cages 45×40×50 cm with at standard temperature condition (23 ± 3ºC), stable air humidity, and a natural day/night cycle. In order to acclimatize them to experimental procedure, rabbits were handled and mock daily during the 14 days before the experiment,. Food and water were given *ad libitum* Animal handling and experimental procedures were performed according to the Guide for the Care and Use of Laboratory animals by the National Institutes of Health (USA) and the current laws of the Iranian government. All protocols for animal experiment were approved by the institutional animal ethical committee in Islamic Azad University, Urmia Branch, West Azarbayjan, Iran.

Plant Material and Extract Preparation

Chamomilla (*Chamomilla recutita*) and flaxseed oil (*Linum usitatissimum seed oil*) were collected from the central region of the Urmia, West Azerbaijan province, Iran in July 2013. Chamomilla and flaxseed species charecteristics were identified in department of Botany Sciences, Agriculture and Natural Resources Research Center, Urmia, Iran. The flaxseed seed oil was extracted by cold pressure, blended with eucerin-vaselin to prepare treatment ointments with doses (0.75 and 1.5%). Chamomilla flower separated by hand, then 600 g of flower dried in shadow on laboratory benches at room temperature (23-24ºC) for six days. Finally, the flower was grinded using an electric blender. A 150 g of the powder suspended in hydroethanolic solution (600 ml) for 96 h at room temperature. The mixture filtered using a fine muslin cloth followed by filter paper (Whatman No. 1). The filtrate placed in oven to dry at 40 ºC. The clear residue obtained were kept at -20 °C until used (Abad *et al.*, 2011).

Topical Wound Remedy Prepare Procedure

In this study 4 various topical ointments were used. All medication consisted base on formulation of comprising: Eucerin and Vaseline in 1:3 ratio (as base formula), respectively. Eighty rabbits were randomly labeled by none toxic color and divided into five experimental groups (n=16 in each). A surgical wound was created to all rabbits. Group 1 animal served as control and receives no administration. Group 2, rabbits received Nitrofurazone ointment 0.2% (NFZ). In group 3, animal treated with *Chamomilla recutita* ointment 2% (CRE). In group 4, rabbits received flaxseed oil 2% (FLO); and finally, combination of flaxseed oil 2% + *Chamomilla recutita* hydroethanolic extract 2% (CRE+ FLO) of

was administrated in 5 group (Hemmati *et al.*, 2002). Experimental ointments were prescribed once a day on the wound area until the wound healed completely. All rabbits were monitored for subsequent fluid accumulation, evidence of infection or other abnormalities in wound area, until complete epithelialization (Trivellato, 2013).

Wound Induction

Animals anesthetized by intraperitoneal (i.p) administration of ketamine 5%, 90mg/kg (Ketaset, Alfasan, Woerden, Netherland) and xylazine hydrochloride 2%, 5mg/kg (Rompun, Bayer, Leverkusen, Germany). The fur was excoriated aseptically and the predetermined area was marked on the back of animals. Each rabbit fixed on the surgery table in ventral posture.

The coordination of experimental wounds (2×2cm dimensions) outlined through clean transparency sheet template and permanent marker on the dorsolumbar region of the rabbits. A square full thickness skin wound including subcutaneous tissue was made using a #11 BP blade on each side of the midline in rabbit.

The wounds were created 2 cm far from the midline at the same location on the trunk. The hemorrhage was controlled by sterile cotton gauze.

The wound area was measured immediately by placing of a transparent paper over the wound and tracing it out; the area of this impression was calculated using the graph sheet. All experimental procedures were performed during 9:00 A.M to 1:00 P.M.

Wound contraction percentage and wound closure times were monitored on 4, 8, 12, 16 and 20 days to assess wound-healing property. The wound healing percentage was calculated by the Walker formula after measuring the wound size (Walker *et al.*, 2008).

Percentage of wound size =Wound area on day X / Wound area on day 0 × 100.

Percentage of wound healing = 100 - Percentage of wound size.

Histopathological Analysis

Animals were anesthetized (as same as mentioned above) and specimens of skin were taken on 3, 7, 14 and 21 days postoperative.

Tissue sample, were dissected out along with 1-2 mm from surrounding normal skin and in an approximately depth of 3mm, pinned on a flat cork surface and fixed in neutral-buffered formalin (10%).

Samples routinely were processed embedded in paraffin wax, sectioned at 5 µm and finally stained with Masson's trichrome and were examined under light microscopy (Olympus CX31RBSF attached cameraman) to assess the predominant stage of wound healing. Three parallel sections were obtained from each tissue specimen.

Quantity of cellular infiltration Polymorph nuclear (PMN), Mononuclear cells (MNC), fibroblasts (Fib) and angiogenesis (number of blood vessels and capillary buds or NV) were evaluated per one mm^2 of each tissue section in ×400 magnification.

Epithelialization (epithelium thickness), collagen production and density were determined and calculated manually. All parameters were analyzed in 5 per high power fields (HPFs).

Statistical Analysis

Data analyzed by two-way analysis of variance (ANOVA) using PASW 18.0 (SPSS, Inc., Chicago, IL, USA), and is presented as mean ± SD.

Model assumptions were evaluated by examining the residual plot. For treatment showing a main effect by ANOVA, means have compared by Dunnett's test. $P<0.05$ was considered as significant differences between treatments.

RESULTS AND DISCUSSION

Table 1: Effects of Flaxseed oil and *Chamomilla* extract compared to Nitrofurazune on subsequent wound healing in Rabbit.

Groups	Wound area (mm^2) ± S.D. 4	8	12	16	20
Control	441.4 ± 29.7(-10.35%) [a]	311.6 ± 52.55(22.1%)[a]	138.6 ±13.88(65.35%) [a]	80.2 ±10.63(79.95%) [a]	14.8 ± 3.27(96.3%) [a]
NFZ	356.6 ± 20.68 (10.85%)[b]	137.6 ± 14.92(65.6%)[c]	78.8 ± 22.01(80.3%) [c]	22 ± 8.09(94.5%) [b]	2 ± 1.58(99.5%) [b]
CRE	416.4 ± 22.45 (-4.1%) [a]	287 ± 32.2 (43.25%) [b]	106 ± 8.68(73.5%) [b]	74 ± 10.58(81.5%) [a]	12.4 ± 2.19(96.9%) [a]
FLO	345.6 ± 35.16 (13.6%) [b]	140.4 ± 59.03(64.9%)[c]	76.2 ± 20.81(80.95%) [c]	12 ± 6.04(97%) [b]	0.2 ± 0.44(99.95%) [b]
CRE+ FLO	337 ± 16.18 (15.75%) [b]	123 ± 66.13(69.25%)[c]	50 ± 8.86(87.5%) [d]	19 ± 3.6(95.25%) [b]	1.2 ± 1.64(99.7%) [b]

Data are presented as the mean ± SD. There are significant differences between groups with different codes in a column (superscript letters a, b, c and d; $P \leq 0.05$).

Table 2: Effects of Flaxseed oil and Chamomile extract compared to nitrofurazune on on polymorphonuclear and mononuclear cells, new vessls and fibroblasts formation on subsequent wound healing in Rabbit

Days	Groups	Mean Distribution PMNI	MNI	NV	Fib
	Control	53.60 ± 7.73[a]	1.00 ± 1.22[a]	8.00 ± 2.00[a]	29.20 ± 2.68[a]
	NFZ	20.60 ± 4.50[c]	0.40 ± 0.54[a]	6.20 ± 1.48[a]	40.40 ± 9.60[a]
Day 3	**CRE**	40.40 ± 9.65[a]	0.00 ± 0.00[a]	9.00 ± 2.73[a]	43.80 ± 8.49[a]
	FLO	26.40 ± 9.98[c]	0.40 ± 0.54[a]	6.00 ± 2.12[a]	16.40 ± 4.21[b]
	CRE+ FLO	31.80±12.43[b]	0.40±0.54[a]	14.60±1.51[b]	33.80±6.41[a]
	Control	28.40 ± 2.40[a]	1.00 ± 1.2[a]	7.40 ± 1.14[a]	43.00 ± 6.74[a]
	NFZ	8.00 ± 2.54[c]	0.60 ± 0.54[a]	9.00 ± 2.23[a]	59.60 ± 8.44[b]
Day 7	**CRE**	17.40 ± 3.36[b]	0.40±0.54[a]	7.60 ± 0.89[a]	48.80 ± 8.61[a]
	FLO	9.40 ± 6.26[c]	1.60 ± 1.14[a]	6.60 ± 1.81[a]	56.20 ± 8.89[a]
	CRE+ FLO	10.20±3.96[c]	2.80±0.83[b]	9.80±1.78[a]	38.60±6.61[a,c]
	Control	14.40 ± 1.14[a]	0.80 ± 1.30[a]	3.60 ± 1.14[a]	63.00 ± 8.48[a]
	NFZ	3.20 ± 2.58[b]	2.00 ± 1.00[a]	13.80 ± 2.28[b]	61.80 ± 13.04[a]
Day 14	**CRE**	2.00 ± 1.58[b]	0.90±0.54[a]	4.80 ± 1.30[a]	55.80 ± 7.56[a]
	FLO	5.20 ± 1.92[b]	1.60 ± 1.14[a]	5.60 ± 1.14[a]	80.80 ± 22.81[a]
	CRE+ FLO	5.20±2.16[b]	2.20±1.30[a]	7.20±1.30[c]	57.00±10.09[a]
	Control	2.60 ± 1.81[a]	0.80 ± 0.83[a]	2.00 ± 1.00[a]	52.40 ± 5.59[a]
	NFZ	1.00 ± 1.22[b]	0.40 ± 0.54[a]	5.40 ± 1.14[b]	51.80 ± 6.53[a]
Day 21	**CRE**	0.00 ± 0.00[b]	0.20 ± 0.44[a]	2.00 ± 0.70[a]	32.20 ± 5.49[b]
	FLO	0.00 ± 0.00[b]	2.00 ± 1.00[a,b]	2.80 ± 0.83[a]	102.20 ± 11.38[c]
	CRE+ FLO	0.60±0.89[b]	0.30±0.00[a]	5.80±1.30[b]	140.20±15.35[d]

PMNI: polymorphonuclear cells, MNI: mononuclear cells, NV: new vessels, Fib: fibroblasts. There are significant differences between groups with different codes in a column (superscript letters a, b, c and d; $p \leq 0.05$). All data are presented in Mean±SD.

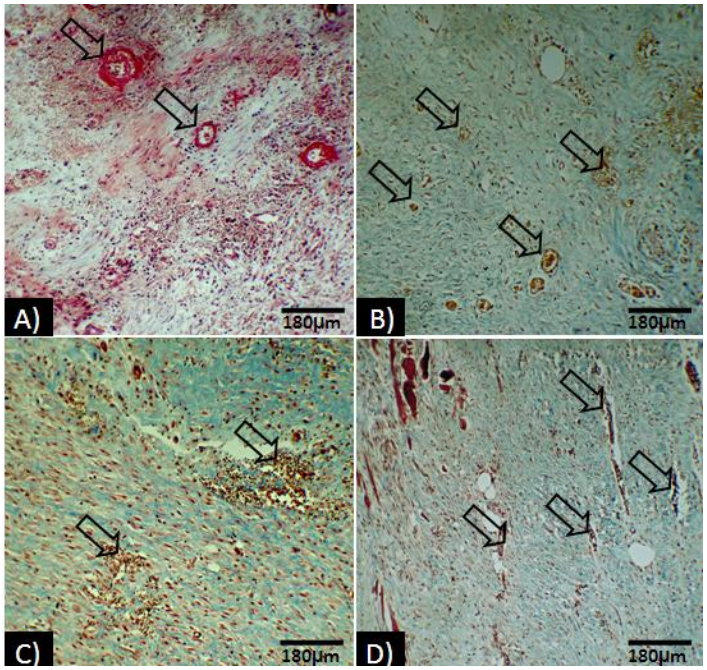

Figure 1: Cross section from dermis on day 7 after wound induction: A) Control-sham, (B) Chamomilla-treated, (C) Nitrofurazone-treated and (D) Chamomilla+flaxseed-treated. Note increased angiogenesis in Chamomilla+flaxseed-treated and control-positive group. The Chamomilla+flaxseed significantly increased vascular distribution (*arrows*) in one mm^2 of the injured area. *Masson-trichrome staining, 600*×

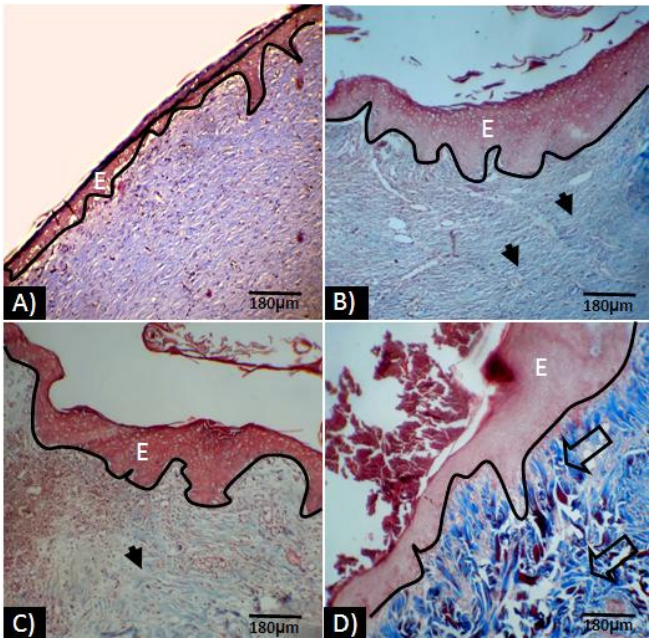

Figure 2: Cross section from re-epithelialization zone: (A) Control-sham, (B) Chamomilla-treated, (C) Nitrofurazone-treated and (D) Chamomilla+flaxseed-treated. See well-formed papillae in epidermis of treated animals. The dermis of Chamomilla+flaxseed-treated group is presented with matured connective tissue and collagen bunds (arrows). Meanwhile the dermis of non-treated control-sham, Chamomilla and nitrofurazone are containing collagen bundles (head arrows). *Masson-trichrome staining (800×)*

Result

Wound Contraction

Effect of flaxseed oil and chamomilla recutita hydroethanolic extract on wound contraction is presented in table 1. According to the data, administration of nitrofurazune, flaxseed and combination flaxseed + chamomilla improved wound healing on all days post injury in rabbit ($P<0.05$). Interestingly, there was no significant effect between nitrofurazune and flaxseed ointment ($P>0.05$) while co-administration of flaxseed + chamomilla had better effects than nitrofurazune or sole flaxseed and chamomilla ointment ($P<0.05$). Obtained results suggest that combination flaxseed + chamomilla oil ointment had prominent effect on wound healing.

Histological Findings

Immune Cells Infiltration

Comparing the groups for ploy-morphonuclear (PMNI) and mon-nuclear immune (MNI) cells showed that, CRE and FLO in alone and simultaneous forms of administrations up-regulated the PMNI cells infiltration on day 3 and exerted no impact on MNI cells infiltration. The CRE significantly ($P<0.05$) reduced the PMNI and MNI cells infiltration on days 7 and 14 after wound induction. The animals in CRE alone-treated and CRE+FLO-received exhibited the lowest PMNI and MNI cells distribution per one mm2 of the wound area on day 21. The data for histopathological analyses are presented in table 2.

Angiogenesis

In order to evaluate the angiogenesis ration in different groups, the vascular distribution per one mm^2 of the wound area was analyzed (Table 2). Observations demonstrated that, 3 and 7 days after wound induction the highest vascularization was manifested in CRE+FLO-treated animals (Figure 1). However, it changed on day 14 and the NFZ-treated animals showed higher vascular distribution versus other groups. Comparing the vessels number per one mm^2 of tissue on day 21 showed that there were no significant differences between animals in NFZ alone-treated and CRE+FLO-treated groups.

Fibroblasts Distribution and Collagen Deposition

Light microscopic analyses showed that administrating the CRE alone resulted in high fibroblasts proliferation on day 3 after wound induction versus the animals in FLO alone and FLO+CRE-treated groups (Table 2). However, the animals in NFZ-treated group exhibited high fibroblast infiltration compared to control-sham, CRE alone and CRE+FLO-treated groups. Meanwhile, comparing the fibroblasts number per one mm^2 of the connective tissue on days 7 and 14 after wound induction showed that, the FLO exerted better results. Accordingly, the animals in FLO alone-treated group exhibited significantly ($P<0.05$) higher fibroblast distribution. Interestingly, on day 21 post-surgery, the animals in CRE+FDX-treated group showed remarkably ($P<0.05$) higher fibroblast distribution versus those in other treated and control-sham groups. Moreover, the result show that increase amount of collagen synthesis and deposition in NFZ-treated and CRE+FDX-treated group, compared to other groups (Figure 2).

Discussion

In present study we showed that CRE and FLO in alone and simultaneous forms of administration significantly reduced the PMNI and MNI cells infiltration in different stages of healing process. Accordingly, the animals in CRE alone treated showed remarkably lower immune cells infiltration on days 3, 7 and 14 after wound induction versus the NFZ-treated ones. Comparing the angiogenesis ratio by estimating the vascular distribution in one mm2 of the tissue showed that combination of CRE and FLO up-regulated the angiogenesis on days 3 and 7.

While the situation reversed on day 14 after wound induction and the animals in NFZ-treated group showed the highest vasculature. Finally, on day 21 post operation the animals in CRE+FLO-treated and NFZ-treated showed the same phenotype in angiogenesis. As an important marker for proliferative stage of healing process, we analyzed the fibroblasts proliferation and distribution in one mm^2 of the tissue. Observations showed that, alone administration of CRE on day 3 and FLO on days 7 and 14 up-regulated the fibroblasts proliferation indicating their rapid reaction through healing process. However, after 21 days the animals in CRE+FLO-treated group exerted better results. Accordingly, these animals exhibited the fibroblast numbers per one mm^2 of the tissue.

Wound healing generally requires support at three levels. First, improving general resistance, stimulating the repairand improves tensile strength or elasticity of the skin (Gutteridge *et al.*, 1993; Patwardhan 2000; Diwanay *et al.*, 2004). Beside these facts the inflammation stage is considered as a main step in order to eliminate the cellular debris from tissue as well as extensive response for microbial infection (Gutteridgde *et al.*, 1995; Gosain and Di-Pietro, 2004; Campos *et al.*, 2008). Rapid PMNI and MNI cells proliferation and infiltrationare considered as an important reaction in healing. Our histopathological observations showed that administrating of the CRE and FLO resulted in rapid immune cells infiltration on days 3 and 7 after wound induction. However, the accelerated shut downing of the inflammatory stage and fast starting of the proliferative phase are reported as an essential features for early wound healing (Patwardhan 2000; Meszaros *et al.*, 2000). In this regard, our light microscopic analyses showed that although the NFZ reduced immune cells (PMNI and MNI) on days 14 and 21, the animals in CRE alone and CRE+FLO-treated groups exhibited more significant decrease in immune cells infiltration accomplished with intensive fibroblasts proliferation. In fact, following inflammatory phase the macrophages (as main interactive immune cells), undergo a phenotypic transition that in turn promotes the fibroblasts proliferation and provokes the angiogenesis (Diwanay *et al.*, 2004; Meszaros *et al.*, 2000). On the other hand, during the proliferative phase, the fibroblasts and the endothelial cells are the most prominent cells that are involved in collagen synthesis, growth of the vascular system and ultimately well formation of the granulation tissue (Meszaros *et al.*, 2000; Gosain and Di-Pietro, 2004; Campos *et al.*, 2008). Considering the CRE and FLO-increased angiogenesis and fibroblast proliferation we can come close to this fact that CRE and FLO promoted the healing process by accelerating the proliferative phase, which in turn shortened the healing time.

Histopathological analyses showed that the CRE up-regulated the fibroblast distribution on day 3 after wound induction while the after 7 and 14 days the highest fibroblast distribution was revealed in FLO-treated group. Ultimately, administrating of the CRE+FLO resulted in significantly higher fibroblast distribution on day 21 after wound induction. Thus, we can conclude that each plant exerts different phenotypes depending on time. At first stages the CRE and in proliferative stage the FLO and in the maturation phase the combination of these two plants exerts better impacts about fibroblasts proliferation. On the other hand, comparing the vascular distribution on different days between different groups showed that, on day 3 after wound induction the CRE+FLO-treated animals exhibited better results for angiogenesis (higher neovascularization). However, the NFZ exerted higher vascular distribution after 7, 14 and 21 days. Thus, here we can suggest that NFZ promotes the angiogenesis at later days while administrating of the CRE and FLO exerts the same feature at earlier period. Considering better results for fibroblasts and taking together, CRE and FLO co-treatment shortened the inflammatory stage and promoted the proliferative phase.

Conclusion

Our data showed that, co-administrating the CRE and FLO shortened the inflammatory phase and enhanced the proliferative stage. Therefore, the CRE and FLO could be considered as appropriate alternative chemicals for NFZ.

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