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Research Article

HISTOPATHOLOGICAL EVALUATION OF THYMUS VULGARIS ON WOUND HEALING

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ABSTRACT
The main purpose of current study was to evaluate effect of Thymus vulgaris essential oil ointment in wound healing. Forty eight male Wistar rats were randomly divided into 3 experimental groups. Experimental wound was induced on the anterior-dorsal side of each rat. Group 1 served as control and received basal formulation ointment. Groups 2 and 3 received 1.5 and 3 % of Thymus vulgaris essential oil. Wound healing activity as a closure percentage was determined on days 4, 12 and 21 post-operation. The number of fibroblast and macrophages, collagen deposition and neovascularization were assessed on 3, 7, 14 and 21 days after wound incision. wound area significantly decreased in Thymus vulgaris ointment treated groups compared to control group on days 4, 12 and 21 post operation, respectively ($P<0.05$). Administrating of Thymus vulgaris ointment, significantly ($P<0.05$) increased fibroblast and macrophages distribution and up-regulated the new vessels formation and collagen deposition on days 3, 7, 14 and 21 after wound induction ($P<0.05$). It is suggested that Thymus vulgaris ointment is useful for healing.

Keywords: *Thymus Vulgaris, Wound, Healing, Rat*

INTRODUCTION
14 species of Thymus L. genus are scattered in Iran (Arzani *et al.*, 2013). Thymus vulgaris is native in Mediterranean countries (Kozics *et al.*, 2013). Essential oil of Thymus vulgaris exerts antimicrobial properties (Bozin *et al.*, 2006). Monoterpenes, phenolic compounds, thymol, eugenol, p-cymene, γ-terpinene, linalool, geraniol and borneol can be extracted from Thymus vulgaris (El Bouzidi *et al.*, 2013). The Thymus vulgaris has antibacterial activity and protects cell against oxidants (Braga *et al.*, 2007; Kozics *et al.*, 2013). Thymus vulgaris is broadly used to treatment asthma, inflammation, hepatotoxicity and atherosclerosis (Stahl-Biskup and Sáez, 2002; Kozics *et al.*, 2013; Chauhan *et al.*, 2014). phenolic and flavonoid compounds in plants are essential in inhibiting of inflammation, collagens synthesis, as well as angiogenesis (Ambiga *et al.*, 2007; Lodhi *et al.*, 2013). One of the problems in wound healing is high risk of infection (Young and McNaught, 2011). To date Thymus vulgaris is used for mastitis, mouth infections and minor researches done on its effect in external wounds (Mohammed and Al-Bayati, 2009).

Considering lower cost, high availability and lower side effects of plants and plant-based chemicals, interests are arising for herbal therapies. properties of different plants, many essential oils and their constituents have been tested for their promoting impact on wound healing. In spite of all the information available in literature, effect of Thymus vulgaris extract on wound healing is not fully studied. Therefore, the aim of current study was to investigate the provoking effects of Thymus vulgaris essential oil ointment on wound healing process. for this purpose the tissue fibroblasts, fibrocytes, macrophages distribution, vessel formation, and collagen and epidermis regeneration in rat model were analyzed.

MATERIAL AND METHODS
Plant Collection and Preparation
Fresh thyme (Thymus vulgaris L.) leaves were collected in Hamadan province, Iran. The plant was identified in department of Botany Sciences, Herb Garden Avicenna, Hamadan, Iran. Plant areal parts (flowers and leaves, 450 g) were dried and cleaned naturally on laboratory benches at room temperature (23-24ºC) for 10 days until they were crisped and powdered by using an electric blender. Then, hydro

distillation via clevenger type apparatus was peformed to obtain essential oil from thyme leaves (Pietrella *et al.*, 2011).

Experimental Animals

To assume wound healing property of Thymus vulgaris ointment, Forty eight male Wistar rats (9 weeks old, 200-210g weight) were used in this study. To adapt rats in new experiment condition, they were kept 5 days prior the study in a standard laboratory condition.

All animals kept in temperature of 22 ±3°C, 60 ± 5%humidity and a 12h light/dark cycle start at 9 A.M. During the study rats had ad libitum chew pellets and fresh water. All experiments were carried out based on the guidelines of the Ethics Committee of the International Association for the Study of pain (Zimmermann, 1983) and current Iranian low for laboratory animal care. All experiments approved by ethic research committee of Tehran Azad University.

Experiential Ointment Preparation

In this study 3 topical ointments were prepared. Control ointment was base formulation comprising Eucerin (25%) and Vaseline (75%).

Two various Thymus vulgaris topical ointments (TVO) were prepared. Ointment 1 was contained 1.5 g of Thymus vulgaris essential oil in base formulation . Ointment 2 was contained 3 g of Thymus vulgaris essential oil in base formulation (Sasidharan *et al.*, 2010).

Skin Irritation Test

The ointment was evaluated for primary skin irritation test on shaved back of the rats for any abnormality of ointment (Laila *et al.*, 2011).

Excision Wound Model

In this study, animals were starved for 12 hours prior to wounding. All animals anesthetized by intraperitoneal (i.p.) injection of ketamine 5%, 90mg/kg (Ketaset 5%; Alfasan, Woerden, Netherlands) and xylazine hydrochloride 2%, 5mg/kg (Rompun 2%, Bayer, Leverkusen, Germany).

The fur was aseptic and predetermined area marked on the back of animals. Rats were fixed on the surgery table in ventral posture. Two circular wounds each about 200 mm^2 were made on the back of each rat (Morton and Malone, 1972).

Thymus vulgaris topical ointments applied for evaluating its impact on wound healing (Shanbhag *et al.*, 2006). Rats were kept individually under hygienic and controlled conditions in polypropylene cages with stainless steel top.

The creams were topically applied once a day, starting 24 hours post-operation, on the wound area until wound completely healed. All rats were monitored for any wound fluid or other abnormalities (Sasidharan *et al.*, 2010).

Wound Healing Activity

Wound contraction percentage and wound closure time were used to assess wound-healing property. A graph paper and permanent marker were used to measure size. The wound size was computed on days 4, 12 and 21 post-operation (Farahpour and Habibi, 2012).

Percent wound contraction was calculated from the days of measurement of wound areas. The wound healing percentage was calculated by the Walker formula (Walker and Mason, 1968).

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

Percentage of wound healing = 100 - Percentage of wound size

Histological Analysis

Specimens from skin were taken on days 3, 7, 14 and 21 after wound infection. Tissue samples were excised and fixed in neutral-buffered formalin 10%. Then the sample tissues were processed routinely.

sections were microscopically evaluated to assess the predominant stage of wound healing include fibroblast, neovascularization, immune cells distribution and macrophages (Sasidharan *et al.*, 2010).

Statistical Analysis

Data analyzed by two-way analysis of variance (ANOVA) using SPSS 22 (SPSS, Inc., Chicago, IL, USA). Result presented as mean ± SEM.

For treatment showing a main effect by ANOVA, means have compared by Dunnett's test. $P<0.05$ were considered as significant differences between treatments.

RESULTS AND DISCUSSION

Result

Wound Contraction

Effect of the Thymus vulgaris essential oil ointment on wound excision area is presented in table 1.

As seen, wound area significantly decreased in TC-treated animals (1.5 and 3% Thym oil) compared to other groups on days 4, 12 and 21 post operation ($P<0.05$).

The wound closure was completed in TVO-treated groups compared to control animals (7 ± 2.31 mm) on day 20.

Table 1: Effect of the Thymus vulgaris essential oil ointment (TVO) on wound excision area (mm^2)

	Wound area (mm^2) ± SEM		
DAY	**Control**	**1.5% TVO-treated animals**	**3% TVO-treated animals**
4	168 ± 7.34[a]	144 ± 7.36 [b]	136 ± 3.29 [b]
12	95 ± 2.56[a]	20 ± 1.36 [b]	30 ± 3.90 [b]
21	7 ± 2.31[a]	0 ± 0.00 [b]	0 ± 0.00 [b]

n= 6 animals in each group. Values are presented in mean ± SEM. There are significant differences between groups with different codes in a the same raws (superscript letters a and b; P<0.05).

Histologic Analysis

The number of fibroblast and macrophages was increased as well as vessel formation compared to control group on days 3, 7, 14 and 21 after incision ($P<0.05$).

Also there was significant difference for fibroblasts and macrophages distribution between 1.5-and-3%treated groups ($P<0.05$).

In treated groups, the collagen bundles were well organized between vessels compared to control group on day 7 ($P<0.05$).

Mature collagen bonds formation and angiogenesis significantly increased in 3% treated group on day 14 post wound induction ($P<0.05$) (Figure 1).

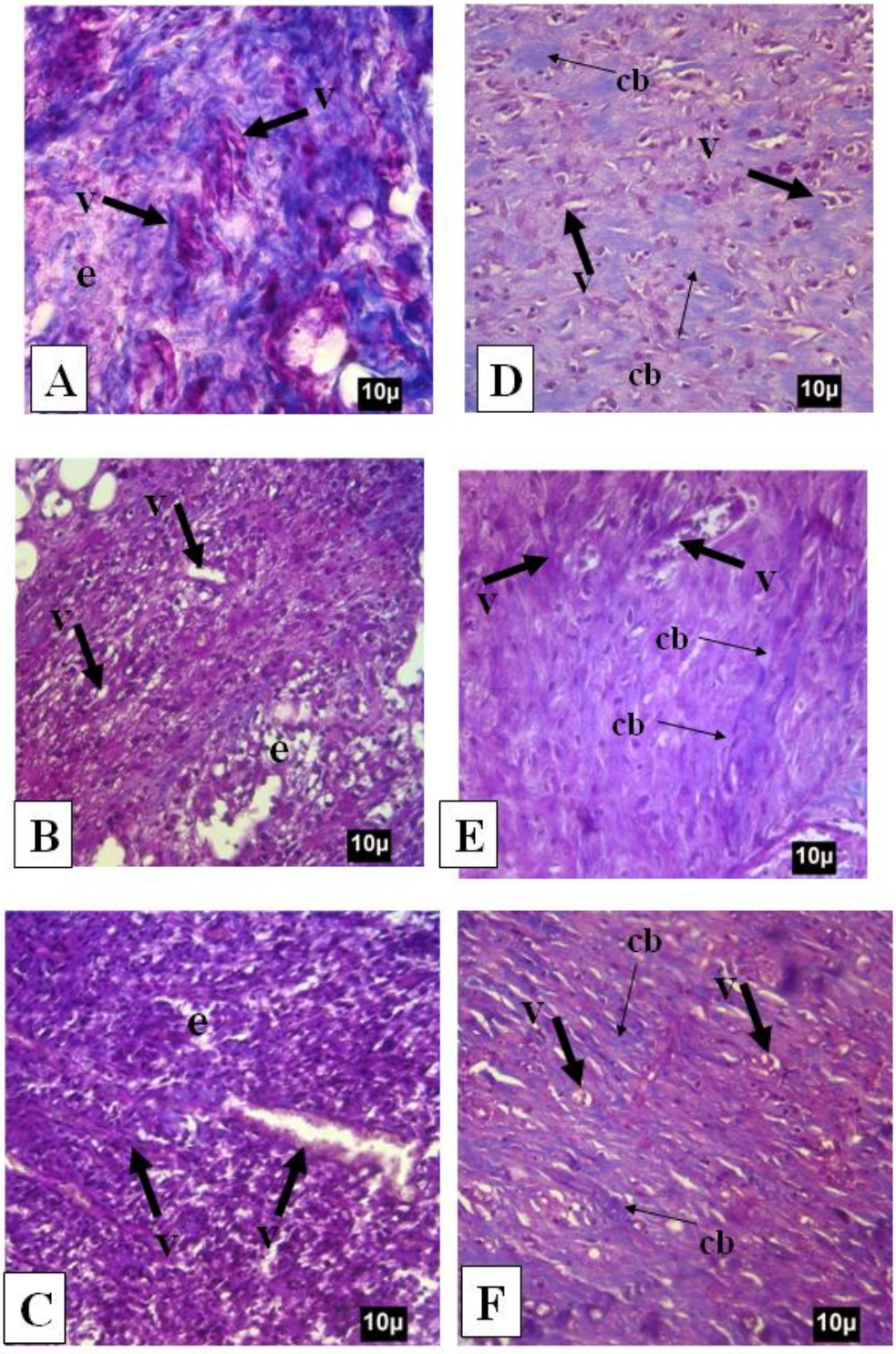

Figure 1: Cross section from wound area on Control, 1.5% and 3% TVO-treated groups for collagen deposition and angiogenesis; A, B, and C represent 7-day-old and D, E and F represent 14-day-old wounds. Eedema and thin walled vasculatures in control group is noteable, which is remarkably inhibited in treated groups on day 7 after wound induction. The mature collagen bonds are organized in 1.5% and 3% thyme treated groups on 14 days after wound induction. e: edema, V: vessels, cb: collagen bondles. Masson-trichrome staining, 400×

Discussion

The present investigation illustrated the wound healing properties of Thymus vulgaris essential oil ointment in rat. To our knowledge few studies done to determine effect of Thymus vulgaris essential oil ointment on wound healing. Because of side effects and high cost of synthetic medicine, recently there is growing interest in the search for new nature wound healing drugs. Our data showed that, the TVO improved wound contraction and is usefull for wound healing.

In this study, different levels of TVO diminished post-operative wound area. Based on previous reports on physiological mechanism(s) for contraction and regeneration of wound area, the accepted pathway among the forerunners of this field is that contraction initiates by the activation of receptor linked and voltage-dependent Ca^{2+} channels or cyclic AMP phosphodiesterase (CAMP) and therefore promotes Ca^{2+} channels or CAMP activity which leads to contract wound area. (Derakhshanfar *et al.,* 2010). Even though Thymus vulgaris widely studied than other Thyme species (Lamiaceae family), but pharmacological and mechanisms action of Thymus vulgaris essential oil is not quite elicited (Derakhshanfar *et al.,* 201l; de Lira Mota *et al.,* 2012). To our knowledge persumably TVO impress its effect through this mechanism or may other mechanism(s) include. Competence studies need to determine direct molecular mechanism pathway(s) to how Thymus vulgaris essential oil promotes tissue contraction in wound area.

Wound healing is a dynamic cellular and physiological process which is mainly depends on rapid reformation of damaged tissue into normal condition. In this regard, down-regulation of inflammation associated with provoked angiogenesis, even in early stages, promotes proliferative machinery such as fibroblasts, fibrocytes physiologic and/or compensatory functions (Kumar *et al.,* 2006).

Macrophages are the main cells in the inflammatory phase of wound healing (Beldon, 2010; Brancato and Albina, 2011). As seen in figure 1, TVO increased number of macrophages and up regulated fibroblasts accumulation in wound area. In fact, the macrophages attack to the wound area to phagocyte invested pathogens and release growth factors as well as cytokines. This leads to precipitate healing phase by migration of fibroblasts and myofibroblasts as well as promoting collagen synthesis in the wound area (Singer and Clark, 1999).

Histological studies showed that, TVO (1.5 and 3%) significantly increased fibroblast and fibrocytes distribution in wound area. Our data showed that mean distribution of mast cells increased in TVO-treated animals where indicating the Thymus vulgaris role in elevating mast cells prolifiration. Thus, we can come close to this fact that TVO by stimulating the mast cells prolifiration and distribution up-regulated the neovascularization and in turn promoted the prolifration and maturation stages.

Morever, mast cells are known for secreting vascular growth factor (VGF) that stimulates endothelial cell proliferation where finally up-regulates the neovascularization in wound area (Younan *et al.,* 2011). Neovascularization is known as a defensive and physiologic structural procedure which leads to reducing free radicals as well as releasing the metabolites to the cells involving in the healing process (Stavrou, 2008). Moreover, the neovascularization is an necessary factor for migration of epithelial cells from adjuscent regions into the central area of the wound area (Adam *et al.,* 1999). Our results mainfested that, there was significant improvement on vessel formation in TVO-treated groups. In correlation, the macrophages increase angiogenesis and endothelial cell proliferation by releasing several growth factors (Beldon, 2010). Proliferation and migration of the epithelial cells and fibroblasts as well as suppressed inflammation, enhances new vessel formation in wound area (Oryan *et al.,* 2010).

With angiogenesis, collagen bundles are organized between vessels in 1.5% TVO-treated group. It is principal to consider that mast cells secret tryptase and chymase which tryptase also eliminated the ability of mast cells to stimulate fibroblast contraction. It is proposed that tryptase secreted by the mast cells may be one of the active mast cell mediators for fibroblast proliferation and promote collagen synthesis (Kumar *et al.,* 2006). Increased viability of epithelial cells plus their successful proliferation and migration from wound bed leads to well-formed epithelialization (Kokh and DiPietro, 2011). High rate re-epithelialization was considered as a indicator for best healing. Therefore, shortened epithelialization time in 3% TVO-treated animals showed its usefulness on epithelial cells proliferation. Whatever, surveillance

of epithelial cells in TVO-treated groups might because of TVO antioxidant property which prevent cellular damage due to oxidative stress and facilitated migration process (Panchatcharam *et al.,* 2006). There is a relation between total phenolic and antioxidant activity which high phenolic component ration of extract is an indicator for better antioxidant activity (Zyad *et al.,* 2007). The antioxidant activities of thymol and carvacrol have been previously reported using different testing systems (Altiok, 2010). Recent researches have shown that phytochemical chemicals such as flavonoids and triterpenoids able to promote wound-healing process against methicillin resistant S. aureus (Tsuchiya *et al.,* 1996). In differentiation, Thymus vulgaris has anti-oxidant activity which prevents lipid peroxidation followed by atherosclerosis (Kaya *et al.,* 2013). Proliferation and migration of the epithelial cells and fibroblasts as well as restrained inflammation, improve new vessel formation in wound area (Oryan *et al.,* 2010).

It seems TVO in one hand regulates fibroblast proliferation and in other hand increase collagen synthesis which terminates to significant wound improvement. Considering these two findings, we conclude that TVO increased fibroblast proliferation and collagen synthesis which finally shortened wound area.

In conclusion, according to the results of the present study, topical administration of Thymus vulgaris essential oil decreased inflammatory cells and afterward increased in fibroblast migration and wound contraction. However, delayed inflammatory phase during healing process increases abnormal cellularity in proliferation phase.

Thymol and carvacrol work as antioxidant, antimicrobial and antifungal agents. So, based on findings of current study we think wound healing properties of Thymus vulgaris was not related only to the antioxidant activity and impress its effect by all mechanisms.We think further studies needed to clear pharmacological properties and physiological mechanisms by those phenolic compounds in Thymus vulgaris essential oil ointment act. Additionally, suitableness studies are needed to distinguish its potential for clinical use in clinical trials.

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