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Plectranthus tenuiflorus (Shara) Promotes Wound Healing: *In vitro* and *in vivo* Studies

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Abstract: The present study proved that both *Plectranthus tenuiflorus* juice and essential oil exerted a healing promoting effect in rat wound model. The effect was shown to be mainly via their ability to stimulate fibroblasts proliferation in addition to an anti-bacterial effect of its thymol content. Leaves of the plant were collected from different regions. The whole leave juice or essential oil were extracted by chemical steam distillation method. Different concentrations were tested for their effects on the proliferation of human foreskin fibroblasts in tissue culture. Its efficiency in enhancing wound healing processes using excision wound model in rat was also designed. The results revealed complete wound healing (100% contraction) at day 14 (10% juice), day 17 (80% juice) and day 18 (10% essential oil) compared to 22 days. Histological studies showed that at day 14 complete epithelization, well formed small sized scar tissue and reappearance of cutaneous appendages were evident in wounds painted with 10% essential oil, followed by 80% juice. *In vitro* study proved a stimulatory effect of plant extracts on human fibroblasts which may explain the speeding of healing process. The healing promoting effect or *P. tenuiflorus* may be attributed to the high content of calcium (903.16333±0.21); zinc (0.37933±0.05). Essential amino acids (Ala, Leu, Glu, Asp, Asn, Phe and His) seemed also to have a role. On the other hand, thymol was known to have an anti-bacterial effect. Thymol found in this study to be the main component (82.16%) of *P. tenuiflorus* extracts.

Key words: *Plectranthus tenuiflorus*, essential oil, leaves juice, whole extract, wound healing

INTRODUCTION

Wound healing is a sequent vital process that usually ends in the production of a healthy scar (Rohrich and Robinson, 1999). The biology of healing has been a concern of physicians throughout the ages. Despite great advances, at present there is no magic bullet that can be used for successful fast management of wounds to obtain a normal healing process.

The key cells in wound healing are fibroblasts where, tensile wound strength depend mainly on its activity and the rate at which it synthesize collagen (Bailey *et al.*, 1975; Kumar *et al.*, 2004), next the ability of the skin epidermal cells to divide, migrate to re-epithelize and cover the wounded area is the second important process for developing a healthy wound scar (Hordichok and Steyger, 2007).

Ancient physicians in Egypt, Greece, India and Europe practiced gentle methods to deal with wounds during healing process. They appreciated the importance of using natural herbal products for protecting injured tissues from the environmental factors and invaded microorganism (De Fatima *et al.*, 2008).

Throughout history, a large variety of plants and plants extracts has been utilized to speed and control wound healing process (Tisserand, 1988).

A good number of these plants was proved to contain significant quantities of aromatic essence, for example bush fuschia (*Eremophila alternifolia*) (Mathews *et al.*, 1988; Rowley *et al.*, 2008), manuka (*Leptospermum scoparium*) (Lis-Balchin and Hart, 1998), yarrow (*Achillea millefolium*) (Kuropka *et al.*, 1991; Tariq *et al.*, 2008) and poplar buds (*Populus candicans*) (Davis *et al.*, 1991).

Plectranthus L. Herit is a large genus of the *Lamiaceae* family widely distributed in tropical regions of Africa, Asia and Australia (Codd, 1985; Ascensão *et al.*, 1999; Abdel-Mogib *et al.*, 2002) that natively grows in Western and Southern region of Saudi Arabia (Collenette, 1998; Rahman *et al.*, 2004). Several

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Plectranthus species are cultivated as ornamentals or as sources of essential oils, whereas other are used as edible tubers, or as food flavorings (Perro, 1944; Ascensão *et al*., 1999). In folk medicine, they are employed for headaches, sores, burns, dermatitis, acute edematous otitis acuta, stomachache, against nausea, scorpion stings and as purgative (Dash and Kashyap, 1987; Cosentino *et al*., 1999; Cateni *et al*., 2003; Chandrasekaran and Venkatesalu, 2004; Kim *et al*., 2004).

Plectranthus tenuiflorus (Euphorbiaceae family), is the generic name for Shara, a perennial succulent herb; having a pleasantly aromatic juice. In Western region, it is used as an eardrop for earache and inflammation of middle ear (Chandrasekaran and Venkatesalu, 2004), whereas it is prescribed in Asia for a remedy sore throat (Rahman *et al*., 2004).

The present study is a trial to test the healing effects of the whole leave extract and the essential oil fraction of *P. tenuiflorus* using experimental wound models. An *in vitro* study on its possible stimulatory effects on skin fibroblasts was also carried on.

MATERIALS AND METHODS

The study was conducted between 2004-2006 in Tissue Culture Unit King Fahd Medical Research Center and Chemistry lab in King Abdulaziz University, Jeddah, KSA.

Plant extracts preparations: *P. tenuiflorus* plant leaves were collected from Taif and Jeddah regions; Saudi Arabia (Fig. 1).

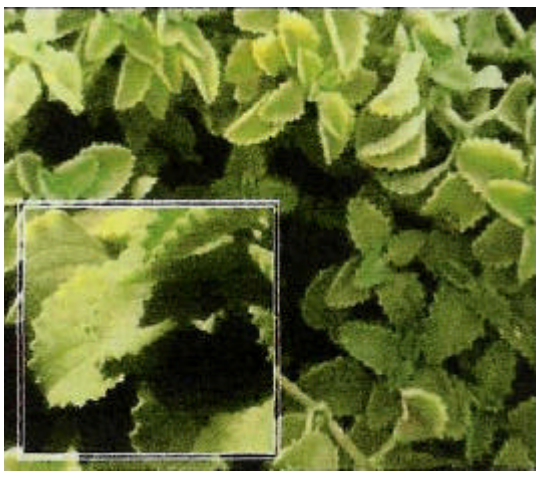

Fig. 1: *Plectranthus tenuiflorus* plant leaves that collected from Taif and Jeddah regions; Saudi Arabia

Steam distillation method was used for preparing the whole juice extract (Alsofyani, 2006).

Essential oil of *P. tenuiflorus* was also separated using syringe from the supernatant solution after steam distillation and subjected to chemical analysis (Alsofyani, 2006).

The stock solutions (in DMSO) from all extracts were prepared, filtered and sterilized through a 0.22 μm filter and store at -4°C. This stock was diluted in MEM at the same time of using.

Preparation of different concentrations of *P. tenuiflorus* extracts for *in vitro* study: Plant extracts (juice and essential oil) were added to MEM media to investigate their effects on fibroblasts proliferation.

Different concentrations of leaves juice of *P. tenuiflorus* (0.05-0.2 w/v) were prepared in MEM. *Essential oil* was prepared in absolute alcohol with a concentrations of 50%, then further diluted in MEM in concentrations ranging from (0.0005 to 0.01 w/v).

Pure Thymol (the major constituent of essential oil) of *P. tenuiflorus*: is prepared as 50% w/v in absolute ethanol then was diluted with MEM in the same concentrations of essential oil.

MEM media containing 10% FCS was used as a control in comparison to the above experimental media.

In vitro model

Human skin fibroblasts cells: Human skin fibroblasts were obtained from human foreskin after circumcision operations (Surgical Clinic King Abdul Aziz, University Hospital, Jeddah, Saudi Arabia). The specimens were transported immediately within 5 min after excision in previously prepared bottles containing MEM media.

Media

Minimal Essential Medium (MEM) (10%FCS): MEM is a rich, multipurpose medium that was used for cultivation of human normal fibroblasts (Eagle, 1977; Pollared and Walker, 1989; Khorshid, 2001).

Tissue culture experiment: The samples were cut for primary culture into small fragments, minced and gently agitated in trypsin solution at a concentration of 0.25, 0.1% glucose and 0.02% EDTA for 15 min (Mather and Roberts, 1998). Trypsin action was then quenched by MEM when, intercellular separation was seen. The supernatant suspension containing the dissociated cells was removed and centrifuged at 100xg for 10 min; cells were re-suspended in MEM containing 20% fetal calf serum heat inactivated (56°C for 30 min). Cells were adjusted to 1×10^5 cells mL^{-1} and plated into tissue culture

flask 25 cm^2 then incubated in a humidified incubator in an atmosphere of 5% CO_2 at 37°C.

Cells were subcultured twice weekly. Cells were used for experimental work after suitable number of cells were obtained in MEM media, which later replaced by the tested media containing different concentrations of plant extracts (n = 3).

Proliferation assays: Cell density in both control and experimental media were estimated at 24, 48 and 72 cell cultures, respectively. The numbers of viable cells were counted using Heamcytometer and Trypan blue exclusion assay (Pollared and Walker, 1989; Mather and Roberts, 1998; Khorshid, 2001, 2005).

The relationship between cell density (growth rate) and cultured period in control and experimental media were analyzed using statistics programs (SPSS and ANOVA) (at $\alpha = 0.05$).

Cultured cells were fixed in 4% neutral buffered formaldehyde and stained with Coomassie blue. The morphology of unstained and stained cells of all groups was examined and photographed using an inverted microscope (Marino *et al*., 2001; Khorshid, 2005).

***In vivo* excision wound model:** Adult male Wister rats, 200-250 g of body weight, purchased from the animal house in King Fahd Medical Research Center, KAU in Jeddah. Animals were housed in standard cages at the animal house and allowed to acclimate to their surrounding for 7 days prior to the experiment. All animals received human care according to ethical requirements approves by the Animals Research Ethic Committee of KAU.

Full thickness skin flap, completely transdermal (2×2.4 cm) was removed from the dorsal surface of the thigh region after subcutaneous injection with 5 mL normal saline to raise the skin and facilitate the excision. All rats were anesthetized by intramuscular injection using kitamine (0.3 mL) and 2% setone (0.1 mL). Animals were allowed to recover and housed individually in cages. The animals were grouped as following:

- **G1:** Served as a negative control where the wounded area was left to heal spontaneously
- **G2:** Served as a positive control, the wounded area was painted daily with 70% ethyl alcohol

The wounded area in other groups were painted daily with the following:

- **G3:** 50% *P. tenuiflorus* essential oil in absolute ethanol
- **G4:** 10% *P. tenuiflorus* essential oil in absolute ethanol
- **G5:** 80% *P. tenuiflorus* whole extract (leave juice) in distilled water
- **G6:** 10% *P. tenuiflorus* whole extract (leave juice) in distilled water

The wounded area in all groups was photographed every 3 days after measuring wound length and width using measuring scale. Results were tabulated and statistically analyzed.

RESULTS

Chemical analysis showed that *P. tenuiflorus* comprises two substances: essential oil, in which thymol (85.3%) is the principle component. Second, the whole substance leaves juice containing oxygenated trypenoids, mono terpinoid substances, seven amino acids (Ala, Leu, Glu, Asp, Asn, Phe and His) and several minerals including Ca, Mg and Zinc (Alsofyani, 2006) (Table 1).

***In vitro* study:** The present study showed that both the whole leave juice extract and essential oil of *P. tenuiflorous* can affect the proliferative activity of human fibroblasts in cell culture.

Effects of leave juice: The juice had a stimulatory effect on fibroblast cell growth after 48 h culture at 0.05-0.1% concentrations compared to control ($p<0.05$). The maximum effect appeared at 72 h cell culture ($p<0.05$).

An inhibitory effect was observed at concentrations higher than IC_{50} (0.1%) at 24 h culture. The more evident effect appeared after 48 and 72 h cell culture (Fig. 2).

Morphological appearance of both control and experimental fibroblasts were shown in Fig. 3a-c.

Effects of essential oil: Essential oil at a concentrations ranged between 0.005-0.01 (w/v) produced a significant stimulatory effect on fibroblasts proliferative activity. The

Table 1: The content of minerals in *Plectranthus tenuiflorus*

Metals	Concentration (ppm)	Metals	Concentration (ppm)
Mn	1.02233±0.22	Ag	0.00033±0.001
Mo	0.01933±0.01	Al	9.15933±0.19
Na	75.07333±0. 17	As	0
Ni	0.08833±0.03	Ba	0.23467±0.07
P	24.87±0.3	Bi	0
Pb	0.09167±0.07	Ca	903.16333±0.21
Sb	0.03433±0.03	Cd	0
Se	0	Co	0.025±0.002
Sr	12.35333±0.97	Cr	0.05567±0.01
V	0.23167±0.001	Cu	0.09333±0.05
Zn	0.37933±0.05	Fe	30.80333±0.23
Mg	367.09333±0.18	Hg	0

effect started after 48 h and continues to 72 h compared to control (Fig. 4). Optimum concentration was found to be near 0.0063% (Fig. 5). Doses higher than IC_{50} (1×10^{-2}) was shown to have an inhibitory effects at 24 h fibroblast cell culture (Fig. 4, 5).

Effect of pure thymol: Control study using pure thymol showed that at low concentration (0.0005%), although it produced an initial decline in cell density at 24 h culture, then it exerted a significant stimulatory effects at 48 h followed by 72 h cell culture ($p>0.05$) compared to control (Fig. 6).

Concentrations more than IC_{50} (0.0063% w/v) has an inhibitory effects on human fibroblasts.

Figure 5a-e showed the morphology of 72 h cultured fibroblasts after addition of different concentrations of essential oil and pure thymol.

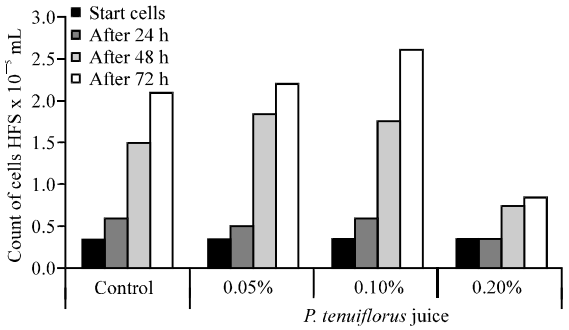

Fig. 2: Showed that activation of cell growth has started at low concentrations of juice (0.05-0.1%), being optimum at 0.1% of juice, whereas the concentrations more than 0.1% resulted in cell growth inhibition

***In vivo* study: Morphology and wound contraction:** In the present study, the degree and extent of wound healing in all studied groups was assessed according to the number of days taken by the wound to be closed which presented in Table 2. The general appearance of the formed scar and the re-appearance of hairs in the healed region showed in Fig. 7.

Essential oil: It was observed that 10% of ethanolic extract of *P. tenuiflorous* essential oil has an effective healing promoting effects compared to 50% concentration (Fig. 7). The effect started on 8th day post wounding, whereas wound size was decreased at the 12th day by 91.23% of the original wound size (4.8 cm^2) and 99.68% at the 16th day (Table 2).

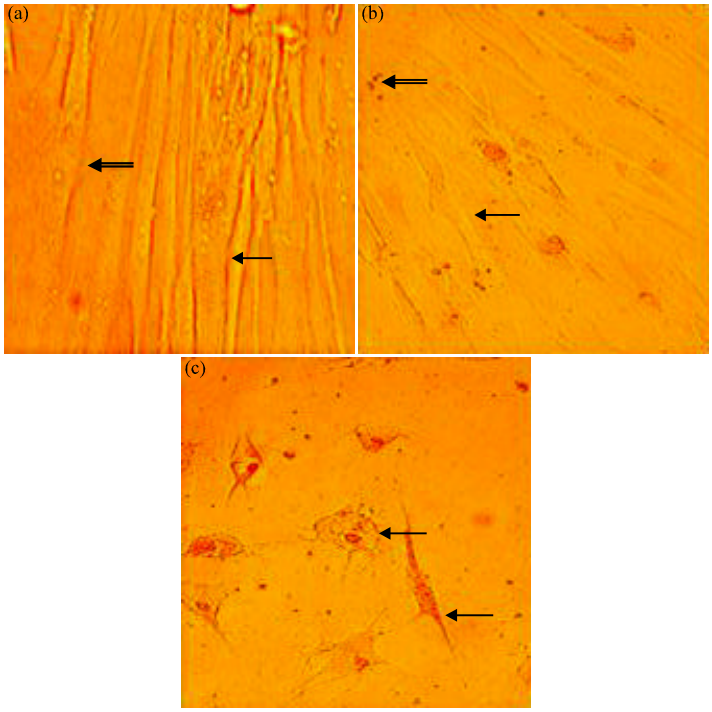

Fig. 3: The morphology of fibroblasts in tissue culture after 72 h (a) Control cells with normal spindle shape (arrow), central nuclei having one or more nucleoli (double arrow) [x40], (b) At 0.1% of juice concentration cells are large (arrow), having central nuclei with prominent nucleoli (double arrow) [x40] and (c) At 0.2% juice concentration, most fibroblast cells were damaged, deformed or swollen (arrows) [x40]

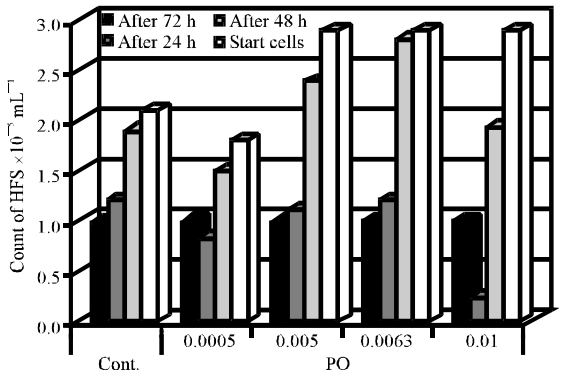

Fig. 4: This diagram showed that essential oil concentrations from 0.005 to 0.01% activated fibroblast cell growth at 48 h until 72 h comparing with control group

However, lacking of hair growth was observed in wounded areas painted with both 10 and 50% concentrations of oil (Fig. 7).

On the other hand, 50% w/v of essential oil produced complete healing on the 20th day.

Complete wound healing in untreated groups and those painted only with ethanol was observed at day 22 post wounding.

Leave juice extract: The healing promoting effects of 10% w/v concentration of the juice started at the 4th day post wounding where, the size of wounded area decreased by 53.30% compared to the original wound size. At the 8th day, the size of wounded area decreased by 88.1% (Table 2, Fig. 7).

Closure of wounds: Complete closure of wounded area painted daily with 10% w/v of juice was observed after

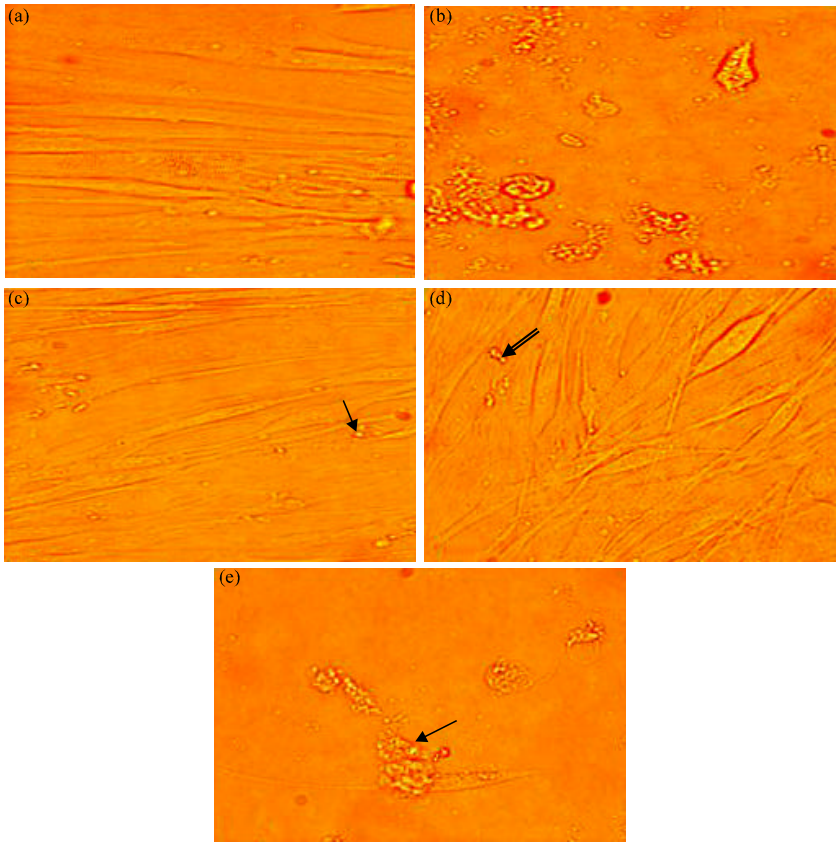

Fig. 5: The morphology of fibroblasts in tissue culture after 72 h. (a) Control cells with normal spindle shape, central nuclei and one or more nucleoli [x40], (b) At low concentration of essential oil (0.0005) damage of most fibroblasts has been occurred, only cell debris was observed [x40], (c) At 0.0063% essential oil of *P. tenuiflorus*, large sized cells with large nuclei and prominent nucleoli were observed (arrow); a sign of increased cell activity [x40], (d) Thymol at very low concentration (0.0005%) leads to an increase of fibroblasts size. Both nuclei and nucleoli are also large (double arrow) [x40] and (f) Thymol concentration more than (0.0005%) has damaged the cells and only cell debris (arrow) were observed in the media

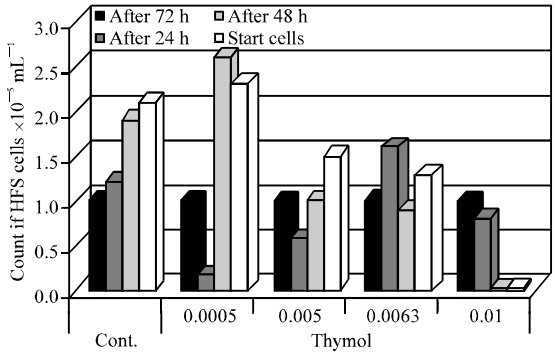

Fig. 6: The effect of Thymol (the major component of essential oil) on the fibroblasts growth density/per ml/72 h compared to the whole essential oil (Fig.4). Notice: the higher stimulatory effect was at low concentration (0.0005%) after 48 h followed by 72 h, while at high concentration (more than 0.0005%) inhibition of cell growth has stared at 48 h

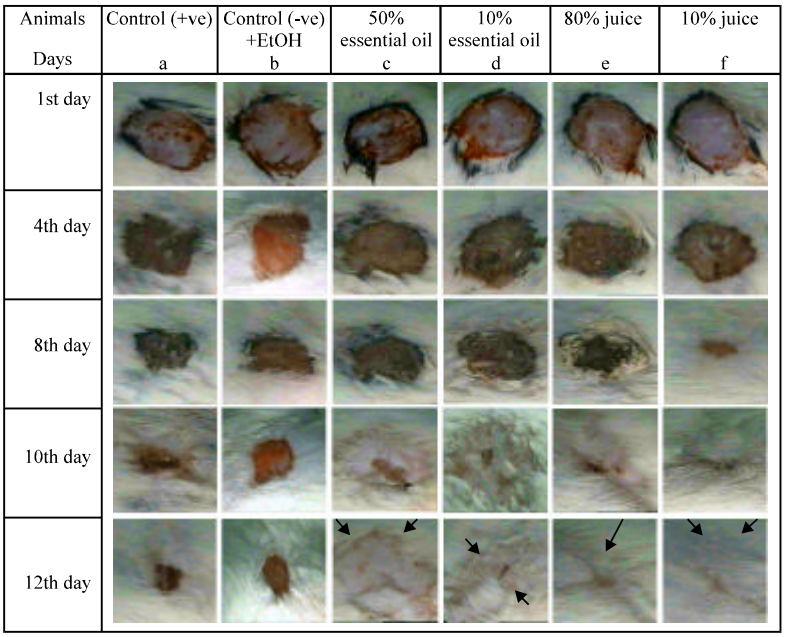

Fig. 7: Photographs of excision rat wounds (treated and non-treated animals) at different stage of healing process. Notice: the enhanced wound healing in treated groups: Control non-treated: showing decrease in wound size without complete closure till 12 days. Slight closure of wounded area in alcohol treated animals. 50% essential oils showed marked wound closure but lack of hair growth (arrow). 10% essential oils: more or less similar to 50% concentration (arrow). 80% juice extract complete healing and closure of wounded area without signs of hair loss (arrow). 10% juice extract: notice that healing start early at the 8th day with complete closure of wounded area and normal scar formation (arrow) at 12th day without signs of hair loss

14 days. A contraction rate 100% was observed after 14 days compared with those painted with 80% (after 17 days). Complete epithelization on which was nearly similar to control, healthy small sized scar (contraction) and reappearance of cutaneous appendages (hair and glands), no increased pigmentation or lack of hair were observed in this group. On the other hand, untreated wounds showed complete wound closure after 22 days. Histological study of wound region showed the normal structure of rat back skin and distribution of collagen fibers (Fig. 8, 9a, b). Figure 10a-d showed histological events occurred during the process of wound healing in rat model and the effect of both leave juice extract and essential oil.

In the present study, it was observed that the *P. tenuiflorus* juice or essential oil enhance healing processes of skin in the area of excision wound of rats *in vivo* and stimulate the growth of fibroblasts *in vitro*.

Several researches (Shukla *et al*., 1999; Thang *et al*., 2001; Kerr, 2003) mentioned the role of some plant extracts in stimulating fibroblast in tissue culture that explained its possible role in enhancing wound healing.

Essential oil constitutes about 0.033% of *P. tenuiflorous* leaves which contains about 64 compounds most are oxygenated trypenoids and oxygenated compounds. Thymol represents an example of the latter; it constitutes about 82.16% of essential oil.

No available literatures concerning the effect of thymol on fibroblast cell growth in cultured media, however, Stammati *et al*. (1999) found that thymol leads to inhibition of cancerous cells isolated from human laryngeal carcinoma, which proved to be of epithelial origin. In the present study, thymol at a very low concentration (5×10^{-4}) has a stimulatory effect on fibroblasts, while at higher concentration it produced inhibition of cell growth. Fibroblasts are considered to be derived from mesenchymal tissue, hence the differences in response to thymol. Since, thymol is considered an oxygenated compound, many literatures described the role of O_2 in stimulating cell proliferation (Stephens and Hunt, 1971; Hunt and Pai, 1972; Silver, 1972), this may explained the observed role of *P. tenuiflorous* in stimulate healing process of wounds.

Extracts of *P. tenuiflorous* leave juice and essential oil were proved to have antibacterial effects against a large number of pathogens normally, present in skin (Awadh *et al*., 2001; Alsofyani, 2006), an effect most probably due to its high thymol content. This may exert a protective effect against contamination of the wounded area here.

Flavonoids were scanty in *Plectranthus* and only two were identified (M10) and (M11). In literature, these substances were proved to have stimulatory effects on fibroblasts both *in vivo* or *in vitro* conditions (Tokuda *et al*., 2001).

In histological point of view, skin wounds involve loss of tissues, cellular damage, alterations in cell-cell relationships, expression of integrins and growth factors receptors on cell surfaces and in extra cellular matrix (Rohrich and Robinson, 1999; Inngjerdingen *et al*., 2004).

Sequential events in wound repair require a conductive environment within the wound bed and a balanced pool of building amino acids and metal ions such as calcium, zinc, magnesium and copper. Amino acids proved to be present in *P. tenuiflorous* extract include (Ala, Leu, Glu, As, Asn, Phe and His). Some of them are considered essential for cell viability. Phenylalanine and histadine are aromatic amino acids, which are reported by many authors to be used in wound healing therapy (Askanazi *et al*., 1980; Chang *et al*., 1983; Li, 1992).

P. tenuiflorous contains about 903.2 mg L^{-1} Ca, 367.1 mg L^{-1} Mg and 0.379 mg L^{-1} Zn. Zn and Ca^{2+} have been reported to play an important role in wound healing (Lansdown, 2002; Khorshid, 2004). Magnesium levels were also reported to have a role in the rat wound (Williams *et al*., 1998).

In the present study, it was observed that *P. tenuiflorous* leave juice and essential oil had a healing promoting effects using rat wound model. Stimulatory effects were also observed on fibroblast proliferative activity in tissue culture. These effects may be due to the high content of the above mentioned minerals.

Calcium is well established as an extracellular regulator and an intracellular modulator of cell proliferation in the mammalian epidermis.

The Cadherins are key cell membrane calcium binding proteins that exhibit a major function in cell motility and migration and cytoskeleton function. It act as calcium sensor in eukaryote cells that modulate intracellular process gene expression (Bailey *et al*., 1975; Howes *et al*., 1929; Byrne *et al*., 1991).

The concentration of calcium in the vicinity of proliferating fibroblast was reported to be needed at least 1.4 mM concentration to retain proliferate activity, while higher concentration leads to an inhibitory effect (Lansdown and Payen, 1994; Doyle *et al*., 1996; Lansdown, 2002). Sotomayor and Schulten (2008) reported that the extracellular repeats of cadherin proteins mediate cell-cell adhesion in a calcium-dependent manner, since the molecular mechanisms behind the influence of calcium in adhesion dynamics and cadherin's mechanical response are not well understood. They show, using molecular dynamics simulations, how calcium ions control the structural integrity of cadherin's linker regions, they concluded that thereby affecting cadherin's equilibrium dynamics, the availability of key residues involved in cell-cell adhesion and cadherin's mechanical response. This may explain the concentration dependant effects of different concentration extracts used in the present study.

The *in vivo* enhancement of wound healing (14 days in wounds painted with 10% juice of *P. tenuiflorous* observed in the current study could be also explained in view of presence of high content of calcium in plant extracts in addition to aromatic and essential amino acids.

Calcium-alginate-based dressing have a proven value in treating moderate to heavily exuding wounds. It has been claimed to show anti-infective properties

both through providing a barrier against infective organisms or by drawing infectious agents into the alginate along with wound exudates and debris (Lansdown, 2002; Lansdown and Payen, 1994; Doyle *et al*., 1996).

Calcium alginate dressings were also used to enrich local calcium during haemostatic phase of wound healing, which have acknowledged clinical value 25, 30. The action is via enhancing clotting blood plasma and aggregation of blood platelets.

Experimental studies with porcine wound have shown that early increase in calcium concentrations (<8 h) were needed as a requirement for effective homeostasis (Kim *et al*., 2005).

Other experimental studies, proved that elevated calcium level in rabbit or rat were reported to improve healing and increase tensile wound strength (Lansdown, 2002).

Topical calcium chloride dressings evoked a significant increase in granulation tissue in guinea pigs (Lansdown, 2002).

Murine wounds treated topically with 100 mg mL^{-1} calcium D-pantothenate, showed 50% increase in fibroblast proliferation and 1.2 to 1.6 fold increase in DNA synthesis 113 especially, in the presence of zinc, insulin and insulin like growth factor (Steenkamp *et al*., 2004; Thang *et al*., 2001; Lansdown, 2002; Brown *et al*., 1989).

Experimental study also showed that a marked increase in calcium concentration within 24 h post wounding with a peak within 5 days. This is coinciding with the period of maximum inflammatory activity within the wound site and high proliferate activity in epidermis and dermis (Lansdown, 2002; Bosisio *et al*., 1997). This may explained the early appearance of scar tissue in 4th days in wounds painted with 10% juice extract in which Ca^{2+} are proved to be abundant.

The role of calcium on keratinocyte cell activity was also reported, proliferation of keratinocytes in normal or regenerated epidermis depends also on appropriate Ca^{2+} signaling (Thakral *et al*., 1979; Marchese *et al*., 1990).

Keratinocytes in tissue culture have been reported to migrate along calcium concentration gradient to form stratified epithelia with increased cellular adhesion and desmosomal contact (Lansdown, 2002).

CONCLUSION

Promoting wound healing by either leave juice or essential oil could be attributed to thymol content, with its well known antibacterial action, to high content of amino acids needed for tissue regeneration and to high content of calcium, zinc that proved to act as antioxidant with enhancement effect of fibroblasts proliferation.

A study of Ca^{2+} adhering or integrals expression on cultured fibroblasts or cellular components during healed wound processes is vital to appreciate the stimulatory or inhibitory role of any new extracts or substances. It also will help understanding the mechanism by which it leads to enhancing wound healing via stimulating either keratinocytes or fibroblast cellular activity.

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