Full Length Research Paper

Evaluation of the wound healing potential of *Protea madiensis* Oliv leaf extract in rats

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***Protea madiensis* Oliv. is a flowering shrub which grows in south eastern Nigeria. Liquids extracted from its leaves are applied on wounds to promote healing. To investigate the effect of *P. madiensis* on wound healing, its methanol extract was applied topically on excision wounds daily. During the experimental period, the rate of contraction, epithelialisation and histology of the wounds were studied. Also, *in vitro* antibacterial study, 1,1-diphenyl-2-hydrazyl (DPPH) scavenging assay and phytochemical analysis were performed using *P. madiensis* extract. Wound contraction and epithelialisation were significantly (p < 0.05) faster in the *P. madiensis* treated rats compared to the control rats. On day 14 post wounding, wound sections of the extract treated rats showed more collagen fibers, more fibroblasts and fewer inflammatory cells compared to the control. At 5, 10 and 20 mg/ml concentrations, *P. madiensis* inhibited the growth of *Staphylococcus aureus* and *Bacillus subtilis*. The extract at 100 µg/ml showed 60.4% DPPH scavenging activity. Phytochemical screening revealed the presence of tannins, saponins, flavonoids, alkaloids and glycosides in the extract. In conclusion, the extract of *P. madiensis* significantly enhanced wound contraction, epithelialisation, fibroblast proliferation and collagen deposition thus authenticating the claim of its efficacy in traditional wound care.**

Key words: *Protea madiensis*, excision wounds, antioxidant, fibroblasts, antibacterial.

INTRODUCTION

Wounds are physical injures which disrupts the normal skin anatomy and function (Rashed et al., 2003). Wounds may be created by physical, chemical or thermal injury to tissues (Raina et al., 2008). In medical practice, the treatment of full thickness wounds with large skin defect continues to be a source of great concern since the healing time is prolonged and often leads to complications (Raina et al., 2008; Hassan et al., 2011). Thus, the use of agents such as hydrocolloid gels, tissue extracts, plants extracts and probiotics which accelerate wound healing is being advocated to ensure rapid healing of full thickness wounds (Udupa et al., 1991; Steed, 1995; Abu-Al-Basal et al., 2010; Nasrabadi et al., 2011).

Ethno medicines made from plants are applied on wounds, burns and ulcers by indigenous West Africans and traditional healers to promote healing (Rashed et al., 2003). Herbal medicines used in wound healing are said to disinfect, debride and provide suitable environment for the healing process (Priya et al., 2002). They are also less toxic and have minimal side effects compared to conventional medicines (Abu-Al-Basal, 2010). Recent studies have shown that extracts obtained from plants accelerated the healing of excision wounds (Shivhare et al., 2010; Olugbuyiro et al., 2010; Abu-Al-Basal, 2010; Ezike et al., 2010). However, many plant species have not been investigated scientifically to confirm their wound healing effect.

Protea madiensis Oliv. is a flowering shrub (3 to 6 m high) belonging to the family Proteaceae (Arbonnier, 2002). The morpohologic features of this plant have been described (Arbonnier, 2002). Not much is known of its medicinal uses. However, while its root is used to prepare

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medicines used in the treatment of epilepsy, ethno medicines prepared from its stem bark are used in treating amenorrhoea, malaria, fever, headache and dysentery (Arbonnier, 2002). The leaves are used for external treatment of fractures (Arbonnier, 2002). Liquids extracted from the fresh leaves of *P. madiensis* are applied on wounds and burns by traditional healers in Ukpabi Nimbo in Uzo Uwani local government area of Enugu state, Nigeria to enhance wound healing. Although its leaf extract is claimed by traditional healers to have potent wound healing activity, no scientific study has been carried out to validate this claim. This present study was conducted to investigate the wound healing effect of the methanol leaf extract of *P. madiensis*. The result of this study will validate the claim by traditional healers of the efficacy of *P. madiensis* leaves in wound healing.

MATERIALS AND METHODS

Fresh leaves of *P. madiensis* were collected from Ukpabi Nimbo in Uzo Uwani Local Government Area of Enugu state, Nigeria and authenticated by a qualified taxonomist at the International Centre for ethno medicine and drug development, Nsukka. A voucher specimen INTERCEDD/866 was deposited at the centre.

Preparation of extract

The leaves (600 g) were air dried, pulverized and macerated in 80% methanol (1200 ml methanol) with intermittent shaking for 48 h. After filtration, the filtrate was concentrated in a rotary evaporator at 40°C to obtain 71.1 g methanol extract (yield: 11.95% w/w). The extract was stored in the refrigerator until use.

Phytochemical analysis

The extract was subjected to phytochemical tests to detect the phytochemical compounds present in it (Evans, 1996). 2000 mg of *P. madiensis* extract was dissolved with 20 ml of distilled water to form a 100 mg/ml test solution. The solution was then filtered. For the following phytochemical tests, 3 ml of the test solution containing 300 mg of the extract was used.

Test for alkaloids

Three tests were performed to confirm the presence of alkaloids. Three drops of Mayer's reagent was added to 3 ml of extract solution in a test tube while three drops of Wagner's reagents was added to 3 ml of test solution in a second test tube. To a third test tube containing 3 ml of test solution was added three drops of Dragendorff's reagent. A creamy precipitate with Mayer's reagent, reddish brown precipitate with Wagner's reagent and brownish precipitate with Dragendorff's reagent indicated the presence of alkaloids.

Test for flavonoids

Two tests were conducted to confirm the presence of flavonoids. To 3 ml of test solution was added three drops of 10% sodium hydroxide (NaOH) while to another 3 ml of test solution was added three drops of 1% NaOH plus three drops of 0.5 N HCl. An intense

yellow colouration of both solutions indicated the presence of flavonoids.

Test for tannins

To 3 ml of test solution was added three drops of 10% ferric chloride. To another test tube containing 3 ml of test solution was added three drops of 10% lead acetate. The presence of bluish black or brownish green precipitate with ferric chloride and brownish precipitate with lead acetate indicated the presence of tannins.

Test for saponins

The frothing and emulsifying tests were carried out to confirm the presence of saponins. In the frothing test, 4 ml of distilled water was added to 1 ml of the test solution while in the emulsifying test, three drops of olive oil was added to 3 ml of the test solution. The mixtures were shaken vigorously and allowed to stand for 10 min. The presence of persistent froth in the frothing test and brown emulsion in the emulsifying test indicated the presence of saponins.

Test for glycoside

To 3 ml of test solution was added 1 ml 10% H_2SO_4 and the solution heated in a water bath for 2 min. On cooling, 2 ml of Fehling's I and II solutions were added followed by boiling for 2 min. A brick red precipitate indicated the presence of glycosides.

Test for terpenoids

0.5 g of the extract was dissolved in 2 ml concentrated chloroform and the solution filtered. Concentrated H_2SO_4 (3 ml) was carefully added by the side of the test tube to form a layer. A reddish brown colouration of the interface indicated the presence of terpenoids.

Experimental animals

The study was carried out using 30 male albino rats weighing 200 to 250 g. They were housed in standard experimental conditions, fed rodent diets and water was provided *ad libitium*. All animal experiments were approved by the Animal Ethics Committee, University of Nigeria, Nsukka.

Infliction of excision wound

Full thickness excision wounds were inflicted on rats as described by Morton and Malone (1972). Rats were anaesthetized with ether and their dorsum shaved and disinfected with chlorhexidine (Hibitane®). Full thickness excision wounds of 4 cm^2 were inflicted on the dorsum of rats. Post wounding, rats were assigned to three treatment groups (n = 10). The wounds of rats in groups 1, 2 and 3 were treated topically with normal saline, 10% *P. madiensis* solution and 10% neomycin-bacitracin (cicatrin®) solution. The rats were housed individually and their wounds treated daily until complete wound healing occurred.

Wound contraction and epithelialisation

The wound diameters were measured on days 0, 3, 6, 9,12,15,18 and 21. Wound contraction was calculated as described by Ezike et al. (2010). The time taken to obtain 50% wound closure (WC_{50}) was

Table 1. Phytochemical constituents identified in *P. madiensis* leaf extract.

Phytoconstituent	Result
Flavonoids	+++
Saponins	++
Alkaloids	++
Tannins	++
Glycosides	+
Terpenoids	-
Arthroquinones	-

-, Absent; +, present; ++, moderately present; +++, highly present.

also calculated (Alqasoumi et al., 2011). Time taken for complete epithelization was recorded (Nayak et al., 2009).

Histology

Tissue biopsies were collected from two rats in each group on days seven and 14 to monitor wound healing. On collection, tissue sections were fixed in 10% formal –saline and later processed as described by Nasrabadi et al. (2011). Haematoxylin-eosin stained sections were examined with light microscope for the presence of macrophages, neutrophils and fibroblasts. The distributions of these cells were qualitatively scored as few, moderate and high. Photomicrographs were captured using a Moticam Images Plus 2.0 digital camera (Motic China group Ltd. 1999 to 2004).

Antibacterial study

The agar diffusion method was used to evaluate the antibacterial effect of the extract (Perez et al., 1990). Pure isolates of *Staphylococcus aureus* (ATCC 25923), *Bacillus subtilis* (NCTC 10073), *Escherichia coli* (MTCC 116), *Salmonella typhi* (ATCC 19430), *Pseudomonas aeruginosa* (ATCC 27853) and *Klebsiella pneumonia* (ATCC 31488) obtained from the stock bacteria kept at the Pharmaceutical Microbiology Laboratory, University of Nigeria, Nsukka were used. A 20 mg/ml stock solution of *P. madiensis* was serially diluted 10, 5, 2.5, 1.25, 0.625 and 0.3125 mg/ml solutions. A single colony of each test isolate was suspended in 2 ml sterile nutrient broth. The suspension was adjusted to 10^8 cfu/ml and used to inoculate Muller Hinton agar. The inoculated agar plate was allowed to dry and the plates properly labeled. A 6 mm cork borer was used to make wells on the agar. With a micropipette, 50 µl of the test extract (20, 10, 5, 2.5, 1.25, 0.625 and 0.3125 mg/ml) was delivered into appropriate wells. The plates were left on the bench for 30 min and then incubated at 37°C for 24 h. After incubation, the inhibition zone diameter (IZD) for each well was measured to the nearest possible millimeter (mm) using a meter rule.

1,1-Diphenyl-2-hydrazyl (DPPH) assay

The free radical scavenging activity of the extract was evaluated using the DPPH radical scavenging assay (Mensor et al., 2001). The extract (10, 50, 100, 200 and 400 µg/ml) were mixed with DPPH and incubated for 30 min. The absorbencies of the mixtures were read after incubation using a spectrophotometer at 517 nm. Ascorbic acid was used as reference drug, DPPH-methanol solution served as control while methanol-extract solution was used as blank. The anti-oxidant activity of the extract was calculated using the formula described by Hsu (2006).

Statistical analysis

The results were expressed as mean ± standard error of mean (SEM) and analyzed using analysis of variance (ANOVA) in SPSS 11.0 software. Least significant difference (LSD) was used to separate variant means. $P < 0.05$ was accepted as significant.

RESULTS

Phytochemical analysis

Phytochemical screening of the extract showed the presence of tannins, alkaloids, flavonoids, saponins and glycosides. Terpenoids and arthroquinones were absent (Table 1).

Wound contraction and epithelialisation

Wound contraction in the extract treated group from day six post wounding was significantly ($p < 0.05$) faster compared to wound contraction in the normal saline treated group (Table 2). The WC_{50} in normal saline, extract and cicatrin® treated groups were 13.4, 7.9 and 7.3 days (Table 3). The epithelialisation was significantly ($p < 0.05$) faster in the extract treated rats compared to the control (Table 3).

Histology

By day seven post wounding, more inflammatory cells (macrophages and neutrophils) were present in the wound sections of the extract and normal saline treated rats compared to the cicatrin® treated rats (Figures 1, 3 and 5). However, more fibroblasts were seen in the wound sections of the extract treated rats compared to the number of fibroblast in the normal saline and cicatrin® treated rats. By day 14, wound sections of the extract and cicatrin® treated rats showed more collagen fibers, high number of fibroblasts and fewer inflammatory cells compared to wounds treated with normal saline (Figures 2, 4 and 6).

Antibacterial study

As shown in Table 4, the leaf extract (20, 10 and 5 mg/ml) inhibited the growth of *S. aureus* and *B. subtilis*. The extract was unable to inhibit the growth of *S. typhi*, *P. aeruginosa* and *K. pneumonia*

DPPH assay

The leaf extract showed DPPH radical scavenging activity with a maximal activity of 60.4% at 100 µg/ml (Table 5).

Table 2. Percentage wound contraction in rats post infliction of excision wounds.

Group	Percentage wound contraction					
	Day 3	Day 6	Day 9	Day 12	Day 15	Day 18
Normal saline	5.0 ± 2.1	16.3 ± 2.1[c]	26.4 ± 7.5[b]	40.9 ± 6.5[c]	59.9 ± 12.3[c]	93.8 ± 0.7[c]
Extract	13.3 ± 4.9	32.1 ± 5.6[a]	59.5 ± 7.1[a]	77.6 ± 12.9[b]	92.8 ± 4.8[b]	97.4 ± 1.0[b]
Cicatrin®	15.5 ± 5.8	30.1 ± 4.1[ab]	85.5 ± 1.8[c]	96.8 ± 0.2[a]	99.8 ± 0.2[a]	99.8 ± 0.2[a]

Table 3. Epithelization time and WC_{50} of rats post infliction of excision wounds.

Group	Epithelization time	WC_{50}
Normal saline	18.2 ± 2.3[b]	13.4
Extract	12.4 ± 1.1 [a]	7.9
Cicatrin®	11.8 ± 1.2 [a]	7.2

Table 4. Inhibitory zone diameter (IZD) of different concentrations of the extract.

Concentration (mg/ml)	Staph.	Bacillus	*E. coli*	Sal.	Kleb.	Pseud.
20	11	4	0	0	0	0
10	10	2	0	0	0	0
5	9	2	0	0	0	0
2.5	0	0	0	0	0	0
1.25	0	0	0	0	0	0
0.6	0	0	0	0	0	0
0.31	0	0	0	0	0	0

Staph, *Staphylococcus aeurus*; *E. coli*, *Escherichia coli*; Sal, *Salmonella typhi*; Kleb, *Klebsiella pneumonia*; Pseud, *Pseudomonas aeuroginosa*.

Table 5. Percentage DPPH scavenging activity of *P. madiensis* extract.

Concentration (mg/ml)	Activity (%)	
	Extract	Ascorbic acid
10	53.9	73.6
20	60.4	73.8
100	65.5	74.6
200	50.2	77.2
400	49.2	79.1

DPPH, 1, 1-diphenyl-2-hydrazyl.

DISCUSSION

In this study, topical application of *P. madiensis* extract significantly accelerated the contraction of excision wounds of rats. Contraction of wounds commences at about a week after wounding due to the action of myofibroblasts (Mohan, 2005; Eichler and Carlson, 2006). Though we did not study the mechanism involved in the contraction of wound by the extract earlier, Shiv-hare et al. (2006), attributed the wound healing effect of *Trichosanthes dioca* to its ability to stimulate interleukin-8 production. This cytokine functions in the recruitment of inflammatory cells, fibroblasts and keratinocytes (Shivhare et al., 2006). Furthermore, Akkol et al. (2009) ascribed the ability of *Arneba densiflora* to cause rapid wound closure to its ability to stimulate the synthesis of collagen, fibronectin and transforming growth factor B1. Therefore we conclude that *P. madiensis* might have accelerated wound contraction by stimulating the release of any of the aforementioned cells known to enhance wound healing.

Wound epithelialisation is the process of epithelial regeneration after wounding and occurs as epithelial cells
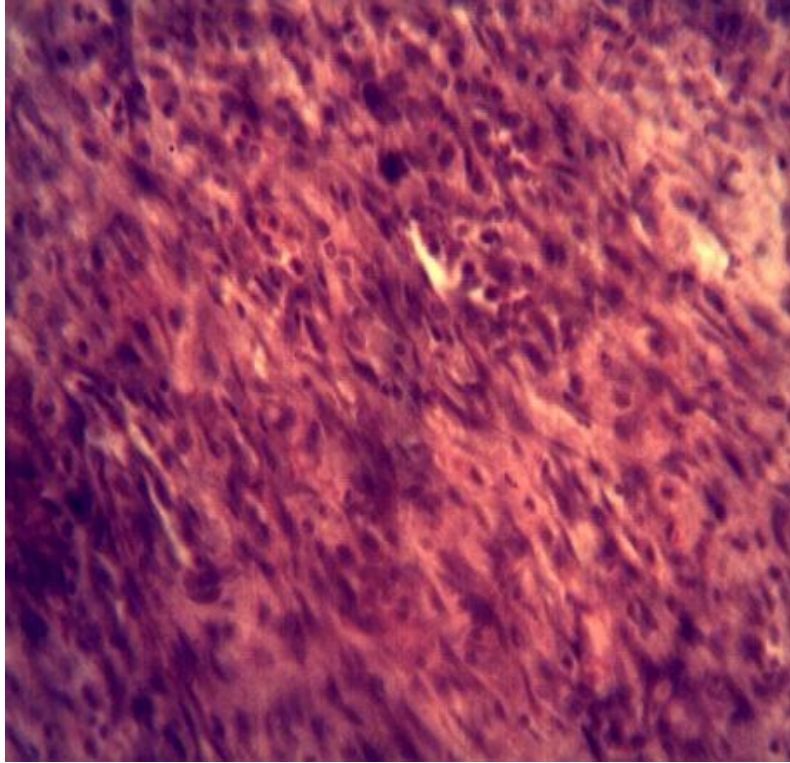
Figure 1. Normal saline treated wound on day 7 showing tissue infiltration with high number of neutrophils, moderate number of macrophages and few fibroblast.
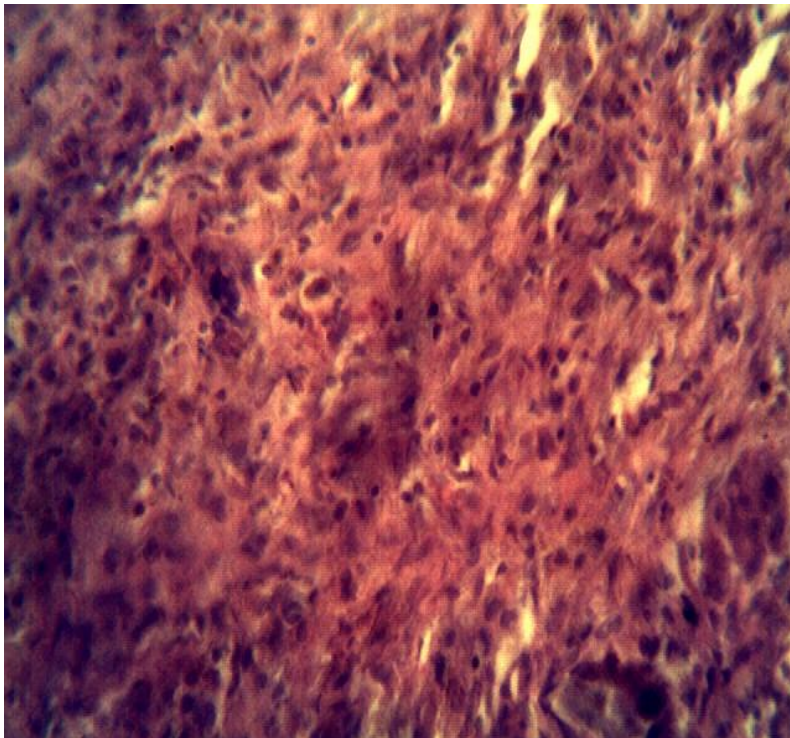

Figure 2. Normal saline treated wound on day 14 showing few fibroblasts, moderate number of neutrophils and high number macrophages.
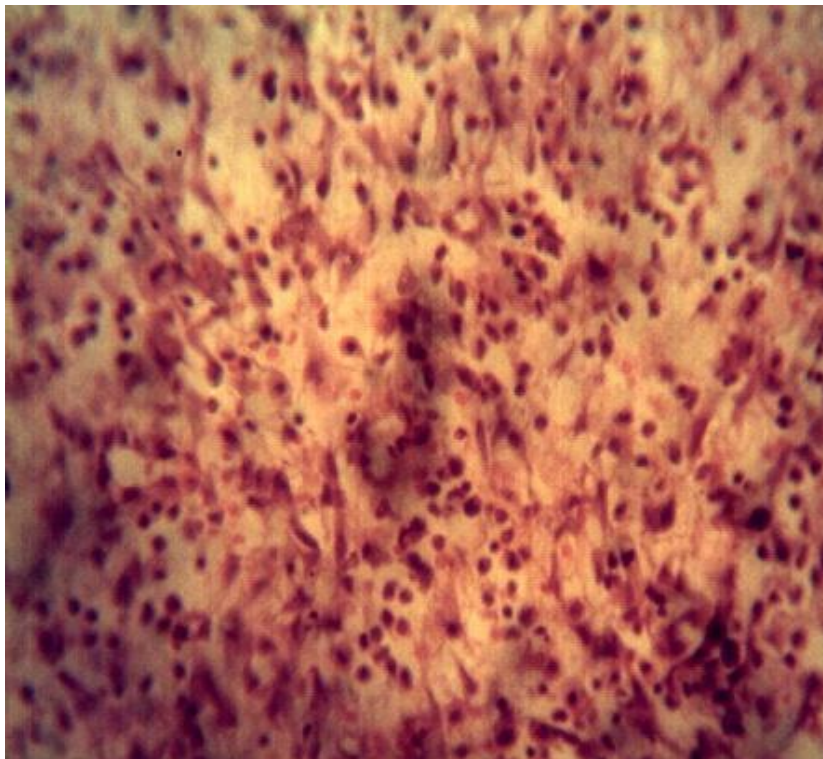
Figure 3. Leaf extract treated wound on day 7 showing moderate number of macrophages, high number of neutrophils and moderate fibroblast proliferation.
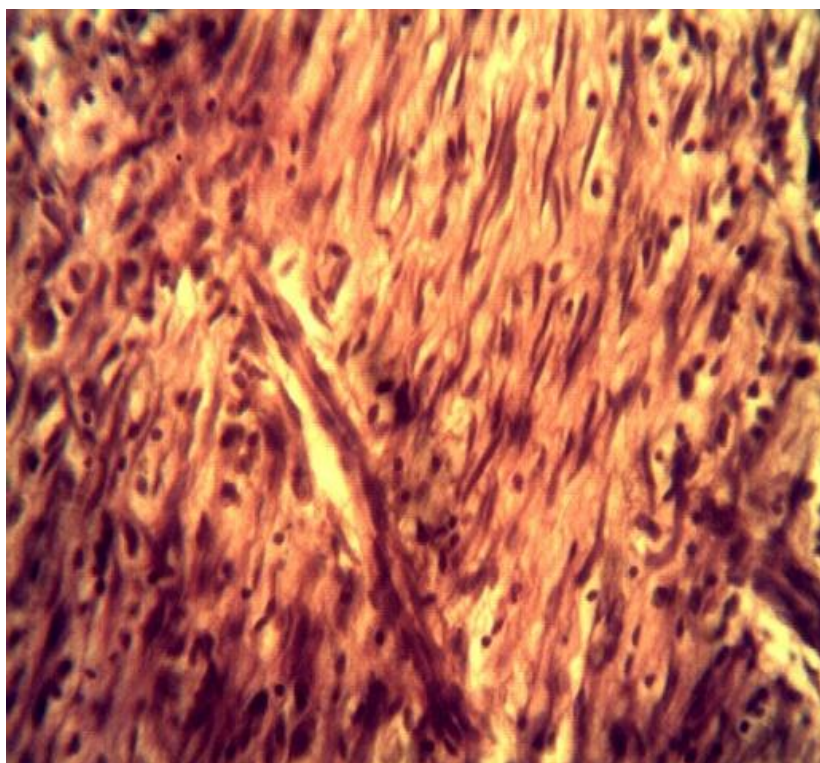

Figure 4. Leaf extract treated wound on day 14 showing few macrophages and neutrophils, high number of fibroblasts and significant collagenization.
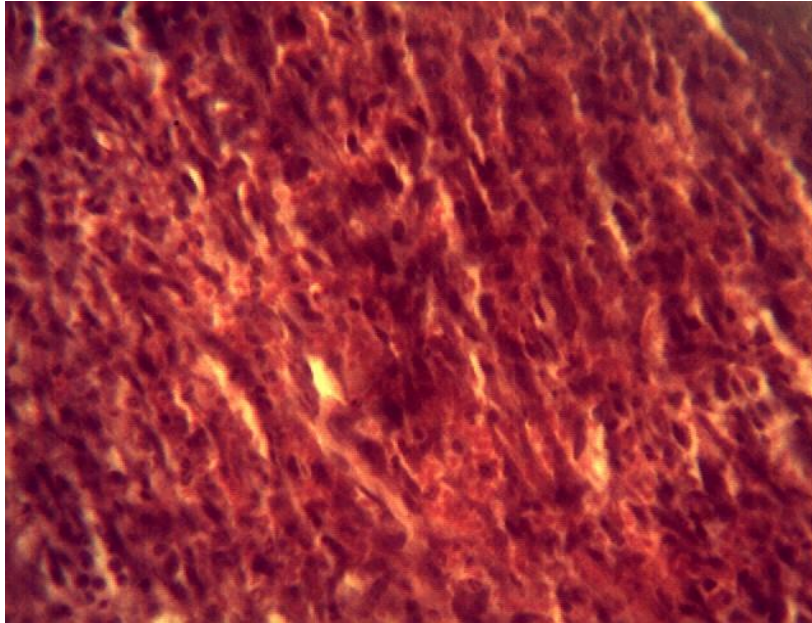
Figure 5. Cicatrin® treated wound on day 7 showing few fibroblasts, moderate number of neutrophils and macrophages as well as newly formed capillaries.
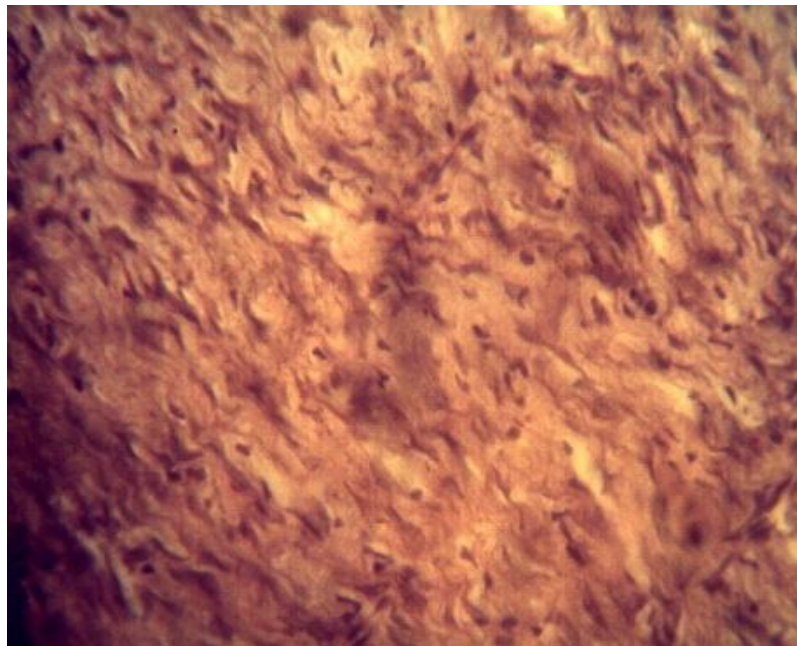

Figure 6. Cicatrin® treated wound on day 14 showing few macrophages and neutrophils, high number of fibroblasts and significant collagenization.

proliferate and migrate over the wound bed providing cover for the newly formed tissues (Cotran et al., 1994). The extract might have enhanced rapid epithelial proliferation due to its ability to promote angiogenesis and collagen synthesis (Cohen et al., 1992; Szabo et al., 1995; Deshmukh et al., 2009). According to Szabo et al.

(1995), angiogenesis in granulation tissues improves circulation providing oxygen and nutrients needed for healing and re-epithelialisation to occur. Collagenation on the other hand plays a central role in wound healing since collagen fibers provide the structural framework for the regeneration of tissues (Cohen et al., 1992).

The presence of fibroblasts in wound site marks the beginning of the proliferative phase of wound healing. Fibroblasts begin to enter the wound site two to five days post wounding as the inflammatory phase ends (Romo, 2010). These cells function in the synthesis of collagen fibers and their penetration of the areolar connective tissue leads to connective tissue maturation and increased wound strength (Nasrabadi et al., 2011). In this study, more number of fibroblasts were seen in the wounds of the leaf extract treated rats by day seven post wounding compared to the fibroblast number in the wounds treated with normal saline and cicatrin®. By day 14 of the study, fibroblasts proliferation and collagenization in the extract treated wounds were comparable to those in the cicatrin® treated wounds. These results show that the leaf extract of *P. madiensis* accelerated fibroblast proliferation and collagen synthesis. This finding adds further credence to our claim that this extract accelerated wound closure and epithelialisation

Post operative wounds are often complicated by microbial infection (Patil and Sunil, 2008; Deshmukh et al., 2009). Studies have shown that wound infection delays wound closure by suppressing the onset of the proliferative phase of wound healing as well as wound remodeling and matrix synthesis (Yates et al., 2007; Eming et al., 2007; Gutierrez-Fernandez et al., 2007). Wound bacteria also produce enzymes and toxins leading to the release of free radical and lytic enzymes at the wound site (Travis et al., 1995). These free radicals contribute to delay in wound healing by destroying lipids, proteins and extracelluar matrix (Calabrese et al., 2000). Studies have shown that plants with antimicrobial (Okoli et al., 2007; Oluwatnyi et al., 2004; Ezike et al., 2010) and anti-oxidant (Calabrese et al., 2000; Moreno et al., 2006; Abu-Al-Basal, 2010) properties promoted wound healing. Thus we infer that both pharmacological properties inherent in *P. madiensis* contributed to its wound healing effect.

Phytochemical screening showed that flavonoids, alkaloids and tannins were highly present in *P. madiensis* extract. Earlier studies showed that, flavonoids, alkaloids and tannins were identified in plants such as *Adhatoda vasica* (Shivhare et al., 2010), *Flabellaria paniculata* (Olugbuyiro et al., 2010) and *Calotropis gigantea* (Deshmukh et al., 2009) with wound healing activities.

Flavonoids and alkaloids isolated from medicinal plants have been shown by various experiments to possess anti microbial (Al-Saleh et al., 1997; Singh and Nath, 1999; Quarenghi et al., 2000; Karou et al., 2006) and antioxidant (Shivhare et al., 2010; Olugbuyiro et al., 2010) activities. Tannins on the other hand aid wound healing by promoting the regeneration and deposition of new tissues (Leite et al., 2002). We are therefore of the opinion that these phytoconstituents acted synergistically to mediate the wound healing effect of *P. madiensis*.

In conclusion, the results obtained in this study show that the leaf extract of *P. madiensis* significantly enhanced wound contraction, epithelialisation, fibroblast proli-feration and collagen deposition. The results of this study provide scientific evidence supporting the use of *P. madiensis* in traditional wound care.

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