

Wound Healing Properties of Tissue-Cultured *Pluchea indica* (L.) Less. Root Extract in Rats

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ABSTRACT

Excision, incision and dead space wound models were used to evaluate the wound-healing activity of tissue-cultured root extract of *Pluchea indica* (L.) Less. (Asteraceae) on Swiss Wistar strain rats of either sex. In each model, animals were divided into four groups of 6 animals each. In the excision wound model, treatment was continued until the complete healing of the wound but in the incision and dead space wound models treatment was continued for 10 days. 10% (w/w) ointment of methanolic root extract of tissue-cultured *P. indica* was prepared and applied topically at 50 mg/day and for oral administration, 200 mg/kgbw/day of aqueous suspensions of methanol root extracts were prepared in 1% Tween 80 solution. The wound-healing activity was assessed by the rate of wound contraction, period of epithelialization, skin-breaking strength, weight of granulation tissue, and collagen content. Both formulations of methanolic root extract of tissue-cultured *P. indica* revealed wound-healing activity significantly in all the wound models studied. A high rate of wound contraction, a decrease in the period of epithelialisation, high skin breaking strength, an increase in dry granulation tissue weight, and elevated hydroxyproline content were observed in animals treated with both formulations. Histological studies of the granulation tissue showed the presence of few inflammation cells, and an increase collagen formation compared to the control. Both the formulation of the root extract of tissue-cultured *P. indica* possesses potent wound-healing activity.

Keywords: epithelialisation, root, skin-breaking strength, wound models

INTRODUCTION

Wound healing is the process of repair that follows injury to the skin and other soft tissues. Due to injury, an inflammatory response occurs and the cell below the dermis begins to increase collagen (connective tissue) production and later the epithelial tissue (the outer skin layer) for their regeneration.

Pluchea indica (L.) Less. (Asteraceae) is a large, ever-green shrub found abundantly in salt marshes and mangrove swamps in Sunderbans (India), Bangladesh, Myanmar, China, Philippines, Malaysia, Tropical Asia and Australia. In Indo-China the decoction of roots is prescribed for fevers as a diaphoretic and an infusion of the leaves is given internally to treat lumbago while the roots and leaves are used as an astringent and antipyretic (Kirtikar and Basu 1999). The plant is also used to cure rheumatoid arthritis (Chatterjee 1996). The root extract has anti-inflammatory (Sen and Nag Chowdhury 1991), antiulcer (Sen *et al.* 1993), and neuropharmacological (Thongpraditchote *et al.* 1996) properties. The hypoglycemic and antihyperglycemic effects of *P. indica* leaves (Pramanik *et al.* 2006) and the antioxidant activity (Sen *et al.* 2002) of roots have already been reported. Four of the pure compounds (R/J/1-stigmasterol (+ β -sitosterol), R/J/2-stigmasterol glucoside (+ β -sitosterol glucoside), R/J/3-2-(prop-1-ynyl)-5 (5,6-dihydroxyhexa-1,3-dienyl)-thiophene, and R/J/4-(-) catechin) isolated from its roots have been reported to possess potent antimicrobial activities (Biswas *et al.* 2005). A eudesmane derivative from the leaves (Mukhopadhyay *et al.* 1983), five terpenic glycosides from aerial parts (Uchiyama *et al.* 1989), three eudesmane-type sesquiterpenes and three lignan glycosides, together with a eudesmane-type sesquiterpene from roots (Uchiyama *et al.* 1991) and two thiophene derivatives, besides two pentacyclic triterpenes of rare occurrence from roots (Chakravarty and Mukhopadhyay 1994) have been isolated

from this plant. A pure compound (R/J/3) isolated from the roots of *P. indica* was found to be very effective against *Entamoeba histolytica* (Biswas *et al.* 2006). *P. indica* was tissue cultured with the purpose of obtaining more secondary metabolites. Tissue-cultured *P. indica* leaves have a diuretic effect (Pramanik *et al.* 2007). The root extract of tissue cultured *P. indica* has antibacterial activity (Pramanik *et al.* 2008) and antitumor activity (Pramanik *et al.* 2008). The objective of this study was to access the wound-healing activity of tissue-cultured *P. indica* root extract of which there are no reports in the main-stream literature.

MATERIALS AND METHODS

Material

The roots of tissue-cultured *P. indica* (8 months matured), based on the protocol by Pramanik *et al.* (2007), were separated, washed, oven-dried at 60°C, powdered by a micropulverizer and sieved through 100 meshes (0.0254 cm diameter). Fibers and unwanted debris were discarded after sieving. The powder was preserved in an airtight container for further use.

Extraction

The pulverized powder (500 g) was soaked overnight with petroleum ether (60-80°C for synthesis, Merck), dried at room temperature and extracted with methanol (AR Grade, Purity 99.8%, Sisco Research Laboratories Pvt. Ltd.) using a Soxhlet extractor to obtain the methanolic extract of *P. indica* (MEPI). The solvent was then evaporated under reduced pressure using a rotary evaporator (Model no. HS-2005V) to obtain a semisolid residue. The yield of the extract was 8.7% (w/w).

Animals

Healthy inbred gender-matched Swiss Wistar rats weighing 200-220 g (supplied Reeta Ghosh) were used for the study. The animals were kept under standard conditions of 12: 12 h light: dark cycle in polypropylene cages and fed with standard laboratory diet and water *ad libitum*. Animals were weighed before and after the experiment. Rats were anaesthetized prior to and during infliction of the experimental wounds. The surgical interventions were carried out under sterile conditions using ketamine anaesthesia (Ket-A-100 from Agrovet Pvt. Ltd., 5-10 mg/kg). Animals were closely observed for any infection and those which showed signs of infection were separated and excluded from the study and replaced. The study was approved by the University Animal Ethical Committee. A previously acute toxicity study in our laboratory revealed that the extract is safe to use orally even at doses of 3.2 g/kg of body weight.

Drug formulation

Two types of drug formulations were prepared from the extracts. For topical administration, 10% (w/w) ointment was prepared in 2% sodium alginate. For oral administration, an aqueous suspension of methanolic root extracts were prepared and applied at 200 mg/kg bw/day orally.

Wound-healing activity

Excision, incision and dead space wound models were used to evaluate the wound-healing activity.

Excision wound

The rats were inflicted with excision wounds as described by Morton and Malone (1972) under light ether anaesthesia. A circular wound of about 500 mm² was made on a depilated ethanol-sterilized dorsal thoracic region of the rats. The animals were divided into four groups of six each. The animals of group I were left untreated and considered as the control, group II served as reference standard and treated with sulphathiazole ointment, animals of group III was treated daily with 50 mg of ointment (10% w/w) prepared from methanol root extract of tissue cultured *P. indica* and animals of group IV was treated with methanolic root extract aqueous suspension of tissue cultured *P. indica* (200 mg/kg bw/day) orally. The ointment was topically applied once a day, starting from the day of the operation, till complete epithelialisation. The parameters studied were wound closure and epithelialisation time. The wounds were traced on mm² graph paper on days 3, 6, 9, 12, 15 and 18 and thereafter on alternate days until healing was complete. The percentage of wound closure was calculated. The period of epithelialisation was calculated as the number of days required for falling of the dead tissue remnants of the wound without any residual raw wound.

Incision wound

In the incision wound model, 6 cm long paravertebral incisions were prepared through the full thickness of the skin on either side of the vertebral column of the rat as described by Ehrlich and Hunt (1968). The wounds were closed with interrupted sutures 1 cm apart. The animals were divided into four groups of six animals each. The animals of group I were left untreated and considered as the control, group II served as reference standard and received sulphathiazole ointment, animals in groups III and IV were treated with 50 mg of ointment (10% w/w) topically prepared from methanol root extract and 200 mg/kg/day methanolic root extract aqueous suspension orally respectively. The ointment was topically applied once in a day. The sutures were removed on the 8th post-wound day. The skin breaking strength of the wounds was measured on the 10th day as described in the method of Lee (1968).

Dead space wound

The animals were divided into four groups of 6 rats in each group. Group I served as the control, which received 1 ml of 1% gum tra-

gacanth/kg bw/po. The group II served as reference standard and received sulphathiazole ointment. The animals of group III and IV received 50 mg of *P. indica* ointment and oral aqueous suspensions of methanol extracts of *P. indica* (200 mg/kg bw/po), respectively. Under light ether anaesthesia, dead space wounds were created by subcutaneous implantation of sterilized cylindrical grass piths (2.5 cm × 0.3 cm), one on either side of the dorsal paravertebral surface of the rat (Turner 1965). The granulation tissues formed on the grass piths were excised on the 10th post-wounding day and the breaking strength was measured. Simultaneously, granulation tissue so harvested was subjected to hydroxyproline estimation following the method of (Stegemann 1958) and histopathological study to evaluate the effect of the extracts on collagen formation.

Determination of wound breaking strength

The anesthetized animal was secured to the table, and a line was drawn on either side of the wound 3 mm away from the line. This line was gripped using forceps one at each end opposed to each other. One of the forceps was supported firmly, whereas the other was connected to a freely suspended light weight metal plate. The weight was added slowly and the gradual increases in weight, pulling apart the wound edges. As the wound just opened up, addition of weight was stopped and the weights added was noted as a measure of breaking strength in grams. Three readings were recorded for a given incision wound, and the procedure was repeated on the contralateral wound. The mean reading for the group was taken as an individual value of breaking strength. The mean value gives the breaking strength for a given group.

Estimation of hydroxyproline content in the granulation tissue

The method of Stegemann (1958) was employed. Granulation tissue of each rat was excised and freed from extraneous tissues, weighed accurately and hydrolyzed at 130°C for 3 h with 5.0 ml of 6N hydrochloric acid in a Pyrex test tube. The contents were decanted into a suitable container after washing thoroughly with glass-distilled water. The pH of the final solution was adjusted to 6-7 by addition of dilute NaOH. 2 ml of solution of each sample was placed in a test tube; 1 ml of 0.05 M solution of sodium-*p*-toluene sulfone chloramide (Chloramine T) was added to each tube, mixed thoroughly and allowed to stand for 20 min at room temperature. 1 ml of 3.15 M perchloric acid was added to it, mixed and again allowed to stand for another 5 min. Finally 1 ml of *p*-dimethyl aminobenzaldehyde solution (20%) was added to the mixture and shaken thoroughly. The tube was then placed in a water bath at 60°C for 20 min and cooled in tap water for 5 min. The absorbance of the solution was measured at 557 nm in a Spectronics 20-spectrocolorimeter.

A series of standard hydroxyproline solution were prepared by adjusting the concentration of standard hydroxyproline solution in such a way that 2 ml solution in the 1st tube contained 10 µg of hydroxyproline and so on up to the 6th tube which contained 60 µg of hydroxyproline. To these test tubes, reagents in the same quantity and in the same order as described earlier were added and the optical density was measured. A standard curve was plotted with concentrations of hydroxyproline versus absorbance. Amount of hydroxyproline present in the test sample were determined directly from the standard curve.

Histopathological study

The healing tissues obtained on the 11th day from all four groups of animals of the dead space wound were processed for histological study. Pieces of granulation tissue of four groups of animals were fixed in 4% formalin (10 ml of 40% formaldehyde made up to 100 ml with normal saline) overnight. Then the tissue were dehydrated with graded concentration of alcohol, cleared with benzene and embedded in a paraffin bath. The paraffin blocks were cut at 5 µm in a rotary microtome (Model: MT-1090A, WESWDX, OPTIK) and sections were stained with vanegason and mounted in Canada balsam on slides to observe the amount of collagen formed.

Table 1 Effect of application of tissue-cultured *P. indica* root extract ointment and suspension on healing of excision wound model.

Group	Post wounding days							Period of epithelialisation
	0-day	3 rd day	6 th day	9 th day	12 th day	15 th day	18 th day	
Control	510.25 ± 1.03 (0.00)	491.75 ± 1.18 (3.62)	421.75 ± 2.68 (17.34)	285.75 ± 2.17 (43.99)	235.25 ± 1.03 (53.89)	153.00 ± 1.08 (70.01)	74.50 ± 1.32 (85.39)	22.00 ± 0.40
Standard	509.75 ± 0.25* (0.00)	421.25 ± 1.49* (17.34)	297.00 ± 3.13* (41.73)	154.75 ± 2.05* (69.64)	84.75 ± 1.70* (83.37)	12.50 ± 0.64* (97.54)	0* (100)	15.50 ± 0.28
<i>P. indica</i> Ointment (10% W/W)	508.00 ± 0.40* (0.00)	433.25 ± 1.97* (14.71)	306.50 ± 1.55* (39.66)	171.25 ± 1.49* (66.28)	91.25 ± 1.49* (82.03)	22.50 ± 1.04* (95.57)	0* (100)	17.25 ± 0.25
<i>P. indica</i> Suspension (200mg/kg/d)	508.00 ± 0.57* (0.00)	446.00 ± 2.08* (12.20)	326.25 ± 1.75* (35.77)	192.50 ± 1.04* (62.10)	99.50 ± 1.84* (80.41)	31.25 ± 1.49* (93.84)	1.75 ± 0.47* (99.65)	18.25 ± 0.14

Values are express as mean ± SEM; *n* = 6 animals in each group; Number in parenthesis indicate percentage of wound contraction; **P* < 0.001 when compared to the control.

Statistical analysis

The data were subjected to one way ANOVA followed by Dunnett's test and the values of *P* ≤ 0.001 were considered statistically significant.

RESULTS

A significant increase in the wound-healing activity was observed in animals treated with both the formulations of tissue-cultured *P. indica* root extract compared with the control. In the excision wound model, the mean percentage closing of the wound area was calculated on the 3, 6, 9, 12, 15, and 18 post wounding days (Plate 1; Table 1) and animals treated with both formulations showed significant reduction of the wound area (*P* < 0.001) compared to the control. The 10% (w/w) ointment of methanolic root extract-treated animals showed faster epithelialization of wound (17.25 ± 0.25) than animals treated with methanolic root extract suspension (18.25 ± 0.144) days. In the case of standard drug sulphathiazole ointment, the period of epitheliali-

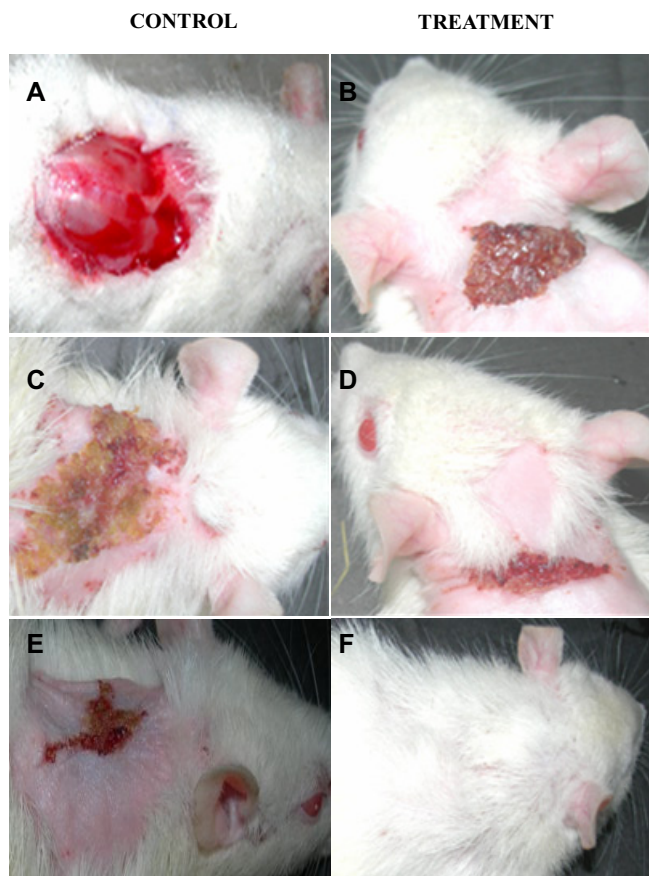


Plate 1 Effect of tissue-cultured *P. indica* root extract ointment (10% w/w) on wound healing. A = Day 0; B = Day 6; C, D = Day 9; E, F = Day 18.

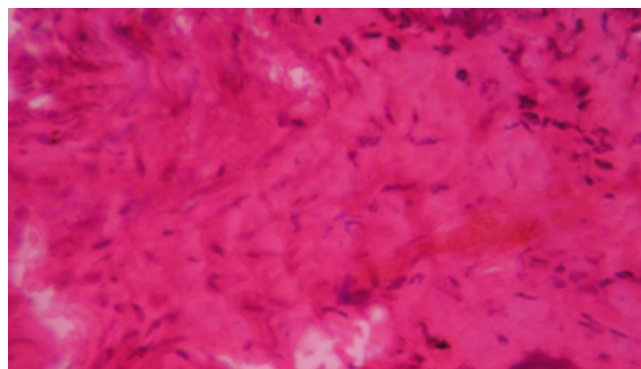


Fig. 1 Granulation tissue of group I animal (Control) showing with less collagen and more macrophages. (Magnification: 400X)

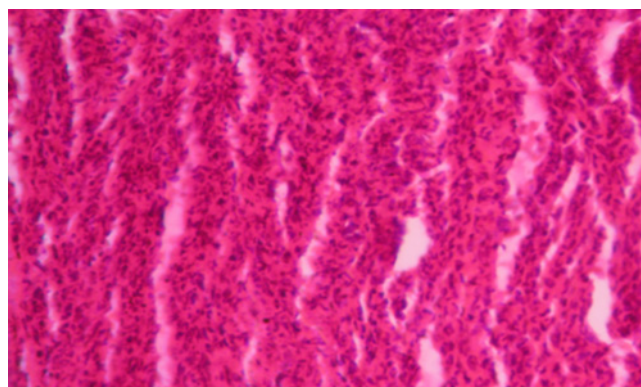


Fig. 2 Granulation tissue of group 2 (standard) animal showing moderate deposition collagen. (Magnification: 400X)

zation was 15.50 ± 0.28 days. In the incision wound model, the ointment and the aqueous suspension of the methanolic extract treated animals showed increased breaking strength (380.00 ± 5.77 and 355.33 ± 2.90, respectively) when compared to the control (312.33 ± 1.45). The mean breaking strength was also significant in animals treated with standard drug (424.33 ± 2.33).

In the case of the dead space wound model both hydroxyproline and granulation tissue dry weight were moderately high (*P* < 0.001) in comparison to the control group of animals. The histological studies of the granulation tissue of the control group of animals (Fig. 1) showed more aggregation of macrophages with less collagen fiber. The two formulations treated group was more or less equal to the animals treated with sulphathiazole (Fig. 2). It was further revealed that the section of granulation tissue obtained from the dead space wound model of 10% (w/w) ointment of *P. indica* extract (Fig. 3) and methanolic root extract suspension (Fig. 4) treated animals showed a significant increase in collagen deposition, few macrophages, tissue edema and more fibroblasts compared to the control group animals (Tables 2, 3).

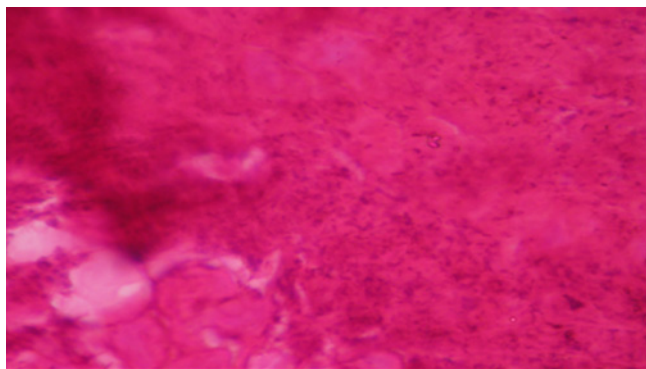


Fig. 3 Granulation tissue of group 3 (10% w/w ointment of tissue cultured *P. indica* root extract) animals showing more collagen and less macrophages. (Magnification: 400X)

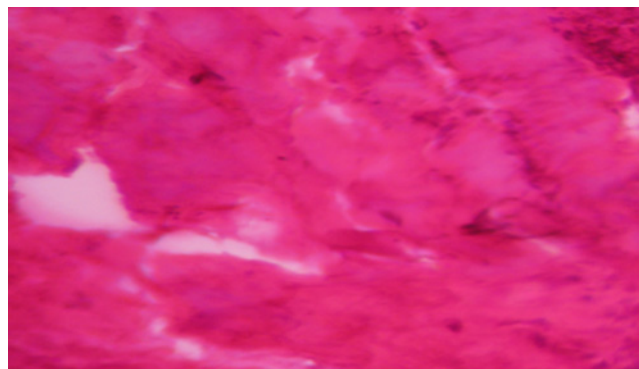


Fig. 4 Granulation tissue of group 4 (200 mg/kg/d methanolic root extract of tissue cultured *P. indica*) treated animal showing moderate deposition collagen. (Magnification: 400X)

Table 2 Effect of application of tissue-cultured *P. indica* root ointment and suspension in incision wound model.

Group	Skin breaking strength (g)	Granulation tissue wet weight (mg)	Granulation tissue dry weight (mg)	Hydroxyproline content (mg/g)
Control	312.33 ± 1.45	75.00 ± 2.30	8.70 ± 0.55	155.33 ± 2.02
Standard	424.33 ± 2.33*	121.33 ± 1.85*	15.86 ± 0.59 *	207.66 ± 1.45*
<i>P. indica</i> ointment (10%)	380.00 ± 5.77*	117.66 ± 1.45*	15.93 ± 0.52*	192.33 ± 1.45*
<i>P. indica</i> suspension (200 mg/kg/d)	355.33 ± 2.90*	112.33 ± 1.45*	14.70 ± 0.92*	85.00 ± 2.88*

Values are express as mean ± SEM; n = 6 animals in each group; *P < 0.001 when compared to the control.

Table 3 Wound-healing effect of tissue-cultured *P. indica* root extract ointment and suspension in dead space wound model.

Group	Granulation tissue wet weight (mg/100 g rat)	Granulation tissue dry weight (mg/100 g rat)	Hydroxyproline content (mg/g)
Control	78.87 ± 1.78	8.45 ± 0.31	29.12 ± 1.59
Standard	127.66 ± 1.45*	18.02 ± 0.46*	62.07 ± 1.59*
<i>P. indica</i> ointment (10%)	112.73 ± 1.45*	16.50 ± 0.76*	69.75 ± 2.13*
<i>P. indica</i> suspension (200 mg/kg/d)	108.33 ± 2.02*	13.83 ± 0.92*	68.25 ± 2.32*

Values are express as mean ± SEM; n = 6 animals in each group; *P < 0.001 when compared to the control.

DISCUSSION

Wound healing is one of the basic biologic phenomena of the entire animal kingdom and is the product of the integrated response of several cell types to injury. The products of wound healing are generative and differentiated. The healing process is physiologic although the sequel of a wound, or aberrations of its repair, may be pathologic. Wound healing is a process by which a damaged tissue is restored as closely as possible to its normal state and wound contraction is the process of shrinkage of area of the wound. It depends upon the reparative abilities of the tissue, type and degree of the damage and general state of the health of the tissue. The granulation tissue of the wound is primarily composed of fibroblast, collagen, edema, and small new blood vessels (Nayak *et al.* 2006). Collagen is a major protein in the intracellular matrix and is the component that finally contributes the wound strength. Breakdown of collagen liberates free hydroxyproline and its peptides. Measurement of hydroxyproline could be used as an index for collagen turnover (Manjunatha *et al.* 2005).

The hydroxyproline content of the granulation tissue of the animals treated with 10% (w/w) ointment of methanolic root extract of tissue cultured *P. indica* and the aqueous suspension of methanolic root extract was significantly increased when compared to the control group of animals (Table 2), indicating an increase in collagen turnover (Nayak *et al.* 2006).

On the other hand an increase in dry tissue weight also indicated the presence of higher protein content. In addition, an increase in breaking strength of the granulation tissue of 10% (w/w) ointment and aqueous suspension of methanolic root extract of tissue-cultured *P. indica* treated animals indicated the enhanced collagen maturation by increased cross-linking.

A preliminary phytochemical investigation of methanolic root extract of tissue-cultured *P. indica* showed the pre-

sence of flavanoids, alkaloids, triterpenoids and glycosides. Flavanoids are known to reduce lipid peroxidation not only the preventing or slowing onset of cell necrosis but also by improving vascularity (Manjunatha *et al.* 2005). Hence any drug that inhibits lipid peroxidation is believed to increase the viability of collagen fibrils by increasing the strength of collagen fibres, increasing circulation, preventing cell damage and promoting DNA synthesis (Manjunatha *et al.* 2005). Flavonoids and triterpenoids (Manjunatha *et al.* 2005) are also known to promote the wound healing process mainly due to their astringent and antimicrobial properties, which seems to be responsible for wound contraction and increase rate of epithelialisation. Thus wound healing potency of tissue-cultured *P. indica* root extract may be attributed to the phytoconstituents presents in it which may be either due to their individuals or additive effect that fasten the process of wound healing.

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