Wound Healing and Cytoprotective Actions of *Paullinia pinnata* L.

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**INTRODUCTION**

Wounds are physical injuries that result in an opening or breaking of the skin. Proper healing of wound is essential for the restoration of disrupted anatomical continuity and disturbed functional status of the skin.¹ Wound healing is a complex process characterized by inflammation, proliferation and migration of different cell types. In cutaneous tissue repair, oxidants and antioxidants play very important roles. Oxidants are known to have the ability to cause cell damage and may function as inhibitory factors to wound healing.² Natural products, particularly those of plant sources, provide a rich source of wound healing remedies in many cultures around the world where extracts, juices and exudates are used. Examples of these include *Ficus asperifolia*, *Gossypium arboreum* and *Chromoleana odorata*.³ These plants, although diverse in terms of taxonomy, contain one or more active constituents that may influence wound healing.

*Paullinia pinnata* L. (Sapindaceae) is a tropical plant which is used traditionally in the treatment of infectious diseases, fractures and as an aphrodisiac.⁴ It is also used in wound healing in the form of a poultice applied externally twice daily for a week in some parts.
of Ghana. Earlier work on *P. pinnata* has established its molluscicidal and antioxidant properties while previous phytochemical investigations have shown the presence of triterpene saponins, catechol tannins, flavone glycosides, paullinsonide A, paullinomide A, β-sitosterol and β-amarin. As a follow up on our earlier report on the wound healing actions of *P. pinnata*, we have investigated the effect of *P. pinnata* extract on excision and incision wound models in vivo and the cytoprotective effect of the extracts on human dermal fibroblast cell line [142BR].

**MATERIALS AND METHODS**

**Plant materials**

Plant materials were collected in the month of August, 2007 and authenticated at the Centre for Scientific Research into Plant Medicine (CSRPM), Akwapim-Mampong, Ghana, where voucher specimen (8/05/17) has been deposited.

**Preparation of extract**

200 gm of dried powdered root was packed into a cellulose thimble (28×100 mm) and soxhlet-extracted with 500 ml methanol over 48 hours until the material was exhausted, and the extract dried in vacuum to give a yield of 4.92% w/w.

**Animals used**

Male Sprague-Dawley rats (160-180 g) were purchased from the animal house of the Central Drug Research Institute, Lucknow, India. They were kept at 26 ± 2°C and relative humidity of 44–55% and light and dark cycles of 10 and 14 hours respectively, for one week before the experiment. Animals were given the rodent diet (Amruth, India) and water ad libitum. All studies were conducted in accordance with the National Institute of Health's guidelines for Survival Rodent Surgery after approval from the institutional ethics committee. All surgical procedures were carried out under thiopentone sodium (25 mg/kg, i.p.) anaesthesia. Animals were allowed to recover and were housed individually in metallic cages containing sterilized paper cuttings.

In the experiment, the rats were divided into three groups (n=6). Group 1 was the control group which received simple ointment BP base, group 2 was treated with the reference standard (0.2% w/w nitrofurazone, a standard antimicrobial agent used in topical wound dressings), group 3 received *P. pinnata* ointment (33.3% w/w *P. pinnata* methanol extract in simple ointment BP) topically, on wounds created on the dorsal skin of rats daily, until the wounds completely healed. 100 mg of ointment was spread over 500 mm² area.

**Excision wound model**

An impression was made on the dorsal thoracic region 1 cm away from the vertebral column and 5 cm away from the ear using a biopsy punch (Acuderm, USA) of 2.5 cm diameter, on the anaesthetized rat. The skin of the impressed area was excised to its full thickness to obtain a wound area of about 500 mm². Haemostasis was achieved by blotting the wound with a cotton swab soaked in normal saline.

**Wound area**

Contractions, which contribute to wound closure in the first two weeks, were studied by tracing the edges of the raw wound. The wound area was measured after specific time intervals by retracing the wound on a millimeter scale graph paper. The difference in the area of the wound indicated the degree of wound healing.

**Collagen estimation**

Hydroxyproline, which is a basic constituent of collagen was measured. Tissues were dried in a hot air oven at 60-70°C to a constant weight and were hydrolysed in 6M HCl at 130°C for 4 hours in sealed tubes. The hydrolysate was neutralized to pH 7.0 and was subjected to chloramine-T oxidation for 20 min. The reaction was terminated by addition of 0.4 M perchloric acid and colour was developed with the help of Ehrlich reagent at 60°C and absorbance measured at 557 nm using the Pye Unicam spectrophotometer.

**Incision wound model**

Rats were anaesthetized and two paravertebral long incisions made through the skin and cutaneous muscles at a distance of about 1.5 cm from the midline on each side of the depilated back of the rat. Full aseptic measures were not applied and no local or systemic antimicrobial was used throughout the experiment. Each of the three groups of animals was treated in the same manner as for the excision wound model. No ligature was used for stitching. After the incision was made, the parted skin was kept together and stitched with black silk at 0.5 cm intervals. Surgical thread [No. 000] and a curved needle [No. 11] were used for the stitching. Continuous threads on both wound edges were tightened for good wound closure. The wound was left undressed and *P. pinnata* L.
extract [PP] ointment along with the water-soluble base ointment [control] and nitrofurazone ointment were applied topically twice daily for 9 days. When wounds were healed completely, the sutures were removed on the ninth day and tensile strength was measured with a tensiometer.

**Tensile strength**

The tensile strength of the healing tissues was measured using the method of Govindarajan.[17] The sutures were removed on the ninth day after wounding and the tensile strength measured on the tenth day. The animals were anaesthetized and healing tissue along with normal skin at two ends was excised for tensile strength measurement using Tensi Testing Machine TKG-20 [from Fine Testing Machines, India]. Strips of 8mm width and 20 mm length were cut out from the excised tissue in treated and control animals and were loaded between the upper and lower holder of the machine in such a way that the effective load bearing size was 8 x 8 mm with the wound remaining in the centre. The total breaking load was measured in Newtons and the tensile strength was calculated by the following equation:

Tensile strength = Total breaking load / Cross-sectional area

The *P. pinnata* ointment along with the standard and control were applied throughout the period, twice daily for 9 days. The mean tensile strength on the two paravertebral incisions on both sides of the animals was taken as the measures of the tensile strength of the wound for an individual animal. The tensile strength of *P. pinnata* ointment treated wounds was compared with the control and nitrofurazone ointment as the standard. Further epithelization period and scar area were measured daily for 25 days after tensile strength determination.[14]

**Protection of fibroblast (142BR) cells against hydrogen peroxide-induced damage**

The fibroblast cells were cultured in minimum essential medium (MEM) supplemented with 10% FBS, 1% penicillin/1% streptomycin and 1% L-glutamine and maintained at 37°C in a 5% CO₂-air atmosphere. Based on the growth profile, the optimal plating density of the cell line was determined to be 5000 cells/well, to ensure almost confluent growth during the period of the experiment. The optimal concentration of hydrogen peroxide that can induce a recoverable damage on fibroblast cells of concentration 5000 cells/well was determined using a modified assay procedure by Murrell.[18] Here, the culture medium was removed and cells exposed to different concentrations from 10⁻⁴ M to 10⁻³ M, of freshly prepared hydrogen peroxide in HBSS for 3 hours at 37°C. The cells were washed again with PBS and MEM containing 10% FBS added. The cell damage was evaluated using Neutral Red assay and also by visual examination. A concentration of 1x10⁻⁴ M was selected as the suitable dose of hydrogen peroxide because cells treated with this concentration of hydrogen peroxide were able to recover from damage and assume an almost perfect shape after treatment and incubation with MEM containing 10% FBS.

**Cytoprotective action of plant extract on human skin fibroblasts**

The method used for the hydrogen peroxide assay was the one described by Yamasaki et al.[19] and modified to evaluate the protective effect of the extracts on the cells against oxidant injury induced by hydrogen peroxide. Fibroblast cells were seeded at 5000 cells/well in a 96-well plate and incubated for five days until almost confluent. The growth medium was then discarded and the confluent cells subjected to three different types of experiments.

In the first experiment, the cells were pre-treated with different concentrations of the extract overnight, after which they were exposed to 10⁻³ M hydrogen peroxide in the standard growth medium and incubated for a further 3 hours.

In the second protocol, fibroblast cells were pre-incubated with the extract at different concentrations overnight, before exposure to the same concentrations of extract together with 10⁻⁴ M hydrogen peroxide in the growth medium.

In the third experiment, different concentrations of extract were applied simultaneously with 10⁻⁴ M hydrogen peroxide in the growth medium and incubated for 3 hours at 37°C. Catalase (250 unit/ml), an antioxidant enzyme was used as positive control in all experiments.

After the incubation period, the fibroblast cells were stained with Neutral Red and observed microscopically for cell damage, followed by the Neutral Red assay to quantify the degree of protection of fibroblast cells by the extract against hydrogen peroxide damage.

**Statistical analysis**

One-way ANOVA was used for the comparison of the means. Results are expressed as mean ± SD (standard deviation) data, using the GraphPad Prism Software, version 5.
RESULTS AND DISCUSSION

The results of the in vivo wound healing tests showed a decrease in the epithelization period from 26.7 days (control) to 12.7 days along with a marked decrease in the scar area of 54.2 mm² (control) to 26.2 mm² upon administration of P. pinnata ointment (Table 1). Significant increase in the tensile strength and hydroxyproline content was also observed as compared to the control and was comparable to the nitrofurazone.

The percentage close of excision wound area, which was an indication of degree of wound contraction, showed that P. pinnata extract significantly stimulated wound contraction with about 69.2% (P<0.01) in 7 days as compared to 46.2% given by the control and 67.5% by the nitrofurazone (Table 2).

The in vitro cytoprotective studies revealed the following results; the first and second protocols when fibroblast cells were pretreated with the extract to assess its effect on hydrogen peroxide induced damage, it was found by both microscopic examination and colorimetric assay that the cells were damaged (Fig. 1) and so were not protected. The cell damage however could be due to the inherent cytotoxicity of the extract rather than the effect of the hydrogen peroxide, due to the relatively high concentrations of the extracts used.

In the third protocol, different concentrations of extract were applied simultaneously with 10⁻⁴ M hydrogen peroxide in the growth medium and incubated for 3 hours at 37°C. Here, it was observed that fibroblast cells were significantly protected against hydrogen peroxide induced damage. The cytoprotective action was found to be 86% at 50 μg/ml, almost comparable with that of catalase (control at 250 units/ml) [Fig. 2].

1. Confluent fibroblast cells without any treatment. Cell density is thick and shapes look more elongated.
2. Fibroblast cells exposed to hydrogen peroxide only. There is reduced cell density and disruption of cell shape.
3. Fibroblast cells protected by catalase. Cell density is thick and cells are more elongated.
4. Fibroblast cells protected by extracts of P. pinnata against hydrogen peroxide damage. Cell density is high and cells look more elongated.

Flavonoids, tannins and triterpenoids have all been found in this specie and wound healing activity is often associated with these compound types. These active constituents promote the process of wound healing by increasing the viability of collagen fibrils and increase the strength of collagen fibres, either by increasing the circulation or by preventing the cell damage or by promoting the DNA synthesis.[20]

As the wound healing process involves various phases, the use of a single model is inadequate and no in vitro experiment exists that can collectively represent the various components of wound healing.[21] Because of this, in vivo assays are highly recommended to confirm the in vitro observations. Some of the in vivo assays of significance

Table 1. Effect of P. pinnata extract ointment on incision wound

<table>
<thead>
<tr>
<th>Topical treatment</th>
<th>Epithelisation period (days)</th>
<th>Tensile strength (g)</th>
<th>Scar area (mm²)</th>
<th>Hydroxyproline (mg/100mg tissue)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>26.7 ± 1.2</td>
<td>287.5 ± 17.3</td>
<td>54.2 ± 3.8</td>
<td>7.22 ± 0.34</td>
</tr>
<tr>
<td>PP ointment (100mg/500mm²)</td>
<td>12.7 ± 1.1b</td>
<td>422.9 ± 19.8a</td>
<td>26.2 ± 3.4a</td>
<td>10.27 ± 0.45c</td>
</tr>
<tr>
<td>Nitrofurazone (2% ointment) [100mg/500mm²]</td>
<td>11.5 ± 1.4a</td>
<td>428.2 ± 21.3a</td>
<td>27.9 ± 2.9c</td>
<td>11.7 ± 0.45b</td>
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Values are mean ± SEM for six rats.
Statistically significant difference in comparison with control group: a p<0.001, b p<0.01, c p<0.02.

Table 2. Effect of P. pinnata extract ointment on excision wound

<table>
<thead>
<tr>
<th>Topical treatment</th>
<th>Percentage of closed excision wound area after days</th>
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<tr>
<td></td>
<td>4</td>
</tr>
<tr>
<td>Control</td>
<td>27.4 ± 2.6</td>
</tr>
<tr>
<td>PP ointment (100mg/500mm²)</td>
<td>36.5 ± 2.9a</td>
</tr>
<tr>
<td>Nitrofurazone (2% ointment) [100mg/500mm²]</td>
<td>37.2 ± 3.1a</td>
</tr>
</tbody>
</table>

Values are mean ± SEM for six rats.
Statistically significant difference in comparison with control group: a p<0.01, b p<0.001.
include the determination of tensile strength, which is an indication of quality of the healing, and the determination of hydroxyproline content. Collagen is a major protein in the extracellular matrix and is the component that ultimately contributes to wound strength. Breakdown of collagen liberates free hydroxyproline. Measurement of
the hydroxyproline could therefore be used as an index for collagen turnover. In the present study, a significant increase in the hydroxyproline content of the granulation tissue of the animals treated with *P. pinnata* extracts was recorded compared to the control group, thus indicating the positive effect of the extract on collagen synthesis and hence, on wound healing. The increase in tensile strength of the granulation tissue indicated enhanced collagen maturation by increased cross-linking. The increase in tensile strength, as well as the epithelialization, could be attributed to the increased hydroxyproline content in the wound tissue.

The cytotoxicity of oxidants and the protective effects of the extract were indirectly assessed via cell viability. It was observed from the experiments with the fibroblast cells that pre-treatment of the cells with the extract gave no protective effect to the cells but rather caused damage to the cells due to the inherent cytotoxicity of the extract. However, the simultaneous application of the extract and oxidant (hydrogen peroxide) caused the cells to be protected to some degree. This is an indication that direct interaction of the extract with the oxidants (peroxide and superoxide) plays a major role in the protection of the cells in *vivo*. Furthermore, these observations suggest that the extract may not have caused any alteration to the cell membrane to protect it against oxidative damage.

**CONCLUSIONS**

*P. pinnata* extract has been shown to exhibit wound healing actions through both *in vitro* and *in vivo* models. The wound healing action is very significant and comparable to conventional wound healing remedy nitrofurazone, and the wound healing action may be attributed to the presence of phytochemicals such as flavonoids, tannins and triterpenoids that have been reported in this species. The increase in tensile strength, as well as the epithelialization, could be attributed to the increased hydroxyproline content in the wound tissue.

**ACKNOWLEDGEMENT**

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**ABBREVIATIONS**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>CO₂</td>
<td>Carbon dioxide</td>
</tr>
<tr>
<td>FBS</td>
<td>Foetal bovine serum</td>
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<tr>
<td>HBSS</td>
<td>Hank’ balanced salt solution</td>
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<tr>
<td>MEM</td>
<td>Minimum essential medium</td>
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<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
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**REFERENCES**