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Evaluation of herbal formulation PACT for wound healing potential

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ABSTRACT

The present manuscript was mainly focused to evaluate the multiherbal formulation PACT (containing extracts of Pongamia pinnata Linn., Artocarpus lakoocha, Cynodon dactylon Pers. and Tridax procumbens Linn.) for wound healing potential employing two common wound models i.e. excision wound and incision wound models. The wound contraction area and epithelialization period was measured in excision model while wound breaking strength and hydroxyproline contents were measured in incision wound model. The PACT significantly increased wound contraction, epithelialization, wound breaking strength and hydroxyproline contents when compared with control and the results were further supported by histopathological studies. This study concluded that this formulation exhibit potent wound healing efficacy in preclinical experiments and can further be studied for clinical purposes.

Keywords: Multiherbal Formulation, Excision wound, Incision wound, Hydroxyproline content, Wound breaking strength

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INTRODUCTION

Use of herbs as medicines is as old as human history. The world health organization estimated that about more than 80% of the world's population still rely on herbal medicines for their primary health care need. Today a huge amount of drugs are being procured from plants having vital therapeutic potential against a number of diseases. Our ancestors made novel discoveries of the healing power of herbal drugs through trial-error methods. Presently a plethora of herbs is used for their wound healing potential as per their traditional values and many of them has been scientifically investigated while a lot of are still waiting [1]. The present study was designed with special emphasis to investigate the wound healing activity of a multiherbal formulation PACT containing methanolic extracts of *Pongamia pinnata* Linn. (Fabaceae), *Artocarpus lakoocha* Roxb. (Moraceae), *Cynodon dactylon* Pers. (Poaceae) and *Tridax procumbens* Linn (Asteraceae).

P. pinnata was found to contain sterols, flavones, chalcones, their derivatives and furanoflavon glucosides etc. It was traditionally used for skin diseases, leprosy, rheumatism, cold, cough, mouth ulcers, bleeding piles, microbial infections, ascites, itching etc. It has also been scientifically investigated for free radical scavenging and antioxidant potential [2-4].

A. lakoocha was found to contain β-sitosterol, cycloartenol, cycloartenone, α-amyrin acetate, lupeol acetate, artocarpin, norartocarpin, norcycloartocarpin, cycloartocarpin, resorcinol, oxyresveratrol etc. and traditionally applied on sores [3]. It was also having skin whitening properties and used in cosmetics [5].

C. dactylon was found to have triterpenoides, alkaloids, proteins, vitamin C, sitosterols, carotenes, furfurals, β-sitosterol-D-glucoside, stigmasterol etc [6]. It was traditionally used as astringent and used for bleeding cuts,

wounds, pruritis, inflammations, microbial infections, menstrual problems, epilepsy, depression, haemorrhagia etc [3, 5, 7-10].

T. procumbens was found to have alkaloids, carotenoids, flavonoids, saponin, tannins etc. and traditionally used for treating wounds, boils, cough, pain etc [11, 12]. It has already been scientifically investigated for analgesic [13], antioxidant [14], immunomodulatory [15] and wound healing properties [16, 17].

In light of above text and detailed review of literature for various herbal drugs used in wound healing, we have selected above mentioned plants for preparing herbal cream PACT and investigated the same for wound healing potential.

MATERIALS AND METHODS

Herbal Formulation

The herbal formulation PACT was prepared by incorporating the 4% methanolic extracts of each *Pongamia pinnata*, *Artocarpus lakoocha*, *Cynodon dactylon* and *Tridax procumbens* in a water soluble cream base. The herbal drugs were collected from BIT campus, Mesra, Ranchi in the month of July 2009 and authenticated by Dr. (Prof) S. Jha, Department of Pharmacognosy, and voucher specimens were also deposited in Department of Pharmaceutical Sciences. The extracts were prepared by cold maceration and the cream base was obtained from White soft paraffin (12 g), Cetostearyl alcohol (8 g), Sodium lauryl sulphate (1 g), Liquid paraffin (8 ml), Paraffin wax (4 g), Methyl paraben (0.02 g), Propyl paraben (0.04 g) and distilled water using standard procedure.

Animals

Inbred Swiss albino mice (25- 35g) of both sexes were procured from animal house of Birla Institute of Technology, Mesra, Ranchi, India (Regd. No.621/02/ac, CPCSEA). The animals were acclimatized to laboratory conditions for 7 days before starting experimental work and kept in standard conditions at room temperature with 12 hrs light-dark cycle. The animals were fed with standard pallet diet and water *ad libitum*. Further, the experimental protocol was duly approved by IAEC (BIT/ PH/ IAEC/09/2010) and animal care was taken as the guidelines of CPCSEA, Govt. of India.

Experimental Protocol

The wound healing potential of PACT was investigated using two models i.e. Excision Wound and Incision Wound model. For each animal model 18 mice were taken and grouped into control (treated with cream base only), standard (treated with Framycetin cream) and test (treated with PACT formulation) groups. Each group in both of the models was containing six animals.

Excision wound model

The animals were anesthetized by an open mask method using anesthetic ether and depilated on the dorsal surface. A predetermined area of 200 mm^2 of full thickness of skin was excised in the dorsal inter-scapular region. The wound area was immediately measured by placing a transparent paper upon the wound and traced out. The area of this impression was calculated using 1mm graph paper and animals were kept in a laboratory environment without any type of dressings for observation. During the observation period, mice were provided with food and water *ad libitum*. The herbal formulation cream PACT and standard drug Framycetin were applied daily until complete healing occurs. The wound areas were measured on every alternate day for 18 days and until the wound healed completely. A graph paper having millimeter scale was used for calculating the wound contraction. The wound contraction was expressed as a percentage reduction in the size of original wounds. The wound area at the time of wounding was considered as 100% and the wounding day was considered as day zero [18, 19].

Incision wound model

The mice were anesthetized using anesthetic ether and an incision wound of about 2-3 cm length and 2 mm depth were made with a sterile scalpel on the shaved back of mice. The parted skin was kept together and stitched with black silk surgical thread at 0.5 cm intervals. The continuous threads on both wound edges were tightened for good closure of wounds. The wounds of animals in the different groups were treated with topical application of creams (control, standard and test) for a period 10 days. The wound day was considered as day zero. When the wounds were thoroughly healed, the sutures were removed on the 8^{th} post wounding day. The wound breaking strength (i.e. Weight in grams required to break/open the wound) was measured on the 10th day by water flow technique [19-22].

Estimation of wound breaking strength

For estimating wound breaking strength, animals were anesthetized using diethyl ether and placed in the middle of the board. The clamps were then carefully fixed to the skin of opposite edges of the wound at a distance of 0.5cm

away from the wound. The longest piece of the fishing line was placed on the pulley and, finally to the polyethylene bottle. The position of the board was adjusted so that the bottle receives water at rapid and constant rate from a large reservoir. The flow of water was regulated with the help of an occlusion clamp on tubing which was connected to the reservoir and raised to a suitable height. As soon as the wound starts to open, the water flow was cut off. The pulling force to the wound was immediately released by lifting up the reservoir to avoid further opening of the wound. The volume of water accumulated in the bottle was measured. The wound breaking strength was expressed as the minimum weight of water necessary to bring out the opening of the wound [23].

Estimation of Hydroxyproline contents

The regenerated tissues from healed wound were collected for the estimation of hydroxyproline content. The wound tissues were excised, weighed and dried in a hot air oven at 60^{o}C for 12 hours to constant weight. The specified quantities of tissue sample were immersed in 2 ml of 6 M HCl and the tubes were sealed without evacuation. Hydrolysis was done for 20 hours at 105^{o}C, and hydrolyzed tissues were filtered through 0.22 µm syringe filter. The filtrate was neutralized to pH 7.0 with sodium hydroxide solution. The 1.0 ml of sample was taken in the sample tube and mixed with 1.0 ml of 0.01M copper sulfate solution followed by the addition of 1.0 ml of 2.5N sodium hydroxide solution and then 1.0 ml of 6% hydrogen peroxide solution. The solutions were occasionally shaken for a period of 5 minutes and then placed in a water bath at 80^{o}C for 5 minutes with frequent vigorous shaking. The heating and shaking destroyed the excess of peroxide. The remaining traces of peroxide decreased the color formation and produced an orange-red hue. The tubes were chilled in ice cold water and, then 4 ml of 3.0N sulphuric acid was added with agitation. After that, 2.0 ml of a p-dimethylamino benzaldehyde solution was added with thorough mixing. The tubes were placed in a water bath at 70^{o}C for 16 minutes and then cooled with tap water. The absorbance was measured at 540 nm and quantity of hydroxyproline was calculated with the help of standard curve prepared with pure L-hydroxyproline [19].

Histopathology

The samples of healed tissues from the skin of mice from different groups were taken and histopathologicaliy studied.

Statistical Analysis

All results were analyzed by one-way-analysis of variance (ANOVA) followed by Dunnet's t-test and values are expressed as mean ± SEM. The $P< 0.05$ was considered as significant.

RESULTS AND DISCUSSION

Wound, unavoidable events of life, is usually a type of injury involving disruption, breaking or piercing of the skin or any other surface tissue that significantly affect physiological aspects. Wounds, if left untreated, may lead to multiple organ failure or death even. Wound healing, involving either regeneration, i.e. the complete restoration of damaged parts, or repair i.e. reconstruction of the injured region, is usually the normal body response to any injury [1, 24, 25]. Wound healing agents are required to enhance and fasten this natural physiological process. Herbal drugs, the natural gift to mankind, comparatively have lesser side effects and give good therapeutic potential at low cost. In light of the above text, the present study involved the evaluation of a multi-herbal formulation PACT for wound healing potential by employing two wound models i.e. Excision wound (for wound contraction and epithelisation phase) and Incision wound (for collagenisation phase) models [25].

The herbal formulation PACT significantly contract the wound in excision model with complete closure of wound on 18th day as compared to control group as shown in table 1. Wound contraction is a vital parameter used for assessing wound healing potential. It involves the deposition and maturation of collagen and is fibroblast dependant [26]. In addition to confer the strength and integrity to the wound matrix, collagen also plays important role in haemostasis and epithelialization stage of wound healing [27]. It healed the excised wound earlier than in control group hence enhanced the process of epithelialization and shortened the epithelialization period. Epithelialization is the process of restoring an intact epidermis after cutaneous injury [28]. Falling of scab without leaving any raw wound is taken as end point of completion of epithelialization and time required for the same is designated as epithelialization period [29].

Table 1: Wound Contraction Studies of Different Groups in Excision Wound Model

Days	Control Group (Cream Base Only) Wound area mm^2 (Mean ± SEM), (% wound contraction)	Test Group (PACT Cream) Wound area mm^2 (Mean ± SEM), (% wound contraction)	Standard Group (1% Framycetin Cream) Wound area mm^2 (Mean ± SEM), (% wound contraction)
0	164.66±8.54, (0.00%)	163.00±2.35, (0.00%)	186.33±9.97, (0.00%)
2	163.00±8.51, (1.00%)	159.00±2.91, (2.45%)	182.16±9.95, (2.23%)
4	155.83±7.80, (5.36%)	157.16±3.06, (3.58%)	167.00±7.78, (10.37%)
6	124.00±8.17, (8.29%)	143.16±3.24, (12.17%)	142.16±7.11, (23.70%)
8	124.00±7.14, (24.69%)	85.66±4.52*, (47.44%)	80.50±5.29*, (56.79%)
10	94.60±4.46, (42.54%)	45.50±2.40*, (72.08%)	57.66±5.99*, (69.05%)
12	63.83±4.20, (61.23%)	24.33±1.97*, (85.07 %)	27.83±3.71*, (85.06%)
14	49.66±4.16, (69.84%)	13.16±1.13*, (91.92%)	10.83±0.91*, (94.18%)
16	31.33±2.50, (80.97%)	3.0±0.73*, (98.15%)	3.00±1.03*, (98.38%)
18	13.83±1.16, (91.60%)	0.00, (100%)	0.00, (100%)
20	6.30±0.08, (96.17%)	---------	---------

*The data was analyzed by one way ANOVA followed by Dunnett's t-test and all the values are expressed as Mean±SEM, (n=6), *p≤0.05*

The wound breaking strength is measured as the force required for opening the healed wound which represents the degree of wound healing. It has commonly been associated with the organization, contents and physical properties of collagen fibril network. The collagen imparts tensile strength and elasticity to healed tissue. It was observed that PACT treated incised wound exhibited an increased wound breaking strength in comparison to control group as shown in table 2. Hydroxyproline is an amino acid and mainly found in collagen fibre of granular tissues. Breaking down of collagen liberates free hydroxyproline and its peptides. Estimation of hydroxyproline contents is used as a marker of collagen synthesis [30, 31]. Higher the concentration of hydroxyproline, faster the rate of wound healing; which is a reflection of increased cellular proliferation and hence increased collagen synthesis. The PACT formulation significantly increased the hydroxyproline contents in comparison to control group which indicated that the granular tissues in wound area were replaced by collagen as described in table 2. The results were further supported by histopathological evidences as shown in figure 1. In PACT treated group, there are clear evidences for epithelialization, new blood vessel formation i.e. neovascularisation and keratinization as compared to control group.

Table 2: Wound breaking strength and hydroxyproline contents in Incision wound Model

Group	Treatment	Wound Breaking Strength gm/mm^2 (Mean ± SEM)	Hydroxyproline contents µg/mg (Mean ± SEM)
Control	Cream having base only	87.5±3.819	248.12±5.96
Standard	1% Framycetin cream	147.0±7.950*	398.33±6.98*
Test	PACT cream	137.6 ± 2.108*	372.53±4.72*

*The data was analyzed by one way ANOVA followed by Dunnett's t-test and all the values are expressed as Mean±SEM, (n=6), *p≤0.05*

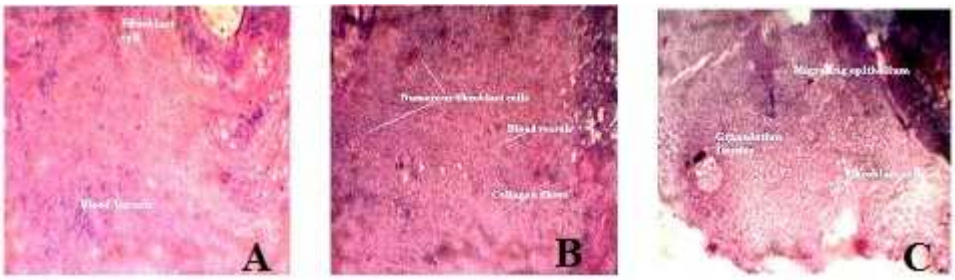

Figure 1: Histopathology of tissues in Control (A), Standard Framycetin (B) and Test PACT Formulation (C). The photographs are clearly giving the evidences for epithelialization, new blood vessel formation i.e. neovascularization, keratinization in PACT treated group as compared to control group.

CONCLUSION

The present research article concluded that the herbal cream PACT having herbal extracts of *P. pinnata*, *A. lakoocha*, *C. dactylon* and *T. procumbens* showed significant wound healing properties in both excision and incision wound models; and can be used clinically for the same after requisite clinical investigations.

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