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Research Article

WOUND HEALING POTENTIAL OF AQUEOUS AND METHANOLIC EXTRACTS OF *PLAGIOCHILA BEDDOMEI* STEPH. - A BRYOPHYTE

G.S.MANOJ, K.MURUGAN*

Plant Biochemistry and Molecular Biology Lab, Department of Botany,University College, Thiruvananthapuram, Kerala.
***Email: harimurukan@gmail.com**

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ABSTRACT

Plagiochila beddomei Steph. a bryophyta is widely used in the form of paste ethnomedicinally by tribe Melghat Region for treating skin diseases. To validate the ethnotherapeutic claims of the plant in skin diseases, wound healing activity was studied, besides antioxidant activity to understand the mechanism of wound healing activit in Wistar albino rats. The rats were divided into four groups of six animals each. Group 1 is normal wounded control and the other groups were treated with alcoholic and aqueous extract. The plant (methanolic and aqueous extract) showed significant wound healing capacity as evident from the wound contraction and increased tensile strength. The wound healing parameters were evaluated by using incision, excision and dead space wounds in extract-treated rats and controls. Both the doses of alcoholic and aqueous extract significantly increased hydroxyproline, hexuronic acid, hexosamines, superoxide dismutase, catalase, reduced glutathione and significantly decreased percentage of wound contraction and lipid peroxidation when compared with the control group. The results suggest that *P. beddomei* has antioxidant properties, which may be responsible and favorable for faster wound healing and this plant extract may be useful in the management of abnormal healing and hypertropic scars.

Keywords: Antioxidant property, *Plagiochila beddomei* , Wound healing and Collagen

INTRODUCTION

Bryophytes are ethno medicinally used by tribes for treating skin diseases in the form of paste or as fresh material externally for the treatment of burns, boil and blister on the body or also applied for the treatment of skin eruption caused due to heat in summer. In recent years, many possible sources of natural antibiotics have been in use for several infectious diseases, mostly bacterial and fungal. In this respect, the most investigated taxa are from angiosperms whereas very little data is currently available about other groups of plants, especially bryophytes[1]. Bryophytes are traditionally used by Chinese, Europe, North American and Indian medicine, to treat other illness such as cardiovascular disorder, tonsillitis, bronchitis, tympanitis, skin diseases and burns. The therapeutic efficacies of many indigenous plants, for various diseases have been described by traditional herbal medicine practitioners. They are still the primary health care system in some parts of the world[2]. There has seen considerable change in opinion regarding ethnopharmacological therapeutic applications. The presence of various life sustaining constituents in plants has urged scientists to examine these plants with a view to determine potential wound healing properties. Dermal wound healing is a coordinated process of tissue remodeling involving an inflammatory response, re-epithelialization, and revascularization. The process is mediated by soluble cytokines and growth factors, which act on multiple cell types including keratinocytes, dermal fibroblasts, and vascular cells. Activated inflammatory cells secrete various matrix proteinases to facilitate breakdown of the extracellular matrix (ECM), which aids in the migration of keratinocytes and fibroblasts into the wound bed[3]. Deposition of provisional matrices such as fibronectin provides a permissive environment for angiogenesis to occur, which ultimately leads to the healing of the wound and restoration of dermal function. Collagen and elastic fibers are important components of the dermal ECM and are essential for the maintenance of skin integrity[4]. So the aim of the present study was to investigate the *in vivo* wound healing activity of *Plagiochila beddomei* Steph. in order to elucidate traditional use of this plant from the scientific point of view. The methanolic and aqueous extracts prepared from the leafy thallus of the plant were tested in mice for wound healing activity using *in vivo* excision and linear incision wound models.

MATERIALS AND METHODS

Materials

Fresh thallus of *Plagiochila beddomei* Steph. was collected from Nilgiri hills of Tamil Nadu, India. Taxonomic identity was confirmed by comparing with authenticated herbarium specimen (MCN 120257) at Department of Botany Herbaria, University of Calicut, Kerala. A voucher specimen of the plant is kept in the Herbarium of Department Botany, University College, Thiruvananthapuram, Kerala.

Preparation of Extracts

Fresh thallus was weighed; chopped and extracted with methanol and water. The extract was prepared with 100 g of fresh thallus for 6 hrs and removing solid matter by filtration. The solvents were removed by rotary evaporation. After lyophilization the methanol and aqueous extracts yielded 4.9 and 4 g dried material respectively and was stored at -20°C .

Preliminary Phytochemical analysis

An attempt was made to observe the presence or absence of different phytochemical constituents, viz., phenols, flavonoids, carbohydrates, glycoproteins, alkaloids, sterols and triterpenes according to Philipson[5].

Animals

Healthy Wistar albino rats (150–250 g body weight) were purchased from the animal breeding laboratories of the Central Drug Research Institute, Lucknow. They were kept in the departmental animal house at 26±2ºC at relative humidity 44–55% and light and dark cycles of 10 and 14 h, respectively, for 1 week before the experiment. Animals were provided with mice diet (Amruth, India) and water *ad libitum*.

Acute Toxicity Studies

P. beddomei extract did not show marked toxicity up to 8 g/kg body weight up to 14 days after administration.

Experimental protocol

The rats were divided into four groups of 6 animals each. Group I served as control; Group II served as standard treated with Madecassol[R] topically; Group III served as test group treated with aqueous extract of ***P. beddomei***; Group IV served as test group treated with methanol extract.

Wound Models

Excision wounds

All studies were conducted in accordance with the National Institute of Health's guidelines for survival rodent surgery[6] after approval

from the Institutional Ethics Committee. Excision model was used to monitor wound contraction, period of epithelization and wound closure time. Adult albino rats were ether anaesthetized prior to and during creation of the wounds. The back of animals were shaved and sterilized with 70% ethanol before excision wound was created by a surgical blade from a pre-determined shaved area on the back of each animal[7]. An impression was made on the dorsal thoracic central region 5 mm away from the ears by using a round seal of 2.5 cm diameter. The skin of the impressed area was excised to full thickness to obtain a wound area of about 500 sq mm. The wound was left undressed to the open environment and no local or systemic microbicidal agents were used. The experimental groups were topically applied with the extracts (methanol and aqueous) twice daily. Reference group treated with Madecassol[R] drug. Progressive decrease in wound contractions were measured by a tracing paper on the wounded margin periodically and calculated as percentage reduction in wound area taking the size of the wound at the time of wounding as 100%. Scar residue area, % of wound closure and time for complete epithelization were measured.

Incision wounds

Two paravertebral straight incisions of 6 cm were made through the entire thickness of the skin on either side of the vertebral column of rats under light ether anesthesia. The wounds were closed with interrupted sutures, which were removed on day 8 of the wounding. Wound breaking strength was measured on day 10[8].

Dead space wounds

Granuloma formation was induced by subcutaneous implantation of sterile cotton pellets[9] and sterilized grass piths (25 × 3 mm) in the groin. On day 10, the granuloma were excised and tested for tensile strength[8]. The hydroxyproline[10], hexosamine[11], and hexuronic acid[12] content of the granulation tissue were estimated. The cotton pellet granuloma excised from dead space wounds were dried overnight at 60ºC and the dry weight was expressed as mg/100 g of body weight[13]. Granulation tissue from the other tube was collected in phosphate-buffered saline for the estimation of antioxidant enzymes like superoxide dismutase (SOD),[14] catalase[15], reduced glutathione (GSH) [16] and tissue lipid peroxidation was read at 535 nm[17].

Histological examination of excised tissue

The excised wound tissue was fixed in 10% neutral buffered formalin, dehydrated in graded ethanol, cleared in xylene, and embedded in paraffin. 5 µ thick sections, including the epidermis, the dermis, and the subcutaneous panniculus carnosus muscle, were mounted on glass slides, dewaxed, rehydrated to distilled water, and stained with hematoxylin and eosin (HE) or Masson's Trichrome (MT). A five-tiered grading system based on degree of re-epithelization, granulation tissue formation and collagen organization was adopted to evaluate the historical differences of different samples[3, 18].

Estimation of collagen in the regenerated tissues

The animals were divided into two groups of six animals each. Excision wound and treatment to all the animals were made in the same manner as mentioned in excision wounds[19]. The regenerated tissues were extracted from the wounds of each group on day 4, 8, 12, 16 and 20 and estimated for collagen content[20].

Statistical Analysis

Results were expressed as mean ± SD and evaluated for statistical significance by unpaired Student's *t* test. Values of $P < 0.05$ were considered statistically significant.

RESULTS

Preliminary phytochemical screening of ***P. beddomei*** thallus showed the presence of flavonoids, saponins, glycoproteins, tannins, phenols, carbohydrates and proteins in both the extracts. The methanolic extract showed remarkable levels of total phenols (19.3 mg/g) and flavonoids (16.9 mg/ g) while the aqueous extract contain 12.5 and 8.76 mg/g phenols and flavonoids respectively.

Histological Evaluation

Number of capillary buds (987 ± 0.49) and fibroblasts (1219. 3 ± 126.08) were higher in methanol treated groups than Madecassol[R] and aqueous treated groups (823 ± 0.02 and 1012 ± 0.08, respectively). The epithelial gap in methanolic and aqueous extracts treated group was significantly less than the control group (Fig. 1). Histological evaluation was carried out for the treated and untreated samples. There was a marked infiltration of the inflammatory cells, increased blood vessel formation and enhanced proliferation of cells as a result of treatment with ***P. beddomei*** extract and Madecassol[R]. There was full thickness re-epitheliasation, in which epidermis was thin and well organized, comparable to the normal adjacent skin which was not involved in the wound generation and healing process. The granular layer was well formed and one cell in thickness. There was a full thickness epidermal regeneration which covered the entire wound area. The epidermis was thick and disorganized, especially when compared with the adjacent normal skin. In all, complete epitheliasation, vasculirisation and hair follicles formation were observed in treated rats (Figs. 2 a-f). Early dermal and epidermal regeneration in treated mice also confirmed that the extract had a positive effect towards cellular proliferation, granular tissue formation and epitheliasation.

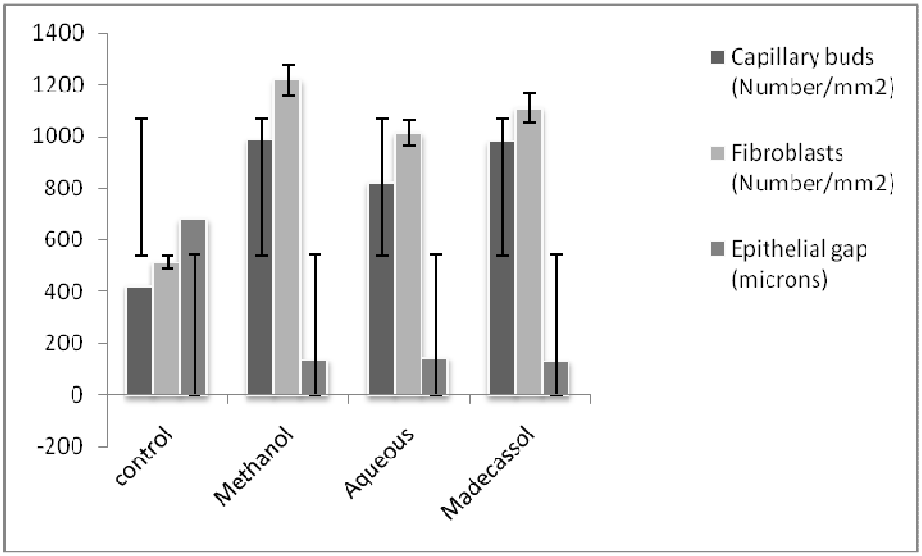

Fig. 1: The number of capillary buds (mm²), fibroblasts (mm²) and epithelial gap (µ) observed in the histopathological samples

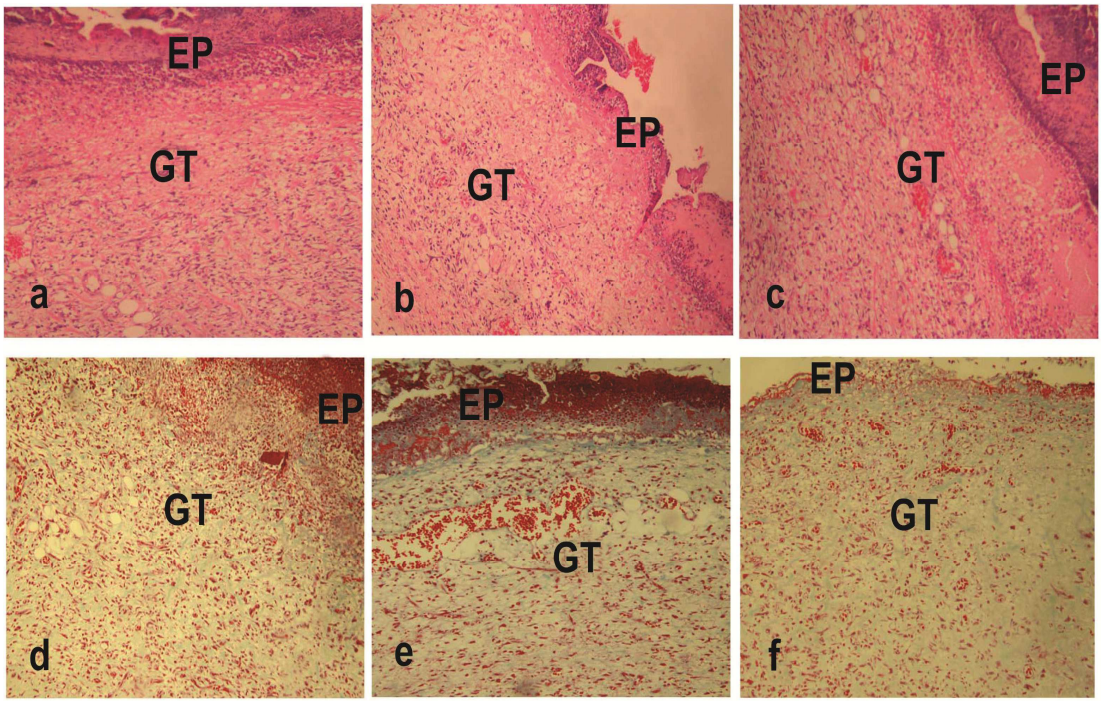

Fig. 2 a-f: Histological comparison of wound healing character on day 3 (100× magnification). A (HE), d (Masson Trichrome-MT) in study group and c (HE), f (MT) in Madecassol^R group showed the thick granulation tissue layer with robust newly-formed vessels as well as plenty of inflammatory and repair cells. B (HE), e (MT) in control group showed massive necrotic substances and a thin granulation tissue layer. EP, epithelium; GT, granulation tissue.

The methanolic and aqueous extracts, in that order, showed statistically significant improvement in the wound breaking strength compared to Madecassol^R treated and control group (P < 0.05) (Table 1). This shows that both extracts of ***P. beddomei*** can be used for incised wounds. Similarly, remarkable increase in the tensile strength (methanolic- 469.9 ± 0.09 g) was observed in methanolic treated group compared with the Madecassol^R and aqueous extract treated groups. Hydroxy proline, hexuronic acid and hexosamine content increased in all the experimental groups compared to control.

Table 1: Effect of *P. beddomei* on wound area in terms of Period of epithelialization, tensile strength, scar area, Hydroxyproline, Hexuronic acid and Hexasamine level in granulation tissue.

Topical treatment	Period of Epithelialization (Days)	Tensile strength (g)	Scar area (mm2)	Hydroxyproline (mg/ g)	Hexuronic acid (mg/g)	Hexoseamine (mg/g)
Control	28 ± 0.42	257.6 ± 0.06	42.2 ± 0.06	9.6 ± 1.4	12	10
Methanolic	12± 0.45	469.9 ± 0.09	24.8 ± 0.12	28.9 ± 1.3	28	21
Aqueous	15 ± 0.45	385.9 ± 0.17	35.1 ± 0.5	25 ± 1.2	24	20
Madecassol^R	13 ± 0.03	466.7±0.01	33±0.01	27±0.5	27	20

Values are mean ± SD of six replications

Table 2: Effect of *P.beddomei* extract on antioxidant enzymes and MDA level in wound area

Topical treatment	% of closed excision wound areas after days				
	4th day	8th day	12th day	16th day	20th day
	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD
control	5.23 ± 7.7	23.6 ± 0.02	36.7 ± 0.06	44.6 ± 0.44	5.4 ± 0.07
Methanolic	38.5 ± 0.05*	63.9 ± 0.07	79.2 ± 0.02	91.7 ± 0.09	96.2 ± 0.49
Aqueous	27.15 ± 4.15*	45.9 ± 2.37	62.6 ± 0.5	72 ± 1.7	89.2 ± 0.9
Madecassol^R	36.08 ± 6.14*	62.87±0.07	83.1±0.03	90.3±0.05	95.6±0.01

Values are mean± SD of six replications

Excision Wound model

Period of Epithelization

Methanolic extract showed statistically significant hastening of epithelization compared to Madecassol^R, aqueous extract and control groups (P < 0.05) (Table 2). This in turn suggests that both the extracts are effective in healing excision wound types.

Percentage of Wound Contraction

The percentage of wound contraction in all the test groups including Madecassol^R treated groups were statistically significant compared to control on the 4th day (P < 0.05, F = 4.65) (Table 2). On the 4th day the percentage of wound contraction ranged from a low of 5.23 ± 1.3 in control to 38.5 ± 0.65 in the methanol extract. There was no statistical significance in the percentage of wound contraction

during the 8th and 12th days. But on 16th day there was a statistically significant difference in percentage of wound contraction ($P < 0.05$, F = 2.94) between the control and the test groups. So the test compounds could be used to hasten healing in excision wounds.

Dead Space Wound Model

Breaking Strength

There was a significant increase in the wound breakings strength (WBS) of granulation tissue in methanolic extract compared to Madecassol[R] treated, aqueous and control ($P < 0.05$) but no statistically significant increase in aqueous extract was observed (Table 3).

On the 1st day, the re-epithelialization, granulation and collagen deposition were no significant difference among the groups with a thin and incomplete re- epithelialization, abundant fibrous exudation, few vessels and trace collagen (Table 4). On the 3rd day, wounds of methanolic and aqueous study group and positive control group displayed a more accumulation of granulation tissue with a high degree of newly-formed micro-vessels and numerous inflammatory cells and fibroblast (Fig. 2). The mean values of parameters related to wound healing in study group (methanolic and aqueous) and positive control group were higher compared to control group *(*$P < 0.05$) except re-epithelialization. On the 7th and 10th day, wounds of all the groups demonstrated

a continuous epithelial line covering the whole wound bed. Moreover, in study groups and positive control group, the granulation was matured showing capillary vertically oriented, robust fusiform fibrocytes and moderate well-arranged collagen. In contrast, in control group, the granulation tissue was unmatured with capillary poorly organized, many fibroblast and slight collagen formation. On the 14th day, all the wounds healed, and there was no significant difference among the groups according to histological examination.

Table 3: Effect of *P. beddomei* on collagen content in terms of wound breaking strength and granulose weight.Values are mean ± SD of six replications

	Wound breaking strength (g)	Granulosa Wt. (g/100 GB.W)
control	196.9 ± 0.24	35 ± 1.7
Methanolic	270.4 ± 3.2	47.8 ± 0.6
Aqueous	260.8 ± 0.32	40.2 ± 0.2
Madecassol[R]	269 ± 0.6	47 ± 0.39

Table 4: Effect of *P. beddomei* on collagen content in wound the area

	4 days	8 days	12 days	16 days	20 days
control	11.56 ± 0.2	22.7 ± 0.8	31.2 ± 0.6	34.3 ± 0.3	38.4 ± 0.4
Methanolic	18.8 ± 0.22	33.2 ± 0.7	38.4 ± 0.42	41.8 ± 0.7	44.2 ± 0.54
Aqueous	16.2 ± 0.3	29.6 ± 0.4	32.4 ± 0.4	39.1 ± 0.55	40.2 ± 05
Madecassol[R]	17.7 ± 0.32	31.4 ± 0.7	37.2 ± 0.2	40.4 ± 0.7	43.7 ± 0.09

Values are mean ± SD of six replications

Significant increased SOD activity was observed in the granulation tissue in the rats treated with methanolic ($P < 0.05$) and aqueous extract compared with control (Table 5). Catalase level in granulation tissue was also significantly increased in the case of both the extract treated groups ($P < 0.05$), compared with control (Table 5). Reduced GSH concentration in granulation tissue was significantly increased in rats treated with different extracts ($P < 0.05$), compared with control. There was significant increase in GSH level in methanolic extract treated group compared with aqueous extract group ($P < 0.05$) in intercomparison statistical analysis (Table 5). Malondialdehyde (MDA) level in granulation tissue was significantly decreased in the case of all the extract-treatment groups ($P < 0.05$) compared with control (Table 5).

Table 5: Effect of *P.beddomei* extract on antioxidant enzymes and MDA level in wound area

	SOD(U/mg)	CAT(k/s/mg protein)	GSG(µg/mg)	MDA(nmol/mg)
control	2	0.05	0.07	0.065
Methanolic	4.6	0.5	0.4	0.01
Aqueous	4	0.38	0.29	0.02
Madecassol[R]	4	0.39	0.34	0.015

Values are mean± SD of six replications

DISCUSSION

Wounds in most tissues heal by repair, by laying down non-specific connective tissue. The results of the present study showed a remarkable increase in wound contraction rate, wound breaking strength (WBS) reflecting increased collagen synthesis. The increase in dry granuloma weight and granuloma breaking strength indicates significant maturation of collagen by increased cross-linking. This is supported by the increase in collagen content in granuloma excised from the wound. Collagen is a fibrous protein component of the connective tissue and provides a structural framework to the tissue consisting of hydroxyproline, hydroxylysine and glycine as principle constituents among which hydroxy proline is considered a specific aminoacid. Hence its estimation in the granulation tissue may trigger on the maturation and healing process[21]. Treatment with ***P. beddomei*** increased the hydroxyproline content which further confirms the involvement of collagen in the present study. The treated wound epithelialised faster and the rate of wound contraction was higher as compared to Madecassol[R] treated and control. The scar residue in the extract treated group was superficial. Histopathological studies revealed a significant increase in epithelialisation in rats by ***P. beddomei*** treated groups.

All these evidences confirm that ***P. beddomei*** enhances the wound healing by acting on various phases of the healing events. Increased fibroblastic proliferation may be due to mitogenic activity of the extract, which might have significantly contributed to healing process. Fibroblasts are the cells in skin connective tissue and are the adhering cells which are thought to play a customary role in wound healing assistance. Early dermal and epidermal regeneration, as shown by reduced epithelial gap and wound surface area and massive angiogenesis in treated rat also confirmed that the extract had a positive effect towards cellular proliferation, granulation tissue formation and reepithelialization. Skin contains collagen fibers, which are arranged in a criss-crossed pattern and characterize the mechanical properties of the tissue [9,10,19]. In the

present study the biomechanical results are consistent with the histopathological results. Increased fibroblastic proliferation results in increased collagen synthesis. Collagens are the main extracellular component of the skin. During the proliferative phase of skin wound healing, the synthesis of\ different proteins of particularly collagen subtype within wounds increases to replace necrotic tissue[13,22]. Collagen not only confers strength and integrity to the tissue matrix, but also plays an important role in homeostasis and epithelialization at the later phase of healing[9,18] Therefore; enhanced synthesis of collagen provides strength to repaired tissue and also healing pattern[13].

Granulation, collagen maturation and scar formation are the major phases of wound healing, which run concurrently, but independent of each other. The use of a single model is inadequate and no reference standard exists that can collectively represent the various phases of wound healing. Hence, different models have been used in the present study to assess the effect of ***P. beddomei*** on the various phases of wound healing. In incision wound, the increase in tensile strength of treated wounds may be due to the increase in collagen concentration and stabilization of the fibers[21]. Increase in WBS and role of antioxidants were experimentally proved by Michel and Fredrickson which inturn supports our results[23].

In excision wound, both the extracts showed faster healing compared with Madecassol[R] treated and control groups and wound contraction rate is faster with methanolic extract as compared with aqueous extract. The faster wound contraction may be due to stimulation of interleukin-8, an inflammatory α-chemokine which affects the function and recruitment of various inflammatory cells, fibroblasts and keratinocytes. It may increase the gap junctional intracellular communication in cultured fibroblasts and induces a more rapid maturation of granulation tissue[24]. The methanolic extract of ***P. beddomei*** increased cellular proliferation and collagen synthesis at the wound site as evidenced by increase in total protein and total collagen contents reflected by hydroxyproline content of granulation tissues. The glycosaminoglycans are a major component of the extracellular matrix of skin, joints, eyes and many other tissues and organs. In spite of its simple configuration, it demonstrates remarkable viscoelastic and hygroscopic properties which are relevant for the functioning of dermal tissue. Biological activities in skin are due to its interaction with various binding proteins. Due to an influence on signaling pathways, hyaluronic acid is involved in the wound healing process and scarless fetal healing. In clinical trials, topical application of hyaluronic acid has improved the healing of wound[25]. In addition, the mucopolysaccharide hyaluronic acid protects granulation tissue from ROS radical damage and thereby stimulates wound healing [22].

Among the glycosaminoglycans, dermatan sulfate and dermatan have also been implicated in wound repair and fibrosis. Their ability to bind and alter protein–protein interactions has identified them as precursors of cellular responsiveness in development, homeostasis and disease[26]. In our study, hexuronic acid and hexosamine concentrations which are the component of glycosaminoglycans were significantly increased with both the extracts when compared with control. Glycosaminoglycans, play a role in stabilizing the collagen fibers by enhancing electrostatic and ionic interactions with it and possibly control their ultimate alignment and characteristic size. Since ***P. beddomei*** methanolic extracts have elevated levels of glycosaminoglycans considerably, it is likely that the observed increase in tensile strength was not only due to increased collagen synthesis but also due to its proper deposition and alignment.

Molecular oxygen plays a central role in the pathogenesis and therapy of chronic wounds. Overproduction of reactive oxygen species (ROS) results in oxidative stress thereby causing cytotoxicity and delayed wound healing. Therefore, elimination of ROS could be an important strategy in healing of chronic wounds[27]. Similarly, estimation of antioxidants like SOD, catalase and GSG in granulation tissues is also relevant because these antioxidants hasten the process of wound healing by destroying the free radicals[21]. The significant alteration in the antioxidant profile accompanied by the reduced levels of MDA, a marker of free radical damage, may be attributed to impaired wound healing in immunocompromised rats. While numerous attempts have been made to identify prognostic biomarkers of wound healing in skin, these have met with limited success. The bioactivities of polyphenols are highly correlated with their chemical structure and action mechanisms, mostly inhibitory on enzymatic systems involved in cellular activations[25].

Results of the antioxidant parameters, mices treated with the methanolic and aqueous extracts of ***P. beddomei*** showed an elevated level in the activity of SOD, CAT and GSH with a decrease in MDA level in granulation tissue compared with controls. These enzymes are known to quench the superoxide radical and thus prevent the damage of cells caused by free radicals[24]. Better collagenation, seen under the influence of this plant extract, may be because of the presence of phenols, which is responsible for the free radical scavenging activity which is believed to be one of the most important components of wound healing[28].

In our study on the effect of methanolic and aqueous extracts of ***P. beddomei*** on wound healing, we found that methanolic extract possesses a better effect than aqurous extract. Since ***P. beddomei*** is ubiquitous and abundantly grown, it could be a fairly economical therapeutic agent for wound management as a prohealer.

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