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Research Article

A Pre-clinical Study on Wound-healing Property of *Calamus floribundus* (Griff.) Leaves Using Mice Excision Model

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ABSTRACT

In the rural areas of Assam, the leaves of *Calamus floribundus* are in use among the traditional healers to treat different skin-related problems. Assessment of *the plant's in-vivo wound healing activity* was undertaken to unearth the extent of its healing potential. Methanolic extracts of the leaves of *C. floribundus* was applied topically on the mice having excision wounds. Different parameters such as rate of wound contraction, epithelialization period, tensile strength, content of DNA, protein, hydroxyproline, expression level of the growth factor and pro-inflammatory cytokines, histopathological and ultrastructural evaluations were carried out for the wound healing potential assessment. The results indicated that the application of the extract lead to the significant increase in the rate of wound contraction, tensile strength, DNA, protein and hydroxyproline content. The expression of PDGF-AA was upregulated and the cytokines levels were downregulated in the extract-treated group. All the values were comparable to that of the reference drug-treated group. Morphometric, histological and ultrastructural observations further supported the results. The findings of our *in-vivo* study indicate that the methanolic crude extracts of the leaves of *C. floribundus* positively affect the healing of excision wounds in swiss albino mice.

Introduction

A wound can be defined as an injury where breakage is created on the body part, mainly in the skin. Wounds are of different types such as incision, ulcers, burn, abrasions, etc.[1] The process of wound healing is a sophisticated and overlapping one, where different types of cells such as keratinocytes, fibroblasts, endothelial cells, and inflammatory cells are responsible for initiating various stages of healing like inflammation, re-epithelialization, angiogenesis, granulation tissue formation which ultimately repairs the damaged tissues.[2]

Wounds are primarily treated to prevent infections at the injury site, as improper management of the wounds leads to delayed healing, ultimately forming chronic wounds. Various antibiotics, such as silver nitrate, povidone-iodine, and bacitracin, are commonly used to prevent infections at the wound sites. But these commercial drugs have been reported to produce unwanted side effects on the patients, thus deteriorating their health status further.[3] So, there is an urgent need for the development of new drugs for the treatment of wounds.

Globally chronic wounds are responsible for the decline in the quality of life among many patients above 60 years old. It has been estimated that approximately 6 million individuals each year suffer from infections due to chronic wounds which has become a major challenge for the medical system worldwide.[4] According to Gupta *et al.*,[5] globally 5 individuals per 1000 people suffer from chronic wounds. Shefali and Bhaduri[6] reported that daily 1000 Dollar is being spent to treat wounds in America.

Since time immemorial, medicinal plants have been used to treat skin diseases. In the texts of Ayurveda, the Indian

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system of traditional medicine, there are descriptions of many plants, plant products, animal products and minerals for their therapeutic potential. In the traditional medicinal systems, kin-related problems such as infections, cuts, burns, allergies, and wounds were used to be cured with traditional therapies.[7] It has been estimated that most Ayurvedic drugs, i.e., approximately 70% are derived from plant resources. The maximum number of plants mentioned as healing agents in the texts is yet to be investigated for their therapeutic properties.[8]

Research conducted to date using natural products is not yet satisfactory as many queries are still unanswered and need to be addressed in the coming future. For example, the mode of action of most of the naturally derived therapeutic agents is still unknown.[9] So, with the recent developments in the field of Science and Technology lot can be done in the field of drug development by incorporating the traditional knowledge of the healers which may lead to better treatment of diseases and will ultimately benefit human society.[10]

Since last few decades, there has been a stiff rise in the development of agents for better curing wounds using plant products and research on evaluating the healing potential of traditional agents. Presently, in Developed counties like USA, UK, Canada etc. people are showing interest in the traditional healing agents as commercially available therapies such as antibiotics, steroids, pain relievers etc. cause negative effects on individuals.[11] So, many pharmaceutical industries are now screening plants and their products for the development of new healing agents.[12]

Traditional healers around the world mainly in countries like India, Bangladesh, China, Japan, Sri Lanka are known for their valuable knowledge about lesser-known wild medicinal plants, which they are using since ages for the treatment of various types of wounds.[13] In Assam, a state in the north-eastern region of India, the use of leaves of *Calamus floribundus* is common for treating small scale external injuries, especially among the rural population.[14] The plant's shots and fruits are edible and also high in nutritional value. The plant has been reported to have anti-oxidant properties.[15] A detailed search of literature revealed that the plant is yet to be investigated for its wound-healing potential. Therefore, the purpose of our present study is to evaluate *in-vivo* wound-healing properties of the leaves of *C. floribundus* in adult swiss albino mice inflicted with the excision wound.

Materials and Methods

Test Plant Material Collection and Crude Extract Preparation

The fresh leaves of *C. floribundus* were collected from the forest areas of Kokrajhar district of Assam and brought to Shillong, Meghalaya. The plant specimen (BSI/ERC/Tech/2019/687) was identified by a Taxonomist (Mrs. Nandita Sharma) from the Botanical Survey of India (BSI), Shillong.

The crude extract of the plant material was prepared following the protocol of Simon *et al.*[16] The traditionally usable part of the plant i.e., the leaves, were separated from the plant, cleaned with water and dried under shade. After completely drying, the leaves were converted into fine powder and soaked in 90% methanol solution. After 10 days,the content was filtered using Whatman filter paper no. 1. The solvent was separated to obtain the methanolic crude extract of the leaves of *C. floribundus*, which was stored at 4°C until further use.

Experimental Animals

Healthy Swiss albino mice (8–12 weeks old and of 25–30 g weight) of both sexes were procured from Pasteur Institute, Shillong. The experimental animals were maintained by strictly adhering to the OECD (Organization of Economic Co-operation and Development) guidelines. They were also supplied with standard mice feed and water *ad libitum*, and acclimatized for two weeks.

Infliction of the Excision Wound

The excision wounds were made on the anesthetized animals by following the method given by Morton and Malone.[17] For the creation of the wound, at the beginning, the dorsal fur of the mice was removed, then the area where the wound needs to made was marked and finally, with the help of a scissor and a forcep an excision wound measuring 10 mm in diameter was created on the animals.

Experimental Design

The animals bearing the excision were divided into three treatment groups having six animals in each group (three males and three females).

First Group

Considered as the control group, where the animals were treated with carboxymethylcellulose (CMC, 100 mg/kg body weight) only.

Second Group

Treatment was performed with methanolic crude extract of the leaves of *C. floribundus* (400 mg/kg body weight) mixed with CMC (100 mg/kg body weight).

Third Group

The animals were treated with the reference drug neosporin (100 mg/kg body weight) mixed with CMC (100 mg/kg body weight) and considered as positive control group.

All the experimental animals of the three groups received topical treatment once a day for 14 days. The wound tissues were collected from the respective groups after 7 and 14 days of treatment for undertaking various studies.

Morphometric Evaluation

The morphometric evaluation was performed by analysis of the images of the wounds of the three experimental groups captured with the help of camera on days 4, 7, 11 and 14 post-wounding.

Measurement of Wound Areas and Calculation of Wound Contraction Rate

The sizes of the wounds of the three groups of animals were measured on days 4, 7, 11 and 14 and noted down. The rate of wound contraction was measured using the formula mentioned below –

$$\text{Rate of wound contraction} = \left(\frac{\text{Healed area}}{\text{Total area}}\right) \times 100$$

Where, Healed area = Total area – Wound area

Epithelialization Period

Epithelialization period is defined as the time needed to completely heal the wounds. For the three experimental groups of animals, the period of epithelialization was noted.

Tensile Strength Measurement

For the measurement of tensile strength, the skin tissue samples were collected after 14 days of tretment from the different experimental groups of animals. The samples were then cut into 5 cm X 1cm strips and the strength was measured with the help of Universal Testing Machine (UTM, Tinius Olsen 5ST).

Estimation of the Level of Protein, DNA and Hydroxyproline

To perform the biochemical estimation, the skin tissue samples of the different groups were collected after 7 and 14 post wounding days and stored in the freezer until further processing.

For the determination of the protein content of tissue samples, the protocol given by Lowry *et al.*,[18] was followed. Quantitative estimation of DNA was carried out following the method of Sambrook and Russell,[19] DNA from the skin tissue samples was isolated and later quantified. The toluene extraction technique suggested by Switzer and Summer[20] was used to measure the tissue samples' hydroxyproline content.

Measurement of the Expression Level of Growth Factor and Pro-inflammatory Cytokines

The level of PDGF-AA, IL-1β, IL-6 and TNF-α was assessed using solid phase sandwich enzyme-linked-immuno-sorbent assay (ELISA) following the instructions given in the kit.

From the different groups, tissue samples were collected after 7 and 14 days of treatment, homogenates were prepared in the phosphate buffer saline (PBS), and centrifuged and the collected supernatants were used as substrate during the assay.

Histological Analysis

For histological study the collected skin tissue samples were first fixed in the 4% paraformaldehyde (PFD) solution. During processing the tissue samples were properly washed to remove the fixative and then sectioning (6–8 µm) of the tissues was done using a microtome (RMT-30).

Later, the tissue sections were passed through different grades of alcohol. To study the extent of epithelialization, the sections were stained with hematoxylin and eosin, and Van Gieson's staining was carried out to evaluate the process of collagen synthesis.

Ultrastructural Study

For the scanning electron microscopic study, the protocol given Roy and Tandon[21] was followed. Briefly, the tissues were first fixed in the neutral buffer formalin (NBF) then dehydrated through different grades of acetone. Finally, the tissues were air-dried using tetramethylsilane (TMS) and the gold-coated samples were observed under JOEL JSM 6360 scanning electron microscope.

For transmission electron microscopic study, the tissue samples were first fixed in the Karnovsky's fixative and then post-fixed in 1% OsO_4. Then the samples were dehydrated using different grades of acetone and embedded in araldite. The obtained ultrathin sections were stained using uranyl acetate followed by lead citrate and finally viewed under JOEL JEM 2100 transmission electron microscope.[22]

Acute Dermal Toxicity Test

For conducting the dermal toxicity test the OECD guideline 404[23] was followed, where a group of five healthy animals were randomly chosen and their dorsal fur was removed. Then an approximate area of 400 mm^2 was marked and the crude extract of the test plant was applied on the marked area. The extract was held in contact with the skin for 24 hours and next day the area was cleaned. The animals were then kept under observation for a duration of 14 days.

Acute Oral Toxicity Test

The oral toxicity test was undertaken by administrating a single dose of 2000 mg methanolic crude extract of *C. floribundus*/kg body weight of mice as described in the OECD guideline 425.[24] Before the dosing, the experimental animals were fasted overnight but water was allowed. After dosing, the animals were observed carefully continuously for 14 days to check for any signs of toxicity.

Statistical Analysis

The results have been expressed as mean ± standard error of mean (SEM). Statistical analysis was performed using one-way ANOVA. The mean differences were considered statistically significant at $p \leq 0.05$ compared to the control value.

RESULTS

Morphometric Evaluation

The detailed analysis of the photographic images of the wounds taken on 4, 7, 11 and 14 post-wounding days revealed that the healing process is faster in the extract-treated group compared with the control. The extract and drug-treated groups showed healed wounds and control animals had the presence of raw wound (Fig. 1).

Wound Areas and Rate of Wound Contraction

Table 1 shows the wound areas of the three experimental groups measured on different post-wounding days. It was recorded that after completion of the treatment period, the extract and drug-treated groups of animals had no wound left whereas, in case of the control group wound area of 7.47 ± 1.15 mm^2 was still present.

Similarly, the rate of contraction of the wounds for the two treated groups was higher than for the control, as shown in Table 2. It has also been observed that the extract and drug treated groups showed significantly higher rate of contraction, i.e., 89.06 ± 0.98% and 95.96 ± 1.04% compared to 74.22 ± 2.39% in the control group after 11 days of treatment.

Epithelialization period

The epithelialization period for the control group was found to be 15.93 ± 0.12 days. Whereas, the extract treated group had shown a significantly lower epithelialization period (12.92 ± 0.15 days) than the control group (Table 3).

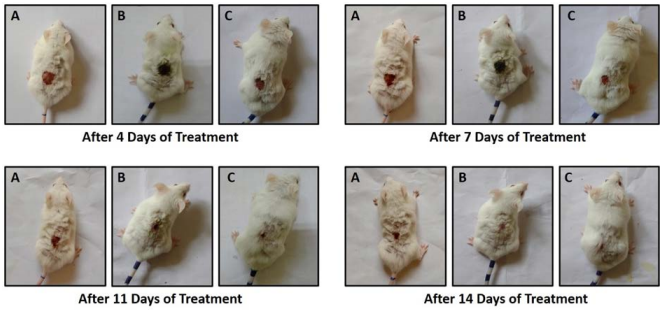

Fig. 1: Photographic representation of the three experimental groups of animals with excision wounds showing sizes of the wounds on different post-treatment days (A. Control, B. Extract treated and C. Drug Treated).

Table 1: Wounds areas (mm^2) of the experimental animals having excision wounds measured after different treatment days. Values have been expressed as mean ± SEM of 3 experiments and analyzed by one-way ANOVA. ⁎*p-values* significant at ≤0.05 compared to the control.

Days	*Control (mm^2)*	*Extract treated (mm^2)*	*Drug treated (mm^2)*
4	49.70 ± 1.69	45.85 ± 1.05	35.14 ± 1.72⁎
7	40.34 ± 1.36	35.98 ± 1.54	24.58 ± 1.93⁎
11	20.24 ± 1.88	8.59 ± 0.77⁎	3.17 ± 0.81⁎
14	7.47 ± 1.15	0 ± 0	0 ± 0

Tensile Strength

The tensile strength of skin tissue samples of the healthy animals was recorded to be 1.89 ± 0.02 MPa. After the completion of treatment period, the strength of newly repaired tissue samples collected from two treated groups were found to be significantly higher (1.44 ± 0.04 MPa for extract treated and 1.78 ± 0.05 MPa for drug treated) compared to the control tissue (Table 3).

Protein, DNA and Hydroxyproline Content

The pre-treatment concentration of protein, DNA and hydroxyproline in the skin tissues of the experimental animals were recorded to be 28.74 ± 0.46, 2.42 ± 0.18, and 3.75 ± 0.22 µg/mg tissue, respectively (Table 4).

The topical treatment of the excision wounds of the experimental mice with methanolic crude extract of the leaves of *C. floribundus* resulted in a significant rise in the levels of the three biochemical parameters in the skin tissues of the animals both after 7 and 14 days of treatment compared to the control group of animals. It was also recorded that the highest level of protein, DNA and hydroxyproline content was found in the neosporin treated group of animals on both post-treatment days (Table 4).

Expression Level of Growth Factor and Pro-inflammatory Cytokines

Table 5 shows the expression level of the growth factor PDGF-AA in the three groups of animals. The growth level of expression has also been increased significantly in the extract and drug treated groups compared to the control. The concentration of the three pro-inflammatory cytokines namely; IL-1β, IL-6 and TNF-α in the healthy group of animals before the experiment were recorded to be 2.89 ± 0.21, 1.41 ± 0.23, and 0.87 ± 0.02 pg/mg tissue, respectively (Table 5).

The treatment with crude extract and reference drug had resulted in significant downregulation in the expression level of the three pro-inflammatory cytokines in both treatment groups after 7 as well 14 days of treatment compared to the control. It was also observed that the concentrations were higher for the extract treated group compared to the drug treated group for all the three cytokines (Table 5).

Table 2: Effects of crude extract of C. floribundus and reference drug on the rate of contraction (Percentage) of excision wounds in three experimental groups of mice. Values are expressed as mean ± SEM of 3 experiments and analyzed by one-way ANOVA. ⁎*p-values* significant at ≤ 0.05 as compared to the control.

Days	*Control (%)*	*Extract treated (%)*	*Drug treated (%)*
4	36.68 ± 2.15	41.59 ± 1.33	55.24 ± 2.19⁎
7	48.61 ± 1.73	54.17 ± 1.96	68.69 ± 2.46⁎
11	74.22 ± 2.39	89.06 ± 0.98⁎	95.96 ± 1.04⁎
14	90.48 ± 1.46	100 ± 0	100 ± 0

Table 3: Effects of methanolic crude extract and Neosporin on epithelialization period (Days) and tensile strength (MPa) of the three experimental groups of animals. Values are expressed as mean ± SEM of 3 experiments and analyzed by one-way ANOVA. **p-values* significant at ≤0.05 as compared to the control.

Groups	*Epithelialization period (Days)*	*Tensile strength (MPa)*
Control	15.93 ± 0.12	0.92 ± 0.10
Extract treated	12.92 ± 0.15*	1.44 ± 0.04*
Drug treated	11.68 ± 0.05*	1.78 ± 0.05*

Histological Analysis

Hematoxylin and eosin staining of wound tissues collected after 7 days of treatment revealed the presence of advanced stage of epithelialization in the drug and extract treated groups. The epithelialization process in the control group tissue section was observed to be delayed (Fig. 2 [A]). Also, the granulation tissue in the control group was observed to be immature whereas the other two treated groups had comparatively matured granulation tissue (Figs. 2 [B] and [C]).

Similarly, with hematoxylin and eosin staining after 14 days the thickness of the epidermal layer in control skin tissue section was observed to be very less compared to the other two treated groups (Fig. 2 [D]). The thickness was maximum in the drug treated group and for extract treated group thickness was comparable to the positive control group (Figs. 2 [E] and [F]).

Similar to our biochemical result, we observed higher hydroxyproline content concentration in the two treated groups compared to the control. Van Gieson's staining of skin tissues revealed that the presence of higher amount of collagen in the tissue sections of two treated groups compared to the control group skin tissue sections on both post-wounding days (Fig. 3).

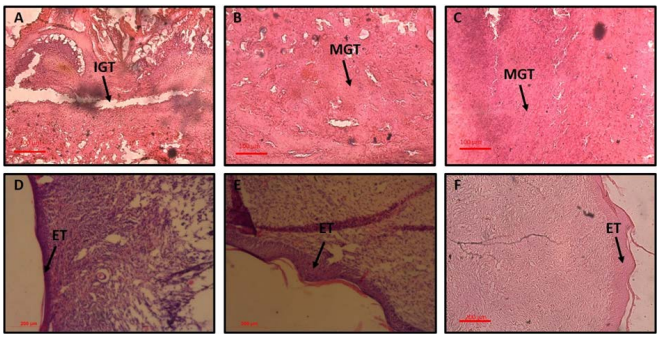

Fig. 2: Histological analysis of the skin tissues of the three groups of experimental animals using hematoxylin and eosin stain after 7 ([A]-[C]) and 14 ([D]-[F]) days of treatment. [A] Control tissue section showing immature granulation tissue (IGT). [B] and [C] Extract and Drug treated skin tissue sections showing mature granulation tissue (MGT). [D] Control tissue section showing thin epithelial layer (ET). [E] Extract treated tissue section showing epidermal thickness comparable to the drug-treated group. [F] Positive control group tissue section showing thicker epithelial layer compared to the control. All scale bar: 100 µm ([A]-[C]) and 200 µm ([D]-[F]).

Ultrastructural Observation

Scanning electron micrographs of the skin tissues collected from the three experimental groups after the completion of the treatment period (14 days) revealed the control group had the presence of irregular surfaces due to the delayed epithelialization process (Fig. 4 [A]). On the other hand, the

Table 4: Concentration of protein (µg/mg tissue), DNA (µg/mg tissue) and hydroxyproline (µg/mg tissue) in the wound tissues of the three groups of experimental mice on various treatment periods. Values are expressed as mean ± SEM of 3 experiments and analyzed by one-way ANOVA. **p-values* significant at ≤0.05 as compared to the respective control.

Period	Protein (µg/mg tissue)		DNA (µg/mg tissue)		Hydroxyproline (µg/mg tissue)	
Before treatment	28.74 ± 0.46		2.42 ± 0.18		3.75 ± 0.22	
Groups	Day 7	Day 14	Day 7	Day 14	Day 7	Day 14
Control	43.12 ± 0.99	17.97 ± 1.24	5.25 ± 0.16	1.85 ± 0.12	6.34 ± 0.39	2.15 ± 0.21
Extract Treated	63.47 ± 0.59*	25.17 ± 0.38*	6.99 ± 0.15*	2.85 ± 0.16*	8.27 ± 0.09*	3.66 ± 0.16*
Drug Treated	74.63 ± 1.85*	30.74 ± 0.71*	8.90 ± 0.21*	3.26 ± 0.13*	10.79 ± 0.31*	4.38 ± 0.10*

Table 5: Expression level of PDGF-AA (pg/mg tissue), IL-1β (pg/mg tissue), IL-6 (pg/mg tissue) and TNF-α (pg/mg tissue) in normal animals and in three treatment groups on 7th and 14th post-treatment days. Values are expressed as mean ± SEM of 3 experiments and analyzed by one-way ANOVA. **p-values* significant at ≤ 0.05 as compared to the respective control.

Period	*PDGF-AA (pg/mg tissue)*		*IL-1β (pg/mg tissue)*		*IL-6 (pg/mg tissue)*		*TNF-α (pg/mg tissue)*	
Before treatment	1.72 ± 0.38		2.89 ± 0.21		1.41 ± 0.23		0.87 ± 0.02	
Groups	*PDGF-AA (pg/mg tissue)*		*IL-1β (pg/mg tissue)*		*IL-6 (pg/mg tissue)*		*TNF-α (pg/mg tissue)*	
	Day 7	*Day 14*	*Day 7*	*Day 14*	*Day 7*	*Day 14*	*Day 7*	*Day 14*
Control	53.73 ± 1.70	7.28 ± 0.80	35.54 ± 0.48	14.42 ± 0.49	51.58 ± 0.68	5.64 ± 0.36	9.28 ± 0.19	2.82 ± 0.06
Extract treated	74.38 ± 1.57*	13.66 ± 0.26*	21.50 ± 0.52*	6.82 ± 0.09*	36.66 ± 1.21*	2.89 ± 0.10*	6.11 ± 0.28*	1.72 ± 0.10*
Drug treated	82.10 ± 0.55*	20.22 ± 1.49*	16.97 ± 0.46*	4.58 ± 0.25*	22.77 ± 0.54*	1.96 ± 0.04*	3.98 ± 0.25*	1.08 ± 0.04*

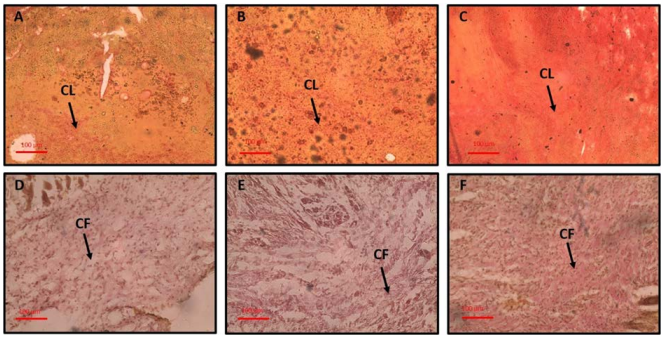

Fig. 3: Histopathological photographs of the wound tissues stained with Van Gieson's stain to observe collagenesis after 7 days of treatment ([A]-[C]) and after 14 days of treatment ([D]-[F]). [A] Control skin tissue section showing less amount of collagen (CL) content. [B] *C. floribundus* treated group tissue section showing moderate amount of collagen. [C] Tissue section of the positive control group showing high level of collagen. [D] Control tissue section showing low collagen fiber (CF) deposition. [E] and [F] Extract treated and Positive control group tissue sections showing well organized collagen fibers compared to the control. All scale bar: 100 µm.

two treated groups sowed the presence of comparatively smooth surface topography (Figs. 4 [B] and [C]).

Similarly, the analysis of transmission electron micrographs of the skin tissues sections revealed the absence of epidermal layers in control tissue section along with the presence damaged nuclei (Figs. 5 [A] and [D]). In drug treated section the epidermal thickness was highest among the three groups and the nuclei was found to be in normal condition (Figs. 5 [C] and [F]). For extract treated section the epidermal layers were present and the shape of nuclei was slightly elongated (Figs. 5 [B] and [E]). Also, in the control tissue section the granular secretion was maximum whereas, for the reference drug treated it was minimum. The plant extract exposed tissue section had lesser secretion compared to the control (Fig. 5 [E]).

Acute Dermal Toxicity

During the 14 days of observation, none of the exposed animals showed any signs of adverse effects. The color of skin and fur were found to be normal, along with normal behavioral pattern.

Acute Oral Toxicity

The LD_{50} value of the methanolic crude extract of the leaves of *C. floribundus* was found to be more than 2000 mg/kgbody weight as none of the extract administered mice died during the observation period. The experimental animals did not show any signs of toxicity, no changes were observed in body weight and all the functions were also performed normally.

Discussion

The process of wound healing is a complicated one whose ultimate goal is the promotion of a faster rate of closure of the wounds and recovery of the functional properties of

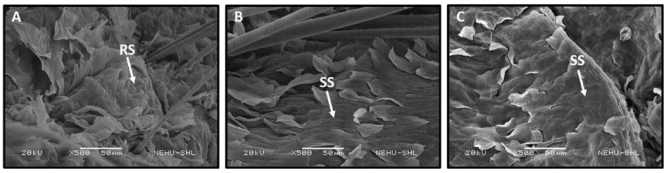

Fig. 4: Scanning electron micrographs of skin tissues of the three groups after 14 days of treatment. [A] Control tissue showing rough surface topography (RS). [B] and [C] *C. floribundus* extract and reference drug-treated groups tissues showing smooth (SS) surface topography. All scale bar: 50 µm.

the damaged tissue. In our study, the excision wound model was chosen for the evaluation of healing potential as the model is suitable for performing physical, morphological, histopathological and ultrastructural evaluations.

The rate of contraction of the wound is very essential step during the process of wound healing as it leads to the closure of the wounds. So, the contraction rate is an important parameter during the evaluation of wound healing process.[25] In our present study, the application of the plant extract resulted in an increased rate of wound contraction compared to the control group. The extract treated group also showed a significant reduction in the epithelialization period compared to the control indicating that the extract has pro-healing properties. The ability of the leaf extract to significantly reduce the contraction rate and period of epithelialization might be due to the anti-oxidant potential possessed by the plant.[26] After the completion of the treatment period, the tensile strength of the extract treated group was also found to be significantly higher than the control. The higher value of tensile strength of the skin tissues collected from extract treated group can be linked to the rise in the level of collagen content and better stabilization of the collagen

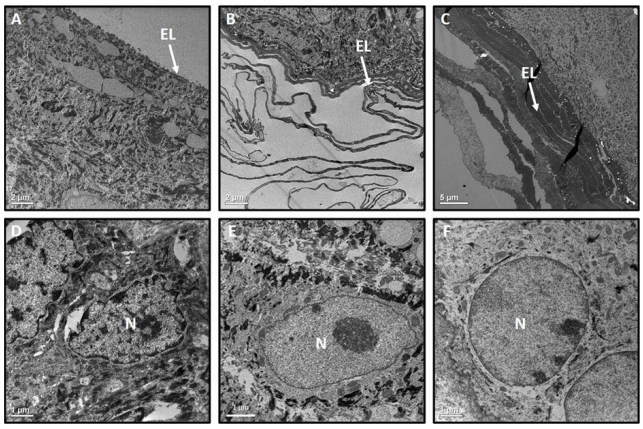

Fig. 5: Transmission electron micrographs of skin tissue of three experimental groups of mice after completion of treatment period. [A] Control section showing absence of epidermal layers (EL). [B] Extract treated group tissue section showing presence of epidermal layers. [C] Positive control group tissue section showing thickest epidermal layer. [D] Control group section showing completely deformed nucleus (N). [E] Plant extract treated group showing a slightly elongated nucleus. [F] Drug treated group showing normal shaped nucleus. Scale bar: 1 µm ([D]-[F]), 2 µm ([A] and [B]) and 5 µm ([C]).

fibers in the regenerated tissues induced by the topical application of *C. floribundus* extract.[27]

In the histological study, we observed that the rate of healing in the *C. floribundus* treated group is comparable to that of the neosporin treated group. The healing period in the control group has been delayed compared to the two treated groups where more collagen deposition, newly generated blood vessels, and matured granulation tissue were observed. The enhanced healing in the two treated groups can be linked with high amount of collagen content and also occurrence of neovascularization.[28] The role of collagen is very essential during the process of healing of wounds, as in healing wounds it is the main constituent of the connective tissue which forms the structural organization during the regeneration of the damaged tissues. In our study, the application of the plant extract has resulted in higher collagen fiber proliferation, as observed in histopathological evaluation where better organization of the collagen fibers in the extract treated group compared to the control is recorded. During the process of healing, angiogenesis is also crucial as it provides nutrients and oxygen to the injury site which ultimately helps in the maturation of the granulation tissue.[29] So, it can be said that the formation of matured granulation tissue in the extract treated group is due to the enhanced angiogenesis initiated by the application of the crude extract.

During wound healing, various cytokines initiate the process of inflammation, re-epithelialization, granulation tissue maturation and angiogenesis, which are responsible for the successful healing of wounds.[30] In the inflammatory phase of healing, the expression levels of the pro-inflammatory cytokines namely; IL-1β, IL-6 and TNF-α are very essential as their prolonged upregulation at the wound sites cause impaired healing which may lead to the development of chronic wound.[31] In our study, we recorded that the expression levels of these cytokines significantly declined in the extract trated group on 7^{th} and 14^{th} post-wounding days. The significant changes in the concentrations of the pro-inflammatory cytokines observed by us are at par with the studies conducted by Trengrove *et al.*,[32] and Muhammad *et al.*,[33] where they also recoded the downregulation in expression levels of these cytokines.

The quantitative estimation of hydroxyproline which is an amino acid found in the collagen, its content in the wound tissues indicate the rate of progression of healing in the damaged tissues.[34] In our experiment, we observed that the levels hydroxyproline levels were significantly elevated in the extract treated group and content was also comparable to the neosporin treated group, further indicating the faster healing rate in the concerned group. The collagen provides mechanical strength to the tissue and plays an essential role during hemostasis, epithelialization and in the later phases of healing.[35]

The significant increase in the levels of DNA and protein content in the extract and drug treated groups compared to the control group can be linked with the hyperplasia of the cells. The higher level of the biochemical parameters in the treated groups is because of the action of topical application of the extract and drug in the process of synthesis of protein. The elevated level of synthesis of protein has resulted in the maturation of granulation tissue leading to the healing of the wounds.[36] Similar to the concentrations of protein, DNA and hydroxyproline the expression of PDGF-AA has also been recorded to be significantly upregulated in the extract and drug treated groups compared to the respective controls on both post treatment days. The rise in the expression level of PDGF-AA further indicates faster healing in the extract treated group as the growth factor is also essential for cellular proliferation, angiogenesis and maturation of the wound granulation tissue.[37]

The ultrastructural observations have well supported the observations recorded by us during physical, biochemical and histopathological evaluations. The appearance of comparatively smoother surface topography in the extract treated group and eroded surface in the control tissues can be linked to the process of delayed epithelialization in the control group. The presence of different epidermal layers and lesser granular secretions as observed in the transmission electron micrographs of the tissue sections of the plant extract treated group indicate that the rate of healing is impaired in the control group compared to the extract and reference drug treated groups.[38]

The result of the acute dermal toxicity test is an indication that the methanolic crude extract of leaves of *C. floribundus* is safe for topical application.[39] The analysis of the results of acute oral toxicity suggests that the leaf extract of the plant is also safe for oral consumption but a detailed sub-acute toxicity study needs to be conducted to fix the dose which would be safe for the purpose of healing if the extract is to be administered orally.[40]

The *in-vivo* study conducted by us was an attempt to perform the scientific validation of the claim of traditional healers that the leaves of *C. floribundus* are a wound healing agent. So, we can finally conclude that the topical application of the crude extract of the plant leaves has shown promising wound healing efficacy in mice excision wound model as the results obtained were comparable to that of the reference drug Neosporin, a very commonly used commercial drug. The pro-healing effect of the plant may be due to the synergistic effect of the different biologically active compounds present in the plant extract.

Ethical Approval

The study was conducted by strictly adhering to all the ethical guidelines (CPCSEA, Government of India). The final approval (NEC/IEC/2018/004) to carry out the wound healing study on Swiss albino mice was granted

by the Institutional Ethics Committee (Animal models), North-Eastern Hill University, Shillong, Meghalaya, India on 1st October 2018.

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Author's Contribution

BR conceptualized the work and revised the manuscript. DD and AS performed the experiment and wrote the manuscript. KM analyzed the results and prepared the final draft. All the authors have read and approved the final manuscript.

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