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Acanthus leucostachyus leaf extracts promote excision wound healing in mice

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ABSTRACT

Objective: To evaluate the *in vivo* wound healing activity of *Acanthus leucostachyus* leaf extracts using an excision wound model in mice.

Methods: Mice were divided into two groups of six animals in each group: the control group and the *Acanthus leucostachyus* extract-treated group. Healing potential was evaluated by determination of physical parameters (contraction rate, epithelialization period, and tensile strength), biochemical parameters (protein, DNA, and hydroxyproline content), the expression of growth factor and pro-inflammatory cytokines, as well as histological and ultrastructural observations.

Results: Treatment with *Acanthus leucostachyus* leaf extracts markedly increased the rate of wound contraction, tensile strength, the concentrations of protein, DNA, and hydroxyproline, and the expression of growth factor, as well as promoted epithelialization, compared to the control. In addition, *Acanthus leucostachyus* leaf extracts significantly reduced the expression of pro-inflammatory cytokines. Histological and ultrastructural studies revealed the presence of thicker epithelial layer and smoother surface topography in the extract-treated group compared to the control.

Conclusions: *Acanthus leucostachyus* leaf extracts show potent wound-healing activity and can be used as a wound healing agent.

KEYWORDS: Chronic wounds; *Acanthus leucostachyus*; Epithelialization period; Tensile strength; Hydroxyproline; Pro-inflammatory cytokine; Wound healing

1. Introduction

The skin, which is made up of epidermis, dermis, and hypodermis, performs various functions like prevention of loss of electrolytes and water as well as thermoregulation, and also serves as a barrier to injuries. Keratinocyte is the most abundant type of cell found in the epidermis and its movement from the basement membrane to the surface of the skin forms various morphologically distinct epidermal layers[1].

Any physical injuries resulting in a breakage of the skin are known as wounds which can be categorized into two types namely: acute and chronic wounds. Ideal healing of the wounds is necessary to restore the normal functioning of the skin[2]. The healing process of wounds is a very sophisticated one that consists of four phases namely; hemostasis, inflammation, proliferation, and remodeling that progress in a highly integrated and overlapping manner leading to the closure of wounds[3]. Delayed and incomplete wound healing leads to the pathogenic inflammatory condition, which is very common all over the world and in the United States itself as many as 3 to 6 million people suffer from non-healing wounds which cost around 3 billion dollars per year for the health care sector[4].

The treatment of external wounds is generally done with the help of commercially available drugs such as antiseptics (iodine, hydrogen peroxide), combined antibiotics (Neosporin), anti-inflammatory

Significance

Due to undesired adverse effects of synthetic wound healing drugs, scientists are looking for new wound healing chemicals of natural origin. The present study shows that *Acanthus leucostachyus* leaf extracts exhibit potent wound healing activity, as evidenced by a significant increase in the levels of biochemical parameters, promotion of epithelialization and downregulation of pro-inflammatory cytokines. *Acanthus leucostachyus* can be further explored as a wound healing agent.

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drugs (ibuprofen), *etc*[5]. The primary reason for performing the treatment of wounds is to reduce the time required for healing and to prevent unwanted effects which may be caused by various external agents[6].

The majority of the population in developing countries like India is dependent on traditional medicine for their basic health care requirements[7]. The World Health Organization is also encouraging the use of traditional medicines in developing countries as these are cheaper than synthetic drugs and also have minimal side effects on the patients. Traditional medicine contributes to around two third of the therapy in the treatment of skin problems and healing of wounds whereas, modern drugs are used in very limited amounts till today all over the globe[8,9].

The shortcomings of modern drugs including undesired side effects and high cost along with their irrational use have increased demand for herbal products all over the world. Thus, a drastic rise has been observed among medical researchers in evaluation of different pharmacological potentials of traditional medicinal plants to justify the claims of the traditional healers, as medicinal plants are important for the present health care system and are also an asset for future drugs[10].

The Northeastern region of India is considered a hotspot for medicinal plants and is inhabited by a large number of tribes of various ethnic groups. These people are very rich in traditional knowledge and have been using many lesser-known wild plants for treating various ailments since time immemorial[11]. *Acanthus leucostachyus* (*A. leucostachyus*) is an unbranched herb belonging to the family Acanthaceae and is being used in traditional practices for various purposes. The leaves of the plant are used for treating toothache and also fever, and the paste of the leaves is used for the treatment of small-scale cuts, burns, and wounds[12]. In light of the use of this plant as a healing agent by a large section of people in Northeast India, the present study was undertaken to evaluate the *in vivo* wound-healing activity of the methanolic crude extract of the leaves of *A. leucostachyus* using an excision wound model in mice.

2. Materials and methods

2.1. Collection of plant material and preparation of extract

The plant *A. leucostachyus* was collected from Kokrajhar district of Assam and identification (BSI/ERC/Tech/2018/69) was done from the Botanical Survey of India (BSI), Shillong, Meghalaya. After collection, the leaves were separated from the plant, washed with water, and dried under shade. The dried leaves were converted into a fine powder using an electric grinder, then the powdered material was soaked in 90% methanol (100 g/L) for 10 d with constant stirring during the first few days. The solution was filtered using Whatman filter paper no. 1 and methanolic crude extract of the leaves was obtained by evaporation of the solvent using a rota-evaporator[13]. The extract was then stored in the refrigerator at 4 ℃ for future use.

2.2. Experimental animals

Adult Swiss albino mice of both sexes (weight 25-30 g, age 8-12 weeks) were purchased from the Pasteur Institute, Shillong. The animals were maintained as per the guidelines of the Organization of Economic Co-operation and Development[14]. The animals were kept in the animal house having uniform temperature (22-25 ℃) and a 12 h light/dark cycle. The animals received standard mice food and water *ad libitum*. For two weeks, the animals were acclimatized before starting the treatment.

2.3. Creation of excision wound

Following the procedure of Kumar *et al*.[15], excision wound was made on the mice. Initially, the fur of the anesthetized animal was shaved off using an electric clipper followed by outlining a circular area of 10 mm diameter with a marker. The skin of full thickness was cut off from the outlined area with the help of a scissor and a forcep. After creating a wound, the area was cleaned with cotton swabs dipped in normal saline.

2.4. Treatment and group division

After infliction of the excision wound, the animals were divided into two groups of six mice in each group (3 males and 3 females) as follows: Group 1: The control group was only treated with carboxymethylcellulose (CMC, 100 mg/kg bw); Group 2: Treatment was done with methanolic crude extract of *A. leucostachyus* leaf (400 mg/kg bw) mixed with CMC (100 mg/kg bw).

The treatment was carried out for 14 d where each mouse of the two experimental groups was treated topically once a day. The skin tissues were collected after 7 and 14 d of treatment to perform various studies.

2.5. Morphometric evaluation

For performing a morphometric study, the photographs of wounds of the two groups were taken on days 4, 7, 11, and 14 with the help of a camera. Morphometric evaluation was done by comparing the photographs of the extract-treated group with that of the control group.

2.6. Rate of wound contraction

The wound areas of the two groups were measured on days 4, 7, 11, and 14. The reduction in wound areas was indicated by wound contraction and expressed as a percentage reduction in the wound size.

$$\text{Rate of wound contraction} = (\text{healed area}/\text{total area}) \times 100$$

Where healed area=total area–wound area.

2.7. Epithelialization period

The number of days required for shedding of eschar with no raw wound being left is defined as the epithelialization period. The period required for healing of the wounds for the extract-treated group was then compared with the control group.

2.8. Tensile strength

This parameter has been used to measure the strength of newly repaired skin tissue in the two experimental groups. For this study, tissue samples were collected from the different groups of animals after 14 d of treatment and stored at −20℃ till further processing. Universal Testing Machine (Tinius Olsen 5ST) was used. During the measurement of the tensile strength, tissue samples were fixed in the clamps and continuous pressure was applied. The pressure at which each sample got torn was noted down and considered as the tensile strength of the specimen.

2.9. Protein, DNA, and hydroxyproline content

The skin tissues were collected from the two groups after 7 and 14 d of treatment and stored at −20℃ for estimation of biochemical parameters. The protein content of the tissues was determined using Folin-ciocalteau reagent following the method of Lowry *et al*[16]. The phenol-chloroform precipitation method by Sambrook and Russell[17] was used for isolation of DNA from tissue samples and the content was then quantified. The hydroxyproline content of skin tissues was determined according to the method of Switzer and Summer[18].

2.10. Determination of growth factor and pro−inflammatory cytokines

Tissue samples were collected from the different groups after 7 and 14 d of treatment. After collection, the samples were washed and then homogenized in phosphate buffer saline. The levels of IL-1β, IL-6, TNF-α, and PDGF-AA in the tissue extracts were measured using commercially available solid phase sandwich enzyme-linked immunosorbent assay (ELISA) as per the kit instructions.

2.11. Histological study

Tissue samples were collected after 7 and 14 d of treatment from the two groups and preserved in a 4% paraformaldehyde solution. Before carrying out the study, the tissues were washed and then sectioned at a thickness ranging from 6-8 µm using a microtome (RMT-30). After sectioning, the sections were passed through different grades of alcohol. For studying epithelialization, the sections were stained with hematoxylin and eosin, whereas for observing collagen synthesis Van Gieson's stain was used.

2.12. Ultrastructural study

Scanning electron microscopic study was carried out for microtopographical observation of the tissues collected from the experimental groups after the completion of treatment. The modified protocol[19] of Dey *et al*.[20] was followed in the study. Briefly, the tissue samples were fixed in neutral buffer formalin and dehydrated in different acetone grades. Subsequently, the tissue samples were air-dried using tetramethylsilane. Finally, the samples were gold coated and viewed under JOEL JSM 6360 Scanning Electron Microscope.

For transmission electron microscopic study, the tissue samples collected after 14 d of treatment were first fixed in the modified Karnovsky's fixative, followed by post-fixation in 1% OSO_4 for 4 h, then dehydrated using different acetone grades and then embedded in araldite. The ultrathin sections of the tissue samples were first stained with uranyl acetate and then with lead citrate. Finally, the skin tissue sections were viewed under JOEL JSM 2100 transmission electron microscope[21].

2.13. Statistical analysis

The values are expressed as mean±standard error of mean (SEM) and evaluated statistically using one-way ANOVA and the differences were considered statistically significant at $P<0.05$.

2.14. Ethical statement

The experimental study on the animals was carried out in compliance with the guidelines issued by the Committee for the Purpose of Control and Supervision of Experiments on Animals, Government of India. The experimental protocol was approved (NEC/IEC/2018/004) by the Institutional Ethics Committee (Animal Models), North-Eastern Hill University, Shillong, Meghalaya, India on 1st October 2018.

3. Results

3.1. Morphometric evaluation

The results showed that the process of healing was accelerated in the *A. leucostachyus* extracts-treated group, as evidenced by no wound left contrary to the control group where a small area of wound was still visible after 14 d of treatment.

3.2. Effect of A. leucostachyus extracts on the rate of wound contraction

The rate of wound contraction was calculated on days 4, 7, 11, and 14. During treatment period, the rate of wound contraction was found to be higher in the *A. leucostachyus*-treated group compared to the control group. A higher rate of wound contraction was observed in the *A. leucostachyus*-treated group from day 7. The treated group showed complete healing after 14 d of treatment while incomplete

healing was observed in the control group (Figure 1).

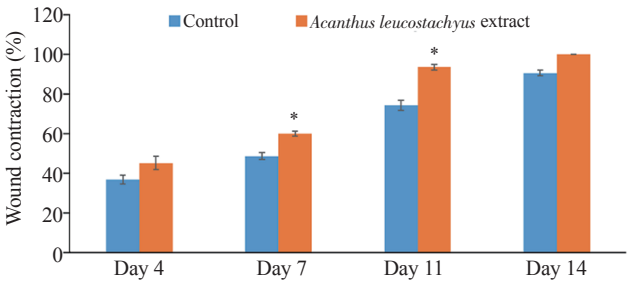

Figure 1. Effects of *Acanthus leucostachyus* extracts on the percentage of wound contraction in mice. Values are expressed as mean±SEM of 3 experiments and analyzed by one-way ANOVA. $^{*}P<0.05$ compared with the control group.

3.3. Effect of A. leucostachyus extracts on epithelialization period and tensile strength

The time required for healing of the wounds in the treatment group was significantly reduced compared with the control group [(12.58±0.10) d for the *A. leucostachyus*-treated group *vs.* (15.93±0.12) d for the control group].

The tensile strength of the *A. leucostachyus*-treated group was found to be higher than the control group [(1.64±0.07) Mpa *vs.* (0.92±0.10) Mpa].

3.4. Effect of A. leucostachyus extracts on protein, DNA, and hydroxyproline content

As shown in Figure 2, the topical application of *A. leucostachyus*

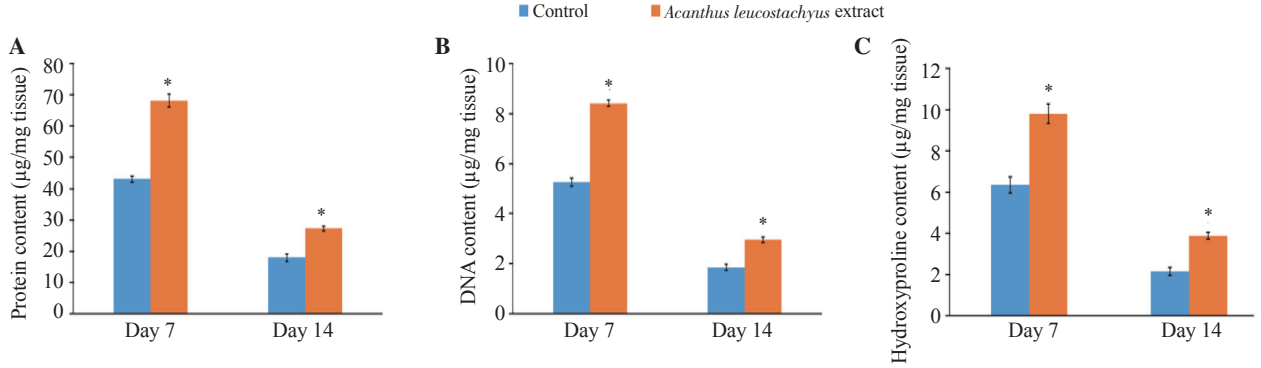

Figure 2. Effects of *Acanthus leucostachyus* extracts on protein (A), DNA (B), and hydroxyproline (C) content in the wound tissues of mice after 7 and 14 days of treatment. Values are expressed as mean±SEM of 3 experiments and analyzed by one-way ANOVA. $^{*}P<0.05$ compared with the control group.

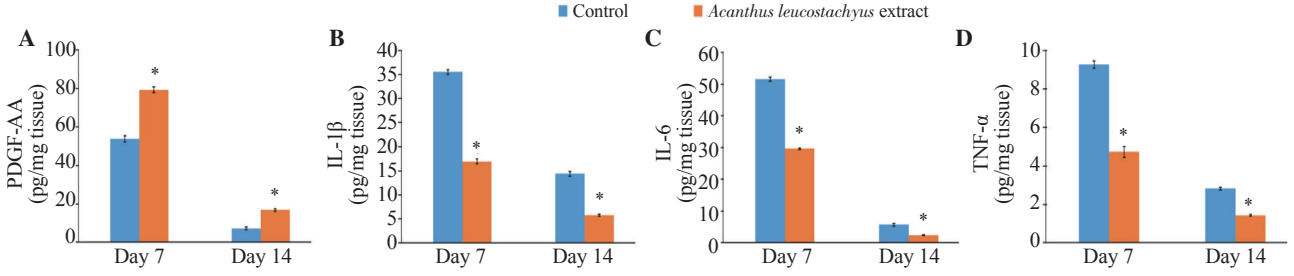

Figure 3. Effects of *Acanthus leucostachyus* extracts on the expression level of growth factor (A) and pro-inflammatory cytokines (B-D) in mice after 7 and 14 days of treatment.Values are expressed as mean±SEM of 3 experiments and analyzed by one-way ANOVA. $^{*}P<0.05$ compared with the control group.

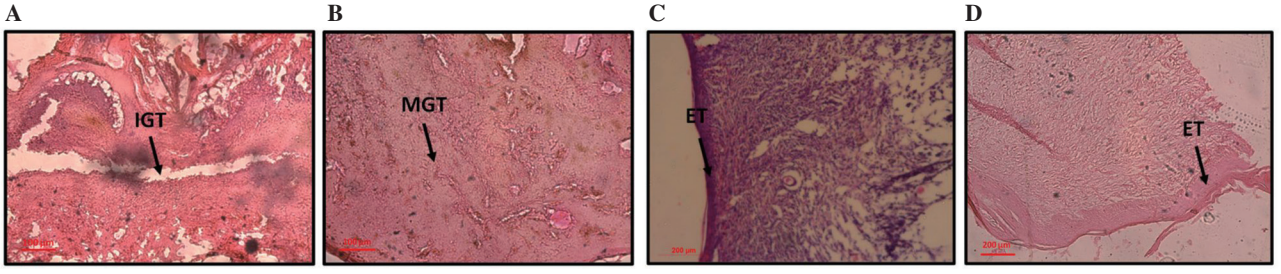

Figure 4. Histological images of skin tissues stained by hematoxylin and eosin after 7 (A-B, 20×) and 14 (C-D, 10×) days of treatment. A: The tissue section of the control group shows immature granulation tissue (IGT). B: The group treated with *Acanthus leucostachyus* extracts shows mature granulation tissue (MGT). C: The tissue section of the control group shows a thin epithelial layer (ET). D: The group treated with *Acanthus leucostachyus* shows a thicker epithelial layer compared to the control. Scale bar: 100 µm (A-B); 200 µm (C-D).

extracts resulted in the elevation of protein, DNA, and hydroxyproline content after 7 and 14 d of treatment compared to the control group.

3.5. Effect of A. leucostachyus extracts on the expression level of growth factor and pro–inflammatory cytokines

The expression level of PDGF-AA was increased significantly in the *A. leucostachyus* extracts-treated group compared to the control after 7 and 14 d of treatment (Figure 3). Moreover, the expressions of the pro-inflammatory cytokines including IL-1β, IL-6, and TNF-α were markedly decreased by treatment with the plant extract (Figure 3).

3.6. Histological observation

Hematoxylin and eosin-stained wound tissues collected from the control group on day 7 revealed the presence of delayed epithelialization and immature granulation tissue (Figure 4A). Treatment with *A. leucostachyus* extracts led to the enhancement of epithelialization as revealed by the formation of matured granulation tissue and more blood vessels (Figure 4B). After 14 d of treatment, a thicker epidermal layer was found in the *A. leucostachyus* extracts-treated group compared with the control group (Figures 4C and 4D).

Van Gieson's stained skin tissues treated for 7 and 14 d showed a

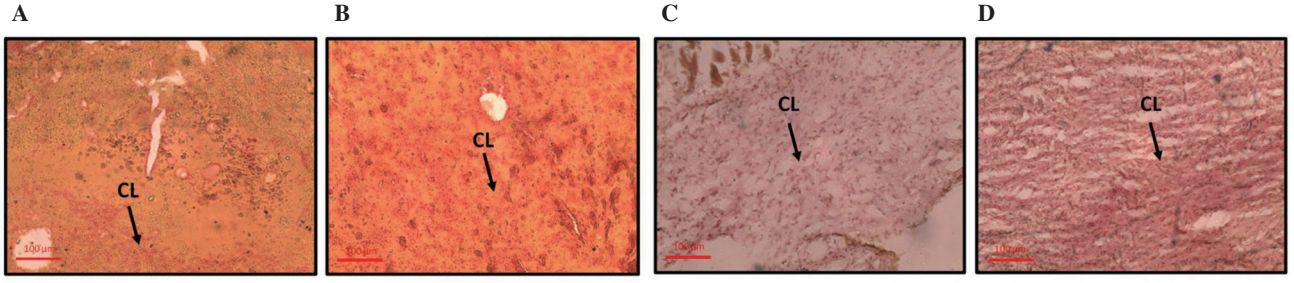

Figure 5. Histopathological images of the wound tissues stained with Van Gieson's stain to observe collagen content after 7 (A-B, 20×) and 14 days of treatment (C-D, 20×). A: The tissue section of the control group shows a small amount of collagen (CL) content. B: The group treated with *Acanthus leucostachyus* extracts shows a higher amount of collagen. C: The tissue section of the control group shows a low amount of collagen deposition. D: The group treated with *Acanthus leucostachyus* extracts shows a higher amount of collagen compared to the control. Scale bar: 100 µm.

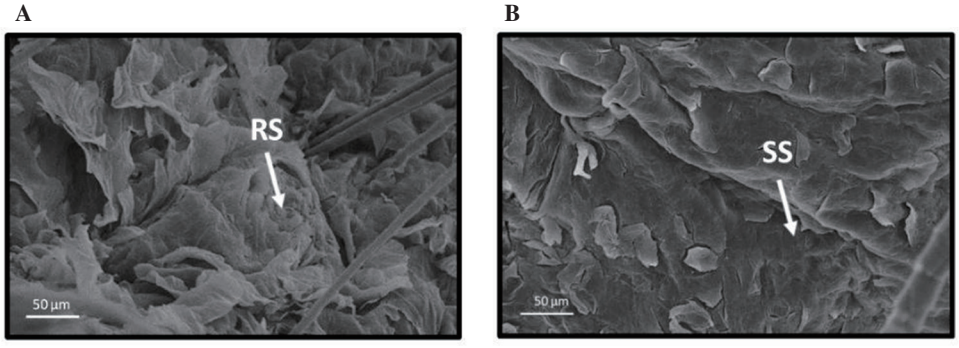

Figure 6. Scanning electron micrographs of skin tissues of two experimental groups showing external healing after 14 days of treatment (500×). A: The tissue section of the control group shows rough surface topography (RS). B: The group treated with *Acanthus leucostachyus* extracts shows smooth surface (SS) topography. Scale bar: 50 µm.

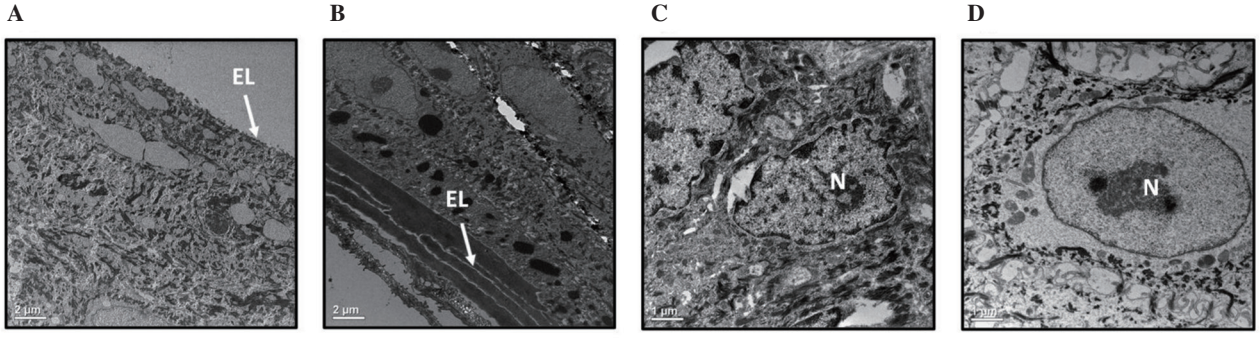

Figure 7. Transmission electron micrographs of skin tissues of two experimental groups of mice after 14 days of treatment (A-B, 1 000× and C-D, 2 000×). A: The control group shows the absence of epidermal layers (EL). B: The group treated with *Acanthus leucostachyus* extracts shows the presence of epidermal layers. C: The control group shows a completely deformed nucleus (N). D: The group treated with *Acanthus leucostachyus* extracts shows that the nucleus is in regular shape. Scale bar: 2 µm (A-B), 1 µm (C-D).

minimum level of collagen deposition in the control tissue section (Figures 5A and 5C). The group that received *A. leucostachyus* treatment showed a higher amount of collagen in the tissue sections (Figures 5B and 5D).

3.7. Ultrastructural study

Scanning electron micrographs of skin tissues revealed the presence of irregular projections (full of flakes) leading to the appearance of rough surface topography in the control group (Figure 6A). However, the tissues of the *A. leucostachyus* extracts-treated group had somewhat polished surface as a fewer number of flakes were present indicating the formation of matured epidermis (Figure 6B).

Transmission electron microscopy also revealed the presence of matured epidermis in the treated group (Figure 7B). The control section was devoid of epidermal layers (Figure 7A), whereas the treated group had the presence of epidermal layers. Also, the nucleus of the control group was deformed with increased granular secretion (Figure 7C) while the organelle was in normal shape in the treated group with low granular secretion (Figure 7D).

4. Discussion

The phenomenon of impaired wound healing is considered a major health crisis globally, in terms of morbidity and mortality[22]. The healing process involves different phases including contraction, re-epithelialization, and fibrosis[23]. The wound-healing process is one of the most important defense mechanisms regulated by the biological response from the body of the organism as the process is essential for restoring the disturbed anatomy of the skin[24].

The findings of our present study have indicated that the topical application of the methanolic crude extract of leaves of *A. leucostachyus* accelerated the process of healing in an excision wound model. In addition, a reduction in wound size and an improved rate of wound contraction were observed in the extract-treated group compared to the control group. The process of wound contraction is responsible for the closure of wounds through the shrinkage of the wounded area. The healing of tissue is also dependent on two other factors *i.e.*, the health condition of the organism and the extent of damage caused[25,26].

Our *in vivo* study showed that there has been a considerable decline in wound size of the extract-treated group from day 7. Similar to our observation, Belachew *et al*.[27] also observed that topical application of methanolic leaf extract of *Hibiscus micranthus* led to a rapid reduction in wound size in experimental animals after 7 d of treatment. Additionally, topical application of *A. leucostachyus* extracts increased the rate of wound contraction and a reduction in the epithelialization period. Similar observations were also recorded in the study of Yiblet *et al*.[28] in which the wound-healing activity of roots of *Stephania abyssinica* was evaluated in mice.

According to Wilkinson and Hardman[29], immediately after the formation of a wound, the fibroblasts that reduce the wound surface area due to their contracting properties migrate to the site of injury. The fibroblasts then produce collagen which is the main extracellular protein of the wound granulation tissue. The collagen imparts elasticity and integrity to the wound matrix and the level of collagen in the wound tissue is directly linked to the tensile strength of the concerned tissue. A higher value of tensile strength of the extract-treated group as observed in the present study compared to the control group suggests that the treatment with the plant extract caused a rise in the level of collagen, resulting in an increase in tensile strength of skin tissue of the extract-treated mice[30]. Collagen is also essential for the process of re-epithelialization at the wound sites, which regenerates the damaged tissue and causes closure of the surface area of the wounds[31].

The process of wound healing includes various processes like inflammation, epithelialization, angiogenesis, formation of granulation tissue *etc.* initiated by the fibroblasts, keratinocytes, and inflammatory cells[32]. Histopathological observation in the present study revealed a higher extent of regeneration of the epidermis in the plant extract-treated group compared to the control group suggesting that the rate of healing has been delayed in the control group. Similar histopathological observations were also recorded by Aboalhaija *et al*.[33] on *Schinus molle*. The delayed healing in the control group can be attributed to the formation of immature granulation tissue as observed in the histopathological study[34]. Scanning electron microscopy showed the presence of eroded surface in the control group compared to somewhat organized surface topography in the extract-treated group indicating that the tissues in the control group are in the early stages of epithelialization[35]. Transmission electron microscopy also revealed the presence of an advanced stage of epithelialization in the treated group as evidenced by a thicker epidermal layer. Also, the treated group had lesser granular secretion along with a regularly shaped nucleus which indicates an advanced stage of healing in the treated group[36].

The process of inflammation, regulated by various pro-inflammatory cytokines is another important event in the process of wound healing. The cytokines including IL-1β, IL-6, and TNF-α stimulate the synthesis of matrix metalloproteinases in the inflammatory cells and fibroblasts. The high concentrations of the abovementioned cytokines prevent the wound from closing leading to a delay in the process of healing[37]. *A. leucostachyus* extract reduced the concentrations of these cytokines which suggest that inflammation is mitigated and thus wound healing is promoted. These findings are consistent with those of Konwar *et al*[38].

Immediately after injury, collagen synthesis gets started at the wound site and is dependent on the availability of proline which is synthesized in the granulation tissue. The breakdown of collagen results in the production of hydroxyproline which is considered an index for measurement of collagen content in the tissues[39]. The level of hydroxyproline was elevated in the *A. leucostachyus*-treated group indicating that *A. leucostachyus* extract helped in the faster maturation of the granulation tissue. This finding was well supported by our histological observation where Van Gieson's staining showed

that the plant extract-treated group had a higher amount of collagen content compared to the control group after 7 and 14 d of treatment.

In the healing tissues, the levels of protein and DNA serve as an indicator for the process of cellular proliferation. The synthesis of protein is also very crucial for the regeneration of damaged tissue[40]. A significant increase in the levels of DNA and protein in the *A. leucostachyus*-treated group also supports our biochemical result, where a higher level of hydroxyproline was recorded. The concentrations of DNA and protein are related to the level of collagen in the wound tissues[41].

Growth factors such as PDGF play a crucial role in the different phases of wound healing process including angiogenesis, formation of granulation tissue, and epithelialization[42]. In the present study, the expression of PDGF-AA was markedly increased in the plant extract-treated group compare with the control group. According to Kutlu *et al*.[43], the lower expression of PDGF-AA in the control group can be linked to the impaired healing of the wound.

Thus, it can be said that the topical application of a methanolic crude extract of *A. leucostachyus* leaf enhanced the rate of healing in the extract-treated group compared to the control group. These findings are consistent with those of the previous studies[44,45]. Therefore, it can be concluded that *A. leucostachyus* has potent wound-healing activity. However, the active components of this plant and their mechanisms of wound-healing action need to be verified in future studies.

Conflict of interest statement

The authors declare that there is no conflict of interest.

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Authors' contributions

BR designed the work, corrected and finalized manuscript. DD and AS carried out the experiment, wrote the manuscript, and prepared the final draft. All authors read and approved the final manuscript.

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