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Original Article

Histological evaluation of wound healing potential of pods of *Acacia nilotica* aqueous and methanol extracts in rats

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ABSTRACT

Background and aim: Wound is a physical trauma that forms as a result of compromise in the integrity of tissue. Wound healing is a physiological mechanism that comprises series of processes, and involves complex biochemical and cellular interactions which result in restoration of functional integrity and structural regain of injured tissues. *Acacia nilotica* pod, bark, and stem extracts have been reported to have enhanced healing of wound by increasing epithelialization, collagen fibers formation, angiogenesis as well as wound contraction. This research aimed to investigate histological wound healing effects of *Acacia nilotica* pod aqueous and methanol extracts in rat model.

Methods: *Acacia nilotica* pod aqueous and methanol extracts were made at different concentrations, wounds were created by skin excision in three groups of rats, wound healing surface areas were assessed, the animals were sacrificed and analyzed histologically.

Results: The results showed that both the aqueous and methanol extracts of Acacia pods has significant effect on wound healing surface area with re-epithelialization, wound contraction, fibroblast and collagen formation.

Conclusion: *Acacia nilotica* pod aqueous and methanol extracts speed up wound contraction and wound healing and both extracts at the concentration of 1% and 2% can be used for the treatment of wound.

Keywords:
Acacia nolitica; fibroblast; collagen; wound contraction

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INTRODUCTION

Wound is the disruption or damage to the normal architecture and function (Robson, 2001). Wounds can originate pathologically and may form externally or internally around the organ involved (Dhivya *et al.,* 2015). Wounds can occur as a result of accident or intentional etiology or as a result of diseases (Dhivya *et al.,* 2015). Wound damages the tissue as well disrupts the area within the vicinity irrespective of the cause. A skin wound occurs as a result of simple break in the epithelial integrity of the skin or it could be deeper, spreading through subcutaneous tissue causing damage to different structures such as vessels, nerves, tendons, muscles, parenchymal tissues and sometimes reaching the bone tissue (Robson, 2001). Wound healing is a complex and dynamic physiological event that involves different coordinated processes such as inflammatory response, compliment activation, bleeding, coagulation, regeneration, migration as well as proliferation of parenchyma and connective tissues, extracellular matrix protein synthesis, new connective and parenchymal tissues remodeling as well as deposition of collagen (Dhivya *et al.,* 2015). Similarly, skin wound healing is a step by step mechanism involving many processes such as inflammation, coagulation of blood after wound formation, formation of new blood vessels as well as deposition of extracellular matrix. These processes can be finely observed histologically (de Moura Estevão *et al.,* 2019).

Acacia nilotica is an important multipurpose plant (Kaur *et al.,* 2005). This plant grown as tall as 5 m to 20 m. The color of its stems and branches is blackish with grey or pink tinge while the bark is fissured. This tree exudes reddish gum. *A. nilotica* is a pantropical and subtropical genus with different species that are abundant throughout Africa, Australia, Asia and America. Plant of *A. nilotica* occurs naturally and is imperative in traditional rural and agro-pastoral systems (Solomon-Wisdom and Shittu, 2010). *Acacia* species possess a secondary metabolites such as

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alkaloids, cyanogenic glycosides, hydrolyzable tannins, amines, cyclitols, fatty acids and seed oils, gums, nonprotein amino acids, terpenes (including essential oils, diterpenes, phytosterol and triterpene genins and saponins), fluoroacetate, flavonoids and condensed tannins (Seigler, 2003). The bark, leaves, pods and flowers of *A nilotica* are used against cold, cough, congestion, cancer, diarrhea, fever, dysentery, gall bladder and hemorrhoids, and they have vasoconstrictor, spasmogenic, anti-hypertensive, mutagenic, carcinogenic, anti-spasmodic, anti-inflammatory, anti-oxidant and anti-platelet aggregatory properties (Singh *et al.,* 2009).

Currently, extract-derived plants products have shown to facilitate wound healing, either through accelerating re-epithelialization, reducing the incidence of in situ inflammation, facilitating proliferation of fibroblast and angiogenesis. Plant extracts have shown effective combined mentioned pathophysiological effects on wound healing (Salehi *et al.,* 2019, Sharifi-Rad *et al.,* 2018).The advantages of plant extract include their easy access, being cheap and having limited side effects. Various pharmacological effects, including modulation of angiogenesis, negative modulation of inflammatory cytokines release, and stimulation of antioxidant enzymes to reduce oxidative stress, are involved in wound healing processes depending on the phytochemical properties of the extract (Hajialyani *et al.,* 2018).

In this research, we hypothesized that *Acacia nilotica* extract could be used to speed up the curing of skin wound by virtue of its anti-inflammatory as well as modulatory effects in cellular physiology. In this research we conducted and described macroscopic as well as microscopic assessment of the skin wound healing process in experimental rat model. We examined the reportedly good method and dosage of administering the extract on an excisional skin wound. The advantages of using this plant for wound healing over standard drugs are: very cheap compared to standard drugs, no more microbial resistance, the plant is available everywhere

MATERIAL AND METHODS

Collection, Preparation and Identification of the Plant Material

Fresh pods of *A. nilotica* were collected from Gummi local government area of Zamfara state and identified by an Herbarium from the Department of Pharmacognosy and Ethnopharmacy, Faculty of Pharmaceutical Sciences, Usmanu Danfodiyo University Sokoto, Sokoto State Nigeria. With a specimen voucher number (PTAAC/An(Ce)/OT/56-23). The pods of the plant were collected and dried under shade for 7 days. The dried pods were milled into fine powder by pounding manually with a clean mortar and pestle. The powder was collected into an airtight water free polythene bag, labelled for easy identification and stored in a cool dry place for further use.

Extraction of the Plant Material

Fifty grams (50 g) of the powdered pod was weighed and extracted with 800 ml of aqueous (Distilled water) and another 50g with 800 ml of methanol using maceration method. Maceration involved soaking the powdered plant material in stoppered containers (Beakers) with their respective solvents (water and methanol). It was then allowed to stand at room temperature for the period of 3 days with frequent agitation. Afterwards, the solvents (water and methanol) were heated off and the extracts were concentrated in an oven to dry residues. The dried extracts were weighed and kept in desiccators to be used in the trial.

Experimental Animals

Twelve (12) healthy matured male Wister rats, 10-12 weeks of age and weighing between 200-250 g were used for this study. The rats were maintained in clean stainless-steel cages at an ambient temperature of 30±2^{o}C with 12 hrs light, 12 hrs dark cycle and standard rat/mouse pellet at Usmanu Danfodiyo University, Department of Pharmacology's Animal house. The study was performed in accordance with the approval of Ethical Committee of animal care and use of the Department of Pharmacology and Toxicology, Usmanu Danfodiyo University Sokoto with Ethical Approval number, AETNO: PTAC/MO/(Ee)/Ta/51-21.

Animals Grouping

Twelve (12) male Wister rats were randomly assigned into four (1-4) groups with 3 rats per group. The groups were as follows:

(1) Negative control - group1 (3 wounded rats on natural healing/untreated)

(2) Positive control - group 2 (3 rats treated with Silver sulfadiazine SSDZ)

(3) Experimental - group 3 (3 rats treated with *A.nilotica* aqueous extract ANWE 0.1%, ANWE 1% and ANWE 2%)

(4) Experimental - group 4 (3 rats treated with *A. nilotica* pod methanol extract ANME 0.1%, ANME 1% and ANME 2%).

Surgical Procedure

Following anesthetization with ketamine and xylazine cocktail, using ether to calm the animals, the dorsum of all the rats was shaved and the exposed skin scrubbed with 70% ethanol. One circular skin wound of 4 cm diameter and full skin thickness of 6mm, was made on the shaved area of each rat's dorsum using a sterile skin biopsy punch (Surgical blade). Hemostasis was achieved by blotting the wound with cotton soaked in normal saline according to Ofori-kwakye, (2009) (Ofori-Kwakye *et al.,* 2009). The animals were numbered on each tail and were kept in their groups. *A nilotica* extracts, ANWE and ANME were applied topically using spatula one time per day to the wounds of groups 3 and 4 respectively and the wounds were left open. Sulfadiazine was applied to the wounds of group 2. Whereas, the wounds of

group 1 were wiped with dry cotton only. The treatment continued daily for five consecutive days. After fourteen days, the animals were euthenized. A square of about 4x4 cm of the wounded skin was taken from each rat of the four groups. The samples were then histologically examined and the results were compared.

Wound Healing Assessment Method

Wound healing was assessed on various parameters including evaluation of wound surface, percentage of wound healing, duration of healing, and complete healing. Wound surface was evaluated as cm^2 unit on days 1,3,5,7,9,11, and 13 after wound creation and the percentage of healing was normalized by the following formula:

$$\text{Percentage of healing (Recovery \%)} = \frac{\text{Wound surface on day 1} - \text{wound surface on day Z}}{\text{Wound surface on day 1}} \times 100$$

Where **Z** is the day on which the wound surface was evaluated (Ofori-Kwakye *et al.,* 2009).

Histological Analysis of Wound Healing

Hematoxylin and Eosin (H and E) Staining was performed. Rats were sacrificed at day 14 post epithelialization using Ether to calm the animal. A square of about 4x4 cm of the wounded skin was taken from each animal of the four groups for histopathological study. The skin samples were fixed in 10 % formalin solution. After fixation, the tissues were washed in running tap water, dehydrated in ascending grades of ethyl alcohol, and cleared in xylene. Paraffin embedded tissue sections of 6 μm thickness were cut using a rotary microtome machine and mounted on glass slides. Histological sections were stained with H & E for histological examination. Digital photomicrographs were captured at representative locations using a digital camera attached to a microscope. Tissue samples were evaluated for the Formation of new blood vessels, hair follicle epidermal regeneration, granulation tissue thickness (formation), angiogenesis, epithelialization tissue formation, collagen contents, the rate of wound closure, fibroblast, and epidermal exfoliation proliferations of fibrous tissues.

Statistical Analysis

Values were expressed as Mean ± Standard Error of the Mean (SEM). Data analysis was performed using Graph Pad Prism Statistical Software version 6.0 and was analyzed using ANOVA. A difference at $p<0.05$ was considered significant.

RESULTS

Wound healing process was assessed by macroscopic and microscopic study. The results of macroscopic study showed the biophysical parameters of wound beds which included; the evaluation of the wound surface area (cm^2) and percentage of healing (wound recovery %) in the study groups compared with the control group as depicted (table 1).The result showed significant differences between the control and treatment groups on days 3, 5, 7, 9, 11 and 13 the ($P<0.05$). There was no significant difference in wound surface on the first day after surgery. However, by the third day of treatment with *Acacia nilotica* extract, the wound area was significantly decreased, in comparison with the positive and negative control groups ($P<0.05$). In the experimental groups, wound healing from the third day to the thirteenth (13^{th}) day of treatment with *Acacia nilotica* extract was significantly greater by percentage (%) than the control group ($p<0.05$) as depicted in (table 1).

The result of microscopic view of open cutaneous wounds of general group has been shown in (Figure 4). The histological findings were epidermal regeneration, fibroblast, collagen, granulation, hair follicle, tissue thickness and angiogenesis. In the 0.5% *A. nilotica* aqueous extract (ANWE) and 0.5% *A. nilotica* methanol extract (ANME) of both treated groups, there was moderate to complete re-epithelialization with improved epidermal regeneration and widening over wound surfaces at day 14.

However, in the control groups, little dermal or epidermal organization was observed on day 14, and a significant reduction in granulation tissue formation, incomplete matrix maturation and remodeling were observed, while the **SSDZ** treated group revealed moderate to complete re-epithelization with little dermal regeneration observed only on day 14. Similar trend was observed in angiogenesis where 1% *A. nilotica* aqueous extract (**ANWE**) and 1% *A. nilotica* methanol extract (**ANME**) in both treated groups showed newly formed capillary vessels in moderate numbers in the dermis of entire wound areas on day 14, and new well-formed capillary vessels were observed disposed vertically toward the wound surface. However, in the negative control and **SSDZ** groups, altered to a few incomplete newly formed capillary vessels were observed on day 14. In terms of granulation tissue thickness, the 2% *A. nilotica* aqueous extract (**ANWE**) and 2% *A. nilotica* methanol extract (**ANME**) of both treated groups were also greater than those of the control groups on day 14

Table1: Comparing wound surface (CM^2) and wound recovery (%) in all study groups in 1st, 3rd, 5th, 7th, 9th, 11th and 13th days of study ($P \leq 0.05$).

Days	Groups	Wound Area (CM^2) Mean ± SD	Wound improvement (%) Mean ±SD
1st Day	UTRD	1.50±.00	11.77±.30
	SSDZ	1.53±.01	11.64±.30
	ANWE	1.86±.06	5.40±.00
	ANME	1.83±.03	7.18±.70
3rd Day	UTRD	1.40±.00	17.83±.30
	SSDZ	1.36±.07	21.10±.00
	ANWE	1.67±.07	15.26±.70
	ANME	1.67±.07	15.16±.70
5th Day	UTRD	1.30±.00	23.73±.30
	SSDZ	1.20±00	32.73±.30
	ANWE	1.46±.07	25.43±.30
	ANME	1.46±.07	25.36±.70
7th Day	UTRD	1.20±.00	29.66±.70
	SSDZ	1.06±.07	38.56±.70
	ANWE	1.26±.07	38.26±.70
	ANME	1.23±.03	37.20±.00
9th Day	UTRD	1.03±.03	39.13±.30
	SSDZ	0.93±.03	46.16±.70
	ANWE	1.06±.07	49.13±.30
	ANME	1.03±.03	47.36±.30
11th Day	UTRD	0.83±.03	51.00±.00
	SSDZ	0.73±.03	57.83±.30
	ANWE	0.76±.07	61.06±.70
	ANME	0.76±.07	60.96±.70
13th Day	UTRD	0.56±.07	68.80±.00
	SSDZ	0.33±.03	76.70±.00
	ANWE	0.36±.07	81.40±.00
	ANME	0.36±.07	82.00±.00
Between-Groups *P*-value		<0.000001	<0.000001
Within-Groups (time) P-value		<0.000001	<0.000001
Time*Group ANOVA P-value		<0.000001	<0.000001

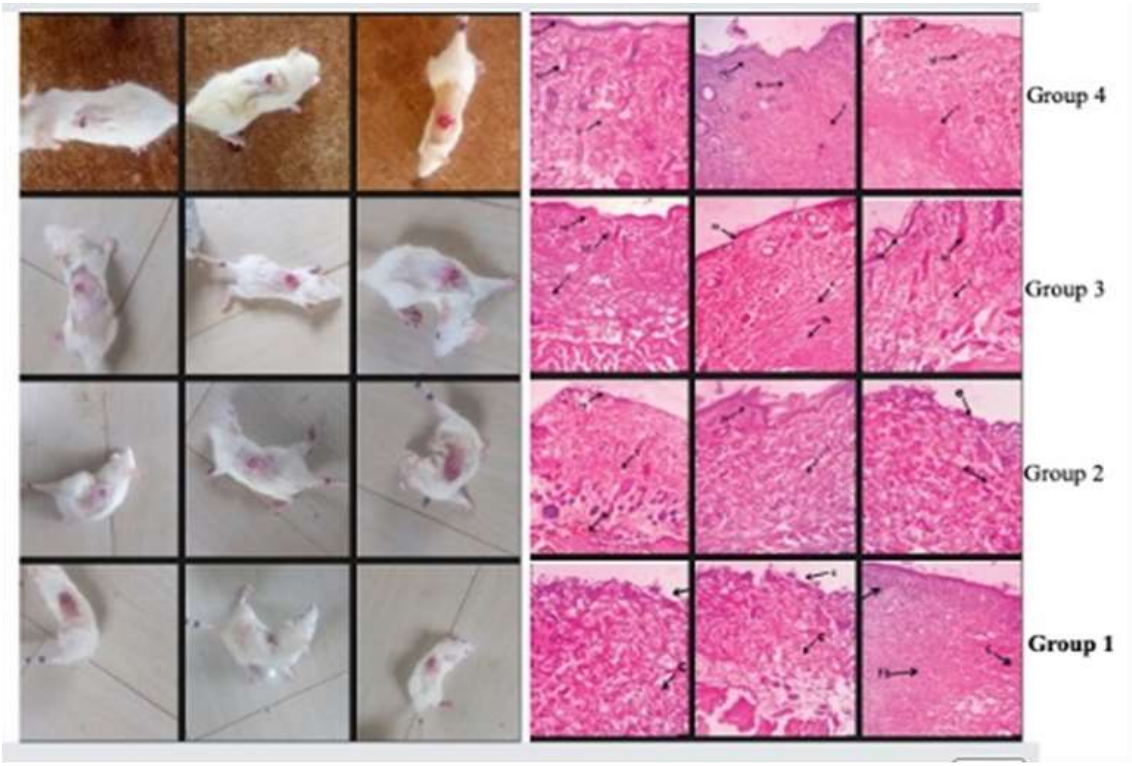

FIGURE 1: Pictures of rats on day 1 compared with H&E stain of histological section of the skin tissue obtained from the 14th day excision wound model. Group 1 (negative control group /untreated. Group 2 (positive control group treated with **SSDZ**). Group 3 (group treated with **ANWE).** Group 4 (group treated with **ANME). A**rrow marks: **e**: epithelial layer; **Fb**: fibroblast cells; **c**: collagen**; re**: re-epithelialization; **hf**: hair follicle.

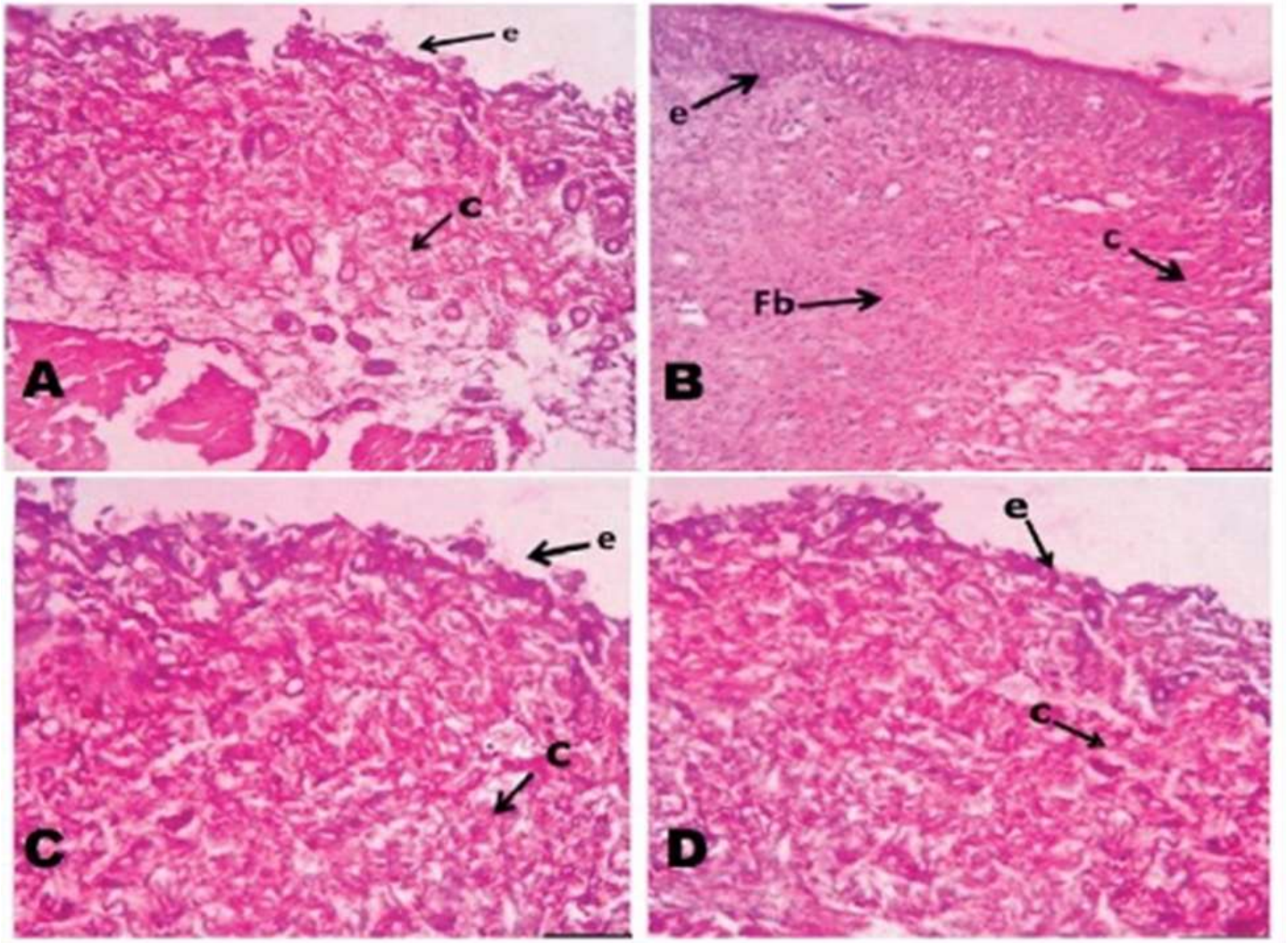

PLATE 1: Photomicrographs of the histological sections of the skin tissue obtained from the 14th day excision wound model. **(A)** Negative control group (untreated). **(B) P**ositive control group (Treated with SSDZ). **(C) G**roup treated with **0.5% ANWE. (D) G**roup treated with **0.5% ANME. H&E (X 100).** **A**rrow marks: **e**: epithelial layer; **Fb**: fibroblast cells; **c**: collagen**; re**: re-epithelialization; **hf**: hair follicle

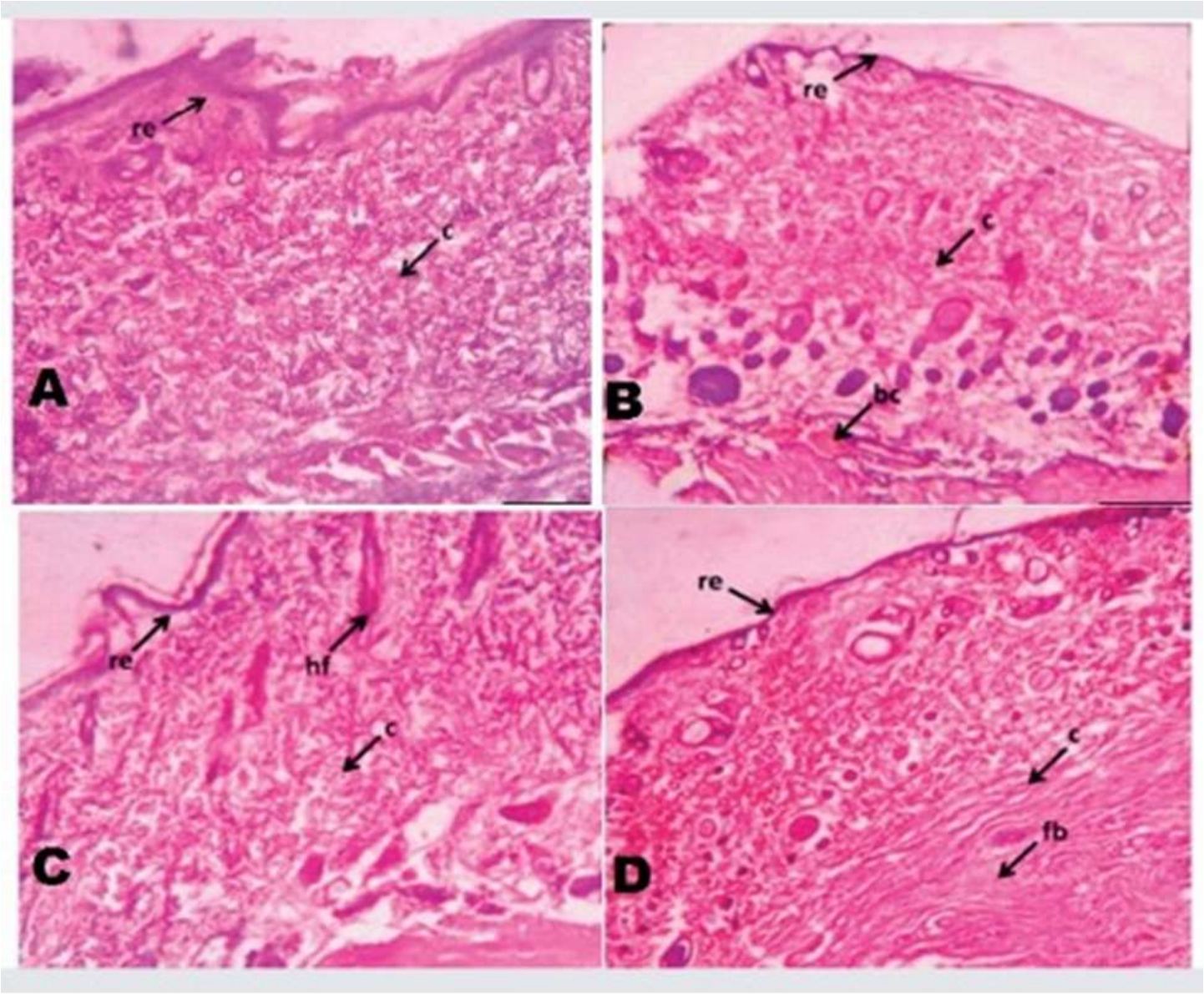

PLATE 2: Photomicrograph of the histological section of the skin tissue obtained from the 14th day excision wound model. **(A)** Negative control group (untreated). **(B)** Positive control group (Treated with SSDZ). **(C) G**roup treated with **1% ANWE. (D) G**roup treated with **1% ANME. H&E (X 100). A**rrow marks: **e**: epithelial layer; **Fb**: fibroblast cells; **C**: collagen**; re**: re-epithelialization; **hf**: hair follicle).

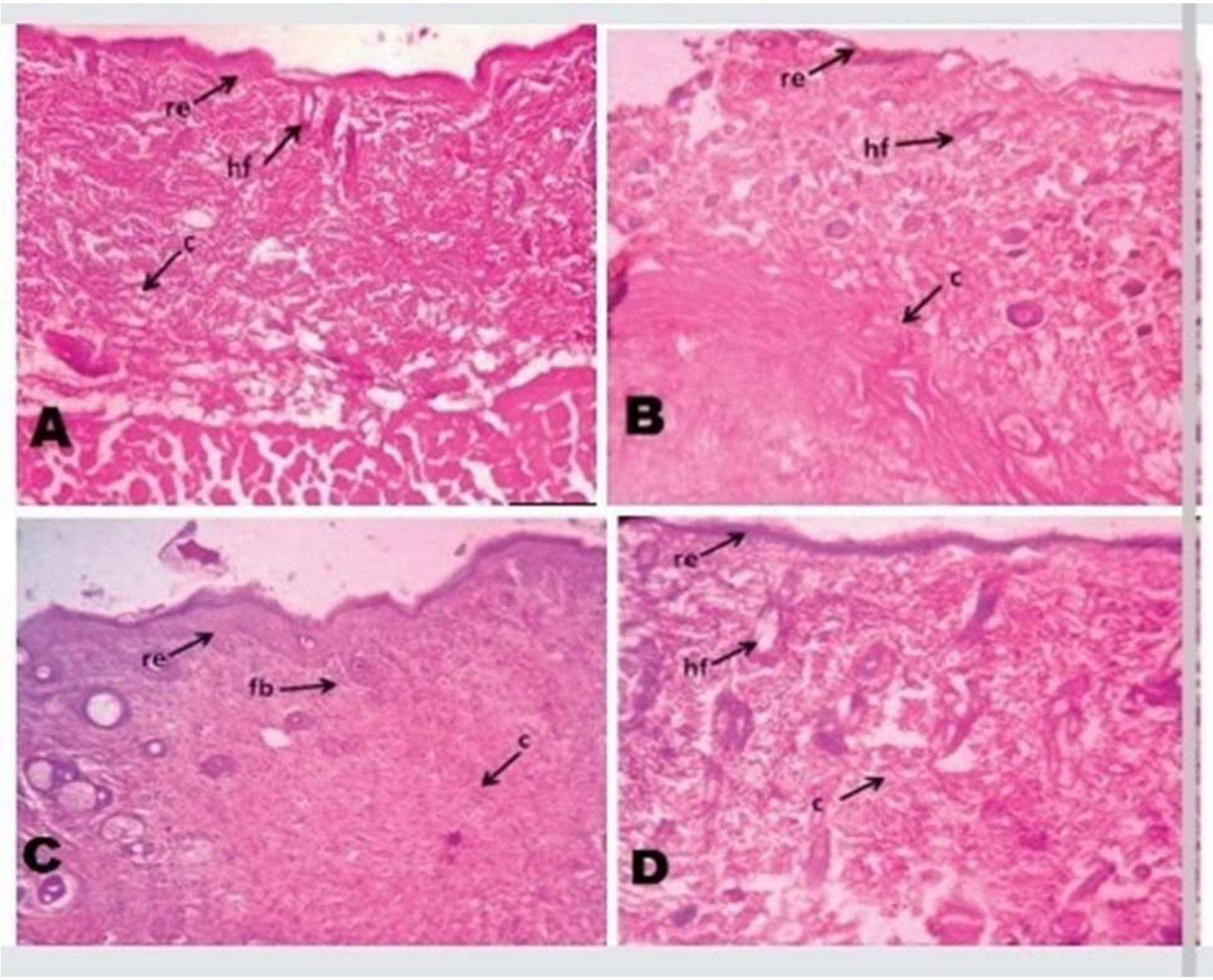

PLATE 3: Photomicrograph of the histological section of the skin tissue obtained from the 14th day excision wound model. **(A)** Negative control group (untreated). **(B) P**ositive control group (Treated with SSDZ). **(C) G**roup treated with **2% ANWE. (D) G**roup treated with **2% ANME. H&E. (X 100).** Arrow marks: **e**: epithelial layer; **Fb**: fibroblast cells; **c**: collagen, **re**: re-epithelialization; **hf**: hair follicle).

DISCUSSION

This study mainly focused on the evaluation of the histological effects of *Acacia nilotica* extract on wound healing using Wister rats. Previous researches show that *A. nilotica* has multiple medicinal effects such as being anti-hypertensive, carcinogenic, anti-inflammatory, wound ulcers, skin diseases and many more (Sultana *et al.,* 2007, Singh *et al.,* 2009).

In this study, analyses of wound healing effect using *A. nilotica* pods aqueous and methanol extracts were conducted and the results revealed increase in wound healing compared to control groups; there was much significant difference in healing between day 11 and 13 when compared to other days in all the groups. The result showed significant differences between the control and treatment groups on days 3, 5, 7, 9, 11 and 13 ($P<0.05$). There was no significant difference in wound surface on the first day after surgery. However, by the third day of treatment with *Acacia nilotica* pods extract, the wound area was significantly decreased, in comparison with the positive and negative control groups ($P<0.05$). In the experimental groups, wound healing from the third day to the thirteenth (13^{th}) day of treatment with *Acacia nilotica* extract was significantly greater than the control group ($p<0.05$). A previous study showed the healing activity of *Acacia nilotica* methanol extract on excision and incision wound models (Suriyamoorthy *et al.,* 2014) which is almost similar to the current study in which we used both methanol and aqueous extract of Acacia pods in wound healing.

Results of the current study show much healing effect on rats treated with *Acacia nilotica* methanol and aqueous extract groups compared with those untreated and with those treated with standard sulphadiazine. It was revealed that Acacia tree, back stem, leaves, roots, gum, flowers and pods were used for treatment of different diseases including wound treatment (Kamil and Abdallah, 2018, Calame and Widgerow, 2017). In the current study we used *Acacia nilotica* pods both methanol and aqueous extract on wound using rat models and it showed a much significant improvement compared to control group especially at 14^{th} day. Phytochemical analysis of Acacia shows the presence of saponosides, tannins, polyphenols and flavonoids which could be responsible for the healing process, by reducing inflammation and pain, increasing re-epithelization, angiogenesis, keratinocyte migration as well as promoting the synthesis of collagens (Sene *et al.,* 2023).

A significant difference was observed in the current study between untreated group and treated group at different percentages both aqueous and methanol extract. But there was no significant difference between the group treated with Acacia aqueous and methanol extract with positive control group treated with standard sulphadiazine. All the percentages of the extract showed a good healing effect but 2% of the extract showed a better healing effect than 1% and 0.5%. It was revealed that Acacia can be used as anti-bacterial as well as homeostatic to help in hastening healing of the wound. The biopolymers seen in Acacia could be an important ingredient which is used for wound dressing. This can be the major agent that help in quicker restoration of damaged tissues (Bhatnagar *et al.,* 2013). The wound healing activity increased in the study groups compared to the negative controls group, while for the rest of the time (11 to 14) days healing activity is more in the groups. A previous study revealed that aqueous extract of *Acacia nilotica* and *Habiscus sabdariffa* were tested for anti-inflammatory effects in wound healing in animal models (Rajvaidhya *et al.,* 2012), which is almost in consistence with the current study in which we only used *Acacia nilotica* made aqueous and methanol extracts for wound healing in rat model and much and speedy healing effect have been observed.

Wound processes begin with hemostasis in which clotting of blood covers the entire wound surface area. The inflammatory cascade causes the releasing of inflammatory infiltrate fluid, fibrin production, immigration of neutrophil, monocytes, polymorphs as well as lymphocytes. Later, formation of new blood vessels (angiogenesis) together with ground substances, tissue granulation, precipitation of collagen, re-epithelization, contraction of wound and finally remodeling and healing occurs (Midwood *et al.,* 2004). Completion of wound healing occurs when collagen connects all the damages on wound surfaces (Abdullah *et al.,* 2022). Histological results of the current study revealed the presence of re-epithelization, collagen appearance, fibroblast that hastened the wound healing process in treated and positive control groups compared to untreated negative control. The appearance of connective tissues was more in 1% and 2% compared to 0.5% in both aqueous and methanol Acacia extract, which concluded from our research that both aqueous and methanol extract of Acacia can be used for wound healing with better prospects in the concentrations of in 1% and 2%.

Application of natural substances possessing medicinal characteristics that can speed up the physiological wound healing is very important. Many studies were conducted on wound healing using different natural crops with anti-inflammatory, antibacterial, antioxidant, pro-collagen synthesis yielding a successful result. Bioactive phytochemical components such as flavonoids, alkaloids, tannins, essential oils, saponins, terpenoids as well as phenolic may play a vital role in healing effects. Different literatures revealed that, these constituents have ability in hastening wound healing (Thakur *et al.,* 2011). Due to the fact that *Acacia nilotica* possessed these constituents, we decided to use *Acacia nilotica* pods extracts aqueous and methanol at different concentrations and the results showed a speedy and good healing effect when compared with the negative control.

Each of the *Acacia nilotica*'s phytochemical constituents has a specific function during wound healing. For example saponin can increase the production of pro-collagen while tannins and flavonoids have anti-bacterial and antiseptic effect on wound healing (Thakur *et al.,* 2011). They are easily absorbed by the skin's superficial layers therefore, they play an important function in wound healing process, also partake in formation of new synthetic substances during wound healing process (Ibrahim *et al.,* 2018). Previous studies show that free radical scavenging enzymes (FRSE) can be triggered and reactive oxygen species (ROS) that possess toxic properties for tissue and can stop healing effect of wound completely are removed (Aliyev *et al.,* 2004). High wound healing effect, tissue reservation from oxidative damage can be maintained through application of materials produced from natural medicinal plants such as *Acacia nilotica* that has free radical scavenging property (Bent, 2008) and the results of our study showed successful and speedy wound healing effect in A. nilotica treated groups compared with control group, which could be as a result of characteristic of *Acacia nilotica* mentioned above.

A study conducted by Abdullah *et al.* (2022) revealed that after application of *Acacia nilotica* extract on topical wounds on rat models epithelium covered the whole surface area of the wound and changed to regular thick skin to normal condition after 14 days shows (Abdullah *et al.,* 2022), this is in agreement with the current study in which we observed much fibroblast, re-epithelialization in treated group compared with control group. Another study conducted by Kankara *et al.,* 2017, shows that *Acacia nilotica* pod extract enhances healing of wound through increasing epithelialization, collagen fibers formation angiogenesis as well as wound contraction when compared with control group (Kankara *et al.,* 2017), this is also in agreement with the current study in which we observed re-epithelialization, angiogenesis, collagen deposition more when compared with control group of rat models. A study conducted by Mathias *et al.* (2022), in which they used the stem back of *Acacia nilotica* methanol extract on rat models, showed a significant wound

healing effect compared to control group (Nefai *et al.,* 2022), this is line with current study except that we used *Acacia nilotica* pod extract while they used stem back extract of *Acacia nilotica*.

CONCLUSION

The outcome of this study revealed that the traditional application of *Acacia nilotica* pods extracts in the treatment of open wound and burn will be successful. Methanol extract shows much wound contraction and recovery when compared with aqueous extract. Also, this plant may have more advantages compared to standard drugs, easy and cheaper found almost everywhere, no microbial resistance or side effects. Topical use of 2% extract of *Acacia nilotica* extract has superior effects in the acceleration of wound healing. The results show absence of any side effects on the rats. The finding of the current study becomes very important due to the fact that many reports revealed that wounds present one of the major world health problems, sometimes proving fatal, putting not only the patients and their families under social and financial pressure but also incurring economic stress on heath institutions. Thus, the current study agrees closely with different previous studies conducted

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