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Antimicrobial and Wound Healing Activity of *Azadirachta indica* and *Neocarya macrophylla* Seed Extracts

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Abstract

Phytochemical and antimicrobial constituents, along with wound-healing activities, of Azadirachta indica and Neocarya macrophylla seed extracts were studied in this work. A. indica and N. macrophylla seeds were subjected to cold extraction using hexane as the solvent. The extracts, labelled as AIHE and NMHE, were then examined for phytochemical and antimicrobial constituents following standard procedures. Investigation of the wound healing activities of AIHE and NMHE was carried out on six groups of seven Wistar rats each (a total of 42), labelled as groups A-F, and incised with a wound area of 550mm^2. 100 % standard ointment was used in treating Group A rats (positive control), 20 % of AIHE and NMHE for Groups B and C with 15 % AIHE and NMHE for Groups D and E respectively. Group F rats (negative control) did not receive any treatment. Parameters such as rate of wound contraction, wound area and epithelialization time at every four-day interval were used in assessing the wound healing activities of AIHE and NMHE. The effect of the incised wound on feed intake, growth parameters, change in body weight, and specific growth rate of the rats was also noted. Phytochemical analysis showed that both AIHE and NMHE contain alkaloids, cardiac glycosides, quinones, reducing sugars, and terpenoids. Flavonoids were found only in AIHE. AIHE and NMHE inhibited B. subtilis and P. aeruginosa. and S. aureus significantly. Wound healing activity of AIHE and NMHE revealed 20 % AIHE as the one with the most potent wound healing capacity out of all the ointments, (epithelialization time: 18.33±1.86 days), after which is 15 % AIHE (epithelialization time: 18.67±2.16 days); 15 % and 20 % NMHE were less potent: 19.29±1.25 and 20.17±2.64 epitheliazation time respectively. AIHE and NMHE regulated the activity of the endogenous enzymes on the scar areas. A. indica and N. macrophylla seeds have potential as sources of wound healing ointment.

Keywords: *A. ndica, N. macrophylla, seeds, phytochemical constituents; antimicrobial activities; wound healing potential.*

1.0 Introduction

Azadirachta indica, known as *Neem* tree and Indian Lilac, is a tree in the mahogany family Meliaceae. It is a tall evergreen tree whose bark is hard, rough and scaly. Its leaves are alternate, and its flowers are small and white in colour. It is one of two species in the genus Azadirachta. Neem trees now grow in islands located in the southern part of Iran. Its fruits and seeds are the source of neem oil. Neem has a strong root system with a deep tap root and extensive lateral roots. It has medicinal properties such as antimalarial activity, antiulcer effect, hypoglycaemic and immune stimulant activities [1].

N. macrophylla commonly known as gingerbread is a food-product, which is sweet in nature and flavoured with ginger typically using honey or molasses (treacle) instead of sugar. N. macrophylla foods could be soft, moist loaf cake to something close to ginger biscuits. The seeds have purgative and anthelmintic properties [2]; they are also a good ointment source. There exist already in the literature some reports on the seed oil and seed cake of *N. macrophylla* [3,4].

This research evaluates the phytochemical constituents and the wound healing activity of *A. indica* and *N. macrophylla* seed extracts at 15 % and 20% in Wistar rats. It is an updated version of the work which has hitherto been presented at a Ghana conference [5] and a continuation of previous work on *A. indica* [6] and *N. macrophylla* [7].

2.0 Materials and Methods

2.1 Preparation of seed extracts

Seeds of *A. indica* and *N. macrophylla* were collected, sun-dried, reduced to coarse powder with an electronic blender and subjected to cold extraction for four days using hexane as the extracting solvent, as described by Omolere *et al.* [7].

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AIHE (*Azadirachta indica* hexane extract) and NMHE (*Neocarya macrophylla* hexane extract) were thereafter stored in universal bottles at 4 °C prior to analyses.

2.2 Phytochemical analysis and screening

The methods of Harbone [8], Sofowora [9] and Solomon [10] were employed in carrying out phytochemical screening of AIHE and NMHE. The determinations were in triplicate.

2.3 Test microorganisms and antimicrobial activity evaluation

Pure bacterial strains of *Bacillus subtilis, Pseudomonas aeruginosa* and *Staphylococcus aureus*, Multi Drug Resistant clinical isolates, obtained from the Department of Pharmacy, University of Ibadan, Nigeria, were used as the tested bacterial organisms. They were cultured overnight in nutrient agar at 37 °C. AIHE and NMHE were subjected to antimicrobial activity and thereby evaluated against a few pathogenic bacteria such as *B. subtilis, P. aeruginosa* and *S. aureus*. A culture of each of the organisms was prepared by taking a loopful of the organism from stock and inoculating it into 5 mL of sterile nutrient broth and incubated for 18-24 h at 37 °C. 0.1 mL of each organism, cultured overnight, was taken and placed into 9.9 mls of sterile distilled water to get a 1:100 (10^{-2}) dilution of the organism. 0.2 mls was then taken from the diluted organism and placed into the prepared sterile nutrient agar at 45 °C, and aseptically poured into sterile Petri dishes and allowed to solidify for about 45- 60 min. An 8 mm diameter sterile cork-borer was used to make wells in which different concentrations of sample were produced; this was done in duplicate. The plates, after being allowed to stay on the bench for 2 h for pre-diffusion to take place, were incubated uprightly in the incubator for 18-24 h at 37 °C.

2.4 Minimum inhibitory concentrations (MIC) of seed extract

MIC for both bacterial and fungal strains were estimated as described by Sarker *et al.* [11].

2.5 Assay of endogenous enzymes

2.5.1 Superoxide dismutate (SOD)

0.2 mL of the sample that has been diluted to 1:10 with distilled water was added to 2.5 mls of 0.05 M carbonate buffer (pH 10.2) in order for it to equilibrate in the spectrophotometer. The reaction was made to start by adding 0.3 mls of freshly prepared 0.3 M sample adrenaline to the mixture which was quickly mixed by inversion. The reference contained 2.5 mL of buffer, 0.3 mls of the substrate (adrenaline) and 0.2 mls of distilled water. The absorbance at 480 nm was monitored every 30 s for 150 s.

2.5.2 Catalase

0.5 mls homogenate was added to the reaction mixture, and incubated for different time period. The reaction was terminated by adding 2 mls acid reagent (dichromate/acetic acid mixture), which was prepared by mixing 5 % potassium dichromate with glacial acetic acid (1:3 by volume). The reagent was added to the enzyme, after which the tubes were heated for 10 mins and the absorbance was read at 610 nm.

2.5.3 Estimation of reduced glutathione

1 ml of the extracted sample was treated with 4.0 ml of metaphosphoric acid precipitating solution. Centrifugation was carried out on the mixture, after which 2.0 ml of protein-free supernatant was mixed with 0.2 ml of 0.4 M Na_2HPO_4 and 1.0 ml of DTNB reagent. Absorbance was read at 412 nm after 2 min.

2.6 Wound healing experiment

2.6.1 Experimental animals

Forty-two female Wistar rats, 60-80 g, properly kept in polypropylene cages and provided with easy access to food and water, were divided into six groups of seven animals each. The study was carried out according to the regulations of the University of Ibadan Ethics Committee on Research in Animals as well as standards that are accepted internationally for laboratory animal use and care.

2.6.2 Oil extracts formulation

15 % and 20 % (v/v) of AIHE and NMHE were prepared by mixing 7.50 mls and 10.00 mls of the extracts in 50 mL of soft yellow paraffin [12].

2.6.3 Excision wound model

The animals were anaesthetized before and during the creation of experimental wounds with ketamine hydrochloride (100 mg/kg, i.e.) [13]. The animals were inflicted with an excision wound [14]. The wound was left open, and all the animals were treated using the ointments that were prepared and formulated. The excision wound model was used to evaluate the wound healing activity of AIHE and NMHE; the 50 mg ointments formulated were applied topically once a day. The animals of group A received ointment base (control), groups B and C animals were treated with 20 % (v/v) of AIHE and NMHE ointments, the ones in groups D and E with 15 % (v/v) of AIHE and NMHE ointments respectively while group F ones were left untreated (negative control; no ointment was applied). The healing of the wound was monitored by tracing and measuring the wound contraction on the 0th, 4th, 8th, 12th, 16th and 20th, after the creation of wound. The wound closure was measured at regular intervals in order to calculate the percentage wound closure and epithelialization time. Epithelialization time indicates the formation of new epithelial tissue to cover the wound [15].

2.6.4 Rate of wound contraction

The rate of wound contraction was measured as the percentage reduction in size of wound inflicted on rats at every 4-day interval. Progressive decrease in the wound size was measured periodically using transparent paper and a marker, and the wound area was measured graphically to monitor the percentage of wound closure, which indicates new epithelial tissue covering the wound. The percentage wound contraction was measured according to Omolere et al. [7]:

$$\text{Percentage wound contraction} = \frac{A_o - A_t}{A_o} \times 100\ \%$$

Where A_0 = Initial area of wound at day "0'' of experiment
A_t = Area of wound at day "t'' of experiment.
The number of days required for filling of the scar without any residual of the raw wound is the period of epithelialization.

2.6.5 Histopathological study

On the 24th day of the experiment, the skin tissues were collected from all the six groups of the animals and processed for histological study to determine the pattern of lay-down for collagen. The skin specimens from the treated animals were collected in 10 % buffered formalin and subjected to sectioning and 6 µm-thick sections were stained with hematoxylin and eosin. The stained slides were visualized for histological changes using a light microscope.

2.7 Statistical analysis

Statistical analysis was carried out on each group, followed by an ANOVA test (GraphPad Prism software), which was used to compare the mean value of each treatment. Significant differences were determined between the means of parameters using Dunn's t-test ($P < 0.05$).

3.0 Results and Discussion

3.1 Phytochemical screening

The results of the phytochemical analysis of 15 % and 20 % (v/v) AIHE and NMHE, including the ointment is shown on Table 1. The same constituents are found to be present in both 15 % and 20 % AIHE and NMHE. Alkaloids, cardiac glycosides, flavonoids, reducing sugars, and quinones were detected in both AIHE and NMHE. Tannins were detected in NMHE only. Steroids was detected in the conventional ointment. These compounds have been proven to be active against pathogens, including those that cause enteric infections [16]; they exhibit antibacterial activities. Compounds such as alkaloids are antimalarial agents and can act as stimulants and as analgesics, while glycoside moieties, such as saponins, anthraquinones, cardiac glycosides and flavonoids, can act as antiparasitic agents. They inhibit tumors growth and are used as antidepressants. It is believed that the metabolites detected in these extracts are one of the major active constituents responsible for wound healing in the experimental rats and as a result, would accelerate the rate of wound contraction. Tannins have astringent properties. Alkaloids are known to have activities such as anticancer, analgesic, antimalarial, etc. It has been known that it acts as a starting material for many drugs in pharmaceuticals. Terpenoids play a major and important role in traditional and herbal medicines. Glycosides are used in medication. Phenols act as drugs, and from study, they are known to act as a good defense against infection [17]. When these constituents are available in high concentration in the extract, they could increase and accelerate wound healing in rats.

3.2 Evaluation of antimicrobial activities

AIHE was found to effectively inhibit the growth of organisms at different concentrations, when compared to those of the negative and positive controls (Table 2). *S. aureus* is slightly more susceptible than *P. aeruginosa. P. aeruginosa* and *S. aureus* have a minimum inhibitory concentration of 25 mg/mL, *B. subtilis* has an MIC of 50 mg/mlL. *S. aureus* has a zone of inhibition diameter of 16.00 mm, *B. subtilis* has 18.00 mm while *P. aeruginosa* has 10.00 mm. NMHE was found to effectively inhibit the growth of organisms at different concentrations. Compared to the negative and positive controls, *P. aeruginosa* was found to be more susceptible to NMHE than *S. aureus* and *B. subtilis*. *P. aeruginosa*, which is more susceptible, has a minimum inhibitory concentration of 50 mg/mL, *B. subtilis* has 100 mg/mL and *S. aureus* has a MIC of 50 mg/mL. *P. aeruginosa* has a zone of inhibition diameter of 16.00 mm, *S. aureus* has 14.00 mm and *B. subtilis* 12.00 mm.

3.3 Minimum inhibitory concentration of the seed extracts

Table 3 presents the minimum inhibitory concentration of AIHE. The extract has good antimicrobial activity. AIHE inhibited the growth of all the microorganisms tested at 50 mg/mL. There was no inhibition of growth in any of the organisms at a concentration of 200 mg/ mL and 100 mg/mL. NMHE showed good antibacterial activity. All microorganisms tested were inhibited at concentrations of 100 mg/mL, 50 mg/mL and 25 mg/mL, respectively. There was inhibition of *B. subtilis* at a concentration of 200 mg/mL, but no inhibition of *S. aureus* and *P. aeruginosa* at this concentration was observed.

3.4 Evaluation of wound healing activities

The initial wound area created was 550 mm^2. As the extracts, which served as medication, were applied on the wound surface, it was observed that experimental animals in Group B (20 % AIHE) had reduced area faster than the animals in other groups, followed by rats in Group D (15 % AIHE*)* (Figure 1). Group C rats had a reduced area faster than Group E; ones the extracts used to treat them were 20 % and 15 % NMHE respectively. All these extracts rats' groups had reduced rate faster than the negative and positive controls. Positive control rats group exhibited reduced wound area faster than the negative control ones. It can be deduced that 20 % AIHE is more effective in the treatment and healing of wounds in rats, followed by 15 % NMHE.

3.5 Wound contraction

Figure 2 shows the wound contraction of experimental animals. When the extracts which served as medication were applied on the wound surface; it was observed that experimental animals in Group B displayed the fastest epithelialization time compared to animals in the other groups, followed by rats in Group D. Ointment used on Group B rats was 20 % AIHE while the one used on Group D rats was 15 % AIHE. Animals in Group C (treated with 20 % NMHE) showed epithelialization time that was faster than those in Group E (treated with 15 % NMHE). All the animals that were treated with the seed extracts reached epithelization time faster than negative and positive controls.

Positive control rats reached epithelialization time faster than the negative control ones. This indicates that 20 % AIHE is more effective in the treatment and healing of wound in rats, after which is 15 % NMHE.

3.3 Epithelialization time

Figure 3 shows the epithelialization time of experimental animals in days. Group B (20 % (v/v) AIHE) had the fastest epithelialization time (18.33±1.86), followed by Group D which was treated with 15 % (v/v) AIHE (18.67±2.16). Group C (20 % (v/v) NMHE had an epithelialization time of 19.29±1.25. Group E treated with 15 % (v/v) NMHE had an epithelialization time of 20.17±2.64. Group A (100 % ointment), which served as the positive control, had epithelialization time of 20.57±1.62. Group F (negative control), which was not treated at all, had an epithelialization time of 24.00±00. According to the definition of Chattopaddhya *et al.* [18], healing of wound is complex in nature and dynamic process which involves tissue structure restoring back to its normal state. Healing depends upon the repairing ability of the tissue, type and extent of damage, and general ability to achieve epithelialization time that is faster than the others. 20 % (v/v) AIHE seemed to be more effective than the other ointments because of its faster wound healing ability.

3.4 Endogenous enzymes

Figure 4 shows the endogenous enzymes present in the experimental animals. Endogenous enzymes are secreted in the body naturally. They serve as wound-healing agents and defense of the body and also help as antioxidants and scavengers. By so doing, they inhibit and control the operations of free radicals in the body, which, when in excess, are dangerous to health. Catalase converts hydrogen peroxide to water and molecular oxygen, while superoxide dismutase neutralizes superoxide ions by going through oxidative and reductive cycles. The low level of superoxide dismutase in the body generally indicates that only a minimum quantity of it is needed by the body to function effectively in comparison to other enzymes. Figure 4 revealed that groups B and D (groups treated with 20 % and 15 % AIHE respectively) have higher values of superoxide dismutase and catalase and lower values of reduced glutathione. The high values implies that the extracts helped to increase the generation of these enzymes in higher rates than those of groups A, C, F and E. This probably explains the reason why rats from groups B and D reached epithelialization time faster than the rest of the rats in the other groups.

3.5 Histological study

Presented on table 4 is the result for histopathological analysis of skin scar' areas of rats used for this study. The epidermis of the skin of rats from group A is intact and thick; the dermis contains numerous hair follicles enmeshed within dense fibrous connective tissue. The epidermis of group B rats is thick; there are numerous mononuclear cells and hair follicles in the dermis. Group C rats have epidermis that is intact; that of the ones from group D is thickened and appears to be regenerating. Skin scars of rats from both Groups E and F have epidermis that is thin and intact. However, the dermis of those from group E is reduced in size and contains numerous hair follicles and sebaceous glands enmeshed within dense fibrous connective tissue while group F ones appear normal with hair follicles.

Table 1: Phytochemicals' constituents of the seed extract

Phytochemicals	AIHE	NMHE	Ointment
Flavonoids	+	-	-
Reducing sugar	+	+	-
Alkaloids	+	+	-
Carbohydrate	-	-	-
Cardiac glycosides	+	+	-
Phenols	-	-	-
Saponins	-	-	-
Steroids	-	-	+
Tannins	-	-	-
Terpenoids	+	+	-
Quinones	+	+	-

- indicates absence of phytochemical and + presence of phytochemical

Table 2: Antimicrobial activity of AIHE and NMHE against flora microorganism

	Zone of inhibition diameter(mm)						
Test organisms	**Concentration of AIHE (mg/mL)**						
	-ve control	200	100	50	25	12.5	+ve control
S. aureus.	-	16	14	12	10	-	38
B. subtilis	-	18	14	10	-	-	36
P.aeroginosa	-	18	14	12	10	-	34
Concentration of NMHE (mg/mL) -ve control 200 100 50 25 12.5 +ve control							
S. aureus.	-	14	12	10			38
B. subtilis	-	12	10	-			36
P.aeroginosa	-	16	14	10			34

-ve control= hexane, +ve control = Getamicin

Table 3: Minimal inhibitory concentration (MIC)

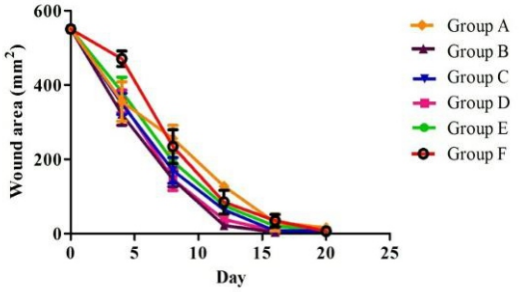
Test organisms	Concentration of AIHE (mg/mL)			
	200	100	50	25
S. aureus.	-	-	+	+
B. subtilis	-	-	+	+
P.aeroginosa	-	-	+	+
Concentration of NMHE (mg/mL)				
	200	100	50	25
S. aureus.	-	+	+	+
B. subtilis	+	+	+	+
P.aeruginosa	-	±	+	+

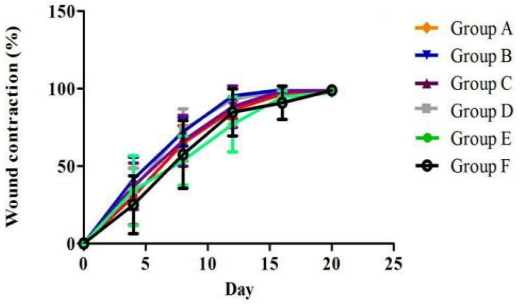
-" indicates no growth of organism, "+" indicated growth of organism

Table 4: Histological examination of the skin scar area

	Group Histological examination of the skin scar area
Group A	The epidermis (thick arrow) is intact and thick. The dermis (star) contains numerous hair follicles (thin arrows) enmeshed within dense fibrous connective tissue.
Group B	The epidermis (thick arrow) is thick. There are numerous mononuclear cells and hair follicles (arrows) in the dermis (star).
Group C	Epidermis is intact. Numerous hair follicles are present. Note the keratin pearl (arrow).
Group D	Epidermis (thick arrow) is thickened and appears to be regenerating. Note the discontinuous portion (star). There is paucity of hair follicles and numerous foci of mononuclear cells (arrows) in the dermis.
Group E	The epidermis (thick arrow) is intact and thin. The dermis (star) is reduced in size contains numerous hair follicles (thin arrows) and sebaceous glands enmeshed within dense fibrous connective tissue. Beneath the dermis is the thick muscle layer (blue arrow) and subcutis (red arrow).
Group F	The epidermis (thick arrow) is thin and intact. Dermis (star) appears normal with hair follicles and sebaceous glands (arrows).

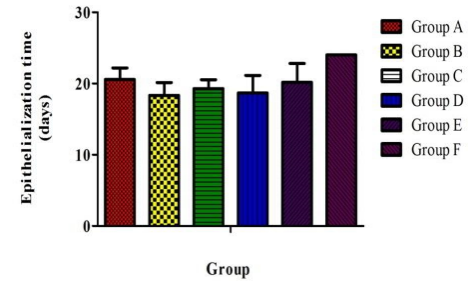
Group A is the positive control; Group B is 20 % AIHE; Group C is 20 % NMHE; Group D is 15 % AIHE; Group E is 15 % NMHE and Group F is the negative control; *Control was used in conjunction with other experiments[5,19,20,21]

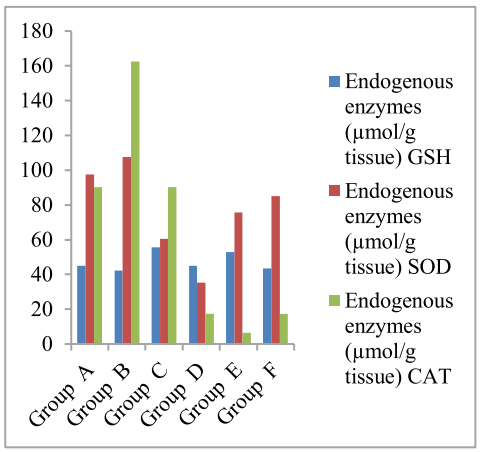




Group A: Positive control Group A: Positive control
Group B: 20 % (v/v) AIHE Group B: 20 % (v/v) AIHE
Group C: 20 % (v/v) NMHE Group C: 20 % (v/v) NMHE
Group D: 15 % (v/v) AIHE Group D: 15 % (v/v) AIHE
Group E: 15 % (v/v) NMHE Group E: 15 % (v/v) NMHE
Group F: Negative control Group F: Negative control

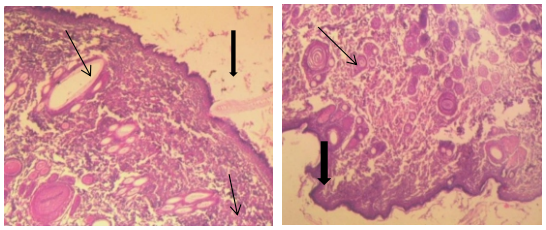
Figures 1 and 2. Graphs showing the rate of wound area (days) and wound contraction (%) respectively. *Control was used in conjunction with other experiments5,19,20,21



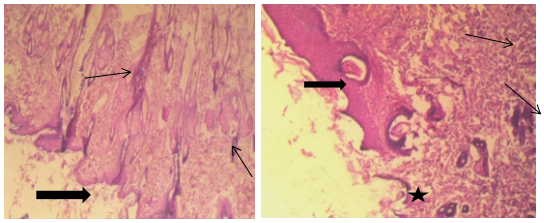


Group A: Positive control Group A: Positive control
Group B: 20 % (v/v) AIHE Group B: 20 % (v/v) AIHE
Group C: 20 % (v/v) NMHE Group C: 20 % (v/v) NMHE
Group D: 15 % (v/v) AIHE Group D: 15 % (v/v) AIHE
Group E: 15 % (v/v) NMHE Group E: 15 % (v/v) NMHE
Group F: Negative control Group F: Negative control

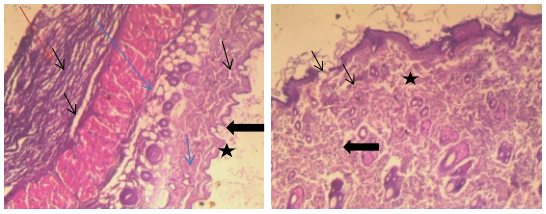
Figures 3 and 4. Histograms showing epithelialization time (days) and endogenous enzymes (µmol/g tissue) respectively. *Control was used in conjunction with other experiments5,19,20,21



Group B: 20 % (w/v) AIHE (H&Ex400) Group C: 20 % (w/v) NMHE (H&Ex400)



Group F: Negative control (H&Ex400) Group D: 15 % (w/v) AIHE (H&Ex400)



Group E: 15 % (w/v) NMHE (H&Ex400) Group A: 100 % ointment (positive control) (H&Ex400)

Figure 5. Photomicrographs showing the histological examination of wound area of test and control rats. *Control was used in conjunction with other experiments5,19,20,21

Conclusion

A. indica and *N. macrophylla* seed extracts have potential as ointments for wound healing, probably due to their free radical scavenging ability and also the possibility of inhibiting the activity of the bacteria that might be on the wound and thereby prevent inflammatory pathway. The extracts might find usefulness in the pharmaceutical and medicinal industries.

Conflict of interest
There are no conflicts of interest in this study.

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