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Amelioration of External Surgical Wound by Topical Application of the Aqueous Formulation of Neem Leaves (*Azadirachta indica*) in Caprine Model

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Abstract

The study was conducted to evaluate the healing potentials of Neem leaf on surgical wounds. A total of sixteen surgical wounds were studied under two groups; Neem (group A) and Normal saline (group B, control). Wound morphology, histopathology, and bacteriological examinations were carried out to assess wound healing potentials of this plant leaves. We have found a remarkably lower swollen area (3.91±0.10mm), suture line levation (2.64±0.19mm) and length of the wound (15.78±0.19mm) in the treatment group than the control (0.36±0.12mm, 3.59±0.12mm, and 17.11±0.08mm respectively). The mean healing period Neem treated wound was significantly ($P<0.05$) lower (12.33±0.42 days) than that of control (18.67±0.33 days). Histopathological study revealed the presence of substantial inflammation with fibroblastic proliferation in samples collected at day 3 in the control group whereas these features were distinctly reduced in the treatment group. There was a distinct thickening of the keratinized layer of the epidermis in Neem treated wound on day 21. In bacteriological study, huge bacterial colonies were found on day 3 in the control group whereas this was markedly reduced in number in the wound of Group-A. Thus, the present study supports the scientific rationale for the use of Neem leaf in the management of wounds.

Key Words: Surgical wound, Neem leaf, Histopathology, Bacteriology, Amelioration

1. Introduction

Wound healing is a complex and dynamic process of several overlapping events following injury, including coagulation, leukocyte infiltration, matrix deposition, epithelialization, and resolution of inflammation with the formation of a mature scar (Sorg et al. 2017). It is dependent on several cell types and mediators that interact in a highly sophisticated temporal sequence. Human and animal being susceptible to bacterial, fungal, and viral infection, skin infection, and tropical wounds require special attention to minimize secondary complications (Mummed et al. 2018). Wounds are considered as one of the major problems in developing countries resulting in severe complications in many cases that lead to high cost for therapy (Alam et al., 2007; Miah et al., 2017; Rassel et al., 2020). Despite advances in controlling

the infection of surgical wounds, bacterial wound contamination is still remaining the most common postoperative complication (Rivandi et al. 2012). The presence of infection in wounds, in addition to interference with the healing process can be resulted in increasing the duration of wound repair, therapeutic period, costs, and even morbidity and mortality rate. Various bacterial species isolated from wounds which can seriously delay the wound healing process by disrupting the normal clotting mechanisms and promoting disordered leukocyte function and poor-quality granulation tissue formation, reduce tensile strength of connective tissue, and impair epithelization (Annan and Houghton 2008; Juyena et al., 2013; Tan et al., 2020).

Nowadays, excessive and inappropriate use of antimicrobial drugs has developed the resistant bacteria and difficulty in the management of infected wounds, so consideration to new antibacterial agents and the least adverse effects seems necessary. Medicinal plants are effective in the treatment of infectious diseases and infections of various types of external wounds and have been used for these purposes in humans and different species of animals (Alam et al. 2005; Tamanna et al. 2020). Many plants are known for related pharmaceutical activities. Among them Azadirachta indica, a member of the Meliaceae family, commonly known as Neem has long been recognized as an excellent therapeutic tool. The Neem leaves contain a mixture of compounds like ascorbic acid, various amino acids, nimbanene, 6-desacetylnimbinene, n-hexacosanol, nimbiol, nimbandiol, nimbolide and several other types of ingredients (Subapriya et al. 2006). Besides this Neem leaves also contain carbohydrates, protein, minerals, calcium, phosphorus, carotene, etc. Neem leaves also contain glutamic acid, tyrosine, aspartic acid, alanine, proline, glutamine, and cystine like amino acids, and several fatty acids (Hossain et al. 2013). Neem leaves have been demonstrated to exhibit immunomodulatory, anti-inflammatory, anti-hyperglycemic, anti-ulcer, anti-malarial, antifungal, antibacterial, antiviral, anti-oxidant, anti-mutagenic, and anti-carcinogenic properties (Alzohairy 2016).

Bangladesh is rich in medicinal plants and Neem is a very well-known and one of the most versatile medicinal plants in the country having a wide spectrum of biological activity as mentioned earlier. Regarding the above mentioned and easy accessibility, we investigated the effect of an aqueous paste of Neem Leaves on postsurgical wound healing in the goat model.

2. Material and Method

2.1. Plant materials and preparation of extract

The branches of Neem plant (*Azadirachta indica*) were collected from the vicinity of Bangladesh Agricultural University (BAU) campus with the verbal permission of the authority. The leaves were separated from the branches, properly cleaned with distilled water, kept in a blender, and blended with some sterile distilled water to make a paste. The paste was kept in a sterile plastic container and stored at 4°C until further use. It was allowed to warm up to room temperature before applying to the wounds.

2.2. Experimental animals

Four apparently healthy goats were purchased from the local market with body weight ranged from 8-10 kg were purchased and used for this experiment. They were kept in the animal shed of the Veterinary Teaching Hospital of BAU, Mymensingh. The animals were kept under standard clinical conditions and veterinary supervision with no restrictions on food and water. Before the study the goats were kept in quarantine for two weeks and vaccinated against PPR (P.P.R Vaccine®, LRI, Dhaka, Bangladesh) and dewormed with Albendazole @15mg/kg body weight. All the animal experiments were carried out in accordance with the

guidelines and approval of the Animal Ethics and Experimentation Committee (AEEC, Permission number: AEEC/DSO-BAU/02/2017) of the Department of Surgery and Obstetrics, BAU, Mymensingh.

2.3. Wounding of animals

To ensure the animals' health, a clinical examination was performed. All surgical interventions were conducted under sterile conditions. After proper restraint and infiltration analgesia with 2% Lidocaine HCl (Jasocaine®, Jayson Pharmaceuticals Ltd., Dhaka, Bangladesh), 20cm long full-thickness cutaneous wounds were made on either side of the vertebral column. Four wounds were prepared in each goat, two on either side of the vertebral column. Goats were divided into two groups. Group-A: Fresh Neem leaf paste was applied locally once daily to eight wounds made in two animals. The animals were maintained carefully to avoid contamination and interference with the healing of wounds. Group-B: Animals of this group were used as control where the wounds were received topically normal saline only (Table 1).

All the wounds were closed using nylon with a simple interrupted pattern. Antibiotic, antihistaminic, and anti-inflammatory drugs were avoided to mitigate their effects on the healing process. Follow-up information was obtained from the day of surgical operation (day 1) up to the end of the experiment (day 21).

2.4. Evaluation of wound healing activity

Morphological characters such as swelling of the wound area, the elevation of the suture line from the skin surface, length of wound area were recorded to determine the healing of the wounds. After surgery the elevation of the suture line was recorded up to 7th days of surgery. Slide Calipers was used to measure the swelling area (mm), the elevation of suture line (mm), and length of suture line (mm) of the wounds on the day-1 (D1), day- 3 (D3), day- 7 (D7), day- 14 (D14), and day- 21 (D21) post wounding. Wounds were closely monitored daily to observe any complications such as swelling, wound dehiscence, suture abscess, local infection, and exudation. The progress of healing in animals of each group was recorded daily. Healing score was categorized as (a) excellent- no inflammation, no exudation, no infection, no dehiscence, gradual decrease of the width of a wound area, (b) good- minimum inflammation with minimum exudation, no dehiscence, gradual decrease of the width of a wound area, and (c) poor- marked inflammation, presence of infection, and exudation.

2.5. Bacteriological study

2.5.1. Sample collection

For the bacteriological study, wound swabs were collected aseptically by using sterile cotton buds from all groups on day 1 and day 3. Cotton buds were moistened first with normal saline solution and applied to the closed wounds by circling to collect the swab samples. Then the cotton buds were quickly transferred into screwed capped test tubes containing nutrient broth.

2.5.2. Culture and staining of bacteriological samples

Culture and staining of collected bacteriological samples were done as the procedure described by Jaman et al. (2018).

2.5.3. Histopathological study

The biopsies were collected from the wound areas of each experimental animal on 3rd, 7th, and 21st days after the creation of wounds using standard surgical procedures. The biopsy tissues contained dermis and epidermis were fixed in 10% neutral buffered formalin for 48 hours for histopathological study.

Table 1. The experimental protocol to study the efficacy of Neem leaf paste in the treatment of artificially produced surgical wounds

Groups	Material used	Form of materials	No. Of animals	No. Of wounds
Group A	Neem	Aqueous paste	2	4 in each animal
Group B	Control (NS)	0.85% NaCl in distilled water	2	4 in each animal

Table 2. The effects of aqueous paste of Neem leaf (Group A) and Normal saline (Group B) on various features of wound healing in goats.

Groups	Area of swelling of wounds (mm)	Elevation of suture line (mm)	Length of wounds (mm)	Healing time (days)
Group A	3.91± 0.10^{a}	2.64± 0.19^{a}	15.78± 0.19^{a}	12.33± 0.42^{a}
Group B	5.36± 0.12^{b}	3.59± 0.12^{b}	17.11± 0.08^{b}	18.67± 0.33^{b}

Values with the different superscript letter in the same column indicate significance ($p<0.05$) Mean± SEM

Slides were prepared and stained in the histopathology lab of the Department of Surgery and Obstetrics, BAU according to the method described by Ashraf et al. (2019). Finally, photomicrography of stained slide was performed.

2.6. Statistical analysis

All the data were expressed as Mean ± SEM (Standard Error of Mean). To compare data among groups, one-way ANOVA (Analysis of variance) factor one analysis was performed using Statistical Package for the Social Sciences (SPSS) version 22.0. Probability $P<0.05$ was considered statistically significant.

3. Results and Discussion

3.1. Morphological change

The wound healing activities of Neem paste are shown in Table 2. The swelling of the wound edges was observed in both the groups of animals. Treatment with aqueous Neem paste (Group-A) and Normal Saline (Group-B, control) resulted in the swelling of wounds at 3.91±0.10 mm and 5.36± 0.12 mm respectively and this difference was significant ($P<0.05$). The elevation of a suture line also differed significantly ($P<0.05$) among the groups. The elevation of the suture line was higher in saline-treated wounds (3.59±0.12 mm) than those treated with Neem paste (2.64±0.19 mm). The higher swelling and elevation of the suture line indicate presence of more inflammation in the wounds of the control group. Treatment with aqueous Neem paste resulted in a prompt reduction of the length of wounds (15.78±0.19 mm) on the other hand it was much higher in the control group (17.11±0.08 mm). The mean healing period was significantly ($P<0.05$) lower in Neem treated wounds (12.33±0.42 days) than those treated with normal saline (18.67±0.33 days). The wound morphology in response to treatment on day 1, day 7, and day 14 are depicted in Figure 1, Figure 2, and Figure 3 respectively.

3.2. Histopathological examination

Day 3: The inflammatory lesions in the regenerating tissues were evaluated based on the infiltration of reactive cells including macrophages, lymphocytes, and neutrophils. Reactive cells decreased gradually in wounds treated with an aqueous paste of Neem.

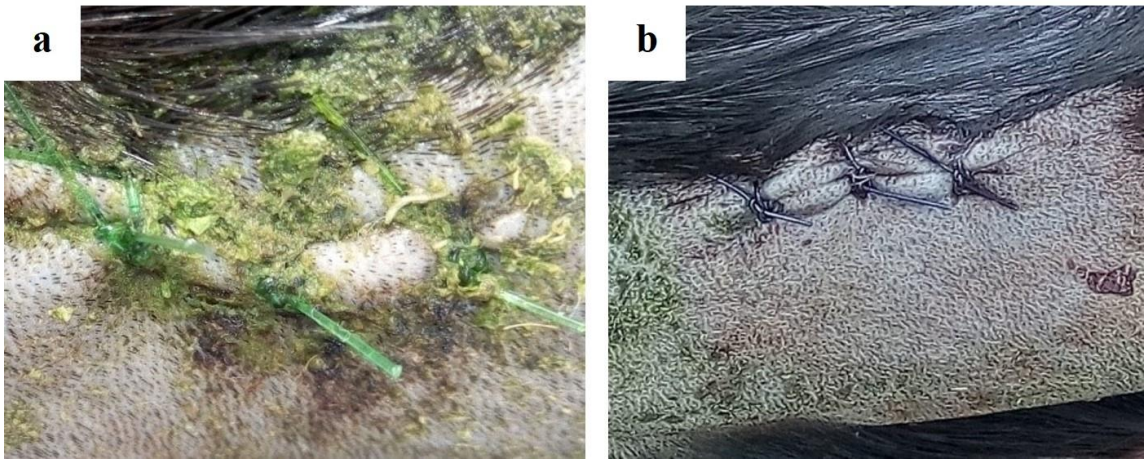

Figure 1. Gross observation of wounds on day 1 treated with (a) Neem, (b) Normal saline (control)

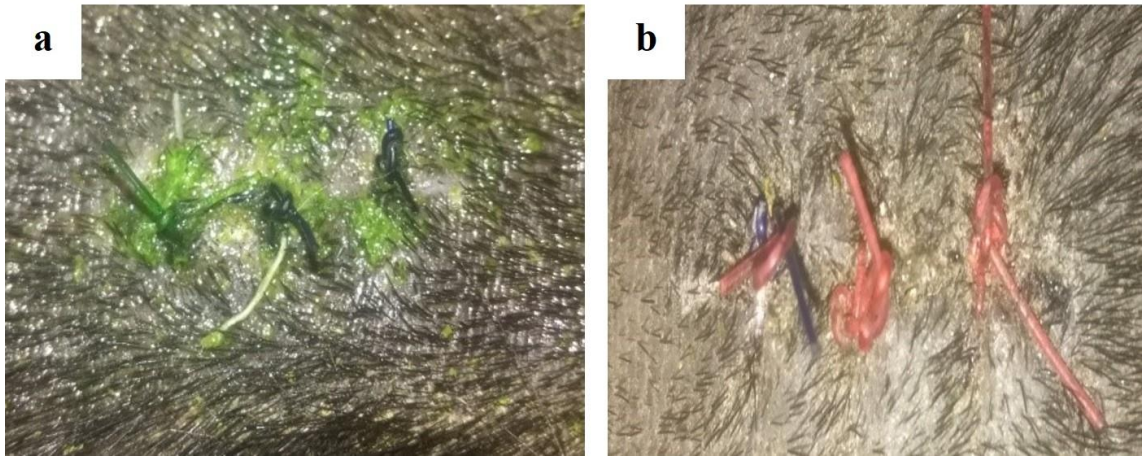

Figure 2. Gross morphology of wounds on day 7 treated with (a) Neem, (b) Normal saline (control)

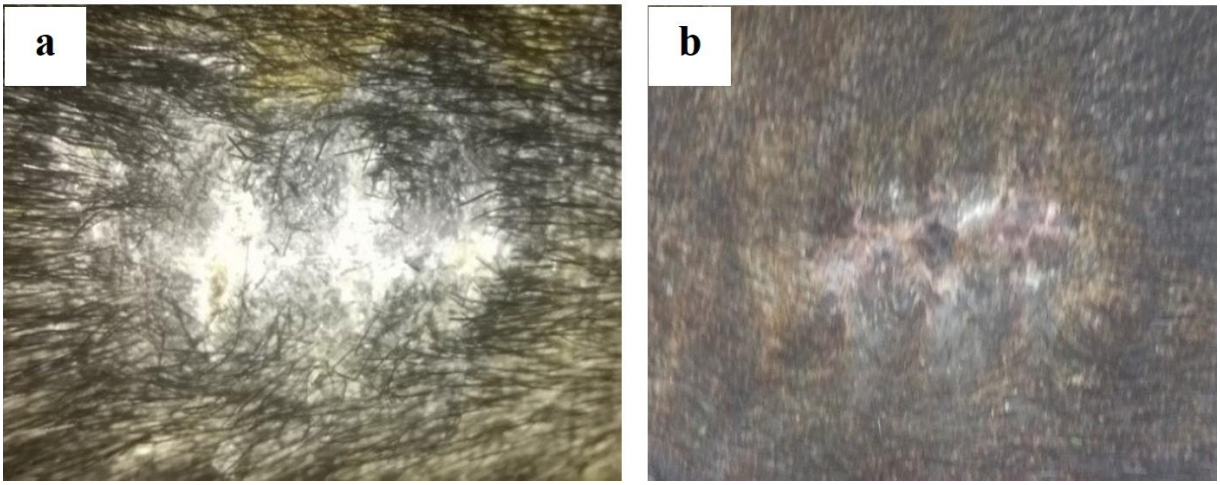

Figure 3. Wound healing on day 21 after treatment with (a) Neem, (b) Normal saline (control)

Moreover, the proliferation of fibrous connective tissue was notably observed in wounds of group A on day 3 (D3) (Figure 4a) while huge inflammation and fibroplasia were encountered along with widespread hemorrhages and congestion in the group B (Figure 4b).

Day 7: Fibroblastic cells were more pronounced than other inflammatory cells and much of the debris and hemorrhages were disappeared and formation of thin keratin layer in group-A (Figure 5a). However, reactive cells are seen in wounds treated with normal saline till day 7 with less fibroblastic proliferation in group-B (Figure 5b).

Day 21: There was the least degree of inflammation in both groups on day 21. Marked thickening of the keratinized layer of the epidermis in wound received aqueous Neem paste (Figure 6a) while a thin keratinized layer of the epidermis in the control group (Figure 6b).

3.3. Bacteriological study

Wound swab samples were spread on the Plate Count Agar (PCA) plates after dilution and incubated overnight at 37°C temperature. The next day cultural characteristics and growth of bacteria were observed. Swab samples collected on day 3 post wounding have demonstrated that wound treated with Neem paste has a remarkably lower colony than those treated with normal saline only (Figure 7a, 7b). Gram's staining confirmed the presence of Staphylococcus spp. characterized by Gram-positive, spherical-shaped, and clustered form bacteria (Figure 7c). Wound healing involves growth factors, cytokines, extracellular matrix (ECM), and relevant enzymes along with the differentiated cells that modify molecular components of the matrix. In developing countries like Bangladesh, the wound is one of the most concerning fact in humans and livestock especially in food and zoo animals (Hoda et al., 2018; Talukder et al. 2018; Sarker et al. 2020). Our study evaluated the efficacy of the aqueous paste of Neem leaves on the healing of surgical wounds in the goat model. Results from the study showed that the mean value of the swelling area, elevation of the suture line, and length of wound was significantly lower in Neem treated wounds than those treated with normal saline (Table 2). This might be because of the presence of active compounds like alkaloids, flavonoids, phenolic compounds, steroids, carotenoids, ketones, and azadirachtin in neem leaves and also the inflammatory response as a foreign body reaction due to traumatic tissue handling and suture placement (Biswas et al. 2002). Furthermore, Neem oil contains fatty acids and maintains the skin's elasticity by building up collagens and provides a moist and soft texture to the skin (Raina et al. 2008). Similarly, average healing time for group A was 12 days while for the control group it was 18 days (Table 2) which is also similar to our previous study with other herbal plants having wound healing effects (Alam et al. 2005; Tamanna et al. 2020). In terms of histopathology, the degree of inflammation and proliferation of blood vessels in the regenerating tissue was higher in the control group at day 3 (Figure 4b) of post wounding compared to Neem treated wounds (Figure 4a). The first inflammatory cells to appear during healing are the neutrophils, which presumably control the microbial growth and sepsis (Sorg et al. 2017). Neutrophilic infiltration in healed tissue was not observed in the present study. This may be because the tissues were collected on day 3 post-operation when the neutrophilic infiltration was replaced by macrophages and lymphocytes. The highest number of monocyte and lymphocyte on day 3 (Figure 4b) of the experiment were seen in the control group compared to group A (Figure 4a). The lymphocyte and macrophage were found to disappear from the Neem treated group on day 7 post wounding (Figure 5a).

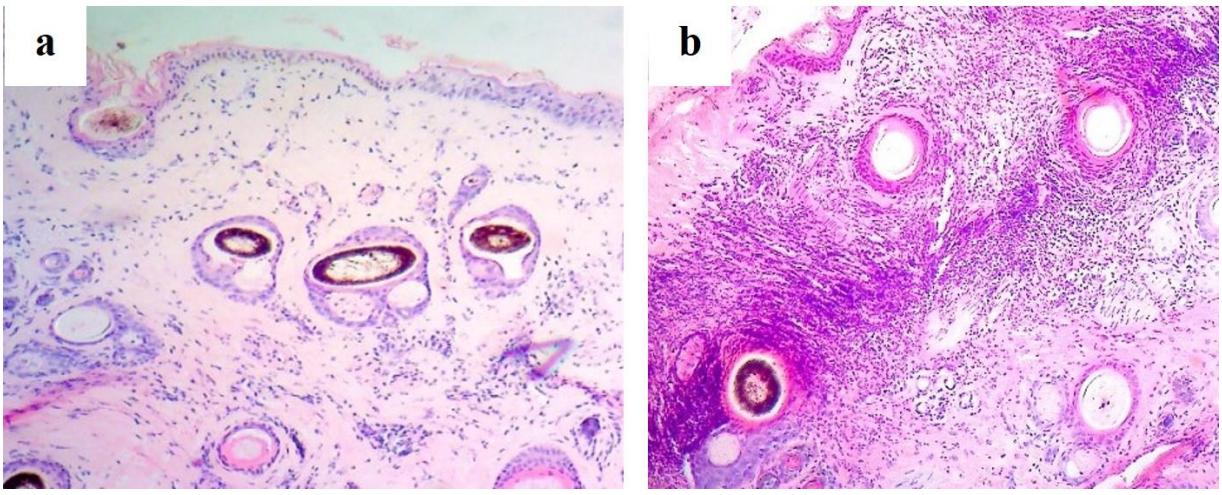

Figure 4. Status of inflammation in the wounds of two groups. (a) Less infiltration of inflammatory cells with marked fibroblastic proliferation in group A, (b) huge inflammation and fibroplasia less intensively found in group B on day 3

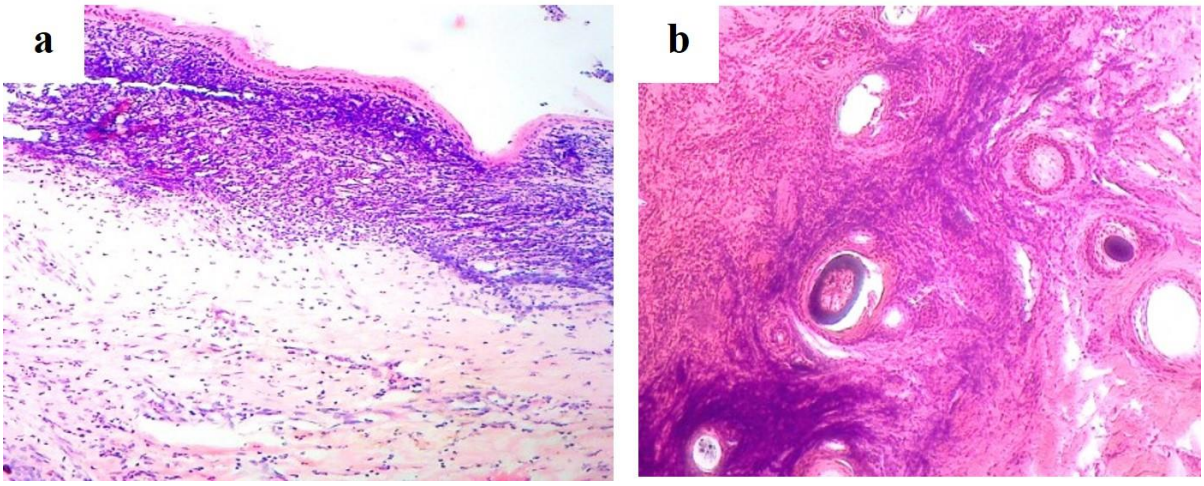

Figure 5. Histological features on day 7. (a) Fibroblastic cells were remarkable than other inflammatory cells in group-A, (b) fibroblastic proliferation with minimal infiltration inflammatory cells in group-B

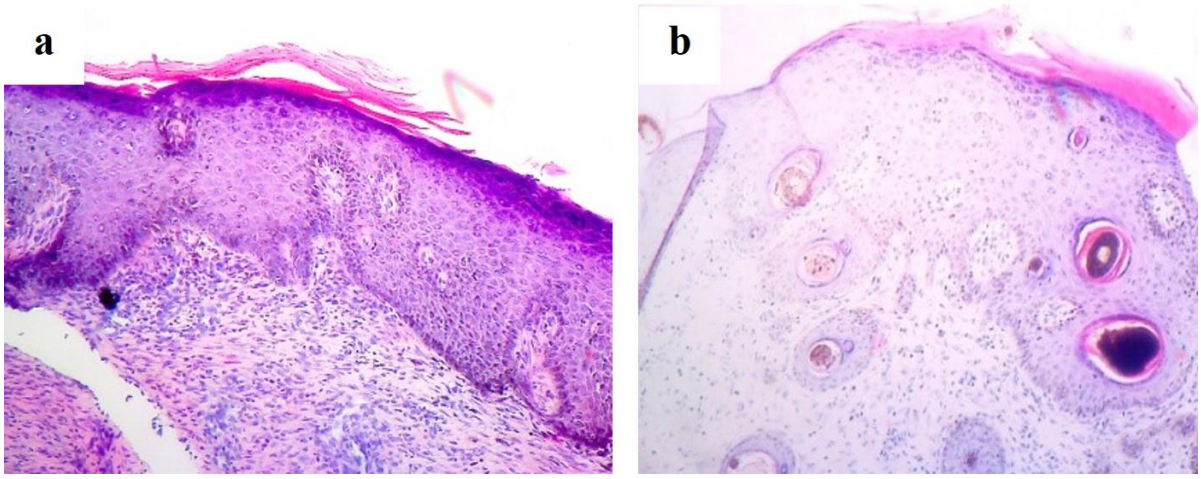

Figure 6. Keratinization of wounds on day 21. (a) Marked thickening of the keratinized layer of the epidermis in group- A (b) thin keratinized layer of the epidermis was observed in group-B

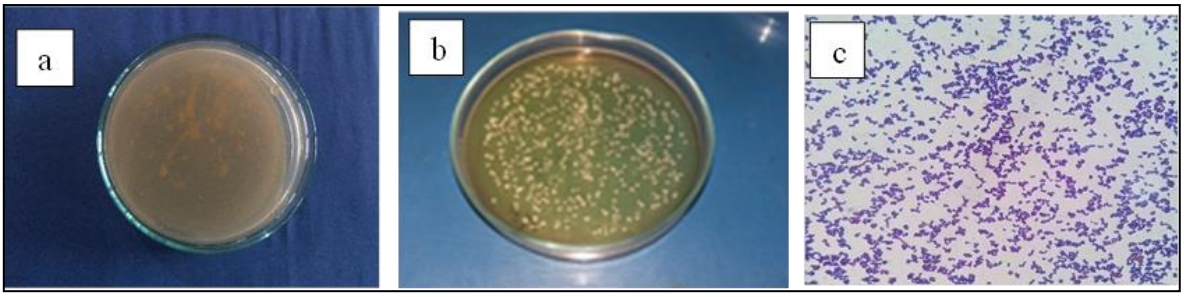

Figure 7. Presence of bacterial colony in primary culture of Mannitol salt agar observed in samples collected from wound treated with (a) Neem leaf paste and (b) normal saline (c) Staining characteristics of isolated bacteria. The bacteria were arranged in grape like structure with spherical shaped indicating Staphylococcus spp.

There was a marked thickening of the keratinized layer of the epidermis in aqueous Neem extract-treated group while a thin keratinized layer in the control group on day 21 post-operation (Figure 6a and Figure 6b). This might be because of the anti-inflammatory properties of Neem which act as effective as cortisone acetate and also helps to accelerate wound healing (Raina et al. 2008). The mean healing period was significantly lower in Neem treated wounds than those of control, which has a clear message that Neem may accelerate the healing of wounds.

Bacteriological studies revealed a remarkably lower number of bacterial colonies in Group-A than that of Group-B. Gram's staining confirmed the presence of Staphylococcus spp. (Figure 7c). The near absence of bacterial colony might be the effects of the antibacterial activity of Neem which has already reported by Patel et al. (2009) against Staphylococcus aureus, Pseudomonas aeruginosa, and Escherichia coli.

4. Conclusion

We have shown that the aqueous paste of A. indica accelerates wound healing in goats. Further investigation is necessary with these findings at subcellular levels including gene studies which could lead to other beneficial effects.

Ackoledgements

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Conflict of Interest

The authors do not have any conflict of interest.

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