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An Evaluation of Wound Repair and Regeneration Potential of the fruits of Phyllanthus Emblica (Amla)

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Abstract : The traditional Indian medicine such as Ayurveda, Siddha and Unani, describes usage of various herbs, fats, oils and minerals forinfected wound healing and management.The fruit of *Phyllanthus emblica*Linn., also known as Amla is one of the most important components in the formulation of various Indiantraditional medicines such as Ayurveda, Unani and Siddha. The fruits of amla are useful in the treatment of various ailments such as diuretic, laxative, liver tonic, refrigerant, stomachic, restorative, anti-pyretic, hair tonic and ulcer preventive. The main bioactive constituent of amla is Ascorbic acid (Vitamin C) that is a co-factor in post translation modification of Collagen biosynthesis and poly phenols. Because of a rich in ascorbic acid in amla, we are motivated to evaluate the dermal and epidermal repair of infected wound in Wister albino rat and find the histological investigation of wound healing patterns. We found that *In vivo* studies and histological analysis of granulated tissue confirm almost >95% regeneration of dermis and epidermis in 16 days in treated groups of Wister Albino rats than that of open wound groups. The data on biochemical analysis of granulated tissue reveal that the newly synthesized components of extracellular matrix in the granulation tissue are more in treated group than open wound group. Moreover, the high amount of type 1 Collagen is synthesized in the granulation tissue of treated group and then better cross-linking of collagen in the granulation tissue was evaluated. Better maturation and cross-linking of Type 1 Collagen was observed in treated rats. Moreover, thealdehydes content of Type 1 Collagen in treated groups is also enhanced and confirmed that the better cross-linking of Type 1 collagen in the treated group. The preliminary investigation confirms that the methanol extract of amla has better wound healing properties in infected environment and useful medicine for wound management.

Keywords: Methanol Extract of Amla, *In vivo* studies, Anti-microbial potential, Wound Pathogens and Type 1 Collagen synthesis and Aldehyde content and cross linking of Type 1 Collagen.

Introduction

The process of dermal and epidermal wound healing consists of several of integrated cellular and biochemical events to reestablishment of structural and functional integrity with regain of strength of injured tissue in the wound. The process included a programmed sequence of coagulation, inflammation, formation of granulation tissue and remodeling of tissue. The most important aim of a novel treatment of wound healing is to shorten the time required for dermal and epidermal regeneration and eradication of microbial film at wound site and produce a quality of wound healing in terms of minimal scar formation and maximum strength of the extracellular matrix at the wound site. Therefore, the drug discovery from numerous herbal plants was developed as alternate for existing therapy for infected wound healing. [1-3]

Topical administration of Drugs or therapeutic agents into the dermal wound is a more practical approach and effective treatment of wound repair. However, Due to the presence of various wound pathogens at wound environment and antimicrobial resistance developed by pathogens, Plant based antimicrobial and

pharmacological agents is required to promote the wound healing and mean time to avoid the existence problem with antibiotics. Past decades, Herbal based agents was useful in treatment of wound and wound management. In this direction, a number of herbal products are being investigated for its potential for wound healing treatment.[4,5]

Plant based therapy not only accelerate healing process and provided anti-microbial action against various wound pathogens and has multiple pharmacological activities to accelerate wound regeneration process. More than 70% of wound healing pharma products are herbal plant based, 20% are mineral based and remaining containing animal products as their base material. The plant base materials are used first aid – antiseptic coagulants and wound washing agents. In recent times, focus on plant researchers has increased allover the world and large body of evidence has collected to show immense potential of medicinal plants used in various traditional systems. More than 13,000 plants were investigated during the last five years period. Even though the wound healing potential of amla was proved, but the lack of information about wound healing progress thro histological and biochemical analysis. [4, 5]

*Phyllanthus emblica*is one of the most important plants in the traditional medicine system of India. It is valued for its unique tannins, flavanoid and vitamin C, which are considered to contain very powerful antioxidant compounds. There are several reports, which showed the antioxidant properties of amla and its constituents, Emblicanin A and B in experimental animals and reported to inhibit lipid per oxidation and reduction of blood sugar levels. [5]

Phyllanthus emblica (L.) or *Emblica officinalis* Gaertn. Commonly called as "amla" (family-Euphorbiaceae) is one of the medicinal plants that were used in ayurvedic medicines for over 2,000 years. Amla Fruits are hard, nearly stemless, round or oblate, indented at the base and smooth on surface. This plant is indigenous to tropical South-East Asia and occurs mainly in dry or moist deciduous forests of Central and Southern India, Nepal, Sri Lanka, Malaysia, Mayanmar etc. It is widely cultivated for its fruits throughout India, Mascareme Islands (Reunion and Mauritius), West Indies (Cuba, Trinidad), central America (Honduras, Costa Rica) and Japan etc. [6]

The bioactive ingredients that have significant pharmacological action in *P. Emblica* are vitamin C, phenolic compounds, including hydrolysable tannins, proanthocyanidins, flavonols, and compounds belonging to other phenolic groups etc. Tannins are found in fruits, leaves and bark at higher concentration[7] and The reported pharmacological activities of amla fruits is reported in the Table 1,

Table-1–Pharmacological activities of Amla

Pharmacological activities of Amla	Reference
Anti-Oxidant activity	[8]
Anti-diabeticactivity	[6]
Wound Healing activity	[9]
Chemo preventive activity	[9]
Anti–inflammatory activity	[10]
Analgesic activity	[12]
Anti-hyperlipidemia, Hypolipedimic Anti-atherogenic activity	[11]

Amla is an important herbal compound and there is limited report about the wound healing activity, it is therefore, we have undertaken to study the wound healing activity in albino Wister rats and histological analysis of wound healing performance.

Materials and Methods

Emblica officinal powder (Amla) was obtained from the local Ayurvedic shop and its 100 % methanolic extract was prepared by the following protocol.

Preparation of alcohol extract of *Phyllanthus Emblica*

100 g of the dried fruit powder was extracted in 500 mL of methanol by stirring overnight and was centrifuged at room temperature. The supernatant was collected and evaporated to dryness under reduced

pressure in a rotary evaporator. The yield of this methanolic extract was 14.5%. The concentrated extract was aliquoted in amber-colored bottles and kept in desiccators for further use. The dried extract was dissolved in 10% Dimethyl Sulfoxide (DMSO) and used to assay the antibacterial activity against wound pathogens.

Determination of antibacterial activity

The minimal inhibitory concentration (MIC) of the extract was determined by the broth tube dilution method. The antibacterial sensitivity test was performed by disc diffusion method. Sterile blank discs (6 mm diameter) were impregnated with minimum inhibition concentrations of amla extract against *Staphylococcus aureus*. Extract impregnated discs were placed in Muller-Hinton agar plates inoculated standard strains and incubated at 37^{o}C for 24-48 hrs. Standard methicillin disc and discs treated with DMSO were used as control. Inhibition zone diameters around each of the disc were measured and recorded at the end of the incubation time. [13]

***In vivo* wound healing activity**

Male Wister albino rats of weights ranging between 150g and 200g were used for the current study. They were housed individually in standardized environmental conditions. The animal experiment was performed according to the approval and guidelines of the Institute's animal ethical committee (466/01/a/CPCSEA). Overall, 64 animals were taken in two groups (control and experimental). Full thickness wounds (1.5x1.5 cm) were created on the dorsal side of the shaved rats using sterile surgical blades and inoculated with the test organisms. The organisms were allowed to infect for 24 hrs. All surgical procedures were carried out under thiopentone sodium (40mg/kg body weight) intramuscularly. The experimental rats were dressed with the formulated ointment, while the control rats were dressed with paraffin alone. Regular application of ointment was performed on all rats.

Group used for In Vivo Studies:

Open Wound Groups with Gauze Dressings	32 Wister Animal Rats
Groups treated with plant extract ointments Formulation	32 Wister Animal Rats

Microorganisms used for infection.

Bacterial strains such as *Staphylococcus aureus* ATCC 29213, *Pseudomonas aeruginosa* ATCC 27853 were collected from King Institute, Chennai, India.

Grouping of Animals:

After wound creation, animals were divided into two groups, each group containing 6animals (n=6). The groups were arranged in the following order.

1. Group 1 – Open wound – (4th Day, 8th Day, 12th day and 16th Day)
2. Group 2 - Rats treated with ointment formulation of methanol extract of *Phyllenthus Emblica*. (4th Day, 8th Day, 12th Day and 16th Day)

Histological analysis of Granulation tissue by Masson's Trichrome staining

Granulated tissues were collected at every 4 days interval and transferred to 10% neutral buffered formalin (NBF) for 24 hours at 4°C. The formalin fixed tissues were dehydrated through grades of alcohol, cleaned in xylene and then embedded in paraffin wax (58-60°C). The molds were labeled and stored for further use. A 5-7µm section was de-paraffinized and Masson's Trichrome Staining was performed for the detection of collagen deposits and its morphology, in the granulation tissue. [14]

Biochemical analysis

Granulated tissues were collected on the 4th, 8th, 12th and 16th days for the estimation of different types of collagen in the granulated tissue.

Estimation of Total collagen content in Granulation Tissue:

Weighed granulation tissue was first hydrolyzed in 6.0 N HCl for 8 hr at 110° C, evaporated to dryness and then made up with a known volume of water .The collagen content was determined by the estimation of hydroxyproline, as described in the paper [15,16].

SDS-PAGE Analysis of Acid soluble Collagen and Pepsin soluble collagen:

Acid soluble and pepsin soluble collagen was prepared from wound tissue as described by Miller and Rhodes *et al*. The α 1(III) chains were resolved from the α 1(I) chains on a 8% separating gel with 5% stacking gel by interrupted electrophoreses with delayed reduction of the disulfide bonds type (III) collagen. [17,18]

Aldehyde Content in the Type 1 Collagen of Granulated Tissue:

The aldehyde content in the pepsin soluble collagen is evaluated by the reported protocol from Berg.R.A *et al* 1973b [19].

Statistical Analysis:

All results are expressed as mean ± S.D and the results were compared statistically by student's independent *t*- test using SPPS software. A statistically significant *p* value <0.05 was considered.

Results

In vitro antimicrobial activity confirmed the activity of fruitamlaextractagainst *S.aureus* and *P.aeruginosa*. The anti-microbial activity evaluated by the disc diffusion study showed a zone of inhibition for *S.aureus* (10±2) mm and *P.aeruginosa* (12±1) mm. The MIC of amla fruit extract is 3925±0.101µg/ml for *S.aureus* and 3925±0.204 µg/ml for *P.aeruginosa*. Complete wound regenerated was observed in treated rats on day 16 where as in control group the wound closure in the animal was taken about 30 days.

Table 2 .Zone of Inhibition for Standard strain Microorganisms

Microorganisms	Amla methanol extract	Std. Antibiotic
S. aureus	11±1.225 mm	34±0.5 mm (methicillin)
P. aeruginosa	12±1.523 mm	30±1.0 mm (ciprofloxacin)

Table 3 .Minimum Inhibitory concentration

Microorganisms	Minimum concentration
S. aureus	3.925±0.0076 mg/ml
P. aeruginosa	3.925±0.0078 mg/ml
Streptococcus pyrogenes	15.25±0.0095 mg/ml

Wound Closure in Albino Wister Rats

Figure 1 shows the rate of wound contraction. Significant difference in the reduction of wound area was observed in treated rats from day 4 onwards and also the wound closer was much faster on later days when compared with control. The wound contraction in treated groups are better than the open wound groups due to the antimicrobial potential of the extracts of amla.

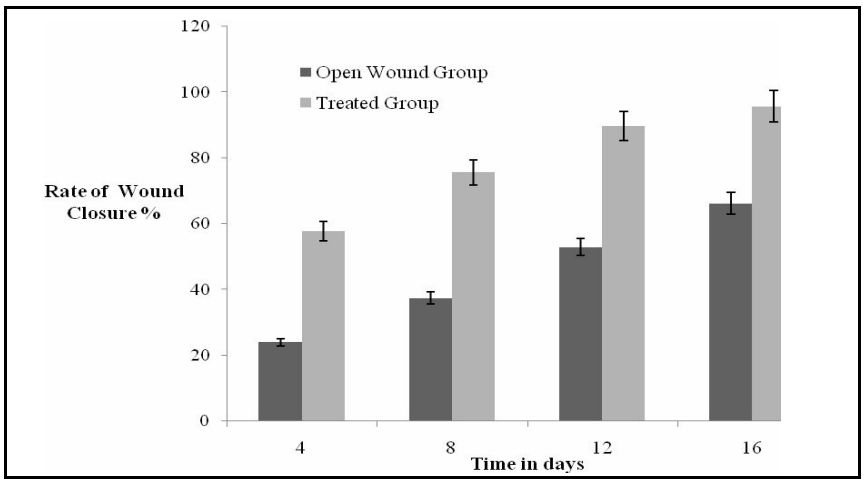


Figure 1 – Rate of Wound Closure in Albino Wister Rats

Biochemical analysis of granulated tissue (Fig.2-3) has exposed a progressive wound healing in the treated group compared with the open wound group. A significant increase in the hydroxyproline content was observed in the treated group than the open wound group. It confirms indirectly that the synthesis of collagen in the granulated tissue from treated group was increased.

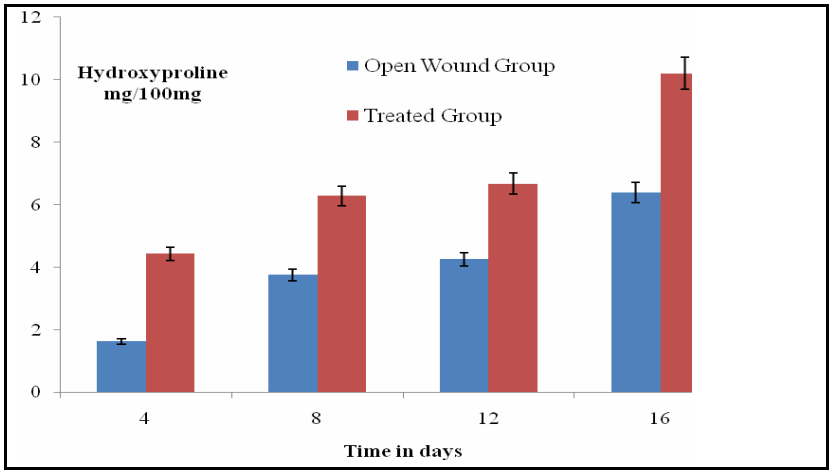


Figure.2. Hydroxyproline content in the granulated tissue

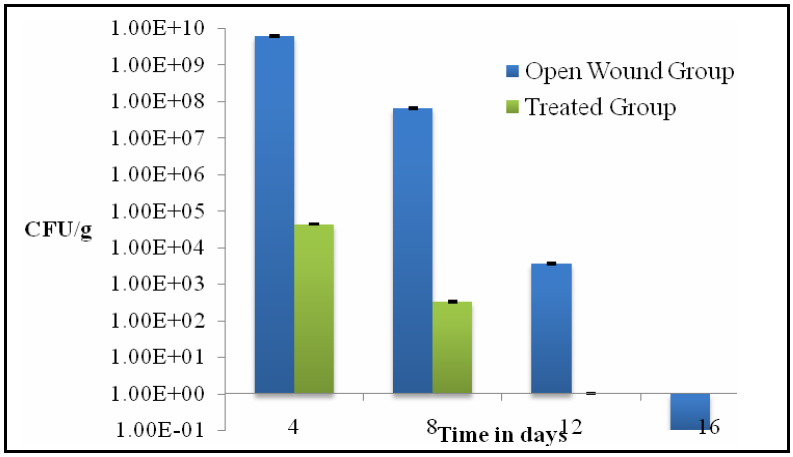


Figure.3.Bacterial Count in the Granulated Tissue

The total bacterial count from the granulation tissue on different days of analysis is shown in figure 2. Application of plant extract based ointment resulted in a diminishing level of total bacterial count in the infected

wound. There was major reduction from 10^9 CFU to 10^4 CFU in treated rats on day 4 when compared to control rats, which records 10^7 CFU.

SDS PAGE Analysis of Acid Soluble Type 1 Collagen in the Granulated Tissue

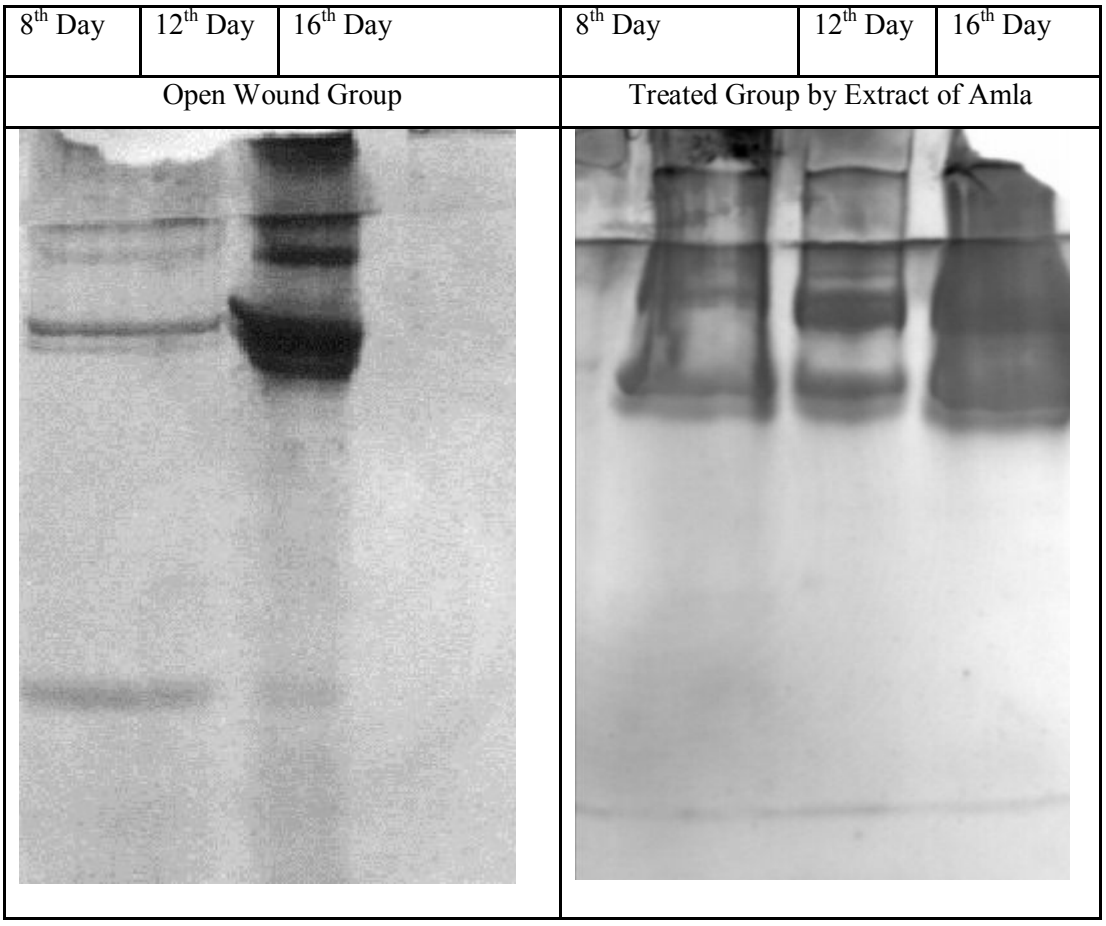


Figure 3. SDS-PAGE Analysis of Type 1 Collagen in the granulated tissue

The above experiment showed the SDS PAGE analysis (Fig.3.) of Type 1 collagen in the granulated tissue. In the treated group, the significant amount of collagen was available in the granulated tissue that is confirmed by bands in SDS PAGE.

Table 4. Aldehyde Content in the Type 1 Collagen of Granulated Tissue:

Aldehyde Content in the Pepsin Soluble Collagen	**Nanomoles of malondialdehyde**		
	8th Day	**12th Day**	**16th Day**
Open Wound Group	610±21.88	760±5.07	1020±12.65
Treated Wound Group	2250±17.85	2897±8.09	3012±2.03

Table 4 shows the aldehyde content of pepsin soluble collagen of all the two groups. From the table, it showed that the group treated by amla extract has higher aldehyde content than in the case of open wound group.

Histological Analysis of Granulated Tissue:

The wound healing progress is evaluated by the histological analysis of granulated tissue such H&E Staining and Masson's Trichrome Staining.

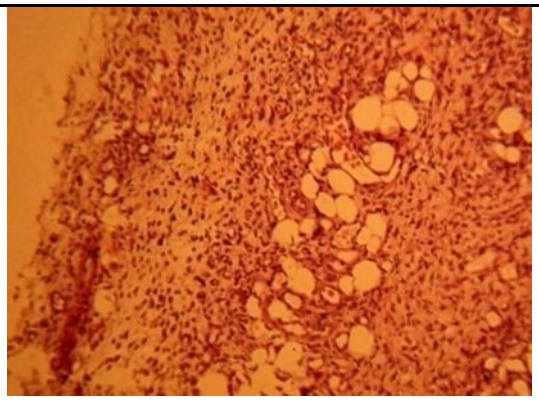
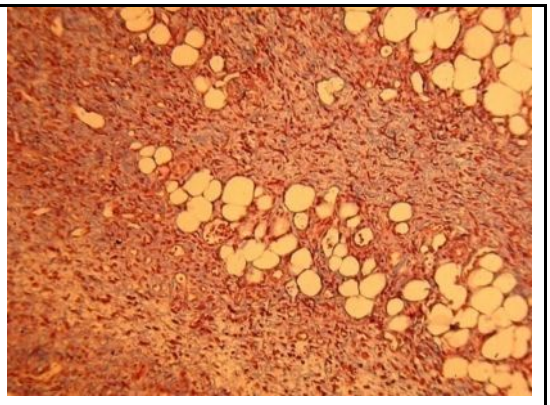
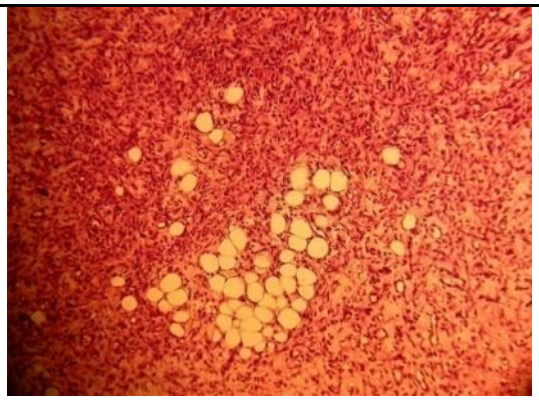
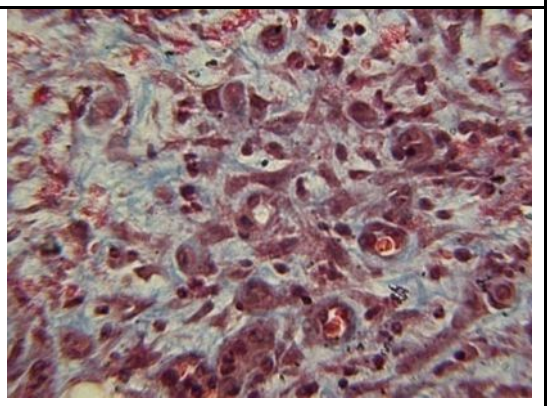
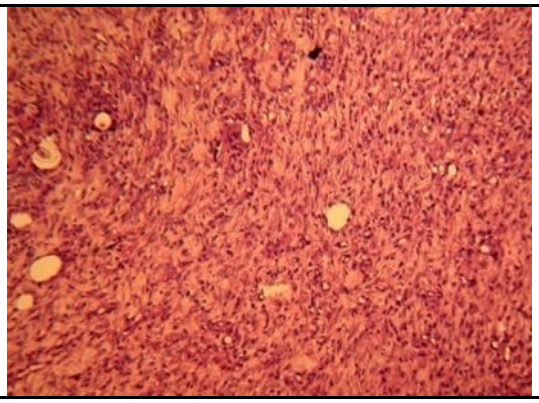
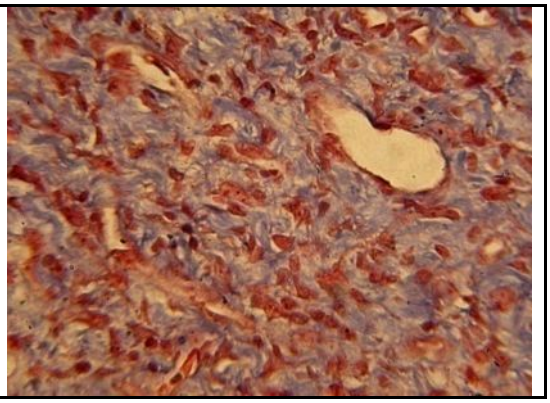
4th Day Open Wound Group		
4th Day Treated Group		

Figure 4. H&E Staining and Masson's Trichrome Staining of Granulated Tissue-4th day

In both H&E staining and Masson's Trichrome staining of granulate tissue from the 4th Day of wound healing, It was observed that there is a presence of neutrophills and absence for formation of epidermis and dermis at the wound site. The wound pathogens at the wound site and moist environment could degrade the extracellular matrix in wound and delays wound closure and regeneration. The topical application of extract of amla effectively eradicates the wound pathogens at the wound site and as a result, the neutrophils in the granulation tissue is started to decreasing in the treated group

8th Day Open Wound Group		

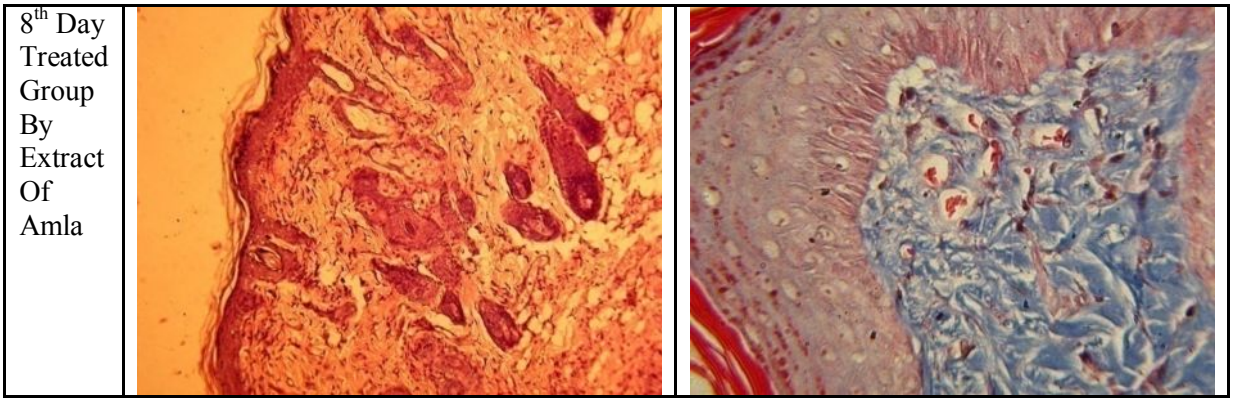


Figure 5 . H&E Staining and Masson's Trichrome Staining of Granulated Tissue-8th day

In the 8th Day of Open wound group, there is still presence of neutrophils and absence for formation of dermis and epidermis. Nevertheless, in the case of 8th Day of Treated group, the absence of neutrophils was seen in the treated group and started to form the epidermis and dermis at the wound site. In Masson's Trichrome Staining, there is an evidence for synthesis of collagen in the wound site in treated group.However, there is no observation of collagen synthesis in the open wound group.

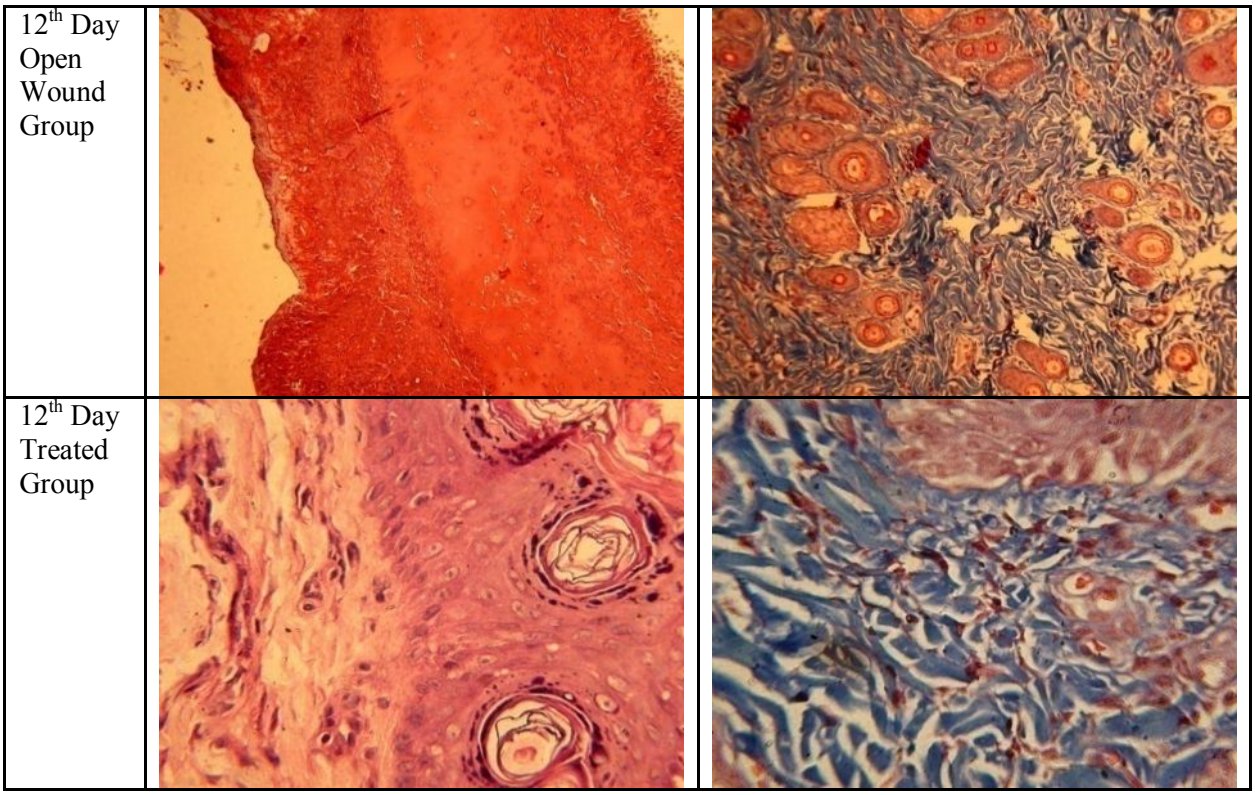


Figure 6. H&E Staining and Masson's Trichrome Staining of Granulated Tissue-12th day

In this figure (Fig. 6.), well-formed dermis and epidermis at wound surface in 12th day treated group was observed and additionally, there is angiogenesis in the wound surface, whereas in 12th day of Open wound group, partial formation of dermis and epidermis was seen. In Masson's Trichrome staining of 12th Day Treated groups granulated tissue, well-formed dermis and epidermis at the wound surface was observed. In addition to that, well formed collagen bundles at wound surface was observed where as in the case of 12th Day Open wound group, loose and less amount of collagen bundles are formed with partial regeneration of dermis and epidermis. In 16th Day of Open wound Group, partial formed dermis and epidermis were observed. The treated group shows complete regeneration of epidermis and dermis are formed in the wound site. (Fig.7)

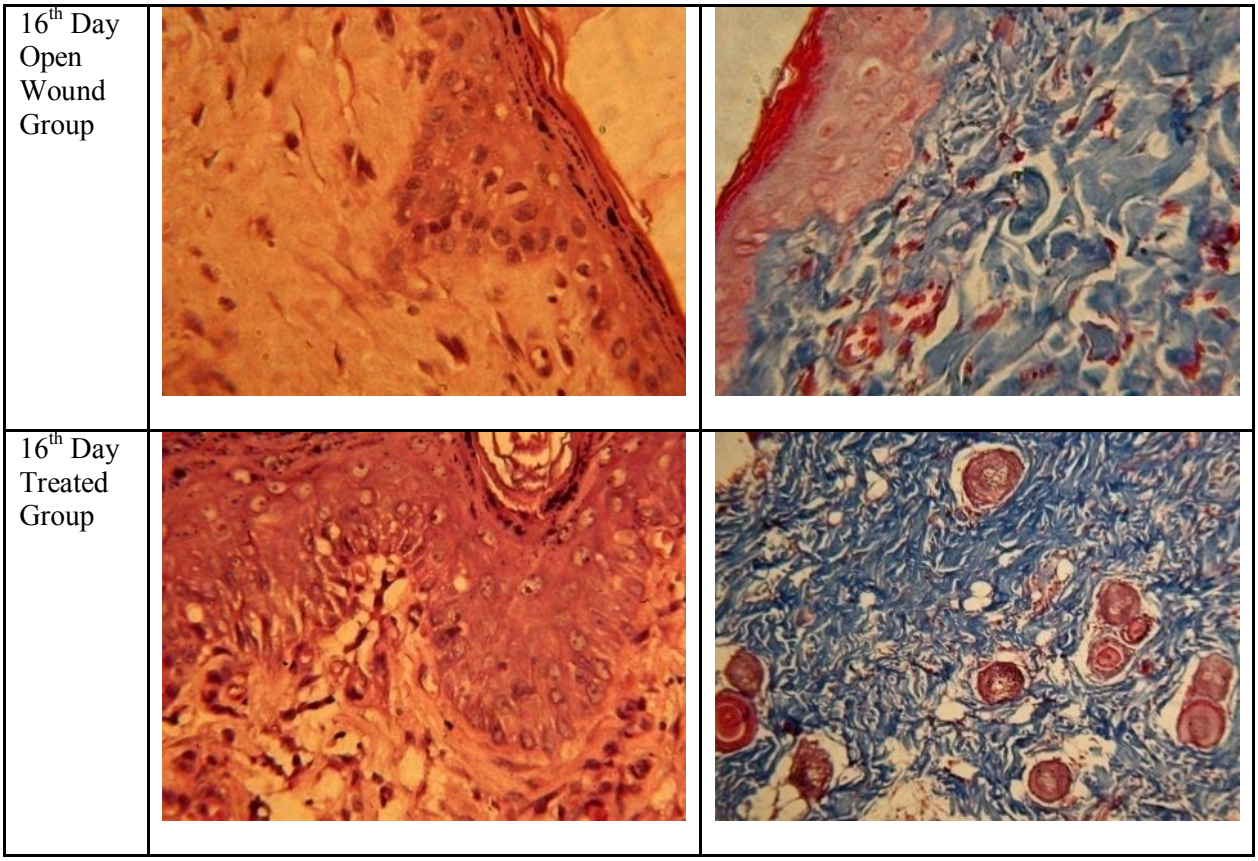


Figure 7. H&E Staining and Masson's Trichrome Staining of Granulated Tissue-16th day

Discussion

The present investigations confirm the wound healing potential of extract of amla thro in vivo studies, biochemical studies and histological studies of wound healing. The topical applications of the ointment formulation of plant extract effectively eradicates the wound pathogens at the wound site and enhance the wound closure in fast manner when compared to the synthetic antimicrobial agents. The extract of Amla contains many potential pytochemicals, which either directly or indirectly involving in the wound healing processes especially vitamin "C" plays a major role in collagen synthesis and poly phenols involved in antimicrobial actions and aligning orientation of collagen fibers in wound site.The short investigation of extract of amla provides various pharmacological activities related to wound healing potential such as anti-microbial activity and collagen synthesis activity for promoting the regeneration of the wound. The wound pathogens effectively eradicated and controlled their growth due to the potential antimicrobial activity of the extract of amla. As a result, wound closure in treated groups is faster than the open wound group. In addition to that, the bacterial count in the granulated tissue is decreased in treated group than the open wound group.

In wound healing with infected, instantaneously after an injury, there is an increased synthesis of extra cellar matrix components in the wound area. Usually, the synthesis of extracellular matrix is delayed by the infection at the wound site and presence of various metabolites secreted by wound pathogens. In addition to that, the wound pathogens and its metabolites trigger the expression of matrix metalloprotease in the skin that affects the balance between tissue inhibitor metalloprotease and matrix metalloprotease. This extreme condition in the infected wound can increase collagen degradation rather than collagen formation and its synthesis. [3]

The bioactive constituents present in the fruits of amlaareascorbic acid (Vitamin C) and Poly phenols such as ECCG (Epigallocatechin Gallate). The Ascorbic acid enhances collagen synthesis at wound site especially in the process of post translation modification of collagen in the extracellular matrix. Collagen contains unique amino acids (hydroxyproline and hydroxylysine) which are the essential amino acids for the stability of the collagen protein and for its complete maturation. Ascorbic acid is insinuated to be specifically required for the decarboxylation of a-ketoglutarate in the prolyl-4-hydroxylase reaction, where it may act as a

compound necessary for the reduction of enzyme-bound ferric iron formed during hydroxylation of proline [20]. In the absence of vitamin C, under-hydroxylated procollagen molecules are not retained within cells, and are less stable and more temperature-sensitive [19]. Procollagens having different hydroxyproline content were shown to have sensitivity towards pepsin digestion at temperatures lower than their physiological condition, which was found to be in direct relation to the extent of hydroxylation. In other words, these bioactive molecules improve the orientation of collagen fibers at the wound site.

After synthesis of collagen by fibroblasts, collagen is secreted by the cell into the matrix where it undergoes cross-linking to form fibers. The initial step in the cross linking reaction is the formation of aldehydic intermediates catalyzed by lysyl oxidase. Collagen molecules that contain aldehydic groups self assemble into fibers and then become cross-linked through reactions that occur between these aldehydic groups and other amino acids of adjoining molecules. It has also been shown that any increase in collagen synthesis leads to an increase in newly formed collagen and is associated with an increase in aldehyde content, the latter leading to a greater potential for crosslink formation [21,22,23]. The present work shows that the collagen obtained from amla treated wounds have a higher content of aldehydic groups (Table 4) than collagen from untreated controls. This indicates that the collagen in treated wounds undergo a higher degree of cross-linking, resulting in an ultimate increase in wound strength. The H&E staining and Masson's Trichrome staining of granulated tissue proved that well starched and tight collagen bundles formed in the granulated tissue from treated group by amla extract ointment and also formed well epithelisation and dermis formed in the treated group proves the effective regeneration of infected dermal wound by amla extract. Due to multiple pharmacological properties of extract of amla, the wound healing in Albino wister rats is effective and better wound closure is observed with eradication of wound pathogens at the wound site. The further research on this investigation is to isolate various bioactive molecules from the extract of amla and its effect of signaling pathway of various stages of wound healing process.

Conclusion

In vivo studies of dermal and epidermal regeneration in infected dermal wound confirmed that the wound healing potential of the methanol extract of fruits of amla is carried out in the infected wound environment. Moreover, the crude methanol extract of amla is not only eradicating the wound pathogens at the wound site and but also enhances the collagen synthesis and increased the cross linking of collagen in the wound site. In conclusion, the ointment formulation from the extract of fruits of *amla* exhibited significant dermal and epidermal regeneration activity in the infected wound when topically applied on rats by modulating various stages of healing process.

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