

## HISTOLOGICAL AND BIOCHEMICAL EVALUATION OF WOUND REGENERATION POTENTIAL OF *TERMINALIA CHEBULA* FRUITS

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### ABSTRACT

The objective of this investigation was to evaluate the histological and biochemical evaluation of wound healing potential of the ethanol extract of *Terminalia chebula* fruits. Antibacterial activity and *in vivo* wound healing properties of the ethanol extract on infected lesions by reference strains of *Staphylococcus aureus* ATCC 25923 and *Pseudomonas aeruginosa* ATCC 27853 in Wistar rats were investigated. A total of 32 rats were divided into two groups such as control group and Group treated with an extract of *T. chebula*. Control groups infected, but not treated with any medicine. The ethanol extract has a real healing potential and antibacterial activity against reference strains used. In addition, Histological analysis of Granulated tissue from treated group confirmed the regeneration of dermal wound with well-formed dermis and epidermis in the skin and then a tight bundle of synthesized collagen in the tissue. Therefore, these antibacterial properties and wound healing activities engage *T. chebula* in the process of developing an improved traditional medicine as alternative for existing therapy.

**Keywords:** Wound infection, *In vivo* studies, Masson's trichrome staining and collagen.

### INTRODUCTION

A variety of limitations is available in synthetic antimicrobial therapy for wound healing such as antibiotic resistance, antiseptics toxic to fibroblast cells, skin irritations, and specific single pharmacological activity. Therefore, the phytopharmaceutical is needed for better treatment for wound healing especially in the highly infectious microorganisms at the wound site. In addition, 450 Phyto-extracts were identified for wound healing treatment [11,24]. The wound healing process has a sequence of coagulation, inflammation, proliferation, formation, accumulation of fibrous tissues, collagen deposition, epithelialization, contraction of the wound with the formation of granulation tissues, remodeling, and maturation. The bioactive constituents of the plant extracts modulate one or more of the above stages at the wound site. In this work, the fruit of *Terminalia chebula* Retz is chosen for evaluation of its wound healing potential using *in vivo* studies. *T. chebula* Retz. This belongs to the family-Combretaceae, commonly known as harde, is a deciduous tree found throughout the Indian forests and plains. Its fruit has 5-6 ribs (Fig. 1) with a variety of pharmacological activities such as astringent, antiseptic, rejuvenative, tonic, anthelmintic, and laxative [8]. It is used in chronic ulcer wound, piles, and stomatitis. Fruit contains about 30-32% of tannin, free tannic acid, Gallic acid and ellagic acid, glucose and sorbitol [3]. A survey of literature revealed (Table 1) that no evidence on histological and biochemical approach has been made to study the wound healing activity of this plant. Thus, the present study

is undertaken to assess the effect of fruits of this indigenous plant on different parameters related to wound healing in rats.

### METHODS

#### Plant material and extraction

The fruits of the *T. chebula* were collected from CLRI institute campus (Central Leather Research Institute, Chennai, India). The fruits were separated and shade dried. The dry fruits were grounded into powder form using the grinder. Extraction was performed by using Soxhlet apparatus with 95% (v/v) ethanol. The resultant extraction was evaporated to dryness under reduced pressure in Rotary vacuum evaporator 40-45°C. The concentration extract was aliquoted in amber-colored bottles and kept in desiccator for further use. The dried extract was dissolved in dimethyl sulfoxide (DMSO) and used to assay the antibacterial activity.

#### Bacteria tested

*S. aureus* ATCC 25923 and *Pseudomonas aeruginosa* ATCC 27853 were obtained from the King Institute, Chennai, India.

#### Culture media and growth

The strains were incubated in Soybean Casein Digest Broth (Hi-Media Pvt. Ltd., Mumbai, India) for overnight at 37°C and adjusted to yield approximately  $1.0 \times 10^5$  CFU/mL.

**Table 1: Reported wound healing activity of *T. chebula* fruits and its shortcomings**

S. No	Types of formulation	Reported bioactive constituents	Lack of information	References
1	Ointment of ethanolic extract of <i>T. chebula</i> fruits	Evaluated on excision and incision model in albino rats	Lack of histological and biochemical test to confirm wound healing stages	[5]
2	Dry fruits powder mixed with water and made paste	Rabbits used for wound model	No biochemical analysis and collagen synthesis in granulation tissue	[9]
3	Extracted with warm water	Cutaneous wound healing in rats	Absence of histological studies on wound healing pattern and information on dermis and epidermis regeneration	[12]
4	Alcoholic extract of the leaves	Performed Biochemical studies	Lack of wound healing potential of <i>T. chebula</i> fruits	[20]
5	Dry powder mixed with water	Wound model on rabbit	Absence of biochemical and histological analysis	[19]

*T. chebula*: *Terminalia chebula*

### Antibacterial susceptibility

The antibacterial sensitivity test was performed by disc diffusion method. *P. aeruginosa* and *S. aureus* grown on Mueller-Hinton agar (MHA, Himedia, India) were suspended in Mueller-Hinton broth (MHB, Himedia, India) and diluted with MHB to  $10^6$  CFU/ml. Sterile blank discs (6 mm diameter) impregnated with *T. chebula* fruit extract were placed in Muller-Hinton agar plates inoculated with the test strains and incubated at 37°C for 24-48 hrs. Standard methicillin disc (5 mcg) and disc with 10% DMSO were used as positive and negative control, respectively. Inhibition zone diameters around each of the disc were measured and recorded at the end of the incubation time. The minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC) of the extract were determined by the tube dilution method. Double dilution was made from higher dilution 100 mg/mL to lower dilution in a series of test tubes. Each tube was inoculated with bacterial suspensions and incubated at 37°C for overnight. The MIC was regarded as the lowest concentration of the plant extract in the series of dilutions, which did not permit the growth of the susceptible bacteria. The MBC of the extract was determined by previously described method with modification. In brief, subcultures were made from tubes, which did not yield any visible turbidity (growth) in the MIC assays on freshly prepared MHA (for *S. aureus* and *P. aeruginosa*). After 24 hrs incubation at 37°C, the MBC was regarded as the lowest concentration of the plant extract that allowed <0.1% of the original inoculums to grow on the surface of the medium. In each experiment, extract was tested in triplicate.

### Ointment formulation

Plain plant extract ointment was prepared by mixing the accurately weighed required quantum of extract with yellow soft paraffin obtained from S.D. fine chem. Pvt Ltd, India.

### In vivo wound healing activity

Male Wister albino rats of weights ranging 150-200 g were used for the present study. They were housed individually in standardized environmental conditions. Totally, 32 animals were taken in two groups (control and experimental) for this study. Full thickness wounds (1.5 cm × 1.5 cm) were created on the dorsal side of shaved rats using a sterile surgical blade and inoculated with the test organisms, allowed to infect for 24 hrs. All surgical procedures were carried out under sodium thiopentone (40 mg/kg body weight, intramuscularly). The experimental rats were dressed with formulated ointment while the control rats were dressed only with paraffin. All dressings were changed regularly every day.

### Rate of wound contraction

The reduction in the size of wound was measured at every 4 days intervals and given as a percentage of wound contraction. The following formula was used to calculate the percentage of wound reduction:

$$\% \text{ wound reduction} = \frac{\text{Wound area day 0} - \text{wound area day (n)}}{\text{Wound area day 0}} \times 100$$

n = 4<sup>th</sup>, 8<sup>th</sup>, 12<sup>th</sup> and 16<sup>th</sup> day

### Bacteriological examination of granulated tissue

Superficial muscles/granulated tissues were excised on days 4, 8, 12, and 16. 1 mg of excised tissue was placed in 10 ml of sterile saline, vortex for few minutes and the total bacterial count was analyzed by serial dilution method.

### Biochemical analysis

About 5 mg of defatted dry granulation tissue was used to estimate the amount of collagen and hexosamine by the method of Neuman and Logan, 1950 [17] and Adamsons, 1964 [2]. Uronic acid content in the granulated tissue was analyzed by the Bitter and Muir method [4,5,10].

### Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of pepsin soluble collagen

Granulated tissues were collected on the 4<sup>th</sup>, 8<sup>th</sup>, 12<sup>th</sup>, and 16<sup>th</sup> days for the estimation of different types of collagen in the granulated

tissue. The pepsin soluble collagen was prepared from wound tissue as described by Miller and Rhodes *et al.* The  $\alpha 1(\text{III})$  chains were resolved from the  $\alpha 1(\text{I})$  chains on a 8% separating gel with 5% stacking gel by interrupted electrophoreses with delayed reduction of the disulfide bonds Type (3) collagen (Sykes *et al.*, 1976, Clore *et al.* 1979) [7,21].

### Histological analysis

Granulated tissues were collected at every 4 days intervals and transferred to 10% neutral buffered formalin for 24 hrs at 4°C. The formalin fixed tissues were dehydrated through grades of alcohol and cleared in xylene and then embedded in paraffin wax (58-60°mp). The molds were labeled and stored until use. A 5-7  $\mu\text{m}$  sections were deparaffinized and stained with hematoxylin



Fig. 1: Fruits of *Terminalia chebula*. The literature revealed that it contains tannins as bioactive constituents which would be responsible for a variety of pharmacological action

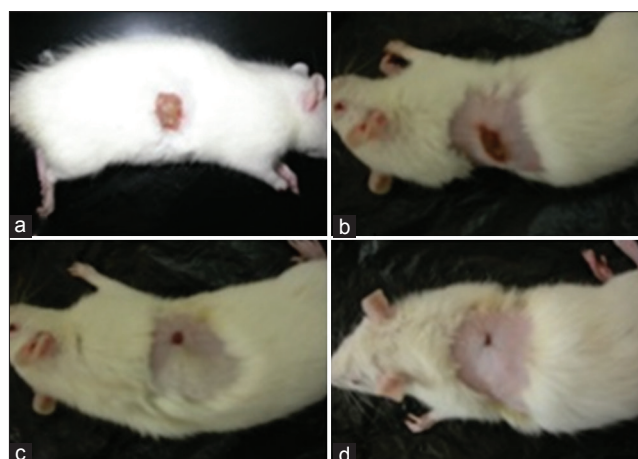


Fig. 2.1: (a-d) Wound contraction in control rats wound closure

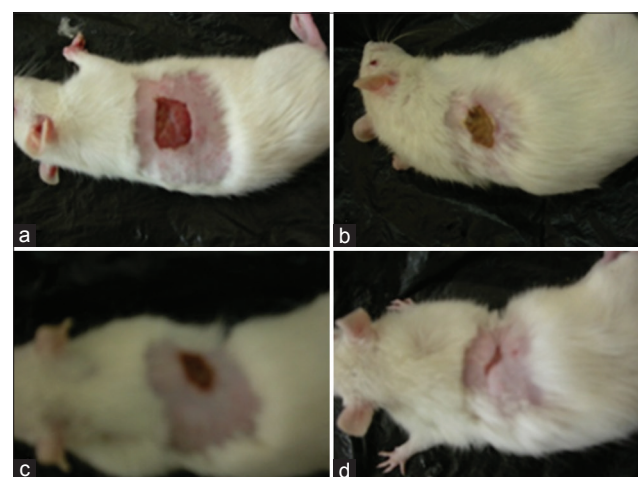


Fig. 2.2: (a-d) Wound contraction in treated rats

following counterstained with eosine and also Masson's trichrome staining for collagen synthesis and its morphology in the granulation tissue [14].

#### Statistical analysis

All results have been expressed as mean  $\pm$  SD and the results were compared statistically by student's independent *t*-test using SPSS software (student version 7.01). The  $p < 0.05$  was considered statistically significant.

#### RESULTS

##### *In vitro* anti-microbial potential of *T. chebula* fruits

The antimicrobial activity of the fruits of *T. chebula* is evaluated by disc diffusion assay and then MIC. The MIC of extract of fruits of *T. chebula* against *P. aeruginosa* and *S. aureus* strains was found to be  $\sim 3.55 \pm 0.0078$  mg/ml. The MBC of fruits of *T. chebula* extract was  $> 3.55$  mg/mL for *P. aeruginosa* and *S. aureus*. All bacterial strains showed susceptibility to *T. chebula* when tested using the disc diffusion method. All strains including control strain exhibit clear zone of inhibition ( $10 \pm 2$  mm) to the methanol extract of fruits of *T. chebula*. No zone of inhibition was observed in DMSO treated disc (Tables 2 and 3).

A significant difference in the wound closure was observed in treated group from day 4 onward and also the rate of wound closure was much faster on later days when compared with control. Complete wound closure was observed in *T. chebula* ointment treated group on day 16, whereas in control group wound closure about 25 days. Moreover, as these fruits have the potential anti-microbial potential to eradicate the wound pathogens, the better regeneration of wound was observed in the treated group (Fig. 6).

##### Histological analysis of granulated tissue

Fig. 3 shows the histological analysis of granulated tissue from control and treated group at 4<sup>th</sup>, 8<sup>th</sup>, 12<sup>th</sup>, 16<sup>th</sup> days. In H and E staining, complete loss of superficial epithelium with inflammatory infiltrates was observed in both the groups on day 4. Accumulation of inflammatory cells/neutrophils was high in control when compared with treated group as inflammatory cells were reduced due to the antimicrobial activity of fruits in the open wound site. Treated group showed well-formed epithelialization with the moderate extracellular matrix on day 8 and day 12 whereas in the control group, incomplete epithelialization with less content of extracellular matrix synthesis and persistence of

inflammatory exudates in the upper dermis with loss of epidermis were observed up to day 16. In day 16, the treated group has shown noticeable epithelialization with the moderate amount of extracellular matrix synthesis and new blood vessel formation. In other words, better angiogenesis and better regeneration of dermis and epidermis were observed than control groups.

Fig. 3 shows the deposit of collagen as violet color observed in Masson's trichrome staining. On day 8, the deposit of collagen was very much less in control group, but the treated group showed moderate deposit of collagen in the granulated tissue whereas in control group, less amount of collagen in the tissue was seen; in case of control group, loose collagenous matrix was found, whereas in treated group more compact and matured collagen deposit parallel to the epidermis was observed on day 12.

In addition, in the open wound group, on the 4<sup>th</sup> day and the 8<sup>th</sup> day, (Fig. 3 Open Wound Group-Masson's Trichrome Staining) neutrophils were observed and bacterial colonies were seen in the injured area. Complete loss of epithelium with inflammatory infiltrates was observed, and less amount of collagen synthesis was seen due to infection. In 12<sup>th</sup> day and 16<sup>th</sup> day (Fig. 5 - 12<sup>th</sup> and 16<sup>th</sup> day), partial epidermis formed and a loose collagen fiber was observed. In the group treated with the ointment of extract, on the 4<sup>th</sup> day (Fig. 5), neutrophils were found and bacterial colonies formed in the injured area. Complete loss of epithelium and no collagen content in tissues were observed. On the 8<sup>th</sup> day (Fig. 5), a bundle of collagen fibers was formed in the granulated tissue.

On the 12<sup>th</sup> day and 16<sup>th</sup> day (Fig. 5), well-formed epidermis and dermis with collagen bundles were seen in the tissues. In Masson's Trichrome stained histological sections of tissue of the 12<sup>th</sup> and the 16<sup>th</sup> days of groups treated by ointment of *T. chebula*, bluish violet color indicates staining of well stretched and deposited collagen bundles formed in the tissue (Fig. 5). In the case of open wound at the end of the 12<sup>th</sup> and 16<sup>th</sup> day, loose collagenous matrix was also seen.

##### SDS-PAGE analysis of collagen

The above experiment showed the SDS-PAGE analysis 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 (Fig. 4).

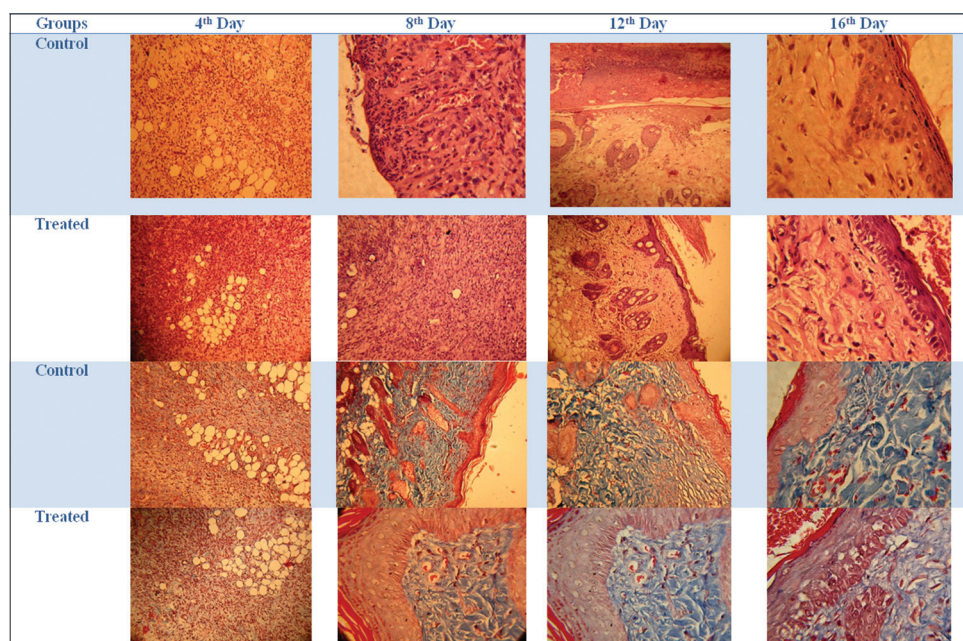


Fig. 3: Histological analysis - H and E staining and Masson's Trichrome Staining - 4<sup>th</sup> day magnification ( $\times 150$ ) and 8<sup>th</sup> 12<sup>th</sup> 16<sup>th</sup> day at  $\times 400$

**Biochemical studies**

Biochemical analysis has shown progressive healing on treated group compared with open wound group. Significant increase in the hydroxyproline content was observed in the treated group than open wound group. It confirms indirectly that the synthesis of collagen in the granulated tissue from the treated group was increased. The hexosamine and uronic acid content was reduced in both open wound

and treated groups, but the amount was comparatively high in the case of the treated group from day 4. Additionally, these data shows synthesis of GAG in the ECM matrix was increased (Fig. 7).

The application of *T. chebula* fruits extract ointment diminished the level of the total bacterial count from  $5 \times 10^9$  CFU/g to  $4.5 \times 10^4$  on day 4 in the infected wound, whereas the control group recorded  $58 \times 10^8$  CFU/g (Fig. 8).

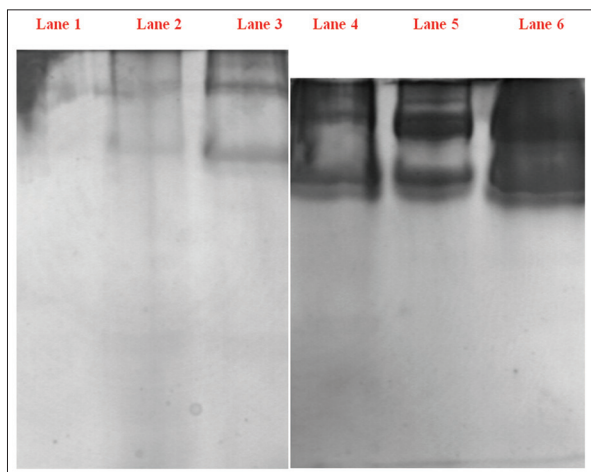


Fig. 4. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of pepsin soluble collagen of granulated tissue from *in vivo* studies. The collagen content in the granulated tissue of open wound group (control) of lane 1 (4<sup>th</sup> day) lane 2 (8<sup>th</sup> day) lane (12<sup>th</sup> day) are shown in SDS-PAGE. It showed that Collagen content in the granulated tissue is less quantity due to infection at wound site

**DISCUSSION**

Repair of infected wound is a fundamental response to tissue injury that results in restoration of tissue integrity [18]. This is mainly achieved

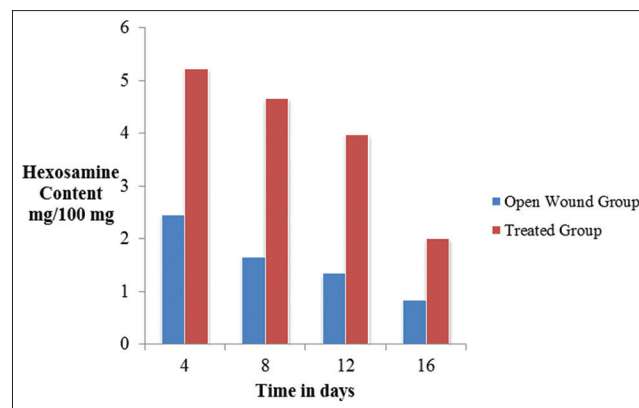


Fig. 7: Effect of extract of fruits of *Terminalia chebula* ointment on synthesis of hexosamine in granulated tissue

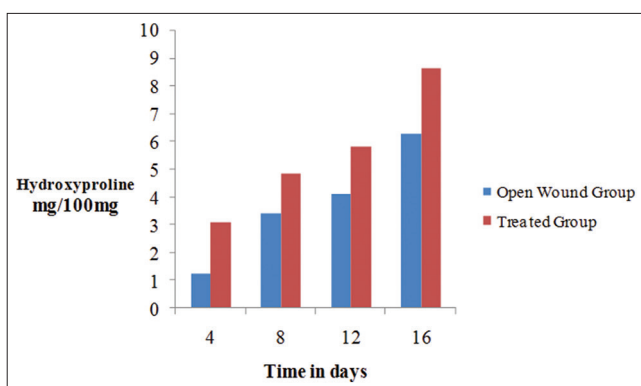


Fig. 5: Effect of extract of fruits of *Terminalia chebula* ointment on synthesis of hydroxyproline in granulated tissue

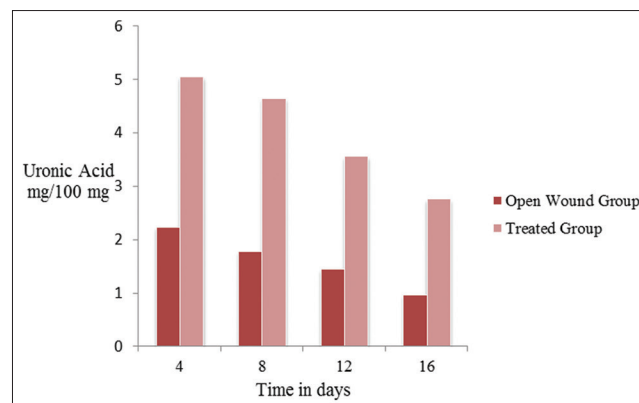


Fig. 8: Effect of extract of fruits of *Terminalia chebula* ointment on synthesis of uronic acid in granulated tissue

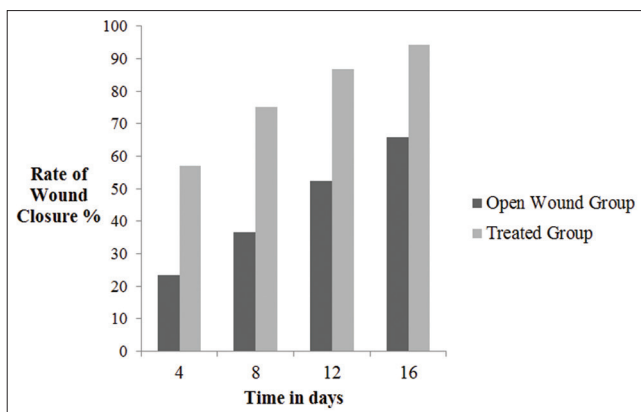


Fig. 6: Effect of extract of fruits of *Terminalia chebula* ointment on rate of wound closure

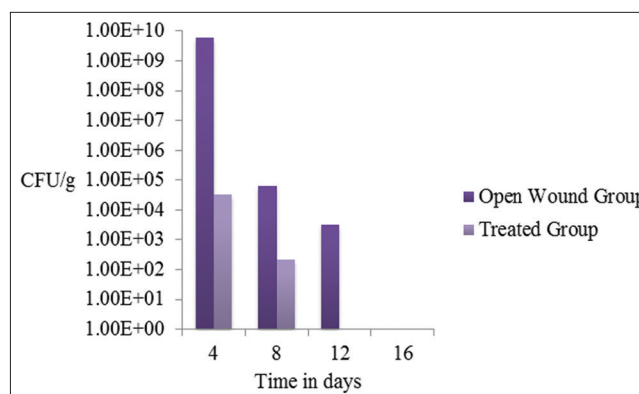


Fig. 9: Effect of extract of fruits of *Terminalia chebula* ointment on control of infection in granulated tissue

by the synthesis of the connective tissue matrix at the wound site. Collagen is a major component of the extracellular matrix and is the fibrous protein that ultimately contributes to wound strength. Topical delivery of drugs is more effective in both antimicrobial and wound healing rate due to its larger availability of therapeutic agents at the infected wound site. The ability of microorganisms in the wound bed creates massive biofilm which cause damage depends on the virulence capacity of the organism, the amount of inoculums present at the wound. *S. aureus*, *S. pyogenes*, and *P. aeruginosa* are the most common wound pathogens with  $\geq 10^3$  CFU/g tissues and its microbial protease degrades the extracellular matrix at wound site [18]. As a consequence, the wound healing delayed (Clark *et al.*) [1,6]. In this study, significant wound contraction was observed in fruits of *T. chebula* treated rats. *In vitro* antimicrobial studies and *in vivo* regeneration of epithelialization in the treated rats support the effect of fruits of *T. chebula* in the infected wound healing.

In addition, Tannins present in the fruits of *T. chebula* was responsible for the antimicrobial action against a variety of wound pathogens [17]. The literature shows that epigallocatechin gallate which is one of the bioactive constituents in the fruits has the specific antimicrobial mechanism for eradication *S. aureus*. The epigallocatechin gallate binds to *S. aureus* than that of Gram-negative bacterium *E. coli* and EGC treated *S. aureus* was more sensitive to high ionic strength and low osmotic pressure. In addition, the epigallocatechin gallate binds to the peptidoglycan layer in the cell wall of *S. aureus*. As a result, it induces its precipitation. Peptidoglycan is a cross-linked complex of polysaccharides and peptides. The cell wall of *Staphylococcus* was composed of 30-50 layers of peptidoglycan where it provides osmotic protection, aids in cell division and serves as a primer for further biosynthesis of peptidoglycan. Therefore, the EGCg-induced damage of the cell wall and interference with its biosynthesis through direct binding with peptidoglycan are the major reasons for the susceptibility of *Staphylococcus* to EGCg. (Yoda *et al.*, 2004; Zhao *et al.* 2002, 2001.). However, it is obscure in the case of antimicrobial action against *P. aeruginosa*.

Significant reduction of bacterial count in the treated rats was observed in the day 8 from  $10^9$  CFU to  $10^2$  CFU/g tissues, further confirms the effectiveness of fruits of *T. chebula* treatment. Increased wound contraction in treated rats might be due to the enhanced activity of fibroblasts in the treated rats. The delayed rate of wound regeneration in open wound rats as control group may be attributable to the presence of microorganisms and their protease, which inhibits wound contraction and impair healing.

Biochemical analysis confirmed that highly significant increase in the hydroxyproline content that revealed the enhanced migration of fibroblast cells, epithelial cells, and synthesis of the extracellular matrix including collagen during the wound healing process in treated rats. The decreased content in hydroxyproline in control rats might be due to the presence of microorganisms which could extend the inflammatory phase of wound healing and then inhibits both epithelial regeneration and proliferation of fibroblast. It results delayed healing. Hexosamine and uronic acid are matrix molecules, which act as ground substratum for the synthesis of the new extracellular matrix at the wound site. The decrease in the hexosamine and uronic acid content was associated with concomitant increase in collagen content which is confirmed by SDS-PAGE. The uronic acid in the wound attracts fibroblast and stimulates collagen synthesis by providing more fluid that facilitates greater cell mobility, early remodeling and helps the wound to heal without scar formation [22].

The biochemical analysis of wound regeneration in treated and control groups were observed with histological examinations. A close observation of granulation tissue sections revealed that the tissue regeneration was much more rapidly in the treated group compared to control wounds. There was marked infiltration of inflammatory cells, increased blood vessel formation, and enhanced proliferation of fibroblasts as a result of *T. chebula* treatment. Increased cellular

**Table 2: Zone of inhibition for standard strain microorganisms**

Microorganisms	<i>T. chebula</i> extract	Std. antibiotic
<i>S. aureus</i>	10±1.25 mm	34±0.5 mm (methicillin)
<i>P. aeruginosa</i>	12±1.58 mm	30±1.0 mm (ciprofloxacin)

*T. chebula*: Terminalia chebula, *S. aureus*: Staphylococcus aureus, *P. aeruginosa*: Pseudomonas aeruginosa

**Table 3: MIC**

Microorganisms	Minimum concentration
<i>S. aureus</i>	3.925±0.0076 mg/ml
<i>P. aeruginosa</i>	3.925±0.0078 mg/ml
<i>S. pyogenes</i>	15.25±0.0095 mg/ml

MIC: Minimum inhibitory concentration, *S. pyogenes*: Streptococcus pyogenes, *S. aureus*: Staphylococcus aureus, *P. aeruginosa*: Pseudomonas aeruginosa

infiltration observed from hematoxylin and eosin staining in *T. chebula* treated rats compared with control may be due to chemotactic effect enhanced by the extract, which might have attracted inflammatory cells toward the wound site. Increased cellular proliferation may be due to the mitogenic activity of the plant extract, which might have significantly contributed to healing process. Early dermal and epidermal regeneration in treated rats also confirmed that the extract had a positive effect toward cellular proliferation, granulation tissue formation, and epithelialization.

The most important bioactive constituents in the extract of *T. chebula* is tannins which promote the wound healing through several cellular mechanism such as chelation of the free radicals and reactive species of oxygen, promoting contraction of the wound and increasing the formation of capillary vessels and fibroblasts and including keratinocyte proliferation and also responsible for wound healing mainly due to their astringent and antimicrobial property.

## CONCLUSION

The above investigation suggested that the topical application of fruits of *T. chebula* ointment on infected dermal wound not only control the risk of infection but also produce better regeneration of dermis and epidermis at the wound site. As the fruits of *T. chebula* possesses various activities and clinically used in several ayurvedic formulations such Triphala [23], our findings may provide scientific rationale for the use of fruits of these herbal plant to promote infected wound healing. Further phytochemical studies are in progress where the *T. chebula* extract will be subjected to further fractionation and purification to identify the active principles responsible for pharmacological activities and their mechanism toward infected wound healing.

## REFERENCES

- Singer AJ, Clark RA. Cutaneous wound healing. N Engl J Med 1999;341(10):738-46.
- Adamsons RJ, Musco F, Enquist IF. The relationship of collagen content to wound strength in normal and scorbutic animals. Surg Gynecol Obstet 1964;119:323-9.
- Bag A, Bhattacharyya SK, Chattopadhyay RR. Therapeutic potential of *Terminalia chebula* Retz. (Combretaceae): The Ayurvedic wonder. Asian Pac J Trop Biomed 2013;3(3):244-52.
- Bitter T, Muir HM. A modified uronic acid carbazole reaction. Anal Biochem 1962;4:330-4.
- Choudhary GP. Wound healing activity of the ethanolic extract of *Terminalia chebula* Retz. Int J Pharm Bio Sci 2011;2(1):48-52.
- Clark RA. Wound repair: Overview and general considerations. In: Clark RA, editor. The Molecular Biology of Wound Repair. New York: Plenum Publishing; 1996. p. 195.
- Clore JN, Cohen IK, Diegelmann RF. Quantitation of collagen types I and III during wound healing in rat skin. Proc Soc Exp Biol Med 1979;161(3):337-40.
- Singh D, Singh D, Choi SM, Zo SM, Ki SB, Han SS. Therapeutical

- effect of extracts of *Terminalia chebula* in inhibiting human pathogens and free radicals. Int J Biosci Biochem Bioinformatics 2012;2:3.
9. Fundter JM. *Terminalia chebula* Retz. In: Lemmens RH, Wulijarni-Soetjipto N, editors. Plant Resources of South-East Asia. No. 3: Dye and Tannin-Producing Plants. Bogor, Indonesia: Prosea Foundation; 1992. p. 122-125.
  10. Hu M, Sabelman EE, Cao Y, Chang J, Hentz VR. Three-dimensional hyaluronic acid grafts promote healing and reduce scar formation in skin incision wounds. J Biomed Mater Res B Appl Biomater 2003;67(1):586-92.
  11. Logeeswari K, Sripathi SK. Wound healing medicinal plants: A review. Int J Chem Environ Pharm Res 2012;3(3):199-218.
  12. Li K, Diao Y, Zhang H, Wang S, Zhang Z, Yu B, et al. Tannin extracts from immature fruits of *Terminalia chebula* Fructus Retz. promote cutaneous wound healing in rats. BMC Complement Altern Med 2011;11:86.
  13. Kumar M, Agarwal RC, Dey S, Rai VK, Johnson B. Antimicrobial activity of aqueous extract of *Terminalia chebula* RETZ. on gram-positive and gram-negative microorganisms. Int J Curr Pharm Res 2009;1(1):56-60.
  14. McManus JG, Mowry RW. Staining Methods: Histological and Histochemical. New York: Harper and Row; 1964. p. 8.
  15. Prasanna G, Jeyamanikandan V. Bioefficacy of *Terminalia chebula* extract against biofilm formation of common pathogens. J Chem Pharm Res 2013;5(6):99-103.
  16. Ghosh PK, Gaba A. Phyto-extracts in wound healing. J Pharm Pharm Sci 2013;16(5):760-820.
  17. Neuman RE, Logan MA. The determination of hydroxyproline. J Biol Chem 1950;184(1):299-306.
  18. Robson MC. Wound infection. A failure of wound healing caused by an imbalance of bacteria. Surg Clin North Am 1997;77(3):637-50.
  19. Shreedevi MS, Sampath Kumar B. Effect of siddha herbal paste on wound healing in induced Derman wounds in rat. Int J Ayurvedic Herbal Med 2011;1(3):86-91.
  20. Suguna L, Singh S, Sivakumar P, Sampath P, Chandrakasan G. Influence of *Terminalia chebula* on dermal wound healing in rats. Phytother Res 2002;16(3):227-31.
  21. Sykes B, Puddle B, Francis M, Smith R. The estimation of two collagens from human dermis by interrupted gel electrophoresis. Biochem Biophys Res Commun 1976;72(4):1472-80.
  22. Tarnuzzer RW, Schultz GS. Biochemical analysis of acute and chronic wound environments. Wound Repair Regen 1996;4(3):321-5.
  23. Tillotson A, Khalsa KP, Triphala CT. Haritaki fruit. Can J Herb 2001;22:16.
  24. Sharma Y, Jeyabalan G, Singh R. Potential wound healing agents from medicinal plants – A review. Pharmacologia 2013;4(5):349-58.