Wound repair and regeneration potential of the fruits of Terminalia bellarica

S. Kirubanandan*, S. Renganathan

ABSTRACT

The infection at the wound site is a severe kind of problem and it delays regeneration of epidermis and dermis in the wound and slows wound closure. Due to the secretion of microbial enzymes by wound pathogens, a variety of extracellular matrix proteins were degraded. Synthetic antimicrobial therapy used in the wound management and eradication of pathogens. However, it has many shortcomings such as anti-microbial resistance, cytotoxicity against host tissue and absence of synergistic activity. In order to overcome these limitations, Pyto-pharmaceuticals extracted from herbal plants were applied to manage the wound infection and treatment. The objective of this work is to evaluate the wound repair and regeneration potential of the fruits of Terminalia bellarica which has a variety of pharmacological activities such as astringent, antiseptic and laxative. The dry fruits of Terminalia bellarica were grounded into powder form using the grinder. Extraction was performed by using Soxhlet apparatus with 95% (v/v) ethanol. The dried extract was dissolved in Dimethyl Sulfoxide (DMSO) and used to assay the antibacterial activity against Staphylococcus aureus ATCC 29213, Pseudomonas aeruginosa ATCC 27853. An ointment was prepared from the ethanol extract (10% w/w) and assessed for its in vivo wound healing potential on infected rat model by rate of healing, bacterial count, biochemical analysis, and expression of matrix metalloproteinase. In addition to that, the collagen content in the granulation tissue was estimated to comment on wound regeneration potential of the fruits of Terminalia bellarica. The treated group has shown significantly improved wound regeneration and well formed epidermis and dermis in the granulation tissue. Furthermore, Assessment of granulation tissue on every fourth day showed significant reduction in bacterial pathogens CFU with significant elevated level of collagen, hexosamine, uronic acid, in the treated group. The reduced level expression of matrix metalloproteinase expression observed in the treated group by gelatin zymography and the synthesis of substantial amount of collagen in the granulation tissue confirms our in vivo assessment. The results showed the antibacterial and wound healing activities of Terminalia bellarica fruits ointment, necessary for the management of infected open dermal wounds. The isolation of bioactive molecules from Terminalia bellarica fruits and its interaction various cells using cell culture studies would be future work.

Keywords: Wound Infection, Terminalia bellarica, MMPs, Collagen, Histology staining.

INTRODUCTION

Wound infections are the most common in the developing countries due to poor hygienic life style and conditions. Staphylococcus aureus, Streptococcus pyogenes, Corynebacterium spp., Escherichia coli and Pseudomonas aeruginosa are the most common wound pathogens causing severe infection in the open dermal wound site. Among the most common microorganisms that cause wound infection are Staphylococcus aureus and β-hemolytic Streptococcus [1], which are considered “transient flora” of the skin [2]. P. aeruginosa infects wounds and these wound pathogens produces microbial protease such as collagenase and elastase. These microbial enzymes degrade the extracellular matrix in wound site. Generally, infected wounds heal more slowly and results an increased incidence of scarring [3]. These microorganisms secrete extra cellular enzymes such as collagenase and elastase which degrades the extra cellular matrix proteins at the injured site. Pseudomonas aeruginosa, the predominant organism causing air born infection and the frequency of infection is more in burn patient. A wide range of antibiotics is being employed at present for treating wound infections, however they are now proved to have adverse effects in the human body such as cytotoxic effects and also these pathogens develop antibiotic resistance. In this point of view, so much recent attention has been paid to extracts of bio active compounds isolated from plant species used in herbal medicine [4].

In India, herbal-based treatments like Ayurveda, Siddha and Unani are used to cure various ailments and physiological abnormalities. As per the report of National Health Experts, numerous plants are used for various medicinal preparations for both internal and external use in India. World Health Organization (WHO) estimates that 80% of the people living in developing countries almost exclusively using traditional medicine. Medicinal plants form the principle component of traditional medicine and around 119 secondary metabolites of plants are used globally as potential drugs [4]. The plant extracts as Drugs
are derived from trees, shrubs and herbs and even from primitive kinds of plants, which do not fall into the above categories. They are made from fruits, flowers, leaves, stems, roots, seeds and even bark. Although hundreds of plant species were used for evaluation for its antimicrobial potential, the vast majority has not yet been adequately evaluated [3]. The fruits of Terminalia bellarica Linn., belonging to the family of Combretaceae widely cultivated throughout India.

The fruit is a drupe, almost globular, 2.5 x 2 cm, covered in soft hairs and obscurely 5-angled. The dried fruit contains about 20% tannins of both condensed and hydrolysable type. Other Constituents identified in the fruit include lipids, (β-sitosterol, saponins, Gallic and ellagic acids) and their derivatives, glycosides and various carbohydrates. The fruit rind (pericarp) of T. bellarica constitutes the Ayurvedic drug vibhitaki. It is described as ‘bitter, acid, astringent, laxative, germicidal and antipyretic’ and is used in a wide range of conditions including cough, tuberculosis, eye diseases, dyspepsia, diarrhoea, dysentery, inflammation of the small intestine, biliousness, flatulence, liver disease and leprosy and also cleansing the blood. The ripe fruit is also used as an astringent, whereas the ‘half ripe’ fruit is used as a purgative. Fruit extracts have significant anti-bacterial activity against Micrococcus pyogenes and Escherichia coli. Oral administration of a water soluble fraction of the fruit demonstrated significant hepatoprotective activity in vivo against carbon tetrachloride induced liver injury. The carbon tetrachloride-induced elevation of lipid peroxidation in the liver was significantly decreased by the extract. Likewise, the accumulation of triglycerides in the liver following exposure to the liver toxin was inhibited and suggests the plant extract may prevent the formation of fatty liver. An alcoholic extract of the fruit was found to have a marked stimulant effect on the secretion of bile in vivo. The increasing of total solid content of the bile was also observed. An aqueous extract had poor activity in the same test model. (T. bellarica) had anti-asthmatic, anti-spasmodic, expectorant and anti-tussive effects. Even though the wound healing activity of Terminalia bellarica is proven and there is no scientific approach on its wound healing potential such as histological and biochemical studies. Therefore, The present study deals with the evaluation of the fruits of Terminalia bellarica for their infected wound healing activity in albino Wister rats through histological and biochemical studies. MATERIALS AND METHODS Plant Material and Extraction The fruits of the Terminalia bellarica were collected from our 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. The resultant extraction was evaporated to dryness under reduced pressure in Rotary evaporator 40°C. The concentration extract was aliquot in amber-coloured bottles and kept in desiccators for further use. The dried extract was dissolved in Dimethyl Sulfoxide (DMSO) and used to assay the antibacterial activity.

Microorganisms tested
The bacterial strains such as Staphylococcus aureus ATCC 29213, Pseudomonas aeruginosa ATCC 27853 were collected from the King Institute, Chennai, India.

Culture media and inoculum
Soyabean Casein Digest Broth (Hi-Media Pvt.Ltd., Bombay, India) was used for the test bacterial strains. Bacterial cultures, freshly grown at 37°C were appropriately diluted in sterile normal saline solution to obtain the cell suspension at 10^7 CFU/ ml.

Determination of antibacterial activity
The antibacterial sensitivity test was performed by disc diffusion method [8]. Sterile blank discs (6 mm diameter) were impregnated with different aliquots of plant extract. Extract impregnated discs were placed in Muller-Hinton agar plates inoculated with the test organisms and incubated at 37°C for 24-48 hrs. Disc with DMSO was used as a control. The minimal inhibitory concentration (MIC) of the extract was determined by the broth tube dilution method. Double dilution was made from higher dilution 1mg/ ml to lower dilution in a series of test tubes. Each tube was inoculated with 10^4 CFU/ ml microbial suspensions. The tubes were incubated at 37°C for overnight observed as turbidity determination by the spectrometer at 620nm and also confirmed by plating. Sample 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 weighs ranging 150-200g were used for the present study. They were housed individually in standardized environmental conditions. Totally 48 animals were taken in two groups (control and experimental) for this study. Full thickness wounds (1.5x1.5 cm) were created on the dorsal side of shaved rats using sterile surgical blade and inoculated with the test organisms, allowed to infect for 24 hrs. All surgical procedures were carried out under Sodium thiopentone (40mg/kg body weight, intramuscularly). The experimental rats were dressed with formulated ointment, while the control rats were dressed only with paraffin. All rats were given regular changes at every day.

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

\[
\frac{Wound\ area\ day\ 0 - wound\ area\ day\ (n)}{Wound\ area\ day\ 0} = \frac{Wound\ area\ day\ 0}{Wound\ area\ day\ (n)} \times 100
\]

n = 4th, 8th, 12th and 16th 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
Granulated tissues were collected on days 4, 8, 12 and 16. 10ml of 5% trichloroacetic acid was added to 100 mg (wt weight) of granulated tissue and kept at 90°C for 30 min in water bath to extract DNA and protein. The DNA content of the extract was estimated by the method of Labarca and Paigen [7] and the protein content was estimated by the method of Lowry et al. [9]. 5 mg of Defatted dry granulation tissue was used to estimate the amount of collagen and hexosamine by the method of Neuman and Logan [10] and Adamsons et al [11]. Uronic acid content in the granulated tissue was analyzed by the Bitter and Muir method [10].

Collagen Content in Granulation Tissue:
Granulated tissues were collected on the 4th, 8th, 12th and 16th 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 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.

MMP Evaluation in Granulated Tissue:

The presence of matrix metalloproteinases (MMPs) in the granulation tissues was evaluated by gelatin zymography. 100 mg (wet weight) of tissue was homogenized with Tris buffer (solute 0.9%, Tris 0.05 mg, Triton X-100 0.25%, and CaCl2 0.02 M) and centrifuged at 6000 rpm for 30 min. Tissue extract was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 10% polyacrylamide containing 0.1% gelatin under non-reducing conditions without prior boiling. After electrophoresis, the gel was washed in 1.5% Triton X-100 for 1 h and subsequently immersed in buffer containing Tris-HCl 50 mM (pH 7.5), 1% Triton X-100, CaCl2 10 mM, and 0.02% sodium azide for 16 h at 37°C. The gel was washed several times with distilled water and stained with 0.25% Coomassie brilliant blue R250/40% methanol/10% acetic acid and de stained in 7% acetic acid. Enzymatic activity was detected as clear bands of gelatin lysis against blue background.

Histological analysis

Granulated tissues were collected at every 4 days intervals and transferred to 10% neutral buffered formalin (NBF) for 24 hours 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 5-7μm sections were deparaffinized and stained with hematoxylin following counterstained with eosine [12] and also Masson’s Trichrome Staining for collagen synthesis and its morphology in the granulation tissue.

Statistical analysis

All results have been expressed as mean ± S.D and the results were compared statistically by student’s independent t- test using SPPS software (student version 7.01). The p value <0.05 was considered statistically significant.

RESULTS

In vitro antimicrobial activity showed the activity of plant extract against S. aureus and P. aeruginosa. The anti-microbial activity determined 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 plant extract is 3925±0.101 μg/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 plant extract is 39.25±0.0076 mg/ml in 34±0.5 mm (methicillin) 30±1.0 mm (ciprofloxacin).

From the above figure, the comparison between open wound group and treated group is commented. In 4th Day of Open wound group, there is no evidence of formation of epidermis and dermis and tissue was contaminated with neutrophils and bacterial colonies. In the case of 4th day treated group, the formation of dermis and epidermis was seen in the wound surface. Furthermore, there is an observance of reduction of neutrophils and bacterial colonies at the wound environment. It is due to anti-microbial potential of fruits of Terminalia bellirica. In the case of Masson’s Trichrome Staining of granulated tissue, No collagen synthesis in 4th day open wound group was seen and also presence of large amount of bacterial colonies and neutrophils at the wound surface. In addition to that, there is no evidence for formation of dermis and epidermis at the wound surface. In the case of 4th Day treated group, Bluish Violet color indicates the formation and synthesis of collagen at wound surface was observed. Moreover, it is seen that there is absence of bacterial colonies and neutrophils at the wound surface.

<table>
<thead>
<tr>
<th>Microorganisms</th>
<th>Terminalia bellirica extract</th>
<th>Std. Antibiotic</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. aureus</td>
<td>10±1.25 mm</td>
<td>34±0.5 mm (methicillin)</td>
</tr>
<tr>
<td>P. aeruginosa</td>
<td>12±1.58 mm</td>
<td>30±1.0 mm (ciprofloxacin)</td>
</tr>
</tbody>
</table>

Table 1: Zone of inhibition for standard strain microorganisms

<table>
<thead>
<tr>
<th>Microorganisms</th>
<th>Minimum concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. aureus</td>
<td>3.925±0.0076 mg/ml</td>
</tr>
<tr>
<td>P. aeruginosa</td>
<td>3.925±0.0078 mg/ml</td>
</tr>
<tr>
<td>Streptococcus pyogenes</td>
<td>15.25±0.0095 mg/ml</td>
</tr>
</tbody>
</table>

Table 2: Minimum Inhibitory concentration

Histological Studies:

Figure 1 shows the histology of control and treated rats at different days of analysis. Complete loss of superficial epithelium and inflammatory exudates were observed in both the groups on day 4. The clumps of bacterial pathogens were high in control when compared with treated rats. Incomplete epithelialization with less collagen synthesis was observed in control rats. The Clumps of degenerating neutrophils, necrotic changes and the persistence of inflammatory exudates in the upper dermis with loss of epidermis were also observed up to day 16. Treated rats have shown well marked epithelialization, increased cellular proliferation, and moderate amount of collagen synthesis in Masson Trichrome Staining and fibro vascular tissue formation.

<table>
<thead>
<tr>
<th>Day</th>
<th>Control Group</th>
<th>Treated</th>
</tr>
</thead>
<tbody>
<tr>
<td>4th Day</td>
<td>H&amp;E Staining</td>
<td>Masson’s Trichrome Staining.</td>
</tr>
<tr>
<td></td>
<td>Figure 1: Histological analysis of granulated tissue from wound environment (4th Day)</td>
<td></td>
</tr>
</tbody>
</table>

Figure 2: Comparative histological analysis of control and treated rats at different days of analysis.
In this figure (Fig. 3), well formed dermis and epidermis at wound surface in 12th day treated group was observed and additionally, there is a formation of blood vessel 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 (Fig. 4), partial formed dermis and epidermis were observed. The treated group shows complete regeneration of epidermis and dermis are formed in the wound site.

Figure 6 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.

Biochemical analysis (Fig. 5-8) 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. 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 treated group from day 4. Additionally, these data show a synthesis of GAG in the ECM matrix was increased.
The total bacterial count from the granulation tissue on different days of analysis is shown in figure 9. Application of plant extract based ointment resulted in a diminishing level of total bacterial count in the infected wound. There was major reduction from $10^7$ CFU to $10^4$ CFU in treated rats on day 4 when compared to control rats, which records $10^7$ CFU.

In contrast with infected normal wound healing processes, Due to presence of wound pathogens at wound site, a particular family of structurally related proteolytic enzymes namely matrix metalloproteinases (MMPs) abnormally elevated in the granulated tissue and retard the progression towards wound closure. Additionally, the presence of wound pathogens and its proteolytic enzymes caused elevation of MMPs in wound site and results in delayed in regeneration of wound closure. In the figure 10, enzymatic activity was detected as clear bands of gelatin lysis against blue background in gelatin zymography. The open wound group shows more lysis of gelatin against blue background in zymography than treated group and it confirms the fruits of Terminalia bellarica controls infection at wound site. As a result, the expression of MMPs in granulated tissue is decreased and maintained a normal level for tissue regeneration.

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

The above experiment showed the SDS PAGE analysis (Fig.11.) 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.

DISCUSSION

In wound management, Topical delivery of potent antimicrobial agents at the wound site are effective in both controlling the growth of wound pathogens and faster wound healing rate due to its larger availability at the infected wound site. The ability of wound pathogens in the wound bed to create a huge damage and infection depends on the virulence capacity of the pathogens, the amount of wound pathogens available at the wound site along with the immune response of the host, Staphylococcus aureus, Streptococcus pyogenes, and Pseudomonas aeruginosa are the most common wound pathogens with $\geq 10^7$ CFU/g tissue has been classified as infection\[1\][11]. In this investigation, A significant wound contraction was observed in rats treated by fruits of Terminalia bellarica. In vitro antimicrobial studies and in vivo short period of epithelialization in the treated rats confirms the effect of fruits of Terminalia bellarica in the infected wound healing. Significant reduction of bacterial wound pathogens in wound site of the treated rats was noted in the day 8 from $10^7$ CFU to $10^4$ CFU/g tissues, further confirms the potential of fruits of Terminalia bellarica treatment. Increased wound contraction in treated rats might be due to the enhanced activity of fibroblasts in the wound environment of the treated rats. Wound contraction is mediated by specialized myofibroblasts found in the granulated tissue\[12\][13]. The slow rate of wound contraction in control rats may be attributable to the presence of wound pathogens and their microbial enzymes such as collagenase and elastase, which inhibits wound contraction and impair healing.
Highly significant increase in the protein and collagen content confirmed the enhanced migration of fibroblast cells, epithelial cells and synthesis of various extracellular matrix including collagen during the healing process in treated rats. The decreased content in both protein and collagen in control rats may be due to the presence of wound pathogens, which can promote and extend the inflammatory phase of wound healing, inhibits both epithelial regeneration and proliferation of fibroblast leads to delayed healing. Hexosamine and uronic acid are the most important matrix molecules of extracellular matrix, which act as ground substrate for the development of newly extracellular matrix. The decrease in the hexosamine and uronic acid content was associated with concomitant increase in collagen content [14]. Uronic acid in the wound tissue attracts fibroblast and stimulates collagen synthesis by providing more fluid that facilitates greater cell mobility, early remodeling at wound environment and helps the wound to heal without scar formation [12].

Well-correlated biochemical studies were observed with histological examinations such as H&E staining and Masson’s Trichrome Staining. A close observation of granulation tissue sections revealed that the tissue regeneration was much faster in the treated group compared to open wounds. There was marked infiltration of inflammatory cells, increased blood vessel formation like angiogenesis and enhanced proliferation of fibroblasts as a result of this fruits treatment. Increased cellular infiltration was observed from H&E staining in treated rats compared with control may be due to chemotactic effect enhanced by the extract, which might have attracted inflammatory cells towards the wound site. Increased cellular proliferation may be due to the mitogenic activity of this fruits 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 towards cellular proliferation, granulation tissue formation and epithelialization. Moreover, Masson’s Trichrome staining confirms the formation of stretched collagen bundles in granulation tissue of treated group than that of open wound group. Gelatin zymography analysis shows the expression of pro and active forms of MMP in the granulated tissue (Fig. 10) at different days of healing (days 4, 8, 12, and 16). The treated group shows less amount of expression of MMPs in the granulated tissue due to control and eradication of wound pathogens at the wound site by the extract of these fruits. The SDS-Page analysis of Type 1 Collagen isolated from the granulated tissue confirms that the treated group contains newly synthesized collagens in the granulation tissue than the open wound group [12][11][15][16].

CONCLUSION

The Ointment formulation from the extract of fruits of *Terminalia bellarica* exhibited significant prohealing activity in the infected wound when topically applied on rats by modulating various stages of healing process. The bioactive molecules present in the fruits of *Terminalia bellarica* are 20-40% Tannins, anthraquinones, volatile oils, and few carbohydrates. Additionally, the tannins present in these fruits might be responsible for numerous pharmacological activities. Apart from this, other properties such as antioxidant, antiinflammatory, hypoglycemic, hepatoprotective, anti-bacterial, hypo-cholesterolaemic and anti-diabetic activities make it as a potential herbal drug. The result of the present investigation confirms the pharmacological evidence on the potential use of fruits of *Terminalia bellarica* for the healing of infected open dermal wounds.

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REFERENCES


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