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Research Article

ACTIVITIES OF TRIPHALA TOWARDS PROMOTING COLLAGEN SYNTHESIS AT WOUND SITE AND INHIBITING METHICILLIN-RESISTANT *STAPHYLOCOCCUS AUREUS* AND ITS ENZYMES

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ABSTRACT

Problem Statement: Infection is a major problem in the management of wounds. Despite the use of synthetic antimicrobial agents, drug resistance and toxicity hinder the activity of these antimicrobial agents; thereby increase the chances of infection. The microbial enzymes produced by wound pathogens degrade the extra cellular matrix at wound site. The present study investigates the influence of Triphala on changes in collagen characteristics during the healing process of an infected cutaneous wound in Albino Wister rats and also how the antimicrobial activity and enzyme inhibition activity of triphala used in wound healing studies.

Approach: A methanol extract of triphala was prepared and its antimicrobial activity was tested against twenty clinical MRSA strains. The activity of triphala extract against serine protease and metalloprotease was studied by Zymography. Enzymatic activities were detected as clear bands of casein/gelatin lysis against a dark background. To measure the relative enzyme levels, clear zones were scanned and the percentage of inhibition was analyzed by Gel Documentation systems. The inhibition of enzymes by Triphala has been expressed in percentage and expressed as mean ± SD of ten experiments. Male Wister albino rats of weights ranging between 150g and 200g were used for *in vivo* wound healing study. Granulated tissues were collected on the 4th, 8th, 12th and 16th days for the estimation of different types of collagen present in the granulated tissue and also for histological studies.

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

Result: The 18±2mm clear zone in disc diffusion assay and minimal inhibitory concentration (MIC) of 7.8125mg/ml against MRSA (as well as methicilin susceptible *Staphylococcus aureus*) control strains clearly showed the antibacterial activity of Triphala. Zymography analysis exhibited greater reduction in serine protease and metalloprotease activity at ≥1500µg/ml. The wound tissues that were removed on the 4th, 8th, 12th and 16th day (post-wound), were used to analyze the biochemical and pathological changes on the injured tissue. Triphala increased cellular proliferation and collagen synthesis at the wound site, as evidenced by increase in type III collagen content of wound tissues. The Masson's Trichrome staining of granulated tissue confirmed that the treated subset of tissues had well-formed epithilization with well stretched bundles of collagen when compared to that of an open wound group (untreated). Better maturation and cross linking of collagen was observed in those rats which were treated with Triphala.

Conclusion: By virtue of the inhibitory effect of Triphalaon different MRSA strains and their enzymes such as serine protease and metalloprotease, it could be, potentially used as a new therapeutic agent for MRSA infected dermal wounds. The results hence highlighted the beneficial effects of the topical application of Triphala in the acceleration of wound healing and its effect on collagen synthesis.

Keywords: Triphala, Collagen, Wound Healing, Infection, *Staphylococcus aureus,* MRSA, serine protease, Metalloprotease.

INTRODUCTION

The process of dermal wound healing is a complex and carefully orchestrated cascade of overlapping events that include phagocytosis, chemotaxis, mutagenesis and the synthesis of an extra cellular matrix component(Gurtner, Geoffrey C et al, 2008). Extra cellular matrix (ECM) is the scaffold that supports cell fate process in both unwounded and wounded states. Its synthesis, a dynamic process, constantly undergoes remodeling during dermal wound healing (Hodde J. P et al, 2007). The infection at the wound site is an imbalance between the bacterial growth and resistance offered by the host.

Wound pathogens which secrete proteases namely collagenase, elastase that degrade collagen, elastin etc., cause slow synthesis of ECM and a delayed deposition of extra cellular matrix proteins at the wound site (S.Kirubanandan,2006). The healing of infected wound results in the formation of a scar, which is morphologically defined as the lack of tissue organization compared to the surrounding normal tissue architecture. Hence the wound site has disorganized collagen deposition. In normal wound healing, new collagen fibers secreted by fibroblasts are present, as early as three days after wounding. The ultimate pattern of collagen in the scar is one of the densely packed fibers and not the reticular patterns found in unwounded dermis. Wounds gradually become stronger with time during remodeling. The tensile strength of the wound increases rapidly during the first eight weeks post wounding. Furthermore, the cross linking of collagen and remodeling of connective tissue results in acquisition of wound strength. Therefore, the synthesis, secretion and subsequent organization of this triple helical protein in the wound granulation tissue are of great significance in the healing process. The regeneration of soft tissue using phytopharmaceuticals appears to be promising in the field of medicine. The various secondary metabolites present in the phytopharmaceuticals assist in the organized manner of wound healing coupled with triggering of the formation of collagen at the wound site. (Venkatanarayana D et al, 2010).

Staphylococcus aureus is one of the most common bacteria isolated from skin infections, surgical wounds etc (Sajna AM et al, 1999). The potential virulence factors include toxins, adhesions and exo-enzymes of this organism help it to colonize on host tissues which in turn lead to serious infections (Arvidson S,2000).*S. aureus* produces a wide array of extra cellular enzymes which are important for pathogenesis and nutritional purposes (Goguen JD et al,1995). The staphylococcal proteases enhance the pathogenesis by a variety of mechanisms such as inactivation of antimicrobial peptides, cleavage of human immunoglobin molecules, and extracellular matrix destruction (Selsted ME et al, 1996,Boyce JM,1971).

The prevalence of methicillin-resistant *Staphylococcus aureus* (MRSA) has been increased in recent times- it was found that more

than 50% of clinical strains in certain parts of the world were MRSA type and it constitutes a major problem. (Hiramatsu K et al, 2001, Espersen F et al, 1998). Recent statistics from Center for Disease Control, Atlanta, USA(Centre for Disease Control and Prevention, 2002) suggest, MRSA has become the most frequent cause of skin and soft tissue infections leads to emergency departments and MRSA related mortality surpasses AIDS annually. During the past decade, there has been a dramatic increase in the proportion of *S. aureus* isolates that are resistant to methicillin and other commonly used antibiotics. The resistant strains are repeatedly multi-resistant, often to erythromycin and tetracycline, with resistance to quinolone developing rapidly (Shovein, J et al,1993). Surgical patients are at high risk for developing postoperative wound infections with MRSA due to prevalence of this organism in the hospital environment and the mechanism of resistance by the synthesis of penicillin-binding protein (PBP2), which was isolated in the early 1960s (Tsuchiya H et al,1996,Hiramatsu K et al,1997). Vancomycin and other glycopeptides are the drugs of choice for the treatment of infections due to MRSA. But in Late 1997, Hiramatsu et al, 1997 isolated the first clinical *S. aureus* with intermediate resistance to vancomycin and became completely resistant to vancomycin. (Hiramatsu K et al,1997). Therefore, the search for novel bactericidal compounds from medicinal plant and determination of their mode of action of phytochemical are the main objects in the current scientific investigation.

Rios et al., (2005)extensively analyzed the past, present and future perspective of the antimicrobial potential of medicinal plants. Triphala, a polyherbal formulation, consists of dried and powdered fruits of three plants, *Terminalia chebula*, *Emblica officinalis* and *Terminalia bellerica* in equal proportions. *Terminalia chebula* and *Terminalia bellerica* are rich in polyphenol and tannins, and *Emblica officinalis* is rich in ascorbic acid. The formulation triphala shows a synergistic action against microbial pathogens. It is a significant medicine of the 'rasayana' group and is believed to promote health, immunity and longevity (Hans Wohlmuth, 2008). In order to search for new antimicrobial botanicals that can be used for the treatment of MRSA infections, this paper studies the possible inhibition of MRSA growth and their enzyme activity by Triphala.

Triphala and/or its constituent plants have been reported to possess numerous biological and pharmacological activities such as antioxidant, antibacterial, antifungal, antiviral, ant malarial, anti-mutagenic, anticancer, radio protective, anti-allergic, cardio tonic, hypocholesterolaemic, capillary strengthening and hepatoprotective. The wound healing potential of triphala and its influence towards collagen synthesis at wound site in male albino Wister rats has been attempted in this present investigation using Wister albino rat model where the infection is induced in open dermal wound using pathogenic organisms such as *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and beta-haemolytic streptococci.

MATERIALS AND METHODS

Isolation and identification of MRSA

Twenty-five strains of *staphylococci* isolated from the wound infection of outpatients in local hospitals, Chennai, India employed in this study. The identification of staphylococcal clinical isolates was conducted according to colony morphology, Gram staining, coagulase positivity and other biochemical tests. The strains were stored as glycerol stock at -20°C. The resistance of isolated strains to methicillin was evaluated by disc diffusion testing according to the guidelines of the National Committee for Clinical Laboratory Standards 2000, using discs containing 10 mg of methicillin and twenty strains were found to be resistant to methicillin.

Culture media and growth

The isolated MRSA strains were incubated in Soyabean Casein Digest Broth (Hi-Media Pvt.Ltd., Mumbai, India) for overnight at 37°C and adjusted to yield approximately 1.0×10^5 CFU/mL. Standard methicillin-sensitive *S. aureus* ATCC 29213 was used as control.

Preparation of alcohol extract of Triphala

100 g of 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 12.5%. 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.

Determination of antibacterial activity

The minimal inhibitory concentration (MIC) of the extract was determined by the broth tube dilution method [18].The antibacterial sensitivity test was performed by disc diffusion method. Sterile blank discs (6 mm diameter) were impregnated with minimum inhibition concentrations of triphala extract against Methicillin-resistant *Staphylococcus aureus* (MRSA). Extract impregnated discs were placed in Muller-Hinton agar plates inoculated with the MRSA strains as well as control strain and incubated at 37°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.

Enzyme inhibition assay

The overnight Methicillin-resistant *Staphylococcus aureus* cultures were transferred in to sterile 50 mL conical tubes and centrifuged at 5000 rpm for 5 minutes. To the culture supernatant, solid ammonium sulfate was added slowly with stirring to achieve 80% saturation. The resulting precipitate was collected by centrifugation and dissolved in 0.02 M phosphate buffer, pH 6.8. From this, 1mL was transferred in to sterile vials and incubated with different concentration of triphala extract (100µg-2000µg/mL) for overnight at 37°C. 1mL of 10% DMSO was used as control. The activity of triphala extract against the serine protease and metalloprotease was studied by Zymography. Enzymatic activities were detected as clear bands of casein/gelatin lysis against dark background. To measure the relative enzyme levels, clear zones were scanned and the percentage of inhibition was analyzed by Biovis Gel Documentation systems. The inhibition of enzymes by Triphala have been expressed in percentage and expressed as mean ± SD of 10 experiments.

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). On the whole, 48 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.

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 triphala. (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 deparaffinized and Masson's Trichrome Staining was performed for the detection of collagen deposits and its morphology, in the granulation tissue.

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 (Woessner J. F. Jr,1961,Neuman RE et al,1950).

Solubility pattern of tissue collagen

The solubility pattern of tissue collagen was determined as described by Miller and Rhodes, 1982.

Extraction of Neutral salt soluble collagen

Granulation tissue was minced well, homogenized in 10 vol of neutral salt solvent (1.0M NaCl, 0.05 M Tris, pH 7.5) containing 20mM EDTA and 2.0 mM N-ethyl maleimide and stirred for 24hr. The suspension was then centrifuged at 35,000g for 1hr at 4°C and the extraction was repeated with the pellet .The supernatants were pooled and an assay of hyroxyproline was done from an aliquot.

Extraction of acid soluble collagen

The residue obtained was resuspended in 10 vol of 0.5 M acetic acid and extracted for 24 hrs and was centrifuged .The pellet was extracted with acetic acid, supernatants were pooled and an aliquot was used for the determination of hydroxyproline. A part of the remaining pellet was used for the study of susceptibility to denaturing agents.

Extraction of Pepsin soluble collagen

The residue obtained after acid extraction was resuspended in 0.5M acetic acid containing 100mg pepsin per g of wet tissue. Digestion was carried out for 24 hr followed by centrifugation and re-extraction. Aliquots of pooled supernatant were used for hydroxyproline measurement and aldehyde content in the collagen.

Insoluble collagen

The residue after pepsin digestion was referred to as insoluble collagen. It was hydrolyzed in 6.0 N HCl and assayed for hydroxyproline content.

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.(Sykes B et al,1976,Clore J. N et al,1979)

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

Determination of antibacterial activity

The MIC of triphala extract against MRSA and methilicin susceptible strains was found as 7.8125± 0.0085mg/ml. The MBC of Triphala extract was ≥7.81 mg/mL for MRSA S.aureus. All bacterial strains showed susceptibility to triphala when tested using the disc diffusion method. All strains including control strain exhibit clear zone of inhibition (18±2mm) to the methanol extract of triphala whereas isolated strains and the control strain showed ≤9mm, 20±2mm of clear zone to methicillin respectively (Figure 1). No zone of inhibition was observed in DMSO treated disc.

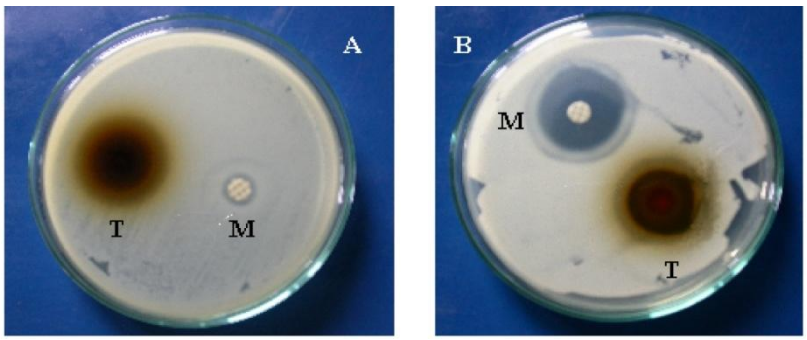


Fig. 1: Agar diffusion test shows the zone of inhibition by triphala (T) and methicillin (M). A – MRSA Clinical isolate, B – *Staphylococcus aureus* ATCC 29213.

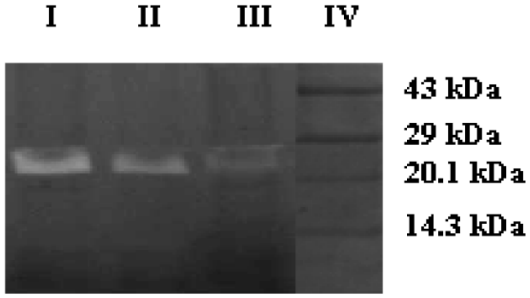


Fig. 2: Casein zymography shows the inhibition of serine proteases from MRSA cultures at different concentrations of triphala extracts. Lane I – Control, Lane II – 1000μg/mL, Lane III – 1500μg/mL and Lane IV – Marker.

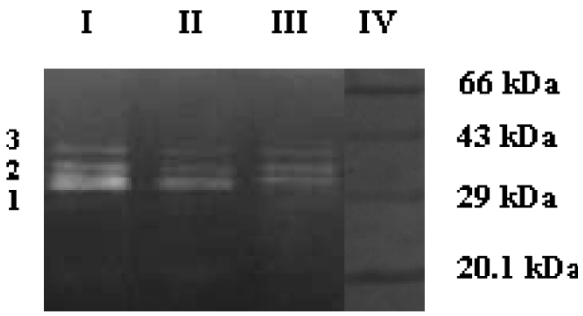


Fig. 3: Gelatin zymography shows the inhibition of metalloproteases from MRSA cultures (1, 2 & 3) at different concentrations of triphala extracts. Lane I – Control, Lane II – 1000µg/mL, Lane III – 1500µg/mL and Lane IV – Marker.

Enzyme inhibition assay

The reduction in the Staphylococcal serine protease and metalloproteases activity was detected as unstained bands on the gels by zymography technique. The band pattern showed 20-29 kDa serine protease in casein zymogram and three distinguished 30-40 kDa metalloproteases in gelatin zymogram. Significant differences were found on serine protease and metalloproteases activity in the triphala treated group compared with control, which shows the inhibitory activity of triphala extract against these enzymes (Figure 2 & 3).

The densitometry analysis revealed the greater enzyme inhibitory activity at the concentration ≥1500µg/mL. The percentage of enzyme inhibition at different concentrations of triphala is given in table 1.

Table 1: Percentage of enzyme inhibition at different concentrations of Triphala

Concentration of Triphala (µg/mL)	Percentage of inhibition			
	Serine protease	Metalloproteases		
		1	2	3
1000	48.21±3.26	44.02±1.96	39.32±2.12	37.32±2.46
1500	75.44±2.87*	84.64±2.06*	68.48±1.86*	64.52±2.60*

Data has shown as mean ± SD; * Significant difference at $p<0.05$.

Table 1: Total Collagen Content

Group	4th Day	8th Day	12th Day	16th Day
Open Wound	1.23± 0.08	3.36 ± 0.12	3.97± 0.15	6.64 ± 0.24
Triphala Ointment	3.1±0.2	4.86 ± 0.18*	5.79 ± 0.19*	8.6 ± 0.28*

Table 2: Solubility Patterns of Different Collagen

Parameters	Types of collagen in the granulated tissue			
	Neutral Soluble collagen	Acid Soluble collagen	Pepsin Soluble collagen	Insoluble collagen
Control Groups	308.19 ± 20.61	2169.44 ± 141.11	3510.98 ± 125.01	54.39 ± 7.32
Treated Groups	222.91 ± 11.11*	3625.19 ± 236.36*	7731.39 ± 245.72*	152.52 ± 25.33*

Table 2 and 3 confirm that the total collagen content in granulation tissue and various types of collagen is increased in Groups treated by triphala ointment.

Table 3: Aldehyde content in pepsin soluble collagen

Aldehyde Content in the pepsin Soluble Collagen	Nanomoles of malondialdehyde		
	8th Day	12th Day	16th Day
Open Wound Group	605±23	768±10	1053±08
Triphala Oinment treated group	2286±12	2756±12	2926±12

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 triphala ointment has higher aldehyde content than in the case of open wound group.

Treated Group Control Group

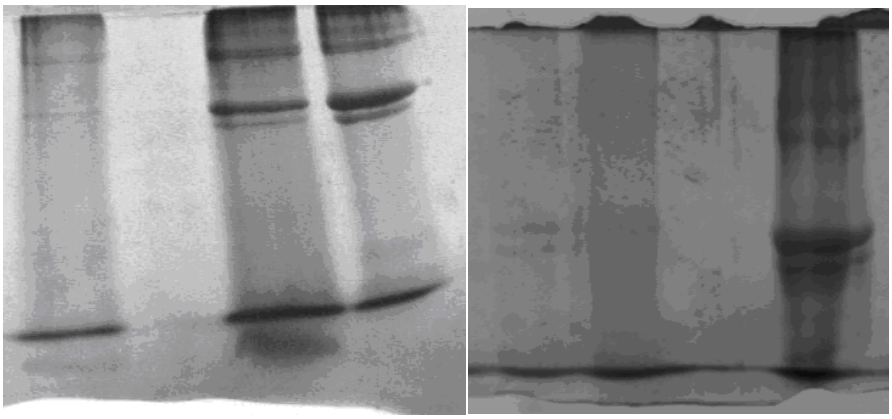


Fig. 4: SDS PAGE Analysis of Collagen

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 has been proved by bands in SDS PAGE.

Open Wound Group:

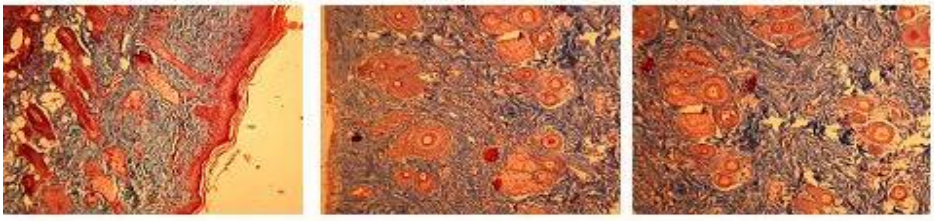
4th Day

In the 4th day of granulated tissue, Neutrophils found on the granulated tissue and No collagen synthesis have seen. Generally, The enzymes of wound pathogens degrade the extra cellar matrix at the wound site and inhibit the collagen synthesis.

8th day

No epithilization formed and slightly Collagen formation has seen. The bluish violet shows the deposition of collagen in the granulated tissue at the wound site.

12th day



Epidermis and Dermis have seen in the granulated tissue. Loose bundles of collagen have seen.

16th Day

The loose collagenous matrix was seen in the granulated tissue. The formation of epidermis and dermis was good at the wound site.

Fig. 5: Masson's Trichrome Staining of Granulated Tissue

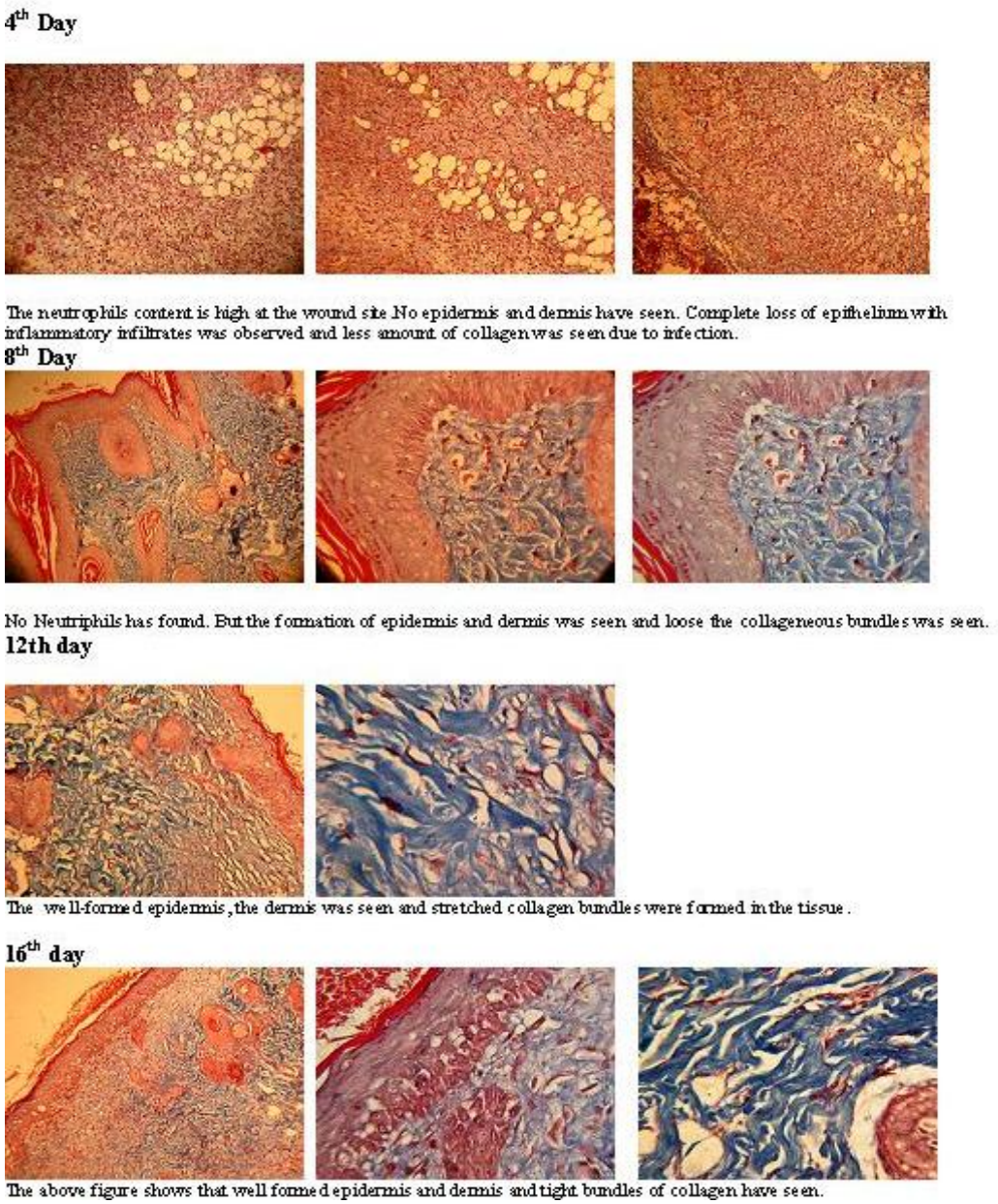


Fig. 6: Treated Group

Histological analysis of Granulation tissue by Masson's Trichrome staining

On the 4th and 8th day in the open wound group**,** (Fig 4) neutrophils and bacterial colonies were found in the injured area. Complete loss of epithelium with inflammatory infiltrates was observed and less amount of collagen was seen due to infection. On the 12th day and 16th day (Fig 4), epidermis was formed partially and a loose collagen fiber was observed.

On the 4th day in the triphala ointment treated group**,** (Fig 5), neutrophils and bacterial colonies were found in the injured area. Complete loss of epithelium with inflammatory infiltrates was observed and less amount of collagen was seen due to infection. On 8th day, (Fig 5), epidermis was formed partially and stretched bundles of collagen fibers were observed in the dermis. On the 12th, day (Fig 5) the sample showed a well-formed epidermis, with the dermis visible and stretched collagen bundles forming in the tissue. In Masson's Trichrome stained histological sections of treated on 12th and 16th days by triphala ointment, bluish violet colored regions were observed, indicating staining of well stretched and deposited collagen bundles formed in the tissue. In the case of open woundat the end of 12th and 16th days, the loose collagenous matrix was seen.

DISCUSSION

From 1940s, an increase in the proportion of beta lactamase enzyme producing MRSA isolates was found and this proportion has subsequently grown to about 80–90% (Barber M et al,1948,Henwood CJ, et al,2000). Now MRSA has spread to nearly every continent, reaching near epidemic proportions in some countries and becoming one of the most common pathogens in hospitals worldwide (Aires de Sousa M et al,2004). Numerous studies have reported that the compounds from botanical origin are effective antimicrobial agents (Basile A et al,2000,Cowan MM,1999). Some phytochemicals have been screened against antibiotic-resistant strains of bacteria (Kone WM et al,2004,Sato Y et al 2000). Inhibitory activity of triphala extract against the growth of MRSA in this study highlights the potential use of triphala against *S. aureus* and provides some scientific rationale for its use as antimicrobial agents on infected dermal wounds.

As an important observation in our study, we have found both methicillin resistant and sensitive *S. aureus* were inhibited at the same concentration of triphala extract. This exhibits that the mechanism of methicillin resistant has not affected the activity of triphala. Moreover, triphala's antimicrobial activity against MRSA seems to be mediated through mechanisms other than that are used by methicillin, which needs to be investigated. Although the triphala has rich in polyphenols and ascorbic acid .The poly phenols might be the responsible for antimicrobial action. In our previous studies, we showed that the triphala contains the presence of EGCG (epigallocatechin gallate) as one of the condensed tannins (Kumar M. S et al,2008). The literature proved that Epigallocatechin gallate has the specific mechanism for antimicrobial activity. More amounts of the epigallocatechin gallate binds to*S. aureus* than that of gram negative bacterium *E.coli* and sensitivity of EGC treated *S. aureus* to high ionic strength was more than towards and low osmotic pressure. The epigallocatechin gallate binds to the pepidodoglycan layer in the cell wall of *S.aureus*. Peptidoglycan is a cross-linked complex formed from various polysaccharides and peptides. Thirty to fifty layers of peptidoglycan forma part of the cell wall of *S.aureus* offering osmotic protection, assisting in cell division and also act as a primer for further biosynthesis of peptidoglycan. EGCg can directly bind to peptidoglycan and induces its precipitation and subsequent damage. Biosynthesis is also halted due to direct binding. This method of cell wall damage and interference with its biosynthesis through direct binding with peptidoglycan are the major reasons for the susceptibility of *Staphylococcus* to EGCg. The above reason might be for responsible for antimicrobial action for triphala. (Yoda, Y et al,2004,Zhao, W. H. et al,2002,Zhao, W. H. et al,2001)

S. aureus serine protease degrades cell surfacefibronectin-binding protein and alimited number of othercell surface adhesions proteins and ligands, which can accelerates thetransition to the invasivephase of infection (McGavin MJ et al,1997). Protease is also essential for activation of a precursor form of the serine protease (Drapeau GR et al,1978).

Furthermore, these staphylococcal exoproteases have beenshown to actively degrade human protease inhibitors, includingα_1-proteinase inhibitor (Potempa J et al,1986,Rapala-Kozik M et al ,1999).The Imbalance between human protease inhibitor and proteases leads to abnormal extra cellular matrix metabolism ultimately resulting into tissue destruction. The reduction in the activity of staphylococcal serine protease and metalloprotease by the triphala extract gives additional support to its potential use as a therapeutic agent against *S. aureus*. Moreover, Inhibitory activity of triphala against human polymorphoneuclear neutrophil collagenase (Matrix metalloproteinase 9) as demonstrated earlier vouch for its use as an effective remedy for *S. aureus* infection. (Sajith Abraham et al,2005)

Triphala has been reported to possess a number of medicinal properties like anti-inflammatory, anti-bacterial, anti-fungal, anti-viral, anti-malarial, anti-mutagenic, radioprotective, anti-allergic, anti-cancer, cardiotonic, hypocholesterolaemic, capillary strengthening, hepatoprotective, immunomodulatory, adaptogenic, analgesic and anti-oxidant activity. In the case of our preliminary studies of triphala, it can be used as potential drug for the treatment of infected wound. Also, the crude extract of each plant posses the lack of cellular toxicity on sheep erythrocyte up to 200mg/mL gives enrichment to our findings. Although Sato et al,1997 reported Gallic acid and ethyl gallate (Polyphenols) in *T. chebula* Retz which was one of the components of Triphala and have shown antibacterial activity of ethanol extracts of this plant against both methicillin resistant and sensitive *Staphylococcus aureus* and other bacteria, the components of *T. chebula* Retz aqueous extracts responsible for the observed bactericidal activity remain unknown (Sato Y et al,1997). Isolation of active constituents, mode of action and in vivo studies in our future analysis make triphala as a potential therapeutic agent. In addition to its antibacterial activity, Triphala's inhibitory activity towards the proteases and collagenase, it has shown to be an instrumental medication in promoting synthesis of collagen, by preventing degradation by the enzymes.

In soft tissue repair process, instantaneously after an injury, there is an increased synthesis of extra cellar matrix components in the wound area. Generally, an infected wound delays the formation of extra cellular matrix in the wound area, and additionally, pathogenic enzymes such as collagenase, elastase secreted by wound pathogens cause extra cellular matrix degradation and triggers 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.

The role of collagen in the healing process starts as soon as the injury is inflicted and continues for many weeks. Though the major function of collagen is to provide strength and integrity to the wound, it also plays a role in other processes such as homeostasis, re- epithilization, and cell –cell and cell – matrix interactions. The degradative products of collagen also assist in the healing process, in that they are chemotactic. Hence, the levels of collagen and its specific types present in a wound, contribute to the healing process. The present investigation showed that treatment of wounds bytopical application of triphala ointmentresulted in increased collagen contents of granulated tissue when compared to the untreated controls. Table 2 describes that the total collagen content in the granulation tissue was increased in treated groups. In addition to that, The various types of collagen also increased in treated group.

The active constituents present in triphala might attribute this increase to increased stimulation and these compounds either directly enhance collagen synthesis or cause increased proliferation of fibroblasts thereby assisting in synthesizing collagen, or both. The most active ingredients in triphala are ascorbic acid (Vitamin C) and Poly phenols such as ECCG (Epigallocatechin gallate). The Ascorbic acid in triphala enhances collagen synthesis at wound site. Collagen contains unique amino acids (hydroxyproline and hydroxyl sine) which are necessary for the stability of the collagen protein and for its complete maturation. Ascorbic acid has been 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 (Kivirikko K. I., et al,1984). In the absence of vitamin C, under-hydroxylated procollagen molecules are not retained within cells , and are less stable and more temperature-sensitive (Berg, R.A. et al,1973b). 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. The significant improvement in the quality of scar formed based on its maturity and orientation of the collagen fibers is brought about by the catechins and other simple polyphenols present in the triphala. In other words, these molecules improve the orientation of collagen fibers at the wound site.

After synthesis 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(Adam M et al,1968,Siegel R. C.,1976,Miller EJ et al,1992). The present work shows that the collagen obtained from Triphala 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.

As cross-linking continues, the solubility of collagen in neutral buffer and acid solution also changes. Highly cross-linked collagen becomes less soluble in the above solutions and can be released only by limited pepsin digestion. From the solubility patterns obtained for collagen of granulation tissues of triphala treated and untreated control rats (Table 2), it could be seen that Triphala treatment resulted in decreased percentage solubility in neutral buffer and in

dilute acid solution. A significantly higher amount was solubilised only by pepsin digestion, and this is an indication of increased levels of cross-linking in treated groups. The insoluble collagen content of treated groups is also greater than that of the untreated control group. The aforementioned formation of cross-links and development of tensile strength is mainly contributed by type I collagen in the wound matrix. The early type III collagen has, in the healing process, important functions such as establishing initial wound structure, guiding inflammatory cells and fibroblasts into the wound site, providing a matrix for the re-establishment of blood supply etc. It also helps in regulating collagen fiber diameter and organization (Tanzer M. L et al,1976,Nakagawa S, et al 1989). Triphala treated wounds synthesize greater amounts of type III collagen when compared to controls, as can be seen from Fig. 4. The presence of higher levels of type III collagen may have beneficial effects on early wound healing process and result in better organization of type I collagen in the final scar.

The Masson's Trichrome staining of granulated tissue proved that well starched and tight collagen bundles formed in the granulated tissue from treated group by triphala ointment and also, well epithelisation and dermis formed in the treated group proves the effective regeneration of infected dermal wound by triphala ointment.

CONCLUSION

Dermal Wound healing consists of a methodical progression of events that re-establishes the integrity of an injured tissue. Usually, infection is a major problem in the management of wounds. Despite the use of synthetic antimicrobial agents, drug resistance and toxicity hinder the activity of these antimicrobial agents, thereby increasing the chances for infection. Methicillin-resistant *Staphylococcus aureus* (MRSA) is the most common pathogenic bacteria responsible for hospital acquired infections. MRSA has almost spread to every continent, reaching near epidemic proportions in some countries. Staphylococcal proteases and its metalloproteases facilitate MRSA to colonize host tissues and play an important role in causing extra cellular matrix destruction. Active ingredients from various medicinal plants have been screened for antimicrobial activity against pathogenic bacteria including MRSA. In the present work, the antimicrobial activity of Triphala, an Indian ayurvedic drug, against MRSA and their pathogenic enzymes was investigated. By virtue of its inhibitory effect on different MRSA strains and their enzymes such as serine protease and metalloprotease, Triphala could be, potentially used as a new therapeutic agent for MRSA infected dermal wounds. However, the antibiotics formulation merely eradicates the microbial growth and has no role in the synthesis of collagen. Collagen is the predominant protein of the extra cellular matrix in the skin, and is the component which ultimately contributes to wound strength. Recent researches show that the natural products from medicinal plants strongly enhance the wound healing process. Triphala increased cellular proliferation and collagen synthesis at the wound site, as evidenced by increase in type III collagen content of wound tissues. The Masson's Trichrome staining of granulated tissue confirmed that the treated subset of tissues had well-formed epithilization with well stretched bundles of collagen than that of open wound group (untreated). Better maturation and cross linking of collagen were observed in the Triphala treated rats. The present investigation concludes that, the topical application of triphala ointment on an infected wound not only effectively heal the wound but also promotes collagen synthesis at the wound site. In regard to antimicrobial activity, this activity of Polyphenols present in the triphala might be exerted by direct binding to peptide structure of bacterial components, and enzymes. By virtue of its inhibitory effect on different MRSA strains and their enzymes such as serine protease and metalloprotease, Triphala could be potentially used as a new therapeutic agent for MRSA infected dermal wounds.

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