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Evaluation of antimicrobial potential of aqueous and alcoholic extract of Triphala against wound pathogens

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Abstract
The healing of an infected wound is still challenging for regeneration of dermis and epidermis in wound site due to the presence of bacterial pathogens and its metabolites such as collagenase and elastase. These metabolites degrade numerous extracellular matrix proteins such as collagen and elastin at wound site. As a result, the wound closure is delayed. Having existing antibiotic therapy for wound healing past decades, it has many disadvantages such as absence of synergistic activity, cyto-toxicity and then development of antibiotic resistance. Therefore, Phyto-pharmaceutical is the best source for potential antimicrobial agents to eradicate the wound pathogens for faster wound healing. Triphala is a kind of poly herbal Ayurvedic formulation and used for evaluation for antimicrobial action against wound pathogens. The aqueous and methanol extract of Triphala is prepared in Soxhlet extraction apparatus. The dried extract was dissolved in 10% Dimethyl Sulfoxide (DMSO) and used to assay the antibacterial potential of these extracts against wound pathogens by minimum inhibitory concentration (MIC) and disc diffusion assay. This investigation showed susceptibility of wound pathogens including clinical isolated from wound environment to the aqueous and alcoholic extract of Triphala. The disc diffusion assay confirmed clear zone of inhibition for *S. aureus* (19±1.5 mm) *P. aeruginosa* (20±1.0 mm) and *S. pyogenes* (15±1.5 mm) in the case of Alcoholic extract. The aqueous extract showed zone of inhibition around ~12 -14 mm for all bacteria. The MIC of Triphala alcoholic extract against *S. aureus* as well as *P. aeruginosa* was found between 3.91–7.81 mg/ml and against *S. pyrogenes* between 15.6–31.25 mg/ml. The alcoholic extract of Triphala has potent antimicrobial activity against wound pathogens and hence prevents wound infection at the wound site. Additionally, this investigation helps to prepare ointment formulation of Triphala for various wound healing including pressure sores and diabetic ulcers.

Keywords: Aqueous and Methanol Extract, Disc diffusion assay, Minimum Inhibitory Concentration and Wound pathogens.

Introduction
Many crude plant extracts from medicinal plants are the best source of valuable antimicrobial agents to cure numerous alignments such as urinary tract infections, cervicitis vaginitis, gastrointestinal disorders, respiratory diseases, cutaneous affections, helmintic infections, parasitic protozoan diseases and also Inflammatory processes. Even though antibiotics therapy is extreme effective, antibiotics are able to induce resistance against the pathogens. Phyto-chemicals and Phyto-pharmaceuticals has many advantages such as synergistic activity, potent anti-microbial agents and meaningful multiple pharmacological activities than synthetic antibiotics. Even though antibiotics were effective against numerous bacterial pathogens including Gram-positive and Gram-negative bacteria, development of resistance against these antibiotics is a great problem now days. Therefore, there are a worldwide movement towards the use of plant based medicines due to the concern over the more invasive, expensive and potentially toxic mainstream practices. Many literature confirmed that the identified and isolated phyto chemical from numerous herbal plants have potential antimicrobial action than the existing antibiotics. Recently, many wound pathogens are developed resistant against a variety of antibiotics especially in the case of MRSA becomes now VRSA. Due to presence of antibiotic resistant wound pathogens at wound site, the regeneration of dermis and epidermis is delayed. Therefore, the phyto pharmaceutical based antimicrobial agents are needed for eradication of bacterial contamination in the wound environment. In this investigation, Triphala is chosen for evaluation of its antimicrobial potential against wound pathogens. It is a traditional alternative medicine herbal formulation, consisting equal parts of three medicinal

plants namely *T. chebula* (Family: Combretaaceae), *T. belerica* (Family: Combretaaceae) and *E. officinalis* (Euphorbiaceae). The active constituents are mostly tannins and Vitamin C. Triphala has been used extensively as a drug against different diseases. Formulations of Triphala is claimed to have anti-viral and anti-bacterial effect. Triphala is prescribed for anti-caries agent, myocardial injury, cancer etc. It is the 'safest and most strengthening'. It is also used as a blood purifier that can improve the mental faculties and is possesses anti-inflammatory, analgesic anti-arthritic, hypoglycemic and anti-aging properties. Although Triphala has numerous pharmacological activities, but the lack of antimicrobial potential against wound pathogens especially MRSA clinically isolated from wounds. This paper provides the preliminary investigation of antimicrobial potential of aqueous and alcoholic extract of Triphala against wound pathogens including clinical isolates.

Materials and Methods

Preparation of Aqueous and Methanol extract of Triphala

The methanol extract was prepared by 100 g of Triphala powder (IMPCOPS Ltd., Chennai, India) in 500 mL of methanol by Soxhelt extraction apparatus and centrifuged at room temperature. The aqueous extract of Triphala was prepared by same protocol. In both case, the supernatant was collected and evaporated to dryness under reduced pressure in a rotary evaporator. The yield of the methanol extract was 12.5%. The concentrated extract was aliquot in amber-coloured bottles and kept in desiccators for further use. The dried extract was dissolved in 10% Dimethyl Sulfoxide (DMSO) and used to assay the antibacterial activity against wound pathogens and also clinical isolates of *S.aureus* from wounds in the patients.

Microorganisms tested

The bacterial strains such as *Staphylococcus aureus* ATCC 29213, *Pseudomonas aeruginosa* ATCC 27853 were collected from the King Institute, Chennai, India. Additionally, the test is performed against Methilicin resistant and sensitive *Staphylococcus aureus* clinically isolated from wounds.

Culture media and inoculums

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 10^5 CFU/ ml.

Determination of antibacterial assay

The antibacterial sensitivity test was performed by disc diffusion method. Microbes 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 Triphala extract were placed in Muller-Hinton agar plates inoculated with the test strains and incubated at 37 ºC for 24-48 hrs. Standard methicillin disc (5mcg) 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 100mg/mL to lower dilution in a series of test tubes. Each tube was inoculated with bacterial suspensions and incubated at 37 ºC 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 h incubation at 37°C, the MBC was regarded as the lowest concentration of the plant extract that allowed less than 0.1% of the original inoculum to grow on the surface of the medium. In each experiment extract was tested in triplicate.

Results

The antimicrobial potential of aqueous and methanol extract of Triphala against standard strains and wound pathogens that clinically isolated from wound site were studied. The antimicrobial potential of aqueous extract was found to be 83.33% with respect to the antimicrobial performance of methanol extract (100%).

Table 1: Antimicrobial activity of Triphala against Standard Strain

Micro organisms	Zone of Inhibition (Water extract)	Zone of Inhibition (Alcoholic extract)
E.coli	15±0.667 mm	18±0.894 mm
S.aureus	13±1.032 mm	15± 0.9486 mm
P. aeruginosa	12±0.8524 mm	14±0.5168mm
S. pyrogenes	12±0.5784 mm	15±1.5 mm

The alcoholic extract has more potent anti-microbial potential than aqueous extract of Triphala. Water is almost universally the solvent used to extract activity. At home, dried plants can be ingested as teas (plants steeped in hot water) or, rarely, tinctures (plants in alcoholic solutions) or inhaled via steam from boiling suspensions of the parts. The most active components are not water soluble and the most commonly used solvents (ethanol and methanol, both used as initial extractants) may demonstrate the greatest sensitivity in yielding antimicrobial chemicals on an initial screening. This disparity should be examined as the search for new antimicrobials intensifies. Based on preliminary screening of extract of Triphala, The methanolic extract has great antimicrobial potential than aqueous extract. Therefore, the methanolic extract of Triphala has studied with numerous clinical isolates of *S.aureus* from contaminated wound and simultaneously the clinical isolated verified with methicillin to find resistant organism.

Table 2: Disc Diffusion Method for Clinical Isolates of *S.aureus*: (Zone of inhibition 10 mg/20 µl)

Sample no	Triphala Alcoholic Extract	Methilicin Antibiotic	Remarks
S1	20±1.172 mm	25±0.408mm	Sensitive
S2	20±0.6648 mm	9±0.6645 mm	MRSA
S3	18±0.6645 mm	8±0.435 mm	MRSA
S4	19±0.6123 mm	9± 0.456mm	MRSA
S5	19± 0.7588mm	8± 0.2456mm	MRSA
S6	17±0.9871 mm	8±0.5868 mm	MRSA
S7	20±0.801 mm	9±1.089 mm	MRSA
S8	13±0.8366 mm	8±0.345 mm	MRSA
S9	24±0.823 mm	8±0.123mm	MRSA
S10	20±1.057 mm	30± 1.089mm	Sensitive

The antimicrobial potential of methanol extract against clinical isolates of S.aureus from human wounds is tabulated in table 5. The data shows that the antimicrobial action against both MRSA and sensitive organism. Therefore, the methanol extract of Triphala is not affected by mechanism of antibiotic resistance of MRSA and gave better zone of Inhibition.

Table 3: Antimicrobial activity of Methanol extract of Triphala against Standard Strain

Microorganisms	Triphala extract	Std. Antibiotic
E.coli	18±0.4089 mm	32±1.2649 mm(ciproflaxcin)
S.aureus	18±0.6324 mm	34± 0.8366mm (methicilin)
P. aeruginosa	20±1.0489 mm	30± 0.3085mm (ciproflaxcin)

Based on preliminary screening of aqueous and methanol extract of Triphala, The methanol extract shows better antimicrobial potential and is compared with standard antibiotic such as ciprofloxacin and methillicin. The methanol extract provides antimicrobial action ~50% when compared with standard antibiotics.

Performance of Methanol Extract of Triphala

The performance of methanol extract of Triphala can be evaluated by the following calculation,

$$Performance = \frac{ZoneofInhibitionofDrugA - ZoneofInhibitionofDrugB}{ZoneofInhibitionofDrugA} * 100 \quad (1)$$

Where, Drug A = Standard antibiotic either methillicin or Ciprofloxacin and Drug B = Methanol extract of Triphala.

Table 4: Performance of methanol extract of Triphala when compared with standard antibiotic.

Microorganisms	Performance of Triphala extract in %
E.coli	~43.75
S.aureus	~47.06
P. aeruginosa	~33.33

Table 5: Minimum Inhibitory concentration (MIC) of Triphala against standard strains

Microorganisms	Minimum concentration
E.coli	31.25± 0.3926 mg
S.aureus	8± 0.223 mg
P. aeruginosa	8± 0.3056 mg

The minimum inhibitory concentration of methanol extract of Triphala against standard strains is tabulated in Table 4. In this case, The MIC of Triphala for *E. Coli* required higher concentration than any other organism. The reason for this deviation is obscure.

Table 5: Minimum Inhibitory concentration (MIC) for clinical isolate of *S. aureus*

Sample number	Minimum concentration
S1	15.625±0.167 mg
S2	8± 0.1601mg
S3	8±0.1603 mg
S4	8± 0.1602mg
S5	8± 0.16mg
S6	8± 0.162mg
S7	8±0.167 mg
S8	15.625± 0.1489mg
S9	8±0.1601 mg
S10	8± 0.1602 mg

Table 6: Minimum Bactericidal concentration (MBC) of Triphala against standard strains

Microorganisms	Minimum concentration (MBC)
E.coli	31.25± 0.3926 mg
S.aureus	8± 0.223 mg
P. aeruginosa	8± 0.3056 mg

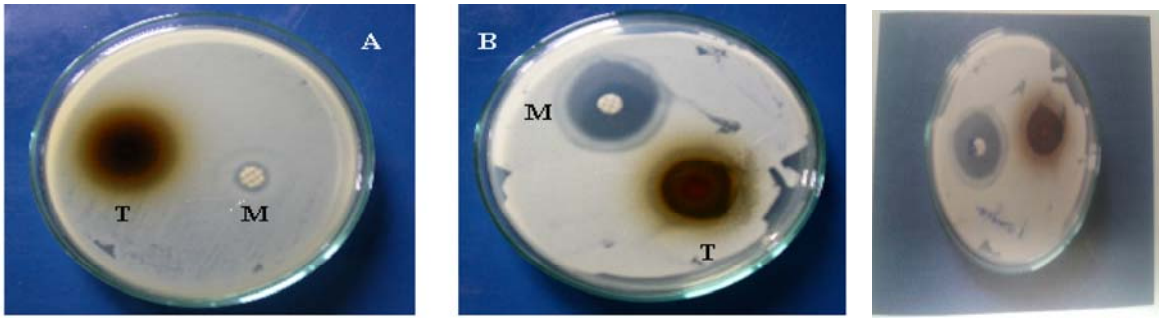

Fig 1: Disc Diffusion Assay for Methanol Extract of Triphala against (A) clinical isolates MRSA, (B) *S.aureus* and (C) *Pseudomonas aeruginosa*.

Discussion

Dermal Wound is contaminated with numerous bacterial pathogens. As a result, wound healing occurs in the presence of wound pathogens. However, certain bacterial appear to aid wound healing. In most case, the wound healing is delayed due to the presence of various bacterial pathogens. It is not the presence of organisms but their interaction with the host tissue that determines their influence on wound healing. In wound Infection, the presence of replicating microorganisms within a wound that cause host injury. Primarily pathogens are of concern here. Examples are *Staphylococcus aureus,* Beta-hemolytic *Streptococcus (S. pyogenes, S. agalactiae), E. coli, Proteus, Klebsiella,* anaerobes, *Pseudomonas, Acinetobacter, Stenotrophomonas (Xanthomonas).*

The eradication of bacterial pathogens at wound site is still challenging for better regeneration of dermis and epidermis at wound site. Even though numerous antibiotics are available for treatment of wound infections, these wound pathogens develop resistance to antibiotics easily and as a consequence, the efficiency of antimicrobial action is decreased. Therefore, Plant based chemicals and pharmaceuticals from the herbal plants are the best source of antimicrobial agents which has multiple pharmacological activity based on concentration. Triphala is used in Ayurvedic medicine in the treatment of a variety of conditions and also forms part of many other Ayurvedic formulations. The conditions for which Triphala is employed include headache, dyspepsia, constipation, liver conditions, ascites, and leucorrhoea. It is also used as a blood purifier and a purgative and to improve the mental faculties and is reported to possess anti-inflammatory, analgesic, anti-arthritic, hypo-glycaemic, and anti-aging properties. In our investigation, Triphala is a potent antimicrobial agent against standard strains as well as methicillin resistant *S.aureus* and sensitive *S.aureus*.

Staph infections are also more common in the wound infection especially methicillin resistant S.aureus in wounds.

Methicillin-resistant Staphylococcus aureus (MRSA) infection is caused by a type of staph bacteria that's become more resistant to many of the antibiotics including vancomycin used to treat ordinary staph infections at wound site. In the mechanism of antibiotic resistance in MRSA, Methicillin resistance requires the presence of the *mec* gene and strains lacking a *mec* gene are not methicillin resistant. The molecular mechanism of methicillin resistance is that mec gene -this presence of the *mec* gene is an absolute requirement for *S. aureus* to express methicillin resistance. The *mec* gene is absent from susceptible strains and present in all resistant strains. The structural component of the *mec* gene, *mecA*, encodes the penicillin-binding protein 2a (PBP2a) that establishes resistance to methicillin and other semi-synthetic penicillinase-resistant beta-lactams. In our study, we observed that the antimicrobial potential of methanol extract of Triphala against MRSA is not affected by methicillin resistance mechanism.

Gram positive organisms were more vulnerable than gram negative organisms. Difference in the cell wall composition in the microorganisms could have affected these results. On comparing our results with other antimicrobial agents such as methilicin and ciprofloxacin, the organisms which were seemingly resistant to other drugs were found to be susceptible to the methanol extract of Triphala. In this investigation, the used aqueous extract of Triphala shows, study could be modified further using alcoholic extract which provided better results and compared with aqueous extracts. The test can be done against clinical specimens of all type of bacterial infections such as contaminated dermal wound etc. or the organisms which are highly resistant to chemical antibiotics. Using many medicinal extract one can effective and can avoid the major problem of antibiotic resistance exhibited by bacteria against antimicrobial agents.

As an important observation in our investigation, we have observed both methicillin resistant and sensitive *S. aureus* were inhibited at the same concentration of Triphala's methanol extract. This exhibits that the mechanism of methicillin resistant has not affected the activity of Triphala. Moreover, Triphala's antimicrobial potential 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 confirmed that the Triphala contains the presence of EGCG (epigallocatechin gallate) as one of the potential condensed tannins (M. Senthil Kumar *et al*, 2008). The literature proved that Epigallocatechin gallate has the specific mechanism for antimicrobial activity. The epigallocatechin gallate more amount 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. The epigallocatechin gallate binds to the peptidoglycan layer in the cell wall of *S.aureus*. Peptidoglycan is a cross-linked complex of polysaccharides and peptides. The cell wall of *Staphylococcus* is 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. EGCg can directly bind to peptidoglycan and induce its precipitation. 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. The above reason might be for responsible for antimicrobial action for Triphala. (Yoda, Y *et al.* 2004; Zhao, W *et al.* 2002, 2001.) [3, 4, 9]

Conclusion

Due to increase of antimicrobial resistance of numerous antibiotics, Phyto-pharmaceutical is an alternative for existing antibiotic therapy and has a potent antimicrobial action. So far there is no evidence for development of resistance against Phyto-chemicals and Phyto- pharmaceuticals in the literature. The work confirms the susceptibility of wound pathogens to the methanol extract of Triphala which combines the anti-microbial effect of three composition of Triphala. Our findings suggest that, an a methanol extract of *Triphala*, traditional Ayurvedic herbal medicine is a potent antibacterial agent against common bacterial pathogens that infect various types of wounds including burns, compared with aqueous extract of Triphala. It can be used in the treatment of wide range of infectious diseases. The results obtained in this study justify the use of *Triphala* in majority of Indian population in treating microbial diseases.

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