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An original research article on enzyme inhibition and antimicrobial potential of triphala against *Pseudomonas aeruginosa*

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Abstract

The controlling and eradication of wound infection is a challenging aspect for the management of open wounds. Despite the use of synthetic antimicrobial agents, drug resistance and cytotoxicity hinder the activity of those antimicrobial agents and thereby increase the chances of infection and delay regeneration of epidermis and dermis at the wound site. Additionally, the microbial enzymes secreted by wound pathogens degrade proteins of extracellular matrix at the wound site. As a consequence, The ECM proteins was degraded and delayed wound closure. The present study investigates the influence of Triphala on antimicrobial activity and enzyme inhibition activity which will be used in wound healing studies. The methanolic extract of triphala was prepared and its antimicrobial activity was tested against *Pseudomonas aeruginosa*. The activity of triphala extract against metalloprotease was studied by Zymography. Enzymatic activities were detected as clear bands of gelatin lysis against a dark background. The 18±2mm clear zone in disc diffusion assay and minimal inhibitory concentration (MIC) of 7.8125mg/ml against *pseudomonas* control strains clearly confirmed the antibacterial activity of Triphala. Zymography analysis exhibited greater reduction in protease activity at ≥1500µg/ml. By virtue of the inhibitory effect of Triphala on *Pseudomonas aeruginosa* and its enzymes such as proteases, it could be potentially used as a new therapeutic agent for *pseudomonas* infected dermal wounds. The results hence highlighted the beneficial effects of the topical application of Triphala would be used in the acceleration of wound healing and its effect on controlling wound infections at wound site.

Keywords: Triphala, Protease, Disc Diffusion Assay and MIC, Wound infection.

1. Introduction

As the development of bacterial resistance to antibiotics and new therapeutic/synthetic antimicrobial agents and controversy regarding the use of topical antiseptics/antimicrobial agents persists, man turned to his prehistory and found literally thousands of Phyto-pharmaceuticals, which has capable to inhibit all types of microorganisms from plants as safe and broadly effective without inducing microbial resistance. According to World Health Organization, nearly 20,000 medicinal plants are present in 91 countries. New compounds inhibiting microorganisms such as benzoin and emetine have been isolated from plants. The antimicrobial compounds from herbal plants may inhibit bacterial growth by different mechanisms of antimicrobial action than those presently used antimicrobial agents and may have a significant clinical value in treatment of resistant microbial strains (Cox, 1994 and Eloff, 1988) [3, 4]. To date, widespread opinion among wound care practitioners is that aerobic or facultative pathogens such as *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and beta-hemolytic *streptococci* are the most important causes of delayed wound healing and infection in both acute and chronic open wounds. Especially, the wound pathogen *Pseudomonas aeruginosa* has capable to secrete virulence proteases that helps establishing and maintaining an infection and thereby controlling and modifying the environment according to the needs of the bacterium within the host tissue. (R.Hoge *et al.*, 2010) [12]

Microbial Proteases are assumed to play a major role during *P.aeruginosa* infection. Usually, this pathogen produces several proteases including elastase and alkaline protease. However, the role of alkaline protease in tissue invasion and systemic infections is still unclear. The ability of *P. aeruginosa* is to destroy the ECM proteins. Elastin, one of the proteins in ECM is a major virulence determinant during acute infection. These are probably involved in initiating and controlling the tissue invasion and necrosis characteristic of *Pseudomonas* infections. In wound healing processes, one of the altered processes is extreme protease activity in the highly

infected wound environment. As a result, the MMP expression was elevated which directs unbalanced regeneration of dermis and epidermis. Matrix metallo proteinases (MMPs) are a family of more than 20 proteases that collectively can degrade most of the components of the ECM (Greener *et al.*, 2005) [17]. Sajith Abraham *et al.* 2005 [16] proved that Triphala has a potential to inhibit the MMPs in the dental tissue. This will be added advantage of Triphala which could help to inhibit MMPs in infected wound environment where the MMPs level elevated by microbial protease.

Triphala is a traditional Ayurvedic herbal formulation consisting of the dried fruits of three medicinal fruits such as *Terminalia chebula*, *Terminalia bellirica*, and *phyllanthus emblica*. Triphala and/or its constituent fruits have been reported to possess a variety of pharmacological activities such as antioxidant, antibacterial, antifungal, antiviral, anti-malarial, anti-mutagenic, anticancer, radio-protective, anti-allergic, cardiogenic, hypo-cholesterolaemic, capillary strengthening and hepatoprotective (Kirubanandan, 2006 and Hans Wohlmuth., 2002.) [15, 8]. However, the lack of antimicrobial activity against wound pathogenic organisms such as *Pseudomonas aeruginosa*, and its microbial enzyme inhibition by extract of Triphala is forced to perform this preliminary work.

2. Materials and Methods

2.1. Preparation of alcohol extract of Triphala

100 g of Triphala powder (IMPCOPS Ltd., Chennai, India) was extracted in 500 mL of methanol by stirring overnight and centrifuged at room temperature. 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 desiccator for further use. The dried extract was dissolved in 10% Dimethyl Sulfoxide (DMSO) and used to assay the antibacterial activity.

2.2. Thin Layer chromatography of Methanol Extract

In order to evaluate antimicrobial potential of Triphala, The presence of a variety of bioactive molecules is identified by TLC with Standard Phyto Chemicals and suggested which bioactive compounds involved in antimicrobial activity.

2.3. Bacteria tested

Pseudomonas aeruginosa ATCC 27853 was obtained from the King Institute, Chennai, India.

2.4. 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.

2.5. Antibacterial susceptibility

The antibacterial sensitivity test was performed by disc diffusion method. *P. aeruginosa* 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 °C 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 °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 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.

2.6. Enzyme Inhibition Assay and Zymography

The overnight microbial 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 metalloprotease was studied by Zymography. Enzymatic activities were detected as clear bands of 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 \pm SD of 10 experiments.

3. Result and Discussion

The Thin Layer chromatography Studies of methanaolic extract of Triphala was confirmed that the organic extract contains a variety of active constituents such as poly phenol, flavanoids and Ascorbic acid, involving in antibacterial action. In this studies, Figure A. Solvent system: Chloroform – methanol-Acetic acid, (90:10:1) and Fig b Solvent system: Petroleum ether – ethyl acetate –formic acid (40:60:1). Detection Agent: alcoholic solution of Ferric chloride. In both cases, the stationary phase is silica gel. The Active constituents in the triphala are Polyphenol – Gallic acid, ellagic acid, Flavonoids – Quercetin, Vitamin C.

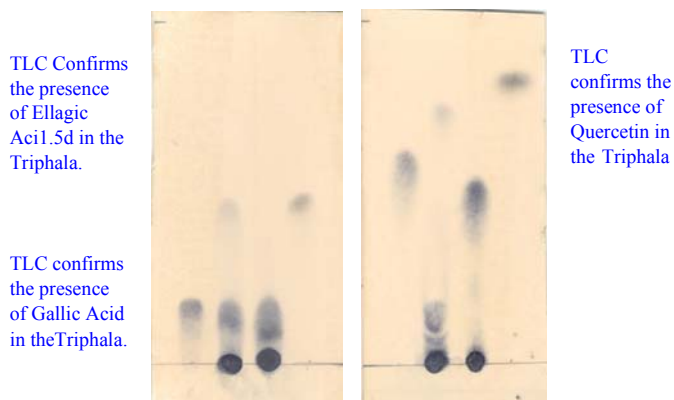


Fig 1: Thin layer chromatographic studies of methanaolic extract of Triphala

Table 1: Zone of Inhibition for Standard strain Microorganisms

Microorganisms	Triphala extract	Std. Antibiotic
<i>S. aureus</i>	18±1.5 mm	34±0.5 mm (methicillin)
<i>P. aeruginosa</i>	20±1.0 mm	30±1.0 mm (ciprofloxacin)

The MIC of Triphala extract against *P.aeruginosa* strains was found as 7.8125± 0.0085mg/ml. The MBC of Triphala extract was >7.81 mg/mL for *P.aeruginosa*. 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. (Figure 2). No zone of inhibition was observed in DMSO treated disc.

Table 2: Minimum Inhibitory concentration

Microorganisms	Minimum concentration
<i>S. aureus</i>	7.8125±0.0085 mg/ml
<i>P. aeruginosa</i>	7.8125±0.0078 mg/ml
<i>Streptococcus pyogenes</i>	31.25±0.0095 mg/ml



Fig 2: Agar diffusion test shows the zone of inhibition by Triphala (T) and methicillin (M) for *Pseudomonas aeruginosa* ATCC 27853.

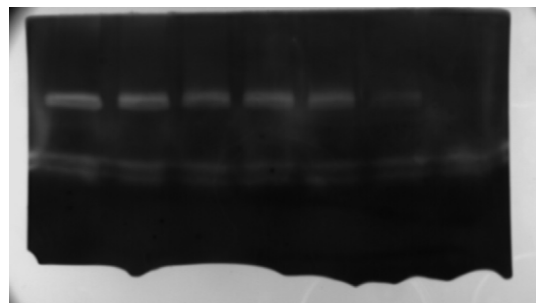


Fig 3: Protease Inhibition by methanolic Extract of Triphala- Case 1: Gelatin zymography shows the inhibition of metalloproteases from *P.aeruginosa* ATCC 27853 cultures at different concentrations of Triphala extracts. Lane 1 – Control, Lane (2-7) – Enzyme inhibition by Triphala extract (from Left to Right).

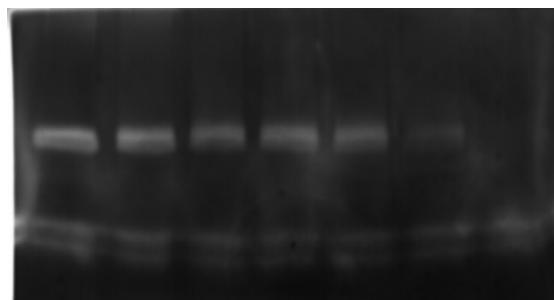


Fig 3: Protease Inhibition by methanolic Extract of Triphala- Case 2: Gelatin zymography shows the inhibition of metalloproteases from *P.aeruginosa* ATCC 27853 cultures at different concentrations of Triphala extracts. Lane1 – Control, Lane (2-7) – Enzyme inhibition by Triphala extract (from Left to Right).

The reduction in the pseudomonas metalloproteases activity was detected as unstained bands on the gels by zymography technique. The band pattern showed 30-40KDa metalloproteases in gelatin zymogram. Significant differences were found on metalloproteases activity in the triphala treated group compared with control, which shows the inhibitory activity of triphala extract against these enzymes (Figure-3).

Table 4: Percentage of enzyme inhibition at different concentrations of Triphala

Concentration Of methnolic extract	Lane 1 Control	Lane 2 200µg	Lane 3 400 µg	Lane 4 600 µg	Lane 5 800 µg	Lane 6 1000 µg	Lane 7 1500 µg
Figure:3.1	100	95.21±3.26	85.02±2.87	70.64±2.06	50.32±2.13	20.07±1.5	>10.21±2.5
Figure:3.2	100	95.44±1.24	85.23±1.45	70.55±1.09	50.48±1.24	20.45±2.1	>10.23±2.1

The presence of Polyphenols and tannins in the methanol extract might be the responsible for inhibition of microbial enzymes and antimicrobial action against wound pathogens. Numerous studies have reported that the compounds from botanical origin are effective antimicrobial agents (Basile A *et al.*, 2000, Cowan MM., 1999) [1, 2]. Some phytochemicals have been screened against antibiotic-resistant strains of bacteria (Kone WM *et al.*, 2004, Sato Y *et al.*, 2000) [9, 18]. Inhibitory activity of Triphala extract against the growth of *Pseudomonas aeruginosa* in this study provides some scientific rationale for its use as antimicrobial agents on infected dermal wounds. In our previous studies, we showed that the Triphala contains the presence of EGCG (Epigallocatechin Gallate) as one of the condensed tannins as bioactive constituents. (M. Senthil Kumar *et al.*, 2008) [10].

Additionally, Triphala have been reported to possess a number of medicinal properties like anti-inflammatory, anti-bacterial, anti-fungal, anti-viral, anti-malarial, anti-mutagenic, radio

protective, anti-allergic, anti-cancer, cardiotoxic, hypo-cholesterolaemic, capillary strengthening, hepatoprotective, immunomodulatory, adaptogenic, analgesic and anti-oxidant activity. In the case of our preliminary investigation of Triphala, it could be used as potential drug for the treatment of infected wound. It is also evidenced that the crude extract of each plant poses the lack of cellular toxicity on sheep erythrocyte up to 200mg/mL gives enrichment to our findings. Although Sato *et al.*, 1997 [17] 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) [17]. Isolation of active constituents, mode of action and *in vivo* studies in our future analysis make triphala as a potential therapeutic agent.

4. Conclusion

To conclude, the antimicrobial activity of a variety of Polyphenols/tannins as bioactive constituents present in the triphala might be exerted by direct binding to peptide structure of bacterial components and its microbial enzymes. By virtue of its inhibitory effect on *Pseudomonas* and their enzymes such as metalloprotease, Triphala can be potentially used as a new therapeutic agent for infected dermal wounds. The investigation of mechanism of inhibiting the enzymes by the isolated bioactive molecules from Triphala would be a recommended and future work.

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