ISSN: 0975-833X

Available online at http://www.journalcra.com

International Journal of Current Research
Vol. 7, Issue, 04, pp.14958-14962, April, 2015

INTERNATIONAL JOURNAL OF CURRENT RESEARCH

RESEARCH ARTICLE

ANTIMICROBIAL ACTIVITY OF METHANOLIC EXTRACTS OF TRIPHALA AND ITS COMPONENTS AGAINST CLINICAL ISOLATES OF MRSA AND VRSA ISOLATED FROM PUS SAMPLES

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ARTICLE INFO

Article History:
Received 26th January, 2015
Received in revised form
22nd February, 2015
Accepted 19th March, 2015
Published online 30th April, 2015

Key words:

Triphala,
Antimicrobial activity,
MRSA, VRSA,
Wound pathogens.

ABSTRACT

Background: Hospital acquired infections and wound infections are a major cause of morbidity and mortality worldwide. The problem is complicated due to the sustained appearance of newer antibiotic resistant strains resulting in higher mortality rates. Herbal medicine offers a promising alternative to treatment of wound infections. The methanolic extracts of Triphala and its components are assessed for their antibacterial activity against antibiotic resistant pathogens, MRSA and VRSA.

Material and Methods: Antibacterial activity of methanolic extracts of Triphala, amla, hirada and behada was tested against multidrug resistant bacterial wound pathogens isolated from pus samples. The eleven selected strains, MSSA, MRSA (confirmed by presence of mec A gene) and VRSA (based on MIC of Vancomycin) were used for testing antimicrobial activity by disc diffusion method.

Results: Methanolic extract of Triphala showed antibacterial activity against MSSA and all ten hospital isolates of MRSA and VRSA. Significantly, all VRSA isolates were also susceptible to methanolic extracts of individual components of Triphala- amla, behada and hirada. Out of the three individual components of Triphala, hirada showed maximum inhibition against one of the MRSA isolate.

Conclusion: Methanolic extracts Triphala and its individual components contain active constituents which show potent antibacterial activity against multidrug resistant *Staphylococcus aureus*- MRSA and VRSA.

INTRODUCTION

Wound and skin infections caused by *Staphylococcus aureus* are becoming increasingly difficult to treat by conventional antibiotics in view of the emergence of antibiotic resistant strains. Antibiotics are used globally as a first line of treatment for any type of infection. Infections caused by methicillin-resistant *Staphylococcus aureus* (MRSA) have been associated with high morbidity and mortality rates. *S. aureus* is a major cause of hospital acquired infections involving skin and soft tissues. In Indian hospitals, MRSA is one of the common causes of hospital-acquired infections and 30 to 80 per cent methicillin resistance in *S. aureus* based on antibiotic sensitivity tests has been reported from different hospitals. In India, the prevalence rate of MRSA infections from Varanasi, Indore and New Delhi is more than 50 %. In a study from Hyderabad in 2011, 79.6% clinical isolates of *S. aureus* were identified as methicillin resistant *S. aureus* (MRSA) (Thati *et al.*, 2011). Vancomycin and other glycopeptides are the drugs of choice for the treatment of infections due to MRSA. But, in late 1997, reported *S. aureus* with intermediate resistance to Vancomycin was reported to become completely resistant to Vancomycin (Hiramatsu *et al.,* 1997). Increasing levels of MIC of Vancomycin in MRSA have been reported by a study from Mumbai (Veer *et al.,* 2010). Recent study from Hyderabad in 2011 reported 2% occurrence of VRSA among the MRSA isolated and resistant to majority of the other antibiotics tested (Thati *et al.,* 2011). The discovery of Vancomycin resistant *S. aureus* (VRSA) and multidrug resistant *S. aureus* has generated worldwide concern. It has thus become evident that there is urgent need for novel antibacterial agent with broader spectrum, lesser side effects, and without cross-resistance to antibiotics in use. The traditional system of medicine in India, Ayurveda, therefore, offers a distinct advantage in terms of efficacy and overall effect over the current approach for treatment of infectious diseases. 'Triphala', an ayurvedic herbal formulation is used traditionally for the treatment of different types of diseases. It is a combination of three potent herbal fruits- *Terminalia chebula, Terminalia bellerica and Emblica officinalis.* Antimicrobial activity of Triphala has been reported against various wound and skin pathogens like *Staphylococcus aureus*, MRSA, *Pseudomonas aeruginosa*, and beta-haemolytic *Streptococci* (Kirubanandan *et al.,* 2013; Mahesh *et al.,* 2008) more ref). Some studies on the antimicrobial effect of Triphala

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on MRSA are reported. (Nayak *et al*., 2015; Kirubanandan *et al.,* 2013). However, there are no reports on the antimicrobial activity of Triphala on VRSA. Alcoholic extract of Triphala have been reported to possess both antimicrobial and wound healing activity (Kirubanandan *et al.,* 2003). The present work was carried out to assess the use of methanolic extracts of Triphala and its individual components against MRSA and VRSA isolated from pus samples.

MATERIALS AND METHODS

Collection, extraction and preparation of methanolic extracts of Triphala and its individual components

Dried fruits of *Emblica officinalis, Terminalia chebula* and *Terminalia bellirica* were obtained from local herbal medicine distributor. The fruits (pericarp and seeds) were oven-dried and macerated into a crude powder. 5 grams of crude powder of each was weighed and filled in a thimble of a Soxhlet apparatus. 200 ml of Methanol was used as the extraction solvent. Each sample was subjected to hot extraction in Soxhlet apparatus for 6 continuous hours. Approximately 10 cycles of extraction were achieved for each. Following extraction, the extracts were concentrated in Rota Vapour at 67ºC and vacuum pressure at 50atm. The dry concentrate obtained was weighed and re-dissolved in 5 ml of DMSO. The extracts were used for further assays.

Micro organisms used

Bacterial wound pathogens isolated from pus sample were used for this study. They were characterized through microscopic examination, Gram staining, growth on Mannitol salt agar, and Coagulase test. Antibiotic resistance profile of each culture was determined by standard disc diffusion assay as per CLSI guidelines. (Performance Standards for Antimicrobial Susceptibility Testing; Twenty-Second Informational Supplement, 2012) The control strain of *Staphylococcus aureus* NCIM 5345 (MSSA) was used. Five Methicillin-resistant *Staphylococcus aureus* (MRSA) strains and five Vancomycin resistant *Staphylococcus aureus (*VRSA) were selected based on following tests.

- Identification of MRSA by growing on Oxacillin and Cefoxitin disc diffusion test The isolates of pus sample were screened for MRSA by using standard discs of Oxacillin (1 µg/disc) Cefoxitin (30 µg/disc) (HiMedia).
- Confirmation of Methicillin-resistant *Staphylococcus aureus* (MRSA) by detecting presence of mec A gene by PCR amplification. PCR conditions were as follows - An initial denaturation at 94^0C for 3 min was followed by 35 cycles of amplification (94^0C for 30 sec, 54^0C for 30 sec and 72^0C for 45 sec) and a final extension step was done at 72^0C for 7 min. The primers used were mec A R & F (Sigma) (Malik *et al*, 2006, Marathe *et al*., 2015).
- Determination of MIC of Vancomycin by strip assay. Estimation of MIC of Vancomycin was done by Vancomycin Ezy MIC strip VAN (0.016 to 256 mcg/ml) (Hi Media). (Flora Grace, 2013), **(**Silvestre Joana *et al*., 2013)

Methodology

Determination of antibacterial activity of methanolic extracts of Triphala and its three components against eleven isolates. (one MSSA, Hospital isolates- five MRSA and five VRSA) Test organism was inoculated on Muller Hinton agar at 37^0c for 24 hrs. Saline suspension of 24 hrs old culture was prepared; (Standard 0.5 McFarland tube). 750 µl saline suspensions was mixed with pre-sterilized, cooled Muller Hinton agar butt and poured in sterile plate. Plates were allowed to solidify at room temperature. Sterile What man filter paper discs were soaked in respective extract prepared as above and kept on agar surface. Plates were kept at 4^0 C for 30 min pre-diffusion and incubated at 37^0 C for 24 hrs. Diameter of zone of inhibition was measured and recorded.

RESULTS

To test the efficacy of Triphala and its individual components; eleven cultures were selected as test organisms. *S aureus NCIM 5345* was found to be Methicillin sensitive (MSSA) by disc diffusion assay of Methicillin and Cefoxitin. The culture showed typical colony on Mannitol salt agar (ability to grow and change the color of the medium around the colony to yellow due to mannitol fermentation) and coagulase test was negative. Ten hospital isolates were grown on Mannitol salt agar and checked for their ability to coagulate plasma by tube coagulase test. All ten isolates showed typical colony on Mannitol salt agar and coagulase test was positive (Table 1). Methicillin resistance arises by acquisition of a staphylococcal cassette chromosome SCC mec, and is conferred by the mecA

Table 1. Pathogens isolated from pus samples

Sr.No	Culture no.	Growth on Mannitol Salt Agar*	Gram staining	Coagulase test	Mec A PCR
1	*Staphylococcus aureus* NCIM 5345	+	Gram positive Cocci in cluster	-	-
2	MRSA 171	+	Gram positive Cocci in cluster	+	+
3	MRSA 278	+	Gram positive Cocci in cluster	+	+
4	MRSA 293	+	Gram positive Cocci in cluster	+	+
5	MRSA 296	+	Gram positive Cocci in cluster	+	+
6	MRSA 297	+	Gram positive cocci in cluster	+	+
7	VRSA 147	+	Gram positive Cocci in cluster	+	-
8	VRSA 162	+	Gram positive Cocci in cluster	+	-
9	VRSA 168	+	Gram positive Cocci in cluster	+	+
10	VRSA 209	+	Gram positive Cocci in cluster	+	-
11	VRSA 213	+	Gram positive Cocci in cluster	+	-

* ' + ' Mannitol fermentation showing yellowing around the colony.

gene, which encodes the low-affinity penicillin-binding protein PBP 2A. (Wielders *et al*, 2002). Presence of mec A gene was detected by PCR (Table 1) Isolates, MRSA 171, MRSA 278, MRSA 293, MRSA 296, MRSA 297 and VRSA 168 showed presence of 675 bp band of amplified mec A gene (Fig 1).

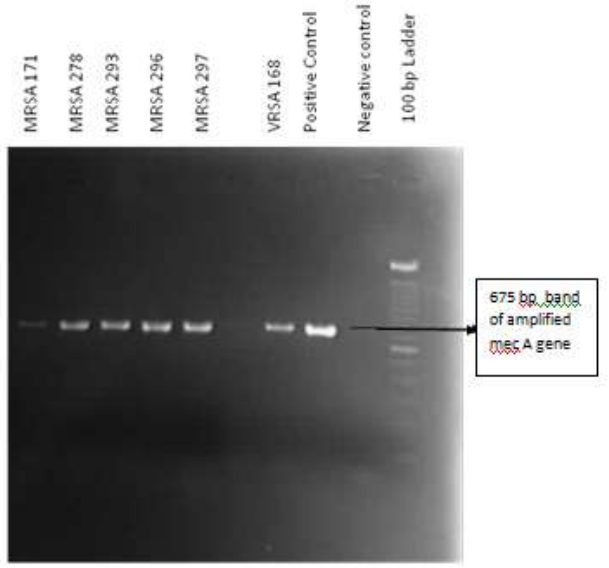


Fig.1. Agarose gel electrophoresis of mec A gene PCR products

VSSA (MIC ≤ 2µg/ml), VISA (MIC 4 to 8µg/ml), VRSA (MIC ≥ 16µg/ml). (BSAC, 2011), (EUCAST, 2011)

Fig 2. Disc diffusion assay for estimation of MIC by Vancomycin Ezy MIC strip showing no zone of inhibition up to 256 µg/ml

The eleven isolates were subjected to determination of antibiotic sensitivity test using standard enzyme strips. (Hi Media) Antibiotic sensitivity profile of selected MRSA showed highest resistance to Penicillin (100% isolates), Cefoxitin (100%), Amikacin, Tazobactum, Levofloxacin and Linezold (60% isolates for every antibiotic mentioned above),

Table 3. Antibiotic Sensitivity profile of eleven selected isolates from pus

Sr. no.	Culture no.	Resistant against	Sensitive against
1	*Staphylococcus aureus* NCIM 5345	Pen	Cx, E, Cd, Sxt, Tcy, Teico, G, Amc, Cot, Cn , Te.
2	MRSA 171	Pen, Cx, E (I), Cd (I), Cn,G, Cr	Cip, Tcy, Van, Teico, Cz, Ctx
3	MRSA 278	Ctx, Pen	Cip, Van Cz, G
4	MRSA 293	Amc, Cot, Le, Pen	Cip, Van, Cz, Ctx, G, Az,Cn, Te, Lz, Rf
5	MRSA 296	Cip, Ctx, Amc, Az, Cot, Cn, Pen	Van, Cz, G, Te, Le, Lz, Rf
6	MRSA 297	Amc , pen	Cip, Van, Cz, Ctx, G, Az,Rf Cot, Cn, Te, Le, LZ
7	VRSA 147	Pen, Cx, E, Cd, Cn, Sxt, Cip, Tcy, Van, Ctx, G	Teico, Cz, Cr
8	VRSA 162	Pen, Cx, E, Cd, Tcy, van Cr	Cn, Sxt, Cip, Teico, Cz, Ctx, G,
9	VRSA 168	Pen, Cx, E, Cd, Cn,Cip, Van, Ctx, Cr	Sxt, Tcy, Teico, Cz, G.
10	VRSA 209	Pen, Cx, E, Cd,Van	Cn, Sxt, Cip, Tcy, Teico,Cz, Ctx, G,Cr
11	VRSA 213	Pen, Cx, E, Cd, Cn,Van, teico, G	Sxt, Cip, Tcy, Cz, Ctx, Cr

Abbreviations –

Pen – Penicillin, Cx – Cefoxitin, E –Erythromycin, Cd - Clindamycin , Cn- Cefoxitin, Sxt- Cotrimazole, Cip- Ciprofoxacin, Tcy- Tetracyclin, Van- Vancomycin, teico- Teicoplanin, Cz- Cefazolin, G- Gentamicin, Cr- Carbenicillin , Amc- Amakacin, Az- Azithromycin, Cot- Ceftolozane, Cn- Tazobactum, Le- Levofloxacin, Lz- Linezolid, Rf- rifampicin, Ctx- Cefotaxime.

All ten cultures except Staphylococcus aureus NCIM 5345 (MSSA), were subjected to determination of MIC of Vancomycin by Vancomycin Ezy MIC strip. (VAN (0.016 to 256 mcg/ml of Hi Media) Out of eleven cultures tested, two were VSSA, three VISA five were VRSA as MIC was ≥ 16 µg/ml. (Table 2, Fig 2)

Table 2. Vancomycin MIC strip assay.

Sr. No.	Culture no.	MIC of Vancomycin (µg/ml)	
1	MRSA 171	1	VSSA
2	MRSA 278	2	VSSA
3	MRSA 293	6	VISA
4	MRSA 296	0.75	VSSA
5	MRSA 297	1	VSSA
6	VRSA 147	>256	VRSA
7	VRSA 162	>256	VRSA
8	VRSA 168	>256	VRSA
9	VRSA 209	>256	VRSA
10	VRSA 213	>256	VRSA

Cefotaxime (40% isolates) and carbenicillin (40% isolates) followed by Ciprofloxacin (20% isolates), Gentamycin (20% isolates), Azithromycin (20% isolates). MRSA showed sensitivity against erythromycin, Clindamaycin, Cotrimoxazole, Tetracyclin, Vancomycin, Teicoplanin, Cefazolin, Teicoplanin, Rifampicin. Among five MRSA, MRSA 171 and MRSA 296 were resistant to seven and six antibiotics among 14 antiobiotics tested, respectively. VRSA showed highest resistance against Penicillin (100% isolates), Cefoxitin (100% isolates) and Vancomycin (100% isolates) followed by Cotrimazole, Ciprofloxacin, tetracycline, Cefotaxime and Gentamycin (40% isolates for all antibiotics mentioned above) and 20 % isolates showed resistance against erythromycin, Clindamycin, teicoplanin and Carbepenicillin. VRSA showed sensitivity against Cefazolin. Among five VRSA, VRSA 147 and VRSA 162 showed resistance to eleven and nine antibiotics respectively out of 14 antibiotics tested.

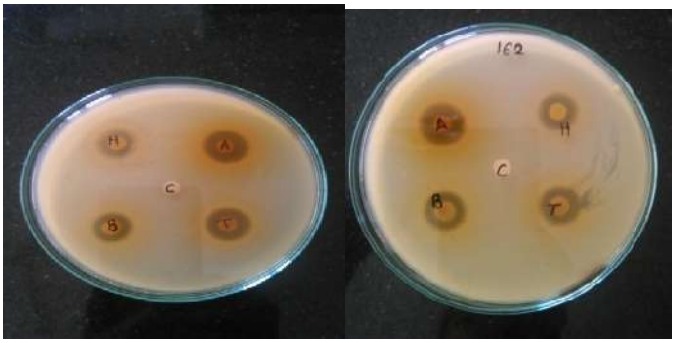
These multidrug resistant (MDR), MRSA or VRSA hospital isolates from pus were selected to check the antimicrobial activity of methanolic extract of Triphala and its components.

the antibacterial activity of methanolic extracts of Triphala, amla, hirada and behada against MDR *S. aureus* including MRSA and VRSA. Formulations of Triphala prepared from

Table 4. Antimicrobial activity of Methanolic extracts of Triphala and its components

Sr.no.	Culture no.	Zone diameter in mm			
		Emblica officinalis (Amla)	*Terminalia bellerica* (Behada)	*Terminalia chebula* (Hirada)	Triphala
1	*Staphylococcus aureus* NCIM 5345 (MSSA)	12±0.76	12±0.66	11±1.1	13±0.33
2	MRSA 171	15±0.88	20±0-84	25±0.1.03	14±0.66
3	MRSA 278	11±0.78	11±0.77	9±0-88	11±0.32
4	MRSA 293	7±0.45	11±0.56	11±0-63	11±0.45
5	MRSA 296	6±0.95	9±0.88	9±0.77	8±0.98
6	MRSA 297	7±1.11	9±0.59	8±0.45	9±0.88
7	VRSA 147	13±0.94	11±0.73	10±0.55	11±0.54
8	VRSA 162	11±0.59	11±0.88	9±0.87	10±0.21
9	VRSA 168	12±1.03	11±0.49	9±0.38	10±0.56
10	VRSA 209	12±0.99	11±0.99	10±0.44	11±0.66
11	VRSA 213	9±0.63	9±0.88	7±0.55	10±0.72

Note: No Zone of inhibition was observed with DMSO as a control.



a. MRSA 296 **b. VRSA 162**

Fig 3. Disc diffusion assay of Triphala and its components using test organism MRSA, VRSA

DISCUSSION

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 (Kirubanandan *et al*., 2013). Approximately 2 – 10% of the U.S. population is now colonized with MRSA. MRSA can cause serious infections of surgical site wounds, bloodstream infections, pneumonia and many more. MRSA has become the most frequent cause of skin and soft tissue infections and MRSA related mortality surpasses AIDS annually (Centre for Disease Control and Prevention USA, 2002). MRSA infections typically are resistant to a variety of antibiotics from other antibiotic classes as well and this can make treatment very difficult. Healthcare-acquired MRSA infections happen frequently in hospitals, rehab facilities, nursing homes and have been increasing in alarming rates for decades. MRSA is becoming more prevalent at healthcare settings due to lapses in infection control. Treatment of MRSA frequently involves the use of Vancomycin, often in combination with other antibiotics given by IV. VRSA infected patients are left with a choice of very few drugs/antibiotics like Linezoid, Trimethoprime and Daptomycin (Micek, 2007). The present work demonstrates methanolic extracts of individual components in different proportions can be evaluated for further improving the antibacterial activity. Ointment preparations of these formulations are recommended for topical application to the infected wounds. Since earlier workers have reported wound healing activity of Triphala, the proposed formulation can serve dual purpose of infection control and wound healing.

Acknowledgement

This work was supported by funding from Indian Council for Medical Research (ICMR), New Delhi, India (project number AMR/12/2011‑ECD 1). We acknowledge Dr. Vaishali Dohe, and Head, Microbiology Department, B. J. Medical College, Pune for providing hospital isolates.

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