In Vitro Assessment of *Terminalia chebula* Retz. Fruits Against Methicillin Resistant *Staphylococcus aureus*

[1*]M. Revathi, [2] G. Senthilkumar,[3] A. Panneerselvam, [4]E.S. Karthy, [5] R. Gopika
[1*,2,3,5] A.V.V.M Sri Pushpam College (Autonomous), Poondi, Thanjavur - 613503, Tamil Nadu, India.
[4] AWECARE, Analytical and Research Laboratories, Agrinagar, Thindal, Erode - 638 012, Tamilnadu, India.
E.Mail: rvthmailbox@gmail.com

ABSTRACT: *Terminalia chebula* is a popular medicinal plant according to Ayurveda for its broad spectrum medicinal value including in the treatment of wound healing. Fruit extracts in aqueous as well as in various solvents like Acetone, Ethanol and Methanol were analyzed to testing its antibacterial activity against 16 Methicillin Resistant *Staphylococcus aureus* (MRSA). The analysis was carried out by taking the extracts at a concentration of 500mg/ml and their activities were recorded by zone of inhibition as produced by disc diffusion method. Maximum zone was observed (20 to 25mm) in all the tested extracts, in microdilution assay revealed that the bactericidal values for in the range of 0.24 to 7.81mg/ml. Phyto-chemical analysis of *Terminalia chebula* extracts showed the presence of secondary metabolites like Alkaloid, Flavonoid, Terpenoid and Tannin. The chromatography analysis has revealed the presence of Aminoacid, Phenolic compound and aromatic compound as functional groups in *Terminalia chebula*. These findings indicate that the *Terminalia chebula* possesses a potential antibacterial activity against MRSA.

Key words: Methicillin resistant *Staphylococcus aureus* (MRSA); *Terminalia chebula*; Antibacterial activity; Minimum Inhibitory Concentration (MIC); Phytochemical.

INTRODUCTION:

Pathogens that infect wounds can be part of normal flora or acquired from the hospital environment or other infected patients. *Staphylococcus aureus*, being the normal microbial flora of the skin, is one of the commonest causes of wound infections. Its increasing incidence is a growing concern with emergence of virulent, antibiotic resistant strains in the community settings. Most infections are caused by aerobic bacteria including *Staphylococcus aureus*., *Pseudomonas* spp., *Klebsiella* spp and *Proteus* spp in wounds (Singh *et al*., 2012). Wound patients have been shown the potential to become colonized and infected more readily than other patients due to deprivation of mechanical barrier provided by the skin and mucous membrane as well as the depression of immunological response (Revathi *et al*., 1998). Infection outbreaks have been reported from burn wards, nurseries, intensive care units as well as in clinical and surgical patients and due to misuse of antibiotics, lack of hand washing, irresponsible nursing care and presence of carriers among the hospital staff (Zermina *et al.,* 2012).

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 drug resistant strains are arising rapidly and thus making the treatment difficult. As a result, Methicillin-Resistant *Staphylococcus aureus* (MRSA) infection is a significant cause of high mortality and morbidity worldwide. Yamamoto *et al*., (2010) reported that the rapid identification of infected patients and interruption of strain transmission is very crucial in controlling the spread of infection. 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.

Terminalia chebula is used in traditional medicine. It is belongs to the family *Combretaceae*. It is commonly called as Black myrobalan, Ink tree (or) Chebulic myrobalan. It is extensively used in unani, ayurvetha and homeopathic medicine. This is used in traditional medicine due to the wide spectrum of pharmacological activities associated with the biologically active chemical present in the plant. The compounds present in *Terminalia chebula* were shown to have anti-cancer, antimicrobial, anti-inflammatory, antimutagenic, antifungal, antiviral, antidiabetic and antiphylactic activities (Chattopadhyay *et al*., 2007). Fruits are valuable in the prevention and treatment of several diseases of the mouth such as dental caries, spongy and bleeding gums, gingivitis and stomatitis. This plant has been extensively used in Ayurveda and Siddha for parasites, tumors, skin diseases, leprosy and wounds (Karthirvel *et al*., 2012). Fruit and leaf extract of this plant also possess wound healing potentiality with improved rate of contraction and a decreased period of epithelization much of the effectiveness of *Terminalia chebula* in many of its medicinal uses is attributed to its antibacterial activity (Saha *et al*., 2011). The aim of this study was to evaluate the possible antibacterial potential of *Terminalia*

chebula extracts (acetone, ethanol, methanol, cold and hot aqueous) against Methicillin Resistant *Staphylococcus aureus* (MRSA).

MATERIALS AND METHODS:

Plant material:

Terminalia chebula fruits were collected from siddha medical center at Erode district, Tamilnadu, India. The seeds were washed with water, then surface sterilized with 10% sodium hypochloride solution, rinsed with sterile distilled water and air dried at room temperature. The dried seeds were powdered and used as a raw material and stored in air tight container for further use.

Extraction of plant material:

10 gram of dried powder of *Terminalia chebula* was suspended in 50ml of Cold water, hot water, ethanol, methanol and acetone. The mixture was soaked for 24hours. The suspended solid was filtered through whatman No.1 filter paper and kept in waterbath at 60-80° C for 2hours. The dried crude extracts were stored at 4° C for further use (Chessbrough, 2000).

Screening of Methicillin Resistant *Staphylococcus aureus* (MRSA):

A total of 132 pus specimens from chronic wound patients were collected for *S.aureus* screening. The samples were obtained from various health care hospitals in Erode District. Wound swabs were streaked on mannitol salt agar and incubated at 37°C for 24 to 48 hours. Growth and fermentation of mannitol on MSA were examined. *Staphylococcus aureus* were identified using gram stain and biochemical tests based on Bergey's manual of systematic bacteriology (Bergey *et al*., 1994). Detection of MRSA by using CHROM agar with oxacillin 4mg/l; Oxacillin resistant Screening Agar Base (ORSAB) with oxacillin 4µg/ml and Blood Agar (BA) with Oxacillin 4µg/ml. The plates were incubated at 37°C for 24 to 48 hours (Karthy *et al*., 2009).

Antibiotic susceptibility test:

The antibiotic resistant and sensitivity test for each isolates was carried out on Muller Hinton Agar by Kirby bauer disc diffusion method. Ampicillin (30µg), cefazolin (30µg), chloramphenicol (30µg), Erythromycin (15µg), Gentamycin (10µg), Norfloxacin (10µg), Rifampicin (5µg), Oxacillin (5µg), Tobramycin (30µg) and Methicillin (5µg) were put on to the bacteria field. The plate was incubated at 37° C and the zone of inhibition was observed after 24hours (Bauer *et al*., 1966).

Disc diffusion method and Minimum inhibition concentration (MIC) – Microdilution:

Antibacterial activity of *Terminalia chebula* extract (Ethanol, Methanol, Acetone, Hot water and Cold water) were tested separately using Disc diffusion method (Kim *et al*., 2004). Minimum inhibition concentration was done to determine the lowest concentration of the *Terminalia chebula* extract, where it can show the bactericidal and bacteriostatic effect. The test was performed in 96 well microtiter plate (Kumarasamy *et al*., 2002).

Phytochemical screening:

Phytochemical analysis of alkaloid, tannin, terpenoid and flavonoid were carried out according to standard methods described by Adetuyi *et al*., 2001; Venkatasan *et al*., 2009.

Identification of amino acid, phenolic compounds and aromatic compounds using column chromatography:

Air dried and powdered *Terminalia chebula* was soaked in methanol for overnight in dark condition. The solvent was removed by water bath and extract was dried. The dried sample was used for column chromatography. Silica gel (mesh 60-120) was used as column packing material. The column was eluted using a series of solvent systems: 100% Acetone and followed by acetone: ethanol (75:25, 50:50, 25:75) finally 100% Ethanol. Each series solvent was added 100ml and fractions were collected 10ml upto 20 fractions. Collected fractions were blended which are having similar Rf value. Blended fractions were applied on TLC plate using ethanol: methanol and acetic acid (5ml: 5ml: 100µl). The compounds were then identified by using iodine for phenolic compounds, ferric chloride for aromatic compounds and ninhydrin for aminoacid (Kumuthavalli *et al*., 2010).

RESULTS AND DISCUSSION:

Out of 132 wound swab samples, *Staphylococcus aureus* accounted for 51 (38.63%) cultures, out of these 16 (31.37%) cultures were found to be MRSA based on CHROM agar with oxacillin 4mg/l; Oxacillin resistant Screening Agar Base (ORSAB) with oxacillin 4µg/ml and Blood Agar (BA) with Oxacillin 4µg/ml. Previous reports have showed variable prevalence of MRSA strains among various cities of Pakistan including 61% in Lahore, 57% in Karachi, 46% in Rawalpindi and 54 % in Peshawar (Ahmad *et al*., 2000, Hafiz *et al*., 2002, Qureshi *et al*., 2004, Shafiq *et al*., 2011). Singh *et al*., (2012) observed out of 100 wound swab samples, *S.aureus* accounted for (62%) of total wound infection. Out of these, 28 (45%) were found to be MRSA in India.

In this study ten antibiotics were used for antibiotic susceptibility test. Maximum strains were showed resistance to Ampicillin, chloramphenicol, Rifampicin, Oxacillin and Methicillin (Table: 1). Lee reported (2003) hospital-acquired MRSA strains are frequently resistant to most common antibiotics including tetracycline, aminoglycosides, macrolides, chloramphenicol and fluoroquinolones. Zermina *et al*., (2012) conducted study in Rawalpindi and found 92% of MRSA were resistant to Ampicillin. Amoxycillin, a derivative of penicillin, has been in use for last two decades, so development of high resistance is obvious. Perveen *et al*., (2013) examined that 67.01% MRSA showed resistance against gentamicin that is lower than the 76.35% of MRSA resistance towards gentamicin and 69.10 % of MRSA were resistant to erythromycin.

Out of 16 strains, 15 different types of patterns were observed. Two isolates (S6, S12) showed 100% of resistant pattern, 4 isolates (S4, S5, S15, S16) showed 90%, 5 isolates (S1, S7, S11, S14) showed 80%, 3 isolates (S3, S8, S10) showed 60% and 2 different isolates (S2, S13) showed 40% and 70% resistant (Table: 2). Mohankumar *et al*., (2012) also observed among 100 isolates 72 MRSA isolates showed more than 50% of resistance and 31 MRSA showed more than 70% resistance, whereas only 2 MRSA showed more than 90% resistance pattern. One isolate possessed maximum resistance of 95.83% and 5 isolates showed 81.8%.

In vitro preliminary screening of the antibacterial activity of the plant extracts from *T. chebula* were studied against MRSA using disc diffusion method (Table: 3). Ethanol extract showed higher inhibition zone (14.30±0.42mm to 25.40±0.56mm), followed by acetone (12.35±0.35mm to 25.30±0.42mm), hot water (11.60±0.84mm to 25.15±0.21mm), methanol (12.40±0.56mm to 25.35mm), and cold water (10.50±0.70mm to 29.75±0.35mm).

The extract was further subjected to the broth microdilution method to determine the MIC. Many strains showed MIC of <0.244 to 1.953mg/ml for all extract (Table: 3). Similarly Sato *et al*., (1997) reported that a fruit ethanol extract of *Terminalia chebula* exhibited antibacterial activity against MRSA. Aneja *et al*., (2009) showed the aqueous extract of *Terminalia chebula* strongly inhibited the growth of *Streptococcus mutans* of salivary bacteria. Phadke *et al*., (1989) concluded 1mg/ml disc of *T. chebula* showed maximum inhibition against *S. epidermidis*, followed by *B. subtilis*. Bag *et al*., (2011) reported ethanolic extract of *Terminalia chebula* fruit showed strong antibacterial activity against multi drug resistant uropathogenic *E. coli.*

Higher percentage of extraction yield was observed in methanol (46.4%) followed by cold water (42.5%), hot water (40.3%) followed by acetone (39.5%) and ethanol (33.7%). The *Terminalia chebula* extracts were found to contain tannins, flavonoid, terpenoid, alkaloid was observed all the five extracts (Table: 4). Amino acid, phenolic and aromatic compounds were identified by using column and TLC chromatography technique. Totally 20 fractions were collected using column chromatography and all three compounds observed from 5^{th} fraction to 16^{th} fraction (Table: 5). The antibacterial activity responsible due to the presence of chemical constituents as tannins, flavonoid, terpenoid, alkaloid, Amino acid, phenolic and aromatic compounds. These results are coinciding with the results revealed by Das *et al.,* 2010, Raju *et al.,* 2009 has also reported on presence of tannins, carbohydrates, glycosides, phenols, alkaloids and flavonoids in *T.chebula.*

In this study, the anti-MRSA potential of *Terminalia chebula* fruits evaluated using different solvent (ethanol, methanol, acetone) and aqueous (hot water and cold water) extraction. The ethanol, methanol and cold water extraction exhibited the highest zone of inhibition than other two extracts. *Terminalia chebula* contains compounds like terpenoids, flavonoids, alkaloids, tannin, phenolic compounds, aminoacid and aromatic compounds which could be responsible for antibacterial activity. We concluded the plants as sources of medicinal compounds have continued to play a dominant role in the maintenance of human health care.

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Table - 1: Antibiotic Resistant and Sensitivity Pattern of Methicillin Resistant *Staphylococcus aureus*:

Antibiotics	No of Resistant Strains	Resistant %	No of Sensitive Strains	Sensitive %
Ampicillin (10µg)	14	87.5	2	12.5
Cefazolin (30µg)	12	75	4	25
Chloramphenicol (30µg)	13	81.25	3	18.75
Erythromycin (15µg)	11	68.75	5	31.25
Gentamycin (10µg)	9	56.25	7	43.75
Norfloxacin (10µg)	11	68.75	5	31.25
Rifampicin (5µg)	15	93.75	1	6.25
Methicillin (5µg)	14	87.5	2	12.5
Oxacillin (5µg)	13	81.25	3	18.75
Tobramycin (30µg)	10	62.5	6	37.5

Table - 2: Multidrug Resistant (MDR) Calculation of Methicillin Resistant *Staphylococcus aureus*:

Isolates	Total No of resistant antibiotics	Resistant %	Total No of Sensitive antibiotics	Sensitive %
S1	A-Cz-C-E-N-R-Ox-Me	80	G-To	20
S2	A-R-Ox-Me	40	Cz-C-E-G-N-To	60
S3	Cz-C-E-N-R-Me	60	A-G-Ox-To	40
S4	A-Cz-E-G-N-R-Ox-To-Me	90	C	10
S5	A-Cz-C-E-G-N-R-Ox-Me	90	To	10
S6,S12	A-Cz-C-E-G-N-R-Ox-To-Me	100	-	-
S7	A-Cz-C-E-G-R-Ox-To	80	N-Me	20
S8	A-C-E-N-R-Ox	60	Cz-G-To-Me	40
S9	A-Cz-Ox-To-Me	50	C-E-G-N-R	50
S10	Cz-C-N-R-To-Me	60	A-E-G-Ox	40
S11	A-Cz-C-G-N-R-To-Me	80	E-Ox	20
S13	A-Cz-C-G-R-Ox-Me	70	E-N-To	30
S14	A-C-E-N-R-Ox-To-Me	80	Cz-G-	20
S15	A-C-E-G-N-R-Ox-To-Me	90	Cz	10
S16	A-Cz-C-E-G-R-Ox-To-Me	90	N	10

A-Ampicillin (10µg), Cz- Cefazolin (30µg), C- Chloramphenicol (30µg), E- Erythromycin (15µg), G-Gentamycin (10µg), N-Norfloxacin (10µg), R- Rifampicin (5µg), Ox- Oxacillin (5µg), To- Tobramycin (30µg), Me- Methicillin (5µg).

Table – 3: Minimum Inhibition Concentration and Antibacterial Bacterial Activity of *Terminalia chebula* Extracts:

S	Ethanol		Methanol		Acetone		Cold Water		Hot Water	
	MIC (mg/ml)	ABA (mm)	MIC (mg/ml)	ABA (mm)	MIC (mg/ml)	ABA (mm)	MIC (mg/ml)	ABA (mm)	MIC (mg/ml)	ABA (mm)
S1	3.91	14.30±0.42	1.95	12.40±0.56	0.48	12.75±0.35	0.48	10.50±0.70	3.91	11.60±0.84
S2	1.95	15.75±0.35	7.81	20.30±0.42	1.95	19.30±0.42	<0.24	20.45±0.63	3.91	19.30±0.42
S3	1.95	23.20±0.28	3.91	23.90±0.41	0.48	24.15±0.21	<0.24	23.35±0.49	1.95	25.15±0.21
S4	1.95	23.45±0.63	7.81	23.20±0.28	0.97	24.35±0.47	0.97	25.70±0.42	7.81	24.80±0.28
S5	1.95	19.85±0.21	7.81	24.45±0.63	<0.24	25.20±0.28	1.95	24.50±0.77	0.97	23.40±0.56
S6	0.48	20.95±0.07	<0.24	19.90±0.14	<0.24	21.85±0.12	1.95	18.85±0.21	<0.24	21.85±0.21
S7	3.91	25.40±0.56	7.81	25.75±0.35	<0.24	25.30±0.42	0.97	29.75±0.35	<0.24	21.50±0.70
S8	7.81	25.35±0.49	7.81	20.85±0.21	0.97	22.95±0.70	3.91	24.30±0.42	3.91	23.45±0.63
S9	1.95	20.35±0.49	1.95	22.90±0.14	0.97	21.35±0.49	0.97	24.40±0.56	1.95	20.90±1.27
S10	<0.24	22.90±0.14	1.95	23.25±0.35	0.97	24.30±0.42	0.97	24.85±0.21	3.91	23.80±0.28
S11	0.48	17.30±0.42	0.97	15.40±0.56	<0.24	17.80±0.28	7.81	18.30±0.42	0.48	16.65±0.91
S12	0.24	20.20±0.28	0.97	18.35±0.49	1.95	17.20±0.28	1.95	19.25±0.35	0.97	18.20±0.28
S13	0.97	18.85±0.21	0.97	17.75±0.35	1.95	20.40±0.56	3.91	17.45±0.63	0.97	17.80±0.28
S14	0.48	20.30±0.42	0.97	18.35±0.49	1.95	18.45±0.63	0.97	16.60±0.56	0.97	19.35±0.49
S915	0.97	24.90±0.14	0.48	23.30±0.42	0.48	24.95±0.07	0.48	25.50±0.63	1.95	23.90±0.14
S16	<0.24	18.45±0.63	0.48	15.10±0.14	<0.24	18.15±0.21	0.48	19.20±0.28	<0.24	17.10±0.14

S: isolates, <: Less than, Values are expressed as Mean deviation ± Standard deviation, MIC: Minimum Inhibition Concentration, ABA: Antibacterial Activity.

Table - 4: Final amount of Extraction yield Phytochemical analysis in *Terminalia chebula* with different extract:

S. No	Name of Extract	Yield of Final Residue (1:5 Ratio)	Phytochemical			
			Terpenoid	Flavonoid	Alkaloid	Tannin
1	Ethanol	33.7 %	+	+	+	+
2	Methanol	46.4 %	+	+	+	+
3	Acetone	39.5 %	+	+	+	+
4	Cold water	42.5 %	+	+	+	+
5	Hot water	40.3 %	+	+	+	+

+ : Present, - : Absent

Table – 5: Phytochemical Analysis by Using Chromatography Technique:

Solvent selection / ratio (%)	No of fraction in column chromatography	Fraction color	Quality analysis of fractionated sample (TLC method)	Presence / Absence
Acetone 100	F1-F4	Colorless	Amino acid	-
			Phenolic compound	+
			Aromatic compounds	-
A+E 75:25	F5-F8	Brown	Amino acid	+
			Phenolic compound	+
			Aromatic compounds	+
A+E 50:50	F9-F12	Brown	Amino acid	+
			Phenolic compound	+
			Aromatic compounds	+
A+E 25:50	F13-F16	Dark Brown	Amino acid	+
			Phenolic compound	+
			Aromatic compounds	+
Ethanol 100	F17-F20	Light Brown	Amino acid	-
			Phenolic compound	+
			Aromatic compounds	-

+ : Present, - : Absent, A+E: Actone+Ethanol, TLC : Thin Layer Chromatography