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Antimicrobial Potential of Plant Seed Extracts against Multidrug Resistant Methicillin Resistant *Staphylococcus aureus* (MDR-MRSA)

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Abstract

Based on ethnopharmacological information, four different varieties of seeds were obtained from authentic seed suppliers. Ethanol, methanol, acetone, chloroform and petroleum ether seed extracts were assessed for antibacterial activity against wound isolates of Multi Drug Resistant - Methicillin Resistant *Staphylococcus aureus* (MDR-MRSA). Ethanol, methanol and acetone extracts of *Moringa oleifera, Elettaria cardamomum* and *Tamarindus indica* seeds showed more effective anti MRSA activity than *Artocarpus heterophyllus.* In addition *Moringa oleifera* seed extracts may have the potential to restore the effectiveness of β-lactam antibiotics against MRSA.

Keywords: *mec*A, Antibacterial, Checkerboard assay, Seed extracts, Multi drug resistant

1. Introduction

Antibiotic resistance is the ability of a microorganism to withstand the effects of an antibiotic. The extensive use of antibiotics over the last 50 years has led to the emergence of bacterial resistance and to the dissemination of resistance genes among pathogenic microorganisms. *Staphylococcus aureus* is one of the most important pathogens that can cause suppuration, abscess formation, a variety of pyogenic infection and even fatal septicemia in human beings. MRSA is still considered as an emerging pathogen and public health threats result from the spread of hospital-acquired as well as community-acquired MRSA (Chambers, 2001).

The heterogeneous expression of methicillin resistance can make it difficult to determine the resistance phenotype definitively (Frebourg et al., 1998), therefore detection of the *mec*A gene remains the "gold standard" (Bignardi et al., 1996). During the last decade, many studies have demonstrated the extremely high capacity of PCR for specifically detecting bacteria and genes of interest (Salisbury et al., 1996). Several authors have already shown the feasibility of the PCR methodology for the identification of *S. aureus* strains and for the detection of antibiotic resistance genes (Cockerill, 1999).

MRSA is resistant to not only methicillin and other β- lactams but also may other antibacterial agents; therefore new agents are needed to treat the MRSA. The treatment of infectious diseases with antimicrobial agents continues to present problems in modern-day-medicine with many studies showing a significant increase in the incidence of bacterial resistance to several antibiotics (Finch, 1998). Many plants have been investigated scientifically for antimicrobial activity and a large number of plant products have been shown to inhibit growth of pathogenic bacteria. Though

pharmacological industries have produced a number of new antibiotics in the last three decades, resistance to these drugs by microorganisms has developed. Medicinal plants are natural resources, yielding valuable products which are often used in the treatment of various ailments. Plant materials remain an important resource for combating illnesses, including infectious diseases, and many of the plants have been investigated for novel drugs or templates for the development of new therapeutic agents (Konig, 1992). Most previous studies on plants for antibacterial activity were mainly performed with the extract of aerial parts of leaves, stem, flowers and ground level roots and rhizomes but meager research was done with seed extracts. The present investigation was conducted to evaluate the antibacterial activity of *Artocarpus heterophyllus* (Palaa), *Elettaria cardamomum* (Ellaykka), *Moringa oleifera* (Murungai) and *Tamarindus indica* (Puli) seed extracts against MDR-MRSA from wound infection.

2. Experiments

2.1 Antibiotic susceptibility test

Staphylococcal strains isolated from Erode district hospitals in Tamilnadu, India from wound infections were used. The antibiotic sensitivity profile of the 12 *S. aureus* isolates were determined according to the method of Bauer-Kirby (Bauer et al., 1966) using 12 antibiotics placed on the surface of MHA medium seeded with the test organism. Antibiotic susceptibility was determined from the size of the inhibition zone, according to the guidelines of the National Committee for Clinical Laboratory Standards (NCCLS, 1997), and the strains were defined as MRSA based on occurrence of the *mec*A gene and their resistance to methicillin.

2.2 PCR for mecA gene

Detection of the *mec*A gene in the *Staphylococcus aureus* isolates was performed by polymerase chain reaction. Total genomic DNA was obtained from *Staphylococcus aureus.* A single colony was taken from a nutrient agar plate that had been incubated overnight and emulsified into 50 µl of lysostaphin (100mg/l). After incubation for 10 min at 37ºC, 50 µl of proteinase K (100mg/l) and 150 µl of TE buffer were added to the suspension and incubated for a further 20 min at 37ºC. Five microlitres were then taken from the suspension and used directly for the PCR (Ubukata et al., 1992). Synthetic oligonuclotide used were *mec*A F primer 1282 (5'–AAA–ATC–GAT–GGT–AAA–GGT–TGG–C–3') and *mec*A R primer 1793 (5'– AGT-TCT-GCA-GTA-CCG-GAT-TTG-C-3') and reaction condition was described by Merlino et al., 2002. A Eppendrof mastercycler was programmed with the initial denaturation 5 min at 94ºC, 30 cycles with a 60 seconds denaturation at 94ºC, 30 seconds annealing at 50ºC and 90 seconds extension at 72ºC and 2 min final extension at 72ºC and a holding at 4ºC until the sample was analyzed. Twenty microlitres of the PCR product was then analyzed by agarose gel electrophoresis. Gels were stained with ethidium bromide and visualized by using UV light. These conditions yielded a 533bp PCR product corresponding to *mec*A gene when compared with standard marker of 100-1000bp ladder.

2.3 Plant material and Extraction

The seeds were collected from Namakkal Dt. Tamilnadu, India, and they were identified by Dr. R. Murugan, Department of Botany, Government Arts and Science College, Krishinagiri, Tamilnadu. Seeds were washed with water, surface sterilized with 10% sodium hypochloride solution, then rinsed with sterile distilled water and air dried using a laminar air flow. The seeds were ground into a fine powder. Powdered air dried seeds (100g) of *Artocarpus heterophyllus*, *Elettaria cardamomum, Moringa oleifera,* and *Tamarindus indica* were soaked separately in 500ml of ethanol, methanol, acetone, chloroform and petroleum ether for 72 hrs at room temperature. Filtered extracts were dried using a rotary evaporator at 45ºC. Then the extract was stored at 4ºC for further use.

2.4 Antimicrobial assay

The agar disc diffusion method was used to determine the antibacterial activity. Sterile discs (6mm, Hi-media, India) were loaded with 50µl of (30mg/ml) seed extracts dissolved in 5% dimethyl sulfoxide (DMSO) and were left to dry for 6 to 10 hrs in sterile condition. Bacterial suspensions were diluted to match the 0.5 McFarland standard scales (approximately $1.5x10^8$ CFU/ml). Muller Hinton Agar (MHA) was poured into Petri dishes to give a solid plate and inoculated with 100 µl of suspension containing $1.5x10^8$ CFU/ml of bacteria, the discs treated with extracts were placed onto petri plates. Methicillin was used as positive control and paper disc treated with DMSO was used as negative control. The plates were then incubated at 37ºC for 24hrs to 36 hrs, inhibition zones diameter around each of the discs were measured and recorded at the end of the incubation time.

2.5 Checkerboard assay

Minimum inhibition concentrations of the plant extracts were tested by the checkerboard assay method (Kumarasamy et al., 2002). The test extracts were dissolved in 5% DMSO to obtain 30mg/ml stock solutions. The 96 well sterile plates were taken and 100µl of seed stock solution was added to row 1. Fifty microlitres of sterile normal saline was added to row 2 to 11. Two fold dilutions were performed by transferring 50 µl of extracts from row 1 to 2 using a multi channel pipette. The above process was repeated up to row 12. Forty microlitres of double strength nutrient broth and 10 µl of

bacterial solutions were added to all the wells, so the final concentrations of inoculum in all the wells were $5x10^6$ CFU/ml. To prevent dehydration, the plates were covered with a sterile plastic cover and then incubated at 37ºC for overnight. Bacterial growth was determined after addition of 40 µl of p-iodonitro tetrazolium violet (0.2mg/ml). The MIC^{INT} was determined as the lowest sample concentration at which no red color appeared. To determine the minimal bactericidal concentration, the broth was taken from each well and inoculated in nutrient agar for 24 hrs at 37°C.

3. Results and Discussion

3.1 Antibiotic susceptibility test

All 12 isolates were shown to be Multi Drug Resistant (MDR) strains; resistant to at least 6, out of 12 antibiotics. Eleven different antibiotic patterns were identified (Table-I), two isolates were resistant to all 12 antibiotics, 2 to 12 (pattern # 1), 1 to 11 (pattern # 2), 2 to 10 (pattern # 3, 4), 3 to 8 (pattern # 5, 6, 7), 4 to 7 (pattern # 8 to 11). The Multiple Antibiotic Resistant (MAR) index was 0.91 in one stain, 0.83, and 1.0 in 2 stains, 0.66 in 3 strains and 0.58 in 4 strains. The MAR index of isolated bacteria was greater than 0.2, which implies that strains of such bacteria originate from an environment where several antibiotics were used (Ehinmidu, 2003). Most of wound isolates showed multiple antibiotic resistances in the study area, which may be due to large portion of the bacteria isolate being previously exposed to several antibiotics.

3.2 Analysis of mecA gene

The genotypic expression of the 12 clinical wound *S. aureus* isolates was examined in this study (Table-I). All the isolates were tested for the phenotypic confirmation for MRSA, hence all were genetically confirmed to be MRSA using PCR. All *S. aureus* were positive for the *mec*A gene in the molecular weight of 533bp. Disc diffusion susceptibility testing of the isolates with some specific antibiotic lacked both sensitivity and specificity, with a large number of strains reported in the intermediate category. Some results were difficult to read because of faint growth at 24 hrs. This was not reported by the studies that showed a high degree of correlation between the disc test and the presence of *mec*A (McDonald et al., 1995). Currently, multiple antibiotic resistant *S. aureus* strains constitute a major healthcare problem, since they are the etiologic agent of several nosocomical and skin infection. For that reason, accurate detection of resistant isolates constitutes a critical goal of clinical microbiology and therefore PCR assays have become an essential tool in laboratory programs.

3.3 Antimicrobial assay

Antibacterial resistance, especially among gram positive bacteria, is an important issue that has created a number of problems in treatment of chronic wounds and necessitates the search for alternative drugs or natural antibacterial agents. The ethanol, methanol, acetone, chloroform and petroleum ether extracts were assayed against 12 MDR-MRSA isolates by agar disc diffusion assay. The control Dimethyl sulphoxide (DMSO) did not inhibit any of the MRSA isolates. The 30mg/ml concentrations of the extracts were found to have a similar or even better effect compared with methicillin.

All seed extracts *Elettaria cardamomum, Moringa oleifera, Tamarindus indica* were more effective than conventional antibiotics except *Artocarpus heterophyllus* with the antimicrobial screening. The antibacterial activity of methanol, ethanol and acetone extracts of all seeds showed considerable efficacy compared with the chloroform and petroleum ether extracts against all the MRSA isolates (data not shown). The results obtained from the screening of the seed extracts, 3 seeds were showed promising results, especially in high polar ethanol, methanol and acetone extracts of *Elettaria cardamomum* (10 to 17 mm zone*), Moringa oleifera* (17 to 22 mm zone) and *Tamarindus indica* (8 to 15 mm zone) against MDR-MRSA (Table -II). To our knowledge, this may be the first report that *Elettaria cardamomum, Tamarindus indica* and *Moringa oleifera* seed extracts were shown to have antibacterial activities against MDR-MRSA isolates from wound infection. Minimum inhibitory zones were observed in all the seed extracts against chloroform and petroleum ether except *E. cardamomum* seed (data not shown). *Artocarpus heterophyllus* seed extracts did not show good inhibitory activity. However, it is interesting to note that *Artocarpus heterophyllus* which have traditionally been used for antibacterial activity, this indicates that the active compounds are mainly distributed in aerial parts, roots and rhizomes, but not in the seeds.

Several studies have revealed that *M. oleifera* had various antibacterial activities (Dayrit et al., 1990). Crude methanolic extract of *M. oleifera* at 10% w/v concentration showed no activity against the bacteria, but column chromatographic fraction showed antibacterial activity against *S. aureus, P. aeruginosa, K. pneumoniae* and *E. coli* tested at 10% w/v (Khesorn, 2006). Doughari et al., (2007) demonstrated that the antibacterial activity of the ethanol extracts of the plant material showed 8mm zone of inhibition at 10mg/ml against *Salmonella typhi* and also found common phyto-constituents saponins, tannins and phenols in both the plant extracts, while alkaloids were only seen in *M. oleifera* and anthrax quinines only in *B. aegyptiaca*. The presence of these constituents has been reported to account for the expression of antimicrobial activity by plants (Pretorius and Watt, 2001).

Similar to our findings, Agaoglu et al., (2005) reported that the extracts of *Elettaria cardamomum* seed displayed a variable degree of antibacterial activity on different microorganisms. *S. aureus* was found to be more sensitive strain

than the others. Some investigators noted that sensitivity of microorganisms to chemotherapeutics differs according to type of strains. Antimicrobial characteristics of herbs are due to various chemical compounds including volatile oils, alkaloids, tannins and lipids that are present in their tissue (Agaoglu et al., 2005). The inhibitory effects of *E. cardamomum* seeds detected in this study may be due to the presence of volatile oils. The chemical composition of *E. cardamomum* varies considerably with variety, region and age of the product. The content of volatile oils in the seeds is strongly dependent on storage conditions (Korikontimath et al., 1999).

Acetone extract of the *T. indica* was more effective than ethanol and methanol extracts. Muthu et al., (2005) reported that the methanolic extracts of *T. indica* alone showed anti *B. pseudomallei* activity and also observed at all concentrations tested that diameter of inhibition zone varied from 10-12 mm chloramphenicol (30µg) and doxycycline (30 µg) inhibited *B. pseudomallei* showed 18 and 21 mm zone respectively. The MIC value of the methanolic extracts of *T. indica* leaves against *B. pseudomallei* was 125µg/ml. The crude ethanol and methanol extracts of the seed of *Artocarpus heterophyllus* exhibited antibacterial activity against MDR-MRSA but minimum zone was observed (<12mm). Acetone, chloroform and petroleum ether extract did not inhibit the tested organisms. Khan et al., (2003) reported the antibacterial activity of *Artocarpus heterophyllus* crude methanolic extract of the stem and root bark, stem and root heart-wood, leaves, fruits and seeds.

3.4 Checkerboard assay

The checkerboard assay is probably the most convenient way of assessing the antibacterial potential of plant extracts. In this method, the test extracts are able to diffuse more easily into the media. Advantage over the agar disc diffusion method includes increased sensitivity for small quantities of extract, ability to distinguish between bacteriostatic and bactericidal effects and quantitative determination of Minimal Inhibitory Concentration (MIC). The use of a colorimetric indicator eliminates the need for a spectrophotometric plate reader and avoids the ambiguity associated with visual comparison. According to the disc diffusion zone, three seeds were selected for MIC test in the checkerboard assay method. The ethanol, methanol and acetone extracts from *M. oleifera, E. cardamomum* and *T. indica* showed MIC between 0.11 mg/ml to 1.87 mg/ml against all MDR-MRSA (data not shown). Two seed extracts of *E. cardamomum* and *T. indica* showed maximum MIC value of 1.87 mg/ml and *M. oleifera* was showed 0.46 mg/ml. The MBC was recorded as the lowest concentration of the extract that did not permit any visible bacterial growth on the appropriate agar plate after the period of incubation. Although the MBC results varied between organisms tested, in most cases the MBC was next to the MIC value.

In conclusion, our results showed that the seed extracts of *M. oleifera* possesses potential antibacterial activity against MDR-MRSA. We believe that these findings will be helpful to many researchers in the field of the evolution of antibacterial activities in plant seeds.

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Table 1. Antibiotic resistant profiles and MAR index of *Staphylococcus aureus*

S. No	Isolates	Class	*mecA* gene	Antibiotic Resistant Pattern	No. of Resistant Ab	No. of Sensitive Ab	MAR Index	% Frequency	Pattern No.	Types of Resistant
1	SaW 1	MRSA	+	T-Of-Cf-R-C-P-M-Ox	8	4	0.666	66.6	5	MDR
2	SaW 2	MRSA	+	T-Of-Cf-Va-E-P-M-Ox	8	4	0.666	66.6	6	MDR
3	SaW 3	MRSA	+	T-G-Of-Cf-R-Va-E-C-P-K-M-Ox	12	0	1.00	100.0	1	MDR
4	SaW 4	MRSA	+	T-Of-Cf-R-Va-E-C-P-M-Ox	10	2	0.833	83.3	3	MDR
5	SaW 5	MRSA	+	T-G-Of-Cf-E-C-P-K-M-Ox	10	2	0.833	83.3	4	MDR
6	SaW 6	MRSA	+	R-Va-E-P-K-M-Ox	7	5	0.583	58.3	8	MDR
7	SaW 7	MRSA	+	G-R-Va-E-C-P-M-Ox	8	4	0.666	66.6	7	MDR
8	SaW 8	MRSA	+	T-G-Of-Cf-R-Va-E-C-P-K-M-Ox	12	0	1.00	100.0	1	MDR
9	SaW 9	MRSA	+	T-Of-Cf-Va-P-M-Ox	7	5	0.583	58.3	9	MDR
10	SaW 10	MRSA	+	T-G-Of-Cf-R-Va-E-P-K-M-Ox	11	1	0.916	91.6	2	MDR
11	SaW 11	MRSA	+	T-Of-Va-C-P-M-Ox	7	5	0.583	58.3	10	MDR
12	SaW 12	MRSA	+	T-G-Va-P-K-M-Ox	7	5	0.583	58.3	11	MDR

SaW: *Staphylococcus aureus* Wound, MRSA: Methicillin Resistant *Staphylococcus aureus*, +: Positive, MAR: Multiple Antibiotic Resistant, Ab: Antibiotics, MDR: Multi Drug Resistant.

T: Tetracycline, G: Gentamicin, Of: Ofloxacin, Cf: Ciprofloxacin, R: Rifampicin, Va: Vancomycin, E: Erythromycin, C: Chloramphenicol, P: Penicillin G, K: Kanamycin, M: Methicillin, Ox: Oxacillin.

Table 2. Antibacterial activity of seed extracts against 12 MDR MRSA

S.No	MRSA Isolates	Mean Zone of Inhibition (mm) 30mg/ml													
		Artocarpus heterophyllus			*Elettaria cardamomum*			*Moringa oleifera*			*Tamarindus indica*			Methicillin	DMSO
		E	M	A	E	M	A	E	M	A	E	M	A	5 mcg	5%
1	SaW1	9	10	8	17	16	16	20	19	20	10	9	14	-	-
2.	SaW2	8	10	-	17	16	16	17	18	17	9	9	14	-	-
3.	SaW3	10	10	-	13	14	12	20	21	22	8	9	14	-	-
4.	SaW4	7	11	-	11	12	11	20	20	18	8	9	15	11	-
5.	SaW5	11	11	8	17	16	16	21	20	18	8	9	13	10	-
6.	SaW6	8	12	7	11	12	13	20	20	20	10	8	14	-	-
7.	SaW7	11	11	-	11	12	13	21	21	20	9	8	13	-	-
8.	SaW8	7	11	7	15	14	10	20	20	20	8	9	13	13	-
9.	SaW9	12	10	-	12	14	14	19	19	19	8	9	12	14	-
10.	SaW10	12	10	-	15	14	16	19	20	18	9	10	14	7	-
11.	SaW11	12	10	-	16	16	16	20	19	18	9	11	15	-	-
12.	SaW12	12	11	-	15	15	15	19	21	20	9	9	15	7	-

MRSA: Methicillin Resistant *Staphylococcus aureus*, SaW: *Staphylococcus aureus* Wound, E: Ethanol, M: Methanol, A: Acetone. -: no inhibition of the concentrated tested, DMSO: Dimethyl sulphoxide.