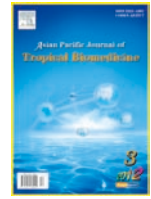




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Isolation of antibacterials from the mangrove, *Avicennia marina* and their activity against multi drug resistant *Staphylococcus aureus*

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ABSTRACT

Objective: leaf extract of *A. marina* was tested on the growth of clinically isolated multi–drug resistant *Staphylococcus aureus* and its bioactive compounds were attempted. **Method:** Clinical strain of *Staphylococcus aureus*, were isolated from sputum, pus and blood of different patients and 22 strains were screened for antibiotic susceptibility. *Avicennia marina* was extracted in different solvents and antibacterial assay was carried out using Kirby–Bauer’s disk diffusion method. Crude methanol extract of the mangrove was loaded on a silica gel column and eluted with chloroform and methanol (9:1 to 1:9) followed by ethyl acetate and methanol (9:1 to 1:9). Based on in vitro assay, the 12th fraction was subjected for minimum inhibitory concentration (MIC). The active fraction was analysed by using a Clarus 500 Perkin Elmer gas chromatography. **Result:** Based on the antibiotic susceptibility test, six strains (RMSA 6, RMSA12, RMSA16, RMSA18, RMSA19 and RMSA21) were resistance against methicillin, vancomycin and ciprofloxacin. The results indicated that the methanolic leaf extract showed the highest antibacterial activity against all the tested strains RMSA 6 (16mm), RMSA12 (15 mm), RMSA16 (13 mm), RMSA18 (10 mm), RMSA19 (17 mm) and RMSA21 (16 mm). The MIC of the partially purified extract showed potential results against all the multidrug resistant strains however, the lowest concentration was recorded against RMSA 6, RMSA19 and RMSA21 strain. In the GC–MS results, 5 bioactive compounds were identified from the partially purified extract of *A. marina*. **Conclusion:** The methanolic extract of *A. marina* has the more potential candidate to inhibit against multidrug resistant *S. aureus*.

1. Introduction

In recent years, drug resistance to human pathogenic bacteria and fungi has been commonly reported from all over the world. Therefore, the increasing prevalence of multidrug resistant strains of microorganisms and the recent appearance of strains with reduced susceptibility to antibiotics raises an urgent need to search for new sources of antimicrobial agents [1]. *Staphylococcus aureus* is one of the most important pathogens that causes suppuration, abscess formation, a variety of pyogenic infections and even fatal septicemia in human beings. Due to the development of methicillin resistance among *S. aureus* isolates, treatment of this infection has become a problematic one [2]. Methicillin–resistant *Staphylococcus aureus* (MRSA) is a frequent cause of nosocomial pneumonia. Inadequate or inappropriate

antimicrobial therapy, often caused by antimicrobial resistance, is associated with increased mortality for these infections. Plants are the richest source of drugs of traditional systems of medicine, modern medicines, nutraceuticals, food supplements, folk medicines, pharmaceutical intermediates and chemical entities for synthetic drugs. The use of plants and plant products as medicines could be traced as far back as the beginning of human civilization [1].

Mangroves are used in traditional medicine for the treatment of many diseases [3]. The mangrove plants have also been proved for antiviral, antibacterial and anti–ulcer properties [4, 5]. Mangroves have been a source of several bioactive compounds and they have been used in folklore medicines and extracts have proven activity against human, animal and plant pathogens. Secondary metabolites like alkaloids, phenolics, steroids, terpenoids have been characterized from mangroves and have toxicological, pharmacological and ecological importance [6, 7]. There is a continuous and urgent need to discover new antimicrobials

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with diverse chemical structures and novel mechanism of action for new and re-emerging infectious diseases. In the present study, biologically active metabolites of mangrove, *Avicennia marina* leaves were extracted and screened against clinically important multi-drug resistant *Staphylococcus aureus*.

2. Materials and methods

2.1. Bacterial culture

Twenty two clinical strains of *Staphylococcus aureus* were obtained from Rajah Muthiah Medical college, Annamalai University. They were cultured in nutrient broth for 24 h and the fresh inoculum was subjected to susceptibility test.

2.2. Antibiotic susceptibility test

The obtained cultures were confirmed for their identification based on gram's stain, catalase test, mannitol fermentation test, and coagulase test. Antibiotic susceptibility was detected by disc diffusion technique using ampicillin, gentamicin, penicillin, vancomycin, erythromycin, ciprofloxacin, tetracycline and methicillin discs [2].

2.3. Collection and extraction of mangrove plant leaves

Healthy leaves of *A. marina* were collected from the Vellar estuary, Parangipettai, Tamil Nadu, India. After washing with distilled water, the leaves were shade dried, powdered and extracted separately in methanol, ethyl acetate and chloroform. Twenty grams of plant powder was taken with 100 ml of solvents and kept in shaker for 24 hrs. After centrifuged at 5000 rpm, the solvent phase was separated and evaporated. The crude was stored at 40 C and used for further studies.

2.4. Antibacterial activity

The antibacterial assay was carried out using Kirby-Bauer's disk diffusion method [8]. The bacterial inoculum (0.5 McFarland) was swabbed over the surface of the media using sterile cotton swab to ensure the confluent growth of the organism. The 5 mm diameter discs were prepared with Whatman No.1 paper along with 100, 200 and 400 μ g of individual fractions. Vancomycin (30 μ g/disc) was used as positive reference standard to determine the sensitivity of the tested strains and Di-methyl sulfoxide (DMSO) was used as negative control. The inoculated plates were incubated at 37°C for 24 h and the inhibition zone was observed. All the experiments were carried out in triplicate.

2.5. Partial purification by column chromatography

Crude methanol extract of *A. marina* was loaded on a silica gel column and elucidated with chloroform and methanol (9:1 to 1:9) followed by ethyl acetate and methanol (9:1 to 1:9). Totally 18 fractions were collected and each fraction was tested for In vitro activity to select the active fractions [9].

2.6. Assessment of minimum inhibitory concentration

Based on in vitro assay, 12th fraction exhibiting antibacterial activity was alone subjected for MIC. It was dissolved in DMSO to obtain 4000 μ g/ml stock solution. About 0.5 ml stock solution was incorporated into 0.5 ml of Muller-Hinton broth to make various concentrations. The stock concentration was changed again and again for analysis the MIC value. Fifty microliter of standard suspension of the test organism (0.5 McFarland) was transferred to each test tube. The crude extract and DMSO was used as control. After 24 h of incubation, the results were evaluated by reading absorbance at 620 nm in spectrophotometer [10].

2.7. Gas chromatography and mass spectroscopy (GC-MS)

The active fraction was analysed using a Clarus 500 Perkin Elmer gas chromatography equipped with an Elite-5 capillary column (5% Diphenyl 95% dimethyl poly siloxane) (30nm \times 0.25mmID \times 0.25 μ mdf) and mass detector turbomass gold of the company which was operated in EI mode. Helium was the carrier gas at a flow rate of 1 ml/min. the injector was operated at 200°C and the oven temperature was programmed as follows; 60°C for 15min, then gradually increased to 280°C at 3min. The identification of components was based on comparison of their mass spectra with those of Wiley and NBS Libraries.

2.8. Statistics

Data were analyzed using one-way analysis of variance (ANOVA) to discover the significant difference at the 5% ($P < 0.05$) level.

3. Results

Leaf extract of *A. marina* was tested against 22 *Staphylococcus aureus* stains Six strains (RMSA 6, RMSA12, RMSA16, RMSA18, RMSA19 and RMSA21) were resistant against methicillin, vancomycin and ciprofloxacin (Table.1). In the present investigation, ethyl acetate, methanol and chloroform extracts of *A. marina* was screened for antibacterial activity against multidrug resistant strains of *S. aureus*. The results indicated that the methanolic leaf extract showed the highest antibacterial potential against all the tested RMSA 6 (16mm), RMSA12 (15 mm), RMSA16 (13 mm), RMSA18 (10 mm), RMSA19 (17 mm) and RMSA21

Table 1. Antibiotic susceptibility patterns of clinical isolates *Staphylococcus aureus*

| | Ampicillin | Gentamicin | Penicillin | Vancomycin | Erythromycin | Ciprofloxacin | Tetracycline | Methicillin |
|--------|------------|------------|------------|------------|--------------|---------------|--------------|-------------|
| RMSA 6 | S | S | I | R | S | R | I | R |
| RMSA12 | I | S | R | R | S | R | I | R |
| RMSA16 | R | I | R | R | R | R | I | R |
| RMSA18 | R | S | S | R | I | R | S | R |
| RMSA19 | I | S | I | R | S | R | I | R |
| RMSA21 | S | I | S | R | S | R | S | R |

S – Sensitive, R – Resistant, I – intermediate

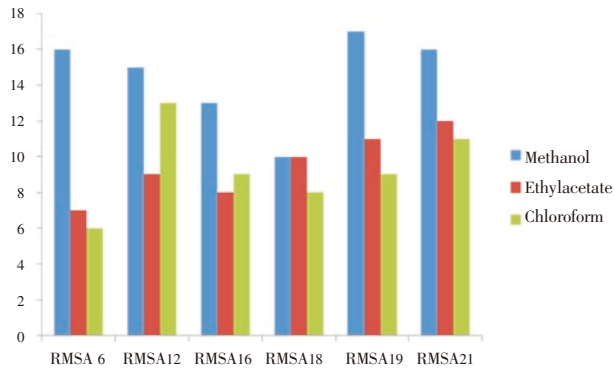


Figure 1. Antibacterial activity of mangrove extract against multi-drug resistant *Staph. aureus*

(16 mm) (Fig.1). *A. marina* extract were partially purified by column chromatography and the same was checked against multidrug resistant strains of *S. aureus*. The MIC of the methanolic extract was found to be the lowest concentration against RMSA6 (150 μ g/ml), RMSA19 (75 μ g/ml) and RMSA21 (150 μ g/ml) strain (Fig.2).

In the GC–MS, 5 bioactive compounds were identified from the partially purified extract of mangrove plant. The identification was performed based on the peak areas, molecular weight and molecular formula. The n-hexadecanoic acid was recorded maximum at the retention time of 30.94 with 10.7026 % of peak value followed by 2-cyclohexen-1-one, 4-hydroxy-3,5,5-trimethyl-4-(3-oxo-1-butenyl) (8.6%), phytol (8.4 %), hexadecanoic acid, ethyl ester (6.2 %) and 3,7,11,15-tetramethyl-2-hexadecan-1-ol (4.9 %) at the retention time of 27.89, 33.27, 31.23 and 28.27 respectively (Fig-3). These five compounds were found active molecules responsible for the inhibition of tested bacteria.

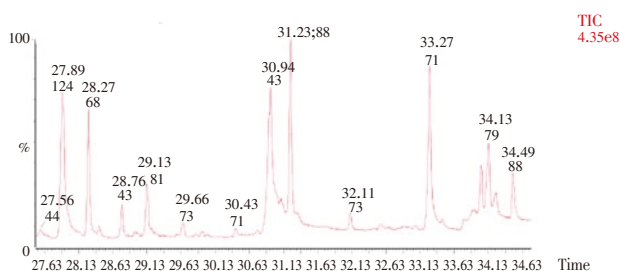


Figure 3. GC–MS chromatogram of the partially purified active fraction of *A. marina* leaf extract

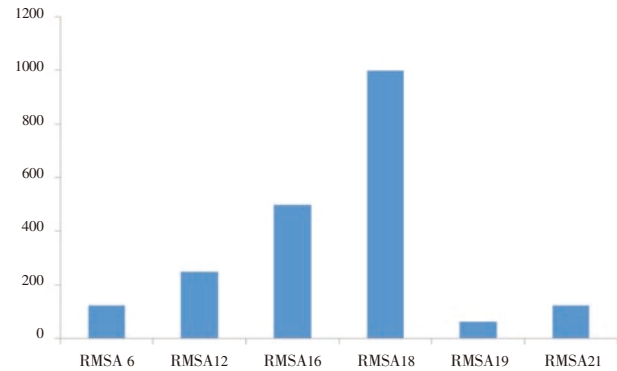


Figure 2. MIC values produced by *A. marina* leaves against multi-drug resistant *Staph. aureus*

4. Discussion

The antibacterial activity exhibited by the mangrove leaf extract could be due to the presence of phytochemicals such as, alkaloids, tannins, flavonoids and sugars, present in the plant extracts [11]. The hypocotyls of *R. apiculata*, *A. marina* and *R. mucronata* appear to have broad spectrum of antibacterial activity [12]. In India, the epidemiology of MRSA is changing over the past few decades. The resistance of MRSA to β -lactams like penicillin and amoxycillin is 100% [13]. Some of the preliminary studies have demonstrated that plant extracts have antibacterial activity against pathogenic bacteria stains such as, *Staph. aureus*, *E. coli*, *Pseudomonas* and antibiotic resistant strains. In the present findings *A. marina* extract showed inhibitory activity against multi-drug resistant *S. aureus*. *Staphylococcus aureus* stains were resistant to ciprofloxacin, methicilin and vancomycin based on the antibiotic susceptible test (Table.1). Mangroves such as *Rhizophora mucronata*, *R. lamarekii* and *Bruguiera cylindrica* have earlier reported for antibacterial activity against MRSA [5]. The antibacterial activities of the *A. marina* extract against 6 multidrug resistant *Staph. aureus* were assayed in vitro by agar well diffusion method. Previous study has reported that the mangrove plants possess higher antibacterial potency than the salt marsh halophytes [2]. The highest activity was recorded with the methanol extract. The methanolic leaf extract showed the highest antibacterial potential, followed by chloroform and ethyl acetate extracts of mangrove extract. The methanol extract showed maximum activity against RMSA19 (17 mm) and minimum activity RMSA18 (10mm). Overall the methanolic extract showed promising activity as compared to other extracts. Compared

with earlier report [9] the crude ethyl acetate extracts of *A. officinalis* shows remarkable antibacterial activity with zone of inhibition of 11mm against *S. aureus*. The higher antibacterial activity of methanolic extract may be its nature of biological active components. The n-hexadecanoic acid was recorded the maximum at the retention time of 30.94 with 10.7026 % of peak value followed by 2-cyclohexen-1-one, 4-hydroxy-3,5,5-trimethyl-4-(3-oxo-1-butenyl) (8.6%), phytol (8.4 %), hexadecanoic acid, ethyl ester (6.2 %) and 3,7,11,15-tetramethyl-2-hexadecen-1-ol (4.9 %) at the retention time of 27.89, 33.27, 31.23 and 28.27 respectively. The previous study [14, 15] suggests the active principle compounds are a mixture of squalene (19.19 %), n-hexadecanoic acid (6.59 %), phytol (4.74 %), 2-cyclohexen-1-one, 4-hydroxyl-3,5 (4.20 %) and oleic acid (2.88 %) in *Avicenia*, species.

In recent years, we search new drug from natural sources against multidrug resistant bacteria. The methanolic extract of *A. marina* has more potential to inhibit multidrug resistant *S. aureus*. The *A. marina* compounds have good antibacterial properties that can be used as anti multi-drug resistant *Staphylococcus aureus* agents.

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Conflict of interest statement

We declare that we have no conflict of interest.

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