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Research Paper

ANTIBACTERIAL AND SYNERGISTIC ACTIVITY OF MANGROVE (*Avicennia marina)* EXTRACTS ON ESBL AND MBL PRODUCING UROPATHOGENS

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Abstract

Growing incidences of β-lactamase producing uropathogens have been observed worldwide. Extended Spectrum β-lactamase (ESBLs) and Metallo β-lactamase (MBLs) are two types of β-lactamases that have emerged as a major threat with limited treatment options. In the current study, the effect of *Avicennia marina* leaf and stem extracts was studied on 10 ESBL and 10 MBL producing uropathogens which were identified and characterized previously in our laboratory. The bioactive components from *Avicennia marina* were extracted at 60°C for 12h with the help of soxhlet apparatus using various solvents. This extract was concentrated at 40°C on water bath to obtain a semisolid mass which was used to carry out qualitative and quantitative analysis. The test isolates showed maximum inhibition (17-27mm) towards ethyl acetate extract of *A. marina.* The MBC of test isolates was found to be 20mg/ml. It also showed synergistic activity with ampicillin by lowering the MBC of ampicillin from over 10mg/ml to 200-300µg/ml. HR-LCMS analysis of ethyl acetate extract of *A.marina* showed the presence of 17 major bioactive components including terpenoids, flavones and anthraquinones which may be responsible for its antibacterial property. These results collectively indicate the possible use of *A. marina* extracts in combination therapy to treat infectious diseases caused by multiple drug resistant pathogens.

Key words: ESBL, MBL, Mangrove, Antibiotic resistance, Bioassay, *A. marina*.

INTRODUCTION

UTI is characterized by the presence of organisms in the urinary tract, which is usually sterile. About 150 million people worldwide are diagnosed with Urinary tract infection (UTIs) each year [1]. They are particularly common among the female population with an incidence of about 1% in school-aged girls and 4% in women through child-bearing years. The most commonly encountered gram negative uropathogens are *E.coli, K.pneumoniae*, *Citrobacter* spp, *P.aeruginosa* and *Proteus* spp [2]. Antibiotic resistance is a consequence of evolution via natural selection. The entire crisis is a result of many years of sustained selective pressure due to the human application of antibiotics, via underuse, overuse and misuse [3].

Extended-spectrum-beta-lactamase (ESBLs) are enzymes that can be produced by bacteria making them resistant to third generation Cephalosporins e.g. Cefuroxime, Cefotaxime and Ceftazidime, which are the most widely used antibiotics in many hospitals [4]. ESBL genes are often located on plasmids that are transferable from strain to strain and between bacterial

species [5]. In view of the increase in ESBL resistance, and the negligible development of antibiotics in the past few years, there is an urgent need for new antibacterial compounds in order to fight the emergence of these new resistant pathogens [6].

Metallo-beta-lactamase (MBL) is another type of β-lactamase reported widely in *P. aeruginosa* and *Acinetobacter* species*.* It is an enzyme that makes bacteria resistant to a broad range of beta-lactam antibiotics. These include the antibiotics of the carbepenem family which are a mainstay for the treatment of antibiotic-resistant bacterial infections [7].

Mangroves are one of the most productive and bio-diverse wetland environments on earth [8]. Mangroves have been a source of several bioactive compounds such as antiviral, antibacterial, and antifungal compounds [9]. Besides, a number of mangrove plant secondary metabolites like gedunin, hydroquinone, xanthone, diterpenes, polyphenol etc. are currently used as anticancer agents in different cell lines; however, not much study has been undertaken [10].

Secondary metabolites like alkaloids, phenolics, steroids and terpenoids have been characterized from mangroves and have toxicological, pharmacological and ecological importance [11]. Also, preliminary studies have demonstrated that the mangrove plant extracts have antibacterial activity against pathogenic bacterial strains *Staphylococcus* sp*., E. coli* and *Pseudomonas* sp. as well as antibiotic resistant bacterial strains *Staphylococcus* and *Proteus* sp*.* Mangrove extracts can also be possible sources of mosquito larvicides, antifungal, antiviral, anti-cancer and anti-diabetic compounds [12]. Pulp of mangrove plants is used as blood purifiers in cases of snake bites. Leaves are useful in rheumatism, diuresis, neuralgia, as hair preservers and as an expectorant. Powdered leaves are used as food for fish and prawns. Roots of Mangrove plants are used for treatment of asthma, paralysis, leucorrhoea and debility [13].

Mangrove plants have been used in folklore medicines and extracts from mangrove species have shown inhibitory activity against human, animal and plant pathogens. For example, *Alanthus illicifolus* plant is used to treat paralysis, asthma, skin disorders and wounds [12]. *Excoecaria agallocha* (*Euphorbiaceae*) is a typical mangrove-associated species which has been used in traditional medicine for the treatment of leprosy and also as an aphrodisiac for decades [13]. *A. marina* has been used in traditional medicine for years by virtue of its anti-oxidant, anti-bacterial, anti-fungal, anti-ulcer, anti-cancer, anti-plasmodial and anti-tumor properties [14]. The barks, leaves, and fruits of this species have been used in traditional medicine in Egypt to treat skin diseases. The stem of *A. marina* is used to treat ulcers, leprosy and also as an aphrodisiac for decades [15]. *A. marina* is abundant in chemical components. Bell *et al.* (2009) reported the bark of *A. marina* contains tri-terpenoids (betulic acid 0.3%, taraxerol 0.06% and taraxerone 0.05%) and traces of hydrocarbon. A lot of other chemical components have been isolated from the different parts of *A. marina* by natural chemists.

The current study was carried out to study the antibacterial activity of *A. marina* on ESBL and MBL producing uropathogens.

MATERIALS AND METHODS

Test organisms

The urine sample isolates were collected from local pathological laboratories and hospitals situated in south Mumbai and characterized for ESBL and MBL production in our laboratory in previous studies [16, 17]. The study was carried out using 10 ESBL and 10 MBL producing uropathogens. It included representative isolates of *Esherichia coli* (2), *Klebsiella pneumoniae* (2), *Pseudomonas aeruginosa* (2), *Citrobacter diversus* (2) and *Proteus mirabilis* (2) respectively. The ESBL and MBL producing isolates were maintained on sterile Nutrient Agar (NA) slants with 100µg/ml ampicillin and stored at refrigerated conditions.

Processing of the *A. marina* plant

A. marina plant was authenticated by an expert botanist. Its stem and leaves was then used for extraction of its bioactive components. The dried plant was crushed into fine powder with the help of a mechanical grinder and refrigerated in sealed vials until further use.

Preparation of the extract

100g of the processed leaves or stem powder was extracted with 200ml of different solvents viz. ethanol, methanol, ethyl acetate and water using soxhlet apparatus for a period of 12h. The extract was further concentrated at 40°C on a water bath to obtain a semisolid mass. This mass was re-suspended in ethanol to get the required concentration of the extract for carrying out further analysis. This concentrated extract was prepared in large volume and preserved at 4^0C in sealed vials until further use to avoid batch to batch variations.

Sterility testing of Plant extracts

The sterility of Mangrove extract was checked by inoculating a loopfull of the extract on Nutrient Agar (NA) and Sabouraud's Agar (SAB) plates, and then checking for growth of bacterial and fungal contaminants respectively after 1 week of incubation at room temperature [18].

Qualitative analysis of antibacterial activity of leaf and stem of *A. marina* by Agar well diffusion method

The crude extracts of the leaf and stem of *A. marina* were subjected to antimicrobial assay using Agar well diffusion method [19]. 20 ml of molten Nutrient agar was inoculated with 0.4 ml of 24h old test cultures (0.1 OD at 540nm), mixed gently and poured into sterile petri dishes. Using a sterile cork borer (8mm diameter), wells was punched in each plate after solidification of the medium and 50µl of the plant extracts was added to the wells. The plates were incubated at 37°C for 24h to observe the zones of inhibition by the different solvent extracts. Control wells were also set up using 50 µl of solvents for each test isolate. The study was carried out in triplicates and the results were reported as mean ± standard deviation (SD).

Detemination of Minimum Bactericidal concentration (MBC) of *A. marina* extracts and ampicillin against test isolates

The agar dilution method was used to determine the MBC of *A. marina* leaf and stem extracts and ampicillin individually. Different concentrations of various solvent extracts of *A. marina* (5-50 mg/ml with an interval of 5 mg/ml), or ampicillin (1-10 mg/ml with an interval of 1 mg/ml) were supplemented into molten NA butts cooled to around 40°C. After solidification of the medium, 5µl of test isolates were spot-inoculated on the plates, and incubated at 37°C for 24h. MBC was defined as the lowest concentration of plant extract/ ampicillin that completely inhibited the growth of test cultures [20].

Determination of the Synergistic action between *A. marina* and ampicillin by Agar dilution method

The agar dilution method was similarly used to determine the synergistic activity between *A. marina* solvents extracts and ampicillin. It was done by incorporating sub-lethal (½MBC) concentrations of *A. marina* extracts into molten NA butt which were cooled to around 40°C along with 100-500 µg/ml of ampicillin with an interval of 100µg/ml [20].

High Resolution-Liquid Chromatography Mass Spectrometer (HR-LCMS):

The bioactive components from Mangrove *A.marina* leaf extract were analysed by HR-LCMS 1290 Infinity UHPLC System, 1260 infinity Nano HPLC with Chipcube, 6550 iFunnel Q-TOF (Agilent technologies). The complete analysis was carried out at IIT Bombay, Mumbai 400076. The compounds of the crude extract were identified by comparison of their retention indices (RI) and mass spectra fragmentation with those on the stored library available with IIT, Bombay.

RESULTS

Identification of Mangroves

Identification of Mangroves was done from the Garden department, Godrej and Boyce Manufacturing Company, Vikroli, Mumbai 79, and it was confirmed as *Avicennia marina.*

Sterility testing of Plant extracts

Leaf and stem extract of *A. marina* was found to be free from bacterial and fungal contaminants as observed by streaking a loopful of extract on Nutrient Agar and Sabouraud's Agar plates and observing after 1 week of incubation at room temperature.

Qualitative analysis of antibacterial activity of leaves of *A. marina* by Agar well diffusion method

Table 1 indicates the zones of inhibition obtained against test isolates using water, ethanol, methanol and ethyl acetate extract as solvents. Best activity was observed against ethyl acetate extracts. The ethyl acetate leaf extracts were found to be more effective in inhibiting the growth of the tested pathogens as compared to the stem extract.

Among various solvents, ethyl acetate extract of *A. marina* showed the maximum activity, with a maximum zone of inhibition of 27 mm. Hence the further study was carried out using ethyl acetate leaf extract.

Table 1: Agar well diffusion test showing zones of inhibition against the test pathogens

Pathogens	Mean zones of Inhibition zone in mm ± SD (n=3)							
	Stem Extract				Leaf Extract			
	Water	Ethanol	Methanol	Ethyl acetate	Water	Ethanol	Methanol	Ethyl acetate
ESBL Producers								
Escherichia coli (2)	-	10.36 ± 0.68	8.20 ± 0.37	11.80 ± 0.48	-	11.67 ± 0.34	11.47 ± 0.52	26.5 ± 0.25
Klebsiella pneumoniae (2)	-	-	8.58 ± 0.63	11.30 ± 0.48	-	-	8.83 ± 0.37	17.4 ± 0.54
Pseudomonas aeruginosa (2)	-	-	7.8 ± 0.45	11. 33 ± 0.52	-	-	8.3 ± 0.39	17. 67 ± 0.52
Citrobacter diversus (2)	-	-	8.33 ± 0.28	12.33 ± 0.58	-	8.33 ± 0.36	10.55 ± 0.36	22.67 ± 0.68
Proteus mirabilis (2)	-	9.8 ± 0.28	8.83 ± 0.27	9.5 ± 0.32	-	10.56 ± 0.43	8.72 ± 0.37	22.17 ± 0.52
MBL Producers								
Escherichia coli (2)	-	8.52 ± 0.67	10.37 ± 0.66	10.79 ± 0.48	-	9.72 ± 0.28	11.5 ± 0.47	21.83 ± 0.27
Klebsiella pneumoniae (2)	-	-	9.63 ± 0.37	8.74 ± 0.36	-	-	11.48 ± 0.69	19.36 ± 0.48
Pseudomonas aeruginosa (2)	-	-	8.33 ± 0.37	8.92 ± 0.52	-	-	11.78 ± 0.74	18.55 ± 0.26
Citrobacter diversus (2)	-	-	8.58 ± 0.63	8.72 ± 0.38	-	-	10.56 ± 0.32	21.67 ± 0.47
Proteus mirabilis (2)	-	-	9.5 ± 0.32	9.63 ± 0.37	-	-	11.48 ± 0.93	23.8 ± 0.47

*****No zone of inhibition was observed for the above mentioned solvents that was used as control

Detemination of Minimum Bactericidal concentration (MBC) of *A. marina* extracts and ampicillin individually and synergistically against test isolates

The MBC of ethyl acetate solvent extracts were carried out for further quantitative analysis of the antibacterial activity of *A. marina* by agar dilution method. The MBC values are tabulated in Table 2. All the test pathogens showed very high MBC for ampicillin (>10mg/ml). The MBC of *A. marina* extracts was found to be 20mg/ml for all the test pathogens (Table 3).

However, in presence of sub-lethal concentrations of *A. marina* extract, the MBC of ampicillin was significantly reduced to 200-300µg/ml. This clearly shows the synergistic effect between *A. marina* ethyl acetate extract and ampicillin.

Table 2: Minimum Bactericidal Concentration of ampicillin and *A. marina* extracts individually and synergistically against the test pathogens

Test pathogens	MBC of ampicillin	MBC of *A. marina* extracts	Synergism:- MBC of ampicillin in presence of sub-lethal concentration of *A. marina* extract
ESBL Producers			
Escherichia coli (2)	>10 mg/ml	20mg/ml	200 µg/ml
Klebsiella pneumoniae (2)			300 µg/ml
Pseudomonas aeruginosa (2)			200- 300 µg/ml
Citrobacter diversus (2)			200 µg/ml
Proteus mirabilis (2)			300 µg/ml
MBL Producers			
Escherichia coli (2)	>10 mg/ml	20mg/ml	300 µg/ml
Klebsiella pneumoniae (2)			300 µg/ml
Pseudomonas aeruginosa (2)			200-300 µg/ml
Citrobacter diversus (2)			300 µg/ml
Proteus mirabilis (2)			300 µg/ml

High Resolution- Liquid Chromatography Mass Spectrometer (HR-LCMS)

The HR—LCMS of identified 17 components listed in Table 3 from ethyl acetate extract of *A. marina*. It was found that coumarin was present in highest concentration followed by Sulfamethoxazole. Deoxyelephantopin, Usnic acid, Genkwanin and Maackiain were present in traces. The corresponding retention times of the identified components are listed in Table 3

Table 3: Components identified from ethyl acetate extract by HR-LCMS analysis

Compound name	Retention Time	Mass	Formula
Coumarin	5.877	146.0347	C9 H6 O2
Rhoifolin	6.552	578.1594	C27H30O14
Rhamnetin	6.553	316.0577	C16 H12 O7
Deoxyelephantopin	7.489	344.1258	C19 H20 O6
Biochanin A	8	284.0662	C16 H12 O5
Kaempherol	8.53	286.0453	C15 H10 O6
Physcion	8.557	284.0683	C16 H12 O5
Purpurin	8.793	256.0359	C14 H8 O5
Sulfamethoxazole	8.798	253.0487	C10 H11 N3 O3 S
(-)-Usnic acid	8.918	344.0905	C18 H16 O7
1,4,5,8-tetrahydroxy-2,6-dimethylanthroquinon	9.722	300.0614	C16 H12 O6
Cantharidin	9.934	196.0719	C10 H12 O4
Genkwanin	10.794	284.069	C16 H12 O5
Maackiain	12.042	284.0686	C16 H12 O5
Rhein	12.301	284.0304	C15 H8 O6
Hederagenin	16.216	472.3556	C30 H48 O4
2-Oxovaleric acid	10.807	116.0467	C5 H8 O3

DISCUSSION

Several studies have demonstrated the qualitative as well as quantitative antibacterial activity of various plants [21, 22, 23]. Traditionally, plants were known to be the main sources of drugs. Interest in this area still continues and many new potent drugs have been screened. Mangroves are one of the most important sources of such discoveries [24]. In the current study, the

antibacterial potential of *A. marina* extracts was explored against multi drug resistant uropathogens.

Very few studies have been carried out previously that shows the effect of plant extracts on Multi-drug resistant pathogens [3, 4, 6, 18]. In the current study, different solvents showed different activity against the test isolates. The variation of the antibacterial activity of the extracts might be due to distribution of extracted antimicrobial components, which varied from solvents to solvents [25]. Qualitative analysis of antibacterial activity showed ethyl acetate leaf extracts to be most effective indicating presence of semi polar bioactive components in *A. marina*. Ethyl acetate is mostly preferred as solvent for extraction of bioactive components from most plants because it is a non-toxic solvent, which can extract large variety of semi polar bioactive components that are mostly found in plants. It is also used in medicine and as an organic acid extracting agent [21]. Other studies have shown methanolic extract of *Pempis acidula* and n-hexane extract of *Lumnitzera littorea* to be effective against *Staphylococcus aureus* [14] and *Bacillus cereus* [26] respectively.

The Minimum Bactericidal concentration (MBC) of ethyl acetate leaf extract was found to be 20mg/ml. Since the extract used in the current study was a crude extract, its potency can be improved by using modified extraction methods and choice of solvents.

Through these qualitative and quantitative studies it is observed that the Mangrove extract provide effective activity against drug resistant organism. This may be due to the difference in modes of action of various compounds present in the extract, to which the organism was never exposed before and thus never had a chance to develop resistance. These activity exhibited by the Mangrove extract could be due the presence of phytochemical like terpenoids, flavones and anthraquinones present in the plant extract. All these extracts suggest that they may possess remarkable therapeutic action in treatment of infectious diseases.

Synergistic activity between *A. marina* leaf extract and ampicillin was also confirmed in the current study where 99% Reduction in MBC of ampicillin was observed in presence of sub-lethal concentration of *A. marina* extract. Hence our study shows the possible use of *A. marina* leaf extract in combination therapy in which the dose of antibiotics can be reduced and the available antibiotics could be used even in cases of infections caused by drug resistant pathogens. The use of antimicrobial agents displaying synergy is one of the well-established indications for combination antimicrobial therapy. Combinations of antimicrobials that demonstrate an in-vitro synergistic effect against infecting strains are more likely to result in successful therapeutic result. Evidence of in-vitro synergism could be useful in selecting most favourable combinations of antimicrobials for practical therapy of serious bacterial infections [17].

This study revealed that the combined use of plant extract and antibiotics could be useful in the treatment of infectious diseases and in fighting the problem of emerging drug resistance. In-vivo experiments however are needed to confirm the phenomenon of synergy between drugs and Mangrove plant extract.

Phytochemical analysis of *A. marina* leaf extract using HR-LCMS revealed 17 major components with coumarin as a major component. In the present study the leaf extract of *A.marina* showed presence of flavonoids such as Kaempferol, Biochanin A, Rhoifolin, Rhamnetin, Genkwanin and Maackian. Previous literature has reported presence of tannin, phenols, alkaloids, xanthoproteins, resins and coumarin as major components of *A. marina* [27, 28]. The LC/MS analysis of aerial part of *A. marina* leaves have shown presence of flavonoids such as luteolin 7-o-methylether 3'-O-β-D-glucoside, galactoside analogue, chrysoeriol 7-Oglucoside, Isorhamnetin 3-O-rutinoside, 5-hydroxy-4, 7-dimethoxyflavone, quercetin, kaempferol, 4'5-dihydroxy-3'-5,7-trimethoxyflavone and 4',5,7-trihydroxyflavone,3',4',5-trihydroxy-7-methoxyflavone [29].

The presence of flavonoids has important effects in plant biochemistry and physiology as antioxidants, enzyme inhibitor, precursors of toxic substances and they are recognized to possess anti-inflammatory, antioxidant, anti-allergic, anticarcinogenic activities [27, 29, 30].

In the present study *A. marina* showed presence of terpenoids such as Hederagenin, Cantharidin. Cantharidin has shown to induce apoptosis of H460 human lung cancer cells [30].

Currently, very little information is available on the study of *A.marina* showing antibacterial activity on drug resistant pathogens either in vitro or in vivo. There is a need for more in-dept studies to be carried out with *A.marina*.

CONCLUSION

In the present study, the effectiveness of *A. marina* extracts was investigated against ESBL and MBL producing pathogens. These results confirm the potential of *A. marina* to be used alone and in synergy with ampicillin against ESBL and MBL producing pathogens.

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