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Therapeutic potential of mangrove and its associate plant extracts from thane creek, against human respiratory tract MDR pathogens

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Abstract

The present study investigates antibacterial activities of four mangrove plants, *Avicennia marina*, *Avicennia officinalis*, *Salvadora persica* and *Sesuvium portulacastrum* against Respiratory tract multidrug resistance pathogens like *Mycobacterium tuberculosis*, Streptococcus *pneumoniae*, *Haemophilus influenzae* and *Staphylococcus aureus*. Mangrove plant extracts were obtained by sequential soxhlet extraction method in methanol and ethyl acetate solvents. These extracts were screened for antibacterial activity, synergistic activity, potentiation effect by agar well diffusion method and phytochemical analysis was also done. Ethyl acetate extract exhibited the highest antibacterial activity than methanolic extract of all the four plants. Extract of *A. marina* and *S. portulacastrum* exhibited highest antibacterial activity as compared to that of other two plant extracts. While in case of synergistic effect, combination of ethyl acetate extracts of all four plants exhibited more potency than other combinations against *M.tuberculosis*. In potentiation activity, *A.marina* and *S. portulacastrum* extracts were individually combined with the antibiotic Gentamicin and exhibited higher activity than other extracts. All extracts were more potent towards *Mycobacterium* as compared to other organisms. According to phytochemical analysis, *A. marina* had almost all bioactive principles (phytochemicals) except saponins and *S. portulacastrum* contained all phytochemicals. GC-MS analysis of *A.marina* also showed the presence of bioactive principles.

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Introduction

Tropical and sub-tropical areas of the World are bestowed with abundant herbs and flora which have untapped properties, such as bacterial, antiviral and antifungal activity. Plants used for traditional medicine contain a wide range of substances that can be used to treat infectious as well as chronic diseases of bacterial, viral or fungal origin (Mouafi *et al.*, 2014; Suganya *et al.*, 2014). Mangroves are facultative halophytic plants found in tropical and sub-tropical areas of inter tidal zones (Shilpi *et al.*, 2012; Shiva *et al.*, 2011; Shanmugapriya *et al.*, 2012). Mangrove vegetation in India covers about 6,749 km² along the 7516.6 km long coast line, including territories of Islands. The mangrove habitats are situated in three zones: (1) West Coast, about 850 km² (2) East Coast, about 4700 km² and (3) Andaman & Nicobar Islands about 1190 km², with Lakshadweep. These three zones have been further categorized into Island, Coastal and Deltaic habitats.

Estimates of mangrove species in the world range from 48 to 90, with 50-60 in India. There are about 82 species of mangroves distributed in 52 genera and 36 families from all the 12 habitats in India The inter tidal vegetation is classified into three categories: 'Major mangroves,' 'Mangrove associates,' and 'Back mangal', on the basis of their morpho-anatomical characters representing adaptation to halophytic condition (Mandal *et al.*, 2008). Many mangrove plants and its associates have medicinal and commercial importance (Prakash *et al.*, 2013). Mangrove and mangrove associates contain biologically active antiviral, antibacterial and antifungal compounds. They provide a rich source of steroids, triterpenes, saponins, flavonoids, alkaloids and tannins (Abeysinghe 2006; Pimpliskar *et al.*, 2012; Harbone, 1984; Sett *et al.*, 2014; Sharief *et al.*, 2011). Many mangrove plants have been used in folklore medicines against human, animal and plant pathogen but investigations have been limited to find out these bioactive compounds (Asha *et al.*, 2012). According to the World Health Organization, plants are a source of compounds that have the ability to combat diseases and possess antimicrobial, antifungal and antiviral activities (Abeysinghe *et al.*, 2010; Pimpliskar *et al.*, 2012).

Due to emerging drug resistance, novel compounds need to be developed from plants (Sharief *et al.*, 2011) as synthetic regular antibiotics are in the process of being labelled out-dated (Sharief *et al.*, 2014) and have exacerbated the treatments with known antibiotics. Respiratory tract infections are the most common type of infections. Nose, pharynx, larynx, middle ear, trachea, bronchus, lungs are associated with the respiratory tract. Diseases like common cold, rhinitis, otitis media, diphtheria, scarlet fever, strep throat, pneumonia, influenza etc are few fatal diseases prevalent in respiratory tract during an infection. As a number of disease causing microorganism for the upper respiratory tract are becoming resistance against the conventional antibiotics, new and effective agents need to be developed (Ansari *et al.*, 2012).

A bit is known about the potential of mangroves conserved in the Soonabai Pirojsha Godrej Marine Ecology Centre, Thane creek, India. The western bank of the Thane Creek is the single largest mangrove belt in Mumbai. After a brief literature review, we didn't find any work of mangrove and its associated plant extracts on the Respiratory tract MDR pathogens of humans, their synergistic activity and potentiation activity against a standard antibiotic, so this work has been reported for the very first time in literature to the best of our knowledge. The present research aims to investigate the antimicrobial properties of these mangroves and their associated plant extracts along with synergistic, potentiation activity with standard antibiotic against Respiratory tract multi drug resistance pathogens (RT-MDR) pathogens and its phytochemical evaluation to search for new source of antimicrobial agents.

Materials and methods

Collection of plant materials

Fresh mangrove plants and their associate species were

collected from Thane Creek Area from the Soonabai Pirojsha Godrej Marine Ecology Centre. The species collected were *Avicennia officinalis*, *Avicennia marina*, *Salvadora persica* and *Sesuvium portulacastrum*. The species were identified by Mr. Hemant Karkhanis from the Soonabai Pirojsha Godrej Marine Ecology Centre. Among these *A.marina* and *A.officinalis* are true mangroves while *S.persica* and *S.portalacastrum* are mangrove associated species.

Preparation of plant extracts

The collected plant materials were completely dried in shade at room temperature for the required number of days until complete dryness was observed and grinded to make powder (Saad ***Et. al.***, 2012). This powdered sample was then subjected to extraction by Soxhlet apparatus with solvents like Methanol and Ethyl Acetate for 6-8 hours. The solvent was evaporated to dryness by rotatory vacuum evaporator to obtain only the extract. The extracts were then dissolved in specific amount of DMSO (Di methyl sulphoxide) to obtain the desired concentration. The concentration of extracts prepared was 100µg/ml.

Pathogenic organism

RT Pathogens were procured from Dr. Vasantrao Pawar Medical College, Adgaon, Nashik and were tested for multidrug resistance profiling against various antibiotics. Method used for this test was multi disc ring antibiotic sensitivity test by use of Combi disc for Gram positive organisms (Hi media), Combi disc for Gram negative organisms (Hi media). Swab plate technique was used for isolation of pathogens. Test organisms isolated were *Streptococcus pneumoniae, Haemophilus influenza, Mycobacterium tuberculosis and Staphylococcus aureus which were used for further activity*.

Antibacterial activity of mangrove extracts on RT-MDR pathogens

The antibacterial effect was tested by agar well diffusion method (Abeysinghe *et al.*, 2006; Sett *et al.*, 2014) using Mueller Hinton agar medium. 24 hours old culture of test organisms was taken and Mc Farland Std. suspension of each microorganism were prepared. These suspensions were swabbed uniformly with sterile cotton swab in aseptic conditions. Wells were made on the seeded plate with the help of a sterilized well borer of 6mm diameter. Wells were then filled with 25µl of the extracts and allowed to diffuse for 60 minutes in refrigerator at 4°C. The plates were then incubated at 37°C for 24 hours. The zone of inhibition were measured with antibiotic zone scale to the nearest mm. Tests were carried out in triplicates.

Synergistic effect of mangrove extracts

If two drugs given together, the chemotherapeutic effect is sometimes greater than the effect of either alone. This is what the synergistic effect is. This test was done by combining different extracts of mangrove plants. Agar well diffusion was the method used for this test. Combinations of extracts were made by mixing equal volume of extract with same concentration. This test was further carried out similarly as antibacterial activity stated in subheading Antibacterial activity of mangrove extracts on RT-MDR pathogens.

Potentiation effect of mangrove extracts

The increase or enhancement of antimicrobial activity of known antibiotics by or in addition with these extracts is known as Potentiation effect. In this method, Mangrove extracts were mixed with antibiotic Gentamicin. Equal concentration and equal quantity of antibiotic was mixed with plant extract. Its effect was tested by agar well diffusion method, the method used for antimicrobial activity as stated in subheading Antibacterial activity of mangrove extracts on RT-MDR pathogens.

Phytochemical analysis of mangrove extracts

Mangrove extracts were screened for Alkaloids, Saponins, Flavonoids, Tannins, Steroids, Anthraquinones, Cardiac glycosides and Triterpenes. The qualitative tests were done by the standard methods described by Kumari *et al.*, Prihanto *et al.*, and Moufai *et al.*, GC-MS analysis of methanolic extract of *A.marina* was also done. The method chosen depended on the availability of chemicals and ease of carrying the

test.

Results and discussions

Antibacterial effect of mangrove extracts

Methanolic extract of *Avicennia marina* showed maximum zone against *M.tuberculosis* and minimum zone against *H.influenzae*. In comparison to *M.tuberculosis*, *Staphylococcus* and *Streptococcus* gave minor differences in inhibition pattern. Ethyl acetate extract of *A. marina* gave higher inhibition zone than Methanolic extract of A. marina.

Table 1. Effect of mangrove extracts on Gram negative and Gram positive respiratory tract MDR Pathogens (MeoH- Methanol and EA- Ethyl acetate; zone diameter to nearest mm with standard deviation).

Gram Character →	Gram negative	Gram positive		
Extracts Pathogens→ ↓	*Haemophilus influenzae*	*Streptococcus pneumoniae*	*Staphylococcus aureus*	*Mycobacterium tuberculosis*
Avicennia marina-MeoH	10.7±0.6	12.7±0.4	12.1±0.4	13.2±0.6
Avicennia marina-EA	12.5±0.2	14.4±0.3	12.8±0.2	14.4±0.5
Avicennia officinalis-MeoH	9.9±0.3	11.6±0.5	11.5±0.3	12.7±0.3
Avicennia officinalis-EA	11.6±0.2	13.2±0.6	13.6±0.4	13.9±0.4
Salvadora persica-MeoH	8.8±0.7	10.5±0.2	10.8±0.5	11.8±0.6
Salvadora persica-EA	9.7±0.6	11.3±0.7	10.4±0.7	12.5±0.7
Sesuvium portulacastrum-MeoH	11.6±0.4	12.4±0.5	12.3±0.3	13.4±0.5
Sesuvium portulacastrum-EA	12.5±0.3	12.6±0.3	12.6±0.4	14.3±0.2

While in case of Avicennia officinalis, Methanolic extract of A.officinalis exhibited maximum zone against *M.tuberculosis*. *H.influenzae* showed least zone diameter. Inhibition pattern decreased following *M.tuberculosis*, *Streptococcus, Staphylococcus* and *H.influenzae*. In case of Ethyl acetate extract of A. officinalis, *M.tuberculosis* gave maximum zone of inhibition following Staphylococcus. *H.influenzae* gave smallest zone of inhibition as compared to other MDR pathogens. Ethyl acetate extract exhibited maximum zone of inhibition than the methanolic extract of *A. officinalis*.

Table 2. Synergistic effect of mangrove extracts (Zone diameter to nearest mm).

Pathogens → Combinations of Extracts ↓	*Streptococcus pneumoniae*	*Haemophilus influenzae*	*Staphylococcus aureus*	*Mycobacterium tuberculosis*
Avicennia marina (MeoH)+*Avicennia officinalis* (MeoH)	12.6±0.3	9.9±0.8	13.5±0.4	15.3±0.4
Avicennia marina (EA)+*Avicennia officinalis* (EA)	14.6±0.5	13.6±0.6	15.6±0.5	17.3±0.2
Avicennia marina (MeoH)+ *Avicennia officinalis* (EA)	13.5±0.2	12.9±0.2	14.4±0.3	16.8±0.2
Avicennia marina (EA)+ *Avicennia officinalis* (MeoH)	14.7±0.3	11.2±0.7	12.2±0.5	15.8±0.4
Salvadora persica (MeoH)+ *Sesuvium portulacastrum* (MeoH)	12.9±0.2	10.2±0.7	12.6±0.3	14.7±0.3
Salvadora persica (EA)+ *Sesuvium portulacastrum* (EA)	13.7±0.6	12.5±0.4	14.2±0.6	16.6±0.4
Salvadora persica (MeoH)+ *Sesuvium portulacastrum* (EA)	12.8±0.6	11.7±0.5	13.6±0.7	14.5±0.5
Salvadora persica (EA)+ *Sesuvium portulacastrum* (MeoH)	12.4±0.5	12.1±0.6	13.5±0.2	14.3±0.5

In case of extracts prepared from *Salvadora persica* maximum zone of inhibition exhibited by methanolic extract was against *M.tuberculosis* while smallest zone of inhibition was against *H.influenzae*. *Staphylococcus* showed greater zone diameter than *Streptococcus pneumoniae and H.influenzae*. Ethyl acetate extract exhibited maximum zone against *M.tuberculosis* followed by *Staphylococcus* and *Streptococcus pneumoniae*. Minimum zone of inhibition was obtained against *H.influenzae*. Ethyl acetate extract of *S. persica* showed maximum zone of inhibition as compared to methanolic extract.

Table 3. Potentiation effect of mangrove extracts (Zone diameter to nearest mm).

Pathogens → / Extracts + Antibiotic ↓	*Streptococcus pneumoniae*	*Haemophilus influenzae*	*Staphylococcus aureus*	*Mycobacterium tuberculosis*
Gentamicin+ Avicennia marina(MeoH)	14.4±0.4	13.5±0.5	12.9±0.5	17.1±0.3
Gentamicin+ Avicennia marina(EA)	18.4±0.2	14.4±0.6	13.8±0.3	19.2±0.2
Gentamicin+ Avicennia officinalis(MeoH)	13.6±0.6	10.5±0.8	12.8±0.6	14.5±0.4
Gentamicin+ Avicennia officinalis(EA)	15.8±0.3	12.5±0.4	14.1±0.7	16.9±0.5
Gentamicin+ Salvadora persica(MeoH)	13.7±0.3	12.7±0.3	11.7±0.6	13.6±0.6
Gentamicin+Salvadora persica (EA)	14.8±0.5	10.7±0.7	12.6±0.5	16.6±0.5
Gentamicin+Sesuvium portulacastrum (MeoH)	15.5±0.5	12.2±0.4	13.4±0.5	14.8±0.6
Gentamicin+Sesuvium portulacastrum (EA)	15.8±0.8	12.8±0.5	12.9±0.2	15.4±0.7
Gentamicin	13.6±0.3	12.4±0.6	11.7±0.4	13.6±0.2

Sesuvium portulacastrum methanolic extract exhibited maximum zone diameter against *M.tuberculosis* and minimum zone diameter against *H.influenzae* while rest of the pathogens were inhibited in the same manner as that using S. persica extract. In case of ethyl acetate extract maximum zone diameter was of *M.tuberculosis* and minimum zone diameter was observed against *H.influenzae*. Here *Staphylococcus* exhibit greater zone as compared to *St.pneumoniae*. Maximum zone diameter was observed using ethyl acetate extract of *S. portulacastrum* than methanolic extract (Table 1).

As a crisis point is reached now due to antibiotic resistance, a more intensified research for smart use of antibiotics and efforts towards design of new antibiotics should be pragmatic. As the result here show susceptibility of MDR pathogens against mangrove and its associate plant extracts, these should further be tested to explore inhibition activities against other MDR pathogens and may prove beneficial.

Synergistic effect of mangrove extracts

Combinations of *A. marina* (methanolic) and *A. officinalis* (methanolic) extract showed low synergistic effect than individual effect against *St.pneumoniae*. While in case of *H.influenzae* (antagonistic effect) it also showed low synergistic effect than individual effect. But the higher or maximum synergistic effect was observed in case of Staphylococcus and *M.tuberculosis* than the individual effects of extracts. Moreover *M.tuberculosis* showed maximum inhibition than other extracts.

While in case of ethyl acetate extract of *A. marina* and *A. officinalis* all organism showed higher synergistic effect than the individual effect but among them *M.tuberculosis* showed maximum synergistic effect than other organism. *Staphylococcus* showed synergistic effect less than *M.tuberculosis*, but greater than *St.pneumoniae* and *H.influenzae*. *H.influenzae* showed lowest synergistic among all organisms (Antagonistic effect).

Combined effect of methanolic extract of *A. marina* and ethyl acetate extract of *A. officinalis* showed increased synergistic effect than individual effect of extracts. Here *H.influenzae* showed lower synergistic effect than all of the test organisms, apart from this

St.pneumoniae showed synergistic effect greater than *H.influenzae*, while *Staphylococcus* showed higher synergistic effect than *St.pneumoniae* and *H.influenzae* and maximum synergistic effect was shown by *M.tuberculosis* than individual effect of extracts among all of the organisms which have been tested.

In case of Ethyl acetate of *A. marina* and methanolic extracts of *A. officinalis*., *M.tuberculosis* showed maximum synergistic effect than other organism while in case of *H.influenzae* and *Staphylococcus* instead of synergistic effect showed antagonistic effect i.e. effect of *A. marina* (ethyl acetate)+ *A. officinalis* (methanolic) is reduced in test of synergistic effect, than individual effect of *A. marina* (ethyl acetate) extract and *St.pneumoniae* showed synergistic effect less than *M.tuberculosis* but greater than *Staphylococcus* and *H.influenzae* respectively. Maximum synergistic effect was shown against *M.tuberculosis* by combined effect of *S. persica* methanolic extract and *S. portulacastrum* methanolic extract, while minimum synergistic effect was shown by *H.influenzae* (i.e. antagonistic) but *Staphylococcus* shows higher effect than *H.influenzae* for these extracts and *St.pneumoniae* also gives higher effect than *Staphylococcus* and *H.influenzae.*

Table 4. Phytochemical analysis of Methanolic extracts ('+' present; '-' absent).

Phytochemicals	*A.marina*	*A.officinalis*	*S. persica*	*S. portulacastrum*
Alkaloids	+	-	+	+
Saponins	-	-	-	+
Tannins	+	+	+	+
Steroids	+	+	+	+
Flavonoids	+	+	+	+
Anthraquinones	+	+	-	+
Cardiac glycosides	+	+	-	+
Triterpenes	+	+	+	-

In case of ethyl acetate extract of *S. persica* and *S. portulacastrum*, compare to all organism *M.tuberculosis* showed maximum synergistic effect than individual effect of extracts. While *H.influenzae* showed normal effect than others i.e. no difference in the zone of individual extract and combined effect in case of S. *portulacastrum* (ethyl acetate) but *S. persica* (ethyl acetate) showed increased zone diameter, *Staphylococcus* showed synergistic effect greater than *H.influenzae* and *St.pneumoniae*.

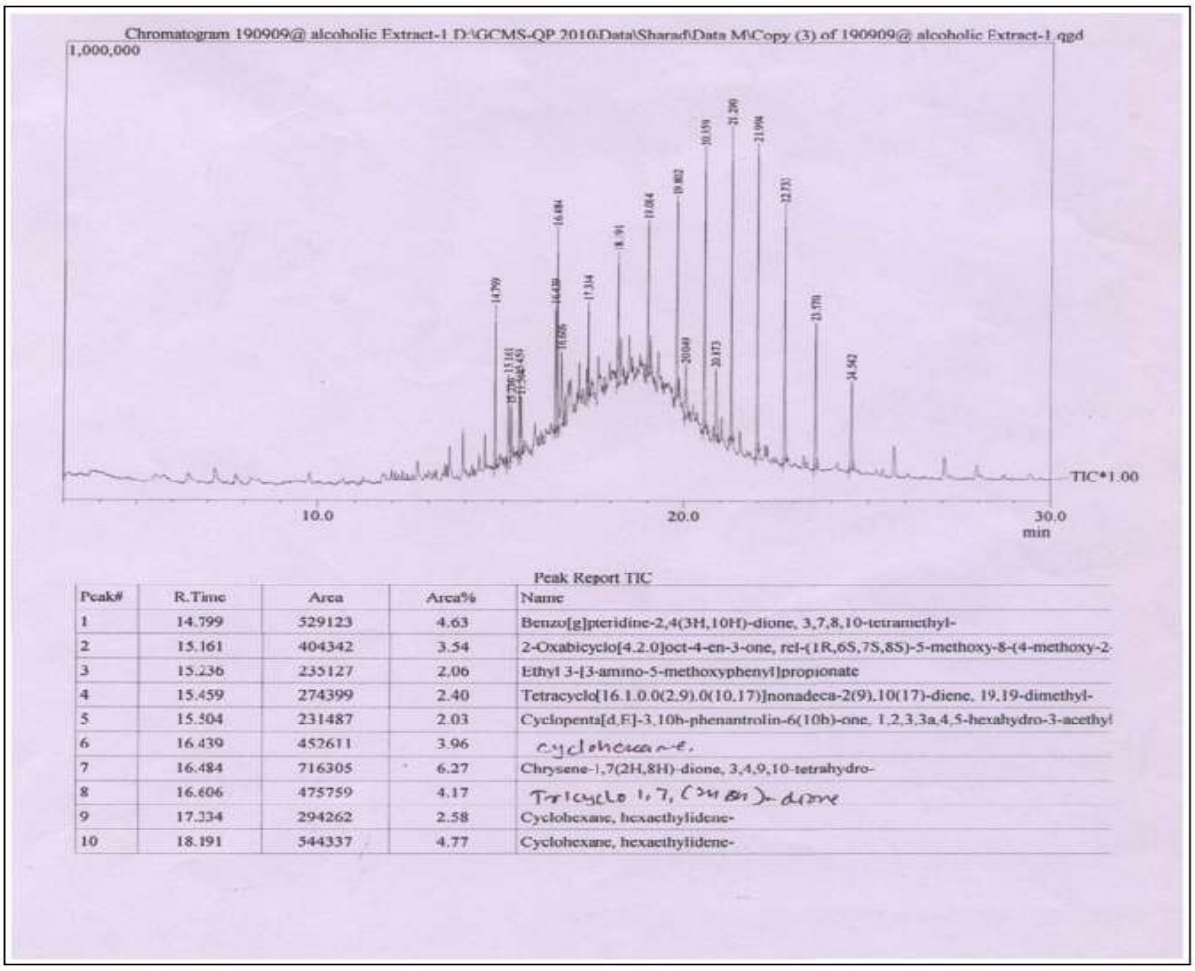
While in case of methanolic extract of *S. persica* and ethyl acetate extract of *S. portulacastrum* *St.pneumoniae* and *H.influenzae* showed decreased synergistic effect than individual effect but *M.tuberculosis* showed maximum synergistic effect. Next to *M.tuberculosis*, higher synergistic effect was shown against *Staphylococcus*. *H.influenzae* showed least synergistic effect *S. persica* (ethyl acetate) + *S. portulacastrum* (methanolic) extracts showed least synergistic effect against *St.pneumoniae* than other organism while *M.tuberculosis* showed maximum synergistic effect and *Staphylococcus* showed effect less than *M.tuberculosis* but more than *H.influenzae* (Table 2).

As these extracts showed a good synergy with the standard antibiotic, they should be checked against other antibiotics also, and if beneficial results are obtained against MDR pathogens, then these plants can be considered as a source of effective antimicrobial agents against the acute and chronic microbial drug resistant pathogens.

Potentiation effect of mangrove extracts

In case of *St.pneumoniae,* Gentamicin in combination

with *A. marina* ethyl acetate extract gave highest activity of potentiation effect, while methanolic extract of *A. officinalis* with Gentamicin showed least potentiation activity. As compared to both extracts of each mangrove plants, maximum activity was observed in ethyl acetate extract of each plant in combination to antibiotic Gentamicin than methanolic extracts of each plant with Gentamicin as compared to effect of individual Gentamicin. Methanolic extract of *A. officinalis* and *S. persica* with Gentamicin showed least activity than other extracts respectively.



Peak Report TIC

Peak#	R.Time	Area	Area%	Name
1	14.799	529123	4.63	Benzo[g]pteridine-2,4(3H,10H)-dione, 3,7,8,10-tetramethyl-
2	15.161	404342	3.54	2-Oxabicyclo[4.2.0]oct-4-en-3-one, rel-(1R,6S,7S,8S)-5-methoxy-8-(4-methoxy-2-
3	15.236	235127	2.06	Ethyl 3-[3-amino-5-methoxyphenyl]propionate
4	15.459	274399	2.40	Tetracyclo[16.1.0.0(2,9).0(10,17)]nonadeca-2(9),10(17)-diene, 19,19-dimethyl-
5	15.504	231487	2.03	Cyclopenta[d,E]-3,10b-phenantrolin-6(10b)-one, 1,2,3,3a,4,5-hexahydro-3-acethyl
6	16.439	452611	3.96	cyclohexane.
7	16.484	716305	6.27	Chrysene-1,7(2H,8H)-dione, 3,4,9,10-tetrahydro-
8	16.606	475759	4.17	Tricyclo 1,7,(2,8)-dione
9	17.334	294262	2.58	Cyclohexane, hexaethylidene-
10	18.191	544337	4.77	Cyclohexane, hexaethylidene-

Fig. 1. GC-MS analysis of methanolic extract of *A.marina*.

The results obtained from each extracts against *H.influenzae* were that ethyl acetate extract of *A. marina* showed highest potentiation effect against it, while least potentiation activity was shown by methanolic extract of *A.officinalis* with Gentamicin. As compared to effect of only Gentamicin all combinations of extracts and antibiotics showed increase activity of potentiation except *A. officinalis* methanolic extract, *S. portulacastrum* methanolic extract and *S. persica* ethyl acetate extract. Here ethyl acetate extract of all plants in combination with antibiotic showed highest activity as compared to methanolic extract of all plants, except ethyl acetate extract of *S.persica* with gentamicin, as increased activity was shown by methanolic extract of S. persica with Gentamicin.

While comparing all extracts against *Staphylococcus*, highest activity or maximum activity was shown by ethyl acetate extract of *A. officinalis* with Gentamicin and least activity was shown by methanolic extract of *S. persica* with gentamicin. But all extracts showed higher activity with gentamicin than individual effect of Gentamicin. Here also, ethyl acetate extract of all plants showed maximum activity of potentiation than

metanolic extract except *S.portulacastrum* ethyl acetate extract with Gentamicin showed least effect than methanolic extract of it with gentamicin.

M.tuberculosis showed higher effect of extracts with Gentamicin than the individual effect of antibiotic Gentamicin. While ethyl acetate extract of all plants showed increased activity in combination with Gentamicin than methanolic extract of plants with Gentamicin. Maximum activity was shown by ethyl acetate extract of *A. marina* with Gentamicin and least activity was shown by methanolic extract of *S. persica* with gentamicin (Table 3).

Phytochemical analysis of mangrove extracts

The preliminary phytochemical analysis of eight extracts revealed the presence of Alkaloids, Flavonoids, Saponins, Tannins, Cardiac glycoside, etc. GC-MS analysis of *Avicennia marina* methanolic extract resulted in the identification of compounds mentioned in the report. The observed activity is due to the presence of potent phytoconstituents in the leaf extracts.

Avicennia marina had all phytochemicals except Saponins. Avicennia officinalis had all phytochemicals except Alkaloids and Saponins. Salvadora persica had all phytochemicals except saponins, anthraquinones and cardiac glycosides. Triterpenes were absent in Sesuvium portulascastrum. (Table 4 and Fig. 1.)

Conclusions

Mangrove plants and its associates were identified, collected and their activities were evaluated successfully. The plants were extracted completely to obtain the antimicrobial principles present in two solvents, ethyl acetate and methanol using Soxhlet apparatus. A total of eight extracts were prepared from four mangrove plants using two solvents. The Respiratory tract MDR pathogens were isolated from infections. The pathogens obtained were *St.pneumoniae, H.influenzae, S.aureus* and *M.tuberculosis*. From all the four tested mangrove plants *Avicennia marina* showed maximum antibacterial activity while the remaining three i.e. *Avicennia officinalis*, *Salvadora persica* and *Sesuvium portulacastrum* showed moderate activity effective against the tested pathogens. *Avicennia marina* can be used to discover bioactive natural compounds that may serve as leads for the development of new pharmaceuticals that address unmet therapeutic needs. Such screening of various natural organic compounds and identifying active agents is the need of an hour, because successful prediction of drug like properties at the onset of drug discovery will pay off later in drug development. The present study provides enough data to show the potential of mangrove extracts for the development of antipathogenic agents against various pathogens.

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