RESEARCH ARTICLE

3 Day National Level Seminar Cum Workshop On Recent Aspect Of Microbiological Techniques(RAMT) During 10-12 February, 2017 By Dept Of Microbiology, Techno India University, West Bengal.

ANTI-MICROBIAL, ANTI-OXIDANT AND PHYTOCHEMICAL PROFILING OF *AVICENNIA MARINA* AND *AVICENNIA ALBA*, THE DOMINANT MANGROVE FLORAL SPECIES OF INDIAN SUNDARBANS.

Pritam Mukherjee[1], Bulti Nayak[1], [*]Madhumita Roy[1] and Abhijit Mitra[2].
1. Department of Biotechnology, Techno India University, West Bengal, EM-4/1 Sector V, Salt Lake, Kolkata-700091, India.
2. Department of Marine Science, University of Calcutta, 35 B. C. Road, Kolkata-700019, India.

Manuscript Info

Manuscript History

Received: 01 January 2017
Final Accepted: 02 February 2017
Published: March 2017

Key words:-
Anti-microbial, anti-oxidant, phytochemicals, multi-drug resistant, true mangroves, alternative drug therapeutics.

Abstract

The Indian Sundarbans represents one of the taxonomically diverse and physico-chemically dynamic ecosystems of the Indian sub-continent supporting around 34 species of true mangrove flora out of which members of the Avicenniaceae family are the second most dominant. This salt-tolerant mangrove community experiences inundation and exposure twice a day during high and low tides, and therefore is unique in comparison to the normal terrestrial flora. In the last decade, there is an outburst of multi-drug resistant (MDR) microbial pathogens owing to antibiotic overuse in disease therapeutics, and therefore the need of the hour is to look for alternative medicine. Interestingly, recent studies have revealed the use of mangroves in traditional therapeutics. In view of this scenario, we investigated the anti-microbial, anti-oxidant and phytochemical profiles of the two major mangrove species (*Avicennia marina* and *Avicennia alba*) of Indian Sundarbans. Although, anti-microbial and phytochemical screening of mangroves were conducted by several researchers in Indian Sundarbans, however, the present study has its own merit in the sense that both clinical and environmental MDR bacterial pathogens have been used to evaluate the potential of the mangrove species in alternative drug therapeutics. Crude solvent extracts of the vegetative tissue parts of the two mangrove plant species were prepared in n-hexane. The anti-bacterial activity of the tissue extracts of *A. marina* and *A. alba* was tested via disc diffusion assay using five environmental MDR pathogenic bacterial strains (*Stenotrophomonas maltophilia, E. coli* strain BGW1, *E. coli* strain BMW1, *Citrobacter sp.* and *Pseudomonas sp.*) and three clinically isolated MDR human pathogens (*Proteus vulgaris*, *Salmonella typhi* and *Staphylococcus aureus*) among others with *A. marina* tissue (stems and leaves) extracts displaying the strongest activity. Consistent with this data, the highest total anti-oxidant activity was also

Corresponding Author:- Madhumita Roy.
Address:- Department of Biotechnology, Techno India University, West Bengal, EM-4/1 Sector V, Salt Lake, Kolkata-700091, India.

accounted in *A. marina* tissue extracts. We also compared essential phytochemicals among the two mangroves and noticed the presence of flavonoids, steroids, tannins, terpenoids, xanthoproteins, saponins, and cardiac glycosides. Together, our findings showed that the members of the Avicenniaceae family as potential sources of biologically active compounds and therefore, suggest their potential especially *A. marina* in alternative disease therapeutics.

Copy Right, IJAR, 2017,. All rights reserved.

……………………………………………………………………………………………………....

Introduction:-

In the recent past, over-exploitation of pharmaceutic drugs or antibiotics has caused the emergence of multi-drug resistant (MDR) microbes, and therefore treatment of such MDR microbial diseases demand a search for novel alternative therapeutic compounds **(Davies, 1994; World Health Organization, 2001)**. The major search focus has thus been shifted towards the natural flora with known anti-oxidant and anti-microbial properties. Several research works have suggested the potential of mangrove floral community in traditional medicines **(Kokpal *et al.,* 1990; Premanathan *et al.,* 1996; Patra and Mohanta, 2014)**. For centuries, the tribal populace employed mangrove plant extracts as their folk medicine for healing several health disorders **(Banerjee *et al.,* 2008; Muthazhagan *et al.,* 2014; Patra *et al.,* 2011)**. However, unlike various herbs, sea weeds and higher medicinal plants, the use of mangroves as an alternative medicine has been comparatively less explored **(Khafagi *et al.,* 2003; Vadlapudi, 2012; Nezhad *et al.,* 2009; Hossain *et al.,* 2010; Green *et al.,* 2011; El Shafay *et al.,* 2015)**.

The Indian Sundarbans, one of the most taxonomically diverse and physico-chemically dynamic ecosystems of the Indian sub-continent, sustains some 34 species of true mangroves among which members of the *Avicenniaceae* family rank second in terms of prevalence **(Chaudhuri and Choudhury, 1994; Barik and Chowdhury, 2014)**. In comparison to the normal terrestrial flora, this halophytic mangrove community gets exposed to high and low tides twice in every 24 hours **(Banerjee *et al.,* 2002)**, and therefore, has developed unique mode of adaptation, which could have enriched their phytochemical repertoire of medicinal importance. **Wu *et al.* (2008)** showed that *Avicennia marina*, a true mangrove floral species (commonly known as gray mangrove) contains few quinone derivatives with therapeutic and anti-microbial properties.

For our present study, we have chosen two major mangrove species namely *Avicennia marina* and *Avicennia alba*, members of the Avicenniaceae family to investigate the anti-microbial potency of their vegetative tissue extracts against few selected clinical and environmental MDR bacterial pathogens (encompassing both Gram-positive and Gram-negative bacterial strains) and identify the minimum inhibitory concentration of these solvent extracts via disc diffusion assay. We have also assessed their anti-oxidant activity and screened the various phytochemicals that are present in their vegetative tissues like the stems and the leaves.

Materials and Methods:-

Description of the sampling site and sample collection:

The Indian Sundarban delta or ISD region lies between 21°40'N and 22°40'N latitude and 88°03'E and 89°07'E longitude. ISD is surrounded by Bangladesh in the east, the Hoogly river in the west, the Dampier Hodges line in the north, and Bay of Bengal in the south. The two true mangrove species used in our study were collected from Namkhana region in western Indian Sundarbans **(Fig 1)**. The vegetative plant parts namely the stems and the leaves were put in clean plastic zip-lock bags, labelled appropriately and brought to lab before storing them under refrigeration until use.

Fig 1 A:- A satellite image of the Western Indian Sundarbans where the Namkhana region is highlighted with blue dotted circle. **B.** The two thriving, major mangrove floral species of the Avicenniaceae family.

Preparation of solvent extracts of vegetative plant parts:-

The stems and leaves of each mangrove species were rinsed separately with distilled water to remove dirt, cut into small pieces and air dried in shade. The dried plant parts were then grounded to a coarse powder using mortar and pestle. 1 g of each of these powdered plant material (either stems or leaves) was mixed with 10 ml of solvent (100% n-hexane) contained in an Erlenmeyer flask and kept under constant agitation for 2-3 days at room temperature. The resulted solvent extracts were filtered using Whatman filter paper No. 1 followed by filter-sterilization. The crude solvent extracts were stored in sterile microfuge tubes, with parafilm seal for further analysis **(Fig 2)**.

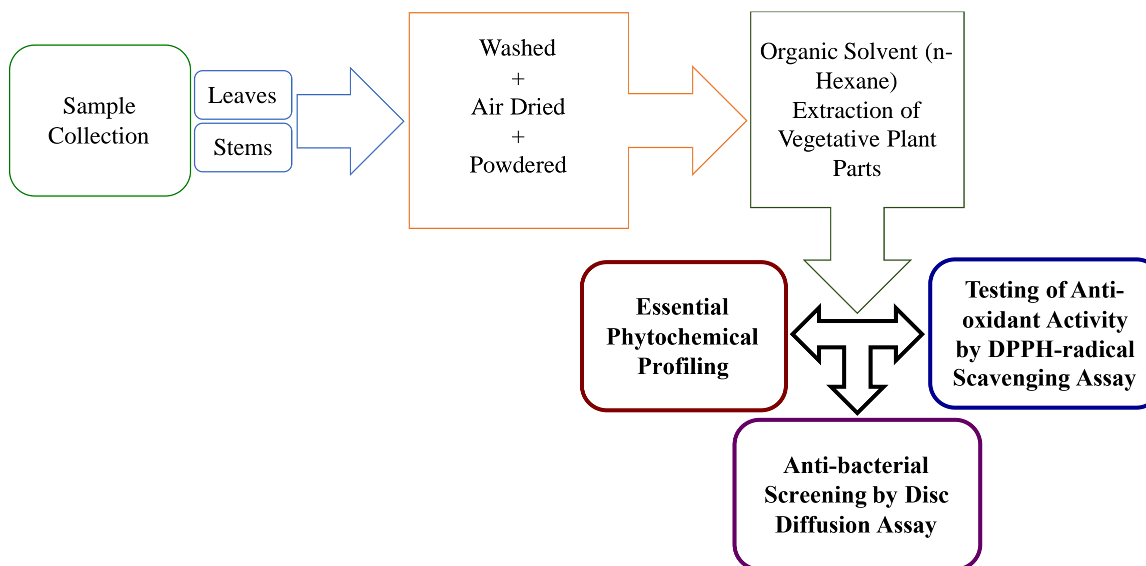

Fig 2:- Schematic representation of the overall experimental approach for this study.

Bacteria used in the study:-

The bacteria under study included both clinical and environmental MDR bacterial pathogens as well as environmental antibiotic-sensitive, Gram-positive and Gram-negative bacterial strains. The three clinical MDR bacterial pathogens used are *Staphylococcus aureus*, *Proteus vulgaris* and *Salmonella typhi.* The five environmental MDR bacterial pathogens used are *Stenotrophomonas maltophilia*, *E. coli* strain BGW1, *E. coli* strain BMW1, *Citrobacter sp.* and *Pseudomonas sp.* The other non-MDR environmental bacterial pathogens that served as control in our study include *Bacillus anthracis*, *Bacillus cereus*, *Kytococcus sp.* and *Staphylococcus sp*. The antibiotic susceptibility status of these bacterial strains along with their type and source of isolation has been summarized in **Table 1.**

Table 1:- Bacterial strains used for screening of anti-microbial activity of the vegetative tissue extracts of *A. marina* and *A. alba* in n-hexane.

	Bacterial strains used	**Type**	**Source**	**Specification**
A.	**Clinical Strains**			
1	*Staphylococcus aureus*	Human pathogen; +	Patient sample	Moderate MDR strain
2	*Proteus vulgaris*	Human pathogen; -	Patient sample	Moderate MDR strain
3	*Salmonella typhi*	Human pathogen; -	Patient sample	Moderate MDR strain
B.	**Environmental Strains**			
4	*Stenotrophomonas maltophilia*	Human pathogen; -	Isolated from RS of mangrove associate	MDR strain
5	*Escherichia coli**	Human pathogen; -	Isolated from GW sample	MDR strain
6	*Citrobacter sp.*	Human pathogen; -	Isolated from GW sample	MDR strain
7	*Pseudomonas sp.*	Human pathogen; -	Isolated from CM sample	MDR strain
8	*Bacillus anthracis*	Human pathogen; +	Isolated from FA sample	Antibiotic-sensitive
9	*Bacillus cereus*	Human pathogen; +	Isolated from FA sample	Antibiotic-sensitive
10	*Kytococcus sp.*	Human pathogen; +	Isolated from FA sample	Antibiotic-sensitive
11	*Staphylococcus sp.*	Human pathogen; +	Isolated from FA sample	Antibiotic-sensitive
12	*Agrobacterium tumifaciens*	Plant pathogen; -	Isolated from CM sample	MDR strain
13	*Bacillus thuringiensis*	Insect pathogen; +	Isolated from FA sample	Antibiotic-sensitive
14	*Halomonas sp. ***	PGPR; -	Isolated from mangrove RS	Indigenous, Antibiotic-sensitive
15	*Paracoccus kamogawaensis*	PGPR; +	Isolated from mangrove RS	Indigenous, Antibiotic-sensitive
16	*Oceanobacillus sp.*	PGPR; +	Isolated from mangrove RS	Indigenous, Antibiotic-sensitive
17	*Brevibacillus agri*	PGPR; +	Isolated from mangrove RS	Indigenous, Antibiotic-sensitive
18	*Paracoccus zeaxanthinifaciens*	PGPR; -	Isolated from mangrove RS	Indigenous, Antibiotic-sensitive
19	*Planomicrobium okeanokoites*	PGPR; +	Isolated from mangrove RS	Indigenous, Antibiotic-sensitive
20	*Bacillus pumilus*	PSB; +	Isolated from mangrove RS	Indigenous, Antibiotic-sensitive
21	*Delftia sp.*	PGPR; -	Isolated from CM sample	Antibiotic-sensitive

*Two different strains used; **Six different strains used; RS: Rhizosphere soil; FA: Fly ash; GW: Ganges water; CM: Coal mine; PGPR: Plant growth promoting rhizobacteria; PSB: Phosphate solubilizing bacteria; "+": Gram-positive; "-": Gram-negative; Mangrove means both *A. marina* and *A. alba* whereas mangrove associate means *Ipomoea pes-caprae.*

Screening of anti-bacterial activity and MIC by disc diffusion assay:-

Screening of anti-bacterial activity was performed by disc diffusion assay based on the method developed by **O'Bryan *et al.* (2008)** with modifications. Briefly, for each petri plate 25 ml of sterilized Luria Bertani (LB) agar medium (Hi Media) was inoculated with the respective freshly grown test bacterial culture (5% v/v). Sterile filter paper disc (with diameter of 0.6 cm) was then placed on the surface of the agar plate previously inoculated with the test bacterium onto which 15 µl of filter-sterilized crude plant extract sample (at a concentration of 100 mg ml^{-1}) was loaded. A separate paper disc loaded with 15 µl of solvent (100% n-hexane) served as a negative control. Additionally, paper discs loaded with Ampicillin (for Gram-positive bacteria) and Kanamycin (for Gram-negative bacteria) at a concentration of 10 µg ml^{-1} and 50 µg ml^{-1} respectively served as positive controls. The agar plates were incubated overnight at 37°C. Anti-microbial activity of the solvent extract samples against each test organism was calculated by measuring the diameter of halo zone (in cm) around the paper disc. Each experiment was done in triplicate and the final values were expressed as mean with standard deviation. The inhibition zones of the solvent extracts of vegetative parts of *A. marina* and *A. alba* were compared. The Minimum Inhibitory Concentration (MIC) of the solvent extracts of vegetative plant parts that inhibits the growth of test bacteria was determined by similar method as mentioned above using serial dilutions of the solvent extracts prepared in 100% n-hexane.

DPPH-free radical scavenging assay:-

The stable radical DPPH (1,1-diphenyl-2-picrylhydrazyl) was used to assess the free radical scavenging activity of the different solvent extracts as a direct read out of their anti-oxidant activity **(Venugopal and Devarajan, 2011)**. To 900 µl of each test sample (100 mg ml^{-1}), 100 µl of 95% methanol and 1 ml of freshly prepared DPPH solution in 95% methanol (1 mM) were added, mixed well and incubated at dark for 30 min. After 30 min, the absorbance was measured at 517 nm using methanol (95%) and deionized water with DPPH solution as reference and control respectively. The ability to scavenge the DPPH radical was measured using the following equation: % DPPH scavenged = {(Ac – At)/Ac} x 100, where 'Ac' is the absorbance of the control and 'At' is the absorbance of the sample (solvent extracts).

Phytochemical screening:-

Qualitative screening of essential phytochemicals was done based on procedures as described by **Lakshmanan *et al.* (2013)** and **Bello Oluwasesan *et al.* (2013)** with modifications.

Test for cardiac glycosides: 100 µl of crude solvent extract was mixed with 500 µl of glacial acetic acid containing 10% $FeCl_3$. Then, 1 ml of concentrated H_2SO_4 was added to the above mixture and observed for a greenish-yellow color at the interface.

Test for saponins: 100 µl of crude solvent extract was mixed with 500 µl of distilled water, shaken vigorously and observed for a stable persistent froth. Then, 3 drops of olive oil were added, shaken vigorously and observed for the formation of an emulsion.

Test for terpenoids: 100 µl of crude solvent extract was mixed with 500 µl of chloroform to which concentrated H_2SO_4 was added carefully to form a layer and observed for a reddish-brown coloration of the interface.

Test for tannins: 100 µl of crude solvent extract was mixed with few drops of 0.1% $FeCl_3$ and observed for brownish-green coloration.

Test for proteins and xanthoproteins: To 500 µl of crude solvent extract, few drops of concentrated nitric acid was added and observed for yellow coloration.

Test for alkaloids: 100 µl of crude solvent extract was stirred with 100 µl 1% HCl on a steam bath, followed by addition of Mayer's and Wagner's reagent, and observed for yellow colored precipitate.

Test for flavonoids: 100 µl of crude solvent extract was mixed with few drops of 1% NH_3 solution and observed for a yellow coloration.

Test for Reducing Sugars: To 100 µl of crude solvent extract, Fehling's solutions A and B were added. The mixture was warmed and observed for a brick red precipitate.

Test for steroids: 100 µl of crude solvent extract was dissolved in 900 µl chloroform. Then, 1 ml concentrated H_2SO_4 was added from the side of the test tube and observed for a brown ring at the interface.

Test for acidic compounds: To 100 µl of crude solvent extract, 100 µl sodium bicarbonate solution was added and observed for the production of effervescence.

Statistical Analysis:-
For determination of the anti-bacterial activity using disc diffusion assay, the results were expressed as mean ± standard deviation (SD) of the triplicate values. Analysis of variance (ANOVA) was done to determine whether anti-bacterial activity of n-hexane extracts varied significantly between *A. marina* and *A. alba* stems and leaves; possibilities ($p < 0.05$) were considered statistically significant.

Results:-

n-Hexane extracts of vegetative tissue parts (leaves and stems) of *A. marina* and *A. alba*, the two thriving major mangrove species of Indian Sundarbans were used to investigate their anti-microbial and anti-oxidant activities along with their phytochemical components.

The main phytochemical constituents screened in our study includes cardiac glycosides, saponins, terpenoids, tannins, xanthoproteins, alkaloids, flavonoids, reducing sugars, steroids and acidic compounds, all of which possess some degree of therapeutic values as shown in **Fig 3**.

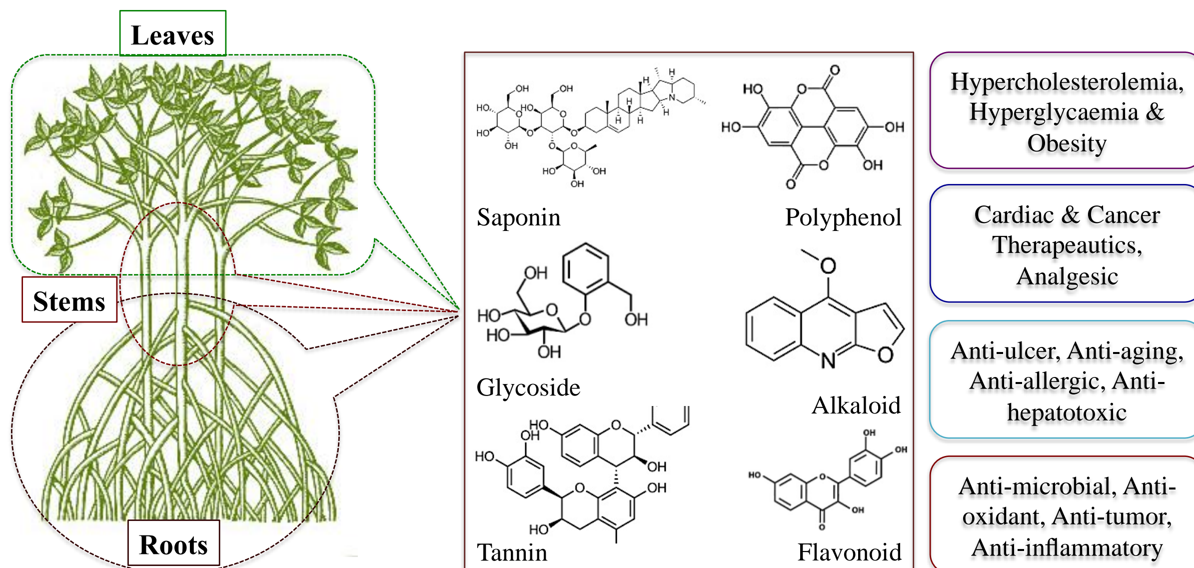

Fig 3:- Schematic representation of the essential phytochemicals typically derived from the vegetative plant parts and their potential medical significance.

Among the two mangrove plant species, the stems and leaves of *A. marina* possess maximum phytochemicals. However, the n-hexane extracts of these mangrove plant parts failed to show the presence of alkaloids, reducing sugars and acidic compounds. Bark extract of Arjuna and leaf extract of Neem in n-hexane along with D-glucose were used as positive controls whereas 100% n-hexane was used as a negative control for the qualitative phytochemical screening **(Table 2)**.

Table 2:- Phytochemical profile of the vegetative tissue parts of *A. marina* and *A. alba*, the dominant mangrove floral species of Indian Sundarbans.

Extract	Phytochemicals	Tests	Positive Observation	Positive Control	Negative Control	Results			
						AMS	AML	AAS	AAL
n-Hexane	CARDIAC GLYCOSIDES	Keller Killiani Test	Greenish-yellow color at the interface	Arjuna Bark in n-Hexane	n-Hexane	+	+	+	+
	SAPONINS	Froth Test	Formation of			+	+	+	+

			foam						
	TERPENOIDS	Solowski Test	A reddish-brown color at the interface			+	+	+	+
	TANNINS	Ferric Chloride Test	Brownish-green coloration			+	+	–	–
	PROTEINS AND XANTHOPROTEINS	Nitric Acid Test	Yellow coloration			+	+	+	+
	ALKALOIDS	Mayer's and Wagner's Test	Yellow colored precipitate	Neem Leaf in n-Hexane		–	–	–	–
	FLAVONOIDS	Ammonia Test	Yellow coloration	Arjuna Bark in n-Hexane		+	+	+	+
	REDUCING SUGARS	Fehling's Test	Brick red precipitate	Dextrose		–	–	–	–
	STEROIDS	Chloroform Test	A brown ring at the interface	Arjuna Bark in n-Hexane		+	+	+	+
	ACIDIC COMPOUNDS	Sodium Bicarbonate Test	Effervescence			–	–	–	–

DPPH-radical scavenging assay, which is a direct read out of anti-oxidant activity revealed significant % reduction of DPPH in case of *A. marina* vegetative tissue parts as compared to the vegetative tissue parts of *A. alba*, and the order of hierarchy is as follows: *A. marina* stems (AMS) > *A. marina* leaves (AML) > *A. alba* stems (AAS) > *A. alba* leaves (AAL). The bark extract of *T. arjuna* was used as a control **(Fig 4)**.

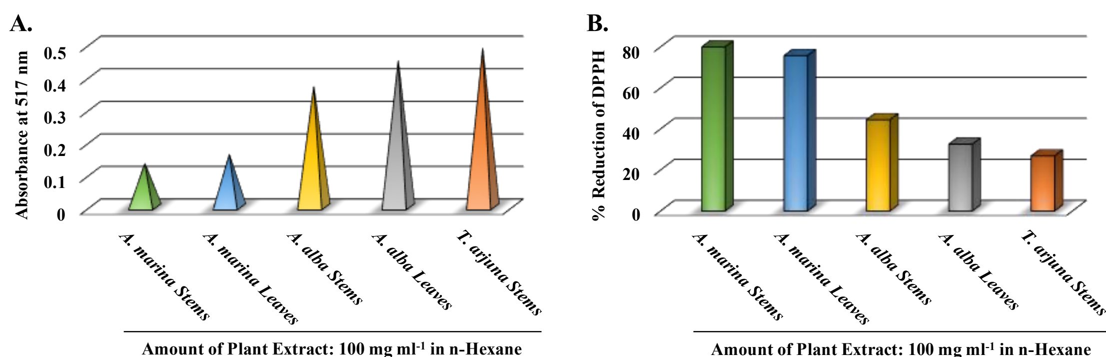

Fig 4:- DPPH-free radical scavenging assay. Bar graph of absorbance at 517 nm (A) and percentage reduction of DPPH (B) versus amount of different reductant (plant extract in n-Hexane) added, for the constant-volume colorimetric titration of DPPH.

For the anti-microbial profiling, both clinical and environmental bacteria were used. We first tested the resistivity profile of the three clinical bacterial pathogens against twenty-two clinically relevant antibiotics. Interestingly, we found that these bacterial strains possess moderate multi-drug resistance **(Table 3)**.

Table 3:- Resistivity profile of the three-clinical pathogenic bacterial strains against twenty-two clinically relevant antibiotics.

ANTIBIOTICS	CONCENTRATIONS	INHIBITION ZONE (IZ) DIAMETER (IN CM)		
		STAPHYLOCOCCUS AUREUS	*PROTEUS VULGARIS*	*SALMONELLA TYPHI*
1. CEFTAZIDIME (CAZ)	30 MCG	0 (R)	1.85 (S)	3.2 (S)
2. CIPROFLOXACIN (CIP)	5 MCG	3.2 (S)	2.4 (S)	4.7 (S)
3. AMIKACIN (AK)	30 MCG	2.9 (S)	1.8 (S)	2.8 (S)
4. NITROFURANTOIN (NIT)	300 MCG	3.5 (S)	0 (R)	0 (R)
5. NETILLIN (NET)	30 MCG	4 (S)	1.9 (S)	3.05 (S)
6. NALIDIXIC ACID (NA)	30 MCG	0 (R)	2.3 (S)	2.9 (S)
7. CO-TRIMOXAZOLE (COT)	25 MCG	0 (R)	1.5 (I)	2 (S)
8. AMOXYCLAV (AMC)	30 MCG	0 (R)	0 (R)	0 (R)
9. GENTAMICIN (GEN)	10 MCG	3.5 (S)	1.03 (I)	2.48 (S)
10. TETRACYCLINE (TE)	25 MCG	4 (S)	0 (R)	0 (R)
11. OFLOXACIN (OF)	5 MCG	2.2 (S)	1.5 (I)	3.35 (S)
12. CEFUROXIME (CXM)	30 MCG	3.4 (S)	0.85 (R)	1.5 (I)
13. CEFOTAXIME (CTX)	30 MCG	4 (S)	1.7 (S)	3.7 (S)
14. LEVOFLOXACIN (LE)	5 MCG	3.4 (S)	2.4 (S)	3.1 (S)
15. AZTREONAM (AT)	30 MCG	3 (S)	2.15 (S)	3.3 (S)
16. IMIPENEM (IPM)	10 MCG	5 (S)	0 (R)	0 (R)
17. CEFTAZIDIME (CAZ)	30 MCG	3.1 (S)	2.0 (S)	2.5 (S)
18. AMPICILLIN (AMP)	10 MCG	0 (R)	1.28 (I)	0 (R)
19. CHLORAMPHENICOL (C)	25 MCG	3.1 (S)	2.1 (S)	2.6 (S)
20. PENICILLIN G (P)	1 UNIT	0 (R)	0 (R)	0 (R)
21. STREPTOMYCIN (S)	10 MCG	3 (S)	1.33 (I)	2.5 (S)
22. SULPHATRIAD (S3)	300 MCG	2.9 (S)	1.88 (S)	3.8 (S)

IZ values are represented as mean of three independent experiments. R: Resistant; I: Intermediate; S: Susceptible (as per the zone of inhibition proposed by CLSI).

The susceptibility or resistivity profile of the other environmental test bacterial strains were also carried out separately against the same set of antibiotics (data not shown). Among the environmental isolates, some are MDR human pathogens, conventional human pathogens, plant pathogen, insect pathogen and indigenous halo-rhizobacterial strains of the Indian Sundarbans **(Table 1)**. In the disc diffusion assay, the control paper disc containing only n-hexane did not show any inhibition zones, confirming that the solvent alone does not have any anti-bacterial activity **(Fig 5)**.

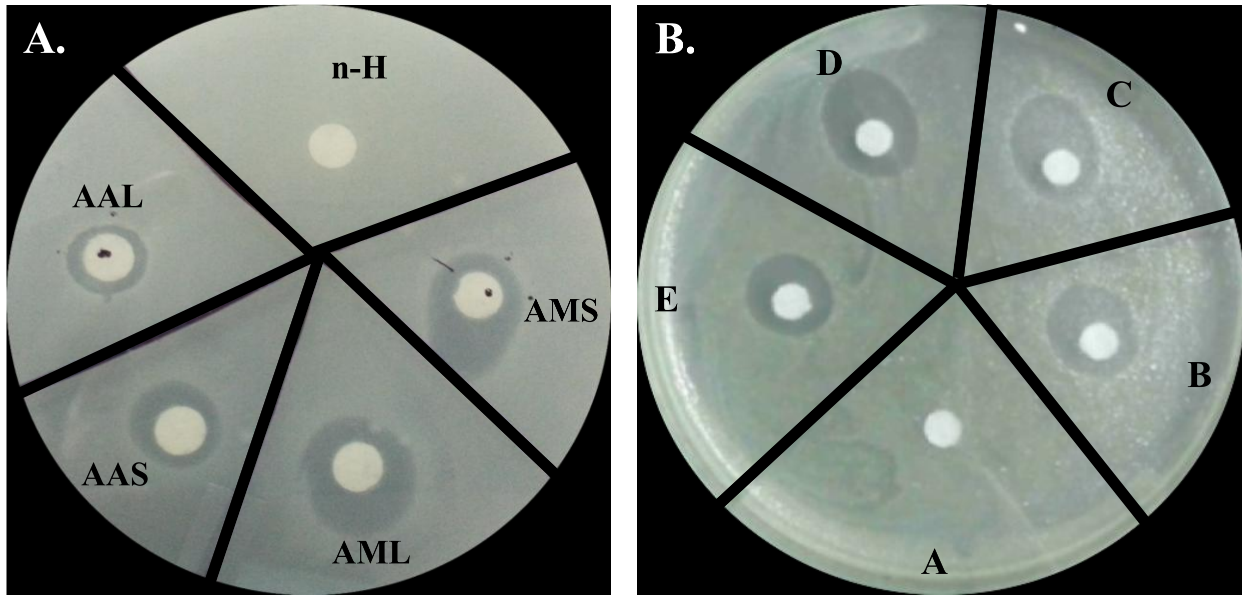

Fig 5:- Representative images of zone of inhibition (A) and minimum inhibitory concentrations of the n-hexane extracts of *A. marina* and *A. alba* stems and leaves against the test bacterial strains (B) from the disc diffusion assay. n-H (n-hexane control), AMS (*A. marina* stems), AML (*A. marina* leaves), AAS (*A. alba* stems), AAL (*A. alba* leaves), A-E (serial dilutions of solvent extracts in n-hexane in descending order; i.e., A = highest dilution and E = lowest dilution).

The anti-bacterial potency of n-hexane extracts of *A. marina* and *A. alba* vegetative aerial parts against all twelve clinical and environmental MDR and non-MDR pathogenic bacterial strains are represented graphically in **Fig 6**.

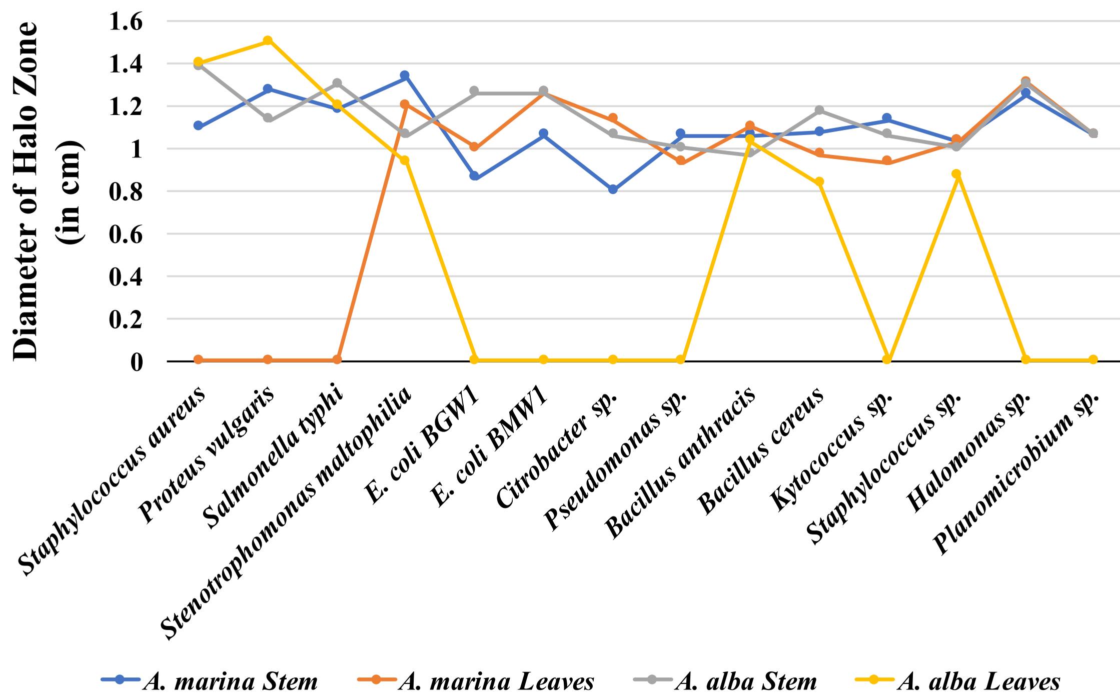

Fig 6:- Linear graph shows the anti-bacterial potency (measured as diameter of halo zones in centimeters) of n-hexane stem and leaf extracts of *A. marina* and *A. alba* against clinical and environmental MDR and non-MDR pathogenic bacterial strains. *Halomonas sp.* and *Planomicrobium sp.* (indigenous mangrove microbiota) served as controls.

The non-pathogenic environmental bacterial strains served as controls. The order of anti-bacterial activity of n-hexane extracts of *A. marina* and *A. alba* against all twenty-seven clinical and environmental bacterial strains is as follows: *A. marina* stems (AMS) > *A. alba* stems (AAS) > *A. marina* leaves (AML) > *A. alba* leaves (AAL) **(Table 5)**. We further calculated the cumulative values and average cumulative values of the halo zones for the solvent extracts of *A. marina* and *A. alba* vegetative parts against all twenty-seven test bacterial strains. We found that the cumulative and average cumulative values of the halo zones were higher in *A. marina* vegetative aerial parts as compared to *A. alba* vegetative aerial parts **(Table 5)**.

Table 5:- Anti-bacterial profile of the vegetative tissue extracts of *A. marina* and *A. alba* against clinical and environmental MDR and non-MDR pathogenic bacterial strains.

	Inhibition Zone (IZ) Diameter (in cm)							
Strain Name	**Strain No.**	**AMS (n-H)**	**AML (n-H)**	**AAS (n-H)**	**AAL (n-H)**	**n-H**	**A**	**K**
A. CLINICAL PATHOGENS								
1) MDR								
Staphylococcus aureus	NMC1	1.1±0.1	0	1.38±0.03	1.4±0	0	0	0.88±0.03
Proteus vulgaris	NMC2	1.27±0.31	0	1.13±0.06	1.5±0.26	0	0	0.9±0.1
Salmonella typhi	NMC3	1.18±0.28	0	1.3±0.26	1.2±0.1	0	0	0.97±0.03
B. ENVIRONMENTAL PATHOGENS								
I. For Humans								
1) MDR								
Stenotrophomonas maltophilia	MDR58	1.33±0.12	1.2±0.2	1.06±0.12	0.93±0.12	0	0	0
E. coli	BGW1	0.86±0.31	1±0.2	1.26±0.12	0	0	0	0
E. coli	BMW1	1.06±0.12	1.26±0.12	1.26±0.12	0	0	0	0
Citrobacter sp.	HGW2	0.8±0.2	1.13±0.31	1.06±0.31	0	0	0	0
Pseudomonas sp.	AS18	1.06±0.12	0.93±0.12	1±0	0	0	0	0
2) Non-MDR								
Bacillus anthracis	B7	1.06±0.06	1.1±0.1	0.97±0.06	1.03±0.06	0	1.6±0	N.A.
Bacillus cereus	A7	1.07±0.12	0.97±0.15	1.17±0.35	0.83±0.06	0	1.6±0	N.A.
Kytococcus sp.	C8	1.13±0.12	0.93±0.12	1.06±0.12	0	0	N.A.	N.A.
Staphylococcus sp.	A5	1.03±0.06	1.03±0.21	1±0.2	0.87±0.12	0	2.0±0	N.A.
II. For Plants								
Agrobacterium tumifaciens	TS14	0.9±0.1	1.13±0.15	0	0	0	0	0
III. For Insects								
Bacillus thuringiensis	B4	1.2±0.2	1.13±0.31	1.06±0.12	0	0	1.6±0	N.A.
C. ENVIRONMENTAL NON-PATHOGENS								
Halomonas sp.	AM1	0	1.15±0.05	1.1±0.09	0	0	0	N.A.
Halomonas sp.	AM4	0.98±0.03	1.17±0.05	1.23±0.06	0	0	0	N.A.
Halomonas sp.	AM5	1.25±0.05	1.31±0.15	1.3±0.05	0	0	0	N.A.
Halomonas sp.	AM7	1.13±0.12	0.93±0.12	0.93±0.31	0.8±0.2	0	0.95±0.05	N.A.
Halomonas sp.	AA3	1.18±0.03	1.3±0.05	1.35±0.05	0	0	1.2±0	N.A.
Halomonas sp.	SW5	1.18±0.03	0.98±0.03	0.98±0.03	0	0	0	N.A.
Paracoccus kamogawaensis	AM2	1.13±0.12	1.2±0.2	1±0.2	0	0	N.A.	N.A.
Paracoccus	SW3	1.26±0.31	1.13±0.12	1.06±0.23	0	0	N.A.	N.A.

ZEAXANTHINIFACIENS								
OCEANOBACILLUS SP.	AM3	0.67±0.12	1±0.2	0	0	0	N.A.	N.A.
BREVIBACILLUS AGRI	AA5	1±0.2	0.86±0.12	1.06±0.12	0	0	N.A.	N.A.
PLANOMICROBIUM SP.	SW7	1.06±0.12	1.06±0.12	1.06±0.31	0	0	N.A.	N.A.
BACILLUS PUMILUS	PSB1	1±0	0.93±0.12	1±0	1.07±0.12	0	N.A.	N.A.
DELFTIA SP.	SR11	1.23±0.06	1±0.17	0.93±0.06	0	0	N.A.	N.A.
CUMULATIVE VALUE*		**28.12**	**25.83**	**27.71**	**9.63**	**0**	N.A.	N.A.
AVERAGE CUMULATIVE VALUE*		**1.04**	**0.96**	**1.03**	**0.36**	**0**	N.A.	N.A.

IZ values are represented as mean ± standard deviation (n = 3). Diameter of filter paper disc = 0.6 cm. AMS: *A. marina* stems; AML: *A. marina* leaves; AAS: *A. alba* stems; AAL: *A. alba* leaves; n-H: n-hexane. Standard antibiotics used: ampicillin (A); kanamycin (K). *The sum and the cumulative average values of the n-hexane extracts of *A. marina* and *A. alba* stems and leaves were derived using ANOVA (two-factor without replication) (N = 27).

The significance of variation in anti-bacterial activity was confirmed through ANOVA (**Table 6**).

Table 6:- ANOVA showing variation of anti-microbial activity in vegetative tissue extracts of *A. marina* and *A. alba.*

	$F_{Observed}$	$F_{Critical}$
ANTI-MICROBIAL ACTIVITY		
1. Between Test Bacterial Strains (Rows)	0.6679	1.6380
2. Between Solvent Extracts of Mangrove Plant Parts (Columns)	18.1427	2.7217

N (No. of test bacterial strains) = 27

The MIC values of the solvent extracts of *A. marina* and *A. alba* stems and leaves ranged from 20 mg ml^{-1} to 40 mg ml^{-1} (**Fig 5**).

Discussion:-

The phytochemical profiling of the vegetative tissue extracts of the two dominant floral species of Indian Sundarbans reveals the presence of several essential phytochemicals with therapeutic importance namely cardiac glycosides, saponins, terpenoids, tannins, xanthoproteins, flavonoids and steroids. Cardiac glycosides are organic compounds with a sugar (glycoside) and non-sugar (steroid) moiety, and are used to treat congestive heart disorders and cancers (**Newman *et al.,* 2008**). Saponins are natural glycosides with cytotoxic activity and several other pharmacological properties (**Podolak *et al.,* 2010**). Terpenoids are derivatives of five-carbon isoprene units, used widely for their aromatic qualities and for their role in traditional herbal remedies (**Patra and Mohanta, 2014**). Tannins are high molecular weight plant polyphenolics with anti-oxidant properties (**Hagerman *et al.,* 1998**). Flavonoids are hydroxylated phenolic substances where a C_6-C_3 unit is linked to an aromatic ring and they are known to have anti-microbial properties against a wide range of microbes including *Streptococcus mutans*, *Vibrio cholera*e and *Shigella* (**Critchfield et al., 1996**). Steroids are four-ringed organic compounds arranged in specific configurations and have anti-cancer properties (**Lubik *et al.,* 2016**). However, alkaloids, reducing sugar and acidic compounds were absent in these solvent extracts. Alkaloids being water soluble might have failed to solubilize in non-polar n-hexane.

The DPPH-radical scavenging assay was used as a direct read out for anti-oxidant activity of the various solvent extracts of *A. marina* and *A. alba* stems and leaves. DPPH (2,2-diphenyl-1-picrylhydrazyl) is a stable free-radical molecule and routinely used to monitor chemical reactions involving radicals. Therefore, DPPH is also known as a free radical scavenger (**Sharma and Bhat, 2009**). Therefore, less is the absorbance of test samples at 517 nm more is the percentage reduction of DPPH and more is the production of free radicals from the leaf and stem extracts of *A. marina* and *A. alba.* Although both *A. marina* and *A. alba* displayed almost similar phytochemical profile (except for tannins, which were missing in the n-hexane fraction of *A. alba* leaves and stems), however, solvent extracts of *A. marina* leaves and stems showed highest anti-oxidant activity, suggesting the role of tannins as biological antioxidants (**Hagerman *et al.,* 1998**).

The clinical and environmental bacterial strains chosen for this study encompass both Gram-positive and Gram-negative bacteria, and most of them are MDR human pathogens capable of causing diseases that ranges from infections of the respiratory tract, urinary tract, skin, stomach, intestine and the brain **(Table 6).**

Table 6:- Potential diseases that are linked with the test pathogenic bacterial strains used.

	Bacterial strains used	**Diseases caused**
A.	**Clinical strains**	
1.	*Staphylococcus aureus*	Skin & Respiratory Infections; Food Poisoning
2.	*Proteus vulgaris*	Wound Infections; Urinary Tract Infections
3.	*Salmonella typhi*	Typhoid Fever
B.	**Environmental strains**	
4.	*Stenotrophomonas maltophilia*	Nosocomial Infections
5.	*Escherichia coli**	Food Poisoning
6.	*Citrobacter sp.*	Infections of the Urinary Tract; Infant Meningitis and Sepsis
7.	*Pseudomonas sp.*	Opportunistic Human Pathogen
8.	*Bacillus anthracis*	Anthrax
9.	*Bacillus cereus*	Foodborne Illness
10.	*Kytococcus sp.*	Opportunistic Human Pathogen
11.	*Staphylococcus sp.*	Food Poisoning; Sialadenitis
12.	*Halomonas sp.***	N.A.
13.	*Planomicrobium sp.*	N.A.

*Two different strains used; **Six different strains used.

Therefore, the fact that *A. marina* and *A. alba* vegetative tissue extracts showed varied degree of anti-bacterial activity with *A. marina* plant extracts showing the highest activity against these MDR and non-MDR bacterial pathogens suggest the potential of *A. marina* in alternative disease therapeutics of human patients. However, this study needs to be further corroborated with additional experiments (including *in vitro* cytotoxicity profile of these solvent extracts together with *in vivo* testing in mouse models) to arrive at a more definitive conclusion. The control non-pathogenic bacterial strains chosen in this study (except for *A. tumifaciens* and *B. thuringiensis*, which are plant and insect pathogens respectively) are also varied in terms of their nature of Gram staining, source, biochemical and physiological properties. These control strains helped us to compare and identify whether the anti-bacterial potency of the *A. marina* and *A. alba* vegetative tissue extracts is specific to only MDR bacterial pathogens or is generic in nature. The ANOVA data suggests the overall generic nature of the plant tissue extracts. We propose that the anti-bacterial activity of the various n-hexane extracts might be due to the presence of some of the essential phytochemicals mentioned above. However, a detailed analysis such as direct bioautography (with the test bacteria) on thin layer chromatograms **(Homans and Fuchs, 1970)** followed by GC-MS of the pure solvent fractions are needed to pin point the key phytochemicals that are responsible for the anti-bacterial activity.

Taken together, our results for phytochemical, anti-microbial and anti-oxidant profiling suggest the potential role of the vegetative aerial parts of the two members of the Avicenniaceae family in alternative disease therapeutics especially *A. marina* as compared to its other counterpart *A. alba*.

Conclusion:-

The vegetative tissue parts (leaves and stems) of *A. marina* and *A. alba* contain essential phytochemicals including flavonoids, steroids, cardiac glycosides, terpenoids, saponins and tannins, all of which have significant medicinal importance. The vegetative tissue extracts (in n-hexane) of *A. marina* showed significant anti-oxidant activity compared to its counterpart. The three clinical bacterial pathogens show moderate multi-drug resistance. The vegetative tissue extracts of *A. marina* also showed significant anti-bacterial activity against the clinical and environmental MDR and non-MDR bacterial pathogens.

Acknowledgements:-

The authors express utmost gratitude to Techno India University, West Bengal for providing us with the instruments and chemicals necessary for our project. We would like to thank Dr. Rajdeep Saha of Calcutta National Medical College, Kolkata for providing us with the clinical human pathogens. We also thank Atanu Roy, Roopali

Roychowdhury and Satarupa Roy for lending their environmental bacterial strains along with Sonia Mukherjee for her contribution in the biochemical assays.

References:-

1. Banerjee, D., Chakrabarti, S., Hazra, A.K., Banerjee, S., Ray, J. and Mukherjee, B. (2008): Antioxidant activity and total phenolics of some mangroves in Sundarbans. Afr J Biotechnol., 7: 805-810.
2. Banerjee, L.K., Rao, T.A., Shastry, A.R.K. and Ghosh, D. (2002): Diversity of Coastal Plant Communities in India. ENVIS & EMCBTAP-Botanical Survey of India, Ministry of Environment & Forests, pp. 524.
3. Barik, J. and Chowdhury, S. (2014): Mangrove Species of Sundarbans Delta, India. Check List., 10(2): 329-334.
4. Bello Oluwasesan, M., Zack Agbendeh, M. and Adikwu Jacob, G. (2013): The comparative studies of phytochemical screening of Piliostigma thonningii root and leaves extract. Asian Journal of Plant Science and Research., 3(6): 74-77.
5. Chaudhuri, A.B. and Choudhury, A. (1994): Mangroves of the Sundarbans. IUCN – The World Conservation Union., 1: 165.
6. Critchfield, J.W., Butera, S.T. and Folks, T.M. (1996): Inhibition of HIV activation in latently infected cells by flavonoid compounds. AIDS research and human retroviruses., 12(1): 39-46.
7. Davies, J. (1994): Inactivation of antibiotics and the dissemination of resistance genes. Science., 264: 375-382.
8. El Shafay, S.M., Ali, S.S. and El-Sheekh, M.M. (2015): Antimicrobial activity of some seaweeds species from Red sea, against multidrug resistant bacteria. The Egyptian Journal of Aquatic Research., 42(1): 65-74.
9. Green, E., Obi, L.C., Samie, A., Bessong, P.O. and Ndip, R.N. (2011): Characterization of n-Hexane sub-fraction of Bridelia micrantha (Berth) and its antimycobacterium activity. BMC complementary and alternative medicine., 11(1): 28.
10. Hagerman, A.E., Riedl, K.M., Jones, G.A., Sovik, K.N., Ritchard, N.T., Hartzfeld, P.W. and Riechel, T.L. (1998): High molecular weight plant polyphenolics (tannins) as biological antioxidants. Journal of Agricultural and Food Chemistry., 46(5): 1887-1892.
11. Homans, A.L. and Fuchs, A. (1970): Direct bioautography on thin layer chromatograms as a method for detecting fungitoxic substances. J Chromatogr., 51: 327-329.
12. Hossain, M.A., Kabir, M.J., Salehuddin, S.M., Rahman, S.M., Das, A.K., Singha, S.K., Alam, M.K. and Rahman, A. (2010): Antibacterial properties of essential oils and methanol extracts of sweet basil Ocimum basilicum occurring in Bangladesh. Pharmaceutical biology., 48(5): 504-511.
13. Khafagi, I., Gab-Alla, A., Salama, W. and Fouda, M. (2003): Biological activities and phytochemical constituents of the gray mangrove *Avicennia marina* (Forssk.) Vierh. Egypt J Bot., 5: 62-69.
14. Kokpal, V., Miles, D.H., Payne, A.M. and Chittarwong, V. (1990): Chemical constituents and bioactive compounds from mangrove plants. Stud Nat Prod Chem., 7: 175-199.
15. Lakshmanan, G., Rajeshkannan, C., Kavitha, A., Mekala, B. and Kamaladevi, N. (2013): Preliminary screening of biologically active constituents of Suaeda monoica and Sesuvium portulocastrum from palayakayal mangrove forest of Tamilnadu. Journal of Pharmacognosy and Phytochemistry., 2(3).
16. Lubik, A.A., Nouri, M., Truong, S., Ghaffari, M., Adomat, H.H., Corey, E., Cox, M.E., Li, N., Guns, E.S., Yenki, P. and Pham, S. (2016): Paracrine sonic hedgehog signaling contributes significantly to acquired steroidogenesis in the prostate tumor microenvironment. International Journal of Cancer., 140(2): 358-369.
17. Muthazhagan, K., Thirunavukkarasu, P., Ramanathan, T. and Kannan, D. (2014): Studies on Phytochemical Screening, Antimicrobial and Anti Radical Scavenging Effect Coastal Salt Marsh Plant of a Suaeda monoica. Res J Phytochem., 8: 102-111.
18. Newman, R.A., Yang, P., Pawlus, A.D. and Block, K.I. (2008): Cardiac glycosides as novel cancer therapeutic agents. Molecular interventions., 8(1): 36.
19. Nezhad, F.M., Zeigham, H., Mota, A., Sattari, M. and Yadegar, A. (2009): Antibacterial activity of eucalyptus extracts on methicillin resistance Staphylococcus aureus. Res J Biol Sci., 4: 905-908.
20. O'Bryan, C.A., Crandall, P.G., Chalova, V.I. and Ricke, S.C. (2008): Orange essential oils antimicrobial activities against Salmonella spp. J Food Sci., 73: 264-267.
21. Patra, J.K. and Mohanta, Y.K. (2014): Antimicrobial compounds from mangrove plants: A pharmaceutical prospective. Chinese journal of integrative medicine., 20(4): 311-320.
22. Patra, J.K., Dhal, N.K. and Thatoi, H.N. (2011): In vitro bioactivity and phytochemical screening of Suaeda maritime (Dumort): A mangrove associate from Bhitarkanika, India. Asian Pac J Trop Med., 4(9): 727-734.

23. Podolak, I., Galanty, A. and Sobolewska, D. (2010): Saponins as cytotoxic agents: a review. Phytochemistry Reviews., 9(3): 425-474.
24. Premanathan, M., Nakashima, H., Kathiresan, K., Rajendran, N. and Yamamoto, N. (1996): In vitro antihuman immunodeficiency virus activity of mangrove plants. Ind J Med Res., 130: 276-279.
25. Sharma, O.P., and Bhat, T.K. (2009): DPPH antioxidant assay revisited. Food chemistry., 113(4): 1202-1205.
26. Vadlapudi, V. (2012): In vitro antimicrobial activity of plant extracts of Avicennia alba against some important pathogens. Asian Pacific Journal of Tropical Disease., 2: S408-S411.
27. Venugopal, S. and Devarajan, S. (2011): Estimation of total flavonoids, phenol and antioxidant activity of local and New Zealand manuka honey. Journal of Pharmacy Research., 4: 464-466.
28. World Health Organization. (2001): WHO global strategy for containment of antimicrobial resistance.
29. Wu, J., Xiao, Q., Xu, J., Li, M.Y., Pan, J.Y. and Yang, M.H. (2008): Natural products from true mangrove flora: source, chemistry and bioactivities. Natural Product Reports., 25(5): 955-981.