BIOSCIENCES BIOTECHNOLOGY RESEARCH ASIA, October 2014. Vol. **11**(Spl. Edn. 1), p. 243-251

Characterization of Antibacterial Phytochemicals from Three Coastal Sand Dunes in Chennai Beaches

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doi: http://dx.doi.org/10.13005/bbra/1417

(Received: 15 August 2014; accepted: 10 October 2014)

***Ipomoea sp* and *Spinifex sp* most widely distributed salt tolerant plants and usually thrive in harsh condition .The present investigation was carried out to determine the antibacterial activity as well as phytochemical analysis using a GC-MS technique.The different solvent extracts (ethyl acetate and methanol) of the plant were tested for antibacterial activity against human pathogens.The result highlighted that the antibacterial activity of ethyl acetate extract of *Spinifex* sp. panicle was especially active against *P. aeruginosa*(mean ; 21mm) and *E. faecalis*(mean ; 17mm). The extract concentration providing 50% inhibition (IC_{50}) was calculated from the graph of inhibition percentage plotted against extract concentration (25-250 μg/mL) and IC_{50} is found to be 100 μg/mL which showed 53.3% inhibition (0.035 O.D) for *E .faecalis.* IC_{50} is found to be 225 μg/mL which showed 55.35% inhibition(0.025 O.D) for *P. aeruginosa.* The phytochemical analysis of the crude extract revealed the presence of alkaloid, flavonoid, sugar, anthraquinones, saponines and phenolic compounds.In TLC, prominent bands were obsevered in Hexane and ethyl acetate combination of concentration 6:4.In bioautography, a clear zone was seen in the *E. faecalis* plate amended with agar.**

Key words: Phytochemicals, Sand dune plants, antibacterial activity.

In recent years, research on biologically active phytochemicals has attracted a lot of attentions globally. Plants produce a diverse amount of secondary metabolites, many of which have antimicrobial activity. Some of these compounds are constitutive, existing in healthy plants in their biologically active forms. Antimicrobials of plant origin have enormous therapeutic potential. They are effective in the treatment of infectious diseases while simultaneously mitigating many of the side effects that are often associated with synthetic antimicrobials. The beneficial medicinal effects of plant materials typically result from the combinations of secondary products present in the plant. In plants, these compounds are mostly secondary metabolites such as alkaloids, steroids, tannins, and phenol compounds, flavonoids, steroids, resins fatty acids gums which are capable of producing definite physiological action on body (Joshi *et al*., 2009). Compounds extracted from different parts of the plants can be used to cure diarrhea, dysentery, cough, cold, cholera, fever bronchitis etc. Decoction or powder of *Xanthoxylum armatum* (Rutaceae) can be taken orally with warm water to treat constipation, stomach pain, toothache and cold (Joshi and Edington, 1990; Manadhar, 1987). The main objective of the research is to screen and evaluate

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antibacterial activity of crude ethanol extract and to find out minimum bactericidal concentration (MBC) against these extracts both gram positive as well as gram negative bacteria (Pokhrel, 2000).. Hence, researchers across the globe are continuously working on the extraction of bioactive metabolites from natural bioresources (Cowan, M.M. 1999).

Coastal Sand Dunes plants are dynamic, but fragile buffer zones of sand and vegetation are formed. Many wild plants have their own unique medicinal values, but majority of them were endemic and are found only in specific ecological niche. CSD are special unique ecologically sensitive niches between terrestrial and marine realms, but their floral composition is poorly understood especially in terms of phytomedicial values. Thus it forms a gap in understanding the diversity, ecological, functional, economical value and conservation of coastal dune vegetation worldwide, especially in Indian coasts. These habitats have been severely affected by natural and anthropogenic activities resulting in loss of habitat and dependent flora and fauna (Padmavathy *et al.*, 2011).

Spinifex is a genus of perennial coastal grasses. They are one of the most common plants that grow in sand dunes along the coasts of Australia, New Zealand and New Caledonia. As they help stabilise the sand, these grasses are an important part of the entire sand dune ecosystem. Of the four species three are indigenous to Australia and one to Indonesia. *Spinifex* is drought tolerant and persists under harsh conditions, growing on poor, shallow soils. Seedlings can enter into a virtual dormant state facilitating survival under poor conditions or periods of intense competition from neighbouring vegetation

Ipomoea is the largest genus in the flowering plant family Convolvulaceae, with over 500 phytochemical extracts from morning glories. It grows on the upper parts of beaches and endures salted air. It is one of the most common and most widely distributed salt tolerant plants and provides one of the best known examples of oceanic dispersal. Its seeds float and are unaffected by salt water. Goat's Foot is a primary sand stabilizer being one of the first plants to colonise the dune. It grows on almost all parts of the dune but is usually found on the seaward slopes sending long runners down towards the toe of the dune. The sprawling runners spread out from the woody rootstock but the large two-lobed leaves are sparse and a dense cover on the sand is rarely achieved except in protected situations. This plant grows in association with sand *Spinifex* grass and is a useful sand binder thriving under conditions of sand blast and salt spray (Daniel F. 2004). With this background the present study was initiated to study the antibacterial activity of *Spinifex* sp. and *Ipomoea* sp. against human pathogen and to partially purify the bioactive metabolite responsible for the antibacterial activity.

MATERIALS AND METHODS

Maintenance of culture

The microorganisms used in the study were two gram positive *E. coli* (MTCC 443),*S. aureus* (MTCC 96), two gram negative *E. faecalis*(MTCC 439), *P. aeruginosa(*MTCC 424) and one yeast *C. albicans*(MTCC 183) .All cultures were obtained from Infectious diseases laboratory YRG CARE , Chennai and maintained in usual laboratory conditions.

Plant collection

Ipomoea sp. and *Spinifex* sp. plants were collected from the coastal sand dunes of Uthandi, Chennai in January, 2012. The plant materials were shade dried at temperature (35°C ± 2) and ground into coarse powder.

Preparation of different parts of plant extract

The plant parts were carefully washed with tap water, rinsed with distilled water, and air-dried for 1 hour. Then it was cut into small pieces & dried in room temperature. Then they were ground into powder and stored in room temperature.

Direct extraction with different solvent

In this method, finely ground material (0.5 gm) was extracted with 5 ml of ethyl acetate and methanol in conical flask in shaking condition or in Soxhlate apaparatus. The extract was decanted in to pre-weighed glass vials. The process was repeated 3 times and the same material but using fresh solvent. The solvent was removed by placing the extracts in front of a steam of air in a fume hood at room temperature. The extracted residues were weighed and re-dissolved in different solvents to yield 1mg/ml solutions ready for further analysis.

Experimental design for bioassays

Four treatments were used in bioassays

Grou 1

Test group consisted of the organism plus different concentration of the extracts (this group determined if the extracts are effective as anti bacterial agents).

Group 2

Positive controls: organism plus a known antibiotic (This ensured that utilized organisms were susceptible to common chemotherapeutics and were not resistant strains).

Group 3

Pure cultures Only the organism in the absence of antibiotics or fruit extract. This was to ensure that the organism was growing properly under the defined laboratory conditions. This was necessary to distinguish poor growth from inhibition of growth.

Group 4

Negative controls: Organism plus the pure extraction solvent (this was necessary to prove that the extraction solvent had no inhibitory action of its own). All determination where quantitative data are important was carried out in duplicate.

Well diffusion assay (Fazeli *et al*., 2007)

Nutrient agar prepared was poured in the Petri dish. 17 h growing culture were swabbed on it. The wells (8mm diameter) were made by using cork borer. The different concentration of the crude extract, and the standard streptomycin were loaded in the wells. The plates were then incubated at 37ºC for 24 h. The inhibition diameter was measured after incubation.

MTT - (3- (4, 5 – Dimethylthiazol-2-yl)-2, 5-Diphenyltetrazolium Bromide) assay (Gabrielson *et al*., 2002)

In the microtitre plate 5µl of the 17 h growing culture with different concentration of crude extract, was added and the plates was kept for incubation at 37ºC for 24 h. 5mg of MTT was weighed and dissolved with 1 ml of milli Q water and 10 µl of this preparation is added to each well and kept for 4 h incubation. The contents were collected and centrifuged at 8000 rpm for 15 min, and the pellets were dissolved with 100µl of Dimethyl sulphoxide. Then the contents were transferred to the appropriate well and read at 570 nm in the ELISA reader. The percentage of viable cells was calculated.

Phytochemical analysis

The concentrated residue from the gradient solvent extracts of the plant material was used to detect the secondary plant metabolites including alkaloids, glycoside, flavanoids, saponins, and anthraquinones based on the methodology described by Asfaw Debella .

Thin layer chromatography

Appropriate size silica plates were taken, crude extract was spotted using capillary tube to TLC plate. Plate were kept into beaker containing solvents in the combinations of hexane, ethyl acetate and hexane, chloroform at different concentrations (9:1, 8:2, 6:4 and 2:8).Compounds were separated and the distance travelled by the solvent and the distance travelled by the compounds were measured.The R_F values, the ratio of the distance travelled by the compound to the distance travelled by the solvent, was calculated as follows.

$$R_f = \frac{\text{Distance travelled by the compound}}{\text{Distance travelled by the solvent front}}$$

Bioautography

Four Developed chromatography plates of ethyl acetate extract of *Spinifex* sp. panicle dried overnight, sprayed with suspension of actively growing cells of test pathogenic bacteria (*P. aeruginosa* and *E. feacalis*) and in two plates a thin layer of agar was poured and incubated at 37°C.After incubation the plates were sprayed with MTT (5mg/ml).The set-up was kept for 4 hrs at 37°C.Clear zones on the chromatogram indicated inhibition of bacterial growth.

Partial purification of compounds – Preparative TLC

In 5 X 5cm TLC plate, crude extract were spotted in a line, the TLC plate was run in a solvent of combination hexane and ethyl acetate. The plate were dried and 0.5cm plate were cutted and developed in an iodine chamber. The bands were observed and marked, the bands were scrapped and taken in an eppendorff. 0.5ml of EA was added and was mixed well in shaker for 10mins. It was centrifuged, and the supernatant was transferred in a fresh eppendorff tube. The compounds were marked as MNP-1, MNP-2, MNP-3 and MNP-4. TLC were run from the above compounds and tested for its purity.

RESULTS

Antibacterial activity of crude extracts of Coastal Sand Dune plants

The antibacterial activity of Ethyl Acetate and methanol extract of *Spinifex* leaf, stem, panicle, *Ipomoea* stem, seed, flower, fruit coat, seed cover and leaf yielded activity against *P. aeruginosa* and *E. faecalis*. The ethyl acetate extract of *Spinifex* panicle produced an inhibitory zone with a mean diameter of 21mm with both the Gram negative and Gram positive bacteria. The ethyl acetate extract of *Spinifex* panicle was especially active against *P. aeruginosa*(mean ; 21mm) and *E. faecalis*(mean ; 17mm)(Fig.1).The ethyl acetate extract was choosen for further studies.

Broth dilution assay of crude extracts of Coastal Sand Dune plants

The extract concentration providing 50% inhibition (IC_{50}) was calculated from the graph of inhibition percentage plotted against extract concentration (25-250 µg/mL) and IC_{50} is found to be 100 µg/mL which showed 53.3% inhibition (0.035 O.D) for *E. faecalis*.IC_{50} for *P. aeruginosa* is found to be 225 µg/mL which showed 55.35% inhibition (0.025 O.D) (Fig. 2).

Phytochemical analysis of crude extracts of Coastal Sand Dune plants

The phytochemical analysis of the crude extract revealed the presence of alkaloid, flavonoid, sugar, anthraquinones, saponines and phenolic were less.

Thin layer chromatography of crude extracts of Coastal Sand Dune plants

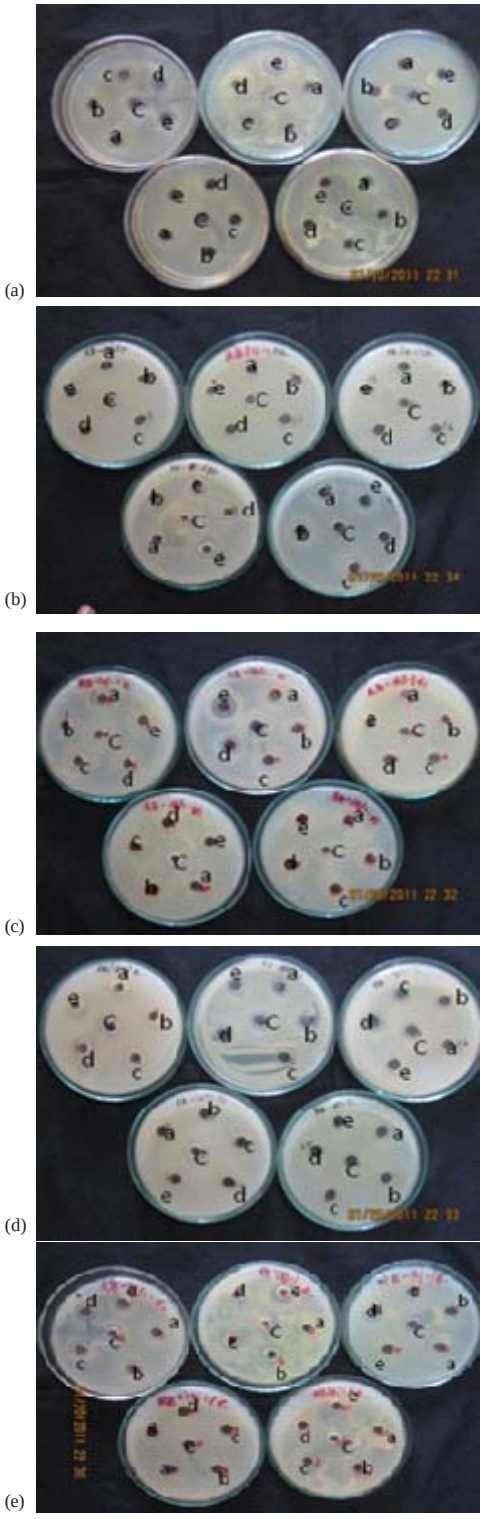
The extract were run on Thin layer chromatography plate using different concentration of solvents (Hexane : Ethyl acetate and Hexane : Chloroform in 9:1, 8:2, 6:4 and 2:8 ratio respectively) and as a result many distinct bands were found in hexane : ethyl acetate, in 6:4 ratio respectively (Fig. 4).

Bioautography of crude extracts of Coastal Sand Dune plants

After incubation of the TLC plate with MTT a clear zone around a particular band is observed and the R_f value is found to be 0.75 for *E. faecalis* respectively (Fig.5).

Gas Chromatography of crude extracts of Coastal Sand Dune plants

The major components and their retention

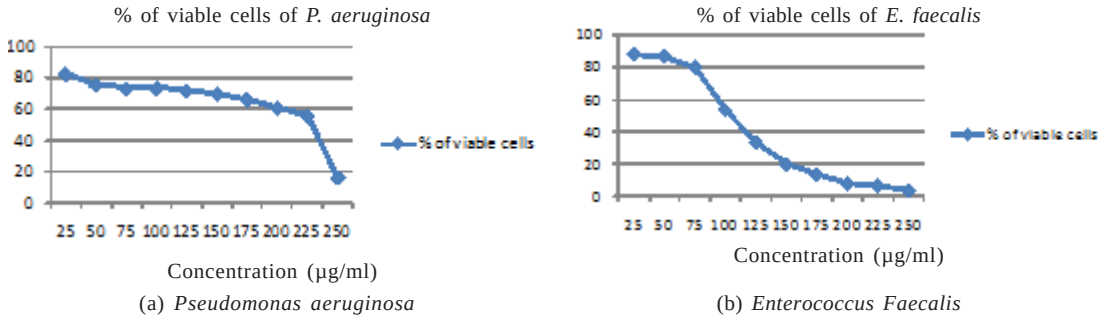


a = control, b = 50µg, c = 250µg, d = 500µg, e = 1000µg, C = empty well

Fig. 1. Antibacterial activity of plant crude extract against *Pseudomonas aeruginosa* and *Enterococcus feacalis*

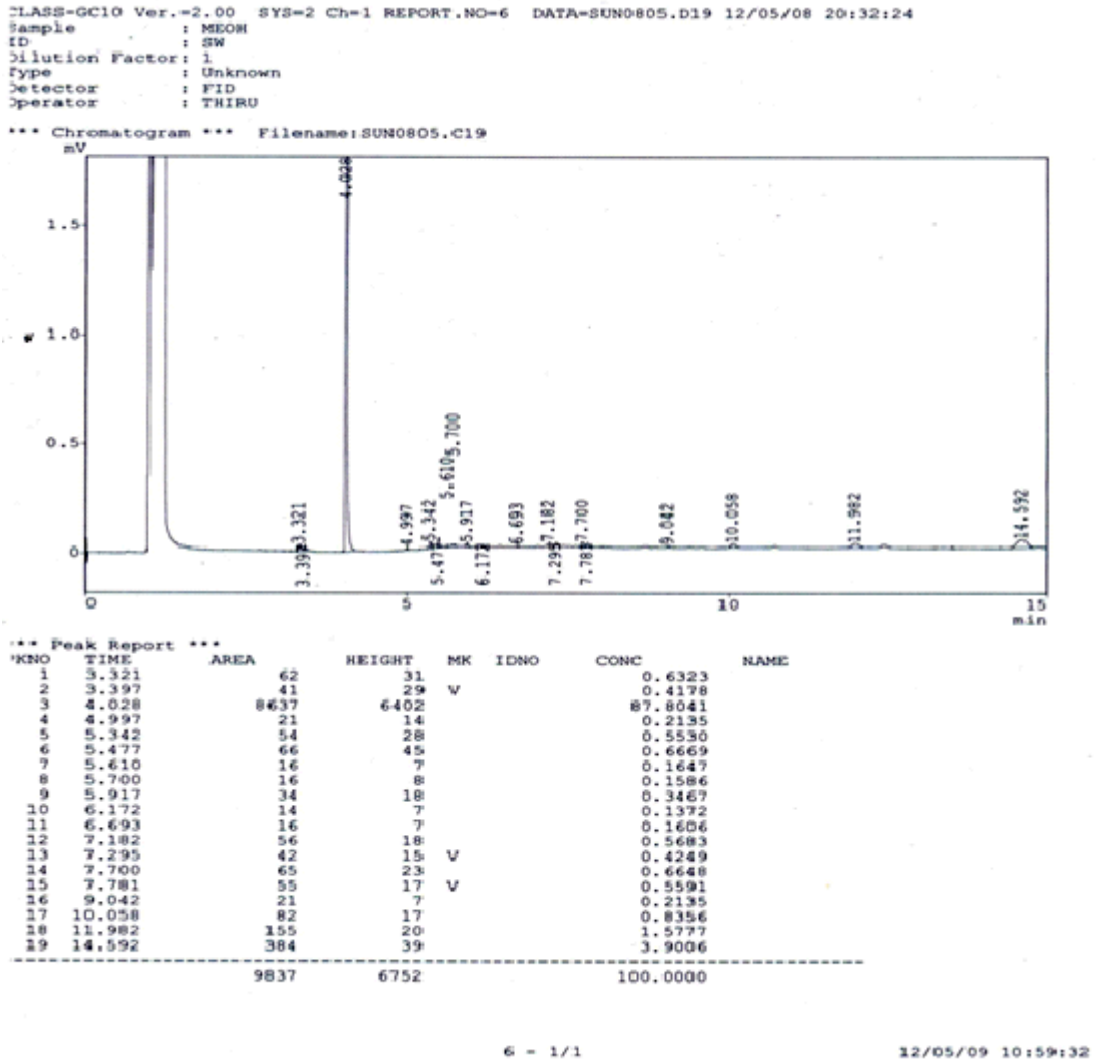
Tables 1. Antibacterial activity of plant crude extracts against *Pseudomonas aeruginosa and Enterococcus faecalis*

S. No	Crude Extract	Concentrations (µg/Ml)	Zone of Inhibition For *Pseudomonas Aeruginosa*(Mm)	Zone of Inhibition For *Enterococcus Faecalis*
1	I-SEED(EA)	50	13	10
		250	14	12
		500	18	15
		1000	20	16
2	I-seed(M)	50	10	12
		250	12	13
		500	12	14
		1000	15	15
3	I-stem(M)	50	10	12
		250	13	13
		500	15	14
		1000	17	16
4	I-flower(M)	50	13	13
		250	14	15
		500	14	16
		1000	15	16
5	I-flower(EA)	50	14	No zone
		250	15	12
		500	16	13
		1000	15	No zone
6	I-seed cover(M)	50	No zone	14
		250	10	16
		500	11	17
		1000	16	18
7	I-fruit cover(EA)	50	No zone	11
		250	13	14
		500	14	13
		1000	16	No Zone
8	I-fruit cover(M)	50	No zone	11
		250	14	12
		500	16	12
		1000	17	14
9	S-stem(M)	50	No zone	12
		250	12	13
		500	13	14
		1000	15	No zone
10	S-leaf(M)	50	No zone	12
		250	13	12
		500	16	11
		1000	17	No Zone
11	S-leaf(EA)	50	No zone	11
		250	10	12
		500	14	13
		1000	16	14
12	S-panicle(M)	50	No zone	No zone
		250	14	14
		500	20	No zone
		1000	20	15
13	S-panicle(EA)	50	10	11
		250	11	13
		500	17	14
		1000	21	17



(a) *Pseudomonas aeruginosa* (b) *Enterococcus Faecalis*

Fig. 2. Broth dilution assay for crude Ethyl acetate extract from panicle of *Spinifex* sp



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CLASS-GC10 Ver.-2.00  SYS-2 Ch-1 REPORT.NO-6  DATA-SUN0805.D19 12/05/08 20:32:24
Sample          : MEOH
ID              : SW
Dilution Factor: 1
Type            : Unknown
Detector        : FID
Operator        : THIRU

*** Chromatogram ***  Filename:SUN0805.C19
mV
1.5
1.0
0.5
0
4.028
3.321
4.997
5.342
5.610
5.700
5.917
6.693
7.182
7.700
9.042
10.058
11.982
14.592
0
5
10
15
min
```

*** Peak Report ***

PKNO	TIME	AREA	HEIGHT	MK	IDNO	CONC	NAME
1	3.321	62	31			0.6323	
2	3.397	41	29	V		0.4178	
3	4.028	8637	6402			87.8041	
4	4.997	21	14			0.2135	
5	5.342	54	28			0.5550	
6	5.477	66	45			0.6669	
7	5.610	16	7			0.1647	
8	5.700	16	8			0.1586	
9	5.917	34	18			0.3467	
10	6.172	14	7			0.1372	
11	6.693	16	7			0.1656	
12	7.182	56	18			0.5683	
13	7.295	42	15	V		0.4249	
14	7.700	65	23			0.6648	
15	7.781	55	17	V		0.5591	
16	9.042	21	7			0.2135	
17	10.058	82	17			0.8356	
18	11.982	155	20			1.5777	
19	14.592	384	39			3.9006	
		9837	6752			100.0000	

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6 - 1/1                                              12/05/09 10:59:32
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Fig. 3. Gas chromatography analysis of Bioactive fractions of *Spinifex sp.* Panicle extract

times are summarized (Fig – 3). Among the identified compounds, some of them are known for their interesting biological active; 3.321- 3,7,11,15-TETRAMETHYLHEXADEC-2-EN-1-OL, 4.029 - Squalene, 4.991 - .alpha.-Tocopherol-.beta.-D-mannoside, 14.592- 2-Cyclohexen-1-one-4-carboxylic acid, 4-(3,7,11-trimethyl-2,6,10-dodecatrien-1-yl)-3-methyl-, ethyl ester, 9.042 – Dotriacontyl trifluoroacetate, 10.058 - Methyl cis-13,16-Docosadienate.

Partial purification of bioactive compounds

The four compounds (MNP-1, MNP-2, MNP-3 and MNP-4) were centrifuged and supernatant was transferred into a fresh eppendorff and TLC were run using concentration of solvents (Hexane : Ethyl acetate, 6:4 ratio). Single bands were observed in all the four compounds and the purity was confirmed

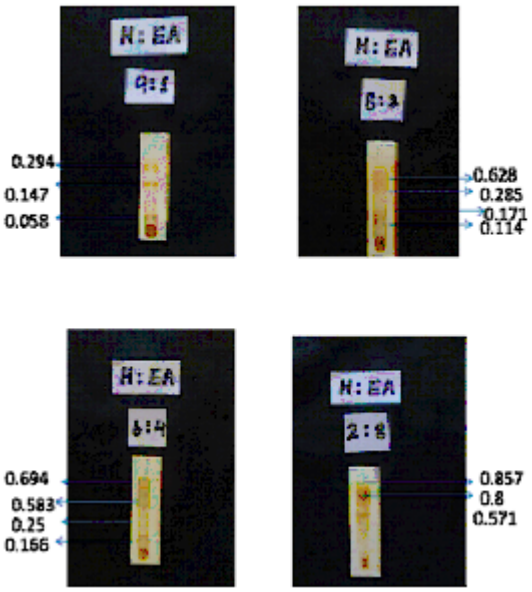

Fig. 4. TLC profile of bioactive compounds

Fig. 5. Autobiography of Bioactive compounds

DISCUSSION

Marine plants and animals are reported to possess a wide spectrum of bioactive substances, which are structurally novel and biologically active substances. Research in the areas of marine natural products has grown geometrically in the recent past(Aswal *et al*., 1984; Rawiwon *et al*., 1990). However, reports on the bioactive substances possessed by coastal plants are very meager. Few reports on the coastal plant *Ipomoea pes-caprae* containing pharmacologically active substances were indicated by de Souza *et al*. (2000), Krogh *et al*. (1999) and Rogers *et al*. (2000).

Different plant parts are used for medicinal purposes i.e., bulb, gel, leaves, roots, barks, peels etc. The use of plants to treat illness is found throughout human culture (Anne-Catherine, 2007). Globally, plant extracts are employed for their antibacterial, antifungal and antiviral activities. It is known that more than 400,000 species of tropical flowering plants have medicinal properties and this has made traditional medicine cheaper than modern medicine (Odugbemi, 2006).

In the present study it was found that The antibacterial activity of Ethyl Acetate and methanol extract of *Spinifex* sp. leaf, stem, panicle, *Ipomoea* sp. stem, seed, flower, fruit coat, seed cover and leaf yielded activity against *P. aeruginosa* and *E .faecalis*. The tested plants showed negative as well as positive activities against tested bacteria. Though the response is not uniform, all plants showed activity against one or more bacterial strain used in this assay. The extract concentration providing 50% inhibition (IC_{50}) was calculated from the graph of inhibition percentage plotted against extract concentration (25-250 μg/mL) and IC_{50} is found to be 100μg/mL which showed 53.3% inhibition (0.035 O.D) for *E .faecalis*. IC_{50} is found to be 225µg/mL which showed 55.35% inhibition (0.025 O.D) for *P. aeruginosa*.

Alkaloids are formed as metabolic by-product and have been reported to having antibacterial activity, as studied in *Tamarindus indica* L.(Doughari, 2006). As far as phytochemical screening is concerned, the plants with the most abundant phytochemical constituents. Medicinal plants have recently attracted the attention of the

biological scientific communities (Das et al., 2010). Aromatic phenolic compounds which have been found to have antimicrobial properties (Alma *et al., 2003*).

The TLC profile of crude extract in chromatogram developed revealed the presence of seven compounds and a prominent spot at R_f value of 0.77. The autobiography of the above chromatogram revealed the presence of clear zone of inhibition at non-polar and also polar regions, a prominent zone of inhibition was seen near polar region at R_f values of 0.75. The compound at the polar region showing effective antibacterial activity was scrapped using sterile needle under sterile condition and profiled for TLC to further confirm its purity. Further, the Gas chromatography revealed the presence of several active metabolite in the crude extract. The compound that was partially purified may be developed as effective antibacterial agent. However, further studies are warranted to purify, characterize and test the active molecule for its antibacterial activity and its mechanisms of action.

CONCLUSION

Plant based antimicrobial compounds have enormous potential as they can serve the purpose without any side effects that are often associated with synthetic antimicrobials. In this endeavor, traditional coastal medicines must perforce to be granted the benefit of modern science and technology to serves further global needs. The drugs derived from sea have the possibility of using in medicine because of its good antimicrobial activity as well as less toxic effects. Overall, the present study indicates the antibacterial activity of ethyl acetate extract of *spinifex* panicle and provides some idea about phyto chemical evaluation on *spinifex* panicle. This study paves the way for further attention and research to identify the active compounds responsible for the antibacterial activity.

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