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SCREENING OF TURBINARIA FOR THE PRESENCE AND STABILITY OF ANTIMICROBIAL COMPOUNDS

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ABSTRACT

Seaweed of Phaeophyceae namely, *Turbinaria* sp was collected from the South Eastern Coast of Tamilnadu (Rameshwaram). The extracts were subjected to antimicrobial assay by agar well diffusion method. Clinical isolates of two gram positive (*Staphylococcus* sp, *Bacillus* sp), five gram negative (*Proteus* sp, *Pseudomonas* sp, *Klebsiella* sp, *E.coli*, and *Salmonella* sp) and one fungal strain Candida albicans) were used for the antimicrobial assay. Large inhibitory zone against *Staphylococcus* sp, *Bacillus* sp, *Proteus* sp, *Klebsiella* sp and *E.coli* was formed by ethanol, chloroform and ethyl acetate extracts of *Turbinaria* sp was and ethanol extracts of *Turbinaria* sp showed a good inhibitory zone against *Candida albicans.* Thin layer chromatography analysis for all extracts were performed for better resolution of the compounds using methanol-chloroform (1:1). Silica gel column chromatography of the chloroform and ethanol extracts of *Turbinaria* sp was done for partial purification of antimicrobial compounds since these compounds have better antimicrobial activity. The different fractions obtained from column chromatography were tested for antimicrobial activity. Fractions 2-11 of ethanol extract and 1-11 of chloroform extract of *Turbinaria* sp was active against various isolates. Stability of the antimicrobial compounds was tested for a period of one and a half months and was found to be better in its activity upon storage also.

KEYWORDS; Phaeophyceae, antimicrobial, *Turbinaria.*

INTRODUCTION

Algae are a large and diverse group of simple, typically autotrophic organisms, ranging from unicellular to multi cellular forms. The largest and most complex marine forms are called seaweeds. The group contains nearly 18000 genera with 21000 species (Alexopoulos and Bold, 1967). They are primitive photosynthetic organisms living in sea or brackish water.

Sea weeds are classified on the basis of their pigment constituents into 4500 species of red algae (Rhodophyta) with phycoerythrin and phycocyanin pigments; another 3000 species of brown algae (Phaeophyta) having pigments like xanthophylls, and fucoxanthins and some 7000 species of green algae (Chlorophyta) with chlorophyll a and b, carotenes and various xanthophylls. Red and brown algae are almost exclusively marine, whereas only 900 species out of 7000 species of green algae are marine (Dring, 1982).

Owing to their possible economic use in various fields such as food, pharmaceutical and textile industries (Chen, 1977; Ruggieri, 1976) seaweed resources have attracted the attention of scientists all over the world. Seaweeds has been a source for the production of a variety of major metabolites such as polysaccharides, lipids, proteins, carotenoids, vitamins, sterols, enzymes, antibiotics and many other fine chemicals (Colwell,1983; Fenical, 1982; Stein and Borden, 1984; Targett and Mitsu, 1979).

The most important use of algae is that they are the primary producers of organic matter in aquatic environment because of their photosynthetic activity (Bold and Wynne, 1978). Animal life in aquatic environment mainly depends on algae because they form the primary source of energy and food for them. More than 100 species, mostly of Phaeophyceae and Rhodophyceae, are used as food by man in different parts of the world. Because of the presence of phosphorus, potassium and some trace elements, the seaweeds in many coastal regions are used as fertilizers. For example, *Fucus* is used as common manure by Irish people.

Seaweeds are not only the source of major metabolites but are an extensive prolific source of secondary metabolites. More than 600 secondary metabolites have been isolated from marine algae (Faulkner, 1984, 1986). Although a majority of these (about 60%) are terpenes, some fatty acids are also common (20%) with nitrogenous compounds (Van Alstyne and Paul, 1988).

Many of these compounds are bioactive and have been extensively studied using bioassays and pharmacological assays.

Most Phaeophyceae (brown algae) are lithophytes. Some of the genera used as human food are *Alaria, Laminaria, Sargassum, Durvillea* and *Pelvetia.* Alginate derivatives and alginic acid are extracted from cell wall of brown algae. Also brown algae contain large amounts of cell-wall polysaccharides (sulphated polysaccharide fucoidans) composed mainly of a (1-3) linked L-fucose residues, as its main constituent. For the last decade, algal sulphated polysaccharides have been extensively studied owing to their numerous biological activities such as anticoagulant, antithrombotic, anti-inflammatory, antitumour and antiviral activities.

Decreased efficiency and resistance of pathogens to antibiotics has necessitated the development of new alternatives (Smith *et al*., 1994). Many bioactive and pharmacologically important compounds such as alginate, carrageens and agar as phycocolloids have been obtained from sea-weeds and used in medicine and pharmacy (Siddhanta *et al*., 1997). Fatty acids are isolated from micro algae that exhibited antibacterial activity (Kellam *et al.*, 1988). Methanolic extracts of sea-weeds collected from South African coast, belonging to Chlorophyceae, Phaeophyceae and Rhodophyceae showed antibacterial activity. Among them, Phaeophyceae members showed highest antibacterial activity (Vlachos *et al*., 1997). Many workers revealed that the crude extracts of Indian sea-weeds are active against Gram-positive bacteria (Rao and Prekh *et al*., 1997). Hence the present study is centered towards the seaweeds of South Eastern Coast of Tamilnadu (Rameshwaram) to rule out the novel antibiotic compounds.

The objectives of the present study are

To screen the brown algae turbinaria for the presence of antimicrobial compounds.

To check the stability of antimicrobial compounds.
To partially purify the antibacterial compounds.

MATERIALS AND METHODS

Collection of Seaweeds

Seaweeds of the class Phaeophyceae namely *Turbinaria* sp was collected fresh from the South Eastern Coast of Tamilnadu (Rameshwaram) in the month of December, 2008. Specimen of the collected seaweeds is preserved in the department of Microbiology, Dr. G.R. Damodaran College of Science for future reference.

PROCESSING OF SEAWEEDS

Seaweeds were cleaned off epiphytes, extraneous matter and necrotic parts. They are washed in fresh water, rinsed with sterile distilled water and shade dried. After drying they were cut into small pieces and powdered in a mixer grinder (Kandhasamy and Arunachalam, 2008). The powdered samples were stored at 4^{o}C until use.

EXTRACTION WITH ORGANIC SOLVENTS

The extraction was carried out with different solvents in the increasing order of polarity (petroleum ether, benzene, chloroform, ethyl acetate, ethanol and water) by soaking the material in the respective solvents thrice overnight at room temperature.

Thirty grams of each of the powdered samples were first soaked in required volume of petroleum ether overnight at room temperature. After 24 hours, the extracts were filtered using a cheese cloth. This procedure was repeated for three times for effective extraction. The extracts from three consecutive soakings were pooled and freed from solvent by evaporation. The same procedure was repeated with benzene, chloroform, ethyl acetate and ethanol after shade drying the sample in each step (Vimala *et al*., 2000). The concentrated extracts were then stored in screw capped tubes and refrigerated until use.

AQUEOUS EXTRACTION AND PRECIPITATION OF POLYSACCHARIDES

After the series of organic solvent extraction the sample was shade dried and soaked in required volume of sterile distilled water for 24 hours. It was then filtered and the extracts were stored.

Polysaccharides from the extracts were precipitated by adding equal volume of ethanol and then centrifuged at 3000rpm for 5 minutes. Precipitated polysaccharides pelleted at the bottom of the centrifuge tube was collected and refrigerated until use (Shanmugam *et al.*, 2000).

ANTIMICROBIAL ASSAY

Test microorganisms

The test organisms used in this study are the clinical isolates which include two gram positive bacteria, *Staphylococcus* sp and *Bacillus* sp, five gram negative bacteria that includes *Pseudomonas* sp, *Proteus* sp, *Salmonella* sp, *Escherichia coli, Klebsiella* sp, and one fungal strain *Candida albicans*.

All the isolates were maintained in the form of pure stock cultures. Bacterial isolates were maintained in Mueller-Hinton agar slants and fungal strain in Sabouraud dextrose agar slant at 4^{o}C.

Preparation of media

A. Mueller-Hinton Agar: pH-7.0

Beef infusion - 300 g.
Acid hydrolysate - 17.5 g. of casein
Starch - 15 g.
Agar - 20 g.
Distilled water - 1000 ml.

B. Sabouraud dextrose agar: pH-5.8

Dextrose - 40 g.
Peptone - 10 g.
Agar - 25 g.
Distilled water -1000ml.

Evaluation of antibacterial activity

Antimicrobial activity was evaluated by agar well diffusion method (Attaie *et al.*, 1987) which is the most widely used method for susceptibility testing. Antibacterial activity of the algal extracts viz petroleum ether, benzene, chloroform, ethyl acetate and ethanol of *Turbinaria* sp determined.

A liquid suspension of the test organism was applied to Mueller-Hinton plates using a cotton swab. After a few minutes (to allow complete absorption of the inoculum), 40µl of the crude extracts were placed in 6mm diameter wells made on the plates with a sterilised cork borer. Antibiotic chloramphenicol was used as positive control for gram negative bacteria and ampicillin for gram positive bacteria. Each organic solvent used for extraction, served as negative control.

After overnight incubation at 37^{o}C, the diameter of the inhibition halo was measured which is an indicative of the bioactivity of the seaweed extracts. Activity was recorded only if the zone of inhibition is greater than 5mm.

Evaluation of antifungal activity

Agar well diffusion method was used for evaluation of antifungal activity. The activity was checked on Sabouraud dextrose agar plates as per the same procedure adopted for antibacterial assay. The fungal plates were incubated at 28^{o}C overnight. Antifungal activity was recorded by measuring the zone of inhibition.

THIN LAYER CHROMATOGRAPHY (Harborne, 1973)

Thin layer chromatography is an easy technique for the separation and identification of organic compounds. The crude organic extracts of the selected seaweeds were taken for thin layer chromatography.

Preparation of glass plates

Dry and clean glass plates were placed on a clean surface. A slurry of the adsorbent in water in the ratio 1:2 (w/v) was prepared. Commonly used adsorbents for the separation of biological compounds in thin layer chromatography are silica gels, aluminium oxide, calcium hydroxide, cellulose, sephadex, magnesium phosphate, polyamide, polyvinylpyrolidones etc. Silica gel GF 250 was used for separation, and the slurry was stirred thoroughly for 1-2 minutes. With the help of applicator, slurry was spreaded on the glass plate uniformly by moving the applicator from one end to another. The plates were allowed to dry at room temperature for 10-30 minutes and then kept in an oven at 100-120^{o}C for 30 minutes to remove the moisture and to activate the adsorbent on the plate.

Saturation of the chromatographic chamber with solvent

The developing container for thin layer chromatography is a jar with a lid. Developing solvent was poured into the beaker to a depth of just less than 0.5cm, covered with lid, and allowed it to stand for some time for saturation of the TLC container.

Application of samples

Organic solvent extracts (40µl) were applied by means of a micropipette or syringe as small spots at 2.5cm away from the end of the glass plate. All the spots are placed equidistant from one end of the plate.

Developing chromatogram

After saturation of the developing container with the solvent (methanol:chloroform), the thin layer plate was placed vertically in the tank. Once the solvent reached the top of the plate, it was removed from the tank, dried and visualized under the UV of short wavelength of 254nm.

COLUMN CHROMATOGRAPHY

Column chromatography was done for the purification of the crude extracts (Choudhury *et al.*, 2005).

Extracts with high antibacterial activity (chloroform and ethanol extract of *Turbinaria* sp) were subjected to column chromatography. Silica gel was activated in hot air oven at 60^{o}C for 8 hours. It was then packed in the column and slurry of the sample was packed. The fractions were eluted with ethanol: chloroform in the ratio 1:1.

ANTIMICROBIAL ASSAY

Antibacterial and antifungal assay of the elutions obtained by column chromatography were carried out as per the procedure mentioned in 3.5.

STABILITY OF ANTIMICROBIAL ACTIVITY

Stability of the bioactive compounds present in the extracts was checked by determining the antimicrobial activity at an interval of 10 days for one and a half months and was recorded.

RESULTS AND DISCUSSION

COLLECTION OF SEAWEEDS

Collection of seaweeds was made from the South Eastern coast of Tamilnadu (Rameshwaram). About one kilogram wet weight of seaweeds were collected and was identified *Turbinaria* sp. The morphological view of the algae has been presented in plate 1.2.

PREPARATION OF BIOMASS FOR EXTRACTION

Epiphytes were removed from the collected seaweeds and were washed gently and repeatedly with sterile

distilled water to remove salt, surface microflora and other adherents. The washed biomass was dried under shade to remove moisture content and powdered.

EXTRACTION OF BIOACTIVE SUBSTANCES

Extraction of the biomass was carried out with solvents of increasing polarity (petroleum ether, benzene, chloroform, ethyl acetate and ethanol) and sterile distilled water. Polysaccharide component of aqueous extract was precipitated with equal volume of ethanol as shown in plate 1.2. The percentage yield of polysaccharides are presented in table 1.1.

Table 1.1: Percentage yield of polysaccharides.

Serial number	Name of the algae	Weight of biomass (g)	Percentage yield of polysaccharide (%)
2	*Turbinaria* sp	15	2.26

SCREENING FOR ANTIBACTERIAL ACTIVITY

Antibacterial activity for all the extracts of *Turbinaria* sp was studied with clinical isolates of two gram positive bacteria, *Staphylococus* sp and *Bacillus* sp, and five gram negative bacteria (*Proteus* sp, *Pseudomonas* sp, *E. coli*, *Salmonella* sp, *Klebsiella* sp) on Muller Hinton agar plates. The wells were impregnated with 40µl of each of the solvent extracts. The mother solvent of 40µl of each extracts served as the negative control, ampicillin and chloramphenicol were the positive for gram positive and gram negative strains respectively. The plates were incubated at 37^{o}C for 24 hours. The zone of inhibition was measured and the results are given in table 1.2.

Table 1.2: Antimicrobial activity of crude extracts of *Turbinaria* sp.

S.NO	Name of extracts	Antimicrobial activity (Zone of inhibition in mm)						
		***Staphylococcus*sp**	***Bacillus*sp**	***Salmonella*sp**	***Proteus*sp**	***Klebsiella*sp**	***Pseudomonas*sp**	***E.coli***
1	Petroleum ether	14	13	-	8	-	-	-
2	Benzene	13	15	13	13	14	13	8
3	Chloroform	14	15	11	12	15	14	10
4	Ethyl acetate	16	16	15	19	12	18	12
5	Ethanol	16	20	14	15	14	13	11
6	Water	-	-	-	-	-	-	-

-Indicates no activity.

Screening for antifungal activity

Antifungal activity of all the extracts of *Turbinaria* sp was studied with *Candida albicans* on sabouraud dextrose agar. The plates were incubated at 28^{o}C for 24 hours. The zone of inhibition was measured and results are presented in table 1.3.

Table 1.3: Antifungal activity of crude extracts of *Sargassum* sp and *Turbinaria* sp against *Candida albicans.*

Serial number	Name of algae	Antifungal activity (Zone of inhibition in mm)					
		Petroleum ether	Benzene	Chloroform	Ethyl acetate	Ethanol	Water
1	*Turbinaria* sp	-	-	-	14	-	-

-Indicates no activity

Thin layer chromatography

Silica gel plates were prepared and a sample volume of 40µl of the various crude extracts of *Turbinaria* sp was loaded as spots and the chromatogram was developed with the solvent mixture methanol-chloroform in the ratio 1:1 for effective separation and better resolution. The detection was done under a short wave length UV lamp (254nm). Results are presented in plate 2.5

Purification by column chromatography

The chloroform and ethanol extracts of Turbinaria sp was fractionated by silica gel column chromatography (methanol: chloroform). Elution was carried out with methanol and chloroform in the ratio 1:1 v/v. The process is presented in plate 2.6.

4.8 Screening for antimicrobial activity

Antibacterial antifungal assay of the purified compounds were carried out. The results are presented in table 1.4.

Table 1.4: Antimicrobial activity of purified ethanol extracts of *Turbinaria* sp.

Serial number	Test organisms	Antimicrobial activity of fractions (Zone of inhibition in mm)										
		1	2	3	4	5	6	7	8	9	10	11
1	*Staphylococcus*sp	-	8	12	13	14	10	12	11	12	9	11
2	*Bacillus* sp	8	7	12	12	13	12	8	8	9	6	10
3	*Salmonella* sp	-	-	-	-	-	-	-	-	-	-	-
4	*Proteus* sp	-	6	8	7	7	6	6	9	6	7	8
5	*Klebsiella* sp	-	-	6	6	6	6	-	-	-	-	-
6	*Pseudomonas* sp	6	6	-	6	7	-	10	-	-	-	-
7	*E.coli*	-	7	7	7	7	-	-	-	-	-	-
8	*Candida albicans*	-	8	-	-	-	-	-	-	-	-	-

- indicates no activity, 1-9 represents the fractions obtained during column chromatography.

Table 1.4: Antimicrobial activity of purified chloroform extracts of *Turbinaria* sp.

Serial number	Test organisms	Antimicrobial activity of fractions (Zone of inhibition in mm)										
		1	2	3	4	5	6	7	8	9	10	11
1	*Staphylococcus*sp	7	8	10	9	9	9	8	9	12	16	12
2	*Bacillus* sp	-	-	-	-	-	-	13	14	14	13	12
3	*Salmonella* sp	-	7	7	7	6	6	6	6	-	-	6
4	*Proteus* sp	8	-	7	7	7	7	11	10	11	9	10
5	*Klebsiella* sp	6	6	7	8	7	7	7	7	7	9	8
6	*Pseudomonas*sp	8	9	9	10	12	12	-	-	-	-	6
7	*E.coli*	9	9	9	9	9	11	8	10	10	9	7
8	*Candida albicans*	-	6	6	-	6	6	6	6	8	6	-

-indicates no activity, 1-9 represents the fractions obtained during column chromatography

Stability of antimicrobial activity

Stability of the antimicrobial activity of the crude extracts of *Turbinaria* sp was studied at ten days interval for a period of one and a half months. The results are presented in figure 3.1.

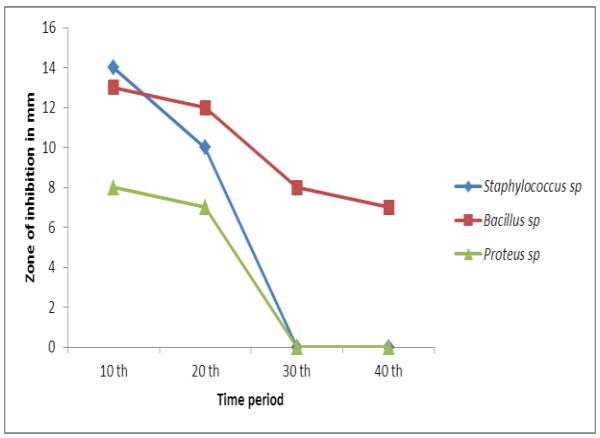

Figure 3.1-a: Petroleum ether extract of *Turbinaria* showing stability of antibacterial activity.

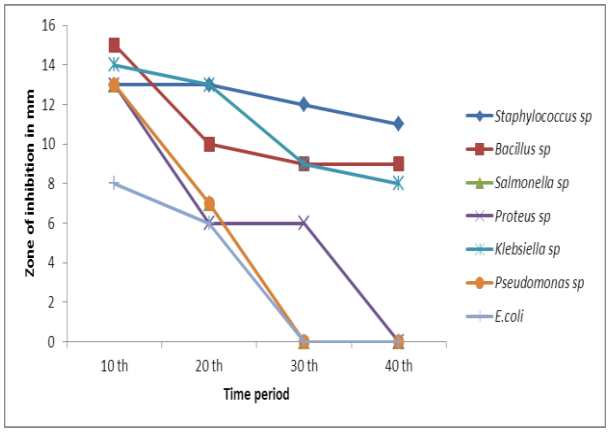

Figure 3.1-b: Benzene extract of *Turbinaria* showing stability of antibacterial activity.

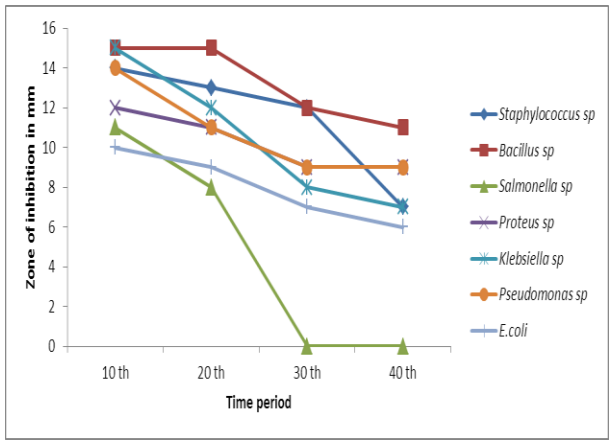

Figure 3.1-c: Chloroform extract of *Turbinaria* showing stability of antibacterial activity.

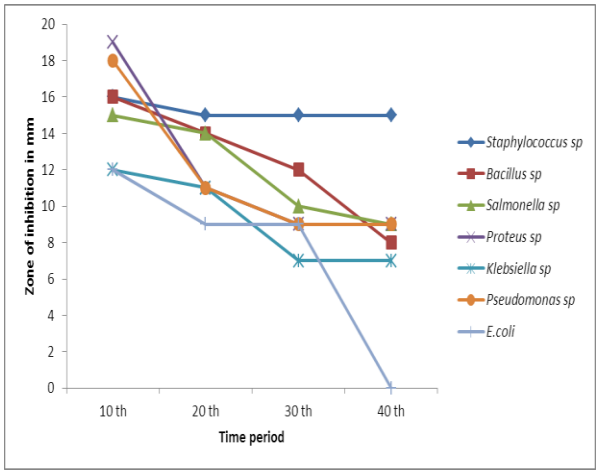

Figure 3.1-d: Ethyl acetate extract of *Turbinaria* showing stability of antibacterial activity.

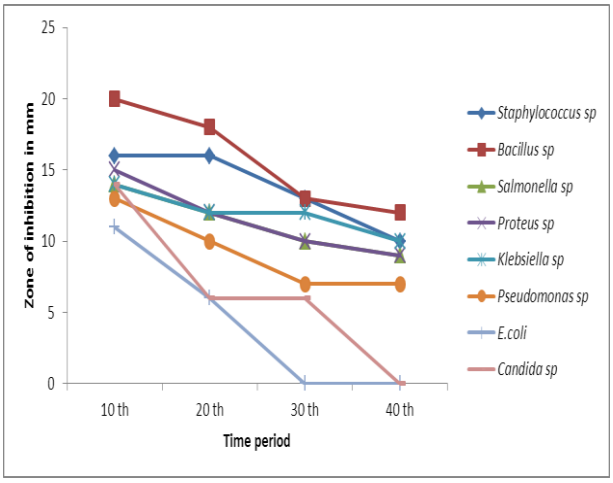

Figure 3.1-e: Ethanol extract of *Turbinaria* showing stability of antibacterial activity.

Search for sources of bioactive compounds is in the race. Every day, a new record of some bioactive substances is being identified or extracted from some biological sample elsewhere in the world. The focus of bioactive substances hunting is on marine algae and wild higher plant materials.

In the present study, the shade dried powdered biomass of *Turbinaria* sp was extracted with solvents of increasing polarity and water. Polysaccharides free aqueous extracts were collected by precipitating with ethanol. With all the above extracts (petroleum ether, benzene, chloroform, ethyl acetate, ethanol and water) antimicrobial assay was performed.

The antibacterial activity of Ethanol, chloroform, ethyl acetate extracts of Turbinaria sp was effective against *Staphylococcus* sp, *Bacillus* sp, *Salmonella* sp, *Proteus* sp, *Klebsiella* sp, *Pseudomonas* sp and *E. coli.* The antifungal activity of ethanol and chloroform extracts of *Turbinaria* sp showed a good inhibitory zone. Suggesting that the algae possess compounds effective not only against prokaryotes but also against eukaryotic cells.

Petroleum ether and benzene extracts of *Turbinaria* sp does not possess any antimicrobial activity against *Salmonella* sp, *Proteus* sp, *Pseudomonas* sp and *Candida albicans.*

The results of the present study revealed that gram positive organisms were more susceptable to the crude extracts of algae used. Tuney *et al.* (2006) also reported that gram positive bacteria were more efficiently controlled by the extracts of algae used in their study compared to gram negative bacteria. Taskin *et al.* (2001) indicated that the more susceptibility of gram positive bacteria to the algal extract was due to the differences in the cell wall structure and their composition. In gram negative bacteria, the outer membrane acts as a barrier to many environmental substances including antibiotics (Tortora *et al.,* (2001).

The variation in antimicrobial activity of the extracts might be due to the species variation, geographical location, ecological parameters, exposure to the test organism and the solvent systems used. Hence for better inhibitory activity extraction has to be carried out with different choice of solvents singly or in combinations so that the antimicrobial compounds will get concentrated. ethanol, chloroform and ethyl acetate extracts of *Turbinaria* sp was effective further analysis was carried out only with those extracts.

In thin layer chromatography with solvent system methanol-chloroform (1:1) all the extracts of *Turbinaria* sp had better resolution of compounds.

Chloroform and ethanol extracts of *Turbinaria* sp was fractionated by silica gel column chromatography (plate 2.6) as they possess better antimicrobial activity. The different fractions obtained were tested for antimicrobial activity. The fractions 2-11 of ethanol extract of *Turbinaria* sp was effective against *Staphylococcus* sp, *Bacillus* sp and *Proteus* sp. Similarly the fractions 1-11 of chloroform extracts of *Turbinaria* sp aws effective against *Staphylococcus* sp, *Salmonella* sp, *Proteus* sp, *Klebsiella* sp, *E. coli* and *Candida albicans* (plate 2.4a, b, c and d). This shows the presence of antimicrobial compounds in the fractions eluted by column chromatography.

For effective and better separation of the purified compounds combination of solvent system and sub fractionation of the fractions has to be carried out. The extracts with antimicrobial principle have got a better shelf life as evidenced by figure 3.1 a-j which is a desirable quality of any antimicrobial substance.

The aqueous extracts and the separated polysaccharides did not seem to possess any antimicrobial activity. As for the effectiveness of the extraction methods, chloroform was better than other solvents (Sastry *et al.*, 1994). The use of organic solvents always provides a higher efficiency in extracting antimicrobial activities,

compared with water extraction (Rosell *et al.*, 1987). However, there are reports of the polysaccharides possessing antiviral properties (Sutapa *et al*., 2002) hence further study could be extended on detecting the antiviral properties of the polysaccharides.

Among the various extracts of the seaweed *Turbinaria* sp's chloroform and ethanol extracts was found to be the best and showed positive results. Finally it is concluded that macroalgae from the South Eastern Coast of Tamilnadu (Rameshwaram) are potential sources of antimicrobial compounds. The further work can be extended towards the determination of minimum inhibitory concentration of the active crude extracts and of the fractions eluted. The active compounds can be further analysed by thin layer chromatagraphy and mass spectroscopic studies for the structure elucidation of the antimicrobial principles. In addition the shelf life of the antimicrobial principle at room temperature can be checked which is a quality preferred by the pharmaceutical studies.

CONCLUSION

Seaweeds of Phaeophyceae namely *Turbinaria* sp were collected from the South Eastern Coast of Tamilnadu (Rameshwaram). The biomass was cleaned, shade dried, powdered and extracted with solvents of increasing polarity (petroleum ether, benzene, chloroform, ethyl acetate and ethanol) and water. Polysaccharides were precipitated from water extract by addition of equal volume of ethanol. The extracts were then subjected to antimicrobial assay by agar well diffusion method. Clinical isolates of two gram positive (*Staphylococcus* sp, *Bacillus* sp), five gram negative (*Proteus* sp, *Pseudomonas* sp, *Klebsiella* sp, *E.coli*, and *Salmonella* sp) and one fungal strain Candida albicans) were used for the antimicrobial assay.

Ethanol, chloroform and ethyl acetate extracts of *Turbinaria* sp was effective against *Staphylococcus* sp, *Proteus* sp, *Klebsiella* sp, *Pseudomonas* sp and *E.coli.* Chloroform and ethanol extracts of *Turbinaria* sp showed a good inhibitory zone against *Candida albicans.* Thin layer chromatography analysis for all extracts were performed for better resolution of the compounds using methanol-chloroform (1:1). Silica gel column chromatography of the chloroform and ethanol extracts of *Turbinaria* sp was done for partial purification of antimicrobial compounds since these compounds have better antimicrobial activity. The different fractions obtained from column chromatography were tested for antimicrobial activity. Fractions 2-11 of ethanol extract and 1-11 of chloroform extract of *Turbinaria* sp was active against various isolates. Stability of the antimicrobial compounds was tested for a period of one and a half months and was found to be better in its activity upon storage also.

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