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Isolation of Bioactive Secondary Metabolites from Seaweeds *Amphiroa anceps* against Chicken Meat Associated Pathogens

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Abstract

The biodiversity of ocean living resources provide an ample scope for the extraction of drugs and chemicals for therapeutic purposes. Sea weed *Amphiroa anceps,* collected from Thondi-Palk Strait region of Tamil Nadu, was studied for antagonistic activity of proteins. Seaweed species were identified based on specula morphology. Ethyl Acetate extracts yielded a total of 0.9 g, 0.12 g, 0.02 g, 0.14 g and 0.17 g from 1.5 g of sponge respectively. The antagonistic activity of crude extract against bacterial pathogens showed clear inhibition zones against *Yersinia* sp., *Streptococcus* sp. and *Vibrio* sp. The extracted Seaweed strain had Minimum Inhibitory Concentrations against all the three chicken meat associated pathogens, *Vibrio* sp., *Yersinia* sp. and *Streptococcus* sp. and Minimum Bactericidal Concentration against two chicken meat associated pathogens, *Vibrio* sp. and *Yersinia* sp.

Keywords: Seaweed *Amphiroa anceps*; *Vibrio* sp.; *Yersinia* sp.; *Streptococcus* sp.; Antagonistic Activity, Minimum Inhibitory Concentration, Minimum Bactericidal Concentration.

Introduction

Seaweeds

Seaweeds can be defined as any large marine benthic algae that are multicellular, macrothallic, and thus differentiated from most algae that are of microscopic size. They are rich and varied source of bioactive natural products and have been widely studied as potential source of biocidal and pharmaceutical agents. Marine plants form an important renewable resource in the marine environment and have been a part of human civilization since the start of time [1] They are commonly found growing in the intertidal, shallow and deep sea areas up to 180 meter depth and also in estuaries and back waters on the solid substrate such as rocks, dead corals, pebbles, shells and other plant materials. They form one of the important living resources grouped under three divisions namely, *Chlorophyceae* (green algae), *Phaeophyceae* (brown algae) and *Rhodophyceae* (red algae). They are abundant on hard substrates and commonly extending to depths of 30-40 m. About 624 species have been reported in India with a potential of 77,000 tons (wet weight) per annum. The red seaweeds contribute 27.0%, brown 0.2% and others 72.8%. About 206 species of algae have been reported from the mangrove environment.

In recent years, most bioactive compounds have been obtained from various marine animals like sea hares, nudibranchs, bryozoans, tunicates, sponges, soft corals, sea slugs and marine microorganisms, etc. [2]. The desire for new metabolites from marine life has resulted in the extraction of more or less 10,000 metabolites till date [3], many of which are gifted with pharmaco-dynamic potentials. More than 28 marine natural products are currently being tested in human clinical trials, with many more in various stages of preclinical development [4]. The rising scientific awareness is now being focused on the potential medical uses of the benthic organisms. These organisms have developed unique adaptations that enable them to survive in dark, cold, and highly pressurized environs. Marine microorganisms harbor new genes, the utilization of which is likely to lead to the detection of new drugs. Secondary metabolites produced by marine bacteria and invertebrates have yielded medicinal products such as novel anti-inflammatory agents (eg: Manoalide, pseudopterosins, topsentins, scytonemin), anti-cancer agents (eg. Eleutherobin, bryostatins, discodermolide, and sarcodictyin) and antibiotics (eg. marinone). Melanins can be exploited for sunscreens, dyes and colouring for its chromophoric properties. Actinobacteria, among the most common microbes on earth are a storehouse of 70% of the world's naturally occurring antibiotics. Metabolites, some with novel chemical structures, and belonging to a diversity of 'chemical classes' have been characterized from mangroves and mangal associates. Chemicals such as amino acids, carbohydrates and proteins, are products of primary metabolism and are vital for maintaining life processes, while others like alkaloids, phenolics, steroids, terpenoids, are secondary metabolites that have toxicological, pharmacological and ecological significance [5].

Metabolites from seaweeds

They contain all major and minor plant nutrients as well as biocontrol properties and contain many organic compounds such as auxins, gibberellins and precursor of ethylene and betaine which affect plant growth [6]. Liquid concentration of brown algae *Ecklonia maxima* significantly reduced the root knot infestation and increased growth of tomato plant. Antimicrobial activity of Canary species of Phaeophyta and Chlorophyta has been reported [7]. Seaweeds occurring at Karachi, Pakistan coast have also shown cytotoxic [8] nematicidal and fungicidal [9] hypoglyceamic [10] and antibacterial [11] activities. Soil amendment with brown seaweeds *Stoechospermum marginatum* and *Sargassum tenerrimum* with or without rhizobia significantly reduced root knot nematode (*Meloidogyne javanica*) and root infecting fungi infections [9].

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Seaweeds are abundant in the intertidal zones and in clear tropical waters. Marine algae have received comparatively less bioassay attention. Presently the seaweed industry consists of two kelps, three *Gelidium* species one *Gracilaria- Gracilariopsis* species, etc.

Significance of endophytic organisms

Presence of endophytic microorganisms in the plant host is in the most cases beneficial for plant. Secondary metabolites produced by endophytes provide a variety of fitness enhancements such as increased resistance to herbivore, parasitism, drought as well as growth enhancements. Endophytes usually produce the enzymes necessary for the colonization of plant tissues. It was demonstrated that most endophytes are able to utilize, at least in-vitro, most plant cell components. Most of investigated endophytes utilize xylan and pectin, show lipolytic activity and produce non-specific peroxidase and chitinnase [12] Microbes, both fungi and bacteria have provided modern medicine with valuable new cures, including penicillin from the fungus *Penicillium notatum* and bacitracin from the bacterium Bacillus subtilis. Additionally taxol, an important chemotherapeutic agent has been discovered to be synthesized by endophytes of the Pacific Yew tree. Endophytes represent a huge diversity of microbial adaptations that have developed in special and sequestered environments, and their diversity and specialized habituation make them an exciting field of study in the search new medicines. The hunt of new drug is particularly important in view of the fact that so many diseases are developing immunity to some of the current treatment.

Seaweed utilization around the world has become a multi-billion dollar industry. Research and utilization of marine algae have increased markedly from last several decades [13]. A number of products based on algae have been developed and applied in many fields like foods, pharmaceuticals, cosmetics and nutritional supplements. Much of this is based on farming of edible species or on the production of agar, carrageenan and alginate. Of all seaweed, hydrocolloids have had the biggest influence on modern western societies. They have attained commercial significance through their use in various industries which exploit their physical properties such as gelling, water retention and their ability to emulsify. However research towards the use of seaweeds for the treatment of various diseases has received less attention.

In the past few years pharmacological industries have exploiting seaweeds for new natural products for pharmacological benefits [14]. In their studies, 3556 Ayesha et al., have reported hypolipidaemic [11] activities of seaweed occurring at Karachi coast, antifungal and nematicidal [15].

Materials and Methods

Description of the study area

Thondi is a small village situated in the Palk Strait region of Tamil Nadu. The study area lies in the latitude of 99°44"N and 79°10' 45" E. The rainfalls in Thondi region are mainly due to north east and south west monsoon. Thondi coast has a very minimal wave action. Turbidity of the seawater is moderately low and also they are rich in nutrients hence, it serves as a treasure house for valuable marine resources like sea grass, seaweeds, and invertebrates like coelenterates, echinoderms and shell fishes. The major occupation of the people is fishing (Figure 1).

Collection of samples

Amphiroa anceps species of Sea weeds were collected during monsoon month (January) for the isolation of endophytic THB strains from Thondi Palk Strait.

Figure 1: Thondi region: location and study sites. Part named S1 represents the coastline where samples were collected.

Isolation of endophytic organisms

One gram of fresh sea weeds species were aseptically weighed and were washed thrice with sterilized distilled water at different time intervals (1 hr, 30 min and 15 min.) After that, the samples were ground well by using mortar and pestle with the addition of 1 ml of sterilized distilled water. Crushed samples were serially diluted with sterilized 50% aged seawater and were plated with the Zobell marine agar medium. One milliliter of the serially diluted sample was pipette out into sterile Petri dishes. The sterile Zobell marine agar medium was poured into Petri dishes aseptically and Swirled for thorough mixing. After solidification, the plates were incubated in an inverted position for 24 hrs at 37°C ± 2°C. All the determinations were carried out in triplicates. The bacterial species developed on the Zobell marine agar medium were counted and the total number of bacterial counts was expressed as Colony Forming Unit (CFU) per gram of sample.

Maintenance of pure culture

Based on the morphological differences and color the different types of THB colonies were chosen. The chosen THB colonies were restreaked thrice in Zobell marine agar and finally the pure culture of THB strains were streaked on Nutrient agar slants. The pure cultures of THB strains were maintained in Nutrient agar slants in refrigerator.

Isolation of chicken meat associated pathogens

In this study the fresh chicken meat samples were collected by using sterile container. The meat sample was placed in a beaker for ten days to rot under room temperature.

Spread plate method: About 1 g of chicken meat is weight from the collected meat sample. Take 5 sterilized test tubes and add 9 ml of distilled water to each tube. Make the master dilution as 10 ml + 1 g

of chicken meat in the first test tube and vortex it for 30 sec. From the 1 st tube transfer 1 ml of chicken meat solution to the 2 nd from 2 nd to 3 rd and hence till 6 th test tube. From the test tube discard 1 ml of the chicken meat solution. Take 0.1 ml of the chicken meat solution from 2 nd tube and spread plate on to the isolation medium nutrient agar, continue to take 0.1 ml of solution from each tube till 6 th tube and spread plate it. Incubate the plate at room temperature for 4-7 days in dark room or at 30°C for 7-10 days. Results were observed.

Gram staining: Smear was prepared in a clean test tube. It was heat fixed by assing through the flame of heat. Add 2-3 drops of crystal violet solution to the repared slide and then allowed to react for a period of 2-3 minutes. This was washed by passing it through running tap water. It was then added with two drops of grams iodine solution and left for some two minutes to react. Wash the slide with dripping tap water

Cultural methods: The colonies from nutrient agar slants were sub cultured in blood agar for *Streptococcus* sp., Macconkey agar for *Yersinia pestis,* TCBS agar for *Vibrio* sp.

***Biochemical test*:** E. Methyl red test, F. Voges Proskauer test (VP)

***Primary screening*:** The antagonistic activity was tested by following cross streak assay method. Single streak (4-6 mm in diameter) of the isolated strains were streaked on the surface of Muller Hinton Agar plates. On obtaining a ribbon-like growth, the overnight culture of antibiotic resistant were streaked at perpendicular to the original streak of isolates and incubated at 37 ± 2°C. The inhibition was measured after 24 hours in the case of bacteria. A control plate was also maintained without inoculating isolates to assess the normal growth of bacteria.

Secondary Screening

Mass cultivation

Ten isolated strains, which showed higher promising antagonistic activity, were selected for mass cultivation for the extraction of antimicrobial metabolites. A loop full of chosen isolated strains were further inoculated into 1000 ml conical flask containing 300 ml of nutrient broth and kept at 28°C for 72 hours with continuous shaking.

Extraction of extra cellular proteins

The mass cultivated broth was filtered by using filter paper. 30 ml of filtrate was mixed with 300 ml of ethyl acetate in separating funnel to extract bioactive compounds. After removing the lower aqueous phase, the upper solvent phase was concentrated in a vaccum evaporator at room temperature for 24 hours bacterial colony on the solid media and crude extract was obtained. This crude extract was used for further secondary screening studies against human pathogens (Figure 2).

Results

Eight THB strains were isolated from sea weed species from these strains. Growth of *Vibrio* sp. increased by the reduction of the concentration amount of the THB strains. There was no growth of *Vibrio* sp. in a concentration of 2000 µg and 1000 µg. Low level of growth was observed on concentration of 500 µg and 250 µg of seaweed strains Sw16 and Sw4 while medium level of growth of *Vibrio* sp. was observed on concentration of 125 µg and 62 µg except for Sw20. There was high level of growth on concentration of 31 µg in all the five seaweed strains (Figure 3).

Minimum inhibitory concentration (MIC)

For MIC determination 0.5 ml of various concentration of extracts

Figure 2: Isolated protein of THB strain.

(31, 62, 125, 250, 500, 1000, 1500, 2000 µg) and mixed with 0.5 ml of nutrient broth. 50 µl of bacterial inoculums serves as positive control. Nutrient Broth alone served as negative control. Whole setup in duplicate was incubated at 37°C for 48 hours. The MIC was the lowest concentration of the extract that did not permit any visible growth after 24 hours of incubation and it was examined on the basis of turbidity.

Minimum bactericidal concentration (MBC)

To avoid the possibility of miss-interpretations due to the turbidity of insoluble compounds. The above selected serial dilutions are plated out on the homologous medium. The MBC are determined by sub culturing the above (MIC) serial dilutions after 48 hours in NB plates using 0.01 ml loop and incubating at 37°C for 24 hours. MBC was regard as the lowest concentration that prevents the growth (Figure 4).

Cross streak assay

Using a clean sterilized inoculation wire loop, the isolated chicken associated pathogen (*Vibrio sp*.) was looped and steaks were made on nutrient agar media plate and there after using another sterile inoculation loop take THB strain isolate and streak against the streaks of the chicken associated pathogen. This was repeated on other nutrient agar plates using the other chicken associated pathogens (*Yersinia* sp. and *Streptococcus* sp.) (Figure 2) and the results were tabulated (Tables 1 and 2).

There was no growth of *Streptococcus* sp. in a concentration of 2000 µg, 1000 µg, 500 µg and 250 µg. Low level of growth was observed on concentration of 125 µg of all other seaweed strains while medium level of growth of *Streptococcus* sp. was observed on concentration of 125 µg and 62 µg. There was high level of growth on concentration of 31 µg in the seaweed strains (Table 3).

There was no growth of *Yersinia* sp. in a concentration of 2000 µg, 1000 µg and 500 µg except on seaweed strain No. Sw20. Low level of growth was observed on concentration of 250 µg of all other seaweed strains except on Sw4 while medium level of growth of *Yersinia* sp. was observed on concentration of 125 µg. There was high level of growth on concentration of 31 µg in all the five sponge strains (Table 4).

Strain No. stw 16 had MIC value of 250 µg against all the three chicken associated pathogens, *Vibrio* sp., *Yersinia* sp. and *Streptococcus* sp. (Table 5 and Figure 5). Sw4 showed MIC value of 125 µg against *Vibrio* sp. and MIC value of 500 µg against *streptoccoccus* sp. and 250 µg *Yersinia* sp. (Table 5 and Figure 5). Sw20 showed MIC value of 125 µg

Figure 3: Cross streak assay against chicken associated pathogens 1 and 4: *Vibrio* sp. 2: *Steptococci* sp., 3 and 5: *Yersinia* sp. A-C are THB strain isolates.

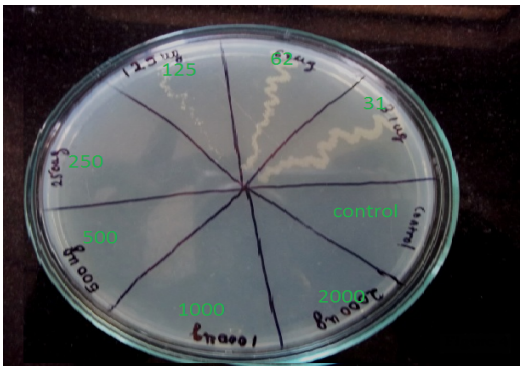

Figure 4: MBC (minimum bactericidal concentration) using seaweed strain 16 against *vibrio sp.*

Strain no	*Vibrio*	*Streptococci*	*Yersinia pestis*
Stw13	-	+	+
Stw14	+	-	+
Stw4	+	+	-
Stw5	-	+	-
Stw6	-	+	-
Stw16	+	+	+
Stw18	-	+	+
Stw20	-	+	-

-: Absence of growth; +: Presence of growth; ++: Medium growth; +++: Large growth; StW: Seaweed.

Table 1: Cross streak assay method against chicken meat associated pathogens.

Strain No	2000 µg	1000 µg	500 µg	250 µg	125 µg	62 µg	31 µg
Stw16	_	_	+	+	+	++	+++
Stw4	_	_	_	_	+	++	+++
Stw20	_	_	_	_	_	++	+++

-: Absence of growth; +: Presence of growth; ++: Medium growth; +++: Large growth; StW: Seaweed.

Table 2: Minimum inhibitory concentration test for antibiotic resistant *vibrio.* Sp.

Strain No	2000 µg	1000 µg	500 µg	250 µg	125 µg	62 µg	31 µg
Stw16	_	_	_	+	++	+++	+++
Stw4	_	_	_	_	+	++	+++
Stw20	_	_	+	+	++	+++	+++

-: Absence of growth; +: Presence of growth; ++: Medium growth; +++: Large growth; StW: Seaweed.

Table 3: Minimum inhibitory concentration test for antibiotic resistant *streptococci sp.*

Strain No	2000 µg	1000 µg	500 µg	250 µg	125 µg	62 µg	31 µg
Stw16	_	_	_	+	++	+++	+++
Stw4	_	_	_	_	+	++	+++
Stw20	_	_	+	+	++	+++	+++

-: Absence of growth; +: Presence of growth; ++: Medium growth; +++: Large growth; StW: Seaweed.

Table 4: Minimum inhibitory concentration test for antibiotic resistant *Yersinia pestis.*

against *Vibrio* sp. (Table 5 and Figure 5). Stw13, Stw14, Stw5, Stw6 and Stw18 did not show any activity (Table 5 and Figure 5).

Discussion

Life originated in the sea and has sustained itself to the present day. The world's oceans comprise the largest part of the biosphere and contain the most ancient and diverse forms of life. The marine biotopes contain an unmatched metabolic and organismal diversity [16]. Seaweeds contain a number of biodynamic compounds of therapeutic value. The chemical compounds provide novel ideas for the development of new drugs against cancer, microbial infections and inflammations [17]. Most of secondary metabolites biosynthesized by the marine plants are well known for their cytotoxic property [18]. An increase in the emergence of multi-drug resistant bacteria in recent years is worrying and that the presence of antibiotic resistance genes on bacterial plasmids has further helped in the transmission and spread of drug resistance among pathogenic bacteria

Marine bacteria being a heterotrophy with simple cell multiplication process, which can be cultivated in large amounts inexpensively. This has prompted the present study, to assess the possible utilization of associated bacteria as resources, to meet the sufficient supply of desired metabolites. The endo symbiotic heterotrophic bacteria have been isolated from sea weed species. Based on the morphological characters, 20 strains were isolated and all of them have been tested for the antimicrobial sensitivity against *Vibrio streptococci* and *Yersinia* sps by cross streak assay (primary screening). Of them, 8 strains were shown sensitivity against one or other pathogenic bacteria. Balagurunathan and Subramanian in 2001 reported that, out of 51 isolates from the littoral sediments of Parangipettai coastal water, 8 strains showed very promising antibiotic activity against bacteria and fungi. Krishnakumar reported that, 33 strains showed antagonistic activity from 63 isolates. It is also interesting to note that, out of 63 isolates, 31 strains showed sensitivity against Candida albicans. Unson et al. reported that, bioactive compounds from Oscillatoria spongelea present in the sponge cells showed sensitivity against both gram positive and gram negative bacteria (*Staphylococcus aureus, E. coli, Bacillus subtilis, Vibrio harveyi*) but did not inhibit the growth of *C. albicans* and *Saccharomyces cerevisea*. Gauthier and Flatau reported that Gram positive bacteria are most susceptible than gram negative bacteria to the antibiotics derived from the marine microorganisms.

All the isolated endo symbiotic strains which shown sensitivity

Strain no	*Vibrio. sp*	*Streptococci. Sp*	*Yersinia. sp*
Stw13	-	-	-
Stw14	-	-	-
Stw4	125 µg	500 µg	250 µg
Stw5	-	-	-
Stw6	-	-	-
Stw16	250 µg	250 µg	250 µg
Stw18	-	-	-
Stw20	125 µg	250 µg	500 µg

-: Absence of growth; +: Presence of growth; ++: Medium growth; +++: Large growth; StW: Seaweed.

Table 5: Antimicrobial MIC_s of thb strains (µg/ml).

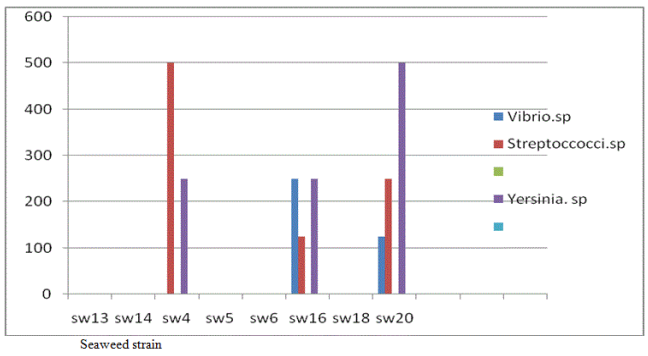

Figure 5: Antimicrobial MICs of sponge strains in µg/ml.

against one or other pathogenic bacteria were subjected for the Minimum Inhibitory Concentration (MIC) assay by following standard methodology. It shows that the strain no Sw4, Sw20 has MIC value of 125 µg against vibrio the Sw16, Sw20 and Sw4 showed MIC value of 250 µg against vibrio, streptococci and *Yersinia pestis*, the Sw16 showed MIC value of 250 µg against vibrio, and streptococci sp., and *Yersinia pestis* but the MIC value was found lower by 125 µg against streptococci.

The Sw20 showed MIC value of 125 µg against vibrio and 500 µg against *Yersinia pestis*, the strain no Sw4 and Sw16 showed MIC value of 250 µg against *Yersinia pestis* (Table 2). The strain no Sw4 and Sw20 showed MBC value of 500 against streptococci, Surprisingly, the strain no Sw4 showed MBC value of 500 µg to 2 of the pathogenic bacteria viz., vibrio and *Yersinia pestis* (Table 3). However the other strains did not showed any MIC and MBC values as mentioned in the tables. The presence and absence of bacterial turbidity and the effective concentration of sensitivity (MIC and MBC) by the isolated bacterial strains against *Yersinia pestis* is mentioned in the Tables 4 and 5 particularly the isolated strains of Sw4 and Sw16 against vibrio and streptococci were also detailed in Tables 2 and 3 for cross reference.

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