Isolation of bioactive compound from marine seaweeds against fish pathogenic bacteria *Vibrio alginolyticus* (VA09) and characterisation by FTIR

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1. Introduction

As more than 70% of the world’s surface is covered by oceans, the wide diversity of marine organisms offer a rich source of natural products. Marine environment contains a source of functional materials, including polyunsaturated fatty acids, polysaccharides, essential minerals and vitamins, antioxidants, enzymes and bioactive peptides[1]. Among marine organisms, marine algae are rich sources of structurally diverse bioactive compounds with various biological activities. Recently, their importance as a source of novel bioactive substances is growing rapidly.
and researchers have revealed that marine algal originated compounds exhibit various biological activities[2]. During the last years, many studies have been made on biological activities of the seaweed and could be potential rich sources of natural antioxidants[3].

Algae are organisms that, because of their unique characteristics, are able to generate a wide range of secondary metabolites (biologically active) that are not found in other organisms; these compounds are produced in response to situations of oxidation and extreme environmental conditions in which they live. Algae, besides being a healthy food due to its low calorie content and its high fiber and mineral content, can be a potential natural source of functional ingredients[4].

Seaweed is the most accessible marine resource of the coastal zone that occupies potential importance source of biochemical compound. Pharmaceutical importance of seaweed is well known all over the world and extensive efforts were given to bring out substances from algae. There are a number of reports regarding the medicinal importance of seaweeds belonging to Phaeophyceae, Rhodophyceae and Chlorophyceae from all over the world[5,6]. Many studies were reported earlier on the antimicrobial study of marine algæ[7].

The revolutionized therapy of infectious diseases by the use of antimicrobial drugs has certain limitations due to changing patterns of resistance in pathogens and side effect they produced. These limitations demand for improved pharmacokinetic properties which necessitate the continued research for new antimicrobial compounds for the development of drugs[8]. There have been a number of reports on antimicrobial activity of marine seaweeds against several pathogens[8–14].

Seaweeds are floating and submerged plants of shallow marine meadows. Marine macro algæ are important ecologically and commercially to many regions of the world, especially in Asian countries. Seaweeds are potential source of bioactive metabolites for the pharmaceutical industry in drug development. Many of the seaweeds possess bioactive components which inhibit the growth of some of the Gram positive and Gram negative bacterial pathogens. They are a valuable food resource which contains low calories, and they are rich in vitamins, minerals, proteins, polysaccharides, steroids and dietary fibers[15].

Seaweeds are one of the most important marine resources of the world and being used as human food, animal feed and raw material for many industries. For centuries, seaweed has been of botanical, industrial and pharmaceutical interest. In recent years research on the chemistry of seaweeds (or more generally marine organisms) has experienced a tremendous increase due to the need for compounds possessing bioactivities of possible pharmaceutical applications or other potential economic properties. Since marine organisms live in a significantly different environment from those of terrestrial organisms, it is reasonable to suppose that their secondary metabolites will differ considerably[16]. After more than 25 years of fruitful research, marine natural product chemistry must now be considered to be approaching maturity. Seaweeds offer a wide range of therapeutic possibilities both internally and externally. Seaweeds are extensive profile source of secondary metabolites. Although a majority of these (about 60%) are terpenes, but some fatty acids are also common (20%) with nitrogenous compounds[14].

2. Material and methods

2.1. Seaweeds collection

Fresh marine seaweeds Gracilaria edulis (G. edulis), Gracillaria verrcosa (G. verrcosa), Acanthospora spicifera (A. spicifera), Ulva facita (U. facita), Ulva lacta (U. lacta), Kappaphycus spicifera (K. spicifera), Sargassum ilicifolium (S. ilicifolium), Sargassum wightii (S. wightii), Padina tetramatica (P. tetramatica) and Padina gymnospora (P. gymnospora) were collected from Mandapam (Rameshwaram, Tamil Nadu) of South East coast of India. Collected samples were washed with tap water to remove epiphytes and other marine organisms and then washed with distilled water. Samples were dried at 45 °C and powdered.

2.2. Seaweeds extract preparation

Each seaweed material mixed with different solvents with increasing polarity (methanol, isopronal, acetone, chloroform and diethyl ether) solvent separately (1:50, w/v) and placed into the soxhlet apparatus. Each extraction was carried out in a soxhlet apparatus for 24 h and after evaporation in vacuum the extracts were stored at −20 °C until use[16].

2.3. Bacterial pathogens

A test organism was isolated from Parangipettai coastal region. Test pathogen was identified as Vibrio alginolyticus (VA09 our culture No.). Colonies were selected from the
bacterial stock and cultured on NA (50% of salt water) and incubated at 37°C for 24 to 48 h. Following incubation, a pure colony of bacteria was selected for each tested organism prior to the antibacterial assays. The strain was subculture into a fresh nutrient agar (50% of salt water) and stored in −20°C for further use.

2.4. Antibacterial activity by disc diffusion method

Antibacterial activities were determined by disc diffusion method. Each seaweed were extracted by using five solvent (methanol, isopropanal, acetone, chloroform, diethyl ether). Whatman No. 1 filter paper disk of 3 mm diameter, and then was sterilized by autoclaving for 15 min at 121°C. The sterile disc was impregnated with each seaweed extracts (50 mg/mL). Agar plates were surface inoculated uniformly from the broth culture of the tested microorganisms (V. alginolyticus). In all cases, the concentration was approximately 1.2x10⁸ CFU/mL. The impregnated disks were allowing the solvent to evaporate and placed on the Muller Hinton medium suitably spaced apart and the plates were incubated at 28°C for 24 h. Paper discs treated with solvent alone served as negative controls. Zones of inhibition were determined as the difference between the disc diameter (5 mm) and the diameter of the inhibition zone. Assays were run in triplicate. The inhibition zones means of the solvent extracts were compared. After incubation the clearance zones around the discs were measured and expressed in centimetre[17].

2.5. Resazurin micro–titre assay

The 96–well micro–titre assay using resazurin as the indicator of cell growth was employed for the determination of the minimum inhibitory concentration (MIC) of the active extracts[18,19].

2.6 Fourier transformed infrared (FTIR)

Cured methanol, di ethyl ether, chlofoform extract of S. wightii were recorded by Fourier–transformed infrared spectra (Shimidzu, UK). IR spectra were recorded in the 400–4000 cm⁻¹ range with a resolution of 1 cm⁻¹. The room was kept at a controlled ambient temperature (25°C) and relative humidity (30%)[20].

3. Results

The methanolic extract S. wightii produced a maximum zone of inhibition (1.95±0.11) cm radius against V. alginolyticus followed by Kappaphycus alvarezii (1.33±0.05) cm, U. lactuca (1.16±0.15) cm, U. facita (1.1±0.1) cm, S. ilicifolium (1.06±0.15) cm, P. tetramatica (1.03±0.11) cm, P. gymonospora (0.93±0.05) cm and minimum was (0.86±0.05) cm radius zone inhibiton produced G. verrcosa but A. spicifera not shown antibacterial activity. Isopropanol extract maximum inhibition was produced by S. wightii (1.93±0.78) cm against Vibrio harveyi, followed by U. lacta (1.76±0.11cm), Acanthospora spicifera (1.53±0.25), K. spicifera (1.33±0.20) cm, P. tetramatica (1.20±0.26) cm, P. gymonospora (1.06±0.11) cm, and minimum inhibition produced by S. ilicifolium (1.05±0.78) cm. G. edulis, G. verrcosa, U. facita not produced any inhibition against test pathogen.

Acetone extract of Gracilaria verrcosa showed maximum zone of inhibition (1.36±0.05) cm radius against test pathogen followed by S. ilicifolium (1.33±0.20), U. facita (1.23±0.05), G. edulis (1.21±0.03), K. spicifera (1.16±0.11), P. gymonospora (1.06±0.11) and minimum inhibition produced by S. wightii (1.04±0.11). A. spicifera, U. lacta, P. tetramatica not produced any inhibition against test pathogen. Chloroform extract of S. wightii produced a maximum zone (1.56±0.25) cm of inhibition, followed by U. facita (1.26±0.20 cm), P. gymonospora (1.23±0.05) cm, A. spicifera (1.16±0.25), P. tetramatica (1.1±0.86), K. spicifera (1.06±0.11), and minimum was 0.86±0.7 cm radius zone inhibition produced U. lacta. Diethyl ether extract of S. wightii produced maximum zone of inhibition (1.86±0.11) cm radius, followed by S. ilicifolium (1.36±0.23), P. gymonospora (1.36±0.05), G. verrcosa (1.20±0.17), G. edulis (1.13±0.23), P. tetramatica (0.80±0.1) (Table 1).

**Table 1**

<table>
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<td>1.16±0.28</td>
<td>1.26±0.20</td>
<td>0.89±0.70</td>
<td>0</td>
<td>1.56±0.25</td>
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<td>Diethyl ether</td>
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<td>0</td>
<td>0</td>
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<td>0.86±0.01</td>
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*Values are mean ± standard deviation.
The *S. wightii* methanolic extract showed a higher MIC values 25 mg/mL were obtained followed by diethyl ether (50 mg/mL), isopropanol and chloroform (100 mg/mL), acectone (200 mg/mL) (Figure 1). The *U. lacta* diethyl ether extract showed a higher MIC values 50 mg/mL were obtained followed by methanolic and isopropanol (100 mg/mL), chloroform (200 mg/mL), acectone (500 mg/mL). The *P. tetramatica* methanolic extract showed a higher MIC values 50 mg/mL were obtained followed by isopropanol (100 mg/mL), acectone and diethyl ether (200 mg/mL), chloroform (200 mg/mL).

Figure 1. Minimum inhibitor activity of *Sargassum* extract against *Vibrio alginolyticus* using modified resazurin assay.

Methanol extract of *S. wightii* was analysed by FTIR (Figure 2). It has carbodiimides–N=C=N– (3452.21–S), alkanes–CH₃ (2931.83–S), phosphate or silane–P–H r Si–H (2353.16–S), Aldehyde and ketone –C=O stretch (1635.64 S), alkanes–CH₃ (1396.46 M), aldehydes and ketones –C=C–C bending (1267.23 M), aldehydes and ketones –C=C bending (1109.07 M), Arenes–CH bending (673.16 M), Chloro compound–C–H stretch (594.08 M) (Table 2).

*S. wightii* di ethyl ether extract was analysed by FTIR (Figure 3). It has the compound of chlorocompound–C–Cl (663.37 M), thiacarbonyl compound–C=S (1049.38 S), alkanes–CH₃ deformation (1112.93 M), aldehyde/ketones–C=C bending (1452.40 M), Isothiocyanates–N=C=S (2044.54 M), Silane–Si–H (2353.16–S), Thios–S–H (2522.89 M), primary amines CH stretch (2831.50 M), alkyl group–C–H stretch (2943.37 strong) alcohol/phenol–O–H stretch (3450.38 S) (Table 3).

*S. wightii* chloroform extract were analysed by FTIR (Figure 4) it has the chlorocompound–C=C=Cl– (667.37 M), strong thiocarbonyl–C=S (1069.89), amines–CN– (1123.93 M), carboxilic acid OH (1450.47 M), aromatic C=C bending (1658.78 M), isothiocyanates–N=C=S (2042.62 M), phosphoryous (phosphine) P–H (2353.16 M), aldehyde/ketones–CH (2831.50 M), alkyl compound–C–H stretch (2943.37 strong) alcohol/phenol–O–H stretch (3450.38 S) (Table 4).

Table 2

<table>
<thead>
<tr>
<th>Frequency ranges (cm⁻¹)</th>
<th>Intensities</th>
<th>Assignment and remarks</th>
<th>Group or functional class</th>
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<td>3452.21</td>
<td>Strong</td>
<td>–N=C=N–</td>
<td>Carbodiimides</td>
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<td>2931.83</td>
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<td>CH₃</td>
<td>Alkanes</td>
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<td>P–H r Si–H</td>
<td>Phosphine or silane.</td>
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<td>1635.64</td>
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<td>Aldehyde ketone</td>
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<td>C=C–C bending</td>
<td>Aldehydes &amp; Ketones</td>
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<td>1109.07</td>
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<tr>
<td>673.16</td>
<td>Medium</td>
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<tr>
<td>594.08</td>
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<td>Chloro compound</td>
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Table 3
Major peak values of the FTIR-di ethyl ether extract of S. Wightii.

<table>
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<th>Frequency ranges (cm(^{-1}))</th>
<th>Intensities</th>
<th>Assignment and Remarks</th>
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<td>666.37 Medium</td>
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<td>Chloro compound</td>
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<td>1049.23 Strong</td>
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<td>1112.93 Medium</td>
<td>CH, deformation</td>
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<tr>
<td>1452.40 Medium</td>
<td>C=C=C bending</td>
<td>Aldehyde/Ketones</td>
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<td>2044.54 Medium</td>
<td>-C=N=S</td>
<td>Isothiocyanates</td>
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<tr>
<td>2353.16 Strong</td>
<td>Si-H</td>
<td>Silane</td>
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<tr>
<td>2522.89 Medium</td>
<td>-S-H-</td>
<td>Thiols</td>
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<td>2831.50 Medium</td>
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<td>2941.44 Medium</td>
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<td>3346.50 Medium</td>
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Table 4
Major Peak Values Of The FTIR - Chloroform Extract Of Sargassum Wightii

<table>
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<tr>
<td>667.37 Medium</td>
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<td>Chloro compound</td>
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<tr>
<td>1069.89 Strong</td>
<td>C=S</td>
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<td>1123.93 Medium</td>
<td>CN</td>
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<tr>
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<td>OH</td>
<td>Carboxylic acid</td>
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<td>1658.78 Medium</td>
<td>C=C bending</td>
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<td>2042.62 Medium</td>
<td>-N=C=S</td>
<td>Isothiocyanate</td>
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<td>2353.16 Medium</td>
<td>PHPHosphate</td>
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<tr>
<td>2831.50 Medium</td>
<td>CH</td>
<td>Aldehyde/Ketones</td>
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<td>2943.37 Strong</td>
<td>C-H stretch</td>
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<tr>
<td>3450.38 Strong</td>
<td>O-H stretch</td>
<td>Alcohol/phenol</td>
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Figure 4. FTIR spectrum of the Chloroform extract of S. wightii.

4. Discussion

The marine environment is incomparable reservoirs of bioactive natural products, many of which exhibit structural features that have not been found in terrestrial natural products. Marine algae are found to be vital source of useful bioactive substances since two decades. Several studies have demonstrated that seaweeds are an excellent source of components with biological activity such as antibacterial.

The objective of this study was to evaluate the antibacterial activity seaweed species from Madapam Costal region. Ten marine Seaweeds were extracted by using five different solvent assayed for the antibacterial activity in vitro by disc diffusion against V. alginolyticus bacteria. The aim to use them in the future as alternatives to antibiotics in aquaculture field.

Genovese et al.[21] reported that the marine biodiversity and associated chemical diversity constitute an unlimited reserve of bioactive substances in the field of bioactive products. Seaweeds provide a rich source of structurally diverse secondary metabolites. Several studies have demonstrated that seaweeds are an excellent source of components such as polysaccharides, tannins, flavonoids, phenolic acids, bromophenols, and carotenoids has exhibited different biological activities[22]. Seaweed extracts showed various bio potential activities such as antibacterial[23].

Antibacterial activity of the nine brown seaweed extracts was analyzed with three types of assays, namely, disc diffusion susceptibility testing, MIC and MBC assays. Disc diffusion susceptibility testing was a preliminary screening of antibacterial activity of the extracted compounds as it can only give qualitative but not quantitative assessment. The size of the inhibition zone may not correspond to their antibacterial activity[24].

Antibacterial activity of red, brown and green algae against both Gram positive and Gram negative bacteria has been established by several scientists[5]. But variation in antibacterial activity may be due to the method of extraction, solvent used in extraction and season at which samples were collected[25].

Hediat et al.[26] reported that different solvents have been reported to have the capacity to extract different phytoconstituents depending on their solubility or polarity in the solvent. In this present study also supported that optimizes their antibacterial activity by selecting the best solvent to extract the active compound from seaweeds. So this suggests that seaweeds should be extracted in different solvent systems in order to optimize their antibacterial activity by selecting the best solvent system. Seaweed extracts in different solvents exhibited different antimicrobial activities[27].

The high and low effect of organic extract against microorganisms could be related to the presence of
bioactive metabolites, which can be soluble in solvent\[^{[10,28]}\]. Vijayabaskar and Shiyamala showed that the marine algae extract of *Turbinaria ornata* and *S. wightii* possessed noticeable activity against positive and negative bacteria and could be utilized as a good source of antimicrobial agent in pharmaceutical industry\[^{[29]}\].

Manilal *et al.*\[^{[14]}\] and Rangaiah *et al.*\[^{[27]}\] showed that methanol extraction yielded higher antimicrobial activity than n–hexane and ethyl acetate which is in supported to our results. The methanolic extract *S. wightii* produced a maximum zone of inhibition 1.95±0.11 cm radius against *V. alginolyticus* and minimum was 0.86±0.05 cm radius zone inhibition produced *G. verrcosa*. In contrast to our results, Ibtissam *et al.*\[^{[30]}\] reported non efficiency of the methanol extracts of *Sargassum vulgarum*, which did not show antibacterial activity against *E. coli* and *S. aureus* growth. Kandhasamy and Arunachalam\[^{[25]}\], who reported that extracts prepared with methanol showed the best activity.

In our study isopropanol extract maximum inhibition was produced by *S. wightii* but *G. edulis*, *G. verrcosa* and *U. facita* not produced any inhibition against test pathogen. In our result contrast with Krishnaveni Eahamban *et al.*\[^{[16]}\] they reported that isopropanol extract of *Gracilaria Corticata* showed a maximum antibacterial activity against the test pathogen (9/12 bacterial pathogens).

Osman *et al.*\[^{[13]}\] reported that, acetone was the best solvent for extracting the bioactive compounds; meantime it gave the highest antimicrobial activity against the selected pathogens. This result agreed with those of Wefky and Ghobrial and Fareed and Khairy\[^{[31,32]}\]. In our result also supported that above study. Acetone extract of *Gracilaria verrcosa* showed antibacterial activity against the test *Vibrio harveyi*. Jebasingh *et al.*\[^{[33]}\] also reported that Acetone extract have good antibacterial activity than other solvent. Kolanj Nathan and Stella indicated that acetone was the best solution for extracting the effective antimicrobial materials from *Sargassum myricystum*, *Turbinaria*\[^{[10]}\].

Rhimou\[^{[34]}\] reported that Chloroform extract had moderate bactericidal activity and a benzene extract had a weak inhibition potential. But in Contrast our result Chloroform *S. wightii* extract showed a good antibacterial activity. Karthikadevi, *et al.*\[^{[35]}\] reported that the extraction by way of diethyl ether minimum activity against *Pseudomonas aeruginosa Vibrio parahaemolyticus* and against Proteus sp. Bibiana\[^{[36]}\] reported that the maximum activity of diethyl ether extract of *S. wightii* and *Kappaphysis alwarezii* showed a good antibacterial activity against test pathogen. In our study diethyl ether extract of *S. wightii* showed a good antimicrobial activity against test pathogens.

Plant materials can be classified as antimicrobial agents based on MIC values of their extracts. Extracts with MIC values less than 100 mg ml\(^{-1}\) are classed as strong inhibitors, at 100–500 mg ml\(^{-1}\) as moderate inhibitors, at 500–1000 mg ml\(^{-1}\) as weak inhibitors and at more than 1000 mg ml\(^{-1}\) as inactive\[^{[37]}\]. According to this classification the methanol, diethyl ether, Isopropanol, Chloroforms extract of *S. wightii*, *U. lacta* and *P. tetramatica* showed as strong inhibitors. The low MIC values of *S. wightii* indication of seaweeds extracts efficacy.

Zubia *et al.*\[^{[38]}\], suggested by the great variation observed in the potential antimicrobial components in seaweeds could be due to the external environmental factors such as herbivory, light, depth, salinity and nutrients of their growing environment. All of these factors could act on the spatiotemporal regulation on metabolic expression of the active compounds leading to marked qualitative and quantitative variations among similar species at a smaller scale than different species. Thus, this might be some of the reasons that led to the higher bacteriostatic activity in *Sargassum polycystum*.

However, crude seaweeds extracts are mixed with many compounds and their active portion may be very low. FTIR major peak showed that it have Phenol, Aldehyde & Ketone groups as a major compound in the seaweeds. Phenolic compounds and exhibited good antioxidant and antimicrobial activities\[^{[39,40]}\]. Further investigations should focus on attempts to purify active compounds and to elucidate their chemical structure. The most active extract resulted nontoxic to fish. The extracts from *S. wightii* could be a source of antibacterial compounds with potential use in aquaculture in order to control fish infections and as fish–feed component.

**Conflict of interest statement**

We declare that we have no conflict of interest.

**Acknowledgements**

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Seaweeds are one of the most important marine resources of the world and being used as human food, animal feed and raw material for many industries. For centuries, seaweed has been of botanical, industrial and pharmaceutical interest. In recent years research on the chemistry of seaweeds (or more generally marine organisms) has experienced a tremendous increase due to the need for compounds possessing bioactivities of possible pharmaceutical applications or other potential economic properties. Since marine organisms live in a significantly different environment from those of terrestrial organisms, it is reasonable to suppose that their secondary metabolites will differ considerably. In this work to find out the seaweed can inhibit the fish pathogen. Few literature only reported the seaweeds inhibit against fish pathogens.

In this study ten different seaweeds were used for screening against fish pathogens. The major fish pathogen are vibrio alginoytics to be used in this study. The seaweeds have high medical values. The seaweeds extract were screened against vibrio alginoitics. Based on the results the S. wightii showed more inhibitory activity against pathogens. So in future it could be characterized the compound level for controlling infectious fish diseases.

Most of the seaweed work to control the multidrug resistant bacteria (Kumaran et al., 2011). In this work they carry out more seaweed samples and its extracted using different solvents. The different extract were tested against V. aginolyticus pathogen. The vibrio is a major fish pathogen (Alagappan et al., 2010) and affected in aquaculture industry.

Ten seaweeds were collected and extracted. The S. wightii showed good inhibitory result against vibrio alginoitics. Based on the result the S. wightii have potential value in aquaculture industry. The tremendous results shown in seaweed FTIR results.

The S. wightii will characterize structural level for drug development. In this work resulted out the S. wightii showed more inhibition against Virbio alginoitics. So the result concluded it have strong anti bacterial effect. The compounds should be strongly inhibiting the bacterial growth. In future it could be helpful to aqua culture industry.

This work shown seaweed has strong potential antibacterial activity. The authors are concluded the S. wightii shown more active than other seaweed. The result pointed out the active compounds of S. wightii has strong correlation with natural drugs. It can control culturing fish infectious disease.

References


[34] Arputha Bibiana M, Nithya K, Manikandan MS, Selvamani P, Latha S. Antimicrobial evaluation of the organic extracts of Sargassum wightii (brown algae) and Kappaphycus alvarezi (red algae) collected from the coast of Meemesal, Tamilnadu. IJPCBS 2012; 2(4): 439–446.


