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Phytochemicals screening and antimicrobial properties of *Sargassum oligocystum* and *Sargassum crassifolium* Extracts

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Abstract

The present study was conducted to investigate the phytochemical contents and evaluate the antimicrobial property of *Sargassum oligocystum* and *Sargassum crassifolium* extracts collected from the coast at Diora-Zinungan, Santa Ana, Cagayan, Philippines. Extracts of powdered seaweed were prepared using sequential extraction with different organic solvents in order to increasing the polarity (Ethanol, n-hexane, dichloromethane and ethyl acetate and aqueous). Five fractions (Ethanol, n-hexane, dichloromethane ethyl acetate and aqueous) were examined for antimicrobial activity by using disc diffusion assay on thirteen (13) strains of aquaculture pathogen. The extracts showed the presence of phytochemical constituents like flavonoids, tannins, phenolics, sterols and terpenoids and the absence of saponins. Among the tested extracts for antimicrobial activity, ethanolic extracts was determined to be the best solvent for isolation of antimicrobial compounds from the tested seaweeds followed by n-hexane, dichloromethane ethyl acetate and aqueous. The extract of the *S. oligocystum* and *S. crassifolium* showed presence of different groups of secondary metabolites such as flavonoids, tannins, phenolics, sterols and terpenoids which are important indicators of the microbial properties of seaweeds.

Keywords: phytochemical, antimicrobial activity, *Sargassum oligocystum*, *Sargassum crassifolium*

1. Introduction

Aquaculture is becoming a more concentrated industry, with fewer, but much larger farms. Infectious diseases are always a risk and may cause significant stock losses and problems with animal welfare. Intensive aquaculture (shrimp and fish farming) has led to growing problems with bacterial diseases [32]. The increasing resistance to of microorganisms to antimicrobial drugs in use has attracted the attention of the scientific community, hence needed to develop a natural alternative agent for the prevention of infections [16]. Over the past decade there has been an explosion of interest in the antimicrobial, particularly antibacterial and antifungal activity of natural products [4].

Sargassum sp., one of the marine macro algae belonging to the class Phaeophyceae, is widely distributed in tropical and temperate oceans. It belongs to the marine family *Sargassaceae* and order Fucales. Wide range of bioactive properties of *Sargassum* has been reported [7]. In addition, The *Sargassum* (Phaeophyceae) like, *Sargassum polycystum* and *Sargassum tenerrimum* [20], *Sargassum latifolium* [6], and *Sargassum glaucescens* [25], have been studied and they showed promising antibacterial and anti-fungal activity.

Phytochemical studies have attracted the attention of researchers due to the development of new and sophisticated techniques. These techniques played a significant role in the search for additional resources of raw material for pharmaceutical industry (phytochemicals) [27]. Seaweeds create a natural source of a variety of drugs for pharmaceutical, food and cosmetic applications including carotenoids, terpenoids, steroids, amino acids, phlorotannins, phenolic compounds, halogenated ketones, alkanes and cyclic polysulphides [39, 10], which can enhance the resistance and immune response against many infectious agents and have been used in traditional medicine for a long time [9]. Phytochemical analysis of the seaweeds will be a good preliminary approach to reveal its secondary metabolite constituents and the resultant medicinal values.

Brown algae are considered as source of bioactive compounds and they are able to produce a great variety of secondary metabolites such as antioxidant, antibacterial, antifungal,

anticoagulant and antiradiation UV-B, wound healing and cell structure regeneration ^[18]. In addition, Seaweeds are naturally renewable sources which are also used as food, feed and fertilizer in many parts of the world. They have been screened extensively to isolate life-saving drugs or biologically active substances all over the world ^[18]. Therefore, antimicrobial properties of seaweeds and their extracts against various pathogens exhibit significant immunostimulatory ^[3]. Due to its availability and bioactive properties the present study aims to evaluate the phytochemical screening and antimicrobial activities of *Sargassum oligocystum* and *Sargassum crassifolium* extracts for various medical and industrial application.

2. Materials and methods

2.1 Sampling and collection site.

The seaweeds were collected by scuba diving and handpicking from the rocky substratum at depth of 1-3 m along the subtidal areas at Diora-Zinungan, Santa Ana (coordinates: 16° 46'79" N latitude, 121° 23'00.48" E longitude) Cagayan, Philippines.

2.2 Seaweeds Extraction.

The collected seaweeds were cleaned of epiphytes and extraneous matters, necrotic parts were removed, and finally washed with clean salt water. The epiphytes collected were separated and were tested for antibacterial activity. The seaweeds were air dried at room temperature for two weeks, cut into a small portions were powdered using a hammer mill. Extract of powdered seaweeds were prepared using sequential extraction with different organic solvents in order to increasing the polarity (Ethanol, n-hexane, dichloromethane and ethyl acetate) by soaking the powder trice in the respective solvents. Each of the pooled extract were filtered and concentrated under vacuum on a rotary evaporator at low temperature to get the crude extracts from each solvent used and reflux method.

2.3 Crude extraction with Ethanol.

Ethanol were added to the pulverized seaweeds. After 48 hours of soaking, the samples were filtered using a Buchner funnel and flask connected to a vacuum source. A fresh solvent will again be introduces into the sample and were soaked for another 24 hours. These parts were done twice. The collected crude ethanol extract from these three soaking were concentrated using rotary evaporator under reduce pressure at 45 °C.

2.4 Extraction with n-hexane.

The aqueous fraction obtained after the extraction with ethanol were subjected to liquid-liquid extraction with 50 ml n-hexane (Malingkrodt) three times. The hexane fraction obtained after the extraction were concentrated using the rotary evaporator under reduced pressure at 45 °C reflux method.

2.5 Extraction with dichloromethane.

The aqueous fraction obtained after the extraction with n-hexane were subjected to liquid-liquid extraction with 50 ml dichloromethane (DCM, Reidel-de Haen) each three times. The DCM fraction obtained after the extraction were concentrated using the rotary evaporator under reduced pressure at 45 °C reflux method.

2.6 Extraction with ethyl acetate. The aqueous fraction obtained after the extraction with DCM were subjected to liquid-liquid extraction with 50 ml ethyl acetate (DCM, each three times. The ethyl acetate fraction obtained after the extraction will be concentrated using the rotary evaporator under reduced pressure at 45 °C reflux method. Aqueous extract was obtained after the extraction to ethyl acetate.

2.6 Phytochemical screening

Suitable amount of sample was extracted using 80% ethanol. The extracts was evaporated to incipient dryness respectively and tested for the presence of secondary metabolites such as flavonoids, tannins, saponins, phenolics, sterols and terpenoids were carried out according to the common phytochemical methods described by ^[11].

2.7 Bacterial and fungal pathogens

For testing the antibacterial activity, the following Gram positive *Staphylococcus aureus* (BIOTECH 1582), *Streptococcus mutans* (BIOTECH 10231), *Micrococcus luteus* (BIOTECH 1061) and *Bacillus subtilis* (BIOTECH 1679) and Gram negative *Aeromonas hydrophila* (BIOTECH 10089), *Escherichia coli* (BIOTECH 1634), *Psuedomonas aeruginosa* (BIOTECH 1335) and *Psuedomonas fluorescens* (BIOTECH 1123) bacteria strain were selected. For fungal activity, the following fungal strains, *Aspergillus parasiticus* (BIOTECH 3167) *Aspergillus niger* (BIOTECH 3080) *Candida tropicalis* (BIOTECH 2085) *Penicillium expansum* (BIOTECH 3097) and *Sacchromyces cerevisiae* (BIOTECH 2096) were used for antifungal activity and they were obtained from the National Institute of Molecular Biology and Biotechnology, University of the Philippines, Los Baños College, Laguna 4031, Philippines.

2.8 Anti-microbial assay

The disk diffusion assay was performed according to ^[33]. Whatman No. I filter paper disk of 6-mm diameter was sterilized by autoclaving for 15 min at 121 °C. The sterile disks were impregnated with the different crude extracts. The bacteria were sub-cultured to Nutrient Agar for 24 h prior to use. One loop of each test organism was suspended in 5 ml Trypticase Soy Broth solution separately. Mueller-Hinton Agar (MHA) was surface inoculated with the suspension of the respective organism. The disks impregnated with the crude extracts of the seaweeds were placed on the MHA medium with suitable apace and the plates were incubated at 32 °C for 24 hours. Chloramphenicol (500 mg/ml) was used as a positive and respective solvents were used as a negative control. The above procedure is allowed for fungal assays, the Saboraud Dextrose Agar (SDA) media were used ^[1], and the Penicillin (500 mg/ml) was used as a standard and the solvents of each extract as a negative control. The diameter of the growth inhibition halos caused by the different extracts of the seaweeds was measured. The antibacterial assay was carried out in triplicate.

2.9 Data analysis.

The experiments have been repeated 3 times. All data were expressed as mean values \pm SD, the mean values being analyzed using Microsoft Excel 2010 software.

3. Results

In this study, the seaweeds *S. oligocystum* and *S. crassifolium* were selected and screened for the presence of phytochemical constituents. The phytochemical constituents such as

flavonoids, tannins, phenolics, sterols and terpenoids are secondary metabolites of seaweed that serve as defense mechanism against different infectious diseases. The present study carried out on the seaweed sample revealed the presence of medicinally active constituents. The phytochemical constituents of the selected seaweed investigated are summarized in (Table 1). The ethanolic extracts of *S. oligocystum* and *S. crassifolium* showed the presence of phytochemical constituents like flavonoids, tannins, phenolics, sterols and terpenoids which could be responsible for the observed antimicrobial property.

Table 1: Phytochemical screening on the ethanolic extracts of the *Sargassum oligocystum* and *Sargassum crassifolium*.

Phytochemical Tested	<i>S. oligocystum</i>	<i>S. crassifolium</i>
Flavonoids	+	+
Tannins	+	+
Saponins	-	-
Phenolics	+	+
Sterols	+	-
Terpenoids	-	+

* + = Presence of constituents; - = Absence of constituents.

Table 2: Zone of inhibition (mm) of the test bacteria against various extracts of *S. oligocystum*.

Pathogens	Solvent used zone of inhibition (mm)					
	Ethanolic	nHexane	Dichloromethane	Ethyl acetate	Aqueous	Chloramphenicol
<i>A. hydrophila</i>	17±5.57	9.33±1.15	8.66±0.58	10±1.15	7±4.58	25.33±3.06
<i>E. coli</i>	18.66±4.16	6.33±0.58	7.33±0.58	8.66±1.15	6.33±1.15	12±1
<i>S. aureus</i>	11.66±1.15	14.66±4.04	10±2	10±1.73	6.33±4.51	22.66±17.93
<i>P. aeruginosa</i>	8.66±0.58	9.33±0.58	7.33±0.58	11.33±3.06	6.66±2.08	26.33±2.08
<i>M. luteus</i>	8.33±1.53	16.33±5.51	12.33±6.66	10.33±1.15	5.66±5.03	33.33±8.74
<i>B. subtilis</i>	8±0	7±1	6.66±0.58	7.66±1.53	6.66±0.58	31±2.65
<i>S. mutans</i>	14±2	8.33±2.52	7±1	8.33±0.58	5.33±3.06	28.66±1.15
<i>P. flourescens</i>	12±4	8±2	9±3.46	7.66±1.53	6.66±3.79	29±2.65

Table 3: Zone of inhibition (mm) of the test fungi against various extracts of *S. oligocystum*.

Solvents	Solvent used zone of inhibition (mm)					
	Ethanolic	nHexane	Dichloromethane	Ethyl acetate	Aqueous	Chloramphenicol
<i>A. parasiticus</i>	15.33±4.04	5.33±4.62	9.66±2.89	3.66±6.35	5.67±4.93	17±6.08
<i>A. niger</i>	15.66±3.06	11.33±4.16	10.66±3.06	12±3.61	7.0±6.56	23.67±4.04
<i>C. tropicalis</i>	10±3.46	2.66±4.62	9±1.73	2.66±4.62	3.33±5.77	18.33±0.58
<i>P. expansam</i>	12±5.29	11.66±2.31	9.33±1.53	7.33±7.02	2.67±4.62	17.67±7.09
<i>S. cerevisiae</i>	9.33±1.15	3.33±5.77	4.66±8.08	8±0	3.0±5.20	19.0±0.58

Sargassum crassifolium: The highest inhibition zone was recorded in ethanolic extract against *A. hydrophila* (24.33±0.58mm). A moderate activity was also seen in ethyl acetate and aqueous extracts against *S. mutans*, *E. coli*, and *B. subtilis* (6.33±2.08 mm, 6.33±0.58). Dichloromethane and

The antimicrobial activity of *Sargassum oligocystum* and *Sargassum crassifolium* using six (6) different solvents were tested against (13) aquaculture pathogenic bacterial and fungi namely: *Aeromonas hydrophila*, *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Micrococcus luteus*, *Bacillus subtilis*, *Streptococcus mutans*, *Pseudomonas fluorescens*, and *Aspergillus parasiticus*, *Aspergillus niger*, *Candida tropicalis*, *Penicillium expansum*, *Sacchromyces cerevisiae* were presented in Table 2-5

Sargassum oligocystum. *S. oligocystum* were active to all of the tested aquaculture pathogens (Table 2 and 3). The highest inhibition zone (18.66±4.16 mm) in ethanolic extract against *E. coli* followed by ethyl acetate, dichloromethane, nHexane and the lowest inhibition (5.33±3.06 mm) in aqueous extract against *S. mutans*. Among the bacterial pathogens *A. hydrophila*, *E. coli*, *S. aureus* and *B. subtilis* were sensitive to all the extracts. For fungal strains, the highest activity was recorded in ethanolic extract against *A. niger* (15.66±3.06 mm) and nHexane and aqueous had a moderate activity against two pathogens such as *S. cerevisiae* and *C. tropicalis* (3.33±5.77 mm).

nHexane had different activity against some pathogens (Table 4). For fungal strain, (Table 5) the maximum inhibition zone was recorded in ethanolic extract against *A. niger* (16.33±4.16 mm) followed by aqueous extract of *A. niger* and *S. cerevisiae* (3±5.20mm) showed moderate activity.

Table 4: Zone of inhibition (mm) of the test bacteria against various extracts of *S. crassifolium*

Solvent	Solvent used zone of inhibition (mm)					
	Ethanolic	nHexane	Dichloromethane	Ethyl acetate	Aqueous	Chloramphenicol
<i>A. hydrophila</i>	24.33±0.58	14±4.36	21.66±3.06	12±1.73	10.66±1.53	22.33±1.15
<i>E. coli</i>	7±0	6.33±0.58	7±1	7±1	6.33±0.58	12.66±1.15
<i>S. aureus</i>	15.33±2.31	14.33±0.58	13.33±1.53	13.67±1.53	7.67±1.15	34±2
<i>P. aeruginosa</i>	23.33±2.08	21.66±1.15	21±1	14.33±2.31	10.33±3.21	33±1.73
<i>M. luteus</i>	8.33±0.58	9.67±2.08	11±2.65	15±1.73	7±1.73	30.67±1.53
<i>B. subtilis</i>	22.67±1.15	15.33±1.53	13.33±1.15	18±1.73	6.33±0.58	35.33±4.93
<i>S. mutans</i>	8.66±2.52	8±3.46	7±1	6.33±2.08	0±0	32±2.65
<i>P. flourescens</i>	9±1	13±4.58	12±2	7.33±1.15	0±0	30.33±2.08

Table 5: Zone of inhibition (mm) of the test fungi against various extracts of *S. crassifolium*

Solvent	Solvent used zone of inhibition (mm)					
	Ethanollic	nHexane	Dichloromethane	Ethyl acetate	Aqueous	Penicillin
<i>A. parasiticus</i>	12.33±4.16	6±5.20	8.66±0.58	7±6.56	9±1	17±5.20
<i>A. niger</i>	16.33±4.16	11.33±3.51	10.66±2.52	11±2.65	3±5.20	19.33±1.53
<i>C. tropicalis</i>	12±5.20	7±6.56	9.66±2.08	6.66±5.86	5.33±4.62	18.67±0.58
<i>P.expanssam</i>	13.66±3.51	11±2.65	11.33±2.52	7.66±7.51	2.66±4.62	18±6.24
<i>S. cerevisiae</i>	7.66±6.81	6.66±5.86	6.33±5.51	6.66±5.86	3±5.20	21.33±2.31

4. Discussions

Because of the increasing development of drug resistance to aquaculture pathogens and the presence of undesirable effects of certain antifungal and antibacterial agents, the search for new antimicrobial agents is of great concern today [38]. However, the evidence of algal bioactive compounds and their antimicrobial activity has been established through many studies. Algae contain different chemical compounds and different extract show different biological activities [19].

Flavonoids are considered as active potentials in the field of medicine. Presence of flavonoids in *Sargassaceae* shows that the seaweed can be used as an antioxidant and as an alternate source in the medicine. Flavonoids are potent antioxidants and have aroused considerable interest recently because of their potential beneficial effects on human health in fighting diseases [36]. Flavonoids are anti-allergic, anti-inflammatory, anti-microbial, and anti-cancer agent. The flavonoids are responsible for antimicrobial activity and activity of the algal extracts [19], which is corroborated with the present investigation.

Phenolic compounds are important in plant defense mechanisms against invading bacteria and other types of environmental stress [13]. In addition, phenolic compound and its derivatives, including simple phenols, flavonoids, phenyl propanoids, tannins, lignins and many other substances, contain aromatic rings and hydroxyl groups that will determine the radical scavenging power of the compound [8]. Seaweeds extracts are considered to be a rich source of phenolic compounds [15]. The results of the present study confirmed the phenolic compounds presence in the tested extracts of studied *Sargassum* species. The antimicrobial activity of *Sargassum* spp. extracts could be attributed to the presence phenolics in the seaweeds.

The importance of tannins in various antibiotics used in treating common pathogenic strains has recently been reported by [22]. In addition, the astringent property of the tannin may induce complexation with enzymes or substrates. Many microbial enzymes in raw culture filtrates or in purified forms are inhibited when mixed with tannins. Atannin's toxicity may be related to its action on the membranes of the microorganisms and complexation of metal ions by tannins may account for tannin toxicity [12]. Tannins has been found to have antiviral, antibacterial, antiparasitic effects, anti-inflammatory, anti-ulcer and antioxidant property for possible therapeutic applications [28]. The present study results confirm the tannin presence only in ethanolic extracts of *S. oligocystum* and *S. crassifolium*.

Antibacterial activity of the different algae against both Gram positive and Gram negative bacteria has been established by several scientists [21]. But the variation in antimicrobial activity might be due to the presence of different antibacterial substances among these species as suggested by [24], and due time and place of sampling collection, capability of extraction protocol to recovered the active metabolites and the assay methods [34], and antifungal activity of seaweeds depends on the species from different division [35]. Several different

organic solvent have been used to screen seaweeds for antimicrobial activity. Early, the chloroform and ethyl acetate extracts *Enteromorpha compressa*, *Chaetomorpha linum* and *Polysiphonia subtilissima* were active against most of the pathogens whereas methanol and ethanol extracts were active only against *Shigella flexneri* [37].

In this study, the ethanolic extracts of *S. oligocystum* and *S. crassifolium* were found more active compare with n-hexane, dichloromethane ethyl acetate and aqueous extracts that confirms the previous findings of [17, 29]. The crude extracts of tested *Sargassum* spp. showed a significant antimicrobial activity against Gram-positive and Gram-negative as well as the fungus. Antimicrobial activity of brown seaweeds includes *Sargassum polycystum* and *Sargassum tenerrimum* showed significant activity against gram-positive, gram-negative and fungal pathogens [20], which confirms in the present investigation. Seaweeds are an excellent source of components such as polysaccharides, tannins, flavonoids, phenolic acids, bromophenols, and carotenoids has exhibits different biological activities [2, 31, 30]. In addition, the species of Phaeophyta showed the strongest activities against [23]. The brown seaweeds contain high amount of flavanoid and phenolic compound could be the reason for antifungal activity [5].

5. Conclusion

Overall, the phytochemical analysis of the extract of the *S. oligocystum* and *S. crassifolium* showed the presence of different groups of secondary metabolites such as flavonoids, tannins, Phenolics, Sterols and Terpenoids which are important indicators of the microbial properties of seaweeds. The study revealed that the extracts of *Sargassum* demonstrated inhibitory effects against the test aquaculture pathogens. This is a promising finding, as there may be a potential to utilize such extracts as antimicrobial agent and as natural immunostimulant for aquaculture.

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