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Seaweed extracts control the leaf spot disease of the medicinal plant *Gymnema sylvestre*

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Abstract: Antimicrobial screening of 12 different seaweeds extracts namely *Chaetomorpha antennina*, *Dictyota dichotoma*, *Enteromorpha flexuosa*, *Laurencia obtusa*, *Gracilaria corticata*, *Gracilaria verrucosa*, *Grateloupia lithophila, Padina boergesenii*, *Sargassum wightii, Turbinaria conoides*, *Halimeda tuna* and *Ulva lactuca* was carried out *in vitro*. The crude extracts were tested against the phytopathogenic bacterium- *Pseudomonas syringae* causing leaf spot disease of the medicinal plant *Gymnema sylvestre*. The methanolic extracts of *Sargassum wightii* showed maximum activity followed by ethyl acetate compared to that of other organic solvent- extracts. Thus, this investigation throws fresh light on the appropriate usage of solvent extraction method in preparing potent biopesticide.

Keywords: *Gymnema sylvestre*, seaweed extracts, plant pathogenic bacteria, leaf spot disease, pathogenicity, *Pseudomonas syringae*

Introduction

Seaweeds have been widely used as food for centuries in Asia (Darcy-Vrillon, 1993; Indergaard & Minsaas, 1991), but in western countries they are generally employed for the production of valuable chemicals. Their main components *viz*. agars, aligns and carrageenans are used as ingredients in food, pharmaceuticals and diverse consumer products and industrial processes (Lewis *et al*., 1988; Skjak- Braek & Martinsen, 1991; Zilinskas & Lundin, 1993; Stephen, 1995; Renn, 1997; Mazumder, 2006). The bulk of the linvestigations pertaining to bioactive compounds from seaweeds deals with human pathogens; studies related to phytopathogens are being restricted to pathogens of commercial crops such as tobacco (Caccamese *et al*., 1980), citrus trees (Kulik, 1995) and rice (Manimala & Rengasamy, 1993; Lourdu Mariadoss & Rengasamy, 1995; Arun Kumar & Rengasamy, 2000a,b; Sultana *et al*., 2005).

The medicinal plant- *Gymnema sylvestre* R. Br. is used for treating diseases such as diabetes, urinary complaints, stomach problems, piles, chronic cough, breathing troubles, colic pain, asthma, bronchitis, cardiopathy, conjunctivitis, constipation, dyspapsia, haemonoids, hepatosplenomegally, inflammation, intermittant fever, jaundice leucoderma and many more diseases.

In this report, we describe the screening of 6 different solvent extracts of 12 different seaweeds collected from the Coastline of Tamil Nadu, India for antibacterial activity against the pathogen *Pseudomonas syringae* isolated from the medicinal plant *Gymnema sylvestre*. This study is part of a programme on screening of seaweeds for a biological activity, with the aim of identifying a novel and potentially useful biopesticides.

Materials and methods

Preparation of seaweed extracts

About 2 kg of fresh and healthy specimens of *Gracilaria corticata* J. Ag., *G. verrucosa* (Hudson) Papenfuss, *Grateloupia lithophila* and *Laurencia obtusa* (Hudson) Lamouroux belonging to Rhodophyceae, *Dictyota dichotoma* (Hudson) Lamouroux, *Padina boergesenii* Allender and Kraft, *Sargassum wightii* Greville and *Turbinaria conoides* (J. Ag.) Kütz. belonging to Phaeophyceae, *Chaetomorpha antennina* (Bory.) Kütz, *Enteromorpha flexuosa* (Wulf.) J. Ag., *Halimeda tuna* (Ellis & Solander) Lamouroux and *Ulva lactuca* Linn. belonging to Chlorophyceae were collected along the coast of Tamil Nadu, India during the year 2006-07. They were brought to the laboratory, washed in seawater to remove the macroscopic epiphytes and extraneous matter, and then rinsed in distilled water. The specimens were shade dried for 10 days, powdered and sieved through 0.8mm^2 sieve plate. Fifty grams of the dried seaweed powder were extracted in 200mL (1:4w/v) of each petroleum ether, ethyl acetate, chloroform, acetone and methanol using shaker for 10 days at room temperature, concentrated and stored at 4°C.

Isolation of bacteria

The leaf spot infected leaves of *Gymnema sylvestre* R. Br. were collected, washed with sterile distilled water and then surface sterilized using 1.0% mercuric chloride for 30 seconds followed by 70% ethanol. A portion of leaf spot cut after the surface sterilization of leaves were ground using a mortar and pestle. A loop full of it was streaked on nutrient agar plates and incubated at 28°C for 48h. The isolated colonies were purified and identified according to the standard biochemical tests outlined in Bergy's Manual of Determinative Bacteriology (Holt *et al*., 1994).

Pathogenicity test

The bacterial strain isolated from medicinal plant, was evaluated for pathogenicity (Koch, 1881). Fifty days old healthy medicinal plant *G. sylvestre* grown in the glass house at the Centre for Advanced Studies in Botany was inoculated with an aqueous suspension of the bacterial isolate (0.5×10^6 cfu/mL, 20mL/20 plants) using hand atomizer (Sonnenwirth & Jarett, 1980; Hindler *et al*., 1990)). Uninoculated plants served as control.

Plants were then covered with polypropylene bags (with scattered holes) to maintain high humidity. Daytime temperatures were 30-35°C with peaks to 37°C in the late afternoon. Night temperatures were 20-22°C. The plants were watered every alternate day for a period of another 10 days (Klement, 1963; Goto, 1992; Boucher *et al*., 2001). The plants remained in the glasshouse for 2 weeks. The bacterial isolate causing the leaf spot symptom was reisolated from the infected leaves and identified based on its morphological and biochemical tests. Representative isolate was preserved at -20°C, or lyophilized, and submitted in the culture collection at Center for Advanced Studies in Botany, University of Madras.

Antibacterial assay

Hundred micrograms of seaweed extracts were mixed with 5% Dimethyl Sulfoxide (DMSO) and loaded on to sterile disc (Himedia) and placed on assay plates containing Muller Hinton Agar spread with over night grown bacterial pathogens (Disc diffusion method - Bauer *et al*., 1966). Inhibition zones around the discs were measured after 24h. Discs loaded with 5% DMSO served as negative control and the antibiotics tetracycline used as positive control. Triplicates were maintained and the mean values are presented.

MIC and MBC

A broth microdilution method was used to determine the Minimum Inhibitory Concentration and Minimum Bactericidal Concentration (Mazzanti *et al*., 2000; Devienne *et al*., 2002; NCCLS, 2003). All tests were performed in Mueller Hinton Agar (Himedia). Serial doubling dilutions were prepared with a solution of maximum active seagrass extract: Dimethylsulfoxide 95:5 in a 96-well microtiter plate over the range of 7-3125µL/L. Overnight broth cultures of the bacterial strain were prepared and the final concentration of the microbe in each well was adjusted to 2×10^3 cfu/ml. Plates were incubated at 37°C for 24h. The MIC was determined as the absorbance of each well using an automatic Elisa tray reader adjusted at 630nm (SLT Spectra). The samples were analyzed in duplicate and the assay was repeated thrice. The antibiotic tetracycline was employed as positive

Table 1. Activity of different seaweed extracts against Pseudomonas syringae

Seaweeds	Zone of inhibition* (mm in diameter)							
	PE	EA	C	A	M	W	NC	PC
Gracilaria corticata	0.00 ± 0.00	0.00 ± 0.00	9.00 ± 0.58	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	12.67 ± 0.33
G. verrucosa	0.00 ± 0.00	8.33 ± 0.33	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00			
Grateloupia lithophila	10.33 ± 0.67	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	9.00 ± 0.58			
Laurencia obtusa	0.00 ± 0.00	8.00 ± 0.58	0.00 ± 0.00	0.00 ± 0.00	12.33 ± 0.33			
Dictyota dichotoma	9.67 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	9.00 ± 0.58	10.33 ± 0.67			
Padina boergesenii	0.00 ± 0.00	11.00 ± 0.58	10.67 ± 0.67	0.00 ± 0.00	9.67 ± 0.67			
Sargassum wightii	0.00 ± 0.00	18.67 ± 0.33	8.00 ± 0.58	7.33 ± 0.33	21.00 ± 0.58			
Turbinaria conoides	8.00 ± 0.58	14.00 ± 0.58	7.00 ± 0.58	0.00 ± 0.00	10.67 ± 0.33			
Chaetomorpha antennina	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00			
Enteromorpha flexuosa	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00			
Halimeda tuna	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	8.00 ± 0.58			
Ulva lactuca	0.00 ± 0.00	10.00 ± 0.58	0.00 ± 0.00	0.00 ± 0.00	12.67 ± 0.67			

***PE**- petroleum ether, **E**- ethyl acetate, **C**- chloroform, **A**- acetone, **M**- methanol, **W**- water, **NC**- negative control (5% DMSO), **PC**- positive control (tetracycline); *100µg per disc ; Mean ± Standard error; Standard deviation P≤ 0.05.*

control. The wells showing complete absence of growth were identified and 10µL of each well were transferred to Mueller Hinton agar plates and incubated at previously mentioned times and temperatures. The concentration where complete absence of growth was observed was considered as the minimum bactericidal concentration.

Statistical analysis

The data were statistically analysed by applying One-Sample T test.

Results

Based on the pathogenicity test, the isolate proved to be the causative agent for leaf spot disease of *Gymnema sylvestre* and the same was identified as *Pseudomonas syringae* based on the morphological and biochemical chacrecteristics.

The results of the antimicrobial screening assays are summarized in Table 1. As evidenced, the methanolic extracts showed significant inhibitory effects followed by ethyl acetate extracts. Maximum activities were recorded in methanolic (21.00 ± 0.58) and ethyl acetate extracts (18.67 ± 0.33) of the brown seaweed *Sargassum wightii* , followed by ethyl acetate extract of *Turbinaria conoides* (14.00 ± 0.58) when compared to commercial antibiotic tetracycline. No activity was recorded in the green seaweeds *Chaetomorpha antennina* and *Enteromorpha flexuosa*.

Among the three groups of seaweeds, maximum activities were recorded in brown seaweeds and minimum activity was recorded in green seaweeds. Methanolic extracts suitably inhibited the *Pseudomonas syringae* followed by ethyl acetate extracts. While, minimum inhibition was recorded in extractions with petroleum ether and acetone. The negative control (5% DMSO) and water extract did not inhibit growth of the pathogen.

Table 2 shows that the MIC and MBC of maximum active seaweed extracts and Tetracycline. Lowest MIC value was recorded in ethyl acetate and methanolic extracts of *Sargassum wightii* followed by ethyl acetate extract of *Turbinaria conoides* when compared to the other extracts and antibiotic Tetracycline.

To conclude, the most effective seaweed were *Sargassum wightii* and *Turbinaria conoides*.

Table 2. MIC and MBC of effective seaweed extracts and antibiotic against Pseudomonas syringae

Extracts	MIC (µg/ml)*	MBC (µg/ml)*
Ethyl acetate extract extract of *Sargassum wightii*	50	25
Methanolic extract of *Sargassum wightii*	50	25
Ethyl acetate extract extract of *Turbinaria conoides*	75	35
Methanolic extract of *Ulva lactuca*	100	50
Tetracycline	100	50

Values are the mean of triplicates using 1 ×10^3 cells of each culture

Discussion

The marine environment has great potential for the discovery of lead compounds that could be used against infectious diseases and parasites (Sultana *et al*., 2005). Substantial investigations revealed that the antibacterial activity was high in Phaeophyta followed by Rhodophyta and Chlorophyta. In the present investigation too, the highest activity observed was in the above order. Pesando & Caram (1984) reported maximum activity in Phaeophyceae followed by Rhodophyceae. In the present investigation 70% of activity was recorded in brown seaweed, 30% in red seaweed and only 15% in green seaweeds.

Sargassum wightii has been an excellent source of antibacterial principles in controlling the bacterial blight of rice caused by *Xanthomonas oryzae* pv. *oryzae* (Arun Kumar & Rengasamy, 2000a, b; Arun Kumar *et al*., 2001). Rao *et al*. (1991) reported the activities of *Padina gymnospora* and *Sargassum wightii* against gram positive and gram negative bacteria. The observed activities of Dictyotalean and Fucalean members are also in accordance with the earlier observations (Caccamese & Azzolina, 1979; Stiger *et al*., 2004). Such records are in agreement with maximum activity recorded in the present observation with *Sargassum wightii* followed by *Turbinaria conoides* and *Padina boergesenii*.

Kulik (1995) indicated the significance of evaluating algae for use in the biological control of plant pathogenic bacteria and fungi. The present study highlights the utilization extracts of seaweed collected from the Coast of Tamil Nadu to control the bacterial leaf disease of important medicinal plant *Gymnema sylvestre*.

In general, gram positive bacteria are more susceptible than gram negative bacteria to algal extracts (Sreenivasa Rao & Parekh, 1981; Pesando & Caram, 1984; Reichelt & Borowitzta, 1984). Lourdu Mariadoss (1998) reported the activity of methanolic extract of *Sargassum wightii* and chloroform: methanol extract of *Enteromorpha flexuosa* against a gram negative bacterium, *Xanthomonas oryzae* pv. *oryzae*, the casual organism of leaf blight of rice. In the present investigation the brown seaweed extracts *Sargassum wightii* and *Turbinaria conoides* proved to inhibit the gram negative bacteria *Pseudomonas syringae* causing leaf spot disease of the medicinal plant *Gymnema sylvestre*.

The MIC and MBC values of the extracts are much higher than those of the positive control substance tetracycline. This is not surprising because extracts are

complex mixtures of many compounds and the portion of active compounds is low.

Seaweeds collected from the Tamil Nadu coast of India have been shown to possess a number of biological activities against the pathogen isolated from the medicinal plant *Gymnema sylvestre*. In our studies, the most interesting species are that of *Sargassum wightii* and *Turbinaria conoides*. To the best of our knowledge, this is the first report demonstrating the antimicrobial activity of the seaweed extracts against the pathogen isolated from an important medicinal plant *Gymnema sylvestre*.

These biologically active seaweed extracts are currently undergoing detailed investigations with the objective of isolating biologically active lead molecules. Furthermore, the encouraging biological activities seen in this study show that the Indian coastline is a potential source of variety of seaweeds worthy of further investigation and conservation from pollution threat.

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