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Antimicrobial Activity of Most Abundant Marine Macroalgae of the Caribbean Coast of Costa Rica

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Abstract

Marine ecosystems have a very large diversity of resources, most of them still partially unknown, and a few others exploited for development of new industrial and toxicological products. Seaweeds are directly exposed and are susceptible to ambient microorganisms such as bacteria, fungi and viruses. In this study, ethanolic and acetonic extracts of 13 marine algae belong to Rhodophyceae (Galaxaura marginata, Gelidiella acerosa, Corallina officinalis, Gracilaria cervicornis, Bryothamnion triquetrum, Laurencia obtusa, Laurencia papillosa), Phaeophyceae (Dictyota mertensii, Padina gymnospora, Sargassum polyceratium) and Chlorophyceae (Codium isthmocladum, Udotea flabellum, Caulerpa racemosa) from the Caribbean Coast of Costa Rica were tested in vitro for their antibacterial and antifungal activities against different types of bacteria: one Gram positive (Staphylococcus aureus) and two Gram negative (Erwinia carotovora and Escherichia coli); and one fungus (Geotrichum candidum) using disc diffusion method. Acetonic extracts of S. polyceratium showed inhibition against all microorganisms tested. The highest inhibition activity among all the acetonic extracts was shown for S. polyceratium against S. aureus. Furthermore, the acetonic extract from S. polyceratium has shown broader activity spectrum against the entire tested organism. On the other hand, the ethanolic extract of S. polyceratium, C. racemosa and G. cervicornis were active against S. aureus, however, no significant response was found against G. candidum, E. carotovora and E. coli, when the ethanolic extracts of other species were applied. Active metabolite of S. polyceratium extract was isolated by High Performance Liquid Chromatography (HPLC) and the molecular structure was preliminarily characterized through Gas Chromatography Mass Spectrometry (GC-MS), Infrared (IR), and Ultraviolet (UV) spectroscopy.

Key Words: Antimicrobial activity, Marine algae, Biological activity, Bioactive extracts
JEL Code: N56

Introduction

Seaweeds serve as an important source of bioactive natural products. They have been used as food stuff in the Asian diet for centuries as they contain carotenoids, dietary fibres, proteins, essential fatty acids, vitamins and minerals (González and Silva, 2001). Many substances obtained from marine algae such as alginate, carrragenean and agar as phycocolliods have been used for decades in medicine and pharmacy (AL-Zereini, 2006). Since algae have been used in traditional medicine for a long time (Fitton, 2006), and also some algal extracts have bacteriostatic and bactericidal activity, they have been extensively studied by several researchers (Mautner et al., 1953; Kobayashi and Kitagawa, 1994; el-Masry et al., 1995; Gonzalez del Val et al., 2001; Nora et al., 2003; Ghosh et al., 2004; Freile-Pelegrin

and Morales, 2004; Salvador et al., 2007; Murti and Agrawal, 2010). The nutrient composition of seaweed is variable and depends by the species, geographic areas, season of the year and temperature of the water. Recently, seaweeds have received significant attention for their potential as natural antioxidants and most of their compounds have shown anti-bacterial activities (Vairappan et al., 2001; Vlachos et al., 1999). The demonstrated antimicrobial activity was considered as an indicator of the capacity of the seaweeds to synthesize bioactive secondary metabolites (Davies-Coleman and Beukes, 2004). Their biostimulant properties are explored for their use in agriculture and the antimicrobial activities for the development of novel drugs, such as antibiotics, laxatives, anticoagulants, anti-ulcer products and suspending agents in radiological preparations (González and Silva, 2001; Aneiros and Garateix, 2004; Murti and Agrawal, 2010).

Also, the wide range of properties of seaweeds represents a great potential for industrial application. For example, the extract or infusion of the active compound of the seaweed, which has been identified with a specific biological activity, can be used either alone or directly as a foundation in the synthesis of organic compounds. It can also be used within the structure of its active sites, in order to develop new substances with predetermined properties (González and Silva, 2001). In addition, chemical research related to the isolation, biosynthesis and structural elucidation of new natural compounds has contributed to pharmaceutical and agricultural progress, for example, the treatment of diseases, pests and the development of new chemicals (AL-Zereini, 2006).

The present study was undertaken in order to examine the antibacterial effects of 13 marine algae belonging to Rhodophyceae (Galaxaura marginata, Gelidiella acerosa, Corallina officinalis, Gracilaria cervicornis, Bryothamnion triquetrum, Laurencia obtusa, Laurencia papillosa), Phaeophyceae (Dictyota mertensii, Padina gymnospora, Sargassum polyceratium) and Chlorophyceae (Codium isthmocladum, Udotea flabellum, Caulerpa racemosa) from the Caribbean Coast of Costa Rica against Gram positive bacteria (Staphylococcus aureus) and Gram negative bacteria (Erwinia carotovora and Escherichia coli) and one fungus (Geotrichum candidum).

Materials and Methods

Algal Materials

The most representative algae were selected based on the latest report from Soto and Ballantine (1986), which reported these species as the most abundant in the southern Caribbean coast of Costa Rica. Seaweeds were collected by hand picking using Scuba diving and snorkeling (1 – 5 m depth) from the Wildlife Refuge Gandoca-Manzanillo and Puerto Viejo, Limón. The zones were investigated from December 2003 to March 2005. Samples were frozen for transport to prevent decomposition, loss of concentration and their normal activity, as recommended by Caccamese et al. (1980) and Rao and Parekh (1981). The taxonomic identification of species was done by experts in these fields, using standard literature and Taylor´s taxonomic key (1979). Voucher specimens of all species tested were deposited in the herbarium of Laboratory of Algology, Department of Marine Biology, Faculty of Natural Sciences at Universidad Nacional, Costa Rica. In field, the algae were cleaned of epiphytes and washed thoroughly in sterile sea water. Then seaweeds were dried for 24 h under an artificial light at 24 ºC and finally in a heater. The dry seaweeds were crushed in an electric mill until a fine powder was obtained (Pesando and Caram, 1984). Every sample was preserved in a freezer until compound extraction.

Chemical extraction

The powder (5 g) of dried algae was extracted in Soxhlet system using ethanol and acetone (200 mL) as solvents (8 h at 80 ºC with ethanol and 8 h at 50 ºC with acetone). The material was filtered using a Büchner funnel and a Whatman filter paper # 1. The remaining extract was centrifuged at 3400 rpm (1000xg) for 15 minutes to remove any solid residue not retained on the filter. The supernatant was concentrated under reduced pressure in a Büchi R-110 model rotary evaporator, with a bath temperature of 95 °F (35 °C) to obtain an extract with viscous consistency. The crystals

obtained were suspended in 2 mL of extraction solvent (Rao and Parekh, 1981).

Biological assays

Antimicrobial activity test was performed by agar plate diffusion method (Burkholder et al., 1960). A total of 15 mL of Mueller – Hinton agar (pH 7.4 ± 0.2 a 25 °C) was added and inoculated with 0.1 mL of bacteria strains, while 15 mL of Potato Dextrose Agar (PDA) were used to inoculate the G. candidum fungus (Fungi) (Rojas et al., 2006). Sterile filter paper was used to construct the sensitivity disks after which them were autoclaved at 161ºC for 1 hour to make them completely sterile, according to Omoruyi and Idemudia (2011).

Sterile discs (BBLTM) of 6 mm were prepared placing 25 µL of algal extract and were introduced into the agar dish for incubation at 37 °C during 48 h in a sterile room (Ballantine et al., 1987). Inhibition results are expressed as width of the clear halo surrounding each disc on cultivated agar dishes (De Lara-Isassi et al., 1996). Ethanol and Acetone solvent (100 %) without algal extract were also used as a negative control. All experiments were performed at least in triplicate. A diameter equal to or greater than 10 mm, in the inhibition halos was defined as the representativeness criteria. Based on the results obtained in the bioassay, the selected extract for purification was the one that showed the highest inhibition zone. The strains of S. aureus, E. carotovora, E. coli and fungus G. candidum were provided by the Laboratory of Microbiology, Department of Biological Sciences, University of Costa Rica.

Isolation, purification and characterization of the metabolite

The extract that showed the most important inhibition was chosen for the isolation and preliminary characterization of the metabolite responsible for this activity. The extract was absorbed onto SiO_2 and purified by flash column chromatography (SiO_2), eluting with gradient solvent system hexane:ethyl acetate to afford eight fractions. Elutions collected from column chromatography were concentrated and screened for antimicrobial activity against Gram-positive bacteria (S. aureus), Gram-negative bacteria (E. carotovora and E. coli) and fungus (G. candidum). One active fraction exhibiting a good antimicrobial potential was selected for a further purification by flash column chromatography (SiO_2), elution with gradient solvent system hexane:THF to obtain three fractions that were bioassay again. The metabolite of the most active fraction was purified with HPLC column [250-46 mm, 5 µm using hexane:ethyl acetate (7:3 v/v), scanned at UV 275 nm] and partially characterized on the basis of Fourier-Transform Infrared (FT-IR) and Gas Chromatography-Mass Spectral (GC-MS) data. Solvents used in the mobile phase were filtered through membrane filters of 0.45 µm in each analysis. Vanillin in H_2SO_4, $FeCl_3$ 1% and ammonia were used as TLC's developers.

Results and Discussion

Antibacterial activity test

Antibacterial activities of crude extracts of 13 marine algae from the Caribbean coast of Costa Rica were tested against bacteria by agar dish diffusion method. The results of screening test against S. aureus and G. candidum are summarized in Table 1. Acetonic crude extracts of seven tested algae (three Rhodophyceae, three Phaeophyceae and one Chlorophyceae) showed inhibition against S. aureus and the extract of S. polyceratium was the most active with an inhibition diameter of 19.00 ± 0.25 mm. Three ethanolic extracts also showed a positive activity against S. aureus. Acetonic extracts of two Rhodophyceae, one Phaeophyceae and one Chlorophyceae were positive against G. candidum. S. polyceratium was the most effective in this case with an inhibition diameter of 12.00 ± 0.25 mm. No positive activity was observed in ethanolic extracts against G. candidum.

Table 2 summarizes the results of the screening tests against E. carotovora and E. coli. Acetonic extracts of three species of seaweeds (one Rhodophyceae and two Phaeophyceae) showed inhibition against E. carotovora. In the other hand, six species of marine algae (three Rhodophyceae, two Phaeophyceae and one Chlorophyceae) showed positive activity against E. coli. In both cases, S. polyceratium showed the highest positive activity with an inhibition diameter of 7.00 ± 0.25 mm against E. carotovora and 9.00 ± 0.25 mm against E.

coli. No activity was observed in ethanolic extracts against Gram-negative bacteria. According Rojas et al. (2006), the inhibition values obtained for S. polyceratium acetonic extract against S. aureus show a high activity, since the inhibition diameters ranged from 16 to 20 mm, while the antimicrobial activity of the same specie against G. candidum, E. carotovora and E. coli is considered moderate because the inhibition diameters ranged from 5 and 15 mm. S. aureus was more sensitive than all the stocks, with the most important inhibition diameter and G. candidum, E. carotovora and E. coli were more resistant against all ethanolic extracts of the seaweeds analyzed. In fact it was reported that the Gram-positive bacterial strains were more susceptible to seaweeds extract than Gram-negative bacterial strains (Padmini et al., 1988; Campos-Takaki et al., 1988; Pesando and Caram, 1984).

The results of the inhibitory effect were greater in the acetone extract than the ethanol extract for all organisms. This indicates that the metabolites responsible for such effect are soluble in this solvent, supporting the results from Rao and Parekh (1981); Ballantine et al. (1987); Vidyavathi and Sridhar (1991); and De Lara-Isassi et al. (1996), who indicate the bioassay conducted with marine algae using acetone as solvent extraction showed a better response as antibiotic in general than those performed with ethanol or any other solvent with higher polarity. This may indicate that the components responsible of inhibitory activity are generally phenol groups, fatty acids and unsaponifiable lipids (Rao and Parekh, 1981).

Non polar solvents usually provide a higher efficiency for the extraction of compounds with antimicrobial activities compared to water methods. It may be some differences between results in difference studies due to several factors. This can be because of the intraspecific variability in the production of secondary metabolites, occasionally related to seasonal variations. Also, there may be differences in the capability of the extraction protocols to recover the active metabolites and differences in the assays methods. This is an inevitable fact for all biochemical research because test materials have trace impurities (Inci et al., 2006) The results revealed slightly less antibacterial activity in individual fraction afford through column chromatography from the crude acetone extract of S. polyceratium compared with the values obtained for crude extract itself S. aureus. This observation is possibly due to the synergic action of secondary metabolites in the crude extract. Future studies should calculate the lowest concentration of each compound (quantitative analysis) that would inhibit the visible growth of the microorganism. This aspect is very important to confirm resistance of microorganisms to an antimicrobial agent and also to monitor the activity of new antimicrobial agents.

Purification and isolation

Based on the results of the antibacterial activity, the purification process of S. polyceratium extract was performed using flash column chromatography, while the isolation and purification of metabolite was conducted using HPLC. Identification of purity was carried out at wavelengths of 275 nm using a diode array detector. HPLC results showed a single peak at a retention time (R_t) of 3.502 min in the chromatogram (Figure 1), indicating the presence of a single active compound responsible for biological activity.

The UV-Visible spectrum of the purified compound showed two maximum absorption bands, approximately 275 nm and 448 nm, typical shifts of compounds with aromatic rings attached to oxygen because the conjugation system allows carrying out transitions such as $\pi \rightarrow \pi^*$ and $n \rightarrow \pi^*$ and a bathochromic shift of the bands occurs (Silverstein et al., 2004). FT-IR spectrum of isolated compound of S. polyceratium (Figure 2) shows a significant band in the 3500 – 2000 cm^{-1} region, indicating the stretching vibration of hydroxyl groups. The lengthening of the double bonds of the aromatic ring is represented by signals in the 1651 cm^{-1} and 1574 cm^{-1} region.

The small band at approximately 3000 cm^{-1} occurs due to the stretching of the CH bond of the sp^2 hybridized carbons belonging to the aromatic ring (Pavia et al., 2009). GC-MS spectrum (Figure 3) shows a molecular ion peak (M^+) at 220 m/z and a base peak at 205 m/z, while the isotopic ratios of x+1 and x+2 provide information to suggest the experimental

molecular formula $C_{15}H_{24}O$. Also, difference of 15 m/z between molecular ion and base peak indicate the fragmentation and presence of methyl group ($-CH_3$), while, the peak at 57 m/z suggests the presence of terc – butyl group (Pavia et al., 2009). Spectroscopic evidence suggests that compound isolated from the seaweed S. polyceratium has the chemical basis of phenolic metabolite or derivatives, which have been reported in literature as compounds based on structures with cytotoxic activity (Rao and Parekh, 1981; Carballo, 2006;

Murti and Agrawal, 2010). This would explain the increased biological activity of the acetone extracts against ethanol extracts. Difference in the inhibitory response is due to the presence of alkyl substituent in the chemical structure of the metabolite, increasing solubility in acetone solvent. Rao and Parekh (1981) reported that the phenolic compounds have been the most common metabolic components with inhibitory activity in marine organisms. Phenolic metabolites have been associated with some properties in organisms, playing a defensive role (Rao and Parekh, 1981). This suggests a possible role of the compound found with the reported antimicrobial activity.

Summary and Concluding Remarks

These results of the antibacterial and spectroscopic analysis of S. polyceratium extracts showed that the samples contained some bioactive substances. These kind of organisms are potential substitutes for drugs being used today such as, ampicillin, which has been known to be evaded by resistant bacteria.

The knowledge that these kinds of seaweeds and its metabolites exhibit vital antimicrobial properties offers a lot to the research world in the search for more drugs that are easily accessible, widely affordable, and highly effective. It also justifies the medicinal uses and claims about the therapeutic values and we therefore, suggest further purification and characterization of the secondary metabolites that would be obtained expecting to find a useful chemotherapeutic agent. This study has shown that the production of antibacterial substances by macroalgae is a regular occurrence among those found on the Caribbean coast of Costa Rica.

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Illustrations

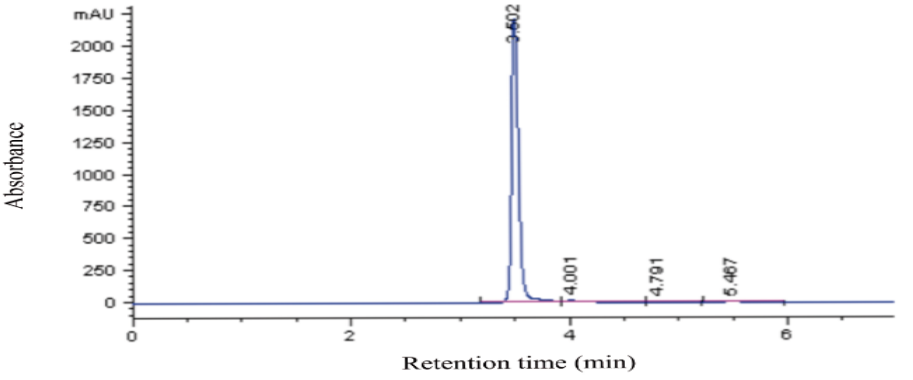

Figure 1. HPLC Chromatogram of acetone fraction of seaweed *S. polyceratium* at 275 nm.

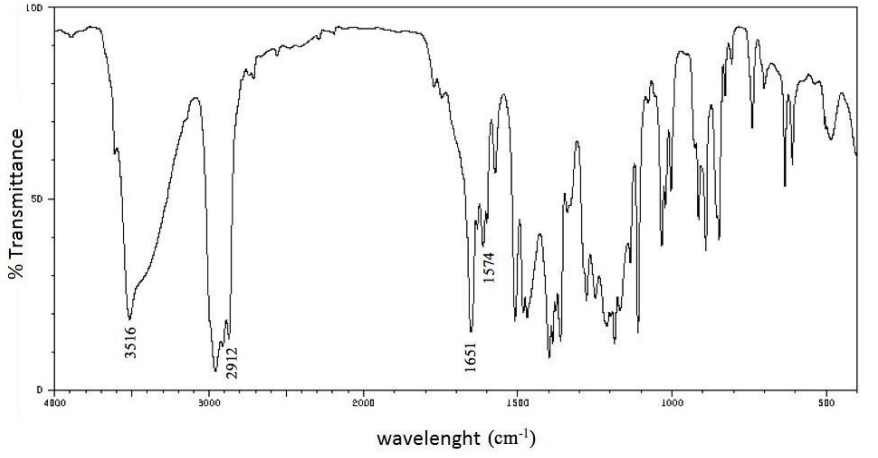

Figure 2. FT-IR spectrum of isolated metabolite of the seaweed S. polyceratium.

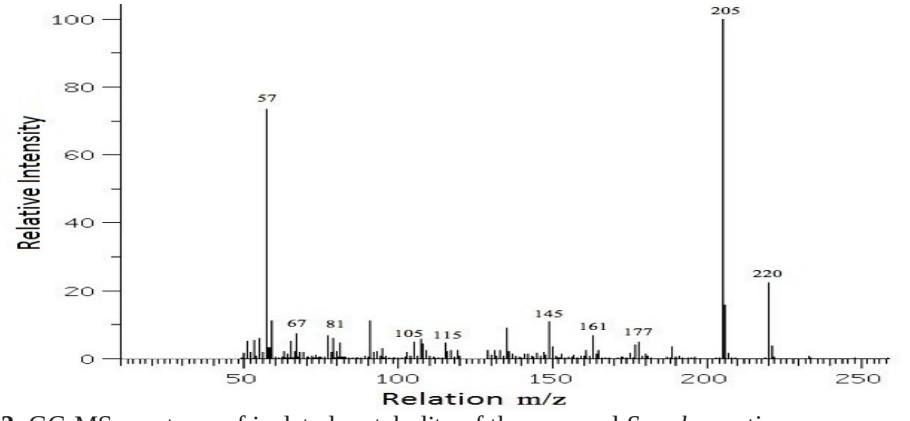

Figure 3. GC-MS spectrum of isolated metabolite of the seaweed *S. polyceratium*.

Tables and Supplementary Materials

Table 1. Antibacterial activity of seaweed species against *S. aureus* and *G. candidum*

	Gram +		**Fungus**	
	S. aureus		**G. candidum**	
Algae	**Ethanolic extract (±0.25 mm)**	**Acetonic extract (±0.25 mm)**	**Ethanolic extract (±0.25 mm)**	**Acetonic extract (±0.25 mm)**
Rhodophyceae				
Bryothamnion triquetrum	-	3.50	-	-
Corallina officinalis	-	-	-	-
Galaxaura marginata	-	-	-	-
Gelidiella acerosa	-	8.00	-	2.00
Gracilaria cervicornis	1.50	7.00	-	2.00
Laurencia obtusa	-	-	-	-
Laurencia papillosa	-	-	-	-
Phaeophyceae				
Dictyota mertensii	-	7.00	-	-
Padina gymnospora	-	2.50	-	-
Sargassum polyceratium	3.00	19.00	-	12.50
Chlorophyceae				
Caulerpa racemosa	2.00	8.50	-	2.00
Codium isthmocladum	-	-	-	-
Udotea flabellum	-	-	-	-

Table 2. Antibacterial activity of seaweed species against E. carotovora and E. coli

	Gram -			
	E. carotovora		**E. coli**	
Algae	**Ethanolic extract (±0.25 mm)**	**Acetonic extract (±0.25 mm)**	**Ethanolic extract (±0.25 mm)**	**Acetonic extract (±0.25 mm)**
Rhodophyceae				
Bryothamnion triquetrum	-	-	-	-
Corallina officinalis	-	-	-	-
Galaxaura marginata	-	-	-	4.50
Gelidiella acerosa	-	3.50	-	8.00
Gracilaria cervicornis	-	-	-	7.00
Laurencia obtusa	-	-	-	-
Laurencia papillosa	-	-	-	-
Phaeophyceae				
Dictyota mertensii	-	-	-	7.00
Padina gymnospora	-	1.50	-	-
Sargassum polyceratium	-	7.00	-	9.00
Chlorophyceae				
Caulerpa racemosa	-	-	-	8.50
Codium isthmocladum	-	-	-	-
Udotea flabellum	-	-	-	-