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Antimicrobial activity of various extracts of *Sargassum* glaucescens on the antibiotic resistant organisms

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Abstract

The antimicrobial activity of brown alga methanol, ethyl acetate, hexane, and chloroform extracts on gram positive and gram negative bacteria, and fungi was evaluated by using nutrient broth macro dilution test. Sargassum glaucescense was collected around the coastal area of Chabahar (south of Iran) the protected marine area of the Oman Sea in April and May 2015. Six pathogenic organisms including; Enterococcus faecium ATCC 51299, Streptococcus mutans ATCC 35668, Shigella boydii ATCC 25923, Pseudomonas aeruginosa ATCC 27853, Klebsiella pneumoniae ATCC 13883, Salmonella enteritidi PTCC, 1709, Candida albicans ATCC 10231 and Aspergillus fumigatus PTCC 5009 were investigated by the broth dilution method. Methanolic extract of six strains showed good activity amongst eight strains. Hexane extract, after methanolic extract has a good effect on the antimicrobial activity against five strains. All bacterial strains in this survey showed resistance against ethyl acetate and chloroformic extracts. S. glaucescens using four various solutions extracts against eight different human pathogens showed an important antimicrobial and antifungal activity. However, more investigation has to be done on separation, purification and detection of the active ingredients in order to recognize their antifungal and antifungal activity.

Keywords: Antimicrobial activity, Sargassum glaucescens, Bacteria, Fungi, Oman Sea.

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Introduction

During last decade. drug therapeutic failure happened due to the inappropriate and extensive use of therapeutic agents (Franceschi et al.. 2004). In some areas. uninformed use of antibiotics and food additives in livestock animals. poultry and household cleaners caused spontaneous mutations and selection pressure in organisms that led to the creation of resistant isolates (Amin et al., 2012; Fallah et al., 2013; Samanta et al., 2014). Antibiotic resistance can occur both environmentally. genetically and resistance spreads both Genetic "vertically," when resistance elements inherited, and are "horizontally." when genetic material transferred other to bacteria (Wright etal., 2007). antibiotic Environmentally, when resistance spread occurs microorganisms transferred are from place to place by airplanes, water and wind. Centers for Disease Control and Prevention (CDC) has assessed that at least two million illnesses and 23,000 deaths occur antibiotic resistant organisms yearly in the USA (Heuer et al., 2011; Blair et al., 2015). There is a continuous and crucial need discover new antimicrobial varied compounds with novel mechanisms of action and chemical structures because there has been an alarming increase in the occurrence of new and re-emerging infectious illnesses, as well as the increasing development of resistance to the

antibiotics in current clinical use (Bhagavathy et al., 2011: Moellering, 2011). Therefore, actions must be taken to control the use of antibiotics. to better comprehend the genetic mechanisms of resistance and to continue studies on developing new drugs (Bhagavathy et al., 2011: 2011: Sasidharan et al.. Savoia. 2012). The use of plant extracts and phytochemicals, both with antimicrobial effects, can be of significance in therapeutic great treatments and control of the infections caused by the multidrugresistance (MDR) strains (Ahmad and Beg, 2001; Nascimento et al., 2000). In the recent years, several studies have been directed different countries to demonstrate such efficiency (Nascimento et al., 2000; Aqil et al., 2006; Betoni et al., 2006; Ahmad and Aqil, 2007; Joshi et al., 2011). Many plants have been used because of their antimicrobial characters, which are due to compounds synthesized in the secondary metabolism of the plant (Aqil et al., 2006; Betoni et al., 2006; Ahmad and Agil, 2007; Joshi et al., 2011). These products are known by their active materials, for instance, the phenolic compounds which are part of the vital oils (Djeridane et al., 2006), as well as in tannin (Hoste et al., source 2006). Algae as a valuable biological diversity has a lot of applications such as food, photography, textile. paint, cosmetic, medical, pharmacy, dental and microbiological media preparation (Cannell, 1993). They can be categorized as three groups, rhodophyta (red algae), phaeophyta (brown algae) and chlorophyta algae) according (green to their chemical nutrient and structure (Davis et al.. 2003). In addition. valuable algae have polysaccharides such as agar, carrageenan and alginate that can used in economic industries (Pulz and Gross, 2004). A chemical study demonstrated compounds such as phenol, tannin, saponin, and steroid the flavin in algae (Kumar et al., 2015). structure Laminaria and Sargassum are two main types of the brown algae (Teas. 1892). Sargassum glaucescens (S. glaucescens) is one of the most important brown algae species from the Oman especially in the Port of Chabahar the Sistan and Baluchestan in Province of Iran (Esmaeili et al., 2015). S. glaucescens has high maximum growth in late autumnearly winter (May-Lin and Ching-Lee, 2013). There are many reports on the antibacterial activity of S. glaucescens extract against aquatic evidence bacteria. but little accessible for human pathogens (Ghaednia et al., 2011). The aim of the present study was to determine antibacterial and antifungal the activities of methanol, ethyl acetate, hexane, and chloroform extracts of S. glaucescens.

Materials and methods

Sampling algae and preparation of the plant extract

Brown algae, S. glaucescense was collected around the coastal area of Chabahar (in the south of Iran), the protected marine area of the Oman Sea in April and May 2015. All transferred samples were the laboratory and washed by distilled water in order to separate sand and epiphytic organisms. Then. algae was air-dried in the shade, at 25°C, and ground to powder with a mortar and pestle. One hundred and fifty grams of each sample were successively extracted by mixing with 800 ml methanol. ethyl acetate, hexane, and chloroform at room temperature. Each extract was the residue filtrated and reextracted. All filtrates were collected to be dried by evaporating under vacuum and re-dissolved in respective methanol, ethyl acetate, hexane, and chloroform.

Testing microorganisms

Antibacterial and antifungal activities of different algal extracts against six pathogenic bacteria (Enterococcus faecium ATCC 51299, Streptococcus mutans ATCC 3566. Shigella ATCC25923, boydii Pseudomonas ATCC27853, Klebsiella aeroginosa ATCC 13883 and pneumoniae enteritidis PTCC1709) Salmonella and two pathogenic fungi (Candida ATCC10231, albicans Aspergillus PTCC5009) were fumigatus investigated by the broth dilution method (Payghami *et al.*, 2014). All isolates were obtained from the department of microbiology Tehran University of medical sciences, (Tehran, IR Iran).

Broth macrodilution test bacterial strain

The MIC values of the different extracts were determined using the broth dilution test as defined by Borah *et al.* (2013). The initial concentration (50 mg ml⁻¹) of the different algae extracts was diluted using double fold serial dilution by transferring 2.5 ml of the sterile different algae extracts stock solutions into 2.5 ml of sterile Mueller Hinton broth (Merck Co., Germany) to obtain a 25 mg ml⁻¹ concentration. The above procedure was repeated several times to get other dilutions: 25 mg ml⁻¹ (1:2), 12.5 mg ml⁻¹ (1:4), 6.25 mg ml⁻¹ (1:8), 3.12 mg ml⁻¹ (1:16), 1.56 mg ml⁻¹ (1:32), 0.8 mg ml⁻¹ (1:64), 0.4 $mg ml^{-1} (1:128), 0.2 mg ml^{-1}$ (1:256), 0.1 mg ml⁻¹ (1:512), 0.05mg ml⁻¹ (1:1024) and finally 0.025 $mg ml^{-1} (1:2048)$. In order prepare different concentrations of extracts. each concentration inoculated with 0.1mlthe standardized bacterial cell (0.5 Mc Farland) of suspensions bacteria in separate sets of tubes and incubation was done at 370 °C for 24 h. The lowest concentration of the different algae extracts that inhibits growth of the organisms, as the lack of detected by visual turbidity, was designated as the

minimum inhibitory concentration (MIC). Two quality control tubes were maintained for each test batch that included an antimicrobial control (the growth medium inoculum without and tube containing extract) and organism control (the inoculum and the tube containing the growth medium). concentration The lowest of the extract that completely inhibited bacterial growth (no turbidity) in comparison to the positive growth control test was observed as MIC. Gentamycin (0.62-5 mg ml⁻¹) was used as drug quality control for microorganisms assay.

For minimum bactericidal concentration (MBC) assessment of different extracts, 0.1ml of each tube content was cultured on the Mueller-Hinton agar plates. After incubation at 370 °C for 24 h. colony count was completed and compared to the number of colony forming units CFU ml-1 in the inoculums. The original lowest concentration of extracts that allowed less than 0.1% of the original inoculums to survive (i.e., 99% killing of bacterial isolates) was determined by MBC.

Fungal strain

Candida albicans

The MIC values were assessed by visual broth macrodilution the Fungal method. suspensions were RPMI-1640 diluted into medium without bicarbonate (pH 7.0 with 0.165 morpholine propane sulfonic acid) broth supplemented with

glutamine, to a concentration approximately 0.5×105 CFU verified by colony count in SDA. A two fold serial dilution of 0.2 ml each of different algae extracts was added to 1.8 ml of the RPMI-1640 The concentrations were medium. 50-0.025 mg ml⁻¹. No antifungal samples were used in the Control group. To compare the results with standard, fluconazole (0.016 to 256 mg ml⁻¹) was used. Tubes were defined as the lowest concentration which did not yield visual growth and MFC were determined as the lowest concentration ofagent resulting in no growth.

Aspergillus fumigatus

different activity of algae extracts against A. fumigatus was determined by the broth macrodilution method (Arikan al., 2001). Dilutions of extracts (50, 25, 12.5, 6.25, 3.12, 1.56, 0.8, 0.4, $0.2, 0.1, 0.05 \text{ and } 0.025 \text{ mg ml}^{-1}$ prepared in **RPMI** 1640 were Medium in 2.5 ml volumes in test tubes. 2.5 ml A. fumigatus with turbidity of 2.5×103 CFU ml⁻¹ added to each test tube. After 48h incubation. MIC and MFC were determined. **MIC** values were determined as the lowest concentration of agent resulting in the maintenance or reduction of the inoculum and **MFC** were determined the lowest as concentration of agent resulting in no growth and then compared with the results of itraconazole (0.002 to 32 mg ml⁻¹) (Alizadeh *et al.*, 2014).

HPLC analysis

Methanolic and hexane extracts of S. glaucescens, due to high antimicrobial and cytotoxic effects, were respectively selected for HPLC analysis. These extracts of S. glaucescens were centrifuged at ×3000 rpm for 12 min and then filtered by Whatmann No.1 filter paper using high pressure vacuum pump. The specimen is diluted to 1:10 with the same solvents. HPLC method was done on a SHIMADZU LC-10AT VP HPLC system (Shimadzu, Kyoto, Japan), equipped with a model LC-10AT pump, UV-Vis detector SPD-10AT, Rheodyne injector fitted with a 20 µL loop and auto injector SIL-10AT. A Hypersil BDS C-18 column (4.6× 250 mm, 5 µm size) with a C-18 guard column was used. The elution was carried out with gradient solvent systems with a flow rate of 1 ml min⁻¹ at ambient temperature (25-28°C). The mobile phase consisted of 0.1% v/v methanol (solvent A) and water (solvent B). The mobile phase was prepared daily, filtered through a 0.45 µm and sonicated before use. Total running time was 15 min. The sample injection volume was 20 µL while the wavelength of the UV-Vis detector was set at 365 nm (Brkljaca and Urban, 2014).

Results

Four different extracts were evaluated against two gram-positive, four gram-negative and two fungi species. Some extracts had a significant activity for gram-positive bacteria but not on gram negative bacteria. Methanolic extract for six strains showed good activity

amongst eight strains. Hexane extract, after methanolic extract had good effect on antimicrobial activity against five strains. All bacterial strains in this survey showed resistance against ethyl acetate and chloroformic extracts. K. ATCC 13883 pneumoniae S. and enteritidis PTCC 1709 were also resistant to all extracts. Furthermore, only methanolic extract had antibacterial activity against Р. aeroginosa ATCC 27853. Methanolic extract showed an MIC of 1.56 mg ml⁻¹ for gram-positive bacteria, while for gram-negative bacteria it showed an MIC of 12.5 mg ml⁻¹ (Table 1). Two fungal *C. albicans* ATCC 10231 and *A. fumigatus* PTCC 5009 strains had a good response to all extracts, although *A. fumigatus* PTCC 5009 in comparison with *Candida albicans* ATCC 10231 had higher MIC and MFC for all extracts. Ethyl acetate extract had the lowest MIC (0.4 and 1.56 mg ml⁻¹) and MBC (0.8 and 3.12 mg ml⁻¹) for *C. albicans* ATCC10231 and *A. fumigatus* PTCC5009 (Table 2). Our findings showed that methanolic extract had a superior effect among four extracts. All strains indicated that they had an MIC range for the quality of control drugs.

Table 1: MIC and MBC (mg ml⁻¹) of various extracts of Sargassum glaucescens and antibiotics.

	Antimicrobials		Microbial isolation								
			Enterococcus faecium ATCC 51299	Streptococcus mutans ATCC 35668	Shigella boydii ATCC25923	Pseudomonas aeroginosa ATCC27853	Klebsiella pneumoniae ATCC 13883	Salmonella enteritidis PTCC1709			
Plant extracts	Chloroformic	MIC	-	-	-	-	-	-			
	Extract	MBC	-	-	-	-	-	-			
	Methanolic	MIC	1.56	1.56	12.5	12.5	-	-			
	Extract	MBC	3.12	3.12	25	25	-	-			
	Hexane Extract	MIC	6.25	6.25	-	-	_	-			
Ĕ		MBC	12.5	12.5	-	-	-	-			
<i>S</i> 2	Ethyl acetate	MIC	-	-	-	-	-	-			
	Extract	MBC	-	-	-	-	-	-			
₽	Piperacillin/	MIC	-	-	2.1	32	4	2			
Antibiotics	Tazobactam	MBC	-	-	3.12	68	8	4.1			
	Gentamycin	MIC	0.25	0.125	-	-	-	-			
CS.	·	MBC	2	2.15	-	-	-	-			

Table 2: MIC and MFC (mg ml⁻¹) of various Sargassum glaucescens extracts and antifungals.

	Plant extracts								Antifungals			
Fungal isolation	Chloroformic Extract		Methanolic Extract		Hexane Extract		Ethyl acetate Extract		Fluconazole		Itraconazole	
	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC
Candida albicans ATCC10231	12.5	25	6.25	12.5	6.25	12.5	0.4	0.8	0.64	0.5	-	-
Aspergillus fumigatus PTCC5009	25	50	12.5	25	12.5	25	1.56	3.12	-	-	0.32	0.64

qualitative **HPLC** fingerprint profile of Hexane extracts of S. selected glaucescens were a wavelength of 365 nm sharpness of the peaks and proper baseline. Hexane extract prepared by cold extraction was subjected to HPLC for the isolation and identification of constituents present in the *S. glaucescens*. Four

compounds were separated at different retention time viz., 6.636, 8.818, 9.167 and 11.267 respectively. The profile displayed one prominent peak at a retention of 8.818 min time and some moderate peaks were also observed at a retention time of 11.267 min, and 9.167 min respectively (Fig. 1). Methanolic of S. extracts glaucescens were illustrated with three compounds with the retention of 16.193, 9.535 and 6.791 time The min respectively. profile displayed one prominent peak at a retention time of 16.193 min (Fig. 2).

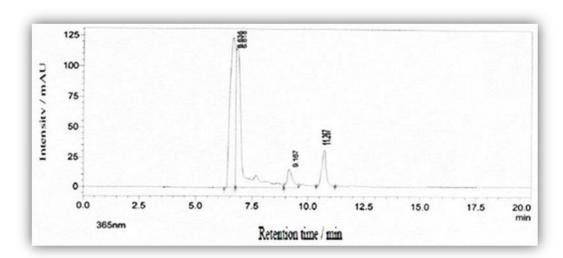


Figure 1: HPLC chromatogram of the Hexane extract of Sargassum glaucescens.

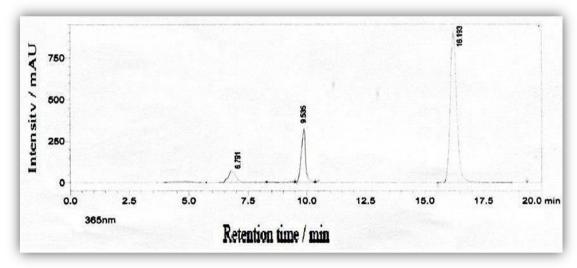


Figure 2: HPLC chromatogram of the Methanolic extract of Sargassum glaucescens.

Discussion

Sargassum species (phaeophyceae) are economically significant brown algae in Sistan O Baloochestan coastline, southern parts of Iran. Marine algae

produce a wide range of new secondary metabolites with numerous biological activities (Noormohammadi *et al.*, 2011). The previous study proved that the *Sargassum* species were the best

sources for components like polysaccharides, flavonoids, tannins. bromophenols, carotenoids and phenolic acids which display different biological activities (14-17). Nowadays, various chemically unique compounds sourced from Sargassum species with different biological activities have been identified and some of them are under examination and are being used to improve novel pharmaceuticals (García-Ríos et al., 2012; Michalak and Chojnacka, 2015). The different cell extracts and active components of several brown algae have been demonstrated to have an in vitro antibacterial (Ibtissam et al., 2009), antifungal (Moreau et al., 1988) and antiviral activity (Barbosa et al., 2004). We evaluated antibacterial and antifungal activities of four extracts of S. glaucescens against eight strains using macrodilution broth. Rare data existed from broth dilution antimicrobial effect of S. glaucescens pathogenic extract against microorganism. However, Turbinaria ornata and Sargassum wightii are two algae that have shown good brown activities against nine microbial pathogens such as Bacillus subtilis, Staphylococcus aureus, Enterococcus Klebsiella faecalis, pneumoniae, Pseudomonas aeruginosa, Escherichia Shigella flexneri, Aeromonas and hydrophila Proteus vulgaris (Vijayabaskar and Shiyamala, 2011). Out of eight strains, 6 strains were susceptible to methanol extract. Our results indicated gram negative bacteria in comparison with gram positive were more resistant to all extracts, because gram-positive bacteria have more their peptidoglycan in cell wall structure while gram-negative bacteria have only a thin layer of peptidoglycan and most of their cell structure is lipoprotein and lip-polysaccharides (Tassou and Nychas, 1995; Ghalem and Mohamed, 2008). Brown algae show different antimicrobial activities because these activities depend on their solubility and polarity in different solvents (Salama and Marraiki, 2010). The methanol extract of S. glaucescens exhibited the strongest antibacterial activity against microorganism. This property is due to the presence of phenolic, alkaloids and amino acids in methanolic S. glaucescens extract which may be responsible for the antimicrobial and antifungal activity (Cox et al., 2010; Srivastava et al., 2010). Mahianeh et al reported that Vibrio harveyi was resistant to extract n-hexane of S. glaucescens, Sub-critical to methanol and chloroform extract and sensitive to extract ethanol, but S. aureus was sensitive to extracts nhexane, chloroform, methanol and ethanol. Also B. cereus was sensitive to methanol and ethanol extract (Mahianeh et al., 2014). They indicated that ethanol extracts of S. glaucescens possess the highest antibacterial activity against all microorganisms. These results were consistent with our results. Plaza et al. (2008) reported that the methanol extract of a species of Sargassum has an antibacterial activity against both gram positive and negative bacteria (Plaza et al., 2008). Both Enterococcus faecium ATCC 51299 and Streptococcus mutans ATCC 35668 were found to be susceptible to the methanolic and Hexane extract of S. glaucescens at both the concentrations 1.56 mg ml⁻¹. MIC value of the methanolic extract for Shigella boydii ATCC25923 and Pseudomonas aeruginosa ATCC27853 was 12.5 mg ml⁻¹. According to table 2, the ethyl acetate extracts of S. glaucescens have high effect against two fungal strains with MIC 0.4 mg ml⁻¹ and 1.56 mg ml⁻¹. The two fungal strains were susceptible to all extracts of S. glaucescens, however Klebsiella pneumoniae ATCC 13883 and Salmonella enteritidis PTCC1709 were resistant to all solution extracts. The previous studies demonstrated that ethanol extract of seaweed species of S. lanceolatum, S. ilicifolium and S. tenerrimum has good effect against root infecting fungi (Ambreen et al., 2012). Bhaskar al.(2005)found antibacterial activity of brown algae of Padinatetra tomatica (46). Our results similar to Manilal et al. (2009) and Rangaiah et al. (2010) clarified that methanol extraction yielded higher antimicrobial and antifungal activity n-hexane and ethyl acetate (Manilal et al., 2009; Rangaiaha et al., 2010). Methanol extract polycystum similar to S. glaucescens showed more activity against bacterial and fungal strains (Kausalya and Narasimha, 2015). HPLC identification test is required to confirm the presence of the active components and molecular weights of the Methanolic Hexanolic extracts of S. glaucescens. In the present study and in line with Marimuthu et al. (2012) the HPLC profile for S. glaucescens exhibited novel markers in standardization as useful analytical tools to check not only the quality of the powder but also the presence of impurity in ayurvedic drugs such as medicinal plant extracts al., 2012). (Marimuthu etThe antibacterial and antifungal activity of the algae extract can be attributed to the various phytochemicals present in the S. glaucescens. Alkaloids, saponins and flavonoids component are found to be related with antimicrobial effects in different studies using algae extracts. Results of the present demonstrated that methanol extract of S. glaucesens can be used as an alternative to antibiotics in gentamycin and fluconazole which is now largely used in human pathogenic infections.

The results of the present study on *S. glaucescens* using four various solution extracts against eight different human pathogens showed an important antimicrobial and antifungal activity. However, more investigation has to be done on separation, purification and detection of the active ingredients in order to recognize their antifungal and antifungal activity.

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