Comparison of antibacterial activities of *Ircinia mutans* extracts in two different seasons from Kish Island, Persian Gulf, Iran

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Abstract

Sponges, which constitute the phylum Porifera, are the most primitive of the multicellular animals, among all marine organisms screened. Marine sponges produce the largest number of structurally diversified natural products. In this study we investigated in vitro antimicrobial activity of *Ircinia mutans* collected from the Kish Island in the Persian Gulf against strains of bacteria *Escherichia coli* (ATCC 15224), *Pseudomonas aeruginosa* (ATCC 25619), *Staphylococcus aureus* (ATCC 1764), and *Bacillus subtilis spizizenii* (ATCC 6633). Diethyl etter, methanol and aqueous extracts of sponge were evaluated by using the Bacterial Broth Dilution Method. The results showed that the aqueous extracts didn't have any antibacterial activity. Minimum Inhibitory Concentrations (MIC) of the winter diethyl etter extract was 2 mg/ml for *E.coli* and 20 mg/ml for *P. aeruginosa*, whereas the summer diethyl etter extract and both of methanol extracts did not show any activity. The MIC and MBC (Minimum Bacterial Concentration) of summer diethyl etter extracts were 2 mg/ml and 3mg/ml against *S.aureus*; and 5mg/ml and 10mg/ml when tested on *B. subtilis*. The MIC and MBC of winter diethyl etter extracts were measured as 1.5 mg/ml and 2mg/ml against *S. aurous*; and 5mg/ml and 10mg/ml when examined on *B.subtilis*. Summer and winter methanol and aqueous extracts of *I.mutans* did not show any activity against these bacteria. Therefore secondary metabolite solutions in diethyl etter contain components with antibacterial properties and can be used as antibiotics products.

Keywords: Sponge, Secondary metabolites, Antibacterial activity, Kish Island, Persian Gulf.

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Introduction

Sponges are the oldest and simplest multicellular animals (Barnes, 1987). In spite of being sessile**,** sponges play a consequential role in marine ecosystem (Bell and Barnes, 2000). Sponges are among the most abundant and diverse sessile animals in many hard substratum marine habitats, including coral reefs, rock walls, and caves (Hooper and van Soest, 2002). However, despite being sessile and having soft bodies, they are consumed by some marine animals, such as turtles (Meylan, 1988), sea urchins (Birenheide, *et al.,* 1993) and sea stars (Lippert *et al.,* 2004). While different parts of sponges such as fibers, skeleton, spicules, appear to have little or no defensive activity gainst predators, their secondary metabolites clearly play a defensive role against predation (Hooper, 2000; Faulkner *et al.,* 2000b). Since sponges contain a large variety of unique bioactive compounds, they have been the focus of many research programs for the application of their natural products. (Faulkner, 2002; Joseph and Sujatha, 2011). Among the phylum porifera (sponges) class Demospongiae is known to produce the largest number and diversity of secondary metabolites isolated from marine invertebrates (Newbald *et al.,* 1999).

Muller *et al.* (2004) have reported that more than 5000 different compounds have been isolated from about 500 species of sponges. Most bioactive compounds from sponges can be classified as antiinflammatory, antitumor, immunosuppressive or neurosuppressive, antiviral, antimalarial, antibiotic, or antifouling (Detmer *et al.,* 2005).

As the sponges are common members of the reefs and reef sediments are one of the main sinks for nutrients, absorbing the mucus and other reef metabolites, and hence providing a substrate rich in organic materials for the bacterial growth and activities (Richman *et al.,* 1975). Therefore these organisms are frequently exposed to intense predation and tissue infection by microorganisms (Faulkner *et al.,* 2000a). So this situation made them one of the important antibacterial activates of secondary metabolites. Variety of antimicrobial extracts and components have been isolated from various species of sponges (Zaro, 1982). Until the last decade more than 800 antibiotic compounds have been isolated from marine sponges that can affect the pathogens causing many human diseases (Urban *et al.*, 1999).

Persian Gulf bordering the south of Iran, is an extension of the Indian Ocean, is the host of many aquatic organisms especially in coral reefs and around its numerous islands. Kish Island with an area of 90 $km^{2,}$ and geographical coordinates of 32° 26′ 0″ N, 58° 53′ 0″ E, is one of the major tourist attraction spots in the Persian Gulf, with a diverse underwater marine life. There have been little studies on the sponges species and their secondary metabolites in the Persian Gulf (Nazemi *et al.,* 2010).

The aim of the present research was to isolate polar extracts (water and methanol solution) semi-polar and non-polar (diethyl etter solution) of *I.mutans* from Kish Island and investigate their antibacterial properties against gram positive and gram negative clinical pathogens.

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Because of the special climatic conditions there are only two distinct seasons in the Persian Gulf, and the secondary metabolites depended on the ecochemistry, therefore we exanimated the antibacterial activities in two seasons.

Materials and methods

Sampling and Identification

The samples were collected by scuba divers in July 2010 and February 2011 from the reef habitats at the depths of 20- 25 meters around Kish Island (Fig. 2). The geographical position of Kish Island is shown in Fig. 1. Samples were frozen (-8°C) as soon as possible and transferred to the laboratory (Science and Research Branch, Islamic Azad University). Taxonomic identification was done based on scanning optical microscope, skeletal slides and dissociated spicule mounts.

Figure 1: Kish Island. Geographical situation of sampling location.

Calcareous and Siliceous Spicules

Small fragments of tissue, both the surface and deeper parts of the sponge, were placed in Erlenmeyer flasks. A small quantity of active bleach (sodium hypochlorite) was added and after a short period, the organic components dissolved, leaving only the mineral skeleton. The bleach has been carefully diluted and tissues were eventually washed out for several times. Then the remaining skeleton was washed with water, followed with ethanol. Finally, a clean calcareous spicule suspension with ethanol was obtained which was allowed to evaporate and mounted (Hooper, 2000). For siliceous spicules fragments of sponges were placed in flasks, directly on glass slides. Several drops of nitric acid were placed on the fragments, gently heated over a flame until bubbling and repeated until all organic matter was digested. Then, mounting was immediately done (Hooper, 2000). These techniques, make the spicules to be useful for both light and scanning electron microscopy.

Tissue preparations

Immediately upon collection sponges were frozen for twenty four hours. This would fix the colour to a certain extent. Then they were defrosted and put in 5% concentration of buffered formaldehyde for 24 hours. After that they were put in ethanol for 5 hours and examined by light microscope (Hooper, 2000).

Extract preparation

The samples were kept in diethyl etter about 24 hours, which resulted in the production of the semi- polar and non-poplar extraction. Then the solution was

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filtered and diethyl etter was evaporated to dryness, at low pressure at 35- 40^{0}C by using Rota-vapor. Then the diethyl etter extract essence analysis was done by GC/MS, followed by identification of the components. Then the sample was put in methanol for 72 hours, the polar extraction was produced. The polar compounds in the phase of methanol- aqueous extracts were separated. The concentrated methanol extracts was then dried to obtain crude semi-solid extracts. The crude extract was then weighted and percentages of extraction from sponge were calculated and then added to DMS. After 72 hours methanol was evaporated to dryness, at low pressure at 40-45^{0}C by using Rota-vapor to obtain dried crude semi-solid extract (Bligh and Dyer, 1959).

Antibacterial assay

Antibacterial activity was determined against *E.coli* (ATCC 15244), *P.aeruginosa* (ATCC 25619), *S.aureus* (ATCC 1764), and *B.subtilis spizizenii* (ATCC 6633) using the Bacterial Broth Dilution Methods. To perform the classic broth dilution susceptibility test. Microorganisms 1.5×10^5 colony forming units[CFU]/ ml, a 1:100 dilution of a suspension of turbidity equal to a McFarland standard 0.5, was added to an equal volume (1ml) of each concentration (50mg/ml ,40mg/ml, 30mg/ml, 20mg/ml, 10mg/ml, 5mg/ml, 3mg/ml, 2mg/ml, 1.5 mg/ml, 0.75mg/ml, 0.5mg/ml, 0.10mg/ml, 0.05mg/ml and 0.01mg/ml) of diethyl etter, methanol and water extracts agent and to a tube of the growth control. An uninoculated tube of medium was incubated to serve as a negative growth control. After overnight, the tubes were examined for turbidity, indicating growth control of the microorganism. After the MIC has been determined, a known quantity, 0.1 ml, of inoculums from each of the tubes of broth that showed on visible turbidity after 22 to 24 hours' incubation is sub cultured to solid agar plates. The number of colonies that grow on the subculture after overnight incubation is then counted and compared to the number of CFU/ml in the original inoclum. Since even bacterial extracts do not always totally sterilize a bacterial population, the lowest concentrate of antimicrobial agent that allowed less than 0.1% of the original inoculums to survive is said to be the MBC (Rosenblatt, 1991).

Results

Sponge Identification:

Mineral skeleton composed of silica spicules and/or spongin fibres [Class Demospongiae]**.**

Lacking mineral spicules, although detritus and contaminating spicules may be acquired; sponges usually tough, difficult to tear, main skeleton a reticulation of spongin Fibres [Order Dictyoceratida].

Poecilosclerida with terminally microspined ectosomal megascleres and up to 5 categories of structural megascleres, most frequently monactinal. Microscleres are palmate chelae, diverse toxas, but sigmas never present [Suborder Microcionina], which is shown in Fig. 3.

Massive, lobate, spherical, digitate, encrusting growth forms, always with a conulose surface, except in forms with an organised superficial sand crust where conules may be reduced to mammiform

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protruberances; fibres making up anastomosing skeleton laminated in cross section with a central pith region, often obscured by large quantities of debris incorporated into fibres and interstitially; skeleton irregularly arranged; almost impossible to tear (Family Irciniidae Gray, 1867). Sponge surface marked by prominent conules, unarmoured; texture extremely tough, difficult to cut or tear; primary fibres are cored and with detritus and frequently attain a great size by being woven into complex fascicles contain no or few spicules but a well-developed spongin fibre system (*I.mutans* Wilson, 1925).

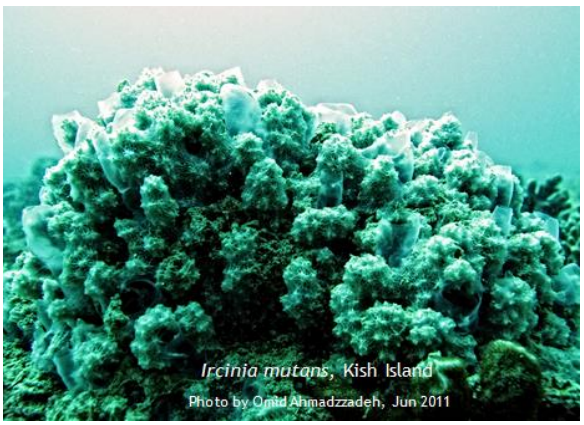

Figure 2: *I.mutans*, Kish Island, Persian Gulf.

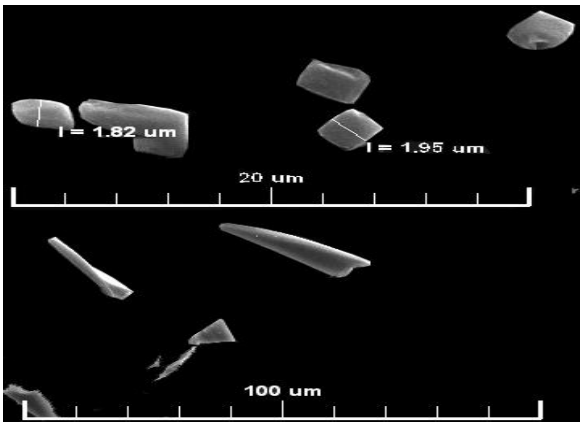

Figure 3: Calcareous spicules of *I. mutans* in scanning electronic microscope.

Identification and Isolation non- polar and semi- polar components from Ircinia mutans

Tridecane (Value 1.98, Quality 99%), Phenol 2,4-bis (1,1-dimethylethyl) (Value 1.45, Quality 99%), Eicosane (Value 10.49, Quality 98%), Ricinoleic acid (Value 20.75, Quality 99%), Bis (2- Ethylhexyl) phthalate (Value 1.41, Quality 97%), Digitoxin (Value 40.78, Quality 99%) were identified in GC/MS in winter diethyl etter extraction (Figs 4 and 6).

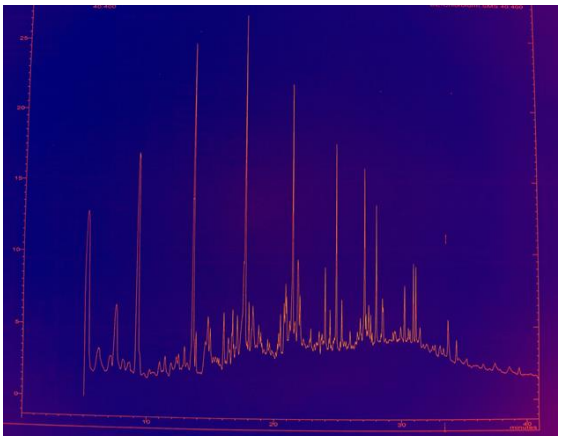

Figure 4: GC/MS in winter diethyl etter extraction.

Eicosane (Value 5.84, Quality 98%), Phenol 2,4-bis (1,1-dimethylethyl) (Value 1.55, Quality 98%), Tridecane (Value 18.78, Quality 99%), Oleyl alcohol (Value 9. 22, Quality 99%), Bis (2- Ethylhexyl) phthalate (Value 1.93, Quality 98%), Digitoxin (Value 18.96, Quality 99%), Cholecalciferol (Value 23.98, Quality 99%) were identified in GC/MS in summer diethyl etter extraction (Figs. 5 and 6).

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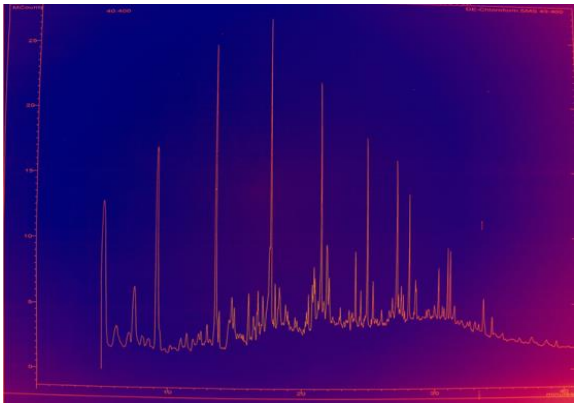

Figure 5: GC/MS in summer diethyl etter extraction.

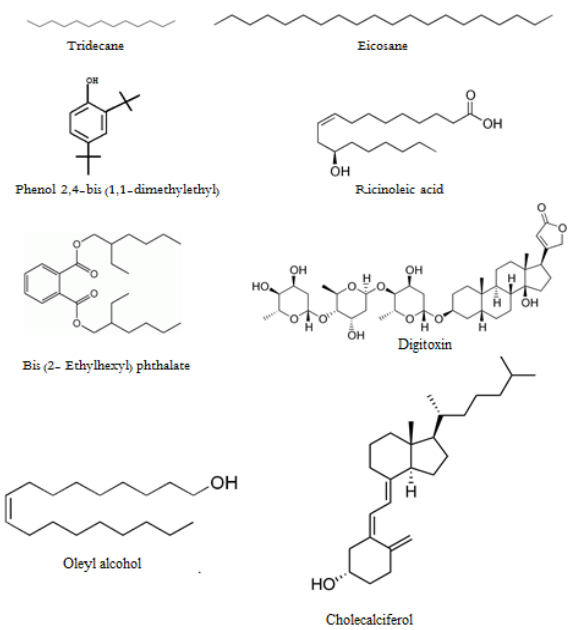

Figure 6: The components which identified of *I.mutans*.

Antibacterial activity of diethyl etter, methanol and water extracts

The results of the Bacterial Broth Dilution Methods of the sponge, *I.mutans*, extracts (methanol, diethyl etter and aqueous) against pathogenic bacteria (*E.coli*, *P.aeruginosa*, *S.aureus* and *B.subtilis spizizenii subdilish spizizenii*) are listed in Tables 1 and 2. As Table 1 shows the MIC values of the extract against four pathogenic bacteria; the MIC values of the winter and summer methanol extract for *S.aureus* was 30mg/ml, for *B.subtilis spizizenii* was 50mg/ml and 30mg/ml, but both of the extracts did not have any activity on *E. coli* and *P. aeruginosa.* Diethyl etter extract of two seasons exhibited significant activity against *E.coli*. (0.05 mg/ml, 20 mg/ml) *S. aureus* (1. 5 mg/ml, 2mg/ml) and *B. subtilis spizizenii* (5mg/ml). The MIC values of the winter diethyl etter extract for *P. aeruginosa* was 5mg/ml, but the summer extract had not any activity on this microorganism. The winter and summer aqueous extracts from *Ircinia mutans* did not have inhibitory activity against different bacterial strains.

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Table 1: Minimum Inhibitory Concentration (MIC) of different extracts from marine sponge, *I. mutans.*

Sponge			*I. mutans, winter*		*I. mutans, summer*
Bacterial strains	***E.coli***	**(ATCC 15224)**	M	R	R
			D	0.05	20
			AQ	R	R
			A	0.75	0.75
			T	0.75	0.75
	P.aeruginosa	**(ATCC 25619)**	M	R	R
			D	5	R
			AQ	R	R
			A	1.5	1.5
			T	1.5	1.5
	S.aureus	**(ATCC 1764)**	M	30	30
			D	1.5	2
			AQ	R	R
			A	0.75	0.75
			T	0.75	0.75
	Bacillus subtilis spizizenii	**(ATCC 6633)**	M	50	30
			D	5	5
			AQ	R	R
			A	1.5	1.5
			T	1.5	1.5

M: methanol extract; D: diethyl etter extract; AQ: aqueous extract; A: ampicillin; T: tetraciclina; R: Resistant. (MIC identify as mg/ ml).

Table 2 show the MBC values of the extract against bacterial strains; winter and summer aqueous and methanol extracts did not have bacterial activity against microorganisms. The MBC values of the winter and summer diethyl etter extracts were determined as (2 mg/ml, 50 mg/ml) for *E.coli;* and (2 mg/ml, 3mg/ml) for *S. aureus* and 10mg/ml for *B.subtilis spizizenii*. The winter diethyl etter extracts exhibited antibacterial activity against *P.aeruginosa* (MBC= 20mg/ml).

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Table 2: Minimum Bacterial Concentration (MBC) of different extracts from marine sponge, *Ircinia mutans.*

Sponge	Bacterial strains											
	***E.coli* (ATCC 15224)**			***P.aeruginosa* (ATCC 25619)**			***S.aureus* (ATCC 1764)**			***B. subtilis spizizenii* (ATCC 6633)**		
	D	A	T	D	A	T	D	A	T	D	A	T
I. mutans, winter	2	1.5	1.5	20	3	3	2	1.5	1.5	10	2	2
I.mutans, summer	50	1.5	1.5	R	3	3	3	1.5	1.5	10	2	2

D: diethyl etter extract; A: Ampicillin; T: Tetraciclina; R: Resistant. (MBC identify as mg/ ml)

Discussion

Persian Gulf is a shallow semi- enclosed sea, where series of islands extending along the western coast have fringing and patches of coral reefs so there is a wide variety of marine life within this ecosystem (UNEP, 1999). One of the most important marine invertebrates are sponges which provide the largest number of marine derived secondary metabolites with pharmacological application (West *et al.*, 2000). One of the most important drugs are antibiotics, more than 5000 different compounds have been isolated from about 500 species of sponges (Rifai *et al.*, 2005), and about 800 antibiotic compounds have been isolated from them (Touati *et al.*, 2007).

In this research, the winter diethyl etter extract of *I. mutans* showed good activity against the Gram-negative bacteria *E.coli* and *P. aeruginosa.*, The MBC of this extract on *E.coli* was 2 mg/ml and 20 mg/ml on *P. aeruginosa*, but summer diethyl etter extract and both of The MIC and MBC of Ampicillin and Tetracycline antibiotics on *E.coli* were 0.75mg/ml and 1.5mg/ml respectively, while on *P. aeruginosa*, were 1.5mg/ml and 3mg/ml.

In another study, sponge *Iophon laevistylus* of Faror Island in the Persian Gulf, methanol and diethyl etter extract showed antibacterial activity on 2 and 3 mg/ml against *E.coli* (Nazemi *et al.*, 2010). Darah and corporation reported the antibacterial activity of *Haliclona spp.* collected from the rocks off the shoreline of Kera Island, Penang, Malaysia, methanol extract values 0.5, 1. 2 mg/ml had not showed antibacterial activity against *E.coli.*

Antibacterial activities of winter diethyl etter extract of *Ircinia mutans* on another Gram-negative bacteria *P. aeruginosa* showed that the MIC was 5 mg/ml and MBC was 20 mg/ml but summer diethyl etter did not show any activity. Winter methanol extract showed that MIC was 50mg/ml.

Antibacterial activity of sponge extracts *Agelas oroides* and *Axinella damicornis* from Tunisian coast was performed using the agar-disk diffusion assay of appeared to be quite promising due to their capacity to

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inhibit the growth of *P. aeruginosa* onto 5 mg/disk (Touati *et al.,* 2007).

In this research, antibacterial activity of summer diethyl etter extracts of *Ircinia mutans* against the Gram-positive bacteria *S. aureus* and *B. subtilis* showed appropriate activity. The MIC and MBC of these extracts were 2 mg/ml and 3mg/ml on *S. aureus*; and 5mg/ml and 10mg/ml on *B. subtilis*. Winter diethyl etter extracts of *Ircinia mutans*, the MIC and MBC of these extract on *S. aureus* were 1.5 mg/ml and 2mg/ml; and 5mg/ml and 10mg/ml on *B. subtilis*. Summer and winter methanol extracts of this sponge did not show appropriate activity against these bacteria. The MIC and MBC of Ampicillin and Tetracycline antibiotics on *S. aureus* was 0.75mg/ml and 1.5mg/ml on *B. subtilis*, was 1.5mg/ml and 2mg/ml.

The results of the agar-disk diffusion assay of the methanol and n-hexane extracts of *S.inconstans* and *I.echinata* from Nay Band Bay, Iran haven't showed activity against *S. aureus* and *B. subtilis*, but methanol extracts of *Gelliodes* spp. inhibited growth of *S. aureus* on 250µg/ml (Safaeian *et al*., 2009).

Most reports showed that Gram-positive bacteria were particularly sensitive to sponge extracts of subtropical and tropical species, while Gram-negative bacteria were not sensitive (McCaffrey *et al.,* 1985). Our research showed that the nonpolar and semi-polar components which dissolve in diethyl etter had appropriate antibacterial activity on S. *aureus* and *B. subtilis.* In conclusion, the Persian Gulf is a potential source of a great variety of marine sponges like *I.mutans*, so it will be subject to detailed research for isolation of antibacterial active molecules with the search for new compounds.

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