Antimicrobial activities of semi polar-nonpolar and polar secondary metabolites of sponge *Dysidea pallescens* from Hengam Island, Persian Gulf

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Abstract

Sponges are the simplest multicellular animals that lack defense mechanisms and rely on chemical defense that have been used by mankind to develop antimicrobial drugs against diseases. The present study was designed to demonstrate the antibacterial and antifungal activities of marine sponge *Dysidea pallescens* semipolar and nonpolar extracts. In this study, *D. pallescens* were collected from Hengam Island in the Persian Gulf. The extracts were produced by Bligh and Dyer method. Broth Dilution Methods were used to check the antimicrobial activity of *D. pallescens* extracts against *Escherichia coli* (ATCC 15224), *Pseudomonas aeruginosa* (ATCC 25619), *Staphylococcus aureus aureus* (ATCC 1764), *Bacillus subtilis pizizenii* (ATCC 6633), *Candida albicans* (ATCC10231) and *Aspergillus fumigates* (PTCC5009). The results showed diethyl ether extract has bactericidal activity against *S. aureus aureus* (MBC=10mg/mL) and *B. subtilis spizizenii* (MBC=20mg/mL). *D. pallescens* diethyl ether extract showed a very weak antifungal activity but methanol extract showed fungicidal activity against *A. fumigates* (MFC=5mg/mL) and *C. albicans* (MFC=1.5 mg/mL). Therefore nonpolar-semipolar secondary metabolites of *D. pallescens* solutions in diethyl ether have shown significant antibacterial activity and polar-secondary metabolites solutions in methanol have shown significant antifungal activity.

Keywords*:* Antibacterial, Antifungal, Marine sponge, Persian Gulf

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Introduction

Marine sponges are a rich source of bioactive compounds; some of which can be useful for the development of new pharmacological tools and medicines as well. Biological activities of sponges such as antimicrobial, haemolytic, hemagglutinating, ichthyotoxic and lethal properties have been studied from various locations (Sepcić *et al*., 2010, Filho *et al*., 2015). Also these organisms are considered as important components in benthic communities, regarding their biomass as well as their potential to influence benthic or pelagic processes (Coppari *et al*., 2016). Sponges (phylum Porifera) are among the oldest multicellular animals (Metazoan) and show relatively little differentiation and tissue coordination (Leys and Meech, 2006). More than 8,000 sponge species were described; they inhabit a wide variety of marine and freshwater ecosystems and are found throughout tropical, temperate and Polar Regions (Hooper and Van Soest, 2002). They are sessile invertebrates with a wide variety of colors, shapes, and consistencies. The presence and abundance of spicules is variable: some species e.g., *Lithistida* and *Astrophoida* have dense or fused siliceous skeletons and therefore a hard consistency, whereas other species have few or no spicules, thus lacking physical defenses (Pawlik, 1995). However, sponges have evolved to develop chemical defenses against predators and larval settlement of organisms (Rohde and Schupp, 2011). In addition, sponges have strategies to defend themselves against foreign prokaryotic and eukaryotic organisms, by production of secondary metabolites (Pawlik, 2011). In fact, marine sponges are among the richest source of interesting chemicals produced by marine organisms. During the past three decades, many efforts have been devoted to isolate numerous biologically active novel compounds from marine sources (Hussain Md *et al*., 2012; Mehbub *et al*., 2014). Many of such naturally occurring compounds have great interest for potential drug development as well as an ingredient of new medicines and commercially successful products for various industrial applications, especially, pharmaceuticals, agrochemicals, functional foods and nutraceuticals.

Little information was recorded on the bioactive potential of marine sponges in the Persian Gulf. The main objectives of this study were to investigate antibacterial and antifungal activities of methanol, aqueous and diethyl ether extracts of *D. pallescens*.

Material and methods

Collection and identification

Samples of the sponge *D. pallescens* were collected by scuba divers in June 2012 from reef habitats at depths of 15-20 m from Hengam Island in the Persian Gulf. The geographical situation of Hengam Island is shown in Fig. 1. Samples were frozen in -20ºC and transferred to the laboratory. The

identification was done based on scanning optical microscope, skeletal slides and dissociated spicule mounts based on Hooper identification key (Hooper and Van Soest, 2002).

Figure 1: Geographical situation of sampling location in Hengam Island.

Extraction

The samples were soaked in diethyl ether for 24 hours and extracted for semipolar- nonpoplar fractions. The soaked sponge in diethyl ether was filtered and the solvent was evaporated by using Rota vapor at low pressure at 35-40ºC till dryness and diethyl ether extract was analyzed by Gas chromatography-mass spectrometry (GC/MS: Agilent7000 Series Triple Quad GC/MS Main Frame), for identifying the compounds.

For polar extraction samples were soaked in methanol for 72 hours in order to produce the polar extract. The polar compounds were separated in the methanol-aqueous phase extraction. Dried crude semi-solid extract was obtained after 72 hours of evaporating to dryness at low pressure at 40-45ºC using Rota vapor. All processes were carried out under dark conditions. The concentrated extract was dried to obtain crude semi-solid extract. The crude extract weight and percentages of extraction from sponge were calculated. Finally, both crude extracts including the semipolar- nonpolar and polar crude extracts were kept in a freezer (Dellai *et al*., 2012; Johnson *et al*., 2012).

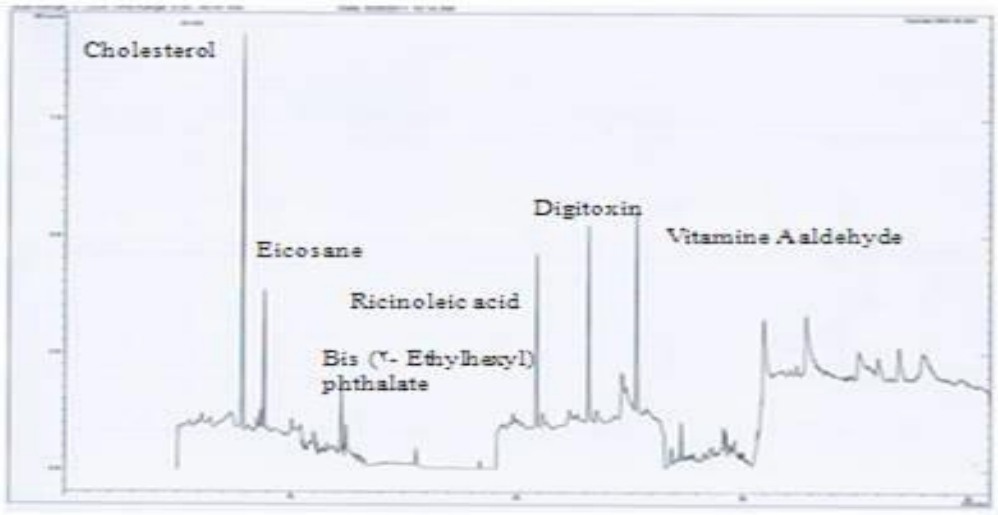

Figure 2: *Dysidea pallescens* diethyl ether extracts GC/MS chromat ogram.

Antibacterial assay

Antibacterial activity was determined by using the Bacterial Broth Dilution Methods (peptone, glucose, yeast extract)(Rosenblatt, 1991) against *Escherichia coli* (ATCC 15224), *Pseudomonas aeruginosa* (ATCC 25619), *Staphylococcus aureus aureus* (ATCC 1764) and *Bacillus subtilis spizizenii* (ATCC 6633). To perform the classic broth dilution susceptibility test, microorganisms 1.5×105 colony forming units [CFU]/mL, a1:100 dilution of a suspension of turbidity equal to a McFarland standard 0.5, was added to an equal volume (1mL) of each concentrations of diethyl ether, methanol and aqueous extracts (50 mg/mL ,40 mg/mL, 30 mg/mL 20 , mg/mL, 10 mg/mL, 5 mg/mL, 3 mg/mL, 2 mg/mL, 1.5 mg/mL, 0.75 mg/mL, 0.5 mg/mL, 0.10 mg/mL, 0.05 mg/mL and 0.01 mg/mL) to a tube of the growth control. An uninoculated tube of medium was incubated as a negative growth control. After overnight incubation, the tubes were examined for turbidity, indicating growth control of the microorganism. The lowest concentration of the extracts that inhibits organism growth, which was detected by lack of visual turbidity (matching the negative growth control) was designated as the minimum inhibitory concentration (MIC).

After the MIC determination, a known quantity of broth that showed visible turbidity after 22 to 24 hours incubation including 0.1 mL of inoculums from each of the tubes was sub cultured to solid agar plates. Following overnight incubation, the number of colonies that have grown on the subculture were counted and compared to the number of CFU/mL in the original inoculum. The lowest concentration of antimicrobial agent that allowed less than 0.1% of the original inoculum to survive is said to be the minimum bacterial concentration (MBC) (Rosenblatt, 1991).

Antifungal assay

Antifungal activity was carried out against *Candida albicans* (ATCC10231) and *Aspergillus fumigates* (PTCC5009) (supplied as Freeze-dried), Persian Type Culture Collection (PTCC).

C. albicans was inoculated on culture medium (20g Agar, 10g Glucose, 5g Peptone, 3 yeast extraction, 1000mL distilled water, pH 6.2±0.2) for 24 hours at 25ºC. *A. fumigates* were inoculated on culture medium (20g potato extract, 20g Glucoses, 15g Agar, 1000 mL distilled water, pH 6.2±0.2) for 72 hours at 26˚C. And then 1 mL of different concentrations (50, 40, 30, 20, 10, 5, 3, 2, 1.5, 0.75, 0.5, 0.1, 0.05 and 0.01 mg/mL) of methanol, diethyl ether and water extracts were added. Niacin was used as a positive control. The inoculate absorbance read between 0.08 and 0.10 AU (equivalent to 0.5 McFarland 108 CFU/mL) adding sterile broth macro dilution, before incorporating the yeast (λ = 530 nm) (Rosenblatt, 1991).

Results

Identification of semi polar and nonpolar compounds

Eicosane (Value 7.84%, Quality 98%), Digitoxin (Value 20.56%, Quality 99%), Bis (2- Ethylhexyl) phthalate)(Value 2.63%, Quality 98%), Ricinoleic acid (Value 18.63%, Quality 99%), Vitamin A aldehyde (Value 28.42%, Quality 99%), Cholesterol (Value 29.16%, Quality 99%) were identified with GC/MS in diethyl ether extract of *D. pallescens*.

Antibacterial activity

The antibacterial activity results for sponge extracts (methanol, diethyl ether and aqueous) and antibiotics (ampicillin and tetracycline) against Gram-positive bacteria (*S. aureus aureus*- ATCC1764 and *B. subtilis spizizenii*- ATCC6333) and Gram-negative bacteria (*E. coli*- ATCC 15224 and *P. aeruginosa*- ATCC 25619) are summarized in Tables 1 and 2. Solvents did not have any effect on microorganisms.

Gram-negative and Gram- positive bacteria had a strong resistance to aqueous extract. Diethyl ether extract of *D. pallescens* was active against Gram-positive bacteria; *S. aureus aureus* (MIC =2mg/mL) and *B. subtilis spizizenii* (MIC =10mg/mL). Diethyl ether extract of sponge showed antibacterial activity against *S. aureus aureus* (MBC = 10mg/mL) and *B. subtilis spizizenii* (MBC=20mg/mL). Methanol extract of sponge showed a very weak antibacterial activity compared to the diethyl ether extract.

Diethyl ether extract of *D. pallescens* had an inhibitor activity against *E. coli* (MIC =20mg/mL). However, both of the extracts were not effective against *P. aeruginosa.*

Table 1: MIC (mg/ml) of *Dysidea pallescens* extracts against bacteria.

Bacterial strains	*Dysidea pallescens* extracts			Control positive	
	D	M	AQ	T	A
Escherichia coli (ATCC 15224)	20±2.3	20±3.1	R	0.75±0.03	0.75±0.008
Pseudomonas aeruginosa (ATCC 25619)	R	R	R	1.5±0.12	1.5±0.08
Staphylococcus aureus aureus (ATCC1764)	2±0.08	R	R	0.75±0.06	0.75±0.04
Bacillus subtilis pizizenii (ATCC6333)	10±1.58	20±2.6	R	1.5±0.09	1.5±0.14

M: methanol extract; D: diethyl ether extract; AQ: aqueous extract; A: ampicillin; T: tetraciclina; R: Resistant. (MIC identify as mg/mL).
Data represents mean±standard deviation (n=3).

Table 2: MBC (mg/mL) of *Dysidea pallescens* extracts against bacteria.

Bacterial strains	*Dysidea pallescens* extracts		Control positive	
	D	M	T	A
Escherichia coli (ATCC 15224)	R	R	1.5±0.09	1.5±0.03
Staphylococcus aureus aureus (ATCC1764)	10±1.7	R	1.5±0.05	1.5±0.07
Bacillus subtilis subsp. spizizenii (ATCC6333)	30±3.8	R	2±0.23	2±0.18

M: methanol extract; D: diethyl ether extract; AQ: aqueous extract; A: ampicillin; T: tetraciclina; R: Resistant. (MIC identify as mg/ mL).
Data represents mean±standard deviation (n=3).

Antifungal activity

The antifungal activity results for sponge extracts (methanol, diethyl ether and aqueous) and antifungal (Nystatin) against fungi *Candida albicans*-ATCC10231 and *Aspergillus fumigatus*-PTCC5009 are summarized in Tables 3 and 4.

According to Table 3 *A. fumigates* and *C. albicans* had a strong resistance to aqueous extract and diethyl ether extract of *D. pallescens* showed a very weak (50±4.2) antifungal activity compared to the methanol extract. However methanol extract of sponge exhibited significant activity against *A. fumigates* (MIC=0.5 mg/mL) and *C. albicans* (MIC=0.75 mg/mL). Methanol extract of *D. pallescens* showed fungicidal activity against *A. fumigates* (MFC=5mg/mL) and *C. albicans* (MFC=1.5 mg/mL).

Table 3:M1C (mg/mL) of *Dysidea pallescens* extracts against fungi.

Fungi	*Dysidea pallescens* extracts			Control positive Nystatin
	D	M	AQ	
Aspergillus fumigatus (PTCC5009)	R	0.5±0.013	R	0.5±0.02
Candida albicans (ATCC10231, PTCC5027)	50±4.2	0.75±0.24	R	0.5±0.02

M: methanol extract; D: diethyl ether extract; AQ: aqueous extract (MIC identify as mg/mL).
Data represents mean±standard deviation (n=3).

Table 4: MFC (mg/mL) of *Dysidea pallescens* extracts against fungi.

Fungi	*Dysidea pallescens* extracts			Control positive
	D	M	AQ	Nystatin
Aspergillus fumigatus (PTCC5009)	R	5±0.33	R	0.75±0.08
Candida albicans (ATCC10231, PTCC5027)	R	1.5±0.21	R	0.75±0.06

M: methanol extract; D: diethyl ether extract; AQ: aqueous extract (MFC identify as mg/mL).
Data represents mean±standard deviation (n=3).

Discussion

Persian Gulf has a unique complex of tropical marine ecosystems with different marine organism such as sponges with potential natural product. During the past decades many efforts have been devoted to isolate numerous biologically active novel compounds from marine sources. Marine sponges are categorized into high biodiverse groups (Van Soest *et al*., 2012). These organisms were successful during evolution and able to avoid extinction. The main reason of this activity might be the fact that sponges, as sessile filter feeders, do not suffer from nutrient shortage and have a strong defense system to defeat foreign invaders. The marine sponge from Dysidea family is a prolific producer of structurally diverse secondary metabolites including sesquiterpenes (Motti *et al*., 2007), sesterpenes, meroterpenes (Blunt *et al*., 2007), and sterols as well (Wei-Hua *et al*., 2014).

In this study *D. pallescens* semipolar and nonpolar extracts were used for compound identification and biological activity. Diethyl ether extract had many compounds including: Eicosane 7.84%, Digitoxin 20.56%, Bis (2- Ethylhexyl) phthalate 2.63%, Ricinoleic acid 18.63%, Vitamine A aldehyde 28.42%, Cholesterol 29.16%. From *Axenella donani* methanol extract 23 different compounds were isolated including Pentadecane, Dodecane, m-Di-tert-butylbenzene, 2,3,7-Trimethyldecane, 5-Isobutylnonane, Nonane, 5-(2-methylpropyl)- CAS) Octane, 4-Butyl-2-methyl- decane, 3,7-Dimethyl - Decane, Nonane, 5-(2-methylpropyl)- (CAS) octane, 4-Butyl-2-methyl-octadecane, 2-ethylhexyl isohexyl ester, Heptadecane, 3-Ethyl-3-methyldecan (Iodice *et al.,* 2003). Nonadecane and Tetradecane volatile compounds were also isolated from suberites domuncula family. The Alpha- Pinene, Sabinene, beta- Cymene, 1-l-Limonene, hendecane, Alpha. Fenchyl Alcohol, Endo- Borneol, Alpha- Terpineol, Verbenone, Naphthalene, 2-methyldodecane, Tridecane, Tridecane, 2- methyl, Tetradecane, Valencene, Pentadecane, Hexadecane, n-Octadecane, 1- methyl tetradecanoate, and Palmitic acid wereisolated from *Iophon laevistylus* (Nazemi *et al*., 2010).

The wide range of variation in antimicrobial activities which is shown by the sponge species might be due to differences in the chemical concentration and composition among the species (Tincu and Taylor, 2004). In the present study *D. pallescens* polar and non-polar extracts were used for determining the biological activity of this species. The results showed that diethyl ether extract of this sponge had significant antibacterial activity against gram-positive bacteria (*S. aureus aureus* (MIC=2mg/mL) and *B. subtilis spizizenii* (MIC=10mg/mL). Diethyl ether extract had only inhibitor activity against gram-negative bacteria (*E. coli*) (MIC =20mg/mL). The result showed in our study was in accordance with Nazemi (Nazemi *et al*., 2014a), who

studied the diethyl ether extract activity of *Haliclona* spp. on gram-positive bacteria (*S. aureus aureus and B. subtilis spizizenii*). Safaeian investigated antibacterial activity of *Gelliodes* spp. and *Spheciospongia* spp (Safaeian *et al*., 2009) extract against *S. aureus* and *B. subtilis*. Nazemi investigated antibacterial activity of *Ircinia mutans,* secondary metabolite solutions in diethyletter against gram positive bacteria (Nazemi *et al*., 2014b). According to MIC and MBC of bacteria studied in this work, diethyl ether extract of *D. pallescens* showed better activity on Gram-positive bacteria than Gram-negative bacteria. Our findings are in agreement with the researchers who mentioned that Gram-positive bacteria are sensitive to the sponge extracts (Marinho *et al*., 2010).

In this work, methanol extract of *D. pallescens* showed very strong antifungal activity on *A. fumigatus* and *C. albicans* (MFC=0.5mg/mL and 1.5 mg/mL). The result was in accordance with Nazemi, who showed that methanol extract of *Haliclona* spp. had a broad spectrum of antifungal activity against *A. fumigatus* and *C. albicans*(Nazemi *et al*., 2014). Dhanalakshmi reported antifungal activity of *Dysidea herbace* chloroform-methanol extract against *A. fumigatus*. In another reported antifungal activity of *Gelliodes carnosa* Polar extract agaisst *Fusarium* sp. (Khakshoor and Pazooki, 2014).

In this study *D. pallescens* collected from Hengam Island in the Persian Gulf were shown to possess significant antimicrobial activity. Furthermore, the Persian Gulf is a potential source of great variety of marine animals as sponges, so it will be subjected for further investigation for isolation of biological active molecules.

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