

Evaluation of antibacterial, antifungal and cytotoxic agents of Ascidian *Phallusia nigra* (Savigny, 1816) from Persian Gulf

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ABSTRACT

This study was carried out in order to screen the antibacterial, antifungal and cytotoxic activity of the bioactive compounds extracted from the test body of the solitary ascidian, *Phallusia nigra* collected from the Iranian coastal water (Persian Gulf) against five human pathogenic microorganisms. Water-methanol extract at 18 µg/ml concentrations produced a maximum inhibition zone of 25 mm against *S. aureus* and the minimum of 20 mm at 16 µg/ml concentrations. The corresponding zones of water-methanol extract produced 25mm against *A. niger* at 18 µg/ml concentration. There is a great scope for finding further novel antimicrobial compounds in the ascidian group, and further research is needed including biochemical and seasonal changes in biologically active peptides in Persian Gulf.

Keywords: Antibacterial, Antifungal, Cytotoxic, *Phallusia nigra*, Persian Gulf.

INTRODUCTION

Natural products and their derivatives contribute more than half of all clinically administered drugs. Of the natural products isolated from marine organisms, only 1% has been examined so far for pharmacological activities [1].

Ascidians (Phylum Chordata, Class Ascidiacea), or sea squirts, are the largest and most diverse class of the sub-phylum Tunicata (also known as Urochordata). They comprise approximately 3,000 species found in all marine habitats [2]. Tunicates have been reported to be rich sources of biologically active compounds and ranked third for their overall activities, next to sponges and bryozoans [3]. Although research on bioactive compounds from ascidians was recently initiated, it is significant that the first marine natural product entering human clinical trials, didemnin B, is an ascidian metabolite. Cytotoxicity of the ascidian metabolite is the most frequently listed agent against a variety of tumor cell lines, followed by antimicrobial, antiviral and anti-inflammatory activities (Davidson, 1993). Ascidians provide a fertile ground for studies in the field of natural products. Ascidians serve as a potential source of new anti-cancer compounds [4]. Ecteinascidin 743, a highly promising, exceedingly potent antitumor agent, isolated from extracts of the ascidian *Ecteinascidia turbinata* (Herdman, 1880) is currently in phase II/III clinical trials [5]. Recently, antimalarial compounds have been isolated from the solitary ascidians *Microcosmus goanus* (Michaelsen, 1918), *Ascidia sydneyensis* (Stimpson, 1855) and *Phallusia nigra* [6].

Phallusia nigra is the most abundant ascidian along the Persian Gulf coast [7]. A review of literature showed that *P. nigra* presents interesting chemical defense mechanisms, such as presence of haemolytic and cytotoxic substances [8], high amount of vanadium in the soft body parts, presence of guanidine based neurotoxins [9] and low pH in the tunic [10].

Even though the Persian Gulf marine environment has diversity, to date, only a fraction of this diversity has been explored for novel bioactive compounds. In Persian Gulf, a preliminary study was carried out on antibacterial, antifungal and cytotoxic activities of some holothurids, *Holothuria leucospilota* [11], *Holothuria scabra* [12].

Though enough information about chemistry and ecological as well as developmental implications of marine natural products is available, there is no information about ascidian metabolites in Persian Gulf, hence this study is a first attempt to screening biological activities of ascidians in this area.

MATERIALS AND METHODS

Sampling and identification

Tunicate samples were collected using SCUBA diving from Larak Island in the Persian Gulf in 2011 at depths of 5–15m. Each sample was cut into small pieces and then immediately frozen and maintained at -20°C Prior to extraction. Information on the organism, the place of collection, date of collection and depth were recorded. Ascidian species identification was carried out using available references.

Extraction

The frozen samples were freeze-dried, macerated with dry ice (CO₂) by a blender and soaked overnight in distilled water. The supernatants were then removed, centrifuged at 10,000 rpm, filtered and collected. The collected water extracts were freeze-dried (aqueous extract). The insoluble solid materials were then successively extracted with ethyl acetate, methanol and water-methanol (50%) successively by percolation (72 h for each solvent) at room temperature. The organic extracts were combined and the solvent removed by rotary evaporation at no more than 40 °C to avoid degradation of compounds. All crude extracts were kept at -20 ° until further processing [12].

Assay of cytotoxicity effect

Cytotoxic activity of extracts was determined by Brine-Shrimp Lethality assay (BSA) as described by Meyer *et al.*, [13]. Simple zoological organism (*Artemia salina*) was used as a convenient monitor for the screening. The cysts of the brine shrimp hatched in artificial seawater (3.8% NaCl solution) for 48 hours to mature shrimp called nauplii. Different concentrations of each extract dissolved in normal saline were obtained by serial dilution. Four concentrations of each extract were prepared with 10, 100, 500 and 1000 µg/ml. Twenty nauplii were added to each concentration of the extracts in 24 well chamber slides. Number of nauplii alive noted after 24 h. The mortality end point of the bioassay was determined as the absence of controlled forward motion during 30 seconds of observation. Seawater and berberine hydrochloride (LC₅₀ = 26 µg/ml) were used as controls. Lethality percentage was determined and LC₅₀ calculated based on Probit Analysis with 95% of confidence interval using computer software “BioStat-2007”.

Antibacterial and antifungal assay

The antibacterial and antifungal activities of *P. nigra* extracts were assessed against *Escherichia coli* (ATCC 1763), *Staphylococcus aureus* (ATCC 25923), *Pseudomonas aeruginosa* (ATCC 25853) *Candida albicans* (ATCC 10231) and *Aspergillus niger* (ATCC 16404) by the Disc Diffusion Susceptibility method [12]. The extracts were tested in the lowest concentration at which no growth was observed, recorded as minimum inhibitory concentration (MIC). Culture media with different concentrations of Gentamycin and Fluconazole were used as positive controls. Antibacterial and antifungal assays were performed in triplicates [11].

RESULTS

The results of antibacterial and antifungal activities of the crude water-methanol extracts of *Pnigra* are given in Table 1. Water-methanol extract at 18 µg/ml concentrations produced a maximum inhibition zone of 25 mm against *S. aureus* and the minimum of 20 mm at 16 µg/ml concentrations. The corresponding zones of water-methanol extract produced 25mm against *A. niger* at 18 µg/ml concentration. The minimum inhibitory concentrations (MICs) of the water-methanolic extracts of *P. nigra* varied between 15-17 µg/ml against bacterial and fungal strains in this study.

Table 1. Antimicrobial activity of *Phallusia nigra* body wall extracts from Persian Gulf

Organism	Extract	Inhibition zone(mm)						
		MIC µg/ml	4 µg/ml	8 µg/ml	10 µg/ml	14 µg/ml	16 µg/ml	18 µg/ml
<i>S. aureus</i>	methanol-water	15	–	–	–	–	20	25
<i>A. niger</i>	methanol-water	17	0	0	0	0	0	25

Table 2. Cytotoxic activity of *Phallusia nigra* extracts from Persian Gulf

Extract	Dose (µg/ml)	Log dose	total	Alive	Death	Lethality (%)	LC50	chi-square	95% Confidence Limits	
									Lower	Upper
BW(methanol)	10	1	20	20	0	0	405.04	2.94	196.41	863.57
	100	2	20	15	5	25				
	500	2.7	20	12	8	40				
	1000	3	20	4	16	80				

DISCUSSION

Chemical antibacterial defense has been suggested as one of an array of defenses potentially available to sessile invertebrates [14]. Advance in molecular methods have shown that some symbiotic microorganisms are responsible for the production of secondary metabolites that serve as chemical defense for their hosts. These metabolites may affect bacteria in a number of ways, ranging from the induction of a chemo tactic response to inhibition of bacterial growth or cell death [15]. On the other hand, infectious diseases represent a serious public health problem and they remain the major cause of death throughout the world [16].

A number of bioactive compounds have also been isolated from ascidians, exhibiting activities such as antiviral [17], cytotoxic, antibacterial [18], and enzyme inhibitory activities [19]. Antibacterial and cytotoxic activity has been previously reported from extracts of some tunicates [20].

Our results showed a low antimicrobial activity of *P. nigra*. This could be justified that the *P. nigra* featured a clear axenic surface. This is in agreement with the results by Meenakshi [6] who reported that among 47 species studied along the Gulf of Mannar, *P. nigra* and *P. arabica* were free of any epibionts on their surface throughout the year.

Our antibacterial and antifungal activities could be attributed to the fact that the test body of *P. nigra* might contain secondary metabolites which inhibit the growth of bacteria and fungi. This view is consistent with the findings of Paul *et al.*, [15] reported that the tunicates have the potential to yield novel compounds of ecological, chemical and also biomedical interest.

Inhibition zone of water-methanol extract was observed in high concentrations and it seems that these activities of *P. nigra* extracts increased with increasing concentrations.

In this study the highest cytotoxic activity was for methanolic extracts with LC50 values about 405 µg/ml from *P. nigra*. The positive results have been recorded in the methanolic extraction and it shows that it is a good solvent system to the solubility of bioactive compounds present in this specie. The values were not as effective as our previous study in sea cucumbers (*H. leocuspilota* and *H. scabra*) [11,12]. It appears that in our study powerful cytotoxic activities were not present maybe due to the unspecific compounds obtained. Thus, further partitioning of different extracts need to be carried out to reveal the potentially active compounds.

In some ascidian species, peptides with antibiotic properties *in vitro* have been shown to have other biological effects such as protection against predation, digestion or prevention of surface epibiosis. There is a great scope for finding further novel antimicrobial compounds in the ascidian group, and further research is needed including biochemical and seasonal changes in biologically active peptides in Persian Gulf.

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