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Evaluation of the anti-microbial properties of *Gelliodes carnosa* sponge alkaloid compounds antimicrobial properties of marine sponge

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Abstract: The collected alkaloid compounds of *Gelliodes carnosa* sponge species from depth of 5-6 meters of Nay band Bay (Iran's coasts of Persian Gulf) were extracted by three different methods The obtained extract was tested on two gram-positive bacteria (*Staphylococcus aureus*, PTCC 1189, *Bacillus subtilis*, PTCC 1156), and five gram-negative bacteria (*Escherichia coli*,PTCC 1763, *Pseudomonas saeroginosa*, PTCC 1310, *Proteus mirabilis*, PTCC 1076, *Serattia marcescens*, *Klebsiella pneumonia*), and six pathogenic fungi (*Candida albicans*, PTCC 5027, *Fusarium solani,* PTCC 5248, *Aspergillus niger*, PTCC 5223, *Saprolegnia* sp., *Fusarium* sp.1, *Fusarium* sp.2). The extracted alkaloid compounds from the three methods had a significant inhibitory activity on the growth of all the bacteria and fungi. The anti-bacterial properties of these compounds were more than their anti-fungal properties. The most anti-bacterial and antifungal properties were from the extracts derived by the second method where it created a halos of above 25mm against all the fungi and bacteria. The lowest activity was from the extracts obtained by the third method against *Klebsiella pneumonia*, *Aspergillus niger*, and *Fusarium* sp.2 where an average of 9mm halos was created. Based on the obtained results this sponge can be considered as a source of bioactive substances which have inhibitory activities against some pathogens.

Key Words: bioactive substances, sponge, Persian Gulf

Introduction

Sponges (Porifera) are bentic simple multi-cellular animals without real organ or tissue layers that are one of the major clusters to create animal communities of coral reef. These animals live in different marine environments (Safaeian *et al.*, 2009; Qaralleh *et al.*, 2010; Galeano and Martinez, 2007). Sponges engage in chemical competition for suitable space with other fixed organisms such as corals and algae (Safaeian *et al.*, 2009). The continuous use of anti-biotic causes the phenomenon of pathogens resistance of which raises the necessity to look for new anti-microbial compounds from natural resources (Qaralleh *et*

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al., 2010; Galeano and Martinez, 2007). Sponges engage in chemical competition for suitable space with other fixed organisms such as corals and algae (Safaeian *et al.*, 2009). The continuous use of anti-biotic causes the phenomenon of pathogens resistance of which raises the necessity to look for new anti-microbial compounds from natural resources (Qaralleh *et al.*, 2010). The marine natural chemical productions in the 1970s were like a child who rapidly evolved during the 1980s and became mature in the recent decade (Chellaram, 2009). About 15000 bioactive substances from marine organisms have been isolated so far, where more than 5000 combination of it are obtained from 500 different species of sponges where there are more than 800 anti-biotic (Lakshmi *et al.*, 2010 ., Kelman *et al.*, 2001). Compounds that have been isolated from the sponges are of a wide variety of chemicals comprising terpens, nucleotides, peptides, usual and unusual fatty acids, and alkaloids (Newbold *et al.*, 1999). Alkaloids are effective biological substances that have medicinal and therapeutic properties and are present in many living organisms. Extraction and evaluation of their properties are of great importance medicine, pharmaceutical and food industries (Talei *et al.*, 2004). Considering the fact that there are no published reports for isolation and evaluation of alkaloid compounds anti-microbial properties from marine sponges of the Persian Gulf, the purpose of this study was to extract the alkaloid compounds of *Gelliodes carnosa* sponge species and to evaluate their anti-microbial properties.

Material and Methods

Sample Collection

The samples were collected from depth of 5-6 meters of Nayband Bay waters located in the Bushehr province in northern coasts of the Persian Gulf and immediately after their acquisition from water were placed and packed inside a polystyrene container with ice and were transported to the laboratory where they were maintained at a temperature of -18 °C for one month before starting the experiments.

Extraction of Alkaloid Compounds

There are different methods to extract the alkaloid compounds where in this study three different methods were employed in order to create the opportunity to compare the different methods and furthermore to select the best method.

- ✓ The first method: 10 g of dried sponge powder was put into a filter paper and placed inside the extractor of the Soxhlet apparatus and was covered by cotton. 300 ml of ethyl acetate were poured in the device's balloon and the extraction was performed within 18 h. Then the obtained solvent was filtered twice using filter paper NO 1 and was dried by rotary evaporator (IKA-Werke) at a temperature of 40 °C and the weight of the resulting substance was obtained (0.41 g).

The resulting substance was dissolved in 50 ml of distilled water, then its pH was changed to 3 using sulfuric acid and the extraction was done by respectively petroleum ether and diethyl ether so that the lipophilic, acidic, and neutral compounds are removed. Moreover, the pH of the solution or the aqueous phase was changed to 10 by ammonium hydroxide 25% m/m and then chloroform was used for extraction. The resulting chloroform phase was washed with distilled water until its pH was neutralized and then the solvent dehydration was done by sodium sulfate. Finally, the obtained pure alkaloid was concentrate by the rotary evaporator and was refrigerated for later use (Djilani *et al.*, 2006).

- ✓ The second method: Similar to the first method, 10 g of dried sponge powder was used for extraction by the Soxhlet apparatus where in this method methanol was used in the device's balloon. After drying the sample by the rotary evaporator, its dried weight was obtained (1.93 g). The obtained sample was dissolved in 100 ml of hydrochloric acid (2 M) to become acidic and then the acidic solution was washed with chloroform and the chloroform phase was isolated. Then the resulting solution was dried by the rotary and the weight of the remaining sediment was obtained (0.40 g). The dried sample was again dissolved in methanol and its pH was raised to 9 by adding ammonia to the solution and again like the previous step the alkaloid compounds were moving to the chloroform phase by adding chloroform to the solution. The extracted substance (chloroform phase) were poured into decanter and washed by water, dehydrated by zinc sulfate, and dried in vacuum with rotary evaporator to obtain the pure alkaloid (Talei *et al.*, 2004).
- ✓ The third method: 10 g of dried sponge powder was soaked in 150 ml of methanol for 24 h and thereafter the solvent was filtered, and this process was repeated for three times. The final extract was dried with rotary evaporator and the weight of the resulting substance was calculated (1.73 g). The obtained dried substance was dissolved in 500 ml of 5% acetic acid and was washed several times with diethyl ether. Moreover, the ether phase was isolated by using decanter and extraction was done by adding chloroform to the ether phase inside decanter. Finally, the solution was dehydrated with sodium sulfate and dried by rotary to obtain the alkaloid (Pérez-Amador *et al.*, 2007).

Anti-microbial test

The obtained alkaloid compounds were tested on 7 bacteria and 6 pathogenic fungi. In this experiment the Nutrient Agar (NA) medium was used for bacteria culture and maintenance, the Mueller-Hinton Agar (MHA) medium was

used for anti-microbial and Minimal Bactericidal Concentration (MBC) tests, the Mueller-Hinton Broth (MHB) medium was used for Minimal Inhibitory Concentration (MIC) test, and the Potato Dextrose Agar (PDA) medium was used for anti-fungal test (the used culture media were of type Merck). The most bacteria and fungi used in this study (Tab. 1) were provided by the Iranian Research Organization for Science and Technology (Persian Culture Type – PTCC). Prior to the experiment, the bacteria were cultured in the NA medium and the fungi were cultured in the PDA medium to obtain fresh and young colonies from the bacteria and fungi. The bacteria were incubated for 24 h at temperature of 37 °C and the fungi were incubated for 48 h at 25 °C to provide a pure fresh culture from bacteria and fungi. The physiological saline method was used to prepare microbial suspension with concentration of 0.5 McFarland (1.2 x 10^8 bacteria per milliliter) (Collee *et al.*, 1999).

Tab. 1: the bacterial and fungal samples in the experiment.

	Bacteria	Fungi
Gram-positive	*Staphylococcus aureus(*PTCC 1189)	*Candida albicans* (PTCC 5027)
	Basilus subtilis (PTCC 1156)	*Fusarium solani* (PTCC 5248)
		Aspergillus niger (PTCC 5223)
Gram-negative	*Escherichia* coli (PTCC 1763)	*Saprolegnia sp*.
	Pseudomonas aeroginosa (PTCC 1310)	*Fusarium sp*.1
	Proteus mirabilis (PTCC 1076)	*Fusarium sp*.2
	Serratia marcescens	
	Klebsiella pneumonia	

Disk-diffusion method

This experiment was carried out using the international standard method provided by NCCLS. First, a solution with identified concentrations from each extract was prepared (Tab. 2) and 200 microliters were injected into each 6mm blank sterile disc and was dried with pump. Discs containing various solvents were used as negative control and Amoxicillin anti-biotic discs with concentrations of 25µg/disc as positive control for the bacteria, and Nystatin anti-biotic with concentration of 30µg/disc was used for fungi. The desired bacteria and fungi with concentration of 0.5 McFarland (1.2 x 10^8 bacteria per milliliter) were prepared and by using sterile swabs on the desired culture medium were cultured in three different directions. Then discs containing the extract together with the negative and positive control discs were placed on the medium by three replicates with specific intervals and the bacteria at 37 °C and fungi at 25 °C were

Tab. 2: the prepared concentrations of alkaloid compounds of different methods whit control

Concentrations	(1)	(2)	(3)	Amoxicillin	Nystatin
Extract concentration in 10cc (mg/ml)	88	52	48		
The amount of substance in 200µl (mg)	17.6	11.2	9.6	25 (µg/disc)	30 (µg/disc)

(1) Extract obtained from first method, (2) Extract obtained from second method (3) Extract obtained from third method

placed in the incubator. The diameter of inhibition zone for bacteria after 24 h and for fungi after 48 h was measured (Forbez *et al.*, 1998).

Statistical Analysis

The SPSS 19 program was used for all the analysis and the Microsoft Excel 2007 program was used to draw the graphs. The Kolmogorov-Smirnov test results revealed that the considered variable did not have a normal distribution. Nevertheless, the logarithm of the variable was used in the two-way ANOVA. The two-way analysis of variance (ANOVA) was used to study the anti-bacterial and anti-fungal effects of alkaloid compounds. The Tukey's test was used for the 2 by 2 comparison of bacteria and solutions.

Results

The efficiency of the first method for alkaloid compounds extraction where the Soxhlet apparatus and ethyl acetate solvent were used was about 1.5 times greater than the other two methods (Tab. 3). The obtained alkaloid extracts were tested on bacteria pathogens and the results are shown in Tab. 4. The results showed that the obtained alkaloid compounds from the third method had less anti-bacterial activity compared to the first and second methods and between the first and second method, the second method had greater growth inhibitory activity. The average halos created by the alkaloid compounds obtained from methods 3, 2, and 1 were 12.28mm, 24.28mm, and

Tab. 3: Alkaloid extraction by three different methods

Method	Primary sponge dry weight (g)	Alkaloid dry weight (g)	Alkaloid percentage	Extraction Temperature (°C)	Solvent firing Temperature (°C)
(1)	10	0.88	0.08%	70	50
(2)	10	0.52	0.05%	25	50
(3)	10	0.48	0.04%	70	50

(1) Extract obtained from first method, (2) Extract obtained from second method (3) Extract obtained from third method

Tab 4: Anti-bacterial activity of the alkaloid extracts of the *G. carnosa* sponge (mean±SD)

	Basilus subtilis	*Staphylococcus aureus*	*Escherichia coli*	*serratia marcescens*	*Proteus mirabilis*	*Pseudomonas aeroginosa*	*Klebsiella pnemonia*
(1)	***	***	***	25±1.5	20±3.61	***	***
(2)	***	***	***	***	***	***	***
(3)	14±1	12±1.5	13.5±1.32	11±0.5	14±1.57	12±1	9.5±0.86
Amoxicillin	14	17	18	11	15	13	16

(1) Extract obtained from first method, (2) Extract obtained from second method (3) Extract obtained from third method

The average bacterial inhibition zones are in millimeters (mm)

The zones diameter with discs is 6mm

*** zones of greater than 25mm

greater than 25mm respectively. 100% of the alkaloid compounds had anti-bacterial properties. 57% of the alkaloid compounds created an aura of greater than 25mm and the anti-bacterial activity of the alkaloid compounds were more on the gram-positive bacteria. Among the tested bacteria, the *Serattia marcescens* and the *Bacillus subtilis* had the lowest sensitivity towards alkaloid compounds, and the rest had roughly equal sensitivity. Fig. 1 depicts some of the bacterial inhibition zones of the extracts.

Fig. 1: Inhibitory zones of alkaloid compounds.

A: *Serattia marcescens*, B: *Staphylococcus aureus*, C: *Eschrichia coli*

The obtained alkaloid compounds were tested on six fungal pathogens where the results are given in tab. 5. As can be seen in the results like the anti-bacterial test, the obtained alkaloid extract from the third method showed less anti-fungal activity compared to the other two methods and the second method had better outcome compared to the first

Tab. 5: Anti-fungal activity of the alkaloid extracts of the *G. carnosa* sponge (mean±SD)

	Candida albicans	*Aspergillus niger*	*Saprolegnia* sp.	*Fusarium solani*	*Fusarium* sp.1	*Fusarium* sp.2
(1)	25±0.86	18±1.5	22±1.7	***	13±0.72	14±1
(2)	25±3.9	24±2.17	***	***	***	***
(3)	10±0.5	9±2	12±1.8	13±0.6	12±3	9±2.1
Nystatin	10	11	15	16	18	17

(1) Extract obtained from first method, (2) Extract obtained from second method (3) Extract obtained from third method

The average bacterial inhibition zones are in millimeters (mm)

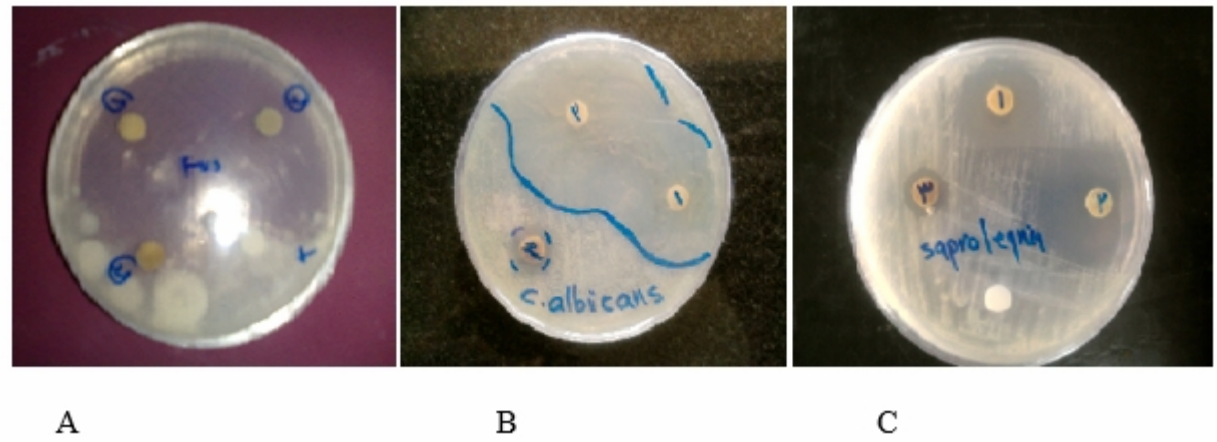
The zones diameter with discs is 6mm

*** zones of greater than 25mm

method. The average aura created by the alkaloid compounds obtained from methods 1, 2, and 3 were 19.50mm, 25mm, and 10.83mm respectively. 100% of the alkaloid compounds had anti-fungal activity where 38% of them created a halo of greater than 25mm. Among the tested fungi *Fusarium* sp.2, *Fusarium* sp.1, and *Aspergillus niger* with average halos of 16mm, 16.6mm, and 17mm had the lowest sensitivity towards alkaloid compounds and *Fusarium solani*, *Candida albicans*, and *Saprolegnia* sp. With average halos of 21mm, 20mm, and 19.66mm had the highest sensitivity towards alkaloid compounds. Fig. 2 depicts some of the fungal inhibition zones of the extracts.

Fig. 2: Inhibitory zones of alkaloid compounds.

A: *Fusarium* sp. B: *Candida albicans*, C: *Saprolegnia* sp.



The analysis of the two-way ANOVA showed that there is a significant difference between the alkaloid extracts from *Gelliodes carnosa* on bacteria and solvents, and the mutual effect of bacteria and solvent (Tab. 6)($p<.05$). Furthermore, the Tukey's test analysis showed that the zones diameter created in the bacterial cultures medium due to the alkaloid extracts from the third method had significant differences comparing *B. subtilis* with *Klebsiella pneumonia*, *S. marcescens*, and *Proteus mirabilis* and also comparing *Escherichia coli* with *S. marcescens*, *Proteu smirabilis*, and *Klebsiella pneumonia* whereas no significant difference was observed between other bacteria ($p<.05$). The data analysis also revealed a significant difference between the three methods used for alkaloid compounds extraction.

The two-way ANOVA analysis showed that there is a significant difference between alkaloid extracts from *G. carnosa* on fungi and solvents, and the mutual effect of fungi and solvent (Tab. 7) ($p<.05$). The Tukey's test analysis showed that the zones diameter created in the fungal cultures medium due to the alkaloid extracts from the third method did not have a significant difference between *C. albicans*, *Saprolegnia* sp., *Fusarium* sp.1, *A. niger, Fusarium* sp.1, *Fusarium* sp.2*, Saprolegnia* sp., *F. solani, Fusarium* sp.1, and *Fusarium* sp.2 fungi ($p<.05$) . Furthermore, the analysis revealed a significant difference between the amount of extracted alkaloid dry substance and the average created inhibitory auras on the cultures medium by alkaloid extracts obtained from the third method.

Tab. 6: the two-way ANOVA analysis results taken from the comparison between anti-bacterial effects of alkaloid extracts obtained from three methods (* p<0.0001)

Source of variation	Mean square	*df*	f
methode	1.188	2	981.536*
bacteri	0.09	6	7.038*
methode*bacteri	0.012	12	9.806*
error	0.01	42	
total		63	
Cprrected total		62	

Tab. 7: the two-way ANOVA analysis results taken from the comparison between anti-fungal effects of alkaloid extracts obtained from three methods (* p<0.0001)

Source of variation	Mean square	*df*	f
fungi	0.044	5	16.706*
methode	0.895	2	342.187*
fungi*methode	0.021	10	8.214*
error	0.003	36	
total		54	
Cprrected total		53	

Discussion

The studies of natural production or secondary marine metabolites have drawn the attention of many researchers of marine biology, biochemistry, chemistry, pharmaceutical, and biotechnology (Periyasamy *et al.*, 2012). The marine sponges have been able to resist against hunters over time due to their simple defense system and have prevented themselves from contamination by pathogenic microbes and formation of bacterial biofilm structures (Newbold *et al.*, 1999, Sonia *et al.*, 2008). Alkaloids are one of the most important groups of secondary metabolites due to different medicinal combinations made from them. The alkaloid distribution on the host depends on age, growth, and as well as ecological and environmental conditions. These factors affect the amount of certain compounds, especially compounds with medicinal properties (Qaralleh *et al.*, 2010). Given that plants were the only source of alkaloid compounds until the past few years, therefore, there is no fixed and applicable method for alkaloid extraction from animals (especially marine animals). Some of the existing methods are not sensitive enough and even some are defective. For instance, some of these methods produce compounds with impurity which results in different spots on the TLC. Highly sensitive methods such as HPLC are not conventional for determining alkaloids in the living organisms because they require special equipment and have high costs (Shamsa *et al.*, 2008). Therefore, three most commonly methods for extraction of alkaloid compounds from plants were utilized in this study where these methods have not yet been used for marine organisms especially sponges. This study can be the first application of these methods for extraction alkaloid from sponges. There are no data for direct comparison with the results of this study, but the results indicated that the Soxhlet method for alkaloid extraction was quantitatively better than the soaking method. Nowadays, the use of various antibiotics on pathogenic bacteria and fungi is common (Sionov *et al.*, 2005). Many of these pathogens have become resistant against these antibiotics over time. Therefore, the researchers are looking for new replacements in the nature and have extracted bioactive compounds from various species. Alkaloids are among these compounds which an extracted marine sponge species and have anti-microbial properties (Touati *et al.*, 2007 and Lakshmi *et al.*, 2010). The obtained alkaloid extracts in this study were used against 7 bacteria and 6 fungi the result showed they had stronger anti-bacterial effects compared to anti-fungal effects which this difference is likely due to cell structure of microorganisms (prokaryotes and eukaryotes). This issue was mentioned by McCaffrey and Endean (1985) and also by Amade *et al.* (1987). Mcclintock and Gauthier (1992) reported

that the sponge extract had weak activity against *Aspergillus niger* and *Candida albicans* fungi which is somewhat compatible with the results of this study. As can be seen in Tab. 4 and Fig. 1, the anti-bacterial effect of alkaloid compounds on gram-positive bacterial is significantly more than gram-negative bacteria. Chakraborty and Brantner (1999) showed that the tested alkaloid compounds have greater inhibitory effect on the growth of gram-positive bacteria rather than the gram-negative bacteria. Furthermore, the studies on the anti-bacterial effects of steroids alkaloid extracted from olive leaves showed that these substances are more effective on gram-positive bacteria than the gram-negative bacteria (Galeano and Martinez, 2007). The microorganisms' sensitivity towards antibiotic compounds depends on the wall structure of gram-positive and gram-negative bacteria. The less sensitivity of gram-negative bacteria is likely to be related to the external protective layer that has surrounded the cell wall. This layer may reduce the access of external compounds to active internal compounds existing in lipopolysaccharide layer, protein, and phospholipids. Sometimes, these conditions increase the sensitivity of bacteria to external compounds (Xue *et al.*, 2004). Also, in another study conducted by Talei *et al.* (2004) the alkaloid extracts obtained from plants in low dilution (MIC = 40, MBC = 600 µg/ml) had growth inhibitory effect on *Bacillus subtilis*, but did not inhibit the growth of *Escherichia coli*, *Pseudomonas aeroginosa*, and *Staphylococcus aureus* which does not match the results of this study. The noteworthy point in most studies is that the bacteriostatic effect is stronger than the bactericidal effect. The difference between the results of this study with other studies could be due to use of different sponge species for conducting the study, the environmental and ecological differences (Touti *et al.*, 2007), the capacity of solvents extraction and extracted compounds (Chakraborty and Brantner, 1999) and the concentration and chemical composition of different species. Also, in the study of Chakraborty and Brantner (1999) it was determined that the anti-microbial activity of alkaloid extract (pure) is more than the methanol extracted (impure). The noteworthy point is that even though the amount of alkaloid dry substance obtained by the first method was more than the second method, but the inhibition zone results show that the anti-microbial property of the extract obtained from the second method was more than the first method. This result may be due to kind of solvent be used in second method which has been able to isolate alkaloid compound with better properties. However, as can be seen in the results table some of the inhibition zones created on the experimented bacteria and fungi are even more than the inhibition zones created by the used antibiotic compound (positive control) which indicates that sponges are

introduced as a new source of biological compounds. The results of this experiment were promised a new source of antibiotics against pathogenic fungi especially the *Saprolegnia* fungus which is one of the most common fungal infections in salmon farms and freshwater pools. The studies on potential natural marine products could eventually lead to production and identification of new medicines

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