Antimicrobial Activity of Some Crude Marine Mollusca Extracts Against Some Human Pathogenic Bacteria

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ABSTRACT:

This study evaluated the antimicrobial of methanol, ethanol and acetone tissue extracts of two molluscs, Pinctada radiate (P. radiata) and Brachidonta variabilis (B .variabilis). Agar diffusion and broth dilution assays were used to test for antimicrobial activity against five nosocomial bacteria including, Staphylococcus aureus, Streptococcus pneumonia, Pseudomonas aeruginosa, Klebsiella pneumonia and Escherichia coli .

Extracts of both molluscs showed significant activity against all the bacteria strains tested. The best antibacterial activity was recorded by methanol extracts of B. variabilis towards Pseudomonas aeruginosa.

Ethanol extracts of B. variabilis had greater overall activity against all test microbes.

Ethanol extracts had higher antimicrobial activity index than their methanol and acetone counterparts.

The findings of this work indicate that the tissue extracts of B. variabilis and P. radiataare promising sources of antimicrobial agents that can be utilized for pharmaceutical and nutraceutical purposes.

Keywords: *Molluscs, Antimicrobial Activity, natural products, Pinctadaradiata, Brachidonta variabilis, Nosocomial Pathogens.*

I. INTRODUCTION

The Mollusca are animals belong to the phylum Molluscs ,there are around 93,000 recognized species extant, making it the largest marine phylum with about 23 % of all named marine organisms. Representatives of the phylum live in a huge range of habitats including marine, freshwater and terrestrial environments. Molluscs are a highly diverse group, in size, in anatomical structure, in behavior and in habitat [1].

Infectious diseases are a major threat to human health due to the unavailability of vaccines or limited chemotherapy. They are responsible for approximately one half of all deaths recorded in tropical countries [2]. Available antimicrobial regimes have substantial limitations in terms of antimicrobial spectrum and side effects. In addition, their promiscuous use has led to increasing trends of resistance among emerging and re-emerging microbial pathogens ([3], [4]). This, in turn, has led to a need to find new therapeutic compounds with preferably novel modes of action. Natural products have led the way in this respect and provided various success stories. Crude natural product extracts have played important roles in the discovery of modern drugs and drug scaffolds for the treatment of various ailments [5].

Aquatic organisms have evolved many different survival mechanisms to thrive in various harsh conditions. These conditions include extreme temperatures, changes in salinity and pressure and actions of pathogenic microbes [6]. The ability of aquatic organisms to adapt and survive in different environments depends both on their physical and chemical adaptive features. Organisms with no apparent physical defense, like sessile organisms, have evolved chemical defenses to protect themselves [7]. Aquatic invertebrates such as bryozoans, molluscs, sponges and others have soft bodies and lead a sedentary lifestyle, making a chemical system of defense absolutely essential for survival. These chemicals, when released into their aqueous habitat, are rapidly diluted. To be effective, the chemicals must be very potent ([8], [9]). The high potency of chemicals used in aquatic defense systems and the requirement for them to be water soluble have attracted many researchers to prospect for biologically active compounds from these ecosystems.

In recent years, a significant number of novel metabolites with potent pharmacological properties have been discovered from aquatic organisms. Many varieties of bioactive substances are being isolated and characterized with great promise for the treatment of many diseases ([10]; [11]). Spongouridine and spongothymidine, from the Caribbean sponge, *Cryptothecacrypta*, were isolated in the early 1950s and approved 15 years later as anticancer and antiviral drugs [11]. So far, over 10,000 bioactive compounds

have been discovered from aquatic sources, with hundreds of new compounds being discovered every year [9].

Prevention and control of these infectious bacteria will require the development of new antimicrobial agents. The secondary metabolites derived from number of marine animals that possess bioactive compounds and extracted from many classes of mollusks exhibit antitumor, antileukemic, antibacterial and antiviral properties ([12], [13]), and antiparasitic activities ([14], [15]). Antimicrobial peptides are important in the first line of the host defense system of many marine species [16].

Most natural products' research programmes in Ghana and Africa have focused on terrestrial plants, with a number of bioactive compounds isolated so far ([17], [18], [19]). Syria's aquatic biodiversity has to this point been explored to only a limited extent. In this study, methanol, ethanol and acetone extracts of the tissues of amarine mollusc, *Pinctada radiate* (*P. radiata*), *Brachidonta variabilis* (*B .variabilis*) were assayed against five test micro-organisms to evaluate their antimicrobial activity. Agar well diffusion and broth dilution assays were used to investigate antimicrobial activities of these extracts.

II. MATERIALS AND METHODS

A. Collection and identification

The two Molluscs (Class Bivalvia), *Brachidonta variabilis* and *Pinctada radiata*, were collected near Afamia region from coastal waters of Latakia city (Lat: 35 35'137 N; long: 35 43'.034E) (Figure 1). They were immediately brought to the laboratory and removed by breaking their shells and washed their soft bodies with distilled water to remove salts and epibionts. The whole body muscle of the sample (50g) was cut into small pieces, the soft bodies powder was prepared according to the method of Narayanasamy [20].

Figure (1): Location of the sampling site.

B. Preparation of B. variabilis and P. radiate Extracts:

Wet samples of *B .variabilis* and *P. radiate* were weighed and exposed to dry air current for remove as much water as possible. Then the sample was soaked with different polar solvents namely: methanol, ethanol and acetone solvents. The amount of each solvent was approximately 100 ml. The samples were soaked in methanol three times overnight, then they were immersed in ethanol three times overnight and once in acetone for 24 hours, filtered through Whatman No.1 filter paper (Kumpulan Saintifik F.E. Sdn. Bhd. (KSFE), Malaysia). After that, the samples were centrifuged at 5000 rpm for 15 minutes and the supernatant was collected. Following each sample was allowed to dry in fume hood to remove any remaining solvent. In the end, three sets of immersion extract one each of methanol, ethanol and acetone were obtained then it was used for the experimental work. Individually, the extract mixtures were rotavapped under vacuum ([21], [22]). The temperature of the water bath was set at 32ºC and the rotation rate was medium. The crude extracts species were placed in small vials and kept at – 20ºC for antimicrobial susceptibility testing using Kirby-Bauer disc diffusion assay [20].

C. Inoculum Preparation for Bacterial Strains

Standard microbial techniques were followed for media preparation and other routine process. Nutrient broth (Himedia, India) was prepared and sterilized in an autoclave at 121°C, 15 lbs pressure for 15 min. The bacterial strains were individually inoculated in sterilized nutrient broth and were incubated at 37°C for 24 hours and used in the test proper.

D. Antibacterial Activity

The crude extracts were used for antimicrobial activity assay against human bacterial pathogens, of the five bacteria, three were Gram negative:
Escherichia coli (E. coli), Klebseilla pneumoniae (K. pneumoniae) and Pseudomonas aeruginosa (P. aeruginosa) and the other two were Gram positive:

Staphylococcus aureus (S. aureus), and Streptococcus pneumoniae (S. pneumoniae).

All these strains used in the study were obtained from the Laboratory of Microbiology at the Hospital of Tishreen University. They were isolated from different clinical specimens of hospitalized patients (Table 1). Then these strains were identified by using API20E.

Table (1): The bacterial strains used in this study and their sources.

Clinical specimen	Bacterial strains used in this study
CSF (Cerebral spinal fluid)	*Klebsiella pneumoniae*
Umbilicus swab	*Staphylococcus aureus*
Urea	*Escherichia coli*
Blood	*Streptococcus pneumoniae*
Gastric secretion (neonate)	*Pseudomonas aeruginosa*

E. Kirby-Bauer Antimicrobial Assay:

Antimicrobial assay for methanol, ethanol and acetone extracts of *B .variabilis* and *P. radiata* were carried out by disc diffusion method followed by re. [23]. 24 hours old nutrient broth cultures of tested bacteria were aseptically seeded on sterile Mueller Hinton agar plates. Punched 6mm sterilized discs (Whatman, No, 1) were impregnated with50 µl of the obtained crude extracts from each crude extract, left to dry at room temperature.

These discs were placed on the inoculated Mueller Hinton agar plates (about 4-5 discs by plate) [24]. Then the plates were incubated at 37°C for 24 hours. Antibacterial activities were evaluated by measuring the diameter of inhibition zone showed in millimeters.

F. Minimum inhibitory concentration:

The broth dilution method was employed to determine the minimum inhibitory concentration (MIC) of the two crude extracts. To a row on a 96-well micro titer plate were added serial dilutions of extract or standard drug, representing different concentrations. Hundred-microliter nutrient broth and 10 µL of a suspension of test micro-organism were then added. Sterile water was added to top each well up to the 200-µL mark. Control wells were prepared using standard ciprofloxacin drug. Plates were incubated at 37°C for 24 h after which a solution of 3-(4,5-dimethylthiazol-2-yl) -2,5 diphenyltetrazolium bromide (MTT) was added to each well. Microbial growth was indicated by wells that changed colour to violet. Wells that were unchanged (in colour) indicated inhibition of microbial growth by the extracts.

G. Antimicrobial activity index

The antimicrobial index (AI) of each extract was calculated as the average of the antimicrobial activity obtained against all test micro-organisms. To do this, the activity of extracts against each test organism was assigned weight ages. For a ZI of 1–10 mm, a weight age of one (1) was assigned. A weight age of two (2) was assigned for ZI of 11–20 mm, three (3) if the ZI was greater than 20 mm and zero (0) for no antimicrobial activity. The AI was obtained by dividing the sum total of weight ages obtained for each individual extract by the total number of test micro-organisms. Separate AI was calculated for Gram-positive bacteria, Gram-negative bacteria to compare the activity of the various extracts ([25]; [26]).

III. RESULTS AND DISCUSSION

A. Taxonomy and description of animals used for extraction:

Description*: B. variabilis* is a small bivalve with a 20 mm shell, externally dark brown-black and internally tinged violet-black. Shell is equivalve, inequilateral, attached to substrate by stout byssus. Sculpture of numerous fine radial bifurcating ribs, which become coarser posteriorly and margin crenulate. The hinge has dysodont teeth. Outline mussel-like with terminal umbones but variable in shape and in its height/length ratio; sometimes greatly expanded posteriorly, sometimes arcuate; occasionally subcylindrical with beaks not quite terminal.

P. radiata is generally between 50 and 65 millimetres in length, though it can reach 106 millimetres. The shell is, thin, compressed, and square-like, with growth rings and ribs on the top surface. Its colouration varies, though it usually displays a brown or red exterior with a pearly interior and a light brown edge. More rarely, the shell may display a green or bronze exterior. Darker brown or red rays may mark the shell, creating darker areas at the margin. The shell's shape and structure also show much variation. Nomenclature of this species follows WoRMS Editorial Board (2017) (WoRMS, 2017).

***Pinctadaradiata* (Leach,1814)** ***Brachidontavariabilis* (Kraus,1962)**

Figure (2*): Morphological Description of Gastropoda*

Table (2): The Classification and description of the Bivalvia samples

Kingdom	**Animalia**	**Animalia**
Phylum	Mollusca	Mollusca
Class	Bivalvia	Bivalvia
Clade	Pteriida	Mytilida
Family	Pteriidae	Mytiloidea
Genus	Pinctada	Brachidontinae
Species	*P. turbinatus*	*Brachidontesvariabilis*

B. Antimicrobial activity

Antimicrobial activity of ethanol, methanol and acetone extracts of two molluscs was evaluated against five strains of bacteria. In general, ethanol extracts seemed to be more active than methanol and acetone extracts (Table 3). Ethanol extracts of *P. radiata* recorded their highest ZI as 18 mm towards the Gram-negative bacteria *P. aeruginosa*. Methanol extracts of *P. radiate* recorded appreciable activity only against *S. aureus*, and *P.aeruginosa*. Acetone extracts of *P. radiate* recorded appreciable activity only against *S. aureus*, and *P.aeruginosa*. Ethanol extracts of *B. variabilis* showed considerable activity towards all test microbes, while methanol extracts of *B. variabilis* exhibited activity against only *S. pneumoniae* and *P. aeruginosa*, When active, high zones of inhibition were recorded by the (17–23 mm). Similarly, Acetone extracts of *B. variabilis* had zones of inhibitions between 12 and 17.43 mm when active.

Table (3). Zones of inhibition (in mm) of ethanol, methanol and acetone extracts of *P. radiata* and *B. variabilis* against test micro-organisms

Test micro-organisms	***P. radiata***			***B. variabilis***			**Positive control**
	Ethanol	**Methanol**	**Acetone**	**Ethanol**	**Methanol**	**Acetone**	
*E. coli**	16.12 ± 0.76	0.00 ± 0.00	0.00 ± 0.00	11.53 ± 0.76	0.00 ± 0.00	0.00 ± 0.00	36.3 ± 1.25
*K. pneumoniae**	11.00 ± 1.00	0.57 ± 0.21	0.00 ± 0.00	12.63 ± 0.40	0.00 ± 0.00	0.50 ± 0.26	35.0 ± 0.09
*P. aeruginosa**	18.00 ± 1.73	11.67 ± 1.15	9.12 ± 0.40	11.53 ± 0.45	23.17 ± 0.76	17.43 ± 0.51	35.3 ± 1.22
*S. aureus***	14.83 ± 0.70	9.67 ± 0.58	7.10 ± 0.76	12.13 ± 0.32	0.00 ± 0.00	0.00 ± 0.00	24.7 ± 0.64
*S. pneumoniae***	0.80 ± 0.26	1.13 ± 0.32	0.00 ± 0.00	11.13 ± 0.40	17.50 ± 0.50	12.00 ± 0.50	20.0 ± 1.74

Notes: Values reported as mean ± standard deviation. Mean of three experiments. Zone in mm indicates the distance from the border of the disc to the edge of the clear zone*Gram-negative bacteria.
****Gram-positive bacteria.**

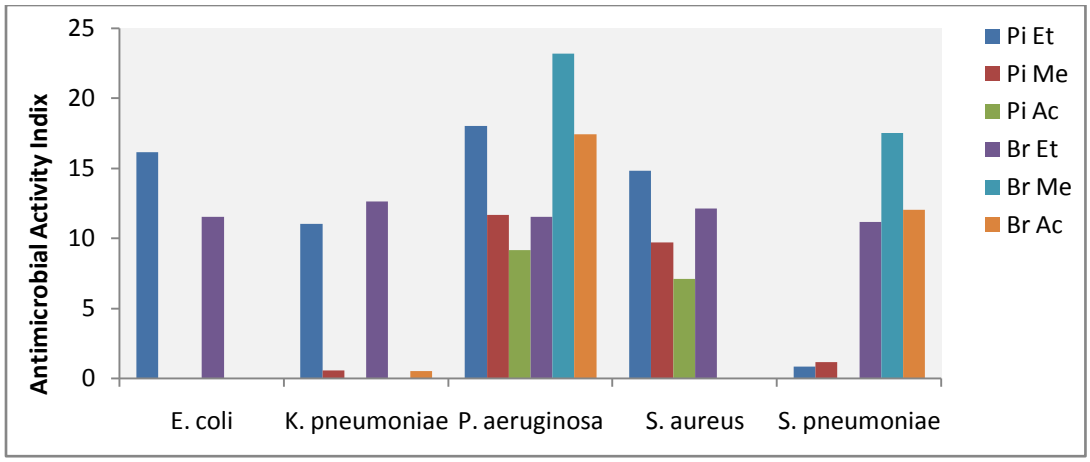

Figure (3). antimicrobial activity indices of *P. radiata* and *B. variabilis* extracts against test micro-organisms. Notes: *PiEt— P. radiate Ethanol extracts, PiMe—P. radiate Methanol extracts, PiAc— P. radiate Acetone extracts, BrEt— B. variabilis Ethanol extracts, BrMe— B. variabilis Methanol extracts, BrAc— B. variabilis Acetone extracts.*

The broth dilution test was used to determine the MIC of the extracts against the test microbes. The results are presented in Table 4. The least MIC of 1 mg/mL was recorded by the Ethanol extracts of *P. radiata* for *E. coli* and *P. aeruginosa.* methanol extracts of *B. variabilis* also recorded low MIC (1 mg/mL) towards *P. aeruginosa*.

A high AI suggests better inhibitory capacity. Methanol extracts of *P. radiate* (AI of 0.9) had lower activity than Ethanol extracts of *P. radiata* (AI of 1.4). In the same vein, methanol extracts of *B. variabilis* (AI of 0.9) was lower in activity when compared with Ethanol extracts of *B. variabilis* (AI of 1.6). Interestingly, Ethanol extracts of *B. variabilis* was slightly more active than Ethanol extracts of *P. radiata*. Methanol extracts from both *B. variabilis* and *P. radiata* had about the same overall activity on the microbes tested. Ethanol extracts of *B. variabilis* had greater overall activity against all test microbes as depicted in Figure 4.

Figure 2 represents the antimicrobial activity index of the various extracts towards the different classes of microbes tested (Gram-positive and Gram-negative bacteria). With respect to Gram-negative bacteria, ethanol extracts were twice as active as their corresponding methanol and Acetone extracts. The same trend is observed in Gram-positive bacteria, but the difference between ethanol and methanol, ethanol extracts is not as pronounced as in Gram-negative bacteria. Statistically, however, there were no significant differences ($p > 0.05$) between the activity indices of all six extracts.

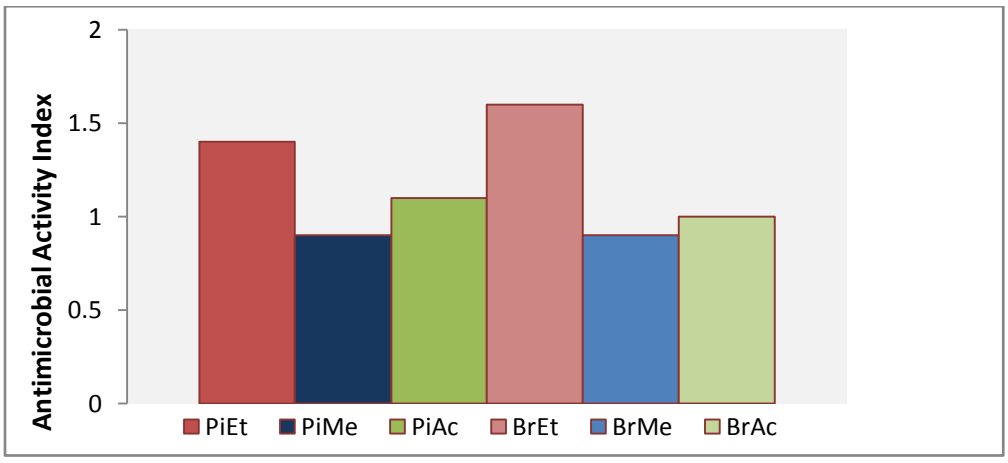

Figure (4). antimicrobial activity indices of *P. radiata*and *B. variabilis* extracts against test micro-organisms. Notes: *PiEt— P. radiate Ethanol extracts, PiMe—P. radiate Methanol extracts, PiAc— P. radiate Acetone extracts, BrEt— B. variabilis Ethanol extracts, BrMe— B. variabilis Methanol extracts, BrAc— B. variabilis Acetone extracts.*

Table 4. MIC of ethyl acetate and methanol extracts of *L. littorea* and *G. paradoxa* against test

Test micro-organisms	*Minimum inhibitory concentrations (mg/mL)*					
	P. radiata			*B. variabilis*		
	Ethanol	**Methanol**	**Acetone**	**Ethanol**	**Methanol**	**Acetone**
*E. coli**	1.0	ND	ND	5.0	ND	ND
*K. pneumoniae**	15.0	15.0	15.0	5.0	25.0	ND
*P. aeruginosa**	1.0	5.0	5.0	5.0	1.0	5.0
*S. aureus***	5.0	5.0	5.0	5.0	15.0	ND
*S. pneumoniae***	5.0	15.0	5.0	5.0	5.0	5.0

Note: ND—no microbial growth inhibition within the range of concentrations tested (*n* = 2).*Gram-negative bacteria. **Gram-positive bacteria.

Antimicrobial inhibitory studies for Gram-negative and Gram-positive bacteria were investigated in this work using six different extracts: three from *G. paradoxa* and another three from *L. littorea*. Because of their benthic nature, many aquatic invertebrates are potentially susceptible to microbial attack. In order to defend themselves, these organisms have evolved a very efficient chemical defense system. Due to the unique environment in which they are produced, secondary metabolites from aquatic sources are particularly well adapted for use in drug discovery programmes. Chemicals released by these organisms are invariably diluted following release into the water and must therefore be very potent to have any significant effect ([8], [9], [7]). Many classes of natural products have been isolated from aquatic sources. These include lipids, amino acids, macrolides, nucleosides and terpenoids, among others. The activity of these compounds ranges from antimicrobial to antitumor to anti-inflammatory to antioxidant [7]. Ethanol extracts showed much greater activity towards the microbes tested. This can be seen in Figure 4 where Ethanol extracts had higher antimicrobial activity index than their methanol and acetone counterparts. Interestingly, Ethanol extracts of *B. variabilis* was slightly higher in activity (AI of 1.6) when compared with Ethanol extracts of *P. radiate* (AI of 1.4). Ethanol extracts of various marine molluscs have been shown to possess significant antimicrobial activity. Re. [27] investigated the antimicrobial activities of various marine cowries and ascidians against some pathogenic micro-organisms. The tests showed both methanol and ethyl acetate extracts to possess some degree of antimicrobial activity, with ethyl acetate fractions slightly more potent [27]. In another test of antimicrobial activity, ethyl acetate extracts of the Sydney rock oyster, *Saccostrea glomerata*, proved to be much more potent than hexane or methanol extracts. Ethanol extracts proved to be rich sources of fatty acids which the authors speculated to be responsible for the observed antimicrobial activity [28]. Because of their lipophilic nature, fatty acids and lipids will most likely be found in the less polar ethanol extracts than polar methanol extracts and probably account for the high AI of ethanol fractions observed in this work. Overall, there was slightly higher activity by ethanol extracts (Ethanol extracts of *P. radiate* and Ethanol extracts of *B. variabilis*) towards Gram-negative bacteria than Gram-positive bacteria. The reverse was true for methanol extracts, where much more activity was observed for Gram-positive bacteria than the Gram-negative ones. *P. aeruginosa* was inhibited the most by the extracts. The highest inhibition zone was recorded by methanol extracts of *B. variabilis* towards *P.aeruginosa*. In a similar study, methanol extracts of *Pernaviridis*, *Neritaalbicilla* and *Oziusrugulosus* yielded the best results towards a set of test micro-organisms, with *P. aeruginosa* being the most susceptible micro-organisms towards the extracts [29].

IV. CONCLUSION

In the present study, the antimicrobial activities of methanol, ethanol and acetone extracts of two molluscs, *P. radiata*, *B .variabilis*, were investigated. The results from the study showed significant antibacterial activity compare with other solvents extraction. These extracts could be further purified to isolate and characterize the compounds responsible for the observed activities. This work further provides credence to the notion that the aquatic environment is a rich source of bioactive compounds

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