IJPPR
INTERNATIONAL JOURNAL OF PHARMACY & PHARMACEUTICAL RESEARCH
An official Publication of Human Journals
ISSN 2349-7203
HUMAN

Human Journals
Research Article
October 2017 Vol.:10, Issue:3

Antimicrobial Activities of the Tissue Extracts of *Phorcus turbinatus* (*Monodonta turbinate*) (Born, 1780) (Mollusca: Gastropoda) from, East Coast of the Mediterranean

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ISSN 2349-7203
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Submission: 27 September 2017
Accepted: 5 October 2017
Published: 30 October 2017

Keywords: Gastropods, Antimicrobial Activity, natural products, *Phorcus turbinatus*, Nosocomial Pathogens.

ABSTRACT

The antibacterial effect of crude extract of marine gastropods (*Phorcus turbinatus)* against six nosocomial bacteria including, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Acinetobacter septicus*, *Proteus vulgaris*, *Klebsiella pneumoniae* and *Escherichia coli* were studied by Kirby-Bauer disc diffusion assay and, the findings of antibacterial activity of methanol and ethyl acetate crude extract of gastropods (*Phorcus turbinatus*)were compared to the efficiency of some marketed antibiotics that were tested against the same bacteria at given concentrations. It was observed that ethyl acetate and methanol crude extract of *Phorcus turbinatus* is effective against *Klebsiella pneumoniae*, *Acinetobacter meningitis*, *Staphylococcus aureus* and *Proteus vulgaris* and except for its ineffectiveness against *Pseudomonas aeruginosa*. It was more active than Amoxicillin and Gentamicin against *Proteus vulgaris* and more efficient than Ciprofloxacin against *Acinetobacter meningitis.* Hexane crude extracts of *Phorcus turbinatus* didn't show any activity against all bacterial pathogens. *Phorcus turbinatus* remains an interesting source of new antibacterial metabolites with better activity than some antibiotics.

1. INTRODUCTION

Around 70% of the Earth's surface is covered by the oceans which occupy 90% of the biosphere. Marine species make up around half of the total global biodiversity and they have been extensively explored in the last decades for potential sources of new bioactive natural products(Aneiros & Garateix, 2004). Marine environment is a huge source to discover bioactive natural products. A wide variety of bioactive substances are being isolated and characterized from the food that is derived from the marine environment, several with great promise for the treatment of human and fish disease. Because of the difficulties in exploring deepwater habitats, many bioactive natural products have yet to be isolated, identified and characterized, thus the oceans constitute a rich source of novel compounds(Wright, 1998). Other selective pressures that could lead to the evolution of bioactive metabolites in the marine environment (Benkendorff, McIver, & Abbott, 2010).The discovery of these bio-regulated roles together with elucidation of the mechanisms of action of the marine peptides would advocate the peptides to be used as potential drugs for cancer, diabetes or hypertension treatment. Marine invertebrates offer a source of potential antimicrobial drugs (Aneiros & Garateix, 2004; Rajaganapathi, 1996).

For the past two decades, pharmaceutical industry has been relatively successful in overcoming problems due to single resistant determinants. However, the advent of multiple resistant mechanism has limited the use of many major classes of antimicrobial compounds. Among the marine invertebrates, the mollusks are the potential source of bioactive substances. The bioactive compounds isolated from the gastropods are considered to have a role in chemical defense of the animals against their predators. Since the 1960's the interest in the marine environment has greatly increased and more or less 10,000 pharmacologically bioactive compounds have been derived from marine invertebrates (Simmons, Andrianasolo, McPhail, Flatt, & Gerwick, 2005).

In recent years, The human pathogenic microorganisms have developed natural resistance in response to the indiscriminate use of commercial antimicrobial drugs commonly employed in the treatment of infectious diseases (Stinson, 1996), and some bacterial strains are resistant to all existing antibiotics (Chellaram, Gnanambal, & Edward, 2004). Moreover, cost of production of synthetic drugs is also high and they cause adverse effect when compared to bioactive naturally defense of the animals derived drugs. There is a vital interest in

discovering new antimicrobial compounds with fewer environmental and toxicological risks and without resistance developed by the pathogens.

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 (Kamiya *et al.*, 1989; Prem Anand, Rajaganapathi, & Edward, 1997), and antiparasitic activities (Grabley & Thiericke, 1999; Simmons *et al.*, 2005),. Antimicrobial peptides are important in the first line of the host defense system of many marine species (Aneiros & Garateix, 2004). The broad bioactivity spectrum of marine peptides medicinal values which attract the attention of the pharmaceutical industry, hoping that they can be used in treatment or prevention of various diseases. Although whole body homogenates of some marine mollusks have been reported contain a variety of antimicrobial compounds. Mantle cavity of many mollusks produces mucus e.g. *Muricid gastropods* (rock snail) which defend the developing larvae against microbial infection (Kumaran, Bragadeeswaran, & Thangaraj, 2011). Most marine mollusks are sessile soft bodies that investigate the antimicrobial effectors like in the extract of marine mollusk *Phorcus turbinatus* against isolated human pathogens.

2. MATERIALS AND METHODS

2.1. Preparation of Gastropoda:

Live individuals of *P. turbinatus* species (Super Family: Trochidae) were collected near Ibn-Hani (lat: 35 35'.173N; long: 35 43'.034E) from coastal waters of Latakia city. 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 (Narayanasamy, 1995).

2.2. Preparation of *P. turbinatus* Extracts:

Wet sample of *P. turbinatus* was 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, ethyl acetate and hexane. The amount of each solvent was approximately 100 ml. The samples were soaked in methanol three times overnight, then they were immersed in

ethyl acetate three times overnight and once in hexane 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, ethyl acetate and hexane were obtained then it was used for the experimental work. Individually, the extract mixtures were rotavapped under vacuum (Becerro, Lopez, Turon, & Uriz, 1994; Wright, 1998). 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 (Narayanasamy, 1995).

2.3. 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.

2.4. Antibacterial Activity:

The crude extracts were used for antimicrobial activity assay against human bacterial pathogens (*Pseudomonas aeruginosa, Klebsiella pneumoniae, Staphylococcus aureus, Escherichia coli* and *Proteus mirabilis*). 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)	*Acinetobacter meningitis*
CSF (Cerebral spinal fluid)	*Klebsiella pneumoniae*
Umbilicus swab	*Staphylococcus aureus*
Blood	*Proteus vulgaris*
Gastric secretion (neonate)	*Pseudomonas aeruginosa*

2.5. Kirby-Bauer Antimicrobial Assay:

Antimicrobial assay for methanol, ethyl acetate and hexane extracts of *P. turbinatus* was carried out by disc diffusion method followed by Kelman *et al.,* (Kelman *et al.,* 2001). 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 with 50 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) (Concepcion, Caraan, Lazaro, & Camua, 1994). 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.

2.6. Collection of Data

The day after, inhibition zone diameters were measured and the results were scored as follow:

1. - (no zone of activity),

2. + (8-10 mm diameter zone of activity),

3. ++ (11-15 mm diameter zone of activity)

4. +++ (>15 mm diameter zone of activity).

On the other hand, the results were compared to reference values currently used antibiotics that belong to different groups:

- Ceftriaxone (30 µg per disc) β-lactam group, Cephalosporin 3rd generation.
- Gentamicin (10 µg per disc) an aminoglycoside.
- Amoxicillin (10µg per disc) as trihydrate.

The antibiotics were chosen according to the pathogen, disease and age (Scherbaum *et al.,* 2014).

3. RESULTS

3.1 Gastropoda Description:

Mediterranean gastropod *P. turbinatus* belongs to the class of (Gastropoda superfamily: Trochidae). The size of the shell varies between 15 mm and 43 mm. The very solid and thick, imperforate shell has a conical shape. Generally, it is heavy and thick and has an elongate-conical shape. It is whitish, tinged with gray, yellowish or greenish, spirally traversed by bands composed of alternating white and black purplish or red spots. The intervals between the bands are longitudinally closely lamellate with blackish. The spire is elevated. The shell contains about 6 whorls. The upper ones are slightly convex, the last generally constricted and concave below the suture, then convex, the base of the shell is eroded in front of the aperture. The aperture is very oblique. The thick, smooth outer lip is beveled to an edge. It is pearly and iridescent within. The columella is flattened on the face, bluntly lobed within, pearly, backed by an opaque white layer (Donald *et al.*, 2012). Nomenclature of this species follows WoRMS Editorial Board (2017) (WoRMS, 2017).

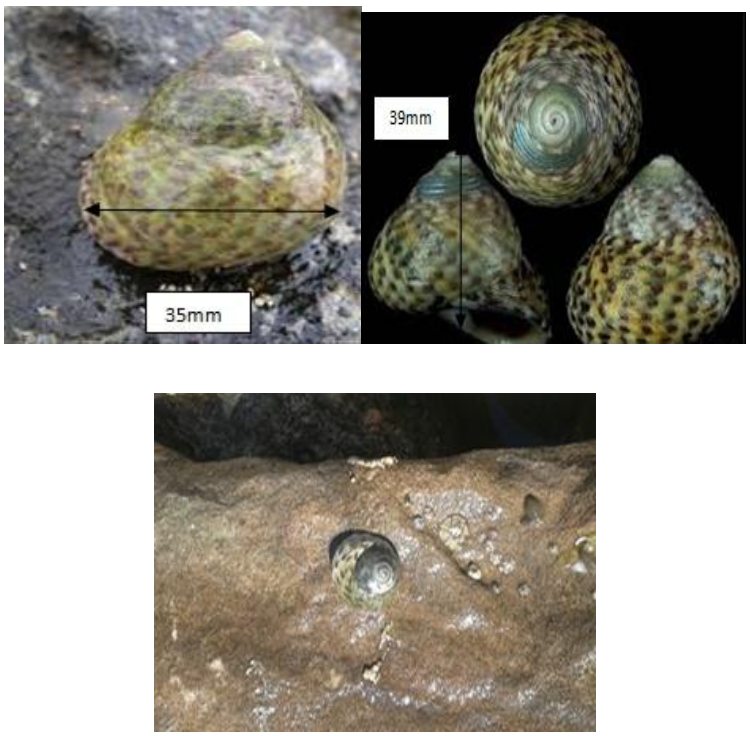

Figure 1*:* Morphological Description of Gastropoda *Phorcus turbinatus (Monodonta turbinatus)* (Born, 1780)

Table 2: The Classification and description of the Gastropoda samples

Kingdom:	**Animalia**
Phylum:	Mollusca
Class:	Gastropoda
Clade:	Vetigastropoda
Superfamily:	Trochoidea (superfamily)
Family:	Trochidae
Genus:	*Phorcus*
Species:	*P. turbinatus*
Binomial name	*Phorcus turbinatus* (Born, 1780)

3.2. Antimicrobial activity

All three crude extracts from gastropod *P. turbinatus* were screened against five human pathogenic bacteria for testing their antimicrobial activities. The inhibition zones of ethyl acetate, methanol and hexane extracts were given in Table 3.

The inhibition zone (25 mm) was observed against *P. vulgaris* with 50 µl from crude ethyl acetate extract of *P. turbinatus* and (9 mm) with 50 µl from crude methanol extract (Figure 6).

The inhibition zone (7 mm) was recorded against *S. aureus* with 50 µl from crude ethyl acetate and methanol extract of *P. turbinatus* (Figure 7). Ethyl acetate extract was able to give an inhibition zone of 22mm against *A. meningitis* and zone of 19 mm against *K. Pneumoniae* (Figure 3). Ethyl acetate extract was able to produce a zone of 9 mm against *P. aeruginosa.* However, only slight activity was shown by the crude extract of hexane *(*Figure 4*)*. Comparatively, as recorded in Table 3, the antibiotic Ceftriaxone showed maximum activity against *P. vulgaris* (20 mm) (Figure 6). The Gentamicin exhibited high activity against *S. aureus* (17 mm) (Figure 7). Also, Gentamicin showed activity against *P. vulgaris* (13 mm) (Table 3 and Figure 6).

Ceftriaxone, Gentamicin and Amoxicillin had weak activity against *P. aeruginosa*, *K. pneumoniae* and *A. meningitis*. Amoxicillin 10µg and Gentamicin 10µg exhibited moderate activity against *P. vulgaris* (12 mm and 13 mm respectively) Table 4 and Figure 4.

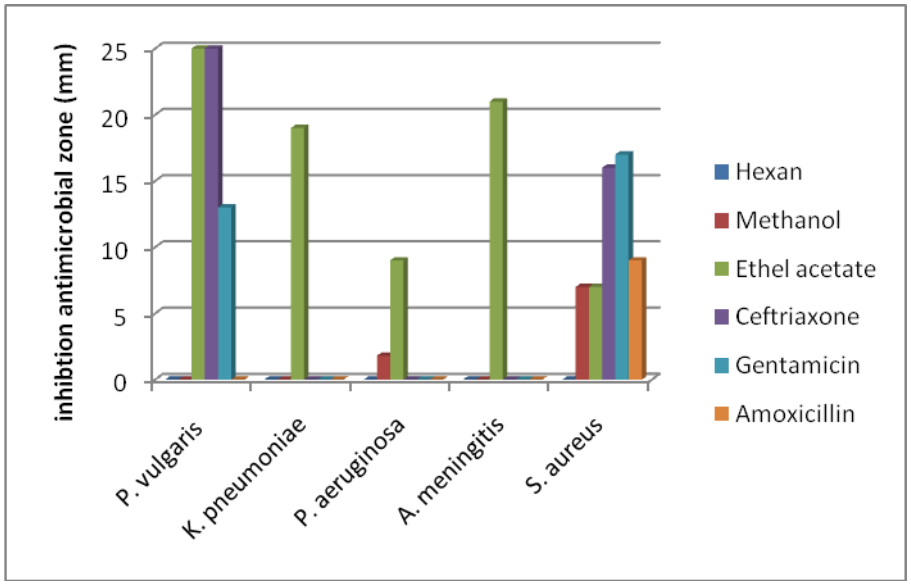

Figure 2: Antibacterial activity of different extracts from *P. turbinatus*

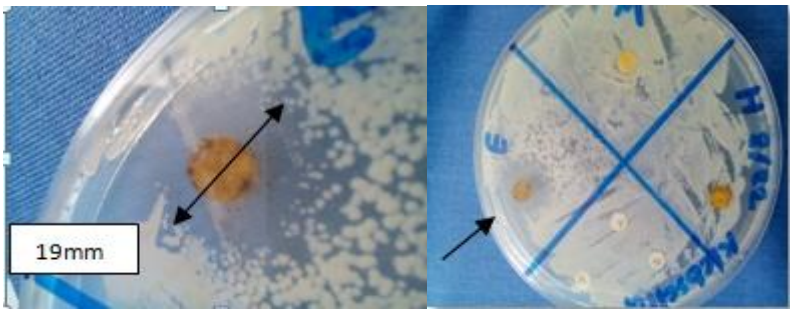

Figure (3): *K. pneumoniae* susceptibility to crude ethyl acetate (E), methanol(M) and hexane(H) extract of *P. turbinatus* Comparatively, as recorded in Figure the antibiotic Ceftriaxone, Gentamicin and Amoxicillin.

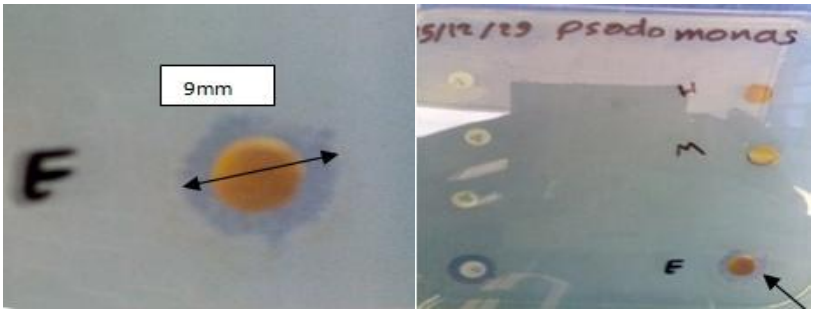

Figure (4): ***P. aeruginosa* susceptibility to crude ethyl acetate (E), methanol(M) and hexane(H) extract of *P. turbinatus* Comparatively, as recorded in Figure the antibiotic Ceftriaxone, Gentamicin and Amoxicillin**

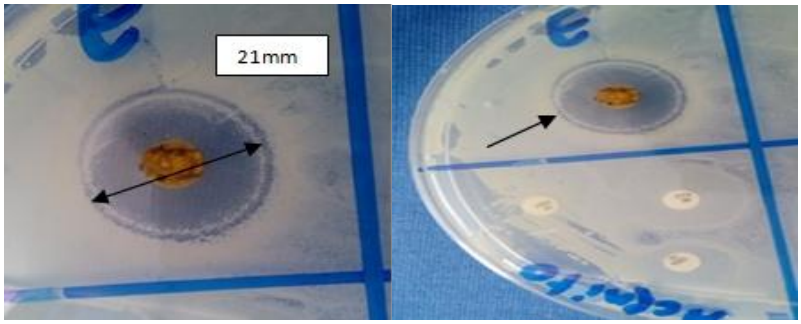

Figure (5): *A. meningitis* susceptibility to crude ethyl acetate (E), methanol (M) and hexane(H) extract of *P. turbinatus* Comparatively, as recorded in Figure the antibiotic Ceftriaxone, Gentamicin and Amoxicillin.

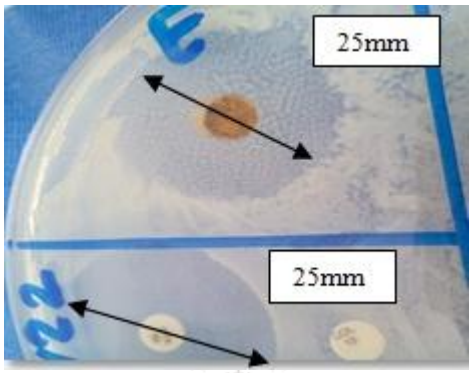

Figure (6): *P. vulgaris* susceptibility to crude ethyl acetate (E), methanol(M) and hexane(H) extract of *P. turbinatus* Comparatively, as recorded in Figure the antibiotic Ceftriaxone, Gentamicin and Amoxicillin.

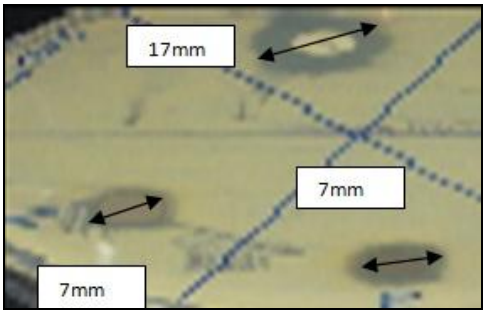

Figure (7): *S. aureus* susceptibility to crude ethyl acetate (E), methanol(M) and hexane(H) extract of *P. turbinatus* Comparatively, as recorded in Figure the antibiotic Ceftriaxone, Gentamicin and Amoxicillin

Table 3: Antibacterial sensitivity of *P. turbinatus* extract and Ceftriaxone, Gentamicin and Amoxicillin against human pathogens.

Pathogens	Hexane	Methanol	Ethel acetate	Ceftriaxone	Gentamicin	Amoxicillin
Proteus vulgaris	–	–	>21 +++	>21 +++	13 ++	–
Klebsiella pneumoniae	–	–	19 ++	–	–	–
Pseudomonas aeruginosa	–	–	9 +	–	–	–
Acinetobacter meningitis	–	–	>21 +++	–	–	–
Staphylococcus aureus	–	7 +	7 +	16 ++	17 ++	9 +

4 DISCUSSION

More than hundred new antimicrobial compounds were isolated every year from marine invertebrates, such as gastropods and bivalves which shows broad spectrum of antimicrobial properties (Bartlett *et al.*, 2002; Kiran, Siddiqui, Khan, Ibrar, & Tushar, 2014). In recent years, significant researches have done antibacterial activities of mollusk. More than thousand new compounds have been categorized from marine invertebrates such as peptides, terpenes, polypropionates, nitrogenous compounds, polypeptides, macrolides, prostaglandins and fatty acid products, sterols and diverse compounds (Kiran *et al.*, 2014).

Many of these organisms have been antimicrobial properties, although most of the antibacterial agents that have been isolated from marine sources have not been active enough to complete with classical antimicrobial obtained from microorganisms (Dhinakaran, Sekar, Sethubathi, & Suriya, 2011).

References show that gastropoda antimicrobial extract attacks some bacteria and retains its activity against resistant bacteria to conventional antibiotics (Mancini, Defant, & Guella, 2007).

In this study, the antimicrobial activity of crude ethyl acetate and methanol extracts of gastropod *P. turbinatus* evaluated against some hospital-acquired pathogens: *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter septicus*, *Proteus vulgaris* and *Pseudomonas aeruginosa*. Different concentrations of various commercial antibiotics, (Ceftriaxone, Gentamicin and Amoxicillin) were used to assay their antimicrobial activity against the bacterial pathogens.

Ethyl acetate extracts showed much greater activity towards the microbes tested. This can be seen in Figure 1, where ethyl acetate extracts had higher antimicrobial activity index than their methanol counterparts. The maximum inhibition zone was observed against *P. vulgaris*. The minimum inhibition zone crude ethyl acetate was recorded against *S. aureus*. However, only slight activity was shown by the crude extract of hexane. Ethyl acetate crude extract of *P. turbinatus* is effective against *Proteus vulgaris*, *Acinetobacter meningitis* and *Klebsiella pneumoniae* and in effective against *Pseudomonas aeruginosa*. While Ethyl acetate crude extract showed the same degree of activity, they were more active than Gentamicin and Amoxicillin against Gram negative bacteria *Acinetobacter meningitis*. In addition, Gram negative bacteria *Proteus vulgaris* revealed high susceptibility against ethyl acetate and methanol extract of *P. turbinatus*. Comparatively, Ceftriaxone had higher antibacterial activity than Gentamicin and Amoxicillin against *P. vulgaris*.

Comparatively, ethyl acetate extract of *P. turbinatus* showed higher activity than Ceftriaxone, Gentamicin and Amoxicillin respectively against *Proteus vulgaris* and *Klebsiella pneumoniae*. This extract was as efficient as Gentamicin, ceftriaxone and Amoxicillin against *P. aeruginosa.*, (Annamalai *et al* (2007)) Green Mussel (*Perna viridis*) against *S. aureus* and *E. coli*.

Similar study was carried out by Anand & Edward (2002), that noticed highest antibacterial activity with extracts of tissue from five species of *Saccostrea glomerata* (Mollusca: Gastropoda), (Tunicata: didemnidae). And Jayaseeli *et al.* (2001) found antibacterial activity of Hemifusus pugilinus against few pathogens and the extracts showed significant activity against *Bacillus subtilis*. Antibacterial activity of gastropods against *S. typhi* was reported by Rajaganapathi (1996). Also supporting present study on antibacterial activity of bivalve extracts (Anand & Edward, 2001).

As an interesting activity has been discovered in these mollusks, the bioactive compound nature needs to be specified through more purification steps and should lead to further studies relating to their antibacterial modes of action. Otherwise, a microbial origin of these active compounds cannot be ruled out.

5 CONCLUSION:

In conclusion in the present study indicates that the good source of antibacterial activity agents and would replace the existing inadequate and cost effective antibiotics.

Commercial antibiotics are highly effective to kill the bacterial pathogens involved in common infection. Methanol, ethyl acetate and hexane extracts of gastropods in this study showed significant antibacterial activity compare with other solvents extraction. It is worthy to note that the product from natural source is good for health and to avoid side effects.

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