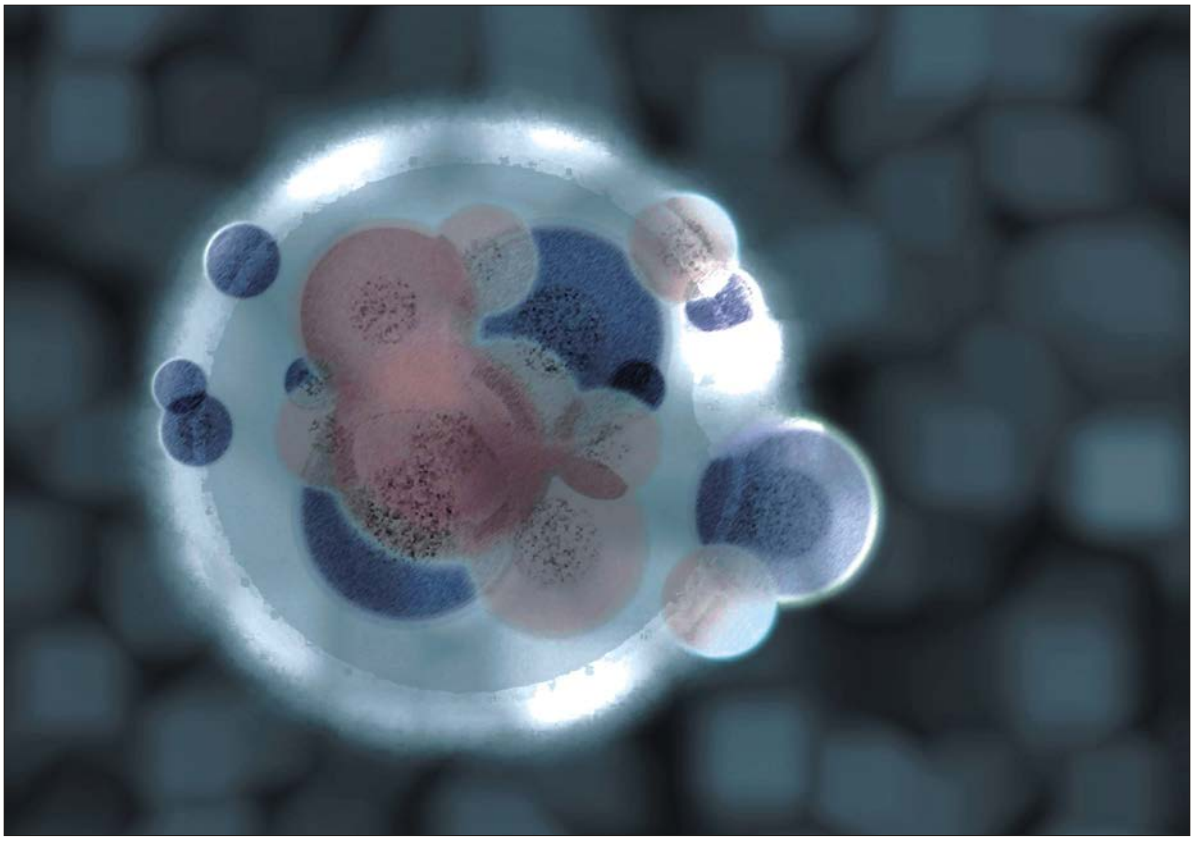

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Antagonistic and Cyto-Toxicity Activity of Mollusc Methanol Extracts

S. Kanchana, R. Vennila, K. Rajesh Kumar, M. Arumugam and T. Balasubramanian
CAS in Marine Biology, Faculty of Marine Sciences, Annamalai University,
Parangipettai, Tamil Nadu, India

Abstract: Marine mollusc paved a way for the isolation of bioactive compounds. Therefore, the present study was to investigate the Antimicrobial and cytotoxicity activities on 25 mollusc samples. The antibacterial activity was conducted against six human pathogens and anti fungal activity was tested against two fungal strains by the disc diffusion method. Further, *in vitro* toxicity assays such as hemolytic activity was done in microtiter plates method and Brine shrimp toxicity assay were tested against *Artemia* µg. The potent antibacterial activities were found against *Staphylococcus aureus*, *Vibrio cholera* and *Salmonella paratyphi* and the high degree of anti fugal activity in *Aspergillus fumigatus* than *Fusarium* sp. The hemolytic activity was detected in *Meretrix casta*, *Sepia officinalis*, *Lophiotoma indica*, *Octopus vulgaris*, *Sepia officinalis* ink, *Ficus ficus*, *Xancus pyrum*, *Natica picta*, *Turrittella attenuata*, *Architectonica* sp. The Brine Shrimp toxicity (LC_{50}) was also found in *Conus betilinus*, *Hemifusus pugilinus*, *Burselltalla leachi*, *Lambis lambis*, *Ficus ficus*, *Conus adustus*. The results revealed that mollusc samples are a promising source for identifying novel drug lead compounds.

Key words: Antibacterial, antifungal, marine mollusca, toxicity studies

INTRODUCTION

The marine environment comprises of complex ecosystem with a vast array of organisms and many of these organisms are known to possess bioactive compounds (Lijima *et al*., 1995). Marine natural products have been explored mostly for their antimicrobial, cytotoxic, antitumor and anti-inflammatory properties (Patterson *et al*., 2000). The biodiversity of marine organism reveals the way to discovery new bioactive substances. Marine invertebrates provide significant antimicrobial drugs (Bansemir *et al*., 2006; Bazes *et al*., 2009; Jayaraj *et al*., 2008; Mayer *et al*., 2007). Among the marine invertebrates the Mollusc constitute many representatives which are widely distributed throughout the world for isolating the biomedical important products (Halstead, 1965). Consequently, reported that mollusc constitute bioactive compounds with promising activities includes antitumor, antileukemic and antiviral (Kamiya *et al*., 1989; Pettit *et al*., 1987; Anand *et al*., 1997; Rajaganapathi *et al*., 2002).

A wide variety of anti-microbial factors have been isolated and characterized from the mollusc which includes chlorinated acetylenes (Walker and Faulkner, 1981), terpenes (Ireland and Faulkner, 1978), Indole derivates (Benkendorff *et al*., 2001), glycerol derivates (Gustafson and Andersen, 1985), macrolides (Matsunaga *et al*., 1986), lysosymes (Nilsen *et al*., 1999) glycoprotein (Yamazaki, 1993), proteins (Kamiya *et al*., 1989), peptides (Iijima *et al*., 2003) and Polysaccharide (Shanmugam *et al*., 2008). Mollusc has being act as a untapped source for bioactive compounds for discovering a promising lead compounds for the discovery of antimicrobial therapeutics for the pharmaceutical use. Many of this anti-microbial therapeutics should be specific for prokaryotes and non-toxic to eukaryotic cells (Hancock and Rozek, 2002) for the development of lead compounds.

Considering the potential use of mollusc the present study has been made to ascertain the antimicrobial activity of whole body extract of bivalves, gastropod, cephalopods and some of the ink fluid of cephalopods. Here screened antimicrobial activity and the cytotoxic potential of the different Mollusc samples.

MATERIALS AND METHODS

Animal collection and sample preparation: Mollusc specimens (25 species) were freshly collected from eight different stations in the east coast of Tamil Nadu at 0.5-2.0 m depths from the sandy areas. The collected animals were immediately kept into ice box at 4°C, transferred to the laboratory, identified and stored in the deep freezer at -40°C until further use.

Corresponding Author: S. Kanchana, Faculty of Marine Science, CAS in Marine Biology, Annamalai University, Parangipettai, Tamil Nadu, India Tel: +9104144-243070 Fax: 04144-243641

The whole body of the animals was cut into small pieces, homogenized with methanol and kept in the deep freezer at -18°C overnight. Samples were centrifuged at 5000 rpm for 15 min and supernatant was collected. The solvent present in the supernatant was evaporated by using rotary evaporator. The condensed samples were lyophilized. The dried crude samples were mixed with Phosphate buffered saline in the concentration of 5 mg mL^{-1} and used for further analysis.

Estimation of protein concentration: Protein concentration was determined by the method of Lowry *et al.* (1951) by using Bovine Serum Albumin (BSA) as standard.

Microorganisms and inoculum preparation: In the present study, the micro organism such as molds (*Fusarium* sp. and *Aspergillus fumigatus*) bacterial strains were used which included two strains of gram positive bacteria *Pseudomonas aeruginosa* and *Staphylococcus aureus*; four strains of gram-negative bacteria *Vibrio cholerae*, *Salmonella paratyphi*, *Shigella dysenteriae*, *Klebsiella pneumoniae*. All micro organisms were obtained from the Department of Microbiology, Annamalai University, India.

Nutrient broth and Sabouraud Dextrose Agar (SDA) was used for growing and diluting the microorganism suspensions. Bacterial strains was inoculated and were grown to exponential phase in nutrient broth at 37°C for 18 h and adjusted to a final density of 10^8 CFU mL^{-1} by diluting fresh cultures by comparing with McFarland density. *Fusarium* sp. and *A. fumigatus* was aseptically inoculated on Petri plates containing sterile SDA medium. After inoculation the petri-plates was incubated at 28°C for 48 h and the colonies were aseptically sub cultured on SDA slants. The mold colonies from SDA slants were suspended in sterilized saline and compared with McFarland solution. The final concentration should be adjusted to 2×10^7 cells mL^{-1}.

Microbial sensitivity test: Inhibition of bacterial growth was determined as earlier method by Bauer *et al.* (1966). Sterile swabs were immersed in the microbial suspensions (10^8 cells mL^{-1}) and evenly applied to Petri dishes containing Mueller Hinton agar. Sterile whatman No. 1 filter paper discs (6 mm in diameter) were fully incubated with 30 μL of 5 mg mL^{-1} concentration of samples and disc were allowed to dry at room temperature and placed over the agar in the plates. Erythromycin 15 μg $disc^{-1}$ and chloramphenicol 30 μg $disc^{-1}$ were used as positive control the plates were incubated overnight at 37°C and then examined for zone of growth inhibition around each disc.

Growth inhibition of isolated samples against *A. fumigatus* and *Fusarium* sp. was determined as described by Roberts and Selitrennikoff (1990). Briefly, agar assay plates were prepared by autoclaved Potato Dextrose Agar (PDA). Sterile swabs were immersed in the microbial suspensions and uniformly applied to Petri dishes containing PDA. Sterile what man No 1 filter paper disc (6 mm) were fully absorbed with 30 μL of the samples (5 mg mL^{-1}) and placed over the agar in the plates. Nystatin was used as positive control and it was incubated at 37°C for 48-72 h. Plates were examined as described by the antibacterial assay.

Hemolytic assay: The hemolytic activity of the crude samples was assessed by the micro hemolytic method (Pamprasad and Venkateshvaran, 1997). In brief, chicken blood was collected in the tube containing 4% Tri sodium citrate solution in 1:9 ratio from slaughter house and brought to the laboratory. The blood was centrifuged at 5,000 rpm for 5 min; the supernatant were discarded, the pellet was suspended in normal saline (pH 7.4). The mixture was further centrifuged at 5,000 rpm for 5 min, the supernatant was discarded and pellet was resuspended in normal saline (pH 7.4). This procedure was repeated thrice. From these, 1% erythrocyte suspension was prepared by adding 99 mL normal saline to 1 mL of packed RBC (Red Blood Cells).

The micro hemolytic test was performed in 96 well 'V' bottom microtiter plate. Normal saline (100 μL) was added in all the wells, crude samples (100 μL) were added in the first well and it was serially diluted up to the last well. RBC suspension of 1% was added into all the wells with appropriate controls were included in the test. RBC suspension of 100 μL was added with distilled water of 100 μL, which served as a positive control and negative control with normal saline (100 μL). The plate was allowed to stand for 3 h at room temperature and the results were recorded. Uniform red color suspension in the wells considered as positive hemolysis and a button formation in the bottom of the wells was considered as lack of hemolysis. Reciprocal of the highest dilution of the crude extract showing the hemolytic pattern was taken as one Hemolytic Unit (HU).

Brine shrimp lethality assay: The toxic effects of isolated samples were tested against *Artemia najplii* (Carvalho *et al.*, 1988). Brine shrimp eggs were hatched in dish filled with sea water. Freshly hatched phototrophic larvae (*Nauplii*) were collected with a pipette and transferred (10 shrimps) to vials filled with sea water (5 mL). The different concentration of sample was added to the vials. In the case of control, a group of vials was filled with sea water. The vials were kept illuminated

during 24 h of contact with the substances and survivals were counted with the aid of a magnifying glass. This assay was carried out three times with five replicates for each fluid concentration. Further, calculated the LC_{50} (mean lethal concentration) values the results were plotted as logit % mortality vs. log concentration. Logit is defined as in (% mortality/% survival).

RESULTS

Antibacterial activity: The *in vitro* anti-bacterial activity of mollusc samples was shown in Table 1. Antibacterial activities with a sample concentration of 5 mg mL^{-1} were compared, the wide differences were observed depends on various samples. Of the six pathogens *V. cholerae* and *S. paratyphi* was the most sensitive than that of *S. aureus*. All other pathogens were resistant to the samples.

Highest antibacterial activity was found in the *C. betulinus* (15 mm) against *S. aureus*. *L. chiragra* and *H. pugilinus* showed the growth inhibition of 13 mm against *S. paratyphi*. The highest activity was exhibited by *L. chiragra* against *V. cholerae*. Moderate activity was observed in *P. virdis* (7 mm), *S. officinalis* (7.5 mm), *T. attenuata* (8 mm), *Architectonica* sp. (8.5 mm) against *V. cholerae* and *S. officinalis* (7.5 mm), *X. pyrum* (8 mm) against *S. paratyphi*. Apart from these no antibacterial activity was detected in the remaining samples.

All pathogens were sensitive to the positive control Erythromycin (15 μg $disc^{-1}$). Chloramphenicol (30 μg $disc^{-1}$) inhibited all the pathogen except *P. aeruginosa* and *S. dysenteria*.

Table 1: Antibacterial activity of methanol extract of Molluscan samples

	Pathogen					
Samples	I	II	III	IV	V	VI
Babylonia zeylanica	-	-	-	-	-	-
Perna virdis	-	++	-	-	-	-
Meretrix casta	-	-	-	-	-	-
Conus loroisii	-	-	-	-	-	-
Conus betulinus	+++	-	-	-	-	-
Lambis chiragra	-	++	-	+++	-	-
Sepia officinalis	-	+	-	-	-	-
Lophiotoma indica	-	++	-	-	-	-
Hemifuses pugilinus	-	-	-	+++	-	-
Strombus urceus	-	-	-	-	-	-
Bursatella leachi	-	-	-	-	-	-
Octopus vulgaris	-	-	-	-	-	-
Strombus listeri	-	-	-	-	-	-
Murex tributa	-	-	-	-	-	-
Ficus ficus	-	-	-	-	-	-
Conus adustus	-	-	-	-	-	-
Sepia officinalis ink	-	-	-	-	-	-
Lambis lambis	-	-	-	-	-	-
Meretrix meretrix	-	-	-	-	-	-
Octopus vulgaris ink	-	-	-	-	-	-
Xancus pyrum	-	-	-	++	-	-
Nantica picta	-	-	-	-	-	-
Turritella attenuata	-	-	-	-	-	-
Architectonica sp.	-	++	-	-	-	-
Aplysia sp.	-	++	-	-	-	-

Pathogens indicates as I: *Staphylococcus aureus*, II: *Vibrio cholerae*, III: *Shigella dyseuteriae*, IV: *Salmonella paratyphi*, V: *Pseudomonas aeruginosa*, VI: *Klebsiella pneumoniae*. Diameters of growth inhibition were used to defined incubation categories, +: Corresponding to the growth of inhibition diameter below 7 mm, ++: Corresponding to the inhibition diameter comprise between 7 and 10 mm, +++: Corresponding to the inhibition diameter above 10 mm and (-) means that no inhibition was detected. Assays were carried out in duplicate

Antifungal activity: The result of anti-fungal activity for the various mollusc samples was shown in Table 2. Most of the samples were inhibited the growth of the fungi *A. fumigatus*. The growth of *A. fumigatus* was inhibited with the highest zone of inhibition by *B. zeylanica* (18 mm), *S. officinalis* (15 mm), *L. indica* (15 mm), *S. urceus* (13 mm), *B. leachi* (13 mm), *C. adustus* (16 mm), *O. vulgaris* ink (14 mm), *N. picta* (12 mm) and *Architectonia* sp. (15 mm).

Moderate growth inhibition was detected in *C. betulinus* (8 mm), *H. pugilinus* (10 mm), *M. tributa* (10 mm), *S. officinalis* ink (9 mm), *M. meretrix* (7 mm) and *Xancus pyrum* (8 mm). Least anti fungal activity against *A. fumigatus* was observed in *O. vulgaris* (6 mm), *F. ficus* (4 mm), *L. lambis* (6 mm). In case of *Fusarium* sp., the highest activity was exhibited by *B. leachi* (13 mm), *Architectonia* sp. (15 mm) and *T. attenuata* (16 mm). The moderate activity was detected in *S. urceus* (8 mm), *S. listeri* (8 mm), *C. adustus* (9 mm). The antifungal control Nystatin inhibited the growth of both *Fusarium* and *A. fumigatus*.

Table 2: Antifungal activity of methanol extract of 25 molluscan samples

Sample	*Fusarium* sp.	*A. fumigatus*
Babylonia zeylanica	-	++
Perna virdis	-	-
Meretrix casta	-	-
Conus loroisii	-	-
Conus betulinus	-	++
Lambis chiragra	-	-
Sepia officinalis	-	+++
Lophiotoma indica	-	+++
Hemifuses pugilinus	-	++
Strombus urceus	++	+++
Bursatella leachi	+++	+++
Octopus vulgaris	-	+
Strombus listeri	++	-
Murex tributa	-	++
Ficus ficus	-	+
Conus adustus	++	+++
Sepia officinalis ink	-	++
Lambis lambis	-	+
Meretrix meretrix	-	++
Octopus vulgaris ink	-	+++
Xancus pyrum	-	++
Nantica picta	-	+++
Turritella attenuata	+++	-
Architectonica sp.	+++	+++
Aplysia sp.	-	-

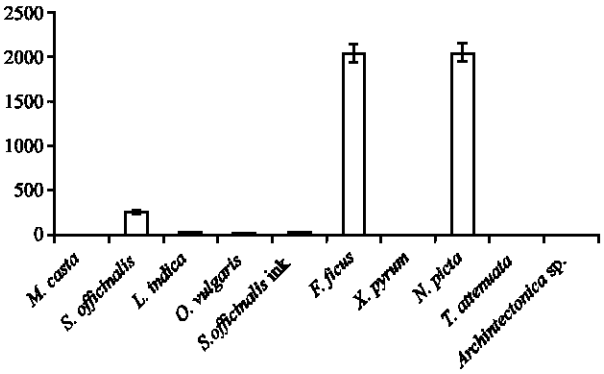

Fig. 1: Hemolytic activity of 10 molluscan samples which exhibited the positive activity out of 25 samples

Table 3: Brine shrimp toxicity of methanol extract of 25 molluscan samples

Sample	LC_{50} (μg mL^{-1})
Babylonia zeylanica	NT
Perna virdis	NT
Meretrix casta	NT
Conus loroisii	NT
Conus betulinus	46.25
Lambis chiragra	NT
Sepia officinalis	NT
Lophiotoma indica	NT
Hemifuses pugilinus	30
Strombus urceus	NT
Bursatella leachi	25
Octopus vulgaris	NT
Strombus listeri	NT
Murex tributa	NT
Ficus ficus	19
Conus adustus	42
Sepia officinalis ink	NT
Lambis lambis	12
Meretrix meretrix	NT
Octopus vulgaris ink	NT
Xancus pyrum	NT
Nantica picta	NT
Turritella attenuata	NT
Architectonica sp.	NT
Aplysia sp.	NT

NT: Indicates no toxicity

TOXICITY STUDIES

Hemolytic activity: The hemolytic properties of mollusc samples were tested using chicken blood RBC. Among the 25 samples, 10 samples exhibited hemolytic activity were shown in Fig. 1. *F. ficus* (7.6 μg) and *N. picta* (17.8 μg) exhibited highest hemolytic activity in the highest dilution with the hemolytic unit of 2048 HU produced 100% hemolysis. Lysis of erythrocytes was dose dependent. *S. officinalis* (11 μg) exhibited 256 HU units. *L. indica* (21 μg), *S. officinalis* ink (7.2 μg) showed 100% hemolysis of 36 HU units. Hemolytic activity with lowest dilution of 16 HU was recorded in *Architectonica* sp. (4.8 μg), *M. casta* (9.1 μg), *X. Pyrum* (8.2 μg), *T. attenuata* (13.5 μg) and *O. vulgaris* (18.2 μg), respectively. The remaining samples were not having the hemolytic activity.

Brine shrimp toxicity: The brine shrimp toxicity test (LC_{50}) results were shown in Table 3. The higher LC_{50} value indicates the low level of toxicity and vice versa. The highest toxicity was detected in *L. lambis* 12 μg mL^{-1}; *F. ficus* 19 μg mL^{-1}; *B. leachi* 25 μg mL^{-1}; *H. pugilinus* 30 μg mL^{-1} and the low level of toxicity were observed from the *C. betulinus* 42 μg mL^{-1} and *C. adustus* 46.25 μg mL^{-1}. No mortality was observed in the other sample.

DISCUSSION

The antibacterial drugs were derived from the marine invertebrates and it serves as potential sources for identification of novel drugs (Bansemir *et al*., 2006; Bazes *et al*., 2009; Jayaraj *et al*., 2008). Molluscs serve as an untapped resource for many bioactive compounds which includes peptide, depsipeptide, sterols, sequiterpene, terpenes, polypropionate, nitrogenous compounds, macrolides, prostaglandins (Carvalho *et al*., 1988; Balcazar *et al*., 2006; Blunt *et al*., 2006).

The result for *in vitro* antibacterial activity was present in 8 samples out of 25 samples, affecting the growth of human pathogen *S. aureus*, *V. cholerea* and *S. paratyphi*. The present study reveals the fact that presence of antibacterial factors in the *P. virdis* (7 mm), *C. betulinus* (15 mm), *L. chiragra* (13 mm), *S. officinalis* (7.5 mm), *H. pugilinus* (13 mm), *X. pyrum* (8 mm), *T. attenuate* (8 mm) and *Architectonica* sp. (8.5 mm).

The higher percentage of antimicrobial activity derives in the animal diet like sponges, bryozoans and algae which stored near the body surface as a chemical defense mechanism (Maktoob and Ronald, 1997; Carte and Faulkner, 1983). Results of the present study do not indicates that the antimicrobial activity originate from the diet-derived metabolites. This may be due to the breakdown of sponges, bryozoans and algae by the digestive enzyme of mollusk.

The presence of anti bacterial activity was due to the presence of protease enzyme. A previous study reported in the digestive organ of *Modiolus* modiolus was sensitive to protease treatment, indicating the presence of protein factor (Okuda and Scheuer, 1985). Proteins and glycoprotein with the antibacterial activity have been characterized from the digestive gland of various molluscs (Haug *et al*., 2004; Iguchi *et al*., 1982; Kamiya *et al*., 1986) and in the hemolymph of the various bivalves

(Pakrashi *et al*., 2001; Anderson and Beaven, 2001), suggesting that this compound is responsible for animal immune system.

The activity detected from present study probably may due to bacterial symbionts living on the surface of the organism. A bacteria associated with the crystalline style of *M. edulis* contains a heat-sensitive anti bacterial agent (Seiderer *et al*., 1987). Barbieri *et al*. (1997) reported the anti bacterial activity was due to the bacterial symbionts *Alteromonas* and *Shewanella* in the nidamental gland of *Loligo pealei*. The presence of antimicrobial activity in mollusc has been reported from the mucous of giant snail (Kubota *et al*., 1985) from the egg mass and purple fluid of sea hare *A. kurodai* (Lijima *et al*., 1995). Antibacterial activity has been reported in unfractioned plasma from the mussel *Geukensia demissa* and from the oyster *Crassostrea virginica* (Anderson and Beaven, 2001).

In the case of antifungal activity, highest zone of inhibition has been identified in the *B. zeylanica* (18 mm), *S. officinalis* (15 mm), *S. urceus* (13 mm), *B. leachi* (13 mm), *L. indica* (15 mm), *C. adustus* (16 mm), *O. vulgaris* ink (14 mm), *N. picta* (12 mm), *Architectonica* sp. (15 mm) against *A. fumigatus*. The highest antifungal activity may due to the presence of potential antifungal compound. The result of antifungal activity in *O. vulgaris* ink (14 mm) and *S. officianalis* ink (9 mm) supports the previous work done by the Lane (1962) who reported the antibiotic effects of the fluid from the ink sac of cephalopods.

The sea hare *B. leachi* (13 mm) exhibited antifungal activity against *Fusarium* sp. and *A. fumigatus*. Lijima *et al*. (1995) isolated Aplysiamin-E, a compound which inhibit the growth of *Yeast* sp. A powerful antifungal agent Halicondramides has been extracted from the marine nudibranch mollusc. High degree of antifungal activity was detected in *T. attenuata* and *Architectonica* sp. against *Fusarium* sp. The antifungal activity probably may due to the presence of antifungal peptide. Charlet *et al*. (1996) characterized an antimicrobial peptide from the *M. edulis*. Mitta *et al*. (2000) isolated a novel antifungal peptide from *M. galloprovincialis* that delays the growth of *Neurospora crassa* and *F. culmonum*.

In the toxicity studies out of 25 Mollusc samples 10 samples lyses the washed erythrocyte. The highest hemolytic activity was recorded in *F. ficus* and *N. picta* which showed the toxic nature and ensures the presence of hemolytic factor. Lattore (1977) isolated hemolytic factor from the venom of *C. textile*. It was suggested that the proteolytic enzyme might contribute to the lysis of the red blood cell membrane by digesting integral protein correspondingly and weakening certain protein of the membrane.

In the brine shrimp *Nauplii* test, high mortality was detected in the *L. lambis* and *F. ficus*. In the present study, toxic activities were found in some sample that also showed antimicrobial activities. However, some samples were shown too toxic without showing any antimicrobial activity, some sample showed little or no toxicity with antimicrobial activity. Haug *et al*. (2004) reported that from a pharmaceutical point of view it is advantageous when antimicrobial drugs have no toxic effect on eukaryotic cells.

CONCLUSION

In conclusion according to the latter point, the sample with antimicrobial activity with no toxicity is considered for isolating a novel antibiotic. In order to identify the chemical nature and evaluate their potential novel drug leads, further purification of the active sample for the isolation of active compounds is necessary.

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