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Research Article

ANTIMICROBIAL ACTIVITY OF THE HEMOLYMPH AND WHOLE BODY EXTRACT OF A FRESH WATER CLAM, *VILLORITA CYPRINOIDES* (GRAY, 1825)

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ABSTRACT

Invertebrates possess some pronounced pharmacological activities or other properties which are useful in the biomedical area. In the present study, antimicrobial activities were observed in a fresh water clam, *Villorita cyprinoides* against a number of pathogens. Antimicrobial activities were carried out by standard disc diffusion method using the hemolymph and whole body extract of the clam *Villorita cyprinoides,* extracted using the solvents ethanol, methanol, butanol, ethyl acetate, chloroform, acetone, hexane and water. The whole body extract showed a high inhibitory action (20 mm) against two gram positive bacteria *Streptococcus mutans* and *Staphylococcus aureus* and minimum zone (7 mm) against *Bacillus subtilis, Escherichia coli* and fungus *Aspergillus niger*. Among the eight different extracts tested, antimicrobial activity was more pronounced in the ethyl acetate extracts. A dose dependent variation was observed in the antimicrobial activity of the hemolymph. The hemolymph at a dose level of 100µl/disc showed the highest inhibitory potency against the fungus *Rhizopus stolonifer*. The antimicrobial potential in the hemolymph and whole body extracts of the clam *Villorita cyprinoides* suggests that the clam possess antimicrobial compounds.

Keywords: Hemolymph, *Villorita cyprinoides*, Pathogens, Antimicrobial activity.

INTRODUCTION

Microbial resistance is one of the adaptations which is well studied in most of the invertebrates. Many invertebrates offer a source of potential antimicrobial drugs (Bazes *et al*., 2009). In recent years, many bioactive compounds have been extracted from tunicates, sponges, soft corals, sea hares, crabs, slugs and snails (Donia and Hamann, 2000; Haefner, 2003). As invertebrates, molluscs lack an adaptive immune system, but have evolved sophisticated strategies and rely exclusively on their innate immunity to defend themselves against a variety of pathogens (Loker *et al*., 2004) whether encapsulated or phagocytosed by blood cells (Charlet *et al*., 1996).

Molluscs which are widely distributed throughout the world have many representatives in the terrestrial and aquatic ecosystem. There are more than thousands of bioactive compounds discovered in molluscs. They are peptide, depsipedtide, sterols, sesquiterpenes, terpenes, polypropionate, nitrogenous compounds, macrolides, prostaglandins, miscellaneous compounds and alkaloids (Blunt *et al*., 2006). The search for new metabolites from invertebrates has resulted in the isolation more or less 10000 metabolites (Fuesetani, 2000). The role of secondary metabolites as a chemical defense against epibiosis has been discussed (Bakus *et al*., 1986; Davis *et al*., 1989; Pawlik, 1993). Many class of bioactive compounds exhibiting cytotoxic, antitumor, antiinflamatory, antileukemic, antineoplastic, antimicrobial and antiviral properties of molluscs (Pettit *et al*., 1991; Anand and Edward, 2002; Rajaganapathi *et al*., 2000; Kamiya *et al*., 1989). Antimicrobial peptides are important in the first line of the host defense system of many animal species. Studies of antimicrobial compounds of invertebrates may provide

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valuable information for new antibiotic discoveries and give new insights into bioactive compounds in molluscs (Suresh *et al*., 2012).

The black clam *V. cyprinoides* (common Indian marsh clam), is the most important edible clam species found in estuaries, rivers, lagoons, manmade canals, marshes and mangroves. Since bivalve have innate immunity and are believed to produce antimicrobial peptides (Li *et al*., 2009) that show strong antibacterial activity against Gram positive and Gram negative bacteria they could be a source for antimicrobial peptides. Hence the present investigation was undertaken to explore the antimicrobial activity of bioactive compounds from the hemolymph and whole body tissue extract of *Villorita cyprinoides*.

MATERIALS AND METHODS

Specimen Collection

Live specimens of clam *Villorita cyprinoides* were collected from the Thamirabharany river near Kappukad area, Kuzhithurai, Vilavancode Taluk, Kanyakumari District, TamilNadu, India. The collected animals were rinsed with river water to remove the associated debris and transported to the laboratory. The animals were carefully removed from the shells.

Collection of hemolymph

The hemolymph was collected from the foot using 0.1ml syringe and 22 guage needle after gently opening the shell using a forceps and knife. Approximately 0.5ml hemolymph was collected per mussel and was pooled in centrifuge tubes placed on ice, centrifuged at 3000rpm for 15 minutes and the supernatant was stored at -20^{o}C.

Preparation of tissue extracts

After remaining the shell, the whole body was weighed and extracted with different solvents such as ethanol, methanol, butanol, ethyl acetate, acetone, chloroform, hexane and distilled water. For extraction, solvents were added at the ratio of 10 g/20 ml and were shaken at regular time intervals for three days at room temperature for thorough extraction of the biologically active compounds from the whole body. The contents were filtered using Whatman No. 1 filter paper and the filtrate was concentrated by allowing the solvent to evaporate at room temperature. The concentrated extract was used as solvent extract of the sample and stored in refrigerator (-20^{o}C).

Microbial strains used

Antimicrobial activity of whole body extract was determined against six human pathogenic bacteria viz., three Gram positive bacteria *Staphylococcus aureus*, *Streptococcus mutans*, *Bacillus subtilis*, three Gram negative bacteria *Klebsiella pneumoniae*, *Escherichia coli*, *Proteus vulgaris* and three fungal strains viz., *Aspergillus niger*, *A. flavus* and *Rhizopus stolonifer*.

Assay of antibacterial activity

Antibacterial activity of the whole body extracts in the different solvents and hemolymph of the clam *Villorita cyprinoides* were tested using standard disc diffusion method of Bauer (Bauer *et al*., 1996). Sterilized Muller Hinton Agar (20 ml) was poured into sterile petriplates and allowed to solidify. After solidification, 100µl of fresh culture of human pathogenic bacteria were spread on the surface of Muller Hinton Agar plates. Sterile disc of 6mm, loaded with 50µl of crude extract /hemolymph (25µl/50µl/ 75µl/100µl) of clam as well as positive and negative control discs for comparison were placed in the plates. For positive control streptomycin disc (25 µg disc) and negative control sterile disc were used. The plates were incubated for 24 h at 37^{o}C. After incubation the diameter of inhibitory zones formed around each discs were measured (mm) and recorded.

Assay of antifungal activity

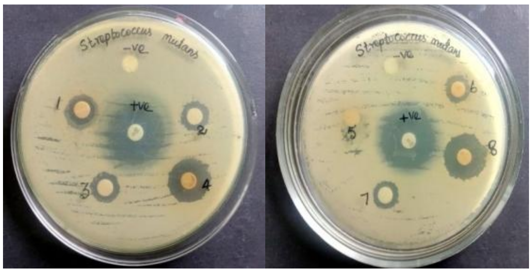
Sterilized potato dextrose agar (20µl) was poured into sterile petriplates and allowed to solidify. After solidification, 100µl of fresh culture of human pathogenic fungal strain was distributed uniformly on the surface of potato dextrose agar plate with the help of sterile cotton swab. The sterile disc of 6mm, loaded with 50µl of crude extract / hemolymph (25 µl / 50 µl / 75 µl /100 µl) of clam and Flucanozole (100 µg / disc) used as positive control and sterile disc used as negative control were placed in the fungal plates and were incubated at 27°C for 48 h and the antifungal activity was measured based on the diameter of zone of inhibition formed around each disc.

RESULTS

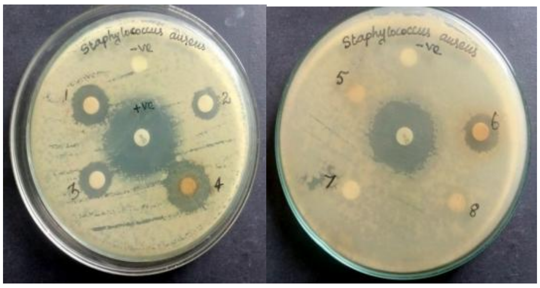
Antibacterial activity of whole body extracts

The whole body extract of the clam *Villorita cyprinoides* showed different antibacterial activity on different bacteria tested (Plate 1). The ethanol extract showed highest inhibition against *Staphylococcus aureus* (16 mm), methanol extract against *Streptococcus mutans* (13 mm), butanol extract against *Staphylococcus aereus* (14 mm), ethyl acetate extract against *Streptococcus mutans* and *Staphylococcus aureus* (20 mm), chloroform extract against *Bacillus subtilis* (14 mm), acetone extract against *Proteus vulgaris* (16 mm), hexane extract against *Streptococcus mutans* (20 mm) and aqueous extract against *Streptococcus mutans* (12 mm). Among the eight different extracts tested, all the bacteria were inhibited by ethyl acetate and butanol extracts (ethyl acetate > butanol), but chloroform extract inhibited *Bacillus subtilis* the growth of just (Table 1).

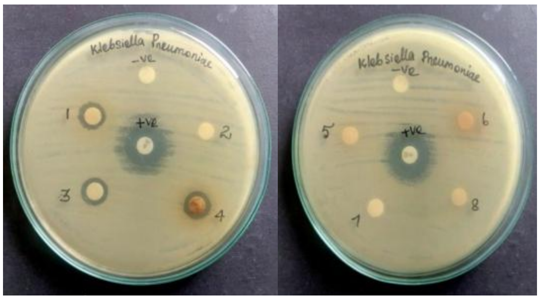
Plate 1. Antimicrobial activity of the whole body extracts of the clam, *Villorita cyprinoides* against pathogenic microbes



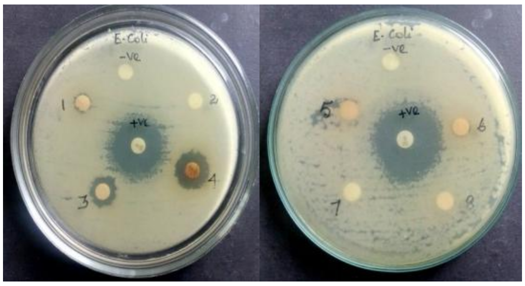
Streptococcus mutans



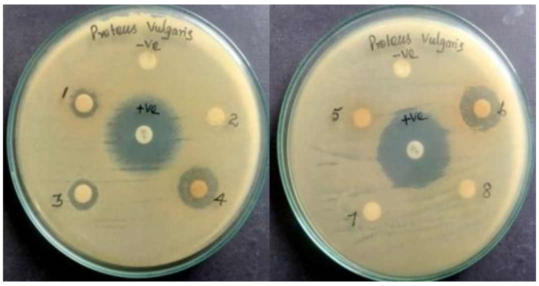
Staphylococcus aureus



Klebsiella pneumoniae

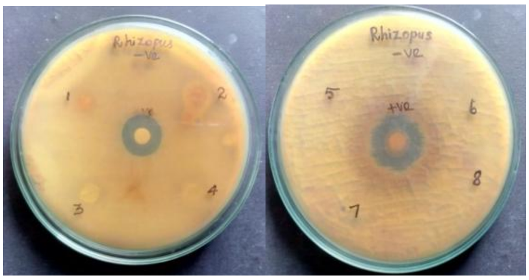


Escherichia coli



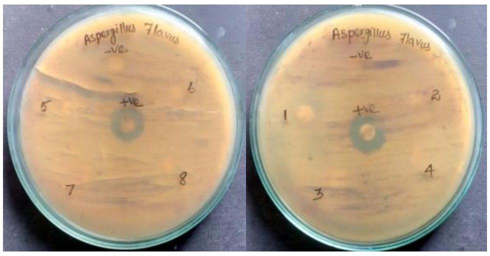
Proteus vulgaris

Bacillus subtilis



Rhizopus stolonifer

Aspergillus niger



Aspergillus flavus

Note: 1. Ethanol extract, 2. Methanol extract, 3. Butanol extract, 4. Ethyl acetate extract, 5. Chloroform extract 6. Acetone extract, 7. Aqueous extract and 8. Hexane extract.

Table 1. Antimicrobial activity of the whole body extracts of the clam, *Villorita cyprinoides* against pathogenic microbes

Pathogens tested		Zone of Inhibition (mm)									
		E	M	B	EA	C	A	AQ	H	NC	PC
Gram positive bacteria	*S. mutans*	15	13	13	20	-	12	12	20	-	26
	B. subtilis	-	-	10	15	14	12	8	7	-	19
	S. aureus	16	12	14	20	-	14	-	-	-	27
Gram negative bacteria	*P. vulgaris*	11	-	13	15	-	16	-	-	-	27
	K. pneumoniae	11	-	11	11	-	-	-	-	-	20
	E. coli	7	-	12	16	-	-	-	-	-	29
Fungus	*A. niger*	-	-	-	-	7	-	-	8	-	20
	A. flavus	-	-	-	-	-	-	-	-	-	14
	R. stolonifer	-	-	-	-	-	-	-	-	-	17

Samples: E- Ethanol, M-Methanol, B- Butanol, EA-Ethyl acetate, C-Chloroform, A-Acetone, AQ- Aqueous, H-Hexane, NC- Negative control (Sterile disc), PC-Positive control (Streptomycin).

Antifungal activity of whole body extracts

Of the three fungal pathogens tested chloroform and hexane extract alone inhibited the growth of the fungus *Aspergillus niger* with a inhibitory zone of 7 mm and 8 mm respectively. Extracts prepared using other solvents failed to inhibit the fungal activity of *A. niger*, *A. flavus* and *Rhizopus stolonifer* (Table 1).

Antibacterial activity of hemolymph

Hemolymph 100 µl of *Villorita cyprinoides* inhibited the growth of just *Bacillus subtilis* with greater potency (14 mm) than the 75 µl hemolymph sample. No inhibition was observed in lower concentration tested. Among the six bacteria tested the hemolymph, failed to inhibit the growth of *Proteus vulgaris* at any tested concentration (Table 2).

Antifungal activity of hemolymph

Hemolymph failed to inhibit the growth of any of the tested fungal pathogens at low concentration (25-50µl/disc). When tested with 75-100µl the highest inhibitory activity was registered on *Rhizopus stolonifer* (14 mm and 15 mm) followed by *A. niger* (12 mm) *A. flavus* (9 mm and 11 mm) and *E. coli* (6 and 10 mm) (Table 2).

Table 2. Antimicrobial activity of the hemolymph of the clam *Villorita cyprinoides* against pathogenic microbes

Pathogens tested		Zone of Inhibition (mm)					
		25µl	50 µl	75 µl	100 µl	PC	NC
Gram positive bacteria	*S. mutans*	-	-	-	10	14	-
	B. subtilis	-	-	9	14	22	-
	S. aureus	-	-	6	9	17	-
Gram negative bacteria	*P. vulgaris*	-	-	-	-	23	-
	K. pneumoniae	-	-	-	6	21	-
	E. coli	-	-	6	10	14	-
Fungus	*A. niger*	-	-	12	12	17	-
	A. flavus	-	-	9	11	22	-
	R. stolonifer	-	-	14	15	20	-

Samples: PC-Positive control, NC-Negative Control.

DISCUSSION

In the present study the ethyl acetate extract of the whole body exhibited a strong antimicrobial activity against both Gram positive as well as Gram negative bacteria when compared to other extracts. The antibacterial activities have been previously reported in several molluscan species such as *Achantina fulica* (Kubota *et al*., 1985; Iguchi *et al*., 1985), *Crassostrea virginica*, *Mytilus edulis*, *Geukensia demissa* (Anderson and Beaven, 2001), *Dicathais orbita* (Benkendorff *et al*., 2001), *Dolabella auricularia* (Vennila *et al*., 2011), *Perna viridis, Nerita albicilla* (Kiran *et al*., 2014), *Babylonia spirata* (Periyasamy *et al*., 2012; Suresh *et al*., 2012) and *Pomacea insularium* (Packia Lakshmi *et al*., 2014). Antibacterial activity of mucus (mucin) of *Achatina fulica* is related to antibacterial factors found in its protein moiety rather than to its activity on the cell surface of bacteria (Iguchi *et al*., 1985). Achacin the antibacterial protein in the mucus of the giant African snail inhibit both Gram positive and Gram negative bacteria (Ehara *et al*., 2002). The presence of antibacterial compounds in the oyster *Pteria chinensis* and bivalve *Perna viridis* have reported using the various solvent extracts (Li *et al*., 2009). Among the bacterial test strain, Gram positive bacteria were more susceptible than Gram negative bacteria. This may be due to the more complex structure of cell wall of Gram negative bacteria (Reichelt and Borowitzka, 1984). Antimicrobial protein/peptides have a broad range of ability to kill microbes. Large antimicrobial proteins (> 100 aminoacids), which are lytic / nutrients binding specifically target microbial cell surface macromolecules and small antimicrobial peptides act by disrupting the structure or function of microbial cell membranes (Gotz, 1988).

Antifungal activity was also reported from the extracts of various bivalve molluscs (Anand and Edward, 2002). Two edible bivalve species, *Perna viridis* and *Meretrix casta* showed antifungal activities (Sumita *et al*., 2009; Jarrar *et al*., 2010). Methanolic extract of *Murex virgineus* exhibited effective antifungal activity against all the tested strains (Lenin, 2011). According to Anand and Edward (2002) the crude extracts of *Cypraea errones* exhibited higher antibacterial and antifungal activities. It may be the antimicrobial protein/peptide/hemolysin like lectin that perturb/alter the structure of chitin or damage to the polymer of the chitin the polysaccharide found on the cell wall of the fungus or it may be agglutinin like sugar binding protein that bind to the chitin of the fungal cell wall to inhibit the fungal activity. The observed results clearly indicate that the extracts of clam shows predominant activity against microbial pathogens as reported on the presence of antimicrobial proteins/peptides in the marine Molluscs (Sumita *et al*., 2009). In the present study, it has been recorded that, a wide spectrum of antibacterial activity is found in whole body extract prepared using various solvents and the hemolymph was effective in inhibiting the growth of pathogenic fungi.

CONCLUSION

This investigation discloses the presence of antimicrobial compounds in the whole body extract and hemolymph of the clam *Villorita cyprinoides* against pathogenic microorganisms. Antimicrobial compounds from the natural resources would be the alternative to overcome the resistance observed to antibiotics. It is promising that the tested clam species synthesize antimicrobial substances to withstand bacterial and fungal infections.

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