Indian marine bivalves: Potential source of antiviral drugs

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Extracts prepared from economically important marine bivalves such as green mussel (*Perna viridis*), estuarine oyster (*Crassostrea madrasensis*), giant oyster (*Crassostrea gryphoides*), estuarine clam (*Meretrix casta*), black clam (*Villorita cyprinoides*) and mud clam (*Polymesoda erosa*) were found to possess high antiviral activity when tested with influenza virus strains type-A (A/Missisipi 1/85/H_3N_2) and type-B (B/Harbin 7/94). Maximum difference in the EID_{50} value was observed in the extract prepared from *P. viridis* for *in vitro* studies conducted with influenza virus type-A (2.50 lg) and virus type-B (3.00 lg). For *in vivo* studies, maximum difference in EID_{50} values was observed in the extracts of *V. cyprinoides* (4.00 lg) and *P. erosa* (4.00 lg) with influenza virus type-A.

MOLLUSCS contribute significantly to the total marine fish catch of the world. Marine bivalves are abundant in coastal and estuarine waters of India[1]. The bivalve fishery is constituted mainly by clams, mussels and oysters[2]. Molluscan fishery is not well-organized along the Indian coast. Molluscs are exploited in large quantities by traditional methods and sold live in the market for human consumption. The economically important species of marine bivalves are green mussel (*Perna viridis*), estuarine oyster (*Crassostrea madrasensis*), giant oyster (*Crassostrea gryphoides*) and clam (*Meretrix casta*, *M. meretrix*, *Paphia malabarica*, *Villorita cyprinoides*).

Recent investigations conducted jointly by Russian and Indian scientists showed that Indian green mussels (*P. viridis*) are not only a rich source of protein for human consumption, but also a potential source of antiviral drug (Chatterji *et al.*, unpublished data). The extract prepared from Indian green mussel showed high antiviral activity when tested with various viral strains such as influenza, hepatitis, RSV and herpes[3]. In continuation with the same investigation, the extracts prepared from six other commercially important marine bivalves were screened for the presence of antiviral properties. The results of the study are presented in this communication.

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Marine bivalves such as *P. viridis*, *C. madrasensis*, *C. gryphoides*, *M. casta*, *V. cyprinoids* and *P. erosa* were collected live from the natural habitats along Goa coast. The bivalves were deshelled with the help of a sharp knife. Meat (300 g) and 50 ml mantle fluid from each of the six species were collected and mixed with equal volume of double-distilled water in a double-necked, round distillation flask. The extract was prepared by enzyme–acid hydrolysis process as developed and patented by Chatterji *et al.*[4].

The antiviral activity of the extracts prepared from marine bivalves was assessed at Pasteur Institute of Epidemiology and Microbiology, St. Petersburg, Russia by using two human influenza virus strains (A/Missisipi 1/85/H_3N_2 and B/Harbin 7/94). The extract prepared from bivalves was diluted to 1/10 times with peptonic solution[5]. Stock solution of influenza viral strain type-A (A/Missisipi 1/85/H_3N_2) and strain type-B (Harbin 7/94) obtained from Institute of Virology, St. Petersburg, Russia was diluted to achieve virus concentrations from 10^{-2} to 10^{-7} for viral strain type-A and from 10^{-1} to 10^{-6} for viral strain type-B. In the present study allantoic fluid of chicken embryo (10–13-day-old) infected with human influenza virus (haemagglutination titre 1 : 512/1 : 1024) was used for *in vitro* and *in vivo* studies.

Gelatin (2 g) was thoroughly dissolved in 100 ml double-distilled warm water. This was followed by the addition of 8 g NaCl, 0.6 g KCl, 0.8 g $CaCl_2$, 0.15 g $MgCl_2$, 0.9 g glucose, 25 ml of 0.1% phenol and 0.1 g antibiotic-levomycitin (100 units/ml media). The final volume was made to 1000 ml with double-distilled water. The pH of the medium for the culture of chorion–allantoic membrane (ChAM) was maintained between 6.5 and 7.0.

The evaluation of the viral inhibition activity of the bivalve extracts with respect to human influenza viruses was conducted *in vitro* by infecting the fragments of ChAM of chicken embryo.

In each of the 96 wells of the three sets of multi-well plates, 0.5 ml peptonic medium and a single piece of a fragment of ChAM (size 5 mm) were transferred so as to completely dip in the peptonic medium. In plate-1, 0.1 ml extracts of N_1 (green mussel) and N_2 (estuarine oyster), in plate-2 extracts of N_3 (common clam) and N_4 (black clam) and in plate-3 extracts of N_4 (mud clam) and N_6 (giant oyster) diluted to 1/10 were added to each well. The plates were kept at room temperature for 2 h. In each well, except those kept for control in plate-1, 0.1 ml of influenza virus type-A (A/Missisipi 1/85/H_3N_2) for the first set of experiments and influenza virus type-B (B/Harbin 7/94) for the second set of experiments were added in quadruplicate in descending dilution from 10^{-2} to 10^{-7} and 10^{-1} to 10^{-6}, respectively. All the three plates were closed properly and kept at a constant temperature of 36 ± 1°C for incubation for

48 h in case of influenza virus type-A and 72 h for influenza virus type-B, respectively.

In each well of all the three multi-well plates, including the control columns 0.05 ml of chicken erythrocyte (5%) was added after removing the piece of the ChAM fragment. All the three plates were kept undisturbed at room temperature for 20–30 min. Infectious virus titre (in lg units) was estimated using the haemagglutination assay (HA). The level of antiviral activity was evaluated by the difference in the infectious titre of the virus, i.e. Expected Infectious Dose (EID_{50}) in the control (extract without virus) and extract with virus. The cytotoxicity in controls (ChAM with no virus) was judged visually.

The evaluation of viral inhibition activity of the extracts prepared from bivalves was conducted *in vivo* in the neutralization test on infected chicken eggs with developing embryos (10–13-day-old). Ten-fold dilution of influenza virus type-A (A/Missisipi $1/85/H_3N_2$) was added with equal volume of undiluted extracts (0.5 ml of virus and 0.5 ml extract) for the neutralization test. The mixture was kept for an hour at room temperature and then inoculated into the chicken embryos. The eggs were sealed with wax and kept for incubation in an oven at a constant temperature of 36°C for 72 h. The eggs were cooled in a refrigerator (4°C) for 18 h immediately after the incubation. About 5 ml of allantoic fluid was removed from each egg with the help of a pipette by making a small hole on the shell and transferred into the respective well of the multi-well plates.

In the neutralization test, HA assay was performed with 1% suspension of chicken erythrocytes. Erythrocyte suspension (0.2 ml) was added to each well of the well-plate and kept for 20–30 min for haemagglutination reaction. The level of antiviral activity was estimated by the difference between EID_{50} in the control and the extract with virus.

The HA showed a high antiviral activity in the extracts prepared from different marine bivalves when the test was conducted using human influenza virus strains for *in vitro* and *in vivo* studies. The reduction of the infectious activity for *in vitro* studies in the extracts of different bivalves was 1.75–2.50 lg EID_{50} for influenza virus type-A (A/Missisipi $1/85/H_3N_2$) (Table 1) and 2.5–3.0 lg EID_{50} for influenza virus type-B (Table 2). The highest reduction in the infectious activity was recorded in the extract of green mussel (*P. viridis*) with virus type-A (2.50 lg EID_{50}) and virus type-B (3.00 lg EID_{50}) compared to the extracts prepared from other bivalves (Tables 1 and 2).

The reduction of infectious activity of influenza virus type-A for *in vivo* studies was 2.75–4.00 lg EID_{50} (Table 3). The extracts prepared from *V. cyprinoides* and *P. erosa* showed maximum reduction in infectious activity (4.00 lg EID_{50}).

The extracts prepared by enzyme–acid hydrolysis of the meat and mantle fluid of commercially important Indian marine bivalves showed high antiviral activity in both *in vitro* and *in vivo* tests. The extracts of black clam (*V. cyprinoids*) and mud clam (*P. erosa*) were found to reduce the infection of influenza virus type-A considerably, when the tests were carried out on chicken embryo *in vivo*. Similar observations were made using the extract prepared from the Russian blue mussels, where influenza virus types A and B was found to reduce infectious activity by 3.0–6.0 lg EID_{50} (ref. 3). The treatment of toxigenic influenza-infected mice with the extract prepared from blue mussel showed 66–84% survival[3]. Administration of extract of mussel in mice, both intranasal and oral, gave significant

Table 1. Result of neutralization reaction with fragments of chicken embryo (VCA) with six different extracts of marine bivalves and virus strain (A/Missisipi $1/85/H_3N_2$), *in vitro*

Sample	Virus dilution (A/Missisipi $1/85/H_3N_2$)						EID_{50} (lg)	Difference (lg EID_{50})
	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}		
Control virus	+ +	+ +	+ +	+ +	– –	– –	5.50*	–
	+ +	+ +	+ +	+ +	– –	– –		
P. viridis	+ +	+ +	+ +	+ +	– –	– –	3.00*	2.50
	+ +	+ +	– –	– –	– –	– –		
C. madrasensis	+ +	+ +	+ +	– –	– –	– –	3.25*	2.25
	+ +	+ –	– –	– –	– –	– –		
M. casta	+ +	+ +	+ –	– –	– –	– –	3.75*	1.75
	+ +	+ +	– –	– –	– –	– –		
V. cyprinoides	+ +	+ +	– –	– –	– –	– –	3.25*	2.25
	+ +	+ –	– –	– –	– –	– –		
P. erosa	+ +	+ +	– –	– –	– –	– –	3.50*	2.00
	+ +	+ +	– –	– –	– –	– –		
C. gryphoides	+ +	+ +	+ –	– –	– –	– –	3.75*	1.75
	+ +	+ +	– –	– –	– –	– –		

+, Shows presence of virus; –, Shows absence of virus; *0.5 added to each value.

Table 2. Result of neutralization reaction with fragments of chicken embryo (VCA) with six different extracts of marine bivalves and virus strain (B/Harbin 7/94), *in vitro*

Sample	Virus dilution (B/Harbin 7/94) 10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	EID_{50} (lg)	Difference (lg EID_{50})
Control virus	+ +	+ +	+ +	+ +	+ +	+ –	5.50*	–
	+ +	+ +	+ +	+ +	+ –	– –		
P. viridis	+ +	+ +	– –	– –	– –	– –	2.50*	3.00
	+ +	+ +	– –	– –	– –	– –		
C. madrasensis	+ +	+ +	– –	– –	– –	– –	2.50*	3.00
	+ +	+ +	– –	– –	– –	– –		
M. casta	+ +	+ +	+ –	– –	– –	– –	3.00*	2.50
	+ +	+ +	+ –	– –	– –	– –		
V. cyprinoides	+ +	+ +	– –	– –	– –	– –	2.50*	3.00
	+ +	+ +	– –	– –	– –	– –		
P. erosa	+ +	+ +	+ –	– –	– –	– –	2.75*	2.75
	+ +	+ +	– –	– –	– –	– –		
C. gryphoides	+ +	+ +	+ –	– –	– –	– –	2.75*	2.75
	+ +	+ +	– –	– –	– –	– –		

+, Shows presence of virus; –, Shows absence of virus; *0.5 added to each value.

Table 3. Reaction of neutralization of chicken embryos with extracts from six marine bivalves and virus strain (A/Missisipi 1/85/H_3N_2), *in vivo*

Sample	Virus dilution (A/Missisipi 1/85/H_3/N_2) 10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	EID_{50} (lg)	Difference (lg EID_{50})
Control virus	+ +	+ +	+ +	+ +	+ +	+ +	– –	8.00*	–
	+ +	+ +	+ +	+ +	+ +	+ +	++ +		
P. viridis	+ +	+ +	+ +	+ –	– –	– –	– –	5.00*	3.00
	+ +	+ +	+ +	+ –	– –	– –	– –		
C. madrasensis	+ +	+ +	+ +	+ –	– –	– –	– –	4.75*	3.25
	+ +	+ +	+ +	– –	– –	– –	– –		
M. casta	+ +	+ +	+ +	+ +	– –	– –	– –	5.25*	2.75
	+ +	+ +	+ +	+ –	– –	– –	– –		
V. cyprinoides	+ +	+ +	+ +	– –	– –	– –	– –	4.00*	4.00
	+ +	+ +	– –	– –	– –	– –	– –		
P. erosa	+ +	+ +	+ +	– –	– –	– –	– –	4.00*	4.00
	+ +	+ +	– –	– –	– –	– –	– –		
C. gryphoides	+ +	+ +	+ +	+ –	– –	– –	– –	4.75*	3.25
	+ +	+ +	+ +	– –	– –	– –	– –		

+, Shows presence of virus; –, Shows absence of virus; *0.5 added to each value.

protective effect. Maximum prophylactic effect has been observed in mice when a dose of mussel extract was given 5 h before inoculation of virus[3] and the mice showed 100% survival.

Marine bivalves are filter-feeding animals, and while feeding they accumulate strains of various diseases along with food from the environment[6,7]. Recent studies on marine bivalves showed the presence of viruses that are virulent for salmonid fishes[8–10]. A number of viruses have also been reported from the tissue of marine molluscs such as iridovirus and Herpes virus[6], plague virus[11], echo, coxsackie and reo[12]. The presence of human pathogens in the body tissue of bivalves has been reported to be much higher than those in the surrounding waters[8]. Enriquez *et al.*[13] reported the presence of Hepatitis A virus 100-fold higher in the mussel tissue than the surrounding water.

The penetration of these virulent viruses in the tissues of bivalves could stimulate the animal to produce special types of antibodies to fight against these viruses. However, when these bivalves are consumed as food, there might not be any active antiviral property in their tissue. The main reason for this could be the presence of many other substances in the tissue of bivalves that inhibit the effect of the antiviral compound. But as soon as these inhibitory substances are removed from the extract by a process of acid hydrolysis, the novel inert substances get activated and show antiviral effects. The present observation confirms that the agglutinins present in viruses show haemagglutination reaction with specific substances that are present in the extract prepared from the tissue of bivalves.

Attempts have also been made during the past several decades to develop effective antiviral drugs from the

natural resources. The recurrence of viral epidemics in tropical and subtropical countries is a common phenomenon where high mortalities were recorded in the past few years. At present, the control of various viral diseases has become a matter of great concern. However, the main constraint in developing an effective drug has been the unique characteristics of antigenic variation of virus resulting in the emergence of new variant virus strains[14]. There are a number of antiviral drugs introduced in the market such as tricyclic symmetric amine, amantadine hydrochloride and its analogue rimantadine, which are effective drugs used for influenza type-A infection[14]. However, though these drugs have been proved non-toxic, sometimes they cause dizziness and insomnia, particularly in elderly persons.

The present investigations showed that marine animals have great potential for developing useful drugs. Extraction of important biologically-active compounds from marine resources will certainly be helpful in protecting and treating various viral diseases in human beings.

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Effect of β-methylcholanthrene on glutathione S-transferases of rat testis

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The glutathione S-transferases (GSTs) of rat testis, purified using S-hexylglutathione linked agarose 4B affinity matrix were fractionated into four cationic and six anionic isoenzymes using the polybuffer exchangers 118 and 94, respectively. Their pI values ranged from 5.4 to 9.2. On electrophoresis, the testis GSTs showed identical subunit pattern to that of brain, i.e. Yc, Yb, Yβ and Yδ To study the induction of these proteins, rat testis treated with 24 mg of β-methylcholanthrene on dot and transblot analyses with antibodies prepared against affinity purified GSTs revealed induction of α-class (Yc subunit) and μ-class (Yβ subunit) GSTs. This observation was further confirmed by using substrate specificity. The β-methylcholanthrene-induced samples have shown more activity with peroxides and 1,2 epoxy 3-(*p*-nitrophenoxy) propane indicated the elevation of Yc and Yβ subunits, respectively, in rat testis. Therefore the Yc and Yβ subunits may be used as marker proteins for chemical toxicity in the testis.

GLUTATHIONE S-transferases (GST.EC 2.5.1.18) are a family of multigene and multifunctional dimeric enzymes that catalyse the nucleophilic attack of the thiol moiety (conjugation) of the glutathione (GSH) on the electrophilic centre of various carcinogens, mutagens and other xenobiotic compounds[1]. The primary step of this reaction is involved in the formation of mercapturic acids, a pathway through which hydrophobic xenobiotics are inactivated and excreted from the body[2]. These enzymes are ubiquitously distributed in a variety of biological systems and in various organs like liver, kidney, intestine, brain and testis. They occur in the cytoplasm, membranes of mitochondria, microsomes and nuclei[3].

Each organ possesses a unique profile of GSTs, the liver and testis having the high GST activity in rodents[4]. The GST enzyme system consists of several isozymes. In rat liver, the GST isozymes are mainly made up of three major subunits, the Ya (Mw 25,600), Yb (Mw 27,000) and Yc (Mw 27,500). Each subunit has a specific function; Yc and Ya catalyse the reduction of hydro peroxides, Ya, Yc and Yδ catalyse the isomerization of prostaglandin, and Yb and Yβ are involved in the formation of leukotrienes and conjugation of GSH to xenobiotics. Yδ subunit is structurally related to the

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