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# A novel DNase like compound that inhibits virus propagation from Asian Green Mussel, *Perna viridis* (Linn.)

Muhammed Zafar Iqbal AN*[,1,#] & Khan MS[1,2]

[1]Biological Oceanography Division, CSIR-National Institute of Oceanography, Dona Paula-403 004, Goa, India
[2]Department of Biotechnology, Integral University, Kursi Road, Lucknow-226 026, Uttar Pradesh, India

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Viral diseases are not only responsible for health related issues but also exert pressure on the State economy. Tropical and subtropical countries have more prevalence of virus associated pathological conditions such as chickenpox, adenovirus related infections, dengue, chickengunya, infectious mononucleosis, etc. Treatment options with effective antiviral drugs are limited and are unfortunately not free from undesirable effects. The Asian Green Mussel, *Perna viridis* (Linn.) (Mytilidae) are not only important for their evolutionary significance, high caloric index, ecological role in the sequestration of environmental pollutants especially heavy metals, but also are potential source for extraction of therapeutic and bioactive compounds. On the other hand, generally in bivalves, virus mediated mortality is not uncommon. In this study, we made a maiden attempt of exploring DNAse like bioactivity for natural non-protenacious compound(s) extracted from *P. viridis*. Crude Methanol Extract (CME) of soft tissue of *P. viridis* and subsequently its partially purified component (PPC) possess exceptional ability to degrade indiscriminately both low and high molecular weight DNAs. *In vitro* digestions for1, 2 and 3 h with CME and PPC were found to be comparable to commercial (Sigma-Aldrich) enzyme, DNase I. Bioactive assays conducted to evaluate antimicrobial property, have shown that CME and PPC exclusively inhibit viral propagation. Nonetheless, CME & PPC have no effect on the propagation of bacteria (0 mm ZOI). These results indicate the possibility of a source of potential antiviral drug against DNA Group I viruses. Although our study does not provide any data to correlate to any physiological functions of these substances but provides a clue towards an important role in the biology of mussels. Any conclusion at this stage is premature. However, taking into consideration the significantly high virus mediated mortality in bivalves and the antiviral bioactivity of these substances, it appears that mussels have evolved some mechanisms to counteract some viruses.

**Keywords:** Antiviral property, Bivalves, DNase-like bioactivity, Plasmid pBR 322

Virus mediated pathological conditions such as Chickenpox (Varicella zoster virus, VZV), Japanese encephalitis, Chickungunya, Dengue, infectious mononucleosis, Human herpes viruses, HHV associated lymphomas and adenovirus infections and mortality in humans is one of the most important issues of concern to humans living in tropical and subtropical countries. Majority of the viral pathogens are classified under Group I (Adenovirus, Herpesvirus and poxvirus) and Group IV (Flaviviridae)[1-6]. Therapeutic approach to the counter the viral diseases are limited to 37 licensed drugs and many reported to be toxic[7,8]. However, natural compounds isolated from various plants and marine organisms have been reported to be safe alternative with lesser toxicity[9,10]. Recently, Chithambaran & David have demonstrated antiviral property and growth promoting potential of alcoholic extract of *Boerhaavia diffusa* in tiger prawns[11].

*Perna viridis* (Linn.), the Asian Green Mussel (Mytilidae) is an important intertidal bivalve species in tropical and subtropical waters of Indian Ocean[12]. *P. viridis* has colossal evolutionary significance as it exhibits marked differences in general anatomy and genetic attributes with other two members of genus *Perna*[12,13]. Most members of Mytilidae including *Perna viridis* are not only important for their high caloric index but also for their ecological role in the sequestration of environmental pollutants especially heavy metals in their soft tissues[14-16]. In addition to this, green mussels are potential source of therapeutic and bioactive compounds. However, investigations on natural products from green mussel are still rare. Grimas reported *P. viridis* to possess a natural substance, which showed bioactivity against bacteria and malignant cells[17].

*Correspondence:
Telefax: +91 838 2226362
E-mail: zafar_ian@yahoo.co.uk; zafarin@gmail.com
[#]Present add.: Dept. of Post Graduate Studies in Zoology, Govt. Arts & Science College, Karwar-581 301, Karnataka, India

DNA specific intracellular nucleases are present naturally in all cells whereas secretion of extracellular nucleases is limited to few bacterial species of eubacteria[18,19]. Within the cells, these specialized enzymes play a central role not only in the degradation of DNA but also in their repair mechanisms as well[20]. DNA specific nucleases are important at least in early stages of apoptosis to degrade DNA while in the later stages of apoptosis, it undergoes complete digestion via oligonucleosomal ladder formation by activating intrinsic endo-nucleases[20,21]. However, there are few reports on the acid and neutral tissue specific DNAse activities in many animals including members of Mytilidae and all of them are associated with DNAse enzymes[22-24]. Until now, no secondary metabolite is reported to possess unique DNAse like bioactivity.

In the present work, we explored Crude methanol extract (CME) and partial purified component (PPC) of soft tissue of the Asian Green Mussel, *Perna viridis* for DNase like bioactivity, if there any. We have also evaluated the DNA degrading ability of CME and PPC on both low molecular weight plasmid pBR 322 and high molecular weight genomic DNA of *P. viridis*. Concomitantly, we have also examined the antimicrobial activity of CME and PPC against a virus (*Bacteriophage M13MP18*) and two strains of bacteria (*Escherichia coli JM 109* and *Pseudomonas aerogenosa*).

## Materials and Methods

### Collection of *Perna viridis*

Green mussels were collected from the natural beds located at Mandovi estuary along coastline of North Goa, India ($15^0 40' N$, $73^0 50' E$). Live animals were transported in 50L capacity plastic containers to the laboratory and immediately sacrificed to obtain soft tissues along with the mantle using sterile surgical instruments.

### Preparation of crude methanol extract

The soft tissue of *Perna viridis* was washed twice thoroughly with distilled water to remove unwanted materials. Of this, 20 g was taken and mixed with 100 mL of methanol and kept under agitation overnight in an arbitrary shaker. The supernatant filtered through a Whatman filter paper of 0.45 μM pore size and tissue debris settled at the bottom after centrifugation discarded. The debris discarded by centrifugation and the supernatant filtered through a Whatman filter paper of the same grade. Methanol was evaporated under reduced pressure and the residue dried completely ($Na_2SO_4$) and concentrated under vacuum pressure. The dried extract quantified by dry weight and dissolved in nanopure water. This preparation was used as Crude methanol extract (CMD) to evaluate DNase like activity and bioactivity.

### Partial purification of DNase like compound(s) from methanol extract

For partial purification, methanol extract was prepared as described with some modifications *viz*. 200 g of animal soft tissue in 1L of methanol. Crude residue of 8.5 g was obtained after methanol evaporation and drying ($Na_2SO_4$). Using an eluotropic series of organic solvents, the dried crude residue was subjected to the solvent extraction (w/v) with *n*-hexane, chloroform, ethyl acetate, *n*-butanol and methanol. Ethyl acetate extract tested positive for DNase like bioactivity. Organic extract (4.3 g remnant after solvent extraction with *n*-hexane and chloroform) was extracted four times with 200 mL of ethyl acetate, and the combined organic extract of the tissue was dried ($Na_2SO_4$) and evaporated under reduced pressure to give a crude residue of 1.7 g. Thin-layer chromatography (TLC) analysis of the residue was performed on silica gel plates (Merck, Germany, Kieselgel 60 F254, 0.25 mm). The spots visualized by exposing the silica gel plates to UV radiation and spraying with 10% $H_2SO_4$ in $CH_3OH$ and followed by heating at 100°C for 5 min. The residue (100 mg) was partially purified by preparative TLC (Merck; Germany, Kieselgel 60 F254, 0.5 mm) to give four fractions A, B, C and D (5.2, 4.5, 6.5 and 24.0 mg, respectively). The fractions were dissolved in nanopure water to check DNase like bioactivity. All four fractions, A–D, were evaluated for the DNAse like bioactivity. Out of four fractions only fraction C (6.5 mg) exhibited DNAse like bioactivity, and hence was designated as partially purified component (PPC). PPC was used to evaluate the DNA degrading ability by taking low (pBR 322) as well as high (genomic DNA of *Perna viridis*) molecular weight DNA

### *In vitro* digestion of low molecular weight DNA (plasmid pBR 322)

The experiment was performed in triplicates with five microfuge tubes of 500 μL capacity for each set of experiment. In each tube 20 μL of reaction buffer comprised of 50 mM Tris/HCl (pH 7.6), 10 mM $MgCl_2$ and 0.5 mM EDTA was taken. To this reaction mixture, 0.25 μg of Plasmid pBR 322 (Cat # D9893, Sigma, USA) was added in triplicates of each of the

five tubes followed by the addition of 5µg of PPC. The reactions mixture incubated at 37°C for 1, 2 and 3 h of duration. Of the remaining tubes, one tube was treated with commercially available DNase I enzyme (Cat # D7291, Sigma, USA) and the other was used as a control. The reactions were terminated by adding 5µL of gel loading buffer comprised of 0.25 % bromophenol blue, 0.25 % xylene cyanol and 30 % glycerol in water to each tube after respective test time intervals. About 10 µL of the reaction mixture from each experimental tubes loaded onto a 1% agarose gel and electrophoresis was carried out at $25^0$C with 60V for 1 h. The gel was viewed under UV light in an AAB gel documentation system and photographed using systems camera.

**Degradation of high molecular weight genomic DNA of *P. viridis***

*Perna viridis* DNA was purified by following the method described by Sambrook *et al*.[25]. DNA degrading ability of the PPC was evaluated by the procedure as explained earlier. After incubation the reaction was terminated by adding 7 µL of gel loading buffer to all tubes.

**Antimicrobial Tests**

*Inhibition of Bacteriophage M13mp18 propagation*

Ten fold serial dilutions of a stock culture, which contained 10 µg lyophilized *M13mp18 Bacteriophage*, procured from Sigma US in 1000 µL liquid, was prepared in 10 labeled test tubes. To each test tube, 100 µL of 6 h old *E. coli JM 109* culture was added after evaluating growth at 550 nm and mixed thoroughly. To this mixture in five test tubes, 250 µg of CME and in another five test tubes, 250 µg of PPC was added. A series of labeled sterile tubes containing 3 mL of melted soft agar made up in LB medium was stored on a heating block at 47°C. About 40 µL of X-GAL and 4 µL of IPTG were added to these tubes containing soft agar. The contents were mixed and poured into the tubes containing bacteria, crude extract and PPC and diluted *Bacteriophage*. The mixture poured onto a readymade hardened LB agar plate. The soft agar was allowed to solidify for 5-10 min and plates were incubated at 37°C. Concomitantly, control experiment with no extract, was carried out simultaneously to check for the pale blue plaques.

*Antibacterial activity by Zone of inhibition Method*

In another experiment, *in vitro* antibacterial bioactivity of CME and PPC was evaluated by ZOI method on sterile Agar plates made up in LB medium. Zone of inhibition (ZOI) was determined by inoculating $1\times10^9$ CFU of *E. coli JM 109* on five sterile Agar plates by spread plate technique to test antibacterial activity of CME and another batch of such five sterile Agar plates to test bioactivity of PPC. After introducing 250 µg each of CME and PPC each on five Agar plates in five punched holes using sterile glass Pasteur pipette with nipple as test area on designated plates, these plates incubated at 37°C for 24 h. The ZOI test was repeated by following the above described protocol for *Pseudomonas aerogenosa*.

## Results

**DNAse like Bioactivity**

In experiment 1, pBR 322 plasmid DNA of about 4.3 kb was selected to evaluate DNase-like activity of PPC. The results showed partial degradation of the plasmid DNA after one hour of incubation (Fig. 1, Lane 1). After 2 h of incubation considerably increased degradation of the plasmid DNA could be seen with a faint band with smear (Fig. 1, Lane 2). However, after 3 h of incubation, complete degradation of the plasmid DNA occurred (Fig. 1, Lane 3). The degradation of the plasmid DNA with the PPC was comparable with the digestion of pBR 322 plasmid DNA with the commercial DNAse I enzyme (Fig. 1, Lane 4) whereas the control experiment showed prominent plasmid DNA band (Fig. 1, Lane 5) and control experiment ruled out any non-specific DNA degradation over a period of 3 h.

A complete loss of small molecular weight DNA was noticed in experiment 1 over a period of 3 h led

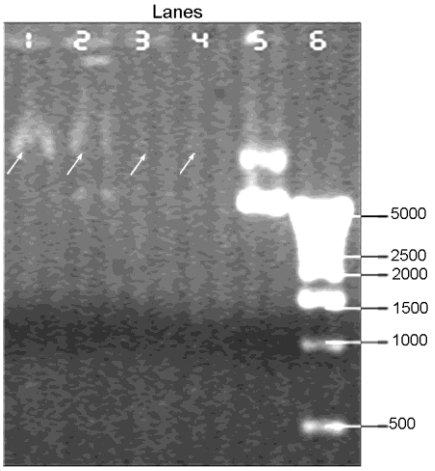


Fig. 1—DNase like bioactivity of PPC on low molecular weight DNA (Plasmid pBR 322). [Lanes 1-3: DNase like activity for 1, 2 & 3 h; Lanes 4: DNase I activity for 3 h; Lane 5: Control DNA; and Lane 6: Marker (5 kb)]

us to use a high molecular weight eukaryotic DNA obtained from *Perna viridis*.

In experiment 2, genomic DNA of *P. viridis*, was chosen as high molecular weight DNA to test the DNA degrading ability of CME and PPC.

After 1 h of incubation with PPC, DNA digestion could be observed as a thick smear in Lane 7 of Fig. 2. Incubation of 2 h, a very faint smear could be observed and the size of the band also became less prominent (Fig. 2 Lane 6). However, after 3 h of incubation, complete digestion of the DNA was observed with clearance of the smear as shown in Lane 5 of Fig. 2. DNA degrading ability was also compared between CME and DNAse I enzyme as shown in Fig. 3. Here, it was much clear to observe the activity of commercial DNAse I and CME, where the band size was almost same after 3 h of incubation in both CME treated (Fig. 3, Lane 6) and DNAse I treated (Fig. 3, Lane 4) experiments. Concomitant control sets with PPC and CME as shown in Fig. 2 (Lane 8) and Fig. 3 (Lane 5), respectively ruled out the possibility of non-specific DNA degradations.

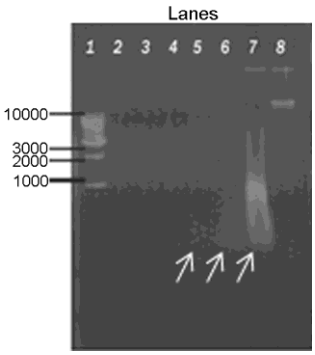


Fig. 2—Comparision of DNase like bioactivity of PPC with commercial DNase I (Sigma, USA) on genomic DNA of *Perna viridis*. [Lanes M: Marker; Lanes 2-4: DNase I activity for 3, 2 & 1 h; Lanes 5-7: DNase like activity for 3, 2 & 1 h, respectively; and Lanes 8: Genomic DNA of *Perna viridis*]

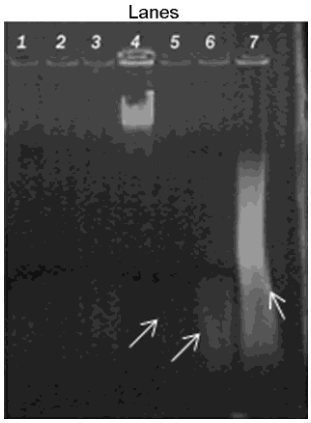


Fig. 3—Comparision of DNase like activity of CME with DNAse I (Sigma, USA). [Lanes 1-3: DNase I activity for 1, 2 & 3 h; Lanes 4: Genomic DNA of *Perna viridis*; Lanes 5-7: DNase like activity for 3, 2 & 1 h, respectively]

These experiments have clearly exhibited the DNase like property to CME & PPC by degrading both low and high molecular DNA.

**Antimicrobial assays**

In experiment 3, proliferation of *bacteriophage M13MP18* in one serotype of *E. coli* was evaluated. In a control experiment, *bacteriophage M13MP18* was allowed to carry out its life cycle with *E. coli JM 109* as its host. In the other experiment with the CME and PPC, it was observed that the CME and PPC had significantly aborted the propagation of the *Bacteriophage M13*. The inhibition of its life cycle could be evidenced by the absence of pale blue plaques. This experiment has shown that these CME and PPC were highly effective in inhibiting the propagation of *bacteriophage M13.*

Experiment 4 was undertaken to evaluate the ability of CME and PPC to prevent the formation of bacterial colonies by Zone inhibition method on two species of bacteria *viz*., *E. coli* and *Pseudomonas aerogenosa*. Incubation at 37°C for 24 h showed no clear zones and colonies were profusely formed throughout the plate including the area where CME and PPC were applied. This test demonstrated no antibacterial bioactivity for CME and PPC.

## Discussion

Marine biotic communities are potential sources yielding new and potent antibiotics and other bioactive compounds. Studies undertaken elsewhere have reported useful bioactivities in natural products obtained from marine organisms, most of the studies focused on identifying compounds with activity against bacteria and cancer cells[10,17,26].

In this study, we have identified potent DNases like bioactivity in CME and PPC from *Perna viridis* which indiscriminately digested both low molecular weight DNA (pBR 322) and high molecular weight DNA (genomic DNA of *Perna viridis*). This DNase like bioactivity found to be almost equally comparable to commercial DNase I enzyme as shown in Figs. 1 and 2. DNAse like activity reported in this paper is different from the DNAse activities described in erstwhile studies done on number of animals including *Mytilus galloprovincialis*[22,23]. These papers discused studies carried out on the neutral and acid

DNAse activities in number of tissues[23]. The DNAse activity identified in the crude mucous extract and its partial purified fraction in coral *Galaxea fascicularis* also happens to be a secretary DNAse[24]. All these studies have revealed that DNAses are nothing but part of intrinsic nuclease enzyme system. We have identified DNAse like activity from the organic compound(s), which is non-protein in nature, residual neutral and acid DNAse enzymes were completely ruled out by heating the extract to 100°C during purification steps. Hence, the CME and PPC are independent of neutral or acid DNAse activity. This report is possibly first in kind to demonstrate DNAse like activity to a non-protein organic compound(s).

We have also evaluated the antimicrobial bioactivity of CME and PPC by conducting antiviral and antibacterial assays. An interesting feature that requires attention is the inhibition of the propagation of the virus (*Bacteriophage M13*) but not of the bacteria. It was found that CME and PPC were effective in inhibiting the proliferation of viruses. This was indicated by the absence of pale blue plaques on *E. coli* cultures. Further, results of antibacterial tests have assigned no antibacterial activity to CME and PPC, this attribute of bioactivity of the extract only on virus but not on the bacteria is certainly a priority for further research. As of now, it appears that bacterial DNA perhaps is not accessible to CME & PPC in the naked form or else bacterial genome might have developed a way to counteract its effect. The mechanism of action of inhibiting the life cycle of the *Bacteriophage M13MP18* is by attacking directly its DNA or the virus itself is not clear at this stage. Whether, the CME and PPC react with proteins as well in addition to DNA is not clear at this stage but absence of antibacterial activity indicates no interaction between these and proteins. From this study, it appears that CME and PPC were more effective on the naked DNA.

Although any conclusion at this stage may be premature, but there must be some key role of DNase like active substances in biology of mussels. Our work essentially provides no data or information to link this bioactivity to a function in green mussels. Ability of these substances to inhibit the propagation of only virus but not of bacteria becomes more pertinent when the viral load and virus-mediated pathogenesis are taken into consideration. The density of virus differs from region to region in oceans and the highest viral density typically occurs closer to coastal and low salinity waters such as intertidal and estuaries[27,28]. Marine and estuarine ecosystems where the green mussels inhabit, have diverse and abundant populations of viruses[29], such as herpes like virus, podovirus and many more have already been reported to be pathogenic to shrimps and intertidal bivalves including *Perna viridis*[29-31]. Viruses mediated mortality is significantly higher in bivalves accounting for 10-30 % with a potential of becoming epidemic[32,33]. Hence, it appears that the bivalves might have evolved some mechanisms to defend themselves from the viruses by synthesizing some metabolites. Corroboration of immune function against virus to this DNase like bioactive substances is an object of future research endeavor.

DNAse like PPC with an antiviral property can be a potential candidate to develop an effective drugs against the DNA viruses like varicella zoster virus and Herpes viruses. Detailed work is underway to further purify the active antiviral component from the PPC involved in DNase-like bioactivity. Secondary metabolites showing DNase like bioactivity is noteworthy and this is the first ever report to demonstrate DNase like activity to natural components obtained from a bivalvia. Although our work essentially provides no data to establish, the exact function of these components but provide important clues for further research to corroborate its function in the biology of mussels. Further, their ability to inhibit the propagation of *bacteriophage M13* can further be refined to develop an effective antiviral drug in future.

## Conclusion

With the results of the present investigation we conclude that the crude methanolic extract (CME) and its partially purified compound (PPC) from the soft tissue of the Asian Green Mussel, *Perna viridis* has potent DNases like bioactivity comparable to commercial DNase I enzyme. Further, the antiviral and antibacterial assays have also confirmed the antimicrobial bioactivity of CME and PPC. Inhibition of the propagation of the virus (*Bacteriophage M13*) but not of the bacteria indicates then need for further research.

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