Antibacterial activity of different solvent extracts of marine bivalve, *Meretrix casta*

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ABSTRACT

Marine biofunctional peptides are protein molecules that promote health by blocking certain harmful chemical pathways in the human body, such as the laying down of cholesterol in blood vessels. Marine bivalves are considered one such rich source of these peptides and bioactive compounds. These, bioactive peptides possess various biological activities such as antibacterial, antioxidant, anticoagulant etc. Thus, in the present study the bioactive potential of marine bivalve *M. casta* was evaluated using different polar and non polar solvents of tissue. The extracts exhibited effective hemolytic and antibacterial activity at different specificity. Among all the samples investigated, Ax extract exhibited potent antibacterial activity against the tested human pathogens, thus ensuring the presence of potent biomedical important compounds.

KEY WORDS: Antibacterial, haemolytic activity, *M. casta*

INTRODUCTION

Marine invertebrates solely dependent on physical barriers and the innate immune system for the protection against pathogenic agents and natural antibiotics have been shown to participate in the immune response (Munoz et al., 2004). The knowledge of the self-defense mechanism of molluscs is extremely limited compared to that of vertebrates and arthropods (Iijima et al., 2003). The immune defense of molluscs is non-specific and lacks inducible immunoglobulins, but the responses against microbial organisms are based on both cellular (phagocytosis, encapsulation, respiratory burst, etc.) and humoral (lectins, agglutinins, lysosomal enzymes, antimicrobial factors, etc.) activities (Canesi et al., 2002; Chu, 1988). In which, humoral immunity is strictly related to the presence of antimicrobial agents in blood cells and plasma. In turn, their cellular immunity is based on cell defense reactions, including encapsulation, nodule formation and phagocytosis (Mydlarz et al., 2006).

Antimicrobial peptides gain importance mainly because of: (i) their innate immunity lies in their ability to function without high specificity or memory, (ii) their small size which makes them easy to synthesize, (iii) their remarkable specificity for prokaryotes with low toxicity for eukaryotic cells (Relf et al., 1999). The involvement of antimicrobial peptides constitutes an original model of anti-infectious defence in invertebrates based on: (i) molecular structures (cysteine array and 3D) similar to that of insect defensins, (ii) constitutive expression in circulating hemocytes and storage in different granules of different hemocytes, and (iii) two routes of involvement, immediate intracellular and later as released in plasma. These small cationic antimicrobial peptides are characterised by their high cysteine content and they have been organised into four groups according to the shared features of their primary structure, mainly their consensus cysteine array: defensins, mytilins, myticins and mytimycin (Mitta et al., 2000).
Thus to understand the importance of bioactive compounds, the study was carried to investigate the presence of antimicrobial compound in the marine bivalve *Meretrix casta* extracted with different types of polar and non-polar solvents.

**MATERIAL AND METHODS**

**Collection of specimen**

The live specimen of *M. casta* was collected from South east coast of Tamil Nadu, India. They were brought to the lab in aerated plastic containers filled with sea water of ambient salinity. Clams of both sexes were selected and washed in distilled water and cleaned thoroughly to get rid of debris.

**Extraction procedure**

150 gm of wet tissue sample of *M. casta* was macerated with 150 ml of following solvent systems: Hexane (Hx), Acetone (Ac), Methanol (Me), Chloroform (Ch), 60 % ACN in 0.1% TFA (Acn) and except for acidic extraction. The mixtures were kept overnight at 4°C and the suspension was centrifuged at 8000 rpm for 20 min. The supernatant was concentrated using vacuum evaporator (35-55°C), lyophilized and stored at -20°C until use.

**Acidic extraction**

Acidic extraction (Ax) was performed by incubating 150 g of wet weight of tissue in hydrochloric acid (HCl) 1 N (1 L) for 10 min at 100 °C (Zatylny et al., 2000). After homogenisation they were centrifuged 20,000 ×g for 30 min at 4 °C. The supernatant was collected and lyophilized to give the acidic extract and stored at -20 °C until use.

**Estimation of Protein**

Amount of protein in the samples were quantified by the method of Lowry et al. (1951) with BSA as a standard.

**Estimation of Carbohydrate**

Content of carbohydrate was quantified using the method of Ashwell (1966).

**Haemolytic assay**

Haemolytic assay was performed following the microtitre plate method of Paniprasad and Venkateshvaran (1997). The chicken blood was collected using EDTA solution (2.7 g/100 ml) as anticoagulant. The mixture was further centrifuged at 5,000 rpm for 5 min, the supernatant was discarded and pellet 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 1ml of packed RBC.

The micro haemolytic test was performed in 96 well ‘V’ bottom microtitre plates. Different rows were selected for the different samples. Serial dilution of the crude extracts (100 µl) was done till the last well and 100 µl was discarded from last well. 100 µl of 1% RBC was added into all the wells with appropriate controls were included in the test. 1% RBC suspension, 100 µl of distilled water was added, which served as a positive control and 100 µl of normal saline which served as negative control. The plate was gently shaken and allowed to stand for 3 hours at room temperature and the results were recorded. The uniform red colour suspension in the wells considered as positive haemolysis and a button formation in the bottom of the wells was considered as lack of haemolysis. The reciprocal of the highest dilution of the crude extract shows the haemolytic pattern was taken as one Haemolytic Unit (HU).

**Determination of Antibacterial activity**

**Microorganisms and inoculums preparation**

The crude extracts of *M. casta* were tested against the bacterial pathogens *Klebsiella pneumoniae, Staphylococcus*
*Staphylococcus aureus*, *Streptococcus pneumonia*, *Escherichia coli*, *Vibrio parahemolyticus*, *Salmonella typhi*, *Klebsiella oxytoca*, *Proteus mirabilis*, *Vibrio cholerae*, *Salmonella paratyphi*. All microorganisms were obtained from the Department of Microbiology, Annamalai University, India.

The bacterial pathogens were subcultured in nutrient agar. The strains were inoculated and grown to exponential phase in nutrient broth at 37°C for 18h and adjusted to a final density of $10^5$ CFU/ml by diluting fresh cultures by comparing with McFarland density.

### Antibacterial activity

Antibacterial activity of crude extracts was determined by agar disc diffusion method (Jorgensen and Turnidge, 2003). About 25 ml of molten Muller Hinton agar was poured into sterile petri plates (Himedia, Mumbai, India) and were allowed to solidify. The plates were then evenly streaked in three different directions with a sterile cotton swab dipped in the bacterial suspension ($10^5$ CFU/ml). Sterile filter paper discs (5 mm diameter) were immersed in 50 µl of respective crude extracts (1.0 mg/ml) and placed over the Muller Hinton agar plates after drying in room temperature. Erythromycin 10 mcg/disc was used as positive control. The plates were incubated overnight at 37°C and the zone of inhibition around the disc was measured.

### RESULTS

#### Percentage of yield

The *M. casta* tissue was extracted with different polar, non-polar and acidic extraction procedure. The yield of extraction was calculated and represented in Table 1.

<table>
<thead>
<tr>
<th>S.No</th>
<th>Solvent system</th>
<th>% of yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Hx</td>
<td>6.809</td>
</tr>
<tr>
<td>2</td>
<td>Ac</td>
<td>1.623</td>
</tr>
<tr>
<td>3</td>
<td>Ch</td>
<td>5.496</td>
</tr>
<tr>
<td>4</td>
<td>Me</td>
<td>7.972</td>
</tr>
<tr>
<td>5</td>
<td>Acn</td>
<td>2.98</td>
</tr>
<tr>
<td>6</td>
<td>Ax</td>
<td>6.809</td>
</tr>
</tbody>
</table>

### Estimation of Protein

The amount of crude protein and carbohydrate present in the extracts varied depending on the solvent system used (Fig. 1).
Haemolytic activity

The haemolytic activity of crude extracts against chicken blood was determined. The crude extracts induced moderate levels of haemolysis on the blood. The haemolytic activity of the crude extracts was tabulated (Table 2). (One HU is defined as the quantity of protein catalyzing lysis of 50% of red blood cells present). Among which the sample Me exhibited highest activity.

Table 2: Haemolytic activity of different crude extracts of *M. casta* using chicken blood

<table>
<thead>
<tr>
<th>S.No</th>
<th>Crude extracts</th>
<th>Protein mg/g</th>
<th>Total haemolysis upto dilution</th>
<th>Haemolytic Titre</th>
<th>Specific haemolytic activity (HT/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Hx</td>
<td>304</td>
<td>2</td>
<td>4</td>
<td>0.013</td>
</tr>
<tr>
<td>2</td>
<td>Ac</td>
<td>521</td>
<td>5</td>
<td>32</td>
<td>0.061</td>
</tr>
<tr>
<td>3</td>
<td>Ch</td>
<td>147</td>
<td>2</td>
<td>4</td>
<td>0.027</td>
</tr>
<tr>
<td>4</td>
<td>Me</td>
<td>786</td>
<td>5</td>
<td>32</td>
<td>0.041</td>
</tr>
<tr>
<td>5</td>
<td>Acn</td>
<td>650</td>
<td>4</td>
<td>16</td>
<td>0.024</td>
</tr>
<tr>
<td>6</td>
<td>Ax</td>
<td>745</td>
<td>7</td>
<td>128</td>
<td>0.172</td>
</tr>
</tbody>
</table>
Determination of Antibacterial activity

The crude extracts of *M. casta* exhibited significant antibacterial activity (Fig.2.). Among all the six samples tested, acidic extraction (Ax) exhibited highest activity against the tested human pathogens specifically against *S. pneumonia* (10 mm). The other solvent extracts Hx, Acn and Ch also exhibited effective antibacterial activity whereas Ac extract did not possess any notable activity.

![Fig.2. Antibacterial activity of different crude extracts of *M. casta* against human pathogens (cm). Values are given as mean ± SD of three experiments](image)

Discussion

Generally, molluscs are widely being used as food and feed supplement around the world. Particularly, clams are one of the important varieties of shellfish and perhaps the most versatile seafood in the world. These edible bivalves are filter feeders; thereby have high conversion efficiency and consequently high levels of biochemical constituents that opens a broad opportunity for the isolation of marine natural products. These MNP’s constituted a survey of marine secondary metabolism where the majority of recent efforts have been focused to a greater extent on the discovery of metabolites with bio-medically relevant activities (Ireland *et al*. 1993; Carte, 1993). They have turned out to be an important resource for the amplification of medicines in the field of pharmaceuticals.

Hence, the present study deals to explore antibacterial activity of different solvent extracts of marine bivalve *M. casta*. The purpose of using different solvents is that they can provide more exhaustive information on the properties of the extracts and hence it could provide maximum results for biological activity (Fernandez *et al.*, 1996). Among the different solvent used to extract the biological active compounds of marine bivalve *M. casta*, the solvent Me yielded higher percentage of 7.972 whereas the solvent Ac yielded the lower percentage of 1.623. The amount of protein and carbohydrate content varied extensively based on the solvent extracted. Me and Ax extract exhibited higher amount of protein 786 µg/mg and 745 µg/mg whereas the Hx and Ch extracts exhibited low protein content of 304 µg/mg and 147
µg/mg respectively. The higher amount of carbohydrate was present in Acn and Ch extracts of 266 µg/mg and 235 µg/mg while low content was observed in Ax and Hx extracts of 95 µg/mg and 75 µg/mg respectively. Similarly, Sarumathi et al. (2012) reported the amount of protein present in the methanol extract to be 200 µg/mg of the sample.

Among the six crude extracts tested for haemolytic against chicken blood, the Ax, Me and Ac extract exhibited the maximum activity of 7, 5 and 5 HU whereas Ch and Hx exhibited minimum activity of 2 HU both. As supporting, the crude methanolic extract of C. tranquebaricus showed 9HU (Sarumathi et al., 2012). Generally, haemolytic activity appears to be common in extracts that show high antibacterial activity (Guzman et al., 1993; Haug et al., 2002). As such, the Ax extract exhibited potent antibacterial activity against S. pneumoniae (10 mm), V. parahemolyticus (8 mm) and exhibited no activity against S. paratyphi where Acn, Ch, Hx and Me extracts also exhibited considerable activity where no significant activity was observed for Ac extract. Similarly, Sri Kumaran (Sri Kumaran et al., 2011) reported that the ethyl acetate extracts of Thais tissoti showed significant activity against the human bacterial pathogen K. pneumonia (10.02 ± 0.11 mm) whereas for Babylonio spirata n-butanol extract exhibited high zone of inhibition against the bacterial pathogen P. mirabilis (7.02 ± 0.04 mm). It was further supported by Sarumathi et al. (2012) that the methanolic extract of C. tranquebaricus exhibited maximum zone of inhibition against V. cholerea (10 mm). Thilaga (2005) also screened the antibacterial activity of a marine mollusc B. spirata against bacterial pathogens revealing efficient activity.

Overall, by studying the different solvent extracts that used to extort the bioactive molecules from the marine bivalve M. casta, the solvent extract Ax (Acidic extraction) was found to exhibit prominent results among the six solvents systems used. In spite of that, the study also clearly depicts the comparative activity of the other solvent extracts. This apparently describes that the bivalve contains active molecules possessing distinct biological properties. Thus, necessitates the need of further investigation and purification of active compounds in order to identify their chemical nature and to evaluate their potential as novel drugs. This investigation signifies that the extraction procedures used to extract the bioactive metabolites should always include various types of solvent system including both polar and non-polar systems which could render effective results for the isolation active compound from marine molluscs by highly recommending acidic extraction for the isolation procedure.

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REFERENCES


