

***In vitro* study of antimicrobial activity in freshwater mussel (*Lamellidens marginalis*) extracts**

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Abstract

Anti-bacterial activity was evaluated in different crude extracts of commercially important, edible freshwater bivalves, namely *Lamellidens marginalis*, collected from the Godavari river of Andhra Pradesh (India). Three different procedures, viz Aqueous, Chloroform and Acetone, were followed to prepare the extracts. The efficacy of the extraction procedure was assessed on the antimicrobial activity. Antimicrobial assay was carried out against 8 bacterial strains (3 gram positive and 5 gram negative bacteria) and 1 species of fungi. The acetone extracts showed significant activity against the tested organisms as compared to aqueous and chloroform extracts. The findings of the present study confirmed that the antimicrobial activity in freshwater bivalves appeared to be dependent on the extraction.

Keywords: *Lamellidens marginalis*; chloroform; acetone; extracts; aqueous; antimicrobial peptides.

Introduction

Due to an alarming rise in the occurrence of antibiotic resistant bacterial strains, the identification of new antimicrobial compounds has become one of the frontier areas in biomedical research. Aquatic (marine and freshwater) invertebrates are known to rely on innate immune mechanisms which include both interacting cellular and humoral components to protect against potential pathogen (Tincu and Taylor, 2004). Innate immune mechanism in freshwater invertebrates is known to protect these organisms against potential pathogens. Moreover, it has been well known that the innate immunity is triggered immediately after microbial infection to produce antimicrobial compounds including small antimicrobial peptides (AMP). In recent years, it has widely been recognized that AMPs are strong defensive weapons against bacteria and/or fungi, viruses, or parasites in multicellular organisms (Zasloff, 2002). Furthermore, AMPs are also known as major components of innate immune defence system in invertebrates (Seo *et al*, 2005). Considering the fact that the aquatic animals can survive in a hostile environment where they are surrounded by various pathogenic organisms, including human pathogens (Bouchriti and Goyal, 1992) and that they are potential sources for bioactive compounds, an attempt has been made in the present study to evaluate the antimicrobial activity in commonly occurring edible bivalve, such as *Lamellidens marginalis*. In continuation with the same

effort, an attempt was also made to assess and compare the efficacy of the extracts prepared using three different extraction procedures.

Materials and Methods

Live bivalves used in the present study were collected from Godavari River (Mancheril) of Andhra Pradesh in India. These bivalves were not collected during the summer months to avoid stress related to disease, elevated water temperature, hypoxia or gametogenesis. Bivalves were brought to the laboratory in water, washed, and de-shelled; tissue and mantle fluids were also collected. Material collected from each animal was divided into three equal parts for the preparation of aqueous, chloroform extracts with protease inhibitors and acetone (Chatterji *et al*, 2000). These procedures yielded four different types of extract from each animal.

All the extracts were quantitatively analyzed using the standard methods for the estimation of total protein (Lowry *et al*, 1951), carbohydrates (Dubois *et al*, 1956) and lipids (Parsons *et al*, 1984). The antimicrobial activity of the bivalve extracts prepared was evaluated against a set of 9 pathogenic micro-organisms (*Bacillus subtilis*, *Escherichia coli*, *Pseudomonas* sp., *Streptococcus pyrogenes*, *Staphylococcus aureus*, *Proteus vulgaris*, *Klebsiella pneumoneae*, *Serratia marganii* and *Candida albicans*) using standard disc diffusion assay (qualitative) and liquid growth inhibition assay (quantitative). The liquid growth inhibition assay was carried out at

32±1°C for 30 hrs. Three sets of sterilized glass vials, with 2.5 ml nutrient broth (NB), were prepared for each pathogenic micro-organism. These sets were inoculated with one bacterial suspension (18 hr old), and out of which, one set each was inoculated with 10 µl of Gentamycin (1 mg/ml). This was treated as the positive control. In another set, all the micro-organisms were inoculated with 10 µl of animal extracts (50 mg/ml). One set was maintained as the negative control to see the

normal growth of the tested organisms. After 2 hrs, an aliquot was taken in a micro titer plate and optical density was measured at 570 nm using an ELISA reader (BioRad, Microplate Reader, Model No. 680). The percentage of the inhibition growth was calculated with absorbance values using the following equation, where CI is the percentage Inhibition index, A = A 570 of bacteria with NB (control) and B = A 570 of bacteria with extract or Gentamycin (experimental).

$$CI = \left[\frac{A - B}{A} \right] \times 100$$

Results

The total protein content was the highest as compared to the total contents of carbohydrate and lipids in all the extracts. The total content of lipid in all the extracts was very less as compared to the total protein and carbohydrate (Table 1).

Bioassay of all extracts of *L. marginalis*, using standard disc diffusion assay, showed that aqueous extract appeared to be the more active one against all tested organisms. In case of the chloroform extracts, *L. marginalis* showed the highest antimicrobial activity against *S. aureus*, *S. pyroenes* and *S. marginii* pathogens. Meanwhile, in the case of acetone and aqueous extract, significant antimicrobial activity was shown against *B. subtilis* and *E. coli* pathogens. On the other hand, the acetone extracts of *L. marginalis* showed a very good activity against *S. pyroenes*, *B. subtilis*, *E. coli*, *Pseud. sp.*, *P. vulgaris* and *C. albicans* organisms (Table 2).

As compared to Gentamycin, all the Aqueous extracts showed more than 30 to 50% antimicrobial activity against *E. coli* when liquid growth inhibition assay was performed. The Aqueous extract of *L. marginalis* appeared to be the most promising extract showing antimicrobial activity against both Gram positive and Gram negative tested organisms for 18-24 hrs (Fig. 1). The activity of the extract of chloroform was better than Gentamycin for 10 hrs. It was interesting to note that Gentamycin did not have very good antimicrobial activity against *S. marginii*, but all the chloroform extracts of freshwater mussel were able to inhibit the growth (>50%) of Gram negative bacterium for more than 6 hrs (Fig. 1). In the group of acetone extracts, inhibited the growth of *S. pyrogenes*, *B. subtilis*, *E. coli*, *Pseud. sp.*, *P. vulgaris* and *C.*

albicans better than Gentamycin for 4–10 hrs (Fig. 1).

All the experiments were carried out in triplicates and the results are expressed as mean values. The results were then compared using the two-way ANOVA test (Statsoft, 1999). p values < 0.05 were considered as significant. In this study, it was observed that when the antimicrobial activity of one extract was compared to all the tested organisms, the p value < 0.05 indicated that the presence of antimicrobial compound/peptide in the extract could have different degrees of activity against various organisms.

Discussion

Most of the work carried out on antimicrobial compounds from marine bivalves deal with *M. edulis*, *M. galloprovincialis*, *G. demissa*, *C. virginica* and *C. gigas* (Tincu and Taylor, 2004; Haug *et al*, 2004; Mitta *et al*, 1999). In this study, an attempt has been made to screen freshwater bivalves, especially the commonly occurring edible ones. The source for majority of the AMPs reported has been from the hemocytes (Charlet *et al*, 1996a; Mitta *et al*, 1999b) epithelial tissues (Marshall and Arenas, 2003; Noriaki *et al*, 2003) and the tissues of gut and respiratory organs (Tincu and Taylor, 2004). Considering this an important aspect, the extracts were prepared using both mantle tissue and mantle fluid of the bivalves.

Presuming that the antimicrobial compounds were either protein or peptide in nature, a combination of 'soft techniques' was selected for the preparation of crude methanol (homogenization with chilled methanol + filtration) and chloroform extracts (homogenization with chloroform + protease inhibitor + centrifugation) to ensure that the functionality and/or the biological activity of the analytes remained intact (Visser *et al*, 2005).

For this, methanol was selected as a suitable solvent as it gave good extraction efficiency. Most of the low molecular weight proteins/peptides (stable at room temperature) were extracted from it and had an added advantage of allowing rapid sample concentration through evaporation. The other solvent/homogenization medium is chloroform as most of the active high molecular weight proteins and peptides are known to be extracted in it. Protease inhibitor simultaneously deactivated proteolytic enzymes in the tissue which would otherwise cause rapid degradation of the proteins/peptides (Conlon, 2007). The results of antimicrobial assay in the present study indicated that these extracts showed high antimicrobial activity against the tested pathogens, indicating that these procedures are capable of extracting the antimicrobial compound(s), with relatively higher activity, without degrading the nature of the compound.

The acetone extraction procedure employed in the present study was very harsh as all the conditions (e.g. highly acidic pH, 100°C temperature) were totally different from the physiological conditions of the biological matrices. Enzyme protosubtilin hydrolyzed high-molecular animal proteins to short peptides and a mixture of free amino acids. The crude extract has been reported to possess short peptides, free amino acids (conjugated with metals like Cu, Zn etc) and minerals. They were also reported to possess low fat and salt contents (Charlet *et al*, 1996a). The antimicrobial activity of the acetone extract might be either due to short peptides, amino acids conjugated with metal ions or both or it could also be due to the generation of artifacts during the extraction process. The standard disc diffusion method is a sensitive and highly accepted method used for the detection of antimicrobial activity, but many feel that it is a qualitative method and should not be used to quantify the activity (Rios *et al*, 1988). Therefore, the results were quantified using the liquid growth inhibition assay. These results largely confirmed the findings of disc diffusion assay and helped in calculating the inhibition percentage of culture growth caused by a particular extract towards the pathogenic organism(s). This assay was carried out for 30 hrs to compare the time when the extracts started inhibiting the growth of the organism and when it lost the activity. It was observed that most of the crude extracts were able to induce inhibition for ± 10 hrs and showed antimicrobial activity at par (or better at some places) with a purified antibiotic like Gentamycin.

The results presented in this paper are the first stage of a bioassay-based baseline survey to achieve the isolation, purification, structure elucidation and biological testing of the active compound(s) from the potent marine bivalves. This data suggests that *L. marginalis* is the potential candidates for the isolation and purification of potent antibiotics. Moreover, the extraction of the antimicrobial compound(s) appeared to be dependent on the extraction procedure and the nature of the solvents used for extraction.

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Table 1. Biochemical analysis of freshwater mussel (*L. marginalis*) extracts.

Extracts	Biochemical analysis		
	Protein ($\mu\text{g/ml/mg}$)	Carbohydrate ($\mu\text{g/ml/mg}$)	Lipid ($\mu\text{g/ml/mg}$)
Aqueous	420	24.48	0.6
chloroform	640	2.31	0.07
Acetone	154	6.54	0.02

Table 2. Antimicrobial activity (zone of inhibition in mm) of different extracts of freshwater mussel (*L. marginalis*).

Test organism	Different extracts of <i>L. marginalis</i>		
	Aqueous	Chloroform	Acetone
<i>S. aureus</i>	11	9	6
<i>S. pyroenes</i>	12	8	11
<i>B. subtilis</i>	13	7	12
<i>E. coli</i>	8	-	12
<i>Pseud. sp.</i>	12	-	11
<i>S. marginii</i>	11	8	6
<i>P. vulgaris</i>	-	-	12
<i>K. pneumon.</i>	-	6	7
<i>C. albicans</i>	10	6	12

Fig 1. Antimicrobial activity of different extracts (A=Aqueous, B=chloroform, and C=Acetone) against pathogenic organisms.

