

Research Article**Investigation for inhibitory effect *Uca triangularis* extracts****Sundaramurthy Kavitha¹, Jayaprakash Jayanthi², Bharathi Ravikrishnan³, Shankar Sharmila¹, Manickavalli Gurunathan Ragunathan¹**¹Department of Advanced Zoology and Biotechnology, Guru Nanak College, Velachery, Chennai-42, India²G.S.Gill Research Institute, Guru Nanak College, Velachery, Chennai-42, India³Department of Biotechnology, Guru Nanak College, Velachery, Chennai-42, India

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Abstract

Objective: There are increased incidences of the multi-drug resistance *Staphylococcus aureus* infections, worldwide, especially in India. In India among the male population the prostate Cancer is high. Thus it results in high mortality rate. Hence this present research is aimed to identify the alternative compounds which generate effective microcidal properties and also the anticancerous properties against the prostate cancer cell line model- PC3. **Material and Method:** The hemolymph of both male and female crab *Uca triangularis* was extracted and screened for the antibacterial activity against *Staphylococcus aureus* and same the same hemolymph of the male and female was subjected to protein purification by ammonium sulphate precipitation, combination of molecular sieve and ion exchange chromatography and the purified protein fraction was subjected to cytotoxicity assay against PC-3 cell line. **Result and Conclusion:** The crude male hemolymph sample was found to generate better antibacterial activity against *Staphylococcus aureus* as 9mm ± 0.02 at 50µl by the well diffusion method. The DEAE cellulose- Sephadex A-25 generated antibacterial activity against *Staphylococcus aureus* as 11 mm ± 0.24. The anticancerous study against PC3 cell line, the IC50 value was determined 120µg/ ml and 29.6% ± 0.87 cells were found to be viable at 200 µl/ ml of the male hemolymph fraction. The antimicrobial peptides extracted from the *Uca triangularis* of the both male and female was able to generate the both antibacterial and anticancerous properties.

Keywords: *Uca triangularis*, antimicrobial peptides, anticancer compounds

Introduction

Prostate cancer occurs in a man's prostate which is a small walnut-shaped gland that produces the seminal fluid that nourishes and transports sperm. Cancer usually grows slowly and initially remains confined to the prostate gland, where it may not cause serious harm. While some types of prostate cancer grow slowly and may need minimal or no treatment, other types are aggressive and can spread quickly. With increase in life expectancy, adoption of newer lifestyles and screening using prostate specific antigen (PSA), the incidence of prostate cancer is on rise. Globally prostate cancer is the second most

frequently diagnosed cancer and sixth leading cause of cancer death in men. The present communication makes an attempt to analyze the time trends in incidence for different age groups of the Indian population reported in different Indian registries using relative difference and regression approaches (Krishnappa *et al.*, 2012).

High incidence rates of these cancers can be attributed to both internal (genetic, mutations, hormonal, poor immune conditions) and external or environmental factors (food habits, industrialization, over growth of population, social etc.) (Ali *et al.*, 2007). Population size increase and with enhanced life expectancy, the population of older men are increasing and thus the number of prostate cancer also. In the occurrence of the prostate cancer androgen receptor, androgen and its related signal transduction plays a very important role. (JsGirling *et al.*, 2007). Crustaceans are huge reservoir of anticancerous products. In crustaceans, the defense system against microbes rests largely on cellular

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activities performed by hemocytes such as adhesion, phagocytosis, encapsulation, nodule formation and melanisation. The potential of marine crabs as a source of biologically active products is largely unexplored.

Materials and methods

Preparation of tissue extract:

In this study both male and female crabs of size: 2.2 x 2.5 (male) and 2.3 x 2.6 (female) were used as the sample. Due to the small size of the animal, the whole animals was used as the sample to prepare the tissue extract. About five animals of uniform size were subjected to homogenization with 5 ml of sterile PBS (7.2±0.2) for 10 minutes at room temperature. The tissue homogenate was transferred into a sterile 15ml centrifuge tube and was subjected to centrifugation at 2500 rpm at 4°C for 15 minutes. The homogenates of both male and female were separated and a pinch of sodium azide was added and stored in refrigerator until further use.

Preliminary screening of antibacterial activity:

Staphylococcus aureus was lawn cultured on to the sterile Muller Hinton agar and six wells were punched with sterile gel cutter (one in the center and five wells over the periphery). To all the periphery wells 10µl, 20 µl, 30 µl, 40 µl and 50 µl of the male tissue homogenate was added. To the center well 10µl of the PBS was added as the control. Similar plates were prepared for female samples also. The plated were incubated at 37°C for 24 hrs in a biological incubator.

Partial purification of Antibacterial proteins

Two ml of tissue homogenate from both the male and female samples were transferred to a clean 50ml beaker and ammonium sulphate was slowly added to achieve 85% of the saturation over a magnetic stirrer and the process was carried overnight. The pellet obtained was subjected to dialysis by using Dialysis membrane-110 (HiMedia). The supernatant was carefully decanted with the help of pasture pipette and the pellets were suspended in 5 ml of 1.0M PBS. The sample was added to the dialysis bag and was subjected to dialysis with 0.1M, 1 liter PBS buffer for overnight with the help of magnetic stirrer. The beaker buffer (0.1M), was changed four times in between the process. After 24 hrs, when the beaker solution is clear, the dialysis process was stopped and the sample from the dialysis bag was transferred to the sterile centrifuge tube.

Ion exchange purification of Antibacterial proteins

Diethyl amino ethyl cellulose (DEAE) -Sephadex A-25 was used as the stationary phase. 1.0 M PBS buffer was used to swell the resin for overnight. The excess of buffer was removed and the resin was washed twice with the sterile PBS buffer and was packed into the column and was equilibrated with 50mM PBS (6.2±0.2). 200µl of the sample were added on to the resin. Initially the column was

eluted with 50mM PBS containing 5% NaCl and after the collection of five fractions of 1ml fractions, the column was eluted with 50mM PBS containing 30% NaCl. The 200µl of 20 fractions were collected. Samples from the male and the female crabs were used.

Antibacterial activity by well diffusion method

All 20 fractions were subjected to antibacterial activity against *Staphylococcus aureus* were determination by well diffusion method. The fractions 7, 8, 9, 10, 11 gave good activity, when compared to the other fractions. These fractions were pooled together and were subjected to SDS-PAGE analysis.

Protein estimation by Lowry's method:

The concentration of the protein present in the female tissue homogenate was estimated by Lowry *et al.*, 1951 method. Bovine serum albumin was used as the standard to detect the concentration of protein present in the pooled sample.

SDS- PAGE

The proteins bands from both the male and female tissue samples were resolved by using 18% resolving gel and 45 stacking gel. The bands were developed using coomassie brilliant blue R-250.

Maintenance of PC3 cell line:

Pc3 cell lines were procured from National center for cell sciences, Pune (NCCS). The cells were maintained in Dulbecco's Modified Eagle Medium Media (DMEM medium) (Kaighn *et al.*, 1979) supplemented with 10% FBS, penicillin (100 U/ml), and streptomycin (100 µg/ml) in a humidified atmosphere of 5% CO₂ at 37 °C. After three days of incubation, the monolayer has been established; the cells are subjected to trypsinisation (0.25% Trypsin and 1mM EDTA in Dulbecco's Phosphate Buffered saline (DPBS), without calcium chloride and magnesium chloride, ± 7.2) and after three minutes of incubation at 37°C in 5% CO₂ the trypsinisation was stopped by the addition of 1 ml of FBS. The cells were then subjected washing process with DPBS (Ian Freshney *et al.*, 2002).

Cell viability assay:

The cytotoxic effect of the bioactive polypeptide extracted from female *Uca triangularis* was tested upon Prostate cancer cells (PC3 cell line). The MTT assay was performed to detect the cytotoxic activity (Mosmann *et al.*, 1983). Cells (1 × 10⁸/well) were plated in 0.2 ml of medium/well in 96-well plates. The microculture plates were incubated at 5 % CO₂ incubator for 72 hours. Then, the sample of various concentrations was added to the each of the well and were further incubated for 24hrs at 5 % CO₂ incubator. After the incubation period, the contents of the wells were discarded

aseptically and 20 μ l/well (5mg/ml) of 0.5% 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl--tetrazolium bromide (MTT) in phosphate- buffered saline solution was added. After 4hrs of incubation in 5 % CO₂ incubator and the cell debris were discarded by centrifugation at 18°C for 1500 rpm for 10 minutes. The supernatant was suspended in 100 μ l of the ethanol to dissolve the formazan crystal and the absorbance was recorded at 540nm. Measurements were performed and the concentration required for a 50% inhibition of viability (IC₅₀) was determined graphically.

Statistical analysis

The research data in this present work were statistically expressed as mean, standard error of mean (SEM). The value of probability was obtained from degree of freedom by using the standard table. The following levels of significance were used P 0.05 for insignificant data. The level of significance (P-value) was calculated for these groups using two tailed paired T-test using the online tool Graphpad-Quickcalcs.

Results

In general the fiddler crabs are the common macrofauna among the coastal regions of South East Asian countries. It has been identified that the carbon, nitrogen and total organic content plays a key role in the population distribution and enhancement of *U. triangularis* (Mohammad *et al.*, 2015). *Uca triangularis* plays an important role in maintaining the geochemistry coastal ecosystem (Subhasish *et al.*, 2014). There is no much of work has been performed related to identify the biopotentials of the species *Uca triangularis*. Hence this present work has been undertaken to identify the biopotential property of the *Uca triangularis*. After the acclimatization of the animal in laboratory condition, the animals (both male and female animals) were sacrificed and subjected to homogenization and the whole tissue extract from the both the gender was prepared and stored. The whole body extract was used as the sample to determine the biopotential property.

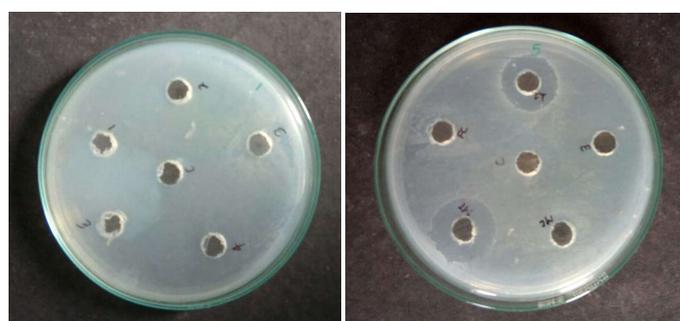


Figure 1. Antibacterial activity of the purified protein fraction: (a) Male tissue sample (b) Female tissue sample

Table 1. Antibacterial activity of the crude tissue homogenate

S. No.	Sample	Zone of inhibition	
		Female crude sample, (mm)	Male crude sample, (mm)
1.	10 μ l	3 \pm 0.92	3 \pm 0.21
2.	20 μ l	3 \pm 0.87	5 \pm 0.28
3.	30 μ l	3 \pm 0.23	5 \pm 0.31
4.	40 μ l	5 \pm 0.54	6 \pm 0.45
5.	50 μ l	7 \pm 0.41	9 \pm 0.02
6.	Ciprofloxacin	5 \pm 0.85	5 \pm 0.78

Mean \pm SE of 3 individual observations of each group, P<0.005 significant

The crude sample was subjected to antibacterial activity identification and the male tissue sample exhibited increased zone of inhibition upto 9mm \pm 0.02 at 50 μ l and the female sample generated 7mm \pm 0.41 of inhibition zone against *Staphylococcus aureus* Figure.1 and Table.1). The total crude protein concentration in male tissue sample was identified as 76.3 mg/ml \pm 4.32 and in the female tissue sample it was determined as 89.25 mg/ml \pm 3.04 (Table.2).

The male tissue sample extract which generated increased antibacterial property, it was subjected to partial protein purification and the purification was performed by DEAE-Sephadex column chromatography A-25 (weak anionic resin), 18% SDS-PAGE gel analysis. The fractions were collected as 200 μ l fractions. The fractions from 7-11 generated good antibacterial activity of *Staphylococcus aureus* upto 11 mm \pm 0.24, with 10KDa, exclusively present in the male sample and absent in the female sample (Figure.2).

Table 2. Total Protein estimation of crude and fractionized protein fraction by Lowry's method of the tissue extract preparation

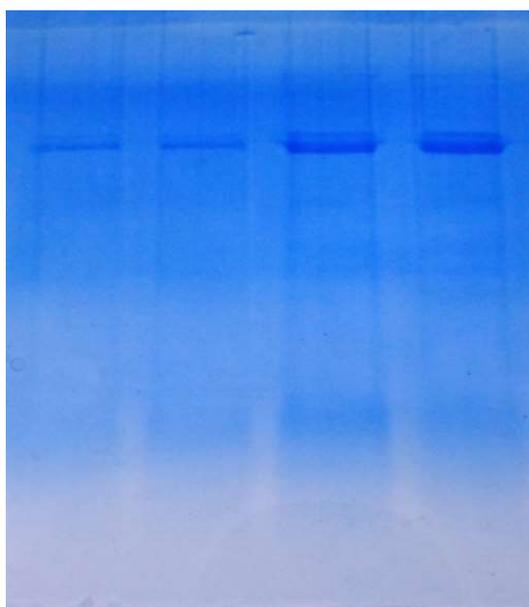
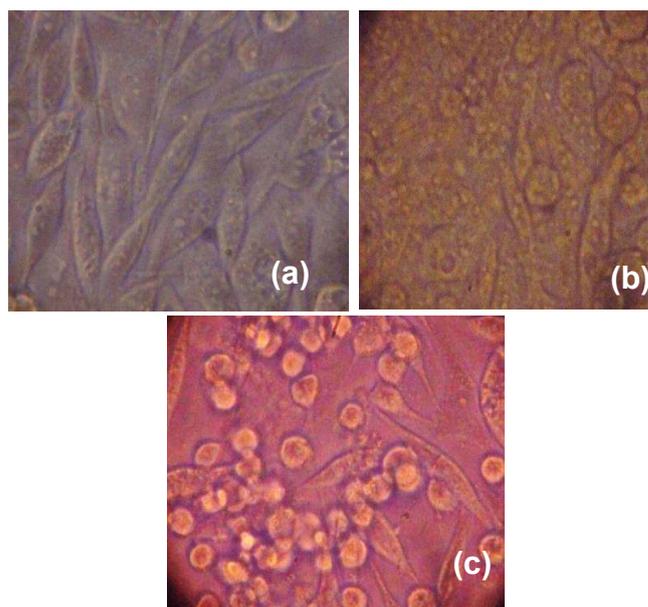
Sample	Crude Protein concentration, mg/ml	Fractionized protein concentration, mg/ml
Purified protein from Male tissue samples	76.3 \pm 4.32	23.4 \pm 3.23
Purified protein from Female tissue samples	89.25 \pm 3.04	17.95 \pm 2.54

Mean \pm SE of 3 individual observations of each group, P<0.005 significant

Table 4. Antibacterial activity of the purified protein fraction

S.No.	Fractions	Zone of inhibition female crude sample, (mm)	Zone of inhibition Male crude sample, (mm)
1.	Fraction 7	No Zone of inhibition	No Zone of inhibition
2.	Fraction 8	No Zone of inhibition	3mm ± 0.34
3.	Fraction 9	3mm ± 0.75	7mm ± 0.22
4.	Fraction 10	3 mm ± 0.43	11 mm ± 0.24
5.	Fraction 11	5 mm ± 0.89	11 mm ± 0.37
6.	Ciprofloxacin	5 mm ± 0.46	5 mm ± 0.39

Mean ± SE of 3 individual observations of each group, P<0.005 significant

**Figure 2.** SDS-PAGE: Resolving gel 18% and Sacking and 4% SDS-Page**Figure 3.** PC3 Cell line: (a) Control cell line, (b) 100µg, (c) 3.12 µg**Table 5.** Cell viability assay

S. No	Concentration, (µl)	Absorbance (540 nm)	% cell Viability
1	200	0.37	29.6 ± 0.87
2	100	0.71	56.8 ± 0.65
3	50	0.98	78.4 ± 0.1.2
4	25	1.07	85.6 ± 0.23
5	12.5	1.17	93.6 ± 0 .87
6	6.25	1.23	98.4 ± 0.41
7	Control cells	1.25	100 ± 0.62

Mean ± SE of 3 individual observations of each group, P<0.005 significant

The total protein present in the pooled fractions was determined by Lowry's method as in male as 23.4 mg/ml ± 3.23 and in female as 17.95 mg/ml ± 2.54 (Table.3). The antibacterial activity for the purified fraction was performed against the lawn culture of *Staphylococcus aureus* and 50µl of the purified extract generated the zone of inhibition upto 5mm (female purified hemolymph)

and 11mm (male purified hemolymph). The male purified protein at concentration of 1000 mg/ ml was subjected to dilution with sterile PBS by syringe sterilization and sample from 200µl to 6.25µl was treated to PC3 cell lines and the LC₅₀ value was obtained as 120µg/ ml and at 200µl/ ml of concentration only 29.6% ± 0.87 cells were found to be viable (Figure 3 and Table 5).

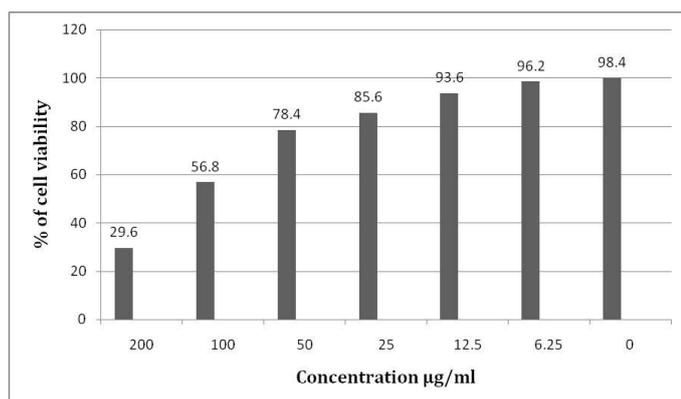


Figure 4. Cytotoxicity assay

Discussion

Joshi et al. (2013), have reported the prevalence of Methicillin resistant *Staphylococcus aureus* (MRSA) is endemic in India and is a most potent pathogen among the nosocomial infection and a total of 26310 isolates were reported among a period of 1 year study from 2008 to (2009). Bouchiat et al., (2015), have reported that from Bengaluru city apart from the methicillin resistance, erythromycin and ciprofloxacin resistant *Staphylococcus aureus* were reported. The methicillin resistance strain of the same bacterial species was reported from the ophthalmological infection (Savitha et al., 2012). Satish and Kurunchi, (2017) have described the importance of methicillin resistance *Staphylococcus aureus* among the dairy cattle. The antibacterial activity of the methanol extract of the hemolymph of marine crab, *O. macrocera* against multi drug resistance, *Staphylococcus aureus* was reported by Ravichandran et al. (2010). Similar results were also reported by Kavitha et al. (2019); as *Uca triangularis* hemolymph also exhibited antibacterial activity against *Staphylococcus aureus*.

Due to the growing drug resistance among the *Staphylococcus aureus* there is a need for the alternative source of antibacterial products. The marine source is good source of these antibacterial products (Haug et al., 2002; Veeruraj et al., 2008; Kiran et al., 2014). The crustacean are of diversified animals, whose immunity solely depends upon the innate immune response, the antimicrobial peptides (Rosa et al., 2010; Zanjani et al., 2018). Hence this present investigation has been performed to screen the presence of antimicrobial peptides against *Staphylococcus aureus*, as it has become a major pathogen of concern.

The cancer patients' accounts for a larger population, as the expenditure involved in the treatment and in the diagnosis is very costly (Saranath et al., 2014; Sharmila et al., 2016). Even those patients', who undergoes the treatment, mortality is increasing due to the side effects. Thus there is always a quench for the development of the cheap, alternative drug source for the cancer. Presently to treat prostate cancer the patients' have to be

administered with different types of drugs like hormonal therapy, anti-inflammatory drugs, serum based vaccines, cryotherapy, by which the patients' undergo stress (Pratap et al., 2013). In this present investigation Diethylaminoethyl cellulose (DEAE) Sephadex A-25, which is a weak anionic exchanger, was used to purify the anionic antimicrobial peptides. To extend this work, the antimicrobial peptide which was isolated was also used for the cytotoxicity research against PC3, prostate cancer cell line model. The IC₅₀ was estimated as 120µg, confirms that the same antimicrobial peptide also has the anticancer properties.

Conflicts of interest

The authors do not have any conflict of interest.

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