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Studies on bioprospecting potential of a gastropod mollusc *Cantharus tranquebaricus* (Gmelin, 1791)

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ABSTRACT

Objective: To study the biological activities of the tissue extract of *Cantharus tranquebaricus* (*C. tranquebaricus*). **Methods:** Crude extract of gastropod was tested for inhibition of bacterial growth. Antibacterial assay was carried out by disc diffusion method and the activity was measured accordingly based on the inhibition zone around the disc impregnated with gastropod extract. Molecular weight of the extract was determined by using SDS-PAGE. Plasma coagulation, Fibrin plate assay and substrate SDS-PAGE were used to determine the effect of sample on plasma coagulation, fibrin (ogen)olytic and proteolytic activity. **Results:** The maximum inhibition zone (10 mm) was observed against *Vibrio cholera* (*V. cholera*) and minimum inhibition zone (2 mm) was noticed against *Proteus mirabilis* (*P. mirabilis*). The molecular weight was determined as 47–106 kDa. The tissue extract shows proteolytic activity above 48 kDa. SDS-PAGE analysis of fibrinogen after incubation with the tissue extract showed fibrinogenolytic activity. In plasma coagulation assay *C. tranquebaricus* tissue extract showed procoagulant property and it coagulated chicken plasma within 150 s, while control took 5 min to clot. The 9 HU hemolytic units were found against chicken blood and also exhibit high level of brine shrimp lethality. **Conclusions:** This study suggests that *C. tranquebaricus* could be used as potential source for isolating bioactive compounds, since it is explored first time and found with promising results.

1. Introduction

The world's oceans, covering more than 70% of the earth surface represent an enormous resource for the discovery of potential chemotherapeutic agents. Since the rich diversity of marine organisms and habitats, marine natural products encompass a wide variety of chemical class, including terpenes, shikimates, polyketides, acetogenins, and peptides, alkaloids of varying structures and multitude of compounds of mixed synthesis[1]. In the past decade alone structures of over 5 000 marine natural products have been published[2]. Thus the marine environment is an exceptional reservoir for bioactive natural products; many of which exhibit structural features that are not found in terrestrial natural products[1]. Many marine natural products have been successfully advanced to the last stages of clinical trials, including dolastatin-10, ecteinascidin-743, kahalaide F and conopeptides (Ziconotide) and a growing number of candidates have been selected as promising leads for extended preclinical assessments[3]. Mollusks, which are widely distributed throughout the world,

have many representatives in the marine and estuarine ecosystem. Many studies on bioactive compounds from mollusks exhibit antitumor, antileukemic, antibacterial and antiviral activities. The rich diversity of marine organisms assumes a great opportunity for the discovery of new bioactive substances. The potential of marine gastropod as a source of biologically active products is largely unexplored in India. Since marine natural products are becoming increasingly attractive due to their potential applications in the pharmaceutical industries, the identification of new sources of these materials could be extremely important. In this connection, it is worth mentioning that through this study on marine gastropod *Cantharus tranquebaricus* (*C. tranquebaricus*) found throughout south east coast of India may provide one of the potential sources for the bioactive compounds. The small and conical shaped shell *C. tranquebaricus* found abundant along the Parangipettai coastal waters; South east coast of India has not been studied for biomedical potential. Hence this species was chosen for the present study with an objective to explore its bioactive potential.

2. Materials and methods

2.1. Collection of animals and preparation of crude extract

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The animals were collected from Parangipettai landing center (Lat 11° 21' N; long 79° 5' E) and brought to the laboratory. The shells were broken and the tissue samples were washed with distilled water. The presences of bioactive compounds were extracted with methanol (5 g of tissue sample was ground well with 5 mL of methanol using mortar and pestle). The extract was centrifuged at 15 000 rpm for 30 min and the supernatants was concentrated by rotary evaporator (VC100A Lark Rotavapor® at 30 °C) with reduced pressure to give predominantly an aqueous suspension and freeze dried to give yellow gummy mass and stored at –20 °C until use.

2.2. Micro organisms and media

Bacterial strains used were as follows: *Escherichia coli* (*E. coli*), *Salmonella typhi* (*S. typhi*), *Klebsiella pneumonia* (*K. pneumonia*), *Vibrio parahaemolyticus* (*V. parahaemolyticus*), *Klebsiella oxytoca* (*K. oxytoca*), *Proteus mirabilis* (*P. mirabilis*), *Vibrio cholerae* (*V. cholerae*), *Streptococcus*, *Staphylococcus aureus* (*S. aureus*), *Salmonella paratyphi* (*S. paratyphi*). The fungal pathogens such as *Candida tropicalis* (*C. tropicalis*), *Aspergillus niger* (*A. niger*), *Penicillium* sp., *Trichophyton mentagrophytes* (*T. mentagrophytes*), *Alternaria alternata* (*A. alternata*), *Candida albicans* (*C. albicans*), *Rhizopus* sp., *Mucor* sp., *Trichophyton rubrum* (*T. rubrum*) were also used. The bacteria and fungi strains were maintained respectively in nutrient agar Potato dextrose agar (PDA).

2.3. Agar disc diffusion method

The agar disc diffusion method was employed to screen the antimicrobial activity of the samples[4]. Inoculum was prepared using fresh cultures of bacterial and fungal strains. A loop of bacterial and fungal culture was inoculated into a nutrient broth and potato dextrose broth. 1 mg/ml concentration of sample were imbibed in paper disc and applied on the test media which were previously inoculated with test strain. All the plates were incubated at 37 °C for bacteria and 28 °C for fungi. Inhibition zones were measured after 24 h of incubation. Standard disk of erythromycin (15 mcg/disc) and nystatin (100 units/disc) served as the positive control for antibacterial and antifungal respectively.

2.4. Estimation of protein

Amount of protein in the samples were determined by the method of Lowry[5] with BSA as a standard.

2.5. Molecular weight determination (SDS–PAGE electrophoresis)

Molecular weight was determined by performing SDS–PAGE (10% gel) analysis by following the method of Laemmli[6]. The molecular weight was observed through their mobility with protein markers such as phosphorylase B (97 400 Da), bovine serum albumin (66 000 Da), ovalbumin(43 000 Da), carbonic anhydrase(29 000 Da), soyabean trypsin inhibitor(20 000 Da), lysozyme (14 300 Da) by using Gel documentation system(Lark Fine Technologies, Version.4.0, India).

2.6. Proteolytic assay

Casein and gelatin were used as substrates and the method of Heussen[7] was adopted. Briefly, 2 mg/mL (w/v) substrates were incorporated in the 10% resolving gel with a 4% stacking gel. The samples (10 µg) were loaded in non-reducing sample buffer. After electrophoresis the SDS was removed by washing the gel twice for 20 min in Triton X–100 before incubation in 20 mM Tris, 0.4 mM calcium chloride pH 7.4 at 37 °C for 16 h and stained with 0.125% Coomassie blue. The clear zone in the gel indicates the regions of enzyme activity and pre-stained molecular weight (Genei) was used as molecular weight marker.

2.7. Fibrinolytic assay

The fibrinolytic activity of the extract was analyzed in a fibrin plate assay method followed by Veiga[8]. Fibrin plate was prepared by a solution containing 25 mg of fibrinogen laid over agarose plates immediately after the addition of 10 µL of thrombin. The plates were incubated at 37 °C for 2 h. Then 20 µL of sample was laid over the fibrin plate. The same volume of water and plasmin (1 mU) were used as negative and positive controls respectively. Further, the plates were incubated at 37 °C for 24 h, dried out, and stained with Coomassie Blue stain. The presence of clear zones in the plates indicates the fibrinolytic activity.

2.8. Fibrinogenolytic activity

The degradation of fibrinogen and fibrin by the extract was analyzed by using sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) (Laemmli UK 1970) by the method of Shacter[9]. For the fibrinogenolytic activity, 50 µL of a bovine fibrinogen solution (2 mg/mL in 10 mM Tris, pH 7.4) was incubated with 50 µL of tissue extract at 37 °C. At the indicated time (1 and 24 h), 20 µL of denaturing solution (10 M Urea, 4% SDS, 4% β-mercaptoethanol) was added to the reaction Mixture. Fibrinogen with 0.85% saline was used as control. The incubated mixtures were subjected to SDS–PAGE on 12% polyacrylamide gel. The protein bands were viewed by staining with 1% Coomassie brilliant blue R250. Fibrinogenolytic activity was monitored by comparing position and appearance of specific bands with that of fibrinogen incubated with 0.85% saline only.

2.9. Coagulation assay

The effect of mollusks extracts upon coagulation was assessed using the re-calcification time assay[10] adopted for the Spectra Max Pro micro plate reader. In a final volume of 150 µL, 50 µL of citrated chicken plasma was incubated with different amount of samples in 90 µL of 20 mM Tris–HCl buffer, pH 7.4. After 5 min at 37 °C, 10 µL of 150 mM CaCl₂ was added and clot formation was monitored at 37 °C for 20 min in the Spectra Max system at 650 nm. To access calcium independents coagulation activity, EDTA was added instead of CaCl₂, to a final concentration of 10 mM.

2.10. Assay of hemolytic activity

Hemolytic assay was performed following the microtitre plate method of Pani Prasad[11]. EDTA solution (2.7 mg/100 mL) was used as an anticoagulant. Chicken blood with

anticoagulant was centrifuged at 5 000 rpm for 5 min, the supernatant discarded and pellet was suspended in normal saline (pH 7.4). This procedure was repeated thrice. From this 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. From 1 mg/mL crude extract serial dilution (100 μ L) was done till the last well and 100 μ L was discarded from the last well. 100 μ L of 1% RBC was added into all the wells with appropriate controls. In 51 % RBC suspension, 100 μ L of distilled water was added, which served as a positive control and 100 μ L of normal saline which served as negative control. The plate was gently shaken and allowed to stand for 3 h at room temperature and the results were recorded. The uniform red color suspension in the wells considered as positive hemolysis and a button formation in the bottom of the wells was considered as lack of hemolysis. The reciprocal of the highest dilution of the crude extract shows hemolytic pattern was taken as one hemolytic unit (HU).

2.11. Brine shrimp toxicity

The freshly brine shrimp eggs were hatched out in a dish filled with of sea water. The freshly hatched phototrophic larvae (nauplii) were collected by a pipette and transferred (10 shrimps) to the vials filled with sea water (5 mL). The extract was added at different concentrations into the vials. As control a group a vials was filled with seawater. The vials were kept illuminated during 24 h of contact with the substances and survival rate was assessed with the aid of a magnifying lens. The assay was carried out three times with triplicates for each fluid concentration tested. To calculate LC₅₀ (Mean lethal concentration) the results were plotted in the graph as % mortality Vs concentration of crude extract.

3. Results

3.1. Antimicrobial assay

The antibacterial activity of extracts of *C. tranquebaricus* was shown in Table.1.

3.2. Protein estimation

The amount of protein present in the methanol extract was found to be 200 μ g/mg of the sample.

Table 1.
Antibacterial activity of CT extract against human pathogens.

S.NO	Microorganisms	Zone of inhibition (mm)
1.	<i>E. coli</i>	9
2.	<i>S. typhi</i>	–
3.	<i>K. pneumonia</i>	5
4.	<i>V. parahaemolyticus</i>	–
5.	<i>K. oxytoca</i>	7
6.	<i>P. mirabilis</i>	2
7.	<i>V. cholerae</i>	10
8.	<i>Streptococcus</i>	2
9.	<i>S. aureus</i>	6
10.	<i>S. paratyphi</i>	6

Figure 1. Electrophoretic profile of the *C. tranquebaricus* extract was analysed using 12% SDS–PAGE under non reducing conditions. Gel was stained with coomassie Blue. Lane 1 corresponds to the position of molecular mass markers.

3.3. Molecular weight determination

In the case of molecular weight determination, the electrophoretic profile of the samples showed the presence of small to high molecular weight protein; upon these some of them are distinct. After SDS–PAGE, under non–reducing conditions 5 bands were seen along the gel in tissue extract. The sample shows intensive bands at 106 kDa and 94 kDa and unclear band also observed at 101 kDa, 71 kDa and 47 kDa (Figure 1).

3.4. Proteolytic activity

C. tranquebaricus crude extract catalyzed the hydrolysis of protein substrates such as gelatin and casein. Protease activity was observed in tissue extracts in both the substrates through the Zymography. Zymograms analysis revealed that the common protease band with the apparent molecular weights ranged from 48 to 120 kDa and 52 to 120 kDa. Proteolysis was detected as colorless bands in the blue gel as shown in Figure 2.

3.5. Fibrinolytic and fibrinogenolytic activity

In the fibrinogenolytic and fibrinolytic activity, the electrophoretic profile of intact fibrinogen incubated with the tissue extracts at 1 h and 24 h by SDS–PAGE analysis. In SDS–PAGE analysis the presence of specific bands of intact fibrinogen were observed at the range of 55 kDa. At the same time, the bands were faint upon incubated with sample for 1 h and almost absent during 24 h incubation (Figure 3). The fibrinolytic activity was also confirmed by fibrin plate assay where samples were laid over the fibrin layer in an agarose plate. The formation of clear area in the plate was 12 mm and therefore, confirms the fibrinolytic activity of the tissue extract (Figure 4)

Figure 2. Caesinolytic and gelatinolytic activities of *C. tranquebaricus* were determined using the technique of substrate SDS–PAGE 12.5%. Clear regions in the gel indicate regions of enzymatic activity.

Figure 3. Fibrinogenolytic activity of *C. tranquebaricus* extract was determined using fibrinogen as substrate in 12% SDS–PAGE technique. Lane 1 shows the three bands responsible for α , β , γ subunits of fibrinogen. Lane 2 shows the partially digested α , β , γ subunits by CT extract after 1 h incubation time. Lane 3 shows the complete digestion α , β , γ subunits by CT Extract after 24 h incubation time.

Figure 4. The fibrinolytic activity of CT extract. Plasmin (P) was used as positive control and double distilled water (DD) was used as negative control.

3.6. Coagulation assay

The effect of samples on blood coagulation was assessed by plasma coagulation assay. To analyze the effect of samples upon clotting, citrated human plasma was incubated with the tissue extract prior to the induction of coagulation by calcium. From Figure 5, it is clear that the tissue extract showing strong pro coagulant effect is capable of shortening the clotting time of human plasma. Plasma incubated with the sample took 150 s to start coagulation while the control took 5 min to clot.

Figure 5. Plasma coagulation assay: The effect of CT extract in the blood coagulation of human plasma was monitored at 650 nm at 37 °C. In the experiment plasma incubated with CT extract ($\diamond$) Plasma incubated with calcium act as control reaction ($\diamond$). Plasma incubated with EDTA instead of CaCl_2 ($\diamond$). Each values represents mean $\pm$ SD of four independent experiments (* $P < 0.05$).

Figure 6. Effect of CT extract on artemia. Newly hatched artemia were incubated for 24 h with increasing doses of CT Extract. Mortality rate of artemia increased with the increasing concentration of toxin. Each value represents the mean of $\pm$ SE of six experiments.

3.7. Hemolytic assay

The crude extract showed promising hemolytic activity on chicken blood. The chicken blood was vulnerable to lysis provoked by the *C. tranquebaricus*. The hemolytic activity of crude extract was found to be 9 HU on chicken blood (One HU is defined as the quantity of protein catalyzing lysis of 50% of red blood cells present).

3.8. Brine shrimp toxicity

In case of toxicity studies, the crude extract showed higher toxicity against brine shrimp nauplii and calculated the LC50 value. The lower LC50 indicated higher toxicity and higher LC50 indicated the lower toxicity. The samples exhibit the

minimum LC50 value of 25 μ g/mL for *Artemia nauplii* (*A. nauplii*) (Figure 6).

4. Discussion

There is a growing interest in marine natural products and marine secondary metabolites. This field of research receives the attention of investigators from various disciplines such as marine biology, marine ecology, chemistry, biochemistry, pharmacology and biotechnology. The studies conducted with marine natural products during the last decades have uncovered many substances with biomedical potential, which has raised the interest of many research groups toward this ecosystem as a source of new drugs^[12].

Many antimicrobial screening studies have shown that the Gram negative bacteria are more sensitive than Gram positive bacterial^[13]. Overall, the gastropod extract caused growth inhibition in gram positive and gram negative bacteria. Thus, indicating that this extract does not selectively inhibit one group of microorganism. In the present study, a pronounced antibacterial activity has been observed against some bacterial strains. The maximum antibacterial activity was observed in *V. cholerae* (10 mm), *E. coli* (9mm), *K. oxytoca* (7 mm) and the minimum activity was recorded in *P. mirabilis* (2 mm). Previously, the maximum antibacterial activity was reported in *T. radiates* against *S. aureus* and *E. coli*^[14]. Further, the antimicrobial activity was reported in four bivalves against few pathogens and found that extracts showed significant activity against *B. subtilis*^[15]. The antimicrobial activity of *Babylona spirata* was also shown mild activities^[16]. The antimicrobial activity from the gill extraction of *Perna viridis*, tissue extract of *Meretrix meretrix*, *M. casta*, *Perna viridis* and edible oyster *Crassostrea madrasensis* were reported^[17–19]. Compared to these studies, the methanolic extract of *C. tranquebaricus* shows higher degree of inhibition indicated that the substance involved in producing the antibacterial effect could be a high polar compound.

The relationship of anti-microbial activity and protease activity of the isolated extract, we have conducted the protease activity through zymography analysis. Protease are considered antimicrobial protein because they are involved in the regulatory production of antimicrobial peptides^[20,21]. In fact, enzymatic activity was more intensive in *C. tranquebaricus* and enzymes with molecular mass above 48 kDa which is responsible for the hydrolysis of the substrate. In tissue extracts, the profile of enzymatic degradation was similar using standard substrates, indicating the presence of proteases with broad substrate specificity. Through the zymography analysis, we confirmed that the presence of anti-microbial property may inflict to the protease activity.

The plasma coagulation assay showed the presence of blood clotting factors in the tissue extract, which shorten the blood clotting time of human blood after the addition of Calcium chloride (CaCl₂), indicated the extract had the effect on blood clotting. The result emphasized that the tissue extract may consist of calcium dependent procoagulant. Procoagulant activity may be due to the presence of compounds which are calcium dependent prothrombin activator^[22]. Recently, the plasma coagulation assays of marine stingray *D. sephen* and *A. narinari* venom found it delayed the clotting period

of human citrated plasma^[33], whereas the earlier report suggested that the stingray *P. falkneri* and *D. guttata* venom extracts incubated with plasma have no significant effect on coagulation time of the human citrated plasma^[23]. The SDS polyacrylamide gel electrophoresis (SDS–PAGE) analysis showed the presence of proteins with the molecular weight of 47 to 106 kDa. These revealed proteins may be responsible for various biological activities in the tissue extracts.

Fibrinolysis dissolves existing thrombin through the action of plasmin on fibrin^[31]. The coelomic fluid (2 mg) of Polychaete *Nereis (Neanthes) virens* (Sars) possessed fibrinolytic activity of about 95mm²^[24]. Whereas the *C. tranquebaricus* tissue extract possesses fibrinolytic activity of about 20 mm represented by the presence of clear area. Fibrinogenolytic activity was analyzed by SDS–PAGE analysis in which, of the three subunit chains A α , B β , and γ of fibrinogen, the A α chain was most easily degraded and the γ chain is most resistant to hydrolysis. After an incubation of 24 h all the three subunit chains were completely degraded.

In the case of hemolytic activity, the highest hemolytic activity was observed in chicken blood toxicity upto 10–9 dilutions (9HU). Earlier, the methanol and aqueous extracts of sponge *C. diffusa* induced pronounced hemolysis on chicken erythrocytes with an activity of 14HU in aqueous extract and 10HU in methanolic crude^[32]. Stempien^[25] reported hemolytic activity of halitoxin obtained from the genus *Haliclona*. Fusetani^[26] reported that the sterol derivatives viz., halistanol sulphate and sokotrasterol sulphate, obtained from *Halichondriidae* sponges showed the similar hemolytic activity. The present study indicates a strong hemolytic activity. The crude extract of *C. tranquebaricus* did not show phospholipase A2 activity and do not require neither lecithin nor serum of blood for complete hemolysis of erythrocytes. Thus, direct lytic factors are involved in the complete hemolysis. A novel dicynthaurin dimer showed hemolytic activity about 21 percents in human red blood cells (100 μ g/mL)^[27]. Therefore, the presence of hemolytic activity indicated that the *C. tranquebaricus* may consist of cytotoxic molecules.

The brine shrimp assay is considered as a reliable indicator for the preliminary assessment of toxicity. The crude extract of *C. tranquebaricus* with brine shrimp lethality test exhibits the LC50 value of about 25 μ g/mL. Extracts resulting in LC50 values of less than 250 μ g/mL were considered significantly active and had the potential for further investigation^[28]. The crude methanol extract of *C. tranquebaricus* indicated high brine shrimp lethality. Previously, the LC50 values of the crude extract of ascidians *P. madrasensis* and *P. nigra* were reported as 92 μ g/mL and 130 μ g/mL respectively^[29]. The LC50 values are given in (Table 1). The crude methanol extract of ascidians (*D. psammatoide*) indicated the highest activity with LC50 value (106.965 μ g/mL) and the ethanol extract of ascidian *P. arabica* exhibited lower brine shrimp lethality with LC50 value of 227.055 μ g/mL^[30]. Compared to these studies *C. tranquebaricus* tissue extract shows higher lethality against brine shrimp larvae. This study suggests that the presence of cytotoxic compounds in the isolated sample.

C. tranquebaricus had not been previously investigated for the presence of bioactive compounds. Hence, the present investigation has been explored the biomedical potential

of this species. From the study, it is clear that this species contains a various active molecules with various biological and physiochemical properties. Even, this is for the first time *C. tranquebariucis*, a gastropod mollusk reported for having biological activities.

Conflict of interest statement

We declare that we have no conflict of interest.

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