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Research Article

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Haemolytic activity and *Artemia* toxicity of jelly fish *Cyanea capillata* (Linneaus, 1758) from Gulf of Mannar, SE Coast India

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ABSTRACT

The lion's mane jellyfish Cyanea capillata consists of proteinaceous compounds with various biological activities such as haemolytic, cytotoxic, neurotoxic, cardiovascular and enzymatic properties. In the present study, crude and DEAE cellulose column fractionated toxin were prepared from the whole body extracts of Cyanea capillata. The toxins were evaluated for protein concentration and were investigated for haemolytic activity and Artemia toxicity. The most active fraction in both the assays was subjected to HPLC analysis to elucidate the bioactive proteins. The crude toxin had 230.15 µg.mL^{-1} protein. Whereas, the protein concentration ranged between 40.85 and 105.62 µg.mL^{-1} in the fractionated toxins. The crude as well as the fractionated toxins exhibited remarkable activity in both the assays with maximum activity in fraction 5. The HPLC analysis of the most active haemolytic fraction reveals a single prominent peak with a retention time of 1.79 min. Further study on the eluted peak would enable us to understand qualitative details of protein responsible for the haemolytic activity.

Keywords: *Artemia* toxicity, Cytotoxicity, *Cyanea capillata*, Haemolysins, Haemolytic activity, HPLC

INTRODUCTION

Haemolysins are proteins capable of lysing erythrocytes by forming pores in phospholipid bilayers or by hydrolyzing the phospholipid bilayer of the cell membrane [1,2]. Jellyfishes, the unique creatures of phylum Cnidaria are well known for their toxic substances principally confined to cytoplasmic capsules, the nematocysts [3]. Lytic effects are a common principal in cnidarian venoms and are described for various species [4-6]. Jellyfish toxins have been extensively examined and revealed to be mostly proteinaceous toxins [7,8] which are novel source of haemolysins with molecular weight ranging from < 70 - 107 kDa [9-12]. Rhizolysin, a haemolysin of 260 kDa has been isolated from the jellyfish *Rhizostoma pulmo* [13]. A haemolysin of 42 kDa has been isolated from the jellyfish *Carybdea alata* [14]. Two haemolytic proteins, CAH1 and CfTX, have been isolated from the nematocyst venom of *Carybdea alata* and *Chironex fleckeri* respectively [14,15]. Hemolytic activity has also been reported from various jellyfishes *viz., Chironex fleckeri* [16,17], *Physalia physalis* [18] *Carybdea marsupialis* [12], *Chrysaora plocamia* [19, 20], *C. quinquecirrha* [21], *C. achlyos* [22, 23], *Chiropsalmus quadrigatus* [24] *Rhopilema esculentum* [25], *Aurelia aurita, Cyanea capillata* and *C. nozakii* [26-29]. Haemolytic active compounds are occasionally cytolytic too. According to the sequence bioinformatic analysis and the investigations of hemolytic drug intervention [26, 30], haemolysin may function as a pore-former on cell membrane both *in vivo* and *in vitro* [31-34]. Earlier studies also evidenced the cytolytic property of jelly fish toxins. Cytolytic activity has been proven in *Chrysaora quinquecirrha* [35], *Carybdea marsupialis* [12], *Chironex fleckeri* [15] and *Cyanea capillata* [36, 37]. Two ctotoxic proteins ranging 10.9 kDa and 11.7 kDa have been isolated from *C.capillata* [37]. Due to the lytic capacity and the possibility to address them to specific tissues, cytolysins have been evaluated as promising anti-tumor agents

[38, 39]. They attract more attention for potential use in treating tumors and killing parasites [40]. These toxins exhibit strong cytotoxicity against U251, NSCLC, BEL 7402 and NIH Swiss mouse fibroblast cell lines [40]. Haemolytic and cytolytic toxins have been reported from other group of cnidarians such as sea anemones also [41]. Hence the present study investigates the haemolytic property of C*yanea capillata* collected from the Gulf of Mannar, India in order to understand its cytolytic potential and toxicity.

EXPERIMENTAL SECTION

Extraction of Jelly fish toxin

Fresh jellyfishes were collected from the Gulf of Mannar area along the Tuticorin Coast and immediately brought to the laboratory in polythene bags kept in ice at 4˚C. Whole body of the jelly fish were homogenized well and centrifuged at 5000 rpm for 15 min. The supernatant was collected and lyophilized. The crude toxin powder was stored at -20˚C for further use.

Partial purification of the crude extract

Crude extract of *Cyanea capillata* was partially purified using DEAE cellulose anion exchange chromatography [42]. Ten fractions were collected in a step-wise gradient with NaCl and stored at -20˚C for further use.

Protein estimation

The crude and partially purified toxins were evaluated for protein concentration following the method of Lowry *et al*. [43] using Bovine Serum Albumin (BSA) as standard.

Haemolytic activity assay

Citrated Human whole blood was used within 24 h after bleeding and washed three times [44] in sterile 0.9 % NaCl saline solution. After each washing, cells were pelleted by centrifugation at 150xg for 5 min and the supernatant was discarded. The final pellet was diluted in sterile 0.9 % NaCl saline solution then in sterile Dulbecco's phosphate buffer saline (D-PBS), pH 7.0 [45] containing 0.5 mM boric acid and 1 mM calcium chloride [46]. Red cell suspensions (1 ml of final volume) were incubated with an aqueous solution [47] of toxin at various concentrations (5, 7.5, 10, and 15 µg/ml). The samples were incubated for 6 h and the incubation temperature was set at 37˚C [46] and during the incubation the samples were occasionally resuspended by inversion. Total lysis of erythrocyte suspension was obtained by incubating the cells with 0.1 % v/v Tween 20. In order to evaluate the degree of spontaneous lysis, also tubes containing exclusively the red blood cell suspension in D-PBS were set. For each concentration and control, the experiments were set in triplicate. After the incubation, the cell suspensions were centrifuged at 900xg for 10 min [47] and the supernatant was carefully collected, by paying attention not to disturb the pellet. The absorbance at 405/540 nm of supernatant was measured with a spectrophotometer (Elico SL-164). The value of absorbance of erythrocytes maintained exclusively in D-PBS has been utilized to set the '0' value before reading the samples that contained toxin extract. Haemolytic levels were expressed by percentage of hemolysis, calculated with the ratio between the value measured for each sample and that registered for the total hemolysis [47]. The assay was done in triplicates and average values were taken for calculation. The effective dose at which 50% haemolysis (ED_{50}) achieved was calculated by conducting repeated haemolytic assays with each toxin at various concentrations.

Percentage of hemolysis was calculated using the following formula:

Haemolysis (%) = $(A_{sample} / A_{Tween20})$ X 100

Artemia Bioassay

Artemia toxicity assay is widely used as a preliminary screening tool for assessment of toxicity [48]. The Artemia bioassay was conducted following Michael *et al*. [49], Vanhaocke *et al*. [50] and Carballo *et al*. [51]. The dried *Artemia* cysts were cultured ssin culture bottles and the cysts were incubated ($1gL^{-1}$) at 28-30˚C with strong aeration, under permanent light conditions. Approximately 24 hrs after hatching the phototrophic nauplii were collected using a sterile pipette from the lighted side and concentrated in a small vial. The nauplii were divided into many groups of 10 individuals each in 10 ml sterile seawater. The extracts at varying concentrations (10, 25, 50, 75 and 100 µg/10ml) were added into the vial with *Artemia* nauplii. Also *Artemia* larvae were exposed to the extracts in another set of experiment. Controls were maintained separately. Toxicity was determined after 24 hrs of exposure. The surviving *Artemia* were counted and % death was calculated. Larvae were considered as dead if they did not exhibit any

internal or external movement during observation. Experiment was carried out in triplicate for each concentration and average was taken and LC_{50} was determined.

High Performance Liquid Chromatographic (HPLC) analysis

The most active haemolytic fraction of the jelly fish toxin was subjected to High Performance Liquid Chromatographic analysis using Varian HPLC C 18 column measuring 250 X 4.6mm X 1/4" (Cyberlab LC-100).

RESULTS AND DISCUSSION

Extraction of crude toxin

Lyophilized whole body extract of *Cyanea capillata* yielded a total amount of 4.8 g of toxin from 500 g of crude extract. An earlier study of Suganthi *et al.* [52] has evolved similar results with 5.50 g and 4.95 g yield extract from 464 g of *Crambionella stuhalmanni* and 585 g of *Chrysaora quinquecirrha*.

Protein estimation

The protein content in crude toxin was 230.15 µg.mL^{-1}. The protein in purified fractions ranged between 40.85 and 105.62 µg.mL^{-1} (Table.1). The protein concentration is found to be higher (105.62 µg.mL^{-1}) in fraction 1 followed by 91.25 µg.mL^{-1} in fraction 5. The protein content of *Cyanea capillata* in the present study was found higher than 144 µg.mL^{-1} in *Chironex fleckeri* [53] and lower than 394 µg.mL^{-1} in *Crambionella stuhalmanni* and 312 µg.mL^{-1} in *Chrysaora quinquecirrha* [52].

Hemolytic activity

In the present study, the crude and the partially purified fractionated toxins of *Cyanea capillata* screened for haemolytic activity were found to exhibit remarkable haemolytic activity as represented in Table.1 & Figure. 1. The activity was found to be dose dependant. Percentage of haemolytic activity in crude toxin ranged between 99.44 ± 4.50 and 392.6 ± 20.62. In the case of fractionated toxins, it was from 6.95 ± 1.35 to 140.2 ± 12.75. Maximum activity was expressed in fraction 5 and minimum activity was in fraction 10.

Table. 1- Protein content and Haemolytic activity of *Cyanea capillata* toxins

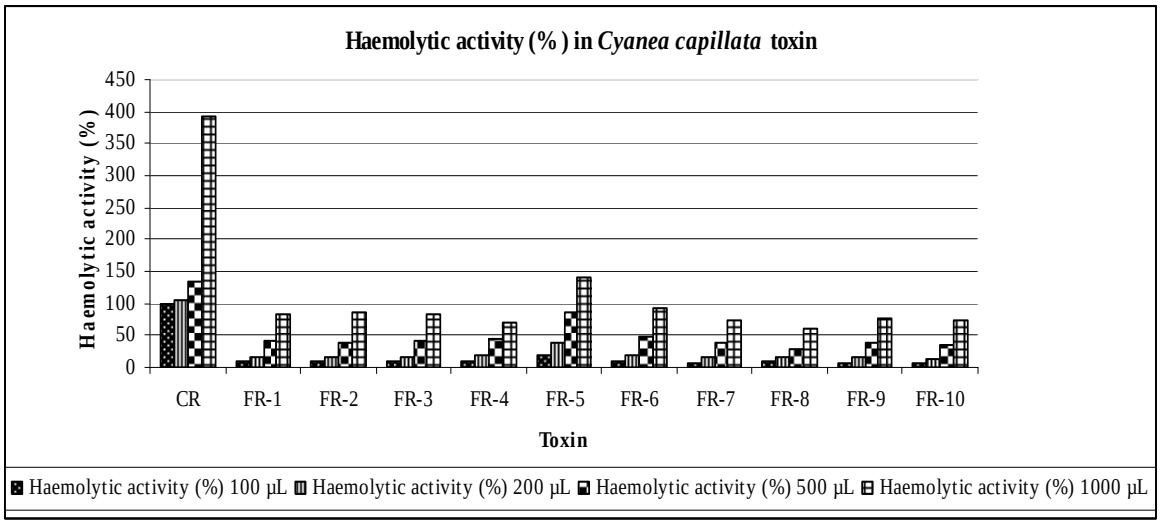
S. No.	Toxin	Protein content (µg.mL^{-1})	Haemolytic activity (%)				ED $_{50}$ (µL)
			100 µL	200 µL	500 µL	1000 µL	
1	CR	230.15	99.44 ± 4.50	106.7 ±10.15	135.6 ± 18.15	392.6 ±20.62	45
2	FR-1	105.62	8.59 ± 1.20	17.18 ± 3.15	41.26 ± 5.62	82.3 ±15.20	580
3	FR-2	86.25	8.24 ± 1.55	16.47 ± 4.60	39.51± 6.21	86.3 ± 11.05	685
4	FR-3	64.52	8.18 ± 1.87	16.36 ± 3.95	40.21± 5.20	81.8 ± 14.35	650
5	FR-4	79.14	9.11 ± 1.35	18.22 ± 4.15	43.25 ± 4.56	69.8 ± 10.27	550
6	FR-5	91.25	19.01 ± 2.56	38.02 ± 4.62	85.26 ± 9.32	140.2 ± 12.75	300
7	FR-6	59.2	9.45 ± 2.11	18.9 ± 3.55	46.98 ± 5.23	92.2 ± 8.54	525
8	FR-7	51.26	7.3 ± 1.20	14.6 ± 2.55	39.12 ± 4.11	73.5 ± 8.22	675
9	FR-8	47.65	8.67 ± 1.88	17.34 ± 3.47	29.56 ± 3.59	60.5 ± 6.55	900
10	FR-9	40.85	7.51 ± 1.42	15.02 ± 3.48	39.81 ± 4.45	75.1 ± 5.64	600
11	FR- 10	42.05	6.95 ± 1.35	13.9 ± 2.35	33.78 ± 4.62	74.6 ± 8.11	700

CR-Crude; FR- Fraction

Crude and partially purified venoms express various biological activities [37]. Hemolytic activity has been described on various jelly fish venoms against erythrocytes of animals such as sheep, chicken, rat, rabbit and human [16- 21, 26-29, 54- 56]. These results suggest that jellyfish venom can have a unique hemolytic activity profile which can be highly variable among different species. The results of the present study reveals that the toxin from the whole body extract of *Cyanea capillata* has potent haemolytic activity and the same has been supported by earlier studies [36,54,57]. The crude and five fractionated venom of *Chrysaora quinquecirrha* have been reported to possess hemolytic activity [58] which is comparable with the hemolytic activity exhibited by the crude and fractionated toxin of *Cyanea capillata.* The nematocyst venom of *Nemopilema nomurai* showed 50% haemolysis at 924µg/mL [34] which also supports the results of the present study. *Cassiopea xamachana* jellyfish venom showed a higher hemolytic activity in human RBCs than sheep RBCs [55]. Cytolytic toxins are generally known to operate by either of two mechanisms. One is an enzymatic mechanism, in which cytolytic components of marine invertebrates bind preferentially to membrane glycolipids or glycoprotein [4]. The other is a stoichiometric mechanism, which contains the binding and insertion of toxin molecules into plasma membrane followed by oligomerization to form transmembrane pores, and resulting in colloid osmotic lysis [59]. It is not clearly understood now how *C. capillata*

jellyfish venom elicits various hemolytic potencies as well as its mechanism of action, which may need further investigation in the future.

Figure 1- Haemolytic activity of *Cyanea capillata* toxins



Artemia toxicity

Artemia toxicity assayed with crude and fractionated venom of *C.capillata* is represented in Table 2. Toxicity was dose dependant. 100% toxicity was exerted in crude venom. Whereas, in fractionated venom, it ranged between 25±1 and 75±10. The LC_{50} for crude venom was 60 µg.mL^{-1} and for the fractions it ranged from 75 to 250 µg.mL^{-1}. Fraction 5 was found to be more effective with LC_{50} of 75 µg.mL^{-1}. Artemia toxicity of *C.capillata* crude toxin is found to be more ssignificant when compared with that of other cnidarian toxins such as sea anemones *viz.*, *Stichodactyla mertensii*, *S. haddoni*, *Lebrunia danae* and *Anthopleura elegantissima* showing LC_{50} values of 0.65 mg/ml, 0.90 mg/ml, 2.82 mg/ml and 5.78 mg/ml respectively [60].

Table. 2- Artemia toxicity of *Cyanea capillata* toxins

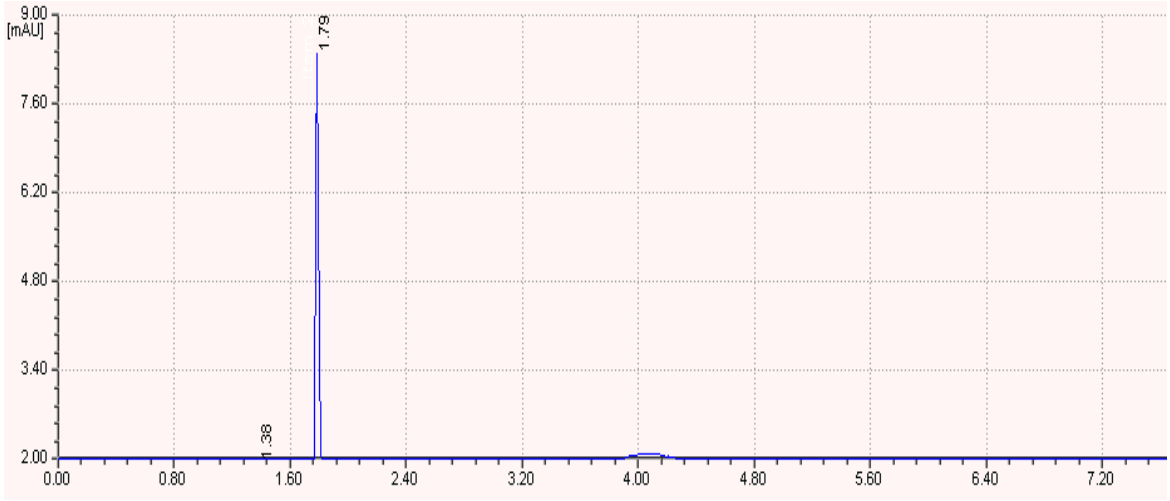
Toxin	Concentration (µg.mL^{-1})	Artemia toxicity (%)	LC_{50} (µg.mL^{-1})
CR	10	0	60
	25	0	
	50	40±5	
	75	50±5	
	100	100±10	
FR-1	100	55±2.3	80
FR-2	100	45±6.6	120
FR-3	100	50±10	100
FR-4	100	40±5	130
FR-5	100	65±5	75
FR-6	100	45±2	135
FR-7	100	25±1	250
FR-8	100	30±5	200
FR-9	100	55±5	90
FR-10	100	30±5	200

CR-Crude; FR- Fraction

High Performance Liquid Chromatographic (HPLC) analysis

HPLC analysis on the active fractionated protein of *C. capillata* revealed one prominent peak with a Retention Time (RT) of 1.79 min (Figure. 2). Similar HPLC result was obtained with a fractionated toxin of *C. capillata* with a retention time of about 1.5 min on ion exchange chromatography [37].

Figure 2- Peaks obtained from HPLC with Retention Time (RT)



The results of the present study showed that the crude as well as the partially purified fractionated toxin of *Cyanea capillata* had significant haemolytic and *Artemia* toxicity. The HPLC analysis of the active fraction has revealed the presence of a prominent haemolytic toxic protein. Further investigation on the structural analysis would lead to isolate the compound responsible for the haemolytic activity of *C.capillata*. The haemolytic protein could be possibly targeted as cytolysins effective in cancer cell abatement after further studies. Many other such pharmacological activities *viz.*, antimicrobial, central nervous system depressant activities have also been recorded from marine invertebrates [61- 63].

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