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Original Article

EFFECTS OF DRUGS AGAINST ANTIOXIDANT AND CYTOTOXIC (HEp 2 CELL LINE) ACTIVITY COMPOUNDS FROM MARINE ANIMALS *CONUS AMADIS* VENOM (GMELIN, J.F, 1791)

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ABSTRACT

Objective: The present study was carried out to explore the hemolytic, antioxidant and cytotoxic activity of conotoxins extracted from the venom of *C. amadis*.

Methods: The crude conotoxin was extracted and tested for cytotoxic, antioxidant activity and cancer cell lines. The cytotoxic activity was studied by brine shrimp cells and i*n-vitro* antioxidant activity were determined by DPPH, Superoxide anion radical scavenging assay and hydrogen peroxide scavenging from active fraction of conopeptides. The active fractions were tested in HEp-2 cancer cell lines at different concentration.

Results: The different dose tested viz 100µg/mL^{-1}, 50µg/mL^{-1}, 25µg/mL^{-1}, 12.5µg/mL^{-1}, 6.25µg/mL^{-1}, and 3.2µg/mL^{-1} cytotoxicity on HEp-2 cell was observed at the 25µg/mL. HEp-2 cell displayed dose dependent decreased in viability detected as early as 48 hrs. The purified conotoxin was showed remarkable cytotoxicity against HEp-2 cell in a dose depend manner. The isolated conotoxin that produced maximum effect at 100µg/mL^{-1} and the half inhibitory concentrations (IC_{50}) was 25µg/mL^{-1} in the concentration of 45.6±0.8%.

Conclusion: The present study revealed that cono-peptides from *C. amadis* have been used as an accessible source of natural antioxidants as well as anticancer after trial with animal and pre-clinical studies.

Keywords: Gastropod, *Conus amadis,* Venom gland, Antioxidant and HEp-2 Cell line and MTT assay.

INTRODUCTION

Oxidation is a chemical reaction that transfers electrons from a substance to an oxidizing agent which reactions can produce free radicals. In this, antioxidants terminate these chain reactions by removing free radical that intermediates and inhibit other oxidation reaction [1]. Uncontrolled assembly of free radicals that outbreak the macromolecules such as membrane lipids, proteins and nucleic acids may lead to many health disorders such as cancer, diabetes, neurodegenerative and inflammatory diseases with severe tissue injuries [2,3,4]. In addition to that, it blocks the oxidation process by neutralizing the free radicals such as, superoxide anion radical (O2-) and hydroxyl radical (OH) which are unavoidable consequence in an aerobic organisms [5]. Reactive oxygen and nitrogen species (ROS/RNS) are continuously produced in the human body and they are controlled by endogenous enzymes (superoxide dismutase, glutathione peroxidase and catalase). However, if there is an over-production of these species, an exposure to external oxidant substances or failure in the defense mechanisms, damage to valuable biomolecules (nucleic acids, lipids and proteins) may occur [6].

Recent scientific studies have reported that the hydrolysis of proteins from plant and animal sources to acquire the compounds which show antioxidant activity has been productive, such examples include capelin protein [7], quinoa seed protein [8], canola [9], egg-yolk protein 10], milk casein [11], hoki frame protein [12], mackerel protein [13]. The bioactive compounds reported in these studies are peptides which are released by the enzymatic hydrolysis of proteins [14].

In this context, the focus of research in antioxidant drugs has now shifted towards antioxidants obtained from natural or organic sources. Venoms are key evolutionary innovations of several animal lineages, consisting of an arsenal of peptides and proteins, designed both immobilizing prey and as defense against predators [15]. The search for cancer cure from natural product (plant and animal) has been practiced for over a century and the use of purified chemicals to treat cancer still continues. As the infectious diseases are evolving and develops resistance to existing pharmaceuticals, the marine environment provides a novel source for the development of lead compounds against fungal, parasitic, bacterial and viral diseases. The earliest efforts in this field derived from the interests of marine biologists and naturalists who found a number of unique toxins that were present in diverse marine life. Cone snails obtain incredibly potent peptide toxins (conotoxins) to immobilize prey fish [16]. A number of these conopeptides reduce pain in animal models and several are now in preclinical and clinical development for the treatment of severe pain often associated with diseases such as cancer [17]. In order to identify a potent molecule, the present study was carried out to explore the hemolytic, antioxidant and cytotoxic activity of conotoxins extracted from the venom of *C. amadis*.

MATERIALS AND METHODS

Preparation of venom extract

Live specimens of *C. amadis* were collected from Mudasalodai landing centre nearby Portonova in the South east coast of Tamil Nadu (11^{o}, 29'N; 79^{o}, 44'E). Snails were captured at a depth of 5–80 m using a trawl net attached fishing gear. The collected snails were dissected and a crude extract was prepared from the venom gland as described by Saravanan *et al*. [18]. The crude extract mixture was centrifuged at 17,200 rpm for 10 min at 4^{o}C. The supernatant (considered to be crude extract) was retained and stored at -20^{o}C for further use [19].

Estimation of radical scavenging activity (RSA) using DPPH Assay

The RSA activity of different extracts was determined using DPPH assay according to the methods of Nenadis and Tsimidou [20]. The decrease of the absorption at 517 nm of the DPPH solution after addition of the antioxidant (Venom extract) was measured in a cuvette containing 3550 µl of 0.1 ml, methanolic DPPH solution was mixed with 40 µl of 15 - 210 µg/mL of venom extract. Blank containing 0.1 ml of methanolic DPPH solution without venom extract and vortexed thoroughly, the setup was left at dark room temperature for 20 min and absorption was monitored after the incubation. The ability to scavenge DPPH radical was calculated by the following equation.

$$\% \text{ of DPPH radical scavenging activity (\% RSA)} = \frac{Abs_{control} - Abs_{sample}}{Abs_{control}} \times 100$$

$Abs_{control}$ is the absorbance of DPPH radical + methanol; Abs_{sample} is the absorbance of DPPH radical + venom extract. Measurements were performed in triplicate. Absorbance values were corrected for radical decay using blank solutions. The IC_{50} (concentration providing 50% inhibition) was calculated graphically using a calibration curve *vs* percentage of inhibition.

Superoxide anion radical scavenging assay

The superoxide assay was followed as described by Nishkimi *et al.* [21]. One ml of NBT solution (156µM NBT in 100 mM phosphate buffer, pH 8) mixed with 1 ml of NADH solution (468µM in 100 mM phosphate buffer, pH8). Then it was mixed with 0.1 ml of sample solution (10mg/ml). The reaction was started by adding 100 µl of PMS solution (60µM PMS in 10 mM, Phosphate buffer, and pH 8). The mixture was incubated at 25ºC for 5 minutes. A control performed with reagent mixture but without the sample. Absorbance was measured spectrophotometrically at 560 nm.

% SOD = from [(A0 - A1)/A0] X100

Where A0 is the absorbance of the control and A1 is the absorbance of the extract/standard.

Hydroxyl radical scavenging activity

The hydroxyl scavenging activity was measured according to the method of Klein *et al.* [22]. Various concentrations (50, 100, 150 and 200µg) of venom extract were added with 1.0 ml of iron-EDTA solution (0.13% ferrous ammonium sulfate and 0.26% EDTA), 0.5 ml of EDTA solution (0.018%), and 1.0 ml of dimethyl sulfoxide (DMSO) (0.85% v/v in 0.1M phosphate buffer, pH 7.4). The reaction was initiated by adding 0.5 ml of ascorbic acid (0.22%) and incubated at 80-90ºC for 15 min in a water bath. After incubation, the reaction was terminated by the addition of 1.0 ml of ice-cold TCA (17.5% w/v). Three milliliters of Nash reagent (75.0g of ammonium acetate, 3.0 ml of glacial acetic acid and 2 ml of acetyl acetone were mixed and raised to 1L with distilled water) was added and incubated at room temperature for 15 min. The reaction mixture without sample was used as control. The intensity of the color formation was measured spectroscopically at 412 nm against reagent blank. The % hydroxyl radical scavenging activity was calculated by the following formula:

% HRSA = from [(A0 - A1)/A0] X100

where, A0 is the absorbance of the control and A1 is the absorbance of the extract/standard.

Cytotoxicity bioassay

The pure fractionated lyophilized venom samples were routinely evaluated in a cytotoxicity test for lethality to brine shrimp larvae with minor modifications. Brine shrimps (*Artemia salina*) were hatched using brine shrimp eggs in a conical flask (1L), filled with purified and sterile seawater under constant aeration for 32 hrs and allowed for the hatching of the eggs and the sufficient maturation of nauplii for experimental purposes as described by Meyer *et al.* [23]. After hatching, active nauplii free from egg shells were collected from brighter portion of the hatching chamber and used for the cytotoxicity assay.

Cytotoxic activity by brine shrimp bioassay

From the stock of the crude and fractionated purified sample (0.9 mg/ml), 10, 20, 40, 80 and 160 µl were placed in different vials and filtered seawater was added to each vial to bring the volume up to 10 ml. The final concentrations of sample in the vials were 2, 4, 8, 16 and 32 µl/ml in the vials labeled A, B, C, D and E, respectively. The test substances added to 10 ml of filtered seawater and maintained at room temperature for 24 hrs under the 60W light lamp and surviving larvae were counted. A series of parallel tests with blank control were always conduced. The vials were then examined under a microscope (10×) and the number of dead nauplii in each well counted. Hundred micro liters of methanol was then added and after 10 min, the total numbers of shrimp in each well were counted and recorded. The lethal concentration for 50% mortality after 24 h of exposure, the chronic LC_{50} values and 95% confidence intervals were determined using the probity analysis method [24] as the measure of toxicity of the extract and fractions.

Cytotoxic studies by HEp-2 cell lines

HEp-2 cell lines were obtained from National Centre for Cell Sciences, Pune (NCCS). The cells were maintained in minimal essential media supplemented with 10% FBS, penicillin (100 U/ml) and streptomycin (100µg/ml) in a humidified atmosphere of 50µg/ml CO_2 at 37 °C.

Reagents

MEM was purchased from Hi-media laboratories, Fetal bovine serum (FBS) was purchased from Cistron laboratories, Trypsin, methylthiazolyl diphenyl- tetrazolium bromide (MTT), and Dimethyl sulfoxide (DMSO) were purchased from Sisco research laboratory chemicals, Mumbai. All of other chemicals and reagents were obtained from Sigma Aldrich, Mumbai.

Assay for Cytotoxicity activity (MTT assay)

The cytotoxicity of samples on HEp-2 was determined by the MTT assay [25]. Cells (1×10^5/well) were plated in 1 ml of medium/well in 24-well plates after 48 hours incubation the cell reaches the confluence. Then, cells were incubated in the presence of various concentrations of the samples in 0.1% DMSO for 48h at 37°C. After removal of the sample solution and washing with phosphate-buffered saline (pH 7.4), 200 µl/well (5mg/ml) of 0.5% 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-tetrazolium bromide cells (MTT) phosphate- buffered saline solution was added. After 4 hrs incubation, 0.04M HCl/ isopropanol were added. Viable cells were determined by the absorbance at 570 nm. Measurements were performed and the concentration required for a 50% inhibition of viability (IC50) was determined graphically. The absorbance at 570 nm was measured with a UV- Spectrophotometer using wells without sample containing cells as blanks. The effect of the samples on the proliferation of HEp-2 cells was expressed as the % cell viability, using the following formula:

(%) cell viability = A_{570} of treated cells / A_{570} of control cells × 100%.

RESULTS

Radical scavenging activity (RSA) using DPPH Assay

The DPPH radical scavenging is used to investigate capabilities of protein hydrolysate. The hydrolysates exhibit the ability to eliminate DPPH radicals at different concentration (15–210µg^{-1} ml) as shown in Fig. 1. Free radical scavenging ability of dose dependent response of cono-toxin was evaluated with reduction in absorbance caused by the DPPH radical. DPPH is stable in DMSO and showed maximum absorbance at 517 nm. The results were indicated that purified extract of cono-toxin showed the hydrolysates at a concentration of 120µg^{-1} ml, the scavenging activity of extract reached (46.2 ± 0.2%), and it was less with respect to commercial standard antioxidant α-tocopherol.

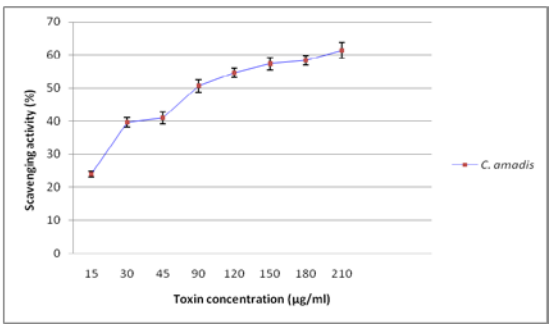


Fig. 1: DPPH radical scavenging assay at different concentration venom of *C. amadis*.

Superoxide anion radical scavenging assay

The Superoxide anion radical scavenging assay of *C. amadis* venom along with the standard ascorbic acid showed in Fig. 2. The venom extract exhibited higher activities in the different concentration. All the activities were relatively higher than that of standard compound. The purified extracts of cono-toxin showed (0.926 ± 0.004%) of SOD activity which was more or less (0.632 ± 0.006%) compared to the standard.

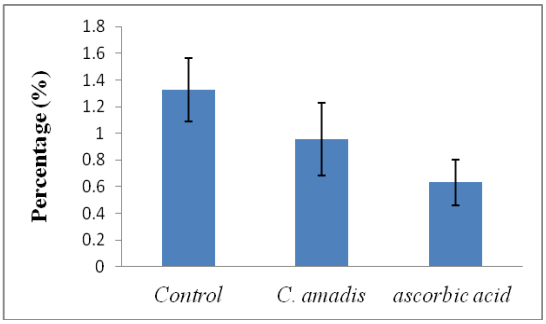


Fig. 2: SOD activity of venom *C. amadis*.

Hydroxyl radical scavenging activity

The activities of the samples to scavenge hydroxyl radical was illustrated in Figure 3. Hydroxyl radical scavenging assay showed that the purified extract was the highest (90.0±0.11%) and lowest (59.9±0.1%) respectively. The standard ascorbic acid used to inhibit hydroxyl radical.

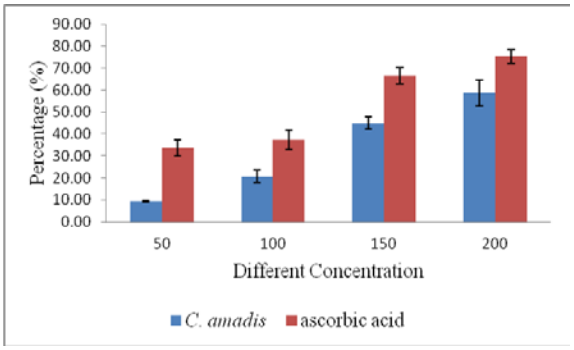


Fig. 3: Hydroxyl radical activity of venom *C. amadis*.

Cytotoxicity activity

The result of the brine shrimp lethality test revealed that the highest level of crude and purified fraction venom of *C. amadis* toxicity was found in 66.66 and 63.33% death at 32µg/ml respectively (Table. 1 and 2). The LC_{50} values of brine shrimp lethality obtained from the both venom samples were found at 14.76 and 11.46µg/ml respectively, and the most predominant activity observed the crude samples of cone snail venom and mostly presented in a neurotoxin compounds.

Table 1: Effect of crude venom from *C. amadis* on brine shrimp lethality bioassay

Samplecode (vialtype)	Conc.of sample (µg/ml)	No.ofbrineshrimp (eachvial)	Numberofbrineshrimpdied			AverageNo. ofdeadanimals	Mortality* (%)	LC50
			Vial 1	Vial 2	Vial 3			
Control	0	10	0	0	0	0	0	14.76
Type-A	2	10	1	1	2	1.33	13.33	
Type-B	4	10	3	2	3	2.66	26.66	
Type- C	8	10	6	4	6	5.33	53.33	
Type-D	16	10	6	6	5	5.666	56.66	
Type-E	32	10	6	7	7	6.66	66.66	

Table 2: Effect of purified venom from *C. amadis* on brine shrimp lethality bioassay

Sample code (vial type)	Conc. of sample (µg/ml)	No. of brine shrimp (each vial)	Number of brine shrimp died			Average No. of dead animals	Mortality* (%)	LC 50
			Vial 1	Vial 2	Vial 3			
Control	0	10	0	0	0	0	0	11.46
Type-A	2	10	1	1	2	1.33	1.33	
Type-B	4	10	3	2	3	2.66	26.66	
Type- C	8	10	4	3	4	3.66	36.66	
Type-D	16	10	6	5	6	5.66	56.66	
Type-E	32	10	6	7	6	6.33	63.33	

* Values are mean of three replicates

MTT assay

The isolated conotoxin was tested to evaluate their cytotoxic potential against the cancer cells and vero cells. Cytotoxicity was assessed by the morphological characteristic of the cells such as rounding of the cell death was observed through phase contrast microscope (Nikon, Japan). The purified conotoxin was showed remarkable cytotoxicity against HEp-2 cell in a dose depended manner (Fig. 4).

Cell Viability

The observations of the cell viability count to determine cytotoxic activity against HEp-2 cell line and Vero cell line were represented in Plate 1. The conotoxin showed cytotoxicity on HEp-2 cells at concentrations viz 100µg/mL, 50µg/mL, 25µg/mL, 12.5µg/mL, 6.25µg/mL, and 3.2µg/mL and cytotoxicity on HEp-2 cell was observed at the 25µg/mL. HEp-2 cell displayed dose dependent decreased in viability detected as early as 48 hrs (Figure. 4). At 48 hrs, the threshold concentration which caused a decrease in HEp-2 cell viability was 25µg/mL. The isolated conotoxin that produced maximum effect at 100µg/mL and the half inhibitory concentration (IC_{50}) was 25µg/mL in the concentration of 45.6±0.8. The effects of the morphology of the cancer cells are given the Plate 1.

DISCUSSION

Venoms of cone snails are among the most complex mixtures of toxin in the animal kingdom. The venom consists in a larger number of functionally different toxins and biologically active small peptide, such as stabilizing agents. Among >50,000 different conopeptides, <0.1 % only pharmacologically characterized [26]. The DPPH radical

scavenging react with suitable reducing agent, the electron became paired off and the satiation lasses color stoichiometric depends on the number of electrons taken up from the present result. It may be postulated that *C. amadis* venom extract reduce the corresponding hydrazine when it reacts with the hydrogen clear in the antioxidant principles. The activity or concentration increased by venom extract of 15 to 210µg/ml should highest scavenging (46%) of that in DPPH radical at 120 µg/ml concentration. Similarly, Sivaperumal *et al*. [27] reported that partial purified protein of *O. macrocera* crab haemolymph exhibited DPPH scavenging activity. Furthermore, Li *et al.* [28] have found that radical scavenging capacity of the porcine collagen hydrolysate improved with increase in DPPH as long as DPPH does not exceed more than 85%. Previously, Jun *et al.* [29] have stated that yellow fin sole hydrolysate prepared from pepsin showed highest DPPH scavenging activity than those produced by other hydrolysate. The effect of antioxidants on DPPH was thought to be done to their hydrogen depends activity [30]. The DPPH radical Scavenging activities of *C. amadis* venom extract are observed at different concentration in the present study. They are significantly comparable to that of ascorbic acid (100%) shows that the extract have proton- donates ability and could serve as free radical inhibitors or scavenger possibly acting as primary antioxidants.

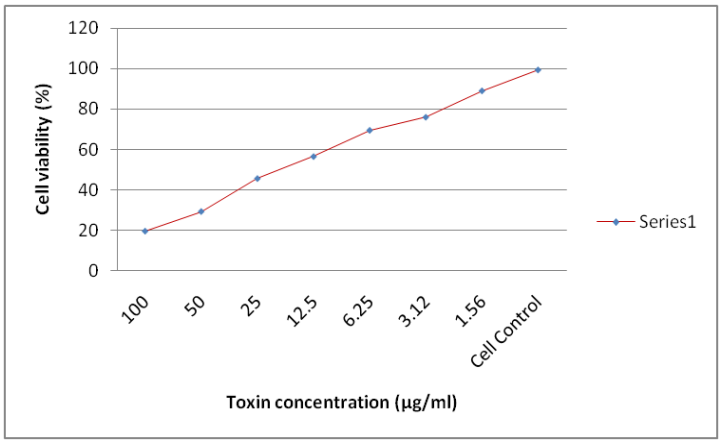
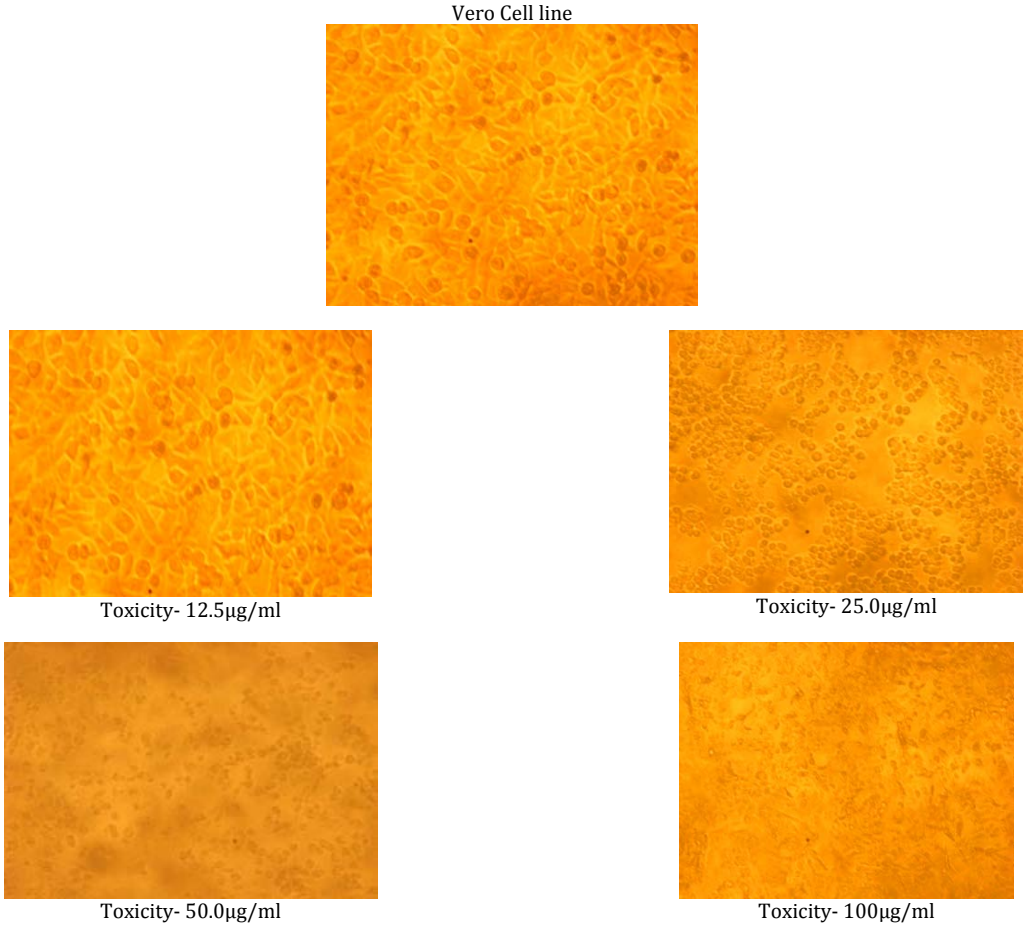


Fig. 4: Showing the cell viability using the MTT assays

Plate 1



Morphological changes on HEp-2 after treating with various concentration *C. amadis* venom.

Superoxide anion radicals are produced endogenously by flavor enzymes like xanthine oxidase, which converts hypoxanthine and subsequently to uric acid. The superoxide anion radicals are derived in PMS-NADH-NBT system, where the decrease in absorbance at 560 nm with both antioxidants [31] indicates the consumption of superoxide anion in the reaction mixture, thereby exhibiting a dose dependent increase in superoxide scavenging activity. In the present study, the low activity of SOD may be related to the elimination of superoxide anion radicals. The present result was supported by earlier study of Abdel-Rahman *et al.* [32]. They have suggested that a decrease in SOD activity and an increase in PCC could explain the induction of free radicals observed after scorpion envenomation.

Hydroxyl radical is an extremely reactive species formed in biological systems. It is capable of damaging almost every molecule found in living cells [33]. This radical has the capacity to join nucleotides in DNA and cause DNA fragmentation which contributes to carcinogenesis, mutagenesis and cytotoxicity. In the present study, the venom extract of 50µg/ml concentration showed the scavenging activity (9.0 ± 0.11%). The activity of the extracts to in each hydroxyl radicals can be related to the prevention of lipid peroxidation and it seems to be good scavenger of active oxygen species thus relation the rate of active reaction. Hydroxyl radical scavenging ability was estimated by generating hydroxyl radicals using ascorbic acid–iron EDTA. Hydroxyl radicals formed by the oxidation react with DMSO to yield formaldehyde, which provides a convenient method for their detection by treatment with Nash reagent [34].

In present work showed significant lethality against brine shrimp, this types of venom has been successfully used as a simple biological test to guide the fractionation process of cone snail venom in order to detect antitumor compounds. The LC_{50} values of the brine shrimp lethality obtained for venom of these sea snail and that of the positive control, purified venom of sea snail showed most prominent activity with LC_{50} -16 and 32 µg/ml. The mortality rate of brine shrimp nauplii was found to increase with increasing the concentration of complexes. This cytotoxicity result was very similar to the brine shrimp lethality testing that the *Aplysia kurodai* egg lectin AKL had the highest levels of toxicity (63.33% and 46.66% death at 32 and 16 µl/mg, respectively) indicating its higher mortality [35].

The MTT assay showed much lower variability and higher signal-to-noise ratios than the LDH assay. However, the highest effective concentration (EC) values were observed for the MTT assay and in contrast to all other three assays, the MTT assay requires the destruction of the cells for the analysis, thus making it impossible to use the cells for other investigations and additionally it is more time consuming. On the other hand, it has the advantage that no washing steps are needed and reagents are added directly to the medium, preventing enhanced variability due to procedural steps. Furthermore, as for the antioxidant assay, they are nondestructive assays, which can be performed within a very short time. Both can be detected spectrophotometrically or fluorometrically [36].

A very sensitive method for testing the toxicity of venom extracts and their degradation products is the assessment of *in vitro* toxicity. *In vitro* cell culture methods have the advantage of relatively well-controlled variables and are generally accepted as very effective for bio-safety testing. Their sensitivity is equal to or greater than that of *in vivo* tests. *In vitro* test methods are based on the extraction of the bioactive compounds. After exposure of cells to extracts, cytotoxicity is assessed by different methods, such as microscopical evaluation of cell morphology, methyl tetrazolium assay (MTT test). However, it has long been realized that while growth in two dimensions is a convenient way of preparing and observing a culture and allows a high rate of cell proliferation, it lacks the cell to cell and cell to substrate interactions characteristic of the whole tissue *in vivo*. The interactions of a cell with a substratum play an important role not only in the development, differentiation and regeneration of multicellular organisms but also in preserving the specific phenotype of cultured cells *in vitro*.

The present study demonstrates that the venom extract of *C. amadis* could significantly suppress that proliferation of liver cancer cells (HEp-2) *in vitro.* Using MTT assay such cytotoxic activity of venom extract was characterized by the close deferent or reflected by the correspondingly low IC_{50} values and the absence of significant effects on normal liver cells. Similar report was observed by Bilberg *et al*. [37] in male zebra fish with a semi static 48 hrs exposure LC_{50} of 84 µg/l and LC_{10} of 57µg/l.

CONCLUSION

In present study, antioxidant and cytotoxic activities of the crude cono-peptides and antioxidant activity of cono-peptides fraction from toxin of *Conus amadis* were investigated. In this study, it is found that the cono-peptides from *C. amadis* have antioxidant activity and cytotoxic activity from brine shrimp. Moreover, these cono-peptides showed good activity in HEp2 cancer cell lines at minimal concentration and which does not affect the normal vero cell lines. Thus it can be concluded that the cono-peptides from toxin of *Conus amadis* can be used as an accessible source of antioxidants with consequent health benefits.

CONFLICT OF INTERESTS

Declared None

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