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## **Inhibition of Cancer Cell Proliferation *in vitro* and Tumor Growth *in vivo* by *Hydrophis spiralis* Sea Snake Venom**

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**Abstract:** The anti-tumor activity of the sea snake venom (*Hydrophis spiralis*) was evaluated against Ehrlich's Ascites Carcinoma (EAC) in Swiss albino mice and HeLa and Hep2 tumor cell cultures. Among the different dose tested  $4.18 \mu\text{g mL}^{-1}$  at 24 h was found to effectively inhibit cancer cell proliferation. The same dose on EAC-bearing mice by i.p. injection significantly reduced the tumor growth and was demonstrated by increased life span of the mice by 182.81%.

**Key words:** Cell line, Ehrlich's ascites carcinoma, antitumor, sea snake venom, *Hydrophis spiralis*

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### **INTRODUCTION**

Sea snakes are the most venomous reptilian group in the world. They are encountered around coasts and reefs, in estuaries, rivers and at sea. Their venoms are more toxic than those of terrestrial snakes. However, they are rarely aggressive or menacing. Bites have become unusual with the advent of modern fishing methods (Senanayake *et al.*, 2005), but potentially serious hazard of the marine environment as their venoms contain potent neurotoxins, more lethal than venoms of many terrestrial snakes (Tu, 1988; Acott and Williamson, 1996). But the venom has biologically active compounds like anti coagulant and anti cancer agent (Yang *et al.*, 2005; Mora *et al.*, 2005).

The anticancer agents search has aroused the interest of scientists since the beginning of this century. Calmette *et al.* (1993) reported an anticancer effect of the venom of *Naja* sp. on adenocarcinoma cells. From that time on, a number of studies have been done by Yeh *et al.* (2001), Sun *et al.* (2003) and Mora *et al.* (2005). Particularly, when used in combination of venoms, snake venom has greatly improved the treatment of several tumors cell lines (Lipps, 1994). Snake venom is effective against tumors *in vivo* and *in vitro*, but causes less toxicity in normal cells (Araya and Lomonte, 2007).

Snake venom had been used as medicine by the traditional people and the antitumor activity of snake venom disintegrin was reported by Lipps (1994), Zhou *et al.* (2000), Yeh *et al.* (2001), Sun *et al.* (2003) and Mora *et al.* (2005). Above observations led us to examine similar activity in the venom of sea snake *Hydrophis spiralis*. To detect cytotoxic antitumor property by MTT [3-(4,5 - Dimethylthiazol-2-yl) -2,5-diphenyltetrazolium Bromide] assay was performed *in vitro* (Alley *et al.*, 1988; O'Reilly *et al.*, 1995) and *in vivo* test using tumor EAC mouse model to detect noncytotoxic antitumor property of sea snake venom.

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## MATERIALS AND METHODS

Sea snake *Hydrophis spiralis* was collected from Kalpakkam to Cuddalore waters of Coromandel Coast (India). Snake Venom (SV) from live snake was squeezed out manually, lyophilized immediately and stored at -4°C until further use (Ali *et al.*, 1999). LD<sub>50</sub> value of crude venom was found to be 0.418 mg kg<sup>-1</sup>.

For *in vitro* experiments Hep2 and HeLa cell lines were maintained in F-12 Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal calf serum, amphotericin (3 µg mL<sup>-1</sup>), gentamycin (400 µg mL<sup>-1</sup>), streptomycin (250 µg mL<sup>-1</sup>), penicillin (250 units mL<sup>-1</sup>) in a carbon dioxide incubator at 5% CO<sub>2</sub>.

Hep2 and HeLa cells were incubated in 96 well plates (1×10<sup>6</sup> cells mL<sup>-1</sup>) in the presence and absence of SV low dose (LD-0.418 µg mL<sup>-1</sup>), median dose (MD-41.8 µg mL<sup>-1</sup>) and high dose (HD-6.27 µg mL<sup>-1</sup>) concentrations for required time periods (12, 24 and 36 h). These venom concentrations tested for cancer cell cytotoxicity (Zhou *et al.*, 2000). At the required time points 50 µL of supernatant was aspirated, added to another well and mixed with 50 µL of the substrate buffer containing MTT dye (provided in the Promega kit). The mixture was incubated for 30 min at 20°C. Care was taken to keep the samples protected from light. After 30 min incubation, 50 µL of stop solution was added and the absorbance was read in an ELISA reader at 490 nm. Raw absorbance readings were used to calculate the percentage cell death in treated wells compared to that released by triton X-100 detergent as:

$$\text{Percent cell death} = \frac{\text{Values from experimental condition} - \text{Values from untreated control}}{\text{Values from triton treated wells} - \text{Values from untreated control}} \times 100$$

Each experiment was performed at least mean of 6 times to ensure consistency and the mean values was plotted in the Fig. 1.

For *in vivo* experiments, adult male Swiss albino mice (22±2 g) were obtained from the Central Animal House, Rajah Muthiah Medical College, Annamalai University. The animals were maintained on commercial standard pellet diet and water *ad libitum*. The first inoculum of Ehrlich's Ascites Carcinoma (EAC) was kindly provided by the Amala Cancer Research Institute, Thirissur, Kerala. EAC was thereafter propagated by weekly intraperitoneal injection of 0.3 mL freshly drawn ascites fluid (diluted 1:5 in sterile saline) from a donor mice bearing ascites tumor of 6-8 days old, into three mice each with a mean body weight of 22±2 g.

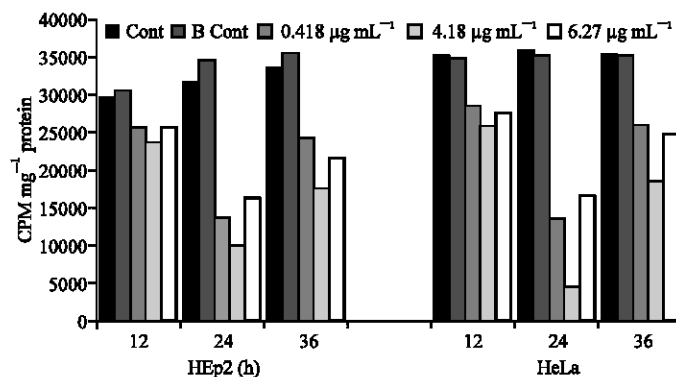


Fig. 1: MTT assay for cytotoxicity of *Hydrophis spiralis* venom

In this experiment, 72 mice were randomly assorted into 6 groups (12 animals per group).

- Group 1:** Normal (received 100  $\mu\text{L}$  of sterile saline by i.p),
- Group 2:** EAC control (received 100  $\mu\text{L}$  of sterile saline by i.p),
- Group 3:** EAC induced mice+ 1% of  $\text{LD}_{50}$  dose of venom in 100  $\mu\text{L}$  of sterile saline (0.418  $\mu\text{g kg}^{-1}$  b.w by i.p - LD),
- Group 4:** EAC induced mice +10% of  $\text{LD}_{50}$  dose of venom in 100  $\mu\text{L}$  of sterile saline (4.18  $\mu\text{g kg}^{-1}$  b.w by i.p - MD),
- Group 5:** EAC induced mice + Standard 5-floururacil (20  $\text{mg kg}^{-1}$  b.w). Group 6: EAC induced mice +15% of  $\text{LD}_{50}$  dose of venom in 100  $\mu\text{L}$  of sterile saline (6.27  $\mu\text{g kg}^{-1}$  b.w by i.p - HD).

Total experimental period was 10 days and after administering the last dose, antitumor effect of venom was assessed by observing the changes with respect to body weight, ascites tumor volume, packed cell volume, viable and non-viable tumor cell count, Mean Survival Time (MST) and percentage increase in life span (%ILS). MST of each group containing six mice was monitored by recording the mortality daily for six weeks. MST and % ILS were calculated using following equation.

$$\text{MST} = \frac{\text{Day of first death} + \text{Day of last death}}{2}$$

$$\% \text{ILS} = \left( \frac{\text{Mean survival time of treated group}}{\text{Mean survival time of control group}} \right) \times 100.$$

## RESULTS AND DISCUSSION

The cytotoxic effect was assessed by the release of lactate dehydrogenase (LDH) from HeLa and Hep2 cells after treating with different concentrations of snake venom. The range of 0.1-10% cytotoxicity at 24 h was observed. Among the doses the MD release of LDH was very less (1.5%) (Fig. 1).

Table 1 shows the changes in the body weight, percentage increase in lifespan, mean survival time, tumor volume, packed cell volume, viable and non-viable tumor cell count was observed in EAC induced albino mice. The EAC bearing mice showed increased body weight due to the increased ascites volumes by actively proliferating peritoneal cells, whereas, the bodyweight decreased from 40.24 $\pm$ 0.3 to 30.16 $\pm$ 0.9 g in treated groups. In the EAC control group, the mean survival time was 16.0 days, whereas, it increased significantly in all treated groups. A better effect was observed in medium dose treatment with an increase in the life span by 182.81%, as compared to the carcinoma control group. Increased life span and decreased bodyweight may be attributed to decreased tumor volume, packed cell volume and viable tumor cell count in a dose dependent manner.

Inhibition of cancer proliferation was measured by the MTT assay and a remarkable inhibition of cancer cell growth was observed after snake venom treatment. The cytotoxic effect was assessed by the release of lactate dehydrogenase (LDH) from HeLa and Hep2 cells after treating with 4.18  $\mu\text{g mL}^{-1}$  venom which showed positive results with a reduced amount of LDH (1.5%). Reduced LDH release showed cell retrieve to normal function and venom has inhibition mechanism against to cancer cells. Poulsen *et al.* (2005) reported that the snake venom protein taipoxin has antiproliferative effect on Small Cell Lung Cancer Cells (SCLC) and also noticed survival rate of normal cell lines treated with taipoxin. The cells were incubated with increasing concentrations of taipoxin for 72 h, after which marked inhibition of SCLC was observed. A similar observation was made by Carvalho *et al.* (2001)

Table 1: Effect of *Hydrophis spiralis* venom on body weight changes and survival time

Parameters	EAC control (10 <sup>6</sup> cells mL <sup>-1</sup> )	SV (LD) + EAC	SV (MD) + EAC	SV (HD) + EAC	5-Flurouracil + EAC
Body weight (g)	40.20±0.3	38.25±0.5	30.16±0.9 <sup>a</sup>	35.55±0.1	25.20±0.02
Mean survival time (day)	16.00±0.1	20.50±0.2 <sup>a</sup>	29.25±0.06 <sup>a</sup>	25.25±0.3 <sup>a</sup>	39.50±0.3 <sup>a</sup>
Increase life span (%)	-	128.13	182.81	157.81	246.88
Tumour volume (mL)	4.50±0.07	2.99±0.06 <sup>a</sup>	1.81±0.02 <sup>a</sup>	2.23±0.01 <sup>a</sup>	-
Packed cell volume (mL)	2.11±0.84	2.01±0.01 <sup>a</sup>	1.45±0.02 <sup>a</sup>	1.94±0.02 <sup>a</sup>	-
Viable tumor cell count (10 <sup>10</sup> cells L <sup>-1</sup> )	12.30±0.07	2.58±0.02 <sup>a</sup>	1.57±0.01 <sup>a</sup>	3.01±0.03 <sup>a</sup>	-
Non viable tumor cell count (10 <sup>10</sup> cells L <sup>-1</sup> )	0.89±0.05	3.02±0.01 <sup>a</sup>	1.02±0.03 <sup>a</sup>	2.55±0.02 <sup>a</sup>	-

Values are mean±SD, n = 6, <sup>a</sup>: p(0.01 vs EAC control group, Body weight of normal mice 22±2 g

where the lectin from the venom of the snake *B. jararacussu* (BJcuL) was found to be a potent inhibitor of growth of some tumor cell lines and an endothelial cell line and it suppress the tumor cell proliferation. This is also supported by Akiyama (1996) and Tang *et al.* (2003). Akiyama (1996) stated that the peptides are known to interfere with integrin dependent cell adhesive events and Tang *et al.* (2003) stated that treatment of osteoblast suspension with venom peptide markedly inhibited cell adhesion to substratum.

In the present experiment, the concentration of the venom was chosen for testing the anticancer potential of snake venom in the *in vivo* model as suggested by Abu-sinna *et al.* (2003). A reliable criterion for assessing the potential of any anticancer agent is the prolongation of life span of the animals (Orsolich *et al.* 2003; Hogland, 1982). Decrease in tumor volume and viable tumor cell count observed in the present experiment can be considered as an important indication for the reduction of tumor burden and enhancement of lifespan of EAC bearing mice. Andreani *et al.* (1983) suggested that an increase in the lifespan of ascites bearing animals by 25% is considered as indicative of significant drug activity. This suggests that the cytotoxic activity of snake venom on the EAC cells might be due to mechanisms other than direct cytolytic effect. In this context, several authors have reported the anti cancer potential of snake venom (Zhou *et al.*, 2000; Yeh *et al.*, 2001; Sun *et al.*, 2003; Orsolich *et al.*, 2003; Lipps, 1994; Silva, 1995; Mora *et al.*, 2005). Considering these present and earlier observations, it is strongly convincing that *L. curtus* venom has deleterious effect on the animal tumor models and cancer cell lines against tumor proliferation.

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