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RESEARCH ARTICLE

ANTICANCER ACTIVITY OF MARINE CEPHALOPOD (*S.PHARAONIS*) FROM GULF OF MANNAR

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ABSTRACT

Anticancer activity of crude and partially purified fractionated ink of *S.pharaonis* (cuttlefish) was evaluated on HepG2 cancer cell line. Different concentration (125µg, 250µg and500µg) of crude and fractionated ink were prepared and the cell viability was measured using MTT assay. Crude ink exhibit significant cytotoxic activity at all concentration. Cytotoxicity increases as the concentration of the sample increases from 125µg to 500µg. Maximum toxicity of 64% was observed at 500µg/ml concentration and IC 50 value obtained at a concentration of 125µg. Anticancer analysis of protein fractionated ink showed potent cytotoxic activity against Hep G2 liver cell line and the toxicity ranged between 56%- 74%. Analysis of fractionated ink resulted in cell death at a concentration of <125. Results indicate that the extracts had a dose dependent inhibitory effect on the growth of the HepG2 liver cell line and the toxicity was higher in the protein fractionated ink of cuttlefish when compared with crude ink. Therefore proteins of cuttlefish ink can be targeted for therapies.

INTRODUCTION

Cancer is a growing health problem around the world. Cancer may affect people at all ages, even foetuses, but the risk for most varieties increases with age and causes about 25% of all human deaths (Hemminkin and Mutanen, 2001,WHO Cancer, 2003). Complete removal of the cancer without damage to the rest of the body is the goal of treatment, but the property of cancers to invade adjacent tissue or any other organs by microscopic metastasis often limits its effectiveness. Liver cancer has become one of the major types of cancer with high mortality (Serag *et al.,* 1999). Liver cancer is not responsive to the current cytotoxic agents used in chemotherapy because of the development of multidrug resistance phenotypes and the presence of p53 gene mutations which tend to be aggressive and are extremely resistant to chemotherapy (Heinze *et al.,* 1999).

The ocean harbour a variety of life forms ranging from microorganisms to vertebrates, which in turn provide mankind with several benefits biologically and medicinally. This feature of wide diversity in marine life forms has been identified as chief source for unique biologically active compounds that exhibit tremendous potential for pharmaceutical applications (Jain, 2009). Cephalopods play an important role in marine ecosystem and are valuable to man as food and in biomedical research (Forsythe and Nanlon, 1988). They also notable for their defences, such as jetting escape movements, changes in colouration, toxic venom and inking (Hanlon *et al*., 1996; Norman 2000). The physical activities of cuttlefish ink such as anti-tumour, immunity promotion, induction of many cytokines, antimicrobial activity have been widely studied in recent years.

Although many compounds of both natural and synthetic origin were discovered which had good activity in the experimental models and only a small number proved to be useful in clinics, hence there is a continuous need for searching more active compounds with novel structures. Thus, the search for drugs (or compounds) extracted from marine source as potential cytotoxic agents for hepatoblastoma is an important line of research in the novel anticancer candidates. Therefore present study was done to evaluate the toxicity of ink against Hep G2 cell using cell proliferation and cell viability assay. Results from both crude ink and purified ink were then compared to determine the selective activity.

MATERIALS AND METHODS

Collection of animals

S.Pharaonis were collected from Gulf of Mannar, Thoothukudi coastal region (Long 78^0 8" to 79^0 30" E and Lat 8^0 35" to 9^0 25" N) by trawl catch, brought to the laboratory, cleaned and washed with fresh sea water to remove all impurities. The ink glands were dissected and ink was collected by gently squeezing the glands with spatula and the ink was diluted immediately with an equal volume of phosphate buffered saline (PBS, pH, 6.8) freeze dried and stored at -80°c.

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The crude ink of *S.pharaonis* showing broad spectrum activity was partially purified by ammonium sulphate precipitation and dialysis (Jadwiga and Sierecka, 1998). Thus the protein fractions corresponding to different precipitation were designated as C1,C2…….C7 (20% to 80%).

Anticancer activity on liver cancer cell lines

The anticancer activity of crude ink, protein fractionated ink (80% obtained through ammonium sulphate precipitation and dialysis showing potent activity) was performed on HepG2 cancer cell lines obtained from National centre for cell science, Pune, India. The cell viability was measured using MTT assay (3-[4,5-dimethylthiazol-2-y]- 2,5-diphenyltetrazolium bromide) (Kang *et al.,* 2004). The cells were grown in a 96-well plate in Delbucco's Minimum Essential Medium (DMEM) (HiMedia, Mumbai)) supplemented with 10% fetal bovine serum (Gibco Laboratories) and antibiotics (streptomycin, penicillin-G, kanamycin, amphotericin B). About 1 ml cell suspension (10^5 cells/ml) was seeded in each well and incubated at 37^0 C for 48 hour in 5% CO_2 for the formation of confluent monolayer. The monolayer of cells in the plate was exposed to various dilutions of extract (125µg, 250µg and 500µg). The cell viability was measured using MTT assay with MTT (5 mg/ml) and DMSO. The tetrazolium salt is metabolically reduced by viable cells to yield a blue insoluble Formazan product measured at 570nm spectrophotometerically. Controls were maintained throughout the experiment (untreated wells as cell control). The assay was performed in triplicate for each of the extracts. The mean of the cell viability values was compared to the control to determine the effect of the extract on cells and % of cell viability was plotted against concentration of the extract. The minimum concentration of the extract that was toxic to liver cancer cells was recorded as the effective drug concentration compared to positive control (PC-Cyclophosphamide).

Percentage of viability = Absorbance of the sample/ Absorbance of control

Percentage of toxicity = 100 – percentage

Morphological studies using a normal inverted microscope were carried out to observe the cancer cell treated with the crude and fractionated purified ink. The untreated cells were used as control.

RESULTS AND DISCUSSION

In the present investigation the crude cuttle fish ink showed potent toxicity against the HepG2 liver cells and the percentage of toxicity ranged from 53%-64% (Table-1). Anticancer analysis of crude ink revealed that 125µg was the minimum inhibitory concentration for cell death. Similar study was carry out by Russo *et al.* (2003) in the melanin free ink of the cuttle fish *Sepia officinalis* on a variety of cell line including *PC 12* cells and active factor was identified as tyrosinase. The antitumour activity of the different peptidoglycan fraction of ink of *Sepia pharaonis* on Dalton's Lymphoma Ascites (DLA) was studied by Sherief *et al.* (2004).

The results of 80% protein fractionated ink of cuttlefish on MTT assay are presented in Table 2. At 125µg concentration partially purified fractionated ink showed 47% viability and at 500µg the extract showed 36% viability. The viability decreases with the increasing concentration of the extract. The toxicity ranged from 53%-64%. At 125µg the Hep G2 cells showed viability of 53%, and 58%, 68 % were observed at a concentration of 250µg, 500µg respectively. Anticancer analysis of protein fractionated cuttle fish ink showed potent toxicity against HepG2 liver cells and perform cell death at a concentration of <125µg. Total number of live cells or dead cells at different concentration was examined using an inverted microscope and compared with the cells serving as control (Plate -1). The decline of cells may be due to the presence of active biological compounds in the ink extract.

Sulfated ink polysaccharide isolated from cuttlefish *Sepiella maindroni* are known for their inhibition potential of MMPs (Koyanagi *et al*., 2003; Isnard *et al.,* 2003; Iida *et al.,* 2007) and especially the metastasis in cancer is strongly inhibited by the O-sulfated polysaccharide (Borgenstrom *et al.,* 2007). But in contrast Priya *et al.* (2006) reported that an uronic acid rich peptidoglycan isolated from the ink of the cuttlefish *Sepia pharaonis* showed cytotoxicity against human cervical cancer *HeLa* cells (CC50 = 135g/ml). Guo-Fang Ding *et al.* (2011) investigated the anticancer activity of peptides isolated from hydrolysates of Sepia ink.

Table 1. Anticancer activity of crude *S.pharaonis* ink on HepG2 liver cancer cell line

S.No.	Concentration (µg)	O.D (mean of three replicates)	Percentage of cell viability	Percentage of cytotoxicity
1	Negative control	1.833	100	0
2	125	0.855	46.64484	53.356
3	250	0.768	41.89853	58.102
4	500	0.662	36.11566	63.89
5	Positive control	0.408	22.25859	77.75

Table 2. Anticancer activity of 80% protein fractionated ink of *S.pharaonis* on HepG2 liver cancer cell line

S.No.	Concentration (µg)	O.D (mean of three replicates)	Percentage of cell viability	Percentage of cytotoxicity
1	Negative control	1.833	100	0
2	125	0.799	43.589	56.411
3	250	0.605	33.006	66.994
4	500	0.479	26.132	73.870
5	Positive control	0.408	22.25859	77.75

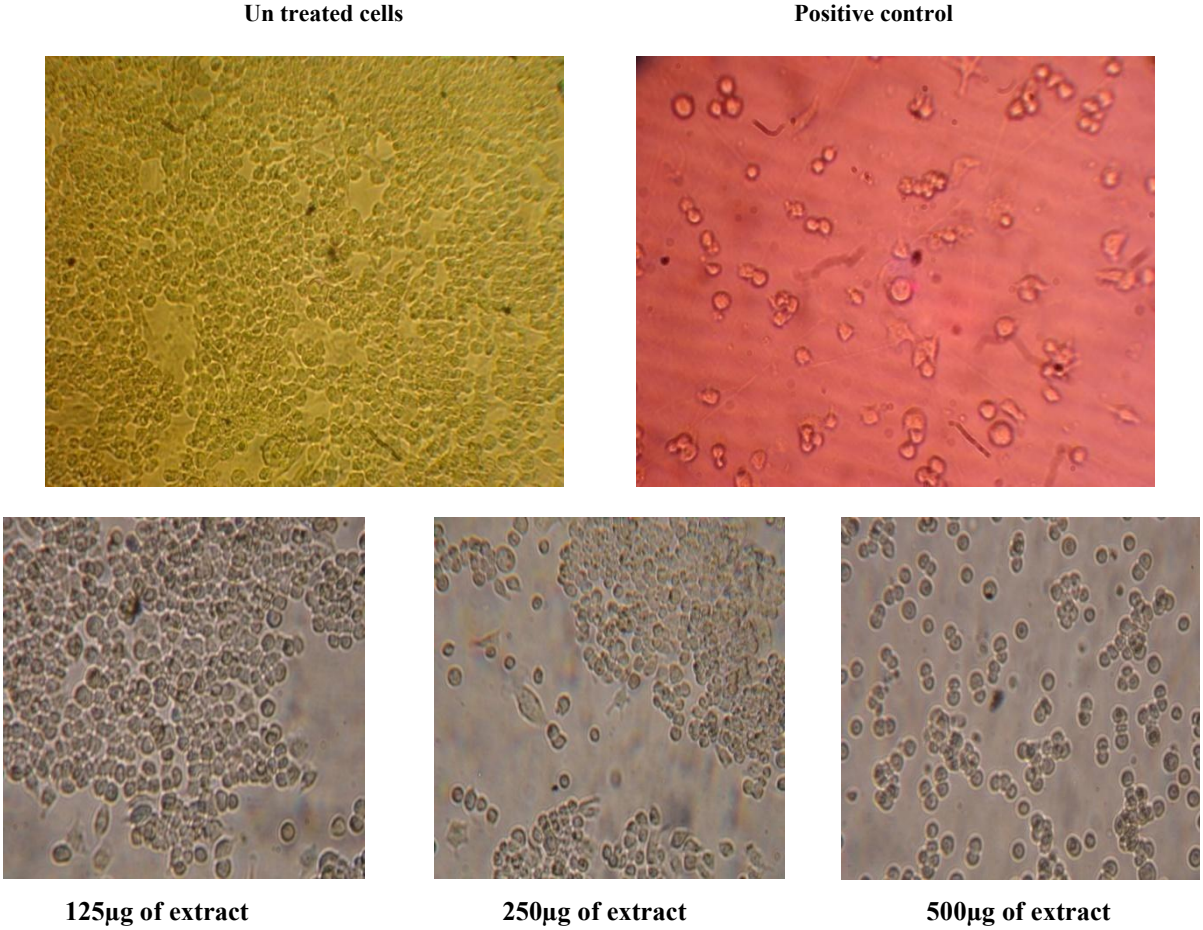

Plate 1. MTT assay – 80% protein fractionated ink of *S.pharaonis*

In this study the partially purified fractionated ink of cuttlefish showed more toxicity than crude ink which indicates that anticancer substances are present mainly in the 80% protein fractionated ink. Further purification, characterization and structural analysis shall pave the way for the development of new drug in the future.

Conclusion

Natural derivatives play an important role to prevent the cancer incidences as synthetic drug formulations cause various harmful side effects to human beings. Marine natural products are potential source of anticancer compounds in medicinal applications that would prove to be beneficial in uplifting human health. Our results give additional support that ink secretions are the source of biologically important compounds of biomedical research and it can be used as an anticancer drug in future.

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