Research Article

Cytotoxic activity of crude and partially purified ink of *L.duvauceli* towards HepG2cell line

*Jemma Hermelin Jesy Diaz[1], R. D. Thilaga[1], V. Sivakumar[2]

1. St. Mary's College (Autonomous), Thoothukudi, Tamil Nadu, India.
2. Research and Conservation 4eindia. NGO, India.

ABSTRACT

The present study aims to evaluate the anticancer activity of the crude and partially purified protein fractionated ink of *Loligo duvauceli* on the Hep G2 cell line using cell viability and cell proliferation assay (MTT assay). Different concentrations of the ink were prepared (125µg, 250µg, 500µg) and the cytotoxic activity was determined. Among the three doses tested, the crude ink of *L.duvauceli* at the concentration of 500 µg/ml was found to inhibit the viability of the cell effectively and the percentage of viability ranged between 33 – 41. Cells viability decreased with increasing concentrations of the samples. HepG2 cells treated with protein fractionated ink, decreased the percentage of viability and the percentage ranged from 30-49. MTT assay revealed that partially purified ink of *L. duvauceli* showed higher activity than that of crude ink. The percentage of cell death was 70 in partially purified protein fractionated ink and 67% in crude ink at a concentration of 500µg. IC 50 value was obtained at a concentration of 125µg. The present findings suggest the profound anticarcinogenic activity of partially purified ink of *L.duvauceli* on HepG2 cancer cell line. Further purified compounds can be used as a potential chemotherapeutic drug for the treatment of Hepatic cancer.

Keywords: Anticarcinogenic, cytotoxicity, Hep G2 cells, MTT assay

Received 21 April 2014 Received in revised form 11 May 2014 Accepted 13 May 2014

***Address for correspondence:**
Jemma Hermelin Jesy Diaz,
Department of Zoology, Assistant Professor, St. Mary's College (Autonomous), Thoothukudi-628002, Tamil Nadu, India.
E-mail: jerrycorera@yahoo.co.in

INTRODUCTION

Cancer is one of the most life-threatening disease and a major public health problem in many parts of the world. Cancer continues to represent the largest cause of mortality in the world and claims over 6 million lives every year [1]. Liver cancer has become one of the major types of cancer with high mortality [2] and is not responsive to the current cytotoxic agents used in chemotherapy because of tumour heterogeneity and the development of multidrug resistance phenotypes. Due to lack of effective drugs, expensive cost of chemotherapeutic agents and side effects of anticancer drugs, cancer can become fatal. Till now the availability of treatments for liver cancer remains unsatisfactory [3]. Several marine derived compounds are currently extracted and synthesized by chemical process for cancer treatment.

Squid ink is a multifunctional marine bioactive material which kills cancer cells. [4-6]. As a safe natural product, ink has potential clinical application in health care and medicine, no specific study had been reported addressing the selective cytotoxic effects of ink on Hep G2 cell lines. Therefore present study was done to evaluate the toxicity of *Loligo duvauceli* ink.

MATERIALS AND METHODS

Collection of animals

L.duvaucelii 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.

The crude ink of *Loligo duvauceli* showing broad spectrum activity were partially purified by ammonium sulphate precipitation and dialysis [7]. Thus the protein fractions corresponding to different precipitation were designated as S1, S2…….S6 (20% to 70%).

Anticancer activity on liver cancer cell lines

The anticancer activity of crude ink, protein fractioned ink (30% 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-diphenyltetra-zolium bromide [8]. The cells were grown in a 96-well plate in Delbucco's Minimum Essential M*edium* (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 spectrophoto-meterically. 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 of viability

Morphological studies using a normal inverted microscope were carried out to observe the cell death in the drug (ink) treated HepG2 liver cancer cells. Concentrations of 125µg, 250µg, 500µg/ml of ink were used for the morphological studies. The untreated cells were used as control.

RESULTS AND DISCUSSION

The results of cell viability and toxicity assay of crude squid ink are given in (**Fig. 1 & 2**). The percentage of cell viability showed variation in between 33 and 41. The percentage of cell viability was decreased with increased concentration (125µg/ml - 41%, 250µg/ml -39%, 500µg/ml - 32%) of the substance and the percentage of toxicity showed variation in between 59 and 67. The maximum toxicity (67%) was attained at 500µg concentration of crude squid ink and minimum (59%) at 125µg concentration of crude ink. A similar study was carried out by Sasaki *et al* [5] for testing the anticancer potential of squid ink in the vivo model.

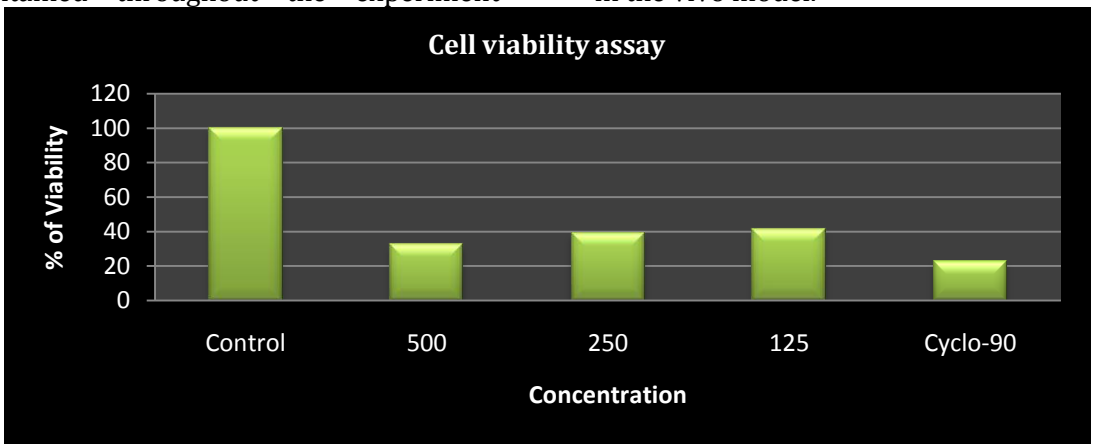


Figure 1: Analysis of cell viability- crude *L. duvauceli* ink

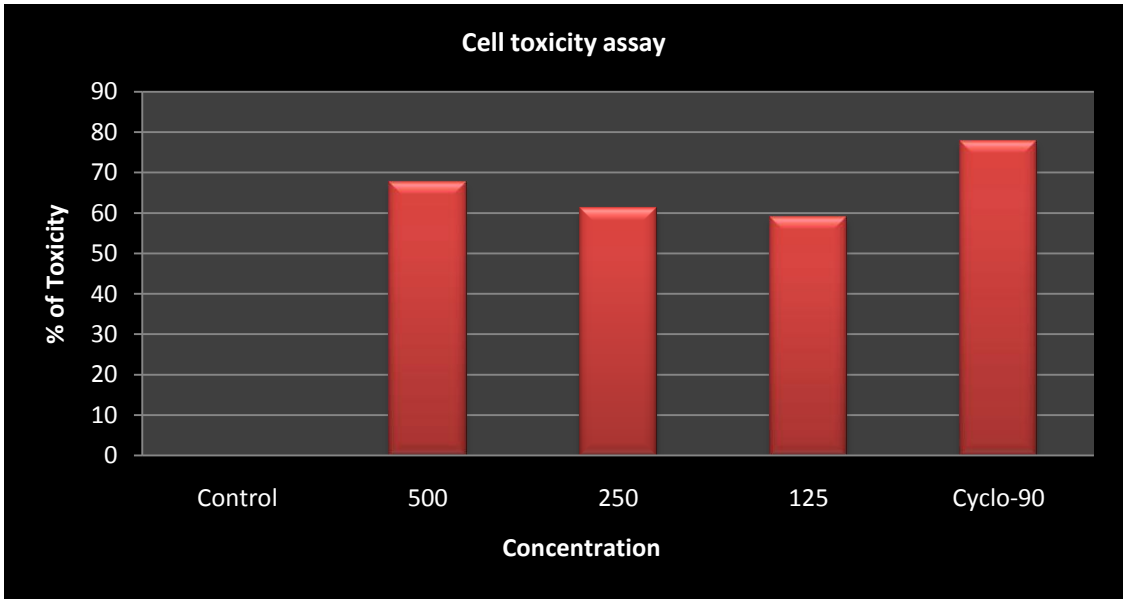


Figure 2: Analysis of cell toxicity – crude *L. duvauceli* ink

Effect of fractionated ink (30%) on Hep G2 cell was shown in Fig.3,4 and in Plate.1. Hep G2 cells experienced a significant decrease in viability at low concentration (125µg/ml -49%) of squid ink, with an eventual decline at the highest concentrations (500µg/ml - 30%) tested. The viability ranged from 30%-49%. The toxicity varied from 51-70%. Maximum toxicity of 70% was observed at 500µg concentration of the protein fractionated squid ink and minimum of 51% was observed at 125µg concentration. IC_{50} value was obtained at a concentration of 125µg. (concentration causing death of 50% of HepG2 liver cancer cell). This infers that 30% of protein fraction possesses higher anticancer activity. There are previous reports on the antitumour [9-14] activity of different species of squid ink. Chen *et al* [15] studied the potential anticancer properties of a sulphated polysaccharide isolated from the ink of the squid *Ommastrephes bartrami* using vitro and vivo models. Our results also indicate that the extracts had a dose dependent inhibitory effect on the growth of the HepG2 liver cell line in vitro, indicating that the ink possess anticarcinogenic activities.

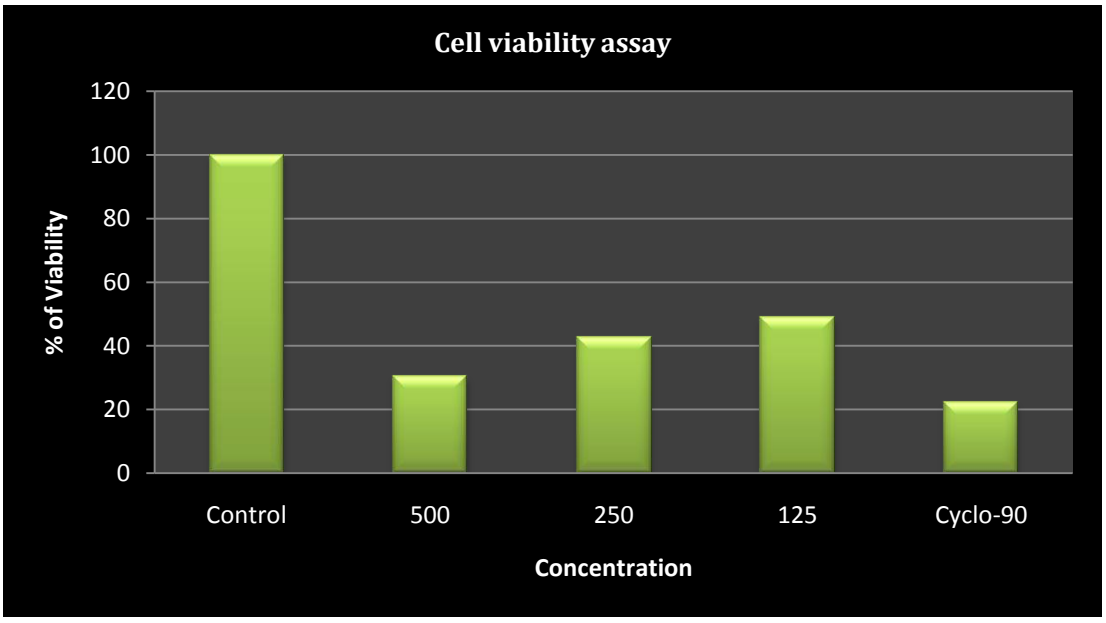


Figure 3: Analysis of cell viability – 30% protein fraction of *L.duvauceli* ink

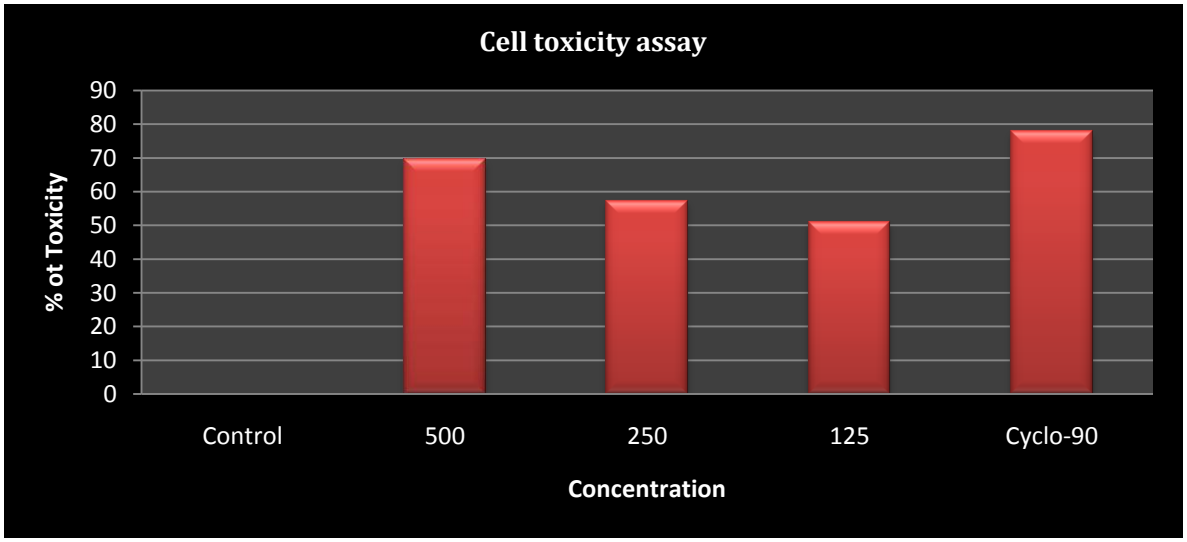
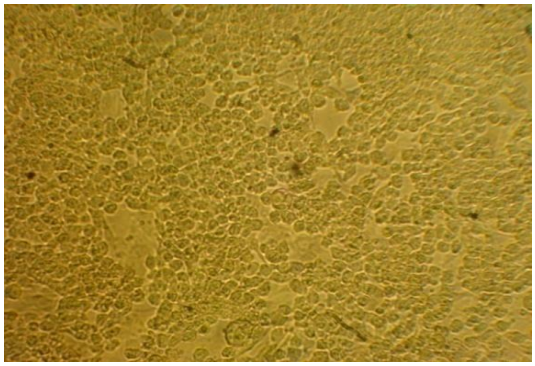
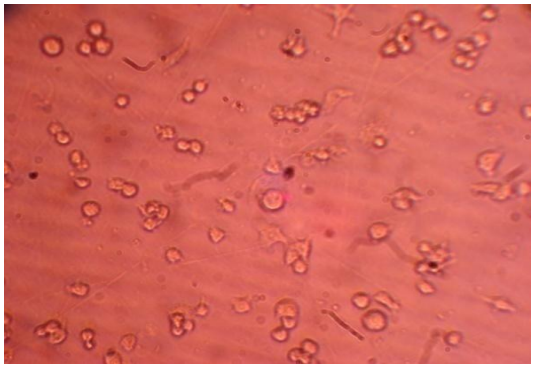


Figure 4: Analysis of cell toxicity – 30% protein fraction of *L. duvauceli* ink

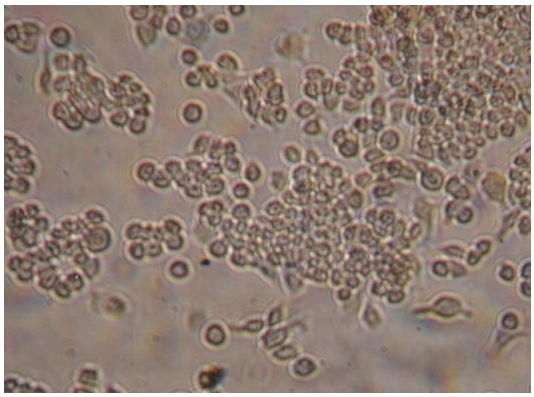
Plate -1



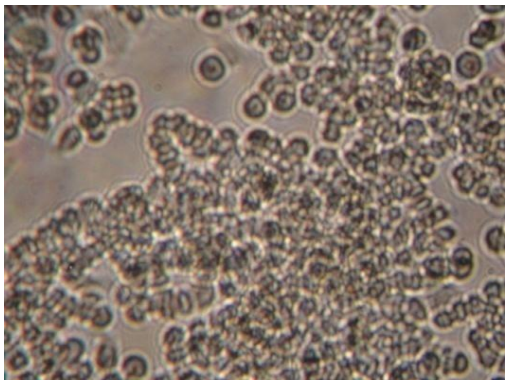
Untreated cells



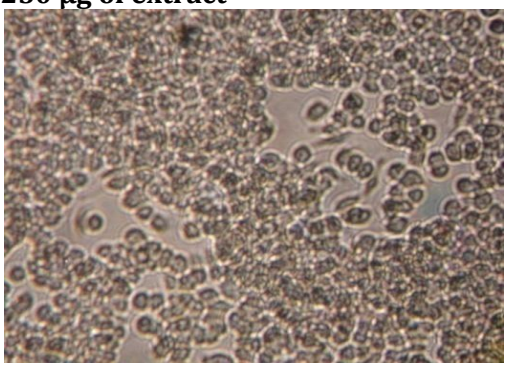
Positive control



500µg of extract



250 µg of extract



125 µg of extract

MTT Assay- 30% protein fraction of *L.duvauceli* ink

CONCLUSION

The result of the present study reveals that the crude ink and partially purified ink had anticancer activity against liver cancer cells. Compared to purified ink crude ink has lesser toxicity and hence proteins could be the best targets for cancer therapy in future. Correct understanding and utilization of ink may lead to its reuse as an anticancer agent.

ACKNOWLEDGEMENT

The authors express their immense gratitude to the directors of Research Centre of Advanced Zoology and Biotechnology V.O.C. College, Thoothukudi for their support and encouragement. The authors also thank Mrs. Florida Tilton of Biozone Technologies, Chennai for her support to carry out this work.

REFERENCES

1. Hemminkin K, Mutanen P. Genetic epidemiology of multistage Carcinogenesis. *Muta. Res.* 2001; 473: 11-21.
2. Serag H.B, Mason AC. Rising incidence of hepatocellular carcinoma in the United States. *New. Engl. J.Med*. 1999; 340: 745 -750.
3. Honda K, Sbisa E, Tullo A, Papero PA, Saccone C, and Poole S*. et al.* Mutation is a poor prognostic indicator for survival in patients with hepatocellular carcinoma undergoing surgical tumour ablation. *Br. J. Cancer* 1998; 77: 776 -782.
4. Takai M, Kawai Y, Inoue N, Shinano H. Comparative studies on microbiological and chemical characteristics of Ika-Shiokara akazukuri and Ika- Shiokara Kurozukuri. *Bull.Jpn Soc. Sci. Fish*. 1992; 58: 2373 – 2378.
5. Sasaki J, Ishita K, Takaya Y, Uchiswa H, Matsue H. Antitumor activity of squid ink. *J. Nutr. Sci. Vitaminol* 1997; 43: 455-461.
6. Rajaganapathy J, Thyagarajan SP, Patterson Edward JK. Study on cephalopod ink for anti retroviral activity. *Journal of Experimental Biology.* 2000; 38 : 519 -520.
7. Jadwiga K, Sierecka A. Purification and partial characterization of a neutral protease from a virulent strain of *Bacillus cerues*. *J. Biochemistry and Cell Biology*. 1998; 30:579-595.
8. Kang BK, Chon SK, Kim SH, Jeong SY, Kim MS and Cho SH *et al*. Controlled release of paclitaxel from microemulsion containing PLGA and evaluation of anti-tumor activity in *vitro* and in *vivo. International Journal of Pharmaceutics.* 2004 ; 286: 147- 156.
9. Takaya Y, Uchisawa H, Matsue H, Narumi F, Sasaki J, Iahada KL. An investigation of the antitumour peptidoglycan fraction from the squid ink. *Biol. Pharm. Bul.* 1994;17 (6) : 846-851.
10. Takaya Y, Uchisawa H, Matsue H, Okuaki B, Narumi F, Sasaki J, Ishida K. An Investigation of the antitumor peptidoglycan fraction from squid ink. *Biol. Pharm. Bull.* 1994a; 17 (6) : 846-851.
11. Zhong JP, Wang G, Shang JH, Pan JQ, Li K, HuangY, Liu HZ. Protective effects of Squid ink extract towards hemopoietic injuries induced by cyclophosphamine. *Mar. Drugs*. 2009; 7(1): 9-18.
12. Naraoka T, Chung HS, Uchisawa H, Sasaki J, Matsue H. Tyrosinase activity in antitumour compounds of squid ink, *Food Sci. Tech. Res*. 2000; 6(3) 171- 175.
13. Venkateshvaran K, Abidi AH, DBT/NBDB sponsored training programme. Aquatic animal Toxins and Pharmacological Bio resources. 2001; Pp. 20-35.
14. Su Wei-ming MA, Run-di, Yu Li-jian, LIAO Ming-neng, He Dong-mei, Hu-lai-zhen, Antitumor effects of extract from the ink of *Loligo chinensis. Chinese Journal of Marine Drugs.* 2005; 2: 47 – 50.
15. Chen S, Wang J, Xue X. " Sulfation of a squid ink polysaccharide and its inhibitory effect of tumor cell metastasis". *Carbohydrate Polymers*. 2010; 81(3) 560-566.