CHAPTER: 6- CYTOTOXIC ACTIVITY

6.1. Introduction

Among various diseases, cancer has become a big threat to human beings globally. As per Indian population census data, the rate of mortality due to cancer in India was high and alarming with about 8,06,000 existing cases by the end of the last century. Cancer is the second most common disease in India responsible for maximum mortality with about 0.3 million deaths per year. All types of cancers have been reported in Indian population including the cancers of skin, lungs, breast, rectum, stomach, prostate, liver, cervix, oesophagus, bladder, blood and mouth etc. The causes of such high incidence rates of these cancers may be both internal (genetic, mutations, hormonal and poor immune conditions) and external or environmental factors such as food habits, industrialization, over growth of population, social etc (Imran Ali, 2011)

Cancer is a group of disease caused by multiple alterations in gene expression resulting in imbalance of cell proliferations and cell death. Abnormal cells can metastasize to other organs and transform the normal cells into cancer cells. These abnormal cells are having specific biological characters called hallmarks of cancer, they include sustaining proliferative signalling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis and activating invasion and metastasis. (Hanahan and Weinberg, 2000)

Liver cancer or hepatocellular carcinoma (HCC) was one of the leading causes of worldwide cancer mortality (El- Serag and Mason, 1999). Hepatocellular carcinoma (HCC) is one of the most common cancers in the world. The curative treatment such as tumor resection and liver transplantation are not feasible in advanced stages of HCC (Herold et al., 2002). The endemic mortalities of HCC were
observed in tropical and subtropical countries. The major risk factors involved in HCC were viral particle Hepatitis B and some hepatocarcinogens such as nitrosamines, aflatoxins etc. The therapeutic options are surgical interventions (tumor resection and transplantation), radiation therapy, liver chemotherapy, immune therapies. But these therapeutic methods are producing adverse side effects. Hence it is necessary to evaluate the new active drugs against HCC with the lack of side effects from a cheaper source.

The natural products isolated from marine organisms has been increased rapidly and hundreds of new compounds being discovered every year. Especially, the marine invertebrates (sponges, mollusc, tunicate, etc.) are producing high amounts of bioactive compounds (Burres and Clement, 1989; Corona et al., 2007; Gao et al., 2007)

Marine natural products (MNP) are organic compounds produced by cyanobacteria, sponges, seaweeds, and other marine organisms. Majority of the marine natural products have been isolated from sponges, ascidians, conus, sea anemone, Coelenterate, tunicates, opisthobranch, molluscs, echinoderms, bryozoans and a wide variety of marine seaweeds (Calvette et al., 2000 and Abel et al., 2003) such as red marine algae (Bryothamnion triquetrum, Solieria robusta) and cyanobacteria.

Opisthobranch molluscs which have attracted the interest of biologists investigating biologically active compounds (Falkner and Stallard, 1973; Yamamura and Terada, 1977). A 250 KDa glycoprotein aplysianin E was purified from the egg of Aplysia kurodai which exhibited anticancer activity against some marine and human tumor cell lines as well as on experimentally tumoral mice (Kisugi et al., 1987) in another study a 320 KDa glycoprotein was isolated from the
albumin gland of *A. kurodai* with antitumor activity (Takamatsu *et al.*, 1995). Dolastatin 10 is another anticancer protein which was isolated from *Dollabella auriculata*, sea hare originating from the Indian Ocean (Turner *et al.*, 1998).

Marine natural anticancer compounds were under various stages of clinical trials bryostatin-1 (polyketide from bryozoans), dolastatin-10 (peptide from sea slug), ILX651 (peptide from sea slug) cemadotin (peptide from sea slug) kahalalide F (cyclic depsipeptide from sea slug and algae) and ET743 alkaloid from sea squirt (Haefner, 2003).

### 6.2. Materials methods

#### 6.2.1. Cell culture

The cell line under investigation was HepG-2 (Human hepatoma cell line) was obtained from National Center for Cell Sciences, (NCCS) Pune, India. The cells were grown in Dulbecco's Modified Eagle's medium (DMEM) (Gibco) media supplemented with 10% (V/V) Fetal bovine serum (FBS, Gibco), with 100 U/ml penicillin and 100 mg/ml streptomycin. All the cell line was maintained at 37°C in a 5% CO₂ atmosphere with 95% humidity.

#### 6.2.2. Determination of cytotoxicity by methyl thiazolyltetrazolium (MTT) assay

MTT assay was done according to method of Carmichael *et al.* (1988) with some modifications. Cytotoxicity effect of sea slug crude and active fraction against HepG-2 cell line was evaluated by MTT assay. Cells were seeded on 24 well plate at density of $10^3$-$10^4$ cells per well, with DMEM supplemented with 10% FBS, penicillin and streptomycin (1%) were added to the medium and maintained in humidified atmosphere 5% CO₂ at 37°C. After 24 hours, the cells were exposed to
different concentration of the crude extract and fractions (2.5, 5.0, 7.5 and 10 mg/ml). Doxorubicin used as standard and DMSO used as control. The cells were further incubated for 24 hours. Morphological changes of the cell culture were examined using an inverted microscope. After 24 hours, cell viability was determined. The IC\textsubscript{50} value was defined as concentration of drug inhibited 50% of cell growth. Experiments were done in triplicate. A cytotoxicity effect of crude extract and fractions were determined by following formula.

\[
\text{Percentage of Cytotoxicity} = \frac{\text{Control OD} - \text{Test OD}}{\text{Control OD}} \times 100
\]

6.2.3. Trypan blue dye exclusion assay

Cell viability assay was calculated by trypan blue dye exclusion assay of (Talwar, 1974), it was used as a semi quantitative method. The cells were incubated with or without antibiotic compound. After 24 hours of incubation, cells were trypsinised, centrifuged for 5 min at 1000 rpm and the pellet was resuspended in 1 ml PBS 10 µl of 0.4% Trypan blue was added with 10 µl of cell suspension and incubated for 3-5 min. After incubation, the cells were exposed to different concentration of the drug. 10 µl of cell mixture was placed in a haemocytometer and a total of 100 cells were counted and the number of viable and non viable cells was recorded.
6.3. Results

The crude extract as well as fractions of *K. ornata* were screened for the *in vitro* cytotoxic activity by using MTT assay. The morphological changes were observed after 24 hrs incubation period (Fig. 32.). The percentage of cell viability was found to be inversely proportional to the concentration of the extracts tested.

The viability of cell culture was determined by trypan blue dye exclusion assay. In this assay the percentage of cell viability gradually decreased according to the extract concentration of both crude and fractions.

Crude extract of the sea slug *K. ornata* showed 17.28±0.57%, 26.91±0.66%, 42.27±1.04% and 63.51±2.13% of cytotoxicity at 2.5 mg/mL, 5 mg/mL, 7.5 mg/mL and 10 mg/mL respectively. In this assay fraction of *K. ornata* exhibited 22.1±0.52%, 37.9±0.47%, 57.86±0.87% and 76.5 ±0.82% of percentage of cell inhibition at 2.5 mg/mL, 5 mg/mL, 7.5 mg/mL and 10 mg/mL respectively. From the obtained results it is evidenced that the fractions of sea slug *K. ornata* revealed high toxicity when compared to that of crude extract. This assay proved that the sea slug *K. ornata* is most prominent source of novel metabolites with promising cytotoxic potentials. IC₅₀ values of sea slug crude and fraction were found to be 8.74 mg/mL and 6.29 mg/mL.
Fig. 32. Percentage of cytotoxic effects of crude and fractions of

*K. ornata* on HepG2 cell line
Normal HepG-2 cell line

Toxicity-10mg/ml

Toxicity- 5mg/ml

Toxicity- 7.5mg/ml

Toxicity- 2.5 mg/ml

Fig. 33. Cytotoxic effect on HepG-2 cell line by crude extract of Sea slug *K. ornata*
Normal HepG2 Cell line

Toxicity-10mg/ml
Toxicity-7.5mg/ml
Toxicity-5.0mg/ml
Toxicity-2.50mg/ml

Fig. 34. Cytotoxic effect on HepG-2 cell line by fraction of Sea slug *K. ornata*
6.4. DISCUSSION

In recent years, researchers have derived numerous chemicals from different marine sources that appears to be more powerful in the application of killing cancer cells and thus it acts as a active and playing a vital role in discovering the potential new drug for anticancer. Majority of bioactive molecules from the sea has been isolated from marine invertebrates (Blunt et al., 2008). Natural products were found to be excellent source of novel and tangible drugs for chemotherapy (Gueritte and Fahy, 2005; Kingston, 2005). There are few of anticancer drugs has derived from the marine resources. However, there is a significant number of compounds presently being further evaluated in the clinical trials for anticancer drugs such as, aplidine which was derived from Aplidium albicans. Discodermolide derived from the Caribbean deep water sponge Discodermia dissolute (Gunasekera and Wright, 2005), bryostatin derived from the bryozoans Bugula neritina (Newman, 2005), eceinascidin 43 isolated from the tunicate Ecteinascidia turbinata, collected initially in the Caribbean (Henriquez et al., 2005) and finally, dolastatin 10 from nudibranch Dollabella auricularia which collected in Indian ocean (Flahive and Srirangam, 2005). Among the marine invertebrates, especially sea slugs are most prominent sources of new compounds with cytotoxic potential effect. Cytotoxic agents like kahalalide F and ES285 have been isolated from the marine mollusc Elysia rufescens, Spisula, Polynyma respectively. Several species of animals in this class are known for rich chemical diversity which accumulated upon their algae feed.

The cell viability assay of the trypan blue exclusion assay was employed to calculate the cell growth inhibition against human HepG2 cell lines. In this assay, the
percentage of cytotoxicity was gradually increased according to the concentration of both crude and fraction obtained from sea slug. The crude extract of *K. omata* showed 17.28±0.57%, 26.91±0.66%, 42.27±1.04% and 63.51±2.13% of cytotoxicity at 2.5 mg/mL, 5 mg/mL, 7.5 mg/mL and 10 mg/mL respectively and all the concentrations, the maximum cell inhibition in human HepG2 was seen in 10 mg/mL. The maximum cell death was found in particular concentration it may due to the development of defence mechanisms of dietary origin including stinging cells procured from the cnidarians prey of eolid nudibranchs and secondary metabolites from the algal diet of ascoglossans and aplysiids (Faircloth and Cuevas, 2006). Surprisingly, some species are able to retain chloroplast from the algae that remain photo-synthetically active with in the animal for prolonged periods of time. However, early experimental results have provided some insight in to the physiological events that correlate with tumour cell killing by this compound (Green et al., 2003). The primary mechanism of the extracted compound was not been identified experimentally and no studies were revealed the mechanism behind the cell death.

The cell viability was determined by trypan blue dye exclusion assay. In this assay the percentage of cell viability was gradually decreased according to the concentration of both crude and fractions of sea slug. IC$_{50}$ values of sea slug crude and fraction were calculated to be 8.74 mg/mL and 6.29 mg/mL. The observed results strongly suggest that the sea slug extract can be used as anticancer or antitumor agent. Several cytotoxic alkaloids have been isolated from sea slug *Jorunna funebris* such as dimeric isoquinoline alkaloid jorumycin (Angelo et al., 2000) tambjamine group of cytotoxic alkaloids that have been isolated from marine invertebrates including bryozoans (*Bugula dentate*) and nudibranchs such as *Tambja ceutae* (Marianna
carbone et al., 2010). Dietary derived terpenes are also presented in sponge feeding nudibrachs (doridoidea) (Karuso et al., 1987; Avila, 1995).

Keivan zandi et al. (2007) reported the 60 KDa protein from opistobranch molluscs Aplysia dactylomela showed antiproliferative and cytotoxicity against four different cancer cell lines (L929, NB4, K562, HL60). The cytotoxic concentration of isolated anticancer metabolites was 1 to 7μg/ml (Wessels et al., 2000). In our present study 8 to 9 mg/ml of the crude and 6 to 7 mg/ml of fraction showed IC50 concentration against HepG2 Cell lines. In another research opistohbranch molluscs Aplysianin P of Aplysia kurodai, the anticancer effect of that protein was determined at 3 to 25 μg/ml for different cancer cell lines (Yamazaki et al., 1989).

The percentage of NB4 cells in 48 hrs post treatment by 0.5μg/ml of purified 60 kda protein was 86% and 0.5 μg/ml of crude protein produced and the percentage of viable cells was 88% in NB4 cell lines. In the present study the crude extract showed 82.72±0.21%, 73.09±0.30%, 57.73±0.82% and 36.49±0.71% of cell viability and the fractions showed 77.9±0.79%, 62.51±0.75%, 42.14±0.43% and 23.5±0.75% of cell viability at 2.5 mg/mL, 5 mg/mL, 7.5 mg/mL and 10 mg/mL respectively. The cytolytic factor (Aplysianin P) from purple fluid of A. kurodai was tested on MM46 and MH134 tumour cells of the eggs of sea hare contain an antineoplastic factor (aplysianin E) that inhibits tumour development in vivo and lyses tumour cells directly in vitro.

The Muricid mollusc extracts inhibits colon cancer cell proliferation and induces apoptosis. The metabolite 6-Bromoisatin inhibited the proliferation of HT29 cells at IC50 223 μM (0.05 mg/mL) and induced apoptosis without increasing
caspase 3/7 activity (Babak Esmaeeli et al., 2014). In particular, based on the results obtained from sea slug *Kalinga ornata* solvent extracts were displayed high cytotoxicity against HepG2 liver cancer cell lines.
CHAPTER 7. INVITRO ANTIVIRAL ACTIVITY

7.1. Introduction

Herpes simplex virus (HSV) was recognised as an infectious agent from the last quarter of the 19th century, and it was the first human virus to be discovered. It is also one of the most commonly studied human viruses due to their ability to cause a range of infections, remain latent in their host, and reactivate to cause lesions at or near the initial site of infection (Binder, 1977). HSV type 1 and herpes simplex virus type 2 are members of a subfamily of the alpha herpes viruses, with common biological activities, but they are different in many aspects. They can infect and establish latency in the neurons of the sensory ganglia. HSV can infect the central nervous system, causing meningitis and encephalitis. Viral latency is a problem in the management of HSV treatment. Lethal infections have also been reported in immune compromised patients (Whitley, 1990; Morfin and Thouvenot, 2003).

The clinical symptoms of primary HSV-1 infections varies greatly, infection can be asymptomatic, combination of fever, sore throat, ulcerative and vesicular lesions, gingivostomatitis, oedema, localised lymphadenopathy, anorexia, and malaise. The onset of recurrent orolabial lesions is distinguished by pain, burning, tingling, or itching, which lasts for usually 6 hrs before a vesicle begins to form. The total area of involvement is usually localised where there may be three to five vesicles. Within 72 to 96hrs, lesions progress to pustular or ulcerative, crusting stages. Viral infections of the eye are usually caused by HSV-1 (Ostler, 1977). Recurrent infections are common, and parallel that of herpes labialis infection. Infections can also occur in the skin. Once viral DNA enters the nucleus, viral transcription, DNA replication, encapsidation, and egress take place. By remodelling the host cell nucleus, the virus