CHAPTER 7

IN VITRO ANTICANCER ACTIVITY OF THE COMPOUNDS
ISOLATED FROM WALSURA TRIFOLIATA

Introduction

Anticancer activity

Plant derived agents are being used for the treatment of cancer. Several anticancer agents including taxol, vinblastine, vincristine, the camptothecin derivatives, topotecan and irinotecan and etoposide derived from epipodophyllotoxin are in clinical use all over the world. The phytoconstituents present in plants may act as anticancer agents either by inhibiting the cell growth or by killing the cancer cells (Indira et al., 2009; Kirankumar et al., 2013). There is a positive relationship between a diet rich in plant foods and reduced incidence of degenerative diseases. Nutrients are consumed through the food that we eat, and through metabolic processes in the digestive system. These nutrients are absorbed at a cellular level in the body. Poor diets have been linked to the occurrence of chronic diseases, including cardiovascular disease, Type-2 diabetes, cancer, osteoporosis and anaemia (Lytle et al., 2002 and Gibney et al., 2009). Low intake of fruit and vegetables increases the risk for developing cancer (Steinmetz and Potter 1996; Mohammad et al., 2009), as well as cardiovascular disease (Hung et al. 2004), whereas low intake of dietary fibre has been linked to being overweight (Patrick et al., 2004). The decrease in the incidence of diseases is customarily attributed to the antioxidant properties of plant foods that may retard or even prevent some of the processes involved in disease development. Plant-derived natural products such as flavonoids, terpenes, alkaloids, alpha-tocopherol, and carotenoids have received
considerable attention due to their diverse pharmacological properties including cytotoxic and chemopreventive effects (Kviecinski et al., 2008).

Hepatocellular carcinoma is one of the most common malignancies worldwide, accounting for nearly 6,00,000 deaths each year. Though there has been several curative methods for the disease, the survival solely depends on the tumour location and the underlying liver disease, cirrhosis (Sharmila and Padma, 2013). Despite surgical management and the use of non surgical therapeutic modalities, the incidence of hepatocellular carcinoma is still on rise (Potduang et al., 2010). The burden of hepatocellular carcinoma has been increasing in Egypt with a doubling of the incidence rate in the past 10 years. According to World Health organization Egypt has one of the highest incidences of hepatitis C, one of the main causes of liver cancer in the world. Factors such as cigarette smoking, occupational exposure to chemicals such as pesticides and endemic infections in the community as schistosomiasis have additional role in the etiology or progression of the disease (Fathya et al., 2013).

Because of the high demand for cancer drugs, the need to less side effects products from natural sources and the constraints of present production technologies for pharmaceuticals, attention has recently been focused on investigation and screening of pharmaceutical anticancer compounds in plants. Indeed, plants have a long history of use in the treatment of cancer. However, the undeniable role of diet containing plants should not be disregarded in the prevention of many diseases such as cancer (Mohammadi-Motlagh et al., 2011).

**Apoptosis**

Apoptosis is the primary goal of chemotherapy. Apoptosis is the cell mechanism that balances between cancer cell proliferation and damage irreparable
cell in DNA damage. Then, dead cells are phagocytosed by macrophages. The advantage of this death mode does not lead to inflammation in neighboring cells as same as necrosis (Brunelle and Zhang, 2010).

**Materials and Methods**

1. MTT (3-[4,5-dimethylthiazol-2-yl] 2,5-diphenyl tetrazolium bromide): 0.5 mg MTT/ml of serum-free DMEM medium.

2. Solubilizing solution: Dimethyl sulfoxide

3. Phosphate buffered saline (PBS) (pH 7.4): As described under cell culture reagents.

**Test Compounds**

Compounds 3,4-trihydroxybenzoic acid and β-sitosterol isolated from *Walsura trifoliata* root were tested for cytotoxicity activities.

**Drug preparation**

The compound was dissolved in different concentrations (10 to 250 µg/ml) in 10% Dimethyl Sulfoxide (DMSO) to give a final concentration of DMSO not more than 0.5% and did not affect cell survival.

**Cell viability test**

The viability of cells was assessed by MTT assay (Mosmann, 1983) using HepG2 cell lines and chang liver cell lines (National Centre for Cell Science, Pune).
Principle

The assay is based on the reduction of soluble yellow tetrazolium salt to insoluble purple formazan crystals by metabolically active cells. Only live cells are able to take up the tetrazolium salt. The enzyme (succinate dehydrogenase) present in the mitochondria of the live cells is able to convert internalized tetrazolium salt to formazan crystals, which are purple in colour. Then the cells are lysed and dissolved in DMSO solution. The colour developed is then determined in an ELISA reader at 570 nm.

Cytotoxicity Studies

The HepG2 cells and chang liver cells were plated separately in 96 well plates at a concentration of $1 \times 10^5$ cells/well. After 24 h, cells were washed twice with 100 µl of serum-free medium and starved for an hour at 37 °C. After starvation, cells were treated with different concentrations of test compound (25-500 µg/ml) and incubated for 24 hours. At the end of the treatment period the medium was aspirated and serum free medium containing MTT (0.5 mg/ml) was added and incubated for 4 h at 37 °C in a CO$_2$ incubator. The 50% inhibitory concentration value (IC$_{50}$) of the compound was identified for normal fibroblast cell line.

The MTT containing medium was then discarded and the cells were washed with PBS (200 µl). The crystals were then dissolved by adding 100 µl of DMSO and this was mixed properly by pipetting up and down. Spectrophotometrical absorbance of the purple blue formazan dye was measured in a microplate reader at 570 nm (Biorad 680). Cytotoxicity was determined using Graph pad prism5 software.
Results

Cancer specific cytotoxic effects of the compounds isolated from *Walsura trofoliata* were studied using HepG2 cells (Human hepatocellular carcinoma cells). The degree of toxicity of the compounds were determined using MTT assay. The assay is a colorimetric based on the ability of the viable cells to reduce a soluble yellow tetrazolium salt (MTT) to blue formazan crystals. The cytotoxic activities were expressed as percentage of cell viability in normal cells and HepG2 cell lines and were compared with Cyclophosphamide. When the cells were treated with the drug Cyclophosphamide the cytotoxic effect of normal cells showed 14.33% of cell viability and also 21.33% in normal cells. β-sitosterol at 10µg/ml concentration showed 78% in HepG2 and 94.33% cell viability in normal cells. β-sitosterol at 250µg/ml showed 39.66% viability in HepG2 and 78% in normal cells (Fig 7.1 and 7.3). 3,4,-5 trihydroxybenzoic acid when treated with 10 µg/ml of the compound 15.33% cell viability was found in HepG2 cells and 18.33% in normal cells. 250 µg/ml of the compound showed 32.33% of cell viability in HepG2 and 59% in normal cells (Fig7.2 and 7.4). As the concentration of the compound increased the cytotoxic effect was also increased so that the percentage of cell viability decreased. When the drug is compared with the compound, cytotoxic effect shows that the drug affected the normal cells more than the cells treated with the compound.
Fig 7.1 Cytotoxicity of different concentrations (10 -250 μg/ml) of β-sitosterol isolated from the extracts of *Walsura trifoliata* root in Chang liver and HepG-2 cells. Values represent the mean ± SEM of triplicate independent experiments. Cyclophosphamide was used as positive control.

Fig 7.2 Cytotoxicity of different concentrations (10-250 μg/ml) of 3, 4-5 trihydroxy benzoic acid isolated from the extracts of *Walsura trifoliata* root in Chang liver and HepG-2 cells. Values represent the mean ± SEM of triplicate independent experiments. Cyclophosphamide was used as positive control.
Fig 7.3. Effect of β-sitosterol on HepG2 cell lines (10 µg/ml to 250 µg/ml)
Fig 7.4. Effect of 3,4,5-trihydroxybenzoic acid on HepG2 cell lines (10 µg/ml to 250 µg/ml)
Discussion

MTT assay helps in quantifying metabolically viable cells through their ability to reduce a soluble yellow tetrazolium salt to blue-purple formazan crystals (Mossman, 1983). By using 96-well microtiter plates and a multi-well spectrophotometer (enzyme-linked immunosorbent assay plate reader), the assay can be semi automated in order to process a large number of samples and provide a rapid, objective measurement of cell number. There is evidence that most anti-cancer agents exert their cytotoxic effects by inducing apoptosis in tumor cells (Kaufman, 1989). Impairments in apoptosis are related to cell immortality, carcinogenesis, and the induction of apoptosis in neoplastic cells (Shinomiya et al., 1994); therefore, induction of apoptosis is vital in cancer treatment. Several chemotherapeutic drugs have been shown to induce apoptosis in vitro, including etoposide, camptothecin, VM26, vincristine, cis-platinum, cyclophosphamide, paclitaxel, 5-fluorouracil, and doxorubicin (Huschtscha et al., 1996). Therefore the potential to induce apoptosis has become an important topic in the study of anticancer drugs. The chloroform extract of Ziziphus jujuba fruit has been reported to cause apoptosis induction effect against hepatocellular carcinoma cells (HepG2) (Huang et al., 2007). Activity against the hepatocellular carcinoma cell HepG2 varied markedly with mean IC$_{50}$ values ranging from 9.67 to 115.47 $\mu$g/ml. The only promising extract was from Zingiber officinale (IC$_{50} = 9.67 \pm 3.91$ $\mu$g/ml) (Mahavorasirikul et al., 2010). The results of Ulmus davidiana planc barks strongly suggest that a 70 % ethanol extract exhibited significant cytotoxicity toward HepG2 liver cancer cells (Guo and Wang, 2009). The exposure of HepG2 cells to lower concentrations of blueberry phenolic extract (6.25-100 mg/ml) for 96 h induced an increase in cell proliferation with a significant peak at 25 $\mu$g/ml, with lower proliferation (comparable to the level of control) at concentrations up to 100 mg/ml (Shafiee-Kermani et al., 2013). The MTT assay for various concentrations of
Asparagus saponins (10⁻¹ mg/ml to 10³ mg/ml) at 72 hours showed a concentration-dependent inhibitory effect on the proliferation of HepG2 cells (Ji et al., 2012). Cha et al., (2008) isolated β-sitosterol from Cyrtandra cupulata which induced apoptosis in MCF-7 cells. Phytosterols have been recognized as cancer preventative biological-active substances together with other secondary plant products such as carotenoids, flavonoids and phytoestrogens (Careri et al., 2001). Methanolic extract of Grewia hirsuta possessed significant antioxidant and anti-proliferative potential when tested against HepG2 cell lines (Ema et al., 2013). Methanolic extract of the leaves of Artemisia vulgaris possessed cytotoxicity and apoptotic properties against HepG2 cells in a dose ranging from 0.01 mg ml⁻¹ to 1 mg ml⁻¹ (Sharmila and Padma, 2013). As per the earlier reports the methanol extract of plants showed significant cytotoxicity. In the present the compounds isolated from the methanol root extract showed significant cytotoxicity upto 39.66% and 32.33% cell viability in 250 µg/ml of β-sitosterol and 3,4,-5 trihydroxybenzoic acid. From this study it was understood that the drug cyclophosphamide was more toxic even to the normal cells when compared to the compounds isolated from plants.