Chapter-5

Determination of in vitro cytotoxicity
Introduction

Around the world, tremendous resources are being invested in prevention, diagnosis, and treatment of cancer. Cancer is a disease in which there is uncontrolled multiplication and spread within the body of abnormal forms of the body’s own cell, is the second leading cause of more than six million deaths each year in the world. Cancer is a worldwide fearful problem causing huge number of mortality in every year (Rang et al., 2007). Discovery and development of anticancer agents are the key focus of several pharmaceutical companies as well as nonprofit government and non-government organizations, like the National Cancer Institute (NCI) in the United States, the European Organization for Research and Treatment of Cancer (EORTC) and the British Cancer Research Campaign (CRC).

Cytotoxicity screening models provide important preliminary data to help select plant extracts/compounds with potential anticancer properties for future work (Cardellina et al., 1999). Identification of cytotoxic compounds led the development of anticancer therapeutics for several decades. The recent growth in molecular sciences and the advances in genomics and proteomics have generated several potential new drug targets, leading to changes in the paradigms of anticancer drug discovery toward molecularly targeted therapeutics. These shifting paradigms have not only resulted in the greater involvement of biological scientists in the drug discovery process but also required changes in the screening and clinical evaluation of drug candidates. Both small and large molecular compounds continue to be investigated as anticancer agents (Schwartzmann et al., 1988). However, many chemotherapeutic drugs are presently placed in a predicament of reduced therapeutic effect due to the problem of drug resistance (Peters et al., 2002). Chemotherapeutic drugs also exert toxicity to normal cells, which in turn causes the unpleasant side effects to the patients. For these reasons, research and development of new classes of anticancer agents which exhibit efficient and selective toxicity in tumour cells is enticing increased attention.

Nature has given indigenous gift to the earth, one of which is plants. Plants have been a source of medicine for thousands of years and phytochemicals continue to play an essential role in medicine (Aggarwal et al., 2003). In recent years, the use of medicinal plant extracts for the treatment of human diseases is an ancient practice has greatly increased. Natural compounds have provided many effective anticancer agents in current use. According to an estimate, 50% of breast cancer and 37% of prostate cancer patients use herbal products (Sini et al., 2012). More
than 60% of currently used anticancer agents are derived in one way or another from natural sources. To date, approximately 100 species of plants have been examined and some active constituents isolated and identified, for instance several of the current chemotherapeutic drugs like vinblastine, vincristin, methotrexate, taxol, topotecan, taxane and so forth, were first identified in plants (Kamkaen et al., 2006).

There are two main strategies for the selection of plants species in anticancer drug discovery: random screening and ethnomedical knowledge. The second approach includes plants used in organize traditional medical systems like herbalism and folklore. The utility of cell lines acquired from tumors allows the investigation of tumor cells in a simplified and controlled environment; uncontrolled proliferation is a universal property of tumor cells. In the cancer drug discovery program, a paradigm based on ethnobotanical and ethnopharmacological data would be more economical and beneficial for identifying potential anticancer molecules than mass screening of plant species (Merghoub et al., 2009). Investigation of the cellular growth control mechanisms has contributed to the understanding of carcinogenesis and identification of compounds with specific antitumor activity. Thus, cytotoxicity screening models provide important preliminary data to help select plant extracts or compound with potential antitumor property for future studies.

**Measuring cytotoxicity**

Cell viability and cytotoxicity assays are used for drug screening and cytotoxicity tests of chemicals. Various reagents used for cell viability detection and they are based on various cell functions such as enzyme activity, cell membrane permeability, cell adherence, ATP production, co-enzyme production and nucleotide uptake activity. Many have established methods such as Colony Formation method, Crystal Violet method, Tritium-Labeled Thymidine uptake method, Tetrazolium cellular viability assay (MTT) and water soluble tetrazolium salts (WST) methods, which are used for counting the number of live cells.

Trypan blue is a widely used assay for staining dead cells. In this method, cell viability must be determined by counting the unstained cells with a microscope or other instruments. However, Trypan Blue staining cannot be used to distinguish between the healthy cells and the cells that are alive but losing cell functions. In the Colony Formation method, the numbers of cell colonies are counted using a microscope as a cell viability indicator. In the Tritium-Labeled Thymidine uptake method, $[^3\text{H}]$-thymidine is involved in the cell nucleus due to the cell growth,
and the amount of the tritium in the nucleus is then measured using a scintillation counter. Though the Tritium labeled thymidine uptake assay is sensitive to determine the influence on the DNA polymerization activity, it requires radioisotope which causes various concerns.

Enzyme-based methods using MTT and WST rely on a reductive coloring reagent and dehydrogenase in a viable cell to determine cell viability with a colorimetric method. This method is far superior to the previously mentioned methods because it is easy to use, safe, has a high reproducibility and is widely used in both cell viability and cytotoxicity tests. Among the enzyme based assays, the MTT assay is the best known method for determining mitochondrial dehydrogenase activities in the living cells (Fotakis and Timbrell, 2006).

**Material and methods**

Cell viability and cytotoxicity assays are widely used in cell biology for the determination of growth expression factors, cytokines and nutrients, as well as for the screening of cytotoxic or chemotherapeutic agents. In this study, trypan blue exclusion and MTT assay was used to determine the cytotoxicity of the methanol extract of *Anthocephalus cadamba* (MEAC) and β-sitosterol glucoside (BSSG).

**Cell lines and culture conditions**

Ehrlich ascites carcinoma (EAC) and Dalton’s lymphoma ascites (DLA) cells were obtained from Chittaranjan National Cancer Institute, Kolkata, India. Ascitic fluid was drawn out from EAC tumor bearing mouse at the log phase (7-8 days of tumor bearing) of the tumor cells. The EAC cells were maintained in vivo in Swiss albino mice by intraperitoneal (i.p.) transplantation of $2 \times 10^6$ cells per mouse after every 10 days and it is used for present study.

Human cancer cell lines namely colon carcinoma (HCT-116), cervical carcinoma (HeLa), breast carcinoma (MCF-7) and hepatocellular carcinoma (HepG2) were obtained from National centre for cell sciences (NCCS), Pune, India. Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% Fetal Bovine Serum, (100 U) 20μg/ml penicillin and 100 μg/ml streptomycin. Incubation was carried out at 37 °C with an atmosphere of 5% CO$_2$.

**Trypan blue exclusion assay**

**Principle**

Trypan blue is diazo dye used to selectively colour dead tissues or cells blue. Live cells or tissues with intact cell membranes are not colored. Since cells are very selective in the
compound that pass through the membrane, in a viable cell trypan blue is not absorbed; however, it traverses the membrane in a dead cell. Hence, dead cells are shown as a distinctive blue colour under a microscope. Since live cells are excluded from staining, this staining method is also described as a dye exclusion method. Cell counting using trypan blue dye can provide both the rate of proliferation as well as the percentage of viable cells.

**Procedure**

Trypan blue exclusion assay of MEAC and BSSG was performed by using EAC and DLA cells. Briefly, $1 \times 10^6$ EAC cells were suspended in 0.1 ml of phosphate buffered saline (PBS, 0.2 M, pH 7.4) and mixed with 100 µl of various concentrations of MEAC (25-300 µg/ml) and BSSG (10-125 µg/ml). Final volume was adjusted to 1 ml with PBS and was incubated at 37 °C for 3 h. After the completion of incubation, the viability of the cells was determined using trypan blue (0.4% in normal saline) and the percentage of cytotoxicity was determined by calculating % inhibition and IC$_{50}$ values (Manojkumar et al., 2009).

**MTT cellular viability assay**

**Principle**

The MTT {3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyl-2H-tetrazolium bromide} is a tetrazolium salt dye used in a colorimetric assay, which measures the mitochondrial conversion or modification of the yellow substrate to an insoluble dark blue/purple formazan product. Substrate modification is brought about by the cleavage of MTT by NADH-generating succinic dehydrogenase present in the mitochondria of living cells, with only living cells containing active mitochondria are able to yield a colour change. As an increase in mitochondrial enzyme activity leads to a linear increase in the production of formazan dye, the measured quantity of
formed formazan dye is directly correlated to the number of metabolically active cells, yielding an accurate measurement of cell viability and thus toxicity (if any). As the formazan dye is insoluble in the reaction medium, it is solubilised by the addition of DMSO or isopropanol and the colour intensity is measured spectrophotometrically.

**Procedure**

Cell viability was determined by using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) on EAC, DLA, HCT-116, HeLa, MCF-7 and HepG2 carcinoma. Briefly, 0.1 ml of cell suspension was seeded in 96-well plates (Greiner, Frickenhausen, Germany) with a seeding density of 1×10⁵ cells/well. The cells were treated with different concentrations of MEAC (25-800 μg/ml) and BSSG (20-300 μg/ml) and incubated for 24 h at 37°C, 5% CO₂ with 98% relative humidity. After incubation, 20 μl of MTT (5 mg/ml) in phosphate buffered saline (PBS) were added to each well and the plates were further incubated for 4 h at 37°C. The colored formazan crystals which were produced from MTT were dissolved in 150 μL of dimethyl sulfoxide (DMSO) and the absorbance was measured at a wavelength of 570 nm by ELISA plate reader (Bhattacharya et al., 2011).

The percentage of cell inhibition was determined using following formula.

\[
\text{% Growth inhibition} = 100 - \left( \frac{\text{Mean OD of individual test group}}{\text{Mean OD of control group}} \times 100 \right)
\]

The inhibitory concentration required for 50% cytotoxicity (IC₅₀) value was analyzed by using GraphPad Prism software.
Results

Trypan blue exclusion assay

In trypan blue cytotoxicity study, MEAC and BSSG showed direct cytotoxic effect on the EAC and DLA cells in a concentration dependent manner. The IC\textsubscript{50} values of MEAC on EAC and DLA were found to be 99.45 ± 6.94 and 120.15 ± 5.03 µg/ml respectively (Figure 5.1A). Similarly the IC\textsubscript{50} values of BSSG on EAC and DLA were found to be 46.50 ± 4.24 and 62.44 ± 6.75 µg/ml respectively (Figure 5.1B).

![Figure 5.1](image)

**Figure 5.1:** Cytotoxic effect of (A) methanol extract of *Anthocephalus cadamba* (MEAC) and (B) β-sitosterol glucoside (BSSG) on Ehrlich ascites carcinoma (EAC) and Dalton lymphoma ascites (DLA) cells using trypan blue exclusion method. Values are represented as mean ± SEM of triplicate determinations.

MTT cellular viability assay

Cell viability of MEAC and BSSG was performed on EAC and DLA cells. MEAC and BSSG were able to reduce viability of the cells in a dose-dependent manner. The IC\textsubscript{50} values of MEAC were found to be 90.45 ± 3.94 and 118.68 ± 6.19 µg/ml on EAC and DLA cells (Figure 5.2A). BSSG have better cell viable property than MEAC extract and the IC\textsubscript{50} values of BSSG were found to be 75.45 ± 2.90 and 85.92 ± 4.12 µg/ml respectively (Figure 5.2B).

Cell viability assay of MEAC and BSSG was performed on colon carcinoma (HCT-116), cervical carcinoma (HeLa), breast carcinoma (MCF-7) and hepatocellular carcinoma (HepG2) cell lines. MEAC and BSSG were able to reduce viability of the cells in a dose-dependent manner. The IC\textsubscript{50} values of MEAC were found to be 76.90 ± 3.90, 106.00 ± 4.16, 127.31 ± 7.22 and 191.08 ± 6.75 respectively against all said human cancer cell line (Figure 5.3A). Similarly
the IC$_{50}$ values of BSSG were found to be 51.90 ± 2.90, 67.50 ± 3.06, 78.29 ± 5.63 and 97.28 ± 8.75 respectively (Figure 5.3B).

![Graph A](image.png)

**Figure 5.2:** Cytotoxic effect of (A) methanol extract of *Anthocephalus cadamba* (MEAC) and (B) β-sitosterol glucoside (BSSG) on Ehrlich ascites carcinoma (EAC) and Dalton ascites lymphoma (DLA) cells using MTT assay method. Values are represented as mean ± SEM of triplicate determinations.

![Graph B](image.png)

**Figure 5.3:** Cytotoxic effect of (A) methanol extract of *Anthocephalus cadamba* (MEAC) and (B) β-sitosterol glucoside (BSSG) on colon carcinoma (HCT-116), cervical carcinoma (HeLa), breast carcinoma (MCF-7) and hepatocellular carcinoma (HepG2) cell lines using MTT assay method. Values are represented as mean ± SD of triplicate determinations.
Discussion

Interest in the pharmacological effects of bioactive compounds on cancer treatments and prevention has increased dramatically over the past twenty years. It has been shown to possess numerous anti-cancer activities in various cancer cells through different forms of cytotoxic effects without exhibiting considerable damage to normal cells (Katiyar et al., 2009; Mantena et al., 2006).

Discovery and development of new anticancer drug that has good efficacy and none of the side effects of present chemotherapeutic drugs can be cumbersome and expensive. Need for time saving, low cost, high throughput drug efficacy testing system has given rise to in vitro cytotoxicity testing model on human cancer cell lines. It is well established that plants have been a useful source of clinically important antitumor compounds and still there have been worldwide efforts to discover new anticancer agents from plants (Kong et al., 2012). On a whole, my goal was to determine whether the extracts of these plants exerted an inhibitory effect on cancer cell proliferation and caused cell death. The results of the present studies suggest that MEAC and BSSG possess the cytotoxic effects on both the animal cancer cell and human cancer cell in a dose dependent manner. The initial screening of plant for its anticancer properties uses cell-based assays and established cell lines, in which the cytotoxic effects of plants extracts or isolated compounds could be measured. To be a good drug candidate, the IC50 value of such agent should be sufficiently low to avoid any possible unspecific effects (Suffness and Pezzuto, 1990). It is important to correct the misguided belief that herbal medicines do not cause adverse effects and even though there may be no evidence of cytotoxicity in vitro, the possibility of in vivo cytotoxicity cannot be excluded. Hence an advanced cancer modeling is necessary to exploit the potentiality of the plant against the cancer.

Conclusion

The present investigations thus suggest that the methanol extract of Anthocephalus cadamba (MEAC) and β-sitosterol glucoside (BSSG) have cytotoxic activity to cancer cells which was worth considering for further exploitation. Cancer cell death appears to be due to induction of apoptosis, perhaps through mitochondrial pathway. This should be explored further on panel of different cancer cell lines.
References:


