Chapter 6

Invitro, Invivo & Insilico Anticancer Studies
6. INVITRO, INVIVO AND INSILICO ANTICANCER STUDIES

INTRODUCTION

A health condition in which, loss of control on cell cycle resulting in the group of diseases is called as cancer. The disease cancer is connected with abnormal and uncontrolled growth of cells (Krishnamurthy, 2007). External as well as internal factors are responsible for the cause of cancer. External factors include tobacco, chemicals, radiation and infectious organisms and internal factors include inherited mutations, hormones, immune conditions, and mutations that occur from metabolism.

Today fighting against cancer has become one of the biggest challenges to mankind. Due to lack of extensive and ample early detection methods, the associated deprived analysis of patients diagnosed in later stages of the disease and its increasing incidence on a global dimension cancer has posed a significant worldwide health (Divisi et al., 2006; Chanda and Nagani, 2013).

Natural products are having a long history for their therapeutic use against cancer, started with folk medicine. From that period to present day it has been incorporated into traditional and allopathic medicine. Several drugs isolated from plant species or derived from their models and several drugs are currently used in chemotherapy. Which include the vinca alkaloids, vinblastine and vincristine, isolated from Catharanthus roseus, etoposide and teniposide, the semisynthetic derivatives of Epipodophyllotoxin, isolated from species of the genus Podophyllum, the naturally derived taxanes isolated from species of the genus Taxus, the semisynthetic derivatives of camptothecin, irinotecan and topotecan, isolated from Camptotheca acuminata, and many other drugs (Wang, 2006).

More than 50% of the drugs which are in clinical trials are being used for treating the cancer or to study the anticancer activity of isolated compound from
natural sources or other effects related to them. From animal and human epidemiological studies it is observed that by altering dietary habits or dietary supplements cancer risk may be modified. Experimental studies indicate that the inhibition of tumour initiation, promotion and progression can be achieved by using phytochemicals with anti-oxidative and anti-inflammatory properties. In prevention and treatment of cancer it is necessary and worthwhile to validate the traditional medicine scientifically for its possible (Priyanka et al., 2009).

Phytochemicals prevent the tumour promotion by the mechanisms like inhibition of genotoxicity effects, increased antioxidants and anti-inflammatory activities, proteases inhibition and cell proliferation, protection of intracellular communications to modulate apoptosis and signal transduction pathways (Soobrette et al., 2006). Based on the ability to modulate one or more specific molecular events several new chemo preventive agents are being identified.

Development of an alternative and complementary method for cancer prevention and treatment can be achieved by discovering effective herbs and elucidating their underlying mechanisms. Our Indian sub-continent has enormous botanical diversity and extensive practice of traditional medicine known as ayurvedic medicine; Though, there is relatively small number of these plants have been used to establish a scientific evaluation for their potential anticancer properties (Krishnaswamy, 2006).

The well-known biological effects of dietary flavonoids include anti-inflammatory, anti-allergic, antimicrobial, hepatoprotective, antiviral, antithrombotic, cardioprotective, capillary strengthening, antidiabetic, anticarcinogenic and antineoplastic effects (Harborne and Williams, 2000; Kappagoda et al., 2000). These dietary antioxidants exert significant immunomodulatory activities and show a
tendency to influence a host of cellular inflammatory processes and immune functions significantly, and cell surface signal transduction, which is in disease states shown as allergic reactions, inflammatory disorders, viral infection, tumor development and vascular dysfunction etc.

Asians have much lower risk of colon, prostate and breast cancers as compared to the populations of west. People of Asia will generally consume more vegetables, fruits and tea. This factor raises the interesting, important and timely question of whether flavonoid components mediate the protective effects of diets rich in these foodstuffs by acting as natural chemo preventive and chemotherapeutic agents. Some phytocompounds, such as isoflavonoids, flavonoids and lignans, have grabbed particular attention as supposed to cancer protective agents in populations with low occurrences of breast and prostate cancer (Adlercreutz et al., 1991). Few of the varied pharmacological and cellular effects have shown by the flavonoids could provide inferences for these activities but the mechanisms underlying in this potential chemo preventive and chemotherapeutic actions of dietary flavonoids need to be evaluated.

Flavonoids from plant origin have the tendency to modify the activities of host of enzyme systems which are crucially involved in cell surface signal transduction, immune function, cellular transformation, tumour growth and metastasis. (Griffiths et al., 1998; Kolibaba and Druker, 1996). Especially the cell protein kinase modulating activities of flavonoids might have the major implications for protecting against cancer. A wide range of cellular effects are shown by flavonoids, several mechanisms might be responsible for their potential anticancer properties. Many food flavonoids not only inhibit the growth of tumour cells, but also capable of inducing induce cell differentiation (Constantinou et al., 1990).
Several reports indicated that there are number of dietary flavonoids which demonstrate potent antitumor activity \textit{in vivo} studies (Edward et al., 1979; Molnar et al., 1981). The ability of flavonoids to inhibit the growth of malignant cells could be a effect of their intervention with the protein kinases activities which are engaged in the regulation of cellular proliferation and apoptosis (Huang et al., 1999; Akiyama et al., 1987). In addition, the modulating cause of flavonoids on the cell cycle and apoptosis are conceivably some of the factors that can mediate their antiproliferative activity (Sato et al., 1996). Limited epidemiological data is being exist on flavonoids and cancers, and we are yet to fully understand the relation between dietary flavonoids and the prevalence of cancers. However, flavonoid-rich foods exhibit some protective characteristics. (Kandaswami et al., 2000).

**Myricetin**

Myricetin is a naturally occurring flavonol with hydroxyl substitutions at the 3, 5, 7, 3', 4' and 5' positions. Its presence in nature is extensive in plants including tea, berries, fruits, vegetables and medicinal herbs (Hertog et al., 1993). Myricetin's present in berries, vegetables and fruits is in the form of glycosides rather than free aglycones and flavonoid content in ripe berries is more than the unripe berries. Myricetin is commonly consumed in our diet through vegetables, fruits and beverages such as tea and wine (Hertog et al., 1993). Since many years, a number of studies have been conducted to investigate its varied therapeutic potential, which includes its use as a potent antioxidant (Londonkar and Kirankumar, 2014), as an anti-carcinogenic agent and in the prevention of platelet aggregation.

**Prostate cancer**

Prostate cancer has the highest prevalence of any non skin cancer in the human body and it is the second most leading cause of cancer related death in men.
(Jemal et al., 2004; Bostwick et al., 2004) Due to aging population and availability of advanced diagnostics the frequency of clinically significant prostate cancers has been increased in the last 50 years. 30% men in their 50s and 70% men over the age of 80 years contain microscopic foci of cancer (‘latent’ cancer). All men with circulating androgens will develop microscopic latent cancer essentially, if they live long enough, but only about 10% men effect by progression of androgens to clinically significant cancer. Prostate cancer causes death about 3%. Throughout the world the incidence of ‘latent’ prostate cancer is similar in all populations. Occurrence of prostate cancer is independent of androgen levels, 5 alpha-reductase activity, diet, environmental factors and ethnicity (Breslow et al., 1977; Gould and Kirby 2006).

**Prostate cancer and testosterone**

Several studies and lot of attention have been offered to the study of the possible relationship between prostate cancer and testosterone levels, but the real physio pathological interaction between prostate cancer and testosterone remains mostly unclear. Huggins and Hodges first noted the sensitivity of prostate cancer to testosterone, and it is now well established, and the use of testosterone ablation or blockade for suppression of disseminated disease is widely accepted. The disease flare that can follow administration of luteinising hormone-releasing hormone agonists, which temporarily stimulate androgen production unless an anti androgen is given, is further is an evidence that advanced prostate cancer is stimulated by androgens. However, less is known about the ability of exogenous testosterone for androgen deficiency to stimulate prostate cancer or increase the risk of prostate cancer development (Vincenzo Mirone, 2007).
MATERIALS AND METHODS

Invitro studies

Cell culture

Human prostate cancer (PC-3) cell lines were cultured in DMEM (2mM L-glutamine, 100 g/ml of streptomycin, 100 U/ml of penicillin) medium supplemented with 10% bovine serum and maintained in a 5% CO₂ humidified incubator at 37°C. Cells were seeded at a density of $1 \times 10^5$ cells/ml.

Bioactive compound

Fruits are subjected to soxhlet extraction and methanol extract was centrifuged at 10,000 by rpm for 15minutes; supernatant was used for the purification and structural elucidation as mentioned in previous chapter. Isolated flavonol dihydroxy myricetin (DHM) was used for the experiment in the present study.

Cytotoxicity assay

**MTT (3-(4,5-Dimethyl thiazol-2-yl)-2,5- di phenyl tetra zolium bromide) assay**

(3-(4,5-Dimethyl thiazol-2-yl)-2,5- di phenyl tetra zolium bromide assay was performed to assess the cytotoxicity of the isolated flavonol. PC-3 cells were cultured in 96-well microtiter plates and are treated with varying concentrations of the compound (50µM and 100µM) for 48 hrs. At the end of treatment period, to each well, 20 µl of (3-(4,5-Dimethyl thiazol-2-yl)-2,5- di phenyl tetra zolium bromide was added. After addition of (3-(4,5-Dimethyl thiazol-2-yl)-2,5- di phenyl tetra zolium bromide, the plates are incubated for 3 h in a dark chamber. Then, 100 µl of DMSO was added to dissolve the formazan crystals. The absorbance was read at 570 nm using micro plate reader.

The percentage of cytotoxicity compared to the untreated cells was determined by the following formula:
OD of treated cell

Cell viability (%) = \( \frac{\text{OD of treated cell}}{\text{OD of control cell}} \times 100 \)

The results are obtained from six individual experiments and each experiment was carried out in 3 sets. The IC\text{50} value was calculated.

**Colony formation assay**

Anchorage-independent colony formation assay was performed on soft agar medium. Cells at the initial density of \( 1 \times 10^5 \) in 2 ml medium was seeded in 6-well plates containing 2 ml of 0.5% agar in medium as the bottom layer, 1 ml of 0.38% agar in medium. The cultures are incubated with 5% and 10% DHM for 7 days. Cultures are maintained at 37\degree C in a humidified 5% CO\text{2} atmosphere. The number of colonies are determined by counting them under an inverted phase-contrast microscope at 400X magnification and a group of ~50 cells were counted as a colony.

**Cell cycle analysis**

Cell cycle analysis assay was done by the flow cytometry method. Cells were washed with ice-cold PBS, detached with 0.25% Trypsin, and fixed with 70% ethanol overnight at 4\degree C. Fixed cells are subsequently washed, treated with 5 µg/mL RNase A (Sigma, USA), and treated with 50 µg/ml, 100 µg/ml and standard drug taxol further stained with 50 µg/mL propidium iodide (Sigma). Stained cells are assayed in a fluorescence-activated cell sorter (Becton Dickinson, USA) and the data are analyzed using the Cell FIT software. A minimum of 10,000 cells were analyzed in each sample.
Invivo studies

Chemicals

Testosterone marketed by Cadilla Pvt. Ltd, was purchased from local pharmacy Kalburgi, DMSO and other chemicals were purchased from Sigma Aldrich Pvt. Ltd, Mumbai.

Dihydroxy myricetin

Purified compound dihydroxy myricetin was obtained from ripe fruits of Ficus glomerata and used for the experimental purpose.

Experimental animals

Healthy young adult male Albino rats of inbred colony weighing about 120 – 135g were used for the study. The protocol followed in this experiment was approved by the Institute’s Animal Ethical Committee (IAEC Reg No. 34800/ CPCSEA Dated: 19.08.2001). Animals were kept in animal house at temperature of 25°C with 45 – 55% humidity, at 12 hour each of dark and light cycles. They were fed with a balanced diet as described by Central Food and Technological Research Institute (CFTRI, Mysore) and water adlibitum. The animals are divided into five groups consisting minimum six animals in each group. Experiment was conducted as per Organization for Economic Cooperation and Development (OECD) guidelines 423 (OECD, 2011). Control group received the vehicle (DMSO) while the experimental groups received doses (1–4 mg/kg) of test compound dihydroxy myricetin orally and were observed for mortality till 48 h and the LD₅₀ was calculated.

Experimental procedure

Group I: Rats of this group received 0.5ml of DMSO/ for 21 days through intraperitoneal mode and maintained as normal control group.
**Group II:** Rats of this group received a single dose of testosterone (1 ml/kg of body weight). The testosterone was dissolved in DMSO and administered through intraperitoneal mode for seven days and maintained as prostate cancer control group.

**Group III:** This group of rats received a single dose of testosterone from day 1 to 7\textsuperscript{th} and 1 mg/kg body weight of isolated myricetin from 8\textsuperscript{th} day to 21\textsuperscript{st} day.

**Group IV:** This group of rats received a single dose of testosterone from day 1 to 7\textsuperscript{th} and 2 mg/kg body weight of isolated myricetin from 8\textsuperscript{th} day to 21\textsuperscript{st} day.

**Group V:** Rats of this group received standard myricetin 0.5 mg/kg/dose of body weight for 7 days after testosterone pre treatment and maintained as standard group.

After 21 days of experimental procedure the animals are fasted over night and sacrificed. Blood was collected in a test tube and plasma was separated by centrifugation at 3000 rpm for 20 min. Prostate tissue was immediately excised from the animals and weighed, 10\% homogenate was prepared in 0.1M, Tris HCl buffer (pH 7.4) using a homogenizer.

The various biochemical activities of Serum glutamate oxaloacetate transaminase (SGOT) and Serum glutamate pyruvate transaminase (SGPT) alkaline phosphatase (ALP) and Superoxide dismutase(SOD), was determined according to the protocol described by Londonkar et al., (2014).
RESULTS

**Invitro anticancer studies**

**MTT Assay :** (Graph 6.1)

Isolated compound dihydroxy myricetin has shown considerable cytotoxic effects against prostate cancer cell lines. When the isolated compound dihydroxy myricetin was added at different concentrations (50 μg/ml and 100 μg/ml) to cultured PC-3 cells, and incubated for 48 hrs, it was observed that the compound was cytotoxic to the prostate cancer cell line yielding 27.39% of inhibition at 50 μg/ml and at 100 μg/ml the inhibition is 46.81% which is comparable with the standard drug vincristine having 58.89% of inhibition. Normal control group treated with dimethyl sulphoxide has shown 97.03% viability.

**Colony formation Assay: (Table 6.1)**

Results of the anchorage dependent colony formation study on soft agar medium has showed that the myricetin has a capacity to inhibit the colony formation ability of PC-3 cells. In dimethyl sulphoxide treated control group 44 colonies per field were observed. Dihydroxy myricetin at the concentration of 50 μg/ml has reduced the colony formation to almost half as that of control group 22 colonies per field. 100 μg/ml of dihydroxy myricetin treatment to PC-3 cells has shown about 14 colonies per field and standard drug taxol treatment has shown only 06 colonies per field.

The number of colonies was recorded after 7 days after treatment. Data represents the mean ± SD of three different assays.

**Cell cycle analysis: (Graph 6.2)**

Using flow cytometry method cell cycle analysis assay was performed. Control group treated with dimethyl sulphoxide has 73% cells in G0-G1 phase, 14%
cells in S phase and 13% cells in G2-M phase. Where as 50 µg/ml of dihydroxy myricetin treatment has shown 49% cells at G0-G1 phase, 24% cells in S phase and 23% cells in G2-M phase. Similarly 100 µg/ml of dihydroxy myricetin treatment has shown 57% cells in G0-G1 phase, 21% cells in S phase and 22% cells in G2-M phase. Standard drug taxol has shown 62% cells in G0-G1 phase, 22% cells in S phase and 25% cells in G2-M phase.

**Invivo studies**

**Physiological studies (Table 6.2)**

After the completion of experimental duration all the animals are sacrificed by cervical dislocation. The reproductive organs are dissected out, freed from fatty tissue and weighed at nearest mg on electronic weighing balance. The weight of testis, epididymis, seminal vesicles and prostate gland were recorded. In dimethyl sulphoxide treated the control group weight of testis was 1.36g, weight of epididymis was 0.37g, seminal vesicle weight was 0.76g and prostate gland weight was about 0.51g. Testosterone induced group has shown testis weight about 1.42g, weight of epididymis was 0.34g, weight of seminal vesicle was 0.62g and weight of prostate gland is 0.63g. 1mg/ml dihydroxy myricetin treatment to testosterone treated group has shown weight of testis 1.27g, weight of epididymis was about 0.33g, the weight of seminal vesicle 0.69g and prostate gland 0.54g. 2mg/ml dihydroxy myricetin treatment to testosterone treated group has shown weight of testis as 1.19g, weight of epididymis was 0.27g, weight of seminal vesicle 0.71g and prostate gland 0.48g. Standard drug vincristine treatment to testosterone treated group has shown weight of testis was about 1.33g, weight of epididymis was 0.39g, seminal vesicle 0.78g and prostate gland 0.53g.
Biochemical studies: (Table 6.3)

The biochemical markers such as, Serum glutamate oxaloacetate transaminase (SGOT) and Serum glutamate pyruvate transaminase (SGPT) alkaline phosphatase (ALP) and lactate dehydrogenase (LDH) estimations are carried out. Normal group serum has shown the recorded values for SGOT (0.45 ± 0.03), SGPT (0.58 ± 0.06), ALP (1.48 ± 0.10), LDH (3.52 ± 0.13). In testosterone induced rat group, serological values of all these enzymes- SGOT (1.98 ± 0.10), SGPT (0.81 ± 0.01), ALP (2.26 ± 0.21), LDH (4.38 ± 0.16) were elevated significantly. Dihydroxy myricetin treatment has reversed the toxic effect of testosterone by decreasing the levels of serum enzymes. Treatment with isolated dihydroxy myricetin to testosterone induced animals at the doses of 1mg/kg body weight resulted in significant recovery of SGOT (0.68 ± 0.02), SGPT (0.70 ± 0.04), ALP (2.02 ± 0.18) and LDH (3.81 ± 0.44) activities. 2mg/kg body weight dose of dihydroxy myricetin to testosterone induced animals has recovered considerably with supporting values of SGOT (1.21 ± 0.10), SGPT (0.60 ± 0.04), ALP (1.80 ± 0.21), LDH (3.61 ± 0.11). Standard drug myricetin has restored the values of biochemical markers to the normal level by exhibiting the value of SGOT (0.48 ± 0.06), SGPT (0.59 ± 0.02), ALP (1.62 ± 0.21), LDH (2.19 ± 0.44).
DISCUSSION

In Present scenario, Cancer is one of the greatest killer across the world and is spreading promptly. The study of Ayurvedic classics has revealed that the symptomatology of the disease entity Arbuda can be correlated to that of tumor or cancer. The most outstanding symptom is that of a swelling which continuously goes on increasing in size but never reaches to the stage of suppuration until and unless complicated by superimposed infection. This swelling is circular, immovable, slightly painful, slowly growing and broad based (Gaidhani, 2013).

Currently, about 50% of drugs are used in clinical trials for treating anticancer activity are isolated from natural sources such as herbs and spices or related plant species to them. A number of active compounds such as flavonoids, diterpenoids, triterpenoids and alkaloids have been shown to possess anticancer activity (Rahman et al., 2011).

Plant origin compounds are promising source of anti-infective and anticancer chemotherapeutic agents. Our research describes that the potential use of *Ficus glomerata* Roxb as a source of anti-cancer drug. The isolated compound dihydroxy myricetin was tested for its cytotoxic and apoptotic properties against the prostate cancer cells in vitro. A dose of 50µg/ml and 100µg/ml was effective in inducing cytotoxicity in the cancer cells. Effective reduction in the viability of cancer cells were seen as determined by the MTT assay. The myricetin also reduced the colony forming ability of prostate cancer cells in soft agar medium. The cell cycle analysis shows the reduction in number of cells in growth phases after treatment with our test compound dihydroxy myricetin in the cancer cells. Study of Vishnupriya et al., (2011) has shown the effect of anti-cancer activity of traditional plant *Tridax procumbens*.
flower crude aqueous and acetone extract on prostate epithelial cancerous cells PC 3 using MTT assay. The plant extracts has successfully shown anticancer activity.

MTT assay involves the cleavage of the soluble yellow coloured tetrazolium salt MTT [3-(4, 5-dimethyl –thiazole-2yl)-2, 5-diphenyl tetrazolium bromide] to a blue coloured formazan by the mitochondrial succinate dehydrogenase. The assay is based on the capacity of mitochondrial enzymes of viable cells to reduce the yellow soluble salt MTT to purple blue insoluble formazan precipitate which is then quantified spectrophotometrically at 570nm.

The study of Cristina et al., (2014) has established the cytotoxic and apoptotic activities of Ficus pseudopalma extracts against PRST2 cells while having no adverse effect on normal hFSE cells. This study also provided the evidence on the pharmaceutical potential of Ficus pseudopalma Blanco leaves as a chemotherapeutic agent against prostate cancer. In our study when the isolated compound dihydroxy myricetin was added at different concentrations (50μg/ml and 100 μg/ml) to cultured PC-3 cells, it was observed that the compound was cytotoxic to the prostate cancer cell line yielding 27.39% of inhibition at 50μg/ml and at 100 μg/ml the inhibition is 46.81% which is comparable with the standard drug vincristine having 58.89% of inhibition. These results of our research are supported by above studies to use plant extract for screening against cancer cell lines under invitro condtions.

Studies of Sharmila et al., (2014), Parviz et al., (1987) and Akbar et al., (2011) have illustrated that the testosterone to induce prostate cancer in animals. Treatment of testosterone stimulates the cell proliferation more and more and leads to the prostate cancer. In the present study we have used commercially available testosterone to induce prostate cancer in albino rats. Treatment with isolated dihydroxy myricetin to testosterone induced animals at the doses of 1mg/kg body
weight resulted in significant recovery of SGOT (0.68 ± 0.02), SGPT (0.70 ± 0.04), ALP (2.02 ± 0.18) and LDH (3.81 ± 0.44) activities. 2mg/kg body weight dose of dihydroxy myricetin to testosterone induced animals has recovered considerably with supporting values of SGOT (1.21 ± 0.10), SGPT (0.60 ± 0.04), ALP (1.80 ± 0.21), LDH (3.61 ± 0.11). Standard drug myricetin has restored the values of biochemical markers to the normal level by exhibiting the value of SGOT (0.48 ± 0.06), SGPT (0.59 ± 0.02), ALP (1.62 ± 0.21), LDH (2.19 ± 0.44).

Sahrmila et al., (2014) have shown that the treatment of quercitin a flavonoid to testosterone induced animals has restored the serum levels in prostate cancer animals. Our compound dihydroxy myricetin which is a flavonoid has also restored the serum enzyme levels in prostate cancer induced animals.