Chapter 7

Anticancer property of *H. enneaspermus*
7.1. Introduction

Cancer is an abnormal growth and proliferation of cells. It is a frightful disease because the patient suffers pain, disfigurement and loss of many physiological processes. Cancer may be uncontrollable and incurable and may occur at any time at any age in any part of the body. It is caused by a complex, poorly understood interplay of genetic and environmental factors. It continues to represent the largest cause of mortality in the world and claims over 6 million people every year. Cancer kills annually about 3500 per million people around the world. One of the major reasons for the quick progression of human cancers is the ability of tumor cells to flee from the immune surveillance mechanism of the body. Cancer cells may secrete immunosuppressive factors that modify the host’s immune responses (Murali and Kuttan, 2014).

When ranked within various age groups, cancer is one of the five leading causes of death among both middle aged males and females and considered as the single largest cause of death worldwide (Jemal et al., 2008). This growing trend indicates deficiency in the present cancer therapies which include surgical operation, radiotherapy and chemotherapy. Since the average survival rates have remained essentially unchanged despite such aggressive treatments, there is a critical need for anti-cancer agents with higher efficacy and less side effects that can be acquired at an affordable cost (Fadeyi et al., 2013).

A large number of chemotherapeutic agents are used to cure various cancers, but they produce side effects that prevent their extensive usage. A major complication of
chemotherapy is its toxicity to normal cells, which is due to the inability of drug to differentiate between normal cells and malignant cells. Therefore, we need to look at cancer chemotherapeutic agents, where one of the requisites is the elimination of damaged or malignant cells through cell cycle inhibition or induction of apoptosis with less or no toxicity to normal cells (Srivastava and Gupta, 2006). Although more than 1500 anticancer drugs are in active development with over 500 of the drugs under clinical trials, there is an urgent need to develop more effective and less toxic drugs where plant resources play an important role (Umadevi et al., 2013). It has been reported that more than 50% of all modern drugs in clinical use are of natural origin, many of which have been recognized to have the ability to induce apoptosis in various cancer cells of human origin (Rao et al., 2000). The use of botanicals when treating cancer patients is considered a natural alternative, because some plants may possess properties to prevent the spread or risk of developing various forms of cancer. In the past, a few highly effective cancer chemotherapeutic drugs have been derived from natural origin (Lee, 2010). These include plant-derived agents, such as the Vinca alkaloids vinblastine and vincristine, isolated from Catharanthus roseus (Gueritte, Fahy, 2005); paclitaxel (Taxol), originally isolated from the bark of Taxus brevifolia and the analogue, docetaxel (Cragg, 1998); etoposide and teniposide, derived semisynthetically from epipodophyllotoxin, an epimer of podophyllotoxin, isolated from roots of Podophyllum species (Lee and Xiao, 2005); and camptothecin, isolated from the bark of Camptotheca acuminata Decne., a precursor to the semisynthethetic drugs topotecan (Hycamptin) and irinotecan (Camptosar) (Rahier, 2005).
Considerable work have been done on medicinal plants to treat cancer and some plant products have been marketed as anticancer drugs, based on the traditional uses and scientific reports. These plants may promote host resistance against infection by re-stabilizing body equilibrium and conditioning the body tissues. Several reports describe that the anticancer activity of medicinal plants is due to the presence of antioxidants in them. In fact, the medicinal plants are easily available, cheap and possess no toxicity compared to the modern (allopathic) drugs. Thus, the various combinations of the active components of these plants after isolation and identification can be made and have to be further assessed for their synergistic effects (Umadevi et al., 2013).

The importance of plant derived compounds for the treatment of malignancy is tremendously increasing day by day due to the severe side effects produced by synthetic drugs. Many naturally occurring compounds have been tested for antitumour activity on animal models, which leads to the availability of so many effective anticancer drugs. Natural Products have long been a prolific source of cures for cancer, which is projected to become the major cause of death in this century. However, there is a continuing need for the development of new anticancer drugs, drug combinations and chemotherapy strategies, by methodical and scientific exploration of an enormous pool of synthetic, biological and natural products.

The Indian sub-continent has great botanical diversity and widespread use of traditional medicine practice known as ayurvedic medicine; however, only a relatively small number of these plants have been subjected to accepted scientific evaluation for their
potential anticancer effects (Krishnaswamy, 2008). Now-a-days, several phytochemicals are used in the treatment of cancer. The continuous search for natural compounds for the treatment of cancer is very significant due to the severe side effects of synthetic drugs. The anti-cancer properties of *H. enneaspermus* have not yet been studied. Hence, the prime focus of this study was to evaluate the cytotoxicity and anticancer properties of the methanol extract of *H. enneaspermus*.

7.2. Materials and Methods

The experiment was conducted at the Amala Cancer Research Centre, Thrissur, Kerala. *In vivo* experiments were carried out with the prior approval of the Institutional Animal Ethics Committee (No. 149/1999 CPCSEA) and conducted strictly according to the guidelines of the committee for Control and Supervision of Experiments on Animals (CPCSEA) constituted by the Animal Welfare Division, Government of India.

7.2.1. Cell lines:

Dalton’s Lymphoma Ascites (DLA) and Ehrlich Ascites Carcinoma (EAC) cell lines were initially collected from Cancer Institute, Madras and maintained as ascites tumours in Swiss albino mice. Tumour cells were aspirated from the peritoneal cavity of the tumour bearing mice before starting the experiment and washed thrice with phosphate buffered saline (PBS).
7.2.2. Animals:

Swiss albino mice (male, 6-8 weeks old, 25±2 g) were procured from Amala Cancer Research Centre, breeding section and maintained under controlled environmental conditions and fed with normal mice chow (Sai Feed, India) and water ad libitum.

7.2.3. Drug preparation:

Methanol extract of *Hybanthus enneaspermus* (Whole plants) extract was resuspended in DMSO and 1% gum acacia respectively for *in vitro* and *in vivo* studies. Detailed procedure for the preparation of extract is given in Chapter 3.8.1.

7.2.4. Determination of antitumour activity:

*In vitro* and *in vivo* methods were utilized to evaluate the antitumour properties of the methanol extract of *H. enneaspermus*.

7.2.4.1. Determination of short term *in vitro* cytotoxic activity using DLA and EAC cells

*In vitro* short term cytotoxic effects of the methanol extracts of *H. enneaspermus* were determined by trypan blue dye exclusion method using Daltons Lymphoma Ascites (DLA) and Ehrlich Ascites Carcinoma (EAC) cell lines (Talwar, 1983). Viable suspension of DLA and EAC cells (1x10^6 cells in 0.1 ml) was added to vials containing various concentrations of the extract (0, 10, 20, 50, 100 and 200 µg/ml) and made up to 1ml using PBS. The vial with only cell suspension was taken as the control. The vials were incubated for 3 hours at 37°C and then treated with 0.1 ml of 1% trypan blue and kept for 2-3 minutes. The number of
stained and unstained cells was counted separately by using a haemocytometer. Dead cells were stained blue by trypan blue while live cells did not take up the dye. Percentage of cell death was calculated to evaluate the cytotoxic effect.

7.2.4.2. Determination of long term *in vitro* cytotoxicity using L929 cells

Long term *in vitro* cytotoxicity was determined according to the method of Campling et al. (1991), using L929 cells and MTT (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide). L929 cells were seeded in 96-well flat bottom titre plates (5000 cells/well) and incubated for 24 hours at 37°C with 5% CO₂ for the attachment of cells. After incubation, different concentrations of the methanol extract (0, 0.5, 1, 2.5, 5 and 10 µg/ml) were added and incubated again for 48 hours. At 44 hours, 20 µl of MTT (5mg/ml in PBS) was added. After the incubation period, the plates were centrifuged, supernatant was removed and 100 µl of DMSO was added to each well. The plate was then incubated at room temperature for 15 min and the optical density was measured at 570 nm. This assay measures cell viability by assessing the cleavage of tetrazolium salt by mitochondrial dehydrogenase. The percentage of dead cells was determined using the formula: (1-OD of drug treated/OD of control) x 100.

7.2.4.3. Effect of *H. enneaspermus* extract on solid tumor development

Swiss albino mice (male, 6-8 weeks old) weighing 20-25g were used for the study. The animals were divided into four groups (6 animals/group) and solid tumor was induced by implanting DLA cells (1x10⁶) on to the right hind limb. The Group II and III test animals
were orally administered, the methanol extract of *H. enneaspermus* (200 and 50 mg respectively per kilogram body weight) after 24 hours of transplantation and were continued for 10 consecutive days. Group I that received only DLA cell line served as control and Group IV was treated with cyclophosphamide (25 mg/kg body weight) served as standard group. Tumor development was determined by measuring the diameter of tumor growth in two perpendicular planes using vernier-calipers on every 3rd day for 4 weeks and comparing it with untreated control (Atia and Weiss, 1966). Tumor volume was calculated using the formula: \( V = \frac{4}{3}\pi r_1^2 r_2 \); where ‘\( r_1 \)’ is the minor radius and ‘\( r_2 \)’ is the major radius (Ma et al., 1991).

### 7.2.4.4 Effect of *H. enneaspermus* on ascites tumor

Swiss albino mice (male, 6-8 weeks old & weighing 20-25g) were divided into four groups (6 animals/group). Viable EAC cells were aspirated from 15 days old EAC tumor in mice and injected into the peritoneal cavity (1x10^6 cells/animal). Group II and III test animals were orally administered methanol extract of *H. enneaspermus* (200 and 50 mg respectively per kilogram body weight) after 24 hours of transplantation and were continued for 10 consecutive days. Group I that received only EAC cell line served as the control and Group IV, treated with cyclophosphamide (25 mg/kg body weight), served as standard group. The death pattern of animals due to tumor burden was noted and the percentage of increase in life span was calculated using the formula:

\[
\frac{\text{Mean survival time of treated group} - \text{mean survival time of control group}}{\text{Mean survival time of control group}} \times 100
\]
7.2.5. Statistical analysis

Data on *in vivo* cytotoxicity studies were expressed as the mean ± standard deviation (SD) of 6 replicates. One-way analysis of variance (ANOVA) was used for the repeated measurements, and the differences were considered to be statistically significant if $P= 0.05$.

7.3. Results and Discussion

Medicinal plants constitute a common alternative for cancer prevention and treatment in many countries across the world. Approximately, 60% of the anticancer drugs currently used have been isolated from plants (Mehta et al., 2010). Plant derived compounds have played an important role in the development of several anticancer agents. The utilization of phytochemical compounds is now considered as an emerging strategy to prevent, impede, delay or cure cancer (Wang et al., 2012). The preliminary phytochemical screening revealed that the methanol extract of *H. enneaspermus* contains flavanoids, coumarins, tannins, phenols, steroids, alkaloids, anthraquinones, quinones, carbohydrates and proteins. The plant has been studied for several pharmacological activities but it has never been studied for anticancer activity. Therefore, the methanol extract of *H. enneaspermus* was initially screened with *in vitro* cytotoxic assays using established cell lines. Since cytotoxicity is one of the chemotherapeutic targets of antitumor activity (Suffness and Pezzuto, 1991), all the clinically used antitumor agents should possess significant cytotoxicity in cell culture systems.
7.3.1. Short term in vitro cytotoxic activity using DLA and EAC cells

*In vitro* short term cytotoxicity of the extract was determined using DLA and EAC cell lines. Methanol extract of *H. enneaspermus* showed significant cytotoxicity in both the cell lines tested (Plate 7.1A). Administration of 200 µg/ml extract showed 44% and 48% cell death in DLA and EAC cells respectively.

7.3.2. Long term in vitro cytotoxicity using L929 cells

The MTT cell viability assay is commonly used in determining drug sensitivity in primary screening of potential chemotherapeutic drugs. Various concentrations of the methanol extract of *H. enneaspermus* (0.5, 1, 2.5, 5, and 10 µg/ml) were tested to find out the cytotoxic effect towards L929 cells in culture. A dose dependent inhibition of L929 cells was noticed (Plate 7.1B). The results revealed that the extract is able to produce metabolic changes in tumor cells which lead to cytotoxicity. The cytotoxic activity of *H. enneaspermus* against DLA and EAC cell lines gave positive hints to its significant antitumor activity against solid and ascites tumor.

7.3.3. Effect of *H. enneaspermus* extract on the solid tumor development

Based on the promising results obtained during the *in vitro* studies, *in vivo* experiments were conducted for screening of antitumor activity in tumor bearing mice. *In vivo* antitumor studies using solid tumour and ascites model revealed the antitumor property of the methanol extract of *H. enneaspermus*. DLA cells induced tumourogenesis in Swiss
albino mice provides a convenient model to study antitumour activity within a short time (Shanker et al., 2000). Methanol extract of *H. enneaspermus* reduced the tumour burden effectively. There is a significant (P=0.05) reduction in the tumor volume in drug treated animals (Plate 7.1C). Tumour volume of control group was 3.034 ± 0.70 mm$^3$ on 30$^{th}$ day while 200 mg extract treated group showed only 0.844 ± 0.14 mm$^3$, on the same day.

### 7.3.4. Effect of *H. enneaspermus* extract on ascites tumour

Ehrlich ascites tumour is a rapidly growing carcinoma with very aggressive behavior (Segura et al., 2000). It’s implantation stimulates a local inflammatory reaction with increasing vascular permeability, which results in an intense oedema formation, cellular migration and a progressive ascitic fluid formation (Fecchio, 1990). The life span of *H. enneaspermus* extract treated animals was found to be significantly increased in the ascites tumour model, (Table 7.1). Test animal groups provided 200 mg extract survived for 20.5 days. However, the control group survived for only 14.8 days after the tumour induction. A reliable criterion for judging the efficiency of any anticancer drug is the prolongation of lifespan in animals. According to National Cancer Institute’s (NCI) criteria, percentage of increase in life span (%ILS) exceeding 25% indicated antitumor effectiveness of a drug (Clarkson and Burchenal, 1965), and thus the data in this study indicate that methanol extract of *H. enneaspermus* has promising anticancer property.
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**Plate 7.1. (A-C) Cytotoxic and antitumour activity of *H. enneaspermus***

(A) *In vitro* short term cytotoxicity of *H. enneaspermus* on DLA and EAC cells. Inhibition rates are expressed as the mean ± S.D.

(B) *In vitro* long term cytotoxicity of *H. enneaspermus* on L929 cells by MTT assay after 48 h. Results are expressed as the mean ± S.D.

(C) Effect of *H. enneaspermus* on solid tumour development. Data were expressed as mean ± S.D (n = 6).
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Table 7.1. Effect of H. enneaspermus methanol extract on the survival of ascites tumour bearing animals.

<table>
<thead>
<tr>
<th>Group</th>
<th>Mean survival days ± S.D</th>
<th>% Increase in life span</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>14.8 ± 3.19</td>
<td>-</td>
</tr>
<tr>
<td>50 mg/kg. b.wt</td>
<td>16.0 ± 3.20</td>
<td>8.1</td>
</tr>
<tr>
<td>200 mg/kg. b.wt</td>
<td>20.5 ± 9.10</td>
<td>38.5</td>
</tr>
<tr>
<td>Cyclophosphamide</td>
<td>26.63 ± 2.7</td>
<td>79.9</td>
</tr>
<tr>
<td>(25 mg/kg body weight)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Values are expressed as mean ± S.D for 6 animals.

The results of the present investigation reveal that H. enneaspermus possesses antitumour activity and the property may be exploited for the treatment of cancer. However, the mechanism of antitumor activity of H. enneaspermus extract is unknown. Correlation between free radical generation and cancer development is well documented (Kumar et al., 2011). Oxidative/electrophilic stress is generally perceived as one of the major causes of carcinogenicity. It was reported that plant-derived extracts containing antioxidant principles showed antitumor activity in experimental animals (Viral et al., 2011). Antioxidant potential of H. enneaspermus has been studied as part of the current thesis and revealed the scavenging
ability of the methanol extract towards various free radicals. Presence of phytochemicals like flavonoids, coumarins, tannins, phenols, steroids, alkaloids, anthraquinones, quinones, carbohydrates and proteins were also noted during the preliminary phytochemical screening of the methanol extract of *H. enneaspermus*. Most of these compounds have been mentioned as antioxidants and consequently involved in antitumor activities. For example, flavonoids have been found to possess antimutagenic and antimalignant effect. Moreover, they have a chemo-preventive role in cancer through their effects on signal transduction in cell proliferation and inhibition of neovascularization (Batra and Sharma, 2013). However, the antitumor activity of *H. enneaspermus* may be due to the additive and synergistic antioxidant activity of phytochemicals present in the species. Antitumor activity of antioxidants is either through induction of apoptosis or by inhibition of angiogenesis. Antioxidants’ protective role in cancer through the differential regulation of transcriptional activators and the redox modulation of gene expression is well documented (Storz et al., 1990). Generation of reactive oxygen species (ROS) combined with potent cytotoxic activity could be exploited for developing novel therapeutic strategies against cancer cells (Pelicano et al., 2003).

In conclusion, it can be said that the methanol extract of *H. enneaspermus* was effective in inhibiting tumor growth in *in vitro* and *in vivo* models. The significant reduction in solid tumor size and increase in life span observed with the treatment of the methanol extract of *H. enneaspermus* substantiates its use as an effective natural chemotherapeutic agent. However, more detailed studies are needed to establish the exact mechanism of action of this natural anti-cancer drug.