CHAPTER- IV

EVALUATION OF ANTITUMOR ACTIVITY
4.1. Introduction:

Cancer is the second leading cause of death worldwide (Amin et al., 2009). Cancer chemoprevention is a relatively new concept. The pioneering work to reduce cancer incidence by chemical intervention was initiated by the groups of Wattenberg and Sporn in the early 1960s and 1970s (Wattenberg, 1985; Sporn, 1993). Later, scientists have embraced the concept of cancer chemoprevention as a distinct new discipline of oncology (Kelloff et al., 1994; Greenwald et al., 1995; Hong and Sporn, 1997; Stoner et al., 1997; Trignali, 2001).

Conventional cancer therapies cause serious side effects and, at best, merely extend the patient's lifespan by a few years. Cancer control may therefore benefit from the potential that resides in alternative therapies. The demand to utilize alternative concepts or approaches to the treatment of cancer is therefore escalating. There is compelling evidence from epidemiological and experimental studies that highlight the importance of compounds derived from plants “phytochemicals” to reduce the risk of colon cancer and inhibit the development and spread of tumors in experimental animals. More than 25% of drugs used during the last 20 years are directly derived from plants, while the other 25% are chemically altered natural products. Still, only 5-15% of the approximately 250,000 higher plants have ever been investigated for bioactive compounds. The advantage of using such compounds for cancer treatment is their relatively non-toxic nature and availability in an ingestive form. An ideal phytochemical is one that possesses anti-tumor properties with minimal toxicity and has a defined mechanism of action. As compounds that target specific signaling pathways are identified, researchers can envisage novel therapeutic
approaches as well as a better understanding of the pathways involved in disease (Amin et al., 2009).

Plants have a long history of use in the treatment of cancer and played an important role as a source of effective anti-cancer agents. Over 60% of currently available anticancer agents are derived in one way or another from natural sources, including plants, marine organisms and microorganisms (Newman et al., 2003; Cragg and Newman, 2005; Cragg et al., 2005). The search for anticancer agents from plant sources started in earnest in the 1950s with the discovery and development of the vinca alkaloids, vinblastine and vincristine, and the isolation of the cytotoxic phodophyllotoxins. As a result, United States National Cancer Institute (NCI) initiated an extensive plant collection program in 1960, focused mainly in temperate regions. This lead to the discovery of many novel chemotypes showing a range of cytotoxic activities (Cassaday and Douros, 1980), including the taxanes and camptothecins, but their development into clinically active agents spanned a period of some 30 years, from the early 1960s to the 1990s. This plant collection program was terminated in 1982, but the development of new screening technologies led to the revival of collections of plants and other organisms in 1986, with a focus on the tropical and sub-tropical regions of the world. It is interesting to note, however that no new plant-derived clinical anti-cancer agents have, as yet, reached the stage of general use, but a number of agents are in preclinical development (Cragg and Newman, 2005).

Oxidative stresses are related with various diseases and pathological conditions such as aging, atherosclerosis and cancer (Cadenas and Davies, 2000; Aviram, 2000; Kogurre et al., 2004). Antioxidants may prevent these chronic diseases by several mechanisms such as enzymatic degradation of free radicals, chelation of
metals which stimulate the production of free radicals and scavenging the free radicals (Penckofer et al., 2002). Medicinal plants are considered to be the important source of antioxidant compounds, and recently there has been considerable interest in finding the natural antioxidants from plant materials to replace synthetic ones (Mehdipour et al., 2006).

Phytotherapeutic products are many times mistakenly regarded as safe because they are natural (Gester, 1992). Nevertheless, those products contain bioactive principles with potential to cause adverse effects (Bent and Ko, 2004). In addition poor pharmacovigilance services of this area make it difficult to determine the frequency of adverse effects caused by the use of phytotherapeutic products (Eisnberg et al., 1998). Thus all the natural products used in the therapeutics must be submitted to efficiency and safety tests by the same methods used for new synthetic drugs (Talay and Talay, 2001; Fères et al., 2006).

In the present study, we report for the first time the, anti-tumor, as well as the acute toxicity studies of leaves parts of methanol extract of Fluggea leucopyrus.

4.2. Materials and Methods:

4.2.1. In vivo Anti-tumor Activity: Determination of Acute Toxicity and Therapeutic Doses:

4.2.1.1. Mice and Tumor Model:

Swiss albino mice of either sex (8-10 weeks old) weighing 20-25 g, were used for the experiment. The animals were maintained under proper environmental conditions i.e., temperature 25 ± 2°C and humidity 50 ± 5% with a 12 h light and dark period. They were housed in polypropylene shoebox type cages with stainless steel
grill top, bedded with rice husk. The animals were provided with pelleted diet (Gold Mohur, Lipton, India) and water ad libitum. Ten animals were used in each control and treated group. Swiss albino mice of either sex were used for implanting Erlich ascitis tumor model.

4.2.1.2. Acute toxicity:

Leucopyrosinol was first dissolved in DMSO (10 mM) and then diluted in 0.9% NaCl. Acute toxicity was assessed on healthy Swiss albino mice of either sex (8-10 weeks old) weighing 20-25 gm, in both single and multiple ip, administration. The drug dosage was fixed according to OECD/OCDE guidelines no.420 (Fig.1.), the mice were observed continuously for 1 h for any gross behavioral changes and deaths intermittently for the next 6 h and then again 24 h after dosing. LD$_{50}$ of the compound was calculated according to OECD guidelines. The behavior parameters observed were convulsion, hyperactivity, sedation, grooming, loss of righting reflex, increased or decreased respiration, food water intake etc.
4.2.1.3. Experimental Chemotherapy:

The study protocol was approved by the ethics committee. Mice (6-8 week old) were challenged with Ehrlich Ascetic carcinoma (EAC) cells ($10^6$ cells/mouse; subcutaneously) on day zero. The treatment began the day after tumor inoculation, and test compound (Leucopyrosinol) and vehicle were administered ip using ten mice per test group. Routinely, the compound was administered four times: on the day after tumor inoculation (D1), on day D5, D9 and D11 (Scheme D1, 5, 9 and 11). The compound was also administered once (D1, single dose). In all chemotherapy trials mice were checked daily, with any adverse clinical reactions noted and deaths recorded. Mice were weighed 2-4 times weekly during treatment and once weekly thereafter. Tumors were measured by calipers twice weekly and tumor volume ($\text{mm}^3$) were estimated as $= 0.5 (\text{Length} \times \text{Width}^2)$. Results are presented for experiments involving ten mice per experimental group.

4.2.1.4. Evaluation of Anti-tumor Activity: Life Span:

Mortality was noted every day and the median life span was calculated as:

$$\text{MLS} = \frac{\text{Dm} + (\text{Mm} - \text{number of mice dead before Dm})}{\text{Number of mice dead on Dm}}$$

The median mouse (Mm) separates into two identical groups (one group, including the mice that died before Mm, the other group including those who died after) and the median day (Dm) is the day Mm died. Mice surviving for at least 45
days were considered as cured and were included in the calculation of the median life span. Compound efficiency was expressed by T/C as follows:

\[ \text{T/C}\% = \frac{\text{MLS of treated animals}}{\text{MLS of control animals}} \times 100 \]

Or by the increase in life span ILS: \[ \text{ILS}\% = 100 \times \frac{\text{T-C}}{\text{C}}. \]

The therapeutic index (defined as the ratio of the dose that kills 10% of tumor-free mice to the dose that gives a 50% increase in life span in tumor-bearing mice) was determined for each experiment. Survival curves of treated and control groups were statistically compared using the Log-rank test.

**4.2.1.5. Tumour Growth:**

Treatment efficiency is assessed in terms of the compound’s effects on the tumor volumes of tumor bearing mice relative to the control vehicle-treated mice. Two evaluation criteria were used in parallel: (i) Specific tumor growth delay (SGD), calculated as follows: for EAC tumor model = \[ \frac{\text{Td (drug-treated group)} - \text{Td (vehicle treated group)}}{\text{Td (vehicle-treated group)}} \], with Td being the tumor doubling time of drug - treated and control groups, defined as the time in days required for the tumor volume to double. (ii) Tumor regressions defined as partial (PR) if the tumor volume decreased to 50% or less of that at the start of treatment, without dropping below measurable size (Plowman et al., 1997).

**4.3. Results:**

**4.3.1. Anti-tumor activity of Leucopyrosinol:**

\( \text{LD}_{50} \) was determined after a single injection to mice by ip route. It was found to be 393 mg/kg body weight. Antitumor activity against EAC was determined for
Leucopyrosinol using single dose and intermittent treatments over two weeks, i.e., four drug administration by the i.p., route on days, 1, 5, 9, and 11 after EAC cells inoculation. The compound and the vehicle were administered ip using 10 mice per test group. Leucopyrosinol exhibited antitumor activity against EAC, with a significant increase in life span. The effect of Leucopyrosinol on the survival of tumor bearing mice is shown in the Table 1. There was a dose-effect relationship and the increase in life span. The ILS% was 14.18, 22.00 and 60.15 % for the single dose and 3.40, 33.75 and 71.00 % for the multiple doses at the dosage of 100, 150 and 200 mg/kg body weight respectively. There was reduction in the tumor volume of mice treated with Leucopyrosinol. The tumor volume of control animal on the 35th day of tumor injection was 2.0 ± 0.59ml, where as the compound treated group it was 1.75 ± 0.47, 1.74 ±0.82, and 0.49 ± 0.98 ml for the single dose and 1.25 ± 0.60, 0.75 ± 0.43, and 0.25 ± 0.83 ml for the multiple dose respectively for the doses of Leucopyrosinol mentioned earlier. Seven or eight out of ten mice were cured with these compounds. The highest therapeutic index was obtained with multiple schedules. The results obtained for these compounds are shown in Figure 3a-b. Present compound Leucopyrosinol was shown to be non-toxic even when the concentration was increased up to 900 mg/kg body weight.
Fig. 2. Measurement of tumor volume by calipers.
Table 1: Antitumor activity of Leucopyrosinol given ip against the sc implanted EAC.

<table>
<thead>
<tr>
<th>Treatment Schedule</th>
<th>MTD * (mg/kg)</th>
<th>TI b</th>
<th>Dose (mg/kg)</th>
<th>SGD</th>
<th>ILS%</th>
<th>Surviv</th>
</tr>
</thead>
<tbody>
<tr>
<td>D1</td>
<td>300</td>
<td>5.70</td>
<td>100</td>
<td>1.10</td>
<td>14.18</td>
<td>9/10</td>
</tr>
<tr>
<td></td>
<td>150</td>
<td>5.72</td>
<td>100</td>
<td>&lt; 4</td>
<td>3.40</td>
<td>6/10</td>
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<tr>
<td></td>
<td>200</td>
<td></td>
<td>100</td>
<td>1.00</td>
<td>71.00</td>
<td>7/10</td>
</tr>
</tbody>
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Tumour cells are inoculated ip as described in Materials and Methods. Drugs are then injected ip with in different schedules, and ILS% are then determined. * MTD – maximum tolerance dose. b TI, therapeutic index.
Figure 3a: In vivo antitumour activity of leucopyrosinol on Ehrlich’s Ascitic carcinoma. Tumors were generated by s.c., inoculation of EAC cells in Swiss albino mice. The compound was administered i.p., once (D1, Single dose) on the day after tumor inoculation. Average tumour volumes of treated (n = 10) and control (n = 10) are shown.
Figure 3b: In vivo antitumour activity of leucopyrosinol on Ehrlich Ascitic Carcinoma. Tumors were generated by s.c., inoculation of EAC cells in Swiss albino mice. The compound was administered i.p., 4 times: on day D1, D5, D9 and D11 (Multiple doses). Average tumour volumes of treated (n = 10) and control (n = 10) are shown.
4.4. Discussion:

Anticancer drugs have well known therapeutic limitations and this has stimulated the search for new agents with enhanced therapeutic efficacy. Considerable efforts have been directed towards medicinal plants, which have been reported to be effective in the treatment of human cancers. Therefore, search for new drugs is required for the treatment of cancers (Xingming et al., 2009). Although the Leucopyrosinol have demonstrated significant in vivo antineoplastic activities against the tumour model, the mechanism of this effect has not been fully examined.

The cytotoxic effects of plant flavonoids are shown to be mediated through apoptosis. Considering the ability of these natural flavonoids to absorb proteins and metal ions, there is a possibility that they can elicit apoptosis signals through various receptors or proteins. Apart from this, they are excellent antioxidants and they thus prevent free radical attack on DNA by acting as scavengers of these free radicals. A number of flavonoids are topo-II poisons inhibiting topo I/II isomerases thus enhancing the DNA cleavage.

Another possible mechanism of reported for anticancer drugs is inhibition of DNA synthesis and thus prevention of cell division. Folic acid supplied from the diet is essential for the production of tetrahydrofolic acid (THF). The conversion of folic acid to THF is carried out by an enzyme folate reductase. Anticancer drugs compete with folic acid for this enzyme thus restricting the production of THF required for synthesis of DNA and consequently for cell replication. Cells, which do not have adequate production of THF eventually, die.
It should be noted that there are a number of reports that the anticancer plant extracts retarded development of ascetic tumour growth and increased the life span of tumour growth and increased the ILS% (Xingming et al., 2009; Abdel-Khader et al., 2007; Rajkapoor et al., 2004; Babu et al., 2002; Latha and Panikkar, 1998). For example, Loranthus extract significantly inhibited Ehrlich's Ascetic Carcinoma (EAC) growth in mice (Mary et al., 1994). Preparations from Solanum trilobatum also reduced the growth of EAC in mice (Mohanan and Devi, 1996). In the present study the methanol extract significantly increased the life span of ascetic tumour bearing mice dose dependently. Moreover, the extracts significantly reduced the solid tumor development in the mice.

In our studies we found that leucopyrusinol posses antioxidant effect. Flavonoids have been shown to posses antimutagenic, antimalignant and antioxidant effects. It was reported that plant derived extracts containing antioxidant principles showed cytotoxicity towards tumor cells and antitumor activity in experimental animals (Babu et al., 2002; Ruby et al., 1995). Antitumor activity of these antioxidants is either through induction of apoptosis or by inhibition of neovascularisation (Putul et al., 2000). The implication of free radicals in different steps of carcinogenesis is well documented (Player, 1982; Frenkel, 1992). The free radical hypothesis supported the fact that the antioxidants can effectively inhibit carcinogenesis and the observed properties may be attributed to the antioxidant principles present in the extract.
4.5. Conclusion:

In summary, this study shows the antitumor potential of Leucopyrosinol isolated from the leaves parts of Fluggea leucopyrus. Leucopyrosinol showed a significant activity against the in vivo tumor models. The high activity of Leucopyrosinol against EAC must be considered as a new class of anti-tumor agents.
4.6. References


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Trignali C. Bioactive compounds from natural sources; Isolation, characterization and biological properties. Boca Raton, FL. 2001; 70-84.
