CHAPTER 6

IN VIVO ANTI-TUMOUR ACTIVITY OF B. PHOENICEA LEAVES
6.1 INTRODUCTION

Cancer is a potentially fatal disease mainly due to environmental factors which mutate genes coding critical cell regulating proteins. The aberrant cell resulted behave abnormally, destroys surrounding normal tissues and spread to vital organs, resulting in dissemination of disease and patient death. Production of free radicals in the body beyond its antioxidant capacity have been related to a number of oxidative stress diseases including cancer (Cune and Johns, 2002). Regardless of the substantial amount of research for the development of precise diagnostic techniques and availability of targeted therapeutic options, cancer remains to be a life threatening disease and is the second leading cause of death following cardiovascular diseases. Chemotherapy is the major treatment modality for cancer therapy. The presently using chemotherapeutics are antimetabolites, alkylating agents and anthracyclin antibiotics, they targets the rapidly proliferating cells and therefore, imparts toxicity towards normal cells especially cells in the bone marrow, hair follicles, mouth, digestive tract and reproductive system. The non-discrimination between normal and cancer cells and the development of multidrug resistance in cancer cells are the main drawbacks in this mode of treatment (Gottesman and Pastan, 1993).

To overcome this situation, research is continuing to find appropriate drugs for cancer therapy. Applications of plant preparations and extracts in medicine have an immense historical legacy amongst people (Duke, 2002). In these years, the use of plant derived chemotherapeutic drugs like vinca alkaloids, taxanes, camptothecin derivatives and podophyllotoxins has increased tremendously. Due to the less toxicity and high effectiveness, more research is focusing on the anticancer activity of natural products (Ozaslan et al., 2011). Diets with plenty of fruits and vegetables are protective against oxidative stress related diseases. It is linked to the presence of antioxidant principles which are responsible for much of their flavour and colour (Plumb et al., 1999).

In our search for new natural sources of antioxidants, we have conducted an antioxidant activity screening and phytochemical profiling of two traditionally important medicinal plants, \textit{B.phoenicea} and \textit{A.catechu}. 
**B. phoenicea** belongs to the family Fabaceae and sub-family Caesalpinioideae. It is a liana, endemic to Southern Westernghats. In the traditional systems of medicine it is using against various ailments including some oxidative stress disorders like diabetes. Many species of Bauhinia have evident anti-diabetic activity (Pepato et al., 2002; Abo and Jimoh, 2004; Silva et al., 2002). According to the results in the previous chapters, leaves and bark of this plant have potent antimicrobial, antioxidant and anthelmintic properties.

**A. catechu** Linn. commonly known as Betel palm or Betel nut tree is a member of the family Areceaceae. Areca palms are growing in India, Malaysia, Taiwan and many other Asian countries for their economically important seed crop. *A. catechu* root is used in the traditional medicines of Kerala for the treatment of urinary tract disorders, skin irritations and worm disturbances. The young leaf sheath is used in the treatment of migraine. The pharmacological activities of *B. phoenicea* and *A. catechu* root are screening for the first time in the present thesis. The aim of this chapter is to determine the potential of the selected medicinal plants as anticancer agents.

### 6.2 MATERIALS AND METHODS

#### 6.2.1 MATERIALS

**6.2.1.1 Cell lines**

Dalton’s Lymphoma Ascites (DLA) cell lines were procured from Amala Cancer Research Institute, Thrissur, Kerala, India. The mice were injected with a suspension of cells (1X10^6) intra peritoneally, and the cells were aspirated from the peritoneal cavity on the 15th day.

**6.2.1.2 Animals**

Swiss Albino mice (Non-pregnant females of 6-8 weeks) were purchased from SABS (Small Animal Breeding Station), College of Veterinary and Animal Sciences, Mannuthi, Thrissur, Kerala. They were kept in well-aerated cages with controlled conditions of light and humidity for 14 days for acclimatisation. The mice were fed with normal mouse chow (Sai Durga Food and Feeds, Banglore, India) and water *ad libitum*. All experiments in the study were carried
out with the prior approval of Institutional Animal Ethics Committee (IAEC) and were conducted as per the guidelines of CPCSEA (Committee for the Purpose of Control and Supervision of Experiments on Animals) constituted by the Animal Welfare Division of Government of India.

### 6.2.2 METHODOLOGY

#### 6.2.2.1 Preparation of plant extract

The plant materials were dried at 45-50°C for two weeks and powdered using mixer grinder. Dried powder sequentially extracted with Petroleum ether, Benzene, Chloroform, Acetone, Ethanol and Distilled water using column chromatography. Rotary evaporator was used to concentrate the extract.

#### 6.2.2.2 Acute toxicity study

Acute toxicity assay was performed in healthy adult non-pregnant female swiss albino mice (25-28g body weight). The mice were divided into two groups of three each and treated with 250 mg/kg drug intraperitoneally. The control group received 2% CMC (Carboxymethyl Cellulose) suspension at the same volume.

#### 6.2.2.3 In vitro cytotoxicity screening

Short term cytotoxic activity of *B.phoenicea* and *A.catechu* sequential extracts were assayed by determining the percentage viability of the DLA cells using Trypan blue exclusion methods (Moldeus *et al.*, 1978). The cells were aspirated from the peritoneal cavity of tumour bearing mice. The collected cells were washed using PBS (phosphate buffered saline) and checked for their viability. Different dilutions of the cells were made (10⁻¹, 10⁻², 10⁻³). The number of cells in the 10⁻³ dilution was counted using haemocytometer and the cell number was adjusted to 1X10⁷ cells/ml. This cell suspension was added to tubes containing various concentrations of test in 1ml PBS and the tubes were incubated at 37°C for 3 hours. 100µl of Trypan blue was added after the incubation period and the percentage of viability were determined.
6.2.2.4 Anti-cancer effect of *B. phoenicea* on ascites tumour bearing animals.

Ascites tumour was induced by injecting DLA cells (1X10^6 cells/animal) in the peritoneal cavity of swiss albino mice. Thirty six animals get divided into six groups, each group consist of 6 animals. Group I was maintained as negative control (not treated with any drug). Group II – V received 50 & 100 mg/kg body weight of aqueous and ethanolic extracts of *B. phoenicea* (which shows highest activity in *in vitro* cytotoxicity screening). Animals in the group VI received Cyclophosphamide (10 mg/kg body weight). The drugs were given intraperitoneally, after 24 hrs of tumour implantation as 5 doses on alternate days. The death of the animals due to tumour burden was noted everyday and the percentage of increase in lifespan (% ILS) was calculated using the formula

\[(T-C/C) \times 100\]

T - mean survival days of treated animals

C - mean survival days of control animals (Kuttan *et al.*, 1985).
6.3 RESULTS

6.3.1 Acute toxicity study

In the toxicity test, dose of 250 mg/kg body weight of the mice did not cause mortality or any signs of toxicity or change in general behaviour during the 14 days of observation. So, both the plants screened are non-toxic to live animals.

6.3.2 In vitro cytotoxicity analysis

The table 6.1 shows the results of the in vitro cytotoxicity screening of *B.phoenicea* leaves. Both polar and non-polar extracts of *B.phoenicea* found to be cytotoxic towards DLA cells. Maximum cytotoxicity (87%) was attained at a concentration of 200 µgL\(^{-1}\) of ethanolic extract. Least IC\(_{50}\) value was showed by aqueous extract (38 µgL\(^{-1}\)). So ethanolic and aqueous extracts were selected for the in vivo anticancer screening.

Table 6.2 given below shows the in vitro cytotoxic potential of *A.catechu* root. When compared with *B.phoenicea* leaves, *A.catechu* root is poor in cytotoxicity based on this assay (Plate 13).

Based on the result of the in vitro cytotoxicity screening, *B.phoenicea* leaves were selected for the in vivo anticancer screening.
Table 6.1 *In vitro* cytotoxic property screening of *B. phoenicea* leaves

<table>
<thead>
<tr>
<th>Sl No</th>
<th>Concentration (µgL⁻¹)</th>
<th>Percentage of inhibition</th>
<th>Petroleum ether</th>
<th>Benzene</th>
<th>Chloroform</th>
<th>Acetone</th>
<th>Ethanol</th>
<th>Distilled water</th>
</tr>
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<tr>
<td>1.</td>
<td>10</td>
<td></td>
<td>7</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>8</td>
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<td>35</td>
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<td>3.</td>
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<td>41</td>
<td>45</td>
<td>56</td>
<td>55</td>
<td>38</td>
</tr>
<tr>
<td>4.</td>
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<td></td>
<td>60</td>
<td>51</td>
<td>60</td>
<td>67</td>
<td>65</td>
<td>58</td>
</tr>
<tr>
<td>5.</td>
<td>200</td>
<td></td>
<td>85</td>
<td>60</td>
<td>80</td>
<td>80</td>
<td>87</td>
<td>75</td>
</tr>
<tr>
<td></td>
<td><strong>IC₅₀ value</strong></td>
<td></td>
<td>40</td>
<td>41</td>
<td>45</td>
<td>56</td>
<td>55</td>
<td>38</td>
</tr>
</tbody>
</table>

Table 6.2 *In vitro* cytotoxic property screening of *A. catechu* root

<table>
<thead>
<tr>
<th>Sl No</th>
<th>Concentration (µgL⁻¹)</th>
<th>Percentage of inhibition</th>
<th>Petroleum ether</th>
<th>Benzene</th>
<th>Chloroform</th>
<th>Acetone</th>
<th>Ethanol</th>
<th>Distilled water</th>
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<td>1.</td>
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<td>2</td>
<td>5</td>
<td>-</td>
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<tr>
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<tr>
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<tr>
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<td></td>
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<tr>
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<td>17</td>
<td>27</td>
<td>15</td>
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<tr>
<td></td>
<td><strong>IC₅₀ value</strong></td>
<td></td>
<td>7</td>
<td>10</td>
<td>3</td>
<td>10</td>
<td>14</td>
<td>7</td>
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</table>
6.3.3 Effect of *B.phoenicea* extracts on ascites tumour development

Animals of the control group survived only for a period of 16 ± 2 days. Treatment of *B.phoenicea* ethanolic and aqueous extracts at different concentrations increased the survival rate of animals (Table 6.3).

One way analysis of variance (ANOVA) was carried out for comparing number of days survived among different treatment groups. F-value was found to be significant at 0.01 level as the p-value is less than 0.01. This shows that there exists significant difference in the number of days survived among different treatment groups. Duncan Multiple range Test (DMRT) was carried out as post hoc analysis to find out which of the groups are homogeneous and which of them are significantly different. Results shows that number of days survived are not significantly different among treatment 3, 5 and 6. ie, at 100mg/kg concentration, both aqueous and ethanolic plant extracts are equally significant in action to the commercial drug Cyclophosphamide. And the number of days survived in these groups is significantly higher than the treatments in group1, 2, and 4. Treatment groups 2 and 4 shows no significant difference in the number of days survived. Number of days survived is significantly lower in the first group compared to all other groups.

From these results it is clear that the plant extracts are highly efficient as an antitumour agents, the percentage of increase in life span is increasing with the increase in concentration of the plant extract (Plates 14&15).
Table 6.3 *In vivo* anti-tumour activity efficiency of *B. phoenicea*

<table>
<thead>
<tr>
<th>Sl No:</th>
<th>Treatment</th>
<th>Number of days survived</th>
<th>% increase in life span.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>DLA cells alone</td>
<td>16.0 ± 2.09&lt;sup&gt;c&lt;/sup&gt;</td>
<td>-</td>
</tr>
<tr>
<td>2.</td>
<td>DLA+ <em>B. phoenicea</em> ethanolic extract (50 mg/kg body weight)</td>
<td>20.7 ± 3.01&lt;sup&gt;b&lt;/sup&gt;</td>
<td>28.75</td>
</tr>
<tr>
<td>3.</td>
<td>DLA+ <em>B. phoenicea</em> ethanolic extract (100 mg/kg body weight)</td>
<td>26.7 ± 2.80&lt;sup&gt;a&lt;/sup&gt;</td>
<td>66.25</td>
</tr>
<tr>
<td>4.</td>
<td>DLA+ <em>B. phoenicea</em> aqueous extract (50 mg/kg body weight)</td>
<td>22.8 ± 2.14&lt;sup&gt;b&lt;/sup&gt;</td>
<td>42.5</td>
</tr>
<tr>
<td>5.</td>
<td>DLA+ <em>B. phoenicea</em> aqueous extract (100 mg/kg body weight)</td>
<td>27.5 ± 2.07&lt;sup&gt;a&lt;/sup&gt;</td>
<td>71.9</td>
</tr>
<tr>
<td>6.</td>
<td>DLA+ Cyclophosphamide (10 mg/kg)</td>
<td>27.7 ± 2.07&lt;sup&gt;a&lt;/sup&gt;</td>
<td>72.5</td>
</tr>
</tbody>
</table>

**F-value** | 22.58**<sup>**</sup>  
**p-value** | < 0.001

**Significant at 0.01 level**  
*Means having same letter as superscript are homogeneous*
6.4 DISCUSSION

Medicinal plants are nature’s gift to human beings to lead a healthy, disease-free life. Most of these plants used today are believed to be much safer and proved as elixir in the treatment of various ailments. Plant derived compounds have played an important role in the development of several clinically useful anticancer agents (Smitha et al., 2014). Oxidative stress induced by an imbalance between production of ROS (Reactive Oxygen Species) and antioxidants are associated with pathogenic disease conditions like carcinogenesis (Niki and Noguchi, 2000). So, radical scavenging activity is very important in the searching of natural sources of cancer drugs.

Cytotoxicity is one of the chemotherapeutic targets of antitumour drugs (Suffness and Pezzuto, 1991). Most of the clinically proved anti-tumour agents possess significant cytotoxic activity in cell culture systems. The cytotoxic activity of B.phoenicea leaf extracts against DLA cell lines partially explains its significant anti-tumour activity. The drug shows toxicity towards the tumour cell line and not toxic to normal cells.

The anti-cancer activity was evaluated using ascites tumour model. Both ethanolic and aqueous extracts of B.phoenicea increased the life span of affected mice effectively. Highest activity was observed in aqueous extract.

The results of the in vitro cytotoxicity screening and antitumour studies of B.phoenicea shows that, it can act as a source of active compounds for the preparation of anti-cancer drugs. The presence of various secondary metabolites like alkaloids, saponins, phenols, steroids and flavonoids provides some scientific evidence for the biological activities and also account for the pharmacological uses. GCMS analysis of the leaf extract shows the presence of large number of compounds with proved medicinal uses. So this unravelled endemic medicinal plant will be a prominent contributor of medicinal compounds in the near future.
Plate 13: *In vitro* cyto-toxicity screening using Trypan blue exclusion method

a) *B.phoenicea* ethanol extract  
b) *B.phoenicea* aqueous extract  
c) *A.catechu* extract
Plate 14: *In vivo* anticancer property screening of *B. phoenicea* leaves

a) Cyclophosphamide treated group b) *B. phoenicea* aqueous extract treated group  
c) *B. phoenicea* ethanolic extract treated group
Plate 15: *In vivo* anticancer property screening of *B. phoenicea* leaves

(Observation of the mice models at the 11th day after inducing cancer)

a) Control group member  b) Cyclophosphamide treated mice

c) *B. phoenicea* aqueous extract treated mice  d) *B. phoenicea* ethanolic extract treated mice