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5.1. Introduction
Cancer is the largest single cause of death in humans and is a cellular malignancy that results in the loss of normal cell-cycle control, such as unregulated growth and the lack of differentiation, can develop in any tissue of any organ, and at any time. Most approaches used in cancer treatment, such as chemotherapy and radiation therapy, kill cancer cells by inducing apoptosis; however, cancer cells often develop resistance to these types of therapies. Apoptosis has been accepted as a fundamental component in the pathogenesis of cancer and the origin of cancer involves deregulated cellular proliferation and the suppression of apoptotic processes, ultimately leading to tumor establishment and growth. Furthermore, many cancer therapies indirectly activate apoptosis by chemically or physically damaging DNA. This may have the unintended effect of generating a pool of heavily mutated cancer cells which increases the chances of developing resistance (Hanahan and Weinberg, 2000; LaCasse et al., 1998; Evan and Vousden, 2001). Recently; resistance to anticancer drugs has also been observed. Therefore, the research and development of more effective and less toxic drugs has become necessary. Many substances derived from dietary or medicinal plants are known to be effective and versatile anticancer agents in a number of experimental models of cancer.

The search for potential anticancer agents from natural products dates back to centuries. Many plant products have proven to be an important source of anticancer drugs (Cragg and Newman, 2005). A number of mechanisms exist by which phytochemicals aid in the prevention of cancer. These mechanisms include antioxidant or free radical scavenging activity and induction of apoptosis. Insight into how apoptotic pathways are deregulated in cancer is therefore critical both to the understanding of how this disease develops and progresses, and for the development of effective and reliable anti-cancer treatments. Thus, therapies that directly and specifically activate apoptosis would be predicted to be both safer and more effective than existing therapies.

A wide variety of plant extracts and plant derived compounds especially terpenes, alkaloids and phenolics have recently aroused considerable interest because of their cancer preventative effects (Williams and Grayer, 2004). A number of mechanisms including apoptosis induction by these compounds for preventing cancer have been reported (Canivence-Lavier et al., 1996; Shih et al., 2000; Moon et al., 2006).
The present study was to evaluate the antitumour activity of *H. furcatus* flower, leaf and root bark extracts and their ability to induce apoptosis in mouse tumour cell lines as the mechanism of antitumor activity.

5.2. Materials and methods

5.2.1. Animals

Male swiss albino mice (20-25gm) were supplied by a small animal breeding station, College of Veterinary and Animal science, Mannuthy, Thrissur. The animals were maintained under sterilized environmental conditions (22 - 28°C, 60 - 70% relative humidity, 12 h dark/light cycle) and fed with standard rat feed (Lipton India Ltd) and water *ad libitum*. All animal experiments were conducted during the present study got prior permission from Institutional Animal Ethics Committee (IAEC) and followed the guidelines of IAEC.

5.2.2. Determination of *in vitro* cytotoxic activity

The extracts were dissolved in minimum volume of double distilled water and concentrations ranging from 10 to 1000 µg/ml were used for the assay. The cytotoxicity was determined by Trypan blue exclusion method (Babu *et al*, 1995) in mouse tumour cell lines Dalton’s lymphoma ascites cells (DLA) and Ehlicers ascites carcinoma cells (EAC). The procedure and requirements were described in Chapter 2 (2.2.5).

5.2.3 *In vivo* antitumor activity of *H. furcatus* extracts

The antitumour activity of *H. furcatus* leaf and root bark extracts were evaluated using Dalton’s lymphoma ascites cells (DLA) induced solid tumour and Ehlicers ascites carcinoma cells (EAC) induced ascites tumour models in Swiss Albino mice. For the experiments both the extracts were prepared in desired volume of double distilled water and used.

5.2.3.1. Effect of *H. furcatus* extracts in reducing Dalton’s Lymphoma Ascites (DLA) cell induced solid tumour.

DLA cells were aspirated from peritoneal cavity of the tumor bearing mice and 0.1 ml containing 10⁶ cells was injected intramuscularly into the right hind limb of all animals. Four groups (10 animals per each group) were used. Group 1 served as
untreated control and received only cell line, group 2 received cyclophosphamide 10 mg/kg body weight as standard reference drug, group 3 received *H. furcatus* extracts 50 mg/kg body weight, group 4 received *H. furcatus* extracts 250 mg/kg body weight. The ability of the extract to reduce tumor volume was compared with tumour volume of mice in group 1 (control group). The drug administration was continued for 10 consecutive days and diameter of the tumor was measured using a vernier caliper at fixed intervals (on each 3\textsuperscript{rd} day) for a period of 30 days and the volume was calculated using the formula,

\[
\text{Tumor volume} = \frac{4}{3} \pi r_1^2 x r_2 \quad \text{(were, } r_1 \text{ is the minor radius and } r_2 \text{ is the major radius)}
\]

The percentage of inhibition of tumor volume in animals =

\[
\frac{\text{Tumor volume of Control on 30\textsuperscript{th} Day} - \text{Tumor Volume of Treated on 30\textsuperscript{th} day}}{\text{Tumor volume of Control on 30\textsuperscript{th} Day}} \times 100
\]

5.2.3.2. Effect of *H. furcatus* extracts on Ehrlich ascites Carcinoma (EAC) cell induced ascites tumour

EAC cells were aspirated from peritoneal cavity of the tumor bearing mice and 0.1 ml containing $10^6$ cells was injected intraperitoneally into all animals. Four groups (10 animals per each group) were used. Group 1 served as untreated control and received only cell line, group 2 received cyclophosphamide 10 mg/kg body weight as standard reference drug, group 3 received *H. furcatus* extracts 50 mg/kg body weight, group 4 received *H. furcatus* extracts 250 mg/kg body weight. The drug administration was continued for 10 consecutive days. The animals were observed for the development of ascites tumor and death due to tumor burden was recorded for 35 consecutive days. The ability of the extract to increase the survival of tumour bearing mice were compared with the survival of mice in group 1 (control group). The life span of animals was calculated using the formula, \(\% \text{ ILS} = \frac{(T - C)}{C} \times 100\), where \(T\) and \(C\) are mean survival of treated and control mice respectively (Mazumdar UK, 1997).

5.2.3.4. Induction of apoptosis by *H. furcatus* extracts in DLA and EAC cell lines

Apoptosis of DLA and EAC cells were assessed by analyzing changes in cell morphology and DNA fragmentation after cell incubation with the presence and absence of extracts (2.2.6 of chapter 2). Morphologically, cells undergoing apoptosis were identified by the following parameters like membrane blebbing, vacuole formation, nuclear condensation and cellular fragmentation into apoptotic bodies.
These changes were readily observed under microscope by preparing slides stained with Geimsa and May Grunwald.

Briefly, for apoptotic assay, the cells were plated at a density of $5 \times 10^6$ in 3 ml culture vials containing 1.5 ml DMEM supplemented with 10% FBS, and antibiotics (100 U/ml benzylpencillin and 100 µg/ml streptomycin). The extract was dissolved in minimum of DMSO and the volume is made up to desired concentrations. The concentrations of extract added were 10 and 50 µg/ml (leaf extract) and 1 and 5 µg/ml (root bark extract). After 24 hrs incubation in a humidified atmosphere at 37°C, the cells were harvested and following characteristics of apoptosis were evaluated.

DNA fragmentation was done by agarose gel electrophoresis. In brief, DNA from control (untreated) and extract treated cells was isolated. Cells were washed in PBS and pellets were lysed (0.2% Triton X-100; 10 mM Tris- HCl, pH 7.4, 10 mM EDTA) for 15 min in ice. Centrifuge at 12,000g at 4°C for 20 min and collect the supernatant, which contains the DNA. Add RNase A to a final concentration of 100 µg/mL and incubate at 37°C for 1 h. An equal volume of saturated phenol, chloroform and isoamyl alcohol (25:24:1) mixture was added. The mixture was subjected to centrifugation 12,000 RPM for 3 min. The above step was repeated four times using the supernatant obtained and to the final supernatant, an equal volume of chloroform: isoamyl alcohol (24:1) was added. Later, precipitate the DNA by adding 25–30 µL of 5 M NaCl to a final concentration of 300 mM and add 2–2.5 volume of ice-cold 100% ethanol. Leave overnight at −20°C. Centrifuge the sample at 12,000 RPM for 30 min at room temperature. Carefully aspirate the ethanol off and wash the DNA pellet with 1 mL of 70% ethanol. Centrifuge sample at 12,000 RPM for 20 min at room temperature. The pellet obtained was dissolved in Tris-EDTA (TE) buffer. DNA was estimated by recording the absorbance at 260 nm. DNA was electrophoresed on 2% agarose gel at 100 V using TBE buffer. DNA was visualized by incorporation of ethidium bromide (0.5mg/ml) in the gel during casting and viewed under UV illumination.

5.3. Statistical analysis

The values were expressed in Mean ± SD, for 7 animals in each group. All groups were analysed for one way anova by Dunnetts test using GraphPad Instat software. The groups with $p<0.05$ were considered significant.
5.4. Results

5.4.1. Cytotoxicity of *H. furcatus* extracts

The flower, leaves and root bark extracts were tested *in vitro* for their toxic effect on mouse cancer cell lines, DLA and EAC. The results summarized in table 5.1 show that the extracts exhibited varying effect on these cell lines. The flower extract didn’t show any cytotoxic effect on these cell lines up to 2 mg/ml concentration. While the leaf and root bark extract showed cytotoxicity on these cell lines. The cytotoxic effect appeared to be concentration-dependent, increasing as the concentration of extract increased. The root bark extract possessed high cytotoxic potential with least IC$_{50}$ values than the leaf extract.

5.4.2. Antitumour activity of *H. furcatus* leaf extract

Administration of the leaf extract for a period of 10 days in DLA tumor bearing mice led to a significant reduction in tumor volume when compared with tumor controls. The reduction in tumour volume was found to be dose dependent when the extract was administrated simultaneously with tumour inoculation. There is about 50.65 % reduction in 50 mg/kg and 65.53 % reduction in tumour volume of animals administered with 250 mg/kg body wt (Table 5.2; Fig 5.1). Of these the higher dosage showed a reduction in tumour volume near to that of standard reference drug cyclophosphamide, which showed 72.58 % only. The effect of the extract in inhibiting the ascites tumour burden and increasing the life span of animals were found to be dose dependent. The extract showed increase in survival of animals up to 44.86 % and 58.37 % for 50 mg/kg and 250 mg/kg body weight treated animals (Table 5.3).

To elucidate the mechanism associated with the antitumour activity the apoptotic assay was done in DLA and EAC cells. This was studied by examining the cells morphological changes and DNA fragmentation. The effect of the extract on the morphological changes in untreated and treated cells with 10 and 50 μg/mL extract for 24 h were analyzed by light microscopy after Geimsa and May Grunwald staining. The extract treated cells showed membrane blebbing, vacuole formation and nuclear condensation, which was not seen in the untreated DLA and EAC cells (Fig 5.3). The DNA fragmentation analysis done by electrophoresis (Fig 5.5).
5.4.3. Antitumour activity of *H. furcatus* root bark extract

The treatment with root bark extract in mice led to a significant reduction in tumor volume when compared with control animals. The administration of the extract with tumour inoculation dose dependently reduced the tumour volume in mice. The percentage of inhibition was found to be 58.74% reduction in 50 mg/kg and 73.62% in 250 mg/kg body treated animals (Table 5.4; Fig 5.2). The 250 mg/kg body treated animals showed a reduction in tumour volume slightly higher than that of standard reference drug cyclophosphamide, which showed 72.58% reduction. The effect of the extract in inhibiting the ascites tumour burden and increasing the life span of animals were also found to be dose dependent. The extract showed increase in survival of animals up to 59.45% and 75.62% for 50 mg/kg and 250 mg/kg body weight treated animals (Table 5.5). The increase in life span associated with the administration of 250 mg/kg body weight extract was higher than that of the cyclophosphamide (75.67%) treated animals.

The mechanism associated with the antitumour activity is evaluated by apoptotic assay, done in DLA and EAC cells. This was studied by examining the changes in cells morphology and DNA fragmentation. The effect of the extract on the morphological changes in untreated and treated cells with 1 and 5 μg/mL extract for 24 h were analyzed by light microscopy after Geimsa and May Grunwald staining. Compared to the untreated DLA and EAC cells (Fig 5.4) cells, the extract treated cells showed membrane blebbing, vacuole formation and nuclear condensation. The DNA fragmentation analysis done by electrophoresis also showed ladder pattern characteristics to apoptosis in both cells (Fig 5.6).

5.5. Discussion

Medicinal plants and their phytochemicals, are reported to possess substantial antioxidant, anti-inflammatory and anticarcinogenic activities. These phytochemicals have been explored extensively for their potential in the treatment of cancer. Well-known compounds like paclitaxel, a diterpenoid from *Taxus brevifolia*, and vincristine, an alkaloid from *Catharanthus roseus* (Wall and Wani, 1996), semi-synthetic derivatives (Topotecan and Irinotecan) of alkaloid camptothecin from *Camptotheca acuminate* (Rahier et al., 2005) are used in chemotherapy today. *Hibiscus* species has been used in traditional and folklore medicines in India for the treatment various ailments (Bindu et al., 1997) and studied extensively for their
pharmacological properties (Lin et al., 2007; Prenesti et al., 2007; Wang et al., 2000; Lee et al., 2007; Rosa et al, 2007; Venkatesh et al., 2008). However the antitumour activity of H. furcatus has not yet been investigated.

The cytotoxicity analysis on mouse tumour cell lines revealed high killing ability of the extracts, especially root bark extract with minimum concentration of drug. Present study also revealed the in vivo tumour controlling ability of leaf and root bark extract. As the administration of extract simultaneous with tumour inoculation, as determined for its tumour preventive ability, showed a remarkable decrease in tumor volume of mice. As with ascites tumour model the extracts had a significant increase in the survival of mice which is comparable to that of standard drug cyclophoshamide. The results with both the extracts has shown their in vitro and in vivo effect on both the tumour cell lines, while the effect was higher with root bark extract and which also possessed high cytotoxicity with least IC\textsubscript{50} values.

In order to study the mechanism of antitumour activity, the ability of the extracts can inducing apoptosis were studied in DLA and EAC cells. Apoptosis is an active regulatory cellular response to certain stimuli, such as loss of trophic factor support (serum deprivation) or exposure to cytotoxic agents (chemotherapeutic drugs), that results in a somewhat stereotyped cell death (Clarke, 1990; Steller, 1995; Thompson, 1995; Vaux, 1993), including membrane blebbing, nuclear condensation, and the formation of apoptotic bodies. A biochemical hallmark of apoptosis is the cleavage of chromatin into small fragments, including oligonucleosomes, that when seen in electrophoresed gels are described as DNA ladders (Wyllie, 1980; Zakeri et al., 1993). During apoptosis a part of the chromatin can be cut between nucleosomes at the linker sites (Arends et al., 1990), producing oligomers that are multiples of about 180 bp (nucleosomal units) which, on agarose gels, appear as characteristic DNA ladders. Although the appearance of a DNA ladder is a good marker of apoptotic cell death (Arends et al., 1990; Montague et al., 1999; Bursch et al., 1990; Gavrieli et al., 1992). The cells treated with both leaf and root bark extracts acquired apoptotic morphological features. The extracted DNA showed a ladder pattern when analyzed by gel electrophoresis.

In conclusion leaf and root bark extracts of H. furcatus was confirmed to exhibit anticancer activities. Their cytotoxic assay and in vivo antitumour activity revealed that these extracts contains anticancer compounds, which exhibits the cytotoxicity against mouse cancer cell lines and are capable of reducing tumour in
mice. The extracts also induced apoptosis in both the cells. However, further studies will be needed to identify the exact molecule for this proposed antitumour activity.
Table: 5.1. *In vitro* cytotoxic activity of *H. furcatus* extracts

<table>
<thead>
<tr>
<th>Extracts</th>
<th>*IC50 Value (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>H. furcatus</em> leaf extract</td>
<td></td>
</tr>
<tr>
<td>1. DLA cells</td>
<td>90 µg/ml</td>
</tr>
<tr>
<td>2. EAC cells</td>
<td>91 µg/ml</td>
</tr>
<tr>
<td><em>H. furcatus</em> root bark extract</td>
<td></td>
</tr>
<tr>
<td>1. DLA cells</td>
<td>13 µg/ml</td>
</tr>
<tr>
<td>2. EAC cells</td>
<td>19 µg/ml</td>
</tr>
</tbody>
</table>

*IC50 Value is the amount of extract needed to cause 50% of cell death

Table: 5.2. Effect of simultaneous administration of *H. furcatus* leaf extract in reducing tumour volume of DLA induced solid tumor bearing mice

<table>
<thead>
<tr>
<th>Groups</th>
<th>Tumor volume in cm³ (30 days after tumor inoculation)</th>
<th>Percentage of inhibition of tumor volume after 30days</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>3.83 ± 0.21</td>
<td>---</td>
</tr>
<tr>
<td>Cyclophosphamide 10 mg/kg</td>
<td>1.05 ± 0.19a</td>
<td>72.58</td>
</tr>
<tr>
<td>Leaf extract 50 mg/kg</td>
<td>1.89 ± 0.08a</td>
<td>50.65</td>
</tr>
<tr>
<td>Leaf extract 250 mg/kg</td>
<td>1.32 ± 0.16a</td>
<td>65.53</td>
</tr>
</tbody>
</table>

Values are mean ± SD, for 10 animals in each group. ‘a’ *p < 0.01; ‘b’ *p < 0.05*, when compared to normal

Table: 5.3. Effect of *H. furcatus* leaf extract in increasing the life span of EAC induced ascites tumor bearing mice

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No: of animals developed tumor</th>
<th>No. of days survived</th>
<th>% increase in life span (%ILS)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>10/10</td>
<td>18.5 ± 3.65</td>
<td>-----</td>
</tr>
<tr>
<td>Cyclophosphamide 10 mg/kg</td>
<td>10/10</td>
<td>31.5 ± 4.97a</td>
<td>70.27</td>
</tr>
<tr>
<td>Leaf extract 50 mg/kg</td>
<td>10/10</td>
<td>26.8 ± 5.27a</td>
<td>44.86</td>
</tr>
<tr>
<td>Leaf extract 250 mg/kg</td>
<td>10/10</td>
<td>29.3 ± 3.19a</td>
<td>58.37</td>
</tr>
</tbody>
</table>

Values are mean ± SD, for 6 animals in each group. ‘a’, *p < 0.01; ‘b’, *p < 0.05*, when compared to normal
Table: 5.4. Effect of *H. furcatus* root bark extract in reducing tumour volume of DLA induced solid tumor bearing mice

<table>
<thead>
<tr>
<th>Groups</th>
<th>Tumor volume in cm$^3$ (30 days after tumor inoculation)</th>
<th>Percentage of inhibition of tumor volume after 30 days</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>3.83 ± 0.21</td>
<td>---</td>
</tr>
<tr>
<td>Cyclophosphamide 10 mg/kg</td>
<td>1.05 ± 0.19$^a$</td>
<td>72.58</td>
</tr>
<tr>
<td>Root bark extract 50 mg/kg</td>
<td>1.58 ± 0.16$^a$</td>
<td>58.74</td>
</tr>
<tr>
<td>Root bark extract 250 mg/kg</td>
<td>1.01 ± 0.32$^a$</td>
<td>73.62</td>
</tr>
</tbody>
</table>

Values are mean ± SD, for 10 animals in each group. ‘a’ $p < 0.01$; ‘b’ $p < 0.05$, when compared to normal.

Table: 5.5. Effect of *H. furcatus* root bark extract in increasing the life span of EAC induced ascites tumor bearing mice

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No: of animals developed tumor</th>
<th>No. of days survived</th>
<th>% increase in life span (%ILS)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>10/10</td>
<td>18.5 ± 3.65</td>
<td>------</td>
</tr>
<tr>
<td>Cyclophosphamide 10 mg/kg</td>
<td>10/10</td>
<td>31.5 ± 4.97$^a$</td>
<td>70.27</td>
</tr>
<tr>
<td>Root bark extract 50 mg/kg</td>
<td>10/10</td>
<td>30.0 ± 2.44$^a$</td>
<td>59.45</td>
</tr>
<tr>
<td>Root bark extract 250 mg/kg</td>
<td>10/10</td>
<td>32.5 ± 1.71$^a$</td>
<td>75.67</td>
</tr>
</tbody>
</table>

Values are mean ± SD, for 6 animals in each group. ‘a’, $p < 0.01$; ‘b’, $p < 0.05$, when compared to control.
Fig: 5.1. Effect of *H. furcatus* leaf extract in reducing tumor volume of DLA induced solid tumor bearing mice

![Graph showing the effect of *H. furcatus* leaf extract on tumor volume.](image)

Fig: 5.2. Effect of simultaneous administration of *H. furcatus* root bark extract in reducing tumor volume of DLA induced solid tumor bearing mice

![Graph showing the effect of *H. furcatus* root bark extract on tumor volume.](image)
Fig: 5.3. Morphology of DLA and EAC cells after apoptosis assay with leaf extract

- Untreated DLA cells
- Untreated EAC cells
- DLA cells treated with 10 μg/ml extract
- EAC cells treated with 10 μg/ml extract
- DLA cells treated with 50 μg/ml extract
- EAC cells treated with 50 μg/ml extract
Fig: 5.4. Morphology of DLA and EAC cells after apoptosis assay with root bark extract

Untreated DLA cells

Untreated EAC cells

DLA cells treated with 1μg/ml extract

EAC cells treated with 1μg/ml extract

DLA cells treated with 5 μg/ml extract

EAC cells treated with 5 μg/ml extract
Fig: 5.5 DNA fragmentation by leaf extract in DLA and EAC cells

Lane 1: Untreated DLA cells
Lane 2: 10 μg/ml extract treated cells
Lane 3: 50 μg/ml extract treated cells

Lane 1: Untreated EAC cells
Lane 2: 10 μg/ml extract treated cells
Lane 3: 50 μg/ml extract treated cells
Fig: 5.6 DNA fragmentation by root bark extract in DLA and EAC cells

Lane 1: Untreated DLA cells
Lane 2: 1 μg/ml extract treated cells
Lane 3: 5 μg/ml extract treated cells

Lane 1: Untreated EAC cells
Lane 2: 1 μg/ml extract treated cells
Lane 3: 5 μg/ml extract treated cells