CHAPTER VI

IN VITRO ANTICANCER ACTIVITY

6.1 INTRODUCTION

It is long-established that plants possess diverse principles, which are of immense nutritional and therapeutic value. Numerous anticancer screening studies have been conducted using traditional medicinal plants with a view to discover new therapeutic agents that are devoid of toxic side effects generally known to be associated with current chemotherapeutic agents. Currently, research is focused more towards developing anticancer drugs and their derivatives from natural sources, especially phytochemicals with improved anticancer potential and minimal systemic toxicity with known mechanism of action. Recent interest in these secondary metabolites has been focused upon their medicinal properties. Studies of plant metabolites have produced the most compelling data for the antitumor activities in various types of cancers, and several metabolites have been shown to inhibit cancer development while exhibiting antioxidant activities in various animal models. Furthermore, some studies suggest that the most promising use of these compounds may be as an adjuvant to currently used therapies. Among the main constituents in *J. tanjorensis* the selected plant drug aglycone flavonoids, flavone glycosides and alkaloids have attracted a special attention. Flavonoids possess numerous physiological activities which are well known and have been extensively studied. Clinical experiments on flavonoids have shown its potent activity against various diseases (Kris-Etherton et al 2004). Flavonoids are well known for its natural non-toxic antioxidant, free radical scavenging and chelating properties, this makes flavonoids as potential bioactive molecules in combating diseases mediated by free radicals such as ischemia, anemia, arthritis, asbestosis and its related secondary diseases like cancer.
Cancer cytotoxicity studies on *J. tanjorensis* has not been carried out so far. Therefore, the present work was aimed to investigate the cytotoxic potential of MEJT against a panel of human and murine tumor cell lines and to elucidate its underlying relative mechanisms using a dual staining method *in vitro*. Present study showcases through various *in vitro* assays remarkable potential role of methanolic extract of *J. tanjorensis* leaves in inhibiting the proliferation of cancer cells mainly Ehrlich Ascites Carcinoma (EAC), Skin carcinoma (A431) and Human epithelial colorectal adenocarcinoma cells (Caco-2).

6.2 MATERIALS AND METHODS

6.2.1 *In vitro* anticancer activity

A431, Ehrlich Ascites Carcinoma (EAC) and Human epithelial colorectal adenocarcinoma cells (Caco-2) were grown and maintained in RPMI-1640 and DMEM high glucose media respectively supplemented with 2nM L-glutamine, 20% heat-inactivated fetal bovine serum (FBS), 1% penicillin and streptomycin at 37°C with 5% CO₂ until 80% confluence level is reached. EAC cells are suspension cell lines hence direct collection using centrifugation at 200 g for 10 min were made, on the other hand Caco-2 cells are adherent type cell lines hence trypsin was used to break the cell protein interactions with surface of culture flask, detachment of cells from flask surface was observed under inverted microscope, upon complete detachment, trypsin inactivation was carried out by adding DMEM with 20% FBS followed by centrifugation at same condition. Cells counted and 0.1 x 10⁶ units were treated with various concentrations of methanolic extract for 48 h under 37°C and 5% CO₂ in CO₂ incubator. MTT assay was performed in accordance with standard textual method (Mossman, 1983). After treating
cells with different concentrations of methanolic extract, absorbance was read at 590 nm using Epoch microplate spectrophotometer (BioTek, USA). Every experiment included a set of negative control (untreated cells) as well as positive control (Cyclophosphamide). All experiments were performed in triplicate. The results obtained were calculated and presented as a percentage of control values.

6.2.2 Double staining fluorescence based apoptosis study

1 x 10^3 EAC and Caco-2 cells were treated with various concentrations of methanolic extract of *J. tanjorensis* for 72 h at 37°C in 5% carbon dioxide. Cells were harvested and stained with 1:1 concentration of acridine orange and ethidium bromide (100 µg/mL) and were incubated further for 15 min at 37°C in 5% carbon dioxide. Cells were then studied with a fluorescence microscope (Axio Scope.A1, Carl Zeiss, Germany) where viable cells will display a green (Acridine orange) fluorescence staining. Early apoptotic cells will show yellow staining in combination with the presence of apoptotic bodies, late apoptotic cells will give a red staining (McGahon et al., 1995 and Liegler et al. 1995). ProgRes software was used to analyze the data. Integrated LED fluorescence illumination served as light source. Green filter was used for acridine orange with 500nm as excitation and 526nm as emission and ethidium bromide red filter was set at 510nm for excitation with emission at 595nm.

6.3 RESULTS AND DISCUSSIONS

Present study clearly demonstrated that *J. tanjorensis* extracts contain several classes of plant flavonoids with anticancer & antioxidant potentials. Mostly flavonoid glycosides and aglycones, as well as biflavonoids were identified in the
methanol extract. Although several publications were focusing on this topic (Silva et al., 2008), there is still a lack of convincing evidence to determine which flavonoid class is mostly responsible for the antioxidant and anticancer activity in the plant drug sources. Hence in the present work attempts were made to detect flavonoids belonging to various classes and their anticancer & anti-oxidant potentials were evaluated.

6.3.1 In vitro anticancer potential

Di Carlo (1999) reports that hydroxyl moieties and 2–3 double bonds are important structural features of flavonoids that are associated with its biochemical and biological activities. Several classes of phytochemicals have been shown to induce apoptosis in cell lines. Anticancer potential of flavonoids may be attributed to the presence of various hydroxyl groups and the degrees of glycosidic linkages. There are many modes of action by which phytochemicals inhibit the growth of cancerous cells, such as initiation of apoptosis through different intracellular cell signaling pathways. Anticancer efficacy of flavonoids rich methanolic extract of J. tanjorensis was evaluated through in vitro cytotoxicity assay against EAC and Caco-2 using MTT reagent. MTT assay has been extensively used to determine the cytotoxic potential of plant extracts against various cancerous cells. The tetrazolium salt 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) is used to determine the cell viability in assays of cell proliferation and cytotoxicity. MTT facilitates this determination, as this gets reduced to yield an insoluble purple formazan product in metabolically active cells. Methanolic extract revealed potent cytotoxic activity against both the cancer cells with an IC\(_{50}\) value 14.57 and 21.00 µg/mL for EAC and Caco-2 respectively. Results are presented in Table 21.
Table 21. Cytotoxic effect of *J. tanjorensis* leaves methanolic extract against Ehrlich Ascites Carcinoma (EAC) and Human epithelial colorectal adenocarcinoma cells (Caco-2).

<table>
<thead>
<tr>
<th>Activity</th>
<th>EAC</th>
<th>Caco-2</th>
</tr>
</thead>
<tbody>
<tr>
<td>IC₅₀ (µg/mL)</td>
<td>14.57</td>
<td>21.0</td>
</tr>
</tbody>
</table>

Each fraction showed almost similar results. They revealed moderate to high activity. EAC cells when treated with different concentration of each collected methanolic extract fraction showed contrasting results from that of antioxidant findings where later fractions are showing better cytotoxic action and comparatively less antioxidant activity. Comparatively fraction D and fraction C showed the highest anticancer activity. Results are presented in Table 22.

Table 22. Efficacy of MEJT fractions against EAC (MTT assay). CPA was used as a positive control

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Fraction</th>
<th>IC₅₀ in µg/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>A</td>
<td>162.4</td>
</tr>
<tr>
<td>2</td>
<td>B</td>
<td>20.13</td>
</tr>
<tr>
<td>3</td>
<td>C</td>
<td>11.38</td>
</tr>
<tr>
<td><strong>4</strong></td>
<td><strong>D</strong></td>
<td><strong>8.03</strong></td>
</tr>
<tr>
<td>5</td>
<td>E</td>
<td>20.84</td>
</tr>
<tr>
<td>6</td>
<td>F</td>
<td>54.53</td>
</tr>
</tbody>
</table>

All fractions were subjected to the cytotoxicity activity against EAC using MTT reagent. Results obtained clearly proved that aglycones and anthocyanidins (kaempferol, Chrysoeriol, Baicalein, 3’,7-Dimethoxy-3-hydroxyflavone and Peonidin) has potent anticancer property than its related flavonoid sugars with various degree of C and O linkages.
It was also noted that fractions containing mono-glycoside flavonoids [Frc D, E and F] and with more di-C-glycosides (Frc C) has shown better cytotoxicity effect than the fractions containing di-glycosides (C-O and O-O linkages). Although Fraction B do contain di-glycosides, but the linkage is through C-O and O-O di-glycoside which may be the reason for decreased cytotoxic potency. IC\textsubscript{50} values of each fraction were calculated using Graphpad Prism software.

6.3.2 Double staining with Acridine orange and Ethidium bromide

Mechanism of cell death induced was assessed normally through morphological changes by staining cells with fluorescent dyes, including acridine orange and ethidium bromide. To understand the mechanism of cell death induced by \textit{J. tanjorensis}, methanolic extract treated and untreated EAC and Caco-2 cells were incubated with acridine orange and ethidium bromide (1:1) at a final concentration of 100μg/mL (AO/EB dual staining). Acridine orange is a vital dye that will stain both live and dead cells, it intercalates into DNA, making it appear bright green (an emission maximum at 526 nm) and binds to cytoplasmic RNA staining it faintly orange. Thus a viable cell will have bright green chromatin in its nucleus and slightly orange cytoplasm. Ethidium bromide is only taken up by nonviable cells or cells undergoing appotosis that have lost their membrane integrity. It intercalates into DNA, making it appear bright red/orange (an emission maximum at 595 nm), but binds only weakly to RNA, which may appear slightly red. Thus a dead cell will have bright red/orange chromatin and its cytoplasm, where ethidium bromide bright red overwhelms the acridine slight orange fluorescence (McGahon et al., 1995).

Two different concentrations were chosen for EAC cells based on the IC\textsubscript{50} values determined by MTT assay, which were 10 and 20 μg/mL for EAC and 20 and 30 μg/mL
for Caco-2 cell line. As a control, both cell lines were cultured in complete media comprising sufficient components of a growth media and stained with AO/EB (Figure 19A and 19A’).

Figure 19. Morphological observation with acridine orange and ethidium bromide (AO/EB) staining at actual magnification 40x. EAC cells (A) and Caco-2 cells (A’) were treated without and with *J. tanjorensis* methanolic extract, 10 μg/mL (B), 20 μg/mL (C), 20 μg/mL (B’) and 30 μg/mL (C’) for 72 h. Each experiment was performed in triplicate (n=3) and generated similar morphological features.
Figure 19B, B’ and 19C, C’ shows that the methanolic extract from *J. tanjorensis* induced apoptosis on both cells. Cells stained with Acridine orange-ethidium bromide showed green fluorescent representing **viable cells**, yellow staining representing **early apoptotic cells**, whereas, reddish or orange staining represented **late apoptotic cells**. Apoptotic cells can be recognized by a set of morphological features that include loss of cell volume (Lockshin and Beaulaton 1981), blebbing of the plasma membrane and compaction of chromatin into dense masses that lie at the periphery of the nucleus or, in other cases, condensation of the entire nucleus into a dense ball with the chromatin distributed evenly throughout the nucleus (Wyllie et al., 1980). As shown in Figure 19B and 19B’, EAC and Caco-2 cells treated with 10 μg/mL and 20 μg/mL respectively of methanolic extract showed changes in cellular morphology like cell shrinkage, membrane blebbing, chromosomal condensation and nuclear fragmentation. On the other hand, Figures 19C and 19C’ showed similar features for cells treated with 10 μg/mL and 20 μg/mL of methanolic extract (Figure 19B and 19B’), but with extra features of late stage apoptotic activity with apoptotic bodies. When cells were treated with 20 μg/mL (EAC) and 30 μg/mL (Caco-2) of methanolic extract of *J. tanjorensis*, were characterized by the condensation and the fragmentation of nuclei with increased brightness.

During apoptosis (programmed cell death), the cell's cytoskeleton breaks up and causes the membrane to bulge outward (Vermeulen et al., 2002). These bulges may separate from the cell, taking a portion of cytoplasm with them, to become known as **apoptotic bodies** as clearly seen in figure 19B, B’, C and C’. This morphological features of treated cancer cells revealed that a stronger apoptosis signal was induced with higher concentration of *J. tanjorensis* extract. This induction of apoptotic signal through *J.
*J. tanjorensis* leaves extract is evidenced and supported by the *in silico* studies also and contributes in providing scientific evidences for the traditional uses of *J. tanjorensis* in tumors.

### 6.4 CONCLUSION

Methanolic extract when subjected to *in vitro* cytotoxic assay exhibited potent anticancer. Present work suggests that the methanolic extract of *J. tanjorensis* induced anticancer activity through an intrinsic apoptotic pathway in EAC and Caco-2 cells. Methanolic extract fractions of *J. tanjorensis* have shown potent anticancer property as proved by MTT bioassay (highest cytotoxicity with IC$_{50}$ of 8.03 µg/mL) against EAC cells. The present investigation constitutes the first comprehensive report on the constituents and anticancer activity of the methanolic extract of *J. tanjorensis* and contributes in providing scientific evidences for the traditional claims of selected plant drug *J. tanjorensis.*