Chapter 8
Summary & Conclusion

Cancer is one of the leading causes of death. Several chemotherapeutical agents have been used to treat cancer, and yet a convincing cure is elusive in most cases. Therefore, there is continued need for development of new anticancer agents. Angiogenesis is one of the vital events for organ development and differentiation during embryogenesis as well as wound healing and reproductive functions in adults. Angiogenesis also contributes significantly to tumour growth and metastasis. Currently, search for effective anti-angiogenesis agents and therapy is an emerging field. Cytotoxic agents with anti-angiogenic activity are designated to control tumour endothelial cell growth and tumour cell survival. Therefore, combinatorial therapy in which anti-angiogenic agents with chemotherapeutic activity administrated in a scheduled manner could result in a much more favourable therapeutic effect. India is one of the richest biodiversity centres with respect to medicinal plants. Many plants have been utilized in the traditional system of medicine for cancer treatment. Plant derived compounds have played an important role in the development of several clinically useful antitumour agents. Lignans are a diverse group of natural products and are considered as potential anticancer agents.

The current study investigated the in vitro cytotoxic potential of petroleum ether and ethanol extracts of four indigenous medicinal plants (J. simplex, M. malabaricum, L. quinqueflora M. smilacifolium) using Brine shrimp lethality bioassay, Trypan blue dye exclusion assay, Antimitotic assay, Micronucleus assay and MTT assay (Breast cancer (MCF 7, MDAMB 231, T47D) cervical cancer (HeLa) and brain glioma (C6) cell lines). A methodological evaluation of detailed cytotoxicity, DNA damaging and anti-proliferative effects in human cancer cells revealed that all the four plant extracts exhibited a dose-dependent cytotoxicity and antiproliferative /cell growth inhibitory activity. Petroleum ether extracts of J. simplex exhibited relatively potent cytotoxic activity in all the preliminary assays when compared with the other selected plants. Therefore, further studies were conducted on this plant.
Acute toxicity studies and the in vivo antitumour studies using EAC induced liquid and DLA induced solid tumour were conducted on Swiss albino mouse. Acute toxicity studies proved that the plant extract is safe up to a dose of 2000 mg/kg. EAC treated groups exhibited a dose-dependent decrease in antitumour activity. JSPE 200 mg/kg was capable of increasing the life span and the mean survival time (32 days), which was comparable with the cisplatin treated group (36 days). The tumour growth responses such as tumour weight, tumour volume, packed cell volume and viable cells decreased with treatment and also showed an increase in non-viable cells. The haematological parameters revealed that the extract, at a dose of 200 mg/kg, was capable of reversing the parameters like Hb, RBC, WBC, especially neutrophils and monocytes to normal. Biochemical parameters revealed the effectiveness of the plant extracts on total protein, albumin, ALT, AST and ALP were also comparable with the standard. Organ weights of the treated group were similar to that of the standard treated group.

Effect of different doses of JSPE and JSOH on solid tumour development, percentage inhibition of tumour growth and histopathological studies were also assessed. JSPE 200 mg/kg exhibited 71.44% inhibition in tumour growth, whereas in the standard drug (cisplatin) caused an inhibition of 95%. Histopathological studies revealed the presence of fibrous cells. Absence of necrosis, inflammatory changes and blood vessels in the high dose treated group proved its efficacy as an anticancer agent. Hence we selected petroleum ether extract of J. simplex for the isolation of the active compounds.

*J. simplex* (bulk quantity 20 kg) was extracted with petrol ether (60-80°C), in batches of 150 g each. Column chromatographic separation (Silica gel column) of individual component was done by gradient elution technique (petroleum ether, Benzene, Chloroform and Methanol); identical fractions were combined based on TLC profile (exposures to UV light and iodine vapour was used for visualization). Crystallization was attempted on the combined fractions using petrol ether. Resultant products were subjected to chemical as well as spectral analysis. The following compounds were identified.
1. Methyl ester of hederagenin
2. 2-Nonadecanone
3. Triacontanoic ester of 5’-hydroxyjustisolin
4. Valeric acid ester of olean-5, 12- dien-1β, 3β, 11α, 28β- tetraol
5. Undecanoic ester of stigmasterol.

Triacontanoic ester of 5-hydroxyjustisolin, methyl ester of hederagenin and valeric acid ester of olean-5, 12- dien-1β, 3β, 11α, 28 β- tetraol were isolated from this species for the first time and are new to the best of our knowledge.

In order to find out the isolated compounds, which are responsible for the cytotoxic action JSPE 03, JSPE 23, JSPE 28 and JSPE 47 were assessed by MTT assay, in both HeLa and MCF 7 cell lines reported the best activity in crude extracts. Though all four compounds exhibited cytotoxicity, JSPE 23 caused the most activity with an IC₅₀ value of 11.852 and 15.15 µg/mL respectively for HeLa and MCF 7 human cancer cell lines. The current study also revealed that, JSPE 23 mediated suppressions in cell viability and growth against normal epithelial cells (HaCaT, immortalized cells) decreased when compared with HeLa and MCF 7 cell line. In HaCat cell line, JSPE 23 showed an IC₅₀ value of 40.97 µg/mL, which indicated the tumour cell specific action of the compound.

The agents, which are capable of inducing selective apoptosis in cancer cells, have received considerable attention in the development of novel cancer chemotherapeutic drugs. The ability of compounds to induce apoptosis was investigated by Acridine orange/ ethidium bromide assay and FITC Annexin V/PI apoptosis assay. The study revealed that isolated and characterised compounds produced apoptosis, and not necrosis, in both MCF 7 and HeLa cells. JSPE 23 showed maximum dead cells both in HeLa and MCF 7 in the Acridine orange/ ethidium bromide assay. Annexin V- FITC apoptosis exhibited that the isolated compounds significantly induced apoptosis both early and late apoptosis against HeLa cells. Total % live cells in the treated group were
less than the control group (89.0%). The total dead cells in the JSPE 23 treated group was 27.20. Therefore, JSPE 23 was selected for further studies.

Activation of cell cycle plays an important role in the regulation of apoptosis and can occur at specific stages of the cell cycle. The DNA content and Cell Cycle Distribution analysis by flow cytometry revealed that the treatment of HeLa cells with JSPE 23 significantly increased the sub G0 phase and showed a cell cycle arrest at G0/G1 phase. Relatively diminished S phase, and G2/M phase indicated the inhibition of DNA replication.

Bcl-2 family includes key regulators of apoptosis and is over expressed in many types of cancer. Reduced Bcl-2 gene expression may promote apoptotic response to anticancer drugs by inhibiting the release of cytochrome c, Apaf-1 and caspase. Thereby increased expression of Bcl-2 causes anti-apoptotic effect, leads to resistance to chemotherapeutic drugs and radiations therapy. Therefore, down-regulation of Bcl-2 by JSPE 23 naturally decreases the resistances towards chemotherapeutic agents. The gene expression studies of JSPE 23 on Bax showed an up-regulation of pro-apoptotic gene Bax. This favours apoptosis by stimulating the release of cytochrome c followed by caspase-3. ELISA test also revealed the direct stimulation of JSPE 23 on caspase-3 enzyme, thus promoting apoptosis.

VEGF (Vascular endothelial growth factor) expression studies on DLA inoculated solid tumour tissues, proved the down-regulation of the same, resulted angiogenic inhibition, which plays an important role in metastasis. Further the activity was confirmed by triacontanoic ester of 5′-hydroxyjustisolin on L929 fibroblast cells using the scratch assay, which showed that there was no significant reduction in wound area, whereas in control group the wound area diminished from 138 to 95 after 48 h of treatment, indicating the inhibition of cell migration and thereby proved as an antiangiogenic agent and prevents metastasis.

IC₅₀ value of the most active extract, the petroleum ether extract of J. simplex was found to be 42.80 µg/mL in HeLa cell line, also increased the mean life span and exhibited 71% of tumour inhibition. The active component isolated (JSPE 23) from the
same could bring down the IC₅₀ values to 11.82 µg/mL. It leads to the conclusion that this is the compound with best activity, contributing to the maximum activity of the extract. In the future for the standardization of the extract for the anticancer activity, this compound could be used as the marker.

In a nutshell, it can be mentioned that the isolated and completely characterized triacontanoic ester of 5”-hydroxyjustisolin heralds a great promise as an anticancer agent, in view of its low IC₅₀ value, coupled with promotion of apoptosis, prevention of metastasis and chemotherapeutic resistance.

**FUTURE DIRECTION**

Promising findings have been obtained during the studies conducted by us, which needs further improvements in terms of long term toxicity and efficacy in many other cell lines. The anticancer activity demonstrated by the extract both in liquid and solid tumour, supported by the presence of active components needs to be further confirmed in nude mice for different types of cancers, and can be extended to clinical studies, if found highly encouraging.