SUMMARY
Cancer is a disease of complex etiology, defined as uncontrolled growth of cells. It is one of the major problems globally, both in developed and developing countries. India is in an epidemiological transition phase and cancer is now one of the leading causes of morbidity and mortality. Medical treatments for cancer viz., surgery, radiotherapy and chemotherapy are yet hard to achieve complete removal of malignant tissue because cancer can return, grow and spread again so large that it may not be curable. Thus, the management of cancer is still not up to the mark and there is still need to search better drugs for the effective management of disease. Natural products, especially plant-based products have frequently been examined as anti-cancer agents. Several plant-derived compounds are successfully employed in cancer treatment e.g., Paclitaxel, Camptothecin, Vincristine, Vinblastine, Vinleurosine, Vinrosidine and Podophyllotoxin. Still, there exists high hope for effective treatment of different cancers by systematic screening of a variety of natural products. Ayurveda, the ancient Indian science of health, is based on natural products including several phytochemicals for the treatment of a variety of diseases. Thus, it provides an important clue to select plant for modern drug discovery. In addition, there are reports to show that plant extracts display anti-tumor/anti-cancer/anti-proliferative effects on cultured human cancer/tumor cell lines. It suggests that there is an urgent need to explore more plants for their therapeutic potential against cancer. In view of the above, the present study has been planned to study anti-cancer effects of *Erythrina suberosa* Roxb. and *Anagallis arvensis* L. which have medicinal value in Ayurveda and are still unexplored for their anticancer potential.

The main objectives of current research are:

- Estimation of anticarcinogenic activity of different isolates of *Erythrina suberosa* Roxb. and *Anagallis arvensis* L. using:
  - Sulforhodamine B Assay
  - MTT assay
  - BrdU incorporation assay
  - *In vivo* studies using murine models:
    - Ehrlich Ascites Carcinoma (EAC)
    - Sarcoma-180 (Ascites)
    - Sarcoma-180 (Solid)
• Lymphoid leukemia (L1210)

 ➢ Mechanistic studies of promising isolates using different techniques:
  • Microscopic studies
    ▪ Light microscopy
    ▪ Fluorescence microscopy
    ▪ Scanning electron microscopy
  • Cell cycle analysis
  • Annexin V/PI labelling
  • Changes in Bcl-2 levels
  • Change in mitochondrial membrane potential ($\Delta \psi_m$)
  • Changes in cytochrome c levels
  • Reactive Oxygen Species (ROS) detection
  • Nitric oxide (NO) production
  • Estimation of Caspases activity
  • DNA fragmentation assay

_Erythrina suberosa_ Roxb. belongs to the family Fabaceae. Plants of the genus are known to have cytotoxic activity. In India, _E. suberosa_ Roxb. has been used as a very important medicinal plant for the treatment of various ailments. The ethanol extract of the leaves has been reported to have anti-tumor activity. Stem and stem bark of _E. suberosa_ were used in the present study to determine their anticancer potential. Three extracts (95% alcoholic, 50% alcoholic and aqueous) from each of stem and stem bark were prepared and evaluated against a panel of human cancer cell lines viz. HCT-15, COLO-205, SW-620 (Colon), DU-145 (Prostate) and MCF-7 (Breast) at 100 µg/ml for 48 h. 95% Alcoholic extract from both the parts were observed to be cytotoxic. 50% Alcoholic and aqueous extract from both the parts showed negligible activity against above stated cell lines at 100 µg/ml concentration. Therefore 95% alcoholic extract of _E. suberosa_ stem bark (ESB) and 95% alcoholic extract of _E. suberosa_ stem (ESS) were identified for further cytotoxicity studies to have better insight into their cytotoxic potential. ESB and ESS were further evaluated at 10, 30, 100 µg/ml and 25, 50, 100 µg/ml respectively against sixteen human cancer cell lines of nine different tissues viz., Colon (502713, COLO-205, HCT-15, SW-620), Lung (A-549, HOP-62), Prostrate (DU-145, PC-3), Breast (MCF-7, T-47D) Ovary (IGR-OV-1, OVCAR-5) Neuroblastoma
(IMR-32), CNS (SF-295), Liver (HEP-2) and Renal (786-0) in time dependant manner (24 and 48 h). It was seen that ESB showed 17-68% cytotoxicity against all the sixteen cell lines used in the study at 100 µg/ml concentration after 24 h incubation. At 10 and 30 µg/ml, cytotoxicity ranged between 0-28% and 4-51% respectively. It was further observed that IC_{50} values were in the range of 32.9 to >100 µg/ml. At 48 h, ESB showed more than 70% cytotoxicity, at 100 µg/ml, against eleven out of sixteen cell lines in the range of 74-100%. Against rest of five cell lines, viz. T-47D, IGR-OV-1, OVCAR-5, 786-0 and IMR-32, activity was found to be between 47-64%. Furthermore ESB showed very prominent activity against 502713, COLO-205, HCT-15, HOP-62, DU-145, PC-3, SF-295 and HEP-2 at lower concentration of 30 µg/ml as the cytotoxicity was between 44-61% against these cell lines. At 10 µg/ml, cytotoxicity against all the cell lines ranged between 0-59%. IC_{50} values were in the range of 22.9 to >100 µg/ml. The in vitro cytotoxicity data showed that the alcoholic extract exhibited the effect in a dose and time dependent manner. The cytotoxicity varied from cell line to cell line and this specificity is expected to be due to different molecular characteristics of these cells. 5-Flourouracil, adriamycin, mitomycin C and paclitaxel were used as positive controls depending on cell lines. Cytotoxicity of ESB against normal human lung fibroblast (WI-38 and MRC-5) and monkey kidney (CV-1) cell lines were also evaluated and it was observed that cytotoxic effect of ESB was negligible, indicating that cytotoxic potential of extract is specific to cancer cell lines. ESB was further subjected to column chromatography and the fractions were tested for their cytotoxic effect.

ESB was also evaluated for its in vivo anticancer potential against Ehrlich ascites carcinoma (EAC) and Lymphoid leukemia tumor models in mice. ESB showed 30.48% and 32.38% tumor growth inhibition against EAC at 125 mg/kg and 150 mg/kg i.p respectively. The Median survival time (T/C) was 103.44% at 200 mg/kg i.p. in Lymphoid leukemia (L1210) model.

The apoptosis-inducing effect of ESB was investigated using human promyelocytic leukemia HL60 cells. Cell viability was estimated by MTT assay and the IC_{50} was found to be 64.9, 45.9, and 39.6 µg/ml after 24, 48, and 72 h of ESB treatment, respectively. ESB inhibited cell proliferation in a dose- and time-dependent manner. A series of well-documented morphological changes, such as cell shrinkage, condensation of nuclear chromatin, and nuclear fragmentation,
were observed by fluorescence microscopy. Scanning electron microscopy was done on HL-60, COLO-205 and SW-620 cells. The scanning electron micrographs showed apoptotic bodies and formation of blebs. Cell cycle analysis showed a significant increase in Sub G₀ population of cells from 6.0% to 46.0% when treated with 0-100 µg/ml. ESB treatment resulted in a dose-dependent increase in annexin V positive cells from 3.44% in control to 26.69% at 75 µg/ml. At 100 µg/ml there was 22.57% apoptotic population with slight increase in necrotic cells (12.52%). Depletion in Bcl-2 levels upto 72% was observed. Dissipation of mitochondrial membrane potential of intact cells (3.4% - 80.1%) accompanied by increase in cytosolic cytochrome c was observed, which was followed by activation of caspase-9 and -3 but not caspase-8. Concentration and time dependant DNA fragmentation analysis revealed typical ladders.

Increase in intracellular ROS production up to six fold was detected in ESB-treated HL60 cells by DCFH-DA assay. As the exposure of ESB was further increased from 12 to 24 h, gradual increase in ROS production was detected. The study thus showed that ESB induced apoptosis through mitochondrial (intrinsic) pathway.

It was seen that growth inhibition ranged from 4-56% at 100 µg/ml against all the sixteen cell lines used in study after 24 h incubation. At 50 µg/ml cytotoxicity ranged between 0-28%. At 25 µg/ml concentration, cytotoxicity ranged between 0-21%. IC₅₀ values were in the range of 90.6 to >100 µg/ml. At 48 h ESS showed more than 70% cytotoxicity against four cell lines out of sixteen cell lines at 100 µg/ml. Against rest of twelve cell lines activity lied between 21-68%. At 50 µg/ml, cytotoxicity ranged between 8-45%. At 25 µg/ml the cytotoxicity observed was between 0-23%. 5-Fluouracil, Adriamycin, mitomycin C and paclitaxel were used as positive controls depending on cell lines. IC₅₀ values calculated was in the range of 56.9 to >100 µg/ml. There was gradual decline in IC₅₀ values against all the cell lines in a time dependent manner from 24-48 h. At 24 h ≥100 µg/ml IC₅₀ was observed in case of fifteen out of sixteen cell lines. At 48h ≥100 µg/ml IC₅₀ was observed in case of five out of sixteen cell lines. Cytotoxicity of ESS against normal human lung fibroblast (WI-38 and MRC-5) and monkey kidney (CV-1) cell lines were also evaluated and it was observed that cytotoxic effect of ESS was negligible, indicating that cytotoxic potential of extract is specific to cancer cell lines.

ESS was evaluated for its in vivo anticancer potential against Sarcoma-
ESS showed 34.55% and 42.80% tumor growth inhibition against Sarcoma-180 (Ascites) at 200 mg/kg and 300 mg/kg i.p. dose level respectively. The Median survival time (T/C) was 91.95 % at 200 mg/kg i.p. in Lymphoid leukemia (L1210) model.

Likewise, the apoptosis-inducing effect of ESS was investigated using human promyelocytic leukemia HL60 cells. Cell viability was calculated by MTT assay in a dose- and time-dependent manner. By AO/EtBr staining it was observed that dose dependant increase in apoptotic cells after treatment with ESS in HL-60 cells. The scanning electron micrographs also showed apoptotic bodies and formation of blebs in HL-60 cells. Cell cycle analysis showed a significant increase in Sub G<sub>0</sub> population of cells and dose-dependent increase in annexin V positive cells. A moderate increase in intracellular ROS production was detected in ESS-treated HL60 cells by DCFH-DA assay. A depletion in Bcl-2 levels up to 49% was observed. Dissipation of mitochondrial membrane potential of intact cells accompanied by increase in cytosolic cytochrome c was observed, which was followed by activation of caspases in a time dependant manner. DNA ladder analysis showed fragmentation of DNA in concentration dependant manner. There was 3 fold increase in caspase 9 cleavation after 24h incubation, which further activates caspase 3 to 1.8 fold. Caspase 8 was increased to 1.5 fold only after 24 h incubation. The study thus showed that ESB had better activity than ESS.

Anagallis arvensis L. belongs to the family Primulaceae and its whole plant extract is reported to have antifungal activities. The main ingredients of Anagallis arvensis L. are Saponins, Triterpenoids, Flavanoids, Arvenins and Tannins. In the present study, whole plant of Anagallis arvensis L. has been used. Three extracts (95% alcoholic, 50% alcoholic and aqueous) from whole plant of A. arvensis were prepared and evaluated against a panel of human cancer cell lines viz. 502713, COLO205, HCT-15, SW-620 (Colon), DU-145 (Prostate) and MCF-7 (Breast) at 100 µg/ml for 48 h. It was observed that there was 70% growth inhibition in four out of fifteen cell lines after 24 h incubation. In total, cytotoxicity ranged between 10-85% against all the cell lines used in the study. At 30 µg/ml, cytotoxicity ranged between 0-74%. 95% alcoholic extract showed more prominent activity against A-549, MCF-7, 786-0 and T-47D, which occurred in the range of 40-74%. At10 µg/ml, cytotoxicity ranged
between 0 to 33%. At 48h 95% alcoholic extract showed more than 70% cytotoxicity against ten out of fifteen cell lines, which were in the range of 73-96%. Against rest of five cell lines activity lied between 14-69%. 95% Alcoholic extract showed a very prominent activity against A-549, HOP-62, MCF-7, T-47D, IGR-OV-1, OVCAR-5, 786-0 and PC-3 at lower concentration of 30 µg/ml, as the cytotoxicity was between 40-84% against these cell lines. At 10 µg/ml cytotoxicity was observed to be between 1-54%. As incubation time was increased to 72h, more than 70% cytotoxicity was observed in eleven out of fifteen cell lines in the study. Cytotoxicity was found to be between 67-98%. 95% Alcoholic extract showed very prominent activity against A-549, HOP-62, MCF-7, T-47D, IGR-OV-1, OVCAR-5, PC-3, HEP-2 and 786-0 cell lines at lower concentration of 30 µg/ml as the cytotoxicity was observed to be between 44-84% against these cell lines. At 10 µg/ml cytotoxicity ranged between 4-64%. Thus in vitro results indicated time and concentration dependent increase in cytotoxicity against cancer cell lines. 5-Flurouracil, adriamycin, mitomycin C and paclitaxel were used as positive controls depending on cell lines. There was a gradual decline in IC₅₀ values against twelve cell lines as the time of incubation was increased from 24-72 h. At 24h IC₅₀ values against these cell lines were between 25.1 to >100 µg/ml and at 48h it declined to 6.0–54.3 µg/ml. At 72h it further decreased from 1.24–45.7 µg/ml. 95% Alcoholic extract was least sensitive against HCT-15, SW-620 and IMR-32 as they showed IC₅₀ values more than 100 µg/ml even after 72h exposure. Cytotoxicity of 95% alcoholic extract against normal human lung fibroblast (WI-38 and MRC-5) and monkey kidney (CV-1) cell lines were also evaluated and it was observed that cytotoxic effect of AAE was negligible, indicating that cytotoxic potential of extract is specific to cancer cell lines.

50% alcoholic extract of A. arvensis showed more than 70% growth inhibition against five out of fifteen cell lines at 24h. In total cytotoxicity ranged between 5-90% against all the cell lines. At 30 µg/ml concentration, cytotoxicity ranged between 0-55%. 50% alcoholic extract showed more prominent (55%) activity against IGR-OV-1 cell line. At 10 µg/ml cytotoxicity was seen to be between 0-25%. At 48h 50% alcoholic extract showed more than 70% cytotoxicity against ten cell lines out of fifteen in the range of 70-94%. Against rest of five cell lines activity lied between 44-65%. 50% alcoholic extract showed very prominent activity against COLO-205, HOP-62, IGR-OV-1 and 786-0 at lower concentration of 30 µg/ml as the cytotoxicity was between 45-66% against these
cell lines. At 10 µg/ml cytotoxicity occurred in the range between 0-57%. As incubation time was increased to 72 h, more than 70% cytotoxicity was observed in thirteen out of fifteen cell lines and cytotoxicity ranged between 73-99%. 50% alcoholic extract showed very prominent activity against COLO-205, SW-620, HOP-62, T-47D, DU-145 IGR-OV-1, PC-3 and 786-0 at lower concentration of 30 µg/ml, as the cytotoxicity was in the range 51-81% against these cell lines. At 10 µg/ml cytotoxicity was observed to be between 4-77%. The *in vitro* results indicated concentration and time dependent increase in cytotoxicity against cancer cell lines. 5-Flourouracil, adriamycin, mitomycin C and paclitaxel were used as positive controls depending on cell lines. Here too, there was a gradual decline in IC$_{50}$ values against all the cell lines in a time dependent manner from 24-72 h. At 24 h IC$_{50}$ values against these cell lines were between 31.4 to >100 µg/ml and at 48 h it varied from 8.2 to >100 µg/ml. At 72 h it further decreased from 2.3 to 94.5 µg/ml. 50% alcoholic extract was least sensitive against IMR-32 as it showed IC$_{50}$ value more than 100 µg/ml even at 48 h exposure and at 72 h it showed 94.5 µg/ml IC$_{50}$ value. Cytotoxicity of 50% alcoholic extract against normal lung fibroblast cell lines (WI-38 and MRC-5) and monkey kidney (CV-1) was also evaluated and it was observed that cytotoxic effect of 50% alcoholic extract was more pronounced and specific to cancer cell lines. Further studies were done with 95% alcoholic extract of *A. arvensis* (AAE).

AAE was evaluated for its *in vivo* anticancer potential against Ehrlich Ascites Carcinoma (EAC), Sarcoma-180 (Solid) and Lymphoid Leukemia tumor models in mice. AAE showed 40.92 % and 87.92 % tumor growth inhibition against EAC at 3 mg/kg and 5 mg/kg i.p. doses respectively. Median survival time (T/C) was 124.02 % at 4 mg/kg i.p. against Lymphoid leukemia (L1210) model. AAE showed 1.70 % and 38.34 % tumor growth inhibition against Sarcoma-180 (Solid) at 3 mg/kg and 5 mg/kg i.p. dose level respectively.

Apoptotic potential of AAE was studied using HL60 cells. Cell viability was calculated by MTT assay in a dose- and time-dependent manner. A series of well-documented morphological changes, such as cell shrinkage, condensation of nuclear chromatin, and nuclear fragmentation, were observed by fluorescence microscopy in HL-60 cells. The features characteristic to apoptosis was observed in HL-60 and A-549 cells treated with AAE. Cell cycle analysis showed a significant
increase in Sub G₀ population of cells from 5.4% in control cells to 39.9% at 20 µg/ml. AAE caused externalization of phosphatidylserine in concentration dependant manner. Increase in intracellular ROS production up to six fold was detected in ESB-treated HL60 cells by DCFH-DA assay. Depletion in Bcl-2 level upto 62% was observed. Mitochondrial membrane depolarization and increase in cytosolic cytochrome c was observed, which was followed by activation of caspase-9, -3 and -6 but cleavage of caspase-8 decreased at higher time periods after initial increase. DNA fragmentation was also observed on agarose gel at 5µ g/ml, which further pronounced at higher concentration of 10 and 15 µg/ml. Intracellular ROS levels were significantly increased to 2.6 fold after 10 µg/ml AAE treatment for first 4h. As the exposure of AAE was further increased from 6 to 12 h, gradual increase in ROS production was detected. The study thus showed that AAE induced apoptosis through mitochondrial (intrinsic) pathway.
Conclusions:

- 95% Alcoholic extract from stem bark (ESB) and stem (ESS) of *E. suberosa* showed promising cytotoxic effect in concentration and time dependent manner against a panel of sixteen human cancer cell lines of various tissue origins.
- ESB induced apoptosis in human leukemia (HL-60) cells through mitochondrial dependent pathway with significant ROS generation.
- ESS induced apoptosis in human leukemia (HL-60) cells through mitochondrial dependent pathway with moderate increase in caspase activity.
- *Erythrina suberosa* stem bark extract had better activity than the stem extract.
- AAE showed *in vitro* cytotoxic effect against a panel of sixteen human cancer cell lines of various tissues origin in concentration and time dependent manner.
- AAE induced apoptosis in HL-60 cells through intrinsic/mitochondrial dependent apoptotic pathway with fair increase in cytochrome c in cytosol and ROS production.
- ESB, ESS and AAE showed relatively much lower activity against normal human lung (MRC-5, WI-38) and monkey kidney (CV-1) cell lines as compared to human cancer cell lines.
- As observed *in vivo* studies, both ESB and AAE have the anticancer potential.

The above studies clearly demonstrated the anticancer potential of stem bark of *E. suberosa*. The chemical constituents responsible for anticancer activity are non-polar and are present in alcoholic extract. The studies also demonstrated the cytotoxic potential of 95% alcoholic extract of whole plant of *A. arvensis* with mitochondrial dependent apoptotic potential. Anticancer and apoptotic potential of 95% alcoholic extract from stem bark of *E. suberosa* and 95% alcoholic extract from whole plant of *A. arvensis* has promising avenues for targeting the mitochondrial target based novel anti-cancer
therapeutics. It can be concluded that *E. suberosa* and *A. arvensis* could be explored as novel source of anticancer agent(s).