SUMMARY AND CONCLUSION

Nature has been a source of medicinal agents for thousands of years and an impressive number of modern drugs have been isolated from natural sources, many based on their use in traditional medicine. Determining the biological properties of plants used in traditional medicine is helpful to the rural communities and informal settlements. Several authors are currently being undertaken to isolate the active compounds from the plant species that showed high biological activity during screening. Therefore, these scientific investigations may be utilized to develop drugs from plant to treat the various diseases. Secondary metabolites of medicinal plants are recognized as sources of natural antioxidants that can protect from oxidative stress and thus play an important role in the chemoprevention of diseases that have their etiology and pathophysiology in reactive oxygen species.

The medicinal properties of folk plants are mainly attributed to the presence of flavonoids, but may also be influenced by other organic and inorganic compounds. Plants accumulate secondary metabolites through evolution as a natural means of surviving in a hostile environment. Herbal medicines derived from plant extracts are being increasingly utilized to treat a wide variety of clinical diseases. There is a growing interest in the pharmacological evaluation of various plants used in Indian traditional system of medicine.

Two medicinal plants namely Cynodon dactylon (Cd) and Terminalia catappa (Tc) were selected for the present study in order to provide systematic study on the antioxidative, antitumorigenic and immunomodulatory effects of their flavonoid constituents.

The research entitled, "Antioxidative, antitumorigenic and immunomodulatory efficacy of flavonoid fractions of Cynodon dactylon
and *Terminalia catappa* leaves in experimentally implanted ELA cells in Swiss albino mice” was carried out and the results are summarized in this chapter.

The study was carried out in five phases.

The first phase involved the extraction of flavonoids, assessment of total flavonoid content in different organic extracts and the selection of flavonoid fractions for the further *in vitro* and *in vivo* studies. In the second phase, the *in vitro* free radical scavenging activity of CdFf and TcFF and the selection of the dose of flavonoid fraction of each plant that showed minimum concentration for 50 per cent cytotoxic activity to ELA tumor cells (EC$_{50}$) were carried out. In the third phase, antioxidative and the antitumorigenic role of CdFf and TcFf were evaluated in ELA induced Swiss albino mice. In the fourth phase, immunomodulatory role of CdFf and TcFf were evaluated in SRBC/ELA tumor induced and Carbon ink induced Swiss albino mice.In the last phase, selected flavonoid fractions of Cd and Tc were characterized by color reactions of flavonoids, chromatographic, spectral analysis and *in silico* docking by GLIDE.

In the Phase I, extraction of flavonoids and quantification of total flavonoid content of Cd and Tc using the organic solvents such as methanol, ethanol and ethyl acetate were carried out. The total flavonoid content of flavonoid fractions of Cd and Tc were found to be 12.17 mg/g leaf and 14.28 mg/g leaf in methanol, 14.51 mg/g leaf and 15.98 mg/g leaf in ethanol and 20 mg/g leaf and 18.02 mg/g leaf in ethyl acetate respectively. Total flavonoid content of Cd and Tc flavonoid fractions were found to be more in ethyl acetate fraction than that of methanol and ethanol fractions. So, the ethyl acetate fraction of *Cynodon dactylon* (CdFf) and *Terminalia catappa* (TcFf) were chosen for the following *in vitro* and *in vivo* studies.

In Phase II, the effect of CdFf and TcFf was assessed on the scavenging of DPPH, NO and H$_2$O$_2$ and compared with the standard BHT. Scavenging of DPPH by CdFf and TcFf was found to be dose dependent and the fifty percent
inhibitory concentration (IC\textsubscript{50}) was found to be 44.9 µg of total flavonoid and 50 µg total flavonoid respectively. Scavenging of NO by CdFf and TcFf was found to be dose dependent with the IC\textsubscript{50} values of 43 µg of total flavonoid and 37 µg of total flavonoid respectively. Similar trend was noticed on the scavenging H\textsubscript{2}O\textsubscript{2} also and the IC\textsubscript{50} was found to be 40 µg of total flavonoid and 43 µg of total flavonoid of CdFf and TcFf respectively. The above IC\textsubscript{50} of CdFf and TcFf were found to be lower than that of the reference compound BHT (75 µg). These results showed the more effective antioxidative potential of CdFf and TcFf than that of BHT.

\textit{In vitro} antitumorigenic effect of flavonoid fractions of Cd and Tc to ELA tumor cells was evaluated by cytotoxic studies using trypan blue exclusion assay. The antitumorigenic effect was found to be dose dependent and the 50 per cent effective concentration (EC\textsubscript{50}) was found to be at 80 µg for CdFf and at 75 µg for TcFf.

In phase III, the antitumorigenic effect of CdFf and TcFf were evaluated by the assessment of the liver marker enzymes, enzymic and non-enzymic antioxidants and TBARS along with the histological analysis of the liver compared to the standard antioxidant, silymarin after 15, 30, 45 and 60 days of the experimental tenure in the 9 groups (group 1 - PBS; group 2 - Paraffin oil; group 3 - DMSO; group 4 - Silymarin; group 5 - CdFf; group 6 - TcFf; group 7 - ELA+CdFf; group 8 - ELA+TcFf and group 9 - ELA) with six Swiss albino mice in each.

The liver marker enzymes namely AST, ALT and ALP were analysed in all the experimental animals to assess the normal functioning of the liver. The activities of all the three liver marker enzymes were found to be significantly increased in ELA tumor induced mice. The increase in the activities of these enzymes in serum might be due to hepatocellular damage which result in the leakage of cytosolic enzymes into the circulation. The activities of the liver test enzymes AST, ALT and ALP were found to be significantly decreased by the
administration of CdFf, TcFf and silymarin. The coadministration of CdFf and TcFf to ELA tumor induced mice showed a significant decrease in the activities of the above enzymes. Activity of the liver function test enzymes reflected the toxicity in ELA induced mice and this was reverted back towards the normal levels by the administration of CdFf and TcFf to ELA induced mice. The above observations of the present study could be attributed to the significant protective effect of the CdFf and TcFf and established the normal functioning of the liver.

Intraperitoneal administration of ELA altered the antioxidant balance of the mice liver and thereby significantly decreased the activities of enzymic antioxidants CAT, SOD, GPx and also the levels of non enzymic antioxidants Vitamin A, E and GSH. CdFf and TcFf administered individually and to ELA tumor induced mice significantly enhanced the enzymic and non enzymic antioxidants status in all treatment periods and this was found to be more significant than that found in silymarin administration. Enzymic and non enzymic antioxidants can protect the cells by quenching the free radicals generated by ELA tumor cells. These results indicated the antioxidative potential of CdFf and TcFf.

Lipid peroxidation status as assessed by the levels of TBARS was found to be significantly increased in the liver of mice transplanted with ELA tumor cells and decreased by the administration of CdFf, TcFf and silymarin and coadministration of CdFf and TcFf to ELA tumor cell treated group. Increase in the concentration of TBARS observed in tumor induced mice is the indication of membrane damage. Thus, it could be inferred that CdFf and TcFf would have strengthened the endogenous antioxidant defense from ROS ravage and restored the optimal balance by neutralizing the ROS. Administration of CdFf and TcFf individually and to the ELA tumor induced mice showed significant decrease in the rate of lipidperoxidation and showed their antilipidperoxidative role.

To follow the antitumor activity of CdFf and TcFf, the in vivo cytotoxic study was carried out in ELA tumor bearing mice treated with the flavonoid
Results and Discussion

Compatibility of Pongamia pinnata biofuel / diesel blends with few industrial metals fractions of Cd and Tc. ELA tumor bearing mice showed an average life span of 19 days whereas administration of CdFf and TcFf to ELA transplanted mice increased the average life span to 60 days and 46 days respectively and indicated their antitumorigenic effect.

Histological examination of the liver of control groups, silymarin, CdFf and TcFf treated groups exhibited normal architecture in all the 3 zones - portal tracts, sinusoids, Kupffer cells and central veins. The histological observations in the ELA treated mice showed severe necrosis, with the peripheral rim of surviving liver cells with focal stasis and balloon degeneration. Liver of ELA+CdFf and ELA+TcFf treated mice showed normal architecture. These observations indicated the reversal of ELA induced membrane damage by the coadministration of CdFf and TcFf. The above results of the present investigation clearly revealed the antioxidative and antitumorigenic role of flavonoid fractions of the Cd and Tc. The histological studies also supported the antioxidative and antitumor activity of CdFf and TcFf in ELA tumor induced Swiss albino mice.

In phase IV, the immunomodulatory role of CdFf and TcFf was evaluated by assessing the humoral immunity, cell mediated immunity and cell mediated non specific immunity.

Humoral immune response of CdFf and TcFf was evaluated in 13 groups of mice (group 1 - PBS; group 2 - Paraffin oil; group 3 - DMSO; group 4 - CdFf; group 5 - TcFf; group 6 - CdFf+Pyrogallol; group 7 - TcFf+Pyrogallol; group 8 - ELA; group 9 - ELA+Pyrogallol; group 10 - ELA+CdFf; group 11 - ELA+TcFf; group 12 - ELA+CdFf+Pyrogallol and group 13 - ELA+TcFf+Pyrogallol) by assessing the haemagglutination antibody titre in the blood of SRBC immunized mice against the immunosuppressor pyrogallol and ELA tumor induced mice. Administration of CdFf and TcFf individually, Coadministration of CdFf and TcFf to pyrogallol, ELA and ELA+pyrogallol treated mice significantly increased the antibody titre in both the primary and
secondary immune responses and reflected their role in the stimulation of humoral immune response.

Effect of CdFf and TcFf on cell mediated immunity was evaluated by assessing the lymphoid organs weight and total leukocyte count in the serum of the above 13 groups of mice and delayed type hypersensitivity in the serum of 5 groups of mice.

Administration of CdFf and TcFf individually, coadministration of CdFf and TcFf to pyrogallol, ELA and ELA+pyrogallol induced mice showed significant increase in the weight of spleen and thymus on 8th day except the spleen weight of ELA +TcFf group of mice when compared to their controls. The weight of spleen and thymus showed no significant difference (except the weight of thymus of TcFf treated mice) on 15th day when compared to 8th day. The above results indicated the stimulation of cell mediated immunity by CdFf and TcFf even in the presence of immune suppressor pyrogallol and ELA tumor cells.

Total leukocyte count was increased in animals treated with CdFf and TcFf on 8th day when compared to the PBS, DMSO and pyrogallol treated mice. The WBC count was found to be increased significantly by the administration of CdFf and TcFf individually and coadministered to pyrogallol and ELA +pyrogallol administered mice in all the treatment periods when compared to pyrogallol and ELA+pyrogallol treated mice. CdFf, TcFf and TcFf+ pyrogallol showed significant increase in the total leucocyte counts on 15th day when compared to the 8th day treatment period.

In the present investigation, SRBC induced delayed type hypersensitivity (DTH) was used to assess the effect of CdFf and TcFf on cell mediated immunity. CdFf and TcFf significantly inhibited SRBC induced DTH response by decreasing the edema size of the mice when compared to pyrogallol treated group and revealed their immunostimulatory effect.
The cell mediated non specific phagocytic activity of CdFf and TcFf were assessed by carbon clearance test in Swiss albino mice. Significant increase in phagocytic index was observed in mice treated with CdFf and TcFf when compared to the control group. The increase in phagocytic index indicated their role in innate immunostimulation.

In phase V, characterization of flavonoid fractions of Cd and Tc was carried out. Analysis of flavonoid constituents of CdFf and TcFf showed the presence flavonones, flavones, flavonols, flavanols and catechin and in addition CdFf showed the presence of chalcones and isoflavones.

The HPTLC analysis of flavonoid fractions of Cd and Tc against the standard quercetin confirmed the presence of quercetin as one of the major flavonoid.

The HPLC analysis of CdFf and TcFf against the standard flavonoids catechin, quercetin and gallic acid showed a major peak which relatively corresponds to the retention time and lambda max of quercetin and TcFf also showed another peak which relatively corresponds to the retention time and lambda max of catechin.

The FT-IR and $^{13}$C-NMR spectra revealed that quercetin is one of the major component in the flavonoid fractions of Cd and Tc. Further studies are warranted to characterize the other flavonoids in CdFf and TcFf.

Since, quercetin is the major constituent of both CdFf and TcFf, it was docked with the active site residues of target protein tubulin (1Z2B). Quercetin showed high score and interaction with the target protein tubulin at the active site residues of tubulin which inturn induces confirmational changes and inhibited microtubule formation. The assessment of ADME properties of quercetin performed using QikProp program showed the drugable property, drug solubility and permeability and also obeyed the Lipinsky rule. So, the quercetin from flavonoid fractions of Cd and Tc can be used as potent
anticancer agent. Further docking studies have to be carried out for designing future pharmaceuticals.

**Conclusion**

To conclude the major flavonoid constituents of CdFf and TcFf offered protective effect against ELA tumor by their *in vitro* and *in vivo* antioxidant and antitumorigenic potential, immunostimulatory role in humoral, cell mediated and non specific immune responses and in the inhibition of microtubule formation. So, CdFf and TcFf can be recommended as antioxidant to the individuals under oxidative stress and individuals suffering from diseases caused by oxidative stress.

**Recommendations for future study**

- Structural elucidation of all the bioactive flavonoids and their mechanism of actions
- Attempt on clinical trials to develop anticancer drug using CdFf and TcFf
- Assessment of other haematological parameters-neutrophil, T and B lymphocytes, Eosinophils, Basophils and Macrophages
- Screening of the mechanism of apoptosis by flavonoid fractions of *Cynodon dactylon* and *Terminalia catappa* in different types of cancer