CHAPTER 8

Summary & Conclusions
SUMMARY AND CONCLUSIONS

During past half a century, scientific community has made an enormous advancement in the identification of latent etiological factors of cancer, particularly its environmental causes, and has imparted rather lucid insights into its mechanism of action. Cancer is believed not to be a single disease but rather a conglomeration of several diseases. It is the uncontrolled growth and spread of cells that may affect almost any tissue of the body. More than 10 million people are diagnosed with cancer worldwide every year. It is estimated that there will be 15 million new cases by the year 2020. Cancer causes six million deaths every year accounting to 12% of the deaths worldwide. Carcinogenesis is a multistage process consisting of apparently three major steps: initiation, promotion and progression driven by genetic and epigenetic alterations that disrupt the regulatory pathways controlling cellular proliferation, programmed cell death (apoptosis), angiogenesis and differentiation.

Utilization of complementary and alternative medicine (CAM) is gaining more popularity as an important and promising strategy for the treatment of disease. With the growing awareness about the potential role of CAM in preventing cancer and other chronic disease, most of the research work in this research area which was ignored for a long time is now focused on validating any of the ancient remedies. Both epidemiological and experimental studies suggest that enormous benefit might be derived from dietary manipulation or supplementation because of their relatively low cost and little or virtually non toxic effects. Chemoprevention is being appreciated as a plausible strategy for the management of neoplasm. Centuries old proverb in English, “An ounce of prevention is worth a pound of cure”, though not said for chemoprevention, is probably the basis behind this emerging concept in cancer management. Chemoprevention, a promising strategy to prevent cancer is the use of either natural or synthetic substances or their combination to block, reverse or retard the process of carcinogenesis. Therefore, it is of interest to explore the possibility of using phytochemicals or other dietary agents as chemopreventive agents.
It is widely accepted that more than two third of human cancer could be prevented through changes in life style and dietary modifications. Plant based phytochemicals have been shown to be excellent chemopreventive agents. Phytochemicals are non-nutritive components in the plant based diet that possess substantial health beneficial properties. Several natural plant products, such as phenolics, indoles and flavonoids, have been shown to alter the initiation phase of carcinogenesis. To assess the cancer chemopreventive effects of these phytochemicals, several bioassay systems are available. Induction in anti oxidants, phase-II-enzymes and inhibition of tumorogenesis using natural plant products represent ideal test systems for such chemopreventive studies.

The present research work described in the thesis entitled "Chemoprevention of chemically induced cancer by phytochemicals -delineation of implicated mechanism" comprises of nine chapters:

Chapter-1 describes the detailed introduction and the extensive review of research work conducted in the area of cancer, oxidative stress and chemoprevention with special emphasis upon Fe-NTA mediated toxicity.

In the introduction section, a general outline of multistage carcinogenesis with its molecular mechanisms has been described in detail. This has been followed by the role of reactive oxygen species (ROS) related oxidative stress in carcinogenesis wherein the biological significance for the involvement of free radicals in carcinogen metabolism and subsequent multistage carcinogenesis has been discussed. Exposure to oxidative damages through a variety of free radicals has led organisms to develop a series of defense mechanisms by which the effect of ROS is balanced including enzymatic and non-enzymatic antioxidants. A detailed description regarding these antioxidants has thus been incorporated. The chemistry and biochemistry of ROS with its potential biological sources and targets have also been documented. Chemoprevention, which is one of the novel approaches of controlling cancer alternative to therapy that possess some drawbacks and limitations in the treatment of patients, has been classified and discussed.
This is followed by the chemoprevention of cancer by phytochemicals derived from various natural sources with the special insight into their mechanistic aspects.

Since major portion of the research work is related with Fe-NTA mediated renal and hepatic carcinogenesis, an extensive review of research work conducted in the field of Fe-NTA mediated carcinogenesis is obligatory and has, therefore, been incorporated in the review of literature section. The role of natural products in Fe-NTA mediated carcinogenesis has also been included. Another portion of the thesis work includes the identification of plant derived inhibitors of farnesyl transferase (FTase) in skin carcinogenesis. FTase is an enzyme that leads to the farnesylation of ras protein. Farnesylation of ras-protein is a mandatory process for its retention of transforming ability (Oh et al., 2005). When a farnesylation of these proteins is blocked, their oncogenic ability is abolished (Oh et al., 2005). The finding that farnesylation of ras-protein is an obligatory step for its transforming activity made FTase a very attractive target for anti-cancer therapy. Therefore, identification and synthesis of FTase inhibitors has become an active area for the development of anti-tumor agents (Oh et al., 2005). A detailed review of literature regarding inhibitors of FTase derived from various natural sources until this time has thus been included in this chapter.

Chapter-2 describes chemicals, instruments and methods used while conducting phytochemical investigations and different experiments carried out on the chemoprevention of chemically induced renal, hepatic and skin carcinogenesis. A general procedure for the preparation of extracts from different plants has been described in this chapter. The characterization of extracts/ fractions/ compounds performed by standard phytochemical and spectroscopic methods has also been documented. Phytochemical characterization was performed by either by phytochemical tests or by HPTLC/ HPLC. GC-MS has also been used for phytochemical composition of various extracts. The structural elucidation of isolated compounds by the chemical and spectroscopic techniques viz., IR, $^1$H NMR, $^{13}$C NMR, DEPT, HMBC, HMQC and mass spectrometry has been described in this chapter.
The general notes on biological experimental work of plants/pure compounds are fully described in this chapter. A series of assays were performed for ascertaining the antioxidant activity and anticancer activity of plants/pure compounds both \textit{in vitro} and \textit{in vivo}. For \textit{in vitro} studies, assays for total phenolics, reducing power, free radical scavenging activities (Scavenging of DPPH, \(O_2^-\), \(H_2O_2\), \('OH\) and NO), lipid peroxidation (LPO) and DNA damage were measured. For all biochemical \textit{in vivo} tests, assays for reduced glutathione (GSH); antioxidant and phase II enzymes such as glutathione-S-transferase (GST), glutathione reductase (GR), catalase (CAT), glutathione peroxidase (GPx), glucose-6-phosphate dehydrogenase (G-6-PD); ornithine decarboxylase (ODC); DNA synthesis; xanthine oxidase (XO); quinone reductase (QR); superoxide dismutase (SOD); lipid peroxidation (LPO); hydrogen peroxide (\(H_2O_2\)), blood urea nitrogen (BUN) and serum creatinine (SCR) and liver function tests (ALT, AST, ALP and bilirubin), were considered. All these assays have been described in detail in this chapter. Assays for FTase activity, measurement of cytokines (IL-6 and TNF-\(\alpha\)) and histopathological procedures have also described in this chapter.

Chapter-3 gives a detailed description of phytochemical investigations of the aerial parts of \textit{Didymocarpus pedicellata} R. Br. (Family Gesneriaceae) and its protection against Fe-NTA mediated renal carcinogenesis. The studies described in this chapter were designed to investigate the bioactive constituents present in the aerial parts of \textit{D. pedicellata}. The phytochemical studies resulted in the isolation of three phytosterols viz., Stigmasterol glycoside (DP-1), Didymosteryl linolenate-A (DP-2) and Didymosteryl linolenate-B (DP-7); four higher aliphatic fatty acids viz., Octacosatetraenoic acid (DP-3), Hexacosenoic acid-A (DP-4), Hexacosenoic acid-B (DP-5) and Hexacosatrienoic acid (DP-6); and a phenolic compound viz., Heptenyl phenol (DP-8). These compounds are reported for the first time from this plant. The phytochemical studies also revealed compounds DP-6, DP-7 and DP-8 as novel compounds to be reported for the first time from a plant/synthetic source. The structural elucidations have been carried using chemical and spectroscopic data. The chemical analysis of petroleum ether extract by GC-MS revealed the presence of a group of oxygenated compounds with a total amount of 49.3% sterols and 30.4% glycerides. The major compounds were \textit{n}-heneicosan-1, 6-}
diol (44.5%), oleoyl glyceraldehydes (22.7%) and linolyl glyceraldehydes (6.8%) observed at retention times of 19.27, 22.50 and 23.45, respectively. This is the first report of composition of petroleum ether extract of *D. pedicellata*.

The biological studies described in this chapter have revealed ethanolic extract of aerial parts of *D. pedicellata* to possess significant antioxidant activity and showed protection against ferric nitrilotriacetate (Fe-NTA) mediated renal oxidative stress, nephrotoxicity and tumor promotion response. *D. pedicellata* extract was found to possess a high content of total polyphenolics, exhibited potent reducing power and significantly scavenged free radicals including several reactive oxygen species (ROS) and reactive nitrogen species (RNS). The extract also significantly and dose dependently protected against Fe-NTA plus H$_2$O$_2$ mediated damage to lipids and DNA. Protective efficacy of the extract was also tested in vivo against Fe-NTA mediated nephrotoxicity and tumor promotion response. Administration of Fe-NTA (9 mg/Kg body weight, i.p.) to Swiss albino mice depleted renal glutathione content and activities of antioxidant and phase II metabolizing enzymes with concomitant induction of oxidative damage. Fe-NTA also incited hyperproliferation response elevating ornithine decarboxylase activity and $[^3]$H-thymidine incorporation into DNA. Elevation in serum creatinine (SCr) and blood urea nitrogen (BUN), and histopathological changes were also evident and suggested Fe-NTA to afflict damage to kidney. Pretreatment of mice with *D. pedicellata* extract (100 to 200 mg/Kg body weight) for 7 days not only restored antioxidant armory near normal values but also significantly protected against renal oxidative stress and damage restoring normal renal architecture and levels of renal damage markers viz., BUN and SCr.

The antioxidant activities of compounds (DP-1, DP-2, DP-7 and DP-8) isolated from *D. pedicellata* have also been performed. Compounds DP-2 and DP-7 were found to be most potent with the activity order GA > AA > DP-2 > DP-7 > DP-1 > DP-8. Such studies were based upon reducing potential and scavenging of free radicals by these compounds and their effect on lipid peroxidation (LPO). In summary, the results of the present study inferred *D. pedicellata* to possess potent antioxidant and free radical scavenging activities and preclude oxidative damage and hyperproliferation in renal tissues. Further, bioactive
compounds such as DP-2 and DP-7 contributed to potent antioxidant activity of the plant were also elucidated. Thus, intake of these compounds (DP-2 and DP-7) may have a potential function in reducing the occurrence of numerous diseases including cancer. Further, these compounds can serve as an important lead for novel drug discovery. The plant needs further phytochemical investigation so as to isolate the potent bioactive compounds responsible for the potent activity of the extract.

Chapter-4 gives a detailed description of phytochemical investigations of the aerial parts of Artemisia maritima L. (Family Asteraceae alt. Compositae) and its protection against Fe-NTA mediated hepatic carcinogenesis. The studies described in this chapter were designed to investigate the bioactive constituents present in the aerial parts of A. maritima. The phytochemical studies resulted in the isolation of five compounds viz., Benzyl octacosene (AM-1), Benzodioxole carboxylic acid (AM-2), Biflavanyl diglycoside (AM-3), Naphthalene diol (AM-4) and Hexdienyl benzenetriol (AM-5). Although these compounds (AM-1 to AM-5) were previously isolated from other plant source, however, their presence has been reported for the first time in A. maritima. The phytochemical studies have revealed the compound, AM-3 as a novel compound. It is reported for the first time from a plant/ synthetic source. The structures have been fully elucidated by chemical and spectral data. The chemical analysis of petroleum ether extract of aerial parts of A. maritima by GC-MS revealed the presence of a group of oxygenated compounds. The major compounds were n-hexatriacont-17-ol-18-ene (13.0%), n-cosan-1, 4, 1-triol (12.4%), n-cosan-1, 6, 18-triol (10.8%) and n-cosan, 1, 10, 17-triol (10.7%) observed at retention times of 23.30, 19.77, 19.55 and 19.82 respectively.

The biological studies described in this chapter have reported ethanolic extract of A. maritima aerial parts to possess potent antioxidant activity and significantly protect against Fe-NTA induced hepatic oxidative stress, hepatotoxicity and tumor promotion response. Intraperitoneal administration of Fe-NTA (9 mg Fe/Kg body weight) to Swiss albino mice depleted renal antioxidant armory (glutathione content and activities of antioxidant and phase II metabolizing enzymes), induced oxidative stress (elevating lipid
peroxidation and H₂O₂ generation) and incited hyperproliferation in kidney apparent from marked induction of ornithine decarboxylase (ODC) activity. Histopathological investigations and liver function tests (LFT) suggested Fe-NTA to afflict substantial damage to liver. Prophylactic treatment of mice with aerial parts of *A. maritima* (100 to 200 mg/Kg body weight) for 7 days not only restored hepatic antioxidant armory close to normal but also significantly precluded oxidative damage restoring normal hepatic architecture and levels of hepatic damage markers close to normal values.

The antioxidant activities of compounds (AM-2, AM-3, AM-4 and AM-5) isolated from *A. maritima* have also been performed. All these isolates were found to be most potent with the activity order GA > AA > AM-2 > AM-3 > AM-5 > AM-4. Such studies were based upon reducing power, scavenging of free radicals by these compounds, and their effect on lipid peroxidation (LPO). In summary, the experimental data of the present study suggest *A. maritima* to possess a potent antioxidant activity and avert chemically inflicted hepatic oxidative damage and tumor promotion response. Further, bioactive compounds such as AM-2, AM-3, AM-4 and AM-5 contributed to potent antioxidant activity of the plant were also elucidated. High reducing power of these compounds with potent free radical scavenging activities and significant LPO inhibitory activities may be directly accountable for the potent antioxidant activity of the plant. Thus, intake of these compounds (AM-2, AM3, AM-4 and AM-5) may have a potential function in reducing the occurrence of numerous diseases including cancer. As a result, *A. maritima* could serve as a new natural source enriched with potent antioxidants and anticancer agents. These isolates (AM-1 to AM-5) may serve as potential lead compounds to be further structurally modified that can manifest into a new and effective therapeutics. Other biological activities of *A. maritima* also need to be investigated.

Chapter-5 documents the antioxidant potential of ethanolic extract of *Rumex patientia L.* (Polygonaceae) roots and its chemopreventive effects against Fe-NTA mediated hepatic oxidative stress, hepatotoxicity and tumor promotion response. The extract exhibited a high polyphenolic content, potent reducing power and significantly scavenged free radicals (including several reactive oxygen species (ROS) and reactive nitrogen species.
(RNS). The extract also significantly and dose dependently protected against oxidative damage to lipids and DNA. These results indicated *R. patientia* root extract to exert a potent antioxidant activity *in vitro*. The efficacy of extract was also evaluated *in vivo* and it was found to exert a potent protective affect in acute oxidative tissue injury animal model: Ferric nitritriacetate (Fe-NTA) induced hepatotoxicity in mice. Administration of Fe-NTA (9 mg/kg body weight, i.p.) to mice led to a significant oxidative stress and allied damage in liver tissues and induced hyperproliferation. A significant depletion was observed in GSH content and enzymes implicated in its metabolism. Attenuation also occurred in activities of other hepatic antioxidant enzymes including SOD, CAT, and GPX. Fe-NTA also incited hyperproliferation response elevating ornithine decarboxylase activity and \[^{3}H\]-thymidine incorporation into DNA. Histopathological investigations and liver function tests (LFT) indicated Fe-NTA to cause extensive hepatic damage. However, prophylactic treatment with *R. patientia* root extract at a dose regimen of 100-200 mg/kg body weight for a week not only restored hepatic antioxidant armory close to normal but also significantly precluded oxidative damage restoring normal hepatic architecture and levels of hepatic damage markers. In summary, the data obtained in the present study illustrates *R. patientia* roots to possess potent antioxidant and free radical scavenging activities and thwart oxidative damage and hyperproliferation in hepatic tissues.

Chapter-6 describes the chemopreventive potential of dietary rutin against DEN-initiated and Fe-NTA promoted renal carcinogenesis. In our present study, Fe-NTA, a known complete renal carcinogen, which generate ROS *in vivo*, was given intraperitoneally to mice and dietary rutin was tested for its ability to inhibit oxidative stress and the activity of ornithine decarboxylase (ODC) as well as histopathological changes in the kidney. Substantial changes in glutathione, antioxidant enzymes as well as changes in phase II metabolizing enzymes were observed in the kidney at 12 h after treatment with Fe-NTA (9.0 mg Fe/kg body weight). Effect of oxidative stress induced by Fe-NTA was also demonstrated by the increase in lipid peroxidation as monitored by formation of thiobarbituric acid reactive substances in kidney. Likewise, the level of protein carbonyl contents, an indicator of protein oxidation was also increased after Fe-NTA
administration. However, the changes in these parameters were restored to normal in rutin-pretreated mice. The ODC activity in the kidney was significantly increased by Fe-NTA, while the increased ODC activity induced by Fe-NTA was normalized in rutin-pretreated mice. In N-diethyl nitrosamine (DEN)-initiated and Fe-NTA-promoted animals, 60% renal tumour incidence was recorded as compared with untreated controls. The rutin pretreatment, however, afforded 80% protection against DEN- and Fe-NTA mediated renal tissue injury in vivo. In addition, rutin pretreatment almost completely prevented kidney biomolecules from oxidative damage and protected the tissue against observed histopathological alterations. In summary, our data suggested that rutin can suppress renal ODC induction and can abrogate the toxic and tumour-promoting effects of Fe-NTA induced by Fe-NTA in kidney of mice and can, therefore, serve as a potent chemopreventive agent to suppress oxidant-induced tissue injury and tumorigenesis.

Chapter-7 describes the identification of plant-derived inhibitors of farnesyl transferase (FTase) in skin carcinogenesis. The present study was designed to test the efficacy of plants with reported anticancer properties in traditional Indian System of Medicine for the inhibition of activity of Farnesyl transferase (FTase). FTase is a cytoplasmic enzyme that plays a crucial role in the farnesylation of ras-p21 protein. Farnesylation of ras-p21 is a mandatory process for retention of transforming ability of ras-p21 protein. When a farnesylation of these proteins is blocked, their oncogenic ability is abolished. The finding that farnesylation of ras is an obligatory step for its transforming activity made FTase a very attractive target for anti-cancer therapy. Therefore, identification and synthesis of FTase inhibitors has become an active area for the development of anti-tumor agents. Three plants were assessed, Azadirachta indica (Neem), Citrus sinensis (Orange) and Aloe barbadensis (Aloe) to study if they inhibit FTase and hence hamper membrane association of ras protein. Our studies revealed C. sinensis to be significant inhibitor of FTase enzyme and was, therefore, selected for further fractionation and assessment of inhibitory activity on FTase enzyme. Fractionation was performed by partitioning whole ethanolic extract of C. sinensis successively with hexane, chloroform, ethyl acetate, and butanol in order of their increasing polarities respectively. Post fractionation results of these studies of C. sinensis showed the chloroform fraction of C.
sinensis to be most potent inhibitor of FTase activity *in vitro*. Thus, this fraction was used for *in vivo* study of prevention of skin tumorigenesis. A significant regression in volume, number of tumor per mouse (5.75 ± 1.87) and percentage of tumor bearing mice (93.33 %) were observed with higher dose of chloroform fraction of *C. sinenesis* (240 mg/Kg b/wt.). This was also confirmed by histopathological results. Taken together, the results of the present study suggested that chloroform fraction of *C. sinensis* can serve as potential source of FTase inhibitors and may, thus, lead to the invention of effective therapeutic and chemopreventive agents.

Chapter-8 involves the summary and conclusions of individual chapters of this thesis.

Chapter-9 involves the relevant bibliography of individual chapters of this thesis.