CHAPTER 10

SUMMARY AND CONCLUSION
SUMMARY

Cancer is the second most common cause of death worldwide after cardiovascular diseases. Cancer can impose a substantial burden through long-term human suffering and high economic loss. During the last decades, carcinogenesis has been an important subject of intense experimental, epidemiological and clinical researches at molecular, cellular and clinical level. But there is no “magic bullet” that completely conquers cancer. Moreover, the leading chemotherapeutic agents used today bring about toxicities to normal tissues. In this thesis, we have evaluated chemopreventive and chemoprotective, radioprotective and hepatoprotective potentials of meso-zeaxanthin-a xanthophyll carotenoid with profound antioxidant activity.

Cancer development is a multi-factorial and multistage process consisting of three distinct phases: initiation, promotion and progression. Oxidative stress has considerable role in all the three stages of this process. During initiation stage, ROS can produce DNA damage by introducing gene mutations and structural alterations to DNA. In promotion stage, ROS can contribute to abnormal gene expression, blockage of cell-to-cell communication and modification of second-messenger systems leading to an increase in cell proliferation or a decrease in apoptosis of the initiated cell population. Finally, oxidative stress can also participate in the progression stage of the carcinogenesis by adding further DNA alterations to the initiated cell population (Klaunig et al., 1998). Since antioxidants can mitigate the damaging effect of oxidative stress imposed by ROS, compounds with antioxidant activity should be analysed for its chemopreventive potential. So in first part of this thesis, we have evaluated the antioxidant potential of carotenoid MZ both in vitro and in vivo. MZ was found to scavenge superoxide radicals and hydroxyl radicals and to inhibit in vitro lipid peroxidation. Concentrations needed for 50% inhibition (IC50) were 27, 3.5, 3.2μg/ml, respectively. MZ scavenged 2,2-azo bis-3-ethyl benz thiazoline-6-sulphonic acid (ABTs) and 2,2-Diphenyl-1-picryl hydrazyl (DPPH) radicals and IC50 values were 46.5 and 6.25 μg/ml respectively. MZ also exhibited significant singlet oxygen quenching activity (IC50 38 μg/ml) and nitric oxide radical scavenging activity (IC50 2.2 μg/ml). Ferric reducing antioxidant power of MZ was found to be 0.23 mM. Oral administration of MZ at doses of 50, 100 and 250 mg/kg b.wt inhibited phorbol-12-myristate-13-acetate (PMA) induced superoxide radical generation in macrophages by 25.22%, 50.12% and 67.18% respectively. Oral administration of MZ to mice for one
month significantly increased catalase, superoxide dismutase, glutathione and glutathione reductase levels in both blood and liver. Levels of glutathione peroxidase and glutathione-S-transferase were also found to be increased in liver in a dose dependent manner. These results showed that MZ has significant antioxidant activity both in vitro and in vivo.

Chronic inflammation has been linked to various steps involved in carcinogenesis including cellular transformation, promotion, survival, proliferation, invasion, angiogenesis, and metastasis (Coussens and Werb, 2002). Anti-inflammatory studies on MZ revealed that it had significant activity against acute inflammation induced by carrageen and dextran and chronic inflammation induced by formalin. MZ showed substantial anti-inflammatory effect against LPS-induced inflammatory model. Excessive production of various proinflammatory cytokines (TNF-α, IL-1β, and IL-6), CRP and nitric oxide (NO) by lipopolysaccharide (LPS) stimulated macrophages were found to be significantly reduced when they were treated with MZ in a dose dependent manner. Moreover, MZ down-regulated the expression of various genes involved in inflammation like COX-2, iNOS and TNF-α confirming its anti-inflammatory potential.

During tumourigenesis, accumulation of multiple gene mutations would lead to the neoplastic transformation of a single cell. These aberrant mutations and over expression of several important genes contribute to the initiation and progression of human malignancies. Antimutagenic activity of carotenoid MZ was tested in Salmonella typhimurium strains TA98, TA100, TA102 and TA1535 by Ames test against direct acting mutagens such as sodium azide (NaN₃), Nitro-O-phenylenediamine (NPD), N-methyl-N-nitro-N-nitrosoguanidine (MNNG), tobacco extract and mutagens needing microsomal CYP450 enzyme activation like acetaminofluorene (AAF). MZ inhibited mutagenicity produced by sodium azide (5 μg/plate). Percentage of inhibition was found to be 46.70, 64.50 and 97% for TA100 strain, 44, 86.6 and 93.80 % for TA102 strain and 46.40, 68.50 and 79.80 % for TA1535 at concentrations 50, 100 and 250 μg MZ/plate respectively. The inhibition of NPD (20 μg/plate) induced mutagenicity by MZ (50, 100 and 250 μg/plate) was 48, 62.7 and 65% respectively for TA98 and 49.1, 57.3 and 62.8% respectively for TA100. Mutagenicity produced by MNNG (1 μg/plate) to S. typhimurium strains TA100 and TA1535 was found to be inhibited by MZ at same doses and the respective inhibition was 39, 51.94 and 56.8% for TA100 and 32.5 56.23 and 63.9 %
for TA1535. Similarly, MZ was found to be anti-mutagenic to carcinogen needing activation like AAF (20 μg/plate). The carotenoid at doses 50, 100 and 250 μg/plate inhibited mutagenicity to strain TA98 by 45.4, 63.7 and 77.4% respectively and to strain TA100 by 47.7, 66.7 and 86.2% respectively. MZ was also found to inhibit the mutagenicity produced by tobacco extract (50 mg/plate) to *S. typhimurium* strain TA102. Percentage of inhibition was found to be 19.8, 51.56 and 79.4% at concentrations 50, 100 and 250 μg MZ/plate respectively. These results indicated that MZ has significant anti-mutagenic activity against both direct acting mutagens and mutagens needing microsomal activation.

MZ was found to be cytotoxic to various transformed cell lines like Dalton’s lymphoma ascites (DLA) cells (IC₅₀ 46 μg/ml), Ehrlich ascites carcinoma (EAC) cells (IC₅₀ 51 μg/ml) and mouse lung fibroblast cells (L929 cells) (IC₅₀ 37 μg/ml). Antitumour activity of MZ was studied using EAC cells induced ascites tumour model and DLA cells induced solid tumour model. MZ administration significantly (P<0.001) increased lifespan of ascites tumour bearing animals and at the same time this carotenoid significantly reduced the solid tumour volume in a dose dependent manner. MZ was found to induce apoptosis in DLA cells as evident from membrane blebbing, chromosome condensation, nuclear fragmentation and formation of apoptotic bodies in MZ treated cells. DNA isolated from MZ treated DLA cells also showed characteristic ladder pattern of apoptosis. MZ was found to down-regulate the expression of anti-apoptotic gene like BCL₂ and at the same time it upregulated the expressions of proapoptotic genes like p53, caspase 3 and caspase 9. Hence the cytotoxic and antitumour effect of MZ on transformed cells is mediated through apoptosis.

Anti-carcinogenic potential of carotenoid MZ was evaluated against different chemical carcinogens. Experimental models used were nitrosodiethylamine (NDEA) induced hepatocellular carcinoma, 3-methylcholanthrene (3-MC) induced sarcoma and DMBA and croton oil induced two-stage skin papillomagenesis. Administration of NDEA to rats resulted in hepatocellular carcinoma formation and the levels of marker enzymes such as gamma-glutamyl transpeptidase, glutamate pyruvate transaminase, glutamate oxaloacetate transaminase and alkaline phosphatase in both serum and liver tissue were drastically elevated. Administration of MZ retarded the tumour growth and significantly lowered the elevated levels of marker enzymes in
both serum and liver tissue in a dose dependent manner. Results of histopathological analysis further confirmed the anticarcinogenic potential of MZ against NDEA induced hepatocellular carcinoma. MZ also inhibited 3-MC induced sarcoma development in mice. Oral administration of MZ significantly increased the tumour latency period and survival rate of mice when compared to those of 3-MC alone treated sarcoma bearing animals. Moreover, MZ exhibited anti-carcinogenic activity against two-stage skin papillomagenesis induced in mice using DMBA as initiator and croton oil as promoter. MZ was applied on the dorsal side of mice prior to DMBA application resulted in significant reduction in both tumour incidence and cumulative number of papillomas per mice and increased tumour latency period when compared to carcinogen alone treated control animals indicating the inhibitory effect of MZ on tumour initiation process. Topical application of MZ before croton oil application also reduced papillomagenesis and cumulative number of papillomas per mice and significantly increased tumour latency period indicating antitumour promotional potential of this carotenoid.

Many chemical carcinogens require metabolic activation to become biologically reactive intermediates which if not detoxified will react with DNA covalently or will lesion DNA through the production of reactive oxygen species. The enzymes involved in bioactivation are often phase I enzymes, while phase II enzymes are involved in conjugation of the functional groups leading to elimination of the carcinogen. It is the balance of these events that determines the carcinogenicity of a chemical in a target tissue. In order to determine the mechanism of anti-carcinogenic action of MZ, effect of MZ administration on various CYP450 isoenzymes and phase II enzymes were studied. MZ was found to inhibit CYP450 isoenzymes both in vitro and in vivo in a concentration dependent manner. On the other hand, the levels of phase II enzymes like UDP-glucuronyl transferase and glutathione-S-transferase were found to be significantly increased by MZ treatment in a dose dependent manner. Possible anticarcinogenic action of MZ is inhibition of phase I carcinogen metabolising enzymes which are involved in carcinogen bioactivation and enhancement of phase II enzymes which are involved in carcinogen detoxification.

Treatment related toxicity to normal tissues is a major problem associated with chemotherapy and radiotherapy. The possible use of MZ as an adjuvant during radiotherapy and chemotherapy was also evaluated. Chemoprotective potential of MZ was
studied against cisplatin induced nephrotoxicity and doxorubicin induced cardiotoxicity. In order to study nephro-protective activity, cisplatin (16 mg/kg b.wt) was injected to BALB/c mice. Kidney function markers like urea and creatinine, which were drastically elevated in serum of cisplatin treated animals, were decreased to normal levels by MZ treatment in a dose dependent manner. MZ also reduced cisplatin induced myelosuppression as evident from the increase in WBC count, bone marrow cellularity and α-esterase positive cells in the MZ treated animals. Oxidative stress markers like lipid peroxide, conjugated dienes and tissue hydroxides were high in kidney of cisplatin challenged animals. MZ treatment decreased them in a dose dependent manner. Activities of antioxidant enzymes like SOD, catalase and glutathione peroxidase as well as glutathione level which were significantly decreased in cisplatin treated kidney were elevated by MZ treatment in a dose dependent manner. In order to study cardioprotective potential of the carotenoid, doxorubicin (30 mg/kg b.wt) was injected to Wistar rats. Serum markers of cardiac injury like LDH, CPK, SGOT and SGPT levels, which were markedly increased by doxorubicin treatment, were decreased significantly by MZ administration in a dose dependent manner. Doxorubicin induced myocardial injury was evident from prolonged PR, RR, ST and QT intervals and decrease in heart beat rate in ECG pattern of DOX alone treated animals; MZ treatment significantly restored ECG changes to normal levels. Oxidative stress markers in doxorubicin challenged heart tissue were high in control animals but were decreased significantly by MZ treatment in a dose dependent manner. Antioxidant enzymes levels like SOD, catalase and glutathione peroxidase as well as glutathione level in the heart tissue were decreased drastically after doxorubicin injection. These levels were significantly elevated by MZ treatment in a dose dependent manner. These results indicate that MZ significantly reduces oxidative stress and thereby toxicity induced by cisplatin and doxorubicin.

Radioprotective potential of oxycarotenoid MZ was studied. MZ administration significantly increased the lifespan and body weight of irradiated mice. Radiation induced myelosuppression was significantly reduced by MZ administration as evident from increases in white blood cell counts, bone marrow cellularity and the number of maturing monocytes in the carotenoid treated irradiated animals. Irradiation reduced the activities of superoxide dismutase, catalase, glutathione peroxidase and glutathione in both liver and intestinal mucosa of radiation control
animals. MZ treatment significantly elevated these activities. The carotenoid treated animals showed a profound reduction in genotoxic activity. Radiation induced micronuclei formation was significantly decreased by MZ treatment in a dose dependent manner. Chromosomal aberrations like chromosome/chromatid breaks and gaps, and other numerical and structural aberrations were markedly elevated by \( \gamma \)-irradiation. These were significantly (P<0.001) decreased by MZ administration. Irradiation also induced damage to cellular DNA as was obvious from increases in comet parameters like tail DNA\%, tail moment, tail length and Olive tail moment in the radiation control group. These parameters were decreased by MZ treatment in a dose dependent manner. All these results indicated a radioprotective potential of MZ.

Paracetamol abuse and ethanol intoxications are the major health concerns of the society. Oxidative stress induced liver damage is the main consequence of these habits. We evaluated hepato-protective potential of carotenoid MZ using in vivo rat models. Paracetamol, ethanol and CCl\(_4\) were used as hepato toxins. Levels of marker enzymes of hepatic injury such as SGOT, SGPT and ALP as well as serum bilirubin, which were drastically elevated by these hepatotoxins, were significantly decreased by MZ treatment in a dose-dependent manner. Oxidative stress markers in liver like tissue lipid peroxidation, conjugated dienes and tissue hydroperoxides, which were very high in paracetamol treated control group animals, were lowered by MZ administration. Levels of glutathione and antioxidant enzymes such as superoxide dismutase, catalase and glutathione peroxidase in liver tissue were increased by MZ treatment when compared to those levels of control group animals during alcohol and CCl\(_4\) induced hepatotoxicity. Hydroxyproline, an indicator of fibrosis in liver tissue, was decreased remarkably by MZ administration despite its notable elevation in ethanol treated rats. Histopathological analysis of liver tissue further supported hepatoprotective potential of MZ. Since all the hepatotoxins used in the study induced oxidative stress, the hepatoprotective potential of MZ can be attributed to its profound antioxidant activity.
CONCLUSION

- MZ exhibited significant antioxidant activity both \textit{in vitro} and \textit{in vivo}.
- MZ showed anti-inflammatory activity against carrageenan, dextran and formalin induced mouse paw oedema models.
- The carotenoid reduced LPS stimulated proinflammatory cytokine levels towards normal levels.
- MZ inhibited the expression of COX-2, TNF-\( \alpha \) and iNOS genes.
- So the anti-inflammatory activity of MZ can be due to the down regulation of these inflammatory genes.
- It exhibited profound anti-mutagenic effect against both direct acting and indirect acting mutagens.
- MZ showed significant cytotoxicity and antitumour effects against DLA and EAC cells induced solid and ascites tumour models.
- MZ was able to induce apoptosis in DLA cells, which was confirmed by DNA ladder analysis.
- MZ inhibited the expression of anti-apoptotic Bcl-2 gene and upregulated the expressions of proapoptotic genes like p53, caspase 3 and caspase 9 i.e. MZ induced p53 dependent caspase 9 mediated intrinsic (mitochondrial) apoptotic pathway.
- So the cytotoxic and anti-tumour effect of MZ is mediated through apoptosis.
- MZ showed significant anti-carcinogenic effect against Nitrosodiethylamine (NDEA) induced hepatocellular carcinoma and 3-Methyl cholangthrene (3-MC) induced sarcoma.
- MZ also showed significant anti-carcinogenic effect against 7, 12-dimethylbenz [a] anthracene (DMBA) and croton oil induced two-stage papillomagenesis. MZ exerted profound inhibitory effect in both initiation and promotion stages of carcinogenesis.
MZ can be called as “dual acting” agent as it inhibited phase 1 enzymes which are involved in carcinogen bioactivation at the same time induced phase 2 enzymes like GST, UDP-glucuronyl transferase which are involved in carcinogen detoxification.

MZ showed significant chemoprotective effect against cisplatin induced nephrotoxicity and doxorubicin induced cardiotoxicity.

MZ revealed its efficacy as an effective radioprotector by reducing radiation induced myelosuppression, gastrointestinal damages and genotoxicity.

Present study also showed hepatoprotective activity of MZ against paracetamol, CCl₄ and ethanol induced toxicity.

Various pharmacological properties exhibited by MZ summarised in figure 10.1.

As healthcare costs being a key issue today, chemopreventive agents should have low cost, high efficacy, oral consumability, no or low toxicity and a known mechanism of action. Present study revealed that MZ from marigold flower possesses most of these features. All these findings support the potential use of carotenoid MZ in the prevention and treatment of cancer.
Figure 10.1. Various pharmacological properties exhibited by MZ