CHAPTER 5

DISCUSSION
Chemoprevention with food phytochemicals is currently regarded as one of the most important strategies for cancer control. Laboratory studies and epidemiological evidence also give credence to chemoprevention strategy in attenuating the risk of developing cancer (Bertram et al., 1987; Boone et al., 1990). Many nutrient and non-nutrient dietary constituents of plant origin have evidence of chemoprevention by inhibiting and/or suppressing or reversing cancer incidence trend evoked by multitude of factors including environmental chemical carcinogens (Block et al., 1992).

In view of the growing interest in the search for food and diet related means of preventing carcinogenesis, the present study has been undertaken to evaluate the chemopreventive potential of the medicinal herb, *Eclipta alba* Linn on DMBA initiated and TPA promoted skin tumor development during the initiation and promotion phases in female Swiss albino mice. Further the effects of the *Eclipta alba* extract in the activities of hepatic biotransformation enzymes i.e., Phase I (Cytochrome P450 and Cytochrome b5) and Phase II enzyme (Glutathione S-transferase) was evaluated at a dose level of 125 mg/kg body weight and 250 mg/kg body weight for 14 days respectively. The antioxidant status of the liver was also assayed by measuring the activities of Superoxide dismutase (SOD) and Catalase (CAT) and compared to the basal level of control values. The decrease in lipid peroxidation, which
is correlated with the concomitant increase of antioxidant enzymes, was also measured as the level of MDA formation as an index of antioxidant property of the modulator. A positive control group was maintained along with the experimental groups using BHA (0.75% in diet) which is a known antioxidant, to ascertain the validity of the assay protocols.

The modulator used in the present study was first tested for any adverse effects on the general body metabolism of the test animals. The modulator is a well known medicinal herb and is traditionally used as a tonic against jaundice (Kirtikar and Basu, 1981; Kumar, 2002). Literature suggests that the LD_{50} value of the *Eclipta alba* extract is 2.0g/Kg body weight when given orally and intraperitonially (Indian Herbal Pharmacopoeia, 2002).

Estimating the activities of serum GOT and GPT, which are enzymes originally present in higher concentration in the cytoplasm can make assessment of the liver function. When there is histopathy, these enzymes leak into the blood stream in conformity with the extent of liver damage. Lack of any significant alteration in the SGOT and SGPT levels (Table: 8, Fig. 6&7) in the modulator treated groups in comparison to the control is indicative of the absence of any sort of histopathy or toxicity of the plant extract at the selected dose levels of 125 mg/kg body weight and 250 mg/kg body weight.
The administration of the hydro alcoholic extract of *Eclipta alba* did not affect the body weight of the animals during the experimental period and also there were no cases of mortality. Further the liver somatic index (i.e., the ratio between liver weights to final body weight) of the modulator treated mice did not show any marked alteration indicating a favorable effect of the extract of *Eclipta alba* (Table: 16).

The skin carcinogenesis model in experimental animals has been found to be a very useful system for investigating the influence of dietary chemopreventors both mechanistically and operationally (Morse and Stoner, 1993). The present study demonstrates the chemopreventive potential of *Eclipta alba* on DMBA induced skin papillomagensis in Swiss Albino mice. Berenblum and Shubik (1947) has suggested that one sub minimal dose of carcinogen initiates the process of carcinogenesis and the treatment with croton oil promotes them to visible tumor stage. The current study also exhibited the same with 100% tumor incidence in the control group (Group I) but there was significant reduction (P<0.05) in the tumor incidence in the modulator treated groups (Groups II, III and IV) (Table: 10, Fig.8). The reason for 100 % incidence in the control group is perhaps due to the accumulation of inflammatory cells such as neutrophils, macrophages and increased
formation of reactive oxygen intermediates leading to skin tumor promotion (Cerruti, 1985). On the other hand, the mice of groups II, III and IV receiving similar treatment of DMBA and croton oil but subjected to a topical application of the hydro alcoholic extract of *Eclipta alba* exhibited a significant reduction (P<0.05) in tumor incidence (Table: 10).

The administration of the hydro alcoholic extract of *Eclipta alba* also showed a significant reduction in tumor yield (Table: 11, Fig. 9), tumor burden (Table: 12, Fig. 10) and cumulative number of papillomas (Table: 13, Fig. 11) in comparison to the control group. The present study also exhibited an elevation in the percentage inhibition of tumor multiplicity in the modulator treated groups (Groups II, III and IV) in contrast with the control mice (Table: 14, Fig. 12).

The histopathology of the skin showed visible reduction in the size of the tumors (Plate - IV & V - C, D and E) in comparison to the control group (Plate - IV-B). The treated groups also have not exhibited any distinct core tissues, thereby ruling out the possibility of newly formed blood vessels.

The chemopreventive efficacy of the extract may be attributed to the presence of the phytoestrogens like flavonoids and coumestans present in the herb, which are considered to have an inhibitory role during initiational
Several natural and dietary compounds from vegetables, fruits, herbs and spices are being considered for the primary and secondary prevention of cancer (Mishra et al., 2003). One such compound is the phytoestrogen, which is a naturally derived compound in plants. Two phytoestrogens (coumestans), wedelolactone and desmethyl wedelolactone were isolated as the main active principles present in *Eclipta alba* (Saxena et al., 1993). Both the constituents showed anti hepato toxic activity in assays using liver enzymes induced cytotoxicity in cultured rat hepatocytes. These constituents also showed a significant stimulatory effect on liver regeneration (Wagner et al, 1986). Evidences also suggest that *Eclipta alba* exerts its protective effect by acting as an anti inflammatory agent. The herb is known to inhibit higher levels of histamine due to chronic inflammation by 58.67% (Reddy et al., 1990).

The herb also contains the flavonoids, apigenin, luteolin, β-amyrin, vitamin A, ascorbic acid etc as minor constituents in addition to the active principles (Indian Medicinal Plants, 1994; Indian Herbal Pharmacopoeia, 2002). Studies have shown that apigenin acts as proteasome inhibitor and apoptosis inducer in human leukemia cells (Chen et al, 2005). Studies have also revealed that apigenin induce cell cycle arrest in activated microglia
(Elsisi et al., 2005). Researches have also shown that apigenin can inhibit pancreatic cell proliferation through G2/M cell cycle arrest (Ujiki et al, 2006) and the expression of vascular endothelial growth factor and angiogenesis in human lung cancer cells (Liu et al, 2005) which may be one of the many reasons that accounted for the lack of distinct core tissues and reduction in tumor size as seen in the histopathological slides in the plant extract treated groups (Groups II, III and IV) (Plate -IV & V: C-E) in comparison to the DMBA and croton oil treated group (control group) (Plate -IV-B).

The use of the herb as a healing and restorative agent against skin diseases, inflammations, wounds and ulcers have led to the supposition that it might have either acted as an anti-inflammatory agent or inhibited the epidermal ornithine decarboxylase. Ornithine decarboxylase (ODC) catalyzes the conversion of ornithine to putrescine, which is the essential building block required for the production of higher polyamines (spermidine and spermine) within the cell. Polyamines are ubiquitous polycationic molecules that are critical for normal and neoplastic cell growth (Pegg, 1988). The importance of ODC in skin tumor development has been investigated in several mouse models (Pegg, 2003). Increased levels of ODC play a crucial role in promoting tumors by driving the continued proliferation and selective clonal expansion of v-Ha-ras–initiated cells (Smith et al., 1998). The present study is
insufficient to assert the direct contribution of ODC in the process of tumor development in mice. But the reduction in tumorigenesis in groups II (where animals were treated with the modulator 7 days before and 7 days after the application of a single dose of DMBA), III (where the modulator treatment was started from the time of croton oil application and continued till the end of the experiment) and IV (where the modulator treatment was continued throughout the experiment) may be due to the inhibition of epidermal ornithine decarboxylase by the *Eclipta alba* extract as virtually all known tumor promoters are potent inducers of ODC (O'Brien et al., 1975). Reduction of tumorigenesis through the inhibition of epidermal ornithine decarboxylase in mice by *Emblica officinalis* (amla), a popular fruit in India (Mou et al., 1988; Sancheti et al., 2005) and the extract of *Rosemarinus officinalis*, which is an evergreen shrub having medicinal properties (Sancheti and Goyal, 2006) have also been reported earlier.

Further it is suggested that aryl hydrocarbon hydroxylase, a cytochrome dependent carcinogen metabolizing enzyme present in the skin appears to play an important role in the activation of polycyclic aromatic hydrocarbons into reactive moieties that can bind to DNA and that may directly induce cancer (Bickers and Kappas, 1978). However it is inadequate to claim the involvement of aryl hydrocarbon hydroxylase in the process of tumor
formation in mice in the present study, but it is presumed that if there is any involvement of aryl hydrocarbon hydroxylase; there is a possibility that *Eclipta alba* might have had an inhibitory influence on the aryl hydrocarbon hydroxylase enzyme system, thereby reducing tumorigenesis in the modulator treated animals. Similar inhibition of aryl hydrocarbon hydroxylase enzyme system in rats using garlic oil have been reported by Siddiqui and Pawar (1984) and also by Sadhana et al (1988) using garlic oil in mice.

Reduction of skin carcinogenesis by various fruits, vegetables and medicinal plants such as lupeol and mango pulp (Prasad et al., 2007), *Rosemarinus officinalis* (Sancheti and Goyal, 2006), *Tribulus terrestris* (Kumar et al., 2006), *Acacia nilotica* (Meena et al., 2006), *Withania somnifera* (Padmavathi et al., 2005), *Emblica officinalis* (Sancheti et al., 2005), *Phyllanthus urinaria* (Bharali et al., 2003) etc are in literature.

Several naturally occurring dietary or non- dietary constituents, as well as parts of several species of edible plants having pharmacological activity, may influence the hepatic biotransformation enzyme profiles that are involved in activation and detoxification of xenobiotic compounds, including chemical carcinogens (Wattenberg, 1983; Bradifield and Bjeldanes, 1984). So, in the present study, the test material was also quantified by measuring any
modulation in the activities of hepatic biotransformation enzymes i.e., Phase
I (Cytochrome P\textsubscript{450} and Cytochrome b\textsubscript{5}) and Phase II (i.e., GST) enzymes with
two selected dose levels of 125mg/kg body weight/day and 250mg/kg body
weight/day of the \textit{Eclipta alba} extract, treated for a duration of 14 days. The
specific activities of the Phase I enzymes i.e cytochrome P\textsubscript{450} and cytochrome
b\textsubscript{5} exhibited a significant increase (P<0.01) in a dose dependent manner
(Table: 17, Fig. 13 & 14). Microsomal cytochrome P\textsubscript{450} is a major electron
transport chain of endoplasmic reticulum that plays a role in oxidative
activation or inactivation of xenobiotics and promotes their excretion from
the body of an individual by modulating the duration and intensity of their
toxicity (Guengerich, 1988; Miller, 1988). In the present study, \textit{Eclipta alba}
could significantly increase the basal level activities of cytochrome P\textsubscript{450}
(P<0.01). The beneficial effect of the herb may be either due to individual or
shared effects of its constituents that embrace the coumestans such as
wedelolactone and desmethyl wedelolactone, apigenin, luteolin, ß-amyrin,
ascorbic acid etc. (Sharma, 2003). Indole - 3- carbinol present in cruciferous
vegetables is known to act as a blocking agent and reduced cancer incidence
through Cyt P\textsubscript{450} dependent metabolism (Kelloff et al., 1996). Similar
induction of Phase I enzyme activity by various phytochemicals have also
been reported by several workers (Singh et al., 2000; Lampe et al., 2000).
Several studies have reported that phytochemicals having chemopreventive properties bring about an elevation in the glutathione S transferase level (Kelloff et al., 1996; Nakamura et al., 2000). GST forms an important component of phase II metabolism and catalyses the conjugation of electrophilic xenobiotics with GSH, thereby eliminating them from the system (Sedlack and Lindsay, 1968; Szarka et al., 1995). The activity of GST, measured as a total of all its isoforms using CDNB as a non specific substrate showed a significant dose dependent increase in the *Eclipta alba* treated groups (P<0.05) (Group II, III and IV) in comparison to the control (Group I) (Table: 18, Fig.15). The elevation in the level of GST suggests that the extract might have improved the conjugation process thereby playing a critical role in chemoprevention. Moreover, the induction of Phase II enzyme system is used for detecting and identifying potential blocking agents in chemopreventive strategies (Nadkarni, 1976) and many phytochemicals having chemopreventive activity are shown to enhance the activity of phase II enzyme (i.e. GST) (Singh et al., 2000; Dasgupta et al., 2001; Trivedi and Rawal, 2001; Bharali et al., 2003).

Oxidative metabolism involving phase I and phase II enzymes plays a pivotal role in altering the toxic manifestation of a wide variety of xenobiotics (Singh et al., 2001). An equilibrium between phase I and phase II enzymes can
impediment carcinogenicity and can fortify against a number of xenobiotics (Guengerich, 1988; Ketterer, 1988; Miller, 1988).

The elevation of phase I and phase II enzymes by the extract of *Eclipta alba* provides a justification for the activation of the innocuous xenobiotics into active substrates followed by their solubilization and excretion from the body (Gibson and Skett, 1994).

Thus, the extract of *Eclipta alba* may be speculative of acting as a bifunctional inducer as it has the ability to induce both phase I and phase II carcinogen metabolizing enzymes which play a critical role in chemoprevention (Wattenberg, 1983).

Moreover a balance between the phase I and phase II enzymes not only protects the body against a wide range of xenobiotic substances including chemical carcinogens and genotoxins but also induces the antioxidant enzymes to rid the body of the harmful substances (Wilkinson and Clapper, 1997). So, in the present study the antioxidant responsiveness has been judged by its efficacy in modulating the basal level enzyme activities of reduced glutathione (GSH), superoxide dismutase (SOD) and catalase (CAT). The antioxidant enzyme profiles are reported to have biological role in eliminating free radicals by dismutation of oxygen radicals and decomposition of hydrogen
peroxide which may otherwise affect the body cells and cause serious diseases including cancer (Gonzales et al., 1984; Saydem et al., 1997).

Reduced glutathione acts as an important antioxidant by promoting the detoxification of xenobiotics and free radicals (Ormeius and Moldeus, 1994). In addition, the elevated level of GSH protects cellular proteins against oxidation through glutathione redox cycle and also directly detoxifies reactive oxygen species (Ketterer, 1998). Evidences also suggest that *Eclipta alba* exerts its protective action through a reduction in GSH depletion (Wagner et al, 1986; Saxena et al, 1993). In the present investigation also the treatment of *Eclipta alba* have shown to modulate the intracellular concentration of GSH, the principal non protein sulphhydril thiol. Since GSH acts as a potent quencher of free radicals, therefore the increased levels of GSH (P<0.01) in the present study indicate the potential role of the extract in chemoprevention (Table: 19, Fig.16).

Free radicals and lipid peroxidation are known to be responsible for initiation and promotion of carcinogenesis (Bauer and Wendel, 1980). The free radicals such as superoxide anions and its derivatives generated during normal cellular metabolism of the system, particularly hydroxyl radical is responsible for peroxidation of cell membrane lipids. The end products of
lipid peroxidation are known to cause cellular damage and in turn are responsible for a number of oxidative free radical induced diseases (Halliwell and Gutteridge, 1989).

Superoxide dismutase (SOD) is an antioxidant enzyme that converts superoxide to hydrogen peroxide and oxygen and thus acts as a first line of defense against injurious effects of superoxides (Han and Rabani, 1981). The free radical scavenging property of SOD is more effective only when it is followed by a concomitant increase in the activity of catalase (CAT) (Sunde and Hoekstra, 1980; Harman, 1991; Moser and Moser, 1996).

Catalase is a major peroxisomal enzyme, which is involved in a number of cellular metabolic processes as well as in detoxification of hydrogen peroxide (Moser and Moser, 1996). The reactive oxygen species produced as a byproduct of normal cellular metabolisms are also checked by catalase (Singh, 1996).

Naturally occurring antioxidants present in plants are the carotenoids (Ashmawy, 1999; Tanaka et al., 2001). Vitamin A has also been found to inhibit the formation of DNA adduct by Aflatoxin B1, a potent hepato-carcinogen in a dose dependent manner (Firozi et al., 1987). The elevated level or activation of the specific activities of SOD (P<0.01) and CAT (P<0.01) in the modulator
treated groups (i.e, Group II, III and IV) indicate that the extract of *Eclipta alba* possess anti oxidant property which may be attributed to the presence of vitamin A and C present in the herb (Indian Medicinal Plants, 1994; Indian Herbal Pharmacopoeia, 2002). This also serves as an explanation for their mode of action in the induction of antioxidant profiles in the present investigation; however, the exact mechanism is yet to be elucidated. (Table: 20, Fig. 17 & 18)

Induction of anti oxidant enzymes by medicinal plants and vegetables, such as *Glycosmis pentaphylla* (Azad et al., 2007), *Tinospora cordifolia* (Singh et al., 2006), *Butea monosperma* (Sehrawat and Sultana, 2006), *Ocimum sanctum* (Kath and Gupta, 2006), *Bauhinia racemosa* (Gupta et al., 2004), *Urtica dioica* (Ozen and Korkmaz, 2003), *Moringa oleifera* (Bharali et al., 2003), *Andrographis paniculata* (Singh et al., 2001) etc are in literature.

The activation of the antioxidant enzymes is also accompanied by a dose dependent depression in the specific activity of lipid peroxidation which is measured as the formation of MDA (Table: 21, Fig.19). Lipid peroxidation is a process responsible for generation of reactive oxygen species (ROS) that is coupled with tissue injury and damage of cellular macromolecules.
(Chung et al., 1996; Wiseman and Halliwell, 1996). Antioxidant activity perpetuated through up-regulation of anti oxidant enzymes and intracellular concentration of reduced GSH, results in a cascade of effects including a reduction in lipid peroxidation (Sun, 1990; Mc Cord, 1993). The depression in the levels of LPO in the present experiment by the *Eclipta alba* extract can be correlated with the induction of the reduced glutathione and the antioxidant enzymes above the basal level.

Studies have revealed that luteolin is effective in the protection of human single cell DNA from oxidative attack which indicates that *Eclipta alba*, which has luteolin as one of its constituents may have a preventive or curative effect on the oxidative stress caused by free radicals which is responsible for a wide variety of clinical disorders including cancers (Horvathova et al., 2004).

Free radicals and lipid peroxidation are known to cause initiation and promotion of carcinogenesis (Bauer and Wendel, 1980). Lipid peroxidation is also believed to increase during the carcinogenic process (Shamberger et al., 1972) and malondialdehyde (MDA), a product of lipid peroxidation was observed to be mutagenic and carcinogenic (Yay, 1979; Apaja, 1980; Basu and Marnett, 1983). It is therefore implied that agents that can reduce generation
of free radicals in vivo may be considered to have the potential for chemoprevention. The present study has shown that the *Eclipta alba* extract treatment could significantly reduce lipid peroxidation (P<0.01) in mice.

The marked modulation in the specific activities of the hepatic Phase I and Phase II drug metabolizing enzyme in female Swiss albino mice by the extract of the medicinal plant, *Eclipta alba* Linn represents a promising chemopreventive strategy, along with enhancement of antioxidant system enzymes which afford protection against cellular damage and inhibits tumor promotion. The biochemical basis of the chemopreventive potency of the *Eclipta alba* extract may be attributed to the individual or synergistic action of the constituents of the extract. However, the mechanisms underlying the action of the modulator (*Eclipta alba* extract) for its chemopreventive efficacy or the role of any of its constituents in mediating the chemopreventive response against DMBA induced skin papillomagenesis, Phase I enzymes, Phase II enzyme (GST), reduced glutathione, antioxidant enzymes (SOD and CAT) as well as lipid peroxidation (LPO) needs further investigation. The realm of study on the present line is very vast and the present work in this field is just a tip of the iceberg. Looking at the potential of the study, there is a lot of scope for in-depth investigations, including exact mechanism and clinical applicability of *Eclipta alba* as a chemopreventor.