Discussion


**DISCUSSION**

Owing to their immense potency, safety and lesser known side effects, medicinal plants have attracted attention as potential remedies for a number of diseases including cancer. Practitioners of traditional forms of medicine have recommended *Azadirachta indica* for the cure of various human ailments including cancer. Substantial amount of literature is available highlighting the pharmacological activities and medicinal applications of the various parts of *Azadirachta indica*. These biological activities have been reported with extracts, active principles and different fractions from leaf, bark, root, seed and oil. *Azadirachta indica* contains multiple active compounds that work simultaneously via different mechanisms. This characteristic explains its effectiveness as a pesticide, spermicide, fungicide etc and hence appears to be responsible for its potent impact on several types of cancers as well.

Although, various reports are available in support of the anti-cancer action of *Azadirachta indica*, it still cannot be recommended for clinical purposes presently. There is a need for carrying out mechanistic studies that could explain the cancer chemopreventive action of *Azadirachta indica* and would strengthen its use as an anticancer agent. Therefore, considering the need of experimental studies addressing the mechanism of action of *Azadirachta indica* mediated chemoprevention against carcinogenesis, the present investigation was designed to identify targets of *Azadirachta indica* leaf extract in the murine skin carcinogenesis model, with a special focus on transcription factors which regulate genes involved in neoplastic transformation (cell proliferation, apoptosis). The present study is also aimed to address the biochemical, histological and morphological changes that occur during the skin carcinogenesis and its intervention using *Azadirachta indica* leaf extract.

**Body Weight, Diet and Water Consumption**

The general health or body status of the experimental animals is one of the essential parameters which should be monitored while studying the effects of
carcinogens and their intervention with known and putative medicinal agents. Monitoring of body weight is essential during the tumorigenesis protocol because it gives an indication of the general health of the experimental animals. Body weight is easy to monitor and is a highly variable parameter as far as the progress of carcinogenesis is concerned. In the present investigation, the body weight of the experimental animals was monitored throughout the treatment period. At the end of the treatment period, it was observed that mice in control and AAILE groups gained weight when compared to their initial body weight. No considerable change was observed in the final body weight of mice in AAILE+DMBA/TPA group when compared to their initial body weight. However, a decrease in body weight was observed in DMBA/TPA group when compared to their initial body weight. Koul et al., (2006b) have earlier reported that DMBA induced skin tumorigenesis, caused reduction in body weight in mice receiving only DMBA treatment. However, mice receiving Azadirachta indica extract along with DMBA was observed to have higher body weights at the end of the treatment period when compared to their initial body weights. The observed decrease in final body weight in DMBA/TPA group may be attributed to the detrimental effects of carcinogen exposure and the subsequent tumorigenesis. Mice were also observed for their diet and water consumption throughout the experimental period. No change was observed in the diet and water consumption in any of the treatment groups.

Characteristics of Squamous Cell Carcinoma

Several models exist to study carcinogenesis and its chemoprevention. One of the better understood and characterised models is the mouse skin tumor promotion model. The tumor induction protocol involves the application of a sub-threshold dose of a carcinogen such as DMBA followed by the repetitive application of a tumor promoter such as TPA. Within 10 weeks, benign papillomas begin to appear and a small percentage of these papillomas eventually progress to malignant SCCs. The papillomas are benign neoplastic lesions comprising of hyperplastic keratinocytes and supporting stromal cells.
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The papillomas which progress to SCC are easily visualized and quantified. A small fraction of these can metastasize to distant sites (Kemp, 2005).

In the present study, skin tumor model was successfully established using DMBA as a carcinogen and TPA as a promoter. Throughout the treatment period, animals were carefully observed for the appearance of any lesions over the areas where DMBA/TPA was applied. The application of DMBA/TPA to the depilated skin of mouse resulted in the formation of SCC in DMBA/TPA and AAILE+DMBA/TPA groups, which were easily visible and counted. The skin underwent various changes such as hardening, roughening and eroding away of the skin surface with appearance of wound like lesions. By the end of seven weeks of DMBA/TPA treatment, small round lesions began to appear which grew in size and number as the treatment period progressed. SCC is a tumor of the epidermal keratinocytes and appeared as a raised, firm, pink to flesh colored keratin papule (Newman and Weinberg, 2007). It must be emphasized that even though the lesions in DMBA/TPA and AAILE+DMBA/TPA began appearing at the same time however, the increase in number and size of tumors was less in AAILE+DMBA/TPA group.

Further, it is worth mentioning that in one of the animals of AAILE+DMBA/TPA group, a tumor grew very large in size, and had a blackish appearance. This tumor got shed off the skin by itself. It is speculated that, in this case the growing size of the tumor was unable to keep pace with the angiogenesis required to nourish this tumor. This outstripping of blood supply may have triggered its loss of contact from the underlying skin and its eventual shedding off. Angiogenesis performs a critical role in the development of cancer and it is known that metastasis and angiogenesis are closely related. The growth of tumors beyond a particular size (1-2 mm³) requires vascularization, because oxygen and nutrients have difficulty diffusing to the cells in the center of the tumor, causing a state of cellular hypoxia which marks the onset of tumoral angiogenesis in growing (large) tumors. Vascularization also influences the dissemination of cancer cells throughout the entire body.
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eventually leading to metastasis or spread of cancer. The vascularization level of a solid tumor is thought to be an excellent indicator of its metastatic potential (Nanus et al., 1993; Toi et al., 1994). Several anti-proliferative agents are effective inhibitors of angiogenesis (Lin et al., 2007). It seems plausible that if new blood vessels are essential for tumor growth, then inhibiting angiogenesis should inhibit tumor expansion. If the tumor vessels regress after treatment, this should cause regression of the tumor (Folkman, 1972).

Chemopreventive Response of *Azadirachta indica* to Skin Tumorigenesis

Tumor incidence, mean tumor volume, mean tumor burden and cumulative number of tumors have been collectively taken as index of chemopreventive response to tumorigenesis. At the end of the treatment period, 100% tumor incidence was observed in DMBA/TPA group, while a tumor incidence of 58.3% was observed in AAILE+DMBA group. Thus, AAILE administration to DMBA/TPA treated animals reduced the tumor incidence by 41.7%. AAILE treatment to DMBA/TPA treated animals reduced the mean tumor volume by 45.6% and mean tumor burden by 54.5%. The chemopreventive response of *Azadirachta indica* was also observed in terms of total number of tumors and percentage of animals surviving without tumors. The total number of tumors at the end of the treatment period was 85 in DMBA/TPA group, while total number of tumors was only 32 in AAILE + DMBA/TPA group. The Kaplan Meir curve revealed that at the end of the treatment period 42% of the animals remained tumor free in AAILE+DMBA/TPA group and none of the animals remained tumor free in DMBA/TPA group. The above mentioned observations of the animal bioassay revealed a marked inhibition in DMBA/TPA induced skin tumorigenesis in response to AAILE treatment as is evident by the decrease in tumor incidence, cumulative number of tumors, tumor volume and tumor burden in AAILE+DMBA/TPA group.

Reports available in literature clearly indicate the anti-oncogenic potential of *Azadirachta indica*. Mahapatra et al (2011) have recently reported that *Azadirachta indica* exhibits anticancer action against prostate cancer due to the
presence of multiple compounds that have potent anti-inflammatory and anti-
oxidant activities. It was revealed that *Azadirachta indica* caused up-regulation of
genes associated with cell death, and drug metabolism, and down-regulated
genes associated with cell cycle, DNA replication, recombination, and repair
functions. Schumacher et al., (2011) have reported that *Azadirachta indica*
modulation of NF-kappa B pathway is associated with its anti-inflammatory,
anti-apoptotic and anti-proliferative activities.

Gangar et al., (2006a) have reported that AAILE exerts chemopreventive
activity against B(a)P induced forestomach tumorigenesis as revealed by the
decrease in tumor incidence, tumor multiplicity and tumor burden. Koul et al.,
(2006b) have reported that *Azadirachta indica* caused an inhibition in DMBA
induced skin tumorigenesis. Arakaki et al (2006) had reported that *Azadirachta
indica* leaf extract inhibited the occurrence of colonic pre-neoplastic lesions
which caused suppression of colon tumors. Studies carried out by Dasgupta et
al (2004) on forestomach and skin tumorigenesis in mice revealed that ENLE
exhibited anti-oncogenic action. Administration of ENLE caused an inhibition
in DMBA induced hamster buccal pouch carcinogenesis (Subapriya et al.,
2005a). *Azadirachta indica* contains a number of phytochemicals such as
flavonoids, limonoids, polyphenols, etc, that have been documented to retard
carcinogenesis at the initiation as well as the promotion stages of
carcinogenesis by virtue of their radical scavenging properties (Makita et al.,
1996; Rice-Evans et al., 1996). Reports indicate that ENLE and AAILE have
the potential to act at the initiation stage of carcinogenesis by repressing
carcinogen activation enzymes and inducing carcinogen detoxification
enzymes in the target and non-target organs (Dasgupta et al., 2004, Gangar et
al., 2006b, Koul et al., 2006a, Gangar and Koul, 2008a). Studies point out that
*Azadirachta indica* leaf extracts modulate genes critical to neoplastic
transformation. ENLE modulated cellular processes such as cell proliferation,
apoptosis, differentiation, etc, in hamster buccal pouch carcinogenesis
(Subapriya et al., 2005b; Subapriya et al., 2006; Manikandan et al., 2008).
**Discussion**

Gangar and Koul (2008b) have reported that AAILE induced apoptosis in forestomach tumors in mice which could have possibly been responsible for its chemopreventive action. The results of the present study are in corroboration with the reports available in literature highlighting the chemopreventive action of *Azadirachta indica* against skin tumorigenesis.

**Histoarchitectural Analysis of Skin Tumors**

Histopathological analysis of skin/skin tumors was conducted at the end of the treatment period. Skin from control animal exhibited uniformly arranged epidermal and dermal layers with the presence of subcutaneous tissue. Over the epidermal layer of the skin lies the layer of keratinocytes which arise by continuous division of cells that form the basal layer of the epithelium. Keratinocytes, in their transit towards the surface synthesize large amounts of a cytoskeletal protein called keratin (Fawcett and Jensh, 2002). Uniformly arranged epidermal and dermal layers with the presence of subcutaneous tissue, were observed in the skin of AAILE group, however a microscopically visible inflammation was observed in the epidermal layer. Application of DMBA/TPA to the mouse skin resulted in well developed skin tumors which were histologically characterised as SCC. Abnormally thickened (acanthosis) and corrugated epidermis was observed in the tumors of DMBA/TPA group. Hyperkeratosis i.e. thickening of keratinized layer of the epidermis and formation of keratin whorls (keratin pearls) and squamous eddies in the dermis was a prominent feature. SCC is a tumor of the epidermal keratinocytes (Preston and Stern, 1992). These results are supported by previous findings that DMBA/TPA results in the formation of keratinized pearls, suggesting invasive SCC (Dhawan et al., 1999). The keratinocytes lay in complete disorder which results in a classic ‘windblown’ appearance of the tissue. ‘Islands of epidermal cells’ encapsulated by keratin were seen invading into the dermis. Keratin whorls encapsulated by layers of cells were also observed. Similar histopathological findings in skin tumors have been reported by several investigators (Dhawan et al., 1999; Prakash et al., 2002; Padmavathi et al., 206
The tumors developed in AAILE+DMBA/TPA group were observed to be similar and diagnosed as SCC. Abnormally thickened and corrugated epidermis was also observed in AAILE+DMBA/TPA tumors; however, the epidermal thickness was less here. Areas with degenerative changes (areas devoid of cells) interspersed with cancer cells were observed in the tumors obtained from animals that were administered AAILE along with DMBA/TPA. It is evident from the histopathological studies that the severity of DMBA/TPA induced skin tumors was reduced markedly by AAILE administration. Based on histopathological analysis and observations of chemopreventive tumor response, previous reports from several laboratories have documented the chemopreventive action of *Azadirachta indica* against several animal models of tumorigenesis (Dasgupta et al., 2004; Koul et al., 2006b; Subapriya et al., 2005a; Gangar et al., 2006a). In the present investigation, the observations of the chemopreventive tumor response along with the histopathological findings strongly support the cancer chemopreventive potential of *Azadirachta indica*.

**Scanning Electron Microscopy of Skin Tumors**

SEM of skin from control animal showed normal epidermis with closely united assembly of squamous cells arranged in layers like tiles. It was observed that cells in the epidermis had well defined outlines and were in close apposition to each other. Such surface morphological features of skin using SEM have been reported previously (Fawcett and Jensh, 2002). The cells of the normal epidermis exhibited smoother topography compared to the cells observed in tumors obtained from DMBA/TPA and AAILE+DMBA/TPA group. In skin tumors of DMBA/TPA group, surface disruption was observed and exhibited rough surface topography. In the tumors of DMBA/TPA group, certain rounded structures were observed. These rounded structures may be regions of hyper proliferation since it is speculated that rapidly dividing tumor cells attain a round contour due to crowding of cells (Kessel and Shih, 1974; Paxton et al., 2000). At higher magnifications it can be seen that cells appeared to have lost
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the orderly arrangement, were disengaged and not in tight contact with each other. The profusely dividing cells gave the appearance of a flower like structure with the cells appearing as petals. Surface modifications were also observed in the tumors of mice that received AAILE along with DMBA/TPA. In tumors of AAILE+DMBA/TPA group, rounded structures were not observed. Areas with degenerative changes i.e. areas being sloughed of cells or areas that have been eroded of cells as a result were observed. These degenerative changes can be correlated with the observed decrease in mean tumor volume during intervention with AAILE. These degenerative changes in tumors of AAILE+DMBA/TPA could be attributed to the cytotoxic effect of the phytochemicals present in Azadirachta indica. Nimbolid, a limonoid present in various parts of Azadirachta indica exhibited cytotoxic effects on leukemic cell lines (Roy et al., 2007). Gangar and Koul (2008b) have documented that SEM view of B(a)P induced forestomach tumors of mice revealed rounded structures which were speculated to be regions of increased mitosis. Tumors of B(a)P+AAILE group revealed absence of rounded structures and presence of degenerative changes which may have resulted due to the differential cytotoxic and/or anti-proliferative activity of phytochemicals present in Azadirachta indica (Gangar and Koul, 2008b).

Modulation of Xenobiotic Metabolising Enzymes during Skin Carcinogenesis and its Amelioration by Azadirachta indica

The ultimate goal of carcinogenesis research is to elucidate the processes involved in the induction of human cancer so that interventions may be developed to prevent the disease. Possible ways of interfering with initiation of carcinogenesis include: modifying carcinogen activation by inhibiting the responsible enzymes or direct scavenging of DNA-reactive nucleophiles and free radicals, enhancing carcinogen detoxification by promoting the activity of carcinogen detoxification enzymes, modulating DNA repair processes, altering the expression of genes involved in cell signaling particularly those involved in cell proliferation, apoptosis, differentiation, invasion etc. It is apt and best

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suited to recognize the specific points along the carcinogenesis pathway that may be amenable to prevention strategies. Therefore, the focus of chemoprevention of cancer should be directed towards stopping carcinogenesis at the earliest possible point in the pathway. Therefore, in the present study we have explored the modulatory effect of AAILE on xenobiotic metabolising enzymes during DMBA/TPA induced skin carcinogenesis.

DMBA, a well known carcinogenic PAH has been reported to induce tumors of various organs in experimental animals and is also linked to the etiology of human cancers. DMBA, like most of the carcinogenic PAHs is a pro-carcinogen and requires metabolic activation to exert its carcinogenic effect. Cytochrome P4501B1 (CYP1B1) oxidizes DMBA to 3, 4-epoxide. This is followed by the hydrolysis of the epoxide by microsomal epoxide hydrolase to the proximate carcinogenic metabolite, DMBA-3,4-diol. This metabolite is then further oxidized by either CYP1B1 or cytochrome P4501A1 (CYP1A1) to the principal ultimate carcinogenic metabolite, DMBA-3,4-diol-1,2-epoxide, which is capable of producing DNA adducts (Dipple and Nebzydoski, 1978; Tierney et al., 1978; Wislocki et al., 1980; Christou et al., 1989). Covalent interaction of the reactive metabolite with DNA results in the formation of DNA adducts which if not repaired may lead to gene mutations, upon replication of the damaged DNA. Certain mutations, particularly those resulting in the activation of proto-oncogenes or inactivation of tumor suppressor genes are thought to play an important role in the initiation phase of carcinogenesis (Dipple, 1991). Although, protein adducts and RNA adducts are also formed on interaction of the reactive metabolite with the nucleophilic sites on these macromolecules, however, DNA adduct formation has been established as a critical phase in the initiation of carcinogenesis (Singh et al., 1998).

Cytochrome P450 is a multi-gene super-family of biotransformation enzymes comprising of numerous structurally related isozymes present primarily in the liver and to some what lesser extent in other organs such as skin, lung, bladder
etc and plays a major role in the metabolism of exogenous and endogenous substrates (Ray et al., 2001). These enzymes function as monoxygenases and catalyze the oxidation of lipophilic substrates such as chemical carcinogens to more polar, hydrophilic, water-soluble metabolites in an attempt to facilitate their excretion from the body. At the same time, induction of phase I enzymes is considered to be a potential risk factor because many of the phase I reactions such as hydroxylation, epoxidation etc leads to the activation of procarcinogens to their ultimate carcinogenic form which is rendered suitable for interaction with nucleophilic sites in DNA (Lampe et al., 2000). Aryl hydrocarbon Hyroxylase (AHH) is a CYP dependent carcinogen-metabolizing enzyme that catalyses the oxidative biotransformation of PAHs to reactive metabolites such as phenols, dihydrodiols, quinones, and epoxides (Sims, 1967; Jerina et al., 1970; Selkirk et al., 1971). AHH is present in several human tissues including liver (Kuntzman et al., 1966; Kapitulnik et al., 1977), lung (Prough et al., 1977), placenta (Juchau et al., 1973), pulmonary alveolar macrophages (McLemore et al., 1977), lymphocytes (Gurtoo et al., 1975), and skin (Levi et al., 1972; Alvares et al., 1973). Inducibility of AHH by environmental carcinogens may correlate with susceptibility to tumorigenesis in experimental animals and in human populations. AHH may be a critical determinant of cutaneous carcinogenic responses to PAHs such as B(a)P by transforming the parent compound into proximately reactive metabolites skin in vivo (Gelboin et al., 1970). Cytochrome b5 is another heme-protein found in the endoplasmic reticulum of eukaryotic cells and has been known to augment some CYP monoxygenase reactions (Porter, 2002). NADPH is used to reduce the oxidized CYP so that there is a continuous source of reduced CYP to metabolize the xenobiotic. Cytochrome b5 can also act as the electron donor to replenish the reduced CYP store.

Treatment of AAILE for two weeks did not cause any appreciable change in CYP and cytochrome b5 content in skin when compared to the control group. AHH activity decreased in skin following two weeks of treatment with AAILE.
DMBA/TPA treatment for eight weeks enhanced the content of CYP, AHH and cytochrome b5 in skin when compared to control group. Administration of AAILE to the DMBA/TPA treated animals caused an appreciable increase in CYP, cytochrome b5 content and AHH activity when compared to control group. AAILE treatment to DMBA/TPA treated animals repressed the carcinogen activation by decreasing the levels of cytochrome P450 without causing any appreciable change in b5 and AHH. Previous studies have revealed that preparations of various parts of neem tree have the potential to reduce the action of phase I enzymes. Repressing the activity of phase I activation enzymes by *Azadirachta indica* may in part be responsible for its anticancer action. Kusamran et al., (1998) have reported that feeding a diet containing *Azadirachta indica* flowers decreased the content of CYP in the hepatic tissues of rats. Dasgupta et al (2004) have reported a down regulatory effect of ENLE on the phase I enzymes in hepatic, forestomach and renal tissues in mice. Koul et al (2006a) have reported a decrease in the content of cytochrome P450 and cytochrome b5 enzymes in hepatic tissue of skin tumor bearing mice after treatment with AAILE. Gangar et al (2006b) have reported a decrease in content of phase I enzymes in hepatic and forestomach tissues after treatment with AAILE.

Flavonoids have been reported to inhibit the activity of cytochrome P450 dependent enzymes that metabolize carcinogens such as PAH (Bueing, 1981). Phytochemicals like tannins and phenols quench the enzymatically generated electrophiles. They also bind irreversibly with the active site of microsomal enzymes or even perform a direct attack on membrane bound phospholipids or cytochrome P450 which are required for bioactivation. They may perform a direct attack on the membrane bound phospholipids or cytochrome P450 or even bind irreversibly with the active site of the enzyme rendering it inactive. Phytochemicals like these and many more are abundantly present in *Azadirachta indica* and could thus explain its suppressive action of phase I enzymes. No change was observed in CYP, cytochrome b5 content and AHH
activity in AAILE group when compared to control group at ten weeks duration.

Glutathione-S-Transferase (GST) is a major phase II xenobiotic biotransformation enzyme that catalyses the conjugation of a variety of endogenous and exogenous compounds with the non-protein thiol, GSH. This conjugation reaction inhibits the reactive cellular nucleophiles from reaching cellular targets such as DNA, RNA and protein and result in the production of a thio-ether linked glutathione conjugate that is less toxic and readily excreted from the body. After the generation of the conjugated moiety it is subsequently eliminated via a GSH-conjugate recognizing transporter (Nakamura et al., 2000). High cancer susceptibility is associated with depletion in GST activity (Abraham and Singh, 1999). The role of GSH in GST mediated conjugation/detoxification of xenobiotic metabolites is most important in modulating the process of initiation of chemical carcinogenesis. Cancer chemopreventive effects of several agents involve the induction of GST activity, facilitating the excretion of DNA damaging entities (Hu and Singh, 1997; Wilkinson and Clapper, 1997). DT-diaphorase is another phase II biotransformation enzyme which catalyses two-electron reduction of a wide variety of substrates including PAH o-quinone to inactive products such as PAH-hydroquinone (Ross and Siegel, 2004). This reaction prevents one electron redox cycling of these groups, thereby preventing the formation of DNA damaging ROS. These quinones can then be conjugated to UDP-glucuronic acid and excreted out of the body (Begleiter et al., 1997). Skin tumors caused by B(a)P and DMBA are increased in DTD knock-out mice, indicating that it is somehow involved in detoxification of PAHs (Long et al., 2001). Another important detoxification pathway is the glucoronidation pathway and is probably the most frequent conjugation reaction. Glucoronidation is a major pathway for detoxification of numerous carcinogens including PAHs, aryl and heterocyclic amines (Hecht, 2002). UDP-glucuronic acid is the glucuronyl donor and glucuronyl transferases present in both the cytoplasm and endoplasmic reticulum are the
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catalysts. Glucuronic acid is conjugated to large number of potentially reactive compounds including phenols, dihydrodiols, quinones and quinols (Mulder et al., 1990). Conjugates can include ether O-glucuronides, ester O-glucuronides, N-glucuronides, S-glucuronides and C-glucuronides (Turesky et al., 1991). Like sulfation and glutathione conjugation, glucuronidation produces polar conjugates that are readily excreted. Glucuronidation catalyzed by glucuronyl transferases is a major pathway of metabolism of estrogens (Zhu and Conney, 1998) and androgens (Belanger et al., 1998) in the breast. The UDP-GT enzyme transforms lipophilic compounds to more water soluble metabolites via conjugation with glucuronic acid. This reaction inhibits reactive electrophiles from reaching cellular targets, hence resulting in the production of less cytotoxic conjugates that may be readily excreted in bile or urine.

AAILE treatment for two weeks enhanced the activity of GST in skin without causing any appreciable change in DTD and UDP-GT activities. DMBA/TPA treatment for eight weeks decreased the activity of DTD in skin and caused no appreciable change in GST and UDPGT activities. Administration of AAILE to DMBA/TPA treated animals enhanced the activities of GST and DTD in skin when compared to control group and remained unaltered when compared to DMBA/TPA group. However, UDP-GT activity increased in skin of AAILE+DMBA/TPA group when compared to DMBA/TPA group. Tumors obtained in DMBA/TPA group exhibited decrease in GST activity when compared to control group. However, administration of AAILE to tumor bearing mice enhanced the activity of GST when compared to tumors obtained in DMBA/TPA group. Earlier reports from our laboratory have revealed that intra-gastric instillation of B(a)P decreased the activity of UDP-GT in forestomach and liver and administration of AAILE to B(a)P treated animals enhanced the UDP-GT activity after 28 and 56 days of initiation of the treatment period (Gangar and Koul, 2008a). Dasgupta et al (2004) have reported an increase in activity of GST and DTD in pulmonary, hepatic and forestomach tissues after treatment with ENLE. Subapriya et al., (2005c) have
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reported an enhancement in GST activity in buccal pouch tumors of hamsters treated with ENLE.

The present observations and reports from the literature reiterate that *Azadirachta indica* has the potential to decrease the activation of carcinogens and increase the detoxification of reactive metabolites and possibly decrease the risk of initiation of carcinogenesis. Thus *Azadirachta indica* serves as a ‘dual’ agent.

**Role of Oxidative Stress in *Azadirachta indica* Mediated Chemoprevention against DMBA/TPA induced Skin Tumorigenesis**

Biotransformation of pro-carcinogens/carcinogens leads to the enhanced generation of reactive oxygen species (ROS)/reactive nitrogen species (RNS) resulting in oxidative stress, which is one of the causative mediators of the tumorigenic process (Perchellet et al., 1995). Excessive ROS production leads to the oxidative modification of cellular macromolecules (lipids, proteins, nucleic acids) with potentially deleterious effects. They cause structural damage to DNA and have the potential to mutate cancer-related genes resulting in the malignant transformation of cells and development of cancer. At the same time, oxidants activate signal transduction pathways and alter the expression of growth and differentiation related genes. There are studies that have established that DNA damage by ROS is responsible for mutagenesis, oncogenesis and aging. ROS induced lesions in DNA include base modifications, strand breaks and abasic sites (Ahmad et al., 2005). The carcinogenic effects of ROS have been primarily attributed to its genotoxic effects, but they are also known to play a significant role in the promotion stage of carcinogenesis. It is known that several oxidants and free radical generators can behave like tumor promoters.

However, now the dual role of ROS/RNS has been recognized in the living systems (Valko et al., 2004), especially in regard to the mechanism of action of anticancer agents. Interestingly, reports on cancer chemopreventive agents
suggest that they enhance peroxidative damage in the tumorous tissue thereby exercising their anti-oncogenic action (Balasenthil et al., 1999; Subapriya and Nagini, 2003; Gangar and Koul, 2008b). The detrimental/beneficial effects of ROS/RNS depend upon the state and the type of tissue and the dose of the phytochemical being used.

Oxidative damage to lipids by ROS leads to LPO forming low molecular entities that have the potential to damage genetic material. ROS formed during metabolism of carcinogens can diffuse from the site of generation to other targets within the cells or at times even out of the cells. ROS entails deleterious effects by initiating LPO directly or by acting as secondary messengers for the primary free radicals that initiate LPO via oxidation of PUFAs. ROS target the carbon-carbon double bond of PUFAs. The double bond on the carbon weakens the carbon-hydrogen bond allowing for easy disassociation of the hydrogen by a free radical. A free radical steals the single electron from the hydrogen associated with the carbon at the double bond. In turn this leaves the carbon with an unpaired electron and hence becomes a free radical. In an effort to stabilize the carbon centered free radical molecular arrangement occurs. The newly arranged molecule is called a conjugated diene (CD). The CD then easily reacts with oxygen to form peroxyl radical. The peroxyl radical steals an electron from another lipid molecule in a process called propagation. This process continues in a chain reaction (Halliwell and Gutteridge, 1999).

Free radicals are extremely reactive and have a very short half life. This sometimes makes it difficult to quantitate them. A common approach of assaying involves measuring markers of free radicals than the actual radical itself. When a fatty acid is peroxidized it is broken down into aldehydes, which are excreted. Aldehydes such as Thiobarbituric Acid Reactive Substances (TBARS) have been widely accepted as a general marker of free radical production (Clarkson, 1995). The most commonly measured TBARS is Malonaldehyde (MDA) (Karlsson, 1997). Therefore, LPO is considered as an index of oxidative stress. During their course of action, chemopreventive
agents produce numerous electrophilic species creating a pro-oxidant milieu in the cell leading to oxidative stress-induced LPO which can then attack key targets in the cell. This oxidative damage can slow cell cycle progression of cancer cells and cause cell cycle checkpoint arrest and also induce apoptosis (Johnson et al., 1996; Conklin, 2004)

In the present study it was observed that skin tumors obtained in AAILE+DMBA/TPA group exhibited enhanced LPO levels when compared to the LPO levels in tumors of DMBA/TPA group. This suggests that *Azadirachta indica* caused damage to the tumors selectively resulting in the inhibitory effect on skin tumorigenesis. Also, at time point of 8 weeks of DMBA/TPA treatment (when the papillomas begin to appear), it was observed that skin of AAILE+DMBA/TPA treated animals exhibited an increase in LPO levels when compared to DMBA/TPA treated skin. Although this increase was statistically non-significant it may be emphasized that *Azadirachta indica* persistently keeps the LPO levels high, in an attempt to prevent the damaged cells from thriving. From our observations and reports available in the literature, it seems that an enhanced LPO level (which is an indication of its pro-oxidant activity) may be one of the key mechanisms by which *Azadirachta indica* exerts its chemopreventive action. DMBA/TPA treatment for eight weeks did not alter the skin LPO levels when compared to control group. However, the tumors in DMBA/TPA group exhibited decreased LPO levels when compared to control group. There are several reports available in literature which indicates that high oxidative stress slows tumor growth (Bartoli and Galeotti, 1979; Masotti et al., 1988).

Earlier reports have revealed that *Azadirachta indica* acts as a pro-oxidant in the tumors which could be responsible for its inhibitory effect on skin tumorigenesis (Koul et al., 2006b) and forestomach tumorigenesis (Subapriya and Nagini, 2003; Gangar and Koul, 2008b). This suggests that *Azadirachta indica* selectively favors the excessive generation of ROS in tumorous tissues, which could have been responsible for the higher apoptotic index observed
Many cancer chemotherapeutic agents have been known to exert their action by behaving as pro-oxidants selectively in the tumorous tissue (Yang et al., 2000; Morin et al., 2001). Several studies have established that anti-neoplastic agents generate ROS in the target tissue which may be involved in the regulation of apoptosis (Gangar and Koul, 2008b; Jeong and Seol, 2008; Kumar et al., 2009), since ROS is considered as a downstream mediator of apoptosis (Johnson et al., 1996). Koul et al (2006b) have reported that administration of \textit{Azadirachta indica} leaf extract during DMBA induced skin carcinogenesis exhibited a significant reduction in the mean tumor burden and tumor volume in comparison with the animals treated with DMBA only. Here, it was observed that mice that received \textit{Azadirachta indica} leaf extract along with DMBA had higher LPO levels in the tumors compared with the tumors of DMBA group. Thus, \textit{Azadirachta indica} may be exerting its protective effect by making the tumor cells more vulnerable to peroxidative damage as was evident through biochemical and histopathological investigations. Diminished LPO in skin tumors may serve to maintain a reduced environment which is conducive for cell proliferation, thus offering a selective growth advantage to the tumor cells (Koul et al., 2006b). In the present study it was observed that administration of \textit{Azadirachta indica} leaf extract induced apoptosis in tumors of AAILE+DMBA/TPA group.

Enhanced ROS generation can also slow cell cycle progression of cancer cells and cause cell cycle checkpoint arrest (Conklin, 2004). In corroboration with other studies, in the present investigation it was observed that \textit{Azadirachta indica} acts as a pro-oxidant in the tumors which could be responsible for its inhibitory effect on skin tumorigenesis and possible regulation of cell cycle culminating in low cellular proliferation. During their course of action, anti-neoplastic agents can generate oxidative stress which can result in damage to important molecules in the cell. This oxidative damage can slow cell cycle progression of cancer cells and cause cell cycle checkpoint arrest (Conklin, 2004). Oxidative damage to cellular DNA in the cancer cells has been proposed...
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as an important mechanism responsible for the anticancer and apoptosis inducing properties of resveratrol (Azmi et al., 2006; de la Lastra and Villegas, 2007). One of the many cellular responses to ROS is that cells leave the cell cycle and may undergo apoptosis. Generation of oxidative stress or inhibition of antioxidant pathways initiates an apoptotic cascade in cells (Hockenberry et al., 1993; Kane et al., 1993).

One of the major effects of oxidative stress is its ability to reduce the rate of cell proliferation by (1) inhibiting the transition of cells from G0 phase to G1 phase, (2) prolonging G1 phase, (3) delaying progression through S phase by inhibiting DNA synthesis, (4) inhibiting cell cycle progression through restriction point and (5) causing cell cycle arrest at checkpoints (Kurata, 2000; Schackelford et al., 2000; Gonzalez, 1992). The cells may be arrested in any of the stages of the cell cycle; G0, G1 or S phase. ROS induces the expression of important cell cycle regulatory proteins like p53, p21 and p27 which block cell cycle progression (Engel et al., 2005). Cell proliferation in normal and cancer cells growing in culture is inhibited in the presence of pro-oxidant milieu. It has also been reported that oxidative stress slows tumor growth in laboratory animals (Bartoli and Galeotti, 1979; Masotti et al., 1988). High growth rate is observed in normal liver tissue (Wolfson et al., 1956; Cheesman et al., 1986) and tumors (Bartoli and Galeotti, 1979; Dianzani, 1993) with low levels of LPO.

Modulation of Antioxidant Defense System during DMBA/TPA Induced Skin carcinogenesis and its Intervention with *Azadirachta indica*

Considering the role of ROS and antioxidant defense system in carcinogenesis and its chemoprevention, components of the antioxidant defense system were analysed at different stages of the treatment period (after two weeks of AAILE treatment, eight and twenty weeks of DMBA/TPA treatment and its modulation by AAILE). Variable reports on the role of various antioxidant enzymes and non-enzymatic components on carcinogenesis and its chemoprevention are available in literature.
Reduced glutathione (GSH) is a tri-peptide (cysteine, glutamic acid and glycine) endowed with the functions of detoxification and antioxidant defense. GSH is an important constituent of intracellular protective mechanisms against a number of noxious stimuli and is known to be a major low molecular weight scavenger of free radicals in cytoplasm. It is present in the cell in free form or bound to proteins. It can be converted to its oxidized form (GSSG) during oxidative stress and can be converted back to GSH by the action of GR. It participates in the detoxification of \( \text{H}_2\text{O}_2 \) and other free radicals. By conjugating with reactive electrophilic moieties, it aids in the detoxification of several unwanted metabolic products which get excreted in the urine or feces in the form of mercapturic acid (Meister and Larsson, 1989; Meister, 1994, Lu, 1999). GSH also serves to maintain the sulphydryl groups of many proteins in their reduced form by preventing oxidation of \(-\text{SH}\) groups or by reducing disulfide bonds induced by oxidative stress and scavenging free radicals, which is essential for their normal cell function (Lu, 1999). The role of GSH in GST mediated conjugation/ detoxification of xenobiotic metabolites is most important in modulating the process of initiation of chemical carcinogenesis. A number of toxic electrophilic xenobiotics are conjugated to GSH by GST and rendered inactive (Schrimer et al., 1989). If the toxic metabolites are not conjugated to GSH they become free to attack the cellular macromolecules such as DNA, RNA and protein with potentially deleterious effects.

In the present study it was observed that levels of GSH increased in skin following treatment with AAILE for two weeks. It may be speculated that enhanced GSH levels may offer protection against the impending carcinogen/genotoxin attack. DMBA/TPA treatment for twenty two weeks produced SCC with low levels of GSH when compared to control group. Gangar and Koul (2008a), have reported a decrease in GSH levels in forestomach of mice after 28 and 56 days of first B(a)P intra-gastric instillation. During the course of action of carcinogens, mutagens, and tumor promoters ROS are produced along with the active reactive metabolites.
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(Cerruti, 1985; Nishigori et al., 2004). GSH pool of the cell serves to scavenge the toxic metabolites and excess ROS produced which may be responsible for the observed decrease in GSH levels in response to treatment of DMBA/TPA. Interestingly, treatment with AAILE caused a decrease in skin GSH levels after eight weeks of DMBA/TPA treatment and increase in GSH levels of tumors after twenty two weeks of DMBA/TPA treatment. The GSH enhancing effect of *Azadirachta indica* leaf extract has been reported in various animal tissues (Dasgupta et al., 2004). In a study carried out on DMBA induced skin tumorigenesis and its chemoprevention with AAILE, an enhancement in GSH levels was observed in tumors of AAILE treated animals (Koul et al., 2006b). Administration of ENLE to buccal pouch bearing tumors also enhanced GSH levels in tumors (Subapriya et al., 2005c). This enhanced GSH levels may serve to detoxify electrophiles in conjunction with GST. Administration of AAILE to tumor bearing mice enhanced the GST activity in tumors when compared to the DMBA/TPA group. However, GSH levels remained unaltered in AAILE group (at ten and twenty two weeks duration) when compared to control group.

Glutathione peroxidase (GPx) catalyses the reaction of hydroperoxides with GSH to form glutathione disulphide (GSSG) and the reduction product of the hydroperoxide (Chance et al., 1979). GPx catalyses the reduction of H$_2$O$_2$ sharing its function with catalase. GPx is responsible for the detoxification of H$_2$O$_2$ in low concentrations whereas catalase comes into play when GPx is saturated with substrate. GPx also reduces lipid hydroperoxides to their corresponding alcohols. The reaction mediated by GPx requires GR and cofactors like GSSG, NADPH to function at high efficiency. GR is an antioxidant defense enzyme that catalyses the NADPH dependent reduction of glutathione disulphide to GSH thus maintaining the antioxidant pool of the cell (Katiyar et al., 1993).

AAILE treatment for two weeks did not cause any change in the skin GPx and GR activities when compared to control group. DMBA/TPA treatment for eight
weeks enhanced the GPx activity in skin while DMBA/TPA treatment for twenty weeks decreased the GPx activity in tumors when compared to the control group. Administration of AAILE to DMBA/TPA treated animals caused a decrease in GPx activity after eight weeks of DMBA/TPA treatment and an increase after twenty weeks of DMBA/TPA treatment. GPx activity remained unaltered in skin of AAILE group when compared to control group. Koul et al., (2006b) have reported that GPx activity increased in tumors of *Azadirachta indica* treated mice. Similar alterations in GPx activity have been reported in hamster buccal carcinogenesis and its intervention with ENLE (Subapriya et al., 2005c). After eight weeks of DMBA/TPA treatment, skin GR activity decreased when compared to control group, however GR activity remained unaltered in tumors after twenty weeks of DMBA/TPA treatment. GR activity increased in skin and skin tumors after treatment with AAILE. Koul et al. (2006b) have reported an increase in GR activity in DMBA induced skin tumors when compared to control group. Following treatment with *Azadirachta indica* GR activity decreased in tumors when compared to DMBA/TPA group.

SOD, a metalloprotein found in both prokaryotic and eukaryotic cells is a part of the primary antioxidant defense system (Fridovich, 1986). SOD catalyses the dismutation of superoxide into oxygen and H$_2$O$_2$. This enzyme is of prime importance as far as free radical mediated damage is concerned. Malignant tumors have been reported to have low MnSOD activity (Oberley and Buettner, 1979; Sun, 1990). Administration of AAILE for two weeks increased SOD activity in skin when compared to control group. DMBA/TPA treatment for twenty weeks decreased SOD activity in skin tumors when compared to the control group and remained unaltered in skin after eight weeks of DMBA/TPA treatment. An enhancement in SOD activity was observed in skin and skin tumors when DMBA/TPA (eight and twenty weeks respectively) treated animals were administered AAILE. Enhanced SOD has demonstrated to inhibit tumorigenesis both *in vivo* and *in vitro* (Oberley and Buettner, 1979; Oberley,
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Enhancement of SOD activity has been reported in hepatic tissue after treatment with ENLE (Dasgupta et al., 2004). Investigations carried on DMBA induced skin tumorigenesis has revealed that SOD activity increased in tumors of mice treated with AAILE in comparison to tumors from DMBA group. This was related with the observed inhibition in DMBA induced skin tumorigenesis in response to AAILE (Koul et al., 2006b). Antioxidant therapies against cancer are focused on enhancing the activities of SOD and catalase (Nelson et al., 2006). At ten and twenty two weeks duration, SOD activity remained unaltered in AAILE group when compared to control group.

Catalase is a hemoprotein which catalyses the decomposition of H$_2$O$_2$ to water and oxygen, thereby protecting the cell from oxidative damage (Deisseroth and Dounce, 1970). However, variable reports are available in literature regarding the involvement of SOD and catalase in carcinogenesis and/or its chemoprevention using several well known and putative chemopreventive agents including Azadirachta indica. In the present investigation, a decrease in skin catalase activity was observed after two weeks of AAILE treatment. After eight weeks of DMBA/TPA treatment skin catalase activity increased when compared to control group, however no alteration was observed in skin tumors after twenty weeks of DMBA/TPA treatment. Catalase activity increased in skin and skin tumors after treatment with AAILE. Koul et al (2006b) have reported no appreciable change in catalase activity in DMBA induced skin tumors and an enhanced catalase activity in tumors of animals administered with AAILE. Catalase activity increased in skin of AAILE treated animals at ten weeks duration but no change was observed at twenty two weeks duration.

Involvement of Transcription Factors in DMBA/TPA Induced Skin Carcinogenesis and its Amelioration by Azadirachta indica

Transcription factors are regulatory proteins that recognize and bind specific DNA sequences and recruit the correct RNA polymerase to carry out RNA synthesis, consequently regulating the expression of genes. The involvement of transcription factors cannot be denied in the process of tumorigenesis because
they regulate the expression of genes involved in cell survival, cell proliferation, cell adhesion, differentiation, cell growth, inflammation, invasion and angiogenesis.

NF-kappa B is a transcription factor that consists of 2 subunits: a 50 kilodalton subunit (p50) and a 65 kilodalton subunit (p65, also known as RelA) (Aggarwal et al., 2006) and is constitutively expressed in a wide variety of tumors, including breast, colon, pancreatic, lung, head and neck, skin etc (Collins et al., 2000). It is activated in response to various inflammatory stimuli such as cytokines, growth factors, hormones, carcinogens, tumor promoters, radiation, oxidative and chemical stress etc. NF-kappa B belongs to the proto-oncogene family and many functions of their encoded proteins have important implications for the development of cancer and its therapy (Luque and Gelinas, 1997).

In the present study, treatment of DMBA/TPA to the skin resulted in formation of tumors with an enhanced mRNA and protein expression of NF-kappa B (p65) when compared to the expression levels in skin from control group. Carcinogens like DMBA, B(a)P diol epoxide (BPDE), 4-(methylnitrosamino)-1-(3-pyridyl)-1-butane and tumor promoters like phorbol ester and okadaic acid have been shown to activate NF-kappa B (Dong et al., 1997; Shishodia et al., 2003). The inhibitory effect of AAILE on skin carcinogenesis was associated with a reduced mRNA and protein expression of NF-kappa B (p65) in tumors of AAILE+DMBA/TPA group when compared to the tumors of DMBA/TPA group. No significant change was observed in the p65 mRNA and protein expression in AAILE group when compared to control group. The results obtained in the present study are in corroboration with other investigations where a decrease in NF-kappa B expression has been observed in tumors following treatment with limonoids isolated from Azadirachta indica (Kumar et al., 2009). Protective effect of Azadirachta indica in HBP carcinogenesis was also associated with a decrease in NF-kappa B expression (Manikandan et al., 2008). NF-kappa B has been implicated in carcinogenesis.
because it regulates genes critical to the process of cancer including cell survival, cell adhesion, inflammation, differentiation, apoptosis, angiogenesis etc (Mitisiades et al., 2002). Besides having implications in tumor growth and progression, NF-kappa B also plays an active role in development of resistance to anticancer drugs and radiation (Luo et al., 2005).

The AP-1 transcription factor functions as a dimeric complex that contains members of the jun, fos, ATF and MAF protein families (Chinenov and Kerppola, 2001; Vogt, 2002). AP-1 proteins exhibit both oncogenic and anti-oncogenic activities. AP-1 can exert its oncogenic or anti-oncogenic effects by differentially regulating genes involved in cell proliferation, differentiation, apoptosis, angiogenesis and tumor invasion. AP-1 activity can be regulated by dimer composition, transcription, post-translational modification and interactions with other proteins (Eferl and Wagner, 2003). The decision as to whether AP-1 is oncogenic or anti-oncogenic depends on the cell type and its differentiation state, tumor stage and the genetic background of the tumor (van Dam and Castellazzi, 2001; Bakiri et al., 2002). Likely as NF-kappa B, AP-1 is important in traversing tumor promotion and progression because of its ability to alter gene expression in response to carcinogens and tumor promoters including TPA, UV radiation and ROS (Dong et al., 1997).

Tumors from DMBA/TPA group expressed higher mRNA and protein levels of c-jun and c-fos when compared to the control group. Interestingly, administration of AAILE to the DMBA/TPA treated animals caused a further appreciable increase in mRNA and protein expression of c-jun and c-fos when compared to the expression levels in tumors from DMBA/TPA group. No significant alteration in c-jun and c-fos expression was observed in AAILE group when compared to the control group. Variable reports are available in literature regarding the involvement of AP-1 in cancer and its chemoprevention. c-jun is essential in the development of skin and liver tumors, as inhibiting c-jun activity in basal keratinocytes of skin or in hepatic cells, interferes with the development of chemically induced papillomas and
liver tumors (Young et al, 1999; Eferl et al., 2003). Some other studies suggest otherwise. Expression of c-jun was shown to be involved in apoptosis induction and growth inhibition of many anti-cancer agents (Liu et al., 2003). Yuan et al., (2004) have reported that over expression of c-jun induced by quercetin and reserveratol inhibits the expression and function of the androgen receptor in human prostate cancer cells. *in vitro* studies have indicated that increased AP-1 activity can lead to apoptosis in specific cell types including human tumor cells (Shaulian and Karin, 002).

AP-1 is a redox sensitive transcription factor which gets up regulated in response to enhanced ROS generation. Application of DMBA/TPA to the mouse skin leads to accumulation of ROS which may explain the observed increase in AP-1 expression. In studies carried out by Murray et al (2007), it has been demonstrated that AP-1 expression in mouse skin increases with an accumulation of peroxidative products. It has been earlier reported that *Azadirachta indica* exerts chemopreventive action in skin tumors by acting as a pro-oxidant and enhancing peroxidative damage in the tumors (Koul et al., 2006b). Therefore it may be possible, that by virtue of the pro-oxidant nature of AAILE and accumulation of peroxidative products in AAILE+DMBA/TPA tumors, there could be an increase in the expression of AP-1. The exact role of AP-1 in *Azadirachta indica* mediated inhibition in skin tumorigenesis needs to be investigated further.

Signal transducers and activators of transcription (STATs) are a family of transcription factors which are localized in the cytoplasm in an inactive form and get translocated to nucleus on activation. STATs are activated in response to a wide spectrum of growth factors, cytokines and hormones to modulate genes involved in cell proliferation, apoptosis, differentiation, survival and other biological functions (Levy and Darnell, 2002). STAT activation is dependent upon tyrosine phosphorylation which induces dimerization between two STAT molecules. Activated dimer STATs translocate to the nucleus and bind to consensus promoter sequences of target genes thereby regulating their...
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Constitutive activation of STAT proteins has been described in a large number of human cancer lines and primary tumors including blood, head, neck, breast, lung, skin etc (Bromberg, 2002). STAT 1 functions as a negative regulator of neoplastic transformation whereas STAT 3 and STAT5 are implicated in the oncogenic transformation of cells. STAT 1 and STAT 3 have antagonistic effects on cell proliferation and apoptosis (Stephanou and Latchman, 2005). The differential functions of STAT proteins is due to their preferential activation by certain ligands, their binding to and activation of specific genes and/or due to distinct functional domains.

In the present study, a decrease in mRNA and protein expression of STAT 1 was observed in skin tumors of DMBA/TPA group when compared to control group. AAILE administration to DMBA/TPA treated animals was accompanied by an increase in mRNA and protein expression of STAT 1 when compared to the DMBA/TPA group. STAT 1 is required for apoptosis in some cell types and seems to act in a pro-apoptotic and anti-proliferative conduct because of its capacity to induce caspase and p21 expression (Bromberg, 2002; Levy and Darnell, 2002). Loss of STAT 1 expression or its inappropriate activation has been observed in malignant cells obtained from tumors of different histological type such as that of breast cancer, head and neck cancer, melanoma, leukemia and lymphoma (Bromberg and Darnell, 2000; Battle and Frank, 2002; Stephanou and Latchman, 2005). Studies have revealed that activated STAT 1 (phosphorylated at serine 727) can positively influence apoptosis in cells (Janjua et al., 2002). Anticancer drugs like doxorubicin and cisplatin which induce apoptosis in malignant cells also potentiate STAT 1 phosphorylation (Thomas et al., 2004; Townsend et al., 2004). No appreciable change was observed in the mRNA and protein expression of skin from AAILE group when compared to control group.

Tumors from the DMBA/TPA group revealed an enhanced mRNA and protein expression of STAT 3 when compared to control group. Interestingly, administration of AAILE to tumor bearing mice also enhanced the mRNA and
protein expression of STAT 3 when compared to the tumors obtained in DMBA/TPA group. Experimental evidences suggest that STAT 3 mediation of apoptosis inhibition may be caused by regulation of proteins involved in apoptosis such as bcl-xl. STAT 3 regulates transcription from the bcl-xl promoter and cells transformed by constitutively active STAT 3 have elevated levels of bcl-xl mRNA (Bromberg et al., 1999). Disruption of STAT 3 function has been shown to induce apoptosis in murine melanoma model (Niu et al., 1999). Grandis et al (2000) have reported increased tumor apoptosis associated with decreased bcl-xl protein expression after intra-tumoral injection of a STAT 3 anti-sense expression construct support. For many cancers, elevated levels of activated STAT3 have been associated with a poor prognosis. In contrast to normal cells where STAT3 phosphorylation is only transient, its constitutive activation has been reported in several primary cancers, in tumors cell lines and in many oncogene-transformed cells. At the molecular level, STAT3 participates in cell transformation through the activation of several genes involved in cell-cycle progression, such as cyclin D1 and CDC25A (Bromberg, 2002; Levy and Lee, 2002; Yu and Jove, 2004). Considering the observed chemopreventive action of *Azadirachta indica* in the present study and reports available, the implications of enhanced STAT 3 expression in tumors of AAILE+DMBA/TPA group need to be looked into more deeply. Also, STAT 3 mRNA and protein expression increased in AAILE group when compared to control group.

p53 is a transcription factor which is considered as one of the important tumor suppressor genes. p53 gets accumulated in an active form in the nucleus in response to external and internal stress signals. The activity of p53 gives rise to a variety of cellular outcomes, most notably cell cycle arrest and apoptosis. Apoptosis inducing effect is essential for tumor suppression. The ability of p53 to induce cell cycle arrest prevents proliferation of cells with damaged DNA or with a potential for neoplastic transformation. p53 also contributes to other vital cellular processes such as differentiation, DNA repair and angiogenesis.
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(Vogt and Haupt, 1999). It has been observed that approximately fifty percent of all human cancers contain cells with mutations or deletions in both alleles of the p53 gene (Vogelstein et al., 2000). These tumors bearing mutated p53 tend to be more invasive with a high metastatic risk and are correlated with a poor prognosis.

In the present investigation, mRNA expression studies revealed an increase in p53 expression in tumors of DMBA/TPA treated animals when compared to their control counter parts, however protein expression studies revealed no change. In a healthy cell, the level of p53 is low. Genetic damage induced by chemical carcinogens, UV radiation etc, triggers p53 expression, probably by means of transcriptional regulation (Lane, 1994; Kaur et al., 2010). The absence of correlation between gene and protein expression of p53 may be attributed to the altered degradation rates of p53. In unstressed cells, p53 levels are kept low through a continuous degradation of p53. A protein called mdm2, binds to p53 and prevents its activity and transports it from the nucleus to cytosol. The mdm2 oncoprotein binds to the N-terminus of p53 and represses its transcriptional activity. Also mdm2 acts as ubiquitin ligase and covalently attaches ubiquitin to p53 and thus marks p53 for degradation by the proteasome (Haupt et al., 1997). mdm2 protein is up regulated in tumors and attaches ubiquitin to p53 marking it for degradation by proteasome. Despite the increase in p53 mRNA no alteration was observed in the protein expression of p53 in DMBA/TPA group. This could be due to the degradation of p53 protein. Administration of AAILE to animals bearing skin tumors, led to an increase in p53 mRNA and protein expression when compared to DMBA/TPA group. This enhanced p53 expression could have initiated a cascade of programmed cell death or cell cycle arrest resulting in the observed inhibition in tumorigenesis. Several anti-tumor agents including *Azadirachta indica* have the potential to enhance the expression of p53. Kumar et al., (2010) have reported an increase in p53 expression following azadirachtin and nimbolide treatment in hamsters bearing buccal pouch tumors. Induction of p53 dependent apoptosis is one of
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the key mechanisms of cancer chemopreventive agents (Huang et al., 1997;
Shin et al., 1997; Kaur et al., 2008). Mihara et al. (2003) have reported that p53
promotes permeabilisation of the outer mitochondrial membrane by forming
complexes with the bcl-xl and bcl-2 proteins thereby inhibiting their anti-
apoptotic effect. No appreciable change was observed in p53 expression in skin
from AAILE group when compared to control group.

Cell Cycle Regulation during DMBA/TPA Induced Skin Carcinogenesis
and its Intervention with Azadirachta indica

Uncontrolled proliferation of cells is the prime hallmark of cancer and
therefore control of cell proliferation is of utmost importance while combating
this hyperproliferative disease. Anticancer agents act at several points along the
cell cycle, differentially regulating the factors involved in progression of cells
through the cell cycle.

Proliferating cell nuclear antigen (PCNA) is a nuclear protein expressed in the
nuclei of proliferating cells during S phase and therefore acts as a marker of
cell proliferation (Subapriya et al., 2006). Overexpression of PCNA has been
reported in a wide range of human tumors (Kohno et al., 2002; Yue and Jiang,
2005). Up-regulation of PCNA accelerates DNA synthesis, promotes cell
proliferation and consequently degradation of PCNA leads to inhibition of
DNA synthesis (Prelich et al., 1987; Engel et al., 2005).

In the present investigation, mRNA and protein expression studies revealed
that treatment of DMBA/TPA to the skin resulted in SCC with an enhanced
expression of PCNA. Enhanced PCNA expression indicates increase in cell
proliferation. Kumar et al (2010) have also reported an enhanced PCNA
expression in buccal pouch tumors. Shin et al (1993) found a gradual increase
in PCNA expression during progression of normal epithelium to hyperplasia
through dysplasia to oral squamous cell carcinoma. However, treatment with
AAILE led to a decrease in mRNA and protein expression of PCNA in skin
tumors of AAILE+DMBA/TPA group when compared to tumors of

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DMBA/TPA group. A decrease in PCNA expression was observed in buccal pouch tumors following *Azadirachta indica* treatment (Subapriya et al., 2006). Kumar et al., (2009) have reported an increase in PCNA expression in human choriocarcinoma cells, which was reduced upon treatment with nimbolide. Down regulation of PCNA has been observed in oral cancer following treatment with azadirachtin and nimbolide (Kumar et al., 2010). Overexpression of PCNA may confer a selective growth advantage to tumor cells in the DMBA/TPA treated group and reflects enhanced proliferation rates in skin tumors. Treatment with *Azadirachta indica* caused a down regulation of PCNA, which is indicative of decrease in cell proliferation. Concurrent with the changes in expression of PCNA in various groups, the cell number is reflected in the histological analysis. No appreciable change was observed in the PCNA expression of AAILE group when compared to control group.

p21 is an important downstream mediator of p53 and regulates the function of several cell cycle proteins like cyclin D1 and cyclin dependent kinases (CDKs), consequently regulating cell cycle (Perkins, 2002). It binds to and inhibits the activity of cyclin-CDK2 or -CDK4 complexes, and thus functions as a regulator of cell cycle progression at G1 stage (Cayrol et al., 1998; Medema et al., 1998). Cyclins and CDKs are important determinants which govern cell cycle progression. Cyclins are positive regulators of CDKs and the selective activation and deactivation of cyclin-CDK complexes regulate the progression of eukaryotic cells through the cell cycle (Morgan, 1995; Sherr, 1996). p21 interacts with cyclin/CDK complex and may facilitate cell cycle arrest (Coqueret, 2003). The expression of this gene is tightly controlled by the tumor suppressor protein p53, through which this protein mediates the p53-dependent cell cycle G1 phase arrest in response to a variety of stress stimuli (Rodriguez and Meuth, 2006). p21 can also interact with PCNA, and plays a regulatory role in S phase DNA replication and DNA damage repair.

In the present study, an increase in p21 mRNA and protein expression was observed in the tumors of DMBA/TPA group when compared to the control.
group. Upregulation of p21 has been reported in several tumors like oral SCCs (Jang et al., 1999; Kuropkat, 2002). However, p21 is also known to be regulated independent of p53 expression (Denis et al., 2005). Treatment with AAILE to skin tumor bearing animals caused a significant increase in p21 mRNA and protein expression in tumors of AAILE+DMBA/TPA group when compared to tumors of DMBA/TPA group. Nimboide treatment to the colon cancer cell line (HT-29) caused cell cycle arrest accompanied by the upregulation of p21 (Roy et al., 2006) The observed increase in p21 expression can be correlated with the observed increase in p53 expression in AAILE+DMBA/TPA group. Expression of p21 correlates with the accumulation of cells in the G1 phase and indicates cell cycle arrest. No appreciable change was observed in the mRNA expression of p21 in skin from AAILE group when compared to control, however protein expression increased significantly. It was observed that skin tumors in DMBA/TPA group exhibited enhanced mRNA and protein expression of cyclin D1 levels when compared to the control group. Overexpression of cyclin D1 is consistent with similar findings in DMBA induced experimental tumors (Letchoumy et al., 2007). However, administration of AAILE to DMBA/TPA treated animals caused an inhibition in cyclin D1 mRNA and protein expression levels when compared to DMBA/TPA group. Inhibition of cyclin D1, GST-P and PCNA with enhanced expression of p21 and p53 by azadirachtin and nimboide suggests its regulatory effects on cell cycle progression (Priyadarsini et al., 2010). No appreciable change was observed in the mRNA expression of cyclin D1 in AAILE group when compared to control, however protein expression decreased significantly.

**Involvement of Apoptosis in Chemopreventive Action of Azadirachta indica against DMBA/TPA Induced Skin Tumorigenesis**

Tumor formation is a result of accumulated mutations in a wide array of genes involved in cell proliferation, survival, differentiation, apoptosis, genomic stability etc (Weeks et al., 1979; Verma et al., 1979). It seems logical that if we
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could induce apoptosis in cells that have accumulated mutations, we could prevent the ‘initiated cells’ from traversing the initiation phase. Induction of apoptosis in ‘transformed cells’ is an important target during cancer therapy (Choedon et al., 2006). Since apoptosis is important in maintaining cell number homeostasis, it has emerged as a key target in cancer intervention (Petkovich et al., 1987; Lotan, 1996).

mRNA and protein expression studies revealed an increase in bcl-2 expression and decrease in caspase 3 expression in tumors of DMBA/TPA group when compared to control group. Overexpression of anti-apoptotic proteins has been documented in tumors (Coultas and Strasser, 2003). Bcl-2, a major anti-apoptotic protein inhibits apoptosis by preventing the mitochondrial release of cytochrome c and eventually resulting in inhibition of caspase activity (Kirkin et al., 2004). Enhanced expression of bcl-2 associated with a diminished expression of caspase 3 in the present study may have facilitated evasion of apoptosis and development of carcinomas. AAILE administration to tumor bearing mice down-regulated the mRNA and protein expression of anti-apoptotic protein bcl-2 and up-regulated the mRNA and protein expression of pro-apoptotic proteins bax, caspase 3 and caspase 9, indicating that it may have apoptosis inducing effects.

The bcl family of anti-apoptotic proteins inhibits cytochrome c release and activation of caspase 3, whereas Bax, is a pore forming pro-apoptotic protein that facilitates cytochrome c release, consequently triggering caspase mediated apoptotic cell death (Daniel et al., 2001). Failure of apoptosis creates a conducive environment for genomic instability resulting in accumulation of mutations that could eventually promote neoplastic transformation. Cancer cells evade apoptosis by malfunctioning of the apoptotic apparatus, i.e. over expression of anti-apoptotic proteins or decreased expression of pro-apoptotic proteins and down-regulation of death receptors (Reed, 1999; Vermeulen et al., 2005). Subapriya et al., (2005b) have reported that Azadirachta indica extract may induce apoptosis by a caspase dependent pathway involving down
regulation of bcl-2 and up-regulation of bim, caspase 8 and caspase 3 expressions. Kumar et al (2009) have reported that nimboïde present in the flowers and leaves of *Azadirachta indica* inhibits proliferation and induces apoptosis in human choriocarcinoma (BeWo) cells by inhibiting bcl-2 expression and enhancing bax and caspase 3 expressions. The limonoids present in *Azadirachta indica* have been demonstrated to exert anti-proliferative and pro-apoptotic activities in an animal model of oral oncogenesis (Kumar et al., 2009). The apoptosis inducing effect of *Azadirachta indica* may be attributed to the enhanced immune surveillance in response to its administration. Reports by Bose and Bose and Baral (2007) and Bose et al (2007) provide evidence that neem leaf induces NK cell mediated death of tumor cells and induce apoptosis of tumor cells by releasing immuno active substances such as cytokines. Gangar and Koul (2008b) have reported that *Azadirachta indica* induced apoptosis in mice bearing fore-stomach tumors.

Survivin belongs to the family of anti apoptotic proteins called inhibitor of apoptosis (IAP) family. It exhibits anti apoptotic function by inhibiting caspase activation. Tamm et al (1998) have shown that survivin inhibits both bax and Fas induced apoptotic pathways. Disruption of survivin induction pathways increases apoptosis and inhibits tumor growth. It is abundantly expressed in tumors and fetal tissue but absent in terminally differentiated cells (Sah et al., 2006). Its aberrant over expression in tumors allows the cancer cells to be resistant to apoptotic stimuli and chemotherapeutic drugs thus behaving as radio and chemoresistance factor (Rodel et al., 2003; Wall et al., 2003). Wild-type p53 has been shown to repress survivin expression at the mRNA level (Mirza et al., 2002). An increase in mRNA and protein expression of survivin was observed in tumors of DMBA/TPA group when compared to control group. Treatment of AAILE decreased the expression levels of survivin in tumors of AAILE+DMBA/TPA group when compared to DMBA/TPA group. Aoki et al (2003) have reported that survivin directly contributed to malignant progression of primary effusion lymphoma and that targeting survivin by
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STAT3 inhibition may provide a novel therapeutic approach for this aggressive lymphoma. Survivin activity results from its phosphorylation on threonine 34 by the mitotic kinase complex cdc2/cyclin B1 (O’Connor et al., 2000; Wall et al., 2003). It promotes mitotic progression and cytokinesis in cancer cells (Honda et al., 2003). A semisynthetic flavonoid, flavopiridol, suppresses the survivin phosphorylation on threonine 34 and enhances cancer cell apoptosis induced by anticancer agents e.g. adriamycin and UVB radiation (Wall et al., 2003). Kumar et al (2010) have reported an increase in expression of cytoplasmic survivin and decrease in expression of nuclear survivin in DMBA induced buccal pouch tumors in hamsters. This was reversed upon the treatment with nimbolide and azadirachtin, however the effects were more pronounced with nimbolide.

One of the important biochemical hallmarks of apoptosis is the degradation of DNA by endogenous DNases, which cleave the inter-nucleosomal regions into double stranded DNA fragments of 180-200 base pairs (Wylie, 1980; Afford and Randhawa, 2000). These fragments contain blunt ends (Alnemri and Litwack, 1990) as well as single base 3’overhangs (Didenko and Hornsby, 1996). Formation of large DNA fragments of the range of 50-300kbp precedes inter nucleosomal fragmentation (Bortner et al., 1995). The DNA fragments formed due to inter-nucleosomal cleavage are detectable as a ladder pattern in the electrophoresis of DNA (Wylie, 1980). In the present investigation, DNA extracted from skin tumors of mice that received AAILE along with DMBA/TPA, upon agarose gel electrophoresis, revealed fragmentation, which indicates apoptosis. However, an intact genomic band was observed in DMBA/TPA group with mild smearing was observed which indicates necrosis. There are reports that indicate that necrosis in tumors potentiates tumor growth (Vakkila and Lotze, 2004). An intact genomic band in control and AAILE groups indicates absence of apoptosis.
Further, terminal deoxynucleotidyl transferase-dUTP nick end labeling (TUNEL) assay was used to identify and localize the apoptotic cells. Apoptosis is characterised by a number of intracellular phenomena such as membrane blebbing, chromatin condensation and nuclear DNA fragmentation. Detection of nuclear DNA fragmentation is a widely accepted method to assay for apoptosis and can be performed in situ by incorporating labeled nucleotides onto the 3’OH ends of DNA fragments using a terminal deoxynucleotidyl transferase enzyme (TdT). The kit utilized in the present study uses brominated nucleotides (BrdU) which are efficiently incorporated at the sites of DNA fragmentation. Incorporated BrdU is detected using a highly specific and sensitive biotinylated anti-BrdU antibody and visualized. DAB generates dark browning staining in cells with DNA fragmentation which is visualized against a green counterstain. Methyl green is a nuclear stain which stains non-apoptotic cells. TUNEL positive cells (brown color) cells were observed only in the skin tumor sections of AAILE+DMBA/TPA group. No apoptotic cells were observed in sections of DMBA/TPA, control and AAILE groups.

Likely as other anti-cancer agents, *Azadirachta indica* also affects apoptosis in tumors. Administration of ENLE significantly inhibited the development of HBP carcinomas by inhibiting cell proliferation and inducing differentiation and apoptosis (Subapriya et al., 2006). Kumar et al., (2009) have provided evidence that nimbolide caused ROS mediated apoptosis in human choriocarcinoma cells. Excessive ROS generation can lead to opening of the mitochondrial permeability transition pore with the consequent release of cytochrome c from the intermembrane space into the cytosol culminating in the activation of the caspase cascade and hence the apoptotic mode of cell death (Jeong and Seol, 2008). In the present study, the inhibition observed in skin tumorigenesis by AAILE may be a result of the enhanced peroxidative damage (discussed later) and ROS mediated caspase dependent cell death. The results suggest that AAILE could induce apoptosis in skin tumor bearing mice, which is an outcome of the modulation in proteins that form the apoptotic apparatus.
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of the cell. Thus, apoptosis may be one of the myriad mechanisms by which *Azadirachta indica* exerts its anticancer effects.

**Hepatic Status of Skin tumor Bearing Mice**

Liver is the primary site for the biotransformation of xenobiotics including carcinogens as well as anticancer drugs. Carcinogens and anticancer drugs after undergoing biotransformation may affect the target as well as non-target tissues directly by active metabolites or indirectly through the generation of ROS. Also, tumor metabolism leads to excessive generation of free radicals, which can cause oxidative stress mediated damage and secondary tumors at distant sites (Santamaria and Bianchi, 1989). Any change in the liver function may lead to altered response of the body towards the tumors and anticancer agents. Therefore, the status of liver in terms of xenobiotic biotransformation enzymes and antioxidant defense system is critical to the process of carcinogenesis and its therapy.

Hepatosomatic index remained unaltered in all the treatment groups at all time points of investigation. Administration of AAILE for two weeks decreased the LPO levels in hepatic tissue when compared to the control group. This was accompanied by an increase in GSH content and increase in the activities of catalase and SOD. No appreciable change was observed in the activities of GPx, GR and GST. Treatment of DMBA/TPA for twenty weeks enhanced hepatic LPO levels in tumor bearing mice when compared to the control group. The enhanced LPO levels were associated with a decrease in GSH content and decrease in the activities of GR and catalase. No change was observed in the activities of GST, GPx and SOD. Hepatic LPO levels increased after eight weeks of DMBA/TPA treatment when compared to control group. Treatment with eight weeks of DMBA/TPA caused a decrease in the activities of GR, GPx, catalase and SOD when compared to control group. No appreciable change was observed in GSH content and activity of GST.

A non-significant decrease in hepatic LPO levels was observed in AAILE+DMBA/TPA group when compared to DMBA/TPA group at the end of the treatment period. This was associated with an increase in GSH levels and
increase in activities of GR, GPx and catalase in AAILE+DMBA/TPA group when compared to DMBA/TPA group. No appreciable change was observed in SOD and GST activities. Administration of AAILE to DMBA/TPA (eight weeks) treated animals caused a non significant decrease in hepatic LPO levels in comparison to DMBA/TPA group. Hepatic tissue of AAILE+DMBA/TPA treated animals exhibited increased GR and GPx activity when compared to DMBA/TPA group. No appreciable change was observed in GSH content and activities of SOD, catalase and GST.

A study by Koul et al (2006a) had revealed that skin tumor induction causes hepatic damage as is evident from the decreased hepatosomatic index and increased activities of hepatic tissue injury marker enzymes. When the tumor bearing animals are treated with AAILE these changes are reversed, indicating the hepato-protective effect of *Azadirachta indica*. Enhanced oxidative stress along with decreased GSH levels and activities of various antioxidant enzymes like GST, GR, GPx was observed in hepatic tissue of tumor bearing animals. AAILE treatment reduced oxidative stress by boosting the antioxidant defense system (Koul et al., 2006b).

Mirunalini et al (2004) have reported enhanced LPO levels in the liver and blood of buccal pouch tumor bearing animals which was accompanied by a significant decrease in antioxidant levels. Topical application of garlic oil effectively suppressed oral carcinomas, decreased LPO, and enhanced antioxidant levels. Subapriya et al (2003) have reported that in contrast to the buccal pouch tumor, the liver and erythrocytes of tumor bearing animals exhibited enhanced LPO levels with compromised GSH dependent enzymes and GSH/GSSG ratio. This indicates enhanced ROS generation and depressed antioxidant defense system. Apart from the target organ (skin), DMBA undergoes metabolism in the hepatic tissue, forming diol epoxides which bind to and damage DNA (Sims and Grover, 1991). Metabolism of carcinogen is known to produce toxic and highly diffusible ROS capable of producing deleterious effects at sites far from the target tissue (Chou et al., 1998). The enhanced LPO levels in tumor bearing animals can be ascribed to the excess
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ROS produced at the target site. Low levels of antioxidants in the liver of tumor bearing animals may be ascribed to increased use to scavenge lipid peroxides.

Administration of AAILE for two weeks reduced the CYP and cytochrome b5 levels and AHH activity in hepatic tissue when compared to control group. DTD and UDP-GT activities increased and GST activity remained unaltered after two weeks of treatment with AAILE. DMBA/TPA treatment for eight weeks increased the contents of CYP and cytochrome b5 and activity of AHH in the hepatic tissue when compared to the control group. DTD activity increased and GST activity remained unaltered after eight weeks of treatment with DMBA/TPA. UDPGT activity decreased in the hepatic tissue after eight weeks of treatment with DMBA/TPA. When the DMBA/TPA treated animals were administered with AAILE, a decrease in the cytochrome P450 and cytochrome b5 contents and AHH activity was observed in the hepatic tissue when compared to the control group. DTD and GST activities increased when compared to control group and remained unaltered when compared to DMBA/TPA group. However, UDPGT activity increased when compared to DMBA/TPA group.

Micronucleus as the name indicates, is a small nucleus (erratic) which is formed during the anaphase of mitosis or meiosis. These are cytoplasmic bodies consisting of acentric chromosomes or whole of the chromosomes which failed to move to the opposite poles of the spindle during anaphase, resulting in missing of part or whole chromosome for the daughter cells eventually formed (Arlett et al., 1998; Fenech et al., 1999). Such left out chromosomes or chromosome fragments develop nuclear membrane and exist as a third nuclei. After cytokinesis, one of the resultant daughter cells formed consist of a single nucleus and the other daughter cell consist of one large and one small nucleus i.e. micronucleus. Clastogenic and aneuploidogenic agents like mutagens, carcinogens, and genotoxins are involved in the generation of micronucleus (Schmid, 1975). Chromosome breakage and mitotic apparatus dysfunctions are involved in the morphogenesis of micronucleus, which are
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generally considered a phenotypic expression of chromosome instability (Norppa and Falck, 2003). Chromosomal changes like rearrangements, aneuploidy etc are linked to tumorigenesis (Sandberg, 1983). These involve structural changes which require chromosomal breaks. It seems plausible that individuals who develop tumors later in their life have a tendency of chromosomal breakages, which may get reflected by higher micronuclei scores. Several researchers have used micronuclei as predictors of carcinogenic risks (Ronen and Heddie, 1984; Stitch et al., 1986; Stitch et al., 1990). The micronucleus test is used for monitoring the genotoxicity of chemical carcinogens and its modulation by known and putative chemopreventive agents (Garg et al., 1992). Oxidative stress and depressed antioxidant defense system has been shown to induce chromosomal breakage and formation of bone marrow nuclei (Simic, 1994).

In the present study, micronucleus assay was performed after eight weeks of treatment with DMBA/TPA in order to assess the genetic damage caused in liver during skin carcinogenesis. In the present investigation, the micronuclei score in liver was low in all the groups and did not exhibit substantial inter-group difference. Spontaneous micronuclei formation has been reported to low in normal healthy liver. Even though high LPO levels indicate an increase in ROS, in the present study it was unable to alter the micronuclei score in DMBA/TPA and AAILE+DMBA/TPA groups. It appears that the burden due to enhanced oxidative stress in DMBA/TPA and AAILE+DMBA/TPA groups (after eight weeks of treatment of DMBA/TPA) was unable to enhance clastogenic damage in the cells.

Mayer et al (2000) have established a positive link between LPO status and genotoxicity as revealed by increased micronuclei score in lymphocytes. There are reports indicating an increase in micronuclei score in hepatic tissue in response to intra peritoneal injection of DMBA (Koul et al., 2010). This was associated with an increase in hepatic LPO levels and depressed antioxidant defense system. Gangar et al (2010) have reported an increase in micronuclei score in spleen of animals who were intragastrically treated with B(a)P. This
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was associated with an increase in hepatic LPO levels. This was reversed upon treatment with *Azadirachta indica* leaf extract. It appears that oxidative stress induced in liver after eight weeks of treatment with DMBA/TPA was unable to cause genetic damage and thus affect the micronuclei score. Subapriya et al (2004) evaluated the effects of pretreatment of ENLE against MNNG induced genotoxicity and oxidative stress in male Swiss albino mice. In MNNG treated mice, enhanced LPO with compromised antioxidant defense system in stomach, liver and erythrocytes was accompanied by an increase in bone marrow micronuclei. This was reversed upon pre-treatment with ENLE. AAILE has demonstrated protective effects on *in vivo* clastogenicity of MNNG in metaphase cells of bone marrow of male Wistar rats (Arivazhagan et al., 2003). Koul et al (2007) have reported that AAILE mediated modulation of diethylnitrosamine induced clastogenicity in hepatocytes. Thus, the reports available in literature suggest that *Azadirachta indica* exerts protection by modulating carcinogen biotransformation as well as antioxidant defense systems at target and non-target organs.

Concluding Remarks

The observations of the present study clearly indicated that *Azadirachta indica* exerted remarkable chemopreventive effects against DMBA/TPA induced skin carcinogenesis. It does so by acting at critical points of along the carcinogenesis pathway. It modulated key processes like carcinogen biotransformation and ROS generation at target and non-target tissues which are crucial in the initial stages of chemical carcinogenesis. Further, *Azadirachta indica* differentially regulated the expression of transcription factors which are important for traversing the promotion and progression stages of cancer. *Azadirachta indica* exercised control over cell cycle associated proteins and proteins that form the apoptotic apparatus of the cell, culminating in low cell proliferation and enhanced apoptosis.