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Predisposition to cancer is governed by environmental and genetic factors. Primarily, cancer is a disease linked to environment however; genetics also influence the risk of some cancers. Epidemiological and experimental studies have revealed that majority of the cancers are linked to the carcinogens present in the environment (Clapp et al., 2007). About 80% of cancers are caused by environmental causes such as tobacco use, alcohol consumption, consumption of high fat diet, exposure to carcinogens at work place, exposure to carcinogens in vehicle exhaust, radiation exposure, viral infections etc (Pitot and Dragon, 2001; Abraham et al., 2004). Polycyclic aromatic hydrocarbons (PAHs) are ubiquitously present environmental pollutants and have been associated with cancer of the lung, mouth, bladder, colon, stomach, liver, skin, esophagus, pancreas etc (Athar et al., 1989; Rubin, 2001). The primary sources of PAHs are combustion of fossil fuels such as coal, shale, petroleum, diesel, volcanic eruption, refuse burning, forest fires, cigarette smoke etc. (Harvey, 1991; Mahadevan et al., 2005). Since PAHs are widely present in the environment therefore exposure to PAHs is possible through inhalation, ingestion and absorption through skin.

Due to its debilitating and devastating nature, cancer is a major cause of health concern in both the developed and developing nations. The International Agency for Research on Cancer (2008) has projected cancer as the leading cause of death in the coming years. Cases of cancer doubled globally between 1975 and 2000 and will further double by 2020 and will nearly triple by 2030. The projected numbers for 2030 are 20 to 26 million diagnoses and 13 to 17 million deaths. Skin cancers account for 80% of the newly reported cancers in Australia (Australian Institute of Health and Welfare, 2008). According to the American Cancer Society, more than 2 million NMSCs are diagnosed annually (American Cancer Society, 2010). Studies suggest that one in every five Americans will develop skin cancer in their life time (Robinson, 2005). The
studies also reveal that one American dies of melanoma almost every hour (World Health Organisation, 2010). There are indications that incidence of skin cancer is also on a rise in India as well (Sivalingam et al., 2009; Teli et al., 2009).

Exposure to UV radiation is one of the leading causes of skin cancer and the incidence of skin cancer is on a rise owing to the depletion in ozone levels (World Health Organization, 2010). Another significant risk factor for the development of skin cancer is the use of tobacco and tobacco related products which are rich sources of potent carcinogens like PAHs. The risk of skin cancer doubles with smoking (Morita, 2008). Skin cancer due to occupation has been diagnosed in people working in industries where the exposure to chemical carcinogens such as PAHs (e.g. from coal tar products) or to metals such as arsenic is high (International Agency for Research on Cancer, 2004). The London surgeon, Sir Percival Pott first was the first person to establish link between occupation and skin cancer in 1775, when he observed the occurrence in chimney sweeps of squamous cell carcinoma (SCC) of the scrotum. Exposure to PAHs (7, 12 di-methyl benz(a)anthracene [DMBA], benzo(a)pyrene [B(a)P] etc.) is mainly by inhalation but also through skin contact (Boffeta et al., 1997). PAH from shale oil, creosote, asphalt and chimney soot have all been associated with skin cancers (Evanoff et al., 1993; Partanen and Bofetta, 1994).

Treatment of cancer poses enough health risks to the patient disrupting their life in more than one ways and often, the patients run the risk of relapse of the disease even after the treatment is over. Also, at times the economical burden of the treatment process makes the treatment inaccessible to the financially weaker sections of the society. Unfortunately, cancer patients suffer a lot and lead miserable lives owing to the deadly nature of the disease. Many a times, especially in advanced stages of the disease, pain management and palliative care are the only means of mitigating the suffering. Therefore, prevention of cancer is a reasonable approach to eliminate this disease.
Primary prevention of cancer attempts to eliminate the disease by protecting
the individuals from the causative agents, mainly by abolishing contact with the
carcinogen. However, this is not possible, since we are constantly exposed to
carcinogens present in our environment and complete isolation is not feasible.
Secondary prevention aims at correcting those pathological conditions which
have already been produced and if allowed to continue, may progress to cancer.
Since it is practically impossible to live in an environment free of carcinogens,
therefore avoiding exposure to carcinogens and adopting certain life style
modifications is a rational approach to prevent cancer.

An important goal of cancer research is to elucidate the processes involved in
the induction of human cancer so that possible therapeutic interventions could
be developed to effectively combat the disease. An equally significant goal is
to recognize points along the carcinogenesis pathway which may be targets for
mechanism based prevention strategies. The major stages of carcinogenesis
were deduced over the past 50 years, primarily from studies conducted using
animal models (particularly mouse skin) (Yuspa and Shields, 1997). These
stages are termed as initiation, promotion and progression. Tumor initiation
begins when DNA in a cell or population of cells gets damaged on exposure to
endogenous and exogenous carcinogens. If this damage is not repaired, it can
lead to gene mutations. There is every possibility that the mutation could occur
in an oncogene or a tumor suppressor gene, which could wreck havoc leading
to uncontrolled proliferation of cells. The response of the mutated cells to their
microenvironment could change under these circumstances giving them a
growth advantage relative to normal cells. The tumor promotion stage is
characterized by selective clonal expansion of the initiated cells, which exhibit
altered gene expression whose products are associated with cell proliferation,
tissue remodeling and inflammation. During tumor progression, pre-neoplastic
cells develop into tumors through a process of clonal expansion that is
facilitated by progressive genomic instability and altered gene expression
(Slaga et al., 1980; Pitot, 1989).
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The possible ways of interfering with the initiation of tumor genesis include: i) modulating carcinogen activation by inhibiting the activity of carcinogen activation enzymes so that less reactive nucleophiles are generated which are capable of attacking the cellular macromolecules such as DNA, RNA and protein; ii) direct scavenging of DNA reactive electrophiles; iii) free radical scavenging; iv) enhancing detoxification of the carcinogenic metabolites by promoting the activity of carcinogen detoxifying enzymes and v) modulating DNA repair processes. Possible ways of interfering with the promotion and progression stages of tumor genesis include: i) scavenging of reactive oxygen species (ROS); ii) altering the expression of genes involved in cell signaling, particularly those regulating cell proliferation, apoptosis and differentiation and iii) decreasing inflammation.

Carcinogens from dietary and environmental sources are subjected to metabolism by phase-I and phase-II carcinogen metabolizing enzymes. Phase-I enzymes primarily oxidize, reduce or hydroxylate the carcinogen to a more polar form suitable for excretion. In this process, sometimes the phase-I metabolic reactions convert pro-drugs or pro-carcinogens to the active form capable of reacting with cellular macromolecules (Vainio et al., 1991). The Phase II reactions comprise of conjugation reactions that convert the active products of phase I reactions to less active or inactive species, which are rendered suitable for excretion in bile or urine. If not detoxified, the active metabolite is then available for interaction with cellular macromolecules. 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). Thus, modulation of carcinogen biotransformation enzymes is a reliable marker in the evaluation of chemopreventive potential of a drug and is an effective anti-initiation strategy.
ROS produced during metabolism of carcinogens may interact with multiple cellular targets including membranes, proteins and nucleic acids with potentially deleterious effects. 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). However, now the dual role of ROS (in carcinogenesis) has been recognized in the living systems (Valko et al., 2004). 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). The detrimental/beneficial effects of ROS depend upon the state/type of tissue and the concentration of the anticancer agent being used. During their course of action, anti-neoplastic agents produce numerous electrophilic moieties creating a pro-oxidant milieu in the cell leading to oxidative stress-induced lipid peroxidation (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 (Conklin, 2004). ROS can induce apoptosis by causing damage to cellular components like DNA and some studies suggest that ROS are downstream mediators of apoptosis (Johnson et al., 1996).

Transcription factors are regulatory proteins that recognize specific DNA sequences, bind them and recruit the correct RNA polymerase to carry out RNA synthesis, consequently regulating the expression of genes. The involvement of several transcription factors including nuclear factor-kappa B (NF-kB), activator protein-1 (AP-1), signal transducers and activators of transcription (STAT), p53 etc can not be denied in the process of tumor genesis because they control the expression of genes involved in cell survival, cell proliferation, cell adhesion, differentiation, cell growth, inflammation, invasion and angiogenesis which are critical to the process of tumor formation. Therefore, transcription factors are considered important targets in cancer prevention and therapy. Transcription factors get activated in response to
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various stimuli such as cytokines, growth factors, hormones, carcinogens, tumor promoters, radiation, oxidative and chemical stress etc (Dong et al., 1997; Shishodia et al., 2003). Constitutive expression of transcription factors has been described in a large number of human cancers including breast, colon, pancreatic, lung, head and neck, skin etc (Collins et al., 2000; Bromberg, 2002). Several anticancer agents like quercetin, black tea polyphenols, lantadene etc exert anti-oncogenic action by modulating the activity of transcription factors (Letchoumy et al., 2007; Kaur et al., 2008; Tanigawa et al., 2008) consequently affecting processes like cell proliferation, differentiation, apoptosis etc.

Cancer chemoprevention may be defined as the use of specific natural or synthetic substances with the objective of reversing, suppressing or preventing carcinogenic progression to invasive cancer (Singh and Lippman., 1998). Chemically synthesized and highly purified medicines exhibit pronounced effects because of their enhanced potency. However, their prolonged use can prove highly disadvantageous to the patient. Non Steroidal Antiinflammatory Drugs (NSAIDs) have proven to be effective in preventing colorectal cancer and several other malignancies like lung, prostate, leukemia, etc. (Holick et al., 2003; Mahmud et al., 2004; Brown and Du Bois, 2005). Chronic use of NSAIDs that inhibit both COX-1 and COX-2 cause toxicities — in particular, gastrointestinal bleeding and renal toxicity (Murray and Brater, 1993; Davies, 1995). COX2-specific inhibitors (COXibs) might be less toxic to the gastrointestinal tract than NSAIDs that target both COX1 and COX2. However, cardiovascular toxicity associated with COXibs raises concerns about their safety as chemopreventive agents. Tamoxifen is an oral selective antiestrogen agent that was used as a chemopreventive agent for breast cancer. Although, its use yielded positive results in terms of reduction in breast cancer incidence, but its use was accompanied by an enhanced risk of invasive endometrial cancer and thrombotic events, with women aged 50 and older (Fisher et al., 2001).
Plants and plant derived products have been considered to be useful as medicinal agents owing to their safety and effectiveness. Extensive studies over the years have supported the use of natural substances from plants, herbs, vegetables, fruits, spices for the chemoprevention of cancer (Shiow et al., 2005; Koul et al., 2006b; Kaur et al., 2008). Traditional forms of medicine were based largely on the use of plants and medicinal herbs and are now serving as the main source of anti-cancer agents (Abdullev, 2001). Ayurveda, the Indian traditional system of medicine uses several herbal extracts to cure a variety of diseases including cancer (Padmavathi et al., 2005). Micronutrients (antioxidant vitamins and trace minerals) as well as certain phenolic compounds present in vegetables and fruits are regarded as the most desirable class of chemopreventive agents (Surh, 2003). Epidemiological studies provide sufficient evidence for the fact that populations consuming low amounts of vegetables and fruits have higher incidence and mortality from cancers (Riboli and Noret, 2003). The protective effect of fruits and vegetables against carcinogenesis may be derived from many different plant components that include vitamins, minerals, fiber, and micronutrients, each acting via a variety of distinct and potentially interactive mechanisms (Greenwald et al., 2001; Sarkar and Li, 2004).

One such plant with profound medicinal value and used abundantly in Ayurveda is *Azadirachta indica* commonly known as ‘Neem’ in the Asian subcontinent. Almost every part of the plant such as leaves, bark, fruit, and flower has been reported to have medicinal value against various human ailments. Extracts of various parts of *Azadirachta indica* have shown immunomodulatory, anti-inflammatory, anti-hyperglycemic, anti-ulcer, anti-fungal, anti-bacterial, anti-viral, anti-oxidant, anti-mutagenic, and anti-carcinogenic properties. Because of its broad spectrum of medicinal properties *Azadirachta indica* has been recognized as the ‘nature’s drug store’ and village pharmacy in India (Puri, 1999; Biswas et al., 2002).

*Azadirachta indica* is a rich source of phytochemicals which contribute to its myriad number of medicinal utilities. Some of the major phytochemicals
present in *Azadirachta indica* are nimbin, nimbolide, nimbidin, quercetin, azadirachtin, gallic acid, catechin, flavonoids, tannins etc. The medicinal utility of *Azadirachta indica* has been described for both crude preparations and compounds isolated thereof. However, extracts have the benefit of being less toxic and being more or equally powerful in combating the disease owing to the presence of myriad number of compounds which when present together act in synergism and enhance the medicinal properties. When used in its entirety, the therapeutic action of the plant or part of the plant is quite different from the medicinal substance or substances isolated thereof (Steinmetz, 1961).

The inhibitory effect of *Azadirachta indica* has been documented in several animal model of carcinogenesis such as forestomach, skin, colon, prostate, buccal pouch etc (Dasgupta et al., 2004; Subapriya et al., 2005a; Gangar et al., 2006a, Koul et al., 2006b, Mahapatra et al., 2011). Reports suggest that *Azadirachta indica* influences several processes critical to tumor formation. 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* mediated modulation of NF-kappa B pathway is associated with its anti-inflammatory, anti-apoptotic and anti-proliferative activities. Gangar and Koul (2008a) in their study on murine forestomach tumorigenesis have reported that intervention with *Azadirachta indica* during the tumorigenesis process mediated the induction of apoptosis in the forestomach tumors. Earlier, Gangar and Koul (2007) have documented that *Azadirachta indica* leaf extract modulated initiation phase of murine forestomach tumorigenesis by altering the activity of carcinogen biotransformation enzymes in liver and forestomach. In a related study *Azadirachta indica* has demonstrated preventive effects on Benzo(a)pyrene–DNA adduct formation in murine forestomach and hepatic.
tissues which was accompanied by modulation of carcinogen biotransformation (Gangar et al., 2006b; Gangar et al., 2007).

Studies point out that *Azadirachta indica* leaf extracts modulate genes critical to neoplastic transformation. Manikandan et al (2008) have demonstrated anticancer activity in various fractions of *Azadirachta indica* leaf extract in hamster buccal pouch (HBP) carcinogenesis model which was associated with modulation in expression of genes associated with carcinogen activation, cell proliferation, apoptosis, angiogenesis etc. The ethanolic extract of *Azadirachta indica* modulated apoptosis, proliferation and differentiation during HBP carcinogenesis (Subapriya et al., 2005b; Subapriya et al., 2006). Neem leaf contains a number of potent antioxidants and anticarcinogens like flavonoids, limonoids, polyphenols etc that have been documented to retard carcinogenesis at initiation as well as promotion stages of carcinogenesis by virtue of their radical scavenging properties (Makita et al., 1996; Rice-Evans et al., 1996).

The reports mentioned above and those available in literature suggest that *Azadirachta indica* can prevent the initial changes that are necessary for initiation of carcinogenesis and regulate the genetic changes that are required for traversing the promotion and progression stages of cancer.

Reports available in literature suggest that there is sufficient evidence in support of the anti-cancer potential of *Azadirachta indica*. However more studies are required to understand its mechanism of action so as to completely understand and validate its use as an anti-cancer agent. Keeping in view the above mentioned facts, the present investigation entitled “Mechanistic studies on the phytomodulatory potentials of *Azadirachta indica* leaf extract on skin carcinogenesis in murine model” was designed to identify targets of *Azadirachta indica* in murine skin carcinogenesis model, with a focus on transcription factors which regulate genes involved in neoplastic transformation (cell proliferation, apoptosis). The present study was also aimed to address the biochemical, histological and morphological changes that occur during skin carcinogenesis and its intervention using *Azadirachta indica* leaf extract.
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The study had been designed with the following specific objectives:

- To induce skin tumors in male LAC A mice using 7,12-dimethyl benz(a)anthracene (DMBA) as a carcinogen and phorbol 12-myristate 13-acetate (TPA) as a promoter and to evaluate chemoprevention, if any, by *Azadirachta indica* leaf extract.

- To study the histopathology of skin tumors using Hematoxylin and Eosin staining in order to investigate the type of tumor formation.

- To explore the morphological changes induced by tumorigenesis in skin using Scanning Electron Microscopy and their modulation by *Azadirachta indica* leaf extract.

- To evaluate the modulation of carcinogen biotransformation enzymes (Phase I and Phase II enzymes) during the process of skin carcinogenesis and its intervention using *Azadirachta indica* leaf extract.

- To determine the modulatory effect of *Azadirachta indica* leaf extract, if any, on the status of antioxidant enzymes and the extent of oxidative stress during the process of skin tumorigenesis.

- To determine the modulatory effect of *Azadirachta indica* leaf extract in gene and protein expression of transcription factors: NF-kB (p65), AP-1 (c-fos, c-jun), STAT 1, STAT 3 and p53.

- To determine the modulatory effect of *Azadirachta indica* leaf extract on the gene and protein expression of cell proliferation associated genes like PCNA, p21, cyclin D1.

- To determine the mode of cell death involved in skin tumorigenesis and its intervention with *Azadirachta indica*.

- To determine the modulatory effect of *Azadirachta indica* leaf extract on the gene and protein expression of apoptosis associated genes like bax, bcl-2, caspase 3, caspase 9 and survivin.