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The present study was designed to determine the chemopreventive potential of Aqueous *Azadirachta indica* leaf extract (AAILE) against DMBA/TPA induced skin carcinogenesis and to investigate the underlying mechanism.

- For the experimental investigation, male LACA mice were used. Depending upon the treatment they received, the animals were divided into four groups: Group I (Control); Group II (DMBA/TPA); Group III (AAILE) and Group IV (AAILE+DMBA/TPA). Skin tumors were raised using a two stage model of tumorigenesis, employing DMBA as a carcinogen and TPA as a tumor promoter. AAILE was used as a chemopreventive agent.

- During the entire treatment period, animals were observed for changes in body weight, diet and water consumption. At the end of the treatment period, mice in control and AAILE groups gained weight when compared to their respective initial body weights. At the end of the treatment period body weight of mice in DMBA/TPA group decreased when compared to the initial body weight while body weight of mice in AAILE+DMBA/TPA group remained unaltered when compared to their initial body weight. No change was observed in the diet and water consumption in any of the treatment groups.

- Throughout the treatment period animals were observed for the occurrence of lesions/tumors. The skin underwent various changes such as hardening, roughening and eroding away of the skin surface with appearance of wound like lesions in response to DMBA/TPA treatment. By the end of seven weeks of DMBA/TPA treatment, small round lesions began to appear which increased in size and number as the treatment period progressed. The lesions in DMBA/TPA and AAILE+DMBA/TPA groups began appearing at the same time i.e. around seven weeks after DMBA/TPA treatment; however the increase in number and size of tumors was less in AAILE+DMBA/TPA group. AAILE treatment to DMBA/TPA animals
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reduced the tumor incidence by 41.7%. Mean tumor burden and mean tumor volume were observed to decrease in mice that received AAILE along with DMBA/TPA. The total number of tumors at the termination of the experiment was 85 in DMBA/TPA group and 32 in AAILE + DMBA/TPA group. The Kaplan-Meir curve clearly depicted that at the end of the study about 42% of the animals remained tumor free in AAILE+DMBA/TPA group. These observations clearly indicated the chemopreventive action of AAILE against DMBA/TPA induced skin tumorigenesis.

- Histopathological analysis of skin/skin tumors was conducted at the end of the treatment period. Uniformly arranged epidermal and dermal layers with the presence of subcutaneous tissue was observed in skin of control and AAILE groups, however the epidermal layer showed a microscopically visible inflammation in AAILE group. Tumors obtained in DMBA/TPA and AAILE+DMBA/TPA groups were histologically identified as squamous cell carcinoma. Abnormally thickened and corrugated epidermis was observed in the tumors of DMBA/TPA group. Hyperkeratosis, formation of keratin whorls (keratin pearls) and squamous eddies in the dermis was a prominent feature. ‘Islands of epidermal cells’ encapsulated by keratin were seen invading into the dermis. Keratin whorls encapsulated by layers of cells were also observed. Abnormally thickened, hyperproliferative and corrugated epidermis was also observed in AAILE+DMBA/TPA tumors; however, the epidermal thickness was less here. Areas of degenerative changes interspersed with cancer cells were observed in the tumors obtained from animals that were administered AAILE along with DMBA/TPA. Empty spaces devoid of cells were observed in tumors of AAILE+DMBA/TPA group.

- Studies were carried out to determine the changes in surface morphology in response to skin tumorigenesis and its intervention with AAILE. SEM of the skin section from control animal revealed that epidermal cells had
smooth topography, well defined outlines and were in close apposition to each other. In skin tumors of DMBA/TPA group surface disruption and rounded structures (regions of hyperproliferation) were observed. SEM view of tumors from DMBA/TPA group showed that cells appeared to have lost the orderly arrangement, disengaged and were not in so tight contact with each other. In tumors of mice that received AAILE treatment along with DMBA/TPA such rounded structures were not observed, however, areas of degenerative changes were observed. The degeneration (sloughing off of surface) observed in tumors of AAILE+DMBA/TPA group can be associated with the observed decrease in tumor volume and tumor burden in comparison to DMBA/TPA group.

- 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 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.

- Levels of CYP and cytochrome b5 and activities of DTD and UDP-GT remained unaltered in skin after two weeks of treatment with AAILE when compared to control group. Skin AHH activity decreased and GST activity increased after two weeks of treatment with AAILE.
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- DMBA/TPA treatment for eight weeks induced the phase I xenobiotic metabolising enzymes like CYP, cytochrome b5 and AHH, without causing any change in phase II enzymes like GST and UDP-GT when compared to control group. However, activity of DTD decreased following DMBA/TPA treatment. AAILE treatment to DMBA/TPA treated animals inhibited the phase I enzymes like CYP without causing any change in cytochrome b5 and AHH when compared to DMBA/TPA group. Activity of phase II enzymes, UDP-GT and DTD increased in AAILE+DMBA/TPA group when compared to DMBA/TPA group. No change was observed in phase I and phase II enzymes in AAILE group when compared to control group. The present observations 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.

- 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). AAILE treatment for two weeks decreased the skin LPO levels when compared to control group. This was associated with an increase in GSH level and activity of SOD. Skin catalase activity decreased and activities of GR and GPx remained unaltered following two weeks of treatment with AAILE.

- No change was observed in the skin LPO and GSH levels and SOD activity in response to DMBA/TPA treatment for eight weeks when compared to control group. DMBA/TPA treatment enhanced the GPx and catalase activities and decreased the GR activity when compared to control group. AAILE treatment to DMBA/TPA treated animals enhanced the skin LPO levels, although statistically non-significant when compared to DMBA/TPA group. This was associated with a decrease in GSH levels and GPx activity.
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when compared to DMBA/TPA group. Activities of GR, SOD and catalase increased in AAILE+DMBA/TPA group when compared to DMBA/TPA group.

- DMBA/TPA treatment for twenty two weeks decreased the tumor LPO and GSH levels when compared to control group. Activities of GPx and SOD decreased and activities of GR and catalase remained unaltered in tumors of DMBA/TPA group when compared to control group. Low LPO levels have been associated with enhanced tumor growth in DMBA/TPA group. Tumors of AAILE+DMBA/TPA group exhibited enhanced LPO and GSH levels when compared to DMBA/TPA group. Activities of GPx, GR and catalase increased and SOD activity remained unaltered in AAILE+DMBA/TPA group when compared to DMBA/TPA group. This suggests that Azadirachta indica caused damage to the tumors selectively resulting in the inhibitory effect on skin tumorigenesis. The peroxidative damage in tumors of AAILE+DMBA/TPA group can be related to low cell proliferation and apoptosis observed in the same group.

- In the present study the modulation in expression of transcription factors was studied during skin tumorigenesis and its intervention with AAILE. Transcription factors are important to carcinogenesis and its chemoprevention because they regulate genes involved in cell proliferation, cell survival, differentiation, apoptosis etc. Tumors obtained in DMBA/TPA group were associated with an increased mRNA expression of NF-kappa B (p65), AP-1 (c-jun, c-fos), STAT 3 and p53 when compared to control group. No change was observed in the mRNA expression of STAT 1. Protein expression studies revealed an enhanced expression of NF-kappa B (p65), AP-1 (c-jun, c-fos) and STAT 3, with no alterations in STAT 1 and p53. The inhibitory effect on tumorigenesis in response to AAILE treatment was associated with a decreased mRNA expression of NF-kappa B (p65) and increased mRNA and protein expression of AP-1 (c-jun, c-fos), STAT 1, STAT 3 and p53 when compared to DMBA/TPA group. Protein
expression of NF-kappa B (p65), AP-1 (c-jun, c-fos), STAT 1 and p53 remained unaltered in AAILE group when compared to control group, however an increase was observed in the protein expression of STAT 3 in AAILE group when compared to control group.

- Control of cell proliferation is of utmost importance while counteracting this hyperproliferative disease. PCNA is cell proliferation marker expressed during the S phase of cell cycle. The high rate of proliferation in tumors of DMBA/TPA group is evident from the enhanced expression of PCNA and the decreased rate of cell proliferation in tumors of AAILE+DMBA/TPA group is indicated by decreased expression of PCNA. Analysis of cell cycle associated genes revealed that tumors obtained in DMBA/TPA group exhibited enhanced mRNA and protein expression of p21, and cyclin D1 when compared to control group. Tumors obtained in AAILE+DMBA/TPA group exhibited decreased mRNA and protein expression of and cyclin D1 and increased mRNA and protein expression of p21 when compared to DMBA/TPA group. These observations indicate low cell proliferation and cell cycle arrest in tumors of AAILE+DMBA/TPA group.

- Induction of apoptosis in transformed cells is an effective way to combat this hyperproliferative disease. Studies were carried out to determine the mode of cell death involved during skin tumorigenesis and its intervention with AAILE. The genomic DNA extracted from each group was run on agarose gel. An intact genomic DNA band was observed in control and AAILE groups indicating the presence of intact DNA. DNA extracted from tumors of DMBA/TPA group showed a band corresponding to genomic DNA with a mild smearing which indicates necrosis. Necrosis in tumors has been linked to hyperproliferation. DNA extracted from skin tumors of mice that received AAILE along with DMBA/TPA, upon agarose gel electrophoresis, revealed fragmentation, which indicated apoptosis. TUNEL assay revealed no apoptotic cells in control, AAILE and DMBA/TPA groups. TUNEL positive cells (brown color) cells were observed in the skin
tumor sections of AAILE+DMBA/TPA group. These observations indicate the apoptosis inducing effect of AAILE in skin tumors. This was associated with a decreased mRNA and protein expression of bel-2 and survivin and enhanced mRNA and protein expression of bax, caspase 3 and caspase 9 in AAILE+DMBA/TPA group when compared to DMBA/TPA group.

- 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. 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.

- AAILE treatment for two weeks repressed the phase I enzymes and induced the phase II enzymes as is evident from the decrease in levels of CYP and cytochrome b5 and AHH activity and increase in activities of DTD and UDP-GT. No change was observed in GST activity.

- DMBA/TPA treatment for eight weeks enhanced the hepatic CYP and cytochrome b5 levels and AHH activity increased when compared to control group. DTD activity increased and UDPGT activity decreased in hepatic tissue of DMBA/TPA group when compared to control group. GST activity remained unaltered. AAILE treatment to DMBA/TPA treated animals enhanced the hepatic UDP-GT activity when compared to DMBA/TPA group. However, the levels of phase I enzymes like CYP, cytochrome b5, AHH and activities of phase II enzymes like DTD and GST remained unaltered in AAILE+DMBA/TPA group when compared to
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DMBA/TPA group. No change was observed in phase I and phase II enzymes in AAILE group when compared to control group.

- AAILE treatment for two weeks decreased the hepatic LPO levels when compared to control group. This was associated with an increase in GSH levels and activities of SOD and catalase. Activities of GPx and GR remained unaltered after two weeks of treatment with AAILE.

- DMBA/TPA treatment for eight weeks increased the hepatic LPO levels when compared to control group. This was associated with a decrease in GPx, GR, catalase and SOD activities. No change was observed in the GSH levels. Hepatic LPO and GSH levels and activities of SOD and catalase remained unaltered in AAILE+DMBA/TPA group when compared to DMBA/TPA group. Administration of AAILE to DMBA/TPA treated animals enhanced the activities of GR and GPx when compared to DMBA/TPA group. An increase was observed in GPx, GR and SOD activities in AAILE group when compared to control group. No change was observed in levels of LPO and GSH and activities of catalase in AAILE group when compared to control group.

- DMBA/TPA treatment for twenty two weeks enhanced the hepatic LPO levels when compared to control group. This was associated with a decrease in GSH level and activities of GR and catalase. Activities of GPx and catalase remained unaltered in DMBA/TPA group when compared to control group. Administration of AAILE to DMBA/TPA treated animals caused an increase in GSH level and increase in activities of GR, GPx and catalase when compared to DMBA/TPA group. LPO level and SOD activity remained unaltered when compared to DMBA/TPA group.

- Micronucleus assay was performed to in liver after eight weeks of DMBA/TPA treatment. Micronuclei score in was low in all the groups and did not exhibit substantial inter-group difference.
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.