Modulatory effects of *Azadirachta indica* leaf extract on cutaneous and hepatic biochemical status during promotion phase of DMBA/TPA-induced skin tumorigenesis in mice

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The modulation in biochemical status of skin and hepatic tissue at the time point of commencement of promotion stage of skin carcinogenesis in mice and its intervention with aqueous *Azadirachta indica* leaf extract (AAILE) were investigated. 7,12-Dimethylbenz(a)anthracene (DMBA, 500 nmol/100 ul of acetone) was applied topically for 2 weeks (twice weekly), followed by phorbol-12-myristate-13-acetate (TPA, 1.7 nmol/100 ul) twice weekly for 6 weeks on the depilated skin of mice and AAILE was administered orally at a dose level of 300 mg/kg body wt thrice a week for 10 weeks. DMBA/TPA treatment upregulated the phase I enzymes in skin and hepatic tissue, as revealed by the increased cytochrome P450 (CYP) and cytochrome b5 (cyt b5) levels and aryl hydrocarbon hydroxylase (AHH) activity when compared to the control group and differentially modulated the activities of phase II enzymes like glutathione-s-transferase (GST), DT-diaphorase (DTD) and uridine diphosphate glucuronosyltransferase (UDP-GT). AAILE treatment decreased the DMBA/TPA-induced increase in cutaneous CYP level and enhanced the DTD and UDP-GT activities when compared with DMBA/TPA group. In the hepatic tissue of AAILE + DMBA/TPA group, an increase in UDP-GT activity was observed when compared to DMBA/TPA group. DMBA/TPA treatment did not alter the skin lipid peroxidation (LPO) level when compared to control group, however, in the animals that received AAILE treatment along with DMBA/TPA, a significant increase in LPO was observed when compared to control group. This was associated with a decrease in cutaneous reduced glutathione (GSH) level of AAILE + DMBA/TPA group. Enhanced LPO level was observed in the hepatic tissue of DMBA/TPA and AAILE + DMBA/TPA groups when compared to control group. However, no alteration was observed in their hepatic GSH levels. The micronuclei score in hepatic tissue did not exhibit significant inter-group differences. The results of the present study suggest that apart from skin, liver may be affected during DMBA/TPA-induced skin tumorigenesis. AAILE treatment has the ability to modulate these changes potentially influencing the process of tumor formation. These findings seem to be important to carcinogenesis and its intervention with anti-cancer agents.

**Keywords:** *Azadirachta indica*, Dimethylbenz(a)anthracene, Phorbol-12-myristate-13-acetate, Xenobiotic biotransformation, Antioxidant defense system

The metabolism of xenobiotics (carcinogens, drugs etc) can be considered to take place in two phases (phase I and II) with each of the phase having distinct functions and special enzymes involved in it. The overall purpose of xenobiotic metabolism is to enhance water solubility and eventually facilitate their excretion out of the body. During the course of metabolism of xenobiotics, they may be either activated or deactivated. Phase I involves reactions that convert xenobiotics from inactive to biologically active compounds. 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.

Amongst all the polycyclic aromatic hydrocarbons (PAHs) tested, 7,12 dimethylbenz(a)anthracene (DMBA) is the most potent mouse skin carcinogen in the conventional mouse skin initiation promotion regime. It is an indirect acting carcinogen and undergoes metabolic activation by cytochrome P450 monoxygenase system to exert its mutagenic and carcinogenic activity. In the two-stage carcinogenesis...
model, active metabolites of DMBA bind to bases in DNA, forming carcinogen-DNA adducts capable of inducing mutations. Phorbol 12-myristate 13-acetate (TPA) treatment that follows DMBA application causes the fixation of mutations in critical genes associated with cell proliferation, apoptosis, differentiation etc. DMBA is known to cause tumors of liver, mammary glands, stomach etc, indicating the potential of these organs to metabolize DMBA to reactive intermediates

Several studies suggest that apart from the target organ, other non-target (where carcinogen/tumor promoter is not applied) organs like liver, blood, spleen, bone-marrow etc get affected during exposure to mutagens and carcinogens. This can have significant implications during carcinogenesis, its prevention and therapy. During the metabolism of carcinogens and anticancer drugs, target and non-target tissues may be directly affected by active metabolites or indirectly through the generation of reactive oxygen species (ROS). Therefore, apart from the target organ, non-target organs should also be monitored. Liver is the primary site for the biotransformation of xenobiotics therefore, any abnormality in the liver function may lead to altered response of the body during carcinogenesis and its intervention with anti-cancer drugs.

The chemopreventive action of *Azadirachta indica* leaf extract has been elucidated in our laboratory and others against several types of cancers including skin, forestomach, hepatic, buccal cavity etc. This may be attributed to its ability to affect biotransformation of carcinogens, oxidant-antioxidant mechanisms, cell proliferation, apoptosis etc. at target and non-target organs. An earlier report from our laboratory has demonstrated that antioxidant mechanism of skin gets affected in the skin tumor-bearing animals receiving only carcinogen treatment and those receiving *A. indica* treatment along with carcinogen treatment. Another report has also shown alterations in hepatic status of tumor-bearing animals as revealed by the changes in hepato-somatic index, liver marker enzymes, xenobiotic metabolizing enzymes and antioxidant defense system enzymes.

Considering the multi-stage nature of tumorigenesis and the possible effect on target and non-target organs, it is of relevance to assess their status at critical points along the carcinogenesis pathway like initiation, promotion and progression. Thus, in the present study, we have investigated on the effect of *A. indica* leaf extract on the modulation in xenobiotic biotransformation enzymes, lipid peroxidation and reduced glutathione levels in skin and hepatic tissue at the time point of commencement of promotion stage of skin carcinogenesis.

**Materials and Methods**

**Chemicals**

7,12-Dimethyl benz(a)anthracene (DMBA) and phorbol 12-myristate 13-acetate (TPA) were obtained from Sigma Chemical Co., St Louis, MO, USA. Reduced glutathione (GSH), bovine serum albumin (BSA), 5,5-dithiobis-2-nitrobenzoic acid (DTNB), NADH, 2,6-dichlorophenolindophenol (DCPIP), 1-chloro-2,4-dinitrobenzene (CDNB) were obtained from Sisco Research Laboratory, India. Other chemicals used were obtained from reputed Indian manufacturers and were of highest purity/analytical grade.

**Preparation of leaf extract:**

Aqueous *Azadirachta indica* leaf extract (AAILE) was prepared according to a method described previously.

**Animal model and treatment**

Male LACA mice weighing 25–30 g each, procured from Central Animal House, Panjab University, Chandigarh (India) were housed in polypropylene cages bedded with sterilized rice husk. Mice in all the groups had free access to standard animal pellet diet (Ashirwad Industries Ltd., Ropar, Punjab, India) and tap water throughout the experiment. The animals in the animal room were maintained at 21°C, humidity 50-60% and a 12 h dark and light cycle. All the experimental protocols were approved by the Institutional Ethics Committee, Panjab University, Chandigarh, India and conducted according to the Indian National Science Academy guidelines for the use and care of experimental animals.

After acclimatization to the experimental conditions for 1 week, mice were randomly divided into four groups (6-8 mice per group): control group (Group I) animals received a topical application of acetone (100 ul/mouse) throughout the treatment period; DMBA/TPA group (Group II) animals received a topical application of DMBA (500 nmol/100 ul of acetone) for 2 weeks (twice weekly), followed by TPA (1.7 nmol/100 ul) twice weekly for 6 weeks; Aqueous *A. indica* leaf
extract (AAILE) group (Group III) animals were administered with AAILE orally at a dose level of 300 mg/kg body weight thrice a week for 10 weeks; and AAILE + DMBA/TPA group (Group IV) animals received a topical application of DMBA (500 nmol/100 ul of acetone) for 2 weeks, followed by TPA (1.7 nmol/100 ul) twice weekly for 6 weeks and were administered with AAILE orally at a dose of 300 mg/kg body weight (on days alternate to DMBA/TPA treatment) for 10 weeks. The first dose of DMBA was given to the animals after 2 weeks of pre-treatment with AAILE.

Biochemical estimation of xenobiotic biotransformation enzymes, lipid peroxidation and GSH levels

At the end of treatment period, animals were kept on an overnight fast before sacrifice. Mice were sacrificed with cervical dislocation under light ether anesthesia. Animals were perfused with 0.9% NaCl after which the skin and hepatic tissues were taken, blot-dried and weighed. The tissues were then homogenized in 100 mM potassium phosphate buffer (pH 7.4) containing 150 mM KCl to obtain 40% homogenate (w/v) and the homogenate was then subjected to centrifugation at 38000 rpm for 1 h at 4°C for preparation of microsomes. The supernatant obtained was discarded and the pellet was re-suspended in potassium phosphate buffer (pH 7.4) to obtain microsomes for estimation of cytochrome P450 (CYP) and cytochrome b\textsubscript{5} (cyt b\textsubscript{5}) levels and aryl hydrocarbon hydroxylase (AHH) and uridine diphosphatase glucuronosyltransferase (UDP-GT) activities. An aliquot of 40% homogenate was diluted with potassium phosphate buffer (pH 7.4) to obtain 10% homogenate which was then subjected to centrifugation at 10,000 g for 30 min at 4°C. The pellet was discarded and supernatant obtained was used for the estimation glutathione-s-transferase (GST) and DT-diaphorase (DTD) activities. An aliquot of 10% homogenate was used for the estimation of GSH and lipid peroxidation levels.

Cytochrome P450 (CYP) and cytochrome b\textsubscript{5} (cyt b\textsubscript{5}) content

The CYP content in the tissues was determined using the carbon monoxide difference spectra (409-490 nm) of dithionate reduced samples\textsuperscript{23} and calculated using an absorption coefficient of 91 mM\textsuperscript{-1} cm\textsuperscript{-1} and was expressed as nanomole of CYP/mg of protein. The cyt b\textsubscript{5} content in the tissues was determined by recording the difference spectrum (409-424 nm) of NADH reduced versus air-saturated samples as described previously\textsuperscript{24}. The cyt b\textsubscript{5} content was calculated using molar extinction coefficient of 185 mM\textsuperscript{-1} cm\textsuperscript{-1} and expressed as nanomole of cyt b\textsubscript{5}/mg of protein.

Aryl hydrocarbon hydroxylase (AHH) activity

The AHH activity in the tissues was estimated following the method described previously\textsuperscript{25}. Benzo(a)pyrene was used as a substrate, which on reaction with AHH is hydroxylated to 3-hydroxy benzo(a)pyrene which can be estimated spectrophotometrically with excitation and emission maxima at 396 nm and 522 nm. The AHH activity was expressed as picomole of 3-hydroxy-B(a)P generated/min/mg protein.

Glutathione-S-transferase (GST)

GST activity in the tissues was determined using a spectrophotometric method\textsuperscript{26}. GST present in the tissue sample catalyzed the reaction between CDNB and GSH forming GSH-CDNB conjugates. The activity of GST was expressed as micromole of GSH-CDNB conjugates formed/min/mg protein using an extinction coefficient of 9.6 mM\textsuperscript{-1} cm\textsuperscript{-1}.

DT-diaphorase (DTD)

DTD activity in the tissues was estimated using DCPIP as a substrate\textsuperscript{27}. The enzyme activity was calculated using an extinction coefficient of 0.021 μM\textsuperscript{-1} cm\textsuperscript{-1} and expressed as micromole of DCPIP reduced/min/mg protein.

Uridine diphosphatase glucuronosyltransferase (UDP-GT)

The UDP-GT activity in the tissues was estimated according to the method described previously\textsuperscript{28}. UDP-GT catalyzed the conjugation reaction between p-nitrophenol and UDP-glucoronic acid and its activity was expressed as micromole of p-nitrophenol-UDP-glucoronic acid conjugates generated/min/mg protein.

Lipid peroxidation (LPO)

The assay for LPO was performed as described previously\textsuperscript{29}. Lipids, mainly polyunsaturated fatty acids are highly susceptible to peroxidation by various oxidizing free radicals that are formed from various cellular enzymatic and non-enzymatic reactions. Cycloperoxides are formed as a result of peroxidation reaction, which give malondialdehyde (MDA) by cleavage. MDA forms a pink colored complex with thiobarbituric acid (TBA), whose absorbance can be read at 532 nm. The amount of MDA formed in the tissues (index of LPO) was calculated using an extinction coefficient of 1.56 x 105 M\textsuperscript{-1} cm\textsuperscript{-1} for...
MDA-TBA chromophore and the levels of LPO were expressed as nanomoles of MDA-TBA chromophore formed/mg of protein.

**GSH**

GSH in the tissues was estimated as the total non-protein sulphydryl groups that react with DTNB to form a yellow colored complex having absorption maxima at 412 nm. GSH was used as a standard to calculate the content of GSH, which was expressed as nanomole of GSH/mg of protein.

**Micronucleus assay**

Micronucleus assay was performed in the hepatic tissue, following the method described previously. The hepatic tissue was washed with chilled homogenizing buffer (24 mM Na₂-EDTA buffer, pH 7.5, containing 75 mM of NaCl) and then homogenized at 500 to 800 rpm. The homogenates were then centrifuged at 7000 rpm for 10 min. The supernatant was removed and fresh homogenizing buffer was poured to re-suspend the hepatocytes. Small drops of suspension prepared were put at one of the pre-cleaned, grease-free microscopic slide. The drops were spread using cover slip held at an angle of 45˚ into a smooth layer. The slides were then air-dried in a dust-free environment for at least 12 h before staining. The hepatocytes were stained with May and Grunwald for 1-2 min, followed by staining with Giemsa for 10 min. The slides were then rinsed twice in distilled water, dried, rinsed in methanol, cleared in xylene and mounted in distyrene plasticizer xylene (DPX). Minimum of 200 cells were counted per mice for the presence of micronuclei using light microscope at 400X.

**Protein estimation**

The protein contents of various samples were estimated by the method of Lowry et al.

**Statistical analysis**

The data were expressed as Mean ± SD. Statistical significance was analysed by One-way ANOVA, followed by post-hoc test.

**Results**

**CYP level**

DMBA/TPA treatment for 8 weeks caused a significant increase in skin (p ≤ 0.05; p ≤ 0.05) and hepatic (p ≤ 0.05; p ≤ 0.05) CYP level, when compared to control and AAILE groups. AAILE treatment significantly decreased the DMBA/TPA-induced increase in skin CYP level (p ≤ 0.05), when compared to DMBA/TPA group. However, an increase was observed, when compared to control (p ≤ 0.05) and AAILE (p ≤ 0.05) groups. Administration of AAILE to DMBA/TPA treated-animals caused a significant increase in hepatic CYP level, when compared to control (p ≤ 0.05) and AAILE (p ≤ 0.05) groups and remained unaltered, when compared to DMBA/TPA group. No change was observed in the skin and hepatic CYP level in AAILE group, when compared with control group (Table 1).

**Cyt b₅ level**

DMBA/TPA treatment for 8 weeks caused a significant increase in skin (p ≤ 0.05; p ≤ 0.05) and hepatic (p ≤ 0.05; p ≤ 0.05) cyt b₅ levels, when compared to control and AAILE groups. Administration of AAILE to DMBA/TPA-treated animals caused a significant increase in skin cyt b₅ level, when compared to AAILE (p ≤ 0.05) group and remained unaltered when compared to control and DMBA/TPA groups. Administration of AAILE to DMBA/TPA treated animals caused a significant increase in hepatic cyt b₅ level, when compared to control (p ≤ 0.05) and AAILE (p ≤ 0.05) groups and remained unaltered when compared to DMBA/TPA group. No change was observed in the skin and hepatic cyt b₅ level in AAILE group when compared with control group (Table 1).

**AHH activity**

DMBA/TPA treatment for 8 weeks caused a significant increase in skin (p ≤ 0.05; p ≤ 0.05) and hepatic (p ≤ 0.05; p ≤ 0.05) AHH activity, when compared to control and AAILE groups. Administration of AAILE to DMBA/TPA-treated animals caused a significant increase in skin (p ≤ 0.05; p ≤ 0.05) and hepatic (p ≤ 0.05; p ≤ 0.05) AHH activity, when compared to control and AAILE groups and remained unaltered, when compared to DMBA/TPA group. No change was observed in the skin and hepatic AHH activity in AAILE group, when compared with control group (Table 1).

**DTD activity**

DMBA/TPA treatment for 8 weeks caused a significant decrease (p ≤ 0.05) in skin DTD activity when compared with control group and remained unaltered, when compared to AAILE group. AAILE treatment significantly increased (p ≤ 0.05) the DMBA/TPA-induced decrease in DTD activity in AAILE + DMBA/TPA group, when compared to DMBA/TPA group and remained unaltered when
compared to control and AAILE groups. DMBA/TPA treatment caused a significant increase in hepatic DTD activity, when compared to control (p≤0.05) and AAILE (p≤0.05) groups. Administration of AAILE to DMBA/TPA treated animals caused a significant increase in hepatic DTD activity, when compared to control (p≤0.05) and AAILE (p≤0.05) groups and remained unaltered, when compared to DMBA/TPA group. No change was observed in the skin and hepatic DTD activity in AAILE group, when compared to control group (Table 1).

Table 1—Effect of DMBA/TPA and/or aqueous A. indica leaf extract on cutaneous and hepatic xenobiotic biotransformation enzymes
[Values expressed as Mean ± SD (n = 6)]

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control</th>
<th>AAILE</th>
<th>DMBA/TPA</th>
<th>AAILE + DMBA/TPA</th>
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<tr>
<td>CYP 450</td>
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<td></td>
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<tr>
<td>Cutaneous</td>
<td>0.15 ± 0.02</td>
<td>0.16 ± 0.01</td>
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<td>0.23 ± 0.02</td>
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<td>Hepatic</td>
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<td>0.36 ± 0.03</td>
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<td>0.41 ± 0.01</td>
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<td>Cyt b5</td>
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<tr>
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<td>0.28 ± 0.01</td>
<td>0.25 ± 0.02</td>
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<tr>
<td>Hepatic</td>
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<td>0.28 ± 0.03</td>
<td>0.35 ± 0.03</td>
<td>0.33 ± 0.01</td>
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<td>AHH</td>
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<tr>
<td>Cutaneous</td>
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<td>43.8 ± 2.70</td>
<td>53.2 ± 3.2</td>
<td>50.6 ± 1.10</td>
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<td>Hepatic</td>
<td>36.3 ± 2.06</td>
<td>33.3 ± 2.62</td>
<td>43.2 ± 3.17</td>
<td>40.6 ± 1.15</td>
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<td>GST</td>
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<tr>
<td>Cutaneous</td>
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<td>1.41 ± 0.15</td>
<td>1.62 ± 0.27</td>
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<tr>
<td>DTD</td>
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<tr>
<td>Cutaneous</td>
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<td>0.02 ± 0.001</td>
<td>0.03 ± 0.005</td>
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<td>0.03 ± 0.005</td>
<td>0.05 ± 0.013</td>
<td>0.05 ± 0.005</td>
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<tr>
<td>UDP-GT</td>
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<tr>
<td>Cutaneous</td>
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<td>12.8 ± 0.29</td>
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<td>24.8 ± 2.50</td>
<td>20.3 ± 3.86</td>
<td>25.5 ± 0.58</td>
</tr>
</tbody>
</table>

Data were analyzed by One-Way ANOVA, followed by post-hoc test.

a p≤0.05 significant with respect to control group; b p≤0.05 significant with respect to AAILE group; c p≤0.05 significant with respect to DMBA/TPA group

Units: CYP: nanomole/mg protein; Cyt b5: nanomole/mg protein; AHH: picomole of 3-hydroxy B(a)P generated/min/mg protein; DTD: micromole of DCPIP reduced/min/mg protein; GST: micromole of GSH-CDNB conjugates formed/min/mg protein; UDP-GT: micromole of p-nitrophenol-UDP-glucuronic acid conjugates generated/min/mg protein

GST activity
DMBA/TPA treatment for 8 weeks was unable to alter the skin GST activity when compared to control and AAILE groups. Administration of AAILE to DMBA/TPA treated animals caused a significant increase (p≤0.05) in skin GST activity, when compared to control group and remained unaltered, when compared to AAILE and DMBA/TPA groups. No change was observed in skin GST activity of AAILE group, when compared to control group. Also, no change in hepatic GST activity was noticed in any of the treatment groups (Table 1).

UDP-GT activity
UDP-GT activity remained unaltered in skin after 8 weeks of treatment with DMBA/TPA, when compared to control group and AAILE groups. AAILE treatment to DMBA/TPA treated animals caused a significant increase (p≤0.05) in skin UDP-GT activity when compared to DMBA/TPA group and remained unaltered, when compared to control and AAILE groups. DMBA/TPA treatment caused a significant decrease (p≤0.05) in hepatic UDP-GT activity when compared to control group and remained unaltered, when compared to AAILE group. AAILE treatment significantly enhanced (p≤0.05) the DMBA/TPA-induced decrease in UDP-GT activity in AAILE + DMBA/TPA group, when compared to DMBA/TPA group and remained unaltered, when compared to control and AAILE groups. No change was observed in skin and hepatic UDP-GT activity in AAILE group, when compared with control group (Table 1).

LPO level
DMBA/TPA treatment for 8 weeks caused a significant increase in skin LPO level when compared to AAILE (p≤0.05) group and remained unaltered, when compared to control group. AAILE treatment to DMBA/TPA-treated animals caused a significant
increase in skin LPO level when compared to control (p ≤ 0.05) and AAILE (p ≤ 0.05) groups and remained unaltered, when compared to DMBA/TPA group. A significant decrease (p ≤ 0.05) in skin LPO level was observed in AAILE group, when compared to control group. DMBA/TPA treatment caused a significant increase in hepatic LPO levels, when compared to control (p ≤ 0.05) and AAILE (p ≤ 0.05) groups. AAILE treatment to DMBA/TPA-treated animals caused a significant increase in hepatic LPO level when compared to control (p ≤ 0.05) and AAILE (p ≤ 0.05) groups and remained unaltered, when compared to DMBA/TPA group. Hepatic LPO level remained unaltered in AAILE group, when compared with control group (Table 2).

GSH level

Skin GSH level remained unaltered after 8 weeks of DMBA/TPA treatment, when compared to control and AAILE groups. AAILE treatment to DMBA/TPA-treated animals caused a significant decrease in skin GSH levels, when compared to control (p ≤ 0.05) and DMBA/TPA (p ≤ 0.05) groups and remained unaltered, when compared to AAILE group. GSH levels remained unaltered in AAILE group, when compared with control group, while hepatic GSH levels remained unaltered in all the treatment groups (Table 2).

Micronucleus assay

Micronucleus assay was performed in liver after 8 weeks of DMBA/TPA treatment. The frequency of micronucleus induction was expressed as percentage number of micronucleated cells to cells having normal nuclei. The micronuclei score was low in all the groups and did not exhibit significant inter-group differences (Fig. 1).

Discussion

Recently, we reported that *A. indica* leaf extract exhibits chemopreventive action against two-stage skin carcinogenesis in murine model\(^{11}\). Papillomas began to appear on the mouse skin surface within 8 weeks of DMBA/TPA treatment and large tumors,
histologically classified as squamous cell carcinoma were obtained after 20 weeks of DMBA/TPA treatment. The appearance of lesions at this time point (i.e. around 8 weeks) could be considered as the beginning of promotion stage of skin tumorigenesis. Benign papillomas begin to erupt on the skin surface during the promotion stage of two-stage skin carcinogenesis model. Studies from our laboratory have revealed that chemopreventive action of A. indica against skin tumorigenesis might be in part attributed to its ability to act as a pro-oxidant selectively in the tumors. A. indica treatment has also been reported to reduce the severity of the hepatic damage in tumor bearing mice.

In the present study, DMBA/TPA treatment on the depilated skin of mice for 8 weeks upregulated the carcinogen activation enzymes in skin and hepatic tissue, as revealed by the increased CYP and cyt b5 levels and AHH activity, when compared to the control group. The induction of phase I enzymes is considered to be a potential risk factor for carcinogenesis, because many of the phase I reactions like hydroxylation, epoxidation etc lead to the activation of pro-carcinogens to their ultimate carcinogenic form, which is rendered suitable for interaction with nucleophilic sites in DNA. AHH may be a critical determinant of cutaneous carcinogenic responses to PAHs by transforming the parent compound into proximate reactive metabolites in skin in vivo. AAILE administration to DMBA/TPA-treated animals caused a decrease in skin CYP level without altering cyt b5 level and AHH activity, when compared to DMBA/TPA group. Repressing the activity of phase I enzymes by A. indica might in part be responsible for its anticancer action. Earlier, it is reported that feeding of a diet containing A. indica flowers decreases the CYP level in the hepatic tissues of rats. A down-regulatory effect of ethanolic extract of leaf of A. indica on the phase I enzymes in hepatic, forestomach and renal tissues in mice is also reported. A decrease in the CYP and cyt b5 levels in hepatic tissue of skin tumor bearing mice and of phase I enzymes in hepatic and forestomach tissues after treatment with AAILE is also documented. A. indica is also found to modulate the initiation phase of forestomach tumorigenesis.

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. DTD activity decreased in skin in response to DMBA/TPA treatment, while GST and UDP-GT activities remained unaltered, when compared to control group. In hepatic tissue, UDP-GT activity decreased appreciably, when compared to control group, while an increase in the DTD activity was observed. No change was observed in the hepatic GST activity in any of the treatment groups.

Earlier report from our laboratory has shown that intra-gastric instillation of benzo(a)pyrene decreases UDP-GT activity in forestomach and liver and administration of AAILE in benzo(a)pyrene-treated animals enhances UDP-GT activity after 28 and 56 days of initiation of the treatment period. Treatment with ethanolic extract of A. indica leaf is also found to increase in GST and DTD activities in pulmonary, hepatic and forestomach tissues. In another study, an increase in GST activity is reported in buccal pouch tumors of hamsters. In the present study, DTD and UDP-GT activities increased in skin of AAILE + DMBA/TPA group, when compared to DMBA/TPA group, while GST activity remained unaltered. In hepatic tissue of AAILE + DMBA/TPA group, an increase was observed in UDP-GT activity, while no alteration was observed in DTD and GST activities when compared to DMBA/TPA group. Thus, it was evident that A. indica served as a ‘dual’ agent, having the potential to decrease the activation of carcinogens and increase the detoxification of reactive metabolites, possibly decreasing the risk of carcinogenesis.

Biotransformation of pro-carcinogens/carcinogens leads to the enhanced generation of ROS and reactive nitrogen species, resulting in oxidative stress which is one of the causative mediators of the tumorigenic process. After 8 weeks of DMBA/TPA treatment, no alteration was observed in the skin LPO level when compared to control group, however, in the animals that received AAILE treatment along with DMBA/TPA, an increase in skin LPO level was observed when compared to control group, although the increase was statistically non-significant when compared to DMBA/TPA group. This was associated with a decrease in GSH levels of AAILE + DMBA/TPA group when compared to control group. Reports from our laboratory and others have provided evidence that A. indica acts as a pro-oxidant in tumors. In our study, high LPO levels were also observed in the hepatic tissue of DMBA/TPA and...
AAILE + DMBA/TPA groups when compared to control group. However, no difference was observed in the hepatic GSH levels in any of the treatment groups.

Metabolism of carcinogens is known to produce toxic and highly diffusible ROS, capable of producing deleterious effects at sites far from the target tissue. Thus, the enhanced LPO levels in the liver of tumor bearing animals can be ascribed to the excess diffusible ROS produced at the target site. During buccal pouch tumorigenesis, the liver and erythrocytes of tumor bearing animals exhibited enhanced LPO levels with compromised GSH-dependent enzymes and GSH/GSSG ratio. This indicated enhanced ROS generation and depressed antioxidant defense system in the non-target tissues. An increase in skin and hepatic phase I enzymes and differential modulation in phase II enzyme was observed in the skin tumor bearing animals. Enhanced LPO levels have also been observed in the hepatic tissue of skin tumor bearing animals.

Micronucleus is a small nucleus which is formed during the anaphase of mitosis or meiosis. These are cytoplasmic bodies consisting of acentric chromosomes or whole of the chromosomes which fail 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. In order to assess the genetic damage that could have been caused in liver in response to enhanced LPO level, micronucleus assay was performed after 8 weeks of treatment with DMBA/TPA. In the present study, the micronuclei score in liver was low in all the groups and did not exhibit substantial inter-group difference. Spontaneous micronuclei formation was low in normal healthy liver. Even though high LPO level 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 appeared that the burden due to enhanced oxidative stress in DMBA/TPA and AAILE + DMBA/TPA groups (after 8 weeks of treatment of DMBA/TPA) was unable to cause clastogenic damage in the cells.

A positive correlation between enhanced LPO levels and genotoxicity (as revealed by increased micronuclei score) in lymphocytes has been established earlier. An increase in micronuclei score has been reported in hepatic tissue in response to intra-peritoneal injection of carcinogens like NDEA and DMBA. This was associated with an increase in hepatic LPO levels and depressed antioxidant defense system. An increase in micronuclei score in spleen of animals intra-gastrically treated with B(a)P has also been reported. In N-methyl-N'-nitro-N-nitrosoguanidine (MNNG)-treated mice, enhanced LPO with compromised antioxidant defense system in stomach, liver and erythrocytes is accompanied by an increase in bone marrow micronuclei, which is reversed upon pre-treatment with ethanolic extract of A. indica leaf. AAILE has also demonstrated protective effects on in vivo clastogenicity of MNNG in metaphase cells of bone marrow of male wistar rats and against clastogenic NDEA-induced damage in hepatocytes of balb/c mice.

In conclusion, the results of the present study along with reports available in literature suggest that target and non-target sites (like liver) may be affected during skin tumorigenesis. AAILE treatment has the potential to modulate these changes, influencing the process of tumor formation. These findings seem to be critical to carcinogenesis and its intervention with anticancer agents.

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