Azadirachta indica leaf extract modulates initiation phase of murine forestomach tumorigenesis

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The effects of aqueous Azadirachta indica leaf extract (AAILE) on benzo(a)pyrene [B(a)P]-induced forestomach tumorigenesis, B(a)P-DNA adduct formation and certain parameters of carcinogen biotransformation system in mice have been reported earlier from our laboratory. In this study, the effects of AAILE on the enzymes of B(a)P biotransformation, which play crucial role in initiation of chemical carcinogenesis — aryl hydrocarbon hydroxylase (AHH) and uridinediphosphoglucuronosyltransferase (UDP-glucuronosyltransferase) have been evaluated in murine forestomach and liver. In addition, lipid peroxidation (LPO) levels in forestomach as well as liver and the activities of tissue injury marker enzymes — lactate dehydrogenase, aspartate aminotransferase and alkaline phosphatase in the serum have also been evaluated. Oral administration of AAILE (100 mg/kg body wt for 2 weeks) reduces the AHH activity and enhances the UDP-glucuronosyltransferase activity in both the tissues, suggesting its potential in decreasing the activation and increasing the detoxification of carcinogens. The LPO levels decrease upon AAILE treatment in the hepatic tissue, suggesting its anti-oxidative and hence anti-carcinogenic effects. Non-significant alterations have been observed in tissue injury marker enzymes upon AAILE treatment, suggesting its safety at the given dose. In conclusion, AAILE appears to modulate initiation phase of carcinogenesis and may be suggested as safe and an effective agent for chemoprevention.

Keywords: Aryl hydrocarbon hydroxylase; Azadirachta indica; Benzo(a)pyrene; Lipid peroxidation; Tissue injury marker enzymes; UDP-glucuronosyltransferase

Azadirachta indica A. Juss (common name: Neem; family: Meliaceae) is an evergreen tree known for its potent insecticidal and medicinal properties. Several parts of tree, in particular the leaves have been used in traditional medicine for various ailments. The leaf extract has shown immuno-modulatory, anti-inflammatory, anti-hyperglycaemic, anti-ulcer, antimalarial, anti-fungal, anti-bacterial, anti-viral, anti-oxidant, anti-mutagenic and anti-carcinogenic properties. Moreover, it also exhibits significant chem-preventive potential against certain malignancies. Recently, we have reported chemopreventive effects of aqueous A. indica leaf extract (AAILE) against benzo(a)pyrene [B(a)P]-induced forestomach tumorigenesis in female Balb/c mice. In our study, animals that received AAILE before, during and after B(a)P treatments have shown reduced tumor incidence, burden and multiplicity when compared to the animals that received only B(a)P. B(a)P, a polycyclic aromatic hydrocarbon (PAH), is a potent pro-carcinogen in the environment. It is metabolized mainly by cytochrome P450 (cyt P450)-dependent aryl hydrocarbon hydroxylase (AHH), an important component of phase I biotransformation reactions into its various derivatives including electrophilic epoxides. One of the primary phase II pathways for B(a)P metabolism is conjugation with glucuronic acid catalyzed by UDP-glucuronosyltransferases and this reaction renders lipophilic metabolites including epoxides to hydrophilic products, hence enhances their excretion out of the cells. Activated carcinogenic metabolites produced by phase I enzymes, if not detoxified by phase II enzymes may induce mutations, leading to initiation of carcinogenesis.

Many chemopreventive agents exert their anti-carcinogenic effects by modulating the status of carcinogen biotransformation. Thus, modulation of carcinogen biotransformation enzymes is one of the reliable biomarkers for evaluating the chemopreventive potential of putative agents in animal
model system\textsuperscript{14,15} and by employing this concept several plant extracts have been screened for their chemopreventive efficacy\textsuperscript{15-17}. In our recent study\textsuperscript{18}, we have found that AAILE decrease B(a)P-DNA adduct formation in the forestomach tissues of mice. It also modulates the activities of cyt P450 and b\textsubscript{5}, glutathione-s-transferase and GSH contents in forestomach and hepatic tissues of mice. These modulations (of carcinogen biotransformation system) have been correlated with the observed decrease in (a)P-DNA adduct formation as well as the reduced extent of tumorigenesis\textsuperscript{7,18}.

In the present study, the effects of AAILE on the initiation phase of B(a)P induced forestomach tumorigenesis have been evaluated. The effects of AAILE on the activities of one of the most crucial enzymes responsible for biotransformation of B(a)P i.e. AHH as well as UDP-glucuronosyltransferase and on the lipid peroxidation levels have been estimated in forestomach and hepatic tissues of female Balb/c mice. To monitor the toxicity of AAILE treatment, the activities of serum lactate dehydrogenase (LDH), aspartate aminotransferase (AST) and alkaline phosphatase (ALP) have also been examined.

Materials and Methods

Chemicals

Benzo(a)pyrene, 3-hydroxy-benzo(a)pyrene, bovine serum albumin (BSA), NADH, NADPH, thiobarbituric acid (TBA), sodium pyruvate and UDP-glucuronic acid were obtained from Sigma Chemical Co. (St. Louis, MO, USA). All other chemicals were obtained from reputed local firms (India) and were of highest purity/analytical grade. Capsules containing 100 mg of spray-dried aqueous \textit{A. indica} leaf extract (Dabur India Pvt. Ltd.) were obtained from local chemist shop. The contents of capsules were reconstituted in double-distilled water immediately before oral administration to the animals to attain the required doses\textsuperscript{2,7}.

Animals and treatments

Female Balb/c mice (6-8 weeks old) procured from Central Animal House, Panjab University, Chandigarh were housed in polypropylene cages bedded with sterilized rice husk and provided \textit{ad libitum} access to clean drinking water as well as standard animal pellet diet (Ashirwad Industries, Tirpari, Kharar, Distt. Roopnagar, Punjab) throughout the experiment. The temperature of animal room was maintained at 21 \pm 1°C, humidity 50-60\% and a 12 h dark and light cycle. The experimental protocols were approved by 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. Animals were acclimatized to experimental conditions for at least 1 week before commencement of treatments. Mice were divided into two groups (6-8 mice in each group) — control and \textit{A. indica} and were given treatments as described earlier\textsuperscript{2,7}. Briefly, \textit{A. indica} group was treated with \textit{A. indica} leaf extract (AAILE) at a dose of 100 mg/kg body weight (thrice a week on alternate days for 2 weeks), whereas control group was not given any special treatment.

Collection of samples

At the end of treatments i.e., at day 14, before their sacrifice mice were kept on an overnight fast. Just before sacrificing, the blood samples were collected by puncturing the retro-orbital venous sinus using sterilized glass capillaries. Blood was allowed to clot at room temperature and the samples were centrifuged at 3000 rpm for 15 min. The serum samples were collected and stored at 4°C until processed for biochemical estimation of tissue injury marker enzymes — LDH, AST and ALP. Mice were sacrificed with cervical dislocation under light ether anesthesia. Perfusion was done with 0.9\% NaCl solution and forestomach and liver were extracted out. They were washed properly in 0.9\% NaCl solution, blotted dry and then weighed carefully. Relative organ wt (absolute organ wt \times 100/body wt at sacrifice time of animals) was calculated for each mouse. For biochemical estimations, the tissues were homogenized in 100 mM potassium phosphate buffer (pH 7.4) containing 150 mM KCl to obtain 10\% homogenate (w/v), which was subjected to cold centrifuge at 10,000 \times g for 30 min. The pellet was discarded and supernatant obtained was used for biochemical estimations of AHH, UDP glucuronosyltransferase and LPO.

AHH activity assay

The activity of AHH in 10,000 \times g supernatant was estimated according to previously described method\textsuperscript{19} with few modifications. B(a)P used as substrate in this protocol on reaction with AHH is hydroxylated to 3-hydroxy benzo(a)pyrene which may be estimated spectrofluorimetrically. In brief, assay mixture
contained 0.4 µM NADPH, 3.0 µM MgCl₂, 0.1 µM EDTA and 50 mg tissue equivalents of 10,000 × g supernatant in 50 mM phosphate buffer (pH 7.4) in a final reaction volume of 1.0 ml. The reaction mixture was pre-incubated at 37°C in a shaking water bath for 5 min and finally the reaction was initiated by the addition of 80 nM of B(a)P in 25 µl of acetone. The reaction was stopped after 15 min by addition of 4.0 ml of chilled acetone: hexane (1:3) mixture. The contents of mixture were vortexed, kept in shaking water bath for 10 min, allowed to settle and 2.0 ml aliquot of organic phase was extracted with 4.0 ml of 1 N NaOH. The upper phase (hexane) was removed and the lower phase [NaOH fraction containing metabolites including 3-hydroxy B(a)P] was washed again with 2 ml of chilled hexane. Finally, hexane phase was discarded and contents of 3-hydroxy B(a)P in lower phase were measured on a fluorimeter at an excitation wavelength of 396 nm and an emission wavelength of 522 nm. Solution of 3-hydroxy B(a)P was used as a standard for calculation of AHH activity. The enzyme activity was expressed as pmols of 3-hydroxy B(a)P generated/mg protein/min.

**UDP-glucuronosyltransferase activity assay**

The activity of UDP-glucuronosyltransferase in 10,000 × g supernatant was estimated as described earlier. Aliquots of the samples were treated with equal volume of digitonin solution (1.5 % w/v) for overnight for denaturation of microsomal membranes. The reaction mixture contained 0.24 mM p-nitrophenol in 50 mM phosphate buffer and appropriate amount of denatured sample. To the test sample, UDP-glucuronic acid (5.5 mM) was added and reaction was allowed to occur at 25°C. The decrease in absorbance due to the formation of UDP-glucuronic acid-p-nitrophenol conjugates was recorded on double-beam spectrophotometer at 440 nm. A standard curve was prepared using different concentrations of p-nitrophenol for calculation of UDP-glucuronosyltransferase activity. The enzyme activity was expressed as µM of p-nitrophenol-UDP-glucuronic acid conjugates generated/ min/mg protein.

**NADPH-Dependent lipid peroxidation**

Quantification of NADPH-dependent lipid peroxidation was carried out by the protocol as described previously. In brief, 62.5 mg tissue equivalent 10,000 × g supernatant in 150 mM KCl/Tris-HCl buffer, pH 7.4 containing 0.3 µM NADPH in a total volume of 1.75 ml was incubated in a metabolic shaker at 37°C for 60 min. Reaction was stopped by the addition of 0.75 ml of cold TCA-HCl mixture (2 M TCA in 1.7 N HCl) and samples were centrifuged (for 10 min at 3,000 × g). Pellet was discarded and to 0.5 ml aliquot of the supernatant, 2 ml of 1% TBA (in glacial acetic acid:water; 1:1) was added. The tubes were covered with glass marbles before keeping them in boiling water for 15 min. Zero time blanks were kept to compensate for any TBA reactivity of the non-enzymatic reaction components. The intensity of pink colored complex formed was measured at 535 nm. The malondialdehyde (MDA) concentration was calculated using an extinction coefficient of 1.56 × 10³ M⁻¹ cm⁻¹ and expressed as nmol MDA formed 60 min⁻¹ mg⁻¹ protein.

**Lactate dehydrogenase (LDH) activity assay**

Serum LDH activity was estimated by recording the rate of oxidation of NADH at 340 nm. The reaction mixture contained 50 mM potassium phosphate buffer (pH 7.5), 0.5 mM sodium pyruvate, 0.1 mM NADH and appropriate amount of serum in a final volume of 1 ml. The reaction was started at 25°C by addition of NADH and rate of oxidation of the same was measured at 340 nm. The enzyme activity was calculated using extinction coefficient 6.22 mM⁻¹ cm⁻¹. One unit of enzyme activity was defined as which caused the oxidation of 1 µmol of NADH oxidized per liter (IU).

**Aspartate aminotransferase (AST) activity assay**

Serum AST activity was estimated by the previously described method. The reaction mixture contained 0.5 ml solution of buffered substrate (200 mM Dl-aspartic acid and 2 mM α-ketoglutaric acid in 200 mM phosphate buffer, pH 7.4) and 100 µl of serum was added, followed by incubation for 60 min at 37°C. Reaction was terminated by the addition of dinitrophenyl hydrazine (DNPH) dissolved in HCl and the reaction mixtures were kept at room temperature for 20 min. Finally, 5.0 ml of 0.4 N NaOH was added to all the tubes and allowed to react for another 10 min before the final color development. Control tubes were also run simultaneously for each test sample and reaction was stopped by prior addition of 0.5 ml of DNPH than serum. The optical density (OD) of all the tubes was read at 510 nm. Sodium pyruvate (4 mM) was used as a standard and the enzyme activity was expressed as µmol of pyruvate formed per liter (IU).
Alkaline phosphatase (ALP) activity assay

ALP activity was assayed using the method of Bergmeyer. In this protocol, \( p \)-nitrophenyl phosphate is used as a substrate, which on reaction with ALP yields \( p \)-nitrophenol having maximum absorption at 420 nm. The enzyme activity was directly proportional to the amount of \( p \)-nitrophenol liberated per unit time. Briefly, 1 ml of buffered substrate (5.5 mM \( p \)-nitrophenyl phosphate in 0.5 M glycine-NaOH buffer \( pH \) 10.5) was taken and incubated at 37°C for 5 min to pre-equilibrate. Thereafter, 0.1 ml serum was added and reaction mixture was incubated again for 15 min at 37°C. The reaction was stopped by addition of 5 ml of 0.1 N NaOH and the intensity of yellow color produced by liberation of \( p \)-nitrophenol was measured at 420 nm. \( p \)-Nitrophenol was used as a standard and the enzyme activity was expressed as µM of \( p \)-nitrophenol liberated/liter (IU).

Protein estimation

Protein contents of 10000 × g supernatants as well as serum samples were estimated by the method of Lowry et al. using BSA as standard at 680 nm.

Statistical analysis

The data were expressed as mean ± SD. Statistical significance was analyzed by unpaired student’s \( t \)-test.

Results

General observations

Non-significant changes were observed in diet and water consumption by the mice in both the groups (data not shown). The treatment did not cause mortality in mice. Also, non-significant changes were observed in final body weights of the groups upon AAILE treatment as seen in mice of A. indica group when compared with the controls (data published earlier). These observations suggested that AAILE at given dose level did not cause adverse effects on normal animal health.

Relative organ weights

Non-significant changes were observed in the relative liver weight (from 4.92 ± 0.61 to 5.24 ± 0.69) as well as relative forestomach weight (from 0.43 ± 0.07 to 0.50 ± 0.8) for 2 weeks of AAILE treatment as seen in mice of A. indica group when compared with the controls (Fig. 1).

Aryl hydrocarbon hydroxylase activity

A. indica treatment reduced the activity of AHH in the forestomach (from 26.3 ± 1.6 to 23.1 ± 1.2 pmols of 3-hydroxy B(a)P generated/mg protein/min) and hepatic tissues (from 40.5 ± 3.2 to 33.6 ± 2.2 pmols of 3-hydroxy B(a)P generated /mg protein/min) of mice as seen in the animals receiving AAILE when compared with the control group (Fig. 2a).

UDP-glucuronosyltransferase activity

A. indica treatment enhanced the activity of UDP-glucuronosyltransferase in the forestomach (from 13.6 ± 2.2 to 17.9 ± 1.0 µmols \( p \)-nitrophenol-UDP-glucuronic acid conjugates generated/mg protein/min) as well as in hepatic tissues (from 8.3 ± 0.4 to 12.5 ± 1.0 µmols \( p \)-nitrophenol-UDP-glucuronic acid conjugates generated/ mg protein/min) of mice as seen in the animals receiving AAILE when compared with control group (Fig. 2b).
**NADPH-Dependent lipid peroxidation**

*A. indica* reduced the levels of lipid peroxidation in the hepatic tissue (from 0.88 ± 0.09 to 0.64 ± 0.08 nmol MDA/mg protein/60 min), whereas in forestomach tissue, a non-significant change (from 1.83 ± 0.14 to 1.84 ± 0.02 nmol MDA/mg protein/60 min) was observed in LPO levels, as seen in the animals receiving AAILE when compared with the control group (Fig. 3).

**Tissue injury marker enzymes**

Non-significant decrease in the activities of ALP, AST and LDH in serum was observed upon *A. indica* treatment as seen in the animals receiving AAILE when compared with control group (Table 1).

**Serum protein contents**

Non-significant changes were observed in the serum protein contents (from 19.5 ± 0.3 to 19.7 ± 0.3) for 2 weeks of AAILE treatment as seen in mice of *A. indica* group when compared with the controls.

**Discussion**

Earlier, we have investigated the chemopreventive efficacy of AAILE against various chemical carcinogens in murine models as well as the mechanism involved in prevention. Recently, we have reported the chemopreventive effect of AAILE against B(a)P-induced murine forestomach tumorigenesis. B(a)P is a pro-carcinogen which undergoes metabolic activation through the action of phase-I biotransformation enzymes for induction of carcinogenesis. Metabolic activation produces many active metabolites of B(a)P, of which (±)-anti-7,8-dihydroxy-9,10-oxy-7,8,9,10-tetrahydroxy B(a)P or [(±)-anti-BaPDE] acts as its major carcinogenic metabolite. (±)-Anti-BaPDE, if not detoxified by phase II biotransformation enzymes may interact with DNA, leading to covalent modification of the same (DNA adduct formation) and mutations may occur upon replication of DNA sequences bearing adducts. Certain mutations, particularly those resulting in activation of proto-oncogenes or deactivation of tumor suppressor genes play a crucial role in the initiation phase of carcinogenesis. Therefore, modulation of biotransformation enzymes as well as DNA adducts formation may be exploited for achieving effective chemoprevention against chemical carcinogenesis.

A number of plant extracts have been screened for their chemoprevention efficacy using carcinogen biotransformation enzymes as markers. Recently, we found that AAILE decrease the B(a)P-DNA adduct formation in the forestomach tissues of mice by modulating the activities of cyt P450 and b5, and glutathione-s-transferase and GSH contents in forestomach and hepatic tissue of mice and these findings have been correlated with the previously observed reduced extent of tumorigenesis. In the present study, we have evaluated the effects of AAILE on the activities of the most crucial enzymes responsible for the biotransformation of B(a)P namely AHH and UDP-glucuronosyltransferases in forestomach and hepatic tissues of mice.

AHH, an isof orm of cyt P450 and an integral component of phase I biotransformation enzymes is primarily found in hepatic tissues and plays a major role in the metabolism of several carcinogens including PAHs. B(a)P, one of the most potent carcinogenic PAHs is mainly metabolized by cyt P450-dependent AHH into its various derivatives including (±)-anti-BaPDE, an electrophilic epoxide of B(a)P that acts as its ultimate carcinogen. The carcinogenic potency and extent of binding of (±)-anti-BaPDE with DNA for formation of adducts is usually correlated with the induction of AHH. Therefore, alteration in AHH activity can be correlated with modulation of carcinogenic process. In our observations, 2 weeks of AAILE treatment has caused decrease in AHH activity in the hepatic (approx 1.14-fold, \( P<0.05 \)) as well as forestomach (approx 1.21-fold, \( P<0.05 \)) tissues. These observations suggest potential of AAILE in decreasing the metabolic activation of carcinogens including B(a)P by reducing the activity of AHH.
Detoxification pathways mediated by phase II (conjugative) enzymes are considered an important factor in determining cellular sensitivity to PAH metabolites. Conjugation with glucuronic acid catalyzed by UDP-glucuronosyltransferase, is one of the primary phase II pathways for B(a)P metabolism. The UDP-glucuronosyltransferase transforms many lipophilic compounds to more water-soluble products via conjugation with glucuronic acid. This reaction inhibits reactive electrophiles from reaching cellular targets and results in production of less cytotoxic conjugates. In our observations, 2 weeks of AAILE treatment has caused increase in the activity of UDP-glucuronosyltransferases in the hepatic (approx 1.32 fold, *P*<0.001) as well as forestomach (approx 1.51-fold, *P*<0.05) tissues. These observations suggest potential of AAILE in increasing the detoxification of activated carcinogenic metabolites by enhancing the activity of UDP-glucuronosyltransferase. To the best of our knowledge, this is the first report on the modulation of AHH as well as UDP-glucuronosyltransferases by *A. indica*. Earlier, *A. indica*-mediated alterations in certain biotransformation enzymes (cyt P450, cyt b5, glutathione-s-transferase, DT-diaphorase etc.) and GSH content have been reported.

Reactive oxygen species (ROS) are generated in biological systems either through normal metabolic pathways or as a result of exposure to chemical carcinogens, leading to oxidative imbalance that may further result in membrane dysfunction, protein inactivation and DNA damage contributing ultimately to carcinogenesis. ROS attack macromolecules, preferentially poly-unsaturated fatty acids, resulting in the lipid peroxidation (LPO) products MDA or lipid peroxides. MDA, owing to its high cytotoxicity and inhibitory action on protective enzymes acts as a tumor promoter and co-carcinogenic agent. Moreover, it possesses mutagenic activity through its reaction with deoxyguanosine to form DNA adducts that may initiate carcinogenesis. ROS-mediated damage to cellular macromolecules. No discernible adverse effects have been observed in *A. indica* group at the given dose of AAILE i.e. 100 mg/kg body wt on alternate days for 14 days, as evident from the fact that no increase has been observed in mortality rate or decrease in body weight of animals, following treatment with AAILE. Furthermore, no alteration in relative organ weight of animals suggest no adverse effect of *A. indica* on general body metabolism. To further confirm the safety of AAILE at cellular level, activities of the marker enzyme and is also an indicator of damage induced by xenobiotic compounds and radiation.

AST catalyzes transfer of α-amino groups of aspartate to the α-keto group of α-ketoglutaric acid, resulting in formation of oxaloacetic acid and pyruvic acid. It has cytosolic and mitochondrial forms and is found in tissues of the liver, heart, kidneys and red blood cells. Phosphatases catalyze the splitting off of phosphoric acids from certain monophosphoric esters, a reaction of considerable importance in several body processes including neoplastic growth. ALP is directly implicated to the activities of tissue injury marker enzymes have remained unaltered. Earlier studies have also found that aqueous extract of *A. indica* leaf extract on oral administration does not impose any toxic effects to experimental animals.

The present study has demonstrated that *A. indica* decrease the AHH and enhance the UDP-glucuronosyltransferase activities, the most crucial enzymes for biotransformation of B(a)P. Modulation of these enzymes might decrease the activation and enhance the detoxification of activated carcinogenic metabolites. Alteration in LPO levels upon 2 weeks of AAILE treatment indicates that it might have lowered the ROS-mediated damage to cellular macromolecules. Non-significant alterations in relative organ weight and activities of tissue injury marker enzymes have established the safety of AAILE at given pharmacological doses (i.e. 100 mg/kg body wt). The observations of the present study strengthen our earlier observations about *A. indica*-mediated modulation of...
initiation phase of murine forestomach tumorigenesis and suggest *A. indica* an effective and safe agent for chemoprevention against carcinogenesis.

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