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NATURAL DRUGS

ANTI-CLASTOGENIC ACTIVITY OF *AZADIRACHTA INDICA* AGAINST BENZO(A)PYRENE IN MURINE FORESTOMACH TUMORIGENESIS BIOASSAY

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Abstract: Present study evaluated the anti-clastogenic efficacy of *Azadirachta indica* (*A. indica*) against benzo(a)pyrene [B(a)P] in murine forestomach tumorigenesis bioassay protocol. Female Balb/c mice were divided into four groups (n = 8). Each mouse from B(a)P and B(a)P + *A. indica* groups received intragastric instillations of B(a)P at a dose of 40 mg/kg b. w. in 0.2 mL olive oil twice a week, starting from 3rd week to the end of 6th week of the experiment. Mice of control and *A. indica* groups received 0.2 mL olive oil in the same schedule as for B(a)P and B(a)P + *A indica* groups. Mice of *A. indica* and B(a)P + *A. indica* groups received oral doses of 100 mg/kg b. w. aqueous *A. indica* leaf extract (AAILE) on alternate days throughout the experiment. Two weeks after the last B(a)P instillation, mice were sacrificed and spleens were processed for micronucleus (MN) assay, while liver tissues were analyzed for lipid peroxidation (LPO), as well as antioxidant defense enzymes, namely: catalase, superoxide dismutase (SOD), glutathione reductase (GR) and glutathione peroxidase (GPx). The incidence of MN formation increased in spleen cells of mice that received only B(a)P instillations. In hepatic tissues, the extent of oxidative stress increased upon B(a)P instillations as was evidenced from enhanced LPO levels with concomitant decrease in antioxidant defense enzyme activities in mice that received only B(a)P instillations. Interestingly, *A. indica* treatment significantly reversed these effects as observed in mice receiving AAILE along with B(a)P when compared to only B(a)P receiving mice. Moreover, in only AAILE receiving mice, enhanced antioxidant defense with slightly decreased levels of LPO as well as MN incidences were observed. Observations of the present study suggest that *A. indica* exert anti-clastogenic effects against B(a)P by modulating oxidative stress and antioxidant defense.

Keywords: *Azadirachta indica*, benzo(a)pyrene, micronucleus, oxidative stress, antioxidant defense

B(a)P is a potent pro-carcinogen/mutagen and perhaps the best characterized member of polycyclic aromatic hydrocarbons (PAHs) family of compounds. With limited human data but sufficient evidence of carcinogenicity in animals, IARC has classified B(a)P as Group I carcinogen i.e., carcinogenic to humans (1, 2). Extensive experimental as well as epidemiological investigations have established that B(a)P exerts immunotoxic, teratogenic, clastogenic (induction of micronuclei and chromosomal aberrations), mutagenic and carcinogenic effects (3–5). Inside biological systems, during its biotransformation, B(a)P is initially metabolized through a series of reactions to trans-7,8-dihydroxy-9,10-oxy-7,8,9,10-tetrahydrobenzo(a)pyrene (BaPDE) which can directly interact with DNA to form adducts for the initiation of carcinogenesis (6, 7). Besides production of activated carcinogenic metabolites, biotransformation of B(a)P also results in generation of reactive oxygen species (ROS) that may lead to oxidative stress, thereby leading to genetic damage through indirect mechanisms (8–10). The latter involves the induction of pro-oxidant status causing LPO which acts as clastogenic factors (low molecular weight components that break chromosomes at the same or remote tissues). LPO and ROS mostly act mainly as secondary agents that produce secondary DNA damage in reactions with cellular molecules other than DNA and are thus potent inducers of chromosomal aberrations (CA) or MN (11).

An association between mutations and cancer has long been evident on indirect ground, such as a correlation between the mutagenicity and carcinogenicity of chemicals, especially in biological systems that have the requisite metabolic activation capabilities. Moreover, human chromosome instability syndromes and DNA repair deficiencies are associated with increased risk of cancer (9, 10).

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Cancer cytogenetics and toxicology have greatly strengthened the association that specific chromosomal alterations, including deletions, translocations, and inversions, have been implicated in many human cancers (12). For assessing the genotoxic damage exerted by chemical compounds and radiations, several bioassays has been designed and certain tests like MN assay, CA assay and comet assay are routinely used in experimental and epidemiological studies. Owing to its several qualities like simplicity, reliability and inexpensiveness, MN test has emerged as an invaluable tool for detecting and quantifying the genotoxic damage (13–15).

MN refers to the fragment of damaged chromosomes that fails to find its way on the spindle during cell division. At anaphase, when the centric elements move towards the spindle pole, the acentric chromatids and chromosomal fragments lag behind. After telophase, the undamaged chromosomal fragments as well as the centric fragments give rise to regular daughter nuclei. The lagging elements are also included in the daughter cells but a considerable portion is transferred into one or several secondary nuclei, which are much smaller than the principle nuclei and are generally called as MN (13, 15). MN formation can be induced by exposing the cells/organisms to chemicals (PAHs, heterocyclic amines, heavy metal ions etc.) and radiations (X-rays, UV rays etc.) (13, 14). In routine acute studies, the MN assay is generally carried in rapidly proliferating cells tissues as the damage caused by the clastogenic agents gets expressed in the new progenies of cells. In chronic administrative studies, it is always desirable to study the MN formation in the spleen, because the irreparable damage to the cellular DNA gets accumulated in the same (16). B(a)P has been observed to be an inducer of MN in several organs including spleen (17–20). Since MN induction in a way is the index of clastogenic/genotoxic damage, MN test is routinely employed for assessing the clastogenicity/ genotoxicity of chemical compounds as well as the preventive effects of putative chemopreventive agents.

In our research laboratory, we have been working on chemopreventive potential of *A. indica* leaf extract against various carcinogens such as B(a)Ps, DMBA and diethylnitrosamine (DEN) in murine models. Recently, we reported the chemopreventive effects of *A. indica* on B(a)P induced forestomach tumorigenesis in Balb/C mice and explored certain underlying mechanisms too (21–25). In continuation to the latter and the facts discussed above, the present study was designed to evaluate anticlastogenic efficacy of *A. indica* against benzo(a)pyrene in murine forestomach tumorigenesis bioassay protocol. In this model, mice are subjected to intragastric instillations of B(a)P at a dose of 40 mg/kg in oil or DMSO as vehicle, twice a week for four weeks and 16–18 weeks after the last carcinogen exposure, the animals develop forestomach tumors. One of the strength of this model is that B(a)P cause carcinogenicity through genotoxic mechanisms (26) and observations especially involving cytogenetic parameters, such as frequency of MN formation, could directly be correlated with the extent of tumorigenesis.

MATERIAL AND METHODS

Chemicals

Benzo(a)pyrene; reduced glutathione (GSH); GR; oxidized glutathione (GSSG); nitroblue tetrazolium (NBT); bovine serum albumin (BSA); thiobarbituric acid (TBA); malondialdehyde (MDA); reduced nicotinamide adenine dinucleotide phosphate (NADPH) and hydroxylamine hydrochloride ($NH_2OH.HCl$) were obtained from Sigma Chemical Co., St. Louis, MO, USA. All other chemicals utilized were procured from reputed local firms (India) and were of the highest purity/analytical grade.

Aqueous *Azadirachta indica* leaf extract

Capsules of spray dried aqueous *A. indica* leaf extract (AAILE) were obtained from a recognized herbal company (Dabur India Pvt. Ltd.). Each capsule contained 100 mg of AAILE in the form of dark green paste like substance. The extract is in crude form and studies from our laboratory as well as others have established AAILE to contain several active components especially polysaccharides and flavanoids (27, 28). The contents of the capsules were reconstituted into double distilled water immediately before its oral administration to the animals, in order to attain the required doses, as described earlier (21–25, 29–31).

Animal model, experimental conditions and treatments

In the present experimental investigation, 6–8 weeks old female Balb/c mice procured from Central Animal House, Panjab University, Chandigarh were utilized. The animals were housed in polypropylene cages bedded with sterilized rice husk and provided *ad libitum* access to clean drinking water and standard animal pellet diet formulated on scientific backgrounds (Ashirwad Industries, Tirpari, Kharar, Distt. Ropar, Punjab, India) throughout the experiment. The temperature of ani-

mal room was maintained at 21 ± 1°C, relative humidity 50–60% and a 12 h dark and light cycle. The animals were cared for according to the "*Principles of Laboratory Animal Care*" of the National Institutes of Health (NIH, USA) and under strict adherence to Indian Animal Ethic Committee (IAEC). Before commencement of various treatments, the animals were kept for acclimatization to experimental conditions for one week and the final day of their acclimatization period was considered as a zero week of the experiment. Mice were randomly divided into four groups (n = 8) as shown below: control; B(a)P; *A. indica*; B(a)P + *A. indica*. For tumor induction, the mice of B(a)P and B(a)P + *A indica* groups were given doses of B(a)P as described by Wattenberg et al. (32), with minor modification. Animals received oral doses of B(a)P (40 mg/kg) in 0.2 mL olive oil twice a week for four weeks, a total of 8 mg of B(a)P per mouse starting from 3rd week to the end of 6th week of the experiment. Also, the animals of control and *A. indica* groups received 0.2 mL of olive oil in the same schedule as for the B(a)P and B(a)P + *A. indica* groups. The animals of *A. indica* and B(a)P + *A. indica* groups were administered with AAILE at a dose of 100 mg/kg b. w. thrice a week on alternate days throughout the experiment as described previously (21–25, 28–30).Two weeks after the last instillation of B(a)P and before their sacrifice, the mice were kept on an overnight fast. Mice were sacrificed with cervical dislocation under light ether anesthesia. Perfusion was done with 0.9% NaCl solution after which the required organs (spleen and liver) were extracted out. They were washed properly in 0.9% NaCl solution, blotted dry and then weighed carefully. The spleen was processed for MN assay, while the livers were analyzed for different biochemical parameters.

Micronucleus assay

For this assay, the spleen tissues were washed with chilled homogenizing buffer (24 mM Na_2-EDTA buffer pH 7.5, containing 75 mM of NaCl), suspended at the rate of 1 mg/mL and were 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 resuspend the spleen cells. Small drops of suspension prepared were put at one end of precleaned, 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 cells were then stained with May and Grunwald stain for 1–2 min followed by staining with Giemsa for 10 min as described by Schmid (13). After the staining was achieved, the slides were rinsed twice in distilled water, dried and then rinsed in methanol. The slides were then cleared in xylene and mounted in DPX. Cells were counted per mice for the presence of micronuclei using light microscope at a magnification of 100×. The frequency of MN induction was expressed as percentage number of micronucleated cells to cells having normal nuclei (31).

Biochemical parameters

For biochemical estimations, the liver tissues were homogenized in 100 mM potassium phosphate buffer (pH 7.4) containing 150 mM KCl to obtain 10% homogenate (w/v). The homogenate was subjected to cold centrifuge at 10000 × g for 30 min. The pellet was discarded and supernatant obtained (10000 × g supernatant) was used for various biochemical estimations, namely: LPO, catalase, SOD, GR and GPx.

NADPH-dependent LPO

NADPH-dependent LPO was assayed by the method of Trush et al. (33). Lipids, mainly polyunsaturated fatty acids (PUFAs), are highly susceptible to peroxidation by means of various oxidizing free radicals, which are formed by non-enzymatic oxidation reactions. Cycloperoxides are formed as a result of peroxidation reactions, which give malondialdehyde (MDA) by cleavage. Lipid peroxidation was measured by the formation of thiobarbituric acid (TBA) reactive metabolites of lipids, such as MDA. NADPH-dependent formation of peroxides leads to the breakdown of PUFAs to MDA and the latter thus formed reacts with TBA to form a pink colored complex, which absorbs maximally at 535 nm. In the present study, 62.5 mg tissue equivalent 10,000 × g supernatant was incubated in 150 mM KCI/Tris-HCl buffer, pH 7.4 containing 0.3 mM NADPH and 9 mM $MgCl_2$ in a total volume of 1.75 mL. The reaction mixture was incubated in a metabolic shaker at 37°C for 60 min. The reaction was stopped by the addition of 0.75 mL of cold TCA-HCl mixture (2 M TCA in 1.7 M HCl). Samples were centrifuged for 10 min at 3000 × g. To 0.5 mL aliquot of the supernatant, 2 mL of 1% TBA (1% TBA in glacial acetic acid : water 1 : 1, v/v) was added and the tubes covered with glass marbles were kept in boiling water for exactly 15 min. Zero time blanks were kept to compensate for any TBA reactivity of the non-enzymatic reaction components. MDA concentration of the 10,000 × g supernatant was measured

at 535 nm using an extinction coefficient of 1.56×10^5 M^{-1} cm^{-1}. The final results were expressed as nM MDA formed min^{-1} mg^{-1} protein.

Antioxidant Defense Enzymes

Glutathione reductase

Activity of this enzyme was determined by the procedure described by Carlberg and Mannervick, (34). NADPH acts as a substrate in this protocol and is oxidized upon GR mediated reduction of oxidized glutathione. The NADPH absorbs maximally at 340 nm and the rate of change of OD upon its (GR mediated) oxidation could be exploited for estimating the activity of the same. The reaction mixture (final volume 1 mL) contained 0.2 M sodium phosphate buffer (pH 7.0), 2 mM EDTA, 1 mM oxidized glutathione (GSSG) and 1.2 mM NADPH. The reaction was started by adding 25 mL of 10000 × g supernatant and the enzyme activity was measured indirectly by monitoring the oxidation of NADPH following the decrease in OD/minute for a minimum 3 min at 340 nm. One unit of enzyme activity was defined as nM NADPH consumed min^{-1} mg^{-1} protein based on an extinction coefficient of 6.22 m M^{-1} cm^{-1}.

Glutathione peroxidase

GPx activity was measured by the protocol as described by Pagila and Valentine (35). This is a coupled assay based on GR mediated oxidation of NADPH (substrate). The rate of change of OD is then monitored at 340 nm for estimating the activity of the GPx. A total of 1 mL of the reaction mixture contained 1 mM EDTA, 0.24 U/mL yeast glutathione reductase, 0.3 mM glutathione (reduced), 0.2 mM NADPH, 1.5 mM H_2O_2 in 50 mM sodium phosphate buffer (pH 7.0) and appropriate amount of 10000 × g supernatant. The reaction was initiated by adding NADPH and its oxidation was monitored at 340 nm by observing the decrease in OD/minute for 3 min. One unit of enzyme activity was defined as nM NADPH consumed $minute^{-1}$ mg^{-1} protein based on an extinction coefficient of 6.22 mM^{-1} cm^{-1}.

Catalase

The method of Luck (36) was used for the estimation of catalase in 10000 × g supernatant. This enzyme catalyzes decomposition of hydrogen peroxide (H_2O_2), which may be measured spectrophotometrically by recording the decrease in absorbance at 240 nm. The concentration of H_2O_2 was determined by recording absorbance at 240 nm and taking 0.071 mM^{-1} cm^{-1} as the extinction coefficient. H_2O_2 at the concentration of 14.12 mM had an approximate absorbance of 0.5. The 3.0 mL reaction mixture in sample cuvette contained 50 mM potassium phosphate buffer (pH 7.0), 1.25×10^{-2} M H_2O_2 and appropriate amount of 10000 × g supernatant. The blanks in reference cuvette lacking H_2O_2 was also run simultaneously. A decrease in absorbance at 240 nm was measured on a double beam spectrophotometer. The enzyme activity was expressed as IU that represents µM H_2O_2 decomposed $minute^{-1}$ mg^{-1} protein.

Superoxide dismutase (SOD)

The activity of SOD was estimated by the method of Kono (37). The method is based on the principle of the inhibitory effect of SOD on reduction of nitroblue tetrazolium (NBT) dye by superoxide anions, which are generated by the photooxidation of hydroxylamine hydrochloride ($NH_2OH.HCl$). The reaction mixture contained 1.3 mL of 50 mM sodium carbonate solution with 0.1 mM EDTA (pH 10.0), 0.5 mL of 96 mM of NBT and 0.1 mL of 0.6% Triton X-100. The reaction was initiated by the addition of 0.1 mL of 20 mM hydroxylamine hydrochloride (pH 6.0) to the reaction mixture and the rate of NBT reduction in the absence of the enzyme source was recorded for about 30 s. Following this, small aliquots of 10000 × g supernatant equivalent to 25–400 mg protein were added to the test cuvette as well as reference cuvette, which did not contain hydroxylamine hydrochloride. Finally, the rate of inhibition of an increase in optical density upon addition of 10000 × g supernatant was calculated and the same was used for further calculating the amount of SOD present in the samples. The specific activity of SOD was expressed as IU and the same represented the amount of protein required to cause 50% inhibition in the increase in optical density per unit time.

Estimation of protein content

Protein contents of different samples were estimated employing the method of Lowry et al. (38). The samples containing protein when treated with alkaline copper tartarate form cupric amino acid complex. Due to the reduction of phosphomolybdic acid and phosphotungstic acid by aromatic amino acids (tyrosine and tryptophan) and cupric amino acid complexes, the intense blue color is produced. The intensity of blue colored complex formed could me measured from 540 to 740 nm (usually measured at 680 nm) and the same comes out to be directly proportional to the quantity/concentration of protein in the sample being tested. In the present piece of

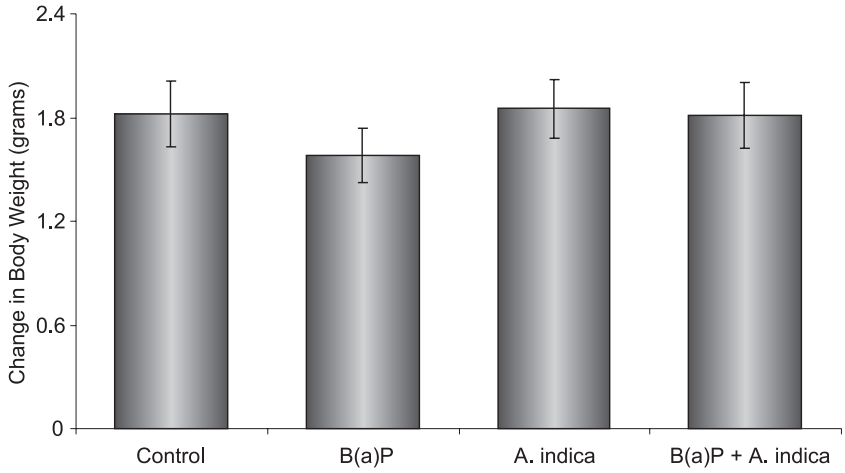

Figure 1. Effects of B(a)P and/or *A. indica* on weight gained by mice. Weight gained was calculated by substracting initial body weight from the final body weight of animals. Values are expressed as the mean ± SD (n = 8)

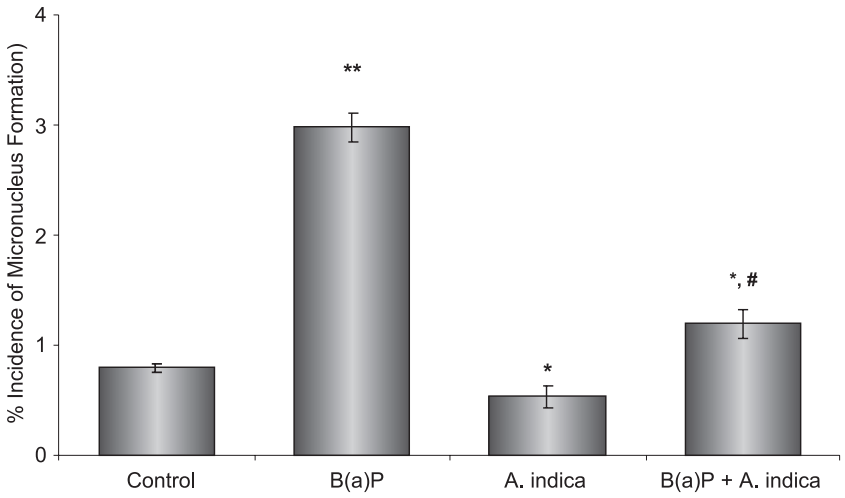

Figure 2. Effects of B(a)P and/or *A. indica* on % incidence of micronucleus formation in spleenocytes. Values are expressed as the mean ± SD (n = 8). Symbols and statistical significance: * and ** represents $p \leq 0.05$ and $p \leq 0.01$, respectively, when test group of mice was compared to control counterparts; # represents $p \leq 0.01$ when B(a)P + *A. indica* group of mice was compared to B(a)P

Table 1. Effects of B(a)P and/or *A. indica* on certain antioxidant defense enzymes in hepatic tissue.

Enzyme	Control	B(a)P	*A. indica*	B(a)P+*A. indica*
Glutathione peroxidase	54.38 ± 3.81	42.08 ± 4.17***	62.82 ± 4.23**	46.17 ± 4.97**, #
Glutathione reductase	36.03 ± 4.42	21.21 ± 3.16***	43.45 ± 2.97**	27.72 ± 3.46**, ##
Catalase	65.95 ± 6.22	43.83 ± 4.81***	73.3 ± 4.74*	52.15 ± 5.53**, ##
Superoxide dismutase	8.13 ± 0.82	4.83 ± 0.62***	9.03 ± 0.75*	6.58 ± 0.66**, #

Values are expressed as the mean ± SD (n = 8); Units: GR and GPx: μM NADPH oxidized minute^{-1} mg^{-1} protein; Catalase and SOD: international units. Symbols and statistical significance: *, ** and *** represent $p \leq 0.05$, $p \leq 0.01$ and $p \leq 0.001$, respectively, when the test group of mice was compared to control counterparts; # and ## represent $p \leq 0.05$ and $p \leq 0.01$, respectively, when B(a)P + *A. indica* group of mice was compared to B(a)P.

research work, 980 mL of double distilled water was added to 10 mL of the sample and after mixing it, 3.0 mL of alkaline reagent (98 mL of 2% Na_2CO_3 in 0.1 M NaOH: 1 mL of 1% $CuSO_4$: 1 mL of 2% sodium potassium tartarate) was added. The contents were mixed and incubated at room temperature for 10 min. After this, 0.30 mL of 1 M Folin's Ciocalteau reagent was added to the tubes. After vortexing, the mixture was incubated for 30 min at room temperature and optical density was noted at 680 nm. BSA (20 mg%) was used for the preparation of standard curve in order to calculate the protein content in the samples.

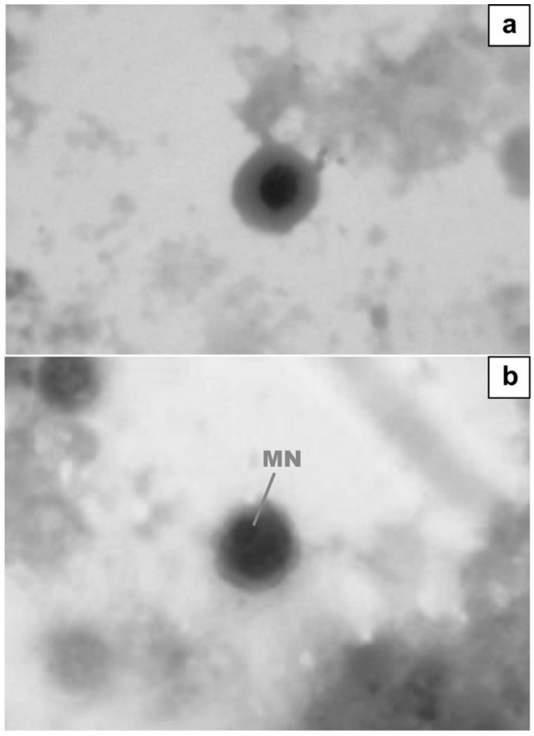

Figure 3. Spleenocutes (a) with normal nucleus and (b) with MN

Statistical analysis

Data were expressed as the mean ± SD. The statistical significance of inter group difference of various parameters were determined by unpaired Student's *t*-test. Comparison was made between the test groups to that of the control group of mice. B(a)P + *A. indica* group of mice were also compared to the mice of B(a)P group.

RESULTS

The body weight gained by mice that received only B(a)P instillations, was observed to be slightly (but non-significantly) lower compared to their respective control counterparts, as well as mice receiving AAILE along with B(a)P (Fig. 1). Non-significant changes were observed in the diet/water consumption by mice in any of the groups studied (data not shown).

A significant increase in the extent of MN induction was observed in the mice that received only B(a)P instillations when compared to control counterparts. However, in the mice that received

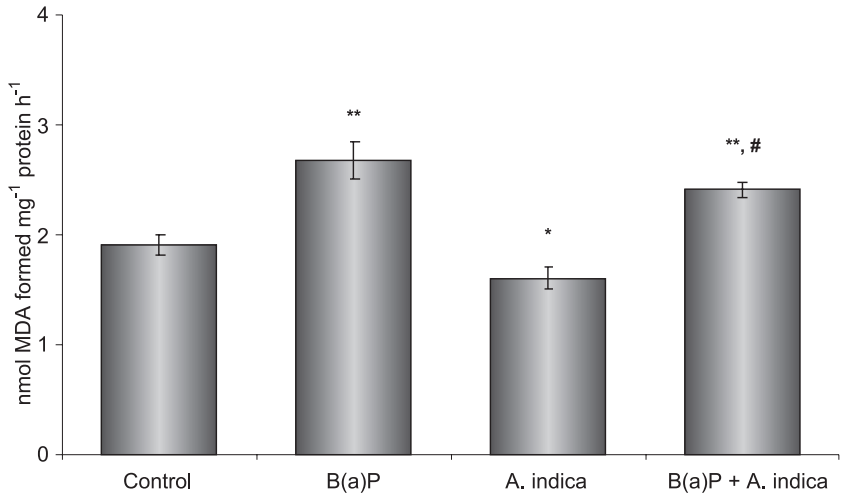

Figure 4. Effects of B(a)P and/or *A. indica* on levels of lipid peroxidation in hepatic tissues. Values are expressed as the mean ± SD (n = 8); units of LPO: nM MDA generated minute^{-1} mg^{-1} protein; statistical significance and symbols: Control *vs.* test, * and ** represents $p \leq 0.05$ and $p \leq 0.01$, respectively; # represents $p \leq 0.05$

AAILE treatments along with B(a)P instillations the levels of micronuclei were found to be much significantly reduced when compared to the animals that received only B(a)P instillations. Moreover, a slightly significant decrease in the extent of MN induction was observed in the mice that received only AAILE treatments (Fig. 2, 3).

An increased levels of LPO were found in mice that received B(a)P intragastric instillations (i.e., B(a)P and B(a)P + *A. indica* groups of mice). However, the mice that received AAILE along with B(a)P i.e., B(a)P + *A. indica* groups of mice, depicted lower levels of LPO when compared to only B(a)P receiving mice. Moreover, a significant decrease in the levels of LPO was observed in only AAILE receiving mice (Fig. 4).

In the mice that received only B(a)P instillations, the activities of antioxidant defense enzymes decreased in liver tissue, whereas in mice that received AAILE along with B(a)P, enhanced activities of antioxidant defense enzymes were found. In mice receiving only AAILE, the activities of antioxidant defense enzymes studied were observed to increase in liver tissues when compared with the control counterparts (Table 1).

Overall, these observations indicate that B(a)P induce micronuclei and cause oxidative stress by lowering the antioxidant defense. On the other hand, *A. indica* prevent MN induction by inhibiting lipid peroxidation and enhancing the activities of antioxidant defense enzymes.

DISCUSSION AND CONCLUSION

Owing of their high efficacy and relative safety, medicinal plants have always been an indispensible part of traditional systems of medicine throughout the world since antiquity. However, the use of medicinal plants in modern medicine suffers from a general lack of scientific evidence supporting their preventive or therapeutic potentials. Therefore, it is necessary to provide scientific proof to justify the use of a plant for medicinal purposes (39). A recent upsurge in identifying non-dietary natural products with high safety margins as cancer chemopreventive agents has been hailed by many investigators to have practical benefits, especially for treating mild to moderate carcinogenic effects. Moreover, the current focus of cancer chemoprevention studies is on intermediate biomarkers capable of detecting early changes that can be correlated with the inhibition of carcinogenesis. Assays such as MN test as well as the assessment of antioxidant status and LPO, are widely used in the detection and evaluation not only of chemical carcinogens but also of presumptive chemopreventive agents (40, 41).

Since last few years, there is a growing interest in evaluation of chemopreventive efficacy of medicinal plants used in Ayurveda, the Indian traditional system of medicine, and one of such plants is *Azadirachta indica* A. Juss. (Family: Meliaceae) commonly called as Neem (40). More than 140 different phytochemicals have been isolated and identified from various parts of *A. indica* and some of the principal constituents of *A. indica* leaves are as follows: 3-acetyl-7-tigloyl-lactone-vilasinin; 3-desacetyl-3-cinnamoyl-azadirachtin; 3-desacetyl-salanin; 4a,6a-dihydroxy-A-homo-azadiradione; 6-desacetylnimbinene; azadirachtanin; azadirachtanin-A; b-sitosterol; hyperoside; isoazadirolide; nimbaflavone; nimbandiol; nimbinene; nimbolide; quercetin; quercitrin; rutin; vlasanin (28, 42). Though several therapeutically valuable phytochemicals have been isolated from *A. indica,* a majority of the pharmacological investigations carried out till date has focused on crude extracts (28, 42). Moreover, crude extract(s) based traditional medicinal preparations, exert better effectiveness and more compatibility in the human body with minimal side effects as compared to their active principles of extracts or their synthetic analogs. Crude extracts show lesser side effects, possibly because of the modulatory activity of some components present in them along with the active component, which neutralizes the side effects and even synergies the medicinal effects of the later (43).

Different parts of *A. indica* have been observed to exert anti-inflammatory, antipyretic, analgesic, immuno-stimulant, hypoglycemic, antiulcer, spermicidal, antimalarial, antifungal, antibacterial, antiviral, hepato-protective, central nervous system depressant, antioxidant and more interestingly, anticancer activity (28, 42). Among all the above listed significant medicinal effects, anti-cancer potential of *A. indica* is on the focus of cancer chemoprevention research since last few years. Earlier, we reported the chemopreventive potential of AAILE on B(a)P induced murine forestomach tumorigenesis (21–25) and the present study addressed the evaluation of AAILE's anticlastogenic efficacy in the same bioassay protocol.

In order to analyze the overall genetic damage that could have been imposed upon carcinogen exposure during the forestomach tumorigenesis bioassay protocol, the status of MN induction was evaluated specifically in the spleen cells two weeks after the last intragastric instillation of B(a)P. A significant increase in the extent of MN induction was

observed in the mice that received only B(a)P instillations when compared to control counterparts. Earlier studies have established that B(a)P exerts immunosupression and shows profound toxicity in the form of MN induction (17, 19, 20, 44). Our observation further substantiated the fact that B(a)P induces genotoxicity in the form of MN in spleen cells. However, interestingly, in the mice that received AAILE treatment along with B(a)P instillations, the levels of micronuclei were found to be much significantly reduced when compared to the animals that received only B(a)P instillations. Moreover, a slightly significant decrease in the extent of MN induction was observed in the mice that received only AAILE treatments. All these observations suggest the preventive effects of *A. indica* against B(a)P induced micronuclei and hence genotoxic damage in spleen cells. These observations are supported by some other recent reports, wherein *A. indica* was reported to exert anti-clastogenic effects against different chemical carcinogens like MNNG, DMBA and DEN (29, 44–46)

Free radicals and other ROS are essential for life, because they are involved in cell signaling and are used by phagocytes for their bactericidal action. In addition to these well-controlled and necessary functions, ROS are also produced in all aerobic organisms as a consequence of mitochondrial respiration, which consumes oxygen in the process of generating ATP, by the coupling of electron transport and oxidative stress. Oxidative stress is potentially harmful to cells, and thus ROS are implicated in the etiology and progression of several pathologies, including cancer (8–10). Oxidative stress results when the balance between the production of ROS overrides the non-enzymatic and/or enzymatic antioxidant defense capability of the target cell (47, 48). Endogenous oxidative stress can be the result of cellular metabolism and oxidative phosphorylation. Exogenous sources of ROS include drugs, hormones and xenobiotics such as chemical carcinogens. ROS thus generated may interact with and modify cellular proteins, lipids. as well as DNA. which results in altered cell functions. Accumulation of oxidative damage has been implicated in almost all the stages of the process of carcinogenesis (8, 49). B(a)P is a potential inducer of ROS, e.g., mitochondrial superoxide anion and hydrogen peroxide (50). ROS formed during B(a)P metabolism can diffuse from the site of generation to other targets within the cells or even propagate the injury to other intact cells. These ROS produce deleterious effects by initiating LPO directly or by acting as second messengers for the primary free radicals that initiate LPO (51).

Antioxidant mechanism that scavenges ROS, by means of low-molecular-weight antioxidants or antioxidant enzyme systems, protects organism from the damaging effects of oxidative stress. Under normal conditions, antioxidant defense mechanism is able to detoxify ROS and protect cellular macromolecules and organelles from damaging effects of oxidative stress. The antioxidant defense system has enzymatic as well as non-enzymatic components. The antioxidant defense system includes enzymes like GPx, catalase, GR, GST and SOD, whereas non-enzymatic components include non-protein thiol GSH, uric acid, some trace metals and certain vitamins such as ascorbic acid and α-tocopherol etc. (8). The enzyme GR is responsible for the regeneration of GSH by the reduction of oxidized glutathione in the presence of NADPH, GPx acts in association with tripeptide GSH, which is present in high concentrations in cells and catalyzes the conversion of hydrogen peroxides (H_2O_2) or organic peroxide to water or alcohol, while simultaneously oxidizing GSH. It also competes with catalase for H_2O_2 as a substrate and is the major source of protection against low levels of oxidative stress. The enzyme GPx is responsible for the detoxification of hydrogen peroxide H_2O_2 in low concentrations, whereas catalase comes into play when glutathione peroxidase is saturated with the substrate. Catalase is present in the peroxisome of aerobic cells and is very efficient in promoting the conversion of hydrogen peroxide to water and molecular oxygen. Catalase has one of the highest turnover rates for all enzymes: one molecule of catalase can convert approximately six million molecules of hydrogen peroxide to water and oxygen each minute. SOD is one of the most effective intracellular enzymatic antioxidants and it catalyzes the conversion of superoxide anions to dioxygen and H_2O_2 (52).

In our observations, an increased levels of LPO were found in mice that received B(a)P intragastric instillations. However, the mice that received AAILE along with B(a)P depicted lower levels of LPO when compared to only B(a)P receiving mice. Moreover, significant decrease in the levels of LPO was observed in only AAILE receiving mice. In the mice that received only B(a)P instillations, the activities of antioxidant defense enzymes decreased in liver tissue, whereas in mice that received AAILE along with B(a)P, enhanced activities of antioxidant defense enzymes were found. In mice receiving only AAILE, the activities of antioxidant defense enzymes studied were observed to increase in liver tissues when compared with the control counterparts. Collectively, these observa-

tions suggest that B(a)P cause oxidative stress by lowering the antioxidant defense whereas *A. indica* prevent and/or reverse these alterations. Our observations are further supported by certain earlier similar reports, wherein different kind of extracts of *A. indica* was documented to exert anti-clastogenic effects through modulation of oxidative stress and antioxidant defense. The modifying effects of AAILE on the *in vivo* clastogenicity of MNNG in metaphase cells from the bone marrow of male Wistar rats was evaluated by Arivazhagan et al. (44). In their observations, pretreatment with AAILE significantly reduced MNNG-induced increase in micronuclei and chromosomal aberrations. Subapriya et al. (50) evaluated the effects of pretreatment with ethanolic *A. indica* leaf extract (EAILE) against MNNG-induced genotoxicity and oxidative stress in male Swiss albino mice. In MNNG-treated mice, enhanced lipid peroxidation with compromised antioxidant defense in the stomach, liver and erythrocytes was accompanied by an increase in bone marrow micronuclei. Pretreatment with EAILE significantly reduced MNNG-induced micronuclei and LPO and enhanced GSH-dependent antioxidant activities. In another study, Subapriya et al. (46) evaluated the effects of pretreatment with EAILE on DMBA-induced genotoxicity and oxidative stress in male Swiss albino mice. In their observations, pretreatment with EAILE was found to significantly reduce DMBA induced micronuclei and LPO and enhanced GSH-dependent antioxidant activities. Recently, we reported AAILE mediated modulation against diethyl nitrosamine induced clastogenicity in hepatocytes. In mechanism, AAILE was suggested to exert its anti-clastogenic effects by modulating carcinogen biotransformation as well as antioxidant defense enzymes (31).

In one of our recent study, we investigated the effects of AAILE on the activities of certain phase I (cytochrome P450, cytochrome b_5 and aryl hydrocarbon hydroxylase) as well as phase II (GST and UDP-glucuronosyl transferase) biotransformation enzymes; and GSH (in forestomach and hepatic tissues) during/after intra-gastric instillations of B(a)P in murine forestomach tumorigenesis bioassay protocol. It was observed that AAILE has a potential to reduce the metabolic activation of xenobiotics/carcinogens by reducing the activity of phase I biotransformation enzymes and to increase the metabolic detoxification of carcinogens by increasing the activity phase II biotransformation enzymes and GSH contents (22, 24). These modulations exerted by *A. indica* on carcinogen biotransformation could also be correlated to the anti-genotoxic potential observed in our current experimental protocol bioassay.

It has been suggested that the chemopreventive agents may be useful in preventing cancer and mutation related diseases, which can be pursued by avoiding exposure to recognized mutagens and/or carcinogens, by fortifying physiological defense mechanisms or by favoring the intake of protective factors (53, 54). Therefore, our observations regarding *A indica* mediated prevention of B(a)P induced clastogenicity could be correlated with the reduction in the extent of tumorigenesis reported earlier (21). The present observations, when taken together with a few earlier reports from peer reviewed literature, strongly suggest (and confirm) the anti-clastogenic activities of *A. indica*.

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