Indian Journal of Biochemistry & Biophysics
Vol. 51, February 2014, pp. 37-45

Azadirachta indica mitigates DMBA-induced hepatotoxicity: A biochemical and radiometric study

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Received 30 May 2013; revised 07 December 2013

The hepatoprotective potential of aqueous *Azadirachta indica* leaf extract (AAILE) was assessed against DMBA-induced hepatotoxicity. DMBA (7,12-dimethylbenz[a] anthracene) treatment (40 mg/kg body weight, ip) to male Balb/c mice resulted in the derailment of liver function as revealed by extremely slow clearance of ^{99m}Tc-mebrofenin from liver, elevated levels of alkaline phosphatase (ALP) and alanine transaminase (ALT), compared to control group. In addition, elevated micronuclei score and high apoptotic index indicated hepatogenotoxicity in DMBA-treated mice. DMBA treatment also upregulated cytochrome P450 (CYP), cytochrome b_5 (Cyt b_5) and decreased glutathione-S-transferase activity in hepatic tissue, compared to control group. Enhanced lipid peroxidation (LPO) levels along with decreased reduced glutathione (GSH) level were also observed in DMBA group, compared to control group. AAILE co-treatment (200 mg/kg body weight, po, thrice a week) for 8 weeks followed by DMBA injection showed significant improvement in hepatic status, as revealed by normalization of ^{99m}Tc-mebrofenin clearance rate, decreased ALP and ALT levels, reduced genotoxicity in terms of micronuclei score and apoptotic index. Levels of LPO were significantly decreased along with increased hepatic GST and GSH levels in AAILE + DMBA group, compared to DMBA group. However, no significant change was observed in hepatic CYP and Cyt b_5 levels, compared to DMBA group. The results indicated that AAILE effectively ameliorated DMBA-induced hepatotoxicity.

Keywords: *Azadirachta indica,* Hepatotoxicity, Carcinogen metabolism, Oxidative stress, 7,12-Dimethylbenz[a] anthracene, Liver function

Polycyclic aromatic hydrocarbons (PAHs) are hazardous chemicals whose major source of prevalence in the environment is through incomplete combustion of coal, oil, leaves, tobacco and other organic materials[1]. 7, 12-Dimethylbenz [a] anthracene (DMBA) is a PAH well-known for its cytotoxic, mutagenic, carcinogenic and immunosuppressive activities[2]. It is a xenobiotic that requires metabolic activation to exert its effect. DMBA is metabolized by phase I and II carcinogen metabolizing enzymes. Reactive oxygen species formed during DMBA metabolism lead to oxidative stress, chromosomal damage and formation of micronuclei[3,4].

DMBA is also known to illicit inflammatory responses by the up-regulation of pro-inflammatory cytokines which initiates a cascade of other cytokines and growth factors[5]. The release of biologically active molecules (e.g., cytokines and reactive oxygen intermediates) from activated Kupffer cells has been implicated in hepatotoxicological and hepatocarcinogenic events[6]. The powerful oxidizing free radicals generated by DMBA are known to play an important role in mutagenesis and initiation of carcinogenesis by damaging cellular macromolecules[7]. Therefore, DMBA has also been widely employed to study clastogenic effects in the bone marrow of experimental animal models[8].

Numerous plant products have been examined against various adverse effects induced by environmental pollutants[9]. There are enough evidences which suggest that phytochemicals present in the plants and plant products are associated with reduced risk of developing chronic diseases[10]. *Azadirachta indica* is one of the medicinal plants known for its enormous beneficial health effects. The leaf of the plant has been demonstrated to exhibit

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Abbreviations: AAILE, aqueous *Azadirachta indica* leaf extract; ALP, alkaline phosphatase; ALT, alanine transaminase; Cyt b_5, cytochrome b_5; CYP, cytochrome P450; DMBA, 7, 12-dimethylbenz[a] anthracene; GSH, reduced glutathione; GST, glutathione-S-transferase; LPO, lipid peroxidation; PAHs, polycyclic aromatic hydrocarbons.

hepatoprotective, immunomodulatory, anti-inflammatory, anti-oxidant, anti-mutagenic and anti-carcinogenic properties[11]. The plant is considered as a store house of natural compounds that can be exploited in the development of drugs against infectious diseases and systemic disorders[12-14]. The hepatoprotective effects of *A. indica* leaf extract (AAILE) in skin tumour bearing mie[15] and N-nitrosodiethylamine-induced hepatotoxicity have been earlier reported by our laboratory[16,17]. The leaf extract has also been found to offer protection against hepatotoxity induced by various agents. such as $CC1_4$, paracetamol and anti-tubercular drugs[18-20]. In the present study, we have investigated the hepatoprotective potential of AAILE against DMBA-induced hepatotoxicity in mice.

Materials and Methods

Chemicals and reagent kits

7,12-Dimethylbenz[a]anthracene (DMBA), 5,5-dithiobis-2-nitrobenzoic acid (DTNB), reduced glutathione (GSH), oxidized glutathione (GSSG), bovine serum albumin (BSA), thiobarbituric acid (TBA), 1-chloro-2,4-dinitrobenzene (CDNB), reduced nicotinamide adenine dinucleotide (NADH) and reduced nicotinamide adenine dinucleotide phosphate (NADPH) were obtained from Sigma (St. Louis, MO, USA). Other chemicals used in the study were obtained from reputed Indian firms. Kits for terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL) assay and liver injury marker enzymes were procured from M/S Trevigen Inc. (Gaithersburg, MD) and RECKON Diagnostic (India), respectively.

Extract preparation and animal treatment

AAILE was prepared according to the method described earlier[21]. Each time fresh solution of AAILE was prepared by dissolving the solid aqueous extract in distilled water to obtain the desired dose.

Male Balb/c mice (25-30 g) were procured from the Central Animal House, Panjab University, Chandigarh. Mice were kept in well-aerated room and the temperature was maintained at 21 ± 2°C. The animals in all groups had free access to standard animal pellet diet and clean drinking water throughout the experiment. The animal treatment protocols were approved by Panjab University Ethical Committee in accordance with guidelines of Indian National Science Academy for the use and care of experimental animals. After acclimatization to the experimental conditions for one week, mice were randomly divided into four groups (n = 5-6). Group I mice served as control while group II mice were administered AAILE at a dose of 200 mg/kg body mass, thrice a week on alternate days for 8 weeks; and group III mice received an intraperitoneal injection of DMBA (40 mg/kg body mass). The animals were sacrificed after 48 h of intraperitoneal injection of DMBA by gaseous anesthesia. Group IV mice received AAILE and DMBA as described for Groups II and III.

Liver function test

^{99m}Tc-labelled mebrofenin hepato biliary test

^{99m}Tc-sodium pertechnetate (185-200) MBq in 3 mL normal saline was mixed with mebrofenin according to the manufacturer's instructions (BRIT, India). The scintillation machine was calibrated using ^{99m}Tc as a source (10% window centered at 140 Kev). Animal was placed on the detector in prone position after intravenous injection of 200 μCi ^{99m}Tc-mebrofenin. The blood pool and hepatic radioactivity were monitored as a function of time. T_{peak}, $T_{1/2peak}$ and the percentage of hepatic retention were calculated in order to detect liver function. T_{peak} was defined as the time at which ^{99m}Tc-mebrofenin radioactivity reached its maximum activity. $T_{1/2peak}$ represented time when the percentage of counts was half the maximum counts (T_{peak} count). The percentage of hepatic retention was calculated as percentage of ^{99m}Tc-mebrofenin remained in liver with respect to maximum activity at any point of time.

Liver injury marker enzymes

Alkaline phosphatase (ALP) and alanine transaminase (ALT)

ALP and ALT activities were estimated according to the manufacturer's protocol ENZOPAK reagent kit (Reckon Diagnostic, India). Briefly, a tablet of ALP was crushed and dissolved in ALP buffer to make buffered substrate. Solution was kept at room temperature for 15 min before use. Solution and sample were mixed in a cuvette and change in absorbance as a function of time was measured at 405 nm. For ALT activity, SGPT powder was transferred and mixed in a bottle containing SGPT buffer to obtain working reagent. Working reagent was mixed with sample and incubated at 37°C for 60 s. The absorbance was

measured at 340 nm as a function of time. Both the activities were expressed as International Units (IU) per litre.

Sample preparation for biochemical analysis

After 48 h of DMBA administration, 25% liver tissue homogenate (w/v) was prepared in 0.1 M potassium phosphate buffer (*p*H 7.4). Aliquots of tissue homogenate were kept at 4°C for the estimation of reduced glutathione (GSH) and lipid peroxidation (LPO) levels. The remaining homogenate was subjected to cold centrifugation (4°C) at 10,000 x *g* for 30 min. The supernatant thus obtained (post-mitochondrial fraction) was used for the estimation of GST activity. Another part of the supernatant was subjected to ultra-centrifugation at 70,000 x *g* for 90 min. The pellet (microsomal fraction) obtained was re-suspended in phosphate buffer for the estimation of cytochrome P450 (CYP) and cytochrome b_5 (Cyt b_5).

Lipid peroxidation (LPO)

The assay for LPO was performed according to the method of Trush *et al.*[22]. Briefly, tissue homogenate was incubated in Tris-HCl buffer (*p*H 7.4) containing 0.3 µmoles NADPH in a metabolic shaker at 37ºC for 60 min. The reaction was stopped by the addition of cold TCA-HCl mixture and centrifuged at 1,500 x *g* for 10 min. Thiobarbituric acid (TBA) was then added to the supernatant and the reaction mixture was boiled at 100°C for 15 min. The absorbance of pink colored MDA-TBA chromophore complex so formed was read at 532 nm. The results were expressed as nanomole MDA-TBA chromophore formed mg^{-1} protein min^{-1} using extinction coefficient of 1.56×10^5 M^{-1} cm^{-1}.

Reduced glutathione (GSH)

GSH was estimated as the total non-protein sulphydryl groups by the method of Moron *et al.*[23]. Liver tissue homogenates were immediately precipitated in cold TCA and centrifuged at 1,500 x *g* for 10 min. The supernatant was diluted in cuvette and DTNB was then added. The absorbance of yellow color formed in the reaction was read at 412 nm on a double-beam spectrophotometer. A standard curve for GSH was plotted and the GSH content was obtained in terms of nanomole of GSH/mg protein.

Cytochrome P450 (CYP)

CYP was determined using carbon monoxide difference spectra, according to the method of Omura and Sato[24]. The concentration of CYP was calculated using molar extinction coefficient 91 mM^{-1} cm^{-1} and expressed as nanomole/mg protein.

Cytochrome b_5 (Cyt b_5)

Cyt b_5 was determined using difference spectra of NADH reduced samples *vs* air-saturated samples according to the method of Omura and Sato[25]. The levels of Cyt b_5 were calculated using molar extinction coefficient 185 mM^{-1} $cm^{-1}$and expressed as nanomole/mg protein.

Glutathione-S-transferase (GST)

Hepatic GST activity was determined according to the method of Habig *et al.*[26]. The reaction mixture was prepared by mixing CDNB and GSH in phosphate buffer (0.1 M, *p*H 6.5). The reaction was started by the addition of post-mitochondrial fraction to the reaction mixture and the increase in absorbance was noted for 3 min at 340 nm. The specific activity of GST was expressed as micromoles of GSH-CDNB conjugates formed min^{-1} mg^{-1} protein using extinction coefficient of 9.6 $mM^{-1}cm^{-1}$.

Micronucleus assay

Liver tissue was homogenized at 100 rpm for 2 min. A drop of tissue homogenate was applied at one end of the slide to make a smear. The slides were dried and stained with May-Grunwald stain for 1 min, followed by staining with Giemsa for 10 min as described by the method of Schmid[27]. The slides were washed twice in distilled water, dried, rinsed in methanol, cleared in xylene and then mounted in DPX. Cells were counted for the presence of micronuclei under light microscope at a magnification of 400X. The frequency of micronuclei induction was expressed as percentage of micronucleated cells to binucleated cells.

TUNEL assay

TUNEL assay was performed on histological slides of liver tissue, according to manufacturer's protocol (TACS-XL DAB, Trevigen, Gaithersburg, Maryland) for *in situ* detection of DNA nicks on formalin-fixed tissues. Briefly, tissue sections were dewaxed, rehydrated in grades of alcohol and permeabilized with 50 µL (20 µg/mL) of proteinase K. The endogenous peroxidase activity was inhibited by treatment with 30% hydrogen peroxide. The labeling of DNA nicks was performed by incubating slides with Tdt enzyme and bromodeoxyuridine (BrdU) for

1 h at 37°C. Biotinylated antibody against BrdU was used for detection of DNA nicks and incubation of this complex with streptavidin conjugated HRP and DAB resulted in the formation of brown colored complex which was visualized under light microscope at 400X. Apoptotic index was calculated as the number of apoptotic cells per 100 normal cells.

Protein estimation

The protein content of the samples was determined by the method of Lowry *et al*[28]. using bovine serum albumin as standard.

Statistical analysis

The results were expressed as mean ± SD (n = 5-6) in each group. For statistical significance, the data were analyzed using one-way ANOVA, followed by least significant difference (LSD) post-hoc test. $P \leq 0.05$ (i.e. 95% confidence interval) was considered statistically significant.

Results

During the entire experimental period, mice were observed at weekly intervals for changes in body weight, diet and water consumption. Non-significant changes were observed in the diet and water consumption by mice in all the groups studied, as compared to their normal counterparts (data not shown).

In order to assess the effect of various treatments on functional status of liver ^{99m}Tc-labeled mebrofenin hepatic biliary clearance test and liver injury marker levels in serum were assessed. After administration of ^{99m}Tc-mebrofenin to control mice, radioactivity started appearing in heart after 20 to 30 s and the T_{peak} in heart was found to be around 130 s and in case of liver it was around 300 s. ^{99m}Tc mebrofenin uptake in both liver and heart was not affected in any of the treatment groups (Fig. 1a,b). The $T_{1/2}$ in case of liver in DMBA-treated mice was not calculated because of extremely slow ^{99m}Tc-mebrofenin clearance in these animals.

^{99m}Tc-mebrofenin metabolic profile in liver and heart for control and AAILE mice is shown in Fig. 2a-d. In liver, ^{99m}Tc-mebrofenin retention was markedly elevated in DMBA-treated mice and even after 60 min of ^{99m}Tc-mebrofenin administration 96% of the radioactivity remained in the liver. In contrast, 26% of ^{99m}Tc-mebrofenin activity remained in the liver of AAILE + DMBA group, indicating clearance rates similar to normal animals. No significant change

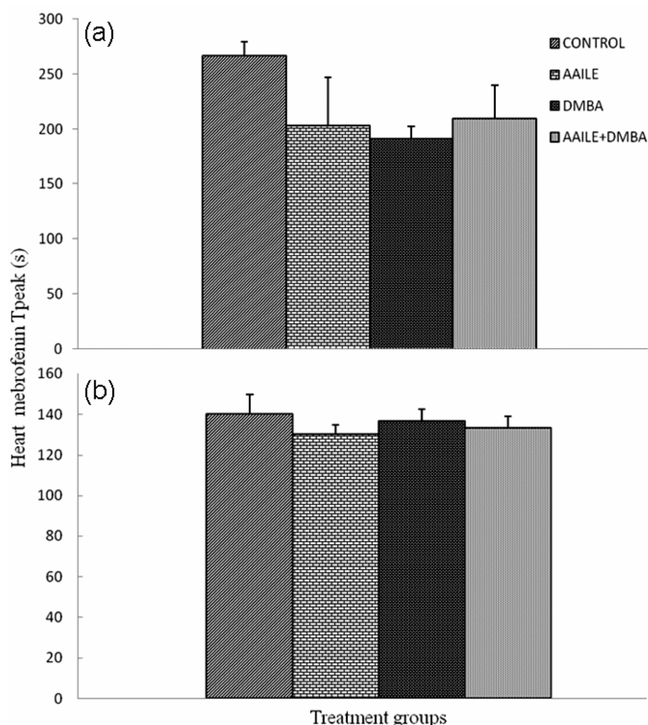

Fig. 1—T_{peak} of ^{99m}Tc-labeled mebrofenin [a, Liver; and b, heart. Values expressed as mean ± SD (n = 5-6). Data were analyzed using one-way ANOVA, followed by post-hoc test]

was observed in ^{99m}Tc-mebrofenin metabolic profile in case of heart in DMBA and AAILE + DMBA groups compared to control group (Fig. 2e-h).

In control mice, at 10, 20, 30 and 60 min after intravenous ^{99m}Tc-mebrofenin administration, 60%, 30%, 28% and 26%, respectively of the peak ^{99m}Tc-mebrofenin activity remained in the liver. The pattern of ^{99m}Tc-mebrofenin clearance in the liver in case of AAILE-treated group was similar to the control group (Fig. 3).

The activity of serum liver injury marker enzymes was also increased after 48 h of DMBA exposure. A significant increase in ALT and ALP activities was observed in DMBA group (p≤0.001), compared to control group. AAILE + DMBA group showed a significant (p≤0.001) reduction in ALT and ALP, compared to DMBA group (Table 1). Non-significant changes were observed in these markers in AAILE group, compared to control group.

The levels of CYP and cytochrome b_5 were observed to increase significantly (p≤0.05) in liver tissue of DMBA group, compared to control group. However, no significant change was noted in the levels of these enzymes in AAILE + DMBA and AAILE group, compared to other groups (Table 2).

Fig. 2—Liver and heart time-activity curves derived for ^{99m}Tc-labeled mebrofenin hepatic biliary clearance test [(a, b) Control; (c, d) AAILE; (e, f) DMBA; (g, h) AAILE + DMBA. Values expressed as mean ± SD (n = 5-6). Data were analyzed using one-way ANOVA, followed by post-hoc test]

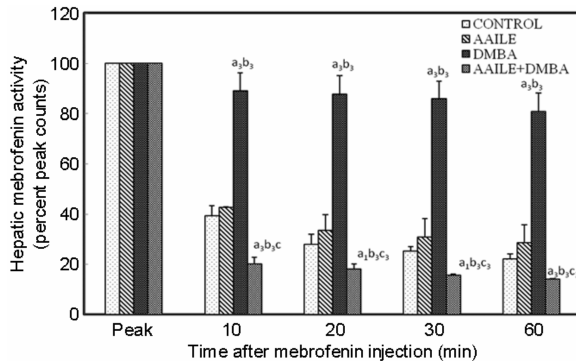

Fig. 3—^{99m}Tc-labeled mebrofenin retention in liver of various treatment groups [Values expressed as mean ± SD (n = 5-6). Data were analyzed using one-way ANOVA, followed by post-hoc test. Statistical significance: $^{a}{}_{1}p\leq0.05$, $^{a}{}_{3}p\leq0.001$ significant with respect to control; $^{b}{}_{1}p\leq0.05$, $^{b}{}_{3}p\leq0.001$ significant with respect to AAILE; $^{c}{}_{3}p\leq0.001$ significant with respect to DMBA]

Table 1—Effect of DMBA and/or AAILE on liver injury marker enzymes in various treatment groups

[Values expressed as mean ± SD (n = 5-6)]

Parameters	Control	AAILE	DMBA	AAILE + DMBA
ALP	28.9 ± 1.09	29.3 ± 2.85	54.2 ± 1.40$^{a}{}_{3}$	29.7 ± 3.09$^{c}{}_{3}$
ALT	36.5 ± 5.66	39.2 ± 6.23	54.2 ± 2.10$^{a}{}_{3}$	23.8 ± 6.34$^{a}{}_{1}{}^{b}{}_{1}{}^{c}{}_{3}$

Data were analyzed using one-way ANOVA, followed by post-hoc test.

$^{a}{}_{1}p\leq0.05$, $^{a}{}_{3}p\leq0.001$ significant with respect to control group; $^{b}{}_{1}p\leq0.05$ significant with respect to AAILE group; $^{c}{}_{3}p\leq0.001$ significant with respect to DMBA group

Units: IU/L

Table 2—Effect of DMBA and/or AAILE on xenobiotic bio-transformation enzymes and antioxidant defense system in various treatment groups

[Values expressed as mean ± SD (n = 5-6)]

Parameters	Control	AAILE	DMBA	AAILE + DMBA
CYP	0.08 ± 0.01	0.09 ± 0.01	$0.14 \pm 0.05^{a}{}_{1}$	0.11 ± 0.03
Cyt b_5	0.014 ± 0.005	0.015 ± 0.002	$0.021 \pm 0.002^{a}{}_{1}$	0.017 ± 0.003
GST	0.68 ± 0.02	$1.35 \pm 0.21^{a}{}_{3}$	$0.35 \pm 0.04^{a}{}_{3}$	$0.39 \pm 0.01^{a}{}_{3}{}^{b}{}_{3}{}^{c}{}_{1}$
LPO	6.16 ± 1.15	$1.84 \pm 0.40^{a}{}_{3}$	$8.24 \pm 0.90^{a}{}_{3}$	$3.51 \pm 0.51^{a}{}_{1}{}^{b}{}_{3}{}^{c}{}_{3}$
GSH	2.03 ± 0.40	$2.56 \pm 0.33^{a}{}_{1}$	$1.07 \pm 0.25^{a}{}_{3}$	$1.71 \pm 0.23\ {}^{b}{}_{3}{}^{c}{}_{1}$

Data was analyzed using one-way ANOVA, followed by post-hoc test.
${}^{a}{}_{1}p \leq 0.05$, ${}^{a}{}_{3}p \leq 0.001$ significant with respect to control group; ${}^{b}{}_{3}p \leq 0.001$ significant with respect to AAILE group;
${}^{c}{}_{1}p \leq 0.05$,${}^{c}{}_{3}p \leq 0.001$significant with respect to DMBA group
Units: LPO: nanomole MDA-TBA chromophore formed/mg protein; GSH: nanomole of GSH/mg protein; CYP: nanomole/mg protein; Cyt b_5: nanomole/mg protein; GST: micromole of GSH-CDNB conjugates formed/min/mg protein

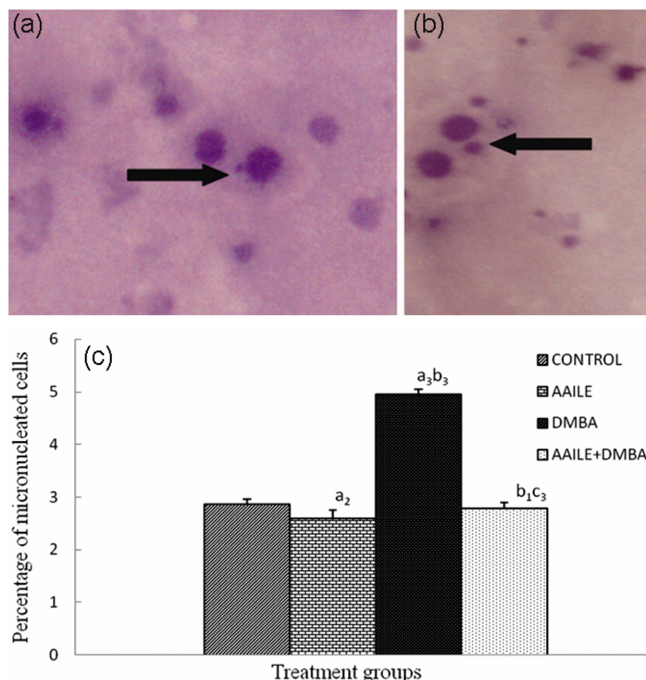

Fig. 4—(a, b): Photomicrograph of dividing cells having micronucleus (arrow); and (c): Effect of DMBA and AAILE on the extent of micronuclei formation in hepatocytes. [Magnification: 400X. Values expressed as mean ± SD (n = 5-6). Data were analyzed using one-way ANOVA, followed by post-hoc test. Statistical significance: ${}^{a}{}_{2}p \leq 0.01$, ${}^{a}{}_{3}p \leq 0.001$ significant with respect to control; ${}^{b}{}_{1}p \leq 0.05$ significant with respect to AAILE; ${}^{c}{}_{3}p \leq 0.001$ significant with respect to DMBA]

Hepatic GST activity was significantly ($p \leq 0.001$) decreased in DMBA group, compared to control group. A significantly increased activity was observed in AAILE + DMBA group with respect to DMBA group. However, a significant ($p \leq 0.001$) decrease was observed in AAILE + DMBA group, compared to control and AAILE groups. AAILE group mice had significantly ($p \leq 0.001$) increased GST activity, compared to control group (Table 2).

The GSH content decreased significantly ($p \leq 0.001$) in DMBA group, compared to control group. A significant ($p \leq 0.001$) increase in GSH levels was observed in AAILE + DMBA group, compared to DMBA group. However, the levels were significantly ($p \leq 0.001$) decreased compared to AAILE group. AAILE group had significantly ($p \leq 0.05$) increased levels of GSH, compared to control counterparts (Table 2).

The extent of LPO, as measured by MDA-TBA levels was significantly ($p \leq 0.001$) increased in DMBA group, compared to control group. However, LPO levels were significantly decreased in AAILE + DMBA group, compared to control ($p \leq 0.05$), DMBA ($p \leq 0.001$) and AAILE ($p \leq 0.001$) groups (Table 2).

A significant ($p \leq 0.001$) increase in the micronuclei score was observed in DMBA group, compared to control group (Fig. 4a,b). However, AAILE co-treatment in AAILE + DMBA group significantly ($p \leq 0.001$) decreased the percentage of micronucleated cells, when compared to DMBA (Fig. 4c).

The DMBA-treated hepatic tissue of mice showed the presence of brown colored apoptotic cells (Fig. 5a-d). A significant ($p \leq 0.001$) increase in the percentage of apoptotic cells was observed in DMBA-treated group, compared to control group. AAILE co-treatment significantly ($p \leq 0.001$) decreased percentage of apoptotic cells in AAILE + DMBA group, compared to DMBA group (Fig. 5e).

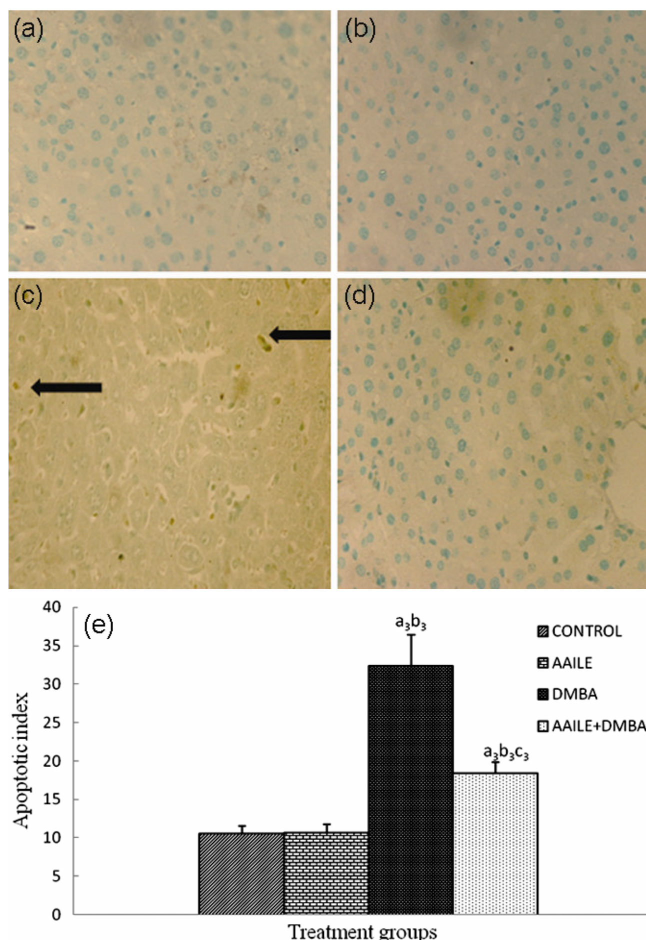

Fig. 5—(a): Photomicrograph of TUNEL stained liver samples of control group showing light blue stained nuclei of normal hepatocytes; (b): AAILE; (c): Brown colored nuclei in DMBA group represent apoptotic cells (arrows); (d): AAILE + DMBA; and (e): Effect of DMBA and/or AAILE on apoptotic index Values expressed as mean ± SD (n = 5-6). Data were analyzed using one-way ANOVA followed by post-hoc test]. Statistical significance: $^{a}{}_{3}p \leq 0.001$ significant with respect to control; $^{b}{}_{3}p \leq 0.001$ significant with respect to AAILE $^{c}{}_{3}p \leq 0.001$ significant with respect to DMBA]

Discussion

The susceptibility of liver to damage by chemical agents is a consequence of its primary role in metabolism of foreign substances. There are numerous classes of chemical toxicants prevalent in the environment and one of the most studied among them is DMBA which belongs to PAH's[29,30]. DMBA is biologically inert molecule and needs bioactivation to form the ultimate cytotoxic metabolite DMBA-3,4-diol-1,2-epoxide[31]. Acute liver injury and inflammatory processes lead to an alteration in the overall physiological status of the liver which could be detected with a variety of liver injury markers or functional tests.

The ^{99m}Tc-mebrofenin clearance test is a routine nuclear medicine test which employs radioactive ^{99m}Tc-labeled mebrofenin to assess the functional status of liver in terms of uptake retention and clearance from the liver. ^{99m}Tc-mebrofenin when injected intravenously, finds its route to liver, where it is metabolized by hepatocytes and excreted in the bile. Any abnormality in its uptake, clearance or retention indicates functional impairment of liver. A correlation has been established between the hepatic extraction efficiency of ^{99m}Tc-mebrofenin and severity of the disease[32].

In the present study, the T_{peak}, $T_{1/2peak}$ and mebrofenin retention calculated at various time intervals provided useful information to assess the liver function. The depletion of ^{99m}Tc-mebrofeninfrom cardiac blood pool was similar in all the groups, indicating that hepatic ^{99m}Tc-mebrofenin extraction was not affected by the exposure of DMBA. T_{peak} analysis of liver from all the groups confirmed rapid extraction of mebrofenin from the blood pool, indicating unaltered liver uptake. The fractional excretion of ^{99m}Tc-mebrofenin in control mice, as judged by the $T_{1/2peak}$ measurement of ^{99m}Tc-mebrofenin excretion, as well as ^{99m}Tc-mebrofenin retention at various times up to 60 min was in agreement with observations by other research groups[33]. ^{99m}Tc-mebrofenin excretion was markedly impaired in animals with DMBA-induced hepatotoxicity. ^{99m}Tc-mebrofenin retention remained elevated even after 60 min, indicating profound perturbations in the regulation of ^{99m}Tc-mebrofenin transport in the bile.

It is reported earlier that in CCl_4-induced liver toxicity, the mebrofenin retention is greatly delayed due to cytokine-mediated inflammation in the liver[34]. The hepatoprotective role of AAILE might be inferred from the fact that ^{99m}Tc-mebrofenin retention and clearance of AAILE + DMBA mice were similar to the normal animals. However, to assess the severity of liver damage, no single test can provide information about global liver functioning. Therefore, along with ^{99m}Tc-mebrofenin clearance, we measured the activities of ALT and ALP. Serum activity of ALT is commonly used biochemical marker of hepatocellular necrosis[35]. The enzyme is located in periportal hepatocytes, reflecting its role in oxidative phosphorylation and gluconeogenesis. ALT is highly specific for liver injury and increased serum activity of enzyme is considered to be the result of cellular

damage and leakage[36]. ALP is related to the catalysis of the hydrolysis of phosphate esters, generating inorganic phosphate. The enzyme is predominantly present in liver, kidney, bones and leucocytes. Serum ALP levels increase to some extent in most types of liver injury[37]. The increased levels of liver injury marker enzymes and impairment of ^{99m}Tc-mebrofenin clearance in DMBA-treated mice indicated liver damage and toxicity. The DMBA-induced alterations in liver function in the present study were effectively modulated by AAILE, as evidenced by normalization of mebrofenin liver metabolism and liver injury marker enzymes.

DMBA metabolism is carried out in two major phases, catalyzed by variety of enzymes. These enzyme systems facilitate the excretion of xenobiotic by increasing its water solubility. The phase I enzymes, of which CYP and Cyt b_5 form an integral part convert DMBA from inactive to biologically active form[38]. The reactive metabolite (epoxides) formed during this reaction may interact with nucleophilic sites in DNA or other macromolecules of the cell. Phase II reactions, on the other hand, may convert active metabolites formed during phase I reactions to less active form[39]. GST is an important phase II enzyme which conjugates reactive metabolites to GSH, resulting in the decrease of its biological reactivity and increases its solubility for excretion in bile. The decrease in the activity of CYP, Cyt b_5 and an increase in the levels of GST (phase II) and GSH in AAILE + DMBA group indicated that AAILE treatment possibly modulated the overall metabolism of DMBA in such a way that lesser amount of reactive metabolite would form which would be eliminated quickly from the cells.

The reactive metabolite formed during the DMBA metabolism leads to oxidative stress which may exert genotoxic effects and cause tissue damage. LPO levels are considered to be important indicator of tissue damage[40]. LPO leads to the production of broad range of reactive species, which are far more damaging than free radicals[41]. It alters membrane integrity, thus impairing major metabolic functions, which are dependent on membrane structure and integrity. Lipid peroxides have also been reported to stimulate cytotoxicity and cell death[42].

The enhanced levels of LPO observed in DMBA group indicated the deleterious effects of DMBA exposure. AAILE co-treated animals showed marked decline in the LPO levels and an increase in GSH content, compared to DMBA and control group, suggesting preventive effects of AAILE against DMBA-induced LPO. GSH is a cellular antioxidant that scavenges ROS generated in biological system and decreases levels of LPO[43]. AAILE alone treatment enhanced levels of GSH compared to control group and thus might contribute to lower levels of LPO in this group. These results were consistent with earlier findings, wherein decrease in LPO levels compared to control group has been reported upon AAILE treatment in various tissues[40, 44,45].

Oxidative stress in many instances can also lead to the induction of apoptosis in different cellular systems, including liver[46]. The increased percentage of apoptotic cells in DMBA-treated group, as assessed by TUNEL assay which detects apoptosis via labeling DNA fragments and strand breaks at 3'-OH free terminal indicated an increase in DMBA-mediated oxidative stress. The decreased percentage of apoptotic cell in DMBA group after AAILE co-treatment indicated the possible modulatory effect of AAILE on oxidative stress and cell death.

Micronucleus induction is considered an effective tool for the study of genomic abnormalities induced by genotoxic exposure[47]. Evaluation of micronucleus frequency *in vivo* is the primary test for genotoxicity and is recommended by various regulatory agencies around the globe to be conducted as part of product safety assessment[48]. The increase in micronucleus frequency observed after DMBA exposure to animals in the present study indicated the genotoxic effects of DMBA and a decrease in its frequency upon AAILE pre-protection might demonstrate the modulatory efficacy of AAILE in DMBA-induced toxicity.

In conclusion, the AAILE pre-treatment to DMBA challenged mice resulted in amelioration of deleterious effects of DMBA on liver tissue, as evidenced by functional studies, serum tissue injury markers and clastogenic assay. Thus, consumption of AAILE may prove effective in mitigating deleterious effects of environmental toxicants.

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