Effect of *Butea monosperma* leaf extracts on cyclophosphamide induced clastogenicity and oxidative stress in mice

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ABSTRACT

Background: *Butea monosperma* is a medium sized deciduous tree of family Fabaceae. It is widely used by rural people in India to cure many disorders. It possesses antioxidant and anticancer activity which is a prerequisite for anticlastogenic activity. **Objective:** To evaluate the effect of *Butea monosperma* leaf extracts on cyclophosphamide induced clastogenicity and oxidative stress in mice. **Materials and Methods:** The present study assessed the role of aqueous and ethanolic leaf extracts of *B. monosperma* (AQEBM and ETEBM) on cyclophosphamide (CP) induced oxidative stress and DNA damage in mice using micronucleus assay for anticlastogenic activity and biochemical estimation of malondialdehyde (MDA) and glutathione (GSH) for antioxidant activity. The frequency of the micronucleated erythrocytes and mitotic index was studied in peripheral blood and bone marrow after 24 and 48 h of clastogenic exposure. **Results:** CP treatment led to a significant ($P < 0.001$) increase in the frequency of micronuclei and decrease in the mitotic index (MI) in bone marrow and peripheral blood cells. Moreover, CP also significantly increased the lipid peroxidation as evidenced by an increase in the MDA content and decreased the antioxidant enzyme (GSH) in mice liver. Pretreatment with AQEBM and ETEBM reduced the frequency of micronuclei and increased the MI in the bone marrow and peripheral blood cells and also restored the MDA and GSH levels in mice liver. **Conclusion:** The AQEBM and ETEBM do contain compounds capable of inhibiting the CP induced oxidative stress and subsequent DNA damage in both the peripheral blood and bone marrow cells in mice.

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INTRODUCTION

Clastogens cause structural chromosomal damage-chromosomal aberration- which may lead to mutation or cancer. Many chemicals, environmental pollutants and dietary substances act as clastogens, e.g. aflatoxin B1, ochratoxin A, pyrrolizidine alkaloids, heterocyclic amines, polycyclic aromatic hydrocarbons (benzo[a] pyrene), etc., Moreover, topoisomerase II enzyme inhibitors (e.g. genistein), DNA repair inhibitors (e.g. caffeine), and mitotic spindle formation inhibitors (e.g. acrylamide) also cause clastogenesis.[1]

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If the damaged DNA is not repaired, it may cause mutations whose cellular expression may lead to diseases.[2] Many pathological conditions, accompanied by oxidative stress, are associated with clastogenic factors, e.g. chronic inflammatory diseases, AIDS, ischemic reperfusion injury, hereditary chromosomal instability syndromes, etc.[3] Hence, there is need of identifying and evaluating therapeutically safe anticlastogens and increasing their use.

Butea monosperma is a medium sized deciduous tree which belongs to family Fabaceae. It is a commonly used Ayurvedic plant with important medicinal values and is widely used by tribal and rural people in different parts of India to cure many disorders. The presence of triterpenes, flavonoids, butein, butin, stigmasterol, ß-carotene, ß-sitosterol, myristic, palmitic, stearic, oleic and linolenic acids, glucoside,[4] etc., in the plant provide

it a vast pharmacological potential. It has been reported to have anticonvulsive, antidiabetic,[5] anti-inflammatory, hepatoprotective, antihelmintic, antioxidant, antistress, antimicrobial activities, etc.[6] Moreover, the leaves of *B. monosperma* possess antioxidant and anticancer activity which is a prerequisite for anticlastogenic activity.

Therefore, the present study was designed to investigate the effects of aqueous and ethanolic extracts of *B. monosperma* leaves against cyclophosphamide (CP) induced oxidative stress and clastogenesis in mice bone marrow and peripheral blood.

MATERIALS AND METHODS

Plant material and extraction

Leaves of *Butea monosperma* were purchased from the local market, authenticated at Guru Nanak Dev University (GNDU), Amritsar, India, and were used for the present study. Shade-dried leaves were crushed and subjected to soxhlet extraction using water and pure ethanol as solvents separately.[7]

Drugs and chemicals

Ascorbic acid and DPPH were procured from Loba Chemie Pvt. Ltd, Mumbai, India and Himedia Laboratories, Mumbai, India respectively. Bovine serum albumin and cyclophosphamide injection IP (brand name Cysmide) were procured from Molychem, Mumbai, India and Samarth Life Sciences Pvt. Ltd., HP, India, respectively.

Experimental animals

Healthy Swiss albino male mice (weighing 20-30 gm) were procured from NIPER, Mohali, India. The animals were fed regularly with diet and water *ad libitum*. The protocol was approved by Institutional Animals Ethics Committee (ATRC/08/2013).

Preliminary phytochemical screening

The AQEBM and ETEBM were screened phytochemically as per the standard methods to investigate the presence of different constituents.[7]

In vitro antioxidant assay

Nitric oxide scavenging activity

The NO scavenging potential of the extracts was determined using method of Naskar *et al.*[8] Briefly, 1 ml of plant extract in varying concentrations was mixed with 1 ml of sodium nitroprusside solution. After incubation, the mixture was mixed with Griess reagent and absorbance of the sample (A_s) was measured. Ascorbic acid was used as standard. The percent NO scavenging activity was calculated as follows:

$$\% \text{ NO scavenging activity} = [(A_c - A_s)/A_c] \times 100$$

where A_c is the absorbance of the control and A_s is the absorbance of test/standard.

DPPH free radical scavenging assay

The DPPH free radical scavenging potential of the extracts was determined using method of Mon *et al.*[9,10] Different concentrations of plant extract were mixed with 0.1 mM DPPH solution and the mixture was kept at room temperature in the dark for 30 min. The decrease in absorbance was measured at 518 nm using spectrophotometer.

The % radical scavenging activity was calculated by equation:

$$\text{DPPH radical scavenging activity } (\%) = [(A_c - A_s)/A_c] \times 100$$

where A_c is the absorbance of control, A_s is the absorbance of extract/ascorbic acid.

In vivo anticlastogenic activity

Experimental design

Six male mice were randomly selected for individual group except for normal group (Group I) in which three mice were used. The group treated with clastogen alone (Group II) was considered as positive control group. Other groups (Group III to VI) were treated with respective extracted drug suspension for 7 consecutive days and on 7th day clastogen was administered after 1 hr of the last dose of extracted drug. The animals were killed by cervical dislocation after 24 and 48 h of clastogen treatment.[11]

Peripheral blood micronucleus test

Peripheral blood was collected from tail vein within 30 s of cervical dislocation and was diluted using bovine serum albumin (BSA) suspending medium. Using a small drop of blood, smear was made on clean glass slide, fixed in methanol and stained with May–Grunwald and Giemsa.[12] A minimum of 2000 PCEs per animal were scored for the incidence of micronucleated polychromatic erythrocytes (MNPCEs) and also the ratio of PCEs to the total erythrocyte (PCEs + NCEs) was estimated by counting a total of at least 1000 erythrocytes.[11-13]

Bone marrow micronucleus test

From the freshly sacrificed animals, both femora were excised and were freed from muscle. The proximal end of the femur was shortened carefully until a small opening to the marrow canal became visible. Using a needle, filled with BSA, marrow sample was aspirated and centrifuged at 1000 rpm for 5 min and the supernatant was removed. A small drop of the suspension was spread on a glass slide, fixed in methanol and stained with May–Grunwald and Giemsa.[12] A minimum of 2000 PCEs per animal were

scored for the incidence of MNPCEs and also the ratio of PCEs to the total erythrocyte was estimated by counting a total of at least 200 erythrocytes.[12,13]

Biochemical estimation

MDA

The MDA content was determined in terms of thiobarbituric acid reactive substances formation.[14] The extent of lipid peroxidation was calculated from the standard curve using 1,1,3,3-tetramethoxy propane and expressed as nM MDA/g of tissue.

GSH

The determination of reduced glutathione level in liver tissue was carried out using method of Ellmann[15] with certain modifications. Briefly, the tissue homogenate was centrifuged and the obtained supernatant was mixed with 10 mM Ellman's reagent. After 20 min, absorbance of mixture was measured at 412 nm. The reduced GSH content was calculated from the standard curve using reduced glutathione and expressed as nM GSH/g of tissue.

Statistical analysis

The experimental results have been expressed as Mean ± SD *(in vitro)* and Mean ± SEM (*in vivo*). The data were analyzed by One-way analysis of variance ($P < 0.05$) followed by Tukey's Multiple Comparison Test.

RESULTS

Preliminary phytochemical screening

The phytochemical screening of extracts revealed the presence of bioactive constituents like alkaloid, carbohydrate, phytosterols, flavonoids, proteins and diterpenes.

In vitro antioxidant assay

Nitric oxide scavenging activity

The plant extracts showed a good nitric oxide scavenging activity between 50 and 800 µg/ml dose dependently [Table 1]. Among the extracts, AQEBM had shown better scavenging power than ETEBM, however, the effect of ascorbic acid was much less when compared to the extracts.

DPPH free radical scavenging assay

AQEBM and ETEBM demonstrated a dose dependent antioxidant activity in the DPPH radical scavenging assay [Table 1]. The standard ascorbic acid possess highest DPPH scavenging activity of 99.216% at 80 µg/ml, whereas, the aqueous and ethanolic extracts possess highest DPPH scavenging activity of 77.07% and 68.79% at 80 µg/ml, respectively.

In vivo anticlastogenic activity

Peripheral blood micronucleus test

A reduced number of cells in mitosis were found in CP treated group at 24 and 48 h. On the other hand, an increase in the number of mitotic cells was observed in the mice treated with AQEBM and ETEBM at 24 h as well as 48 h [Table 2].

The frequencies of MNPCEs induced by CP in peripheral blood of mice were extremely significant ($P < 0.001$)

Table 1: Percent NO scavenging activity and percent DPPH scavenging activity of ASA, AQEBM and ETEBM

Conc. (µg/ml)	Mean±SD		
	ASA	AQEBM	ETEBM
Percent NO scavenging activity			
50	19.278±0.621	62.987±0.367	58.481±0.813
100	30.084±0.770	66.326±0.642	62.538±0.703
200	38.449±0.700	70.113±0.867	66.828±0.763
400	48.914±0.647	74.026±0.971	71.280±0.859
800	62.071±0.770	77.042±0.889	74.098±0.899
Percent DPPH scavenging activity			
5	86.601±0.589	48.312±0.998	38.290±0.839
10	90.087±0.411	58.007±0.980	46.678±0.822
20	92.647±0.589	64.325±0.998	54.031±0.929
40	96.623±0.340	71.405±0.910	62.091±0.589
80	99.216±0.134	77.070±0.680	68.791±0.589

Values were expressed as mean±SD, ASA=Ascorbic acid; AQEBM=Aqueous extract of *B. monosperma* and ETEBM=Ethanolic extract of *B. monosperma;* NO=Nitric oxide

Table 2: Percent mitotic index and percent micronucleated polychromatic erythrocytes in peripheral blood cells of mice at different recovery times

Groups (dose mg/kg)	Mitotic index %		MNPCEs %	
	24 hr	48 hr	24 hr	48 hr
I. Normal	8.17±0.50	8.17±0.50	0.17±0.04	0.17±0.04
II. CP (50)	3.23±0.56***a	3.17±0.39***a	2.01±0.12***a	2.30±0.11***a
III. AQEBM (200)+CP (50	5.70±0.61*a,*b	5.67±0.43**a,**b	1.09±0.28*a,*b	1.23±0.20**a,**b
IV. AQEBM (400)+CP (50)	7.13±0.35***b	7.33±0.43***b	0.57±0.12***b	0.60±0.09***b
V. ETEBM (200)+CP (50)	4.70±0.27**a	4.93±0.33***a	1.55±0.13***a	1.67±0.13***a
VI. ETEBM (400)+CP (50)	6.20±0.49**b	6.16±0.09*a,**b	0.88±0.16**b	0.92±0.18*a,***b

Values were expressed as mean±SEM (n=3), ***P<0.001, **P<0.01, *P<0.05, [a]compared with normal, [b]c7ompared with CP (50), CP=Cyclophosphamide; AQEBM=Aqueous extract of *Butea monosperma*; ETEBM=Ethanolic extract of *Butea monosperma*; MNPCEs=Micronucleated polychromatic erythrocytes

to that of the normal group [Table 2]. In mice, fed with AQEBM (200 mg/kg and 400 mg/kg) and ETEBM (400 mg/kg), % MNPCEs was significantly decreased as compared to CP treated group. But in ETEBM (200 mg/kg) treated mice, the decrease in %MNPCEs was insignificant as compared to CP treated group.

Bone marrow micronucleus test

The MI in bone marrow cells of normal mice was found to be 11%. The mice administered with CP showed an extremely significant ($P < 0.001$) decrease in MI up to 3.5 and 2.83% at 24 hand 48 h respectively as that of the normal mice [Table 3]. In case of AQEBM (200 mg/kg and 400 mg/kg) and ETEBM (400 mg/kg) treated mice, a significant increase in MI was observed as compared to the CP treated mice. However, the increase in mitotic index in ETEBM (200 mg/kg) treated mice was found to be insignificant as compared to the CP treated mice.

The effect of AQEBM and ETEBM on the frequency of MNPCEs in bone marrow cells after 24 h and 48 h of CP treatment has been expressed in Table 3. As compared to the normal mice, an extremely significant ($P < 0.001$) increase in MNPCEs was observed in CP treated mice. The frequency of MNPCEs was significantly lowered in the AQEBM (200 mg/kg), AQEBM (400 mg/kg) and ETEBM (400 mg/kg) treated mice as compared to the CP treated mice. However, in case of ETEBM (200 mg/kg) treated group, the decrease in the frequency of MNPCEs was found to be insignificant when compared to the CP treated mice.

Biochemical estimation

MDA

The lipid peroxidation in CP treated mice was higher as compared to normal mice, as evidenced by a significant ($P < 0.001$) increase in the MDA level in the mice liver. This indicated the induction of oxidative stress in hepatic tissue [Table 4].

However, oral administration of AQEBM (200 and 400 mg/kg) and ETEBM (400 mg/kg) for one week resulted in a significant reduction of MDA content as compared to CP treated group. But administration of ETEBM (200 mg/kg) resulted in non-significant decrease in MDA content as compared to CP treated group.

GSH

The hepatic GSH level in normal mice was found to be 94.47 nM/g of tissue. A single i.p. administration of CP caused an extremely significant ($P < 0.001$) reduction in hepatic GSH content as compared to the normal mice [Table 4]. Pre-treatment with AQEBM (200 mg/kg), AQEBM (400 mg/kg) and ETEBM (400 mg/kg) significantly increased the hepatic GSH content at 24 h and 48 h when compared to CP treated group. On the other hand, ETEBM (200 mg/kg) treated

Table 3: Percent mitotic index and percent micronucleated polychromatic erythrocytes in bone marrow cells of mice at different recovery times

Groups (dose mg/kg)	Mitotic index %		MNPCEs %	
	24 h	48 h	24 h	48 h
I. Normal	11±0.87	11±0.87	0.23±0.06	0.23±0.06
II. CP (50)	3.50±0.50***a	2.83±0.44***a	2.77±0.38***a	3.95±0.31***a
III. AQEBM (200)+CP (50	7.17±0.73*a,*b	6.33±0.84**a,*b	1.56±0.16*a,*b	2.20±0.50**a,*b
IV. AQEBM (400)+CP (50)	8.83±0.88**b	8.64±0.63***b	0.98±0.09**b	1.25±0.19***b
V. ETEBM (200)+CP (50)	5.33±0.44**a	5.00±0.76***a	2.25±0.33***a	2.50±0.35**a
VI. ETEBM (400)+CP (50)	7.67±0.93*b	7.50±0.58*a,**b	1.43±0.09*a,*b	1.73±0.26*a,**b

Values were expressed as mean±SEM (n=3), ***P<0.001, **P<0.01, *P<0.05, [a]compared with normal, [b]compared with CP (50), CP=Cyclophosphamide; AQEBM=Aqueous extract of *B. monosperma*; ETEBM=Ethanolic extract of *B. monosperma* MNPCEs=Micronucleated polychromatic erythrocytes

Table 4: Levels of lipid peroxidation and GSH in livers of mice following treatment with AQEBM, ETEBM and CP

Groups (dose mg/kg)	MDA (nM/g of tissue)		GSH (nM/g of tissue)	
	24 h	48 h	24 h	48 h
I. Normal	3.38±0.46	3.38±0.46	94.47±2.93	94.47±2.93
II. CP (50)	7.64±0.34***a	8.23±0.48***a	54.74±4.96***a	47.40±4.93***a
III. AQEBM (200)+CP (50	5.68±0.06**a,**b	5.56±0.07**a,**b	75.40±0.65**a,**b	76.42±2.12*a,**b
IV. AQEBM (400)+CP (50)	4.78±0.20***b	4.64±0.32***b	88.97±1.55***b	89.03±3.35***b
V. ETEBM (200)+CP (50)	7.15±0.38***a	6.81±0.1***a	63.65±2.42***a	64.09±5.84**a
VI. ETEBM (400)+CP (50)	5.54±0.22**a,**b	5.36±0.5*a,**b	78.28±1.59*a,***b	79.33±1.11***b

Values were expressed as mean±SEM (n=3), ***P<0.001, **P<0.01, *P<0.05, [a]compared with normal, [b]compared with CP (50), CP=Cyclophosphamide; AQEBM=Aqueous extract of *B. monosperma*; ETEBM=Ethanolic extract of *B. monosperma*

mice showed an insignificant increase in hepatic GSH content as compared to CP treated group.

DISCUSSION

The phytochemical screening of extracts revealed the presence of various bioactive phytoconstituents like alkaloid, carbohydrate, phytosterols, flavonoids, proteins and diterpenes.

Free radicals are continuously produced inside the living system as metabolic by product during respiration, cell-mediated immune functions and other processes utilizing oxygen.[16] The level of antioxidants is sufficient to protect the body against the deleterious effect of free-radicals generated at physiological rate. Any additional burden of free-radicals, either from environment or produced within the body, induces ROS leading to oxidative stress.[17] Oxidative stress induces DNA damage and micronuclei formation in bone marrow cells.[18] Overproduction of NO leads to DNA damage as represented in Figure 1.[19] Scavenging of reactive molecules (free radicals) represents one of the most important techniques of anticlastogenesis.[20] The result indicated that both AQEBM and ETEBM have significant effects on scavenging free radicals. Therefore, the protective effect of AQEBM and ETEBM against the clastogenesis generated by CP could arise from the ability to scavenge free radicals.

CP exhibit severe clastogenicity in humans and experimental animals. CP is rapidly metabolized in liver to generate cytotoxic metabolites, viz., 4-hydroxycyclophosphamide, phosphoramide mustard, and acrolein.[21] Phosphoramide mustard is associated with the anticancer effect of CP. However, acrolein induces oxidative stress by producing ROS which leads to DNA damage of normal cells.[22] Moreover, it induces nitric oxide production (directly or through NF-κB and AP-1).[23]

In vivo MN assay, developed by Schmid, is a well established method to evaluate the ability of test agent to induce/prevent chromosomal damage.[12] It detects chromosomal damage by analyzing the frequency of MNPCEs as sampled in bone marrow and/or peripheral blood cells of rodents. When a bone marrow erythroblast develops into a PCE, its main nucleus is extruded. However, any MN that has been formed may remain behind in the anucleated cytoplasm, and can be easily visualized because the cell lacks a main nucleus.[13] An increase in the frequency of MNPCEs (up to 6% or more) in the treated animals is a measure of chromosomal damage.[13]

The rodent micronucleus test demonstrated an inhibitory effect of AQEBM and ETEBM on the incidence of MN induced by CP. In the positive control group, there was an extremely significant ($P < 0.001$) increase in the percentage

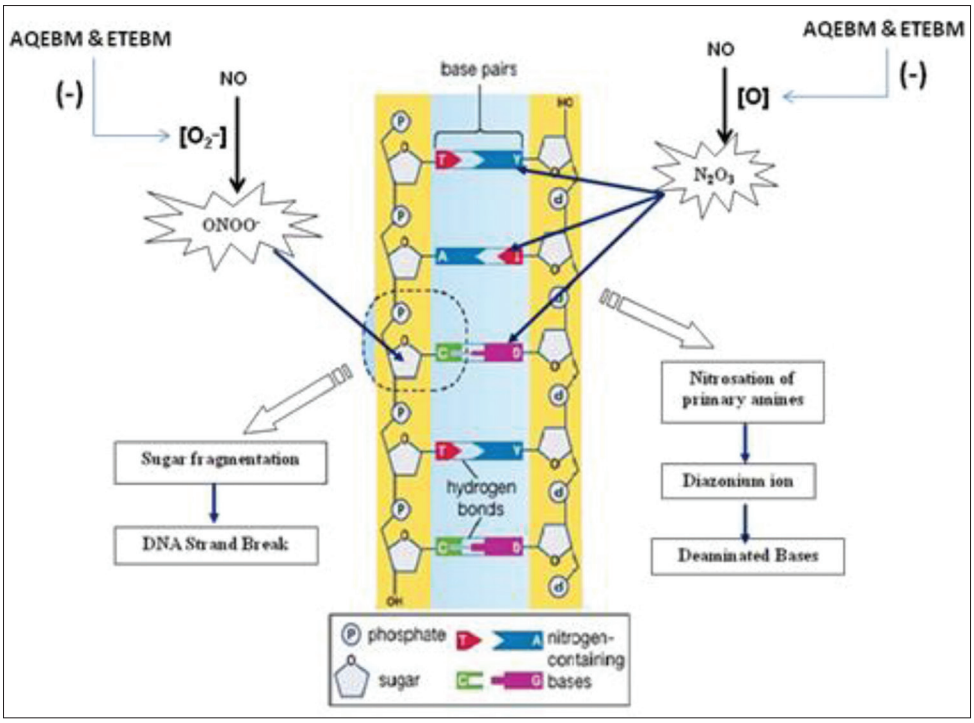

Figure 1: Mechanism of DNA damage induced by NO and its prevention by plant extract. Nitrous anhydride (N_2O_3), an auto oxidation product of NO, causes nitrosation of primary amines in the DNA bases. While, peroxynitrite ($ONOO^-$), formed from the reaction of NO with superoxide, attacks sugar-phosphate backbone causing single strand breaks. The plant extract competes with the oxygen to react with NO, inhibiting the formation of nitrous anhydride and peroxynitrite

of MN in PCEs after the administration of CP as compared to the normal group. In AQEBM (400 mg/kg) treated mice, the increase in MNPCEs was found to be insignificant as that of the normal mice showing its pronounced effect against CP induced clastogenesis. Moreover, a significant reduction in the frequency of MNPCEs induced by CP in animals pretreated with AQEBM and ETEBM indicated either prevention of CP induced chromosomal damage or enhancement of DNA repair by the extract. Furthermore, MI was also analyzed in order to investigate the protective effect of plant extracts against CP induced cytotoxicity. The MI is the number of PCEs as a percentage of total number of erythrocytes. A decrease in the MI in the blood and bone marrow cells could be interpreted as cellular death or a delay in cellular proliferation kinetics.[24] The cytotoxicity of CP is evident from the reduction of MI in comparison to normal group. However, there was an increase in the number of mitotic cells in animals treated with AQEBM and ETEBM at 24 and 48 hour.

Previous results obtained from *in vitro* antioxidant assay of plant extracts revealed that AQEBM and ETEBM posses ability to prevent oxidative and nitrosative stress. Based on these findings, it was speculated that AQEBM and ETEBM, being anti-oxidative and anti-nitrosative, prevented the interaction of acrolein and DNA in the nucleus resulting in reduced frequency of micronucleated cells in the erythrocyte population.

Lipid peroxidation is a biomarker of oxidative stress. It leads to the production of oxygenated compounds especially aldehydes like malondialdehyde and conjugated dienes which damage the cell membrane resulting in the loss of membrane fluidity, decreased membrane potential and increased permeability of membrane to ions.[25] Moreover, malondialdehyde reacts with deoxyguanosine to form a major endogenous adduct with DNA in human livers.[26] In the present study, significantly ($P < 0.001$) increased levels of MDA was observed in CP treated mice as compared to the normal mice. However, pretreatment of mice with AQEBM and ETEBM decrease the formation of lipid peroxidation byproduct MDA.

GSH is a tripeptide normally present in the cytoplasm and constitutes the major reducing capacity of the cell.[27] It is obvious that a severe GSH depletion makes the cell more vulnerable to attack by free radicals.[28] Acrolein directly reacts with GSH to form an adduct glutathionylpropionaldehyde, which induces oxygen radical formation.[21] That is why, a marked decrease in the hepatic GSH was observed in the CP treated mice. It was observed that the pretreatment with AQEBM and ETEBM increased the GSH content in the liver of mice.

CONCLUSION

Based on the obtained data, it could be concluded that AQEBM and ETEBM pre-treatment attenuate the CP-induced oxidative stress and subsequent DNA damage in both the peripheral blood and bone marrow cells of mice. AQEBM and ETEBM may be a potential antioxidant, antigenotoxic, and chemopreventive agent and could be used as an adjuvant in chemotherapeutic applications.

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