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Effect of *Buchanania lanzan* Spreng. bark extract on cyclophosphamide induced genotoxicity and oxidative stress in mice

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ABSTRACT

Objective: To elucidate the effect of ethanolic extract of *Buchanania lanzan* Spreng. (*B. lanan*) bark against cyclophosphamide induced genotoxicity and oxidative stress in mice. **Methods:** The prevalence of micronuclei in bone marrow, the extent of lipid peroxidation, reduced glutathione and the status of the antioxidant enzymes, superoxide dismutase and catalase in liver of mice were used as intermediate biomarkers for chemoprotection. Lipid peroxidation and associated compromised antioxidant defenses in cyclophosphamide treated mice were observed in the liver. **Results:** Pre-treatment with *B. lanzan* 250, 500 and 1 000 mg/ kg, *p.o.*, daily for 7 days significantly reduced the chromosomal damage and lipid peroxidation with concomitant changes in antioxidants and detoxification systems. **Conclusions:** These results point out the presence of chemopreventive phytoconstituents in the crude extract offering protection against cyclophosphamide induced genotoxicity and oxidative stress in mice.

1. Introduction

Exposure to various environmental factors leads to free radical formation. The most common form of free radicals is oxygen. When an oxygen molecule (O_2) becomes electrically charged, it tries to steal electrons from other molecules, causing damage to the DNA and other molecules and therefore cause mutagenesis. A critical factor in mutagenesis is cell division[1]. When the cell divides, an unrepaired DNA lesion can give rise to mutation. To protect against oxidative damage, animals have many different types of antioxidants defenses, these antioxidants decrease mutagenesis and carcinogenesis, in two ways: by decreasing oxidative DNA damage and by decreasing cell division[2].

Naturally occurring substances of plant origin and dietary components that have been widely studied for their antimutagenic activity were studied, which includes carotenoids, flavonoids, tea-polyphenols, vitamins, cucuminoids, tannins, coumarins, chlorophyllin, porphyrins and alkylresorcinols from cereal grains. It is now well recognized that the antioxidants and antimutagenic phytochemicals also play an important role in prevention of cancer. Hence, there is a need to establish the relations between antioxidants and antimutagenic agents[3].

Cyclophosphamide is an alkylating agent widely used in cancer chemotherapy. Its cytotoxic effects are the result of chemically reactive metabolites that alkylate DNA and protein, producing cross-links. The injury of normal tissues is the major limitation of using cyclophophomide, which gives rise to numerous side effects. It has been reported that oxidative stress mediated disruption of redox balance after cyclophophomide exposure generates biochemical and physiological disruptances[4]. Several studies suggest that antioxidant supplementation can influence the response to chemotherapy as well as the development of adverse side effects that result from treatment with antineoplastic agents[5].

Buchanania lanzan Spreng. (*B. lanzan*) belongs to the Family Anacerdiaceae, is commonly known as 'Chaar' in India and 'Almondette tree' in English. In ayurvedic medicine various part this plant used as astringent,

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depurative, constipating, brain tonic, cardiotonic and for glandular swelling[6]. This plant is used in treatment and prevention of cancer by traditional healers and herbalists of the Chhattisgarh state of India[7]. Furthermore, antioxidant activity of its bark has been described by us and other authors[8-10]. The phytoconstitute reported in bark of this plant tannins, quercetin, gallic acid and glucoside[6, 11]. High content of polyphenoles, in vitro antioxidant activity and traditional use of this plant in prevention of cancer, compel to investigate the effect of ethanol extract of Buchanania lanzan bark against cyclophophomide-induced genotoxicity and oxidative stress in mice.

2. Materials and methods

2.1. Plants

The bark of *B. lanzan* were collected from forest of Pendra, district Bilaspur Chhattisgarh, India during the month of December 2008. The plant was identified and authenticated by Dr. H. B. Singh, Scientist, National Institute Scientific Communication and Research (NISCAIR) New Delhi, India. The voucher specimen has been stored in herbarium of SLT institute of Pharmaceutical Sciences, Bilaspur India for future reference (Specimen No. 03/HSBL). The bark of *B. lanzan* was dried in shade and powdered. Dried powder was extracted with petroleum ether (60-80 ℃) and filtered. The residue was extracted with 90% ethyl alcohol by using Soxhlet extraction apparatus. Then solvent was completely removed under reduced pressure and controlled temperature. The crude extract obtained was a dark brown solid (yield of the extract was 18.5%).

2.2. Chemicals

Folin-Ciocalteu reagent, cyclophophomide, bovine serum albumin, thiobarbituric acid (TBA), sodium azide, nitro blue tetrazolium (NBT), NADH, 5,5′-dithio-bis(2- nitrobenzoic acid) (DTNB), phenazine methosulphate were obtained from HIMEDIA, Mumbai, India. All other chemical used were of analytical grade.

2.3. Phytochemcal analysis and total polyphenol content

The ethanol extract *B. lanzan* was subjected to preliminary phytochemical screening to identify the presence of various phytoconstituents present in the extract[12].

Content of total polyphenol was determined according to method of folin-ciocalteu reaction[13]. Different concentrations of Gallic acid (10-50 μg/mL) were prepared for standard curve. 0.1 mL of these different concentrations of Gallic acid was taken in different test-tubes and to it 0.5 mL undiluted Folin-Ciocalteu reagent was added. After 1 min, 1.5 mL 20 % (w/v) anhydrous sodium carbonate were added and volume was made up to 10 mL with water. After 1 hour incubation at 25 ℃, the absorbance was measured at 760 nm. 0.1 mL of extracts (1 mg/mL in methanol) was taken and followed as above. Absorbance of this sample was taken and Gallic acid equivalent was determined by extrapolation of the standard curve. The total phenolic content was expressed as Gallic Acid Equivalents (GAE) in microgram per milligram of extract.

2.4. Animals

Male albino mice [10 week old, 20-25 g, body weight (bw)] were used from animal house facilities of School of Pharmacy, CEC, Bilaspur, India. The animals were fed with a standard pellets diet and drinking water ad libitum. The study was approved by Animal Institutional Ethics Committee (IACE) (CPCSEA Reg.: 1275/ac/09/CPCSEA)

The animals were divided into 7 groups (Group I-VII) of six animals each:

Group I: Normal control received distilled water (10 mL/kg, bw) for 7 days by gavage.

Group II: Vehicle control received 2% acacia (10 mL/kg, bw) for 7 days by gavage.

Group III: Positive control, treated with single dose cyclophosphamide (i.p. 75 mg/kg, bw) in saline, 1 h after the last dose of distilled water (10 mL/kg, bw) for 7 days by gavage.

Group IV: Treated with single i.p. dose of cyclophosphamide, 1 h after the last dose of *B. lanzan* extract (250 mg/kg, BW for 7 days) in 2% acacia (10 mL/kg, bw) by gavage.

Group V: Treated with single i.p. dose of cyclophosphamide, 1 h after the last dose of *B. lanzan* extract (500 mg/kg, BW for 7 days) in 2% acacia (10 mL/kg, bw) by gavage.

Group VI: Treated with single i.p. dose of cyclophosphamide, 1 h after the last dose of *B. lanzan* extract (1 000 mg/kg, BW for 7 days) in 2% acacia (10 mL/kg, bw) by gavage.

Group VII: Extract control treated with *B. lanzan* extract (1000 mg/kg, bw for 7 days) in 2% acacia (10 mL/kg, bw) by gavage.

2.5. Determination of micronuclei in bone marrow

After treated with cyclophophomide for 30 h, mice were sacrificed by cervical dislocation. Bone marrow cells of both femurs were flushed through a 21-gauge needle using a disposable syringe into a centrifuge tube containing 1 mL of 5 % bovine albumin solution. This suspension was centrifuged at 1 000 rpm for 5 min. The pellet of cells was resuspended in a drop of fresh bovine albumin solution and smears were prepared on clean glass slides and air-dried. Four slides were prepared for each mouse. The dried, evenly spread bone marrow smears were fixed with methanol for 5 min and stained with May-Grunwald and Giemsa as

described by Schmid[14]. A total of 1 000 polychromatic erythrocytes (PCE) were screened per animal by the same observer for determining the frequency of polychromatic erythrocytes (PCE) containing micronuclei (MNPCE) and the ratio of PCE: NCE was also determined by counting the number of PCEs among 300 erythrocytes.

2.6. Lipid peroxidation and antioxidant enzymes

The effects of treatments on lipid peroxidation and antioxidant enzymes were determined by corresponding assays in every group. Livers were removed from mice and perfused immediately with ice-cold saline (0.9% sodium chloride) and homogenized in chilled phosphate buffer (0.1 M, pH 7.4) using tissue homogenizer. The homogenate was centrifuged at 4 000 rpm for 5 min at 4 ℃. Products of lipid peroxidation (LPO) were estimated by measuring the concentration of malondialdehyde (MDA) expressed as thiobarbituric acid reactive substances (TBARS) according to the methods of Ohkawa *et al.*[15] and expressed as nMole MDA/mg of protein. Total superoxide dismutase activity (SOD) was determined by the phenazine methosulphate method using nitro blue tetrazolium (NBT) and NADH to generate superoxide radicals, and a unit of activity was defined by Kakkar *et al.*[16] and expressed as (Unit/mg of protein). Catalase activity (CAT) was measured spectrophotometrically by measuring the decrease of absorbance at 240 nm due to H_2O_2 decomposition according to the methods of Aebi[17] and expressed as (Unit/mg of protein). Reduced glutathione (GSH) was estimated spectrophotometrically by determination of dithiobis-2-nitrobenzoic acid (DTNB) reduced by SH-groups, as described by Moron *et al.*[18] and expressed as nmol/mg of protein. Protein content of homogenates was determined by reaction with coomassie Blue dye, using bovine serum albumin as the standard, in a spectrophotometer.

2.7. Statistical analysis

Values are expressed as mean ± SEM from 6 animals. Statistical difference in mean were analyzed using one way ANOVA followed by Tukey's multiple comparison tests, $P < 0.05$ will be considered statically significant.

3. Results

3.1. Phytochemical analysis and total polyphenol content

Preliminary phytochemical study of the ethyl alcohol extract of Buchanania lanzan indicated the presence of flavonoid, sterol, terpenoids, glycosides and tannins. The total polyphenol content was found 30.12 ± 0.78 μg GAE /mg of extract.

3.2. Micronuclei in bone marrow

The results obtained for mice treated with different concentrations of *B.lanzan* in combination with cylcophoshphamide as well as *B. lanzan* alone are shown in Table 1. No significant difference in the frequency of MNPCE was observed between mice treated with 1 000 mg/kg of Buchanania lanzan (Group-VII) and the normal control (Group-II). A high increase in the frequency of MNPCE was detected in mice treated with 75 mg/kg of cyclophophomide (group-III) compared to the normal control (Group-II) ($P<0.001$). Simultaneous treatments with different concentrations of *Buchanania lanzan* and cylcophoshphamide (Group IV, V & VI) led to reduction in the frequency of MNPCE compared to cylcophoshphamide alone (group-III), which was significant for the treatments using 250, 500 and 1 000 mg/kg of *B. lanzan* with the addition of cylcophoshphamide (**$P < 0.01$ and ***$P < 0.001$), corresponding to decreases of 37.2%, 56.14 and 69.99% in the frequency of MNPCE, respectively. These results indicate a dose-response correlation, *B. lanzan* was found to be effective and a gradual increase in concentration is proportional increase in the reduction of mutagenicity. Evaluation of erythropoietic cytotoxicity is a key component of safety assessment in new drug development. The occurrence of fewer immature erythrocytes (PCE) relative mature or normochromatic erythrocytes (NCE) is considered to be an indicator of mutagen-induced cytotoxicity. Therefore, the ratio of PCE to NCE is one index of cytotoxicity that is routinely included in micronucleus tests to assess the mutagenicity of chemicals to mammals. No significant differences in the PCE/NCE ratio were observed when comparing mice treated with 1000 mg/kg of *B. lanzan* (Group VII) and the normal control (Group II). This result indicates a lack of toxicity to bone marrow.

3.3. Effect on lipid peroxidation and antioxidant enzymes

Table 2 represents the extent of lipid peroxidation as evidenced by the formation of MDA as well as the levels of GSH and activities of the antiperoxidative enzymes SOD and CAT in the livers of control and experimental animals. The lipid peroxidation levels in mice treated with cyclophophomide alone (group III) were significantly higher when compared to controls (group I & II), with concomitant decrease in the GSH and activities of SOD and CAT. Oral pretreatment with *Buchanania lanzan* at all doses tested (group IV, V & VI), significantly prevented these adverse changes and maintained the animals at near normal status in a dose-dependent manner.

Table 1

Frequency of MNPCE and PCE/NCE ratio in mice treated with *B. lanzan* and/or cyclophashamide after 30 h.

Group	Treatment	MNPCE				PCE/NCE
		Individual data	No.	%	Mean±SEM	Mean±SEM
I	Distilled water	04–02–03–04–04–03	19	0.31	3.33±0.33	0.84±0.01
II	Vehicle (2% acacia)	02–03–04–03–04–03	20	0.33	3.16±0.30	0.82±0.02
III	Positive control (CP 75 mg/kg bw i.p.)	18–20–22–24–24–22	130	2.16	21.66±0.95^{a***}	0.57±0.02^{a*}
IV	BL extract 250mg/kg + CP	16–13–14–12–12–14	81	1.35	13.50±0.61^{b**}	0.69±0.03
V	BL extract 500mg/kg + CP	10–09–11–10–08–09	66	1.10	9.5 0±0.42^{b***}	0.55±0.05^{b}
VI	BL extract 1 000mg/kg + CP	09–06–08–05–04–07	39	0.65	6.50±0.34^{b***}	0.62±0.13^{b}
VII	BL extract 1 000mg/kg	04–03–02–03–02–03	17	0.28	2.83±0.30^{a}	0.91±0.12^{a}

BL: *B. lanzan*. CP: cyclophashamide; Comparison: (a) group II *vs*. III & VII, (b) group III *vs*. IV, V & VI; Statistical significance *P< 0.05), ** P<0.01, *** P<0.001; PCE, polychromatic erythrocytes; NCE, normochromatic erythrocytes; MNPCE, micronucleated polychromatic erythrocytes.

Table 2

Levels of lipid peroxidation and antioxidants in livers of mice following treatment with different doses of *B. lanzan* against CP.

Group	Treatment	LPO (nmole MDA/mg of protein)	GSH (nmol/mg of protein)	SOD (U/mg of protein)	CAT (U/mg of protein)
I	Distilled water	1.77±0.17	54.16±4.73	12.37±1.25	7.21±0.44
II	Vehicle (2% acacia)	1.67±0.20	53.56±1.64	12.82±1.52	7.15±0.38
III	Positive control(CP 75 mg/kg wt i.p.)	5.12±0.29^{a***}	25.33±1.63^{a***}	4.69±0.40^{a***}	3.68±0.41^{a**}
IV	BL extract 250mg/kg + CP	4.06±0.40^{b}	28.36±3.42^{b}	7.83±0.93^{b}	5.65±0.57^{b*}
V	BL extract 500mg/kg + CP	3.23±0.42^{b**}	41.66±3.44^{b*}	9.55±1.10^{b*}	6.05±0.25^{b**}
VI	BL extract 1000mg/kg + CP	2.71±0.48^{b***}	44.44±3.87^{b**}	10.82±0.71^{b**}	6.91±0.29^{b**}
VII	BL extract 1000mg/kg	1.52±0.12^{a}	54.48±2.48^{a}	12.24±0.95^{a}	7.28±0.40^{a}

Comparison: (a) group II *vs*. III & VII, (b) group III *vs*. IV, V & VI; Statistical significance * P<0.05, ** P<0.01, *** P<0.001.

4. Discussion

Based on the results of the micronucleus assay, chromosomal damage was the major cause for the appearance of micronuclei due to exposure to cyclophophomide and as an indicator of genotoxic insult to nuclei[19]. The fact that administration of 75 mg/kg bw i.p. (group III) resulted in a significantly greater frequency of MNPCE in bone marrow cells than did distilled water or Vehicle groups (I and II) (Table 1) demonstrates the genotoxicity of cyclophophomide. Administration of *B. lanzan* for 7 days inhibited formation of micronuclei in mouse bone marrow cells by cyclophophomide. *B. lanzan* significantly reduced the frequency of MNPCE and exhibited protection and anticlastogenic effects against the effects of cyclophophomide. Several studies indicate that cyclophophomide has a prooxidant character, and generation of oxidative stress after cyclophophomide administration leads to decrease in the activities of antioxidant enzymes and increase in lipid peroxidation (LPO) in liver, lung and serum of mice and rats[20-21]. The greater lipid peroxidation (LPO), which is considered to be an indicator of increased oxidative damage, suggests that this was a primary effect caused by cyclophophomide in mouse liver. Some anticancer drugs including cyclophophomide are known to exert their cytotoxic effects by a free radical mediated mechanism[22]. We noted significant increases of MDA in liver after cyclophophomide treatment. Pretreatment and concomitant treatment with *Buchanania lanzan* resulted in significant decrease in MDA levels, and this may be due to the free radical scavenging potential of the extract. cyclophophomide metabolism produces highly reactive electrophiles and the decreased value of GSH in cyclophophomide-treated group most probably due to the electrophilic burden on the cells and also due to the formation of acrolein, which deplete GSH content[23-30]. Treatment with *Buchanania lanzan* reduced the electrophilic burden and thereby increased GSH levels in liver. Administration of *B. lanzan* significantly elevated the SOD and CAT activities, suggesting that it had the ability to restore the activities of these two enzymes. Oxidative stress arising from overproduction of ROS and the breakdown of antioxidant defenses is documented to induce chromosomal breakage and formation of bone marrow micronuclei[31]. It has been suggested that clastogenic factors are released by cells exposed to oxidative stress[32]. Free radical scavengers, including naturally occurring compounds such as vitamin C, have been found to reduce or neutralize the activity of such reactive oxygen species[22]. These results support the hypothesis that cyclophophomide-induced genotoxicity and cytotoxicity to bone marrow cells may be partially repaired by antioxidant activity. The protective activity of the *Buchanania lanzan* may be due to its inhibitory action on several enzymes or it's blocking effect on oxidative damage.

In summary, the results of this study demonstrate that *B. lanzan* protects against cyclophophomide-induced genotoxicity in mice. The chemopreventive actions of *B. lanzan* in this effect may be partially attributed to its elevation of the levels of enzymatic antioxidants (SOD and CAT). The present findings imply that *B. lanzan* may be a potential antigenotoxic, antioxidant and chemopreventive agent and could be used as an adjuvant in chemotherapeutic applications.

Conflict of interest statement

We declare that we have no conflict of interest.

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