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Pluchea lanceolata attenuates cadmium chloride induced oxidative stress and genotoxicity in Swiss albino mice

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Abstract

Cadmium intoxication induces lipid peroxidation and causes oxidative damage to various tissues by altering antioxidant defence system enzymes. At 24 h after treatment with a single intraperitoneal dose of cadmium chloride (5 mg kg^{-1}), Swiss albino mice showed a significant increase in the levels of malanodialdehyde and xanthine oxidase ($P < 0.001$), and a concomitant depletion of renal glutathione, catalase ($P < 0.001$) and other antioxidant enzymes. $CdCl_2$ also led to a simultaneous increase in micronuclei formation ($P < 0.001$) and chromosomal aberrations ($P < 0.05$) in mouse bone marrow cells. Oral pre-treatment with *Pluchea lanceolata* extract at doses of 100 and 200 mg kg^{-1} for 7 consecutive days before $CdCl_2$ intoxication caused a significant reduction in malanodialdehyde formation and xanthine oxidase activity ($P < 0.001$). A significant restoration of the activity of antioxidant defence system enzymes such as catalase, glutathione peroxidase ($P < 0.05$), glutathione-*S*-transferase and glutathione reductase ($P < 0.001$) was observed. A significant dose-dependent decrease in chromosomal aberrations and micronuclei formation was also observed ($P < 0.05$). The results indicate that pre-treatment with *P. lanceolata* attenuates cadmium chloride induced oxidative stress and genotoxicity by altering antioxidant enzymes and reducing chromatid breaks and micronuclei formation.

Introduction

Cadmium, a well-known environmental pollutant (Prozialeck & Lamar 1993), is a non-essential transition metal that has carcinogenic potential (Sunderman 1986) and affects reproductive processes, causing retardation of growth, sterility and teratogenic effects (Dalton et al 1996). Cadmium has many toxic manifestations, including lung fibrosis, kidney tubular dysfunction, osteoporosis and cancer (Peters et al 1986; Nordberg & Nordberg 1988). Cadmium intoxication appears repeatedly in both humans and experimental animals (Stowe et al 1972). The toxicity of cadmium may also involve reactive oxygen species (Manca et al 1991). Free radicals may react with lipid and carbohydrate moieties of lipoproteins and glycoproteins, leading to oxidation of lipids and proteins. Hence, suppressing or enhancing the metabolic enzymes (Sun 1990) and cadmium leads to the formation of micronuclei and chromosomal aberrations (Hartwig 1998). Cadmium is reported to increase chromosomal aberrations in lymphocytes after occupational exposure (Deaven & Campwell 1980) and dose-dependently induces micronuclei formation in mouse bone marrow cells (Jagetia & Adiga 1994), DNA strand breaks, and sister chromatid exchanges in plant mammalian and human cells (Forni 1994). Little is known about the mechanisms of metal induced carcinogenesis, but one possible pathway may involve the interaction with macromolecules such as DNA, either directly or indirectly (Hartmann & Speit 1994).

Natural herbs and shrubs have been used since ancient times for the treatment of human diseases (Steinmetz & Potter 1991). Earlier studies have shown an inverse correlation between the incidence of cancer and the intake of dietary antioxidants and plant phenols (Archer 1988; Steinmetz & Potter 1991; Bronzetti 1997).

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Therapeutic intervention in cadmium toxicity involves chelating cadmium bound to metallothionein or scavenging free radicals by antioxidant defence mechanisms. *Pluchea lanceolata* is an important medicinal plant widely used in indigenous medicine (Ayurveda) for rheumatism and allied disorders, diseases of the abdomen, dyspepsia, bronchitis and inflammation (Prasad et al 1966). The decoction of *P. lanceolata* has been used traditionally for the treatment of arthritis (Chaturvedi & Singh 1965). The leaves are aperients and are used as laxatives, analgesics and antipyretics. Quercetin and isorhamnetin have been identified in the air-dried leaves of *P. lanceolata* (Chawla et al 1991; Dixit & Tewari 1991). Flavonoids are reported scavengers of free radicals (Bors et al 1990; Zhou & Zheng 1991) and are a potent inhibitor of lipid peroxidation. Flavanols such as quercetin and rutin are known for their antihistamine, anti-inflammatory and antiviral activities (Soleas et al 1997). Quercetin has been shown to mediate down-regulation of mutant P^{53} in human breast cancer cell lines (Avila et al 1994) and to inhibit chemically induced carcinogenesis (Havsteen 1983). Various studies have reported the isolation of tertiary bases and a large number of water-soluble quarternary bases, including pluchine, which has been identified with betaine hydrochloride. Quercetin and isorhamentin are present as aglycones in the leaves of *P. lanceolata*. Glycosides and tannins were absent (Srivastava et al 1990).

The protective effects of *P. lanceolata* extract against cadmium chloride ($CdCl_2$) induced renal oxidative stress and genotoxicity were studied in mice. Since quercetin and isorhamnetin have been reported to be active constituents of *P. lanceolata* in previous studies (Chawla et al 1991; Dixit & Tewari 1991), we hypothesized that they would be the major constituents of our plant extract.

Materials and Methods

Chemicals

Oxidized and reduced glutathione, NADPH, H_2O_2, dithionitrobenzene, 1-chloro-2,4-dinitrobenzene (CDNB), glutathione reductase, cadmium chloride ($CdCl_2$) and colchicine were purchased from Sigma Chemical Co. (St Louis, MO, USA). Stains were purchased from Hi-Media Labs (Mumbai, India). All other chemicals and reagents used were of the highest purity commercially available.

Plant material and extraction

Total plant material (*P. lanceolata*) was purchased from Saiba Industries (Mumbai, India). The material is claimed to possess all the active ingredients of the plant. The methanolic fraction was used for the present study after preliminary in-vitro tests.

The plant was extracted by the method of Didry et al (1998). Powdered dried plant (500 g) was extracted in a round-bottomed flask with 2000 mL petroleum ether (60–80°C) and then repeatedly with methanol using a Soxhlet apparatus. The ethanol extract was recovered and evaporated to dryness by distillation under reduced pressure in a rotatory evaporator (Buchi Rotavapor; Buchi, Switzerland). The required amount of the concentrated ethanolic fraction obtained (35 g) was dissolved in aqueous suspension at the time of dosing.

Animals

Eight-week-old adult male Swiss albino mice (20–25 g) were obtained from the Central Animal House Facility of Hamdard University (New Delhi, India). They were housed in a ventilated room at 25 ± 2°C under a 12-h light–dark cycle. The animals were allowed to acclimatize for 1 week before the study and had free access to standard laboratory feed (Hindustan Lever Ltd, Bombay, India) and water.

Ethical approval for the study was granted by the Committee for the Purpose of Control and Supervision of Experimental Animals (173/CPCSEA).

Experimental design

For study of biochemical parameters and micronuclei assay, 25 male Swiss albino mice were divided into five groups. Group 1 was the saline treated control group. Group 2 served as the treatment control and was given a single intraperitoneal dose of $CdCl_2$ ($5\,mg\,kg^{-1}$) freshly dissolved in distilled water. Groups 3 and 4 were pretreated orally with *P. lanceolata* at doses of 100 and $200\,mg\,kg^{-1}$, respectively, for 7 consecutive days. Group 5 received $200\,mg\,kg^{-1}$ plant extract orally for 7 consecutive days. On Day 7, a single intraperitoneal dose of $CdCl_2$ ($5\,mg\,kg^{-1}$) was given to Groups 2, 3 and 4. All the animals were killed 24 h after $CdCl_2$ intoxication. The kidneys and femur bones were removed and processed for enzyme estimation and micronuclei assay. The doses of plant extract were selected based on preliminary studies.

Mutation assays

Chromosomal aberration test

The treatment schedule described above was followed for the chromosomal aberration test. Mice were killed by light ether anaesthesia 24 h after treatment with a single intraperitoneal dose of $CdCl_2$ ($5\,mg\,kg^{-1}$). A single intraperitoneal dose of colchicine ($4\,mg\,kg^{-1}$) was administered to the animals approximately 90 min before killing. The time of killing was chosen based on preliminary experiments as optimal for scoring chromosomal aberrations. The slides of bone marrow cells were prepared and stained according to the routine hypotonic acetic acid/methanol flame-drying Giemsa schedule for metaphase plate analysis. At least 75–100 well-spread intact metaphases were scored per animal under $100 \times$ oil immersion using a light microscope (Olympus BX 50). The types of chromosomal aberration included chromatid and chromosome breaks and chromosomal rearrangements. All aberrations were considered as equal regardless

of the number of breaks involved; gaps were not included. A single observer carried out blind scoring.

Micronucleus test
Mice were killed 24 h after treatment with a single intraperitoneal dose of $CdCl_2$ (5 mg kg^{-1}). Femur bones for bone marrow were collected for the micronucleus assay and kidney tissue was used for enzymatic assays. The time of killing was chosen based on preliminary assays as the time of peak micronuclei induction. A preliminary assay was done to select an appropriate dose of $CdCl_2$ that did not suppress cell proliferation in combination with the highest dose of plant extract. The micronucleus test was carried out according to the method of Schimd (1975). The air-dried slides were stained with May-Grunwald and Giemsa as described by Schimd (1975), made permanent and coded. A total of 2500–3000 polychromatic erythrocytes were scored per animal by the same observer to determine the frequency of micronucleated polychromatic erythrocytes.

Biochemical assays

Tissue processing and preparation of post-mitochondrial supernatant was done as described by Athar & Iqbal (1998). All the biochemical estimations were completed within 24 h of killing the animal.

Estimation of lipid peroxidation
The microsomal lipid peroxidation assay was done according to the method of Wright et al (1981). The reaction mixture consisted of 0.58 mL phosphate buffer (0.1 M, pH 7.4), 0.2 mL microsomes, 0.2 mL ascorbic acid (1 mM) and 0.02 mL ferric chloride (100 mM) in a total volume of 1 mL. The mixture was incubated at 37°C in a shaking water bath for 1 h. Then, 1 mL 10% trichloroacetic acid and 1 mL 0.67% thiobarbituric acid were added. All the tubes were placed in a boiling water bath for 20 min. The tubes were placed in an ice bath and then centrifuged at 2500 *g* for 10 min. The amount of malanodialdehyde formed in each of the samples was assayed by measuring the optical density of the supernatant at 535 nm. The results were expressed as nmol malanodialdehyde formed h^{-1} (g tissue)$^{-1}$ at 37 °C using a molar extinction coefficient of 1.56×10^5 M^{-1} cm^{-1}.

Xanthine oxidase activity
Xanthine oxidase activity was assayed by the method of Stripe & Della Corte (1969). The reaction mixture consisted of 0.2 mL post-mitochondrial supernatant incubated for 5 min at room temperature with 0.8 mL phosphate buffer (0.1 M, pH 7.4). Then, 0.1 mL xanthine (9 mM) was added to the reaction mixture and kept at 37°C for 20 min, followed by the addition of 0.5 mL of 10% perchloric acid and 2.4 mL of double-distilled water in a total volume of 4 mL. After 10 min, the mixture was centrifuged at 1467 *g* for 10 min. Xanthine oxidase activity was recorded at 290 nm and expressed as μg uric acid formed min^{-1} (mg protein)$^{-1}$.

Catalase activity
Catalase activity was measured by the method of Claiborne (1985). The reaction mixture consisted of 2 mL phosphate buffer (0.1 M, pH 7.4), 0.95 mL hydrogen peroxide (0.019 M) and 0.05 mL post-mitochondrial supernatant in a final volume of 3 mL. Changes in absorbance were recorded at 240 nm. Catalase activity was calculated as nmol H_2O_2 consumed min^{-1} (mg protein)$^{-1}$

Estimation of reduced glutathione
Reduced glutathione was determined by the method of Jollow et al (1974). A 1.0-mL sample of post-mitochondrial supernatant was precipitated with 1.0 mL sulfosalicylic acid (4%). The samples were kept at 4°C for 1 h and then centrifuged at 1200 *g* for 20 min at 4°C. The assay mixture contained 0.4 mL supernatant, 2.6 mL sodium phosphate buffer (0.1 M, pH 7.4) and 0.2 mL dithionitrobenzene (100 mM) in a total volume of 3.0 mL. The yellow colour that developed was immediately read at 412 nm using a spectrophotometer.

Glutathione-S-transferase activity
Glutathione-*S*-transferase activity was estimated by the method of Habig et al (1974) as modified by Athar et al. The reaction mixture consisted of 1.425 mL sodium phosphate buffer (0.1 M, pH 7.4), 0.2 mL reduced glutathione (1 mM), 0.025 mL CDNB (1 mM) and 0.3 mL post-mitochondrial supernatant (10% w/v) in a total volume of 2.0 mL. The changes in the absorbance were recorded at 340 nm and enzyme activity was calculated as nmol CDNB conjugate formed min^{-1} (mg protein)$^{-1}$ using a molar coefficient of 9.6×10^3 M^{-1} cm^{-1}.

Glutathione reductase activity
Glutathione reductase activity was assayed by the method of Carlberg & Mannervick (1975) as modified by Mohandas et al (1984). The assay system consisted of sodium phosphate buffer (0.1 M, pH 7.4), 0.5 mM EDTA, 1 mM oxidized glutathione, 0.1 mM NADPH and post-mitochondrial supernatant (10% w/v) in a total mixture of 2.0 mL. The enzyme activity was measured at 340 nm and calculated using a molar extinction coefficient of 6.22×10^3 M^{-1} cm^{-1}.

Glutathione peroxidase activity
Glutathione peroxidase activity was assayed by the method of Mohandas et al (1984) as described by Athar & Iqbal. The assay mixture consisted of sodium phosphate buffer (0.1 M, pH 7.4), 1 mM EDTA, 0.2 mM NADPH, 1 mM sodium azide, 1 IU mL^{-1} glutathione reductase, 0.25 mM H_2O_2, and post-mitochondrial supernatant (10% w/v) in a total volume of 2.0 mL. The enzyme activity was recorded at 340 nm and calculated using a molar extinction coefficient of 6.22×10^3 M^{-1} cm^{-1}.

Protein estimation
The protein content in all samples was estimated by the method of Lowry et al (1951) using bovine serum albumin as standard.

Statistical analysis

Significant differences between groups were determined using analysis of variance followed by the Dunnett's *t*-test.

Results and Discussion

$CdCl_2$ treatment alone produced gaps and chromosomal breaks in the cells; cells with multiple chromosomal aberrations and exchanges were observed infrequently and are not considered good indicators of chromosomal damage. Table 1 shows the reduction in frequency of chromosomal aberrations induced in mouse bone marrow cells after oral pre-treatment with *P. lanceolata* (100 and 200 mg kg^{-1}) for 7 consecutive days before $CdCl_2$ (5 mg kg^{-1}) compared with the group treated with $CdCl_2$ alone. A significantly greater incidence of chromosomal aberrations was observed when the control group was compared with the $CdCl_2$ only treated group. In all cases, the frequency of chromosomal aberrations was reduced in the $CdCl_2$ treated mice pre-treated with the plant extract. Table 2 shows the protective effect of *P. lanceolata* against $CdCl_2$ induced mutagenicity as assessed by the bone marrow micronucleus test. $CdCl_2$ produced significant micronuclei formation when compared with the control group, and there was significant inhibition in $CdCl_2$ induced micronuclei formation after pre-treatment with *P. lanceolata*.

Tables 3 and 4 show that $CdCl_2$ (5 mg kg^{-1}) caused an overproduction of cellular oxidants and concomitant enhancement of xanthine oxidase, with a decrease in content of renal glutathione and inhibition of catalase and other glutathione dependent enzymes. Pre-treatment with *P. lanceolata* at doses of 100 and 200 mg kg^{-1} resulted in a restoration of the antioxidant system, with significant inhibition of malanodialdehyde formation and a marked decrease in xanthine oxidase levels. Only plant extract treated groups showed lower levels of malanodialdehyde and a decrease in xanthine oxidase activity; antioxidant levels were found to be near control values.

$CdCl_2$ induces oxidative damage by inducing lipid peroxidation and suppression of antioxidants and other metabolic phase II enzymes (Muller 1986). $CdCl_2$ causes

Table 1 Effect of pre-treatment with *Pluchea lanceolata* extract on $CdCl_2$ induced chromosomal aberrations in mouse bone marrow cells

Treatment	Total metaphases	Abnormal metaphases	Breaks	Chromatid gaps	Exchanges	Multiples	Ring formation	Fragments	% Abnormal metaphases (mean ± s.e.)
Control (0.9% saline)	389	21	6	8	3	3	–	1	5.3 ± 0.65
$CdCl_2$ (5 mg kg^{-1})	440	42	9	18	4	6	3	2	9.5 ± 0.60[a]
P. lanceolata (100 mg kg^{-1}) + $CdCl_2$ (5 mg kg^{-1})	411	32	7	14	4	4	2	1	7.7 ± 0.53[c]
P. lanceolata (200 mg kg^{-1}) + $CdCl_2$ (5 mg kg^{-1})	392	25	6	12	3	3	1	–	6.3 ± 0.28[b]
P. lanceolata (200 mg kg^{-1})	373	20	6	7	3	3	1	–	4.5 ± 0.28

There were five mice in each group; 75–100 metaphases were observed for each mouse. [a]$P < 0.05$, significantly different compared with the saline treated group. [b]$P < 0.05$, significantly different compared with the $CdCl_2$ treated group. [c]$P < 0.01$, significantly different compared with the $CdCl_2$ treated group.

Table 2 Effect of pre-treatment with *Pluchea lanceolata* extract on $CdCl_2$ induced micronuclei formation in mouse bone marrow cells

Treatment	No. of counted nucleated cells	No. of polychromatic erythrocytes	No. of polychromatic erythrocytes with micronuclei	% Polychromatic erythrocytes (mean ± s.e.)	% Polychromatic erythrocytes with micronuclei (mean ± s.e.)
Control (0.9% saline)	1963	236	10	12.0 ± 3.64	4.2 ± 0.489
$CdCl_2$ (5 mg kg^{-1})	2007	302	29	15.0 ± 2.96[b]	9.6 ± 0.769[a]
P. lanceolata (100 mg kg^{-1}) + $CdCl_2$ (5 mg kg^{-1})	1987	286	18	14.3 ± 2.21	6.2 ± 0.357[b]
P. lanceolata (200 mg kg^{-1}) + $CdCl_2$ (5 mg kg^{-1})	1974	265	13	13.4 ± 1.670[b]	4.9 ± 0.357[b]
P. lanceolata (200 mg kg^{-1})	1967	242	09	12.3 ± 1.56	3.7 ± 0.334

There were five mice in each group; 2500–3000 nucleated cells were observed for each group. [a]$P < 0.01$, significantly different compared with the saline treated group. [b]$P < 0.05$, significantly different compared with the $CdCl_2$ treated group.

Table 3 Effect of pre-treatment with *Pluchea lanceolata* extract on $CdCl_2$ induced stress on malanodialdehyde formation and xanthine oxidase activity in mouse kidney

Treatment	Xanthine oxidase activity (μg uric acid formed min^{-1} (mg protein)$^{-1}$)	Malanodialdehyde (nmol malanodialdehyde formed h^{-1} (g tissue)$^{-1}$)
Control (0.9% saline)	0.5779 ± 0.0187	5.41 ± 0.0040
$CdCl_2$ (5 mg kg^{-1})	0.8476 ± 0.00792[a]	9.69 ± 0.0185[a]
P. lanceolata (100 mg kg^{-1}) + $CdCl_2$ (5 mg kg^{-1})	0.666 ± 0.00239[b]	8.877 ± 0.0331[b]
P. lanceolata (200 mg kg^{-1}) + $CdCl_2$ (5 mg kg^{-1})	0.559 ± 0.001080[b]	7.647 ± 0.0040[b]
P. lanceolata (200 mg kg^{-1})	0.539 ± 0.0026	5.39 ± 0.380

Results are presented as the mean ± s.e. of five mice per group. [a]$P < 0.001$, significantly different compared with the saline treated group. [b]$P < 0.001$, significantly different compared with the $CdCl_2$ treated group.

Table 4 Effect of pre-treatment with *Pluchea lanceolata* extract on $CdCl_2$ induced stress on catalase, glutathione content and glutathione dependent enzymes in mouse kidney

Treatment	Reduced glutathione (nmol glutathione (g tissue)$^{-1}$)	Glutathione-*S*-transferase (nmol CDNB conjugate formed min^{-1} (mg protein)$^{-1}$)	Glutathione peroxidase (nmol NADPH oxidized min^{-1} (mg protein)$^{-1}$)	Glutathione reductase (nmol NADPH oxidized min^{-1} (mg protein)$^{-1}$)	Catalase (nmol H_2O_2 consumed min^{-1} (mg protein)$^{-1}$)
Control (0.9% saline)	0.256 ± 0.0165	268.8 ± 0.09912	1525.007 ± 6.24	1718.007 ± 30.15	198.9 ± 4.82
$CdCl_2$ (5 mg kg^{-1})	0.126 ± 0.0024[a]	204.2 ± 2.145[a]	1212.52 ± 41.93[a]	1251.8 ± 14.65[a]	165.5 ± 2.82[a]
P. lanceolata (100 mg kg^{-1}) + $CdCl_2$ (5 mg kg^{-1})	0.180 ± 0.00043[b]	242.3 ± 6.4213[b]	1292.3 ± 12.615[c]	1377.7 ± 30.79[b]	173.58 ± 2.504[c]
P. lanceolata (200 mg kg^{-1}) + $CdCl_2$ (5 mg kg^{-1})	0.206 ± 0.0040[b]	225.6 ± 8.2144[c]	1309.6 ± 24.187[c]	1502.6 ± 30.79[b]	188.04 ± 2.238[b]
P. lanceolata (200 mg kg^{-1})	0.208 ± 0.00043	309.7 ± 6.4086	1366.32 ± 10.908	1540.8 ± 26.16	193.40 ± 3.85

Results are presented as the mean ± s.e. of five mice per group. [a]$P < 0.001$, significantly different compared with the saline treated group. [b]$P < 0.001$, significantly different compared with the $CdCl_2$ treated group. [c]$P < 0.05$, significantly different compared with the $CdCl_2$ treated group.

DNA strand breaks and lipid peroxidation (Berlett & Stadtman 1997). Cadmium produces hydroxyl radicals in the presence of metallothionein (O'Brien & Salacinski 1998). Studies have shown that the potent antitumour promoting activities of many natural herbs and their compounds may be because of their antioxidant constituents (Hayatsu et al 1988; Dhir 1989). The potential of *P. lanceolata* as a potent chemopreventive and antigenotoxic agent is evident from results that show that pre-treatment with *P. lanceolata* before $CdCl_2$ intoxication leads to significant decrease in lipid peroxidation and xanthine oxidase levels, with enhancement of glutathione and dependent enzymes. The antigenotoxic properties of *P. lanceolata* were also demonstrated in the present study: pre-treatment with *P. lanceolata* before $CdCl_2$ administration led to a dose-dependent decrease in the number of chromosomal aberrations and micronuclei formation. In the plant extract treated groups, the enhanced catalase activity may be a protective mechanism against cadmium toxicity. The glutathione level is considered to be the basic defence mechanism against oxidative damage as glutathione works as a free radical scavenger and as a cofactor in detoxification of toxins (Bray & Taylor 1993). A significant increase in the glutathione level and its dependent enzymes was noted in the groups pre-treated with the plant extract. Thus, we conclude that *P. lanceolata* extract ameliorates $CdCl_2$ induced clastogenic effects in mice.

Conclusion

Our results suggest that *P. lanceolata* has potent chemopreventive activity against $CdCl_2$ induced renal oxidative stress and genotoxicity. The overall antimutagenic and anticlastogenic efficacy of *P. lanceolata* is probably due to the presence of flavanols such as quercetin and isorhamnetin. The levels of glutathione and catalase were restored to control levels, with a decrease in lipid peroxidation, in the plant extract treated groups. The reduction in micronuclei formation and chromosomal aberrations indicate the antigenotoxic effects of *P. lanceolata*. The induction of various antioxidant

enzymes to suppress oxidative stress may be the possible mechanism of action of *P. lanceolata* in modulating the clastogenecity of $CdCl_2$.

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