AMELIORATIVE EFFECT OF CURRY LEAF AQUEOUS EXTRACT AGAINST LEAD ACETATE-INDUCED OXIDATIVE STRESS IN RAT KIDNEYS

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ABSTRACT

Objective: Aim of the study is to find therapeutic potentials of aqueous extract of Murraya koenigii (CuLE) against lead induced oxidative damage in renal tissues of rats.

Methods: Rats were intraperitoneally (i.p.) injected with lead acetate (15mg/kg body weight), another group was pre-treated with CuLE (50 mg / kg, fed orally), the positive control group was fed CuLE (50 mg / kg) and the control animals received vehicle treatment (i.p.) for 7 consecutive days. Concentration of lead in renal tissue was quantified using AAS study. The changes in the activity of different bio-markers of renal damage, biomarkers of oxidative stress, activities of the antioxidant and some of the mitochondrial enzymes were studied. Histomorphology was studied through H-E staining and alteration in tissue collagen level was studied through Sirius red staining and confocal imaging.

Results: Lead acetate caused changes in all the parameters evaluated. All these changes were ameliorated when the rats were pre-treated with CuLE. Concentrations of lead in kidneys were also decreased following pre-treatment of rats with CuLE.

Conclusion: CuLE has the potentiality to protect against lead acetate-induced oxidative stress mediated nephrotoxicity in rat possibly through its antioxidant activity. CuLE may have future therapeutic relevance in lead acetate-induced nephrotoxicity in human who are environmentally or occupationally exposed to lead.

Keywords: Antioxidant, Murraya koenigii, Oxidative stress, Nephrotoxicity.

INTRODUCTION

Murraya koenigii Linn (Rutaceae) is a popular spice herb, grown often in kitchen gardens in South East Asian countries. The leaves possess antioxidant, antibacterial, antifungal, anti-diabetic, larvicidal, anticarcinogenic, hypoglycemic, anti- lipid peroxidative, hypolipidemic and antihypertensive activity [1].

The reported potent antioxidant phytoconstituents isolated so far from the leaves are alkaloids i.e., mahanine, koenine, koenigine, koemidine, gininimbioi, gininimbine, koemimbine, O-methyl murrayamine A, O-methyl mahanine, isomahamine, bismahamine, bispyranofole and coumarin glycoside i.e., scopotin, murrayamine. Some other compounds recognized from the curry leaves are thiamine, riboflavin, niacin, vitamin C, carotene and oxalic acid. It is also reported to contain 5,8-dimethyl furanoocumin, 1,3- [6', 6' dimethyl 5-hexene] carbazole and β-sitostol etc [2]. The leaves have potent ROS scavenging activities also [3,4]. The leaves are used in cooking, hence they undergo thermal processing and are often used in fried and dried forms. Some of the phytoconstituents present in the leaves are reported to be thermostable and hence retain their structural and functional forms even often being used in cooking. And there has been no scientific exploration of the polar constituents of the curry leaves and their biopotency in respect to antioxidant and free radical scavenging capacity. The leaves contain flavonoids and Clifford and Cuppert [5] classified the antioxidant mechanisms of flavonoids into free radical chain breaking, metal chelating, and singlet oxygen quenching, with the inhibition of enzymatic activity.

Lead is an environmental pollutant, widely distributed, naturally occurring toxic heavy metal. Lead is also recognized as an occupational hazardous metal [6] which if enters the body accumulates in soft organs and cannot be metabolized by the body. Heavy metals like lead, cadmium etc. have very long half life and are severely toxic at a very low dose. Studies revealed and confirmed the involvement of oxidative stress as an important mechanism for heavy metal toxicity [7]. Several studies suggest that the toxic effects of lead on brain, heart, liver and kidney tissues have a direct correlation between these effects and lead-induced oxidative damage [3,8]. Kidney is recognized to be the prime target of heavy metal toxicity [6]. It is reported that lipid peroxidation is a key contributor to the progression and perhaps to the origin of acute and chronic renal failure [7]. Heavy metals accumulate in kidney and thereby impair normal renal functions [9]. Instances of heavy metal induced nephrotoxicity, where chelation therapy has limited applicability and success, the inclusion of antioxidants in the treatment of metal-induced toxicity may be a useful treatment. Antioxidants of herbal origin are safe to use cause of their no reported cytotoxic effect. These alternative therapies are devoid of any kind of side effect. These are potent, easily available and less expensive and can be adapted as nutraceuticals or may find a place in pharmacology after further experimental and clinical confirmations. Aqueous extract of Murraya koenigii leaves (CuLE) has already been found to provide protection against cadmium and lead induced cardiac toxicity and hepatotoxicity [3,4,8]. The curry leaves grown in the Indo Gangetic plain have not yet been investigated to possess any protective effect against oxidative stress in kidneys. The aqueous extract of the leaves contain all polar phytoconstituents which may include some vitamins, micro and macro elements and some polyphenolic compounds which may be the prime players in the antioxidant property of the aqueous extract of curry leaves. Here we investigated the ameliorative effect of CuLE against lead induced oxidative stress in kidneys of male Wistar rats.

MATERIALS AND METHODS

Plant Material

Fresh, green Curry leaves [Murraya koenigii (L) Spreng] were collected from different parts of West Bengal, i.e., from the districts of Burdwan, Hooghly, South 24 Parganas and Kolkata metropolitan area throughout the year during the course of the study. The identity of the plant was confirmed by Mr.P. Venu, Scientist ‘F’, Botanical...
Survey of India, Central National Herbarium (Government of India, Ministry of Environment and Forests), Botanic Garden, Howrah 711 103, West Bengal. The Herbarium of the plant was deposited in the BSI against voucher specimen no. CNH/1-1/41/2010/Tech.II/232.

Preparation of aqueous extract of the Curry leaves

The Curry leaves were separated, washed thoroughly in normal tap water and kept at room temperature in Borosil tray for one hour with its bottom covered with a piece of blotting paper to soak any excess water. The leaves were then dried in a hot air oven at 50 °C for two hours till they were dry and crispy and crushed into a coarse dust with mortar and pestle. Then they were grinded in a mechanical grinder to fine dusts and were stored in air tight Tarson bottles at 20 °C until further use.

For the preparation of the aqueous extract, the dried leaf dusts were soaked overnight in double distilled water (7.5g per 100 ml), filtered through loin cloth and the filtrate was centrifuged at 5000 rpm for 10 min (using a REMI cold-centrifuge). The supernatant, thus obtained, was filtered again through loin cloth, collected in sterile polypropylene tubes and frozen at -20 °C. The contents of the tubes were then lyophilized and the resulting lyophilized material (a dry powdery material) [herein after referred to as the aqueous extract] was stored at -20 °C until further use. A definite amount of the aqueous extract was always freshly dissolved in double distilled water to give a particular concentration and the resulting solution was used in our in vivo studies. Any leftover of this solution was always discarded.

Chemicals used

All chemicals used in the present studies were of analytical grade. Anhydrous DTNB, Folin Ciocalteu phenol reagent and Hematoxylin were procured from SRL, India Limited. TEP was procured from SIGMA, ALDRICH, MO, USA. Sodium carbonate (Na2CO3), cupric sulfate pentahydrate (CuSO4·5H2O), hydrochloric acid (HCl) was obtained from Merck (Darmstadt, Germany).

Animals

Male Wistar rats of body weight 160-180 gm were used throughout the experiments. The animals were handled as per the guidelines of institutional animal ethics committee (IAEC) of department of Physiology, University of Calcutta in accordance with the committee for the purpose of control and supervision of experiment on animals (CPSEAA), Ministry of Environment and Forest, Government of India. All the experimental protocols had the approval of Institutional Animal Ethics Committee (IAEC) [IAEC/PROPOSAL/DB-2/2010, APPROVAL DATE:16/11/2011 ] of the Department of Physiology, University of Calcutta. Prof. P. K. Samanta, M. Sc. (Vet.), Ph. D., Professor and Veterinary Surgeon and CPSEAA Nominee to Department of Physiology, University of Calcutta, acted as the advisor for animal care and handling and continuously monitored animal experiments.

Lead (Pb) acetate induced oxidative stress in kidneys in vivo and protection with CuLE

After acclimatization to laboratory conditions, the rats were divided into four groups, with 6 rats in each group:

Group I: Control
Group II: CuLE
Groups III: Lead treated
Groups IV: CuLE + Lead acetate treated

Thirty minutes after CuLE was fed, the animals of the lead acetate and the CuLE + lead acetate treated groups were injected with lead acetate solution, intraperitoneally, at a dose of 15 mg kg⁻¹ body weight (LD₅₀ = 150 mg/kg BW) for 7 consecutive days. The animals of the control group received the vehicle only. Each day the body weight of the animals were measured and recorded.

Collection of blood and tissues, and preparation of the serum

After the treatment period, animals of each group were kept fasted overnight. The body weight of the animals of each group were measured and recorded. The animals were sacrificed through cervical dislocation and the abdominal cavity was carefully opened and blood was immediately collected from hepatic vein in two different sets of tubes, one was used for blood analysis and the other for measurement of serum parameters. The blood in the latter tube was allowed to clot for serum to separate out and then centrifuged at 2500 rpm for 15 minutes. Serum was collected carefully with auto pipette in individual microfuge tube and stored at -20 °C. The heart was excised carefully and washed several times in ice cold saline, and bottled dry, immediately weighed and stored at -20 °C until analysis.

Determination of Lead content of renal tissue

The tissue samples were prepared and the lead content was measured as per the protocol mentioned in the cook book of the Varian AA240 Atomic Absorption Spectrophotometer, GTA 120 (Graphite tube atomizer) available at the Chemical Engineering Department of University College of Science and Technology, University of Calcutta. The tissue samples were incubated overnight at 37°C and their respective dry weight was recorded. Then the tissue was placed in a conical flask containing measured volume of double distilled water. Concentrated nitric acid was carefully added to it and the conical flask with its contents were placed on the hot plate and heated at 65-70°C for digestion of the tissues. Then, perchloric acid was added for the precipitation of the protein and heated until white fumes come out. The contents of the conical flasks were then carefully and quantitatively transferred into 25 ml volumetric flasks, and, finally the volume was made up to 25 ml with double distilled water. The lead content of the samples was then measured using an atomic absorption spectrophotometer. The lead content was expressed in μg/g of rat tissue.

Histological studies

Immediately following sacrifice of the animals, kidneys were surgically extirpated and fixed in 10% formalin and embedded in paraffin following routine procedure as described earlier [3,4]. Tissue sections (5 μm thick) were prepared and stained with hematoxylin-eosin.

Quantification of fibrosis by confocal microscopy

The rat kidney tissue sections (5 μm thick) were stained with Sirius red (Direct Red 80; Sigma Chemical Co., St. Louis, MO, USA) and imaged with a laser scanning confocal system (Zeiss LSM 510 META, Germany) and the stacked images through multiple slices were captured. Four slides were prepared for each rat from each group and only the representative images are presented. The digitized images were then analyzed using image analysis system (Image], NIH Software, Bethesda, MI) and the total collagen area fraction of each image was measured and expressed as the % collagen volume.

Preparation of homogenate and measurement of lipid peroxidation level

The kidney tissues were separately homogenized (10%) in ice-cold 0.9 % saline (pH 7.0) with a Potter Elvehjem glass homogenizer for 30 s and lipid peroxides in the homogenate were determined as thiobarbituric acid reactive substances (TBARS) [10] with some modification as adopted [11]. In brief, the homogenate was added to thiobarbituric acid–trichloro acetic acid (TBA–TCA) reagent with thorough shaking and was heated for 20 min at 80°C. The samples were then cooled to room temperature. The absorbance of the pink chromogen present in the clear supernatant after centrifugation at 1200 g for 10 min at room temperature was measured at 532 nm using a UV–Vis spectrophotometer (Bio-Rad, Hercules, CA, USA). Tetraethoxypropane (TEP) was used as standard. Values were expressed as nmole of TBARS/mg protein.

Measurement of protein carbonyl (PCO) content

Protein carbonyl content was estimated by DNPH assay [12]. About 0.1 g of liver was rinsed in 10 mM PBS buffer (pH 7.4) and homogenized and centrifuged at 10,000g for 10 min at 4°C. After centrifugation, 0.5 ml of tissue supernatant was taken in each tube and 0.5 ml DNPH in 2.0 M HCl was added to the tubes. The tubes were vortexed every 10 min in the dark for 1 h. Proteins were then

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precipitated with 30% TCA and centrifuged at 4000g for 10 min. The pellet was washed three times with 1.0 ml of ethanol: ethyl acetate (1:1, v/v). The final pellet was dissolved in 1.0 ml of 6.0 M guanidine HCl in 20 mM potassium dihydrogen phosphate (pH 2.3). The absorbance was determined at 370 nm. The protein carbonyl content was calculated using a molar absorption coefficient of 2.2 

\[ \text{X} \times 10^4 \text{M}^{-1} \text{cm}^{-1}. \]

The values were expressed as nmols of carbonyl/mg protein.

Measurement of the activities of cytosolic (Cu-Zn type or SOD1) and mitochondrial (Mn-type or SOD2) superoxide dismutases, catale (CAT)

Copper-Zinc superoxide dismutase (Cu-Zn SOD or SOD1) activity was measured by hematoxylin autooxidation method [13] with some modifications as adopted [15]. In brief, the tissues were homogenized (10%) in ice-cold 50 mM phosphate buffer containing 0.1 mM EDTA, pH 7.4. The homogenate was centrifuged at 12,000 g for 15 min. The supernatant was collected. Inhibition of hematoxylin auto-oxidation by the cell free supernatant was measured at 560 nm using a UV–Vis spectrophotometer. The enzyme activity was expressed as U / mg of tissue protein. Manganese superoxide dismutase (Mn-SOD or SOD2) activity was estimated by pyrogallol autooxidation method [13]. A weighed amount of tissue was homogenized in ice-cold 50 mM Tris–HCl buffer containing 0.1 mM EDTA, pH 7.4. Centrifuged at 2000 rpm for 5 min. The supernatant was carefully collected and centrifuged again at 10,000 rpm in cold for 20 min. The supernatant was discarded and the pellet was suspended in 50 mM Tris–HCl buffer, pH 7.4. One ml of assay mixture contains 50 mM of Tris–HCl buffer (pH 8.2), 30 mM EDTA, 2 mM of pyrogallol and suitable volume of the mitochondrial preparation as the source of enzyme. An increase in absorbance was recorded at 420 nm for 3 min in a UV/VIS spectrophotometer. The enzyme activity was expressed as units/min/mg of tissue protein.

Catalase was assayed using hydrogen peroxide [14] with some modifications as adopted [13]. Briefly, weighed amounts of the tissues were homogenized (5%) in ice-cold 50 mM phosphate buffer, pH 7.0. The homogenate was centrifuged in cold at 12,000 g for 12 min. The aliquots of the supernatant serving as the source of enzyme were incubated with 0.01 ml of absolute ethanol at 4°C for 30 min. Then 10% Triton X-100 was added to have a final concentration of 1%. The sample, thus obtained, was used to determine the catalase activity by measuring the breakdown of H₂O₂ spectrophotometrically at 240 nm. The enzyme activity was expressed as μM H₂O₂ consumed / min / mg protein.

Measurement of reduced glutathione (GSH) level, oxidised glutathione (GSSG) level, GSSG/GSH ratio and total sulphhydril group (TSH) content

GSH content (as acid soluble sulfhydryl) of the liver was estimated by its reaction with DTNB (Ellman’s reagent) [16] with some modifications adopted [15]. The tissues were homogenized (10%) in 2 mM ice-cold ethylenediaminetetraacetic acid (EDTA). The reaction mixture contained 0.1 mM sodium phosphate buffer, EDTA, NADPH and 0.14 units per ml glutathione reductase. The absorbance was measured at 340 nm using a UV–VIS spectrophotometer to determine the GSSG content. The values were expressed as nmols GSSG/mg protein. GSSG/GSH ratio was evaluated.

Total sulphhydril group content was measured [16]. The values were expressed as nmols TSH/mg protein.

Measurement of the activities Glutathione reductase (GR), Glutathione peroxidase (GPx), and Glutathione-S-transferase (GST)

Glutathione reductase activity was estimated using NADPH [17]. The final volume of 3 ml assay mixture contained 50mM phosphate buffer, 200 mM KCl, 1mM EDTA and water.0.1 mM NADPH was added together with suitable amount of homogonate (enzyme) into the assay mixture. The reaction was initiated with 1mM oxidized glutathione (GSSG). The decrease in NADPH absorption was recorded at 340 nm. The specific activity of the enzyme was calculated as units/min/mg tissue protein.

Glutathione peroxidase activity was measured using NADPH [18]. Cardiac tissue was homogenized (10%) in ice-cold 50mM phosphate buffer containing 2mM EDTA (pH 7.0) A volume of 1ml of the assay mixture contained 0.5 M phosphate buffer with 2 mM EDTA, pH 7.0, 0.025 mM sodium azide, 0.15 mM glutathione, and 0.25 mM NADPH. The reaction was started by the addition of 0.36 mM H₂O₂. Linear decrease of absorbance at 340 nm was recorded using a UV /VIS spectrophotometer. The specific activity was expressed as nmoles of NADPH produced / min / mg tissue protein.

Glutathione-S-transferase activity of was measured at 340nm [19]. The enzymatic activity was recorded observing the conjugation of 1-chloro, 2,4-dinitrobenzene (CDNB) with reduced glutathione (GSH). One unit of enzyme conjugates 1.0 nmols of CDNB with reduced glutathione per minute at 25°C. The rate where the reaction is linear is noted at 340nm. The molar extinction of CDNB is 0.0096 μM⁻¹/ cm⁻¹.

The enzyme activity was expressed as Units/min/ mg of tissue protein.

Measurement of xanthine oxidase (XO) and xanthine dehydrogenase (XDH) activities

Xanthine oxidase activity was estimated by the conversion of xanthine to uric acid [20]. The tissues were homogenized in cold (10%) in 50 mM phosphate buffer, pH 7.8. Then centrifuged at 500 g for 10 min. The supernatant thus obtained was again centrifuged at 12,000 g for 20 min. The supernatant was used for spectrophotometric assay at 295 nm, using 0.1 mM xanthine in 50 mM phosphate buffer, pH 7.8, as the substrate. The enzyme activity was expressed as milli units/min/ mg protein.

Xanthine dehydrogenase activity was measured by following the reduction of NAD⁺ to NADH [21]. The weighed amounts of rat liver and kidney tissues were homogenized in cold (10%) in 50 mM phosphate buffer with 1 mM EDTA, pH 7.2. The homogenates were centrifuged in cold at 500g for 10 min. The supernatant was further centrifuged in cold at 12,000 g for 20 min. The final supernatant was used as the source of the enzyme, and the activity of the enzyme was measured spectrophotometrically at 340 nm with 0.3 mM xanthine as the substrate (in 50 mM phosphate buffer, pH 7.5) and 0.7 mM NAD⁺ as an electron donor. The enzyme activity was expressed as milli units/min/ mg tissue protein.

Measurement of the activities of the pyruvate dehydrogenase (PDH) and some of the mitochondrial Kreb’s cycle enzymes

The kidney tissues were homogenized (10%) in ice-cold 50 mM phosphate buffer, pH 7.4, with a Potter Elvehjem glass homogenizer (Belco Glass Inc., Vineland, NJ, USA) for 30s. The homogenate was then centrifuged at 500 g for 10 min. The supernatant was again centrifuged at 12,000 g for 15 min to obtain the mitochondrial fraction. The mitochondrial pellet, thus obtained, was re-suspended in the buffer and used for assaying the mitochondrial enzymes.

Pyruvate dehydrogenase (PDH) activity was measured spectrophotometrically [22], following the reduction of NAD⁺ to NADH at 340nm using 50mM phosphate buffer, pH 7.4, 0.5 mM sodium pyruvate as the substrate, and 0.5mM NAD⁺ in addition to enzyme. The enzyme activity was expressed as Units/ mg protein.

Isoctirate dehydrogenase (ICDH) activity was determined by measuring the reduction of NAD⁺ to NADH at 340nm with the help of a UV–VIS spectrophotometer [23] One ml assay volume contained 10mM phosphate buffer, pH 7.4, 0.5mM isocitrate, 0.1mM MnSO₄, 0.1mM NAD⁺ and enzyme. The enzyme activity was expressed as units/mg protein.

Alpha-ketoglutarate dehydrogenase (α-KGDH) activity was measured spectrophotometrically [23] by measuring the reduction of 0.35mM NAD⁺ to NADH at 340nm using 50mM phosphate
buffer, pH 7.4, as the assay buffer and 0.1mM α-ketoglutarate as the substrate. The enzyme activity was expressed as units/mg protein.

Succinate dehydrogenase (SDH) activity was measured spectrophotometrically by following the reduction of potassium ferricyanide (K₃FeCN₆) at 420nm [24] with some modifications. One ml assay mixture contained 50mM phosphate buffer, pH 7.4, 2% (w/v) BSA, 4mM succinate, 2.5mM K₃FeCN₆, and the enzyme. The enzyme activity was expressed as units/mg protein.

**Measurement of some of the mitochondrial respiratory chain enzymes**

The NADH-cytochrome c oxidoreductase activity was measured spectrophotometrically by following the reduction of oxidized cytochrome c at 565nm [25]. 50mM phosphate buffer, 0.1 mg BSA, 20mM oxidized cytochrome c, and 0.5 mM NADH was contained in 1.0 ml assay mixture along with enzyme. The enzyme activity was expressed as Units / mg protein.

Cytochrome c oxidase activity was determined spectrophotometrically by following the oxidation of reduced cytochrome c at 550nm [25]. 50 mM phosphate buffer, pH 7.4, 40 mM reduced cytochrome c, and a suitable aliquot of the enzyme was contained in 1 ml assay mixture. The enzyme activity was expressed as Units / mg protein.

**Measurement of tissue protein content**

Protein was estimated by using bovine serum albumin (BSA) as the standard [26].

**Statistical evaluation**

Each experiment was repeated at least three times with different rats. Data are presented as means ± S.E.M. Significance of mean values of different parameters between the treatments groups were analyzed using one way analysis of variances (ANOVA) after ascertaining the homogeneity of variances between the treatments. Pair wise comparisons were done by calculating the least significance. Statistical tests were performed using Microcal Origin version 7.0 for Windows.

**RESULTS**

**Lead content of renal tissue**

Accumulation of lead in renal tissues following treatment of rats with lead acetate at a dose of 15 mg / kg bw (i.p.) for a period of seven consecutive days compared to control (p<0.001 Vs. control) was observed. However, when the rats were pre-treated with CuLE at a dose of 50 mg / kg bw (fed orally), the tissue lead content was found to be reduced significantly compared to lead acetate treated group (40.88% p<0.001 vs. Pb-treated group) (Figure 1).

**Biomarkers of oxidative stress**

Treatment of rats with lead acetate at a dose of 15 mg/Kg body weight for a period of 7 consecutive days caused generation of oxidative stress in rat kidney as evident from significantly increased level of lipid peroxidation compared to control (Table 1) (3.2 folds in renal tissue ; *P< 0.001 vs. control group). Pre-treatment of rats with CuLE significantly protected the lipid peroxidation level from being increased (50 % kidney tissues ; **P<0.001 vs. Pb-acetate-treated group). However, CuLE alone has no effect on the lipid peroxidation level of tissue.

Table 1 also shows that there occurred a significant increase in the level of protein carbonyl of kidney tissues of rat following lead acetate treatment (2.38 fold kidney tissues ; *P< 0.001 vs. control group). Pre-treatment of rats with CuLE almost completely protected the tissue protein carbonyl level from being increased (41.83% kidney tissues **P< 0.001 vs. Pb-treated group). However, CuLE alone has no significant effect on the protein carbonyl level of kidney tissues.

**Status of antioxidant enzymes**

Table 1 reveals that treatment of rats with lead acetate at the indicated dose increased the activities of cytosolic Cu-Zn-SOD, the mitochondrial Mn-SOD and the catalase of the rat renal tissues (1.9 folds, 2 folds and 1.7 folds increase in renal tissue vs control, * P < 0.001 vs. control). Pre-treatment of rats with CuLE was found to protect the activities of these antioxidant enzymes from being increased in the tissue studied (42.21%, 37.18% and 37.57% decrease respectively, **P < 0.001 vs. Pb-treated group). However, CuLE alone did not significantly alter the activity of any of the enzymes studied.

**Table 1: Effect of aqueous extract of Murraya koenigii (CuLE) on the levels of lipid peroxidation, protein carbonyl, the activities of Cu-Zn superoxide dismutase, Mn superoxide dismutase and catalase in kidneys of the experimental rats.**

<table>
<thead>
<tr>
<th>Parameters Studied</th>
<th>CON</th>
<th>CuLE</th>
<th>Pb</th>
<th>CuLE + Pb</th>
</tr>
</thead>
<tbody>
<tr>
<td>LPO (nmoles of TBSA/mg protein)</td>
<td>0.320±0.010</td>
<td>0.306±0.008</td>
<td>0.643±0.011*</td>
<td>0.403±0.008**</td>
</tr>
<tr>
<td>Protein carbonyl (nmole per mg protein)</td>
<td>5.35±0.025</td>
<td>5.37±0.024</td>
<td>12.72±0.025*</td>
<td>7.4±0.028**</td>
</tr>
<tr>
<td>Cu-Zn SOD activity (units/min/mg protein)</td>
<td>1.70±0.159</td>
<td>1.73±0.168</td>
<td>3.20±0.167*</td>
<td>1.85±0.153**</td>
</tr>
<tr>
<td>Mn SOD activity (units/min/mg protein)</td>
<td>1.5±0.173</td>
<td>1.5±0.187</td>
<td>3.12±0.189*</td>
<td>1.96±0.156**</td>
</tr>
<tr>
<td>Catalase activity(nmoles H₂O₂ consumed/min/mg protein)</td>
<td>21.25±1.012</td>
<td>20.92±0.910</td>
<td>35.5±1.041*</td>
<td>24.51±1.321**</td>
</tr>
</tbody>
</table>

*Fig. 1: Effect of aqueous extract of the leaves of Murraya koenigii on the concentration of lead in renal tissues of experimental rats. CON = control, injected with vehicle; LEAD= 15 mg/kg bw Pb (CH₃COO): injected i.p.; MUR = 50 mg/kg bw of Murraya; MUR+LEAD= 50 mg/kg bw of Murraya (fed orally) + 15 mg/kg bw Pb(CH₃COO); injected i.p.; the values are expressed as Mean ± S.E.M. of six rats in each group; *P < 0.001 compared to control values. **P < 0.001 compared to lead treated values.*
Status of GSH, GSSG, GSSG: GSH and TSH

Exposure to lead caused an increase in the level of reduced glutathione and oxidised glutathione and a marked increase in the ratio of oxidised and reduced glutathione. Fig. 2 (A, C and D) shows that there occurred a significant increase in GSH, GSSG level and in the GSSG: GSH ratio of kidney tissues of rat following lead acetate treatment (1.9 folds, 19.28% and 16.66% respectively; *P< 0.001 vs. control group). Pre-treatment of rats with CuLE almost completely protected the tissue GSH and GSSG levels and thus the GSSG: GSH ratio also from being increased in renal tissue (28.18%, 19.71% and 14.29% respectively; **P< 0.001 vs. Pb-treated group). However, CuLE alone has no significant effect on the GSH and GSSG levels of kidney.

Treatment of rats with lead acetate decreased the total thiol (TSH) level significantly (19.75%, *P< 0.001 vs. control group). Pre-treatment of rats with CuLE almost completely protected the TSH from being decreased in renal tissue (21.68%, **P< 0.001 vs. Pb-treated group). However, CuLE alone has no significant effect on the TSH level of kidney (Fig.2B).

Fig. 2: Effect of aqueous extract of Murraya koenigii against lead-induced alteration in the value of GSH (A), TSH (B), GSSG (C) and GSSG: GSH (D) in rat renal tissue. Values are expressed as Mean ± SE of 6 animals in each group. Data were analyzed by using one way analysis of variances (ANOVA) using Microcal Origin version 7.0 for Windows. *P<0.001 compared to control; **P< 0.001 compared to lead treated group; CON = Control; MUR = Curry leaf extract; Pb=Lead; MUR +Pb= Curry leaf extract +Lead.

Status of the activities of glutathione peroxidise (GPx), glutathione reductase (GR) and glutathione –S- transferase (GST)
Treatment of rats with lead acetate for seven consecutive days at a dose of 15 mg / kg body weight increased the activities of glutathione peroxidase (GPx) and glutathione reductase (GR) in renal tissue. However, CuLE alone has no significant effect on the activities of those two enzymes in all of the organs studied when the rats were pre-treated with CuLE (46.51% , **P < 0.001 vs. Lead acetate -treated group). However, CuLE alone has no effect on the activity of the enzyme in the renal tissues, which is needed for the regeneration of mitochondrial GSH or thioredoxin. The activity of the enzyme in the renal tissue was found to be protected significantly from being decreased when the rats were pre-treated with CuLE at a dose of 50mg/kg body weight for the similar period of time (46.44%, 39.13% and 40.24%, **P< 0.001 vs. Lead acetate -treated group). However, CuLE alone has no significant effect on the activities of these enzymes in renal tissue.

Status of pro-oxidant enzymes

Table 2 shows that the activities of renal XO, XDH, XO + XDH and XO/XDH in the kidneys of the experimental rats were found to be completely protected when the rats were pre-treated with CuLE at a dose of 50mg/kg body weight for the similar period of time (46.44%, 39.13% and 40.24%, **P< 0.001 vs. Lead acetate -treated group). However, CuLE alone has no effect on the activity of isocitrate dehydrogenase in renal tissue.

Table 2: Effect of aqueous extract of Murraya koenigii on the activities of XO, XDH, XO+XDH and XO/XDH in the kidneys of the experimental rats

<table>
<thead>
<tr>
<th>Parameters</th>
<th>CON (milliunits/min/mg protein)</th>
<th>CuLE</th>
<th>Pb</th>
<th>CuLE + Pb</th>
</tr>
</thead>
<tbody>
<tr>
<td>XO</td>
<td>0.11±0.0013</td>
<td>0.01±0.0014</td>
<td>0.04±0.0012</td>
<td>0.01±0.0013**</td>
</tr>
<tr>
<td>XDH</td>
<td>0.022±0.0012</td>
<td>0.019±0.0013</td>
<td>0.059±0.0012</td>
<td>0.026±0.0012**</td>
</tr>
<tr>
<td>XO + XDH</td>
<td>0.31±0.0002</td>
<td>0.28±0.0003</td>
<td>0.084±0.002*</td>
<td>0.03±0.002**</td>
</tr>
<tr>
<td>XO/XDH</td>
<td>0.409±0.020</td>
<td>0.421±0.030</td>
<td>0.511±0.020*</td>
<td>0.384±0.020**</td>
</tr>
<tr>
<td>XO/(XO+XDH)</td>
<td>0.29±0.0012</td>
<td>0.28±0.0013</td>
<td>0.345±0.012*</td>
<td>0.27±0.012**</td>
</tr>
</tbody>
</table>

Values are expressed as Mean ± SE of 6 animals in each group. Data were analyzed by using one way analysis of variances (ANOVA) using Microcal Origin version 7.0 for Windows. *P<0.001 compared to control; **P< 0.001 compared to lead treated group; CON = Control; CuLE = Curry leaf extract; Pb=Lead; CuLE + Pb= Curry leaf extract +Lead.

Status of the activities of pyruvate dehydrogenase (PDH) and some of the mitochondrial Kreb’s cycle enzymes

Table 3 reveals that treatment of rats with lead acetate inhibits pyruvate dehydrogenase activity (4.65 times decrease , *P < 0.001 vs. their control in kidney tissues). Treatment of rats with CuLE significantly protected the enzyme activity from being decreased in (4.2 folds increase respectively in kidney tissue **P < 0.001 vs. Lead acetate -treated group). However, CuLE alone was found to have no effect on the activity of this enzyme in the tissue.

Table 3 further reveals that treatment of rats with lead acetate significantly decreased the activity of isocitrate dehydrogenase in renal tissues (54.00% decrease, *P < 0.001 vs. their respective control). Isocitrate dehydrogenase is a key enzyme in cellular defense against oxidative damage as it provides NADPH in the mitochondria, which is needed for the regeneration of mitochondrial GSH or thioredoxin. However, the enzyme activity of this enzyme in any of the tissue studied.
Table 3: Effect of aqueous extract of Murraya koenigii on the activities of PDH, ICDH, α-KGDH and SDH in kidneys of the experimental rats

<table>
<thead>
<tr>
<th>Parameters Studied</th>
<th>Control</th>
<th>CuLE</th>
<th>Pb</th>
<th>CuLE + Pb</th>
</tr>
</thead>
<tbody>
<tr>
<td>PDH (units/min/mg protein)</td>
<td>0.93±0.028</td>
<td>0.94±0.029</td>
<td>0.20±0.022*</td>
<td>0.84±0.026**</td>
</tr>
<tr>
<td>ICDH (units/min/mg protein)</td>
<td>0.05±0.002</td>
<td>0.05±0.004</td>
<td>0.023±0.004*</td>
<td>0.043±0.003**</td>
</tr>
<tr>
<td>α-KGDH (units/min/mg protein)</td>
<td>0.035±0.014</td>
<td>0.036±0.015</td>
<td>0.015±0.001*</td>
<td>0.029±0.0014**</td>
</tr>
<tr>
<td>SDH (units/min/mg protein)</td>
<td>2.30±0.042</td>
<td>2.20±0.052</td>
<td>0.653±0.042*</td>
<td>1.63±0.042**</td>
</tr>
</tbody>
</table>

Values are expressed as Mean ± SE of 6 animals in each group. Data were analyzed by using one way analysis of variances (ANOVA) using Microcal Origin version 7.0 for Windows.

*P<0.001 compared to control; **P< 0.001 compared to lead treated group; CON = Control; CuLE = Curry leaf extract; Pb=Lead; CuLE + Pb= Curry leaf extract +Lead.

Status of the activities of mitochondrial respiratory chain enzymes

Treatment of rats with lead acetate for similar period of time also decreased cytochrome c oxidase activity in renal tissues (67.57 % decrease respectively, *P< 0.001 vs. control group). The activity of this enzyme was found to be significantly protected from being decreased compared to lead acetate treated group when rats were pre-treated with CuLE (3.91 folds increase in kidney tissues, **P< 0.001 vs. Lead acetate -treated group). CuLE alone, however, has no effect on the activity of this enzyme (table 4).

Table 4: Effect of aqueous extract of the leaves of Murraya koenigii on the activities of Cytochrome c oxidase and NADH cytochrome c oxido-reductase in kidneys of the experimental rats

<table>
<thead>
<tr>
<th>Parameters Studied</th>
<th>Control</th>
<th>CuLE</th>
<th>Pb</th>
<th>CuLE + LEAD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytochrome c oxidase activity</td>
<td>3.70±0.091</td>
<td>3.71±0.013</td>
<td>1.2±0.016*</td>
<td>3.5±0.014**</td>
</tr>
<tr>
<td>NADH cytochrome c oxido-reductase</td>
<td>0.088±0.004</td>
<td>0.087±0.005</td>
<td>0.025±0.0027*</td>
<td>0.076±0.0036**</td>
</tr>
</tbody>
</table>

Values are expressed as Mean ± SE of 6 animals in each group. Data were analyzed by using one way analysis of variances (ANOVA) using Microcal Origin version 7.0 for Windows. *P<0.001 compared to control; **P< 0.001 compared to lead acetate treated group. CON = Control; CuLE = Curry leaf extract; Pb=Lead; CuLE + Pb= Curry leaf extract +Lead.

Histopathological Studies of the kidneys

Figure 4 (upper panel) shows severe degeneration of tubular epithelial cells and collapse of tubules indicating severe acute tubular necrosis (ATN) and increased inter tubular gap in renal tissue of lead acetate treated rats as evident from hematoxylin and eosin stained tissue sections compared to control. Pre-treatment of rats with CuLE protected the tissue damage to some extent. CuLE alone, however, has no effect on renal tissue morphology.

Furthermore, the renal tissue sections depicted in figure IV (middle panel, magnification 400X) show collagen deposition around glomeruli and renal tubules following treatment of rats with lead acetate for seven consecutive days. Pre-treatment of rats with CuLE prevented the deposition of renal tissue collagen. Fig IV (lower panel) shows similar images of the renal tissue captured by confocal laser scanning microscope (magnification 400X). Figure IV depicts quantification of fibrosis as percent collagen volume.
oxidized proteins are functionally inactive and unnecessary for living system. Some of these oxidised proteins may accumulate and add to further oxidative damage and also may interfere with normal function of the organ [29].

The results indicate that oxidized proteins that are capable of causing damage are produced in kidneys, following exposure of experimental rats to lead. The aqueous Curry leaf extract seems to be potent in either scavenging the toxic free radicals and other reactive intermediate(s) or chelating the heavy metal cations and thus protecting the critical cellular proteins from getting oxidized.

Superoxide dismutase, cytosolic i.e., Cu-Zn SOD and mitochondrial i.e., Mn-SOD are one of the prime antioxidant enzymes. They scavenge superoxide anion radical and produce hydrogen peroxide at their respective site of occurrence through dismutation reaction [30]. Catalase decomposes hydrogen peroxide to water and oxygen and thus neutralises the ROS [31]. CAT, and SOD are metalloproteins and they enzymatically detoxify the H₂O₂ and O²⁻, respectively. As the activity of these antioxidant enzymes depend on various essential trace elements and prosthetic groups for proper molecular structure, they are potential targets for lead toxicity [31].

The reports on the effect of lead on SOD activity are divergent. Decreased SOD activity in erythrocytes has been reported by more than one group of investigators, in studies on animals with high exposure to lead [32, 33]. Whereas others [34] did not notice any changes in the SOD activity in the brain. Some other investigators noted decreased activity of SOD [35, 36, 37, 38] and according to them this may be by interaction between lead and copper, a metal necessary for the proper functioning of the SOD cytosol enzyme. Our finding is compatible with the research of many investigators who found increased SO D activity after lead exposure [39, 40, 41,42,43]. Increased SOD activity was also observed in the female rat liver and in both female and male rat kidney [31]. In stress conditions, normal capacities of the endogenous antioxidant mechanisms are insufficient, triggering cells to increase and expand their antioxidative network [44]. Increased superoxide anion radical caused by lead in the cell may cause an increased activity of the superoxide dismutase enzyme type. Increased SOD activity lead to formation of increased H₂O₂ which in turn caused increased activity of catalase. CuLE probably removed lead by chelation or otherwise and thus inhibited the lead-induced generation of the ROS (s) which caused the activities of the antioxidant enzymes to be maintained near normal.

Glutathione i.e., GSH is oxidised to GSGG in combating stress. In other words we can say that GSH detoxifies free radicals and itself gets oxidised to GSGG in the process. Thus, it may be inferred that exposure to lead caused induction of generation of free radicals in kidneys which in turn caused utilization of GSH for detoxifying them. As a result of which, GSSG level we observed to be increased. On the other hand GSH level also we found to be increased compared to control animals which may be interpreted considering the relation “increased demand lead to increased supply”. TSH level i.e., total sulphhydril group content was observed to be decreased in kidney tissue with lead acetate treatment which may be due to denaturation of other sulphhydril group entities by lead. CuLE was found to be protective in respect to all GSH, GSGG and TSH. CuLE neutralised the effect of lead by probably removing lead by chelation of and scavenging the ROS formed due to lead exposure. Earlier also we observed such alteration in GSH and GSGG and TSH levels in heart and liver tissues of lead exposed experimental rats [8].

Increased utilization and demand of GSH in situation of lead induced generation of reactive oxygen species probably stimulated the increase in the activity of GR. GR being the prime enzyme that catalyses the conversion of GSSG to GSH and thus supports the antioxidant defense system indirectly. We observed an enhanced activity of glutathione peroxidase (GPx) also. It is also considered in the same queue with the other two antioxidant metals-enzyme namely SOD and catalase. GPx accomplishes their antioxidant function by enzymatically detoxifying peroxides. It is probable that increased generation of ROS like superoxide anion radical and hydroxyl radical etc. causes enhanced activity of GR, GPx and GST. Studies by other investigators support our finding. GST activity is
reported to be enhanced in the hepatic and renal tissues of male rats at a high dose of lead [31]. Some studies have also showed that a single injection of lead nitrate significantly increased renal GSTs, and acute exposure to triethyl lead chloride induced GSTs in rat kidney [45, 46]. While chronic exposure to inorganic lead during development, acute exposure produces cell-type specific increases in particular isoforms GST in the rat kidney [47]. These changes in GSTs are a result of lead-mediated oxidative injury or are as a protective adaptation needs more exploration.

Xanthine oxidase and xanthine reductase catalyze the oxidation and reduction of xanthine primarily and produces superoxide anion radical as a by-product in the process. Under normal condition, the enzyme, Xanthine oxidoreductase, exists in dehydrogenase form and uses NAD+ and there is no or very little production of superoxide anion radical. Studies reveal that in conditions of decreased yield of ATP, loss of membrane Ca2+ gradient occurs. Ca2+ dependent proteases get activated in presence of increased Ca2+ levels. These proteases cause selective proteolysis of the dehydrogenase to convert it into xanthine oxidase (XO) which catalyses the oxidation of both hypoxanthine and xanthine and produces superoxide anion free radical [48]. Thus, in oxidative stress situation if there is reduced production of ATP, XO may play a significant role in producing superoxide anion free radical and subsequent oxidative damage. A significant increase in the activity of xanthine oxidase and xanthine dehydrogenase activity, in the tissue, causes generation of reactive oxygen species in the kidneys of experimental rats following exposure to lead. CuLE thus has a potent radical scavenging role as well as antioxidant capacity, the prime contributors being the polar antioxidant phytoconstituents present in the aqueous extract.

It is now a well known fact that the Mitochondria are the major source of ROS production in cells [49]. The electrons leak from the ETC and ROS are produced in presence of molecular oxygen. We observed a considerable decrease in the activities of pyruvate dehydrogenase and the Kreb’s cycle enzymes like Isocitrate dehydrogenase, alpha-keto glutarate dehydrogenase and succinate dehydrogenase following treatment of experimental rats with lead. The activities of all these TCA enzymes were protected when the rats were pre-treated with the aqueous Curry leaf extract. Heavy metals are known to affect respiratory chain complexes [50]. The impairment of electron transfer through NADH:ubiquinone oxidoreductase and ubiquinone:cytochrome c oxidoreductase may induce superoxide anion radical formation.

Lead stimulates and enhances the production of ROS in mitochondria. Similar observations were earlier reported in presence of lead and other heavy metals like Cadmium in heart, liver and kidneys of experimental rats [4,12]. We are reporting for the first time that CuLE has the capacity to protect the mitochondrial metabolic enzymes in kidneys of experimental rats against lead induced oxidative damage. This strongly indicates that the extract possesses some chelating property and removes lead from the organ or is simply able to prevent mitochondria from ROS production by itself being a quencher of reactive oxygen species.

Lead exposure caused functional compromise of the respiratory chain enzymes in the kidneys of rats and we found a potent protective effect of the CuLE against lead induced reduction of the two prime enzymes of the ETC. CuLE probably has very potent capacity to remove lead and as well as the ROS. Histomorphological study revealed that lead caused acute tubular necrosis which was observed to be protected when the rats were pre-treated with CuLE. Collagen deposition around glomeruli and the renal tubules was observed to be enhanced following treatment of rats with lead acetate. Collagen deposition was prevented when pre-treated with CuLE. CuLE alone had no effect on collagen deposition. The results indicate a protective effect of CuLE against Pb-induced damage in rat renal tissue.

CONCLUSIONS

Treatment of male Wistar rats with lead acetate at a dose of 15 mg/kg bw i.p. for seven consecutive days caused accumulation of lead in the kidneys. Lead in turn induced generation of free radicals in the tissue. Thus, exposure to lead acetate for seven consecutive days, caused oxidative stress mediated damage in the rat renal tissues as reflected from the alterations in the biomarkers oxidative stress, antioxidant and pro-oxidant enzymes with simultaneous increases in lipid peroxidation and other oxidative stress biomarkers oxidative stress in the renal mitochondrial Kreb’s cycle and respiratory chain enzymes. These biochemical findings were supported by our histological studies of the rat kidney sections. Pre-treatment of rats with the aqueous Curry leaf extract at a dose of 50 mg/kg bw, fed orally, protected against lead-induced alterations of the biochemical parameters as well as tissue morphological status of the kidneys. Aqueous Curry leaf extract also decreased the level of lead in the tissue. Thus, this aqueous extract seems to provide protection to rat renal tissue against lead-induced oxidative stress through its direct as well as indirect antioxidant activity and also through its possible lead chelating property. Our observations may be of future therapeutic significance particularly in the geographical areas where people gets chronic exposure to the toxic heavy metal, lead, either occupationally or environmentally. The Curry leaf extract contains an abundance of polyphenols and flavonoids, specifically the ones which are polar to some extent and got extracted in the aqueous extract of the leaves (CuLE) which are responsible for its antioxidant and radical scavenging activity. Polyphenols present in other plant foods have been reported to possess potent antioxidant activity by some investigators [30,51,52,53]. As Curry leaves are almost regularly used in cooking in Indian and South East Asian dishes, as well as in many recipes around the globe, it may serve as a possible nutritional intervention. The Curry leaves can be considered to be a mocktail of potent phyto-constituents which have pronounced ROS scavenging and antioxidant activities. The aqueous Curry leaf extract also seemed to have lead chelating potential. The aqueous Curry leaf extract, containing mainly the polar constituents, may be considered for development of some nutritional supplement to combat oxidative stress-induced tissue damage in the people exposed to lead occupationally or environmentally.

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