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Research Article

PROTECTIVE EFFECT OF AQUEOUS LEAF EXTRACT OF *Murraya koenigi* AGAINST LEAD INDUCED OXIDATIVE STRESS IN RAT LIVER, HEART AND KIDNEY: A DOSE RESPONSE STUDY

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ABSTRACT

The current studies evaluated whether an aqueous extract of the leaves of *Murraya Koenigii* (Curry leaves) has the ability to protect against lead – induced oxidative stress in the liver, heart and kidneys of experimental rats. The studies revealed that the extract dose dependently protected the biomarkers of tissue damage, oxidative stress and antioxidant enzymes from getting altered in the rat tissues following treatment with lead acetate. The results indicate that the aqueous curry leaf extract (CuLE) might have protected the rat tissues from lead acetate induced oxidative stress through antioxidant mechanism(s).As curry leaves form one of the important component of regular diet.

Keywords: *Murraya koenigii,* lead,liver, kidney, heart, antioxidant.

INTRODUCTION

Toxicity due to heavy metals like lead, cadmium, mercury, arsenic etc.is well documented in the literature.[1]. Mechanism of heavy metal toxicity may be multifactorial. One of the primary mechanisms behind heavy metal toxicity has been attributed to heavy metal induced generation of the reactive oxygen species (ROS) leading to oxidative stress induced organ damage. Literature provide evidence that metals may interact with nuclear proteins and DNA causing oxidative deterioration of biological macromolecules.[2]

Lead poisoning is one of the oldest and widely studied occupational and environmental hazards.[1]Lead because of its dense, ductile, malleable and corrosion resistant nature has found its pronounced use in human civilization.[3]Lead is used extensively in building materials, pigments, to glaze ceramics, water pipes and glass, paints, dyes, artificial jewellery, cosmetics, protective coatings, acid storage batteries and also as gasoline additives. Exposure of humans to lead and its derivatives in day-to-day life is unavoidable because of its extensive use and application.[3]

It has been reported that lead has deleterious effects in experimental animals including rat and human.[4,5]Lead induces a broad range of physiological, biochemical, and behavioral dysfunctions in laboratory animals and humans[6-8]i.e., central and peripheral nervous systems[9], haemopoietic system[10], cardiovascular system[11], kidneys[12], liver[13], and male[14], and female reproductive systems[15]. It has been suggested that lead exerts its toxic effects through lead-induced oxidative stress and the pathogenesis of lead poisoning is due to oxidative stress induced disruption of the delicate prooxidant/antioxidant balance that exists within mammalian cells.[16]

The leaves of *Murraya koenigii* (L) Spreng (*Rutaceae*) are reported to have great medicinal value such as antibacterial, anti-inflammatory etc.[17]The plant is native to India and is distributed in most of southern and South-East Asia including India. The leaves of this plant are popularly known as curry leaves and is an important spice herb. The different parts of *Murraya koenigii* plant have popular use in folk medicine. The leaves are reported to enhance digestive secretions and relieve nausea, indigestion, and vomiting.[18]As is evident from the unchanged blood parameters/constituents and normal histopathology of hepatic tissue in *Murraya koenigii* fed rats, It was concluded that Curry leaves have no adverse effects.[19]

Curry leaf extracts has been shown to possess antioxidant and free radical scavenging activity *in vitro.*[21] The extract has also been shown to protect against oxidative stress induced in diabetes.[20] Furthermore the Curry leaves been shown to reduce lipid peroxidation and to reduce cellular damage, thus protecting liver from ethanol induced toxicity.[22] Various carbazoles from curry leaf have been isolated and are known to possess antioxidative properties.[18]

Recently, the probable toxic side effect of using synthetic antioxidants has been addressed.[2,32] It is assumed that regular consumption of plant-derived phytochemicals from vegetables, fruits, and herbs may cause shift of the balance toward an adequate antioxidant status. The interest in natural antioxidant, especially of plant origin has greatly increased in recent years.[23]

Herein, we provide evidence that the aqueous extract of curry leaf provides protection to hepatic, cardiac and kidney tissues from oxidative injury following exposure of the experimental rats to lead acetate in a dose dependent manner. The results of the current studies indicate that this aqueous extract provides protection against heavy metal-induced oxidative onslaught of the rat tissues possibly through its antioxidant mechanism(s) and point toward its therapeutic usefulness as a protective anti-oxidant against situations arising out of exposure of the humans to heavy metals environmentally or occupationally and where chelation therapy has limited success.

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, Hoogly, 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 ^{o} 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 ^{0}C until further use.

For the preparation of the aqueous extract preparation, the dried leaf dusts were soaked over night in double distilled water (7.5g per 100 ml), filtered through loin cloth and the filtrate 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 ^{0}. 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 ° 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) were 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 (CPCSEA), Ministry of Environment and Forest, Government of India. All the experimental protocols had the approval of Institutional Animal Ethics Committee (IAEC) of the Department of Physiology, University of Calcutta. Prof. P. K. Samanta, M. Sc. (Vet.), Ph. D., Professor and Veterinary Surgeon and CPCSEA Nominee to Department of Physiology, University of Calcutta, acted as the advisor for animal care and handling.

Induction of oxidative stress *in vivo* with lead acetate and protection with different doses of CuLE

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

Group I: Control

Group II: Lead treated

Groups III: Lead treated + CuLE at a dose of 12.5 mg/kg BW

Group IV: Lead treated + CuLE at a dose of 25 mg/kg BW

Group V: Lead treated + CuLE at a dose of 50 mg/kg BW)

Group VI: Lead treated + CuLE at a dose of 100 mg/kg BW)

30 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 (LD_{50} is 150 mg/ kg BW) for the 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 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 liver and the kidneys were excised carefully and washed several times in ice cold saline, and bottled dry, immediately weighed and stored at -20 ° C until analysis. The heart was surgically removed after opening the thoracic cavity and immersed in ice-cold 0.9% saline for proper washing, blotted dry and weighed.[24]

Determination of blood creatinine level

Blood creatinine level was estimated by the method of Folin Wu with slight modification. In a conical flask, 3.5 ml double distilled water, 0.5ml blood, 0.5 ml sodium tungstate and 0.5 ml 2/3 N sulfuric acid were added and mixed, and allowed to stand for 10 minutes and filtered. To the filtrate, alkaline picrate was added in 2:1 ratio, allowed to stand for 15 minutes and the absorbance was recorded at 530 nm. A blank and a standard were prepared using distilled water and creatinine was dissolved in acid solution.[25]

Measurement of the activities of serum glutamate oxaloacetate transaminase (SGOT) and serum glutamate pyruvate transaminase (SGPT)

Serum GOT and GPT activities were measured by standard routine methods. The enzyme activities were expressed as IU/L.[26]

Preparation of homogenate, measurement of lipid peroxidation level and reduced glutathione content in rat liver, heart and kidney tissues

The liver, heart and the kidney tissues were separately homogenized (10%) in ice-cold 0.9%saline (pH 7.0) with a Potter Elvenjem glass homogenizer for 30 s and lipid peroxides in the homogenate were determined as thiobarbituric acid reactive substances (TBARS) according to the method of Buege and Aust[27]with some modification as adopted by Bandyopadhyay et al.[28]Briefly, the homogenate was mixed with thiobarbituric acid–trichloro acetic acid hydrochloric acid (TBA–TCA) reagent with thorough shaking and 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 nmoles of TBARS/mg protein.

Reduced GSH content (as acid soluble sulfhydryl) of the above mentioned tissues were estimated by its reaction with DTNB (Ellman's reagent) following the method of Sedlak and Lindsey[29] with some modifications [28]. The tissues were homogenized (10%) in 2 mM ice-cold ethylenediaminetetraacetic acid (EDTA). The homogenate was mixed with Tris–HCl buffer, pH 9.0, followed by DTNB for color development. The absorbance was measured at 412 nm using a UV–Vis spectrophotometer. The values were expressed as nmoles/mg protein.

Measurement of the activities of superoxide dismutase (SOD; Cu-Zn type) and catalase (CAT)

Copper-zinc superoxide dismutase (SOD1) activity was measured by hematoxylin autoxidation method of Martin et al.[30]Briefly, 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 and the supernatant, thus obtained, 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.[30]

Catalase was assayed by the method of Beers and Sizer[31]with some modifications as adopted by Chattopadhyay et al.[32]. 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 supernatant, thus obtained, was then collected and 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, after which 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 H_2O_2 spectrophotometrically at 240 nm. The enzyme activity was expressed as μmoles H_2O_2 consumed / min / mg protein.

Measurement of tissue protein content

Protein was estimated by the method of Lowry *et al* (1951) [33]using bovine serum albumin (BSA) as the standard.

Statistical analysis

Each experiment was repeated at least three times with different sets of male Wistar rats. Data are presented as means ± S.E. Significance of mean values of different parameters between the treatment groups were analyzed using one way analysis of variances (ANOVA) after ascertaining the homogeneity of variances between the treatments. Pairwise comparisons were done by calculating the

least significance. Statistical tests were performed using Microcal Origin version 7.0 for Windows.

RESULTS AND DISCUSSION

The present studies investigated whether CuLE has a protective effect against lead-induced oxidative stress in experimental rats.

Table1 shows the levels of the bio-markers of organ damage i.e., serum glutamate pyruvate transaminase (SGPT), serum glutamate oxaloacetate transaminase (SGOT) and blood creatinine for hepatic, cardiac and renal damages respectively.

Table 1 : Table shows activities of SGPT, SGOT and the levels of blood creatinine in lead acetate treated and CuLE protected rats.

Groups	SGPT (IU/L)	SGOT (IU/L)	Blood Creatinine (mg%)
Control	10.2 ± 0.61	8.90 ± 0.65	1.72 ± 0.086
Lead	22.2±1.23*	19.2± 0.86*	5.01 ±0.065*
Lead+CuLE (12.5mg/Kg BW)	17.6±0.98	14.3 ± 0.68	3.53 ±0.068
Lead+CuLE (25mg/Kg BW)	13.9±0.84**	12.6 ± 0.80	2.31 ± 0.046
Lead+CuLE (50mg/Kg BW)	10.9± 0.75**	9.01 ± 0.68**	1.84 ± 0.098**
Lead+CuLE (100mg/Kg BW)	10.3±0.76**	8.92 ± 0.70**	1.80 ± 0.085**

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;

Table 2: Table shows the levels of lipid peroxodation, reduced glutathione content as well as the activities of superoxide dismutase and catalase of the hepatic tissues in lead acetate treated and CuLE protected rats.

Group	Lipid perxidation (nmoles of TBARS / mg protein)	Reduced Glutathione content (nmoles/mg protein)	Cu-Zn SOD activity (Units/min/mg protein)	Catalase activity (Units/min/mg protein)
Control	0.121 ± 0.006	27.4 ± 0.414	3.85 ± 0.182	201.5±10.0
Lead	0.335 ± 0.005*	48.1 ± 0.369*	5.98 ± 0.008*	278.3± 11.2*
Lead+CuLE (12.5mg/Kg BW)	0.248 ± 0.006	38.1 ± 0.335	5.23 ± 0.044	241.2± 12.2
Lead+CuLE (25mg/Kg BW)	0.194 ± 0.007	29.7 ± 0.301	4.25 ± 0.207	228.4± 10.1
Lead+CuLE (50mg/Kg BW)	0.144 ± 0.008**	28.2 ± 0.395**	3.93 ± 0.223**	218.1± 11.2**
Lead+CuLE (100mg/Kg BW)	0.130 ± 0.008**	27.9 ± 0.303**	3.85 ± 0.248**	205.5± 10.7**

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;

Table 3: Table shows the levels of lipid peroxidation and reduced gluthione as well as the activities of superoxide dismutase and catalase of cardiac tissue in lead acetate treated and CuLE protected rats.

Group	Lipid peroxidation (nmoles of TBARS / mg protein)	Reduced gluthione (nmoles/mg protein)	Superoxide dismutase activity(Cu-Zn SOD) (Units/min/mg protein)	Catalase activity (Units/min/mg protein)
Control	0.147 ± 0.008	13.6 ± 0.073	3.595±0.064	18.2±0.677
Lead	0.550 ± 0.008*	21.7 ± 0.618*	6.063±0.163*	25.6±0.633*
Lead+CuLE (12.5mg/Kg BW)	0.418 ± 0.006	17.4 ± 0.478	5.315±0.196	21.5±0.658
Lead+CuLE (25mg/Kg BW)	0.328 ± 0.009	14.1 ± 0.215	4.907±0.085	20.3±0.577
Lead+CuLE (50mg/Kg BW)	0.181 ± 0.008**	13.9 ± 0.343**	3.975±0.112**	19.1±0.684**
Lead+CuLE (100mg/Kg BW)	0.154 ± 0.005**	13.3 ± 0.138**	3.525±0.132**	18.2±0.668**

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;

Table 4: Table shows the levels of lipid peroxidation and reduced gluthione as well as the activities of superoxide dismutase and catalase of renal tissue in lead acetate treated and CuLE protected rats.

Group	Lipid peroxidation (nmoles of TBARS / mg protein)	Reduced gluthione (nmoles/mg protein)	Superoxide dismutase activity(Cu-Zn SOD) (Units/min/mg protein)	Catalase activity (Units/min/mg protein)
Control	0.127± 0.008	16.5 ± 0.368	1.792±0.159	14.34±0.549
Lead	0.302± 0.007*	26.1 ± 0.265*	3.895 ±0.148*	18.91±0.453*
Lead+CuLE (12.5mg/Kg BW)	0.242± 0.006	20.9 ± 0.288	3.243 ±0.156	16.32±0.639
Lead+CuLE (25mg/Kg BW)	0.182± 0.007	17.4 ± 0.425	2.392 ±0.223	15.22±0.324
Lead+CuLE (50mg/Kg BW)	0.135± 0.004**	16.6 ± 0.246**	1.827±0.179**	14.65±0.356**
Lead+CuLE (100mg/Kg BW)	0.130± 0.006**	16.5 ± 0.395**	1.782±0.146**	14.4±0.254**

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;**

All the three parameters studied were found to be significantly increased compared to control. However, when the rats were pre-treated with CuLE, a dose dependant protection of the activities of SGOT and SGPT was observed. The levels of blood creatinine were also found to be protected dose-dependently. The results indicate that CuLE is capable of stabilizing the plasma membrane as well as repair of hepatic, cardiac and renal tissue damage caused by lead acetate.

SGPT increases in infections, hepatitis, etc. In hepatocellular disorders SGPT rises sooner, faster and higher than SGOT. All the three parameters studied were found to be significantly increased compared to control. SGOT increases in myocardial infarction but is not specific for this condition. Moderate increase in SGOT is observed in hepatitis, portal and biliary cirrhosis etc. In cirrhosis of liver it increases more than SGPT. SGOT also increases in renal insufficiency. The level of blood creatinine level increases in pathological situation like nephritis and other renal diseases and elevated creatinine is evident of impairment of kidney function.[26, 34]

Treatment of rats with lead acetate at a dose of 15 mg/kg BW intraperitoneally for a period of 7 consecutive days caused oxidative stress-induced damage in the hepatic, cardiac and renal tissues as reflected from the alterations in the levels of biomarkers of oxidative stress and the status of antioxidant enzymes.[26]

Table 2,3 and 4. demonstrate the levels of lipid peroxidation, reduced glutathione and also the activities of the two most important antioxidant enzymes i.e., SOD and catalase in the liver, heart and kidney tissues of the rats of different experimental groups.Glutathione protects the cells from free radical induced oxidative damage.[35, 36]

Exposure of rats to lead acetate cause an increase in the level of reduced glutathione (GSH) concentrations. Species and individual variation in sensitivity to Pb poisoning among animals may be due to differential resistance to oxidative stress.[2, 6]

Pre-treatment of rats with CuLE was found to protect dose-dependently the alterations in the level of GSH in all three tissues. In our experiment, a dose-dependent protection of the lipid peroxidation levels of all the three tissues was observed when the rats were pre-treated with CuLE. Lipid peroxidation is considered as one of the primary biomarkers of oxidative stress.[27]The results indicate that CuLE at a dose of 50 mg/Kg BW almost completely protected the GSH levels in liver, heart and kidney tissues of rat.

The production of oxidants and the protection against them is intrinsic to every living cell.[2] To minimize oxidative damage, organisms developed antioxidative mechanisms thought to be triggered by increased ROS production.[2] Oxidants such as ROS are taken care of by antioxidative defense system that consists of enzymes and metabolites in all subcellular compartments.[24, 32] In stress conditions, normal capacities of these mechanisms are insufficient, triggering cells to increase and expand their antioxidative network. In this context, catalase and SOD are the two important antioxidant enzymes that are affected by lead acetate. Superoxide oxide dismutase (SOD) and catalase (CAT) together constitutes an important defense against reactive oxygen species (ROS). Catalase catalyzes the conversion of hydrogen peroxide to water and oxygen. Thus, catalase reduces the tissue injury by removing the H_2O_2. Superoxide dismutase (SOD) catalyzes the conversion of superoxide anion free radical to H_2O_2 through dismutation reaction.[30, 31]

The activity of SOD and CAT have been found to be increased in heart, liver and kidney tissues following administration of lead acetate in rats. Pre-treatment of rats with CuLE prevented the increase of the activity of both the enzymes in a dose dependent manner. Here also the activities of these enzymes were found to be protected completely at a dose of 50 mg/kg BW. This further confirms that CuLE is capable of providing protection against lead acetate induced oxidative stress in liver, heart and kidney tissues of rat.

In conclusion, CuLE seems to provide protection to rat hepatic, cardiac and renal tissues against lead-induced oxidative stress through its antioxidant activity. The antioxidant activity might be exerted through modulation of antioxidant enzymes in the form of oxidative stress although possibility of the extract acting as a ROS scavenger cannot be ruled out and needs through investigation. The results presented here may be of future therapeutic significance particularly in the areas where man is chronically exposed to lead either occupationally or environmentally. As Curry leaves are part of a regular diet in India and many parts of the world, it may also serve as a possible nutritional intervention and the extract itself or fractions obtained there from may be used also as a future nutritional supplement to combat oxidative stress-induced tissue damage in the people exposed to lead.

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