Aqueous Curry leaves extract protects against lead induced oxidative stress in rat spleen: a new insight

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ABSTRACT

Background: Lead is a toxic heavy metal and a persistent environment pollutant. Exposure to lead induces oxidative stress and causes oxidative stress mediated damages in various organs including spleen in a living system. Murraya koenigii is a popular Indian spice herb with established antioxidant properties. We have earlier checked the potential of the Murraya koenigii leaves extract against lead induced oxidative stress in organs like heart, liver and kidneys. Aims and objectives: Aim of the study is to find therapeutic potentials of aqueous Curry Leaf (Murraya koenigii) Extract (MUR) against lead induced oxidative damage in spleen tissue using rat as an animal model. The objectives are to study the alterations of various stress parameters in lead induced toxicity in spleen and amelioration of the same with MUR. Methods: Rats were intra-peritoneally injected with lead acetate (15mg/kg body weight [bw]). Another group was pre-treated with MUR (50 mg / kg, fed orally). The positive control group was fed MUR (50 mg / kg bw), and the control animals received treatment with vehicle i.p. for 7 consecutive days. The alterations in the activity of the different bio-markers of organ damage, biomarkers of oxidative stress, activities of the antioxidant and some of the mitochondrial enzymes were studied. Histo-morphology was studied through routine H-E staining and concentration of lead in spleen tissue was estimated using atomic absorption spectrophotometry (AAS). Detailed changes in splenic cyto-architecture were studied using scanning electron microscopy (SEM). Results: Lead caused alterations in all the parameters studied. All these changes were mitigated when the rats were pre-treated with MUR. Also, the concentration of lead in tissues was found to be significantly lower in animals treated with MUR along with lead. Histo-morphological changes induced by lead exposure in spleen were significantly ameliorated with pre-treatment of the animals with MUR. Conclusions: The results indicate that the MUR ameliorates lead-induced oxidative stress mediated damage in spleen in experimental rats. The antioxidant and free radical scavenging mechanisms may be responsible for such protective effects. MUR may have future therapeutic relevance in the prevention of lead-induced toxicity and damage in spleen in humans exposed occupationally or environmentally to lead and may be used for development of new drug formulation of herbal origin with less cytotoxic effects.

KEY WORDS: Antioxidants, atomic absorption spectrophotometry, curry leaves, lead, oxidative stress, scanning electron microscopy, spleen, rat

1.INTRODUCTION:

Lead is a highly toxic metal found on the crust of the earth. Soluble salts of lead are easily absorbed into the blood stream from the intestine which is highly poisonous and act as a cumulative poison. Total amount of lead in the body is called ‘Body Burden’, which is found to be less with increased age. Lead binding Protein binds to lead and thus helps in its absorption. Adult bones and teeth contain 95% of ‘Body Burden’ that impairs erythropoeisis. After being absorbed the first load of lead passes through and accumulates in the vascular bed of the digestive tract, followed by the liver. From liver the lead laden blood passes to the heart, then lung, back to the heart and then onwards to different parts of the body. Lastly, the kidneys face with reduced load of lead. While passing through the kidney, lead from the blood gets partially deposited in the renal tissue and partially gets secreted in the renal tubule and gets excreted in the urine. Lead burden of the body is gradually cleared through excretion in urine, if a further increase in the burden is avoided by restricting exposure to lead.
Lead enters spleen cells from circulation and gets deposited there, i.e., accumulates and causes generation of free radicals which damage the different cellular elements leading to their respective functional impairment which ultimately culminates to severe oxidative damage of the organ with consequential disruption of functions. There has been report of lymphocyte damage and inhibitory effect on erythropoiesis can be seen in spleen of lead poisoned animals.

Studies suggest that following lead exposure, there occur inhibition of phagocytosis, nitric oxide release, induction DNA fragmentation in spleen suggesting the apoptotic death of the target cell. Evidence has also been presented that inhibition of macrophage functional responses implicated alteration of humoral and cell mediated immunity. The mechanism being the destruction of the membranes of the cells of the spleen due to free radical attack to the membrane lipid components and occurrence of lipid peroxidation. The free radical generation is stimulated by the lead deposited in the cellular matrix[1].

Therapeutic potentials of plant extracts and derived molecules are being investigated by researchers all around the world with great interest for development of new drugs of herbal origin with less cytotoxic effects[2,3].

According to World Health Organization (WHO), almost 80% of the world population uses herbal medicine for basic aspect of primary health care (WHO, 2008). Antioxidants protect against oxidative stress and thus prevent organs from oxidative damages. Environmental or occupational or even accidental exposure to certain heavy metals (e.g. lead, cadmium, mercury etc) might lead to elevated concentration of the same in living body, which might in turn can induce generation of free radicals and cause oxidative stress and lead to oxidative stress induced damages[4-6]. The curry leaves (Murraya koenigii L.) are used widely in South-East Asia including India as a spice herb and consumed as one of the important dietary component. The leaves of Murraya koenigii are known to possess various types of pharmaceutically important components and are in use for ages in folk and traditional medicines[7,8]. Herein, we demonstrate that administration of lead to rats cause oxidative stress-mediated damages to spleen and pre-treatment of rats with MUR ameliorates such damages to the organ possibly through its antioxidant mechanisms.

2. METHODS AND MATERIALS

2.1. Animal Selection and Conditioning
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) [IAEC/PROPOSAL/DB-2/2010] 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 and continuously monitored animal experimentations.

2.2. Treatment and collection of tissue samples
The animals were marked and divided into four experimental groups in such a way that the average weight of animals in each group remained similar. Each group of animals comprised of 6 rats. The groups were as follows:

GROUP I: Control;
GROUP II: Lead treated;
GROUP III: Murraya treated (Curry leaf extract treated);
GROUP IV: Murraya + Lead treated;

2.3. Preparation of Lead Acetate [Pb (OOCH)₂] Solution
150 mg of lead acetate was dissolved in 10 ml of distilled water[4].

2.4. Plant Material
Fresh, green Curry leaves [Murraya koenigii (L.) Spreng] were collected. 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/1-41/2010/Tech.II/232.

2.5. Preparation of an aqueous extract of the Curry leaves
The 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 °Celsius for two hours till the leaves were dry enough and crispy so as they could be crushed into a coarse dust with mortar and pestle and then they were grinded in a mechanical grinder to fine dusts and were stored in air tight Tarson bottles at -20°Celsius until further use. For aqueous extract preparation, the dried leaf dusts were soaked...
overnight in double distilled water (7.5g per 100 ml), filtered through loin cloth (fine cotton cloth). 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 °Celsius. The contents of the tubes were then lyophilized and the resulting lyophilized material (a dry powdery material) [herein referred to as the aqueous extract] was stored at -20 °Celsius until further use. A definite amount of the lyophilized material was always freshly dissolved in double distilled water to give a particular concentration and this solution was used in our assay systems. Any leftover of this solution was discarded.

2.6. Animal Treatment
After acclimatization to laboratory condition, animals of the Murraya group and the ones of the Murraya + lead group were fed the aqueous extract of Curry leaves made in normal drinking water, at a dose of 1ml kg⁻¹ body weight of the rats for 7 consecutive days. An hour after the Curry leaves treatment, animals of the lead treated group and the ones of the Murraya+lead groups were injected, lead acetate solution, intra-peritoneally, at a dose of 15mg kg⁻¹ body weight (LD₅₀ is 65 mg/ kg bw) on consecutive seven days. The control group and the ones of the Murraya+lead groups were injected, lead acetate solution, intra-peritoneally, at a dose of 1ml kg⁻¹ body weight of the rats for 7 consecutive days. An ml of supernatant was then mixed with 2 ml of 0.8(M) TRIS-HCl (pH-7.4). The mixture was then treated with 100 µL DTNB and mixed well. The optical density was noted in a UV/VIS spectrophotometer at 412 nm.

2.7. Animal Sacrifice and Collection of Blood & Tissue Samples
At the end of the treatment period, the animals of each group were kept fasted over night. Body weight of animals of each group were checked and recorded. Each animal was anaesthetized using mild ether, sacrificed following cervical dislocation, and blood was collected from hepatic vein and was allowed to clot for serum to separate out and then centrifuged at 2500 rpm for 15 minutes. Serum was collected with auto-pipette in individual microfuge tube and stored at −20°C. Organ i.e. spleen was collected separately from each animal being removed, washed in ice cold saline, and bottled dry, immediately weighed and stored at -20° C until analysis.

2.8. Preparation of Tissue Homogenate
A 10% tissue homogenate of spleen was prepared in two separate buffer solutions, ice cold 0.1 M phosphate buffer (ph 7.4) and 1mM EDTA buffer respectively (as per the biochemical analysis required), using a glass homogenizer. The homogenates were prepared freshy and kept in ice and processed for further biochemical analysis.

2.9. Tissue Lipid Peroxidation (LPO) Level
LPO level was determined according to the method of Buege and Aust (1978) [19]. One millimolar EDTA was used in the reaction mixture to chelate iron and reduce its interference in the peroxidation reaction of unsaturated fatty acids. After completion of the total reaction, the optical density was read at 533nm. The molar extinction coefficient, 1.56 x 10⁵ cm²/m mole was used to calculate the malonaldehyde production.

2.10. Reduced Glutathione (GSH) Content
GSH content of spleen tissue was estimated by the method of Lindsay and Sedlak (1968) [10]. Accordingly, a 10% tissue homogenate was prepared and treated with 10% TCA containing 2mM EDTA to allow precipitation of proteins. The mixture was centrifuged at 500 rpm at 4°C. One ml of supernatant was then mixed with 2 ml of 0.8(M) TRIS-HCl (pH-7.4). The mixture was then treated with 100 µL DTNB and mixed well. The optical density was noted in a UV/VIS spectrophotometer at 412 nm.

2.11. Estimation of Cu-Zn Superoxide Dismutase Activity (SOD 1)
Copper-zinc superoxide dismutase (Cu-Zn SOD or SOD1) activity was measured by hematoxylin autooxidation method of Martin et al., 1987 [11]. One ml of 10% tissue homogenate (made in 50 mM Phosphate buffer) were taken in centrifuge tubes, to which 1 ml of 0.1 M. phosphate buffer was added, centrifuged for 15 min at 7000 rpm, then the supernatants were taken in microfuge tubes, marked and placed in ice bath. In the cuvette, 2.95 ml 50 mM phosphate buffer and 50 ul Hematoxylin solution were taken, mixed well by closing the mouth of the tube with parafilm and recorded the optical density (O.D.) using a photoelectric colorimeter at 250 nm by setting the instrument at zero density with distilled water for 10 min. at every 30 sec interval. Thus, after estimating the auto oxidation of hematoxylin, each sample supernant was taken 50 ul along with 2ml 50 mM Phosphate and 50 ul Hematoxylin and their optical densities (O.D. s) were recorded as before.

2.12. Estimation of Mn Superoxide Dismutase Activity (Mn-SOD or SOD2)
Manganese superoxide dismutase activity was assayed by method of Marklund and Marklund [12] which involves inhibition of pyrogallol autooxidation in the presence of EDTA at pH 8.2. Briefly, a weighed amount of spleen tissue was homogenized (10%) in ice-cold 50 mM Tris-HCl buffer containing 0.1mM EDTA, pH 7.4 and centrifuged first, at 2,000 rpm for 5 min, and the supernatant thus obtained, 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. To 50 µL of the suspended pellet, 50 mM of Tris-HCl buffer (pH8.2), 30 mM EDTA and 2mM of pyrogallol were added. An increase in absorbance was recorded at 420 nm for 3
min in a UV / VIS spectrophotometer. One unit of enzyme activity is 50% inhibition of the rate of autooxidation of pyrogallol as determined by change in absorbance/min spectrophotometrically at 420 nm. The enzyme activity was expressed as units/min/mg of tissue protein.

2.13. Estimation of Catalase Activity (CAT)
Catalase activity was measured by the method of Beers and Sizer (1952) [15]. A weighed amount of tissue was homogenized (5%) in ice-cold 50 mM phosphate buffer, pH 7.0. The homogenate was centrifuged in cold at 12,000g for 12 min. The supernatant, thus obtained, was then collected and incubated with 0.01 ml of absolute ethanol at 4 °C for 30 min, after which 10% Triton X-100 was added so as to have a final concentration of 1%. The sample, thus obtained, was used to determine catalase activity by measuring the breakdown of H₂O₂ spectrophotometrically at 240 nm. The enzyme activity was expressed as umoles of H₂O₂ consumed/min/mg tissue protein.

2.14. Estimation of Lead Metal (Pb) Content in the Rat Spleen Tissues By Atomic Absorption Spectrophotometry (AAS)
The tissue samples were prepared and the lead content was measured as per the protocol mentioned in the book of the Varian AA240 Atomic Absorption Spectrophotometer, GTA 120 (Graphite tube atomizer) at Chemical Engineering Department of University college of Science and Technology, Kolkata. The tissue samples were incubated over night at 37°C and subsequently the dry weight was taken and placed in a conical flask containing measured volume of double distilled water. Thereafter, 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 [8].

2.15. Determination of activities of the Xanthine Oxidase (XO) and Xanthine Dehydrogenase (XDH)
Xanthine oxidase activity of the rat tissues was assayed by measuring the conversion of xanthine to uric acid following the method of Greenlee and Handler (1964) [14]. Briefly, the weighed amount of spleen tissue was homogenized in cold (10%) in 50 mM phosphate buffer, pH 7.8. The homogenates were centrifuged at 500g for 10 min. The supernatant was further centrifuged at 12,000g for 20 min in cold. The new supernatant, thus obtained, was collected and used for spectrophotometric assay of the enzyme 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 tissue protein.

The XDH activity was measured by following the reduction of NAD+ to NADH according to the method of Strittmatter (1965) [19] with some modifications. In brief, the weighed amount of spleen tissue was 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, thus obtained, was further centrifuged in cold at 12,000g 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.

2.16. Measurement of the Activities of the Mitochondrial Kreb’s Cycle Enzymes
Spleen tissue was homogenized (10%) in ice-cold 50 mm phosphate buffer, pH 7.4 with a Potter Elvenjem glass homogenizer (Belco Glass, Inc., Vineland, NJ, USA) for 30 s. The homogenate was then centrifuged at 500 g for 10 min, and the supernatant was again centrifuged at 12,000g for 15 min to obtain the mitochondrial fraction.

The pellet thus obtained was re-suspended in the buffer and used for assaying the mitochondrial enzymes. Pyruvate dehydrogenase activity was measured spectrophotometrically according to the method of Chretien et al. [14] with some modifications by following the reduction of NAD+ to NADH at 340 nm using 50 mm phosphate buffer, pH 7.4, 0.5 mm sodium pyruvate as substrate, and 0.5 mm NAD+ in addition to enzyme. The enzyme activity was expressed as Units/mg protein. Isocitrate dehydrogenase (ICDH) activity was measured according to the method of Duncan et al. [14] by measuring the reduction of NAD+ to NADH at 340 nm with the help of a UV–VIS spectrophotometer. One milliliter assay volume contained 50 mm phosphate buffer, pH 7.4, 0.5 mm isocitrate, 0.1 mm MnSO₄, 0.1 mM NAD+, and the suitable aliquot of the enzyme. The enzyme activity was expressed as Units/mg protein. Alpha-ketoglutarate dehydrogenase activity was measured spectrophotometrically according to the method of Duncan et al. [17] by measuring the reduction of 0.35 mM NAD+ to NADH at 340 nm using 50 mm phosphate buffer, pH 7.4 as assay buffer, and 0.1 mM a-ketoglutarate as substrate. The enzyme activity was expressed as Units/mg protein. Succinate dehydrogenase activity was measured spectrophotometrically by measuring the reduction of NAD+ to NADH with some modifications by following the method of Duncan et al. [14] by measuring the reduction of 0.35 mM NAD+ to NADH at 340 nm using 50 mm phosphate buffer, pH 7.4 as assay buffer, and 0.1 mM a-ketoglutarate as substrate. The enzyme activity was expressed as Units/mg protein.
by following the reduction of potassium ferricyanide (K$_3$Fe(CN)$_6$) at 420 nm according to the method of Veeger et al. [18] with some modifications. One ml assay mixture contained 50 mM phosphate buffer, pH 7.4, 2% (w/v) BSA, 4 mM succinate, 2.5 mM K$_3$FeCN$_6$, and a suitable aliquot of the enzyme. The enzyme activity was expressed as Units/mg protein. The measurement of mitochondrial respiratory chain enzymes NADH-Cytochrome c oxidoreductase activity was measured spectrophotometrically by following the reduction in oxidized cytochrome c at 565 nm according to the method of Goyal and Srivastava [19]. One milliliter assay mixture contained in addition to enzyme 50 mM phosphate buffer, 0.1 mg BSA, 20 mM oxidized cytochrome c, and 0.51 M NADH. 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 550 nm according to the method of Goyal and Srivastava [18]. One ml assay mixture contained 50 mM phosphate buffer, pH 7.4, 40 mM reduced cytochrome c, and a suitable aliquot of the enzyme. The enzyme activity was expressed as Units/mg protein.

2.17. Tissue Protein Content

Protein content of the spleen tissue was estimated by the method of Lowry et al (1951) [20] using bovine serum albumin (BSA) as the standard protein. 0.1(M) 200 μL NaOH was used as the blank. 20 μL homogenate was mixed with 180 μL of 0.1(M) NaOH and was marked as Sample. Then, 165 μL Folin-Ciocalteau reagents was added to all the tubes and mixed. After waiting for 30 minutes, the optical density was measured using a photoelectric colorimeter using green filter (570nm) after setting the zero density with blank. A standard curve was prepared by taking aliquots of standard BSA ranging from 10 μL to 120 μL.

2.18. Tissue morphological and histochemical studies

2.18.1. Staining of tissue sections using hematoxylin-eosin (H & E)

Immediately following sacrifice of the animals, spleen was surgically extirpated and fixed in 10% formalin and embedded in paraffin following routine procedure as described earlier [8]. Tissue sections (5 μM thick) were prepared and stained with hematoxylin-eosin. The stained tissue sections were examined under a Leica microscope and the images were captured with a digital camera attached to it.

2.19. Scanning electron microscopy (SEM)

Small pieces of spleen tissue were fixed overnight with 2.5% glutaraldehyde. After washing three times with PBS, the pieces were dehydrated for 10 min at each concentration with a graded ethanol series (50, 70, 80, 90, 95 and 100%). The dehydrated pieces were immersed in pure tert-butyl alcohol and were then placed into a 4°C refrigerator until the tert-butyl alcohol solidified. The frozen tissue pieces were dried by placing them into a vacuum bottle. The tissue surface morphology was evaluated by scanning electron microscopy (SEM; Zeiss EVO 18 model EDS 8100).

2.19. Statistical Analysis

Each measurement was repeated at least three to four times. 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. Pairwise comparisons were done by calculating the least significance. Statistical tests were performed using Microcal Origin version 8.0 for Windows.

3. RESULTS

3.1. Animal Behavior

Lead treated rats became less active and drowsy. During the last 2-3 days of the treatment it was observed that the rats of the lead treated group, became more restless and aggressive. The rats of the control group and the Murraya leaves extract treated group were observed to exhibit a calm and normal behavior. The rats of the Murraya leaves extract +lead group were not much aggressive and but remained active.

3.2. Body Weights

It was found that the animals of the four groups showed normal growth pattern before and during the period of study.

3.3. Tissue Weight

There was no significant change in the weights of the spleen tissues of the individual animals from different groups.

3.3. Level of lipid peroxidation (LPO) and reduced glutathione content (GSH)

Lead treatment significantly increased the level of LPO and GSH values in the spleen of rats (83.33% and 54.85% Vs. control) compared to that in control (°P < 0.001). Oral treatment with MUR alone did not cause any significant change in the values of LPO and GSH compared to that of control. Pre-treatment of the animals with MUR significantly lowered the values of LPO and GSH compared to that of lead treated group (49.78% and 26.17 % respectively higher Vs. lead) than the lead treated values. (##P< 0.001) [Fig.1 A & B].

A large number of antieplipetic drugs (AEDs) are available in market including phenytoin, carbamazepine, valproic acid, etc.[2] However,
Fig. 2. Effect of aqueous extract of Curry leaves (50mg/kg BW) against lead-induced alteration in the activities of cytosolic Cu–Zn–SOD [A], the mitochondrial Mn–SOD [B] and the catalase [C] in rat splenic tissue.

Values are expressed as Mean ± SE of 6 animals in each group. #P<0.001 compared to control; ##P< 0.001 compared to lead treated group; CON = control, injected with vehicle; LEAD= 15 mg/kg bw Pb (CH3COO)2 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)2 injected.

3.4. Status of tissue lead content

Figure 3 demonstrates accumulation of lead in spleen tissue following treatment of rats with lead acetate at a dose of 15 mg / kg bw (i.p.) for a period of seven consecutive days. However, when the rats were pre-treated with MUR at a dose of 50 mg / kg bw (fed orally), the tissue lead content was found to be reduced significantly in (48.75%, P<0.001 Vs. Pb-treated group) [Fig 3.A]. The accumulation of lead acetate in spleen of lead treated rats was also evident from the pictographic representation of spleen tissues of rats after the period of treatment. And it was also evident that pre-treatment with MUR caused a significant decrease in the tissue lead content (reduced visible patches) [Fig.3.B.].
3.5. Status of activities of pro-oxidant enzymes

The activities of splenic xanthine oxidase (XO) and xanthine dehydrogenase (XDH), as well as the total enzyme activity, i.e., XO plus XDH and XO : XDH and the ratio, all increased significantly following treatment of rats with lead acetate (0.96 folds, 1.22 folds, 1.15 folds and 29.37% increase respectively in splenic tissue, *P < 0.001 Vs. control). All these parameters were significantly protected from being increased when the rats were pre-treated with MUR (45.89%, 62.50%, 58% and 9.45% decrease respectively in splenic tissue vs. lead acetate treated groups, **P < 0.001 Vs. Lead acetate-treated group). However, MUR alone has no effect on the activities of xanthine oxidase (XO) xanthine dehydrogenase (XDH), the total enzyme activity, i.e., XO plus XDH and, XO: XDH [Fig. 4.A, B, C and D].
3.6 Status of the activities of pyruvate dehydrogenase and some of the Kreb’s cycle enzymes

**Figure 5A.** reveals that treatment of rats with lead acetate inhibits rat splenic pyruvate dehydrogenase activity (43.24% decrease, \#P < 0.001 vs. their respective control). Pre-treatment of rats with MUR significantly protected the enzyme activity from being decreased in spleen tissue (68.28% increase \##P < 0.001 vs. Lead acetate-treated group). However, MUR alone was found to have no effect on the activity of this enzyme.

**Figure 5B.** also reveals that treatment of rats with lead acetate significantly decreased the activity of isocitrate dehydrogenase in splenic tissue (83.93%, \#P < 0.001 vs. control). The activity of the enzyme in the spleen was found to be protected significantly from being decreased when the rats were pre-treated with MUR (4.63 folds, \##P < 0.001 Vs. Lead acetate-treated group). However, MUR alone has no effect on the activity of isocitrate dehydrogenase of the splenic tissue.

**Figure 5C.** reveals that treatment of rats with lead acetate inhibits alpha-keto glutarate dehydrogenase (\(\alpha\)-KGDH) activity in splenic tissue (75%, *P < 0.001 vs. control). The activity of the enzyme was found to be significantly protected from being decreased in the spleen when the rats were pre-treated with MUR at the above mentioned dose (2.71 folds, **P < 0.001 Vs. Lead acetate-treated group). However, MUR alone has no significant effect on the activity of \(\alpha\)-KGDH.

**Figure 5D.** reveals that treatment of rats with lead acetate significantly decreased the activity of the enzyme succinate dehydrogenase in splenic tissue (27.72%, \#P < 0.001 Vs. control). The activity of the enzyme in the spleen was found to be protected significantly from being decreased when the rats were pre-treated with MUR (33.9%, \##P < 0.001 Vs. Lead acetate-treated group). However, MUR alone has no effect on the activity of isocitrate dehydrogenase in the splenic tissue.

*Fig.5. Effect of aqueous extract of Curry leaves (50mg/kg BW) against lead induced alteration in activities of pyruvate dehydrogenase [A], isocitrate dehydrogenase [B], alpha keto glutarate dehydrogenase [C] and succinate dehydrogenase [D] in rat splenic tissue.*

Values are expressed as Mean ± SE of 6 animals in each group. *P<0.001 compared to lead treated group; CON = control, injected with vehicle; LEAD= 15 mg/kg bw Pb(CH\(_3\)COO)\(_2\), 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(CH\(_3\)COO)\(_2\), injected.
3.7. Histopathological studies of spleen tissue

Histological studies with routine eosin and haematoxylin stain shows marked damage in spleen of rats exposed to lead acetate (15 mg/kg bw Pb(CH₃COO)₂ injected i.p. for the above mentioned time-period). The integrity of the red and white pulp in spleen tissues of lead exposed rats was observed to be affected adversely in animals who got only lead treatment. Neutrophil infiltration was also observed in spleen of lead exposed rats. Such observations indicate lead induced marked damage and disruption of normal tissue architecture of spleen of rats. No such marked damage was observed in rats who were pre-treated with MUR (50 mg/kg bw [fed orally] + 15 mg/kg bw Pb(CH₃COO)₂ injected for the time-period mentioned in methods section) . The condition of the tissue architecture was maintained and protected compared to that of lead treated group indicating protective impact of MUR against lead induced damage in rat spleen. No significant differences were observed in the sections of the spleen tissues of only MUR treated (i.e., positive control) and control group of rats [Figure 6].

![Figure 6](image1)

**Figure 6.** Histopathological studies of splenic tissue [200X]

3.8. Studying spleen tissue through scanning electron microscopy (SEM):

Figure 7. demonstrates images obtained through SEM of spleen tissue sections. Images of lead acetate (15mg/kg bw. i.p.) treated sections showed damages of spleen tissue surface architecture indicating almost complete disruption. But this was found to be partially ameliorated when the rats were pre-treated with MUR [50 mg/kg bw (fed orally) + 15 mg/kg bw Pb(CH₃COO)₂ i.p. for the time-period mentioned in methods section]. No significant changes were found in groups treated with MUR alone, i.e., positive control group [50 mg/kg bw (fed orally) for the time-period mentioned in the methods section].

![Figure 7](image2)

**Figure. 7.** Scanning electron microscopic images of the rat spleen tissue. Red arrows indicate the damaged portion of the spleen tissue following treatment with lead acetate (15mg/kg bw. i.p. for the time-period mentioned in methods section) ; First row represents scanning electron microscopic image of spleen tissue captured at 5KX magnification [i.e., 5000 K], second row represents images captured at 10 K X magnification [i.e., 10,000 X] and third row represents images captured at 25 K X magnification [i.e., 25,000 X]. Groups: Control, MUR [50 mg/kg bw of Murraya (fed orally)], Lead [15 mg/kg bw Pb(CH₃COO)₂, i.p.], MUR + Lead [50 mg/kg bw MUR (fed orally) + 15 mg/kg bw Pb(CH₃COO)₂ i.p.].

4. DISCUSSION

Spleen is a soft organ located right under the rib cage in the upper left side of the abdomen. It is covered by fibrous tissue called the splenic capsule that supports the constituent blood vessels and tissues. This organ is primarily responsible for enhancing the immune system of the body. Spleen is made of two tissues: the white pulp and the red pulp. The white pulp produces white blood cells (lymphocytes) which generate antibodies to fight invasion of any foreign substances [21].

Lead (Pb) enters our circulation through various routes and travels to various essential organs of our body and gets deposited therein. Lead is known to alter the functions of numerous organs and organ systems [22], including the hematopoietic and immune system [23, 24]. Lead is known to affect the expression of various genes associated with immune system [24]. Deposition of lead in spleen leads to change in the normal activities of spleen and as a result the immunogenic capacity is greatly hampered. Earlier studies reveal that Pb exposure
induces the generation of reactive oxygen species (ROS) and impairs the antioxidative system of the body [13]. Lead is absorbed in spleen cells from circulation and gets deposited there, i.e., accumulates there and causes generation of free radicals as well as various toxic reactants which damage the different cellular elements leading to their respective functional impairment which ultimately culminates to severe oxidative damage of spleen with consequential loss of function. Our present investigation reveals that exposure of experimental rats to lead caused elevation in the level of lipid peroxidation in the spleen tissue of the animals, alteration in the level of reduced glutathione, activities of antioxidant enzymes like superoxide dismutase (cytosolic and mitochondrial) and catalase also. Elevation in lipid peroxidation is considered as a significant marker of oxidative damage [8, 25, 26]. On the other hand, changes in the level of reduced glutathione and activities of the antioxidant enzymes indicate lead induced disruption of the natural antioxidative system of the spleen tissue and the overall body at large [8, 22, 23, 26].

We observed that pre-treatment of the animals with aqueous extract of Murraya koenigii [MUR] mitigated the changes brought about by lead. The level of lipid peroxidation and reduced glutathione content were protected from being altered by lead in spleen of rats who were pre treated with MUR. And also the activities of the antioxidant enzymes were protected from being altered by lead exposure in the animals who were pre-treated with MUR. We had such observations in our earlier studies also in rat organs like liver, kidneys and heart [4, 5]. Our studies also show that lead exposure caused increase in the activities of the pro-oxidant enzymes, xanthine oxidase and xanthine dehydrogenase in spleen of rats. Elevated activities of these enzymes are considered as indirect marker of generation of superoxide anion free radicals within the spleen tissue. Lead induced increase in their activities may be considered to be indicator of increased generation of superoxide anion free radicals and other reactive oxygen species in spleen of rats. Atomic absorption spectrophotometric study showed accumulation of lead in spleen of rats treated with lead. Earlier studies also report possibilities of accumulation of lead in spleen of lead exposed experimental animals [27] but, our experiments, have provided direct evidence of such lead accumulation in spleen. We also observed marked reduction in the activities of the various key mitochondrial enzymes involved in energy production and respiratory chain reactions. These enzymes are primarily involved in production of NADH as well as NADPH and thus help in energy production and consequential cellular defense against oxidative damage. The NADPH is utilized for regeneration of mitochondrial GSH (reduced glutathione) or thioredoxin. This reduced glutathione is a very important part of our body’s natural antioxidative system [8, 22]. Disruption of spleen tissue architecture was observed in lead treated animals while the damage was found to be markedly protected when the rats were pre-treated with aqueous extract of Murraya koenigii [Fig. 6 and 7]. All the adverse changes following lead treatment were found to be ameliorated in the experimental animals which were pretreated with MUR further establishing the fact that MUR has protective potential against lead induced oxidative stress mediated damages in rat spleen. Our histological studies support our biochemical observations.

5. CONCLUSION
Thus, we conclude from this investigation that curry leaves can be considered as a potent natural antioxidant food material, and also can be considered for development of some potent and cost effective drug formulation with minimum or no side effects against lead induced oxidative stress mediated damage in spleen for people who get occupationally, environmentally or accidentally exposed to this toxic heavy metal.

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Conflict of interests
The authors declare that they do not have any conflict of interest.

REFERENCES


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