Debosree Ghosh. *et al.* / *International Journal of Phytopharmacology*, 4(2), 2013, 119-132.

e- ISSN 0975 – 9328
Print ISSN 2229 – 7472

International Journal of Phytopharmacology

Journal homepage: www.onlineijp.com

IJP

AQUEOUS LEAF EXTRACT OF *Murraya koenigii* PROTECTS AGAINST LEAD-INDUCED CARDIO TOXICITY IN MALE WISTAR RATS

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ABSTRACT

Murraya koenigii is a popular spice herb in India and South East Asia. The leaves have uses in folk medicine and ayurvedas. Lead is a widely distributed, toxic environment pollutant. Aim of the study is to find therapeutic potentials of aqueous extract of *Murraya koenigii* (CuLE) against lead induced oxidative damage in cardiac tissue of rats. The objectives are to study the alterations of various stress parameters in lead induced cardiotoxicity and amelioration of the same with CuLE.Rats were intraperitoneally 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 cardiac tissue was quantified using AAS study. Histomorphological changes and alteration in tissue collagen level was studied through H-E staining and Sirius red staining respectively. Treatment of rats with lead acetate for seven consecutive days caused accumulation of lead in cardiac tissue, alterations of the biomarkers of organ damage and oxidative stress and caused injury to the cardiac tissue. There were alterations in the activities of the antioxidant as well as pro-oxidant enzymes and some of the enzymes of the Kreb's cycle following lead acetate treatment. All these changes were ameliorated when the rats were pre-treated with CuLE at a dose of 50 mg / kg (fed orally) for a similar period of time. The observations indicate that CuLE has the potentiality to protect against lead induced oxidative stress mediated cardiotoxicity in rat possibly through its antioxidant activity and may have future therapeutic relevance in human exposed to lead environmentally or occupationally and in situations where chelation therapy has limited success.

Key words: Antioxidant, Lead acetate, *Murraya koenigii*, Oxidative stress, Cardiotoxicity.

INTRODUCTION

Lead toxicity has a story of lead induced generation of ROS leading to oxidative stress (Ghosh *et al*., 2012). Studies suggest that lead toxicity mediated immune suppression also has a background of oxidative stress (Dipti *et al*., 2003). Lead does not have any detectable beneficial physiological roles. There are reports by several investigators documenting toxic and deleterious effects of heavy metals including lead on

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physiological, biochemical, and behavioral aspects of man and other experimental animals (Goyer *et al*., 1979; Ruff *et al*., 1996).

Accumulation of lead in body affects the central and peripheral nervous systems (Dressier *et al*., 1999), haemopoietic system, cardiovascular system, renal system, liver and reproductive systems (Patra *et al*., 2011).

The mechanism of lead-induced oxidative stress involves an imbalance between generation and removal of ROS (reactive oxygen species) in tissues and cellular components causing damage to membranes, DNA and proteins. The effect on the antioxidant defense systems of cells is the second mechanism for lead-induced oxidative stress (Patra *et al*., 2011).

The ethnomedicinal plant *Murraya koenigii* (popularly known as Curry plant) is a spice herb with promising medicinal value. It is widely and regularly used as a spice and condiment in India and other tropical countries. Different parts of the curry plant have use in traditional medicine formulation in ayurvedic and unani medicine (Joshep *et al*., 1985). Naturally occurring herbal antioxidants and nutraceuticals are devoid of the cytotoxicity and unwanted side effects unlike synthetic antioxidants and thus has the potential to be used extensively for the treatment or prophylaxis of various oxidative stress-related diseases (Malothu *et al*., 2012). CuLe has components like polyphenols and flavonoids which imparts it the strong antioxidant potential. CuLE has been found to provide protection in cadmium induced cardiac damage (Mitra *et al*., 2012).

Herein, we provide evidence that a CuLE provides protection to cardiac tissue of rats from lead-induced oxidative injury. The results of the current studies indicate that CuLE provides protection against lead induced oxidative onslaught of the rat cardiac tissue possibly through the radical scavenging and antioxidant activities of its potent antioxidant components. CuLE may have future therapeutic relevance in the prevention of lead-induced cardiotoxicity in humans exposed occupationally or environmentally to this toxic heavy metal and may be used for development of new cardioprotective drugs of herbal origin with less cytotoxic effects.

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, the dried leaf dusts were soaked over night 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 ^{0} 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 ^{o} 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 (Na_2CO_3), cupric sulfate pentahydrate ($CuSO_4.5H_2O$), 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 (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, 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 CPCSEA Nominee to Department of Physiology, University of Calcutta, acted as the advisor for animal care and handling and continuously monitored animal experimentations.

Pb induced cardiotoxicity *in vivo* and cardioprotection 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 treated

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 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^{o}C. The heart was excised carefully and washed several times in ice cold saline, and bottled dry, immediately weighed and stored at -20 ^{o} C until analysis (Mitra *et al*., 2012).

Determination of Lead content of cardiac 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^{0}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^{0}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 ug/g of rat tissue.

Histological studies

Immediately following sacrifice of the animals, hearts were surgically extirpated and fixed in 10% formalin and embedded in paraffin following routine procedure as described earlier (Mitra *et al*., 2012). Tissue sections (5 µM thick) were prepared and stained with hematoxylin-eosin.

Quantification of fibrosis by confocal microscopy

The rat heart tissue sections (5 µm thick) were stained with Sirius red (Direct Red 80; Sigma Chemical Co., St. Louis, MO, USA) (Mitra *et al*., 2012) 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 (ImageJ, NIH Software, Bethesda, MI) and the total collagen area fraction of each image was measured and expressed as the % collagen volume.

Serum biochemical estimations

Serum GOT activities was measured by standard routine methods. The enzyme activities were expressed as IU/L (Mitra *et al*., 2012). The activity of lactate dehydrogenase1 (LDH1) was obtained according to the method of Strittmatter (Strittmatter *et al*., 1965) with some modifications (Mitra *et al*., 2012) using a UV–Vis spectrophotometer (Bio-Rad, Hercules, CA, USA).

Preparation of homogenate, measurement of lipid peroxidation level

The liver 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) according to the method of Buege and Aust (1978) with some modification as adopted by Bandyopadhyay et al. (2004) . 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^{0}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.

Measurement of protein carbonyl content

Protein carbonyl content was estimated by DNPH assay (Levine *et al*., 1994) . 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 4ºC. 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 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 $X10^4$ M^{-1} cm^{-1}. The values were expressed as nmoles of carbonyl/mg protein.

Measurement of the activities of cytosolic (Cu-Zn type) and mitochondrial (Mn-type) superoxide dismutase (SOD), catalase (CAT)

Copper-Zinc superoxide dismutase (Cu-Zn SOD or SOD1) activity was measured by hematoxylin autooxidation method of Martin *et al* (Martin *et al*., 1987) with some modifications as adopted by Mukherjee *et al* (Mukherjee *et al*., 2010). 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 (Marklund *et al*., 1974). A weighed amount of tissue was homogenized (10%) 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 by the method of Beers and Sizer (1952) with some modifications as adopted by Chattopadhyay et al (Chattopadhyay *et al*., 2003). 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.Then10% 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 μm H_2O_2 consumed / min / mg protein.

Measurement of reduced GSH level, GSSG level, GSSG:GSH ratio and total sulfhydryl group content

GSH content (as acid soluble sulfhydryl) of the liver was estimated by its reaction with DTNB (Ellman's reagent) following the method of Sedlak and Lindsey (1968) with some modifications (Mitra *et al*., 2012). 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 addition of DTNB for colour development. Using a UV–Vis spectrophotometer (BIORAD, Smart Spec Plus), the absorbance was recorded at 412 nm and the values were expressed as nmoles/mg protein.

GSSG content was measured by the method of Sedlak and Lindsay, 1968 with some modifications (Mitra *et al*., 2012). Cardiac tissue was 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 nmoles GSSG/mg protein. GSSG:GSH ratio was evaluated.

Total sulfhydryl group content was measured following the method as described by Sedlak and Lindsay (1968). The values were expressed as nmoles TSH/ mg protein.

Measurement of the activities Glutathione reductase, Glutathione peroxidase and Glutathione-S-transferase

Glutathione reductase activity was estimated using the method of Krohne-Ehrich et al (Krohne-Ehrich *et al*., 1977). 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 homogenate (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 as per the method of Paglia and Valentine (1967) with some modifications as adopted by Chattopadhyay et al (Chattopadhyay *et al*., 2000). 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 0.05 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 H_2O_2. 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 according to the method of Habig et al (Habig *et al*., 1974). The enzymatic activity was recorded observing the conjugation of 1-chloro, 2,4-dinitrobenzene (CDNB) with reduced glutathione (GSH). One unit of enzyme conjugates 10.0 nmoles of CDNB with reduced glutathione per minute at 25ºC. The rate where the reaction is linear is noted at 340nm. The molar extinction of CDNB is 0.0096 μM^{-1}/cm. The enzyme activity was expressed as Units/min/ mg of tissue protein.

Measurement of xanthine oxidase and xanthine dehydrogenase activities

Xanthine oxidase activity was estimated by the conversion of xanthine to uric acid following the method of Greenlee and Handler (1964). 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 according to the method of Strittmatter (1965) with some modifications (Mitra *et al*., 2012). 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 12,000g 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.

Measurement of the activities of the pyruvate dehydrogenase and some of the key mitochondrial Kreb's cycle enzymes

The liver 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 according to the method of Chretien et al (Chretien *et al*., 1995), with some modifications as adopted by Mitra *et al*., 2012, following the reduction of NAD^+ to NADH at 340nm using 50mM phosphate buffer, ph 7.4, 0.5mM sodium pyruvate as the substrate, and 0.5mM 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 (Duncan *et al*., 1979) by measuring the reduction of NAD+ to NADH at 340nm with the help of a UV-VIS spectrophotometer. One ml assay volume contained 50mM phosphate buffer, pH 7.4, 0.5mM isocitrate, 0.1mM $MnSO_4$, 0.1mM NAD+ and enzyme. The enzyme activity was expressed as units/mg protein.

Alpha-ketoglutarate dehydrogenase (α-KGDH) activity was measured spectrophotometrically according to the method of Duncan et al (Duncan *et al*., 1979) 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 (K_3FeCN_6) at 420nm according to the method of Veeger et al (Veeger *et.al*., 1969) with some modifications. One ml assay mixture contained 50mM phosphate buffer, pH 7.4, 2% (w/v) BSA, 4mM succinate, 2.5mM K_3FeCN_6 and the enzyme. The enzyme activity was expressed as units/mg protein

Measurement of some of the mitochondrial respiratory chain enzymes

The NADH-cytochrome coxidoreductase activity was measured spectrophotometrically by following the reduction of oxidized cytochrome c at 565nm according to the method of Goyal and Srivastava (1995). 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 according to the method of Goyal and Srivastava (1995). 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 the method of Lowry *et al*., (1951) using bovine serum albumin (BSA) as the standard.

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. Pairwise comparisons were done by calculating the least significance. Statistical tests were performed using Microcal Origin version 7.0 for Windows.

RESULTS

Status of tissue leads content

Figure 1. demonstrates accumulation of lead in cardiac 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 compared to control ($p<0.001$ Vs. control). 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 (48.90%, $p<0.001$ vs. Pb-treated group).

Status of heart size and heart weight body weight ratio

Figure 2a reveals a reduction in heart size in the rats treated with lead acetate compared to control. However, pre -treatment of rats with the present dose of CuLE protected the heart size from being reduced. Figure 2b reveals a significant decrease in heart weight: body weight ratio compared to control. Here also, pre-treatment of rats with CuLE significantly attenuated the heart weight: body weight ratio. However, CuLE, at the present dose, alone was found to have no effect either on heart size (Figure 2a) or heart weight: body weight ratio (Figure 2b).

Histological studies

Figure 2c (upper panel) reveals that there occurred focal ischemia and tissue damage in lead acetate treated rats as evident from hematoxylin and eosin stained tissue sections compared to control. However, in rats pre-treated with CuLE, there was no sign of ischemia or tissue damage. CuLE alone, however, has no effect on cardiac tissue morphology. Figure 2c (middle panel) shows a depletion of cardiac tissue collagen following treatment of rats with lead acetate. Pre-treatment of rats with CuLE protected the cardiac tissue from being depleted of tissue collagen. CuLE alone was found to have no effect on tissue collagen content as evident from the microscopic examination of the cardiac tissue sections stained with acid Sirius (Figure 2 c). Fig. 2 c (lower panel) shows similar images captured by confocal laser scanning microscope. Figure 2 d represents quantification of fibrosis as % collagen volume. The results further indicate a protective effect of CuLE against Pb-induced damage in rat cardiac tissue.

Biomarkers organ damage

Table 1 shows that treatment of rats with lead acetate caused a significant elevation in the level of activity of SGOT (70.50%, $p<0.001$ vs. control) and LDH1, a specific marker enzyme of cardiac damage (3.4 times, $p<0.001$ vs. control). However, when the rats were pre-treated with the present dose of CuLE, the activities of both SGOT and LDH1 in the serum were found to be similar to that observed in the control animals (28.46% and 65.55% respectively **P< 0.001 vs. Pb acetate-treated group). CuLE alone, however, was found to have no significant effect on the activities of these marker enzymes for organ damage.

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 heart as evident from significantly increased level of lipid peroxidation compared to control [Table 2] (1.76 folds , *P< 0.001 vs. control group). Pre-treatment of rats with CuLE significantly protected the lipid peroxidation level from being increased (62.18%, **P< 0.001 vs. Pb acetate-treated group). However, CuLE alone has no effect on the lipid peroxidation level of tissue.

Table 2 also shows that there occurred a significant increase in the level of protein carbonyl of heart tissues of rat following lead acetate treatment (2.1 folds, *P< 0.001 vs. control group). Pre-treatment of rats with CuLE almost completely protected the level of protein carbonyl from being increased in cardiac tissue (62.42%, **P< 0.001 vs. Pb-treated group). However, CuLE alone has no significant effect on the GSH level of heart.

Status of antioxidant enzymes

Table 2 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 cardiac tissues (1.4 folds, 95.64%

and 1.1 times increase respectively 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 (53.00%, 39.79% and 54.51% decrease respectively, **P < 0.001 vs. Pb-treated group). However, CuLE alone did not significantly alter the activity of any of the enzymes studied.

Status of GSH, GSSG, GSSG: GSH and TSH

Fig. 3 (a, c and d) shows that there occurred a significant increase in GSH, GSSG level and in the GSSG: GSH ratio of heart tissues of rat following lead acetate treatment (47.54%, 83.33% and 28.57% 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 cardiac tissue (32.22 %, 45% and 22.22% respectively, **P< 0.001 vs. Pb-treated group). However, CuLE alone has no significant effect on the GSH and GSSG levels of heart.

Treatment of rats with lead acetate decreased the total thiol (TSH) level significantly (20.12%,*P< 0.001 vs. control group). Pre-treatment of rats with CuLE almost completely protected the TSH from being decreased in cardiac tissue (22.50%, **P< 0.001 vs. Pb-treated group). However, CuLE alone has no significant effect on the TSH level of heart (fig.2b).

Status of the activities of glutathione peroxidase, glutathione reductase and glutathione –S- transferase

Treatment of rats with lead acetate for seven consecutive days at a dose of 15 mg / kg body weight increased the activities of glutathione peroxidise (fig. 3a), glutathione reductase (fig.3b) and glutathione –S-transferase (3 c) (43.48%, 80.00% and 33.33% respectively,*P< 0.001 vs. control group). However, the enzyme activities 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 (21.21%, 40.00% and 25.21%,**P< 0.001 vs. Lead acetate -treated group). However, CuLE alone has no significant effect on the activities of these enzymes in cardiac tissue.

Status of pro-oxidant enzymes

Table 3 shows that the activities of cardiac XO, XDH, the total enzyme activity, i.e., XO plus XDH, XO :XDH ratio and XO/(XO+XDH) all increased significantly following treatment of rats with lead acetate (88.11%, 2.1 folds, 1.28 folds, 19.67% and 1.16 folds increase respectively vs control, *P < 0.001 vs. control) . All these parameters were significantly protected from being increased when the rats were pre-treated with CuLE, indicating, the ability of CuLE to neutralize free radicals *in vivo* (53.60%, 72.50%, 61.25%, 22.01% and 50% decrease respectively in cardiac tissue vs lead acetate treated group, **P < 0.001 vs. Lead acetate-treated group). However, CuLE alone has no effect on the activities of those two enzymes and their ratios.

Status of the activities of pyruvate dehydrogenase and some of the mitochondrial Kreb's cycle enzymes:

Table 4 shows that treatment of rats with lead acetate significantly decreases the activities of rat cardiac PDH (64.97% decrease, *P < 0.001 vs. control), ICDH (57.89%, *P < 0.001 vs. control),α KGDH (75.00%, *P < 0.001 vs. control) and SDH (77.67%, *P < 0.001 vs. control). This might result in interference of the metal in electron transport chain (ETC) and thus generate copious amounts of superoxide anion free radicals in the tissue mitochondria. Pre-treatment of rats with CuLE significantly protected the PDH activity from being decreased (1.7folds increase, **P < 0.001 vs. Lead acetate -treated group). ICDH 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. The activity of cardiac ICDH was protected significantly from being decreased when the rats were pre-treated with CuLE (1.9 folds, **P < 0.001 vs. Lead acetate -treated group). Alpha KGDH was found to be able to generate ROS during its catalytic function, which is regulated by the NADH/NAD+ ratio (Tretter and Adam-Vizi, 2005). The activity of α-KGDH was found to be significantly protected from being decreased when the rats were pre-treated with 50 mg/kg body weight of CuLE (2.9 folds in cardiac tissue, **P < 0.001 vs. Lead acetate-treated group). Pre-treatment of rats with CuLE significantly protected the SDH activity from being decreased (1.96 times, **P < 0.001 vs. Lead acetate-treated group). However, CuLE alone has no significant effect on the activity of any of those enzymes in enzymes studied rat cardiac tissues.

Status of the activities of mitochondrial respiratory chain enzymes

Table 5 further shows that treatment of rats with lead acetate also significantly decreased the activities of cytochrome c oxidase (86.50%,*P< 0.001 vs. control group) and NADH cytochrome c oxidoreductase (71.81%, *P< 0.001 vs. control group) in cardiac tissue. The activity of cytochrome c oxidase was found to be significantly protected from being decreased compared to lead acetate treated group when rats were pre-treated with CuLE (4.6 folds), **P< 0.001 vs. Lead acetate-treated group). NADH cytochrome oxido-reductase activity was also found to be completely protected when the rats were pre-treated with CuLE at the indicated dose (2.4 folds, **P< 0.001 vs. Lead acetate -treated group). CuLE alone, however, has no significant effect on the activity of these enzymes.

Table 1. Effect of CuLE on the activities of SGOT and LDH1 in lead acetate treated rats

BIOMARKERS	CONTROL	CuLE	LEAD	CuLE + LEAD
SGOT(IU/L)	10.10±0.412	9.96±0.865	17.22±0.547*	12.32±0.654**
LDH 1(IU/L)	1.333±0.056	1.099±0.096	5.986±0.098*	2.032±0.095**

Values are expressed as Mean ± SE of 6 animals in each group.*P<0.001 compared to control; **P< 0.001 compared to lead acetate treated group;

Table 2. Effect of aqueous extract of the leaves of *Murraya koenigii* on the levels of lipid peroxidation, the activities of Cu-Zn superoxide dismutase, Mn superoxide dismutase and catalase in heart of the experimental rats

Parameters Studied	CON	CuLE	Pb	CuLE+Pb
LPO (nmoles of TBARS/mg protein)	0.113±0.007	0.112±0.007	0.312±0.008*	0.118±0.007**
Protein carbonyl (nmoles per mg protein)	6.212±0.115	6.121±0.118	18.98±0.118*	7.132±0.116**
Cu-Zn SOD activity (units/min/mg protein)	7.050±0.164	6.933±0.118	16.62±0.117*	7.811±0.124**
Mn SOD activity (units/min/mg protein)	6.241±0.215	5.981±0.324	12.21±0.321*	7.351±0.251**
Catalase activity (µmoles H_2O_2 consumed/min/mg protein)	15.13±0.784	15.54±0.354	31.24±0.456*	14.21±0.245**

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.

Table 3. Effect of aqueous extract of *Murraya koenigii* on the activities of XO, XDH, XO+XDH and XO/XDH in heart of the experimental rats

PARAMETERS	CONTROL	CuLE	LEAD	CuLE +LEAD
XO(milliunits/min/mg protein)	0.0075±0.0012	0.0075±0.0012	0.02±0.0059*	0.0075±0.0015**
XDH(milliunits/min/mg protein)	0.020±0.0021	0.020±0.0022	0.051±0.0022*	0.030±0.0019**
XO+XDH	0.027±0.0015	0.027±0.0016	0.071±0.0016*	0.031±0.0014**
XO/XDH	0.303±0.025	0.302±0.019	0.421±0.016*	0.279±0.013**
XO/(XO+XDH)	0. 277±0.0012	0.277±0.0011	0.281±0.0012*	0.241±0.0013**

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; CuLE = Curry leaf extract; Pb=Lead; CuLE +Pb= Curry leaf extract +Lead.

Table 4. Effect of aqueous extract of the leaves of *Murraya koenigii* on the activities of PDH, ICDH, α-KGDH and SDH in heart of the experimental rats

Parameters Studied	CONTROL	CuLE	LEAD	CuLE + LEAD
PDH (units/min/mg protein)	0.925±0.012	0.898±0.045	0.324±0.031*	0.888±0.024**
ICDH(units/min/mg protein)	0.057±0.0014	0.057±0.0030	0.024±0.0032*	0.050±0.0017**
α-KGDH (units/min/mg protein)	0.060±0.0021	0.061±0.00243	0.015±0.0030*	0.058±0.0027**
SDH(units/min/mg protein)	1.505±0.205	1.504±0.042	0.336±0.062*	0.996±0.067**

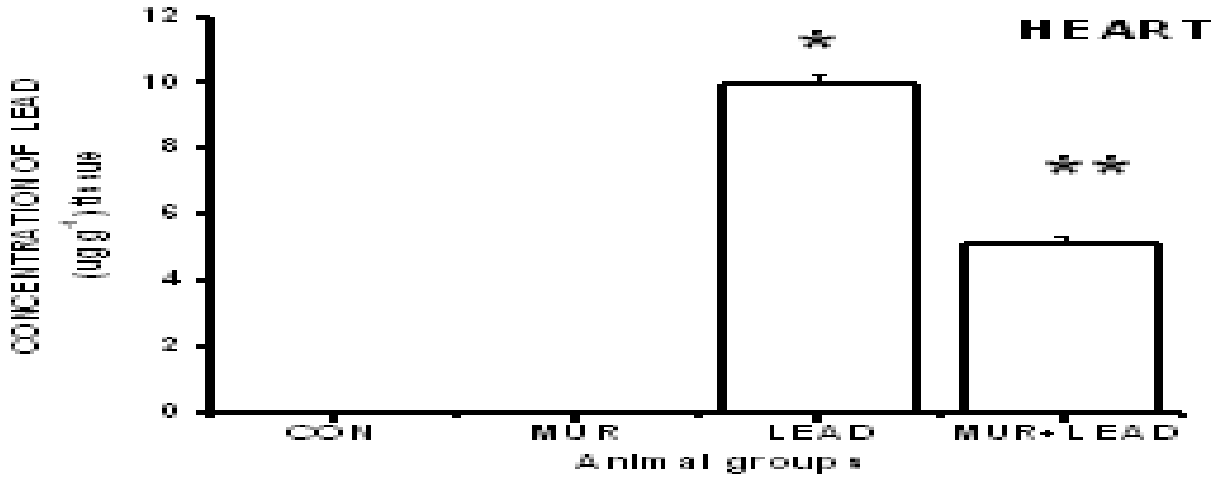
Values are expressed as Mean ± SE of 6 animals in each group.**P< 0.001 compared to lead treated group; CON = Control; CuLE = Curry leaf extract; Pb=Lead; CuLE +Pb= Curry leaf extract +Lead.

Table 5. Effect of aqueous extract of the leaves of *Murraya koenigii* on the activities of Cytochrome c oxidase and NADH cytochrome c oxido-reductase in heart of the experimental rats

Parameters Studied	CONTROL	CuLE	LEAD	CuLE + LEAD
Cytochrome c oxidase activity(units/min/mg protein)	0.126±0.011	0.129±0.0011	0.017±0.0028*	0.095±0.0035**
NADH cytochrome c oxido-reductase activity(units/min/mg protein)	7.121±0.036	8.072±0.045	2.007±0.012*	7.013±0.0058**

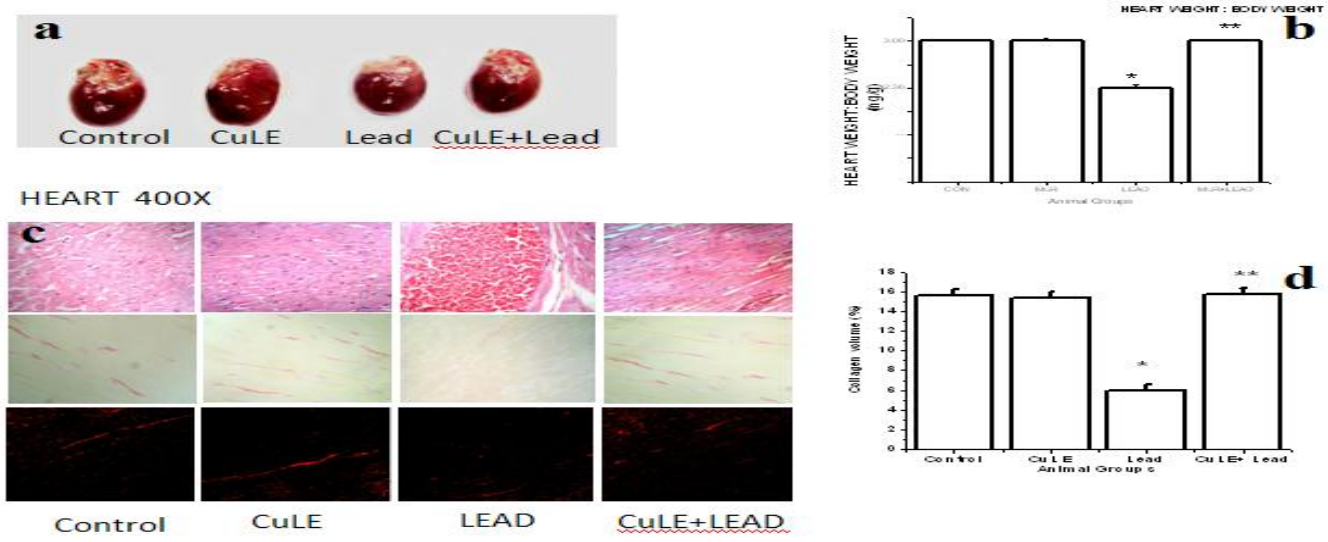
Values are expressed as Mean ± SE of 6 animals in each group.*P<0.001 compared to control; **P< 0.001 compared to lead acetate treated group. CON = Control; CuLE = Curry leaf extract; Pb=Lead; CuLE +Pb= Curry leaf extract +Lead.

Fig.1. Effect of CuLE on lead content in cardiac tissue of male wistar rats estimated by AAS;



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.

Figure 2. 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.



a. Effect of CuLE against lead-induced reduction in the heart size of the rats

b. Effect of CuLE on heart weight: body weight ratios

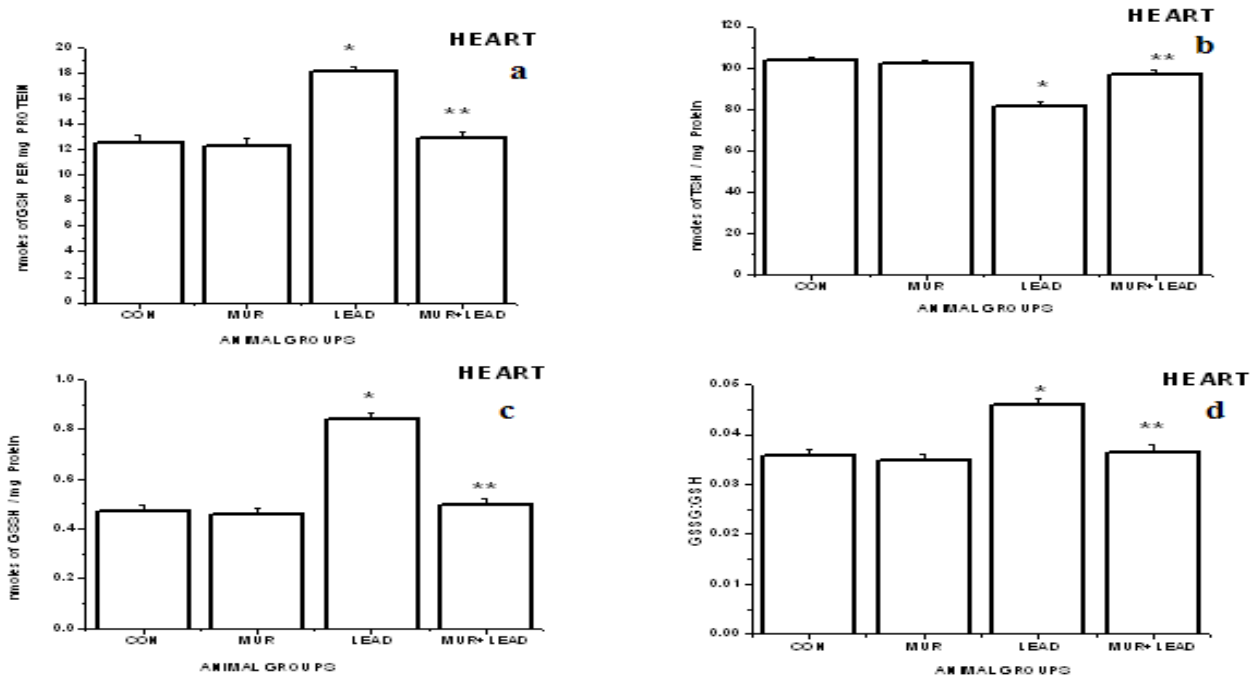
c. **upper panel :** Effect of CuLE on changes in the rat cardiac tissue morphology (Hematoxylin and Eosin stained , 400X magnification)

middle panel : Effect of CuLE on changes in the rat cardiac tissue morphology (Sirius red stained sections, 400X magnification)

lower panel : Similar images captured by confocal laser scanning microscope for quantification of fibrosis. Arrow heads indicate collagen fibres in b and c.

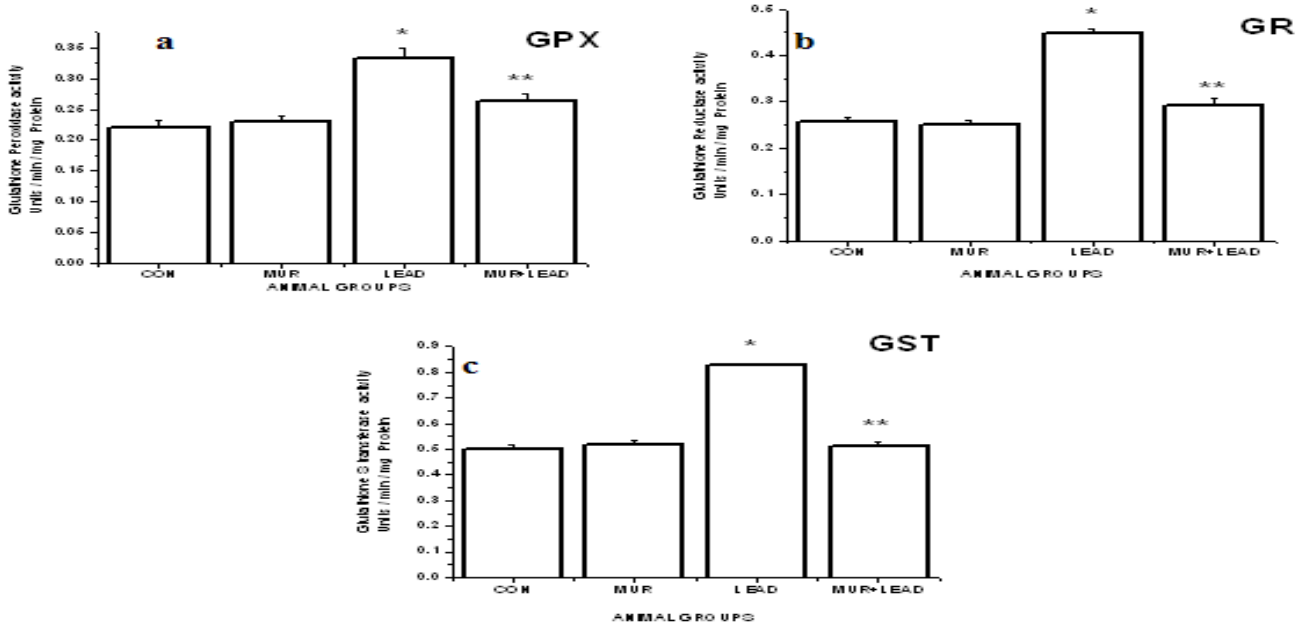
d. Graph showing collagen volume % of the cardiac tissues . Con = control, injected with vehicle; Lead = 15 mg/kg bw, $Pb(CH_3COO)_2$ injected i.p.; CuLE = 50 mg/kg bw of CuLE; CuLE + Lead = 50 mg/kg bw of CuLE + 15 mg/kg bw$Pb(CH_3COO)_2$ injected i.p.

Fig.3. Effect of aqueous extract of the leaves of *Murraya koenigii* against lead-induced alteration in the value of GSH (A), TSH (B), GSSG (C) and GSSG: GSH (D) in rat cardiac 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; MUR = Curry leaf extract; Pb=Lead; MUR +Pb= Curry leaf extract +Lead.

Fig.4. Effect of aqueous extract of *Murraya koenigii* against lead-induced lead-induced alteration in the activities of glutathione peroxidises [GPX](a), glutathione reductase [GR] (b), glutathione S transferase [GST] (c) in rat cardiac 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 acetate treated group. CON = Control; CuLE = Curry leaf extract; Pb=Lead; CuLE +Pb= Curry leaf extract +Lead.

DISCUSSION AND CONCLUSION

This investigation was undertaken to ascertain the cardio-protective activity of aqueous extract of the leaves of *Murraya koenigii* (CuLE) on lead-induced cardio-toxicity in male albino wistar rats. There have been several studies on the mechanisms by which lead induces oxidative stress (Gurer *et al*., 2000). Till date, the exact mechanisms involved in lead induced oxidative stress have not been completely understood (Sugawara *et al*., 1991). Evidence indicates that lead induced oxidative stress is a multi-mechanistic event. (Solliway *et al*.,1996). Some protection against lead induced oxidative stress has been investigated and reported (Sokkary *et al*., 2003; Flora *et al*., 2004) but there has been no study on the potentiality of CuLE in providing protection against lead induced oxidative stress mediated cardio-toxicity. *Murraya koenigii* is an easily available, widely cultivated, popular spice herb of South East Asia with pronounced medicinal values recognized for combating diabetes and hypoglycemic situations (Arulselvan *et al*., 2007). Other investigators have suggested phyto-pharmacological potentials in combating situations of various pathological states (Devi *et al*., 2012; Singh *et al*., 2012).

Our AAS study revealed a highly significant content of Pb in the cardiac tissue of the experimental rats following treatment of those with lead acetate for seven consecutive days. The accumulation of the toxic heavy metal in the tissues might have brought about oxidative stress-induced damages.The cardiac tissues of control and CuLE only (positive control) treated rats had zero lead concentration and the most unique and significant new finding we had was that pre- treatment with CuLE reduced the concentration of metal lead significantly in the cardiac tissues. Mitra *et al*., 2012 has reported reduction of tissue cadmium content in CuLe pre-treated rats. We observed a reduction in the heart size and in heart weight: body weight ratio of the lead treated rats. This reveals that probably subchronic lead induced cardiotoxicity leads to cardiac hypotrophic sitiation in the experimental rats.

Histological examination of haematoxylin-eosin stained sections of cardiac tissues of lead treated animals showed some significant alterations. However, the tissue sections from the rats pre-treated with CuLE did not show any such changes. The results indicate the ability of the aqueous extract to provide protection against lead induced tissue injury. Picrosirius stain of the tissue sections show that there was depletion of collagen in lead acetate treated animals. Pre-treatment of rats with CuLE was found to protect the depletion of collagen content indicating a protective role of the extract in maintaining the tissue integrity. There was not much difference between collagen content of the cardiac tissues of the control and the CuLE only treated group. The lead induced cardiac damage in our experimental situation, is due to generation of oxidative stress as is evident from elevated levels of tissue LPO and protein carbonyl content and GSSG level and the bio-markers of oxidative stress.

The basis for clinical diagnosis of pathological symptoms depends on comparing the levels of a number of cytoplasmic enzymes, which are released as a result of physicochemical alterations in the tissues. It is evident from our studies that there occurred a significant increase in the levels of the cardiac damage marker enzymes i.e., SGOT and LDH1 due to lead acetate treatment in experimental rats. The present dose of lead acetate i.e., 15 mg/kg bw, for seven consecutive days not only produced significant changes in the parameters studied in comparison to control animals. High levels of activity of SGOT and LDH 1 indicate myocardial damage in the experimental rat. Oral administration of CuLE at the present dose (i.e., 50 mg/kg bw) attenuated the lead acetate -induced elevation of the serum levels of the activities of these marker enzymes indicating that CuLE may have the capacity to provide protection against lead – induced cardiac damage.

The increased levels of LPO, protein carbonyl, GSH and GSSG are indicative of occurrence of oxidative stress (Mitra *et al*., 2012, Ghosh *et al*., 2012). Hsu J M reported in 1981 that concentrations of GSH in the erythrocytes, liver and kidney were significantly increased after 4 weeks of lead-acetate feeding in both males and females.

We observed a significant increase in the level of TBARS in the cardiac tissue following treatment of rats with lead acetate. Lead is known to produce oxidative damage in the tissues by enhancing peroxidation of membrane lipids, a deleterious process solely carried out by free radicals. However, pre-treatment of rats with CuLE prevented the enhancement in the levels of TBARS in the cardiac tissue. Protein CO is used as a popular biomarker of oxidative stress (Mitra *et al*., 2012). Protein CO groups are used extensively as biomarkers of oxidative stress because it is advantageous compared to the measurement of other oxidation products. Protein CO groups are formed relatively early are relatively stable and thus easy to estimate. We found in an enhanced level of protein CO as well as level of lipid peroxidation in cardiac tissues of lead treated rats. Both were prevented from being increased on pre-treatment with CuLE.

Superoxide dismutase (SOD) catalyzes the conversion of superoxide anion free radical to H_2O_2 through dismutation reaction. Catalase catalyzes the conversion of H_2O_2 to water and oxygen. The activity of SOD and CAT in cardiac tissue was found to be increased following administration of lead. Pre-treatment of rats with CuLE protected activities of these antioxidant enzymes from being altered. Super oxide anion radicals are generated *in vivo* and are increased with condition of oxidative stress. Increased level of super oxide anion

radical causes enhanced activity of the enzyme SOD while increased SOD activity leads to increased level of hydrogen peroxide. Thus the level of Catalase activity is also increased in response to the increased hydrogen peroxide.

Glutathione is tripeptide made up of L-cysteine, L- glutamic acid and glycinecysteinyl moiety. Reactive Oxygen Species (ROS) are reduced by GSH in the presence of GSH peroxidase. GSH is oxidized to GSSG, which in turn is rapidly reduced back to GSH by GR at the expense of NADPH. The GSH redox cycle consist of four prime components i.e., GSH, GPx, GR and GST. These are integral part of the antioxidant defence system. GSH plays important role in the detoxification of a variety of electrophilic compounds and peroxides via catalysis by glutathione S-transferases (GST) and glutathione peroxidases (GPx). Lipid peroxidation can generate large amounts of electrophilic and oxidizing reactive species which can lead to a variety of DNA and tissue damage (Awasthi *et al*., 1995).

Glutathione reductase (GR) reduces oxidized glutathione (GSSG) to biologically active GSH and the cofactor of GR is NADPH. GPx detoxifies peroxides using GSH as an electron donor, producing GSSG as an end product. We observed increased activities of all the three enzymes in rat cardiac tissues on treatment with lead. Increased level of GSH is probably the inducer for enhanced activity of GR. On the other hand increased oxidation of GSH to GSSG leads to increased level of GSSG and the ratio of GSSG: GSH in lead treated animals compared to control. GSH is increased to meet the increased demand of the same for combating the situation of increased lipid peroxidation. This increase in the level of lipid peroxidation is caused by lead induced generation of ROS. All these were prevented from being increased on pre-treatment with CuLE.

The activities of XO and XDH are highly significantly increased in the cardiac tissues of lead acetate treated experimental rats compared to control with a concomitant increase in the XO plus XD, XO/XD ratio, XO/XO + XD ratio. Metabolic reactions involving these two enzymes do serve as the source of superoxide anion free radical generation. XO and XDH in oxidative stress conditions may play an important role in contributing free radical mediated damage. Earlier workers have also indicated the involvement of XO in free radical production (Reghuvanshi *et al*., 2007; Desco *et al*., 2002).

We have studied the effect of lead acetate on the mitochondrial enzymes related to energy metabolism. The impairment of electron transfer through NADH: ubiquinone oxidoreductase (complex I) and ubiquinol: cytochrome c oxido-reductase (complex III) may induce superoxide formation. Mitochondrial production of ROS is thought to play an adverse role in many pathologic states of heart (Chen *et al.,* 2003). Heavy metals are also known to affect respiratory chain complexes and there is substrate specificity (Belyaeva *et al*., 2011).

In our present study, lead acetate treatment inhibits PDH, ICDH, SDH, α KGDH of TCA cycle and NADH cytochrome c oxidoreductase and cytochrome c oxidase enzymes of ETC of mitochondria of rat cardiac tissue. The activities of these enzymes were found to be protected when the rats were pre-treated with CuLE. This strongly indicates that the extract possesses either some chelating property or is simply able to prevent mitochondria from ROS production by itself being a quencher of reactive oxygen species. Thus, lead acetate induced alteration in mitochondrial redox metabolism and respiratory functions may lead to the increased production of ROS in cells, which is effectively alleviated by CuLE.
This study establishes for the first time in the literature, the cardio-protective potential of the CuLE in lead acetate induced oxidative stress mediated cardio toxicity. From these studies it is concluded that the aqueous extract of the leaves of *Murraya koenigii* protects rat heart against lead-induced oxidative damage. *Murraya koenigii*, very well known as Curry leaves may find its extensive use against lead induced cardiotoxic situation at a specific pharmacological dose. Based on our earlier CuLE dose-response studies (Ghosh *et al*., 2012), we have carried out this detailed investigation to invent the fact that CuLE has the potential to provide protection against lead acetate-induced oxidative stress mediated damage in the rat heart. Till date there has been no report on the side effects of CuLE. Hence, we may conclude from this investigation that CuLE is a potent cardio-protective agent and can be used as an effective protector against lead induced cardiac damage. Further works are needed to identify the active principle (s) present in the leaves of the plant and elucidate its possible mode of action. The CuLE appears to provide protection against lead induced cardio-toxicity through its antioxidant activity and these may be attributed to the presence of phenolics and flavonoids (Ghosh *et al*., 2012).

ACKNOWLEDGEMENTS

Debosree Ghosh gratefully acknowledges the receipt of a Junior Research Fellow (JRF) under INSPIRE program of Department of Science and Technology, Government of India. SBF is a URF of UGC, under University of Calcutta. EM is a Project Fellow under a major research project of UGC awarded to Dr. D.B., Government of India. Dr. A.C. is supported from the funds available to her from a Minor UGC project, Govt. of India. Dr. S.K.P., Dr. S.D. and Dr.K.J. are supported from the funds available to them from their respective institutes. This work is also partially supported by UGC Major Research Project Grant awarded to Dr. DB [F. No. 37-396/2009 (SR)]. Technical help from Parthabrata Roy (Technical Assistant, Chemical Technology Department,

C.U.), Barindra Nath Mandal (Technical Officer B, Div of Mol Med, Bose Institute) and Sumanta Ghoshal is also gratefully acknowledged.

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