Debosree Ghosh et al. / Journal of Pharmacy Research 2015,9(12),618-634

Research Article
ISSN: 0974-6943

Available online through
www.jprsolutions.info

Melatonin and aqueous curry leaf extract in combination protects against lead induced oxidative stress mediated injury to rat heart: a new approach

Debosree Ghosh[1,2], Sudeshna Paul[1,3], Aindrila Chattopadhyay[3], Debasish Bandyopadhyay[1*#]

[1] Department of Physiology, University of Calcutta, University College of Science and Technology, 92, APC Road, Kolkata 700 009 India.
[2]Department of Physiology, Hooghly Mohsin College, P.O. - Chinsura, Dist.- Hooghly, Pin-712 101, West Bengal, India.
[3]Department of Physiology, Vidyasagar College, 39, Sankar Ghosh Lane, Kolkata 700 006, West Bengal, India.
#Principal Investigator, Centre with Potential for Excellence in a Particular Area (CPEPA),
University of Calcutta, University College of Science and Technology, 92 APC Road,Kolkata 700 009 India.

Received on:23-09-2015; Revised on: 27-10-2015; Accepted on: 30-11-2015

ABSTRACT

Objective(s): The objective(s) of the present study was to investigate whether a combination of melatonin and aqueous curry leaf extract is capable of providing protection against lead acetate induced oxidative stress mediated injury to cardiac tissue of experimental rats. **Methods:** Male Wistar rats were used as an animal model for the present study. After acclimatization to laboratory condition, in the first set of experiments rats were divided in to different groups. In the second, rats were divided in to four groups, i.e., control, positive control, lead acetate treated and melatonin + curry leaf extract in combination protected. Rats were treated intraperitoneally (i.p.) with lead acetate (15 mg / kg body weight) for a period of seven consecutive days. Rats of the protected group were pre-treated with melatonin also for seven days. The control rats were treated with vehicle only while the positive control rats were treated with melatonin + aqueous curry leaf extract only. After the treatment period, rats were sacrificed; blood and cardiac tissue collected and processed for analysis. **Results:** Treatment of rats with lead acetate caused accumulation of lead in the cardiac tissue and alterations in the biomarkers of organ damage and oxidative stress. It caused deteriorative changes to the cardiac tissue morphology and collagen content which were evaluated using light microscopy, electron microscopy and confocal microscopy. Involvement of oxidative stress is evident from the alterations in the level of lipid peroxidation and protein carbonyl content, activities of the antioxidant as well as pro-oxidant enzymes and some of the enzymes of the citric acid cycle and Electron Transport Chain (ETC) following lead acetate treatment. All changes were protected when the rats were pre-treated (fed orally) with melatonin (10 mg / kg body weight) and aqueous extract of curry leaves (50 mg/kg body weight) in combination for seven days. **Conclusion:** The results of the current studies indicate protective effect of the combination of melatonin (a natural antioxidant in pure form) and the aqueous extract of curry leaves to mitigate lead acetate-induced oxidative stress in experimental rats possibly through their synergistic antioxidant mechanism(s) .This study opens up avenues for development of an effective drug formulation against lead induced oxidative stress mediated cardiac damage in people who get environmentally or occupationally exposed to lead.

KEY WORDS: Curry leaves, lead acetate, melatonin, oxidative stress, tissue injury, synergistic antioxidant mechanism(s).

INTRODUCTION:

Metal lead is a ubiquitous highly toxic environmental pollutant[1]. Air, water and soil get contaminated by the toxic metal through processes like mining, metallurgy, its extensive uses in industries (i,e., paint, dye, pottery, jewellery, mint, water pipeline manufacturing, battery, bullets etc.) and recycling. The heavy metal is unnecessary in living system and if it enters the body, causes extensive range of physiological, biochemical deteriorative changes[1]. The toxic metal is known from long time and the mechanism of lead induced toxicity i.e., 'plumbism' has been addressed by many and has been concluded to be multifactorial[1]. Recent studies revealed that lead induces toxicity by mediating oxidative stress [2, 3, 4]. Our studies also reveal the same [5, 6]. Lead induced oxidative stress may be held responsible for the reported occupational health hazards experienced by workers who get exposed to lead regularly in industries. It is an area of extensive research around the globe.

***Corresponding author.**
Prof. Debasish Bandyopadhyay
Oxidative Stress and Free Radical Biology Laboratory,
Department of Physiology,University of Calcutta
University College of Science and Technology
92, APC Road, Kolkata 700 009 India.

Melatonin is a neuro-hormone secreted from the pineal gland. It is present in unicells as well as in human and plants. It helps to maintain circadian rhythms [7], boosts immune system [7], induces sleep, regu-

lates blood pressure and seasonal reproduction [8]. Melatonin has also been reported to be anti-depressive [8]. Studies reveal that this small indole possesses potent antioxidant activity [9, 10, 11] . Our earlier studies revealed melatonin's ability to mitigate lead induced oxidative stress [5, 6]. Melatonin has been recognized to be protective against lead induced neurotoxicity [12], DNA damage [13] and hematotoxicity [14]. Melatonin's ability to act synergistically with other natural antioxidants at low doses in a number of models of oxidative stress has been identified recently [15].

Curry leaves (*Murraya koenigii*) are popular spice herb used extensively in South-East Asian cooking. The leaves contain certain useful, nontoxic phyto-constituents which have the potential to boost the body's endogenous antioxidant system [16, 17]. The antioxidant phyto-chemicals found in the curry leaves (*Murraya koenigii*) have been reported to scavenge reactive oxygen species (ROS) and thus can protect against metal-induced oxidative stress, damage and subsequent health hazards[18]. The phyto-components present in curry leaves, so far reported, have no cytotoxic or adverse side effects, if not over consumed. The leaves have been reported to provide cardio-protective, anti-diabetic, anti-microbial, anti-ulcer as well as potent antioxidative activity [19]. Aqueous curry leaf extract (CuLE) has already been found to provide protection against cadmium and lead-induced cardio toxicity and hepato-toxicity [20,21]. The aqueous CuLE contain all polar phyto-constituents which primarily are some polyphenolic compounds and these may be the prime contributors in its anti-oxidant property [19,20,21].

Studies reveal that melatonin enhances the protective activities of vitamin E, vitamin C and GSH against free radical-induced oxidative stress, *in vitro* [22]. Our earlier finding reveals that aqueous extract of curry leaves can provide protection against lead induced oxidative stress[20]. Antioxidant potential of the herbal extracts in the amelioration of metal-induced oxidative stress need thorough investigation because these natural antioxidants form part of our regular diets and are not cyto-toxic and do not possess side-effects. Melatonin is present in all organisms and in many food items i.e., cereals, green vegetables and fruits[23]. Pharmacologically administered melatonin is well tolerated in humans with no reported side-effects[24]. Earlier we have demonstrated that melatonin and aqueous CuLE individually can ameliorate lead induced cardio-toxicity and cardiac tissue damage [5, 6, 20]. The current study was further extended to examine whether the small indole exhibits any synergism with aqueous CuLE in mitigating lead acetate-induced oxidative stress. The present studies demonstrate that pre-treatment of the rats with melatonin and CuLE in combination protected the rat cardiac tissues against lead-induced oxidative stress. The protection offered by this combination was found to be better compared to when these were tested individually, in one of our earlier work[5, 6, 20, 25] . The protection may possibly be exerted through their synergistic antioxidant activity. The results of the current studies seem to have relevance in the context of developing a unique pharmacological combination against lead induced cardio toxicity keeping in mind their high antioxidant potential and minimum or no side effects for humans who are exposed to lead environmentally or occupationally regularly.

MATERIALS AND METHODS

Chemicals used

All chemicals used in the present studies were of analytical grade. Anhydrous sodium carbonate (Na_2CO_3), cupric sulfate pentahydrate ($CuSO_4.5H_2O$), ferric chloride hexahydrate ($FeCl_3$. 6H2O), Folin–Ciocalteu phenol reagent, melatonin and lead acetate were obtained from SRL, India. Anhydrous DTNB and hematoxylin were procured from SRL, India Limited. Tetra ethoxy propane (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).

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.

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) 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.

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

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

Group I: Control

Group II: Lead acetate treated
Groups III: Melatonin (at a dose of 5 mg/kg BW) + lead acetate treated
Group IV: Melatonin (at a dose of 10 mg/kg BW) + lead acetate treated
Group V: Melatonin (at a dose of 20 mg/kg BW) + lead acetate treated

One hour after melatonin was fed the animals of the lead acetate and the melatonin + 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.

Preparation of an aqueous Curry Leaf Extract [CuLE]

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 ° Celsius for two hours till they were dry and crispy and crushed into a coarse dust with mortar and pestle. They were then grinded in a mechanical grinder to fine dusts and were stored in air tight Tarson bottles.

To prepare the aqueous extract, the dried leaf dusts were soaked overnight in double distilled water (7.5g per 100 ml), filtered through loin cloth (fine cotton 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} 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 *in vivo* experiments as well as also in *in vitro* assay systems. Any leftover of this solution was discarded.

Induction of oxidative stress *in vivo* with lead acetate and protection by melatonin and CuLE in combination

In a separate set of experiment and after acclimatization of the rats to laboratory conditions, the animals were divided into four groups, with six rats in each group:
Group I: Control
Group II: Melatonin (at a dose of 10mg/kg BW) + CuLE (at a dose of 50 mg/kg BW) (Positive control)
Groups III: Lead acetate treated (at a dose of 15 mg/kg BW)
Groups IV: Melatonin (at a dose of 10mg/kg BW) + CuLE (at a dose of 50 mg/kg BW) + lead acetate treated (at a dose of 15 mg/kg BW)

The rats of the melatonin + CuLE and the melatonin + CuLE + lead acetate group were fed melatonin dissolved in normal drinking water, at a dose of 10 mg kg^{-1} body weight, for 7 consecutive days. Half an hour after melatonin was fed, the animals of the melatonin + CuLE and the melatonin + CuLE + lead acetate group were fed CuLE at a dose of 50 mg/kg BW. After half an hour from then, i.e., an hour after melatonin was fed, the animals of the lead acetate and the melatonin + CuLE and the melatonin + CuLE + lead acetate treated groups were intraperitoneally injected with lead acetate solution (15 milligram per kg body weight, which is 10% of LD_{50} for lead acetate) for the 7 consecutive days. Animals of the control group received the vehicle only. Each day the body weight of the rats were recorded.

Collection of blood and tissues, and preparation of the serum

After the treatment period, the animals were sacrificed through cervical dislocation following mild ether anesthesia. The thoracic cavity was surgically opened and the blood was collected through cardiac puncture and the serum was prepared following a standard protocol. The hearts were surgically extirpated and rinsed immediately in cold saline, soaked properly, weighed and stored in plastic vials at -20^0Celcius for further biochemical analysis. A small portion of the cardiac tissue was processed for estimation of tissue cadmium through atomic absorption spectrophotometry. Another portion of the tissue was used for histological studies and the rest of the tissue was used for estimation of different biochemical parameters.

Estimation of the biomarkers of organ damage

(a) Measurement of the activities of serum glutamate oxaloacetate transaminase (SGOT)

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

(b) Measurement of serum total LDH and lactate dehydrogenase 1(LDH1) activity

Serum total lactate dehydrogenase (TLDH) activity was obtained by measuring the oxidation of NADH (0.1mM) to NAD^+ at 340 nm using 1.0 mM sodium pyruvate as substrate according to the method of Strittmatter *et al.*, [27].

The serum activity of lactate dehydrogenase1 (LDH1) was obtained by measuring the oxidation of NADH (0.1 mM) to NAD^+ at 340 nm using 1.0 mM sodium pyruvate as substrate, after incubating the serum samples at 65°C for 30 min, which destroys all isoforms except LDH1 according to the method of Strittmatter *et al.*, [27] using a UV–Vis spectrophotometer (Bio-Rad, Hercules, CA, USA). The enzyme activity was expressed as IU/L.

Estimation of the lead (Pb) content in the rat heart tissue by Atomic Absorption Spectrophotometry (AAS)

The cardiac 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 cardiac tissue.

Preparation of rat cardiac tissue homogenate and measurement of biomarkers of oxidative stress, viz., lipid peroxidation (LPO) level and protein carbonyl (PCO) content

The rat cardiac 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 (LPO) in the homogenate were determined as thiobarbituric acid reactive substances (TBARS) according to the method of Buege and Aust[28] with some modification[14]. 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^{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.

Protein carbonyl content was estimated by the method of Levine *et al.* [29]. A small piece of tissue (0.1g) 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 reduced glutathione content (GSH), oxidised glutathione content (GSSG), GSSG: GSH ratio and total sulfhydryl group content (TSH)

GSH content (as acid soluble sulfhydryl) of the heart was estimated by its reaction with DTNB (Ellman's reagent) following the method of Sedlak and Lindsey [30].The tissues were homogenized (10%) in 2 mM ice-cold ethylenediamine tetra-acetic 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 Ikediobo *et al.*, [31] with some modifications [32]. Tissues were homogenized (10%) in 2 mM ice-cold ethylenediaminetetraacetic acid (EDTA).The reaction mixture contained 0.1 mM sodium phosphate buffer, EDTA, NADPH and 0.14 units per ml glutathione reductase. The absorbance was measured at 340 nm using a UV-VIS spectrophotometer to determine the GSSG content. The values were expressed as nmoles GSSG/mg protein. GSSG: GSH ratio was evaluated.

Total sulfhydryl group content was measured following the method as described by Sedlak and Lindsey [30]. The values were expressed as nmoles TSH/ mg protein.

Measurement of the activities of the antioxidant enzymes, viz., the activities of cytosolic copper zinc superoxide dismutase (Cu-Zn SOD) and mitochondrial superoxide dismutase (Mn-SOD), catalase (CAT), glutathione reductase, glutathione peroxidase (GR), and glutathione-S-transferase (GST)

Copper-Zinc superoxide dismutase (Cu-Zn SOD or SOD1) activity was measured by hematoxylin autooxidation method of Martin *et al.*,[33].

Manganese superoxide dismutase (Mn-SOD or SOD2) activity was estimated by pyrogallol autooxidation method[34]. A weighed amount of cardiac 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 activity of rat cardiac tissue was assayed by the method of Beers and Sizer [35] . The GR activity of rat cardiac tissue was estimated using the method of Krohne-Ehrich *et al*,.[36]. 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 (as the source of enzyme) into the assay mixture. The reaction was initiated with 1mM oxidized glutathione (GSSG). The decrease in absorbance of NADPH was recorded at 340 nm. The specific activity of the enzyme was calculated as units/min/mg tissue protein.

Glutathione peroxidase activity of the rat cardiac tissue was measured according to the method of Paglia and Valentine [37]. The 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. A 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.

The activity of rat cardiac GST was measured according to the method of Habig et al., [38]. 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 the activities of pro-oxidant enzymes, viz., xanthine oxidase (XO) and xanthine dehydrogenase (XDH) activities of rat cardiac tissue

The activity of XO in rat cardiac tissue was measured by the conversion of xanthine to uric acid following the method of Greenlee and Handler [39]. The cardiac tissue was homogenized in cold (10%) in 50 mM phosphate buffer, pH 7.8. The homogenate was then centrifuged at 500 g for 10 minutes. The supernatant, thus obtained, was again centrifuged at 12,000 g for 20 min. The final supernatant was used, as the source of enzyme, 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) [27]. The weighed amount of rat cardiac tissue was homogenized in cold (10%) in 50 mM phosphate buffer with 1 mM EDTA, pH 7.2. The homogenate was 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.

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

The rat cardiac tissue was 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 of rat cardiac tissue was measured spectrophotometrically according to the method of Chretien et al.,[40] 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 of rat cardiac tissue was measured according to the method of Duncan *et al.*,[41] 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 of rat cardiac tissue was measured spectrophotometrically according to the method of Duncan et al.,[41] 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 a-ketoglutarate as the substrate. The enzyme activity was expressed as units/mg protein.

Succinate dehydrogenase (SDH) activity of rat cardiac tissue was measured spectrophotometrically by following the reduction of potassium ferricyanide (K_3FeCN_6) at 420nm according to the method of Veeger et.al.[42]. 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 the activities of some of the mitochondrial respiratory chain enzymes

The NADH-cytochrome c oxidoreductase activity of rat cardiac tissue was measured spectrophotometrically by following the reduction of oxidized cytochrome c at 565nm according to the method of Goyal and Srivastava[35]. One ml of assay mixture contained 50mM phosphate buffer, 0.1 mg BSA, 20mM oxidized cytochrome c, and 0.5 mM NADH along with a suitable aliquot of the mitochondrial suspension as the source of enzyme. The enzyme activity was expressed as Units / mg protein.

Cytochrome c oxidase activity of the rat cardiac tissue was determined spectrophotometrically by following the oxidation of reduced cytochrome c at 550nm according to the method of Goyal and Srivastava[35]. One ml of assay mixture contained 50 mM phosphate buffer, pH 7.4, 40 mM reduced cytochrome c, and a suitable aliquot of the mitochondrial suspension as the source of enzyme. The enzyme activity was expressed as Units / mg protein.

Measurement of tissue protein content

The protein content of various samples was estimated by the method of Lowry *et., al.*,[36] using bovine serum albumin (BSA) as the standard.

Histological studies

Routine hematoxylin-eosin staining of rat cardiac tissue sections

Immediately following sacrifice of the rats, hearts were surgically extirpated and a small portion of the ventricular tissue was fixed in 10% formalin and embedded in paraffin following routine procedure as described earlier [5]. Tissue sections (5 µM thick) were prepared and stained with hematoxylin-eosin.

Quantification of fibrosis by confocal microscopy

Another set of the tissue sections were stained with Sirius red (Direct Red 80) and the stained tissue sections were examined under Olympus BX51 (Olympus Corporation, Tokyo, Japan) microscope and images were captured with a digital camera attached to it. The same tissue sections were further imaged with laser scanning confocal system (Leica TCS, SP2, and Germany) and the stacked images through multiple slices were captured. The digitized images were then analyzed using image analysis system (Image J, NIH Software, Bethesda, MI) and the total collagen area fraction of each image was measured and expressed as the % collagen volume.

Study of left ventricular surface using scanning electron microscopy [SEM]

Tissue sections obtained from the left ventricle were fixed using 3% glutaraldehyde followed by dehydration using graded concentration of ethanol and imaged with a scanning electron microscope (EVO 18, Special Edition, Germany) at 3.00 KX.

Assessment of protection of DNA degradation by DNA gel electrophoresis

Rat cardiac tissue genomic DNA was extracted according to the method of Wu et al., [45]. Briefly, tissue samples suspended in phosphate buffered saline (PBS) were homogenized and the homogenate was suspended in lysis buffer in a 1:1 ratio (100 mM NaCl, 10 mM TRIS-HCl, pH- 8.5, 25 mM EDTA, 0.5 % SDS, 20 µg/ ml RNAse A, 0.1mg/ µl Proteinase K). The microfuges were kept at 0-4 °C and then were incubated 8 hours at 57°C. After digestion, an equal volume of phenol: chloroform: isoamyl alcohol [25:24:1] was added to each microfuge tube and mixed by gentle rocking. The mixture was then centrifuged at 10,000 rpm for 10 minutes. The aqueous portion was collected with auto-pipette and it was again washed with an equal volume of phenol: chloroform: isoamyl alcohol mixture. The aqueous portion was collected and washed with 100% ethanol and ammonium acetate [1:0.5:2].Then, the mixture was centrifuged at 14,000 rpm for 35 minutes in cold. The supernatant was discarded and the pellet was washed with 70% cold ethanol and was centrifuged at 10,000 rpm for 10 minutes. The tubes were dried and the white precipitates were dissolved in Tris EDTA buffer. Five microlitre of DNA sample was added to 995µl water and the absorbance were taken at 260 and 280 nm simultaneously for DNA and protein respectively. The DNA, thus obtained, gave an average 260/280 absorbance ratio of > 1.6. The obtained DNA samples were then mixed with loading dye [reaction stop mix dye] and resolved in an 1% agarose gel. The gel was stained with ethidium bromide and DNA bands were detected in a Gel-Doc apparatus (Biorad, Hercules CA).

Statistical evaluation

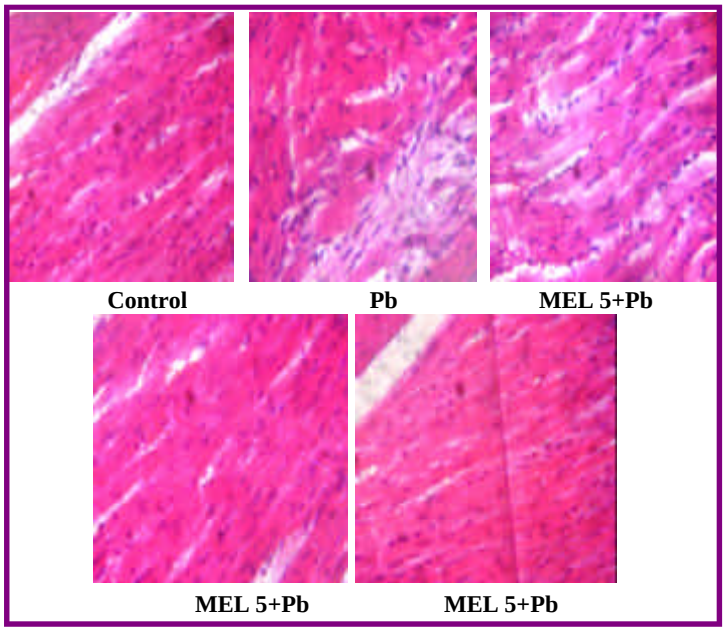
Each experiment was repeated at least three times with different rats. Data are presented as means ± S.E.M. Significance of mean values of different parameters between the treatments groups were analyzed using one way analysis of variances (ANOVA) after ascertaining the homogeneity of variances between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

RESULTS:

Selecting the dose of melatonin and CuLE

From the dose response studies, the dose of 10 mg /kg BW of melatonin was found to be the minimum effective dose which demonstrated statistically significant change (P<0.001 vs. lead acetate treated rats) against lead induced oxidative stress in rat heart (Figure 1and Table 1). Our previous studies have demonstrated that a dose of 50 mg/kg BW of CuLE have produced changes that were found to be the statistically significant (P<0.001 vs. lead acetate treated rats) against lead induced oxidative stress in rat heart[20].

Figure.1. Histopathological Studies of heart.



Effect of Melatonin at different doses against lead-induced changes in the rat cardiac tissue morphology (Hematoxylin and Eosin stained , 400X magnification)

Table 1. Table shows the levels of lipid peroxidation and reduced glutathione as well as the activities of superoxide dismutase and catalase of cardiac tissue in lead acetate treated and CuLE protected rats.

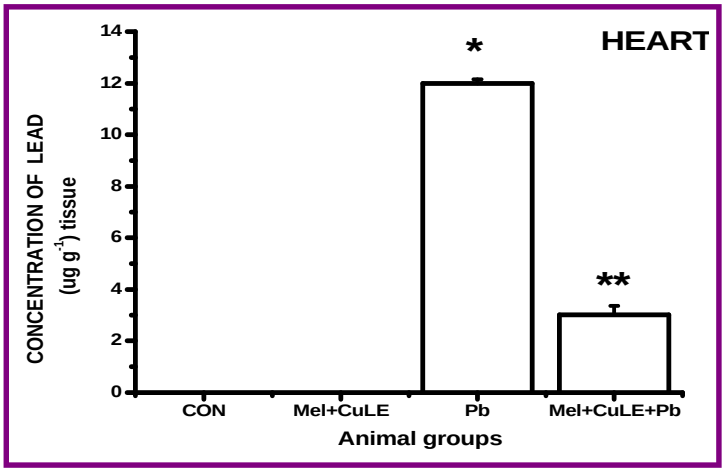
Group	Lipid Peroxidation (nmoles of TBARS / mg protein)	Reduced Gluthione (nmoles/mg protein)
Con	0.1299±0.0354	12.12833±0.124
Pb	0.5636±0.0457*	18.66333±0.654*
Mel (5mg/Kg BW) +Pb	0.4521±0.0245	17.0214±0.541
Mel (10mg/Kg BW) +Pb	0.1792±0.0321**	14.3333±0.514**
Mel (20mg/Kg BW) +Pb	0.1723±0.0443	13.98721±0.074
Group	**Superoxide dismutase activity (Cu-Zn SOD) (Units/min/mg protein)**	**Catalase activity (Units/min/mg protein)**
Con	4.443±0.331	15.51±0.672
Pb	10.98±0.434*	31.98±0.822*
Mel (5mg/Kg BW) +Pb	8.552±0.659	28.98±0.976
Mel (10mg/Kg BW) +Pb	4.335±0.324**	15.67±0.222**
Mel (20mg/Kg BW) +Pb	4.112±0.284	15.55±0.453

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; Mel= Melatonin; Pb=Lead; Mel(5mg/Kg BW) + Pb= Melatonin(5mg/Kg BW) +Lead; Mel(10mg/Kg BW) + Pb= Melatonin(10mg/Kg BW) +Lead; Mel(20mg/Kg BW) + Pb= Melatonin(20mg/Kg BW) +Lead.

Status of cardiac tissue lead content

Figure 2 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. However, when the rats were pre-treated with melatonin + CuLE in combination at a dose of 10 mg/ kg BW and 50 mg / kg BW (both fed orally) respectively, the tissue lead content was found to be reduced significantly (62.13% ; **P<0.001 vs Pb-treated group) .

Figure.2. Effect of melatonin+ Curry leaf aqueous extract (CuLE) on lead content of 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; Mel+ CuLE = Melatonin + Curry leaf extract; Pb=Lead; Mel+ CuLE +Pb= Melatonin +Curry leaf extract +Lead.

Biomarkers of organ functions and organ damage

Table 2 shows that treatment of rats with lead acetate caused a significant elevation in the level of activities of SGPT (2.21 folds, *P<0.001 vs control), of SGOT (1.83 folds, *P <0.001 vs control) and serum total LDH (1.84 folds, p<0.001 vs. control) , LDH1(3.9 folds, *P <0.001 vs control; a specific marker enzyme of cardiac damage). However, when the rats were pre-treated with the present dose of melatonin + CuLE in combination, the activities of all the four enzymes in serum were found to be significantly protected from being increased (SGPT:53.31%, **P < 0.001 vs Pb-treated group; SGOT : 45.64% , **P < 0.001 vs. Pb-treated group ; TLDH:45.55%, **P < 0.001 vs Pb-treated group ; LDH1: 72.80% , **P < 0.001 vs Pb-treated group. Melatonin + CuLE in combination, alone, was found to have no significant effect on the activities of these marker enzymes for organ function and damage.

Table 2. Effect of melatonin (10 mg/kg BW) and aqueous Curry leaves extract (CuLE) (50 mg/kgBW) on the activities of ALKP, the levels of serum bilirubin, SGPT, SGOT, TLDH and LDH 1 of the experimental rats.

Parameters Studied	Con	Mel + CuLE	Pb	Mel + CuLE +Pb
SGOT(IU/L)	10.21±0.324	10.54±0.754	18.65±0.458*	10.25±0.564**
TLDH (IU/L)	4.654±0.211	4.425±0.214	8.547±0.312*	4.654±0.354**
LDH 1(IU/L)	1.451±0.054	1.654±0.078	5.684±0.084*	1.546±0.078**

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; Mel+ CuLE = Melatonin + Curry leaf extract; Pb=Lead; Mel+ CuLE +Pb= Melatonin +Curry leaf extract +Lead

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 3) (3.1 folds in cardiac tissue; *P< 0.001 vs control group). Pre-treatment of rats with melatonin + CuLE in combination significantly protected the lipid peroxidation level from being increased (68.08% in heart; **P< 0.001 vs Pb acetate-treated group). However, melatonin + CuLE in combination had no effect on the lipid peroxidation level of tissue.

Table 3 also shows that there occurred a significant increase in the level of protein carbonyl of cardiac tissue of rat following lead acetate treatment (2.9 folds in heart; *P< 0.001 vs control group). Pre-treatment of rats with melatonin + CuLE in combination almost completely protected the tissue protein carbonyl level from being increased (65.03%; **P< 0.001 vs Pb-treated group). However, melatonin + CuLE in combination have no significant effect on the protein carbonyl level of rat cardiac tissue.

Status of the activities of the antioxidant enzymes

Table 3 also reveals that treatment of rats with lead acetate at the indicated dose significantly increased the activities of cytosolic Cu–Zn–SOD, the mitochondrial Mn–SOD and the catalase of the rat cardiac tissue (2.3 folds, 1.95 folds and 2.02 folds increase respectively vs control, * P < 0.001 vs control). Pre-treatment of rats with melatonin + CuLE in combination was found to protect the activities of these antioxidant enzymes from being increased.(54.84%, 46.92% and 59.51% decrease respectively, **P < 0.001 vs Pb-treated group). However, melatonin + CuLE in combination did not significantly alter the activities of any of the enzymes studied.

Table 3. Effect of Melatonin (10 mg/kg BW) + CuLE (50 mg/kg BW) on the levels of lipid peroxidation, protein carbonyl, the activities of Cu-Zn superoxide dismutase, Mn superoxide dismutase and catalase in heart of the experimental rats.

Parameters Studied	Con	Mel+CuLE
LPO (nmoles of TBARS/mg protein)	0.113±0.006	0.111±0.005
Protein carbonyl (nmoles per mg protein)	6.546±0.123	6.455±0.121
Cu-Zn SOD activity (units/min/mg protein)	7.121±0.123	7.101±0.122
Mn SOD activity (units/min/mg protein)	6.321±0.354	6.254±0.356
Catalase activity (μmoles H_2O_2 consumed/min/mg protein)	15.65±0.684	15.55±0.558
Parameters Studied	**Pb**	**Mel +CuLE+Pb**
LPO (nmoles of TBARS/mg protein)	0.354±0.007*	0.113±0.006**
Protein carbonyl (nmoles per mg protein)	19.02±0.123*	6.652±0.126**
Cu-Zn SOD activity (units/min/mg protein)	16.21±0.114*	7.321±0.115**
Mn SOD activity (units/min/mg protein)	12.32±0.345*	6.54±0.365**
Catalase activity (μmoles H_2O_2 consumed/min/mg protein)	31.65±0.287*	15.98±0.245**

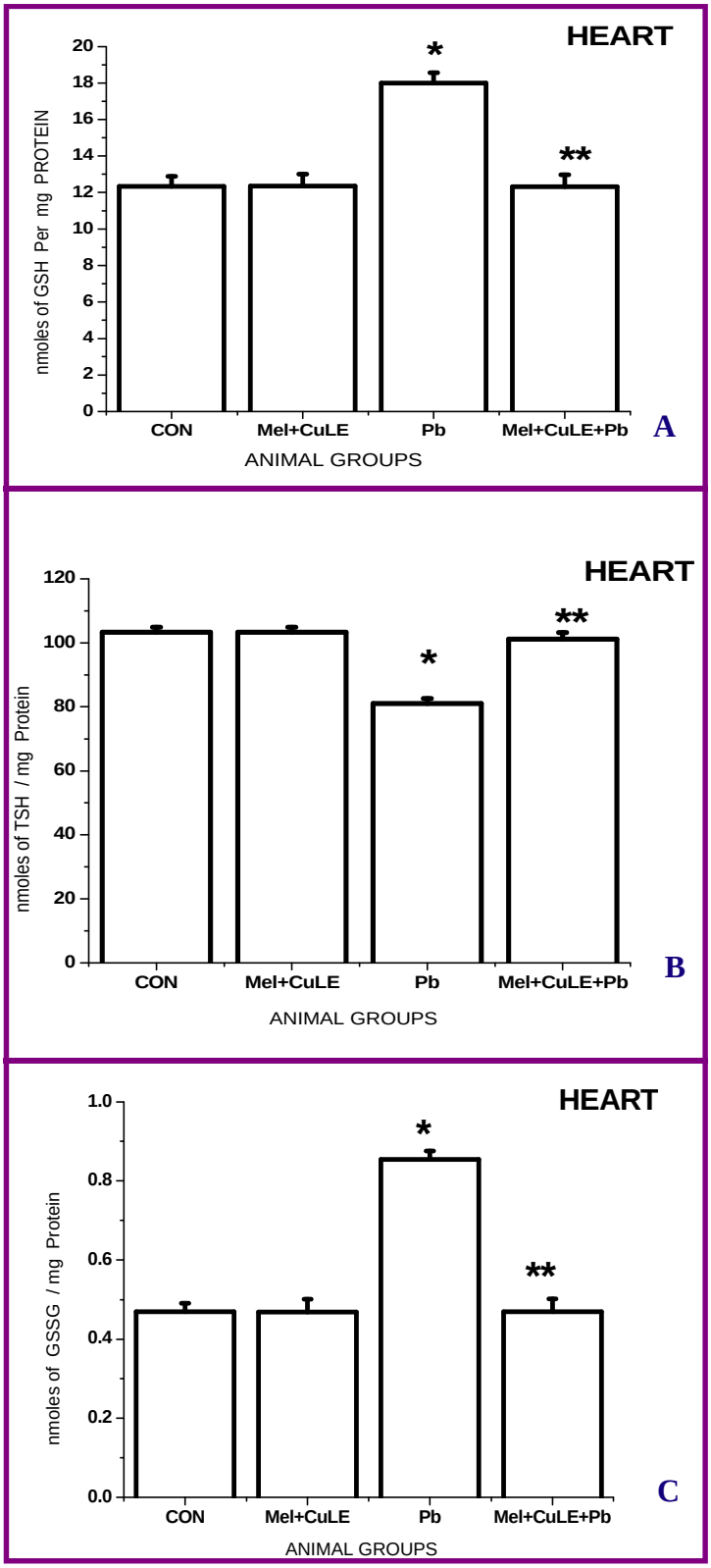
Values are expressed as Mean ± SE of 6 animals in each group. Data were analyzed by using one way analysis of variances (ANOVA) using Microcal Origin version 7.0 for Windows.*P<0.001 compared to control; **P< 0.001 compared to lead treated group; Con = Control; Mel+ CuLE = Melatonin + Curry leaf extract;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

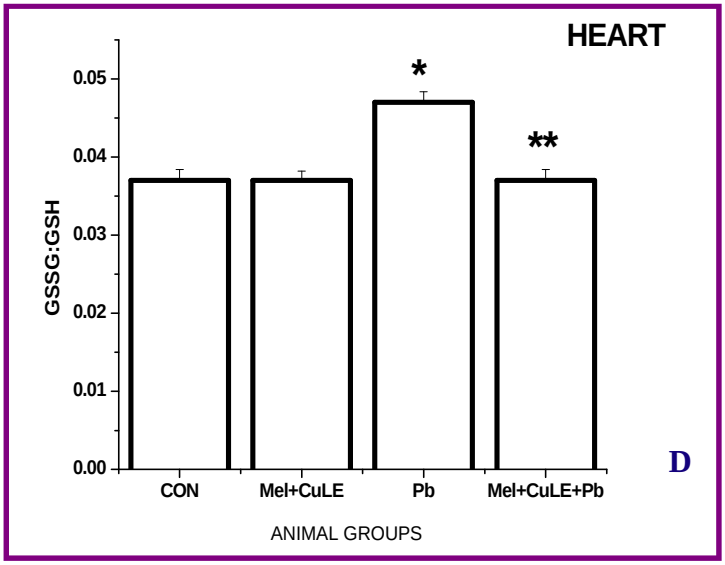
Status of the levels of the tissue GSH, GSSG, TSH and the ratio of GSSG to GSH

Figure 3 (a,c and d) shows that there occurred a significant increase in the level of cardiac tissue GSH and GSSG as well as in the GSSG: GSH ratio of cardiac tissue of rat following lead acetate treatment (45.78%, 82.82% and 27.02% respectively,*P< 0.001 vs control group). Pre-treatment of rats with melatonin + CuLE in combination almost completely protected the tissue GSH and GSSG levels and thus the GSSG: GSH ratio also from being increased in cardiac tissue (31.51 %, 44.97% and 21.27% respectively, **P< 0.001 vs Pb-treated group). However, melatonin + CuLE in combination has no significant effect on the GSH and GSSG levels of rat cardiac tissue.

Treatment of rats with lead acetate decreased the total thiol (TSH) level significantly (21.64%,*P< 0.001 vs control group). Pre-treatment of rats with melatonin + CuLE in combination almost completely protected the TSH from being decreased in cardiac tissue (24.97%, **P< 0.001 vs Pb-treated group). However, melatonin + CuLE in combination have no significant effect on the TSH level of heart (Figure 3b).

Figure.3. Effect of Melatonin (10 mg/kg BW) + CuLE (50 mg/kg BW) against lead-induced alteration in the value of GSH (A), TSH (B), GSSG (C) and GSSG: GSH (D) in rat cardiac tissue.



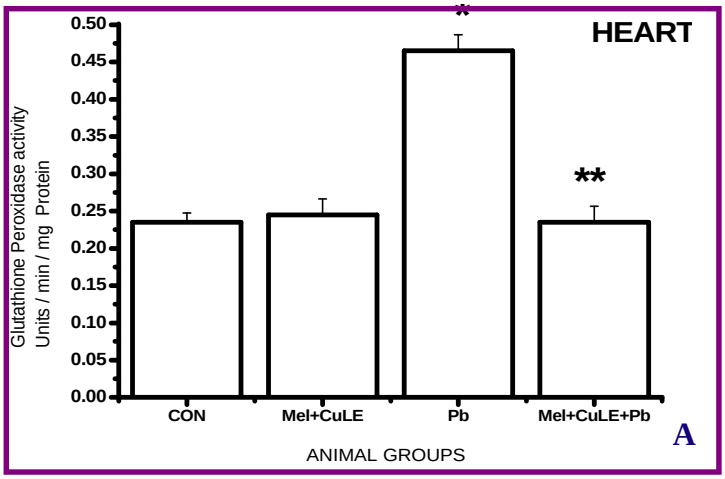


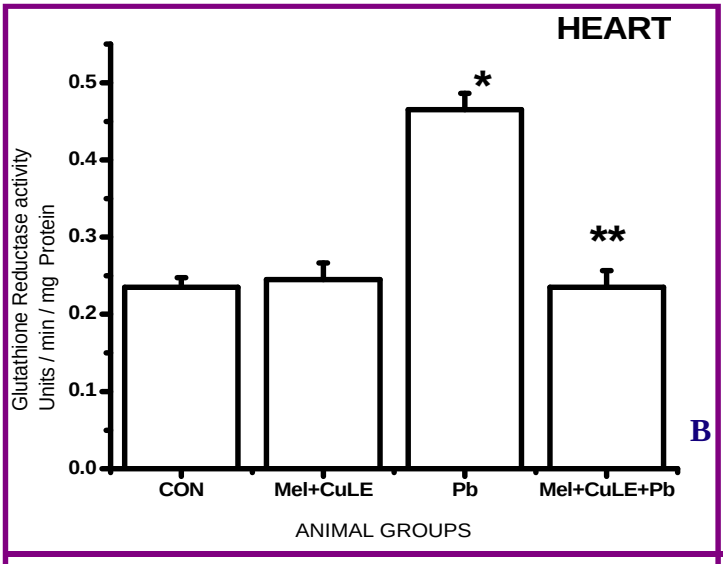
*Values are expressed as Mean ± SE of 6 animals in each group. *P<0.001 compared to control; **P< 0.001 compared to lead treated group; CON = Control; Mel+ CuLE = Melatonin + Curry leaf extract; Pb=Lead; Mel+ CuLE +Pb= Melatonin +Curry leaf extract +Lead.*

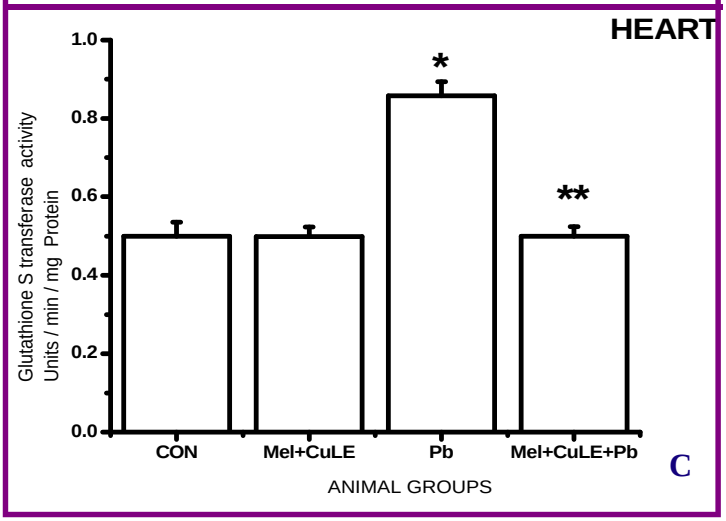
Status of the activities of glutathione peroxidise (GPx), glutathione reductase (GR) and glutathione –S- transferase (GST) of the rat cardiac tissue

Treatment of rats with lead acetate for seven consecutive days at a dose of 15 mg / kg body weight increased the activities of GPx , GR and GST (Fig. 4a,b and c) [1.34 times, 0.97 times and 0.71 times respectively,*P< 0.001 vs control group] in cardiac tissue. However, the enzyme activities were found to be completely protected from being increased when the rats were pre-treated with melatonin + CuLE in combination for the similar period of time (56.23% , 49.46% and 41.85%,**P< 0.001 vs lead acetate -treated group). However, melatonin + CuLE in combination, alone (positive control), have no significant effect on the activities of these enzymes in the rat cardiac tissue.

Figure.4. Effect of Melatonin (10 mg/kg BW) + CuLE (50 mg/kg BW) against lead-induced alteration in the activities of glutathione peroxidises (A), glutathione reductase (B), glutathione S transferase (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 treated group; combinationCON = Control; Mel+ CuLE = Melatonin + Curry leaf extract; Pb=Lead; Mel+ CuLE +Pb= Melatonin +Curry leaf extract +Lead.*

Status of the activities of the pro-oxidant enzymes of rat cardiac tissue

The activities of cardiac xanthine oxidase (XO) [Table 4] and xanthine dehydrogenase (XDH) as well as the total enzyme activity, i.e., XO plus XDH and, XO : XDH ratio, XO/(XO+XDH) all increased significantly following treatment of rats with lead acetate (2.8 folds, 2.7 folds, 2.8 folds , 1.02 folds and 1.0 fold increase respectively in cardiac tissue vs. control, *P < 0.001 vs control). All these parameters were significantly protected from being increased when the rats were pre-treated with melatonin + CuLE in combination (64.76%, 62.07%, 62.06%, 1.07 folds and 1 fold decrease respectively in cardiac tissue vs. lead acetate treated groups, **P < 0.001 vs lead acetate-treated group). However, melatonin + CuLE in combination have no effect on the activities of XO and XDH, the total enzyme activity, i.e., XO plus XDH, XO: XDH ratio as well as XO/(XO+XDH).

Table 4. Effect of Melatonin (10 mg/kg BW) + CuLE (50 mg/kg BW) on the activities of XO, XDH, XO+XDH, XO/XDH and XO/ (XO+XDH) in heart of the experimental rats.

PARAMETERS	Con	Mel+CuLE
XO(milliunits/min/mg protein)	0.0074±0.0012	0.0074±0.0014
XDH(milliunits/min/mg protein)	0.021±0.0020	0.021±0.0022
XO+XDH	0.028±0.0014	0.028±0.0015
XO/XDH	0.352±0.021	0.352±0.019
XO/(XO+XDH)	0. 264±0.0014	0.250±0.0013
PARAMETERS	**Pb**	**Mel + CuLE +Pb**
XO(milliunits/min/mg protein)	0.021±0.0054*	0.0074±0.0017**
XDH(milliunits/min/mg protein)	0.058±0.0020*	0.022±0.0015**
XO+XDH	0.079±0.0015*	0.029±0.0016**
XO/XDH	0.362±0.016*	0.336±0.015**
XO/(XO+XDH)	0.266±0.0013*	0.255±0.0014**

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

Status of the activities of pyruvate dehydrogenase (PDH) and some of the Kreb's cycle enzymes:

Table 5 reveals that treatment of rats with lead acetate caused a decrease of pyruvate dehydrogenase activity (64.497% decrease , *P < 0.001 vs their control in heart tissues respectively). Pre-treatment of rats with melatonin + CuLE in combination significantly protected the enzyme activity from being decreased (2.8 folds protection in heart tissue; **P < 0.001 vs lead acetate -treated group). However, melatonin + CuLE in combination, alone (positive control), were found to have no effect on the activity of this enzyme in the cardiac tissue.

Table 5 further reveals that treatment of rats with lead acetate significantly decreased the activity of isocitrate dehydrogenase in cardiac tissue (56.89% decrease respectively, *P < 0.001 vs their respective control). Isocitrate dehydrogenase is a key enzyme in cellular defence against oxidative damage as it provides NADPH in the mitochondria, which is needed for the regeneration of mitochondrial GSH or thioredoxin. The activity of the enzyme in the heart was found to be protected significantly from being decreased when the rats were pre-treated with melatonin + CuLE in combination (3.8 folds , **P < 0.001 vs lead acetate -treated group). However, melatonin + CuLE in combination, alone (positive control), have no effect on the activity of isocitrate dehydrogenase in the cardiac tissues.

Treatment of rats with lead acetate decreased alpha keto glutarate dehydrogenase (α-KGDH) activity in cardiac tissue (73.77% decrease, *P < 0.001 vs control) (Table 4). The activity of the enzyme was found to be significantly protected from being decreased when the rats were pre-treated with melatonin + CuLE combination (3.8 folds increase in heart tissues **P < 0.001 vs Lead acetate -treated group). However, melatonin + CuLE in combination, alone (positive control), have no significant effect on the activity of α-KGDH in the tissues (Table 5).

Treatment of rats with lead acetate for seven consecutive days decreased the activity of succinate dehydrogenase (SDH) in cardiac tissues significantly (77.38% decrease, *P < 0.001 vs control) (Table 4). 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. However, pre-treatment of rats with melatonin + CuLE in combination significantly protected the SDH activity from being decreased in cardiac tissues (4.3 folds, **P < 0.001 vs lead acetate -treated group). Melatonin + CuLE in combination, alone (positive control), has no effect on the activity of this enzyme in rat cardiac tissue (Table 5).

Table 5. Effect of Melatonin (10 mg/kg BW) + CuLE (50 mg/kg BW) on the activities of PDH, ICDH, a-KGDH and SDH in heart of the experimental rats.

Parameters Studied	Con	MEL+CuLE
PDH (units/min/mg protein)	0.921±0.014	0.932±0.015
ICDH(units/min/mg protein)	0.058±0.0015	0.057±0.0028
α-KGDH (units/min/mg protein)	0.061±0.0023	0.061±0.0025
SDH(units/min/mg protein)	1.512±0.212	1.511±0.032
Parameters Studied	**Pb**	**MEL+CuLE+Pb**
PDH (units/min/mg protein)	0.318±0.016*	0.901±0.023**
ICDH(units/min/mg protein)	0.025±0.0035*	0.057±0.0027**
α-KGDH (units/min/mg protein)	0.016±0.0031*	0.060±0.0028**
SDH(units/min/mg protein)	0.342±0.043*	1.498±0.045**

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

Status of the activities of mitochondrial respiratory chain enzymes

Treatment of rats with lead acetate for similar period of time also decreased cytochrome c oxidase activity in cardiac tissues (77.95% decrease, *P< 0.001 vs control group). The activity of this enzyme was found to be significantly protected from being decreased compared to lead acetate treated group when rats were pre-treated with melatonin + CuLE in combination (6.8 folds increase respectively in heart , **P< 0.001 vs Lead acetate -treated group). Melatonin + CuLE in combination alone (positive control), however, have no effect on the activity of this enzyme (Table 6).

Treatment of rats with lead acetate for seven consecutive days at a dose of 15 mg / kg body weight decreased NADH cytochrome-c-oxido reductase activity of rat cardiac tissue (77.95% cardiac tissue,*P< 0.001 vs control group). However, the enzyme activity was found to be completely protected from being decreased when the rats were pre-treated with melatonin + CuLE in combination for the similar period of time (6.8 folds heart tissues, **P< 0.001 vs Lead acetate -treated group). However, melatonin + CuLE in combination alone (positive control), have no significant effect on the activity of this enzyme in rat cardiac tissue (Table 6).

Table 6. Effect of Melatonin (10 mg/kg BW) + CuLE (50 mg/kg BW) on the activities of Cytochrome c oxidase and NADH Cytochrome c oxido-reductase in heart of the experimental rats.

Parameters Studied	Con	MEL+CuLE
Cytochrome c oxidase activity (units/min/mg protein)	0.127±0.013	0.127±0.0016
NADH cytochrome c oxido-reductase activity (units/min/mg protein)	7.121±0.043	7.112±0.032
Parameters Studied	**Pb**	**MEL+CuLE+Pb**
Cytochrome c oxidase activity (units/min/mg protein)	0.018±0.0029*	0.123±0.0032**
NADH cytochrome c oxido-reductase activity (units/min/mg protein)	2.007±0.013*	7.013±0.0054**

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

Macroscopic and microscopic (histological) studies

Figure 5a reveals a reduction in heart size (macroscopic study) in the rats treated with lead acetate. However, pre-treatment of rats with melatonin + CuLE in combination protected the heart size from being reduced. The heart size did not get affected in the rats treated with melatonin + CuLE only (positive control). Figure 5b reveals a significant decrease in heart weight: body weight ratio compared to control. Here also, pre-treatment of rats with melatonin + CuLE in combination significantly attenuated the heart weight: body weight ratio. However, melatonin + CuLE in combination alone (positive control), was found to have no effect on heart weight: body weight ratio (Figure 5b).

Figure 5c (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 melatonin + CuLE in combination, there was no sign of ischemia or tissue damage. Melatonin + CuLE in combination alone (positive control), however, has no effect on cardiac tissue morphology. Figure 5c (middle panel) shows a depletion of cardiac tissue collagen following treatment of rats with lead acetate. Pre-treatment of rats with melatonin + CuLE in combination protected the cardiac tissue from being depleted of tissue collagen. Melatonin + CuLE in combination alone (positive control) 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. Fig. 5c (lower panel) shows similar images captured by confocal laser scanning microscope. Figure 5d represents quantification of fibrosis as percent collagen volume. The results further indicate a protective effect of melatonin + CuLE in combination against lead acetate-induced damage in rat cardiac tissue. However, melatonin + CuLE in combination alone (positive control), was found to have no effect on tissue collagen content.

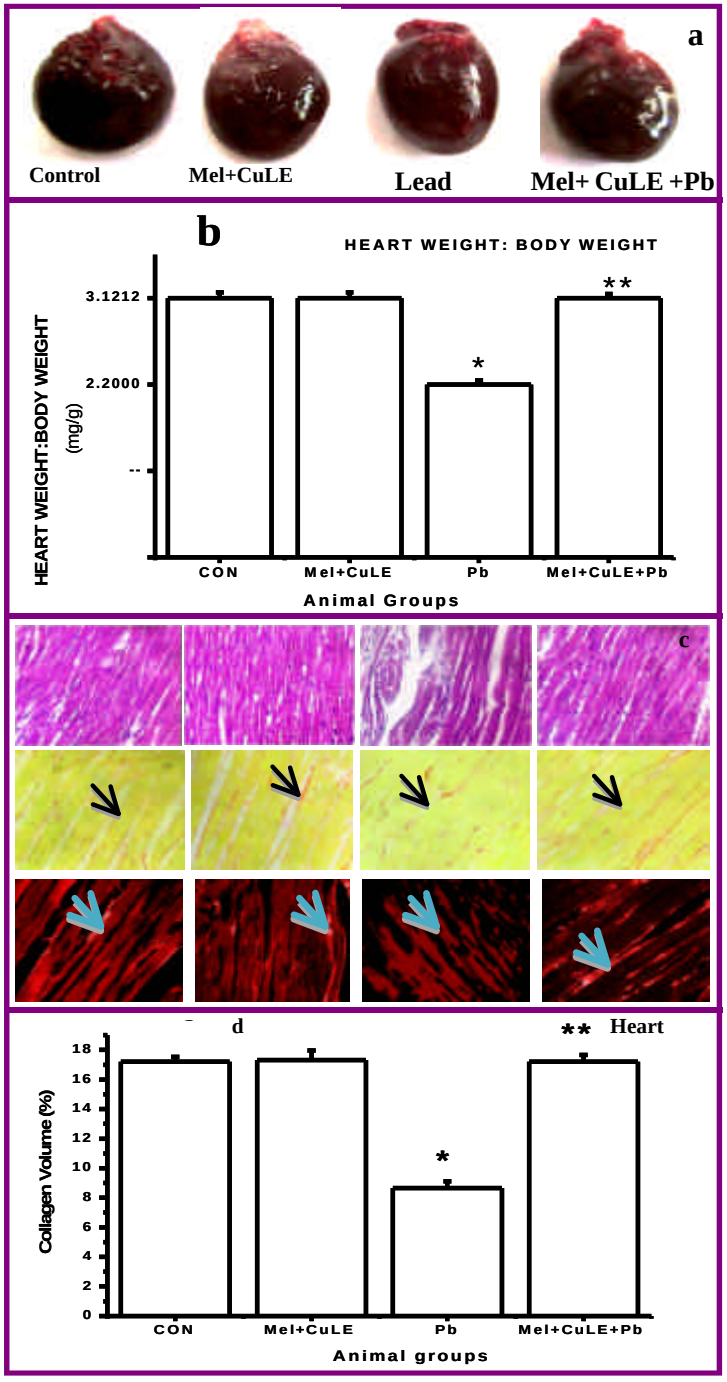


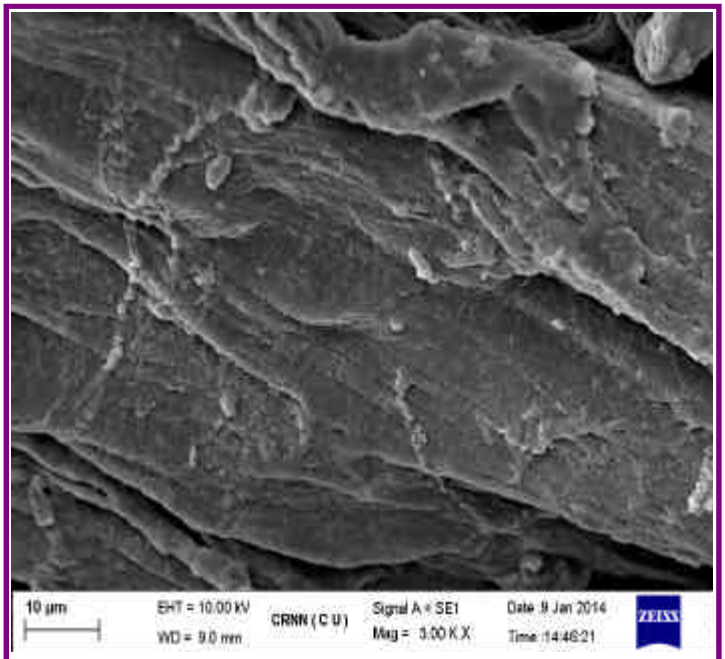
Figure.5. a. Effect of Melatonin + CuLE against lead-induced reduction in the heart size of the rats **b**. Effect of Melatonin + CuLE on heart weight: body weight ratios **c, first panel :** Effect of Melatonin + CuLE on changes in the rat cardiac tissue morphology (Hematoxylin and Eosin stained , 400X magnification) **c, second panel :** Effect of Melatonin + CuLE on changes in the rat cardiac tissue morphology (Sirius red stained sections, 400X magnification) **c,third panel :** Similar images captured by confocal laser scanning microscope for quantification of fibrosis.

d. Graph showing collagen volume % of the cardiac tissues.

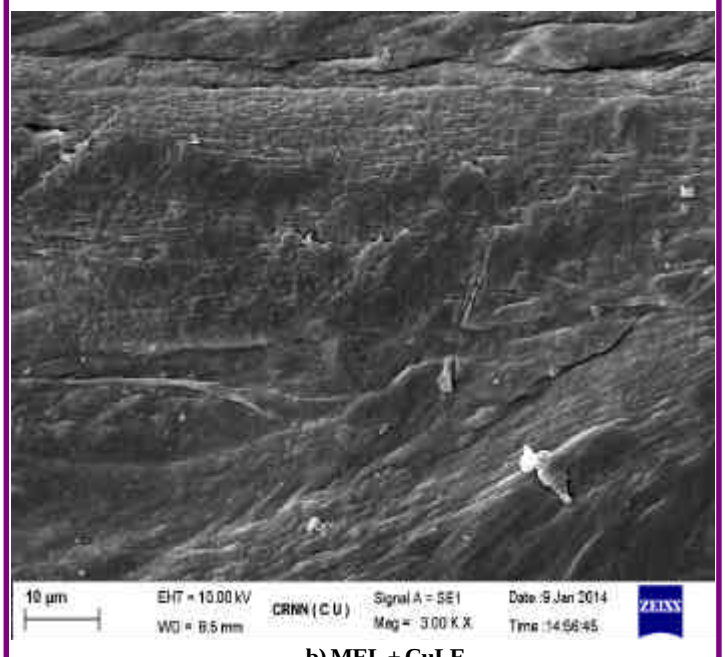
Values are expressed as Mean ± SE of 6 animals in each group. Data were analyzed by using one way analysis of variances (ANOVA) using Microcal Origin version 7.0 for Windows.*P<0.001 compared to control; **P< 0.001 compared to lead treated group; CON = Control; Mel+ CuLE = Melatonin + Curry leaf extract;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

The architecture of the surface of the tissue from the inner side (i.e., from the cavity side) of the left ventricle was studied using scanning electron microscopy. The ventricular surface was found to be deformed in lead acetate treated rats (Figure 6). The changes observed were found to be protected in the rats pre-treated with melatonin + CuLE in combination (Figure 6). However, melatonin + CuLE in combination alone (positive control), was found to have no effect on ventricular tissue surface.

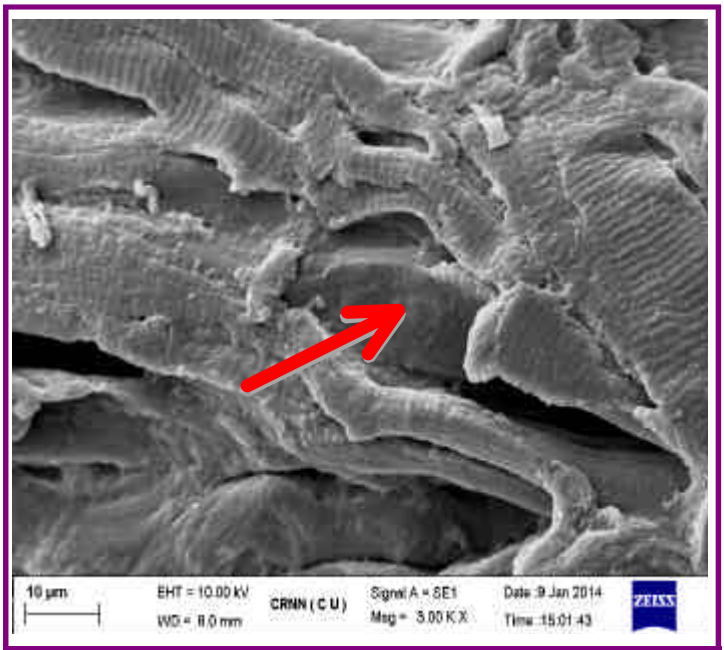
Figure. 6. Pictomicrograph of left ventricular surface using scanning electron microscopy [SEM]



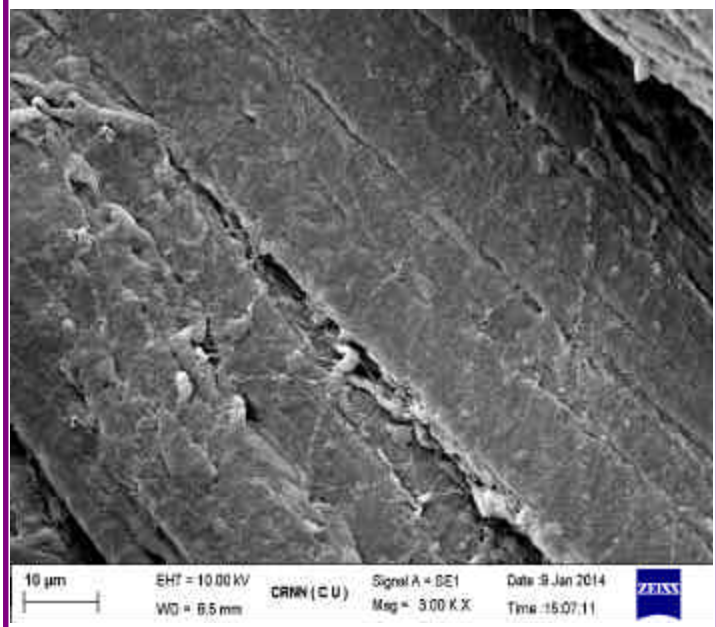
a) CONTROL



b) MEL + CuLE



c) LEAD



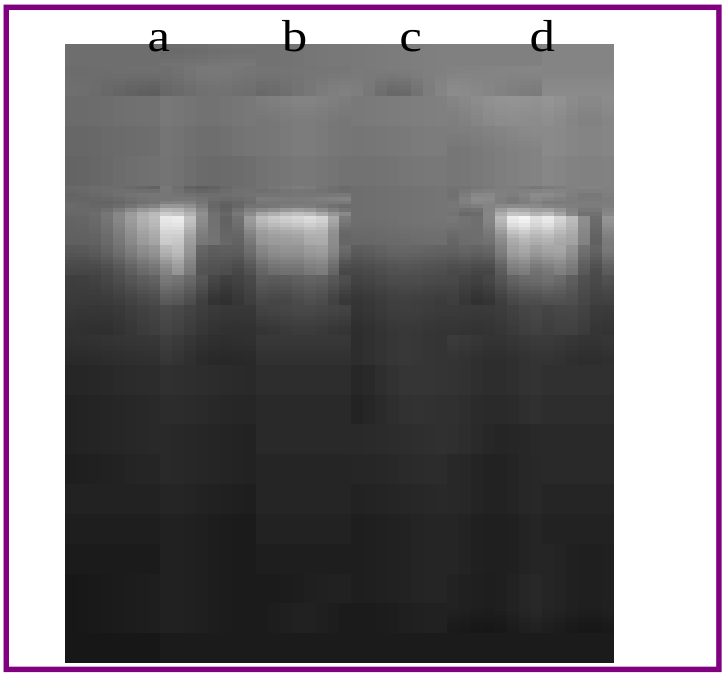
d) MEL + CuLE + LEAD

Control (a), Melatonin+CuLE trteated (b), lead acetate-treated (c) and Melatonin+CuLE+Lead acetate treated (d).

Assessment of protection of DNA damage in cardiac tissue by DNA gel electrophoresis

Treatment of rats with lead acetate for seven consecutive days at a dose of 15 mg / kg body weight caused DNA damage. DNA damage by lead acetate was indicated by formation of smear in gel electrophoresis of DNA extracted from the cardiac tissue. No such smear was detected in electrophoresis of DNA extracted from heart tissues of control, melatonin + CuLE (positive control) and melatonin + CuLE +lead acetate treated rats (Figure 7).

Figure. 7. Analysis of cardiac genomic DNA degradation by lead treatment and protection of the same by Mel+CuLE pre treatment using agarose gel electrophoresis of genomic DNA from cardiac tissue of rat.



From left, control (a), Melatonin+CuLE trteated (b), lead acetate-treated (c) and Melatonin+CuLE+Lead acetate treated (d). The extraction of genomic DNA and agarose gel electrophoresis was repeated at least 3 times and best representative blot given.

DISCUSSION

It is evident from our studies that there occurred a significant increase in levels of the marker enzymes due to treatment of rats with lead acetate and we did not observe any animal mortality during the entire treatment period. Increased level of serum lactate dehydrogenase and serum lactate dehydrogenase 1 are indicative of cardiac damage. Increased level of SGOT also indicates cardiac damage. LDH1 is a specific marker of cardiac tissue damage. We observed increased level of TLDH, LDH1 and SGOT on treatment with lead acetate indicating cardiac damage. All these were protected from being increased on pre-treatment of rats with the combination i.e., melatonin + CuLE. Thus, our results provide conclusive evidence about cardio-protective ability of the melatonin and CuLE when they are used in combination against lead acetate induced oxidative stress situation.

Membrane lipids are highly susceptible to free radical damage [46]. Lipids when react with free radicals can undergo the highly damaging chain reaction of lipid peroxidation [28]. The other mechanism is that a molecule can indirectly induce oxidative stress by increasing the vulnerability of membranes to the attack of ROS[47]. The major constituents of biological membranes are lipids and proteins. Membrane lipid peroxidation is a deleterious process solely carried out by free radicals. Lead is known to produce oxidative damage in the tissues by enhancing the process of peroxidation of membrane lipids[48]. Protein carbonyl content is used as a biomarker of oxidative stress[49]. The usage of protein CO groups as biomarkers of oxidative stress is advantageous compared to the measurement of other oxidation products because of the relative early formation and the relative stability of carbonylated proteins[49]. We found, in our studies, an enhanced level of protein CO as well as level of lipid peroxidation in cardiac tissues of lead acetate treated rats. When the rats were pre-treated with melatonin + CuLE combination both level of LPO and protein CO were observed to be protected from being increased.

Glutathione and glutathione-related enzymes play a key role in protecting the cell against oxidative stress. Reactive Oxygen Species (ROS) are reduced by GSH in the presence of GPx. GSH is oxidized to GSSG, which in turn is rapidly reduced back to GSH by GR at the expense of NADPH. Reduced glutathione plays a role in the detoxification of a variety of electrophilic compounds and peroxides via catalysis by glutathione-S-transferases and glutathione peroxidases. Glutathione-S-transferases are a family of ubiquitous enzymes that can catalyze the formation of GSH electrophile thioether conjugates and the GSH-linked reduction of lipid hydroperoxides [50]. Glutathione reductase reduces oxidized glutathione (GSSG) to biologically active GSH. The NADPH is the cofactor of GR. Glutathione peroxidase 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 tissue on treatment with lead acetate. Increased level of GSH is probably the inducer for enhanced activity of GR [51]. On the other hand, increased oxidation of GSH to GSSG leads to increased level of GSSG and the ratio of GSSG: GSH in lead acetate treated animals compared to control. Reduced glutathione is increased to meet the increased demand of the same for combating the situation of increased oxidative stress. The elevated levels of oxidative stress due to increased generation of ROS by lead acetate caused increased lipid peroxidation in rat cardiac tissue. All these parameters were protected from being increased on pre-treatment of the rats with melatonin + CuLE in combination.

To minimize oxidative damage, organisms developed antioxidative mechanisms[52]. Often it is observed that these antioxidative defense systems of cells are triggered by increased ROS production [52]. Superoxide anion free radicals are generated *in vivo* and are increased with condition of oxidative stress [46]. Increased level of superoxide anion free radical causes enhanced activity of the enzyme SOD while increased SOD activity leads to increased production of hydrogen peroxide[53]. Thus, the level of catalase activity is also increased in response to the increased hydrogen peroxide[53]. We observed enhanced activities of both these crucial antioxidant enzymes in the cardiac tissues of lead acetate treated rats. The activities of both the enzymes

were found to be protected from being increased when the rats were pre-treated with melatonin + CuLE in combination.

Xanthine oxidoreductase, under normal conditions, exists in dehydrogenase form and uses NAD+ and there is no or very little production of superoxide anion free radicals. Under ischemic conditions, there is depletion of ATP and subsequent loss of membrane Ca^{2+} gradient. Increased Ca^{2+} levels activates Ca^{2+}- dependent proteases which cause selective proteolysis of the dehydrogenase to convert it into xanthine oxidase (XO) which acts both on hypoxanthine and xanthine at the expense of molecular oxygen to produce superoxide anion free radical [54]. Thus, XO in oxidative stress conditions may play a major role in contributing free radical mediated damage[54]. A significant increase in the ratio of activity of xanthine oxidase: xanthine dehydrogenase, and also increase in their individual activity in the tissues indirectly confirms lead acetate- induced oxidative stress mediated generation of reactive oxygen species. We observed enhanced activities of both these pro-oxidant enzymes and their sum and ratios in the cardiac tissue of lead acetate treated rats. The activities of both of these enzymes were found to be prevented from being increased when the rats were pre-treated with melatonin + CuLE in combination. Melatonin +CuLE in combination (positive control) alone had no significant effect on the activities of both of the enzymes.

Mitochondria, the 'power house' of the cell are the major source of ROS production in cells[55, 56]. In our study, we found that there has been considerable decrease in activities of pyruvate dehydrogenase and the Kreb's cycle enzymes like isocitrate dehydrogenase, alpha-keto glutarate dehydrogenase and succinate dehydrogenase in cardiac tissues following treatment of rats with lead acetate for seven consecutive days. The activities of all these enzymes were protected from being decreased when the rats were pre-treated with melatonin + CuLE in combination. Earlier researchers have reported that other heavy metals markedly inhibit uncoupler-stimulated oxidation of various NADH-linked substrates as well as that of succinate [55]. Heavy metals are also known to affect respiratory chain complexes[55].We have also observed effect of lead on respiratory chain complexes[57]. The impairment of electron transfer through NADH: ubiquinone oxidoreductase (complex I) and ubiquinol: cytochrome c oxidoreductase (complex III) may induce superoxide anion free radical formation. Mitochondrial production of ROS is thought to play an adverse role in many pathologic states of organs [55, 56]. In our present study, lead acetate treatment decreased the activities of NADH cytochrome c oxidoreductase and cytochrome c oxidase of ETC of rat cardiac tissue. The activities of these enzymes were found to be protected when the rats were pre-treated with melatonin + CuLE in combination. This strongly indicates that the extract as well as melatonin possess either some chelating property or is simply able to prevent mitochondria from ROS production or by acting as quenchers of reactive oxygen species. Aqueous curry leaf extract and melatonin when used in combination exhibited an excellent protective action against lead acetate-induced oxidative stress mediated decrease in activities of the Kreb's cycle and Electron Transport Chain (ETC) enzymes of the cardiac tissue. This may contribute toward a reduced generation of mitochondrial ROS. Melatonin and the active components of the CuLE may have the opportunity to act through synergistic mechanism(s).

Histological examination of haematoxylin-eosin stained sections of the cardiac tissue of lead acetate treated rats showed some significant adverse alterations as indicated in the result section. However, the cardiac tissue sections from the rats pre-treated with melatonin + CuLE in combination did not show any such changes. The results indicate the ability of the melatonin + CuLE in combination to provide protection against lead acetate induced tissue injury. Picrosirius staining of the cardiac tissue sections showed that there was depletion of collagen in cardiac tissues in lead acetate treated animals. The collagen content of the cardiac tissue was found to be protected from being depleted when the rats were pre-treated with melatonin + CuLE in combination. The results indicate again that the combination is effective in providing protection against lead acetate-induced cardiac tissue injury in rats and lead-induced oxidative stress appears to be associated with induction of such tissue injury. Moreover, in our study, there was not much difference between collagen content of the cardiac tissues of the control and the CuLE + melatonin only (positive control) treated group. The lead acetate induced cardiac damage in our experimental situation, is due to generation of oxidative stress as is evident from elevated levels of tissue LPO, protein carbonyl content and GSSG level as well as the bio-markers of oxidative stress. Besides, SEM studies of the cardiac tissue from the inner wall of the ventricle revealed damage of the tissue following lead acetate treatment. Such ventricular tissue damage was found to be protected when the animals were pre-treated with melatonin + CuLE in combination.

Lipid peroxidation can generate large amounts of electrophilic and oxidizing reactive species which can lead to a variety of DNA and tissue damage[58]. Oxidative damage of DNA following accumulation of high concentrations of lead in tissues due to treatment of rats with lead acetate suggests that lead-induced oxidative stress plays a role in lead-induced toxic effects. Lead induced enhanced generation of ROS leads to this oxidative damage of nucleotides [58] . We observed DNA damage by lead which was indicated by formation of smear in gel electrophoresis of DNA extracted from the rat cardiac tissue. No smear was observed in electrophoresis of DNA extracted from the rat cardiac tissue of control, positive control and melatonin + CuLE protected groups. Formation of smear in DNA gel electrophoresis is indicative of the fact that the DNA is partially degraded [59].Smear on DNA gel electrophoresis also occurs due to necrotic damage induced

by lead which is in agreement with our histological observations.

Thus, our studies reveal that the aqueous curry leaf extract (CuLE) [a native indigenous spice herb; *Murraya koenigii*] and melatonin both possess a potent protective activity against lead acetate-induced damages in cardiac tissue of male Wistar rats[5, 6]. Our detailed studies further enlighten us on the fact that a combination of CuLE and melatonin possess a better protective effect against lead acetate-induced oxidative stress mediated damages in the cardiac tissue of rats. The results of our present studies indicate toward a possible future combinatorial therapeutic relevance in handling with situations arising out of exposure of mankind to lead occupationally or environmentally. The possibility of synergism between melatonin and the components of CuLE may not also be ruled out and needs further investigation.

CONCLUSION

Melatonin and aqueous curry leaves extract (CuLE) in combination can provide almost complete protection against lead-induced oxidative stress mediated damages in rat heart. Till date there has been no report on the side effects of CuLE and melatonin also has no reported cytotoxic effect. Hence, we may conclude from this investigation that CuLE and melatonin if used in combination may give better results and almost complete protection in situations of lead-induced oxidative stress mediated cardio-toxicity in experimental rats. Further works are needed to identify the active principle (s) present in the aqueous extract of the curry leaves and elucidate its possible mode of action. The CuLE and melatonin in combination appear to provide protection through their antioxidant activities. These may be attributed to the presence of phenolics and flavonoids in CuLE which appears to work synergistically and wonderfully in presence of and may complement the free radical scavenging activity and antioxidant capacity of melatonin.

ACKNOWLEDGEMENTS

Debosree Ghosh gratefully acknowledges the receipt of a Senior Research Fellowship (SRF) under INSPIRE program of Department of Science and Technology, Government of India. SP is a UGC Non-NET JRF under University of Calcutta. This work was also partially supported from the funds available to Dr. DB under a UGC Major Research Project. Further, Dr. DB also extends grateful thanks to UGC, Govt. of India, for the award of a Major Research Project under Centre with Potential for Excellence in a Particular Area (CPEPA) Scheme at University of Calcutta. Technical help from Parthabrata Roy (Technical Assistant, Chemical Engineering Department, C.U.), Tridib Das, CRNN, University of Calcutta and Barindra Nath Mandal (Technical Officer B, Div of Mol Med, Bose Institute) and Dr. Roy's Diagnostics and Imaging Centre, Kolkata, is also gratefully acknowledged.

REFERENCES

1. Gurer H & Ercal N, Can Antioxidants Be Beneficial in The Treatment of Lead Poisoning? *Free Radic Biol Med*, 29 (2000) 927.
2. Somashekaraiah B, Padmaja K & Prasad ARK, Lead-induced lipid peroxidation and antioxidant defense components of developing chick embryos, *Free Radic. Biol. Med.*, 13 (1992) 107.
3. Sandhir R & Gill KD, Effect of lead on lipid peroxidation in liver of rats, *Biol.Trace Elem. Res*, 48 (1995) 91.
4. Adanaylo VN & Oteiza PI, Lead intoxication: antioxidant defenses and oxidative damage in rat brain, *Toxicology* ,135 (1999) 77.
5. Ghosh D, Mitra E, Dey M, Firdaus S B, Ghosh AK, Mukherjee D, Chattopadhyay A, Pattari S K, Dutta S & Bandyopadhyay D, Melatonin Protects Against Lead-Induced Oxidative Stress In Rat Liver And Kidney, *AJPCR*, 6 (2013) 137.
6. Ghosh D, Mitra E, Firdaus S B, Ghosh AK, Chattopadhyay A , Pattari PK & Bandyopadhyay D, Melatonin Protects Against Lead-Induced Cardio Toxicity: Involvement of Antioxidant Mechanism, *Int J Pharm Pharm Sci*, 5 (2013) 806.
7. Asayama K, Yamadera H, Ito T, Suzuki H, Kudo Y & Endo S, Double blind study of melatonin effects on the sleepwake rhythm, cognitive and non-cognitive functions in Alzheimer type dementia, *J Nippon Med Sch*., **70** (2003) 334.
8. Maldonado MD, Perez-San Gregorio MA & Reiter RJ,The Role Of Melatonin In the Immune-Neuro-Psychology of Mental Disorders, *Rec Patents Cns Drug Disc* , 4 (2009) 69.
9. Miller SL, Yawno T, Alers NO, Castillo-Melendez M, Supramaniam VG, VanZyl N, Sabaretnam T, Loose JM, Drummond GR, Walker DW, Jenkin G & Wallace EM, Antenatal antioxidant treatment with melatonin to decrease newborn neurodevelopmental deficits and brain injury caused by fetal growth restriction, *J Pineal Res*, 56 (2014) 283.
10. Tamura H, Takasaki A, Taketani T,Tanabe M, Kizuka F, Lee L, Tamura I, Maekawa R, Aasada H, Yamagata Y & Sugino N, The role of melatonin as an antioxidant in the follicle, *Journal of Ovarian Research*, 5 (2012) 1.
11. Khdheir FA, Al- Sabbagh M & Al-Rubeiee S, An evaluation of melatonin as antioxidant in Iraqi patients with hyperthyroidism, *G.J.B.B.*, 3 (2014) 51.
12. El-Sokkary GH, Kamel ES & Reiter RJ. Prophylactic effect of melatonin in reducing lead-induced neurotoxicity in the rat.CellMol Biol Lett. 2003; 8: 461.
13. Martínez-Alfaro M, Hernández-Cortés D, Wrobel K, Cruz-Jiménez G, Rivera-Leyva JC, Piña-Zentella RM & Cárabez Trejo A. Effect of melatonin administration on DNA damage

and repair responses in lymphocytes of rats subchronically exposed to lead. *Mutat Res.* 2012; 742: 37.

14. Othman AI, al Sharawy S & el-Missiry MA. Role of melatonin in ameliorating lead induced haematotoxicity. *Pharmacol Res*, 50 (2004) 307.
15. Khaldy H, Escames G, Leon J, Bikjdaouene L & Acuna-Castroviejo D, Synergistic effects of melatonin and deprenyl against MPTP-induced mitochondrial damage and DNA depletion, *Neurobiol Aging*, 24 (2003) 491.
16. Mitra E, Basu A, Ghosh D, Ghosh AK, Chattopadhyay A, Pattari SK, Datta S & Bandyopadhyay D, Ameliorative Effect of Aqueous Tulsi Leaf (*Ocimum Sanctum*) Extract Against Cadmium-Induced Oxidative Stress In Rat Liver, *Int J Pharm Pharm Sci*, 5(2013) 557.
17. Ningappa MB, Dinesha R & Srinivas L, Antioxidant and free radical scavenging activities of polyphenol-enriched curry leaf (*Murraya koenigii L.*) extracts. *Food Chem*, 106 (2008) 720.
18. Ajay S, Rahul S, Sumit G, Paras M, Mishra A & Gaurav A, Comprehensive review: *Murraya koenigii Linn*. *Asian Journal of Pharmacy and Life Science*, 1 (2011) 425.
19. Bonde SD, Nemade LS, Patel MR & Patel AA, *Murraya koenigii* (Curry leaf): Ethnobotany, Phytochemistry and Pharmacology - A Review, *Int.J.Pharm.Phytopharmacol. Res.*1 (2011) 27.
20. Ghosh D, Firdaus SB, Mitra E, Dey M & Bandyopadhyay D, Protective effect of aqueous leaf extract of *Murraya koenigi* against lead induced oxidative stress in rat liver, heart and kidney: a dose response study, *Asian J Pharm Clin Res*, 5(2012)58.
21. Mitra E, Ghosh AK, Ghosh D, Mukherjee D, Chattopadhyay A, Dutta S, Pattari SK & Bandyopadhyay D, Protective Effect of Aqueous Curry Leaf (*Murraya Koenigii*) Extract Against Cadmium-Induced Oxidative Stress In Rat Heart, *Food Chem Toxicol*, 50 (2012) 1340.
22. Gitto E, Tan DX, Reiter RJ, Karbownik M, Manchester LC, Cuzzocrea S, Fulia F, & Barberi I, Individual and synergistic actions of melatonin: Studies with vitamin E, vitamin C, glutathione and desferoxamine in liver homogenates, *J Pharm Pharmacol*, 53 (2001**)**: 1393.
23. Hardeland R & Fuhrberg B, Ubiquitous melatonin – presence and effects in unicells, plants and animals. *Trends Comp Biochem Physiol*, 2 (1996) 25.
24. Seabra ML, Bignotto M, Pinto LR, Jr & Tufik S, Randomized, double-blind clinical trial, controlled with placebo, of the toxicology of chronic melatonin treatment. *J Pineal Res*, 29 (2000) 193.
25. Ghosh D, Firdaus SB, Mitra E, Dey M, Chattopadhyay A, Pattari S K, Dutta S, Jana K & Bandyopadhyay D. Aqueous leaf extract of *Murraya koenigii* protects against lead-induced cardio toxicity in male Wistar rats, *IJP*, 4(2013) 119.
26. Reitman S & Frankel S, Determination of serum glutamic oxaloacetic and glutamic pyruvic transaminase, *Am J Clin Pathol*, 28 (1957) 56.
27. Strittmatter C, Studies on avian xanthine dehydrogenases: properties and patterns of appearance during development, *J Biol Chem*,240 (1965), 2557.
28. Buege JA & Aust SG, Microsomal Lipid Peroxidation, *Methods Enzymol*, 52 (1978) 302.
29. Levine RL, Williams JA, Stadtman ER & Shacter E, Carbonyl assays for determination of oxidatively modified proteins, *Methods Enzymol*,233 (1994) 346.
30. Sedlak J & Lindsay RH, Estimation of total, protein-bound, nonprotein sulfhydryl groups in tissue with Ellman's reagent, *Anal Biochem*,25 (1968) 192.
31. Ikediobo C, Badisa VL, Takem LA, Latinwo L &, West J, Response of antioxidant enzymes and redox metabolites to cadmium-induced oxidative stress in CRL-1439 normal rat liver cells, *Int J of Mol Med*, 14 (2004) 87.
32. Mitra E, Ghosh D **,** Ghosh AK, Basu A, Chattopadhyay A**,** Pattari SK, Datta S & Bandyopadhyay D, Aqueous Tulsi Leaf (*Ocimum Sanctum*) Extract Possesses Antioxidant Properties And Protects Against Cadmium-Induced Oxidative Stress In Rat Heart**,** *Int J Pharm Pharm Sci*,6 (2014) 500.
33. Martin Jr JP, Daily M & Sugarman E, Negative and positive assays of superoxide dismutase based on hematoxylin autooxidation, *Arch Biochemn Biophys*, 255 (1987) 326.
34. Marklund S & Marklund G, Involvement of the superoxide anion radical in the autoxidation of pyragallol and a convenient assay for superoxide dismutase *Eur J Biochem*,47 (1974) 469.
35. Beers Jr RF & Sizer IW, A spectrophotometric method for measuring the breakdown of hydrogen peroxide by catalase, *J Biol Chem*, 195 (1952) 133.
36. Krohne-Ehrich G, Schirmer RH & Untucht-Grau R, Glutathione reductase from human erythrocytes, Isolation of the enzyme and sequence analysis of the redox-active peptide, *Eur J Biochem* ,80 (1977) 65.
37. Paglia DE & Valentine WN, Studies on the quantitative and qualitative characterization of erythrocyte glutathione peroxidase, *J Lab Clin Med*, 70 (1967) 158.
38. Habig WH, Pabst MJ & Jakoby WB, Glutathione-S-transferases, the first enzymatic step in mercapturic acid formation, *J Biol Chem*, 249 (1974) 7130–7139.
39. Greenlee L & Handler P, Xanthine oxidase. VI. Influence of pH on substrate specificity, *J Biol Chem*, 239 (1964) 1090.
40. Chretien D, Pourrier M, Bourgeron T, Séné M, Rötig A, Munnich A & Rustin P, An improved spectrophotometric

assay of pyruvate dehydrogenase in lactate dehydrogenase contaminated mitochondrial preparations from human skeletal muscles, *Clin Chim Acta*, 240 (1995) 129.

41. Duncan M &, Fraenkel D G, Alpha-ketoglutarate dehydrogenase mutant of Rhizobium meliloti, *J Bacteriol*, 137 *(*1989) 415.
42. Veeger C, DerVartanian D V & Zeylemaker W P, Succinate dehydrogenase, *Methods Enzymol*,13 (1969) 81.
43. Goyal V& Srivastava M, Oxidation and reduction of cytochrome c by mitochondrial enzymes of *Setaria cervi*, *J Helminthol*, 69 (1995) 13.
44. Lowry OH, Rosebrough NJ, Farr AL & Randall RJ, Protein measurement with the Folin phenol reagent, *J Biol Chem*,193 (1951) 265.
45. Wu Q, Chen M, Buchwal M & Philips RA, A simple, rapid method for isolation of high quality genomic DNA from animal tissues, *Nucleic Acids Res*, 23 (1995) 5087.
46. Rahman K, Studies on free radicals, antioxidants, and cofactors, *Clin Interv Aging*, 2 (2007) 219.
47. Adly AMA, Oxidative stress and disease: an updated review, *Res J Immunol*, 3 (2010) 129.
48. Sandhir R & Gill KD, Effect of lead on lipid peroxidation in liver of rats, *Biol Trace Elem Res*, 48 (1995) 91.
49. Dalle-Donne I, Rossi R, Giustarini D, Milzani A & Colombo R, Protein carbonyl groups as biomarkers of oxidative stress, *Clin Chim Acta*,329 (2003) 23.
50. Awasthi YC, Zimniak P, Singhal SS & Awasthi S, Physiological role of ghlutathione S- transferases in protection mechanisms against lipid peroxidation : A commentary, *Biochem Arch*, 11 (1995) 47.
51. Kocsy G, Balimoos PV, Ruegsegger A, Szalai G, Gakiba G & Brunoid C, increasing the Glutathione content in a Chilling-Sensitive Maize Genotype Using Safeners increased protection against Chilling-Induced Injury, *Plant Physiol*, 127 (2001) 1147.
52. Sharma P, Jha AB, Dubey RS & Pessarakli M, Reactive Oxygen Species, Oxidative Damage, and Antioxidative Defense Mechanism in Plants under Stressful Conditions, *Journal of Botany*,2012(2012) 1.
53. Patil AJ, Bhagwat VR, Patil JA, Dongre NN, Ambekar JG, Jailkhani R & Das KK, Effect of Lead (Pb) Exposure on the Activity of Superoxide Dismutase and Catalase in Battery Manufacturing Workers (BMW) of Western Maharashtra (India) with Reference to Heme biosynthesis, *Int J Environ Res Public Health*, 3 (2006**)** 329.
54. Reghuvanshi R, Kaul A, Bhakuni P, Mishra A & Mishra MK, Xanthine oxidase as a marker of myocardial infarction, *Indian J Clin Biochem*, 22 (2007) 90.
55. Miccadei S & Floridi A, Sites of inhibition of mitochondrial electron transport by cadmium, *Chem Biol Interact* 89 (1993)159.
56. Pandya JD, Nukala VN & Sullivan PG, Concentration dependent effect of calcium on brain mitochondrial bioenergetics and oxidative stress parameters, *Front Neuroenergetics*, 5 (2013)10.
57. Ghosh D, Firdaus S B, Mitra E, Dey M, Chattopadhyay A, Pattari SK, DuttaS·, Jana K & Bandyopadhyay D, Hepatoprotective activity of aqueous leaf extract of *Murraya koenigii* against lead-induced hepatotoxicity in male Wistar rat, *Int J Pharm Pharm Sci*, 5, (2013) 285.
58. Douki T, Onuki J, Medeiros MH, Bechara EJ, Cadet J & Di Mascio P, Hydroxyl radicals are involved in the oxidation of isolated and cellular DNA bases by 5-aminolevulinic acid, *FEBS Lett*, 428 **(**1998**)** 93.
59. Lieberthal W, Triaca V, & Leviene J, Mechanisms of Death induced by Clisplatin in proximal tubular epithelial cells: apoptosis vs necrosis, *Am J Physiol*, 270 (1996) F700.

Source of support: Nil, Conflict of interest: None Declared