Protection against lead-induced oxidative stress in liver and kidneys of male Wistar rats using melatonin and aqueous extracts of the leaves of *Murraya koenigii* - A novel combinatorial therapeutic approach

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**ABSTRACT**

Treatment of rats with lead acetate (15 mg / kg body weight) intraperitoneally for seven consecutive days caused significant damage in rat liver and kidneys indicated by the altered levels of lipid peroxidation, reduced and oxidised glutathione content, the activities of hepatic and renal antioxidant, pro-oxidant enzymes, mitochondrial Kreb’s cycle and respiratory chain enzymes. Histomorphological changes were also induced in both the tissues by lead acetate which was evaluated using histological studies and microscopy. Collagen content of the liver and kidney tissues were also altered with lead treatment and those were evaluated using acid sirius stain and confocal microscopy and quantified using Image J software. All these changes were ameliorated when the rats were pre-treated with melatonin (10 mg / kg BW, fed orally) and aqueous extract of curry leaves (CuLE) (50 mg / kg BW, fed orally) in combination. The current studies indicated that co-treatment of melatonin and CuLE protected the rat hepatic and renal tissues against lead-induced oxidative stress possibly through their antioxidant activity. The results of the current studies may have future applications in developing a potent pharmaceutical agent with minimum or no cytotoxic side effects against lead induced hepatotoxicity and renal toxicity.

**KEY WORDS:** lead acetate, rat, liver, kidneys, melatonin, curry leaves, co-treatment

**INTRODUCTION**

The largest glandular organ in the body is the liver. It performs many vital functions to eliminate toxins and harmful substances from the body. Almost every organ in the body is supported by this vital organ. A person cannot survive without a healthy liver. Weight of an average adult liver is about three pounds. The liver receives about 1.5 quarts of blood every minute via the hepatic artery and portal vein.¹

On the other hand, the kidneys are a pair of vital organs that do many functions and maintain the blood clean and chemically balanced. In humans each kidney is about 4 or 5 inches long. Each kidney contains around a million nephrons. Each nephron acts as a microscopic filter for blood. As much as 90% of kidney functional disorder can occur without experiencing any symptoms or problems.²

Our earlier studies reveal that heavy metals like lead, cadmium, arsenic accumulate in highly perfused soft organs and induce generation free radicals therein.³,⁴,⁵ Lead has been recognised as the most common cause of heavy metal poisoning. Lead is number 2 on the ASTDR’s “Top 20 List.” Now most people are aware that lead is a poison yet people in occupations like paint, dye, crayon, cosmetics, battery, metallurgy, bullet manufacturing and glamour industries get regularly exposed to lead occupationally. While others get exposed unknowingly, environmentally. The mechanism of lead toxicity has been revealed to be oxidative stress in earlier studies including studies from our laboratory.⁶,⁷, ⁸, ⁹

Antioxidant potential of the herbal extracts in the amelioration of metal-induced oxidative stress need thorough investigation because these natural antioxidants are components of many edible substances and has the potential for safe future use by humans. Melatonin is present in all organisms and in many food items i.e., cereals, green vegetables and fruits.¹⁰ Pharmacologically administered melatonin is well tolerated in humans with no reported side-effects.¹¹ Melatonin’s ability to act synergistically with other natural antioxidants at low doses in a number of models of oxidative stress has been identified recently¹²,¹³. The current study is aimed to examine whether melatonin exhibits any
synergism with aqueous curry leaf extract (CuLE) in mitigating lead acetate-induced oxidative stress.

MATERIALS AND METHODS

Chemicals used
All chemicals used in the present studies were of analytical grade. Anhydrous sodium carbonate (Na$_2$CO$_3$), cupric sulfate pentahydrate (CuSO$_4$·5H$_2$O), ferric chloride hexahydrate (FeCl$_3$·6H$_2$O), Folin–Ciocalteu phenol reagent, hydrochloric acid (HCl), glacial acetic acid, methanol (AR), potassium chloride (KCl) were obtained from Merck (Darmstadt, Germany). Melatonin, lead acetate, thiourea, bismuth nitrate and vanillin were obtained from SRL, India. Gallic acid was procured from Sigma (St. Louis, USA). Anhydrous DTNB, Folin Ciocalteu phenol reagent and Hematoxylin were procured from SRL, India Limited. Tetra ethoxy propane (TEP) was procured from SIGMA, ALDRICH, MO, USA. Sodium carbonate (Na$_2$CO$_3$), cupric sulfate pentahydrate (CuSO$_4$·5H$_2$O), 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, Hooghly, South 24 Parganas and Kolkata metropolitan area throughout the year during the course of the study. The identity of the plant was confirmed by Dr. 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 an aqueous extract of the Curry leaves [CuLE]
Aqueous curry leaves extract was prepared following the standard protocol of our laboratory.\textsuperscript{14, 15, 16}

Induction of oxidative stress with lead acetate and protection by melatonin + CuLE
After acclimatization to laboratory conditions, the rats were divided into four groups, with 6 rats in each group:

- **Group I:** Control
- **Group II:** Melatonin + CuLE (Positive control)
- **Groups III:** Lead acetate treated
- **Groups IV:** Melatonin + CuLE + lead acetate treated

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.

Determination of Lead content of liver and kidneys
Lead content was determined 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 calibration curves were constructed by adding known amount of lead standards. The lead content was expressed in µg/g of tissue.

Histomorphological studies

Compound microscopic evaluation
Immediately following sacrifice of the animals, liver and kidneys were surgically extirpated and fixed in 10% formalin and embedded in paraffin following routine procedure as described earlier.\textsuperscript{15} Tissue sections (5 µM thick) were prepared and stained with hematoxylin-eosin. Besides, a small portion of the tissue was fixed in 10% neutral buffered formalin acetic acid alcohol fixative and processed further for Per-iodic Acid Schiff (PAS) staining for glycogen.

Confocal microscopic evaluation
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 confocalsystem (Leica TCS, SP2, 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.

Scanning electron microscopic evaluation
Another set of liver and kidney tissues were fixed using 3 % glutaraldehyde and the images were recorded using Scanning Electron Microscopy.

Biochemical estimation of hepatic glycogen content
Hepatic glycogen content was evaluated biochemically in hepatic tissue of the rats of the four experimental groups by routine method.

Assessment of biomarkers of oxidative stress and activities of the antioxidant enzymes
The tissues were homogenized (10%) in ice-cold 50 mM phosphate buffer for measurement of LPO, and for GSH estimation, in 2 mM
EDTA, pH 7.4, with a Potter Elvehjem glass homogenizer (Belco Glass Inc., Vineland, NJ, USA) for 30s. Lipid peroxides were determined as thiobarbituric acid reactive substances (TBARS) according to the method of Buege and Aust\(^{16}\). GSH content (as acid soluble sulphydryl) of the tissues was estimated by its reaction with DTNB (Ellman’s reagent) following the method of Sedlak and Lindsey\(^{17}\) with some modifications. \(^{18}\) Copper-zinc superoxide dismutase (Cu-Zn SOD or SOD1) activity was measured spectrophotometrically by hematoxylin auto oxidation method of Martin et al (2012). \(^{19}\) Manganese superoxide dismutase (Mn-SOD or SOD2) activity was also measured spectrophotometrically by pyrogallol autooxidation method. \(^{20}\) Catalase was assayed spectrophotometrically by the method of Beers and Sizer (1952). \(^{21}\)

Measurement of xanthine oxidase and xanthine dehydrogenase activities, the pro-oxidant enzymes

Xanthine oxidase (XO) was assayed by measuring the conversion of xanthine to uric acid \(^{22}\). Xanthine dehydrogenase (XDH) activity was measured by following the reduction of NAD\(^+\) to NADH. \(^{23}\)

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

The 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 and the resulting 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. \(^{24}\) Isocitrate dehydrogenase (ICDH) and alpha-ketoglutarate dehydrogenase activities were measured spectrophotometrically according to the method of Duncan et al (1979). \(^{25}\)

Succinate dehydrogenase (SDH) activity was measured spectrophotometrically according to the method of Veeger et.al (1969). \(^{26}\)

Measurement of some of the mitochondrial respiratory chain enzymes

The NADH-cytochrome c oxidoreductase activity and Cytochrome c oxidase activity was measured spectrophotometrically by the method of Goyal and Srivastava (1995). \(^{27}\)

Measurement of tissue protein content

Proteins of the different samples were estimated by the method of Lowry et al (1970) \(^{28}\) using bovine serum albumin (BSA) as the standard.

Statistical analysis

Data are presented as means ± S.E.M. Significance of mean values of different parameters between the treatment groups were analyzed using one way analysis of variances (ANOVA) after ascertaining the homogeneity of variances between the treatments. Pairwise comparisons were done by calculating the least significance. Statistical tests were performed using Microcal Origin version 7.0 for Windows.

RESULTS

Status of tissue lead content

Fig. 1 A, B demonstrates accumulation of lead in liver and kidneys 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 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 in liver and kidney tissues (74.90%, 62.13% and 53.72% respectively **P<0.001 vs Pb-treated group).

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.

Fig.1. Effect of melatonin+ Curry leaf aqueous extract (CuLE) on lead content of rat hepatic and renal tissue.
Histological studies
Figure 2A shows deposition of lead acetate as a white crust on the surface of liver tissue of lead treated rats. Pre treatment of melatonin prevented the deposition and hence accumulation of lead acetate on the surface of liver.

Figure 2 B (upper panel) documents H and E stained sections of hepatic tissue (magnification 400X) showing unchanged portal veins, scattered dead hepatocytes, dilated sinusoids and focal hepatic necrosis, in lead acetate treated rats compared to control. Whereas, pre-treatment of rats with Melatonin + CuLE protected the tissue from being damaged and we find normal sinusoids. Melatonin + CuLE alone, however, have no effect on hepatic tissue morphology. Figure 2 B (middle panel) depicts Sirius red stained hepatic tissue section (magnification 400X) which reveals deposition of collagen around the central vein region following treatment of rats with lead acetate. Pre-treatment of rats with Melatonin + CuLE prevented the deposition of hepatic tissue collagen. Melatonin + CuLE alone were found to have no effect on tissue collagen content. Fig. 2 B (lower panel) shows similar images (magnification 400X) captured by confocal laser scanning microscope. Figure 3 B represents quantification of fibrosis as percent collagen volume. The results further indicate a protective effect of Melatonin + CuLE against lead acetate-induced increase in collagen deposition around central vein of rat hepatic tissue. Melatonin + CuLE, however, have no effect on tissue collagen content. Figure 2 C shows the status of hepatic glycogen content studied by PAS staining of the tissue sections. Here also, pre-treatment of rats with Melatonin + CuLE was found to have a protective effect on tissue glycogen content compared to lead acetate treated rats. Melatonin + CuLE alone, however, have no effect on tissue glycogen content. Figure 2 D demonstrates a reduction of hepatic glycogen content, measured biochemically, following treatment of rats with lead acetate for seven consecutive days. However, pre-treatment of rats with melatonin significantly protected the tissue glycogen content from being reduced compared to lead acetate group. However, Melatonin + CuLE alone were found to have no effect on hepatic tissue glycogen content compared to control. Figure 2 E represents scanning electron microscopic photo of sections of liver tissue. Treatment with lead caused dilated central vein which was protected when pre-treated with melatonin+CuLE.

Fig.2. Histopathological Studies of Liver.

Fig.2A. Pictographic representation of deposition of lead in liver tissue

Fig.2B. Graph showing collagen volume % of the hepatic tissues.

Fig.2C. Changes in the glycogen content in rat liver (PAS stained, 400 X magnifications).
Fig. 2D. Graph showing glycogen content of the hepatic tissues.

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.

Fig. 3. Histopathological Studies of kidneys.
A, first panel: Effect of Melatonin + CuLE on changes in the rat renal tissue morphology (Hematoxylin and Eosin stained, 400X magnification)
A, second panel: Effect of Melatonin + CuLE on changes in the rat renal tissue morphology (Sirius red stained sections, 400X magnification)
A, third panel: Similar images captured by confocal laser scanning microscope for quantification of fibrosis.

B. Graph showing collagen volume % of the renal 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.

B. Graph showing collagen volume % of the renal tissues

Figure 3 A (upper panel) documents severe degeneration of tubular epithelial cells along with collapse of tubules indicating severe acute tubular necrosis (ATN) and increased inter tubular gap in renal tissue of lead acetate treated rats as evident from hematoxylin and eosin stained tissue sections compared to control. However, pre-treatment of rats with Melatonin + CuLE combination prevented the tissue damage to some extent. Melatonin + CuLE in combination, however, has no effect on renal tissue morphology.

Furthermore, the renal tissue sections depicted in figure 3 A (middle panel, magnification 400X) show collagen deposition around glomeruli and renal tubules following treatment of rats with lead acetate for seven consecutive days. Pre-treatment of rats with Melatonin + CuLE combination prevented the deposition of renal tissue collagen. Fig. 6A (lower panel) shows similar images of the renal tissue captured by confocal laser scanning microscope (magnification 400X). Figure 6 B depicts quantification of fibrosis as percent collagen volume. The results further indicate a protective effect of Melatonin + CuLE combination against lead acetate-induced damage in rat renal tissue.
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 liver and kidney as evident from significantly increased level of lipid peroxidation compared to control [Table 1 A, B ] (1.23 folds in hepatic tissue and 2.0 folds in renal tissue; *P< 0.001 vs control group). Pre-treatment of rats with Melatonin + CuLE combination significantly protected the lipid peroxidation level from being increased (53.92% and 47% respectively in liver and kidney tissues; **P< 0.001 vs Pb acetate-treated group). However, Melatonin + CuLE in combination has no effect on the lipid peroxidation level of tissue. Table 1 also shows that there occurred a significant increase in the level of protein carbonyl of liver and kidney tissues of rat following lead acetate treatment (2.4 folds and 2.21 folds respectively in liver and kidney tissues; *P<0.001 vs control group). Pre-treatment of rats with Melatonin + CuLE combination almost completely protected the tissue protein carbonyl level from being increased (68.31% and 48.36% respectively in liver and kidney tissues **P<0.001 vs Pb-treated group). However, Melatonin + CuLE in combination has no significant effect on the protein carbonyl level of liver and kidney tissues.

Table 1 reveals that treatment of rats with lead acetate at the indicated dose increased the activities of cytosolic Cu–Zn–SOD, the mitochondrial Mn–SOD and the catalase of the rat hepatic tissues (2.4 folds, 1.9 folds and 1.9 folds increase respectively vs control, * P < 0.001 vs control). Pre-treatment of rats with Melatonin + CuLE combination was found to protect the activities of these antioxidant enzymes from being increased in liver (56.40%, 45.05% and 46.24% decrease respectively, **P< 0.001 vs Pb-treated group). However, Melatonin + CuLE in combination did not significantly alter the activity of any of the enzymes studied.

Table 1. 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 liver and kidneys of the experimental rats.

<table>
<thead>
<tr>
<th>Parameters Studied</th>
<th>Liver</th>
<th></th>
<th>Kidney</th>
<th></th>
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<tbody>
<tr>
<td></td>
<td>CON Mel + CuLE</td>
<td>Pb Mel + CuLE +Pb</td>
<td>CON Mel + CuLE</td>
<td>Pb Mel + CuLE +Pb</td>
</tr>
<tr>
<td>LPO (nmoles of TBARS/mg protein)</td>
<td>0.131±0.008</td>
<td>0.132±0.007*</td>
<td>0.293±0.007*</td>
<td>0.135±0.007**</td>
</tr>
<tr>
<td>Protein carbonyl (nmoles per mg protein)</td>
<td>6.02±0.132</td>
<td>6.03±0.145*</td>
<td>20.65±0.165*</td>
<td>6.54±0.147**</td>
</tr>
<tr>
<td>Cu-Zn SOD activity (units/min/mg protein)</td>
<td>7.02±0.166</td>
<td>7.11±0.175*</td>
<td>16.54±0.195*</td>
<td>7.21±0.167**</td>
</tr>
<tr>
<td>Mn SOD activity (units/min/mg protein)</td>
<td>5.64±0.321</td>
<td>5.59±0.365*</td>
<td>10.89±0.132*</td>
<td>5.98±0.124**</td>
</tr>
<tr>
<td>Catalase activity(μmoles H₂O₂ consumed/min/mg protein)</td>
<td>152.3±5.321</td>
<td>154.2±5.214*</td>
<td>296.3±5.654*</td>
<td>159.3±6.214**</td>
</tr>
<tr>
<td></td>
<td>0.331±0.010</td>
<td>0.330±0.009*</td>
<td>0.668±0.012*</td>
<td>0.354±0.008**</td>
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<tr>
<td>Protein carbonyl (nmoles per mg protein)</td>
<td>0.365±0.032</td>
<td>0.371±0.026*</td>
<td>0.732±0.025*</td>
<td>0.378±0.026**</td>
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<tr>
<td>Cu-Zn SOD activity (units/min/mg protein)</td>
<td>1.712± 0.165</td>
<td>1.715±0.154*</td>
<td>3.65±0.145*</td>
<td>1.798±0.135**</td>
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<tr>
<td>Mn SOD activity (units/min/mg protein)</td>
<td>1.54± 0.124</td>
<td>1.54± 0.168*</td>
<td>3.54± 0.179*</td>
<td>1.68± 0.162**</td>
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<tr>
<td>Catalase activity(μmoles H₂O₂ consumed/min/mg protein)</td>
<td>25.21± 1.032</td>
<td>25.32± 0.654*</td>
<td>36.65± 1.051*</td>
<td>25.84± 1.542**</td>
</tr>
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</table>

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

*P<0.001 compared to control; **P< 0.001 compared to lead treated group;

CON = Control; Mel+ CuLE = Melatonin + Curry leaf extract; Pb=Lead; Mel+ CuLE +Pb= Melatonin +Curry leaf extract +Lead.
Status of GSH, GSSG, GSSG: GSH and TSH

Fig. 4 (A, C and D) shows that there occurred a significant increase in GSH, GSSG level and in the GSSG: GSH ratio of liver tissues of rat following lead acetate treatment (1.6 folds, 0.95 folds and 24.24% respectively, *P< 0.001 vs control group). Pre-treatment of rats with Melatonin + CuLE combination almost completely protected the tissue GSH and GSSG levels and thus the GSSG: GSH ratio also from being increased in hepatic tissue (33.97%, 48.93 %, and 21.95% respectively, **P< 0.001 vs Pb-treated group). However, Melatonin + CuLE in combination has no significant effect on the GSH and GSSG levels of liver.

Treatment of rats with lead acetate decreased the TSH level significantly (18.03%, *P< 0.001 vs. control group) (fig. B). Pre-treatment of rats, however, with Melatonin + CuLE combination almost completely protected the TSH from being decreased in hepatic tissue (20.00 %, **P< 0.001 vs. Pb-treated group). However, Melatonin + CuLE in combination has no significant effect on the TSH level of liver (fig.3 B).

Treatment of rats with lead acetate decreased the total thiol (TSH) level significantly (20.13%, *P< 0.001 vs control group). Pre-treatment of rats with Melatonin + CuLE combination almost completely protected the tissue GSH and GSSG levels and thus the GSSG: GSH ratio also from being increased in kidney tissue (45.43 %, 54.39% and 11.11% respectively, **P< 0.001 vs Pb-treated group). However, Melatonin + CuLE in combination has no significant effect on the GSH and GSSG levels of kidney.

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.

Fig. 5 (A, C and D) shows that there occurred a significant increase in GSH, GSSG level and in the GSSG: GSH ratio of kidney tissues of rat following lead acetate treatment (1.8 times, 54.39% and 12.5% respectively, *P< 0.001 vs control group). Pre-treatment of rats with Melatonin + CuLE combination almost completely protected the tissue GSH and GSSG levels and thus the GSSG: GSH ratio also from being increased in kidney tissue (45.43 %, 54.39% and 11.11% respectively, **P< 0.001 vs Pb-treated group). However, Melatonin + CuLE in combination has no significant effect on the GSH and GSSG levels of kidney.

Treatment of rats with lead acetate decreased the total thiol (TSH) level significantly (20.13%, *P< 0.001 vs control group). Pre-treatment of rats with Melatonin + CuLE combination almost completely pro-
Detected the TSH from being decreased in cardiac tissue (25.32%, **P<0.001 vs Pb-treated group). However, Melatonin + CuLE in combination has no significant effect on the TSH level of kidney (fig.5B).

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.

Fig.5. 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 renal tissue.

Status of the activities of glutathione peroxidase, glutathione reductase and glutathione –S- transferase of the rat hepatic and renal tissues

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.10A), glutathione reductase (fig.10B) and glutathione –S- transferase (6 C) (0.95 times, 1.34 times and 0.95 times respectively,*P< 0.001 vs control group) in hepatic tissue. However, the enzyme activities were found to be completely protected when the rats were pre-treated with Melatonin + CuLE combination for the similar period of time (48.50%, 55.52% and 48.83 %, **P< 0.001 vs lead acetate -treated group). However, Melatonin + CuLE in combination has no significant effect on the activities of these enzymes in hepatic 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.

Fig.6. Effect of Melatonin (10 mg/kg BW) + CuLE (50 mg/kg BW) against lead-induced alteration in the activities of glutathione peroxidides (A), glutathione reductase (B), glutathione S transferase (C) in rat hepatic tissue.

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.12A), glutathione reductase (fig.7B) and glutathione –S- transferase (7 C) (1.19 times, 0.80 times and 1.19 times respectively,*P< 0.001 vs control group) in renal tissue. However, the enzyme activities were found to be completely protected when the rats were pre-treated with Melatonin + CuLE combination for the similar period of time (54.39%, 44.51% and 53.64%, **P< 0.001 vs Lead acetate -treated group). However, Melatonin + CuLE in combination has no significant effect on the activities of these enzymes in renal 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.

Fig7. 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 renal tissue.
Status of pro-oxidant enzymes
The activities of hepatic xanthine oxidase (XO) (Table 3) and xanthine dehydrogenase (XDH) as well as the total enzyme activity, i.e., XO plus XDH and, XO : XDH ratio, all increased significantly following treatment of rats with lead acetate (4.1 folds, 2.7 folds, 2.9 folds and 1.5 folds increase respectively in hepatic 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 combination (75.75%, 63.09%, 64.63% and 34.72% decrease respectively in hepatic 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 xanthine oxidase (XO) and dehydrogenase activity (72.52% and 76.95% decrease , *P < 0.001 vs their control in liver and kidney tissues respectively). Pre-treatment of rats with Melatonin + CuLE combination significantly protected the enzyme activity from being decreased in (3.6 folds and 4.3 folds increase in liver and kidney tissues respectively **P < 0.001 vs Lead acetate -treated group). However, Melatonin + CuLE in combination have no effect on the activity of isocitrate dehydrogenase in the hepatic, cardiac and renal tissues.

Treated rats with lead acetate inhibits alpha keto glutarate dehydrogenase (α-KGDH) activity in hepatic and renal tissues (79.66% and 52.63 % decrease respectively, *P < 0.001 vs control) (Table 3). The activity of the enzyme was found to be significantly protected from being decreased in all the organs studied when the rats were pre-treated with Melatonin + CuLE combination (4.8 folds and 4.3 folds increase in liver and kidney tissues respectively **P < 0.001 vs Lead acetate -treated group). However, Melatonin + CuLE in combination have no significant effect on the activity of α-KGDH in hepatic and renal tissues.

Status of the activities of pyruvate dehydrogenase and some of the Kreb’s cycle enzymes
Table 3 reveals that treatment of rats with lead acetate inhibits pyruvate dehydrogenase activity (72.52% and 76.95% decrease , *P < 0.001 vs their control in liver and kidney tissues respectively). Pre-treatment of rats with Melatonin + CuLE combination significantly protected the enzyme activity from being decreased in (3.6 folds and 4.3 folds increase respectively in liver and kidney tissue **P < 0.001 vs Lead acetate -treated group). However, Melatonin + CuLE in combination were found to have no effect on the activity of this enzyme in the tissue.

Table 3 further reveals that treatment of rats with lead acetate significantly decreased the activity of isocitrate dehydrogenase in hepatic and renal tissues (80.33% and 52.63% 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 liver was found to be protected significantly from being decreased when the rats were pre-treated with Melatonin + CuLE combination (4.9 folds and 4.3 folds, **P < 0.001 vs Lead acetate -treated group). However, Melatonin + CuLE in combination have no effect on the activity of isocitrate dehydrogenase in the hepatic, cardiac and renal tissues.

### Table 2. 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 liver and kidneys of the experimental rats.

<table>
<thead>
<tr>
<th>Parameters Studied</th>
<th>Liver</th>
<th>Kidney</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CON</td>
<td>Mel + CuLE</td>
</tr>
<tr>
<td>XO (milliunits/min/mg protein)</td>
<td>0.02±0.007</td>
<td>0.02±0.007</td>
</tr>
<tr>
<td>XDH (milliunits/min/mg protein)</td>
<td>0.25±0.081</td>
<td>0.24±0.065</td>
</tr>
<tr>
<td>XO+XDH</td>
<td>0.275±0.054</td>
<td>0.273±0.075</td>
</tr>
<tr>
<td>XO/XDH</td>
<td>0.09±0.065</td>
<td>0.09±0.052</td>
</tr>
<tr>
<td>XO/XO+XDH</td>
<td>0.024±0.015</td>
<td>0.019±0.0014</td>
</tr>
</tbody>
</table>
Table 3. Effect of Melatonin (10 mg/kg BW) + CuLE (50 mg/kg BW) on the activities of PDH, ICDH, α-KGDH and SDH in liver and kidneys of the experimental rats.

<table>
<thead>
<tr>
<th>Parameters Studied</th>
<th>Liver</th>
<th>Kidney</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CON Mel + CuLE Pb</td>
<td>Mel + CuLE +Pb</td>
</tr>
<tr>
<td>PDH (units/min/mg protein)</td>
<td>1.612±0.029</td>
<td>1.610±0.026</td>
</tr>
<tr>
<td>ICDH(units/min/mg protein)</td>
<td>0.061±0.002</td>
<td>0.061±0.002</td>
</tr>
<tr>
<td>α-KGDH (units/min/mg protein)</td>
<td>0.059±0.0022</td>
<td>0.059±0.0029</td>
</tr>
<tr>
<td>SDH(units/min/mg protein)</td>
<td>1.600±0.025</td>
<td>1.601±0.026</td>
</tr>
</tbody>
</table>

Values are expressed as Mean ± SE of 6 animals in each group. Data were analyzed by using one way analysis of variances (ANOVA) using Microcal Origin version 7.0 for Windows. *P<0.001 compared to control; **P< 0.001 compared to lead treated group; CON = Control; 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 hepatic and renal tissues (70.32%, and 67.74 % decrease respectively , *P< 0.001 vs control group). The activity of this enzyme was found to be significantly protected from being decreased compared to lead acetate treated group when rats were pre-treated with Melatonin + CuLE combination (3.4 folds and 3.1 folds increase respectively in liver and kidney tissues, **P< 0.001 vs Lead acetate -treated group). Melatonin + CuLE in combination, however, have no effect on the activity of this enzyme.

Assessment of protection of DNA damage in liver and kidney tissues 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 two tissues, i.e., liver and kidney tissues. No smear was observed in electrophoresis of DNA extracted from liver and kidney tissues of control, Mel+ CuLE and Mel+ CuLE+Pb treated rats.
DISCUSSION

Lead is known to produce oxidative damage in the tissues. The major constituents of biological membranes are lipids and proteins. Lipids when react with free radicals can undergo the highly damaging chain reaction of lipid peroxidation. Protein Carbonyl is used as a biomarker of oxidative stress. The usage of protein CO groups as biomarkers of oxidative stress is advantageous the relative stability of carbonylated proteins. We found, in our model, an enhanced level of protein CO as well as level of lipid peroxidation in hepatic as well as in renal tissues of lead acetate treated rats. Both were protected from being increased on pre-treatment with CuLE as well as with melatonin. When the rats were pre-treated with melatonin + CuLE combination both level of LPO and protein CO were observed to be prevented from being increased and were maintained near normal levels in all the three tissues.

Glutathione and glutathione-related enzymes play a key role in protecting the cell against oxidative stress. The GSH redox cycle consist of GSH, GPx, GR and GST, which are the major components of the antioxidative defense system. Coordinated activities of these enzymes maintain intracellular thiol status. GSH plays a 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. Glutathione reductase (GR) reduces oxidized glutathione (GSSG) to biologically active GSH. 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 hepatic and renal 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 protected from being increased on pre-treatment with melatonin + CuLE.

Oxidants such as ROS are balanced against this antioxidative defense system that consists of enzymes and metabolites in all sub cellular compartments. In conditions of oxidative stress, normal capacities of these mechanisms are insufficient, triggering cells to increase and expand their antioxidative network. Increased level of super oxide anion free radical leads to enhanced activity of the enzyme Superoxide dismutase (SOD) while increased SOD activity leads to increased level of hydrogen peroxide. Hydrogen peroxide is the substrate for the enzyme catalase which catalyses the process of conversion of hydrogen peroxide to water. The level of catalase activity is also increased in response to the increased hydrogen peroxide. We observed enhanced activities of both these antioxidant enzymes in liver and kidney tissues of lead acetate treated animals. The same was protected when pre-treated with melatonin + CuLE.
Xanthine oxidoreductase, under normal conditions, exists in dehydrogenase form and uses NAD+ and there is no or very little production of superoxide anion. XO in oxidative stress conditions may play an important role in contributing free radical mediated damage. We observed enhanced activities of both these pro-oxidant enzymes and their sum and ratios in liver and kidney tissues of lead acetate treated animals. The same were protected when pre-treated with melatonin + CuLE.

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 hepatic and renal tissues following treatment of rats with lead for seven consecutive days. This finding is as per with our previous observations. The activities of all these enzymes were protected in all the tissues when the rats were pre-treated with melatonin + CuLE combination. Mitochondrial production of ROS is thought to play an adverse role in many pathologic states of organs. In our present study, lead acetate treatment inhibits NADH cytochrome c oxidoreductase and cytochrome c oxidase enzymes of ETC of rat hepatic and kidney tissue. The activities of these enzymes were found to be protected when the rats were pre-treated with melatonin + CuLE 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 by themselves being quenchers of reactive oxygen species. CuLE and melatonin when used in combination as pre-treatment exhibited a better and excellent protective action against lead induced oxidative stress mediated inhibition of the Kreb’s cycle and Electron Transport Chain (ETC) enzymes of the organs thereby reducing mitochondrial ROS generation. Thus they both complement each other.

Histological examination of haematoxylin-eosin stained sections of the tissues of lead treated animals showed marked adverse alterations. However, all the tissue sections from the rats pre-treated with melatonin + CuLE did not show any such changes. The results indicate the ability of the melatonin + CuLE combination to provide protection against lead induced tissue injury. Picrosirius stain of the tissue sections show that there was increased deposition of collagen around the central hepatic vein, around glomerulus in lead treated animals. We had similar observation earlier also.

We observed that treatment with lead acetate caused dilated central veins in hepatic tissue which was protected when the rats were pre-treated with melatonin + CuLE combination. Pre-treatment of rats with melatonin + CuLE in combination showed a better protective action on the organs studied against lead induced oxidative stress mediated damages. There was not much difference between collagen content of the hepatic and renal tissues of the control and the positive control rats. The lead acetate induced tissue damage in our experimental situation, is oxidative stress mediated which is established from elevated levels of tissue LPO and protein carbonyl content, GSSG level. Earlier studies from our laboratory and others suggest that lead and other heavy metals causes hepatotoxicity and deplete the glycogen content of liver. We also observed that lead acetate deplete the glycogen reserve of liver and we have observed that pre-treatment of the rats with melatonin + CuLE combination can protect the depletion of glycogen reserve much more efficiently than we had earlier observed with melatonin as well as CuLE alone pre treatment.

Studies reveal that lead induces oxidative DNA damage leading to death of hepatocytes. Oxidative damage of DNA following accumulation of high concentrations of lead in tissues due to occupational or experimental exposure 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. We observed DNA damage by lead which was indicated by formation of smear in gel electrophoresis of DNA extracted from all the three tissues. No smear was observed in electrophoresis of DNA extracted from liver and kidney tissues of control, Melatonin+CuLE combination and Melatonin+CuLE+Pb treated rats. Formation of smear in DNA gel electrophoresis is indicative of the fact that the DNA is partially degraded. Smear on DNA gel electrophoresis also occurs due to necrotic damage induced by lead which is in agreement with our histological observations.

Our detailed studies enlighten us on the fact that a combination of CuLE and melatonin possess a better protective effect against lead induced oxidative stress mediated damages in the tissues of rats. Thus, a clinically well standardised, perfect pharmacological dose of the blend of the CuLE and melatonin in a right proportion can be of great application in future world of pharmaceutical and medical sciences. The findings, evidences and inferences deduced from our experimental studies may be a landmark in the arena of Pharmaceutical and Physiological research. The combinatorial therapy of the two non-cytotoxic, potent antioxidant may evolve as a great weapon in the war of mankind against lead induced oxidative stress mediated hepatic and renal damages.

CONCLUSION

Hence, we may conclude from this investigation that CuLE and melatonin both if used in combination, may give almost complete protection in situations of lead-induced oxidative stress mediated hepatotoxicity and nephrotoxicity. The CuLE and melatonin appear to provide protection through their antioxidant activities. These may be attributed to the presence of phenolics and flavonoids in CuLE which works wonderfully in presence of and complements the free radical scavenging activity and antioxidant capacity of melatonin.

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