

## Protective effect of aqueous garlic extract against lead-induced hepatic injury in rats

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Effect of aqueous extract of garlic on hepatic injury due to lead-induced oxidative stress in experimental rats has been investigated. Lead acetate (LA) at a dose of 15 mg / kg body wt was administered ip to rats for 7 consecutive days to induce hepatic injury. Freshly prepared aqueous garlic extract (AGE) at a dose of 50 mg /kg body wt was fed orally to rats 1 h before LA treatment for similar period. LA treatment caused hepatic injury as evident from increased activities of serum glutamate pyruvate transaminase (SGPT) and alkaline phosphatase (ALP), increased serum bilirubin level and damage in the tissue morphology. Lead-induced oxidative stress in liver was evident from increased levels of lipid peroxidation and reduced glutathione. The decreased activity of superoxide dismutase (SOD) and an increased activity of catalase as well as an increased activity of xanthine oxidase (XO) indicate generation and possible accumulation of reactive oxygen intermediates. Furthermore, altered activities of lactate dehydrogenase (LDH), isocitrate dehydrogenase (ICDH), alpha-keto glutarate dehydrogenase ( $\alpha$ -KGDH) and succinate dehydrogenase (SDH) also indicate an impaired substrate utilization and generation of oxidative stress. All these changes were found to be mitigated when the rats were pre-treated with the AGE. Results indicate that AGE has the potential to ameliorate lead-induced hepatic injury due to oxidative stress in rats. The protective effects may be due to the antioxidant properties of AGE and may have future therapeutic relevance.

**Keywords:** Antioxidant, Garlic, Lead, Liver, Oxidative stress

Human exposure to heavy metals has risen dramatically in the last 50 years as a result of an exponential increase in the use of heavy metals in industrial processes and products<sup>1</sup>. Many metals play important role in the functioning of enzymes, cell-signaling processes, and gene regulation<sup>2</sup>. Heavy metals, like lead is not known to have any biological role. Increasing concern has been expressed about the rapidly rising level of chemicals in the environment, particularly lead, which has well-known hazardous effects. It affects each and every organ and system in the body<sup>1</sup>. In India, the range of sources of lead exposure is extensive. This ubiquitous environmental pollutant enters the atmosphere from production of coal, oil, iron, steel, and batteries, as well as from smelters, solid waste, and tobacco smoke. Lead exposure occurs mainly through the respiratory and gastrointestinal systems. Liver is a frequent target for many toxicants. The currently approved treatment for

lead intoxication is to give chelating agents, such as meso-2, 3-dimercaptosuccinic acid (DMSA) and monoisoamyl DMSA (MiADMSA), which form an insoluble complex with lead and shield it from biological targets, thereby reducing its toxicity. However, these chelators are potentially toxic and often fail to remove lead from all the body tissues<sup>3</sup>. Moreover, because of their hydrophilic nature, they cannot cross the cell membrane to capture intracellular lead<sup>3</sup>. Thus, drugs with lipophilic properties are needed.

Use of natural antioxidants in the treatment and/or control of a number of stress-induced diseases are currently being favoured. The role of antioxidants in ameliorating oxidative damage brought about by the environmental or occupational exposure to heavy metals has been reported<sup>4,5</sup>. The human diet, which contains many natural compounds, is thought to have the capability to provide protection to the body against the development of many diseases. Garlic (*Allium sativum* L.), which comprises an important component of the regular diet of the people in South East Asia including India and China, has been shown

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to have a broad spectrum of activities like, antibacterial, anticarcinogenic, hypolipidemic, hypoglycemic, antifungal, antiatherosclerotic, and antioxidant<sup>6,7</sup>. Due to its low cost garlic is easily available even to economically poor and disadvantaged people of the society. In practice, garlic cloves are consumed either in raw form or in cooked form along with other food stuffs in different parts of India and other countries. Thus, the aim of the present investigation is to explore whether garlic has any potential protective effect against lead-induced oxidative stress.

Antioxidant potential of the extracts of the herbs and different plant parts in the amelioration of metal-induced oxidative stress need thorough investigation because these natural antioxidants are component of many edible substances and has the potential for safe future use for humans. The current work demonstrates the protective effects of the aqueous garlic extract (AGE) against lead-induced hepatic injury using rat as an experimental animal model.

## Materials and Methods

**Chemicals**—Lead acetate was procured from Merck India Ltd. All other reagents used were of analytical grade and were either procured from SRL, India, Bombay or from Sigma-Aldrich, USA or from Merck, India. Fresh garlic was purchased from the local Kolkata Municipal Corporation approved vegetable market.

**Animals**—Male albino rats of Charles Forster strain, weighing 180-200 g, procured from an authorized breeder were used for all the experiments. Rats were housed in Tarson's polypropylene autoclavable cages and kept under standard laboratory conditions with sufficient food and water *ad libitum* throughout the experimental period. All the experiments were carried out as per the guidelines of the Institutional Animal Ethics Committee [IAEC (No:820/04/ac/CPCSEA, dt. 06/08/04)], Department of Physiology, University of Calcutta. After acclimatization to laboratory conditions, the rats were divided into 4 groups:

Group-I: Vehicle control

Group-II: LA treated

Group-III: AGE pre-treated

Group-IV: AGE only treated (positive control)

Both the effective doses of LA and the AGE have been standardized in our laboratory condition through our dose-response studies (results not shown). The effective dose for LA was found to be 15 mg/kg

body wt administered intra-peritoneally (ip) and that for the extract, 50 mg/kg body wt administered orally.

**Preparation of aqueous garlic extract**—The AGE was prepared with slight modification of the method of Ide *et al.*<sup>8</sup>. Fresh garlic cloves were weighed, minced properly in cold and homogenized in a sterilized Teflon glass homogenizer using aqua guard purified cold drinking water for 1-2 min in cold. The homogenate was centrifuged in cold at 3,000 rpm for 5 min to remove any debris. The homogeneous suspension, thus obtained, (referred to herein after as aqueous garlic extract [AGE]) was brought to room temperature before administration. This method of preparation of the garlic extract was also used by other investigators<sup>7,8</sup>.

**Treatment of animals, preparation of serum and collection of hepatic tissue**—The rats of the group I constituted the vehicle treated control. The rats of the group II served as the positive control which was fed with freshly prepared AGE at a dose of 50 mg /kg body wt at a fixed time of the day. The rats of the group III were injected with LA solution intra-peritoneally (ip) at a dose of 15 mg/kg body wt/day for 7 consecutive days at the same time of the day. The rats of the group IV were fed with freshly prepared AGE at a dose of 50 mg/kg body wt 1 h prior to LA injection. Care was always taken so that the volume of the AGE fed never exceeded 0.8 ml-1.0 ml. The animals were kept at the temperature and humidity controlled room for the entire period of treatment and were sacrificed 24 h after the last treatment on the seventh day, by cervical dislocation. The abdomen and the thoracic cavity were opened carefully and the blood was collected from the animals by cardiac puncture, kept in vials with or without an anticoagulant which was processed for the preparation of serum and the other portion of the blood used for the estimation of hemoglobin and other blood parameters. Immediately after collecting blood, the liver tissue was excised, properly rinsed in cold-saline, soaked in tissue paper, small pieces of the tissue preserved for the histological and histochemical studies in appropriate fixatives and the rest of the portions finally stored at -20°C for further biochemical analyses.

## Measurement of bio-markers of hepatic injury

**Estimation of Serum Glutamate Pyruvate Transaminase (SGPT) activity**—Non-hemolyzed serum (0.05 ml) was mixed with 0.25 ml of glutamate pyruvate transaminase substrate and incubated for

30 min at 37°C. Then 0.25 ml of 2, 4-dinitrophenyl hydrazine (DNPH) solution was added, mixed and kept for 20 min at room temperature. Then, 2.5 ml of 0.4(N) NaOH was added, mixed and kept at room temperature for 10 min. The intensity of the developed colour was noted at 540 nm after setting the UV/VIS spectrophotometer (Biorad; Model SmartspecPlus) to zero with water. The decrease in absorbance represents the decrease in the concentration of  $\alpha$  - keto-glutarate from which the activity was calculated. The level of the enzyme activity was expressed as IU/L<sup>11</sup>.

*Estimation of Serum Alkaline Phosphatase (ALP) activity*—Serum alkaline phosphatase (ALP), at an alkaline pH, hydrolyses di-sodium phenyl phosphate to phenol. The phenol formed, reacts with 4-aminoantipyrine in the presence of potassium ferricyanide, as an oxidising agent, to form a red coloured complex. The intensity of the colour formed is directly proportional to the activity of ALP present in the sample. ALP activity was quantitatively determined using ALP-kit, based on the method as described by Kind & King<sup>16</sup>, and the kit manufactured by Span Diagnostic Ltd, Plot No. 336.338.340. RD. No. 3 G.I.D.C. New code no. 75MB100-40. The values of the enzyme activity were expressed as KA (King-Armstrong) Units which was used after their name<sup>12</sup>.

*Estimation of serum bilirubin level*—The principle of formation of a pink coloured azo-bilirubin by the reaction between bilirubin and the diazo reagent was utilized. The total bilirubin level was measured using a previously reported method<sup>13</sup>.

### **Processing of the hepatic tissue for histological and histochemical studies**

The extirpated liver tissue was fixed in 10% formalin and embedded in paraffin following routine procedure. The tissue sections (5  $\mu$ m thick) were prepared and either stained with hematoxylin–eosin or acid-sirius or PAS according to the previously reported method<sup>14</sup>. The tissue sections were examined under the Leica microscope and images were captured with a digital camera attached to it. The digitized images were then analyzed using image analysis system (Image J, NIH Software, Bethesda, MI) for the total collagen area fraction.

### **Measurement of blood parameters**

*Estimation of haemoglobin concentration*—Haemoglobin concentration in the whole blood were spectrophotometrically measured by the

cyanomethemoglobin method<sup>15</sup>. Blood samples (20  $\mu$ l) were mixed with 5 ml Drabkins solution (0.1% sodium bicarbonate, 0.005% potassium cyanide, and 0.02% potassium ferricyanide) for the determination of haemoglobin.

*Estimation of blood glucose level*—Blood glucose level was estimated by the method of Nelson & Somogyi. In separate 10 ml marked test tubes, 0.5 ml of blood filtrate and 0.5 ml of working standard glucose solution was taken. To each of these tubes, 1 ml of alkaline copper reagent was added. The contents of the tubes were mixed thoroughly and heated in a boiling water bath in an upright position for 20 min. After cooling to room temperature, 1 ml of arsenomolybdate colour reagent was added to each of the tubes and the contents of each tube were diluted up to 10 ml with distilled water. The intensity of the developed colour was noted at 540 nm using a UV/VIS spectrophotometer after setting the instrument to zero density with blank<sup>16</sup>.

*Estimation of blood lactate level*—Lactate level of the whole blood was estimated by nova lactate plus test strips with the nova lactate plus meter of Nova Biomedical Corporation, 200 Prospect Street, Waltham, MA 02454-9141, USA. This lactate biosensor has been constructed based on the immobilisation of the enzyme lactate dehydrogenase and its cofactor nicotinamide adenine dinucleotide (NAD) using a cellulose acetate membrane cast in situ. These components were deposited on to a screen printed carbon electrode containing 2% Meldola's Blue (by weight of carbon). The instrument is calibrated with lactate standards between 0 and 15 mM/L. The lactate sensor is composed of a peroxide sensor and an enzyme transducer membrane. The sensor, a polarographic enzyme electrode, gives a current which is a linear function of the lactate concentration. The lactate is stoichiometrically converted to pyruvate and hydrogen peroxide by lactate oxygen oxidoreductase derived from *Pediococcus*. The oxygen required for the enzymatic oxidation is supplied via an air-permeable silicone elastomeric membrane used for stirring. Each nova test strip contains the enzyme lactate oxidase greater than or equal to 0.03 IU; additional ingredients (mediator, buffer etc) greater than 15  $\mu$ g<sup>17</sup>.

*Measurement of the glycogen content of liver*—Liver tissue (200 mg) was weighed on a torsion balance and finely homogenized with 20 ml. of 5% TCA in an all glass homogenizer (Potter & Elvehjem, 1936).

The precipitate of the proteins was filtered off and the clear filtrate was subjected to analysis. Iodine reagent was prepared (16.5 ml of Lugol's solution, prepared by dissolving 1 g of iodine and 2 g of KI in 20 ml. of water, was added to 983.5 ml. of an aqueous solution, containing 25% (w/v) of KCl. In a colorimeter tube (1.2 cm diameter) 2 ml of the clear filtrate was added to 3 ml. of the iodine reagent. After mixing, the optical density is noted in a photometer at 650 nm against a blank obtained by adding 2 ml. of 5% TCA to 3 ml of reagent in the same way<sup>18</sup>.

#### Measurement of biomarkers of oxidative stress

**Lipid peroxidation level**—The weighed amount of the hepatic tissue was homogenized (10%) in ice-cold 0.9% saline (pH 7.0) with a Potter Elvehjem all glass homogenizer (Belco Glass Inc., Vineland, NJ, USA) for 30 sec and the levels of the lipid peroxidation products in the homogenate was determined as Thio-Barbituric Acid Reactive Substances (TBARS) according to the method as employed by Chatyopadhyay *et al*<sup>19</sup>. In brief, the homogenates were mixed with trichloro acetic acid-thiobarbituric acid-hydrochloric acid (TBA-TCA-HCl) reagent and mixed thoroughly and heated for 20 min at 80°C. The tubes containing the samples were then cooled to room temperature. The absorbance of the pink chromogen present in the clear supernatant obtained after centrifugation at 1200 × g for 10 mins at room temperature was measured at 532 nm using a UV-VIS spectrophotometer (SmartSpec Plus, BioRad, Hercules, CA, USA). Tetraethoxypropane (TEP) was used as standard. The values were expressed as nmoles of TBARS per mg protein.

**Reduced glutathione (GSH) content**—Reduced GSH content (as an acid soluble sulfhydryl) was estimated by its reaction with DTNB (Ellman's reagent) following the method of Sedlac and Lindsey with some modifications. The weighed amounts of the hepatic tissue were homogenized (10%) in 2 mM ice-cold ethylenediaminetetraacetic acid (EDTA). The homogenates were mixed with Tris-HCl buffer, pH 9.0, followed by the addition of DTNB for colour development. The absorbance was measured at 412 nm using a UV-VIS spectrophotometer to determine GSH content. The values were expressed as nmoles of GSH / mg protein<sup>20</sup>.

#### Assay of antioxidant enzymes

**Superoxide dismutase (SOD)**—Superoxide dismutase (Cu-Zn SOD) activity was measured by hematoxylin auto oxidation method as employed earlier by Mukherjee *et al.*<sup>20</sup>. In brief, the weighed amounts of the hepatic tissue were homogenized (10%) in ice-cold 50 mM phosphate buffer containing 0.1 mM EDTA pH 7.4. The homogenates were then centrifuged at 12000 × g for 15 min and the supernatant was carefully collected. The inhibition of hematoxylin auto oxidation by the cell free supernatant was measured at 560 nm using a UV-VIS spectrophotometer. The enzyme activity was expressed as Units per mg tissue protein.

**Catalase**—Catalase was assayed by measuring the breakdown of hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>). The weighed amounts of the hepatic tissue were homogenized in 5% ice-cold 50 mM phosphate buffer pH 7.2. The homogenates were then centrifuged at 12000 × g for 12 min. The supernatant thus obtained was then carefully collected and incubated with 0.01 ml of absolute ethanol at 4°C for 30 min. Thereafter, 10% Triton X-100 was added to have a final concentration of 1%. The sample, thus obtained, was used to determine the catalase activity by measuring the breakdown of H<sub>2</sub>O<sub>2</sub> spectrophotometrically at 240 nm. The enzyme activity was expressed as micromoles of H<sub>2</sub>O<sub>2</sub> consumed per min per mg protein<sup>20</sup>.

#### Assay of a pro-oxidant enzyme

**Xanthine oxidase (XO)**—Xanthine oxidase was assayed by measuring the conversion of xanthine to uric acid following the method as employed by earlier workers<sup>20</sup>. The weighed amounts of the hepatic tissue were homogenized (10%) in 50 mM phosphate buffer pH 7.8. The homogenates were centrifuged at 500 × g for 10 min. The supernatant thus obtained was further centrifuged at 12000 × g for 20 min. This final supernatant was carefully collected and assayed for the enzyme activity spectrophotometrically 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 Units per mg tissue protein.

#### Assay of the metabolic enzymes

**Lactate dehydrogenase (LDH)**—Lactate dehydrogenase was assayed by measuring the formation of NAD from NADH during the reduction

of pyruvate according to the method of Strittmatter with some modifications. In brief, the weighed amounts of the hepatic tissue were homogenized (10%) in 50 mM Phosphate buffer with 1 mM EDTA, pH 7.2. The homogenates were centrifuged at  $500 \times g$  for 7 min. The supernatant thus obtained was further centrifuged at  $12000 \times g$  for 20 min. This final supernatant was carefully collected and used for the enzyme assay at 340 nm with 10 mM sodium pyruvate as the substrate, 50 mM Phosphate buffer, pH 7.5, as the assay buffer and 0.2 mM NADH. The enzyme activity was expressed as Units per mg tissue protein<sup>21</sup>.

*Alpha-ketoglutarate dehydrogenase*—The mitochondrial enzyme, alpha-ketoglutarate dehydrogenase ( $\alpha$ -KGDH) was assayed by following the reduction of  $\text{NAD}^+$  to NADH according to the method of Duncan *et al.*<sup>22</sup> with some modifications. In brief, the weighed amounts of the hepatic tissues were homogenized (10%) in 50 mM Phosphate buffer with 1 mM EDTA, pH 7.2. The homogenates were centrifuged at  $500 \times g$  for 10 min. The supernatant thus obtained was further centrifuged at  $20,000 \times g$  for 20 min. The  $20,000 \times g$  supernatant was discarded and the pellet was re-suspended with the same buffer and the aliquots were prepared for all the subsequent enzyme assays. The assay of this enzyme was carried out at 340 nm using a UV/VIS spectrophotometer with 0.1 mM  $\alpha$ -ketoglutarate as the substrate, 0.1 M phosphate buffer, pH 7.5, as the assay buffer and 1.0 mM  $\text{NAD}^+$  as an electron donor. The enzyme activity was expressed as units/ mg tissue protein.

*Isocitrate dehydrogenase*—Isocitrate dehydrogenase (ICDH), another mitochondrial enzyme, was assayed following the method of Duncan *et al.*<sup>22</sup>. The method is based on the measurement of the increase in optical density at 340 nm due to the production of NADH from  $\text{NAD}^+$  with the consequent oxidation of  $\text{D}_s$ - isocitrate, used as the substrate. The assay was performed at pH 7.6, which is the optimal pH for the reaction. Specific activity of the enzyme was expressed as the Units of enzyme activity per mg of protein.

*Succinate dehydrogenase*—Succinate dehydrogenase (SDH), another important mitochondrial enzyme, was assayed by a slight modification of the method of Veeger *et al.*<sup>23</sup>. The method is based on the measurement of the decrease in optical density at 420 nm spectrophotometrically due to reduction of  $\text{K}_3\text{Fe}(\text{CN})_6$  by succinate used as the substrate. The assay was carried out with 0.3 M phosphate buffer,

pH 7.6, 30 mM EDTA at pH 7.6, 0.03 M KCN, 0.4 M succinate, 3% bovine serum albumin in  $\text{H}_2\text{O}$  and 5 mM  $\text{K}_3\text{Fe}(\text{CN})_6$ . Specific activity was expressed as the units of enzyme activity/mg of protein<sup>23</sup>.

*Measurement of the protein*—Protein contents of the serum, tissue homogenates, cell free homogenates, post-mitochondrial supernatants and the mitochondrial suspension were determined by the method of Lowry *et al.*<sup>24</sup>.

*Measurement of the lead content of the hepatic tissue by atomic absorption spectrophotometry*—The tissue samples were processed and the lead content was measured as per the protocol mentioned in the cook book of the SAIF (Sophisticated Analytical Instrument Facilities) Thermo Scientific iCE 3000 Series AA Spectrometer available at the Bose Institute, Kolkata, India. The method of Kostial *et al.* (1999) was also used for our estimations with slight modification. The tissue samples were incubated overnight at 37°C. The dry weight of the tissues was subsequently taken. The dried tissues were then placed with nitric acid and double distilled water in the conical flask. The conical flasks with their contents were then placed on the hot plate and heated at 65-70°C for digestion of the hepatic tissue. Then, perchloric acid was added for the precipitation of the protein and heated until white fumes came out. The contents of the conical flasks were then transferred quantitatively into 25 ml marked volumetric flasks and the volume was finally made up to 25 ml. The samples thus prepared were used for the measurement of lead content using an atomic absorption spectrometer<sup>25</sup>.

*Statistical analysis*—Data were expressed as Mean  $\pm$  SE. Microcal Origin version 7.0, a computer program was used to compute statistical analysis. Difference between means was assessed by two tail Student's *t* test and the statistical significance was accepted at  $P < 0.001$  or  $P < 0.01$ .

## Results

Body weight of rats in all the groups remained the same before the experiment. After the treatment period, the rats of the control and the AGE only treated groups showed no significant change in their body weight. However, the rats of the LA treated group showed a significant decrease in body weight compared to the control group of rats. The body weight of the rats pre-treated with AGE exhibited an improvement but this change was not statistically significant (Table 1).

**Hepatic biomarkers**—Following LA treatment (ip) of rats, the level of SGPT activity significantly increased by 3 fold in comparison to the activity level measured in the control animals. But when the rats were pre-treated with AGE, a reduction (26%) in the activity level of SGPT was observed compared to the level of activity in LA treated group. However, the level of SGPT activity in the control as well as the AGE only treated groups did not show any change (Table 1).

ALP activity was significantly increased by 62% in comparison to the activity measured in the control animals following LA treatment. However, pre-treatment of rats with the AGE for 7 consecutive days prevented the ALP activity from getting increased. The enzyme activity, however, in the control and the AGE extract only treated groups exhibited no change (Table 1).

Rats when treated with LA for 7 consecutive days significantly increased the serum bilirubin level by 38% in comparison to the values obtained in the control rats. Pre-treatment of rats with AGE for the similar period of time prevented the serum bilirubin from rising. However, the serum bilirubin level in the control as well as in the AGE alone treated rats showed no change (Table 1).

**Haemoglobin level**—Rats when treated with LA (ip) for 7 consecutive days, the haemoglobin content of blood significantly decreased by 25% in comparison to the values observed in the control rats. But when the rats were pre-treated with AGE for a similar period of time, the haemoglobin content of blood was found to be significantly higher by 15% in comparison to the animals treated with lead acetate. However, the haemoglobin content of the control and the AGE only treated rats showed no such change (Table 1).

**Tissue morphology**—Histological studies have been carried out using hematoxylin and eosin staining of the liver tissue sections which reveal that the treatment of rats with the present dose of LA lead to severe damage of the cells surrounding the central vein when compared to the liver sections of the control rats. The cellular damage and disruption of the cytoarchitectural features extend deep into the hepatic parenchymatous tissue. However, when the rats were pretreated with 50 mg/kg/day of the AGE for the similar period of time, such changes were found to be at their minimum. The hepatic tissue morphology in AGE only treated rats resembles that observed in the control rats (Fig. 1A).

The histological studies also reveal that there occurred a large deposition of collagen in the extracellular matrix following injury of hepatocytes and other cells due to treatment of rats by LA for the 7 consecutive days when compared to control. The deposition of collagen was intense around the central vein. The collagen deposition was decreased to near control level when the rats were pre-treated with AGE also for the similar time-period. When compared, there was not much difference in collagen deposition around the central vein in the livers of the control as well as the AGE only treated group (Fig. 1B & Table 3).

**Bio-markers of oxidative stress**—Treatment of rats with LA at the present dose resulted in a significant increase in the level of lipid peroxidation in the liver tissue by 8 fold in comparison to the level of LPO measured in the control animals. However, pre-treatment of rats with the AGE for the same time period protected the hepatic tissue from getting peroxidized as evident from a significantly reduced level of lipid peroxidation products (45%) when

Table 1—Protective effect of aqueous garlic extract against lead-induced changes in body weight and biomarkers of hepatic function in rats  
[Values are mean  $\pm$  SE of 6 animals in each group]

Parameter studied	Control	Garlic treated	Lead treated	Lead + Garlic
Body weight (gm)				
Pre-experiment	177.33 $\pm$ 7.77	176.66 $\pm$ 5.78	178.33 $\pm$ 6.01	177.33 $\pm$ 5.21
Post-experiment	184.00 $\pm$ 7.37	179.66 $\pm$ 9.31	148.00 $\pm$ 6.40 *	172.66 $\pm$ 7.15
SGPT (IU/Lit)	6.98 $\pm$ 0.35	7.34 $\pm$ 0.26	19.83 $\pm$ 0.25#	14.58 $\pm$ 0.58**
ALP	3.62 $\pm$ 0.063	3.74 $\pm$ 0.11	5.88 $\pm$ 0.034*	3.94 $\pm$ 0.092**
Bilirubin (Mg/dl)	0.55 $\pm$ 0.014	0.54 $\pm$ 0.014	0.76 $\pm$ 0.011#	0.52 $\pm$ 0.028**
Haemoglobin (Gm %)	14.7 $\pm$ 0.086	14.55 $\pm$ 0.11	11.05 $\pm$ 0.072#	12.75 $\pm$ 0.192**

Data were analysed by Student's one-tail *t*-test

\**P* < 0.01 compared to control; #*P* < 0.001 compared to control; \*\**P* < 0.001 compared to lead-treated group

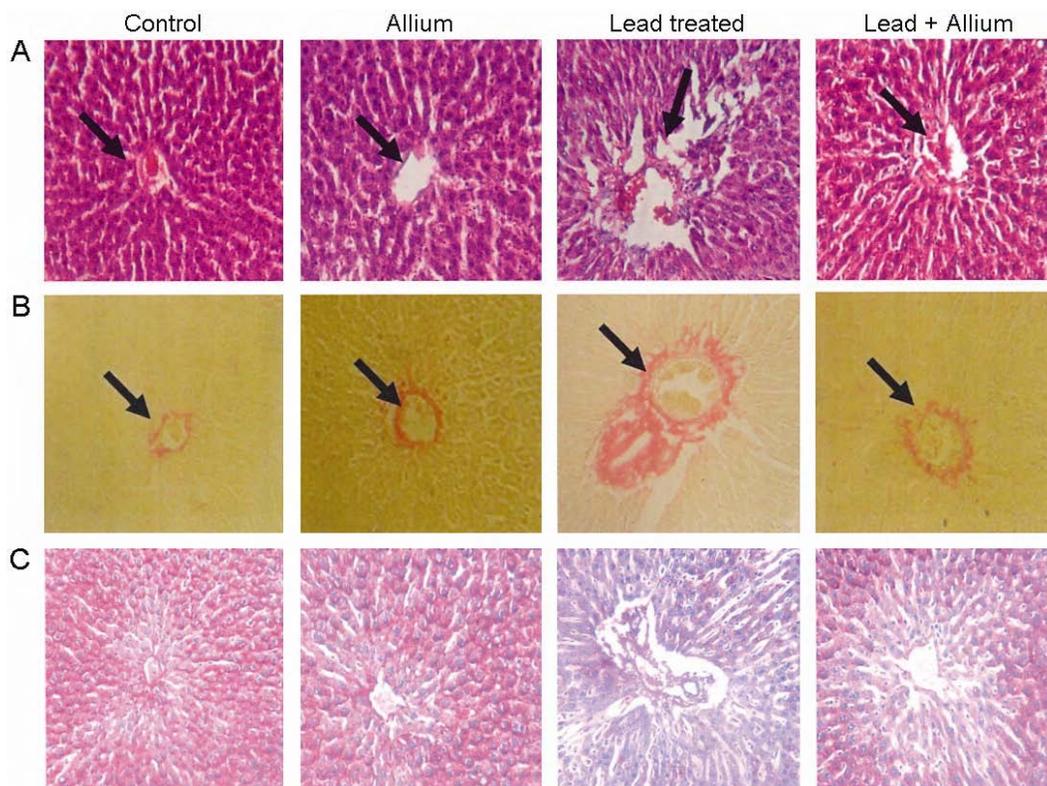


Fig. 1—Effect of different treatments on hepatic tissue morphology. [(A)-arrow indicate the central vein, H & E staining 20 ×, (B)-arrow indicate collagen deposition around the central vein, acid-sirius staining 20 ×. (C)-tissue glycogen content, PAS staining 20 ×]

compared to the LPO levels measured in the LA treated group. The LPO level in the control and the AGE only treated group remained unaltered (Table 2).

Following treatment of rats with LA for 7 days caused a significant elevation in the reduced glutathione level of the liver tissue by 6 fold in comparison to the levels measured in the control animals. Pre-treatment of rats with the AGE for the similar period of time, caused a significant reduction in the GSH level by 36%. However, the rats of the control and the AGE only treated group showed no change in the level of this antioxidant (Table 2).

**Antioxidant enzymes**— Following treatment of rats with LA for 7 consecutive days, the Cu-Zn SOD activity of the liver tissue decreased significantly by 4 fold in comparison to the activity of this enzyme measured in the control animals. However, pre-treatment of rats with the AGE for the similar period of time prevented the enzyme activity from decreasing. However, the activity of the hepatic Cu-Zn SOD in rats of the control as well as the AGE only treated groups remained unaltered (Table 2).

Catalase is an important antioxidant enzyme which helps in converting the hydrogen peroxide to water and oxygen and thus, has the ability to control the

concentration of this ROS within the cells. An enhancement in the activity of catalase indicates oxidative stress. The results presented reveal that rats when treated with lead acetate for 7 consecutive days, increased the catalase activity in liver significantly by 54% in comparison to the activity measured in the control animals. Pre-treatment of rats with AGE similar period of time caused a significant (18%) reduction in the level of activity of the enzyme but did not reach the activity measured in the tissues of the control animals. However, the activity of the enzyme in the hepatic tissue of the control and the AGE only treated rats exhibited no change (Table 2).

**Xanthine oxidase activity**—Xanthine oxidase is a pro-oxidant enzyme and an enhancement in the activity of this enzyme is considered to indicate an increased level of oxidative stress<sup>20</sup>. An increased xanthine oxidase activity indicates increased formation of superoxide anion free radical. The results reveal that the rats when treated with LA for 7 consecutive days, increased the xanthine oxidase activity of the liver tissue significantly by 2.5 fold in comparison to the activity measured in the control rats. However, the rats when pre-treated with the AGE for the similar period of time exhibited a

significant reduction (40%) in the activity of the xanthine oxidase compared to the activity measured in the hepatic tissues of the LA treated rats. However, the activity of this enzyme in the hepatic tissue of the control as well as the AGE only treated group did not differ significantly among themselves (Table 2).

**Blood glucose and lactate levels**—Treatment of rats with LA for 7 consecutive days showed a significant decrease (19%) in blood glucose level in comparison to the level measured in the control animals. However, when the rats were pre-treated with AGE for a similar period of time, the reduction in the blood glucose level was found to be less (only 12.7% vs. control) compared to control values. Animals of the control and AGE only treated group, however, showed no change in the blood glucose level (Table 2).

Rats treated with LA for 7 consecutive days showed a highly significant increase (56%) in blood lactate level in comparison to the levels measured in the control animals. However, when the rats were pre-treated with present dose of AGE during the treatment period, the blood lactate level significantly decreased (69%) compared to the values obtained in the LA

treated rats. Blood lactate level, however, showed no change when it was measured in the rats of the control and the AGE only treated group (Table 2).

**Activities of the enzymes related to energy metabolism**—Activities of several enzymes associated with the energy metabolism following treatment of rats with the LA were studied in order to understand the effect of Lead on these enzymes and whether the AGE has any protective effect on the activities of these enzymes.

**Lactate dehydrogenase (LDH)**—Rats when treated with LA for 7 consecutive days caused a significant elevation of the lactate dehydrogenase activity in liver by 48% in comparison to the activities measured in the control animals. However, pre-treatment of rats with the AGE for a similar period of time prevented the lactate dehydrogenase activity from getting increased. Animals of the AGE only treated group, however, showed a highly significant decrease in the LDH activity by 34% compared to the control (Table 2).

**Isocitrate dehydrogenase (ICDH)**—Rats when treated with LA for 7 consecutive days, the isocitrate dehydrogenase activity of the liver tissue increased

Table 2—Protective effect of aqueous garlic extract against lead-induced alterations in the biomarkers of oxidative stress, antioxidant enzymes and pro-oxidant enzymes, hepatic glucose metabolism and total lead content  
[Values are mean  $\pm$  SE of 6 animals in each group]

Parameter studied	Control	Garlic treated	Lead treated	Lead + Garlic
LPO (nmoles TBARS/ mg protein)	0.146 $\pm$ 0.021	0.165 $\pm$ 0.024	0.786 $\pm$ 0.013#	0.428 $\pm$ 0.009**
GSH (nmole GSH/ mg protein)	44.73 $\pm$ 1.84	45.5 $\pm$ 2.54	66.47 $\pm$ 4.34#	169.46 $\pm$ 5.80**
SOD (Units/ mg protein)	1.51 $\pm$ 0.17	1.56 $\pm$ 0.185	0.486 $\pm$ 0.040#	1.39 $\pm$ 0.125**
Catalase ( $\mu$ moles H <sub>2</sub> O <sub>2</sub> consumed/min/ mg protein)	83.33 $\pm$ 0.665	82.55 $\pm$ 0.411	128.01 $\pm$ 1.21#	104.51 $\pm$ 3.57**
Xanthine Oxidase (mUnits/ mg protein)	8.42 $\pm$ 0.06	7.41 $\pm$ 0.33	20.8 $\pm$ 0.31#	12.4 $\pm$ 0.76**
Blood Glucose level (Mgm/dl)	93 $\pm$ 0.28868	91.92 $\pm$ 1.02	74.95 $\pm$ 0.344#	84.5 $\pm$ 1.3**
Blood Lactate (nmole/lit )	2.6 $\pm$ 0.22	2.6 $\pm$ 0.12	5.9 $\pm$ 0.245	1.8 $\pm$ 0.22
LDH activity (Units / mg protein)	1.40 $\pm$ 0.03	0.92 $\pm$ 0.04#	2.1 $\pm$ 0.042#	1.315 $\pm$ 0.12**
$\alpha$ - KGDH activity (Units / mg protein)	0.23 $\pm$ 0.002	0.24 $\pm$ 0.005	8 $\pm$ 0.0003#	0.2 $\pm$ 0.005**
ICDH activity (Units / mg protein)	0.042 $\pm$ 0.004	0.041 $\pm$ 0.0006	0.15 $\pm$ 0.005#	0.05 $\pm$ 0.003**
SDH activity (Units / mg protein)	1.48 $\pm$ 0.009	1.45 $\pm$ 0.021	0.76 $\pm$ 0.019#	2.26 $\pm$ 0.08**
Lead Content (ppm)	1.022 $\pm$ 0.045	1.00 $\pm$ 0.042	10.65 $\pm$ 0.96*	10.03 $\pm$ 1.12

Data were analysed by Student's one-tail *t*-test

\**P* < 0.01 compared to control; #*P* < 0.001 compared to control; \*\**P* < 0.001 compared to lead-treated group

Table 3—Protective effect of aqueous garlic extract against lead-induced changes in collagen area and glycogen content of hepatic tissue of rats  
[Values are mean  $\pm$  SE of 6 animals in each group]

Parameter studied	Control	Garlic treated	Lead treated	Lead + Garlic
Area of collagen (%)	0.021 $\pm$ 0.0002	0.021 $\pm$ 0.0002	0.024 $\pm$ 0.0002#	0.021 $\pm$ 0.00015**
Glycogen Content (Mg/ml)	1.95 $\pm$ 0.03	1.89 $\pm$ 0.013	0.53 $\pm$ 0.036#	0.65 $\pm$ 0.024**

Data were analysed by Student's one-tail *t*-test

#*P* < 0.001 compared to control; \*\**P* < 0.001 compared to lead-treated group

significantly by 3.5 fold in comparison to the activity measured in the control rats. However, on pre-treating the rats with the AGE for similar time period, the isocitrate dehydrogenase activity was almost completely prevented from getting increased when compared to the activity measured in the rats treated with LA. No significant change in the activity of the enzyme, however, was noted in the control and the AGE only treated rats (Table 2).

*$\alpha$ -keto-glutarate dehydrogenase ( $\alpha$ -KGDH)*— $\alpha$ -KGDH is one of the important enzymes of the TCA cycle and is associated with the production of ATP. The results reveal that rats when treated with LA for 7 consecutive days, the  $\alpha$ -KGDH activity of the liver tissue decreased significantly by 3.5 fold in comparison to the activity measured in the control animals. However, pre-treatment of rats with the AGE for a similar period of time protected the enzyme activity from getting decreased to the level of enzyme activity observed in the hepatic tissue of the LA treated animals although the protection provided by the extract was not complete. The activity of the enzyme, however, in the hepatic tissue of the control and the AGE only treated group of rats showed no significant change when compared to each other (Table 2).

*Succinate dehydrogenase (SDH)*—Succinate dehydrogenase is also another very important enzyme of the TCA cycle associated with the generation of ATP. The results reveal that rats when treated with LA for 7 consecutive days, exhibited a significantly decreased SDH activity in liver tissue by 2 fold in comparison to the activity measured in the control rats. However, the activity of this enzyme was found to be completely protected when the rats were pre-treated with the AGE for the similar time-period. It is interesting to note that the activity of this enzyme in the AGE protected rats was found to be even higher than the activity observed in the hepatic tissue of control rats. The reason for this elevated level of activity of this enzyme in allium protected rats remains unclear and needs further investigation. The activity of this enzyme, however, in the control as well as the AGE only treated group showed no significant change when compared to each other (Table 2).

*Glycogen reserves*—Glycogen reserves are important in energy metabolism as they provide glucose for ATP production following their hydrolysis. We have measured the glycogen content

biochemically as well as assessed the situation histochemically. The results are presented below.

Rats when treated with LA for 7 consecutive days caused a significant depletion of the hepatic glycogen content by 3.5 fold in comparison to control animals. Rats were pre-treated with the AGE for a similar time-period, the glycogen content increased slightly but not significantly (Table 3). However, rats of the control and the AGE only treated group showed no significant change in the hepatic glycogen content when compared to each other. Why there occurred almost no improvement in the glycogen content in the AGE protected rats remains an interesting issue for future investigation.

In addition to biochemical estimation for tissue glycogen content, we have also performed histochemical studies using PAS staining for glycogen (Fig. 1C). The figure reveals a severe depletion of tissue glycogen content accompanied by intense tissue damage following treatment of rats with LA for seven consecutive days compared to the control rats. However, pre-treatment of rats with AGE not only prevented the hepatic tissue from getting damaged but also tissue glycogen content was restored only to some extent supporting our biochemical observation. Moreover, tissue glycogen content of AGE only treated rats did not differ much from that observed in the control rats (Table 3).

*Hepatic lead content*—To determine whether this AGE has the potential to bind with lead we have measured the lead content of the rat tissues through atomic absorption spectrophotometry. The lead content in the liver was found to be significantly increased in comparison to the control animal groups following injection of LA for the 7 consecutive days. However, when the rats were pre-treated with AGE for the similar time-period, the lead content of this group of animals did not show a significant decrease in comparison to the LA groups. Again, the lead content of the AGE alone treated group also remains unchanged when compared to the control group of animals (Table 2).

## Discussion

Treatment of rats with LA for 7 consecutive days caused a significant decrease in the body weight of rats. The results are in agreement with several other studies<sup>26</sup>, which suggested that the reduced growth was due to reduced food consumption via lead effects on the satiety set-point. This change in the body weight

was prevented from occurring when the rats were pre-treated with the AGE at a dose of 50 mg/kg body wt orally for the similar time-period (Table 1). However, the extract alone has no effect on the body weight.

Treatment of rats with lead acetate for 7 consecutive days caused a significant increase in the levels of SGPT and serum alkaline phosphatase activity and an elevation in the level of serum bilirubin content indicating serious hepatic tissue damage. These changes were mitigated when the rats were pre-treated with the effective dose (50 mg/kg body wt) of the AGE indicating that the extract has the capability of providing protection to the hepatic tissue against lead-induced injury<sup>27</sup>. SGPT is an important biomarker of hepatic function. A significant decrease in the hemoglobin content following treatment of rats with lead acetate may be due to the effect of lead on the hemoglobin biosynthesis or this decrease may also be due to breakdown of hemoglobin in to its downstream products. However, pre-treatment of rats with the AGE, has significantly protected the hemoglobin level from getting decreased although a complete protection has not been detected. Furthermore, treatment of rats with the AGE only, has no effect on the serum GOT and ALP activity as well as hemoglobin and serum bilirubin content indicating clearly that the extract by itself does not cause any adverse effect on the hepatic tissue.

Results indicate that the rats when treated with LA with the present dose for the 7 consecutive days brought about significant injury to tissue morphology in the liver which was most evident around the central vein of the hepatic lobule with parallel hepatocyte damage extending deep into the parenchymatous tissue of the hepatic lobule. These findings are in conformity with the findings of other researchers<sup>28</sup>. Earlier researchers have also shown that the liver of lead-treated rats revealed remarkable degenerative alterations<sup>29</sup>. However, pre-treatment of rats with the effective dose of AGE was found to provide protection against LA-induced tissue injury in liver. However, treatment of another set of rats with AGE only was found to have no effect on tissue morphology indicating that the extract not only has the ability to protect the tissues in the face of oxidative stress but also do not injure the tissue in the control animals as well indicating again that the aqueous extract may be considered safe for future human consumption. Moreover, the damage to tissue morphology following LA treatment was further evident from our studies on tissue collagen

distribution. It was revealed that a large amount of collagen was found to be deposited in the intercellular matrix in the section of the liver obtained from the LA treated rats. The collagen was particularly concentrated around the central vein of the hepatic lobule. When the rats were pre-treated with the effective dose of AGE, the deposition of tissue collagen was found to be significantly decreased indicating again a protective effect of this aqueous extract against LA-induced tissue injury. Treatment of another set of rats, however, with the effective dose of AGE was found to have almost no effect on tissue collagen content in the liver tissue.

That the tissue injury observed following treatment of rats with LA was brought about due to oxidative stress is evident from a highly significant increase in the levels of lipid peroxidation products and also of reduced glutathione of the hepatic tissue. These two parameters are considered as the primary bio-markers of oxidative stress. Pre-treatment of rats with AGE at a dose of 50 mg/kg body wt per os for the 7 consecutive days prevented the levels of lipid peroxidation and reduced glutathione to rise when the animals were challenged with LA (Table 2) indicating that this dose of the extract is fully capable of mitigating the oxidative stress induced following treatment of the animals with LA. It is also interesting to note that the extract when given alone to another group of rats has almost no effect on these classical bio-markers of oxidative stress. This indicates that the extract may be considered safe for future human consumption, as evident at least from the results of our experiments although further studies in this area will only strengthen our views.

Several studies have reported alterations in antioxidant enzyme activities such as SOD, catalase, and changes in the concentrations of some antioxidant molecules, such as glutathione (GSH) in lead-exposed animals<sup>26,29</sup> and workers<sup>30,31</sup>. Although these findings suggest a possible involvement of oxidative stress in the pathophysiology of lead toxicity, it is not clear whether these alterations are the cause of the oxidative damage or a consequence of it. However, as lead toxicity affects the components of the antioxidant defense system, it might cause impairment in pro-oxidant/antioxidant balance of cells, resulting in oxidative damage.

Superoxide dismutase (Cu-Zn type) activity in the hepatic tissue was found to be almost completely protected from getting decreased when the rats were pre-treated with the AGE at the present effective dose

(Table 2). Here also, the AGE alone has no significant effect on the activity of this enzyme in the hepatic tissue indicating not only that the extract has the potential to alleviate the oxidative stress but also may be considered safe for future human consumption as a natural antioxidant although further studies are required for adjudging the long-term effects of the extract on animals or the humans. Similarly, the extract was also found to have a protective effect on another important antioxidant enzyme, catalase in the hepatic tissue. Here again, the extract alone has no effect on this enzyme activity (Table 2).

It was also revealed from our studies that the activity of xanthine oxidase, a pro-oxidant enzyme, responsible for the generation of superoxide anion free radical, was increased significantly in the liver tissue of rats treated with the LA for seven consecutive days indicating generation of reactive oxygen intermediates *in vivo* that may lead to oxidative stress. This elevated level of activity of xanthine oxidase was restored to near normal activity in liver tissue when the rats were pre-treated with AGE. Again, the activity of this enzyme was not affected when the rats were treated with the AGE only also for the seven consecutive days. Formation of superoxide anion free radical *in vivo* following LA treatment of rats is clearly indicative of generation of oxidative stress. This superoxide anion radical can lead to the formation of hydrogen peroxide, another important reactive oxygen species, which in turn may lead to formation of hydroxyl radical in presence of redox-active transition metals like iron and/or copper, and other radical species which may bring about the oxidative damage to biomacromolecules like lipid, proteins, carbohydrates and nucleic acids leading to tissue injury and consequential malfunctions. The AGE while protecting the tissues from lead-induced oxidative stress seems to have the potentiality to either decrease the formation of reactive oxygen species by controlling the activities of the antioxidant enzymes or by directly scavenging the superoxide anion free radical. Whether this AGE has the ability to scavenge the superoxide anion free radical remains to be investigated.

The results presented so far clearly demonstrate that treatment of rats with LA induces oxidative stress in liver. Sterling<sup>32</sup> reported that chronic lead ingestion affects the glucose metabolism in the CNS. Likewise, in our studies, treatment of rats with the present dose of LA for the 7 consecutive days not only induces oxidative stress but also blood glucose level was

significantly decreased with a concomitant increase in the blood lactate level. This situation is ameliorated when the rats were pre-treated with the AGE. However, treatment of rats with the AGE alone has no effect on these parameters. The possible reason behind the decreased blood glucose level and an increased blood lactate level following LA treatment of rats may be due to the fact that mitochondria remains in a reduced state in oxidative stress situations with consequential shift of metabolism from aerobic state to anaerobic state. The results reveal that AGE may have the potential to prevent this metabolic shift from occurring.

Treatment of rats with LA for seven consecutive days significantly elevated the activity of lactate dehydrogenase (LDH) in the hepatic tissue which was found to be restored to normal level of activity when the rats were pre-treated with the AGE. However, treatment of another set of rats with the AGE only seems to have a slight inhibitory effect on the enzyme activity in hepatic tissue. Further, the results also indicate a shift of metabolism from aerobic to anaerobic state which happens in situations of oxidative stress. It seems that the AGE has the capability of preventing this shift of metabolic status although detailed studies in future will throw light on the exact underlying mechanism.

The studies further reveal that treatment of rats with LA for a period of 7 consecutive days caused serious alterations in the oxidative metabolism as is reflected in a significant increase in the activity of isocitrate dehydrogenase and a decrease in the activity of alpha-keto glutarate dehydrogenase and succinate dehydrogenase, the Krebs's cycle enzymes, in the liver tissue critically responsible for the generation of ATP though the intermediate formation of NADH and FADH<sub>2</sub>. It is firmly believed that mitochondrial electron transport chain is a rich source of ROS<sup>33</sup>. The effect of ROS (superoxide anion radical, hydrogen peroxide and hydroxyl radical) could be damaging on virtually all cellular components but membrane lipids are particularly sensitive to free radicals due to the presence of polyunsaturated fatty acids, which preferentially undergo lipid peroxidation. The fragmentation products of lipid peroxidation are highly toxic aldehydes, of which 4-hydroxy-2-nonenal (HNE) is the major and probably the most reactive product<sup>34</sup>. Exposure of cardiac mitochondria to HNE resulted in a reduced  $\alpha$ -KGDH activity<sup>35</sup>. In the elevated conditions of oxidative stress, there occurs a reduction in the activities of the components

of the electron transfer chain of the mitochondria<sup>36</sup>. However, when the rats were pre-treated with AGE, the activities of these enzymes were restored to near normal indicating that this aqueous extract has the potential to protect the tissues from oxidative damage by protecting enzymes of the Krebs' cycle. The activity of these enzymes in the AGE only treated group was found to be unaffected under our experimental conditions. However, out of these three enzymes,  $\alpha$ -KGDH and SDH were found to be most sensitive to H<sub>2</sub>O<sub>2</sub> which were inhibited by slight increase in H<sub>2</sub>O<sub>2</sub> but the vulnerability of these enzymes to H<sub>2</sub>O<sub>2</sub> and the metabolic consequences of their impaired function were markedly different as reported by other researchers. They have also reported that  $\alpha$ -KGDH is a crucial target of reactive oxygen species and a generator of oxidative stress in the mitochondria<sup>37</sup>. A normal rate of Krebs' cycle within the cell will maintain a normal concentration of ATP which in turn will help to maintain normal cellular metabolism. The AGE only treated group, however, exhibited normal enzyme activity when compared to the activities observed in the control rats indicating again that the extract may be considered in future as a safe preparation for human consumption to combat heavy metal-induced oxidative stress.

Earlier studies reported that lead hepatotoxicity led to vacuolization of the cells, polymorphism of the nuclei, and decrease in glycogen content of the hepatocytes<sup>38</sup>. The results also indicate that treatment of rats with LA for seven consecutive days depletes the hepatic glycogen content which was found to remain in a depleted state even if the animals were pre-treated with the AGE for the similar period of time. However, the AGE only appears to have no effect on the glycogen content when compared to the control animals.

The biochemical observations were further supported by our histochemical findings (PAS staining) which also indicate a depletion of glycogen content in the liver tissue following treatment of animals with LA for 7 consecutive days. However, hepatic glycogen content did not return to the control level when the rats were pre-treated with AGE. Furthermore, the AGE only treated rats did not exhibit any change in the tissue glycogen content of the hepatic tissue. The results indicate that exposure of the animals to lead put much higher constraints on carbohydrate metabolism in the liver tissue. This might be the reason that hepatic glycogen content

gets depleted to provide the tissue with more glucose to be utilized through anaerobic metabolism in our experimental conditions.

The generation of free radicals may also be prevented/decreased if the aqueous extract has the potential to quench the divalent cation, namely, lead. To ascertain this, the content of lead in the liver tissue of control, LA-treated, AGE protected as well as AGE only treated rats was estimated through atomic absorption spectrophotometry. The results reveal clearly that this AGE does not quench lead as the contents of lead in the hepatic tissue of control and the AGE only treated rats as well as LA-treated and AGE protected group of rats were found to be the more-or-less same. Although several studies reported that garlic decreases lead contents in tissues of lead exposed rats and chicken, respectively<sup>39,40</sup>, their observations could not be confirmed and suggest that this difference may be due to difference in the preparation procedure of the extract and different experimental conditions.

Results of the present study indicate that treatment of rats with the present dose of LA brings about oxidative stress-induced hepatic tissue injury due to alterations in the balance between antioxidant/pro-oxidant system and affecting the enzymes related to energy metabolism. The AGE may exert its protective actions against lead-induced hepatic injury in rats possibly through its antioxidant mechanisms although involvement of mechanisms other than this may not be ruled out. The results raise the possibility of garlic being considered as one of the component of the regular diet of the people in the areas who may have chances of exposure to lead occupationally or environmentally.

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