

# Hepato Ameliorative Effect of *Azadirachta indica* Leaves Extract against Mercuric Chloride Environmental Pollution.

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**Abstract:** Mercury is a highly toxic metal which induces oxidative stress in the body. The present study aimed to investigate the possible protective effect of neem (*Azadirachta indica*) leaves extract (NLE) as an antioxidant to protect against mercury-induced oxidative stress and hepatotoxicity. Forty male albino Sprague Dawley rats were classified into four groups, Control group received distilled water. Mercury-treated group was administrated 2 mg/Kg b.wt., mercuric chloride (HgCl<sub>2</sub>). Mercury and NLE-treated group was treated with 200 mg/Kg b.wt of NLE simultaneously with the same dose of HgCl<sub>2</sub>. NLE-treated group was given NLE only with the same dose. The daily treatments were administered orally for 30 days. Hepatotoxicity was assessed by increased tissue malondialdehyde (MDA), nitric oxide (NO) concentrations, glutathione peroxidase (GPx) activity, serum total bilirubin, direct bilirubin concentrations, serum activities of alanine aminotransferase (ALT), aspartate aminotransferase; (AST), and alkaline phosphatase (ALP) enzymes and decreased hepatic content of reduced glutathione (GSH), vitamin C, vitamin E and serum albumin concentration. Moreover, the histopathological changes revealed destruction of the normal hepatic architecture and severe pathological alterations post HgCl<sub>2</sub> treatment. Carbohydrates, DNA and proteins contents were decreased in the liver tissue. The ultrastructural alterations were represented by degenerative features which covered all the hepatic cells and their cytoplasm contained extensive necrotic areas. Many lipid droplets dispersed within the hepatic cells in addition to the disintegration of most cellular contents. On the other hand, treatment of rats with NLE reversed all these biochemical indices, histopathological, histochemical and ultrastructural alterations. The levels of various serum and tissue parameters for organ toxicity after neem treatment at the dose of 200 mg/kg.b.wt. remained more or less close to the normal values suggesting no significant adverse effects of NLE. The present results implicate that mercury-induced oxidative damage in hepatic tissues was improved by neem leaves extract, with its antioxidant effects. [Journal of American Science 2010; 6(9):735-751]. (ISSN: 1545-1003).

**Keywords:** Liver function, Mercuric chloride, Neem, Oxidative stress, Rat, Liver Ultrastructure.

**Abbreviations:** ALT, Alanine aminotransferase; ALP, alkaline phosphatase; AST, aspartate aminotransferase; GSH, reduced glutathione; GPx, glutathione peroxidase; MDA, malondialdehyde; HgCl<sub>2</sub>, mercuric chloride; NLE, neem leaves extract; NO, nitric oxide; ROS, reactive oxygen species

## 1. Introduction

Heavy metals are natural constituents of the Earth's crust and are present in varying concentrations in all ecosystems. Human activities play a major role in polluting the environment by toxic and carcinogenic metal compounds. Hence, industrial pollution of the environment with metal compounds is becoming a serious problem (Sarith Babu et al., 2007). Unlike most organic pollutants, heavy metals are not degraded and they have a tendency to accumulate in the soil, water sources and food chain (US EPA, 1987).

Although, mercury has been recognized as a hazardous widespread environmental and industrial pollutant (WHO, 1991), and the exposure to this metal is associated with several adverse health effects

in humans, it is commercially important in many industries, and their occupational and environmental exposures continue to occur (Järup, 2003) and is even increasing in some parts of the world. Mercury is widely used in agriculture as fungicide, in medicine as topical antiseptic, disinfectant and parasiticide (ATSDR, 1999), as amalgam fillings in dentistry (Jagadeesan, 2004). Metallic mercury is used in thermometers, barometers and instruments for measuring blood pressure. The largest occupational group exposed to mercury is dental care staff (Järup, 2003). The most frequent chemical forms to which humans and animals are exposed are elemental mercury vapour, mercurous salts as mercuric chloride (HgCl<sub>2</sub>) and organic mercury compounds such as methyl mercury (CH<sub>3</sub>Hg) (Drasch et al., 2001). All of which are toxic to animals and humans.

From the side of nutrition, seafood, and fresh-water fish are further important sources of human exposure (Magos and Clarkson, 2006; Grotto et al., 2010). Poisoning can result from inhalation, ingestion, or absorption through the skin (Goyer, 1991). It is poorly absorbed from the gastrointestinal tract; however it tends to rapidly accumulate mainly in the kidney and in the liver as well (Endo et al., 2002; Kenow et al., 2008).  $HgCl_2$  is one of the most toxic forms of mercury because it easily forms organomercury complexes with proteins (Wargovich et al., 2001), leading to functional and structural alterations in many organs, such as kidney, liver, lung, central nervous system, and testis (Mahboob et al., 2001).

Over the last decade, evidence has accumulated for a role of reactive oxygen metabolites as a mediator of tissue injury in several animal models of toxicity including  $HgCl_2$ . Although the exact mechanisms of free-radical generation are not yet completely understood, it is postulated that the antioxidant GSH depletion by mercury may be a trigger for the production of reactive oxygen species (ROS) that induce lipid, protein, and DNA oxidation. Generation of ROS in the cytoplasm of cells may increase the mitochondrial hydrogen peroxide production and lipid peroxidation of cell and mitochondrial membranes, resulting in loss of membrane integrity and finally cell necrosis or apoptosis (Valko et al., 2006).

It is worth mentioned that, most of the studies related to mercury toxicity have been carried out in kidney (Sener et al., 2007); brain (Jedrychowski et al., 2006; Cao et al., 2010) or cardiovascular system (Fillion et al., 2006). However, few studies have been performed in liver, even though this organ is the most involved in detoxification and the hepatic function has to be necessarily implicated in the incorporation and transport of that element in the kidney itself.

In recent years, much attention has been focused on the protective effect of naturally occurring antioxidants in biological systems against toxic heavy metals. The present work uses a non-conventional agent developed from a well known bioresource-the leaves of the *Azadirachta indica* that was commonly known as Neem tree. It has attracted world wide prominence in recent years, owing to its wide range of medicinal properties. Alkaloids, tannins, proteins, coumarin, flavonoids, polyphenols, saponins and sugars have been isolated from the neem tree and reviewed (Nair et al., 1997; Ross, 2003). The leaves of neem tree are traditionally used as medicinal preparations for their immunomodulatory, antiinflammatory, antihyperglycemic, antiulcer, antimalarial, antifungal, antibacterial, antiviral,

antimutagenic and anticarcinogenic properties (Subapriya and Nagini, 2005), antioxidant, hepatoprotective (Yanpallewar et al., 2003) and cardioprotective effects (Peer et al., 2008). Moreover, in Thailand, the young leaves and flowers of that plant are consumed as bitter tonic vegetables in order to promote good health due to very high antioxidant content (Clayton et al., 1996; Sithisarn et al., 2005).

In view of these considerations, this plant was considered to be interesting for a more detailed study. Therefore, the present study has been designed to elucidate whether the NLE when administered with mercury can ameliorate the oxidative stress-mediated hepatic dysfunction caused by mercury using biochemical, histopathological, histochemical and ultrastructural approaches.

## 2. Materials and Methods

### 2.1. Animals

Fourty adult male albino Sprague Dawley rats weighing 120-150g were used. Animals were maintained in cages with food and water *ad libitum* under controlled conditions of light, humidity and temperature. They were obtained from Animal House Colony of the National Research Centre, Doki, Giza. All experimental procedures were conducted in accordance with the guide for the care and use of laboratory animals and in accordance with the local Animal Care and Use Committee.

### 2.2. Preparation of neem plant extract

Fresh matured leaves of neem tree (*Azadirachta indica*) were collected from different locations in Beni-Suef governorate. The samples were identified in Botany Department, Faculty of Science, Beni-Suef University. The leaves were cleaned, dried in shade, powdered and labeled, the powder was used for the preparation of crude ethanolic extract according to the procedure described by Manikandan et al. (2008) based on the method of Chattopadhyay (1998).

Air-dried powder (1kg) of *Azadirachta indica* leaves were extracted by percolation at room temperature with 70% ethyl alcohol and kept at room temperature for 36 hours. Leaves extract of *Azadirachta indica* (NLE) was concentrated under reduced pressure (bath temperature 50°C) and dried in a vacuum desiccator. The residue was dissolved in distilled water and filtered. The filtrate was evaporated to dryness. The dried mass (yield=50.2 g) was diluted with distilled water and used in experiments.

### 2.3. Chemicals

Glutathione peroxidase (GPx) and nitric oxide (NO) kits were purchased from *Biodiagnostic Company*. Albumin, total bilirubin, ALT, AST and ALP enzyme kits were purchased from *Greiner Diagnostic GmbH-Bahlingen, Germany*. Other non-mentioned chemicals used in the present experiment were purchased from *Sigma, USA*.

### 2.4. Experimental design

Rats were divided into four groups of 10 rats each and treated as follows:

The 1<sup>st</sup> group served as (control group) and received distilled water. The animals of the 2<sup>nd</sup> group (Mercury-treated group) were daily administered 2 mg/ Kg body weight HgCl<sub>2</sub>. The animals of the 3<sup>rd</sup> group (Mercury and NLE-treated group) were given daily 200 mg / Kg b.wt. NLE simultaneously with the same dose of HgCl<sub>2</sub>. The animals of the 4<sup>th</sup> group (NLE-treated group) were given NLE only with the same dose as in the 3<sup>rd</sup> group. The treatments were administered orally by stomach tube and daily for 30 days. The dosage of HgCl<sub>2</sub> has been determined from the study performed by (Jaquadeesan and Sankarsami Pillai, 2007) as sufficient to elicit mild or moderate oxidative stress. NLE dose used in the present study (200 mg/kg b.wt.) was reported to be effective for antioxidant potential (Jaiswal *et al.*, 1994).

### 2.5. Sampling

Twenty four hours after the last dose(s), the animals were sacrificed by decapitation after an overnight fast. Blood samples were obtained and sera were separated by centrifugation and kept at -20°C for biochemical analysis.

Liver tissues were excised after dissection from the animals and designated for biochemical analysis. Fresh liver tissue sample, 0.5 g was homogenized in ten volumes of ice cold phosphate buffer (pH 7) until a uniform suspension was obtained. The homogenate was centrifuged at 20000×g for 10 min at 4 °C. The supernatant was collected and monitored for oxidative stress parameters (MDA, NO, GSH, GPx enzyme activity, vitamin C.). The hepatic vitamin E content was measured in the lipid extract of another fresh liver tissue sample which was prepared by the method of Folch *et al.* (1957) in which the chloroform-methanol mixture would disrupt the lipoprotein complex, precipitate the proteins and dissolve the lipids in the organic phase.

For light microscopic preparation, small pieces of the liver were immediately fixed in

alcoholic Bouin's solution for 24 hours, then embedded in paraffin wax, sectioned and prepared for staining by Harris Haematoxyline and Eosin (Bancroft and Gamble, 2002) for general histopathology, Periodic Acid Schiff's technique of Hotchkiss (1948) for carbohydrates, Bromophenol Blue technique of Mazia *et al.* (1953) for proteins and Feulgen reaction of Feulgen and Rossenbeck (1924) for DNA.

For electron microscopic preparation, specimens from liver were cut into small pieces measuring about 1 mm<sup>3</sup> and fixed in 2.5 % glutaraldehyde for 4 hours and 2 % paraformaldehyde in 0.1 M cacodylate buffer (pH 7.4). The samples were post-fixed in a buffered solution of 15 osmium tetroxide at 4 °C for 1-5 hr. This was followed by dehydration in ascending grades of ethyl alcohol, clearing in propylene oxide for two changes, 5min each, and embedding in Epon-epoxy-resin. Semi thin sections were stained with toluidine blue and investigated under a bright field microscope. Ultra thin sections were cut with a diamond Knife on the ultra microtome and mounted on formvar-coated grids, stained with uranyl acetate and lead citrate (Weakly, 1981). Sections were examined in a Joel transmission electron microscope at an acceleration voltage of 60-80-Kv. at the Faculty of Science, Ain shams University.

### 2.6. Biochemical assays

#### 2.6.1. Liver oxidative stress biomarkers

MDA concentration in liver homogenate, which is a good indicator of the degree of lipid peroxidation, was determined according to the method described by Albro *et al.* (1986). Nitric oxide (NO), an unstable reactive nitrogen free radical, was determined in the present study using biochemical method of Montgomery and Dymock (1961) where the production of NO is expressed by endogenous nitrite and nitrate metabolite. GSH concentration was assayed according to the method of Beutler *et al.* (1963). GPx enzyme activity was assayed according to Paglia and Valentine (1967). Tissue vitamin C was estimated by the method of Roe and Kuether (1943). Tissue vitamin E was estimated by the Baker *et al.* (1980). All concentrations and enzyme activities were spectrophotometrically determined using a Hitachi U-2000 Automatic Analyzer.

#### 2.6.2. Liver function biomarkers

Liver function was assayed by measuring serum albumin and bilirubin (total, direct, indirect) according to Dumas and Biggs (1972) and Tolman and Rej (1999) respectively. ALT, AST enzyme

activities according to Young (1990) and ALP enzyme activity according to Thomas (1998).

### 2.7. Statistical Analysis:

Statistical analysis for biochemical assays was carried out using one way analysis of variance (ANOVA) followed by least significant difference (LSD) test (Rao et al., 1985). The values are expressed as mean  $\pm$  standard error (SE). The p values below 0.05 were considered statistically significant.

## 3. Results

### 3.1. Biochemical assays

Our results revealed that HgCl<sub>2</sub> intoxication caused significant increase ( $P \leq 0.05$ ) in MDA, NO concentrations, significant decrease in GSH, vitamin C and vitamin E content in liver tissues, while GPx enzyme showed a slight higher activity in hepatic tissues compared to control group (Table, 1). Regarding the effect of NLE, the data reported in HgCl<sub>2</sub> and NLE-treated group showed a significant increase in liver GSH, liver vitamin C and vitamin E content and significant decrease in liver MDA and NO content with slight change of GPx activity compared to HgCl<sub>2</sub>-treated group.

The liver function tests revealed a significant increase ( $P \leq 0.05$ ) in serum ALT, AST and ALP activities, total, direct bilirubin concentrations and a significant decrease ( $P \leq 0.05$ ) in serum albumin concentration in HgCl<sub>2</sub>-treated group compared to the control group (Table, 2). Treatment of mercuric-intoxicated rats with NLE significantly modulated the hepatic functions concerning ALT, AST, ALP, total bilirubin concentrations, with a slight increase in serum albumin level and slight decrease in direct bilirubin level compared to HgCl<sub>2</sub>-treated group. There were no significant variations between control group and NLE-treated group (Tables, 1, 2).

### 3.2. Histopathological examination

Microscopic examination of the liver obtained from rats treated with HgCl<sub>2</sub> showed destruction of the normal hepatic architecture and severe pathological alterations (Figs.1b-1e) compared to control group (Fig.1a). Many hepatocytes showed vacuolar degenerative changes in their cytoplasm (Figs. 1b & 1c). Focal necrotic areas infiltrated with mononuclear leukocytes could be observed containing pyknotic and karyolytic nuclei of necrotic hepatocytes (Fig.1c). The central veins, portal veins and sinusoids were severely damaged; they appeared dilated and congested (Figs.1d, 1e). Some of the Kupffer cells were pushed into the

sinusoidal lumens (Fig. 1d). The administration of NLE simultaneously with HgCl<sub>2</sub> revealed marked restoration of the hepatic configuration. Most nuclei exhibited normal shape, being rounded and centrally located except for few pyknotic ones. No inflammatory changes were observed (Fig. 1f). There were no histological alterations in the liver of NLE alone treated rats when compared to control.

### 3.3. Histochemical Study

Mercury induced great depletion of glycogen, DNA and protein contents in the hepatocytes (Figs.2b, 3b and 4b respectively) compared with control group (Figs.2a, 3a and 4a respectively). However, the simultaneous administration of NLE plus HgCl<sub>2</sub> resulted in moderate increase of glycogen content (Fig.2c), retaining near normal distribution of DNA (Fig.3c) and protein (Fig.4c) content in hepatocytes compared to HgCl<sub>2</sub>-treated rats. No obvious variations were determined in glycogen, DNA and protein contents as a result of treatment with NLE only.

### 3.4. Electron microscopic study

The normal structure of the hepatic cells as revealed by electron microscopy is shown in Figs. (5a, 5b). The liver of rats which treated with HgCl<sub>2</sub> showed severe damage, these degenerative features had apparently covered all the hepatic cells and their cytoplasm contained extensive necrotic areas (Figs. 5c, 5d & 5e). Many lipid droplets dispersed within the hepatic cells in addition to the disintegration of most cellular contents. The endoplasmic reticulum was broken and was seen devoid of ribosomes. The mitochondria together with a few arrays of endoplasmic reticulum formed electron dense clusters that lie near the cell membrane and around the nucleus (Fig. 5e). The nuclei showed alterations of the nuclear envelope and accumulation of heterochromatin (Fig.5e). Also, the cytoplasmic organelles were devoid of glycogen (Figs. 5c, 5d & 5e). The phagocytic Kupffer cells were hypertrophied and appeared distinctly activated as symptomized by the presence of many lysosomes in their cytoplasm (Fig. 5f). Rats treated with HgCl<sub>2</sub> and NLE revealed that hepatocytes regained their normal appearance as their cytoplasm contained numerous mitochondria of variable shapes and sizes, well developed rough endoplasmic reticulum in the form of parallel and flattened cisternae studded with ribosomes, few vacuoles and lipid droplets (Fig. 5g). The nucleus contained homogenous chromatin distribution and there was marked restoration of the glycogen compared to HgCl<sub>2</sub> -treated rats (Fig. 5g). The sinusoids contain little or no debris (Fig. 5h).

**Table (1): Hepatic concentrations of MDA, NO, GSH, GPx, vitamin C and vitamin E in different groups.**

parameters Groups	MDA(n.mol /g wet tissue)	NO (n.mol/g wet tissue)	GSH (u.mol/g wet tissue)	GPx (mU/ g wet tissue )	Vitamin-C (mg/100g wet tissue )	Vitamin-E (mg/100g wet tissue)
<b>control group</b>	20.95±2.07 <sup>b</sup>	25.48±4.02 <sup>c</sup>	10.49±0.40 <sup>a</sup>	67.62±2.67	6.67±0.38 <sup>a</sup>	4.39±0.25 <sup>b</sup>
<b>HgCl<sub>2</sub>-treated group</b>	38.39±2.05 <sup>a</sup>	59.22±8.07 <sup>a</sup>	7.40±0.81 <sup>c</sup>	77.65±5.29	4.06±0.20 <sup>c</sup>	2.57±0.08 <sup>d</sup>
<b>HgCl<sub>2</sub> and NLE-treated group</b>	25.17±1.91 <sup>b</sup>	42.71±3.83 <sup>b</sup>	8.94±0.47 <sup>b</sup>	65.69±4.28	5.11±0.17 <sup>b</sup>	3.53±0.19 <sup>c</sup>
<b>NLE-treated group</b>	21.81±1.72 <sup>b</sup>	22.84±3.44 <sup>c</sup>	9.58±0.19 <sup>ab</sup>	63.54±4.10	6.05±0.19 <sup>a</sup>	5.27±0.08 <sup>a</sup>
F-probability	0.0001	0.0001	0.001	0.1	0.0001	0.0001
LSD at 5%	5.60	14.98	1.49	-	0.72	0.48
LSD at 1%	7.54	20.17	2.01	-	0.97	0.65

- Data are expressed as Means ± SE. (n=10).

- a, b, c, d superscript indicate significant difference at P < 0.05.

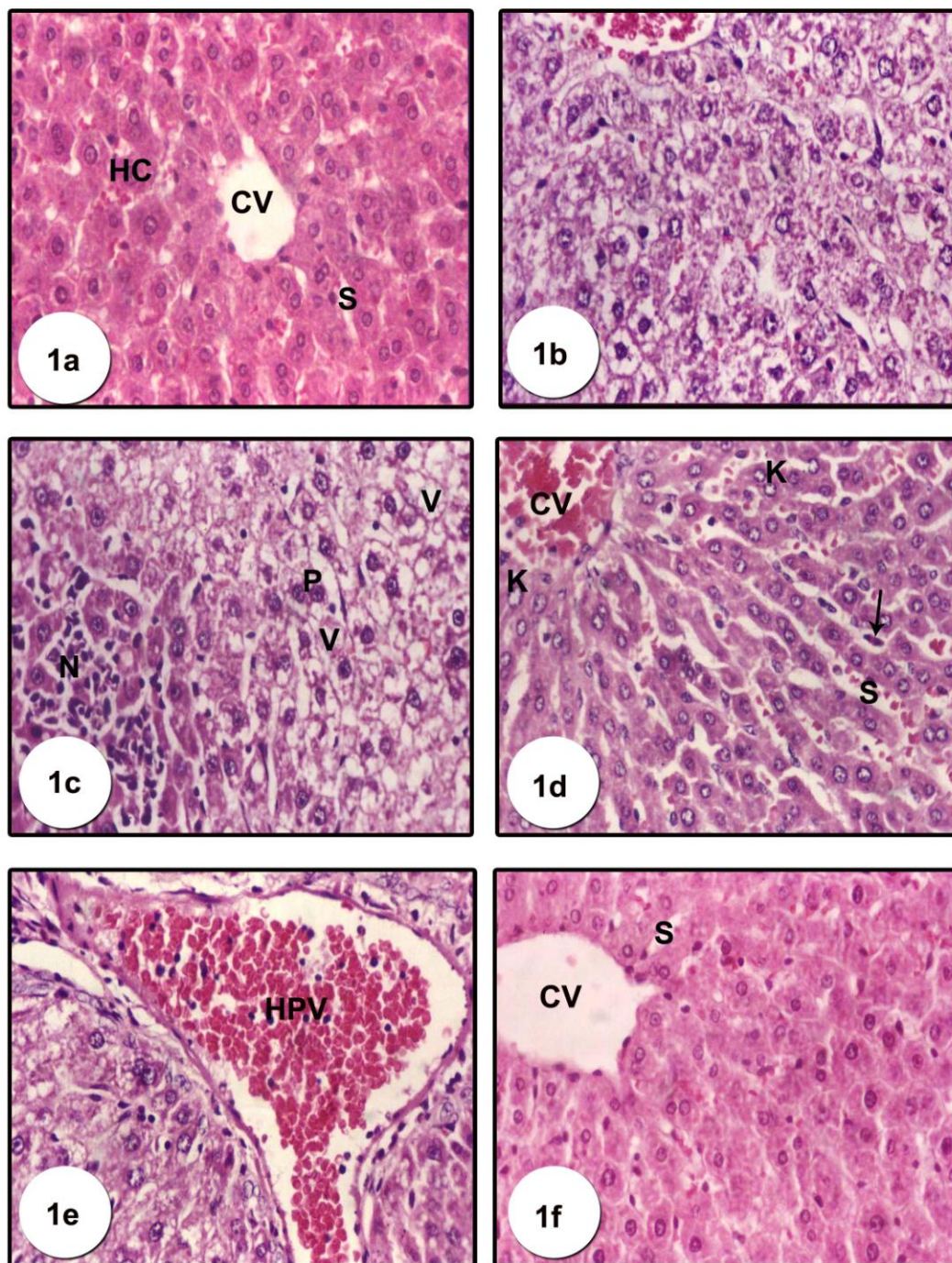
**Table (2): Serum ALT, AST, ALP activities, total bilirubin, direct bilirubin, indirect bilirubin and albumin concentrations in different groups.**

Parameters Groups	ALT(U/L)	AST(U/L)	ALP(U/L)	Total bilirubin(m g%)	Direct bilirubin(m g%)	Indirect bilirubin (mg%)	Albumin (g %)
<b>control group</b>	24.69±1.67 <sup>c</sup>	9.98 ±0.85 <sup>c</sup>	71.89±5.37 <sup>b</sup>	0.722±0.02 <sup>b</sup>	0.181±0.03 <sup>b</sup>	0.541±0.03	3.53 ±0.06 <sup>a</sup>
<b>HgCl<sub>2</sub>-treated group</b>	34.98 ±1.30 <sup>a</sup>	38.58±1.15 <sup>a</sup>	112.36±6.28 <sup>a</sup>	0.883±0.03 <sup>a</sup>	0.313±0.03 <sup>a</sup>	0.570±0.05	3.15 ±0.15 <sup>b</sup>
<b>HgCl<sub>2</sub> and NLE-treated group</b>	28.65±0.47 <sup>b</sup>	35.14±0.87 <sup>b</sup>	78.01±5.82 <sup>b</sup>	0.786±0.03 <sup>b</sup>	0.281±0.02 <sup>a</sup>	0.505±0.02	3.32 ±0.06 <sup>ab</sup>
<b>NLE-treated group</b>	25.13±1.59 <sup>bc</sup>	8.19±0.70 <sup>c</sup>	78.16±7.35 <sup>b</sup>	0.745±0.01 <sup>b</sup>	0.187±0.02 <sup>b</sup>	0.558±0.06	3.49 ±0.07 <sup>a</sup>
F-probability	0.0001	0.0001	0.0002	0.0008	0.0007	0.611	0.018
LSD at 5%	3.88	2.61	18.03	0.078	0.072	-	0.25
LSD at 1%	5.22	3.52	24.29	0.105	0.097	-	0.33

- Data are expressed as Means ± SE. (n=10)

- a, b, c, d superscript indicate significant difference at P < 0.05.





**Figure 1. Light micrographs of liver sections (a) control group, (b, c, d ,e); HgCl<sub>2</sub>-treated group, (f); HgCl<sub>2</sub> and NLE- treated group (H& E; X 400).**

Abbreviations: Hepatocytes (HC), Central Vein (CV), Sinusoids (S), Vacuolation (V) , Pyknosis (P), Necrosis (N), Karyolysis (K), Kupffer cell (arrow), Hepatic Portal Vein (HPV) .



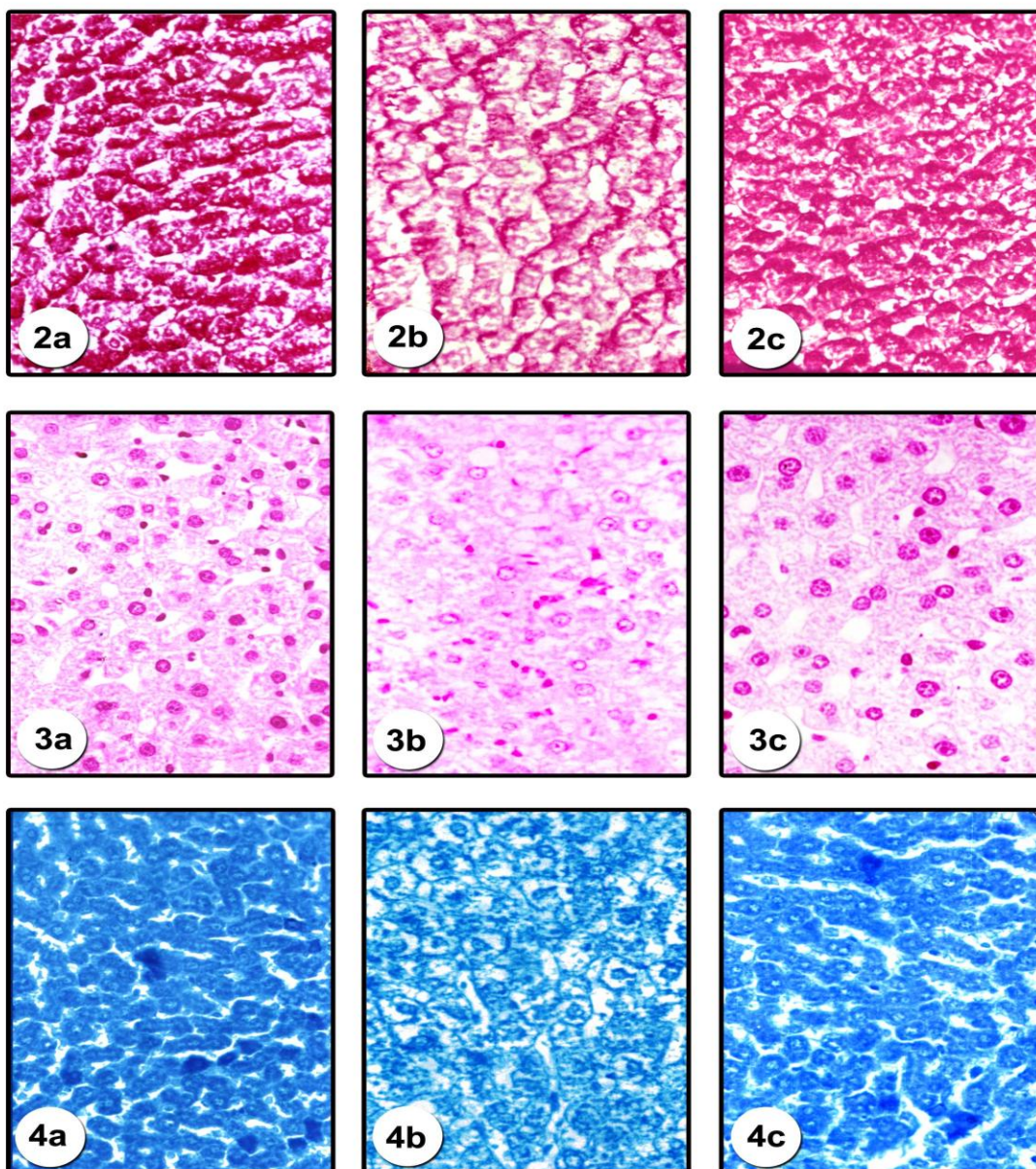
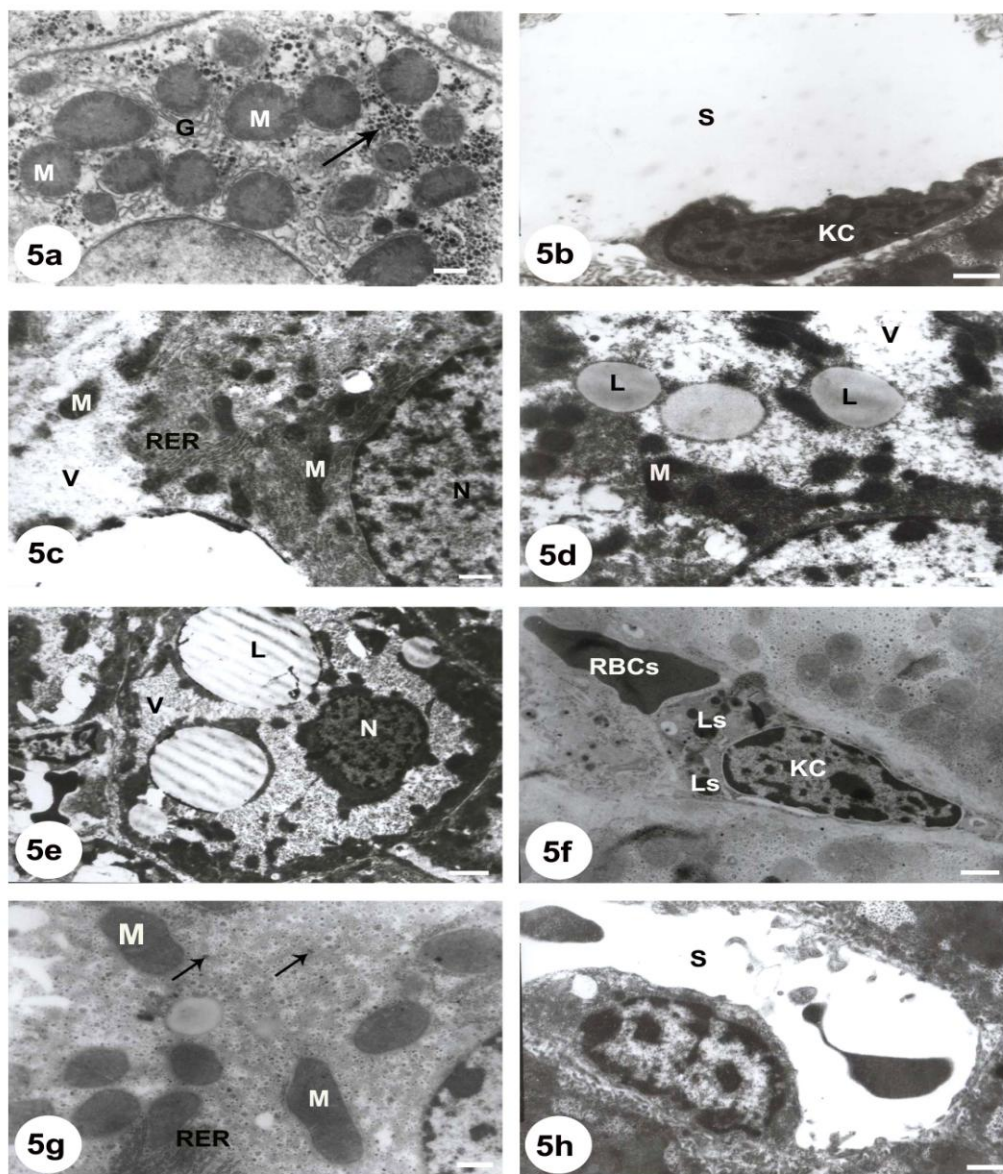


Figure 2. Light micrographs of liver sections (a) control group (b) HgCl<sub>2</sub>-treated group (c) HgCl<sub>2</sub> and NLE-treated group (PAS; X 400).

Figure 3. Light micrographs of liver sections (a) control group (b) HgCl<sub>2</sub>-treated group (c) HgCl<sub>2</sub> and NLE-treated group (Feulgen; X 400).

Figure 4. Light micrographs of liver sections (a) control group (b) HgCl<sub>2</sub>-treated group (c) HgCl<sub>2</sub> and NLE-treated group (Bromophenol blue; X 400).



**Figure 5. Electron micrographs of liver sections (a, b); control group, (c, d, e, f); HgCl<sub>2</sub>-treated group, (g, h); HgCl<sub>2</sub> and NLE- treated group.**

Abbreviations: Golgi complex (G), Mitochondria (M), Glycogen (arrows), Sinusoids (S), Kupffer cells (KC), Rough Endoplasmic Reticulum (RER), Vacuolation (V), Lipid droplets (L), Nucleus (N), Red Blood Cells (RBC), Lysosomes LS.

Scale bars: a= 500 nm

d= 500 nm

g= 500 nm

b= 1 μm

e= 2 μm

h=1 μm

c= 500 nm

f= 1 μm



#### 4. Discussion

Toxicity with mercury is associated with oxidative stress in which mercury induces the formation of free radicals including ROS and RNS, and alters the antioxidant capacity of the cells (Lund et al., 1993; Hussain et al., 1997). This likely occurs in the mitochondria, where mercury can uncouple oxidative phosphorylation and electron transport (Mori et al., 2007). Living organisms possess intrinsic antioxidant defense mechanisms against free radicals, which are sufficient to prevent oxygen radical cytotoxic effects (Betteridge, 2000). These mechanisms include (i) catalytic removal of free radicals and reactive species by cytoplasmic and mitochondrial enzymatic scavengers such as catalase (CAT), superoxide dismutase (SOD), glutathione reductase (GR) and GPx; (ii) reduction of free radicals by electron donors, such as GSH, vitamin E, vitamin C, selenium, zinc, bilirubin, and uric acid (Halliwell and Gutteridge, 1999). The imbalance between free radicals and antioxidant defense system may increase the oxidative burden and leads to the damage of macromolecules, which play a role in pathological process (Sies and Stahl, 1995). We chose MDA as a lipid peroxidation indicator and some major antioxidants to carry out our study. GSH, GPx, non-enzymatic antioxidants like vitamin C and vitamin E were detected because these four major antioxidants play important roles in the defense systems (Mourente et al., 2002; Zhang et al., 2004).

The ability of mercury to produce oxidative stress was indicated in our study by the increased amount of hepatic MDA and NO, and the depletion of GSH stores which can account for the diminished vitamin C and vitamin E potential and enhanced lipid peroxidation. Moreover, Mercury induced liver injury was detected by a significant increase in serum ALT, AST and ALP activities and by the enhanced levels of total bilirubin, direct bilirubin and a significant decrease in serum albumin concentration.

Our results concerning the oxidative imbalance in mercury intoxication, agreed with many studies (Sharma et al., 2005; Sener et al., 2007; Grotto et al., 2010), Where elevated values of MDA with decreased GSH contents in liver and other tissues (Jagadeesan and Sankarsami Pillai, 2007; de Freitas et al., 2009), were reported in mercury-induced oxidative damage. In addition, Aydin, et al. (2004) and Chia et al. (2008) stated that the occupational exposure to metals is associated with increased lipid peroxidation, increased DNA oxidation, and decreased levels of vitamin C and vitamin E. With respect to NO content, Ji et al. (2006) reported that NO content in the liver tissue significantly increased during 20-day period of

dietary exposure of environmental mercury, and kept increasing with longer exposure. The obtained results in the current investigation concerning ALT, AST and ALP enzyme activities were in accordance with data reported by Jagadeesan and Sankarsami Pillai (2007); Sener et al. (2007); El-Shenawy and Hassan (2008). In addition, Reus et al. (2003) reported higher serum levels of total bilirubin in rats exposed to mercury toxicity.

Mercury is one of the most potent thiol-binding agents. Both inorganic mercury and methyl-mercury have a great affinity for thiol (SH-) groups, especially those on endogenous biomolecules, such as GSH, cysteine, homocysteine, metallothionein, and albumin (Zalups, 2000; Patrick, 2002). The metal-GSH conjugation process is desirable in that it results in the excretion of the toxic metal into the bile (Rana, 2008; Watanabe et al., 2009). However, it depletes the GSH, the main antioxidant in mammalian cells, from the cell such as human erythrocytes, mammalian glial, renal and hepatic cells (Queiroz et al., 1998; Zalups, 2000) and thus decreases the antioxidant potential. The decreased glutathione levels, leads to an increase of free radicals, like superoxide radical, hydrogen peroxide, hydroxyl radical, nitric oxide and peroxynitrite (Stoys and Bagchi, 1995; Perottoni et al., 2004). Following radical-radical interaction with superoxide, NO produces another potent and powerful long-lived oxidant, peroxynitrite anion (ONOO<sup>-</sup>), which can interact with nucleic acids, proteins and lipids aiding substantially the cellular redox state (Radi et al., 2001), since not only ROS but also RNS are involved in oxidative stress (Hamano et al., 2002). Moreover, Carmeli et al. (2009) suggested that the high serum level of NO might induce toxicity and lipid peroxidation. Accordingly, mercury exposure has been demonstrated to induce membrane lipid peroxidation detected by increased MDA content in many tissues (Emanuelli et al., 1996; Kim and Sharma, 2003) as well as increased hepatic nitric oxide content (Ji et al., 2006; Cheng et al., 2010). Since the liver is the major site of GSH synthesis, so a significant depletion of hepatic GSH was reported (Sharma et al., 2002). Organisms have developed many defense mechanisms to protect themselves from injuries by ROS. The small molecule antioxidants, such as vitamin E ( $\alpha$ -tocopherol) and vitamin C (ascorbic acid) are able to interact with oxidizing radicals directly (Jones et al., 1995). Vitamin E terminates the chain reaction of lipid peroxidation in biomembranes and lipoproteins (Dieber-Rotheneder et al., 1991), whereas vitamin C scavenges aqueous-phase ROS by very rapid electron transfer and thus inhibits lipid peroxidation (Halliwell et al., 1987), as well as reduces the oxidized

tocopheroxyl radicals. Therefore, vitamin C and vitamin E function together to protect membrane lipids from damage (Frei, 1991). The decreased level of hepatic GSH in HgCl<sub>2</sub>-treated rats of the present study may be due to enhanced utilization during detoxification of mercury. It is well established that the non-enzymatic antioxidants such as vitamin C and vitamin E concomitantly decreased along with GSH in heavy metal toxicity (Chia et al., 2008). GPx is well known to defend against oxidative stress in the cellular environment. So, the enhanced activity reported in the present investigation occurred probably as an adaptive cellular response against hydroperoxides generated by HgCl<sub>2</sub>, as described by Santos et al. (1997) and Augusti et al. (2008).

ALT and AST are of value indicating the existence of hepatic dysfunction and damage as these enzymes are present in large quantities in the liver. ALT and AST are intracellular transaminases, ALP is membrane bound biomarker enzyme, their increase in serum of rats as detected in the present study might be due to leakage of these enzymes into the blood stream as a result of mercury-induced tissue oxidative damage with hepatocellular necrosis and increased permeability of plasma membrane (Rana et al., 1996; Sharma et al., 2002). Considering that albumin is the most abundant protein in plasma, we assayed the concentration of albumin in serum of HgCl<sub>2</sub>-treated rats. The results reported a significant reduction of albumin synthesis by injured hepatic tissue. The decreased albumin level detected in the present study could be a consequence of the effect of mercury on albumin molecule, since albumin possess a free sulfhydryl group on a terminal cysteinyl residue to which mercuric ions can bind (Reus et al., 2003). The elevated levels of total bilirubin observed in this study is due in fact to the elevation of direct bilirubin (conjugated bilirubin diglucuronide) formed from unconjugated bilirubin (indirect bilirubin) in the presence of UDPGA (uridine diphosphate glucuronic acid) catalyzed by microsomal bilirubin-UDP-glucuronyl transferase in Kupffer cells (Okuda et al., 1983). Active excretion occurs in the canaliculi at the canalicular membrane, by means of cytoplasmic binding transport proteins (Meier, 1995; Jedlitschky et al., 1997). So, the injured hepatocyte observed in the present study might be less able to form the transporter proteins required for transporting of conjugated bilirubin to bile and consequently it was returned back to blood elevating its level in serum. On the other hand, the pioneering studies of Stocker et al. (1987) introduced the concept that bilirubin, which was until then regarded as a toxic waste product of heme catabolism, has a beneficial role at low, "physiological" plasma concentrations by acting as a potent intrinsic antioxidant that scavenges

peroxyl, hydroxyl and hydrogen peroxide radicals which are produced under several inflammatory conditions (Minetti et al., 1998; Kaur et al., 2003). Bilirubin synthesis is regulated by the rate-limiting enzyme, heme oxygenase-1 which is rapidly induced by oxidative stress and inflammatory reactions caused by factors such as cytokines, ischemia, NO (Naughton et al., 2002; Yamamoto et al., 2007) and peroxynitrite (ONOO<sup>-</sup>) (Foresti et al., 1999). Accordingly, the observed hyperbilirubinemia in the present study may have a protective role against ROS-induced hepatocellular damage. This gains support from Dennery et al. (1995) who found a reduced oxidative injury to serum proteins and lipids in the first days of life in hyperbilirubinemic neonatal Gunn rats exposed to hyperoxia. This result was then confirmed by Granato et al. (2003) who postulated that the unconjugated and conjugated bilirubin, both inhibited bile acid-induced apoptosis in rat hepatocytes and suppressed the generation of ROS by these cells.

Previous studies revealed that HgCl<sub>2</sub> caused histopathological and ultrastructural lesions evidenced by preportal fatty degeneration and cell necrosis in the liver (Stacchiotti et al., 2003; 2004). Recent studies have demonstrated cause-to-effect links between exposure to pollutants and the development of hepatic lesions. The presence of necrosis is in fact one of the most visible damages in tissues affected by a pollutant (Rabbito et al., 2005). In addition, Manahan (1991) and Orr and Blakley (1997) considered that such necrotic lesions may be due either to a progressive degenerative action of intracellular enzymes of the injured cells or to the metabolic disturbance and inhibition of protein and carbohydrate synthesis in the hepatic cells. The histochemical results of this study revealed a decrease in glycogen, DNA and protein content in hepatocytes of HgCl<sub>2</sub>-treated rats. The decrease in glycogen content may probably be correlated with the liver injury by xenobiotics (HgCl<sub>2</sub>) which in turn affects the capacity of the hepatocytes to metabolize glycogen normally. This gains support from Patel and Rao (1999) and Gajawat et al. (2005) who also observed a fall in glycogen after heavy metal intoxication. The fall in glycogen content may also be due to requirement of greater amount of nervous and muscular energy, which is provided by liver glycogen reserve (Ramalingam et al., 1999). Mahour and Saxena (2007) also reported reduction in the amount of glycogen and protein in hepatic lobules after acute and sub-acute HgCl<sub>2</sub> intoxication.

In fact, DNA was a vital molecule in the cell activities and was the main target for HgCl<sub>2</sub>-induced cell injury (Schurz et al., 2000). HgCl<sub>2</sub> is a potential genotoxicant even at low doses and has a high

affinity for macromolecules and binds to DNA *in vitro* leading to alterations in DNA structure (Ariza and Williams, 1996). It was found that the changes observed in the nucleus and heterochromatin distribution of hepatocytes could be the result of the metal accumulation in that organelle, as previously proposed by Heath (1995). The author also, suggested that the morphological effects observed in nuclei are evidence that this organelle can accumulate metals more intensely than other cellular compartments. These results agreed with those of Homma et al. (1999), who observed that HgCl<sub>2</sub> caused DNA fragmentation in the liver and El-Shenawy and Hassan (2008), who reported a decrease in DNA density in the liver cells. Furthermore, Carey (1994) concluded that the abnormal DNA was a primary consequence of tumor growth and development. Junquera et al. (1995) explained the nuclear degeneration of liver cells as a result of inhibition of the dihydrofolate reductase enzyme that interferes with the synthesis of DNA.

Our biochemical and histochemical findings confirmed the decreased protein synthesis by mercury-intoxicated hepatocytes that were reflected by low serum albumin level and low protein content in hepatocytes concomitant with the ultrastructural changes noticed in rough endoplasmic reticulum. This may be attributed to the formation of mercaptides formed by interaction between mercury and intracellular thiol group of terminal cysteinyl residue of albumin molecule, and the formation of less stable complexes with other amino acid side chains (Ramalingam et al., 1999; Mahour and Saxena, 2007) which in turn can lead to enzymatic inactivation and inhibition of protein synthesis (Bohets et al., 1995).

The ultrastructural alterations noticed in the present study were necrosis of most hepatocytes, disintegration of most cellular contents and the rough endoplasmic reticulum were broken and devoid of ribosomes. Nuclear alterations, increased lipid droplets, lysosomes and glycogen depletion were also observed. These above mentioned results suggested mercury-induced hepatotoxicity and oxidative stress in animals. Fatty changes observed in the present study could be attributed to impaired protein synthesis as a result of rough endoplasmic reticulum damage and therefore inhibition of lipoprotein manufacture and its inhibition results in the accumulation of fats in the cytoplasm. The rough endoplasmic reticulum is particularly liable to the free radical attack, not only because it is considered as a site of radical production but also due to the enrichment of its membrane with polyunsaturated fatty acids (Slater, 1984). Leedle and Aust (1990) demonstrated also the relationship between

peroxidation of membrane phospholipids and GSH concentration. Moreover, the role of GSH in the mitochondria is believed to be critical in the maintenance of vital mitochondria and cellular functions through the metabolism of reactive oxygen derivatives generated within the electron transport chain and through the regulation of mitochondrial inner membrane permeability by maintaining Ca<sup>2+</sup> homeostasis (Martensson and Meister, 1989; Martensson et al., 1989). The observed progressive dilatation of the vessels in the liver could be considered as a reactive change that may be related to the inhibitory effect on the vascular smooth muscles which induce relaxation and consequent vasodilatation (Jeremy et al., 1990). This vasodilatation and increased vascular permeability should lead to loss of fluid from the blood so the vessels are engorged with blood cells with consequent slowing down of the blood stream which result in degeneration and necrosis in the liver cells (More and Brown, 1994; El-Shrief et al., 2002). Burk et al. (1995) attributed necrosis to the depletion of glutathione in the liver tissue. Nitric oxide is an important reactive nitrogen free radical serving as a messenger molecule involved in many physiological and pathological processes within the mammalian body (Hou et al., 1999). Appropriate levels of NO production are important in protecting an organ such as the liver from ischemic damage. However sustained levels of NO production result in direct tissue toxicity and contribute to the vascular collapse associated with septic shock (Tylor et al., 1997). NO, known as the endothelium-derived relaxing factor, is biosynthesized endogenously from arginine and oxygen by various nitric oxide synthase (NOS) enzymes and by reduction of inorganic nitrate. NO once synthesized, it results in phosphorylation of several proteins which triggers the signal to the endothelium smooth muscle to relax (Tanaka et al., 2006), thus resulting in vasodilatation and increasing blood flow with increased vascular permeability (Yoon et al., 2000). Therefore, NO may be expected to aid in the observed vasodilatation. This insight might confirm the results obtained by Golpon et al. (2003) who reported that HgCl<sub>2</sub> induces an endothelial-dependent vasorelaxation which was totally blocked by the NO inhibitor and also, alters structure and function of vascular endothelial cells.

Therefore, the excess availability of free radicals and the decreased levels of antioxidants, indicate the aggravation of hepatic oxidative status after mercury exposure. So, the observed dramatic ultrastructural changes may be attributed to the increased oxidative stress, determined biochemically in the present study. Thus, it is reasonable that administration of some antioxidant should be one of



the important therapeutic approaches in mercury intoxication. Indeed, HgCl<sub>2</sub>-induced injury can be ameliorated by *Azadirachta indica* (Meliaceae), a biological antioxidant that occur naturally. *Azadirachta indica* can effectively scavenge lipid peroxide radicals, thereby protecting lipids and biological membranes from oxidative damage (Yanpallewar et al., 2003), also, neem leaves extract can *in vitro* scavenge the ROS as measured by various antioxidant assays (Sithisarn et al., 2007; Sultana et al., 2007). The radical scavenging activity is normally attributed to the phenolic, flavonoid compounds and carotenes which were previously isolated and identified in neem fractions (Van Der Nat et al., 1991, Subapriya and Nagini, 2005; Manikandan et al., 2008). Chemical analysis revealed that the neem leaves extract contains quercetin and rutin compounds which are the most frequently studied bioflavonoid in the class of flavonols (Chattopadhyay, 1998). Manikandan et al. (2009) suggested that the antioxidant and protective effects of active neem leaves fractions could be attributed to their ability to inhibit various free radicals. Flavonoids are natural phenolic substances present in vegetables and in the red wine (Goldberg et al., 1995) that can act as antioxidants in biological systems. It is well established that quercetin, one of the most abundant flavonoids, is a more potent antioxidant than other antioxidant nutrients such as vitamin C, vitamin E and β-carotene (Rice-Evans et al., 1995) and can chelate transition metal ions, including iron (Ferrali et al., 2000). In that respect, the treatment with flavonoids quercetin has been shown to prevent liver damage and suppress the hepatitis-induced increase in the intensity of NO radical generation (Timoshin et al., 2005; Crespo et al., 2008). In addition, it was found that; peroxynitrite-induced oxidative hepatotoxicity is protected by the quercetin (Yokoyama et al., 2009). Epidemiological studies have suggested that the intake of food containing flavonoids may be associated with reduced risk of coronary heart disease, hypercholesterolemia, atherosclerosis and heart failure (Stoclet et al., 2004). Other research indicated that rutin protected the intracellular GSH antioxidant system and prevented H<sub>2</sub>O<sub>2</sub>-induced apoptosis through regulating ROS-mediated mitochondrial dysfunction pathway (Gong et al., 2010).

The present results showed that treatment of mercuric-intoxicated rats with NLE counteracted the effect of HgCl<sub>2</sub> where it significantly increased liver GSH, vitamin C and vitamin E content and significantly decreased liver MDA and NO contents, serum ALT, AST, ALP activities, total bilirubin, but slightly decreased hepatic GPx activity and direct bilirubin with slight increase in serum albumin levels.

It moderately increased hepatic glycogen, DNA and protein content; markedly improved the hepatic histological as well as ultrastructural configuration compared to HgCl<sub>2</sub>-treated group. Such finding reflects the ameliorative effect and efficiency of NLE to scavenge the free radicals and support the biochemical results concerning oxidants, antioxidant defenses and hepatic function. In the present study, we obtained a high content of these scavengers in the extract that may explain its potent radical scavenging activity against mercury-generated ROS. This may be expected to aid in decreasing oxidative stress, and preventing the further consumption of endogenous non-enzymatic antioxidants thus restoring the GSH, vitamin C and vitamin E contents in hepatic tissues. The non hepatotoxic nature of neem was proved in the study performed by Haque et al. (2006) who found unaltered and normal activities of serum ALT, AST, ALP as well as retained architecture of liver after neem treatment. Also, Yanpallewar et al. (2003) reported the hepatoprotective role of neem leaves extract against paracetamol-induced hepatic damage in albino rats as indicated by stable serum activity of ALP, ALT, AST and histopathological observations of liver tissues. In addition, Peer et al. (2008) reported the cardioprotective effect of neem leaves extract on isoprenaline-induced myocardial infarction in rats as evidenced by significant decrease in serum cardiac marker enzymes, lactate dehydrogenase and AST.

The observed low GSH, vitamin E and vitamin C and the high tissue MDA and NO levels in rats treated with HgCl<sub>2</sub> implicate the oxidative stress and failure of antioxidant defense system to overcome the influx of ROS that consequently led to cellular damage. *Azadirachta indica* leaves extract afforded protection from such HgCl<sub>2</sub>-induced oxidative tissue damage. Possible mechanisms of action seem to underlie the protection of HgCl<sub>2</sub> - induced tissue damage by *Azadirachta indica* leaves extract include the following (i) *Azadirachta indica* leaves extract by itself could act as a free radical scavenger intercepting those radicals involved in HgCl<sub>2</sub> metabolism. Thus, by trapping oxygen related free radicals *Azadirachta indica* leaves extract could hinder their interaction with polyunsaturated fatty acids and would abolish the enhancement of lipid peroxidative processes. Flavonoids and polyphenols components of the plant extract seem to be responsible for its antioxidant activity. (ii) *Azadirachta indica* leaves extract treatment exhibited a novel effect on the glutathione status of the liver cells. *Azadirachta indica* leaves extract significantly increases the tissue content of GSH. These results suggest that a higher content of glutathione in liver would afford the tissue a better protection against an

oxidative stress, thus contributing to the abolishment of HgCl<sub>2</sub>-induced hepatotoxicity.

Thus, from the above findings, it could be concluded that the mercury-exposed population exhibited cell injury, which may have dramatic consequences and many pathological effects on the liver with ultimately cell death. The use of *Azadirachta indica* leaves extract is a promising hepatoprotective agent and this protective activity of *Azadirachta indica* leaves extract may be due to its antioxidant and normalization of impaired membrane function activity. This was manifested by improvement in the biochemistry, histology, histochemistry, and ultrastructure of liver of the studied animals. The present study demonstrated the effect of crud NLE on mercury deposition in the liver tissues and we suggest the use of NLE supplementation which could be beneficial for workers exposed to mercury for long time.

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