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Protective Effects of Latex of *Ficus carica* L. against Lead Acetate-Induced Hepatotoxicity in Rats

Falah M.Aziz*

Salahaddin University, College of Science, Biology Department, Erbil-Iraq

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Abstract

Ficus carica L. latex is used traditionally for many therapeutic purposes. Oxidative stress may be the main reason behind most histological and cellular effects of lead. The aim of this study was to investigate the possible hepatoprotective role of *Ficus carica* L .latex against lead acetate-induced oxidative stress in rats. In the present investigation, lead acetate (500 mg Pb/L) was given orally to male rats for 28 days to induce hepatotoxicity. The *Ficus* latex was found to contain high total phenols and flavonoids. The levels of hepatic markers such as alanine transaminase (ALT), aspartate transaminase (AST) and alkaline phosphatase (ALP) were significantly ($P<0.05$) increased in blood serum following lead acetate administration. Lead-induced oxidative stress in liver tissue was indicated by a significant ($P<0.05$) increase in the level of liver Malondialdehyde (MDA) and decreased levels of liver reduced glutathione (GSH) and superoxide dismutase (SOD). Histologically and ultrastructurally, the liver showed several histological alterations such as degeneration of hepatocytes by necrosis and apoptosis, fatty changes and inflammatory cells infiltration. *Ficus* latex (1mL of 1:50 diluted latex/kg b.wt) markedly attenuated the previous lead-induced biochemical alterations in serum and liver tissues ($P<0.05$) as well as the histological and cellular changes. From this study, it can be concluded that the *Ficus l*atex showed effective hepatoprotective and antioxidative action against lead acetate-induced hepatotoxicity in rats.

Keywords: Ficus Latex, Lead Acetate, Hepatotoxicity

1. Introduction

Lead (Pb) exposure is considered to be a major public health problem, therefore it has been paid attention by researchers in probing further into its toxicity. This heavy metal has been found to induce a wide range of behavioral, biochemical and physiological effects (Jackie *et al*., 2011).The liver, kidneys, and brain are considered to be the target organs for the toxic effects of lead (Sharma and Street, 1980; Jackie *et al*., 2011). Toxicity of lead is mainly attributed to the induction of oxidative stress by elevation of reactive oxygen species (ROS) such as superoxide radicals, hydrogen peroxide and hydroxyl radicals and lipid peroxides, therefore, increased interest among phytotherapy investigators to use medicinal plants with antioxidant activity for protection against metal, especially lead, toxicity has been noted (Sharma and Street 1980; Xu *et al*.,2005; El-Nekeety *et al*., 2009; Haleagraha *et al*.,2010a; Jackie *et al*., 2011). In addition, the studies regarding protection against lead toxicity included some chelating agents (Lim 2001; Massó-González and Antonio-García 2009; Jackie *et al*., 2011) and certain antioxidants such as vitamin C, E, methionine, N-acetylcysteine, homocysteine and α-lipoic acid (Dalley *et al.,* 1989; Chaurasia and Kar, 1997; Patra et *al*., 2001; Flora *et al*., 2003; Caylak *et al*., 2008; Upadhyay *et al*., 2009) .

Ficus carica latex has been traditionally used in the treatment of gout, ulcers and warts, among other (Senapati *et al*., 2001; Hsu and Guo, 2002; Noweg *et al*., 2003; Habsah *et al*., 2005), given its proteolytic and keratolytic effects, associated with its viscosity (El-Nekeety *et al*., 2009). *Ficus* fruit latex (FFL) contains significant amounts of polyphenolic compounds (Wang *et al*., 2008; Oliveira *et al*., 2010a;Yadav *et al.,* 2011) in addition to several types of fatty and amino acids (Oliveira *et al*., 2010b). A number of chemical examinations of *Ficus carica* L. have shown the presence of psoralen, bergapten, umbelliferone (Seong-Kuk *et al*., 1995, Louis *et al*., 2000), β-sitosterol, campesterol, stigmasterol, fucosterol, fatty acids (Jeong and Lachance, 2001), 6-(2-methoxy-Z-vinyl)-7-methyl-pyranocoumarin and 9,19-cycloarlane triterpenoid as an anticancer (Weiping *et al*., 1997a, Weiping *et al*., 1997b) and antiproliferative agent: 6-O-acyl-β-Dglucosyl-β-sitosterol (Shai *et al*., 2001), calotropenyl acetate, and lupeol acetate (Saeed and Sabir, 2002). Krishna Mohan *et al*., (2007) reported that the leaf extracts of *Ficus carica* possessed a significant hepatoprotective activity against carbon tetrachloride-induced hepatotoxicity in rats. They related this hepatoprotective activity to the presence of coumarins in the methanolic extract of their leaves. Gond *et al*. (2008) reported significant hepatoprotective activity

* Corresponding author. e-mail: inaamfalah@yahoo.co.uk.

with the petroleum ether (60-80˚) leaf extract of *Ficus carica* Linn. in response to rifampicin induced hepatic damage in rats.

The *Ficus* latex was found to reveal chemotherapeutic effects (El-Shobaki *et al*., 2010; Aref *et al*., 2010; Khodarahmi *et al*., 2011; Chawla *et al*., 2012) due to its antioxidant activity and several other pharmacological actions.

As far as our literature survey could ascertain, no attempt has been made to study the protective role of *Ficus* latex against lead toxicity. The aim of the present work was to investigate the hepatoprotective action of *Ficus* latex against lead acetate-induced hepatotoxicity in rats and finding the exact mechanism for this protection.

2. Materials and Methods

2.1. Latex Collection

Latex was collected directly from the neck of *Ficus carica* L. fruit before ripening in June of 2011 from Erbil city, north of Iraq. The *Ficus* latex was collected in sterile screw bottles and kept in cool boxes until transported to the laboratory. Before administration, the latex was diluted 1:50 with distilled water without any further treatment. The identity of *Ficus carica* L. was confirmed by a plant taxonomist.

2.2. Experimental Animals

The present study was conducted using 32 mature male Wistar albino rats (*Rattus norvegicus*). All rats were healthy, weighing 200 - 270 gm. and 8-10 weeks old at the time when the experiment started. The animals were bred and housed in plastic cages (56 x 39 x 19 cm) bedded with wooden chips in groups of seven rats per cage in a room with controlled temperature of 24 ± 3 ˚C, in animal house of Biology Department -College of Science-Salahaddin University-Erbil-Iraq. The animals were kept on 12/12 hours light/dark schedule during the experimental study. They were fed with standard laboratory chow containing 0.5% NaCl, 22% protein and 4-6% dietary fat and allowed to drink water *ad libitum*.

2.3. Total Phenol Content

Total phenolic compounds were determined using the Folin–Ciocalteu method (Lamien-Meda *et al*., 2008). One mL of the *Ficus* latex (without dilution) was added to 10.0 mL distilled water and 2.0 mL of Folin–Ciocalteu phenol reagent (Merck-Schuchardt, Hohenbrun, Germany). The mixture was allowed to stand at room temperature for 5 min and then 2.0 mL sodium carbonate was added to the mixture. The resulting blue complex was then measured at 680 nm. A standard calibration curve was plotted using gallic acid concentrations at 0.02-0.1mg/mL and the results were expressed as mg of gallic acid equivalents (GAE)/mL of latex (W:V). The data obtained were the means of three determinations.

2.4. Total Flavonoid Content

For the determination of total flavonoid, modification of Lamien-Meda *et al*., (2008) was used. $AlCl_3$ (2%, w/v) was mixed with 0.5 ml of undiluted latex. After 10 min, the absorbencies were measured at 415 nm against a blank on a UV/visible light spectrophotometer (CECIL CE 2041, CECIL Instruments, England) and compared to quercetin. The calibration curve was plotted by dissolving 100 mg pure quercetin in 100 ml double distilled water then further dilution was made in five different concentration solutions such as 10µl, 20 µl, 30 µl, 40 µl, 50 µl respectively. The data obtained were the means of three determinations. The amounts of flavonoids in plant extracts were expressed as mg of quercetin equivalents (QE)/mL of latex.

2.5. Experimental design

The thirty two male rats were divided randomly into the following groups with eight animals in each group. Group 1: control rats received only 1mL of distilled water by gavage; Groups 2: received daily dose of 1mL of diluted latex/kg b.wt by gavage. Group 3: rats exposed to lead acetate (500mg/L) in drinking water Groups. 4: received lead acetate in drinking water and latex (1mL of diluted latex/kg b. wt) for 28 day. The selected dose of *Ficus* latex was based on a preliminary test for the histological effect of 1mL/kg b.wt. of different dilutions of latex in distilled water,1:30, 1:40, 1:50, and 1:60, in which the third dilution (i.e. 1:50) was more effective in normalizing liver histological features. The administration of latex in the second and forth groups was started one day earlier.

2.6. Biochemical Study

2.6.1. Preparing The Liver Homogenate

Liver was sliced into pieces and homogenized with a blender in ice-cold 0.1 M sodium phosphate buffer (pH-7.4) at 1-4 ˚C to give 10% homogenate (w/v). The homogenate was centrifuged at 10,000 rpm for 15-20 min at 4 ˚C twice and the resulting supernatant was separated and used for various biochemical estimations such as malodialdehyde (MDA), reduced glutathione (GSH) and superoxide dismutase (SOD) in various groups of rats.

2.6.2. Malondialdehyde (MDA) Determination

The method reported by Utley *et al. (*1967) was followed. Aliquots of homogenate (1 mL) were incubated at 37 ºC for 3 h in a metabolic shaker. Then, 1 mL of 10% aqueous trichloroacetic acid (TCA) was added and mixed. The mixture was then centrifuged at 800g for 10 min. Then, supernatant (1 mL) was mixed with 1 mL of 0.67% thiobarbituric acid and placed in a boiling water bath for 10 min. The mixture was cooled and diluted with 1 mL distilled water. The absorbance of the solution was then read at 535 nm. The content of malondialdehyde (MDA) (nmol/g wet tissue) was then calculated, by reference to a standard curve of MDA solution.

2.6.3. Determination of Superoxide Dismutase Activity

Superoxide dismutase was assayed as described by Misra and Fridovich (1972). The assay mixture contained 0.5 ml of hepatic PMS, 1 ml of 50 mM sodium carbonate, 0.4 ml of 25 µm nitroblue tetrazolium and 0.2 ml of freshly prepared 0.1 mM hydroxylaminehydrochloride. The reaction mixture was mixed quickly by inversion followed by the addition of clear supernatant of 0.1 ml of liver homogenate (10% w/v). The change in absorbance was recorded at 560 nm. Percentage inhibition was calculated using this equation:

% superoxide dismutase inhibition = [(normal activity – inhibited activity)/(normal activity)] × 100%.

2.6.4. Determination of Liver Reduced Glutathione (GSH)

Reduced glutathione (GSH) was estimated as follow: One mL of 10% of liver homogenate was taken and 1 ml of 5% TCA (w/v) was added. After 30 min, the mixture was centrifuged at 2500 rpm for 15 min. Also, 0.5 ml of supernatant was taken and 2.5 ml of 5,5'dithiobis (2-nitobanzoic acid) (DTNB) was added, mixed thoroughly and absorbance was recorded at 412 nm. The results were expressed as µmol/g tissue (Ellman, 1959).

2.6.5. Determination of Liver Homogenate Lead Level

Accurately measured samples of liver (250 mg) were digested in 10 ml concentrated nitric acid (HNO_3) by using Microwave Digestion System. After evaporation of HNO_3, dried samples were dissolved in 10 ml of distilled water. Lead content was estimated by Atomic Absorption Spectrometer (AAS, Perkin Elmer model A Analyst 100, Uberlingen, Germany) against suitable standards processed identically.

2.6.6. Determination of Liver Enzymes

For determination of liver enzymes, blood samples were collected from the heart into clean and dry tubes. Sera were obtained by centrifugation of the blood samples at 3000 rpm for 15 min at 4°C and stored at -20°C until assayed for the biochemical parameters. Aspartate aminotransferase (AST), alanine aminotransferase (ALT) and alkaline phosphatase (ALP) were measured using special kits (obtained from BIOLABO SA, Maizy, France) following the method described by Reitman and Frankel (1957) for AST and ALT and the method described by King and King (1954) for ALP. The absorbance for ALT and AST were measured at 505 nm, while for ALP was measured at 405nm.

2.7. Histological Examination

Liver samples were removed from the anesthetized animals, they were immediately fixed in Bouin's fluid for 24 hours, followed by a dehydration using a series of graded ethanol in ascending concentrations (50%, 70%, 95%, and 100%), immersed in xylene for clearing, infiltrated in paraffin wax, and finally embedded in paraffin wax. Four micrometer thick paraffin sections were obtained by using rotary microtome (Bright, MIC) and stained by hematoxylin and eosin (H&E) (Bancroft *et al*., 1977). The specimens were examined and photographed under light microscope (digital binocular compound microscope 40x-2000x, built-in 3MP USB Camera).

2.8. Electron Microscopy

Samples of liver ($1mm^3$) were fixed in 2.5% glutaraldehyde in 0.1M cacodylate buffer pH 7.2 - 7.4 for 24 hours, washed by cacodylate buffer 0.1M, postfixed in 1% Osmium tetroxide for one hour, dehydrated through a graded series of ethanol (50%, 70%, 95%, and 100%), and then cleared in propylene oxide for 15 minutes (twice), infiltrated with propylene oxide plus resin mixture and finally embedded in resin. The semi-thin sections were stained by 1% toluidine blue in 1% borax, while the ultrathin sections were mounted on copper grids and stained by uranyl acetate and lead citrate (Hayat 1974) and examined by TEM (LE1CA/ EM FC6, CM12 Philips).

2.9. Data Analysis and Statistics

All data were expressed as means ± standard error of mean (M ± SE) and statistical analyses were carried out using statistically available software of statistical package for social science (SPSS) version 11.5. One-way analysis of variance (ANOVA) was performed to test for significance followed by Duncan's multiple range comparison tests for comparisons between the groups. *P* values ≤ 0.05 and 0.01 were considered statistically significant.

3. Results

The present work revealed that the latex of *Ficus* had significant quantity of phenolic compounds and flavonoids. (Table1). The total phenolic content was 149.33 mg GAE/mL of the latex, while the total flavonoid content was 43.23 mg QE/mL of the latex.

Table 1. Total phenol and flavonoid in *Ficus* latex

Chemical content	latex
Total phenol mgGAE/ml	149.33±7.04
Total flavonoidmg QE/ml	43.23±3.35

Total phenol was expressed as mg of gallic acid equivalents (GAE)/mL of latex.

Total flavonoids were expressed as mg of quercetin equivalents (QE)/mL of latex.

Values given are means of 3 determinations ± SEM

It was found that the mean serum AST, ALT, ALP levels in control animals were 66.8, 31.1 and 445.3 IU/L respectively whereas in lead acetate treated rats, the level rose to 319.6, 192.8 and 809.3 IU/L respectively. When *Ficus* latex was administered to the lead acetate treated group, it reduced the AST, ALT and ALP levels to 55.5, 36.5 and 489 IU/L respectively (Table 2).

Table 2. Serum liver enzymes in different groups of treated rats

Biochemical analysis	Control	Latex	Lead acetate	Lead plus latex
ALT(IU/L)	31.1 ± 2.0^{b}	20.62 ± 1.11^{a}	192.8 ± 16.0^{c}	36.5 ± 3.6^{b}
AST(IU/L)	66.8 ± 4.2^{b}	51.72 ± 0.22^{a}	319.6 ± 22.7^{d}	55.5 ± 5.13^{ab}
ALP (IU/L)	445.3 ± 23.1^{b}	320.9 ± 0.68^{a}	809.3 ± 65.3^{c}	489.6 ± 43.9^{b}

As shown in Table 3, the liver mean values of MDA, GSH and SOD in control rats were 7.11 nmol of malondialdehyde/ g of liver, 2.13 µmols of GSH/g of the liver and 13.25 IU of SOD/gm of the liver respectively. In lead acetate treated animals, the MDA, GSH and SOD levels were 15.5, 1.02 and 6.27 respectively. *Ficus* latex reduced MDA to 8.84 nmol MDA/g of the liver and increased GSH and SOD levels to 1.91 and 15.62 respectively. The liver tissue of the control was found to contain 0.59 µg of lead/g of the liver. This quantity was significantly raised to higher amounts in the other groups in comparison to the control. The highest amount was recorded in the lead acetate treated group (3.79±0.17). The *Ficus* latex was found to cause a significant reduction of

the quantity of lead when administered to the lead acetate group (Table 3).

Table 3. Biochemical analysis in liver homogenates of different treated groups of rats

Biochemical analysis	Control	Latex	Lead acetate	Lead plus latex
lead μg/g liver	0.59± 0.06[a]	0.98± 0.01[b]	3.79± 0.17[d]	1.34± 0.09[c]
MDA nmol /gm liver	7.11 ± 0.17[b]	5.25± .34[a]	15.5± 0.59[d]	8.84± 0.23[c]
GSH(µmols/gm liver)	2.13 ± 0.13[b]	2.73± 0.32[d]	1.02± 0.12[a]	1.91 ±0.44[c]
SOD(U/g protein)	13.25± 1.14[c]	10.61± 0.07[b]	6.27± 0.16[a]	15.62 ±0.13[c]

Different letters in the same row refer to significant changes, while similar letters refer to non- significant changes at (p<0.01) and (p<0.05).

The most characteristic feature of lead toxicity after 28 days of lead acetate exposure as revealed by the present investigation was the fatty degeneration of hepatocytes, pyknosis of nuclei and dilatation of blood sinusoid lumen in comparison to control sections (Fig 1.A-D). In the plastic sections, slightly split nuclei were also detected in addition to infiltration of few inflammatory leucocytes. The liver sections of *Ficus* latex treated rats were showed approximately attenuated histological alterations (Fig 1E &F).

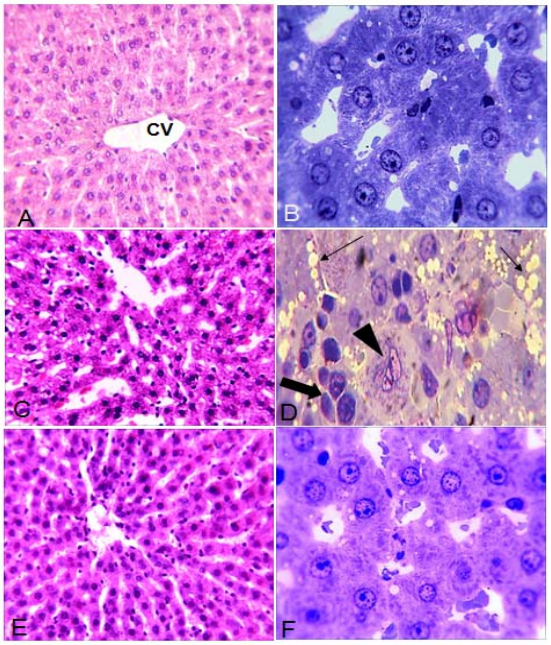

Figure 1. Sections through rats livers: A) showing normal structure,H&E,400X, B) control, plastic section, toluidine blue,1000X, C) lead acetate treated rat liver showing nuclear pyknosis, fatty change, H&E,400X, D) Plastic section revealing large lipid droplets (thin arrows) and early fragmented nuclei(arrow head), notice the appearance of few infiltrated inflammatory leucocytes(thick arrow), toluidine blue,1000X, E) Ficus latex plus lead acetate treated rat liver appears close to the control structure, H&E, 400X, F) Plastic section of Ficus latex plus lead acetate treated rat liver, notice the very low quantity of lipid droplets and the approximately normal nuclei appearance, Toluidine blue,1000X, CV means central vein.

The ultrastructural figures (Fig 2A-D) confirmed the histological observations and gave further information. The lead acetate treated rat liver showed accumulation of a high quantity of lipid droplets in comparison to a normal hepatocyte cytoplasm of the control. Furthermore, characteristic apoptotic cells containing a fragmented nucleus were detected (Fig 2C&D). Electron micrographs of the liver of Ficus latex plus lead acetate treated rats showed approximately normal ultrastructural feature of nucleus and cytoplasmic organelles especially mitochondria(2E&F).

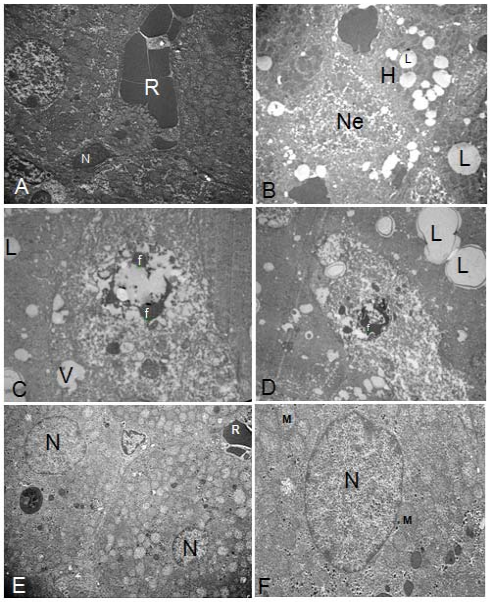

Figure 2. Ultrastructure of the liver of rats: A) Control section showing healthy hepatocyte containing normal mitochondria structure, 2000 X, B) Lead acetate treated rat liver section showing necrotic hepatocyte (Ne) adjacent to lipid accumulated hepatocytes(H), 2000X, C) An apoptotic hepatocyte containing a fragmented nucleus (f), 2000 X, D) Another apoptotic hepatocyte condensed and is going to fragment, 2000 X, E) & F) Healthy hepatocytes in the latex plus lead acetate treated group in two different magnifications, 2000 X and 5000 X respectively. L: lipid droplet, N: hepatocyte nuclei, n: blood sinusoid lining cell nuclei, M: mitochondria, R:RBC, V:vacuole**.**

4. Discussion

The present investigation revealed increase of MDA and decrease of GSH and SOD levels in the liver of lead acetate treated rats in comparison to control. This means that it increased the oxidative stress in the treated rats. It is known that lead-induced oxidative stress tissue damage could be caused by two mechanisms: increased generation of ROS, and by causing direct depletion of antioxidant reserves (Gurer *et al*.,1998; Upasan *et al*.,2001; Ercal *et al* 2001; Hamadouche *et al*.,2009). Intense lipid peroxidation caused by lead exposure may affect the mitochondrial and cytoplasmic membranes causing more severe oxidative damage in the tissues and consequently releasing lipid hydroperoxides into circulation (Shabani and Rabbani 2000; Abdel-Wahhab *et al*.,2008) which reflects the induction of oxidative stress (Dringer 2000; Newairy and Abdou 2009; Bokara *et al*., 2009).

The histological study of the present investigation showed several changes such as degeneration of liver cells especially hepatocytes, fatty changes, blood sinusoidal lumen dilatation and the appearance of infiltrated inflammatory leucocytes. Several publications have mentioned the lead-induced liver damage (Sharma and Pandey 2010; Sharma *et al*., 2011; Liu *et al*., 2011). Liver steatosis includes macrovesicular and microvesicular steatosis. Macrovesicular steatosis is characterized by a single large cytoplasmic vacuole of triglycerides within the hepatocyte that displaces the nucleus peripherally, while microvesicular steatosis is characterized by the presence of multiple small droplets of triglycerides within the hepatocyte, which do not displace the nucleus (Sturgill and Lambert 1997). It appeared that the microvesicular steatosis was more prominent in the present work. Lead was recently found to cause increase of serum triglycerides (Liu *et al*.,2011).

The current work revealed an increase in the level of ALT, AST and ALP in lead acetate treated rats in comparison to control and this may be due to the degeneration of hepatocytes by necrosis which causes leakage of these enzymes into blood circulation (Jensen *et al*., 2004). This elevation was attenuated after giving latex to the lead acetate treated rats. Gond *et al*., (2008) have reported a significant reduction in serum ALT and AST levels in rifampicin treated rats after administrating *Ficus carica* Linn. leaf extract.

Despite its chemical, biological, and ecological importance, *Ficus carica* latex is still poorly studied.(Oliveira *et al* 2010). In the present work, *Ficus* latex was found to contain high phenol and flavonoid contents. Recently, bioflavonoids and polyphenols of plant origin have been used extensively for free radical scavenging and to inhibit lipid peroxidation (Xu *et al*., 2005; Newairy and Abdou 2009). These antioxidant compounds could have played a major role in scavenging the reactive oxygen species induced by lead acetate in the serum (Haleagrahara *et al* 2010b).

Treatment with *Ficus* latex along with lead acetate treatment decreased the lead induced changes in MDA and antioxidant enzyme levels. Results on liver lead levels (LLL) showed that lead acetate alone showed a significantly higher LLL compared to all other groups. The detected decrease in LLL in latex plus lead acetate treatment group may refer to the possible chelating effect of *Ficus* latex. However, this property of *Ficus* latex requires further study. Relatively few data were available regarding the antioxidant effect of *Ficus* latex (Oliveira *et al*., 2010a; Joseph and Justin 2011; Yadav *et al*., 2011). Histologically, the *Ficus* latex when administered to lead acetate treated rats attenuated the previous histological changes.

It has been previously reported that zinc and ascorbic acid treatment showed moderate therapeutic efficacy when administered individually, whereas more pronounced protective effects were observed after combined therapy of zinc and different doses of ascorbic acid (Upadhyay *et al*., 2009). As compared with latter work, it seemed that *Ficus* latex gave better therapeutic results. Cylak *et al*.,(2008) have obtained good antioxidant results when methionine, alpha-lipoic acid, N-acetylcysteine and homocysteine were administered individually, although the present investigation used higher dose of lead (twice and half time). Haleagrahara and his co-workers (2011) detected significant antioxidant effect of alpha-lipoic acid against lead-induced oxidative stress when lead was given at same present dose but with shorter duration. It can thus be concluded that, the *Ficus* latex used in the present work for attenuating lead hepatotoxicity introduces an easiest antioxidant agent compared with other antioxidants.

Electron micrographs revealed the appearance of apoptotic hepatocytes with its characteristic feature, fragmentation of the nucleus (Ziegler and Groscurth, 2004).This type of programmed cell death was induced due to the oxidative stress (Buttke and Sandstrom 1994) caused by lead acetate as revealed by the current results.

5. Conclusion

Lead is considered as a strong hepatotoxic agent, caused its toxicity, at least in part, through inducing oxidative stress. The *Ficus* latex was highly successful in attenuating lead hepatotoxicity due to its high total phenol and flavonoid contents. Three mechanisms were suggested for this attenuation: first: lowering the oxidative stress, second: increasing the oxidant enzymes level and third: acting as chelating agent for lead ions.

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