In vitro and In vivo Studies on Mulberry Extracts: Evaluation of Chemical and Anticancer Activities and Attenuation of Lead Toxicity

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Authors’ contributions

This work was carried out in collaboration between all authors. All authors designed the study and wrote the protocol. Author ESR managed the experiment of the study, managed the literature searches and wrote the discussion part. Authors GMH and KMES wrote the remaining parts of manuscript, managed the analyses of the study and performed the statistical analysis. All authors reviewed and approved the final manuscript.

ABSTRACT

Lead (Pb) is one of the environmental pollutants. There has been a serious concern in the recent past regarding natural source for protection or curing from lead. The aim of the present study is evaluation and comparing of mulberry leaves and fruits extracts in protection from lead toxicity on the brain and testes of rats, as well as determination of their chemical activity and anticancer effect on hepatocarcinoma (HEPG2) and colon cancer cell line (HTC). Leaves extract showed higher results than fruits extract in total phenols, total flavonoids and antioxidant activities in both radical scavenging activity (DPPH) and ferric reducing power tests (FRAP). Leaves and fruits extracts exerts almost have the same killing effect on (HEPG2). Leaves extract has slight better killing power on (HTC) than fruits extract. In the experiment on rats; leaves showed more efficiency in a time dependent manner in ameliorating the harmful effect of lead on blood parameter like white and red blood cells, hemoglobin, cholesterol, triglycerides and glucose. Both leaves and fruits extracts have protective effect from injury induced by lead on tissues of rat testeses, however, they showed little protective effect on brain tissue. In conclusion, mulberry leaves and fruits have considerable active compounds and antioxidant properties which are useful as anticancer agents. Moreover they showed protective effect against lead toxicity which induced anemia, metabolic disorder and tissue injury.

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Keywords: Lead acetate; mulberry (Morus alba L) fruits and leaves; chemical activity hepatocarcinoma (HEPG2); colon cancer cells (HTC); biochemistry; histopathology.

1. INTRODUCTION

Exposure to low levels of environmental toxicants can influence the development of chronic metabolic diseases [1]. One environmental toxicant of interest is lead (Pb). Lead has widespread industrial uses in alloys, pigments, batteries and other applications. Because of its low melting point and high vapor pressure, industrial uses of inorganic lead may cause extensive local environmental pollutions. Childhood lead intoxication is still a serious problem in some countries [2].

Exposure to lead, even at low levels, has several functional implications in the development of the central nervous system, causing medical conditions such as cognitive impairment, learning deficiency, encephalopathy and degenerative diseases [3]. Lead crosses the blood-brain barrier and accumulates in the brain, causing alterations in the function of cerebral enzymes leading to behavioral changes and decline cognitive ability in both human and experimental animals. In addition, Lead causes brain damage of some special areas, such as hippocampus, the forebrain of the cerebral cortex and cerebellum [4,5].

Researchers from worldwide institutes have been investigating the mechanisms of lead toxicity, such as cytotoxicity, apoptosis, energy metabolism disorders, oxidative stress and the pathogenesis of inflammatory processes [6].

Male reproductive system has been found to be highly sensitive to lead. Studies have shown that exposure to lead even at low doses induced testicular and epididymal toxicity in rats represented by decreased steroidogenesis and spermatogenesis, the weight of the testes and epididymis, the daily sperm production, epididymal sperm count, sperm motility and sperm viability [7, 8, 9,10]. Lead also decreased serum testosterone and testicular hydroxysteroid dehydrogenases with increasing the serum follicle stimulating hormone levels [11, 12]. These effects were reported in association with increasing lipid peroxidation levels and a decrease in catalase and superoxide dismutase activity levels in the testes of lead treated rats [13, 14,15].

Mulberry belongs to the genus Morus of the family Moraceae. It is widely distributed in Asia, Europe, North America, South America, and Africa. After in vitro gastro-intestinal digestion, the digest of mulberries possesses a high antioxidant capacity. Thus, the mulberry fruits may protect against brain damage and memory impairment in vascular dementia [16].

Mulberry (Morus sp.) fruits contain high levels of anthocyanins, quercetin glycoside, and chlorogenic acid [17]. Extracts of mulberry fruits were found to possess a wide scope of biochemical activities such as scavenging free radicals, anti-hyperlipidemia and anti-atherogenic properties in a cholesterol-fed-rabbit model[18]as well as neuroprotective effects in PC12 cells in vitro [19] and in mouse-brain-injury model [20]. Also, they have anti-cancer effects of human lung cancer cell line A549[21], human gastric carcinoma cell lines (SNU-601) [22] and AGS gastric cancer xenograft model cells [23].

The aim of the present study is evaluation and comparing of mulberry leaves and fruits extract in protection from lead toxicity on blood components, some biochemical parameter and histopathology of the brain and testes of rats. As well as studying the efficiency of mulberry extracts through knowing their chemical activities and anticancer effect on hepatocarcinoma (HEPG2) and colon cancer cell lines (HTC).

2. MATERIALS AND METHODS

2.1 Chemicals

The used lead was in the form of lead acetate and all other chemicals and reagents used for chemical analysis were purchased from Sigma Chemical Co. (St Louis, MO, USA).

2.2 Plant Materials

Mulberry (Morusalba L) leaves and fruits were bought from a market of Giza, Egypt. The plant's accession number in The Orman garden herbarium is 06-04-03-05. The mulberry leaves and fruits were washed with tap water and dried in a hot air oven at 40°C. The dried material was ground to a fine powder with electric blender, and kept at -4°C until further use.
2.3 Preparation of Mulberry Leaves and Fruits Extracts

The dried leaves and fruits of mulberry (15 g) were extracted overnight with 100 ml of 60% methanol in a mechanical shaker at room temperature. The extracts were filtered with Whatman No.1 filter paper. The filtrate was evaporated at 45°C in a rotary evaporator to concentrate the solution, then lyophilized in order to obtain the dry extract and stored at -4°C until use [24].

2.4. Total Phenolic Content

Total phenolic content of Mulberry leaves and fruits were determined using the method of Singleton and Rossi [25] with some modification by Akanitapichat et al [26]. Diluted test extracts (200 µL) were mixed with 125 µL of Folin–Ciocalteu followed by the addition of 250 µL of 7% aqueous sodium carbonate. Water was then added to adjust the final volume to 2 mL. After standing in the dark at room temperature for 40 min, the absorbance of the mixture was read at 760 nm against reagent blank using spectrophotometer (SCHIMADZU spectrophotometer, UV–Vis. 1201). A standard curve was plotted using different concentrations of gallic acid. The total phenolic content was expressed as Gallic acid equivalents (GAE) in mg/100 g extract.

2.5 Total Flavonoid Content

Total flavonoid content of Mulberry leaves and fruits were measured using the aluminum chloride colorimetric method as described by Dewanto, et al. [27]. Briefly, 500 µL of each extract were mixed with 1.25 mL of distilled water followed by the addition of 75 µL of a 5% sodium nitrite solution. After 6 min, 150 µL of a 10% aluminium chloride solution was added and allowed to stand for another 5 min before adding 500 µL of 1 M sodium hydroxide. The reaction volume was brought to 3 mL with the addition of distilled water. The absorbance was measured immediately versus the blank at 510 nm. The total flavonoid content was expressed as catechin equivalents (CE) in mg/100 g extract from a calibration curve of catechin standard solution.

2.6 Evaluation of Antioxidant Activity

2.6.1 DPPH radical scavenging activity

Radical scavenging activity of Mulberry leaves and fruits were estimated using a stable DPPH radical (DPPH•) assay according to Brand-Williams, et al. [28]. Briefly, 100 µL of various concentrations (2-16 mg/mL) of the tested extract were distributed into different test tubes and then 3.9 mL of a DPPH solution (25 mg/L methanol) was added to each tube. After incubation for 30 min in the dark at room temperature, the absorbance was recorded at 517 nm. A control solution, without a tested compound, was prepared in the same manner as the assay mixture. All the analyses were done in triplicate. The degree of de-colorization indicates the radical-scavenging efficiency of the extract. The DPPH radical-scavenging activity was calculated using the following formula:

\[
\text{Radical scavenging activity (\%) } = \left[ \frac{1 - A_1/A_0}{} \right] \times 100
\]

A_0 is the absorbance of the control (DPPH solution), and A_1 is the absorbance of the sample.

2.6.2 Ferric Reducing Antioxidant Power (FRAP)

The reducing ferric power of each extract was determined according to the method of Oyaizu [29]. 1 mL of extract concentrations (leaves 100-1000 µg/mL) (fruits 2-16mg/mL), 1.0 mL of sodium phosphate buffer (0.2 M, pH 6.6) and 1.0 mL of potassium ferricyanide (10 mg/mL) were mixed and incubated at 50°C for 20 min. Then, 1.0 mL of 10% trichloroacetic acid was added to the mixture and centrifuged at 13,400×g for 5 min. 1 mL of supernatant was mixed with 1.0 mL of water and 0.1 mL of 0.1% ferric chloride, and then the absorbance was measured at 700 nm.

2.7 Anticancer Activity Using Sulforhodamine-B Assay

Cytotoxic potential of Mulberry leaves and fruits was tested against colon cancer cell line (HCT)
and liver cancer cell line (HEPG2) using the method of Nahata et al. [30]. Cells were plated in 96-well plate (10^4 cells/well) for 24 hrs before treatment with the tested compounds to allow attachment of the cells to the wall of the plate. Different concentration of each compound under test (0, 12, 25, 50 and 100 µl/ml culture media) were added to the cell monolayer triplicate wells prepared for each individual dose. Monolayer cells were incubated with each compound for 48hrs at 37°C and in atmosphere of 5% CO₂. After that cells were fixed, washed and stained with Sulforhodamine B stain. Excess stain was washed with acetic acid and attached stain was recovered with Tris EDTA buffer. Color intensity was measured in an ELISA reader. The relation between surviving fractions and compound concentration is plotted to get the survival graph of tumor cell line after the specified compound.

2.8 Animal Experimentation

Thirty-six healthy male albino rats weighing about 135±5 g were used in this experiment. During the acclimation period (1 week) and experimental period (4 weeks), the normal basal diet was supplied adlibitum. All lead–treated groups were given lead acetate (equivalent to 500 mg lead/L) in drinking water. Rats were divided into six groups as shown in Table 1.

After 2 and 4 weeks of the treatment, blood samples were collected from the retro-orbital venous plexus into two tubes, one contains heparin which used freshly for hematological parameters. The second tube didn’t contain anticoagulant to obtain serum. The separated serum stored at −20°C until analysis. Rats were scarificed at the end of the experiment; testes and brain were removed and preserved in 10% formalin for histological examination.

2.9 Biochemical Assays

The (ACE) Alera Clinical Chemistry System Automatic Analyzer (Alfa Wasserman Corporation) was employed to measure glucose, cholesterol and triglycerides. Whole blood was analyzed for white blood cells (WBCs), red blood cells count (RBCs), hemoglobin (Hb), hematocrit (HCT), mean corpuscular volume (MCV) and mean corpuscular hemoglobin concentration (MCHC) using automated Hematology Analyzer 5 (XT-2002, sysmex corporation, KOBE, JAPAN).

2.10 Histopathological Techniques

Parts of the brain and testes of each rat were fixed in 10% formalin for 2 days, washed in tap water, dehydrated in ascending grades of ethyl alcohol and finally cleared with xylene and embedded in paraffin wax. The paraffin blocks were Five-micron cute and stained by haematoxylin and eosin as described by Bancroft and Stevens [31].

2.11 Statistical Analysis

The results of the blood analysis tests were represented as mean ± standard error (SE) and statistically analyzed using the least significant difference test (LSD) when calculated probability value (p) was less than 0.05 as described by Gomez and Gomez [32] and Waller and Duncan [33]. Statistical analysis was performed by computer Duncan test institute program.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Parameters</th>
<th>Treatments</th>
</tr>
</thead>
<tbody>
<tr>
<td>G1</td>
<td>Control</td>
<td>Citrate buffer 0.5ml/100 gram body weight by stomach tube 5 days a week [34].</td>
</tr>
<tr>
<td>G2</td>
<td>Lead</td>
<td>Lead in drinking water and administrated citrate buffer 0.5ml/100 g. b.wt. by stomach tube 5 days a week.</td>
</tr>
<tr>
<td>G3</td>
<td>Fruit</td>
<td>20 mg fruit extract dissolved in 0.5 ml citrate buffer /100 g.b.wt. by stomach tube 5 days a week.</td>
</tr>
<tr>
<td>G4</td>
<td>Fruit + lead</td>
<td>Lead in drinking water and administrated 20 mg fruit extract dissolved in 0.5 ml citrate buffer /100 g. b.wt. by stomach tube 5 days a week.</td>
</tr>
<tr>
<td>G5</td>
<td>Leaves</td>
<td>20 mg leaves extract dissolved in 0.5 ml citrate buffer /100 g. b.wt. by stomach tube 5 days a week.</td>
</tr>
<tr>
<td>G6</td>
<td>leaves + lead</td>
<td>Lead in drinking water and administrated 20 mg leaves extract dissolved in 0.5 ml citrate buffer /100 b.wt. by stomach tube 5 days a week.</td>
</tr>
</tbody>
</table>
3. RESULTS AND DISCUSSION

3.1 Total Phenolic and Total Flavonoid Content

The results (Table 2) revealed that total phenolic content of leaves is double that of fruits and the total flavonoid of leaves is much more than that of fruits. In agreement with our results, Memon, et al. [35] reported the TP of leaves (8.33) is approximately double that of fruits (4.56 mmolGAEq/100 g) in Pakistan, and reported variations in TP of *Morus alba* assayed in Turkey, China, Korea and India, which may be attributed to variation in the growing conditions and the species (genetically modified) [24].

![Table 2. Total phenols (TP) and total flavonoids (TF) content in fruits and leaves extract](image)

<table>
<thead>
<tr>
<th>Extract</th>
<th>Total phenols (mg GAE/100g)</th>
<th>Total flavonoids (mgCE/100 g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fruits extract</td>
<td>7</td>
<td>3.5</td>
</tr>
<tr>
<td>Leaves extract</td>
<td>14.4</td>
<td>20.4</td>
</tr>
</tbody>
</table>

3.2 Evaluation of Antioxidant Activity: DPPH Radical Scavenging Activity (RSA) and Ferric Reducing Antioxidant Power (FRAP)

In the present results (Fig. 1), radical scavenging activity of leaves was much more than that of fruits extracts. Memon, et al. [35] reported similar variation between RSA of leaves (48.13) and that of fruits (22.85) in Pakistan.

These results coincident with the present results of total phenolic content indicating that antioxidant activity of mulberry leaves and fruits might be due to the role of flavonoids in the reduction of free radical formation, decomposition of hydrogen peroxide, quenching active singlet oxygen by trapping and quenching radicals [36,37]. Similar studies reported mulberry fruits to contain high content of phenolics and antioxidant enzymes [38,39] and display high antioxidant activities [40].

The results of reducing power of leaves and fruits extracts (Figs. 2&3) were coincident with that of RSA and TP where leaves exhibited much far activity than fruits extracts where the concentration of fruits used to exert a detectable FRAP were in milligrams while that of leaves was in micrograms.

3.3 Cytotoxic Effect of Mulberry Leaves and Fruits Extracts

As shown in figures 4&5, leaves and fruits extracts of mulberry have a dose dependent effect on hepatocarcinoma (HEPG2) and colon cancer cells (HTC). Leaves and fruits extracts approximately exhibited the same activity against hepatocarcinoma, while leaves extract is slightly more efficient in killing colon cancer cells. This may be due to resveratrol, a polyphenol found in mulberries, which have antioxidant, anticancer, and anti-inflammatory properties and play substantial role in curing a wide range of cancers including breast, colorectal, liver, pancreatic, prostate cancer and lung cancer [41].

![Fig. 1. Radical scavenging activities of fruits and leaves extract (DPPH)](image)
Several studies indicating the possible anticarcinogenesis mechanisms including antioxidant activity, detoxification activity, induction of apoptosis, antiproliferation, and antiangiogenic activity due to the presence of hydroxycinnamic acid derivatives, chlorogenic acid, anthocyanins, cyanidin and pelargonidin [17].

### 3.4 Animal Experiment

#### 3.4.1 Effect of Different Treatments on blood components

The average values of some blood components including WBCs, RBCs, Hb, HCT, MCV and MCHC in both control and treated rats are presented in Table 3. For mulberry leaves or fruits extracts-treated groups, there were no significant differences (P <0.05) of all tested parameters compared to control group.

Administration of 500 mg lead/L in drinking water significantly (P <0.05) induced time dependent increase in WBCs and decrease in RBCs, Hb, HCT, MCV and MCHC values compared to control and all other treated groups. Similarly, Quinlan, et al. [42] reported lead as inhibitor of porphobilinogen synthase and ferrochelatase preventing both porphobilinogen formation and the incorporation of iron into protoporphyrin XI, the final stage in heme synthesis. This causes ineffective heme synthesis and subsequent microcytic anemia. Additionally, lead poisoning reported to induce reduction of red blood cell counts, hemoglobin levels and hematocrit values in rats [43] and rabbits [44].

Intake of leaves or fruits extract with lead caused time-dependent diminution of the effect of lead, where G4&G6 showed no significant difference (P <0.05) of MCHC values and also G6 showed no significant difference (P <0.05) for RBCs values compared to control after 4 weeks. Similar protective effect of *Morusnigra* leaves extract in controlling anemia and WBCs in extract-treated diabetic rats was reported by Hassanalilou, et al. [45].
### Table 3. Effects of different treatments on certain blood components

<table>
<thead>
<tr>
<th>Parameters</th>
<th>WBCs (10³/mm³)</th>
<th>RBCs (10⁶/mm³)</th>
<th>Hb (g/dl)</th>
<th>HCT (%)</th>
<th>MCV (μm³)</th>
<th>MCHC (g/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>groups</td>
<td>2weeks 4weeks</td>
<td>2weeks 4weeks</td>
<td>2weeks</td>
<td>4weeks</td>
<td>2weeks</td>
<td>4weeks</td>
</tr>
<tr>
<td>G1 control</td>
<td>12.30± 0.55</td>
<td>7.61± 0.12</td>
<td>13.73±</td>
<td>47.83±</td>
<td>66.67±</td>
<td>29.27± 0.70</td>
</tr>
<tr>
<td>G2 Lead</td>
<td>22.93± 0.49</td>
<td>6.44± 0.09</td>
<td>10.93±</td>
<td>38.70±</td>
<td>58.33±</td>
<td>26.00± 0.42</td>
</tr>
<tr>
<td>G3 Fruit</td>
<td>12.30± 0.46</td>
<td>7.62± 0.12</td>
<td>13.63±</td>
<td>46.90±</td>
<td>63.33±</td>
<td>29.17± 0.24</td>
</tr>
<tr>
<td>G4 Fruit + lead</td>
<td>19.73± 0.90</td>
<td>7.08± 0.04</td>
<td>12.70±</td>
<td>44.93±</td>
<td>63.33±</td>
<td>28.03± 0.13</td>
</tr>
<tr>
<td>G5 Leaves</td>
<td>12.43± 0.27</td>
<td>7.66± 0.07</td>
<td>13.83±</td>
<td>47.13±</td>
<td>67.33±</td>
<td>29.27± 0.43</td>
</tr>
<tr>
<td>G6 Leaves + lead</td>
<td>19.07± 0.52</td>
<td>7.19± 0.10</td>
<td>12.03±</td>
<td>45.03±</td>
<td>64.00±</td>
<td>28.23± 0.43</td>
</tr>
<tr>
<td>LSD₀.₀₅</td>
<td>1.46</td>
<td>0.33</td>
<td>0.39</td>
<td>1.69</td>
<td>1.69</td>
<td>1.29</td>
</tr>
</tbody>
</table>

The various superscript letters (for each parameter between groups for the two experimental periods) statistically indicate significant differences in the Duncan test, with P <0.05.
3.4.2 Effect of different treatments on cholesterol, triglycerides and glucose biochemical parameters

The average values of cholesterol, triglycerides and glucose of treated rats in all groups are presented in Table 4. Administration of 500 mg lead/L in drinking water significantly (P < 0.05) induced time dependent elevation in cholesterol, triglyceride and glucose compared to control and all other treated groups after 4 weeks of treatments. Similar deleterious effect of lead on metabolism were reported by Yokoyama, et al. [46] Hamadouche, et al. [47] and Newairy and Abdou [48] where lead at different doses and intervals was shown to reduce the rate of glucose metabolism and elevate cholesterol and triglycerides. In present work, Intake of leaves or fruits extracts induced significant (P < 0.05) time-dependent reduction in these three parameters. In addition, intake of leaves or fruits extracts with lead caused time dependent diminution of lead effect in cholesterol, triglyceride and glucose. Furthermore, G6 (lead and leaves extract) showed no significant difference (P < 0.05) compared to control in these parameters after 4 weeks indicating good protection from lead effect.

These results were in agreement with the present results of total phenols and antioxidant activity assays where leaves extracts contains more phenols and showed high antioxidant activity than fruits extract. Also, these results were in agreement with Andallua and Varadacharyulu [49] and Singab, et al. [50] who reported mulberry leaves extract as good glycemic controller in diabetic rats and decreased the lipid peroxidation and catalase activity of erythrocytes. In addition, several studies have reported mulberry fruits to possess multiple biological activities such as hepatoprotective, antioxidative, and hypolipidemic activities due to high amounts of phenolics and anthocyanins [40, 6, 37, 51].

3.4.3 Histopathological results

3.4.3.1 Brain

Microscopically, brains of control, untreated rats revealed no histological changes. Meanwhile, brains of rats from G2 (treated with lead) showed necrosis of neurons associated with neuronophagia (Fig. 6), focal hemorrhage (Fig. 7) and pyknosis of neurons (Fig. 8). The mechanism by which lead can induce oxidative stress was explained by Ercal, et al. [52] who mentioned that, lead induced free radical damage which could be resulted from disruption of haem biosynthesis leading to accumulation of delta-aminolevulonic acid dehydratase (δ-ALAD), direct interaction with biological membranes and induction of lipid peroxidation or indirect oxidative damage via δ-ALAD generated reactive oxygen intermediate. Moreover, the disruption of the prooxidant/antioxidant balance in Lead burdened tissues could contribute to tissue injury via oxidative damage to critical biomolecules (i.e., lipids, proteins and DNA). In addition significant (P < 0.05) accumulations of malondialdehyde (MDA), a byproduct of lipid peroxidation or indirect oxidative damage.

Table 4. Effect of different treatments on cholesterol, triglycerides and glucose biochemical parameters (means ± SE)

<table>
<thead>
<tr>
<th>Parameters Groups</th>
<th>Cholesterol (mg/dL) 2weeks</th>
<th>4weeks</th>
<th>Triglycerides (mg/dL) 2weeks</th>
<th>4weeks</th>
<th>Glucose (mg/dL) 2weeks</th>
<th>4weeks</th>
</tr>
</thead>
<tbody>
<tr>
<td>G1</td>
<td>50.33 ±1.76&lt;sup&gt;gh&lt;/sup&gt;</td>
<td>55.33</td>
<td>61.67 ±2.03&lt;sup&gt;gh&lt;/sup&gt;</td>
<td>65.67</td>
<td>143.33 ±2.33&lt;sup&gt;ef&lt;/sup&gt;</td>
<td>147.00</td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G2</td>
<td>81.67 ±1.86&lt;sup&gt;c&lt;/sup&gt;</td>
<td>98.00</td>
<td>94.67 ±2.19&lt;sup&gt;c&lt;/sup&gt;</td>
<td>108.67</td>
<td>180.00 ±1.15&lt;sup&gt;c&lt;/sup&gt;</td>
<td>195.33</td>
</tr>
<tr>
<td>Lead</td>
<td>49.33 ±1.68&lt;sup&gt;b&lt;/sup&gt;</td>
<td>44.67</td>
<td>59.33 ±2.20&lt;sup&gt;df&lt;/sup&gt;</td>
<td>59.00</td>
<td>141.00 ±3.18&lt;sup&gt;df&lt;/sup&gt;</td>
<td>137.67</td>
</tr>
<tr>
<td>Fruits</td>
<td>69.33 ±1.76&lt;sup&gt;gh&lt;/sup&gt;</td>
<td>61.67</td>
<td>80.67 ±2.20&lt;sup&gt;gh&lt;/sup&gt;</td>
<td>70.67</td>
<td>167.33 ±2.60&lt;sup&gt;gh&lt;/sup&gt;</td>
<td>159.67</td>
</tr>
<tr>
<td>G6</td>
<td>46.33 ±1.76&lt;sup&gt;gh&lt;/sup&gt;</td>
<td>41.33</td>
<td>60.33 ±1.20&lt;sup&gt;ghi&lt;/sup&gt;</td>
<td>53.67</td>
<td>139.33 ±1.76&lt;sup&gt;ghi&lt;/sup&gt;</td>
<td>132.00</td>
</tr>
<tr>
<td>Leaves</td>
<td>65.33 ±1.86&lt;sup&gt;ghi&lt;/sup&gt;</td>
<td>58.67</td>
<td>75.67 ±1.20&lt;sup&gt;ghi&lt;/sup&gt;</td>
<td>68.00</td>
<td>157.67 ±1.45&lt;sup&gt;ghi&lt;/sup&gt;</td>
<td>149.33</td>
</tr>
<tr>
<td>Lead+ Leaves</td>
<td>5.830 ±2.03&lt;sup&gt;ef&lt;/sup&gt;</td>
<td>5.617</td>
<td>6.280 ±1.88&lt;sup&gt;ef&lt;/sup&gt;</td>
<td>6.180</td>
<td>1.45&lt;sup&gt;ghi&lt;/sup&gt;</td>
<td>1.45&lt;sup&gt;ghi&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

The various superscript letters (for each parameter between groups for the two experimental periods) statistically indicate significant differences in the Duncan test, with P < 0.05.
Fig. 6. Brain of rat from G2 showing necrosis of neurons associated with neuronophagia (H and E X400)

Fig. 7. Brain of rat from G2 showing focal hemorrhage (H and E X400)

Fig. 8. Brain of rat from G2 showing pyknosis of neurons (H and E X400)

Fig. 9. Brain of rat from G3 showing no histopathological alterations (H and E X400)

Fig. 10. Brain of rat from G4 showing necrosis of neurons associated with neuronophagia (H and E X400)

Fig. 11. Brain of rat from G5 showing no histopathological alterations (H and E X400)

Fig. 12. Brain of rat from G6 showing necrosis of neurons associated with neuronophagia (H and E X400)
Brains of rats from G3 and G5 which treated with mulberry fruits or leaves extract respectively, showed no histopathological changes (Fig. 9 & 11). However, brains of G4 (treated with lead and mulberry fruits extract) and G6 (treated with lead and mulberry leaves extract) revealed necrosis of neurons associated with neuronophagia (Fig. 10 & 12). Indicating that mulberry extracts didn’t fully protect brain tissue from injury induced by lead; they only prevent focal hemorrhage induced by lead. The extracts of mulberry fruits were reported to protect brain cells (PC12) when it was in direct contact in vitro [19] and ameliorates Parkinson's-disease-related pathology [54,55]. The cyanidin-3-O-β-d-glucopyranoside isolated from mulberry fruits showed neuroprotective effects against cerebral ischemia [20].

3.4.3.2 Testes

Microscopically, testes of control untreated rats (G1) revealed normal seminiferous tubules. Meanwhile, testes of rats from G2 (treated with lead) showed vacuolization and desquamation of germ cells lining seminiferous tubules (Fig. 13) as well as degeneration of spermatogonial cells lining seminiferous tubules (Fig. 14). The same destructive effects of lead were reported by Priya and Reddy [15] such as mild degenerative changes, centrally atrophied seminiferous tubules with increasing in intertubular spaces, evidence of disturbed spermatogenesis and loss of structural integrity as evidenced by rupture of the epithelium. This may be due to increased oxidative stress and reduction of testosterone concentration associated with lead treatment [11, 12]. However, no histopathological changes were noticed in the testes from the other groups (G3, G4, G5 and G6) (Fig. 15 and Fig. 16). This indicates that both leaves and fruits mulberry extracts have a protective effect from injury induced by lead. This coincides with our results of the powerful antioxidant activity of mulberry extracts. In addition, mulberry extracts were reported to have high antioxidant and anti-inflammatory effect due to its high polyphenols content [17]. Resveratrol is one of these phenols that found in mulberries and reported to maintain structurally and functionally active seminiferous tubules [56].

![Fig. 13. Testis of rat from G2 showing vacuolization and desquamation of germ cells lining seminiferous tubules (H and E X 400)](image)

![Fig. 14. Testis of rat from G2 showing degeneration of spermatogonial cells lining seminiferous tubules (H and E X 400)](image)

![Fig. 15. Testis of rat from G4 showing normal seminiferous tubules (H and E X 400)](image)

![Fig. 16. Testis of rat from G6 showing normal seminiferous tubules (H and E X 400)](image)
4. CONCLUSION

The results of this study indicate that leaves extract is potent powerful antioxidant than fruits. But generally, intake of mulberry leaves and fruits extracts act as a suppressor against metabolic disorder, anemia and testes damages induced by lead toxicity. They also have considerable anticancer activity against hepatocarcinoma (HEPG2) and colon cancer cell line (HTC).

ETHICAL APPROVAL

“All authors hereby declare that "Principles of laboratory animal care" (NIH publication No. 85-23, revised 1985) were followed, as well as specific national laws where applicable. All experiments have been examined and approved by the appropriate ethics committee”.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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