THE EFFECT OF MULBERRY (MORUS SP.) TEA SUPPLEMENT ON ACETAMINOPHEN INDUCED RENAL FAILURE IN RATS

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ABSTRACT

Mulberry (Morus sp.) is a rich source of antioxidants and phenolic compounds. Its leaves possess many bioactive compounds, such as deoxymycine and anthocyanin, and high levels of calcium, magnesium, potassium, sulfate, zinc and vitamins. Regular consumption of mulberries thought to provide potential against cancer, aging, neurological diseases, inflammation, diabetes and bacterial infections. This study has investigated the effect of mulberry tea supplement on the cellular damage of kidney (nephrotoxicity) induced by supratherapeutic acetaminophen administration. Three groups of rats (6 animals each) used, treated group (rats were fed with mulberry tea extract supplemented), positive control and negative groups (rats were fed with normal pellet diet). After 7 days animals of treated group and positive control injected with acetaminophen, 1000 mg/kg bw dissolved in 5 ml of normal saline, while rats of negative control injected with 5 ml of normal saline only. Kidneys removed, processed, sectioned, and stained for histological examination. Glomerular morphometric analysis (GMA) has performed to evaluate kidney glomerular morphology. Food intake monitored along the experiment, biological value of diet intake assessed, by its effect on body weight gain (BWG) and feed efficiency ratio (FER). Aqueous extraction of mulberry tea has prepared and total phenolic compounds in the sample quantified. Results showed mulberry tea extract supplement did help to maintain the kidney closer to normal and serve to protect it from severe damage due to nephrotoxicity, compared to animals that didn’t receive the
supplement. Mulberry extract supplement could serve as a candidate for developing safe, complaisance and promising nutraceutical product for the management of nephrotoxicity.

KEYWORDS: Mulberry extracts supplement, kidney failure, acetaminophen, nephrotoxicity

INTRODUCTION
Mulberry (Morus sp.) is a flowering plant belongs to the family Moraceae. There are estimated of 10 to 16 species of this deciduous tree, which commonly known as mulberries. Its fruits grow as multiple numbers in a single stem, 2 to 3 cm long, with color of white green or pale yellow for immature fruits, and (In most species) pink then red while ripening, and finally turn to dark purple or black for matured fruits. It is well known that mulberry is a good source of polyphenols, especially anthocyanins and micronutrient. Mulberry fruits have reported to be rich in many bioactive components, such as alkaloids, carotenoids and flavonoids, vitamins, fats (mainly linoleic acid, palmitic acid, oleic acid), sugars (glucose and fructose), minerals, antioxidant content, hypolipidemic effect, and macrophage activating effect. The antioxidant compounds (e.g. anthocyanins, water-soluble pigments) present in berry fruits have potential effects in reducing the risk of cardiovascular diseases and cancers via their antioxidant, anti-inflammatory, anti-metastasis and chemoprotective properties. The Tea from mulberry leaves indicated to provide rich antioxidant properties and to help preventing diabetes mellitus. Mulberry leaves extract has shown to produce anti-obesity effect and inhibitory effect on melanin biosynthesis. There are various phenolic compounds have been identified from mulberry leaves, such as flavanoid and other derivatives; these compounds are responsible for the most of potential health benefits of mulberry leaves and help to maintain the body against cellular injuries, cancer, aging and neurological disease, inflammation, diabetes and bacterial infection. Acetaminophen, also known as paracetemol, is widely use as analgesic and anti-pyretic, prescribed as pain reliever and fever reducer. It is commonly used to relieve headache and other minor muscle aches, pain and major ingredient in numerous cold and flu remedies, and broadly use in the management of more severe pain such as post-surgical pain and providing palliative care in advanced cancer patient. However, over dosage of paracetemol has the tendency to produce dangerous side effects that may result mainly in liver and kidney toxicity. Therefore, the present study has designed to evaluate the effect of mulberry (Morus sp.) tea extract towards the reduction of the cellular damage of kidney (nephrotoxicity) induced in rats with supratherapeutic acetaminophen.
MATERIALS AND METHODS

Animal Sampling
The experiment was carried out using male Sprague dawley rats, weighing 200-250 g, hosted under controlled conditions (temperature 22-24°C, humidity, 50%-±5%, 12 hours light/dark cycle) with free access to food and water. Total of 18 rats were chosen and divided randomly into three groups of 6 animals each. The treated (experimental) group, animals were fed with Mulberry tea Supplemented instead of normal pellets for 7 days prior to acetaminophen induction, and at the 8th day they injected intraperitoneally with a single dose of acetaminophen 1000 mg/kg bw dissolved in 5 ml of normal saline. The positive control group, animals were fed with normal diet and monitored for 7 days prior to induction, at the 8th day they injected intraperitoneally with a single dose of acetaminophen 1000 mg/kg bw dissolved in 5 ml of normal saline. The negative control group, animals were allowed to feed normally with standard pellet diet, monitored for 7 days, and at the 8th day they injected with 5 ml normal saline only.

Mulberry tea Supplemented Diet Preparation
Approximately 1.5 kg to 2 kg of basal diet pallets weighed and minced using laboratory blender till completely turned into a powder form which mixed completely with 960 ml to 1000 ml of brewed mulberry tea. The mixture of basal diet and mulberry tea was shaped back to a desired shape and allowed to dry using oven at 40°C for 2-3 days.

Aqueous Extraction of Mulberry Tea
Mulberry tea was extracted using the aqueous extract technique.\textsuperscript{[23]} Approximately 2 gram of mulberry tea leaves were brewed with 100 ml distilled water. The brewing process was allowed to steep for 5 minutes with stirring accruing continuously. The brewed tea then filtered using filter paper to remove any debris or particulates.

Measurement of Phenolic Compound
In order to quantify the amount of total phenolic compounds contained in mulberry tea extract sample, Folin-Ciocalteu method has used, 1 ml of the tea extract mixed with 75 ml of distilled water, added 5ml of Folin-Ciocalteu phenol reagent and mixed gently. After 5 minutes, 15 ml of sodium carbonate added and time was noted. The total volume of the solution made up to 100 ml with distilled water and mixed up thoroughly by inversion. After 2 hours, the sample diluted with the factor of 4 and absorbance was recorded using spectrophotometer at 760 nm.
Induction of Kidney Failure by Acetaminophen

To induce kidney failure (nephrotoxicity) by supratherapeutic acetaminophen we followed the procedure provided in Gulnaz et al.\textsuperscript{[24]} An intraperitoneal administration of acetaminophen, 1000 mg/kg bw dissolved with 5 ml normal saline, injected to the rats of the treated group and positive controls. Negative controls injected intraperitoneally with 5 ml normal saline only.

Biological Determination

Food intake (FI) was monitored along the 7 days of the experiment. The biological value of diet was assessed on its effects on body weight gain (BWG) and feed efficiency ratio (FER) \textsuperscript{[25]}. The formula to calculate the biological determination used as follow.

\[
BWG = \text{final weight gained} - \text{Initial body weight}
\]

\[
FER= \frac{BWG (g)}{\text{food consumed}}
\]

Tissue Preparation and Histological Examination

Preparation of tissue was done using laparotomy and excised, at the last day of the experiment. Both kidneys of each animal were removed and fixed in formaldehyde at room temperature for 48 hours. Kidney tissue dehydrated in graded alcohol (70%, 95%, 95%, and 100% respectively) for 1 hour each. Dehydrated tissue samples immersed in chloroform for 3 times, for 1 hour each, then, immersed in xylene twice, for 1 hour each, followed by embedding in paraffin wax for 2 times (1 hour then 30 minutes). Kidney samples blocks stored in a freezer until used. Sectioning has done using the microtome with 5\(\mu\)m thickness. Histological slides stained with H&E and observed under the microscope (Olympus DP72) and micrographs were captured using Olympus CellSens Standard software.\textsuperscript{[26]}

Statistical Analysis

The results expressed as Mean ± SEM. Normality of data were analyzed using Shapiro-Wilk normality test. The significance of differences (P-value) were confirmed using one-way ANOVA and multiple Dunnett t-tests, where P<0.05 is considered to have a significant difference, and P<0.001 is considered to be highly significant.
RESULTS

Rat Reaction towards Acetaminophen Induced Toxicity

Physical examination of rats, along the 7 days of the experiment, showed reduced in feed intake with limited movement. After injection with acetaminophen (at the 8th day) a drastic decrease in the average amount of feed intake of the injected rats, treated group and positive control group from 112.25g and 144.66g to 28.24g and 27.97g respectively, compared to negative control group (Fig. 1) after 12 hours of nephrotoxicity induction during the 8th day of the experiment.

![Feed Intake Chart](image)

Fig.1: Food Intake by the Three Groups Studies (g/day)

Mortality Observation

Mortality observation has noted after 12 hours of nephrotoxicity induction, all positive control group rats were recorded died after 12 hours (100%), while only one third of the rats (33%) of the treated group recorded dead within the same period of time. The animals of the negative control group all of them survived.

Body Weight Gain of Rats (g)

Daily body weight of each rat has been recorded and the mean of body weight gained has calculated. There weren’t significant differences observed in animals’ body weight among the three groups studied (table 1).
Table 1: Body Weight Gained for All Groups (Mean±SD)

<table>
<thead>
<tr>
<th>Group</th>
<th>No.</th>
<th>BWG (g)</th>
<th>F-value</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative Control</td>
<td>6</td>
<td>27.03 ± 7.14</td>
<td>0.779</td>
<td>0.476</td>
</tr>
<tr>
<td>Positive Control</td>
<td>6</td>
<td>28.03 ± 5.35</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Treated Group</td>
<td>6</td>
<td>23.86 ± 5.42</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Body Weight Gained (BWG) = Final Weight Gain – Initial body weight

Feed Efficiency Ratio (%)

Results did not show significant differences for feed efficiency ratio among the three groups. A comparison of the feed efficiency ratio illustrated in table 2.

Table 2: Feed Efficiency Ratio (Mean±SD)

<table>
<thead>
<tr>
<th>Group</th>
<th>No.</th>
<th>FER (%)</th>
<th>F-value</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative Control</td>
<td>6</td>
<td>0.33 ± 0.06</td>
<td>2.69</td>
<td>0.100</td>
</tr>
<tr>
<td>Positive Control</td>
<td>6</td>
<td>0.40 ± 0.06</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Treated Group</td>
<td>6</td>
<td>0.39 ± 0.04</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Feed Efficiency Ratio (FER) = Body Weight Gained (BWG) / food consumed

Relative Percentage of Kidneys Weight

Kidneys weight of animals of each group did not show significant differences among or within the groups studied. Table 3 shows the mean of the relative percentages of kidneys weight for the rats of treated group compared to the rats of the positive and negative groups.

Table 3: Relative Percentage of Kidneys of Rats (Mean±SD)

<table>
<thead>
<tr>
<th>Group</th>
<th>No.</th>
<th>Kidney %</th>
<th>F-value</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative Control</td>
<td>6</td>
<td>2.23</td>
<td>0.829</td>
<td>0.456</td>
</tr>
<tr>
<td>Positive Control</td>
<td>6</td>
<td>2.30</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Treated Group</td>
<td>6</td>
<td>2.31</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Relative percentage of kidneys weight = kidney weight x 2 / body weight x 100.

Histological Findings

Fig. 2 shows a section in the kidney of negative control rats. It showed normal structure with dense and round renal corpuscle, comprises of glomeruli (G) surrounded by Bowman’s capsule and lined with squamous epithelial cells. The cortical tubule, which mainly consists of proximal convoluted tubules (PCT) and distal convoluted tubules (DCT), lined with simple cuboidal epithelium. Well defined and clear lumen with nucleus can be seen in every section of the cells.
Fig. 2: Section in the Kidney of Negative Control Rats (H&E 40X)

$G$=glomeruli, $PCT$= proximal convoluted tubules, $DCT$= distal convoluted tubules

Whereas, kidney structure of the induced nephrotoxicity (positive control) showed small and unequal size of glomeruli (G) structure observed with severe loss of brush border of proximal convoluted tubule. Desquamation of epithelial lining on the proximal (PCT) and the distal convoluted tubule (DCT), with severe necrosis and degeneration in both tubules scattered fine granule in the cytoplasm (Fig. 3).

Fig. 3: Section in the Kidney of Positive Control Rats (H&E 40X)

$G$=glomeruli, $PCT$= proximal convoluted tubules, $DCT$= distal convoluted tubules

The section in kidney of treated group showed reduction of glomeruli changes and increases in number of normal size of glomeruli (G). Proximal (PCT) and distal convoluted tubule
(DCT) were more resemblance to normal kidney structure morphology that showed in negative control group, with no sign of vacuolation or epithelial desquamation (Fig. 4).

![Fig.4: Section in the Kidney of Mulberry Tea Supplemented Treated Rats (H&E 40X)](image)

G=glomeruli, PCT= proximal convoluted tubules, DCT= distal convoluted tubules

**Glomerular Morphometric Analysis (GMA)**

The morphology of rats kidney tissue were analyzed using glomerular morphometric analysis based on area (µm²), perimeter (µm), diameter (µm) and roundness of the glomerular. Table 4 shows larger mean values of glomeruli area of treated group (1767.98±470.36) compared to positive control group (962.78±137.48) and negative control group (1837.38±271.34). There were high significant differences (P<0.001) for the glomerular perimeter of the treated group (158.35±9.94) compared to positive control group (120.27±10.22), and negative control group (156.27 ± 9.94). Glomerular diameter of the treated group showed significant increases (58.84±5.667µm) (P<0.05) compared to positive control (43.80±2.66) and negative control (55.84±5.67). Glomerular roundness of kidney showed insignificant differences between treated group (0.75±0.24), compared to positive control (0.73±0.62) and negative control group (0.79±0.72).

**Table 4: Glomerular Morphometric Analysis (Mean±SD)**

<table>
<thead>
<tr>
<th></th>
<th>Negative Control</th>
<th>Positive Control</th>
<th>Treated Group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Area (µm²)</td>
<td>1837.38 ± 271.34 b</td>
<td>962.78 ± 137.48 ac</td>
<td>1767.98 ± 470.36 b</td>
</tr>
<tr>
<td>Perimeter (µm)</td>
<td>156.27 ± 9.37 b</td>
<td>120.27 ± 10.22 ac</td>
<td>158.35 ± 9.94 b</td>
</tr>
<tr>
<td>Diameter (µm)</td>
<td>55.34 ± 1.94 b</td>
<td>43.80 ± 2.66 ac</td>
<td>58.84 ± 5.67 b</td>
</tr>
<tr>
<td>Roundness</td>
<td>0.79 ± 0.72 a</td>
<td>0.73 ± 0.62 b</td>
<td>0.75 ± 0.24 c</td>
</tr>
</tbody>
</table>
Same letter indicates no significant different between GMA at $P<0.05$ level using Turkey test. $P<0.05$ indicates high significant differences. $P<0.001$ indicates high significant differences.

**Antioxidant Activity**

The test has performed using Folin-Ciocaltue method. Total phenolic content of mulberry tea measured spectrophotometrically in order to compare the concentration of phenolic contained in the extract (measured as Gallic acid equivalent mg/l). Thus, Gallic acid standard curve has drawn in the laboratory. Gallic acid concentration, absorbance, and standard curve with its corresponding equation are shown in Fig. 5.

![Gallic Acid Standard Curve mg/ml](image)

**Fig. 5: Gallic Acid Standard Curve mg/ml**

The absorbance of mulberry tea extract at different concentration ratio factor 4, based on the Gallic acid standard curve counted, and the total phenolic content of mulberry tea has calculated using the equation ($y = 0.009x - 0.0033$, $R^2 = 0.9973$).

**Table 5: Total Phenolic Content (TPC) in Mulberry Tea Extract**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Absorbance 760nm</th>
<th>Gallic Acid Equivalent-GAE (mg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mulberry tea</td>
<td>0.287</td>
<td>64.51</td>
</tr>
</tbody>
</table>

**DISCUSSION**

**Feed Efficiency Ratio and Body Weight Gain**

Feed efficiency ratio (FER) used to measure animal’s efficiency in converting feed mass into an increase of desired outputs. Specifically FER is the mass of food eaten divided by the output all over a specified period. In this experiment the desired output was the mass or body
weight gain by animals of each group. Increase feed intake may enhance energy balance or caloric storage, hence increase body weight observed in rats. However, it has been reported that body weight gain may occur independent of increased feed intake.\textsuperscript{[27]} Our results showed insignificant differences of FER between treated group and control groups. These results indicated that treated animals with supplement of mulberry tea extract did not gain an extra weight compared to the animals fed with normal diet.

**Rat Reaction on Acetaminophen Induce Toxicity**

Reaction of rats toward administration of acetaminophen results into high level of mortality in animals of positive control group and treated group, this may be indicated to the over dosage of acetaminophen. Toxicity of acetaminophen is closely related to its metabolism in liver and extra-hepatic tissues, due to the metabolism of cytochrome p450 enzyme system which produce a metabolite (N-acetyl-p-benzo-quinone imine ‘NAPQI’) that is toxic to the liver and kidney.\textsuperscript{[24]} It has been reported also that the drastic decreasing in the amount of feed intake (noted on rats of positive control group and treated group) due to the over dosage of acetaminophen, increases blood urea level that cause an acute kidney failure, may contribute to changes of taste and loss of appetite.\textsuperscript{[28]}

**Histology Assessment**

Glomerular morphometric analysis of the positive control group showed smaller size of glomeruli, reduction of perimeter and diameter may due to bleeding of glomerular, as evidence of damage due to the epithelial ruptured in bowman capsule, or due to rupture of the mesangial cells of the glomerulus. Fine granule scattered and vacuole formation has been observed in the cytoplasm of the proximal and distal tracts of the kidney; and these findings have agreed with a previous study.\textsuperscript{[29]} Acetaminophen treatment induced massive proximal tubular necrosis associated with luminal necrotic debris and interstitial vascular congestion and extravasation of RBC’s, as well as, severe loss of brush border of proximal convoluted tubule and necrosis of tubule have observed in the positive control group, and this supports the nephrotoxicity by acetaminophen referred by other authors who uses different doses, such as 1000 mg/kg\textsuperscript{[24]}, 2500 mg/kg\textsuperscript{[30]}, 500 mg/kg\textsuperscript{[31]}, 750 mg/kg\textsuperscript{[32]}, those authors also reported severe tubular necrosis with dilation, degeneration and desquamation of epithelial cell with a single intraperitoneal administration of high dosage of acetaminophen.
Phenolic content of Mulberry tea extraction

Phenolic compounds are constituents of fruit, vegetable, nus plant-derived beverages such as tea, and in traditional Eastern medicines. Phenolic compounds usually produced by plants as secondary metabolites and involved in diverse process, such as growth, lignification, pigmentation, resistance against pathogen and environmental stresses.\[33-34\] Mulberry fruit dry extract is known to be one of richest sources of phenolic compounds.\[25\] The level of the total phenolic contents obtained in this study has considered very rich (64.51 mg/g) compared to other studies that reported lower levels, like 17.66-34.88 mg/g (Turkey)\[35\], 14.22 mg/g (Turkey)\[3\], and 8.80 mg/g (Pakistan).\[36\] Different levels of phenolic contents in different studies might be related to the different genus of mulberry tested, different solvent used for extraction, or due to different geographic area and soil condition. Using of mulberry tea as supplement has been reported earlier to give positive effects (due to its phenolic compounds) to prevent Reactive Oxygen Species (ROS) damage, caused by scavengers, and protect cells from free radicals\[37,38\], in addition, it can inhibit the production of oxidative stresses, which induces various diseases\[39\], and also it reduces the post-prandial hyperglycemia type-2 diabetes patients.\[40\]

CONCLUSION

Our histological and morphological findings have shown that the use of mulberry tea extract supplementation provides a protective effect to kidney’s tissue against nephrotoxicity caused by acetaminophen, which supported by the morphometric analysis of glomerular assessment, that indicates a significant improvement and reduction of glomerular damages in the treated group. These findings suggested that mulberry’s high phenolic levels (antioxidant) have a potential to reduce or maintain renal failure. So that, it could suggest mulberry tea extract supplement to serve as a candidate for developing safe, complaisance and promising nutraceutical product for the management of renal failure.

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