Corchorus Olitorius L. Leaf Extract Protects Rats from Acrylamide-Induced Hepatic Injury

ASSAM IBRAHIM ABDULLAH AL-KHALIFAH¹ and FAIYAZ AHMED²*

¹Department of Radiology, College of Medicine and Medical Sciences, Qassim University, Al Qassim Region, Unaizah 56219, Saudi Arabia.
²Department of Clinical Nutrition, College of Applied Health Sciences in Ar Rass, Qassim University, Al Qassim Region 51921, Saudi Arabia.

Abstract
Acrylamide is a water-soluble compound that forms during the high-temperature cooking of starchy foods and has carcinogenic, neurotoxic, and genotoxic properties. Also, short-term exposure to acrylamide has been shown to cause significant hepatic injury in laboratory animals, along with disruption of antioxidant defense mechanisms due to excessive ROS production. Therefore, dietary antioxidants are believed to be useful in combating the negative effects of acrylamide. Corchorus olitoris L., also known as molokhia in Arabic, is a leafy vegetable which is shown to possess potent antioxidant and organ oprotective properties. In this study, rats were administered with an aqueous extract of molokhia leaves to see if it could protect them against acrylamide-induced hepatic damage. Hepatic injury markers included serum total protein, total bilirubin, ALT, AST, and ALP, while oxidative stress markers included MDA, GSH, CAT, and SOD after dosing with three levels of extract (100, 250, and 500 mg/kg) for 21 days. Results indicated that the extracts substantially reduced elevated levels of bilirubin, ALT, AST, ALP, and MDA to normal levels at all doses. The extracts also brought serum protein, GSH, CAT, and SOD levels back to normal. Although the restoration of serum hepatic enzyme levels was dose dependent, no specific dose dependent relationship was found for serum proteins, MDA, GSH, CAT, or SOD activities. The study's findings show that molokhia leaves extract protects against acrylamide-induced hepatic damage by virtue of its good radical scavenging and anti-lipidperoxidative properties conferred by phenolics, flavonoids, and alkaloids.
Abbreviations
MLE - Molokhia leaf extract  
MDA - Malonaldehyde  
GSH - Glutathione  
CAT - Catalase  
SOD - Superoxide dismutase  
CCl4 - Carbontetrachloride  
TBA - Tetramethoxypropane  
DTNB - 5,5-dithiobis 2-nitrobenzoic acid  
NBT - Nitro blue tetrazolium

Introduction
Acrylamide is a well-known environmental toxin that causes a spectrum of systemic adverse effects in humans when they are exposed to it through occupation or food.1 Because of its widespread usage in numerous industrial processes including but not limited to the manufacture of plastics, paper, and cosmetics, acrylamide possess occupational hazard. It also makes it way in to the human system through the consumption of fried starchy foods.1 The margin of exposure to dietary acrylamide are stated to be 300 μg/day/kg body weight.2 Acrylamide, known to exert carcinogenic, neurotoxic, genotoxic, and hepatotoxic effects is typically formed by high-temperature cooking (>120°C) of starchy foods when asparagine and reducing sugars or reactive carbonyls are combined. This condensation, accompanied by dehydrogenation, yields N-(D-glucos-1-yl)-L-asparagine, which is pyrolyzed to generate acrylamide.1,3 Formation of acrylamide increases with temperature, duration of cooking and low moisture content of food. In general, the darker the color of fried food, the higher is the acrylamide content. French fries, potato chips, crackers, bread, cakes and pastries are the major sources of dietary acrylamide.3

The liver is the organ most affected by acrylamide since it is metabolized via the cytochrome P450 pathway into glycidamide, a highly reactive genotoxic and mutagenic epoxide that may cause changes in cellular functions and signal pathways.4 Glycidamide is hepatotoxic because it induces oxidative stress and weakens antioxidant defenses, as demonstrated by increased lipid peroxidase and decreased glutathione peroxidase activity in experimental animals' liver tissues.5,6 Glycidamide is known to cause cancers of the lung, kidney, brain, uterus, and testicles in addition to the potential injury to liver, since high acrylamide intake leads to an increase in reactive oxygen species (ROS) and lipid peroxides, and a decrease in glutathione.7,8 Acrylamide has also been shown to lower hepatic protein levels, likely due to reduced protein synthesis or hepatocytic leakage of protein stores9 as a result of altered membrane structure and permeability10 induced by excessive ROS production.11 As a consideration, dietary antioxidants with potential radical scavenging activity could be effective in combating the adverse effects of acrylamide.

In Middle Eastern countries, Corchorus olitorius L. (Tillaceae) is known as molokhia, a common green leafy vegetable valued for its nutrient composition and medicinal properties. Molokhia leaves have been shown to contain triterpenes, sterols, glycosides, saponins, tannins, and phenolic compounds, along with mucilaginous polysaccharides and lignin,12-14 which give the leaves a viscous consistency when cooked and are commonly consumed as soup in Middle Eastern countries.15 In addition to its culinary uses, the leaves are used as a herbal treatment for fevers, enteritis, dysentery, chronic cystitis, and aches and pains.16 The leaves are also reported to exhibit a range of pharmacological effects including antioxidant, antimicrobial, antidiabetic, cardioprotective, hepatoprotective, nephroprotective, anticonvulsant, antiestrogenic and antimalarial effects.16 Different extracts of molokhia leaves have shown significant antioxidant and hepatoprotective activity in different models of hepatotoxicity including carbon tetrachloride,17,18 sodium arsenite,19 streptozotocin,20 hydrogen peroxide21 and thioacetamide22 induced hepatotoxicity in rats. Thus, the present research investigated the potential of molokhia leaf aqueous extract to protect rats from acrylamide-induced hepatic injury.

Materials and Methods
Chemicals and Reagents
Thiobarbituric acid (TBA), tetramethoxypropane, 5,5-dithiobis 2-nitrobenzoic acid (DTND) and nitro blue tetrazolium (NBT) were procured from Sigma-Aldrich. Laboratory diagnostic kits from Sigma-Aldrich were used to determine total bilirubin, total protein, alanine aminotransferase (ALT), alkaline phosphatase (ALP) and aspartate aminotransferase (AST) in serum. Finest analytical grade chemicals were used to prepare reagents for all assays.
Preparation of Aqueous Extract
Molokhia leaves were powdered using a portable cyclonic laboratory blender and sieved using a 60-mesh sieve after being washed with distilled water and dried in the shade. Molokhia leaf powder was mixed with hot distilled water at 70°C (1:8 w/v) and extracted over a 24-hour period using a mechanical shaker. The extract was then filtered, and the residue was re-extracted for 12 hours with hot water and filtered again. Both filtrates were combined and concentrated in a flash evaporator set to 60°C to obtain molokhia leaf extract (MLE), which was kept in an airtight container in the refrigerator for later use.

Experimental Design
Male Wistar rats weighing 140±10 g and aged 8-9 weeks were housed in polycrylic cages in an air-conditioned animal house maintained at a temperature of 25±2°C and relative humidity of 45–60% using a Beurer (LB-88) room humidifier. The animals received a daily pellet diet and free potable water. A 12-hour photo period was maintained throughout the research. The research ethics committee approved the study (CHR/RU/REC-001/1441-42), and standard animal experimentation guidelines were followed. The rats were categorized by randomized block design into five groups, each with six animals. All of the experimental groups were given acrylamide (25 mg/kg) by gavage for 21 days, while the control group received potable drinking water.

MLE was given to the extract-treated groups in doses of 100, 250, and 500 mg/kg by gavage as per the following groups.

- **Group 1** – Control: received 1 mL water.
- **Group 2** - Acrylamide: received acrylamide (25 mg/kg) dissolved in 1 mL water.
- **Group 3 - MLE100**: received acrylamide (25 mg/kg) + MLE (100 mg/kg) dissolved in 1 mL water.
- **Group 4 - MLE250**: received acrylamide (25 mg/kg) + MLE (250 mg/kg) dissolved in 1 mL water.
- **Group 5 - MLE500**: received acrylamide (25 mg/kg) + MLE (500 mg/kg) dissolved in 1 mL water.

After the study duration (21 days), the rats were fasted overnight to reduce metabolic differences upon being scanned with a high-frequency ultrasound under diethyl ether anesthesia. Animals were returned to the laboratory after the scan and sacrificed by cervical dislocation. Direct cardiac puncture was used to draw blood into EDTA tubes, which was then centrifuged (2500 g; 20 min) to separate plasma, which was used for the estimation of total protein, total bilirubin, alkaline phosphatase (ALP), alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities as per the protocols supplied with assay kits. The livers were immediately removed, and a small portion of the liver was fixed in 10% formaldehyde solution for histopathological studies. The remainder of the liver was homogenized in phosphate-buffered saline (1:5 w/v; pH 7.4) using a high-speed tissue homogenizer in ice cold conditions and used for malonaldehyde (MDA), glutathione (GSH), catalase (CAT), and superoxide dismutase (SOD) estimation as markers of oxidative stress.

Malonaldehyde was estimated by mixing 0.5 mL of liver homogenate with 3 mL H3PO4(1%), and 1 mL thiobarbituric acid (0.6%) and heated on boiling water bath. After 45 min, the resultant MDA was extracted in 4 mL of n-butanol and the absorbance was read at 535 nm against n-butanol (blank). The standard curve was created with tetramethoxypropane, and the results were represented as nano-moles of MDA per mg of protein.

GSH in liver homogenates was measured according to the method of Ellman. The absorbance of the yellow-colored reaction mixture developed with the chromogen 5,5-dithiobis-2-nitrobenzoic acid was read at 405 nm. A test containing all reagents without sample was used as blank and the results were expressed as µM of glutathione per milligram of protein.

Catalase activity in liver homogenates was determined using hydrogen peroxide, whose reduction in absorbance by the presence of catalase was measured at 240 nm against phosphate buffer used as a blank for a period of 1 minute. The results were presented as units per milligram of protein. Superoxide dismutase activity in liver homogenates was determined as per earlier method. The blue color produced by the reduction of nitro blue tetrazolium was read at 560 nm against distilled water used as blank. The results were presented as units per milligram of protein.

Histopathology
Portions of the formaldehyde-fixed livers were dehydrated with increasing concentrations of alcohol (50-100 percent) and washed with xylene. The portions were then embedded in paraffin to create blocks. Using an automated microtome, the paraffin blocks were cut into 4 mm thick sections
and dyed using hematoxylin and eosin dye. Under the microscope, the stained slides were examined for morphological changes, such as fatty infiltration, necrosis, and hydropic degenerative changes.

**High Frequency Ultrasound Scan**

To minimize ultrasound attenuation, the anesthetized animals were positioned in supine positions with their abdomens shaved and normal ultrasound gel applied on the probe prior to its use to achieve coupling. The single element probe (Vevo 770) was used to conduct ultrasound imaging with a center frequency of 40 MHz having 6-mm focal depth, 40 μm axial resolution, and a field view of 14.6 mm. Ultrasound observations included liver parenchyma echotexture variability.27

**Statistical Treatment**

The biochemical results were presented as mean ± Stdev (n=6). The statistical software SPSS 20.0 (IBM, USA) was used to analyze the data with one-way ANOVA. Duncan’s post-hoc test was used to differentiate values at 95% confidence level (p≤0.05).

**Table 1: Effect of MLE on serum biochemical parameters**

<table>
<thead>
<tr>
<th></th>
<th>Total protein (g/dL)</th>
<th>Total bilirubin (mg/dL)</th>
<th>AST (U/L)</th>
<th>ALT (U/L)</th>
<th>ALP (U/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>5.7±0.13</td>
<td>0.3±0.01</td>
<td>102±6.1</td>
<td>37±0.7</td>
<td>210±7.5</td>
</tr>
<tr>
<td>Acrylamide</td>
<td>4.3±0.13</td>
<td>2.3±0.15</td>
<td>266±3.2</td>
<td>351±10.1</td>
<td>573±15.3</td>
</tr>
<tr>
<td>MLE100</td>
<td>5.1±0.11</td>
<td>0.7±0.01</td>
<td>191±2.5</td>
<td>263±10.3</td>
<td>341±17.1</td>
</tr>
<tr>
<td>MLE250</td>
<td>5.5±0.09</td>
<td>0.6±0.02</td>
<td>130±2.3</td>
<td>172±7.8</td>
<td>269±10.5</td>
</tr>
<tr>
<td>MLE500</td>
<td>5.9±0.05</td>
<td>0.4±0.02</td>
<td>110±5.7</td>
<td>53±7.7</td>
<td>250±10.9</td>
</tr>
</tbody>
</table>

*Values were represented as mean ± SD (n=6). Values with different superscript letters in columns differ significantly from each other at p ≤ 0.05. MLE100: molokhia leaf extract 100mg/kg; MLE250: molokhia leaf extract 250mg/kg; MLE500: molokhia leaf extract 500mg/kg.

**Results**

**Effect of MLE on Serum Biochemical Parameters**

Table 1 shows the levels of total protein, total bilirubin, alkaline phosphatase (ALP), and serum transaminases (AST and ALT) in serum of different groups. When acrylamide-treated rats were compared to control rats, significant hypoproteinemia (p≤0.05) was observed. The use of different doses of MLE resulted in a significant (p≤0.05) increase in serum protein levels. At both 250 and 500 mg/kg dose of MLE restored total protein levels to control levels. In the case of total bilirubin, oral administration of MLE reduced elevated serum bilirubin levels substantially (p≤0.05), suggesting a protective effect against acrylamide-induced hepatic damage/injury. The extracts at all levels were able to effectively restore total bilirubin levels to near normal levels. Though no significant (p≤0.05) dose dependent correlation was established with respect to total protein and bilirubin levels, the extracts demonstrated dose dependent attenuation of increase in AST, ALT and ALP. The extracts effectively restored the activities of AST, ALT and ALP to basal levels indicating a strong dose dependent hepatoprotective activity.

**Effect of MLE on Hepatic Oxidative Stress**

Table 2 shows the levels of malonaldehyde (MDA), glutathione (GSH), catalase (CAT), and superoxide dismutase (SOD) in liver tissues. Administration of acrylamide significantly (p≤0.05) increased MDA and decreased GSH associated with a decrease in the activities of CAT and SOD indicating higher oxidative stress leading to depletion of tissue antioxidant defenses. The extracts at all dosage levels resulted in significant reduction of oxidative stress as indicated by restoration of MDA, GSH, CAT and SOD activities to near normal levels. MLE at 500 mg/kg had a slightly higher but statistically significant (p≤0.05) antioxidant effect than MLE at 100 mg/kg, but at the dosage levels tested, a consistent dose dependent restoration of antioxidant defenses was not identified.
Effect of MLE on Histopathology of Liver

Histopathological evaluation of liver sections confirmed MLE’s hepatoprotective effect. The liver sections from control animals showed normal hepatic architecture in terms of normal cytoplasm, prominent nucleus and uncongested central vein, while acrylamide-treated animals’ liver sections showed necrosis, fatty infiltration, and neutrophil infiltration associated with inflammation and cell vacuolation. The inflammatory changes induced by acrylamide administration were countered effectively with MLE administration as reflected by significantly well-preserved hepatic architecture. However, histopathological examination could not establish dose dependent effect as the changes observed in the liver sections of 3 doses of MLE showed similar observations. In all MLE treated liver sections, a mild degree of vacuolation of hepatic cells was seen. It is noteworthy that MLE extracts were found to be effective in preventing severe necrosis, neutrophil infiltration, and hydropic liver tissue degeneration caused by acrylamide administration.

Effect of MLE on High-frequency Ultrasound Imaging of Liver

High-frequency ultrasound examination of livers was undertaken since it is an excellent technique to monitor changes in liver echotexture to substantiate biochemical and histopathological findings. Liver ultrasound images revealed that the normal rat liver parenchyma had a homogeneous, sponge-like texture of low-level echogenicity with blood vessels that could be traced to portal of hepatic veins. However, in acrylamide group, the livers parenchyma showed a course echotexture and appeared as irregular or dotted line along with fatty changes. These changes induced by the administration of acrylamide were effectively reversed in MLE treated groups as revealed by a relatively homogeneous echotexture with smooth spongy texture of low-level echogenicity compared to acrylamide group. Similar to histopathological examination it was not possible to conclusively ascertain or differentiate between the degree of protection offered by 3 different MLE dosage levels viz, 100, 250, 500 mg/kg.

Discussion

Acrylamide is an important environmental pollutant with potential systemic toxicity in human beings as a result of dietary exposure through carbohydrate-rich foods cooked at high temperatures. The astounding toxicity of acrylamide is attributed to its high-water solubility which enables it to easily diffuse across body tissues to damage vital organs including liver.28 Therefore, in the present study the potential of molokhia leaf aqueous extract to protect from acrylamide-induced hepatic injury was investigated in rats. Acrylamide administration resulted in elevated total bilirubin, ALT, AST and ALP indicating liver damage as elevated ALT suggests hepatic necrosis,29 AST suggests acute liver damage or liver cytolysis,26 while elevated ALP suggests loss of plasma membrane integrity.31 In MLE treated groups, the levels of these hepatic enzymes and bilirubin were normalized suggesting a potential hepatoprotective effect. These results are in good agreement with earlier studies, wherein different solvent extracts of molokhia leaves significantly reduced serum bilirubin and liver enzymes in carbontetrachloride,17,18 sodium arsenite,19 streptozotocin,20 hydrogen peroxide21

<p>| Table 2: Effect of MLE on hepatic oxidative stress |</p>
<table>
<thead>
<tr>
<th>Malonaldehyde (nM/mg P)</th>
<th>Glutathione (µM/mg P)</th>
<th>Catalase (U/mg P)</th>
<th>Superoxide dismutase (U/mg P)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.6± 0.03</td>
<td>6.4± 0.55</td>
<td>97d ± 1.1</td>
</tr>
<tr>
<td>Acrylamide</td>
<td>1.7± 0.11</td>
<td>2.7± 0.51</td>
<td>45± 4.3</td>
</tr>
<tr>
<td>MLE_{100}</td>
<td>1.0± 0.01</td>
<td>5.1± 0.41</td>
<td>73± 4.7</td>
</tr>
<tr>
<td>MLE_{250}</td>
<td>0.7± 0.02</td>
<td>5.4± 0.47</td>
<td>85± 1.9</td>
</tr>
<tr>
<td>MLE_{500}</td>
<td>0.7± 0.01</td>
<td>6.1± 0.39</td>
<td>93± 2.5</td>
</tr>
</tbody>
</table>

*Values were represented as mean ± SD (n=6). Values with different superscript letters in columns differ significantly from each other at p ≤ 0.05. MLE_{100}: molokhia leaf extract 100mg/kg; MLE_{250}: molokhia leaf extract 250mg/kg; MLE_{500}: molokhia leaf extract 500mg/kg.
and thioacetamide\textsuperscript{22} induced hepatotoxicity in experimental rats. Lowering of total bilirubin and liver enzymes levels may be attributed to the presence of gallic acid, caffeic acid, quinic acid, myricetin, luteolin and quercetin, quercetin and vitamin C\textsuperscript{21} which are known to exhibit significant hepatoprotective activity mediated through strong free radical scavenging properties.\textsuperscript{32-34}

The hepatoprotective potential of molokhia leaf extracts exerted via antioxidant effect was confirmed by measuring the levels of malondialdehyde, glutathione and the activities of catalase and superoxide dismutase in liver homogenates. GSH is converted to glutathione disulfide and degraded during oxidative stress, resulting in lipid peroxidation and elevated MDA levels. As a consequence, GSH is regarded as a key predictor of oxidative stress.\textsuperscript{28,35} It is well known that even short-term exposure to acrylamide can induce severe hepatic damage accompanied by disruption of antioxidant defense systems due to ROS accumulation in liver cells.\textsuperscript{36} As expected, acrylamide administration caused notable cellular damage in the liver evidenced by the elevated biochemical markers including serum AST, ALT, ALP and total bilirubin. Consequently, the levels of antioxidant enzymes CAT, SOD and glutathione were decreased leading to hyperoxidative environment. Treatment with molokhia extracts effectively balanced depletion of antioxidant defenses by restoring the levels of GSH, CAT and SOD in liver tissues.

Similar observations were reported in an earlier study, wherein aqueous leaf extract of molokhia at 50 and 100 mg/kg dosage not only restored the levels of hepatic enzymes, CAT and SOD but also reduced DNA fragmentation in liver tissues.\textsuperscript{19} Similar findings were reported by earlier\textsuperscript{17} wherein, aqueous extract at the dose of 500 and 1000 mg/kg exhibited significant hepatoprotection against CCl\textsubscript{4} induced hepatotoxicity in rats. The extract restored elevated levels of liver enzymes and MDA to normal levels. The activity of glutathione peroxidase which was increased upon administration of CCl\textsubscript{4} also restored to normal levels with extract treatment. The hepatoprotective effect was dose dependent and 1000 mg/kg dose showed higher effect mediated through strong antioxidant activity. However, in the present study such a strong dose dependent restoration of key antioxidant parameters was not demonstrated though the observed hepatoprotective effect was higher at 500 mg/kg dosage than 100 mg/kg, no statistical proportional relationality was established with respect to MDA, GSH, CAT and SOD. Similar observations were made in histopathological and ultrasound imaging studies, wherein no clear differentiation could be established between the histopathological sections of rats dosed at three different level. The sections showed clear improvement in hepatic architecture compared to acrylamide group. In ultrasound imaging the hepatic echotexture of MLE treated rats was more homogenous compared to acrylamide treated rats.

The hepatoprotective effect shown by MLE could be attributed to the presence of phenolics, flavonoids, glycosides, steroids and alkaloids as established by a number of studies ascribing strong antioxidant activity in terms of radical scavenging activity, reducing power and anti-lipidperoxidative activity to various extracts of molokhia leaves both \textit{in vitro} and \textit{in vivo}.\textsuperscript{37-43} Phenolic antioxidant compounds such as chlorogenic acid, 3,5-dicaffeoylquinic acid, quercetin 3-galactoside, quercetin 3-glucoside, quercetin 3-(6-malonylglucoside), and quercetin 3-(6-malonylgalactoside) have been isolated from molokhia leaves.\textsuperscript{44}

\textbf{Conclusion}

The findings of the study demonstrate that molokhia leaves extract offers notable protection against acrylamide induced hepatic damage through strong radical scavenging and anti-lipidperoxidative properties by the virtue of phenolics, flavonoids and alkaloids.

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\textbf{Conflict of Interest}

The authors declare no conflicts of interest.
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