Investigation of the nephroprotective activity of *Moringa peregrina* leaves aqueous extract in mice

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Abstract

Traditional remedies for *Moringa peregrina* leaves have a variety of uses with confirmed biological and therapeutic effects, as per published reports. The current study aims to evaluate the ability of the leaves aqueous extract to protect from nephrotoxicity in gentamicin-treated mice. Phytochemical analysis for the aqueous extract was performed using DPPH (2,2-Diphenyl-1-picrylhydrazyl) assay for antioxidants, Folin-Ciocalteu, AlCl₃ and HPLC-MS/MS analysis, focusing on phenol and flavonoid content. The nephroprotective activity of the prepared extract was evaluated by means of variable biochemical parameters including Creatinine (Cr), Uric Acid (UA), and Urea (Ur). In addition, histological examination of renal tissues was performed in all mice groups (control, gentamicin-induced (150 mg/Kg i.p) and aqueous extract-orally treated groups (500 and 1000 mg/Kg)). Findings reveal that the prepared extract has total phenols (555.57±0.92mg/g, equivalent to gallic acid), flavonoids (40.08±1.56 mg/g, equivalent to quercetin), and DPPH IC₅₀ (3.10 µg/ml). HPLC-MS/MS analysis revealed the presence of 10 phenols and flavonoids compounds. In vivo studies showed a significant (P < 0.05) reducing effect for the high-dose treatment, on serum and urine concentrations for UA, Cr, and U, among the nephrotoxicity induced mice. Low-dose treated group showed significant reduction on serum concentration of UA, Cr and U, but only for Cr concentration in urine. The histological examination showed an improvement in the image of the renal tissue among the induced-nephrotoxicity mice, which was treated with high-dose extract. In conclusion, leaves aqueous extract of *M. peregrina* have shown potential protective effect to counteract some of the gentamicin consequences on kidney functions.

Keywords

*Moringa peregrina*, nephroprotective, anti-oxidants, traditional medicine, kidney function test, phenols, flavonoids
**Introduction**

Herbal Medicine, often known as Ethnic Medicine, a branch of the Complementary and Alternative Medicine, is an old form of health care used to prevent and treat physical and mental disorders around the world (Kpobi et al. 2019; Randeepraj et al. 2020). Nowadays, using natural products, in particular herbal, for treating either mild or serious illnesses is in an increasing global trend (Tabatabaee 2011).

Acute and chronic nephrotoxicity might result after exposure to hazardous substances, medication, or simply a reflection of an unbalanced diet (Wimalawansa 2015). The renal proximal tubule is highly vascularized thus; it would be more susceptible to drug-induced nephrotoxicity (Cohen et al. 2021). Globally, more than 5 million people die each year due to a lack of access to crucial kidney disease therapies (Luyckx et al. 2021). Variable drugs could induce nephrotoxicity. Reports confirmed that around 20% of renal complaints have been induced by drugs side effects. The elderly is the most affected with a 66% prevalence rate of nephrotoxicity was recorded (Kim et al. 2012). Aminoglycoside antibiotics have confirmed nephrotoxic effects. That’s why their use is limited to treating life-threatening Gram-negative infections (Ali 2005; Al-Azzam et al. 2010; Tavafi 2013).

Recently, the nephroprotective activity of variable herbal remedies was clinically approved (Khatoon et al. 2019). Examples of these remedies include Cryptocoryne auriculata and Annona reticulata. The extracts, of these medicinal plants, were investigated in in-vivo study against the nephrotoxic effects of gentamicin and cisplatin. The medicinal effect was related to the antioxidant activity of the extracts, that was determined by the phenols content (Negi et al. 2020).

The Moringaceae family of flowering plants includes only one genus “Moringa” with 13 species native to tropical and subtropical climates. The two most studied species are Moringa peregrina and Moringa oleifera (Al Khateeb et al. 2013). Generally, Moringa species contain large amounts of flavonoids, phenols, vitamins, amino acids and minerals (Mansour et al. 2014). These compounds are well known for their role as antioxidants and as nutrients (Mir et al. 2022).

The crude extract of M. peregrina leaves was found rich in antioxidants such as phenolic compounds and flavonoids (Al-Ouaihi et al. 2014). Accordingly, confirmed activities against microbes, inflammation, hyperglycemia, hypertension, hyperlipidemia, and cytotoxicity were reported (Said-Al Ahl et al. 2017; Senthilkumar et al. 2018; Dhanaleshkimi et al. 2022). The spasmolytic and memory enhancement properties were also approved (Senthilkumar et al. 2018). Additionally, the hepatotoxicity induced by doxorubicin, was alleviated with M. peregrina extracts in experimental animals (Sliai et al. 2016).

Previous studies have shown that M. oleifera has a nephroprotective effect, when tested in animal experiments with gentamicin and acetaminophen-induced nephrotoxicity (Ouédraogo et al. 2013; Sheikh et al. 2014). Therefore, the overall aim of the current study is to evaluate the nephroprotective activity of M. peregrina leaves extract based on the well-known antioxidant rich components. This in-vivo study lines up with the justification purpose of the use of this herbal drug by people in Jordan for the biological activity of interest.

**Materials and methods**

**Chemicals**

Gentamicin was purchased from Medscheme (Cyprus), Quercetin, 1,1-diphenyl-2-picrylhydrazyl (DPPH) and gallic acid were purchased from Sigma-Aldrich, USA. Folin- Ciocalteu reagent was obtained from Merck, Germany. All other chemicals and solvents used in the study were of analytical grade procured locally.

**Collection and extraction of plant material**

Fresh M. peregrina leaves were collected in August 2021 by citizens in the Ghor of Jordan valley (60 Km south Amman). Authenticated voucher was deposited at the Phytochemistry laboratory, Faculty of Pharmacy, Mutah University. Leaves were picked then washed thoroughly under tap water. The washed leaves were dried under shade and stored in dried conditions until used.

Dried leaves were crushed into coarse pieces; 10 g of the crude plant was macerated in 100 ml distilled water. The mix was kept in a shaker water bath (Memmert shaker waterbath) at 25 °C for 16 hr. Then, the solution was filtered for the aqueous part. The process was repeated for three days until the filtrate was pale in color. The filtrate then was concentrated under low-pressure using rotary evaporator at 60 °C. The extract was further dried using a Benchtop Manifold Freeze Dryer from Millrock Technology, UK for 72 h until fine powder of the crude extract was obtained. The powder was then stored at -20 ºC for further experiments.

**Determination of total phenols**

Total phenol content was measured using Folin-Ciocalteu method described by Sadeghi et al. (2015). Briefly, a 5 ml of plant extract solution (or gallic acid as standard phenolic compound), was mixed with Folin-Ciocalteu reagent and aqueous Na2CO3 (5%). After 30 min, the phenolic contents were determined calorimetrically at 765 nm. The total phenolic content (mg/ ml) ±SD was determined as gallic acid equivalent.

**Determination of total flavonoids**

Total flavonoids quantification was performed using colorimetric method based on the formation of a complex flavonoid–aluminum as described by Pękal and Pyrzynska (2014). Briefly, 5 ml of plant extract solution was mixed with aqueous NaNO3 (5%) and aqueous AlCl₃ (10%).
After 30 min, the flavonoid content was determined colorimetrically at 510 nm. The total flavonoid content (mg/ml) ±SD was determined as quercetin equivalent.

Determination of antioxidant activity using the DPPH assay

The reduction of DPPH• free radical solutions assay was performed following a modified reported protocol by Proestos et al. (2013). Briefly, the DPPH was dissolved in ethanol solution (1mM) to create the free radical that poses a deep violet color. Dry plant extract, was dissolved in ethanol at different concentrations ranging from (0.5–7 µg/ml) and used as reducing agents. A 2 ml of DPPH• free radical solution was mixed with 2 ml of the plant extract solution. This mixture was then incubated for 1 hr in a dark place at room temperature. The absorption for each sample was measured at λmax= 517 nm using a plate reader. The effective concentration required for scavenging of 50% of the free radicals (IC50) was calculated using the plotted graph of scavenging activity against the extract and used to compare the antioxidant activity relative to ascorbic acid.

Identification of phenols and flavonoids using HPLC/MS-MS

Extract samples have been dissolved in DMSO (2 ml), diluted with Acetonitrile (up to 50 ml), then centrifuged individually (4000 rpm for 2.0 min). 1.0 ml of each sample was transferred to autosampler and injected (3.0 µl).

A Bruker Daltonik (Bremen, Germany) Impact II ESI-Q-TOF System equipped with Bruker Daltonik Elute UPLC system (Bremen, Germany) was used for screening compounds of interest against an integrated library. Standards were used for identification of m/z with high resolution Bruker TOF MS and exact retention time of each analyte after chromatographic separation.

The instrument was operated using the Ion Source Apollo II ion Funnel electrospray source. Chromatographic separation was performed using Bruker solo 2.0 C -18 UHPLC column (100mm × 2.1 mm × 2.0 µm) at a flow rate of 0.51 mL / min and a column temperature of 40 °C. Solvents:(A) water with 0.05% formic acid and (B) acetonitrile. Gradient elution of B (80–95%) was performed for a total analysis time of 35 min.

Experimental animals

Male BALB/c albino mice, average weight of 26.5 ± 2.76 g, were obtained from the animal house, Faculty of Pharmacy, Mutah University. The mice were transferred from the animal house to the animal laboratory, a week before the experiment to be acclimatized.

All procedures were performed in accordance with international regulations for the care and use of laboratory animals. Ethical approval on the study was obtained by the ethical committee at Mutah University, Al-Karak, Jordan, (4/2021/2022).

Induction of nephrotoxicity

Nephrotoxicity induction was performed following the protocol performed by Edeogu et al. (2020), using gentamicin solution prepared by dissolving the required weight of the powdered drug in distilled water, vortexed and used immediately.

Experimental animals (in vivo study)

Treatment with the plant extract was performed at two doses (500 & 1000 mg dry extract/ kg animal body weight). Therefore, extract stock solution was prepared by dissolving dry extract in distilled water to obtain a solution with final concentration of (100 mg/ml), vortexed and used immediately. The volume of the extract solution used for treatments was calculated accordingly to each animal body weight.

Experimental animals were distributed into 4 groups of 6 mice each as follow:

Group 1: the normal control; treated with vehicle only.
Group 2: the negative control, treated daily with intraperitoneal (i.p) doses of gentamicin in a concentration of 150 mg/Kg, for 7 consecutive days.
Group 3: treated daily with herbal extract solution equivalent to (500 mg/kg) via oral gavage for the prepared experimental animals, for 7 consecutive days. Starting from the 8th day and for 7 consecutive days, intraperitoneal doses of gentamicin (150 mg/kg) were added to the previous medication regimen. A two-hour gap between the antibiotic and the herbal remedy were let.
Group 4: the same medication protocol for group 3 was applied. Animals were treated daily with the herbal extract solution equivalent to (1000 mg/kg).

The experimental animal was confirmed to develop renal injury after histological and biochemical measurements including Creatinine (Cr), Uric Acid (UA), and Urea (Ur). All biochemical tests were performed in triplicates.

The experimental animals have access to water and were deprived of food 2 hr. prior to the experiment. No anesthesia was needed either during the intraperitoneal or the oral treatments.

Urine, blood and kidney samples collection

Immediately before scarifying the animals for blood and tissues samples collection, urine samples were collected and stored at -20 °C until used. The animals were let to move freely over a clean glass slap. After spontaneous urination, samples were immediately collected via aspirated pipette tips to avoid contamination.

Blood samples were collected from the heart ventricles of the experimental animals under mild anesthesia. The anesthetic agent used was diethyl ether. The mouse was constrained on the anatomical plate a cotton pad filled with the anesthetic agent covering the nostrils. Blood samples were collected in gel tubes, centrifuged for 5 min
The supernatant was collected and kept at -20 °C for further biochemical analysis.

After sacrificing the experimental animals, on the anatomical board, a longitudinal cut was performed in order to collect the kidneys. Then, the organs were immediately kept in previously prepared 10% formalin.

**Biochemical data measurements**

Serum and urine samples were assayed at the end of the experiment period using liquicolor-HUMAN kits for the concentrations of (Cr, UA, and U). The absorbance for each sample was measured at \( \lambda_{max} = 490-510, 340, \) and 520 nm, respectively.

**Histological studies**

The tissue was immersed in formalin solution (10%), dehydrated, and then prepared into the microscopical sections. The paraffin sections were then cut at 5 \( \mu \)M thickness using Leica microtome (Germany), stained with hematoxylin-eosin stain to be used later for the histopathological examination. Sections were examined by two histopathologists who were blind to the treatments. A thin slice of each part was subjected to a light microscope with a magnification power of 40X, microscope. Microscopic images were taken for further detailed examination of glomerulus, and kidney tubules in cortex.

**Statistical analysis**

Data analysis was performed using SPSS version 23. Numerical data were analyzed using ANOVA test. Values of \( P \leq 0.05 \) were considered significant. Microsoft Excel sheet was used to calculate the average± SE or SD for the numerical data.

**Results**

**Extraction yield**

The phytocomponents were extracted from the plant by maceration using water. The extraction yield for *M. peregrina* leaves extract was calculated as 40% w/w dry weight.

**Quantification of total phenol content in the *M. peregrina* leaves extract**

The total phenol content was calculated using the following linear regression equation obtained from the standard plot of gallic acid:

\[ Y = 0.0004x + 0.0042, R^2 = 0.968 \]

\( Y= \) absorbance and \( x=\)concentration

It was found that the prepared extract contains total phenols 555.6±0.92 mg/g equivalent to gallic acid.

**Quantification of total flavonoid content in the *M. peregrina* leaves extract**

The total flavonoid content was calculated using the following linear regression equation obtained from the standard plot of quercetin:

\[ Y = 0.0037x - 0.0035, R^2 = 0.999 \]

\( Y= \) and \( x=\)concentration

It was found that the prepared extract contains total flavonoids 52.27±2.18 mg/g equivalent to quercetin.

**In vitro antioxidant activity**

The antioxidant activity of *M. peregrina* leaves extract was investigated by commonly used radical scavenging method DPPH. Although the IC_{50} value of ascorbic acid (1.37 \( \mu \)g/mL) was much lower than the aqueous extract (3.10 \( \mu \)g/mL), it is still considered as a rich plant extract with antioxidant compounds.

**Identification of phenols and flavonoids using HPLC/MS-MS**

Of the available library, out of the 43 screened compounds, 10 different components (supplementary 1) were detected using the HPLC/MS-MS in the aqueous extract (Fig. 1). Only 3,6,2’,4’-tetrahydroxyflavone was not previously detected in this genus.

**Animal study**

**Biochemical parameters**

A remarkable decrease in UA, U, and Cr were measured in both serum and urine samples after treatment with *M. peregrina* aqueous extract (Fig. 2). Serum concentrations of UA, U, Cr in the groups received only gentamicin (negative control) and the group that received gentamicin with low-dose aqueous extract (500 mg/Kg), was significantly low (\( P\)-value < 0.05). Only for Cr, a significant reduction in the urine samples was observed.

In a comparison between the nephrotoxic group and those treated with a high dose of the aqueous extract (1000 mg/Kg), a significant decrease in UA, U and Cr values (\( P\)-value < 0.05) was obtained in both serum and urine samples.

**Histological analysis**

Histological examination of the kidneys for the normal control group showed normal glomeruli with abundant podocytes, mesangial cells with a healthy mesangial matrix in between and normal capsular space (Fig. 3A). Moreover, tubules were not dilated and proximal tubules appeared filled, because of the long microvilli of the brush border, while lumens of distal tubules appeared empty.
After inducing nephrotoxicity by gentamicin (negative control), a clear reduction in area and cellularity was observed in the kidney’s glomeruli compared to the normal control group (Fig. 3B). In addition, heavy leucocyte infiltration was also detected (Fig. 3C). Histological images show an extensive globular degeneration occurred after treating the experimental animal with a nephrotoxic agent (Fig. 3D).

After 7-days of oral prophylactic doses administration of the aqueous extract of *M. peregrina* in a concentration of 500 mg/Kg (low does), it was followed by further 7-days of conjugated gentamicin administration. The histological examination for the kidney tissues showed a remarkable improvement in the overall histology, less damaged glomeruli, mild leucocyte infiltration, and less tubular degeneration (Fig. 3E).

High doses treatment of the herbal aqueous extract (1000 mg/Kg), showed a clear improvement in the overall health status of the experimental animals in all biological replicates. Findings of the histological studies support the biochemical findings, with fewer leucocytes infiltrations, and proximal, distal tubes damage (Fig. 3F).

**Discussion**

The phytochemical analysis for the aqueous extract for *M. peregrina* revealed that content of phenols and flavonoids, with considerable antioxidant effect, would be considered as a potential herbal treatment, which supports its traditional use as a nephroprotective agent. These findings agree with several previous reports on the *Moringa* species (Siddhuraju et al. 2003; Dehshahri et al. 2012).

In this study, it is the first time to investigate the nephroprotective effect for the aqueous extract, aiming to justify its tradition use. Therefore, the prepared extract was evaluated on gentamicin induced nephrotoxicity mice model. Findings agree with previous reports. In a study by Azim et al. (2017) acetaminophen was used to induced oxidative damage. Findings shows that improved status by *M. peregrina* leaves ethanol extract treatment, due to its antioxidant potential.

Similarly, a study by Alkhudhayri et al. (2021) investigated the ethanolic *M. peregrina* leaf extract, in rats fed with a high-fat diet. Leaf extract reduced hepatic triglycerides and cholesterol levels, and increased serum high-density lipoproteins levels and triglycerides and cholesterol levels in feces. Moreover, improved liver architecture, reduced fat accumulation, reduced hepatic malondialdehyde, tumor necrosis factor-α, and interleukin-6 levels. Hepatic glutathione peroxidase, superoxide dismutase, and catalase activities were significantly increased. These effects were mediated by reduced lipid absorption, anti-hyperlipidemic effects, and hepatic antioxidant effects. These findings would partially justify the results of the current study, and relate the proposed hepatoprotective effect of the plant extract to the antioxidant activity, supported by its content of phenols and flavonoids.
The HPLC-MS/MS analysis of *M. peregrina* aqueous extract resulted in the identification of a total of 10 phytochemical components with previously reported nephroprotective effect, such as for Succinic acid (Cienfuegos-Pecina et al. 2020), Benzoic acid (Pandit et al. 2018), 4-Hydroxybenzoic acid (Wang et al. 2012), 2,5-Dihydroxybenzoic acid (Moreno et al. 2018), p-Coumaric acid (Rafiee et al. 2020), Caffeic acid (Rjeibi et al. 2017), 3,6,2',4'-Tetrahydroflavone...
(Gollen et al. 2018), Chlorogenic acid (Dkhil et al. 2020), ISO-Orientin (Ali et al. 2018), and Rutin (Radwan et al. 2017). Compared to a previous similar study conducted by Jaffal et al. 2020, different phenols and flavonoids derivatives were detected, as the solvent used for extraction was methanol. These findings are important, as alcoholic preparation in the Arabic and Islamic traditional medical system is not in use, for religious believes. In addition, alcohol is known for its kidney damage potential. Therefore, studies that mimic the traditional methods for preparation of herbal remedies are important.

As expected, these compounds were found to play a potential nephroprotective effect, supported by the in vivo findings of this study. Results suggest a remarkable improvement in the kidney’s anatomy and functions at cellular and biochemical levels, which were measured after treatment with *M. peregrina* aqueous extract, compared to the untreated animals. The biochemical measured data was concomitant with the histological examination, which showed fewer tubular necrosis, inflammation signs such as leucocytes infiltrations, and other markers were observed in the plant extract-treated mice with gentamicin induced nephrotoxicity.

**Conclusion**

The promising nephroprotective activity that has been concluded from the current study, could be strongly correlated to the enriched polyphenol and antioxidants in *M. peregrina* leaves aqueous extract. These findings would support its traditional use as a nephroprotective agent.

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