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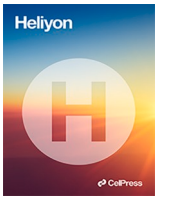

The nephrotoxicity of *Aristolochia rotunda* L. in rats: Mitochondrion as a target for renal toxicity of Aristolochic acids-containing plants

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A R T I C L E I N F O

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A B S T R A C T

In recent years, there has been a growing trend in the usage of traditional medicine and herbal treatments. However, the misconception that they are completely safe resulted in irreversible complications and damages. The present study was conducted to investigate the potential renal toxicity of a commonly used drug in Iran's traditional medicine and pharmacy, known as Zaravand Gerd or Nokhod Alvand (*Aristolochia rotunda* L.). In Iranian traditional medicine, Zaravand Gerd is used as a remedy for respiratory system ailments, back pain, anxiety, headache and septic wounds. Fifty-six male rats were divided into seven groups (n = 8). The first group served as the control and received normal saline, while the second to seventh groups were administered varying doses of the aqueous extract of Zaravand Gerd (0.1, 0.5, 1.25, 2.5, and 5 g/kg) for a period of three weeks. Various parameters were measured to evaluate the potential kidney damage caused by the extract, including serum creatinine and BUN levels, as well as urine protein and glucose levels, which were analyzed using an autoanalyzer. Additionally, kidney tissue samples were examined pathologically, and mitochondria from the kidney tissue were isolated to assess mitochondrial parameters. The results of this study revealed that high doses of Zaravand Gerd extract led to a significant increase in urinary glucose and protein excretion compared to the control group. Pathological examination of the isolated kidney tissues indicated that the concentrations of 2.5 and 5 g/kg of Zaravand Gerd extract resulted in kidney damage and dilation of proximal convoluted tubules. Furthermore, the study demonstrated that high doses of the extract (2.5 and 5 g/kg) caused damage to the mitochondria. Based on the findings of this study, it can be concluded that the administration of high doses of Zaravand Gerd extract, which are not commonly used in traditional medicine, can have toxic effects on the kidneys in rats as an animal model. These results highlight the importance of considering the potential risks associated with herbal medicines and the necessity of usage based on scientific evidence.

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1. Introduction

The utilization of herbal medicine has witnessed a significant expansion, both in developing and advanced nations [1]. Despite the burgeoning popularity of traditional herbal remedies, there often exists a paucity of comprehensive information concerning their potential adverse effects [2]. Kidney damage can occur as a result of exposure to various agents, encompassing drugs, chemicals, or botanical products. The renal toxicity attributed to herbal medicines may be related to a multitude of factors, including the intrinsic toxic nature of herbal compounds, contamination by nephrotoxic agents such as heavy metals, fraudulent adulteration of medicinal products, misidentification of botanical species, interactions with concomitant medications, or improper utilization [3]. The kidneys constitute one of the principal organs responsible for the excretion of pharmaceutical compounds, toxins, and their metabolites, thereby being subjected to a substantial blood flow, approximately 20–25 % of cardiac output, to facilitate the elimination of nephrotoxic compounds. The extensive surface area provided by tubular epithelium and endothelium within the renal structure serves as sites for drug absorption, interactions and potential injury [4]. Two primary mechanisms, namely acute tubular necrosis and acute interstitial nephritis, are commonly associated with nephrotoxicity [5].

Aristolochic acids are an important group of compounds that have been associated with kidney damage. These acids are commonly found in the roots of plants belonging to the *Aristolochiaceae* family [6]. The genus *Aristolochia* is particularly significant, comprising over 400 species that have both numerical abundance and therapeutic potential. Different species of *Aristolochia* are utilized in local and traditional medicine across various countries [7]. In Iranian traditional medicine, two widely-used species from this genus are Zaravand Tawil (*Aristolochia longa*) and Zaravand Madharij or Gerd (*Aristolochia rotunda* L.). Traditional medicine texts mention numerous uses for these species, either individually or in combination [8]. The majority of *Aristolochia* species contain aristolochic acid, which is known to be responsible for the reported toxicity associated with them [9].

In traditional medicine, Zaravand Tawil has been utilized topically to treat gum infections and body lice [10]. Research has demonstrated that the aqueous extract of Zaravand Tawil root has toxic effects on liver and kidney function in Wistar rats [11]. Zaravand Gerd is primarily used as an antidote to eradicate infections, as well as a remedy for respiratory system ailments like coughs and shortness of breath, nervous system disorders, and purulent wounds [12]. Despite its usage in traditional medicine and the current market of medicinal plants, there have been very few studies conducted on the compounds, effects, and toxicity of Zaravand Gerd. This lack of knowledge can potentially lead to irreversible issues among consumers. Therefore, the objective of this study was to assess the potential nephrotoxicity of Zaravand Gerd in rat as an In vivo model.

2. Materials and methods

2.1. Chemicals

Bovine Serum Albumin (BSA), Hank's Balanced Salt Solution (HBSS), N-(2- hydroxyethyl) piperazine-N′ -(2-ethanesulfonic acid) (HEPES), Rotenone, D-mannitol, Sucrose, 2′,7′ -Dichlor-fluorescein (DCF), Rhodamine123, Ellman's reagent (5,5′ -dithiobis-(2-nitrobenzoic acid, 3-morpholinopropane-1-sulfonic acid (MOPS), 2-Amino-2-hydroxymethyl-propane-1,3-diol (TRIS), 4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Dimethyl sulfoxide (DMSO), Sodium succinate, Potassium chloride, Monopotassium phosphate and Magnesium chloride were purchased from Sigma (St. Louis, MO USA).

2.2. Preparation of root extract of Zaravand Gerd

The root of Zaravand Gerd was obtained from a local store and then verified by a botanist. A voucher specimen was deposited in the Herbarium of the Medical Plants Research Center at Shahrekord University of Medical Sciences, Shahrekord, Iran (SKUMS-1037). The extraction of the root was conducted using the maceration method. To provide the extract, the powdered root of Zaravand Gerd was mixed with distilled water at a ratio of 1–5, and the mixture was allowed to soak at room temperature for 48 h. Afterward, the soaked root was filtered, and the resulting extract was dried in an incubator at 37 °C.

2.3. Measurement of AA content

The determination of AA I and AA II content in the root extract of Zaravand Gerd was conducted using High-Performance Liquid Chromatography (HPLC) [13]. The extraction process involved treating 1 g of powdered sample with 30 ml of n-hexane using an ultrasonic extractor to remove non-polar components. After solvent evaporation, the residue was subjected to two extractions with methanol (30 ml, 30 min and 15 ml, 15 min). The combined extract was then centrifuged at 3000×*g* for 5 min, and the resulting supernatant was adjusted to a final volume of 50 ml with methanol. The analytical sample for HPLC was obtained by filtering this solution through a 0.45 mm pore size filter. The identification of AA I and AA II was accomplished by comparing their retention times with standard data. The quantification of these compounds was performed at a wavelength of 250 nm. The recovery rates of AA I and AA II were found to be 96 % and 92 %, respectively. The HPLC instrument used in this study was a Shimadzu LC 20Ap equipped with a diode array detector (Shimadzu SPD-M20A). The HPLC conditions employed were as follows: a VP-ODS column (Shim-pack C18, 150 × 4.6 mm), eluted with a mixture of 1 % acetic acid and methanol (6:4) at a flow rate of 1.0 ml/min. The retention time observed for AA I was 29.4 min, while the retention time for AA II was 18.3 min.

2.4. Approval from animal's ethics committee

This study was conducted at Shahrekord University of Medical Sciences and received approval from the Animals Ethics Committee of the university. Ref. No. IR.SKUMS.AEC.1401.036.

2.5. Laboratory animals and grouping

The present study obtained approval from the Research Ethics Committee of Shahrekord University of Medical Sciences. Male wistar rats weighing between 200 and 250 g were housed in a controlled environment, maintaining a temperature of 21 ± 2 °C. The rats were subjected to a 12-h light-dark cycle and were given ad libitum access to food and water. Subsequently, the animals were randomly assigned to seven groups, with each group consisting of 8 rats. The first group (control) received a gavage of normal saline (1 ml/kg) once a day for 3 weeks [34]. The second to the sixth groups received daily gavage doses of Zaravand Gerd extract (0.1, 0.5, 1.25, 2.5, and 5 g/kg respectively) for 3 weeks [11].

2.6. Blood and urine sample collection

After receiving an intra-gastric administration of extract on the 21st day of the experiment, the animal subjects were individually housed in metabolic cages and subjected to a fasting period of 12 h. Following this fasting period, urine samples were collected from the rats. Subsequently, anesthesia was induced by administering 40 mg/kg of ketamine and 15 mg/kg of xylazine and blood samples were collected directly from their hearts. The collected blood samples were transferred to clotting tubes and maintained at 25 °C for 30 min to allow for clot formation. Then, the serum fraction was separated from the blood samples by performing centrifugation at 2000×*g* for 10 min. The obtained serum samples were subsequently stored at −70 °C for future biochemical analyses.

2.7. Biochemical analysis

Serum creatinine and blood urea nitrogen (BUN) levels, along with urinary protein and glucose excretion, were assessed using commercial assay kits provided by Parsazmun Co., (Tehran, Iran). These measurements were conducted utilizing the BT3000 autoanalyzer.

2.8. Histopathology assessment

After extracting blood from the hearts of the animals, the kidneys were surgically excised and divided into two separate portions. One portion was immediately immersed in a fixative solution containing 10 % formalin, to prepare the kidney samples for subsequent processing and sectioning. The other portion of the kidney tissue was used for mitochondrial isolation. In order to study the kidney tissue, a series of steps were followed. First, the kidney samples were subjected to dehydration by ethanol. Next, the dehydrated tissue was clarified using a xylene-alcohol mixture. Once the tissue was adequately dehydrated and clarified, it was then molded. After molding, tissue sections with a thickness of 5 μm were prepared using a rotary microtome. The prepared sections were then transferred onto slides and dried. The prepared sections were stained with a combination of Hematoxylin and Eosin (H&E). The stained sections were finally observed using a microscope, which allows for the detailed examination and analysis of the kidney tissue. The histological evaluations of the renal tissue were graded as described in the study of Medeiros et al. [14] (Table 1). For histological evaluation, EGTI scoring system, which was developed especially for animal studies in kidney tissues in the context of injury, was also used [15], (Table 2).

2.9. Isolation of kidney mitochondria

After the removal of fat and connective tissue, the kidneys were meticulously washed and subsequently chopped into smaller pieces. The tissue fragments were then homogenized in an isolation buffer, composed of 0.2 mM EDTA, 225 mM D-mannitol, and 75 mM sucrose, adjusted to a pH of 7.4. The homogenization process was performed using a glass homogenizer. Following homogenization, the resultant tissue homogenate was centrifuged at 1000×*g* for 10 min. The supernatant fraction which contains the mitochondria was collected and subjected to a second centrifugation step at 10,000×*g* for another 10 min. The whole process was carried

Table 1
Scoring system for renal histopathology.

Score	Histopathological pattern
0	Normal
0.5	Small focal damaged areas
1	<10 % Cortical damaged zone
2	10–25 % Cortical damaged zone
3	25–75 % Cortical damaged zone
4	>75 % Cortical damaged zone

Table 2
The EGTI histology scoring system.

Tissue	type Damage	Score
Tubular	No damage	0
	Loss of Brush Border (BB) in less than 25 % of tubular cells. Integrity of basal membrane	1
	Loss of BB in more than 25 % of tubular cells, Thickened basal membrane	2
	(Plus) Inflammation, cast formation, necrosis up to 60 % of tubular cells	3
	(Plus) Necrosis in more than 60 % of tubular cells	4
Endothelial	No damage	0
	Endothelial swelling	1
	Endothelial disruption	2
	Endothelial loss	3
Glomerular	No damage	0
	Thickening of Bowman capsule	1
	Retraction of glomerular tuft	2
	Glomerular fibrosis	3
Tubulo/Interstitial	No damage	0
	Inflammation, haemorrhage in less than 25 % of tissue	1
	(Plus) necrosis in less than 25 % of tissue	2
	Necrosis up to 60 %	3
	Necrosis more than 60 %	4

out on ice (4 °C) and the isolated mitochondria were used for assessment of toxicity parameters within 4 h [16]. To quantify the concentration of mitochondrial protein, the Bradford method was employed, using BSA as a standard protein reference.

2.10. Succinate dehydrogenase activity

Mitochondrial functionality was assessed by performing an MTT assay to measure succinate dehydrogenase (SDH) activity. Isolated kidney mitochondria were suspended in mitochondrial assay buffer (0.5 mmol/L KH2PO4, 20 mmol/L HEPES, 0.5 mmol/L EGTA, 10 mmol/L NaCl, 140 mmol/L KC, 2 mmol/L MgCl2; supplemented with 1 mg/ml rotenone and 10 mmol/L succinate). To initiate the assay, 100 μL of isolated mitochondria (at a concentration of 100 μg/well) were combined with 25 μL of MTT solution (0.5 mg/ml) in 96-well plates. The mixture was then incubated at 37 °C for 30 min. Following incubation, the formazan crystals formed were dissolved by adding 100 μL of dimethyl sulfoxide (DMSO) to each well. The absorbance of the resulting solution was measured at 570 nm using an ELISA reader [17].

2.11. Mitochondrial swelling

The swelling of isolated mitochondria was evaluated by measuring light scatter at a wavelength of 540 nm. A decrease in absorbance at 540 nm indicates mitochondrial swelling [18]. The isolated kidney mitochondria were incubated in a freshly prepared mitochondrial swelling buffer (0.5 mmol/L KH2PO4, 20 mmol/L HEPES, 0.5 mmol/L EGTA, 10 mmol/L NaCl, 140 mmol/L KC, 2 mmol/L MgCl2; supplemented with 1 mg/ml rotenone and 10 mmol/L succinate). The absorbance of isolated kidney mitochondria (100 μg/well of 96-well plates) was recorded at 540 nm.

2.12. Mitochondrial membrane potential collapse

A fluorescence dye known as Rhodamine 123 was employed to measure the mitochondrial membrane potential. Mitochondria, having a protein concentration of 1 mg per milliliter (1 mg protein/mL), were incubated in mitochondrial membrane potential (MMP) buffer. The MMP buffer consisted of the following components: 68 mM D-mannitol, 5 mM KH2PO4, 10 mM HEPES, 220 mM sucrose, 2 μM rotenone, 2 mM MgCl2, 50 μM EGTA, 10 mM KCl, 5 mM sodium succinate, and 5 μM Rhodamine 123. The incubation took place at 37 °C for 15 min. Following incubation, the MMP buffer was replaced with Rhodamine 123 free MMP buffer, and further incubated for an additional 15 min. Finally, the fluorescence intensity of Rhodamine 123 was measured at an excitation wavelength of 470 nm and an emission wavelength of 540 nm using a fluorescence spectrophotometer (Shimadzu RF5000U) [19].

2.13. Assessment of mitochondrial ROS formation

2′,7′-dichlorodihydrofluorescein diacetate (H2DCF) was employed as a probe for assessing mitochondrial reactive oxygen species (ROS) production. Mitochondria (1 mg protein/mL) were incubated in a respiration buffer (consisting of 0.5 mM MgCl2, 50 mM EGTA, 0.32 mM sucrose, 10 mM Tris, 0.1 mM KH2PO4, 20 mM MOPS, 5 mM sodium succinate, and 10 μM H2DCF) at 37 °C for 15 min. Subsequently, the respiration buffer containing H2DCF was replaced with a H2DCF-free respiration buffer, and the sample was allowed to incubate for an additional 15 min, shielded from exposure to light. The fluorescence intensity was measured with a fluorescence spectrophotometer (Shimadzu RF5000U) with excitation and emission 495 and 530 nm [19].

2.14. Statistical analysis

Statistical analysis of the data was performed using GraphPad Prism 6 software. The normality of the data was assessed using the Kolmogorov-Smirnov test. One-way analysis of variance (ANOVA) with Tukey's post-test was conducted to determine if there were any significant differences between the treatment groups. The results were presented as mean ± standard error of the mean, and $p < 0.05$ was considered as statistically significant.

3. Results

3.1. Aristolochic acids (AA) content

To determine the AA (AA I + AA II) concentration, we quantified the concentrations of two distinct forms of Aristolochic Acids, AA I and AA II, within the root extract of Zaravand Gerd. The measured concentration of AA II was found to be 0.033 mg/g, while that of AA I was determined to be 0.096 mg/g. Consequently, the overall concentration of Aristolochic Acids in the Zaravand Gerd root extract was calculated to be 0.129 mg/g (Fig. 1).

3.2. Serum creatinine and urea levels

Renal Function was assessed by measuring the levels of Serum creatinine and urea in control and extract treated animals. The results of serum creatinine levels analysis indicated that there was a reduction in serum creatinine levels across the tested groups. However, this reduction was not statistically significant ($P < 0.05$) when compared to the control group (Fig. 2A). Similarly, the analysis of BUN (Blood Urea Nitrogen) levels in the tested groups revealed that the administration of various doses of the aqueous extract of Zaravand Gerd led to changes in BUN levels, but these alterations were not statistically significant ($P < 0.05$) when compared to the control group (Fig. 2B).

3.3. Glucosuria

Glucosuria was assessed to reveal the possible impairment of renal glucose absorptive capacity by zaravand Gerd extract. The results of the urine glucose levels assay in rats treated with different doses of Zaravand Gerd extract demonstrated that the doses of 2.5, and 5 g/kg led to a statistically significant ($P < 0.05$) increase in urine glucose compared to the control group. On the other hand, the extract at doses of 0.1, 0.5, and 1.25 showed a trend towards a decrease in urine glucose levels compared to the control group; however, this difference was not statistically significant ($P < 0.05$) (Fig. 3A).

3.4. Proteinuria

Proteinuria which is elevated protein in the urine was measured to determine the possible kidney damage by Zaravand Gerd extract in treated animals. Quantitative analysis of urinary protein levels was performed in rats treated with varying doses of Zaravand Gerd extract. The results showed a dose-dependent increase in protein excretion through urine following administration of the extract. Specifically, doses of 2.5, and 5 g/kg demonstrated statistically significant ($P < 0.05$) increases in urinary protein excretion compared to the control group, as depicted in Fig. 3B.

3.5. Histopathological assessment

Tissue damage in rat's kidney was determined by histopathological assessment. The histopathological analysis of kidney samples

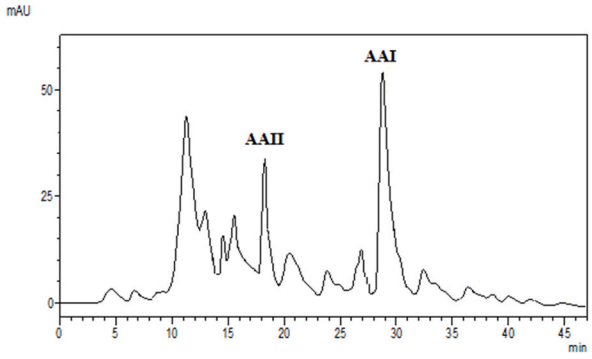

Fig. 1. HPLC chromatogram obtained from the analysis of AA I and AA II in the root extract of Zaravand Gerd. The retention times of AA I and AA II were 29.4 and 18.3 min, respectively.

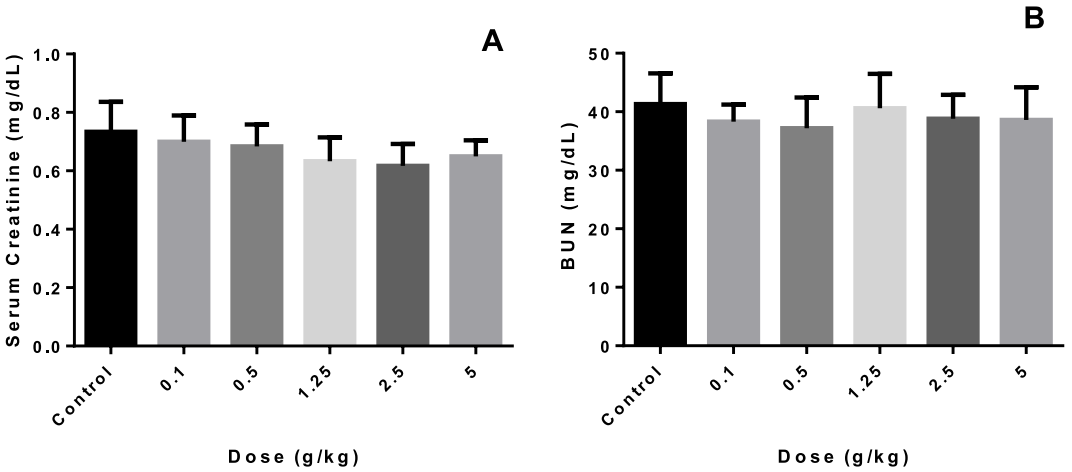

Fig. 2. The effect of different doses of aqueous extract of Zaravand Gerd on serum creatinine (A) and BUN (B) levels in treated animals. Different doses of the aqueous extract of Zaravand Gerd caused non-significant ($P < 0.05$) changes in serum creatinine (A) and BUN (B) levels compared to the control group.

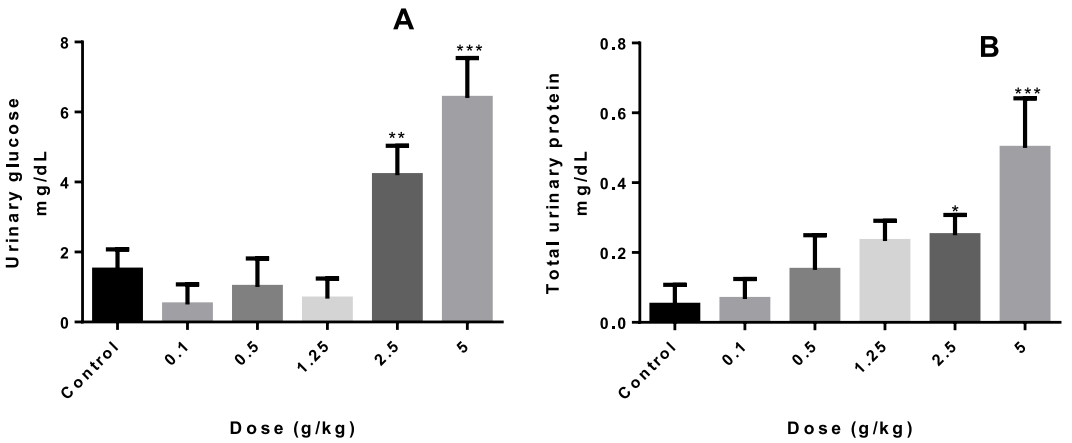

Fig. 3. The effect of different doses of the aqueous extract of Zaravand Gerd on the levels of glucose and proteins in the urine of treated animals. Zaravand Gerd extract in doses of 2.5 and 5 g/kg caused a significant ($P < 0.05$) increase in urine glucose (A). Statistically significant ($P < 0.05$) increase in levels of urine proteins was observed with 1.25, 2.5 and 5 g/kg of extract (B). *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$.

revealed the absence of tissue inflammation and pathological alterations in animals administered doses of 0.1 and 0.5 g/kg. However, in rats receiving doses of 1.25, and 2.5 g/kg, there was observed a brief dilation of the proximal convoluted tubules along with the presence of scattered inflammatory cells. Remarkably, the administration of Zaravand Gerd extract at a dose of 5 g/kg resulted in significant pathological changes in the kidney tissue. Fig. 4 demonstrates the presence of inflammatory cells surrounding the fat cells, indicating the occurrence of renal toxicity upon exposure to this particular dose (5 g/kg). Tables 3 and 4 show the results of the kidney damage quantification.

3.6. Mitochondrial function

Mitochondrial function was assessed through measuring SDH activity using MTT assay. According to the experimental results presented in Fig. 5, the mitochondrial succinate dehydrogenase (SDH) activity was observed to decrease in a dose-dependent manner in rats exposed to the aqueous extract of Zaravand Gerd. This decrease in SDH activity was found to be significant ($P < 0.05$) in the mitochondria of rats treated with 2.5 and 5 g/kg of the extract compared to the control group.

3.7. Mitochondrial swelling

Mitochondrial swelling was assessed as a hallmark of mitochondrial dysfunction. Fig. 6 demonstrates the renal mitochondrial swelling in rats that were treated with the aqueous extract of Zaravand Gerd. The results indicate that there was a significant increase

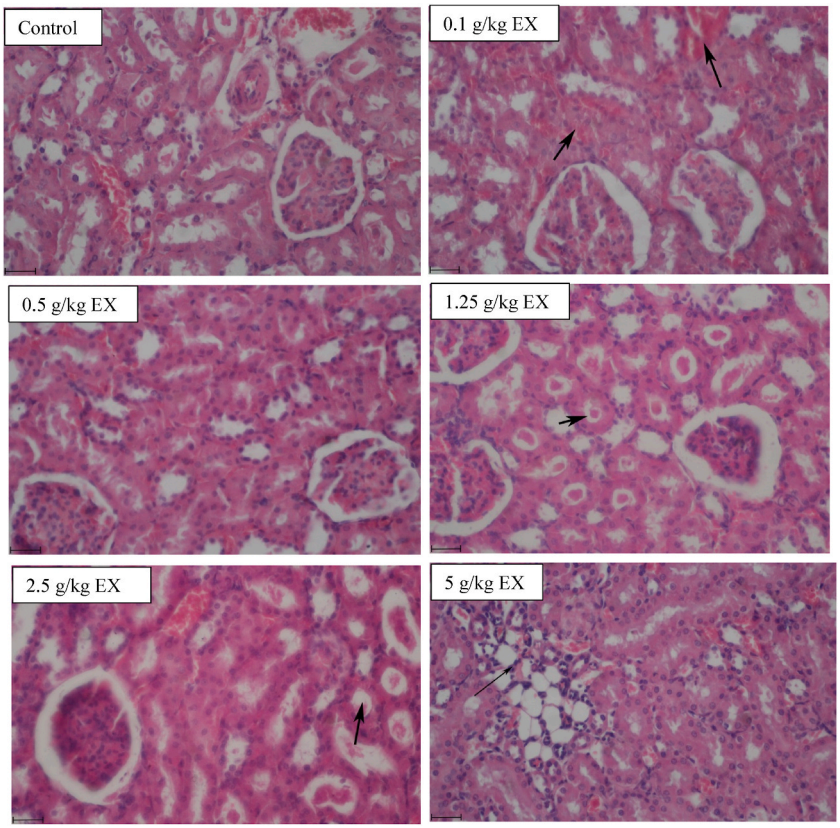

Fig. 4. Histopathological changes of the kidneys of rats following treatment with different doses of the aqueous extract of Zaravand Gerd for 21 days. Brief dilation of the proximal convoluted tubules and scattered inflammatory cells was found with 1.25, 2.5 and 5 g/kg of Zaravand Gerd extract. As shown in Fig. 4, there are inflammatory cells surrounding the fat cells in kidneys of animals which treated with 5 g/kg of Zaravand Gerd extract. (EX: extract, 1.1, 5.1, 1.25, 2.5 and 5 g/kg of different concentrations of the extract) (scale bar: 25 μm).

Table 3
Histopathology scoring of cortical damage in rats treated with different doses of Zaravand Gerd.

Treatment groups	Control	0.1 g/kg EX	0.5 g/kg EX	1.25 g/kg EX	2.5 g/kg EX	5 g/kg EX
Kidney pathological score	0	0	0	1	2	4

Table 4
Comparison of rats treated with different doses of Zaravand Gerd in terms of EGTI scoring.

Treatment groups	Control	0.1 g/kg EX	0.5 g/kg EX	1.25 g/kg EX	2.5 g/kg EX	5 g/kg EX
Tubular	0	0	0	1	1	3
Endothelial	0	0	0	1	1	2
Glomerular	0	0	0	0	0	0
Tubulo/Interstitial	0	0	0	1	1	3

($P < 0.05$) in mitochondrial swelling in animals exposed to 2.5 and 5 g/kg of the extract in comparison to the control group. However, no significant ($P < 0.05$) alteration in mitochondrial swelling was observed in rats that received 0.1, 0.5 or 1.25 g/kg of the aqueous extract of Zaravand Gerd for a duration of 21 days when compared to the control group.

3.8. Mitochondrial ROS formation

Generation of mitochondrial ROS mainly takes place at the electron transport chain located on the inner mitochondrial membrane during the process of oxidative phosphorylation. The impact of the aqueous extract of Zaravand Gerd on the formation of Reactive Oxygen Species (ROS) in renal mitochondria is demonstrated in Fig. 7. Notably, a significant ($P < 0.05$) increase in DCF fluorescence intensity, serving as an indicator of ROS generation, was observed in rats treated with aqueous extract of Zaravand Gerd. 2.5 and 5 g/

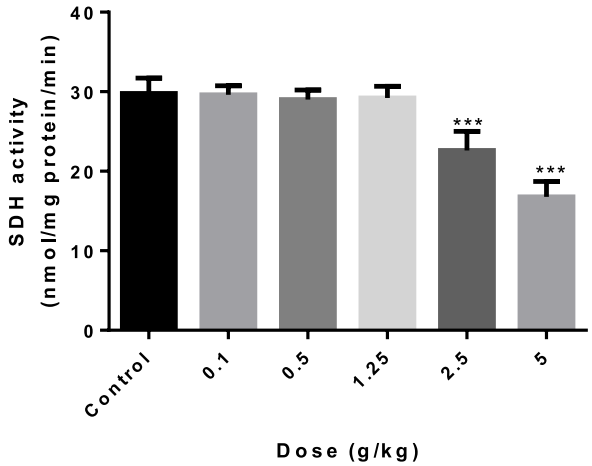

Fig. 5. SDH activity in renal mitochondria following treatment of rats with different doses of Zaravand Gerd for 21 days. Aqueous extract of Zaravand Gerd significantly ($P < 0.05$) reduced mitochondrial SDH activity at doses higher than 1.25 g/kg in a dose-dependent manner. ***$P < 0.001$.

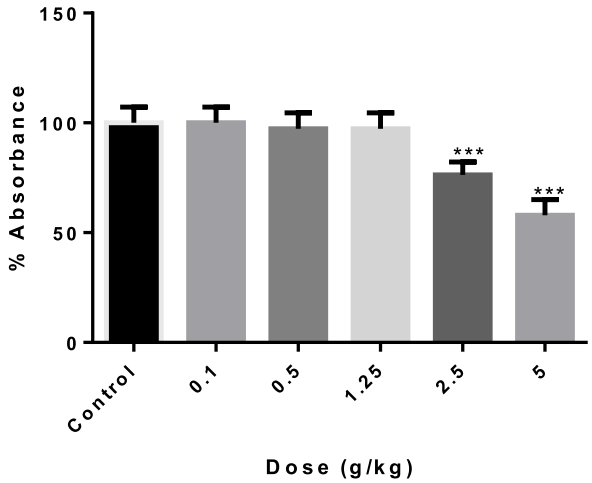

Fig. 6. Mitochondrial swelling in kidneys of rats following treatment with aqueous extract of Zaravand Gerd for 21 days. 2.5 and 5 g/kg of Zaravand Gerd extract induced significant ($P < 0.05$) swelling in kidneys mitochondria in comparison with the control group. ***$P < 0.001$.

kg of Zaravand Gerd extract caused a significant ($P < 0.05$) increase in mitochondrial ROS formation when compared with the control group.

3.9. Mitochondrial membrane potential (MMP) collapse

Mitochondrial membrane potential was measured to investigate the effect of extract on capacity of mitochondria to maintain an electrochemical gradient required to produce ATP. According to the findings shown in Fig. 8, the aqueous extract of Zaravand Gerd was found to induce a dose-dependent collapse in the mitochondrial membrane potential (MMP) in rats. After treating the rats with different doses of the extract for a period of 21 days, it was observed that the collapse of MMP was not significant ($P < 0.05$) in rats treated with doses of 0.1, 0.5, and 1.25 g/kg of the aqueous extract. However, higher doses of the extract (2.5, and 5 g/kg) caused a statistically significant ($P < 0.05$) collapse in the MMP of the treated animals compared to the control group.

4. Discussion

The *Aristolochia* genus is known to contain a toxic compound called aristolochic acid, which has been associated with reported adverse effects [6]. However, there is currently a lack of research addressing the potential renal toxicity of Zaravand Gerd, a widely used herbal medicine in Iran's traditional medicine and pharmacy. Notably, in the Iranian herbal Pharmacopoeia, none of the herbs originating from the *Aristolochia* genus have been accepted [20]. In the context of Iranian traditional medicine, the recommended

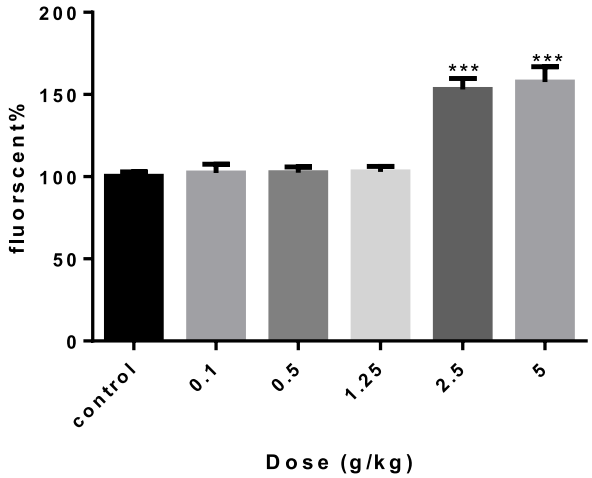

Fig. 7. ROS generation in renal mitochondria after administration of aqueous extract of Zaravand Gerd to animals for 21 days. Dichlorofluorescein diacetate (DCFH-DA) and fluorescence spectrophotometer were used for determination of ROS production in renal mitochondria. Generation of ROS was significant ($P < 0.05$) with 2.5 and 5 g/kg of Zaravand Gerd extract when compared with the control group. ***$P < 0.001$.

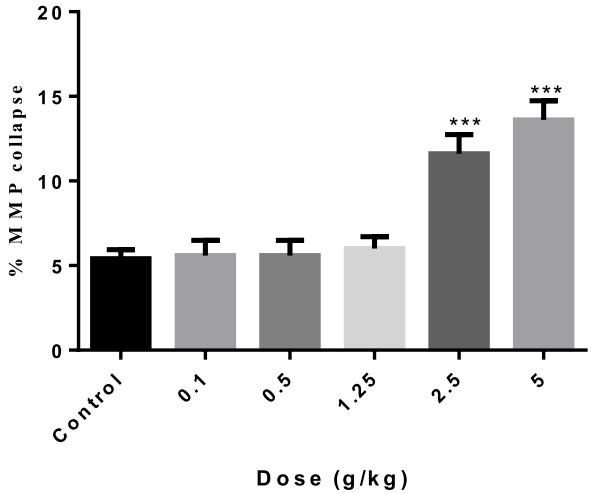

Fig. 8. Collapse of mitochondrial membrane potential (MMP) in rat's kidneys following administration of aqueous extract of Zaravand Gerd for 21 days. Zaravand Gerd significantly ($P < 0.05$) decreased mitochondrial membrane potential at doses of 2.5 and 5 g/kg *$P < 0.05$ and ***$P < 0.001$.

dosage for Zaravand Gerd usage is approximately 5–7 g for an individual weighing 70 kg [21]. In this study, we have selected a dose of 0.1 g/kg, in accordance with traditional Iranian medicine recommendations. Additionally, we have included higher doses, up to 5 g/kg, in order to determine both the highest safe dose and the lowest toxic dose in a 21-day repeated-dose toxicity study. Our results demonstrate that the elevated dosages of the Zaravand Gerd extract (specifically, doses of 2.5, and 5 g/kg) have the potential to cause adverse effects on the renal system of laboratory rats. However, the doses of Zaravand Gerd which is used in Iranian traditional medicinal practices did not show any observable renal toxicity.

The measurement of kidney function and early detection of kidney diseases relies heavily on the results of blood tests. Elevated levels of creatinine and urea in the blood can be indicative of impaired kidney function in eliminating toxins from the body. The results of an animal study demonstrated that intraperitoneal injection of aristolochic acid at a dose of 15 mg/kg for 3 days led to a significant increase in BUN and blood creatinine levels in C57BL/6 mice [22]. In a study by Liang et al. the nephrotoxic effects of *Aristolochia fangchi* extract were investigated. The oral administration of this plant extract at a dosage of 8 mg/kg for a duration of 4 weeks resulted in a significant increase in BUN and creatinine levels compared to the control group [23]. Similarly, Liu et al. conducted a study examining the effects of *Aristolochia manshuriensis* alcoholic extract. They found that the oral administration of this extract at a dosage of 4 mg/kg for a period of 14 days led to a significant increase in blood creatinine and urea levels [24]. Contrarily, in the present study, various doses of the aqueous extract of Zaravand Gerd did not show a significant impact on BUN and serum creatinine levels when compared to the control group. These results could be attributed to the very low concentrations of aristolochic acid present in the Zeravand Gerd extract. It is important to note that the evaluation of kidney toxicity cannot rely solely on BUN and creatinine levels, as

they may not change significantly during the initial stages of kidney toxicity, as highlighted in various other studies [25].

A previous study reported that the administration of 4 g/day of *Aristolochia manshuriensis* for 5 days resulted in glucosuria in the treated animals [24]. Additionally, it has been observed that the administration of 50 and 100 mg/kg of arasticholic acid for 3 days caused kidney damage and a significant increase in the levels of glucose and protein in the urine [26]. In accordance with these previous investigations, the current study investigated the effects of zaravand Gerd extract on urine glucose levels in treated animals. Interestingly, the results indicated a significant elevation in urine glucose levels at doses of 2.5, and 5 g/kg of zaravand Gerd extract compared to the control group.

Urine protein analysis is a classic test to assess the nephrotoxicity of chemicals in laboratory animals. Researchers have investigated the nephrotoxicity of *Aristolochia fangchi* extract and demonstrated a substantial increase in urine protein levels following oral administration of 8 mg/kg of the extract for 4 weeks [23]. Additionally, another study showed that proteinuria resulted from the oral administration of 4 mg/day of the alcoholic extract of *Aristolochia manshuriensis* over a 5-day period [24]. In alignment with these prior investigations, our study found that the administration of Zarvand Gerd extract led to a significant elevation in urine protein levels. Notably, this increase was observed at doses of 1.25, 2.5, and 5 mg/kg. Proteinuria, as an outcome, arises from two underlying mechanisms: firstly, the abnormal passage of proteins through the glomerulus, attributed to increased permeability of the glomerular capillary wall; and secondly, subsequent disruption of protein reabsorption by epithelial cells within the proximal tubules [27]. It is important to note that assessments relying solely on total protein determinations offer limited insight into the nature and location of the renal lesion. Therefore, we emphasize the necessity of employing electrophoretic or chromatographic techniques for urinary protein analysis. These methods are crucial in enhancing our comprehension of the nature and localization of renal damage [28].

Several studies have investigated the effects of aristolochic acid and *Aristolochia* genus extract on kidney tissues in experimental animals. Intraperitoneal injection of aristolochic acid at a dose of 15 mg/kg for 3 days resulted in a significant increase in the filtration of inflammatory cells and atrophy of tubules in C57BL/6 mice [22]. Another study found that the administration of 10, 50, and 100 mg/kg of aristolochic acid for 3 days induced necrosis of kidney tubule epithelium cells [26]. Additionally, oral administration of *Aristolochia manshuriensis* alcoholic extract for 14 days led to kidney tissue damage, necrosis, and apoptosis of tubule epithelial cells [24]. In the present study, histopathological assessments of kidney tissue were conducted following the administration of Zarvand Gerd extract. It was observed that doses of 0.1 g/kg and 0.5 g/kg did not cause any pathological changes. However, at doses of 1.25 g/kg and 2.5 g/kg, brief dilation of the proximal convoluted tubules was detected in the kidney tissue. Notably, at a dose of 5 g/kg of Zarvand Gerd extract, both the filtration of inflammatory cells and the dilatation of the proximal convoluted tubule were observed. This expansion of the lumen was attributed to the reduction of microvilli in the epithelial cells of the proximal convoluted tubule, as depicted in Fig. 4.

Renal cells possess various functions that collectively establish the kidney as one of the most energy-demanding organs in the human body. This high energy demand is primarily met through the process of adenosine triphosphate (ATP) production, and the kidney's reliance on ATP underscores the presence of a substantial number of mitochondria within its cellular architecture, particularly within the proximal tubule cells [29,30]. It is well-established in previous research that mitochondria within renal cells constitute a crucial target for damage induced by toxins or toxicants, leading to a spectrum of renal impairments, including nephrotoxicity, acute renal damage and chronic renal damage [31,32]. Adverse effects on kidney mitochondria can result in the failure of the electron transport chain, a decline in ATP production, the generation of oxidative stress and ultimately, cell death [33,34]. The present study focused on the impact of Zarvand Gerd extract on renal health. The results revealed that this extract led to renal toxicity through several mechanisms, including a reduction in succinate dehydrogenase (SDH) activity, the collapse of mitochondrial membrane potential, mitochondrial swelling and the generation of reactive oxygen species (ROS), which manifested in biochemical and histopathological alterations as ultimate outcome. In alignment with our findings, recent researches have also highlighted the significant role of mitochondria in the toxic effects of aristolochic acid on various kidney cell lines, including HK-2, NRK-52E and cultured human kidney cells. This further underscores the notion that mitochondrial dysfunction may be a key contributor to aristolochic acid-induced nephrotoxicity [35,36,and37]].

In a study conducted by Mengs and Stotzem in 1993, the renal toxicity of Aristolochic Acid (AA I and AA II) in rats was investigated. They found that administering a single oral dose of 100 mg/kg of AA resulted in widespread necrosis affecting nearly all nephrons in the rats' kidneys. However, no necrotic lesions were observed when a lower dose of 10 mg/kg was administered [38]. A 4-week toxicity study with AA in rats demonstrated that an oral dose of 25.0 mg/kg of AA led to degenerative lesions primarily in the kidneys, urinary bladder, and testes. Notably, a non-toxic effect level was identified at 0.2 mg/kg of AA [39]. In the present study, it was determined that the content of both AA I and AA II in Zaravand Gerd was relatively low. However, the non-toxic level of AA which was found to be 0.2 mg/kg, equates to approximately 1.54 g/kg of Zaravand Gerd in this study. No toxicity signs were observed at doses of 0.1 g/kg and 0.5 g/kg, while moderate signs of toxicity were found at doses of 1.25 g/kg and 2.5 g/kg of the Zaravand Gerd extract. Interestingly, a dose of 5 g/kg, which corresponded to approximately 0.65 mg/kg of AA, caused significant biochemical and pathological changes in the treated animals. These results suggest the safety of the recommended doses for Zaravand Gerd in Iranian traditional medicine. However, it's important to note that natural compounds in plants can vary significantly, even within different regions of Iran. Therefore, further investigations are needed to assess the AA content in Zaravand Gerd across various geographic locations. This scientific approach helps provide a clearer understanding of the research findings regarding the renal toxicity of AA in rats and its implications for traditional medicine.

5. Conclusion

According to the findings of this research, it can be concluded that elevated dosages of the Zaravand Gerd extract (specifically,

doses of 2.5, and 5 g/kg) exhibit the potential to induce adverse effects on the renal system of laboratory rats. Conversely, these results also suggest the absence of any observable renal toxicity when considering the customary doses of Zaravand Gerd employed in traditional medicinal practices. However, it's imperative to acknowledge that the scope of our conclusions should be limited to the data provided within the confines of this study. To definitively assert the non-toxic nature of the doses employed in traditional medicine, it is incumbent upon us to undertake additional investigations. In conclusion, while this study offers valuable insights into the potential nephrotoxicity of Zaravand Gerd, it is imperative to conduct further research encompassing different animal species, in vitro models and the analysis of specific compounds such as aristolochic acid to provide a more comprehensive understanding of its safety and potential medicinal applications.

Data availability statement

Data supporting this study are included within the article and/or supporting materials.

CRediT authorship contribution statement

Zohreh Abolhasanzadeh: Project administration, Writing – review & editing. **Simin Ansari:** Investigation, Writing – original draft. **Zahra Lorigooini:** Data curation, Software. **Maryam Anjomshoa:** Conceptualization, Investigation. **Elham Bijad:** Investigation. **Pantea Ramezan Nejad:** Formal analysis, Resources. **Mohammad Hadi Zarei:** Conceptualization, Project administration, Supervision.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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