Phytochemical analysis, antioxidant activity, and nephroprotective effect of the *Raphanus sativus* aqueous extract

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Abstract: In Morocco, Raphanus sativus is a widespread traditional medicinal plant used to treat various kidney diseases such as nephropathy.

Objective: The present study aims to evaluate the protective effect of the *R. sativus* aqueous extract against the gentamicin-induced acute nephrotoxicity in rats.

Methods: Rats were randomly separated into four groups (n=6; ♂/♀=1). The control group was treated only with distilled water (10 mL/kg; *p.o*). The gentamicin group was treated with distilled water (10 mL/kg; *p.o*) and injected intraperitoneally by the gentamicin (80 mg/kg; *i.p*). *R. sativus* groups were treated with the aqueous extracts of this plant at a dose of (200 or 400 mg/kg; *p.o*) and injected by the gentamicin (80 mg/kg; *i.p*). The plasma Creatinine, Urea, Uric Acid, Albumin, Total Protein, Alanine transaminase, Aspartate transaminase, and Calcium levels were measured. The urinary creatinine, urinary Calcium, urinary volume, water intake, creatinine clearance, body weight gain, relative right kidney weight, and kidney malondialdehydes were determined.

Results: This study showed that the daily pretreatment with *R. sativus* aqueous extract at two doses of 200 and 400 mg/kg, p.o prevented the rats from the gentamicin-induced nephrotoxicity. Moreover, the *R. sativus* aqueous extract showed a high amount of polyphenols and flavonoids and a significant antioxidant activity.

Conclusion: *R. sativus* aqueous extract, as a novel natural product, may have preventive properties against gentamicin-induced nephrotoxicity in rats. The present study describes new areas of investigation to introduce better therapeutic agents for renal disorders and dysfunction.

Keywords: *Raphanus sativus*; nephrotoxicity; gentamicin; rats.

1. Introduction

Nephrotoxicity is one of the most prevalent kidney problems among all populations, caused by many substances, such as toxic chemicals and medicines [1,2]. Many environmental xenobiotics, heavy metals, drugs, and chemicals influence the kidneys function [3,4].

Renal failure is quite often associated with sepsis, diabetes, and cardiovascular diseases [5]. Latest studies have documented that oxidative stress is highly prevalent in patients with renal disease [6]. Drugs are known to cause nephrotoxicity, and it exerts their toxic effects by one or more common pathogenic mechanisms. Drug-induced nephrotoxicity appears to be more frequent in some patients and particular clinical conditions [7]. Many antibiotics, including penicillins, tetracyclines, and aminoglycosides, can adversely affect kidney function [8–10].

Gentamicin (GM) is an aminoglycoside used to treat Gram-negative bacterial infections in particular infections of the abdomen and urinary tract [11]. However, its use is restricted because of the development of nephrotoxicity [12]. Nephrotoxicity has been related to a selective accumulation of gentamicin in the renal cortex [13]. GM causes renal morphological changes and an overall syndrome very similar in humans and experimental animals [14]. Although many hypotheses have been proposed and tested, the exact mechanisms of GM-induced nephrotoxicity remain unclear. It has been shown that the GM nephrotoxicity involves the renal free radical generation, reduction in antioxidant defense mechanisms, alterations include degenerative

changes (e.g., focal necrosis and apoptosis), and an increase of monocyte/macrophages infiltration [15,16]. GM nephrotoxicity leads to decreased urine concentration capacity, tubular proteinuria, lysosomal enzymatic, mild glucosuria, decreased ammonium excretion, and lower glomerular filtration rate (GFR) [7]. Many researchers link these mechanisms to increased peroxidation of membrane lipids [17], protein denaturation, and DNA damage by generating reactive oxygen species (ROS) [4]. Several experiments have shown that therapy using natural antioxidants reduces the nephrotoxicity of GM in rats [18,19].

R. sativus belonging to the *Cruciferae* family originated in Europe and Asia; it contains many essential vegetables of economic importance. It grows in temperate climates at altitudes ranging from 190 to 1240 m [20]. It is commonly used in folk medicine to treat various diseases such as gall bladder trouble, diabetes, hepatitis, and gastrointestinal disorders [20,21]. Furthermore, *R. sativus* was tested for its antiurolithiatic and diuretic effect [22,23]. The leaves and roots of *R. sativus* were reported to possess various biological activities like antioxidants [24], hepatoprotective [25], antifungal [26], antiurolithiatic [27], cardioprotective [28], and gastrodynic pains [28]. So far, limited studies exist concerning the beneficial effects of *R. sativus* against GM-induced kidney damage. Therefore, this research aimed to investigate the nephroprotective effect of *R. sativus* aqueous extract (RSAqEx) against GM-induced renal damage in rats.

2. Experimental

2.1. Chemicals and reagents

GM ampoules (80 mg/2 mL, IM) were purchased from the pharmacy (Cooper Pharma Company, Morocco). Alanine aminotransferase (ALT), Aspartate aminotransferase (AST), Albumin, Total Protein, Urea, plasma Calcium, plasma Creatinine, and Urine Creatinine kits were purchased from Biosystems, Spain. All other chemicals and reagents used in this study were of high quality and analytical grade.

2.2. Plant material

The plant has been obtained from a local market in Oujda, Morocco. The plant was taxonomically identified, and voucher specimen HUMPOM457 was deposited at the Faculty of Sciences, University Mohammed First, Oujda, Morocco.

2.3. Aqueous extract preparation

Plant material was prepared according to the traditional technique used by the Moroccan population. The fresh plant was ground with a blender and decoct in water. The mixture was then filtered, and the filtrate was air-dried at 40°C to get the extract in powder form. After that, the crude extract was stored at 4°C until analysis.

2.4. Total polyphenols quantification

The amount of the total phenols of RSAqEx was determined by the Folin-Ciocalteau method with some modifications [29]. Briefly, 200 µL aliquots of RSAqEx (1000 µg/mL) were mixed with 1000 µL Folin-Ciocalteu reagent and 800 µL (75 g/L) sodium carbonate (Na_2CO_3). The mixture was incubated for 1 hour, and the absorbance was measured at 765 nm against methanol as blank. A standard curve was plotted using gallic acid as a standard. For the preparation of calibration curve, methanolic gallic acid solutions (0.78; 1.56; 3.12; 6.25; 12.5; 25; and 50 µg/mL) were used. All samples were performed in triplicate. Data for the total phenol amount was presented as mg gallic acid equivalents (GAE) per mg dry matter (DM).

2.5. Flavonoids quantification

The total flavonoids were assessed according to the method described by Kim et *al.* (2003), with some modifications [30]. In brief, 200 µL of RSAqEx (1000 µg/mL), 1000 µL distilled water, and 50 µL of $NaNO_2$ (5 %) were mixed. After 6 min, 120 µL of AlCl3 (10 %) was added, followed by 5 min of incubation. Then, 400 µL of NaOH (1M) and 230 µL of distilled water were added to the mixture. The calibration curve was made using quercetin standard solution with different concentrations (1.56; 3.12; 6.25; 12.5; 25; 50 and 100 µg/mL). The absorbance was measured at 510 nm using methanol as blank. All trials were performed in triplicate. The data for the concentration of total flavonoids was expressed as quercetin equivalents (QE) per mg dry matter (DM).

2.6. Antioxidant assessment

The 2,2-diphenyl-1-picryl-hydrazyl (DPPH) scavenging activity was measured according to the method described by Bouhrim et *al.* (2020) [31], with some modifications. Briefly, 2.5 mL of methanolic solution of the free radical DPPH (4%) was added to 0.1 mL of increased concentrations of RSAqEx (0.62; 1.25; 5; 10; 20; 40; 80 and 120 µg/mL). The reaction mixture was incubated for 30 min in obscurity at room temperature, and the absorbance was measured at 517 nm. Ascorbic acid was used as a standard reference. Methanol was used as a negative control. Redundant testing was performed three times with each sample (n=3). The scavenging activity of the samples was calculated according to the following formula:

DPPH scavenging effect (%)=$[1-(Abs_0 - Abs_1)/Abs_0)] \times 100$

Abs0 is the absorbance of the control reaction, and Abs1 is the absorbance of all of the extract samples and standard.

The antioxidant activity is expressed as IC_{50} values (µg/mL of *R. sativus*).

2.7. Animals

Twenty-eight *Wistar* rats (M/F=1) weighing between 170 and 280g and twenty-four Swiss albino mice weighing between 20 and 30g were obtained from the animal house, Faculty of science, University Mohammed First, Oujda (Morocco). Animals were acclimatized for 15 days before the treatment and housed 6 per cage with food freely available and water ad libitum and maintained under a 12-h light/dark cycle (light on 07:30 – 19:30 h) and a constant temperature (23 ±1°C).

2.8. Acute toxicity study of *R. sativus* extract in mice

The single-dose acute oral toxicity study was evaluated following the recommendations by OECD Guidelines (425) [32]. Acute toxicity test was carried out in healthy Swiss albino mice, weighing 20–28g each, using a single dose, administered by oral gavage. All animals have fasted overnight, with water *ad libitum*. The animals were randomly divided into 4 experimental groups. Each group of 6 mice (M/F=1) received, respectively, a single oral dose of 1000, 3000, or 5000 mg/kg body weight of RSAqEx, while the control group was treated with distilled water. The general behavior of mice and signs of toxicity were observed continuously for 1h after the oral treatment, and then intermittently for 4h and after that throughout 24h [33]. The mice were further observed once a day up to 14 days following behavioral changes and toxicity and/or death signs. During this study, the mice's body weight was measured and recorded on days 1, 7, and 14. At the end of the 14th day, the Median Lethal Dose (LD_{50}) was calculated according to MILLER and TRAINER (1944) [34].

2.9. Experimental procedure

All experiments were executed following the internationally accepted Guide for the Care and Use of Laboratory Animals, published by the US National Institutes of Health (NIH publication no. 85-23, revised in 1985). The animals were divided into four groups, with each group consisting of six rats. Group 1; received vehicle (distilled water; *p.o*) for 14 days consecutively (control group). Group 2; received GM (80 mg/kg; *i.p*) intraperitoneally for 14 days consecutively (gentamicin group). Groups 3 and 4; received GM (80 mg/kg; i.p) and RSAqEx with doses of 200 and 400 mg/kg simultaneously; *p.o* administered orally for 14 days.

2.10. Biochemical parameters

Several biochemical parameters have been measured in urine and plasma: albumin by the Bromocresol Green method [35], Calcium by the NM-BAPTA method [36], urea by the enzymatic process [37], creatinine by Jaffe method [38], AST and ALT by IFCC method without pyridoxal-5-phosphate [39], and protein by Biuret method [40], Uric acid by the enzymatic colorimetric method [41]. All tests were performed with the COBAS INTEGRA® 400-Plus analyzer.

2.11. Determination of creatinine clearance

Glomerular filtration was assessed by creatinine clearance relying on serum and urinary creatinine levels, with values expressed in mL/min, calculated using the following formula:

Creatinine Clearance (mL/min) = Urine creatinine/ Serum creatinine

Urine flow was computed dividing 24 hours of urine volume by 1,440, which corresponding to the number of minutes in 24 hours (60 min x 24h = 1,440):

Urine flow (mL/min) = value of urine volume (24h)/1,440.

2.12. Relative kidney weight

On the 14th day of the experiment, the fasting rats and the live body weight (g) were recorded on the 15th day before euthanasia. The kidneys were separated and weighed (g) (absolute renal weight) against every animal's body mass.

Relative kidney weight (g) = (Absolute kidney weight (g)/Bodyweight of the rat on sacrifice day (g)) x100

2.13. Determination of MDA

Lipid peroxidation was evaluated by the TBARS assay using Ohkawa's method [42]. Kidneys were cutout, weighed, and homogenized. 0.5 mL of this homogenate was added to 0.5 mL of trichloroacetic acid (30%) and then centrifuged for 10 minutes (3500 rpm) at 4°C. Then, 1 mL of the supernatant obtained after centrifugation was homogenized with 1 mL of thiobarbituric acid (0.67%) and placed in boiled water at 100°C for 10 minutes and then immersed in ice to stop the reaction. The absorbance has been read at 532nm using a spectrophotometer. Results were expressed in nmoles of MDA kidney produced per mg of tissue at 37°C, using the molar above extinction coefficient:

$1.56 \times 10^5 \, M^{-1}.cm^{-1}$

2.14. Statistical analysis

Data expressions were presented as means ± Standard Error of Measurement and were analyzed statistically by ANOVA (One-way-analysis of variance with Tukey post hoc test). $p < 0.05$, $p < 0.01$ and $p < 0.001$ were considered statistically to be significant.

3. Results and Discussion

3.1. Total phenols and flavonoids quantities

The determination of the total polyphenols and flavonoids in RSAqEx was studied using the Folin-Ciocalteu and aluminum trichloride methods. The results showed that the complete phenol content of

RSAqEx was 320.20 ± 39.08 µgEAG/mg DM and the flavonoid content was 114.29 ± 4.79 µgEQ/mg DM (Table 1). These results were consistent with data from Goyeneche et *al.* (2015), which showed that the total polyphenol and flavonoid contents of *R. sativus* methanolic extract were 3.41 ± 0.06 µgEAG/mg and 2.67 ± 0.06 mg EQ/mg respectively [43].

Table 1. Polyphenols and flavonoids contents of RSAqEx.

	Total polyphenols (mg EAG/mg DM)	Flavonoids (mg EQ/mg DM)
RSAqEx	320.20 ± 39.08	114.29 ± 4.79

3.2. Antioxidant activity

Figure 1 shows the RSAqEx (A) antioxidant capacity and ascorbic acid (B). The results revealed a moderate free radical scavenging capacity of RSAqEx with an IC_{50} equal to 30.04 ± 0.07 µg/mL, which is still lower compared to the ascorbic acid (IC_{50} =3.36 ± 0.09 µg/ml). However, the methanolic extract of *R. sativus* showed a significant ferric reducing ability, a moderate metal chelating activity, and a strong radical scavenging activity [44,45]. The polyphenols and flavonoids found in the *R. sativus* aqueous extract could be responsible for this antioxidant activity.
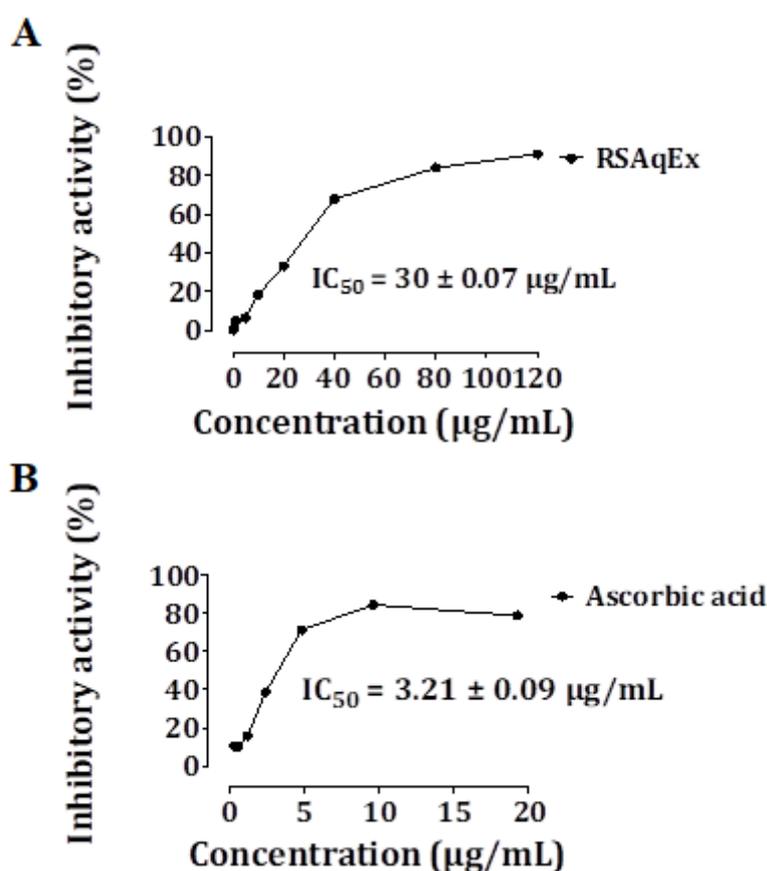

Figure 1. DPPH Free radical scavenging activity of RSAqEx **(A)** and ascorbic acid **(B)**

3.3. Acute toxicity

In the sighting study, the animals were monitored for 14 consecutive days following single oral administration of RSAqEx at 1000, 3000, and 5000 mg/kg, and no deaths were observed. The animals did not show any symptoms of toxicity during the period of study. The animals' body weight was not significantly different between days 1, 7, and 14 (data are not shown). Moreover, the LD_{50} values for the oral administration of RSAqEx were greater than 5000 mg/kg. According to Hodge and Sterner's (2005) classification, the tested RSAqEx falls in the fifth class (substance with LD_{50} higher than 5000 mg/kg body weight) and is considered practically non-toxic [46].

3.4. Effect of RSAqEx on the water intake and urine volume

In this study, the effect of RSAqEx on water intake and urinary volume for the rats exposed to GM was evaluated (Figure 2). No significant increase in the water intake and a significant (p ≤0.05) increase in the urinary volume were observed in the rats exposed to GM than the control group. However, the administration of RSAqEx at doses of 200 and 400 mg/Kg concomitantly with GM produced a no

significant decrease in water intake and a substantial decrease of urinary volume in the highest dose. This could be explained by the fact that GM accumulates in the renal tissue, leads to the loss of proximal

tubular cells and Henle's loop to reabsorb water, which causes dehydration and increased urinary volume. Therefore an increase in water intake is necessary for compensation [47].
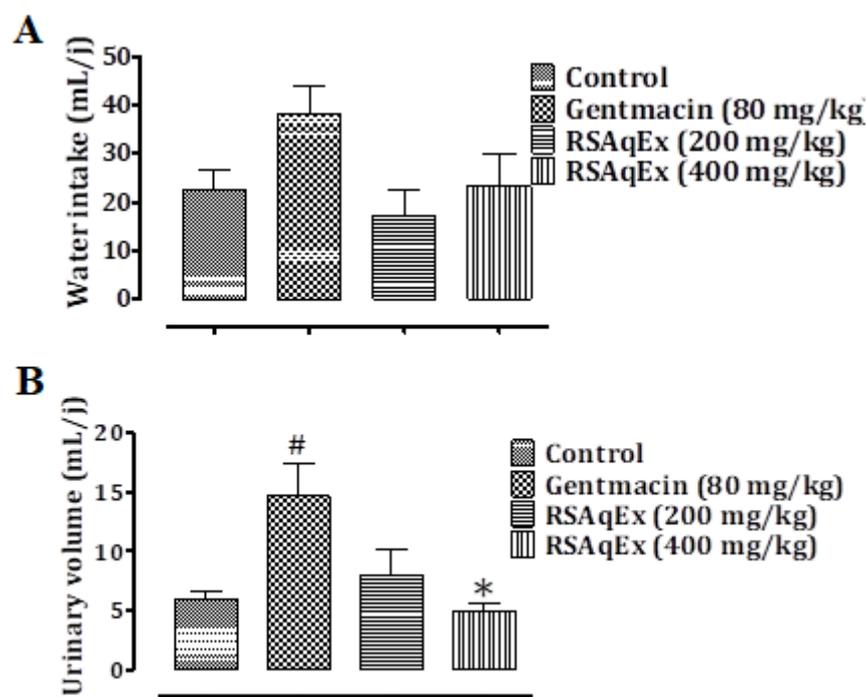

Figure 2. Effect of *Raphanus sativus* aqueous extract on water intake (A) and urinary volume (B) in GM exposed rats. Data are mean ± SEM, n=6. # P≤0.05 versus the control group. *P<0.05versus GM group

3.5. Effect of RSAqEx on variation in relative right kidney weight and body weight gain

The effect of RSAqEx on weight gain and relative kidney weight in GM-poisoned rats was presented in Figure 3. Daily intraperitoneal shot of the rats by the GM (80 mg/kg; i.p), produced a significant decrease (p < 0.05) in body weight gain and increase (p< 0.05) in relative kidney weight, compared to normal control rats. However, the daily pretreatment of the rats by the RSAqEx at doses 200 and 400 mg/Kg, 3 hours before the injection of the GM, was induced a no-significant increase in body weight gain and a no-significant diminish in the relative weight of the kidneys, compared to the rats exposed only to GM (80 mg/kg; i.p). These results confirm the results of other work already published [47,48]. This could be due to the GM's accumulation in the kidneys, resulting in swelling of the kidneys and reduced dietary intake, thus body weight due to kidney injury [47]. Besides, GM's accumulation in the kidney tissue leads to the loss of tubular cells to reabsorb the water, which causes dehydration and, therefore, loss of body weight [47]. After treatment with GM, inflammation and edema may be responsible for increasing his kidneys' relative weight indices [49]. Therefore, our extract's effect on

the kidneys' relative weight may be due to our plant's anti-inflammatory impact.

3.6. Effect of RSAqEx on the plasma and urinary Calcium

In the present study, the daily intraperitoneal injection of GM (80 mg/kg; i.p) in the experimental rats caused a significant increase (p<0.05) in the plasma level of Calcium and a decrease in the level of urinary Calcium in a not significant manner, compared to the rats in the control group (Figure 4). On the other hand, administering RSAqEx to rats exposed to GM resulted in a reduction in plasma calcium concentration and an increase in calcium concentration in the urine. The reversal of GM's deleterious effect on the plasma calcium concentration was significant (p< 0.01) at the dose of 400 mg/kg and was not significant for urinary calcium concentration. This effect can be explained by gentamicin's action on the membrane supports of both the brush border and the basolateral membranes leading to electrolyte abnormalities [47]. Since the nephrotoxicity caused by reactive oxygen species generated by gentamicin injection, it appeared that the nephroprotective effect of RSAqEx on gentamicin-induced renal damage might be due to the antioxidant activity of RSAqEx.
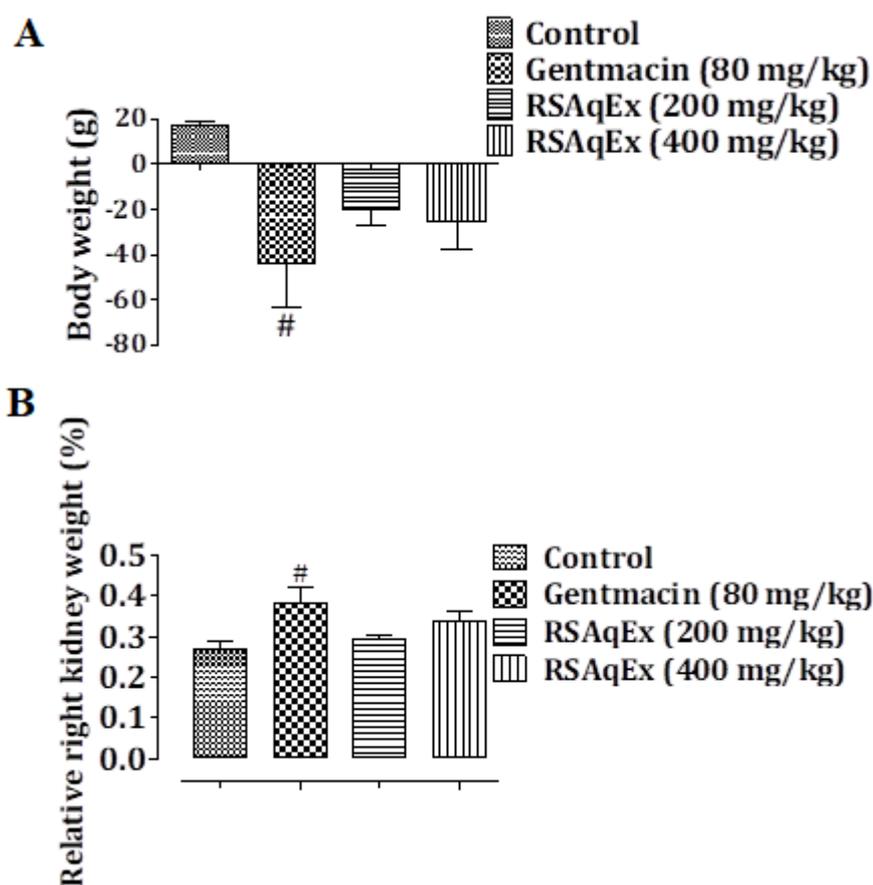
Figure 3. Effect of the RSAqEx on variation in body weight gain (A) and relative kidney weight (B) in GM exposed rats. Data are mean ± SEM, n=6. # P≤0.05 versus the control group. *P<0.05 versus GM group
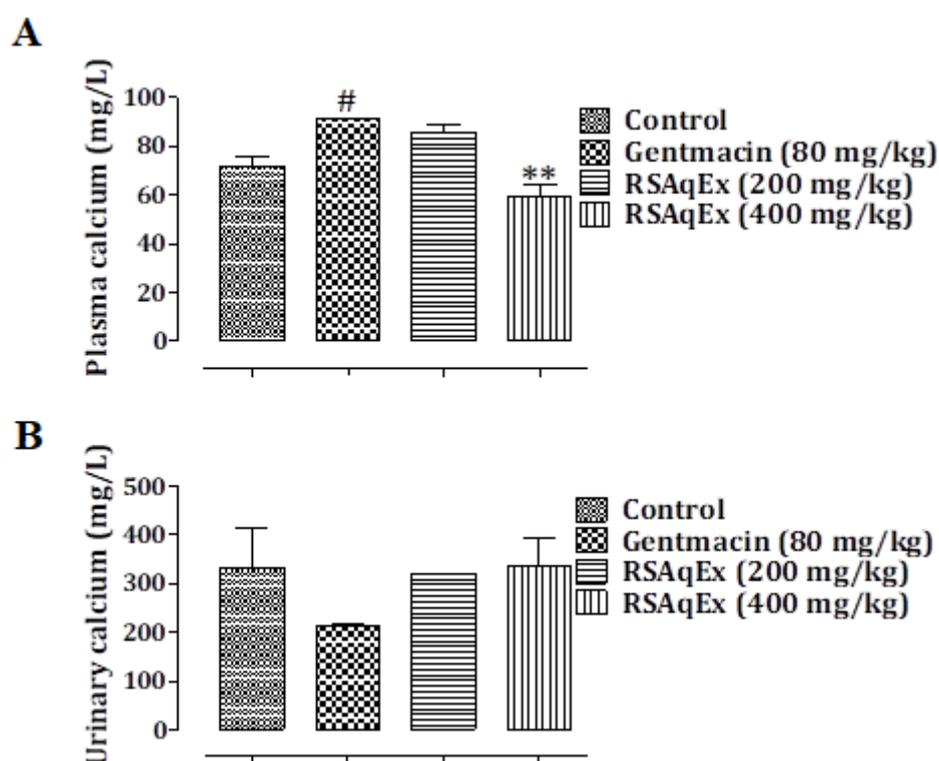

Figure 4. Effect of the RSAqEx on the plasma (A) and urinary (B) calcium in GM exposed rats. Data are mean ± SEM, n=6. # P≤0.05 versus a control group. **P<0.01 versus GM group

3.7. Effect of RSAqEx on plasma urea, plasma creatinine, urine creatinine, and creatinine clearance

The effect of various doses of RSAqEx on plasma urea in GM intoxicated rats was showed in Figure 5. Results showed that intraperitoneal injection of GM (80mg/kg; i.p) to the rats increased urea plasma levels significantly compared with the control group. These results were consisted of the results of Erdem et al. (2000) [50], indicating altered glomeruli and tubular functions in the rats. Furthermore, there was no significant decrease in plasma levels of urea in rats treated with RSAqEx at doses 200 and 400 mg/kg compared with the GM group. The effect of RSAqEx could be due to the antioxidant properties of its polyphenols and flavonoids compounds [50]. And maybe using a high dose of RSAqEx can produce significant results.
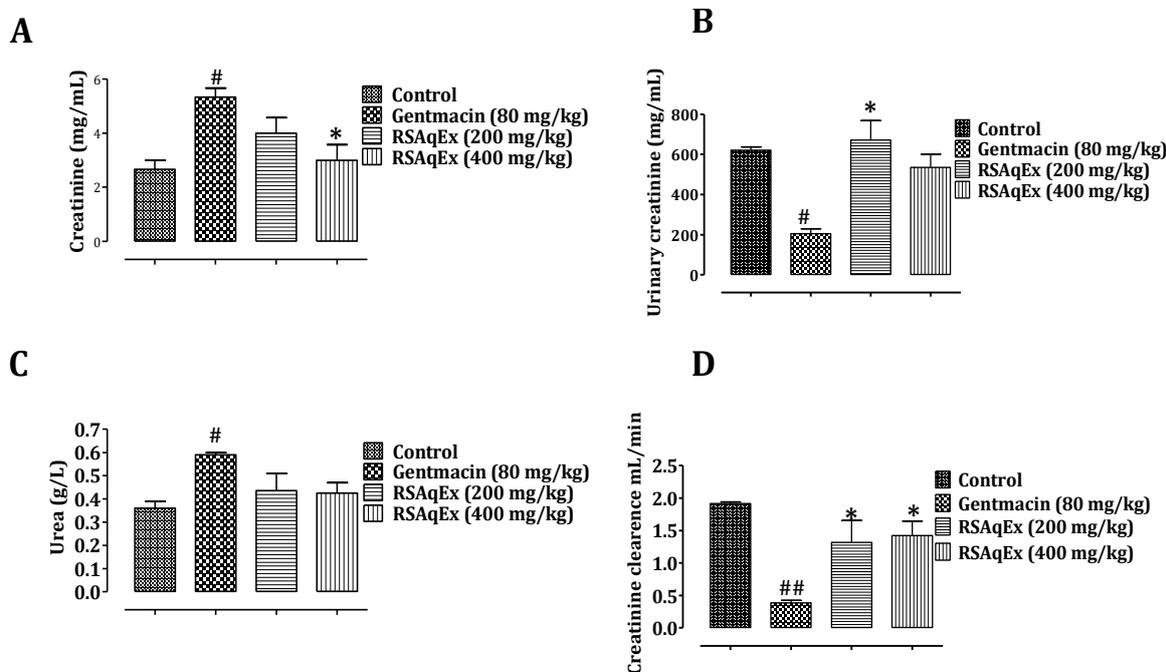

Figure 5. The effect of RSAqEx at 200 and 400 mg/kg on the plasma creatinine (**A**) and urinary creatinine (**B**) plasma urea (**C**) and creatinine clearance (**D**) in GM intoxicated rats. The results were expressed by mean ± SEM (n=6). # P≤0.05, ##P≤0.05 versus a control group *P<0.05 versus GM group

As shown in Figure 5, the effect of RSAqEx on serum and urine creatinine levels for all treated groups was evaluated. The marked increase in serum and urinary creatinine has been considered as a significant functional impairment of the kidneys in GM-induced renal failure [51]. A significant increase in serum creatinine (p<0.05) and a significant decrease (p< 0.05) in urinary creatinine were observed in the rats exposed to GM (80 mg/kg; i.p) compared to the animals in the normal controlled group. However, a significant decrease in the serum creatinine level (p <0.05) at the dose of 400 mg/Kg and a significant increase (p <0.05) in the urinary creatinine level at dose 200 mg/Kg were observed in rats pretreated with RSAqEx, compared to the GM group. The dose of 400 mg/Kg did not induce a significant increase in the urinary creatinine level; this data may be produced by how the molecules interfere with each other to induce this biological effect. These results were in good agreement with the results of other previously published work [49]. Several studies have reported that aminoglycoside (gentamicin) is a classic antibiotic able to cause nephrotoxicity by inducing reactive oxygen species [52,53]. Natural antioxidants such as polyphenols and flavonoids are known to trap free radicals in vivo [54]. The characteristic phytochemical constituents in R. sativus are flavonoids and phenolic acids. It is noteworthy to suggest that RSAqEx can ameliorate renal function in GM-intoxicated rats by neutralizing the free radicals caused by gentamicin. The effect of RSAqEx on creatinine clearance was evaluated in all rats in the study groups (Figure 5, D). GM caused a significant decrease (p< 0.01) in creatinine clearance. Administration of RSAqEx at doses 200 and 400 mg/kg significantly (p< 0.05) decreased creatinine clearance in the rats exposed to GM (80 mg/kg; i.p). These results were concordant with those found by Hosaka et *al.* (2004) and Govindappa et *al.* (2019) [47,55]. Effect of RSAqEx of the plasma AST and ALT.

The effect of various doses of RSAqEx on ALT (A) enzymatic activity (**A**) and AST (**B**) in GM intoxicated rats was shown in Figure 6. Data showed that GM (80 mg/kg; i.p) did not significantly influence ALT and AST's enzymatic activity than the control group. Elevated serum ALT activities, AST after treatment with gentamicin in agreement with the study of sally et al., 2016 [56]. They were indicating hepatic damage to rat hepatocytes [57].

Besides, there was no significant effect on ALT and AST's enzymatic activity in rats treated with RSAqEx at 200 and 400 mg/kg compared with the GM group. The decreased ALT activity and AST in rats treated with RSAqEx might be due to bioactive molecules with antioxidant properties.
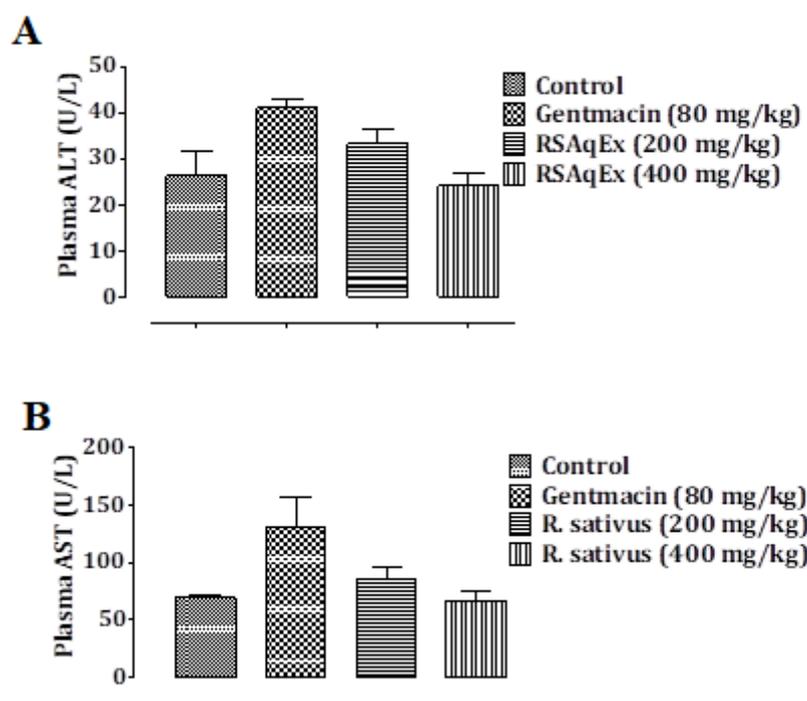

Figure 6. The effect of RSAqEx at 200 and 400 mg/kg on plasma ALT (A) and AST (B) in GM intoxicated rats. The results were expressed by mean ± SEM (n=6)

3.8. Effect of RSAqEx on the plasma albumin, total protein, and lipid peroxidation

The levels of plasma total proteins and albumin in control, GM, and RSAqEx treated animals were showed in Figure 7. The treatment of rats with GM induced a significant increase in plasma total proteins and albumin levels ($p<0.05$ and $p<0.01$, respectively). However, gentamicin treatment causes this variation in total protein and albumin, useful liver function markers, might be depressed due to defective protein synthesis [58]. These findings were in good agreement with the results of other previously published work [47]. The administration of RSAqEx extract at 400 mg/kg decreased significantly the total plasma proteins and albumin levels ($p<0.01$ and $p<0.001$, respectively) compared to the GM group. The RSAqEx at 200 mg/kg was produced a significant decrease in plasma albumin levels ($p<0.05$). Still, there was no significant effect on the total plasma proteins compared to the GM group. The enhancing effect on plasma total protein and albumin might due to the bioactive compounds of RSAqEx. This result suggests that these natural compounds have an overall protective effect on the hepatic and renal cells.

The kidney MDA levels in control, GM, and treated RSAqEx animals were showed in Figure 7. The injection of rats with GM induced a significant elevation in kidney MDA levels ($p<0.01$), The GM is assumed to be associated with the production of reactive oxygen species in the form of superoxide anion (O_2), hydrogen peroxide (H_2O_2), and hydroxyl radical ($OH•$) of renal cortical mitochondria, which are accompanied by an increase in lipid peroxidation [59]. Changes in the lipid composition of the membrane could be induced by the lipid peroxidation initiated by free radicals with a subsequent increase in MDA, one of the products of lipid peroxidation [51]. In this investigation, the GM's daily administration by rats was caused an increase in the lipid peroxidation, which is indicated by the elevation of renal MDA. Our results agreed with Kumar et al. (2019), who reported that the treatment with gentamicin increase renal MDA level [47]. Whereas a significant decrease in kidney MDA levels was observed on intoxicated rats treated with RSAqEx at 200 and 400 mg/kg ($p<0.05$, $p<0.01$, respectively). The mechanism of this protective effect might be due to the antioxidant potential of RSAqEx that is responsible for the diminution of the oxidative damage in the renal tubular cell membrane.
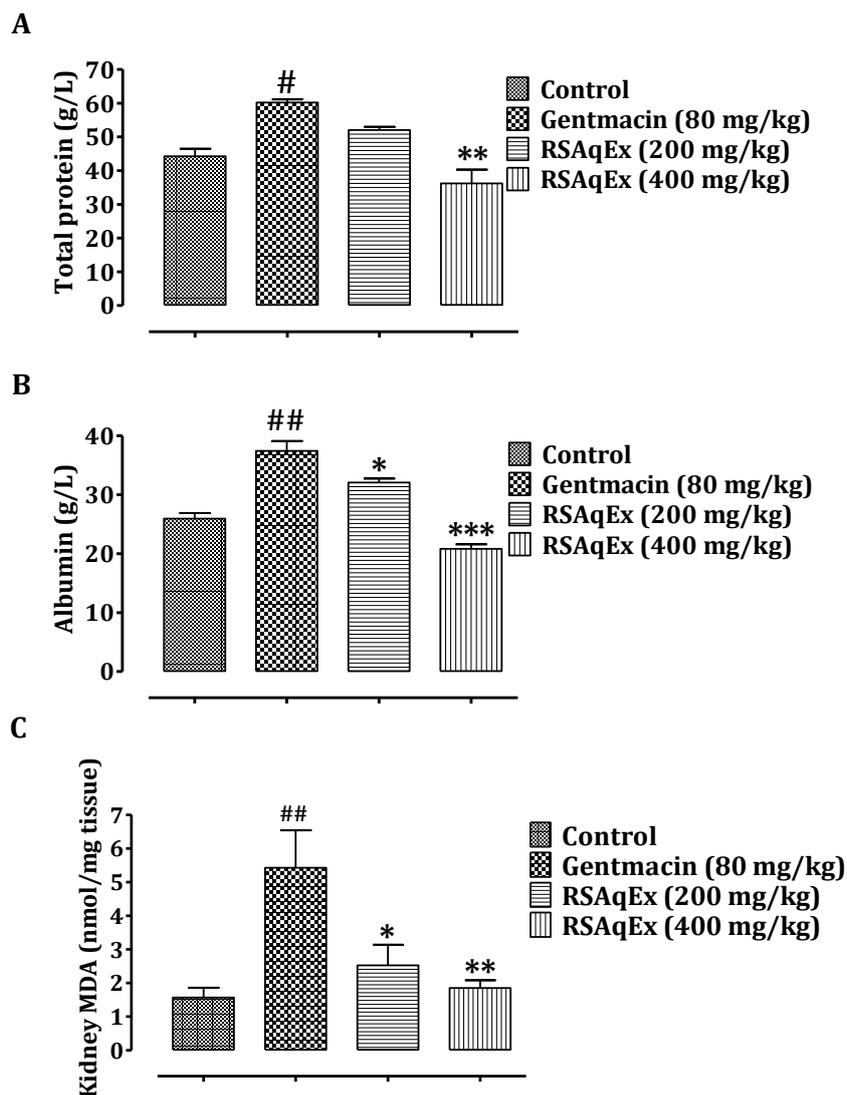
A

B

C

Figure 7. The effect of RSAqEx at 200 and 400 mg/kg on the total protein **(A)** albumin **(B)** and kidney levels of MDA **(C)** in GM intoxicated rats. The results were expressed by mean ± SEM (n=6); #p < 0.05; ##p < 0.01 compared with control; *p<0, 05; **p < 0, 01; *** p <0,001 compared with GM group

4. Conclusion

In conclusion, our results showed that the oral administration of *R. sativus* exerted favorable nephroprotective activity against gentamicin-induced renal damage in rats. This effect could be due to the presence of flavonoids, polyphenols, and other bioactive compounds. Furthermore, complementary studies will be necessary for precise mechanisms involved in *R. sativus* -induced renal protection.

Conflict of interest

The authors declare that there is no conflict of interest.

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