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Studying the protective role of resveratrol against D-galactose-induced renal damage in male albino rats

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Abstract
Background: Chronic kidney disease is a significant global health issue with a sharply rising incidence, although modern medicine has made enormous strides, there are still several limitations to kidney disease prevention and therapy. The objective of this research was to investigate resveratrol's potential protective results against d-galactose-induced kidney injury in male albino rats.
Methods: Forty adult male albino rats were used in this study (weighing 150 – 200 g). All rats were split into four equal groups, as follows: group I (Control group): injected with 0.9% saline subcutaneously every day for eight weeks, group II (D-gal group): received 120 mg/kg D-gal in 0.9% saline subcutaneously every day for eight weeks, group III (Res group): received resveratrol at a dose of (50 mg/kg/day) dissolved in saline 0.9 % via oral gavage for eight weeks, group IV (D-gal + Res group): received D-gal subcutaneously and oral resveratrol daily for eight weeks in the same doses as in groups II and III.
Results: group II (D-gal group) demonstrated significant rise in serum creatinine level, BUN, TNF-α level and renal MDA level with significant drop in renal GPx level while group IV (D-gal + Res) revealed significant decline in serum creatinine, BUN, TNF-α and renal MDA levels with significant increase in the renal GPx level.
Conclusions: Resveratrol effectively protects the kidney tissues against d-galactose-induced injury in aging rats.

Keywords: Resveratrol, D-galactose, renal damage, male albino rats

Introduction

The frequency of chronic kidney disease is drastically rising, making it a major global health concern. Although modern medicine has made enormous strides, there are still several limitations to kidney disease prevention and therapy. One of the organs most prone to age-related tissue deterioration is the kidney [1].

Aging is a slowly progressive process exhibiting a decrease in ability of numerous organs to function and an increase in susceptibility to stress [2]. The aging kidney experiences intricate alterations that put it at risk for renal disease and kidney injury [3].

The primary cause of age-related damage is oxidative stress, which is brought on because of a mismatch between the production of reactive oxygen species (ROS) and antioxidant defenses [2].

By interacting with DNA, lipids, and proteins, an accumulation of ROS can cause cellular damage. This inflammation then leads to cellular damage and apoptosis [4].

It is widely known that oxidative stress and inflammation contribute to kidney impairment. As a result, a viable preventive method for kidney damage is to reduce oxidative stress and inflammatory response [1].

A typical lowering sugar in body is called D-galactose (D-gal). Galactose-1-phosphate uridylyl transferase and galactokinase typically convert it into glucose at the normal level. However, at high concentrations, it may result in an accumulation of ROS and oxidative stress. Animal models of oxidative damage have been created using d-gal-injected rodents [5].

In grapes and berries, there is a naturally occurring polyphenolic substance called resveratrol (3,5,4′-trihydroxystilbene). It is a dietary supplement known for its numerous beneficial health effects and is said to have defensive properties on cardiovascular and renal system due to its antioxidative properties [6].

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The potent anti-inflammatory and antioxidant impact of resveratrol is well established [7].

When resveratrol was given orally to animals with kidney damage, it reduced albuminuria, elevated levels of oxidative stress, and inflammation. According to studies, resveratrol can directly neutralize ROS while also regulating the production and activity of antioxidant enzymes like glutathione peroxidase. In addition to neutralizing ROS, resveratrol may provide a variety of anti-ageing benefits [8]

This research's objective was to examine the protective function of resveratrol against d-galactose-induced renal damage in male albino rats.

Material and Methods

Experimental Animals: For this investigation, 40 mature male albino rats were used (weighing 150 - 200g). All of the rats were kept in regular animal laboratory conditions (25±3 °C) with unlimited access to a standard meal and water.

After receiving approval from ethical committee at Tanta University Hospitals in Egypt, the study was executed. (approval code: 34440/2/21).

Animal groups

Four equal groups of all rats were created:

Group I (Control group): Animals obtained a subcutaneous injection of 0.9 % saline once each day for eight weeks.

Group II (D-gal group): Animals were given 120 mg/kg D-gal in 0.9 % saline subcutaneously every day for 8 weeks [9, 10].

Group III (Res group): Resveratrol was administered orally to animals for 8 weeks at a dose of (50 mg/kg/day) in saline 0.9 % [10].

Group IV (D-gal + Res group): In the same dosages as in groups II and III, animals got D-gal subcutaneously and resveratrol orally every day for eight weeks [10].

Drug preparation

D-galactose was purchased in the form of powder, 120 mg/kg and then dissolved in 0.5 ml sterile normal saline solution and administrated daily by subcutaneous injection.

Resveratrol was purchased from in the shape of white powder, 50 mg/kg and then dissolved in 0.5 ml saline solution to be given daily by oral gavage.

Blood and tissue sampling: Blood samples were taken utilizing a cardiac puncture 24 hours following the last treatment, and by centrifugation, serum was isolated for 15 minutes at 3000 rpm, transferred into clean storage tubes and stored at -30 ºC for estimation of Serum Creatinine, Serum Blood Urea Nitrogen, Serum TNF-α, After sacrificing the animals, The kidney was removed right away, weighed, and perfused with ice-cold isotonic saline while still in place. It was then blotted dry on filter paper and chopped into little pieces. A single piece of each specimen was weighed and homogenized in a Potter Elvehjem tissue homogenizer in 10 volumes of ice-cold, 50 mμ of phosphate buffer saline, pH 7.4, comprising 1.15 % kcl. Homogenates were centrifuged at 10,000 r/min for 20 minutes at 4 ºC to pellet tissue debris and resultant supernatant was kept at -80 ºC till utilized for estimation of Malondialdehyde (MDA), Glutathione Peroxidase (GPx).

Histopathological examination: Other kidney tissue samples underwent hematoxylin and eosin (H & E) staining and light microscopy analysis after being embedded in paraffin wax, fixed in 10% formalin, and stained.

Determination of serum creatinine level: In an alkaline environment, creatinine in the sample interacts with picric acid to produce a color complex (Jaffè reaction), which absorbs light at a wavelength of 492 nm. The rate of color formation is proportional to the sample's creatinine content.

The reagents (R) set is stored at room temperature, stable for 2 days at room temperature and for one week at 2-8°C R1(Creatinine standard (2.0 mg/dl)), Mix equal volumes of R2 (Picric acid(38mmol/l)) +R3 (Sodium hydroxide (0.4mol/l)). 30 seconds, 2 minutes later, absorbance A1 A2 (respectively) of the standard or sample were read. Working reagent (1.0ml), Standard (100 μl).

Calculation: $A2 - A1 = A$ sample or A standard Concentration of creatinine in serum were Normal values (0.6-1.4 mg/dl). Creatinine (mg/dl) = A sample \ A standard $\times 2$

Determination of serum blood urea nitrogen (BUN) level: When urea is hydrolyzed with urease and water present, ammonia and carbon dioxide are generated. Urea + $H2O \xrightarrow{Urease} 2NH3 + CO2$. Free ammonia reacts with an indicator to generate a colored complex proportionate to the amount of urea present in the material when the pH is alkaline.

R: Standard urea = primary standard, 50 mg/dl. (8.33 mmol/l). R1; Buffer: Phosphate buffer in pH 8.0 (100 mmol/l), Sodium salicylate (80 mmol/l), Sodium nitroprusside (6.0 mmol/l), EDTA (30.0 mmol/l). R2; Enzyme: Urease (> 6000 U/l). R3; Alkaline) Sodium hydroxide (400 mmol/l), Sodium hypochlorite (20.0 mmol/l).

Procedure: For at least three minutes, blend and incubate at 37 °C or for 5 minutes at 20-25 °C. Using Urease colorimetric method at Wavelength 578 (578-623) nm. Serum BUN concentration (mg/dl) = (A specimen)/ (A standard) ×n. Where n = 50.0 mg / dl (8.33 mmol/l) and Normal value (15–22 mg/dL).

Determination of serum TNF-α level: Rat TNF-levels in samples were determined utilizing ELISA. TNF- α antibodies tagged with biotin and mixed with streptavidin-HRP to generate immune complexes are added to monoclonal antibody enzyme wells, which have already been pre-coated with rat TNF- α monoclonal antibodies, and are then incubated and washed to remove the enzyme that has not yet been combined. When you add Chromogen Solution A or B, the liquid turns blue. When acid has its effect, color finally turns yellow. Samples: Only Chromogen solutions A and B and stop solution are permitted in the blank well. Test wells should first contain sample 40 μl, followed by standard 50 μl, Streptavidin-HRP 50 μl, and finally TNFα-antibody 10 μl. The sealing membrane was then sealed, and the mixture was gently shaken for 60 minutes at 37 °C. Confection: As a backup, dilute the washing concentration 30 times with distilled water. After washing, add 50 μl each of chromogen solutions A and B to each well. Gently stir, then let sit for 10 minutes at 37 °C without light. To halt the reaction (the blue quickly turns yellow), add Stop Solution (50 μl) to each well. Then, treat

the blank well as zero and measure optical density (OD) at 450 nm. Applying sample's OD values and standard curve linear regression equation to determine corresponding sample's concentration within the (8ng/l–1000ng/l) range.

Determination of Renal malondialdehyde (MDA) level:
At a temperature of 95 °C for 30 minutes, thiobarbituric acid (TBA) and MDA react in an acidic media. The pink product that is produced can be tested for absorbance at 534 nm to produce Thio barbituric acid reactive product. Sample: To eliminate any RBCs and clots, tissue was perfused with phosphate buffered saline solution with pH 7.4 and 0.16 mg/ml heparin. After homogenizing the tissue in 5-10 ml cold buffer (50 mM phosphate buffer, pH 7.5) per gram tissue and centrifuged at 4000 rpm for 15 minutes. Supernatant was taken out for analysis and kept on ice. At least a month was spent keeping the sample frozen at -80°C. R: Standard (10 nmol/l), Chromogen (Thiobarbituric acid) and Detergent (Stabilizer) (25 mmol/l).
Procedure: Sample and Standard 0.2 ml, Chromogen1.0 ml. Mix well, add 0.2 ml of Blank after cooling the test tube and covering it with a glass bead. Within six hours of mixing, measure the sample's (A sample's) absorbance at 534 nm against a blank and a standard against distilled water.

Determination of renal glutathione peroxidase (GPx) activity: Erythrocytes were freed by filtration through a column of microcrystalline cellulose and alpha cellulose. Glutathione peroxidase, glutathione reductase and catalase activity were determined. Utilizing this method, one unit of enzyme was used to oxidize one μ mole of NADPH per minute under normal circumstances. The enzyme concentration was measured in units per milliliter. Method: The 1.5 ml standard test combination included 50 mM HCL, 0.1 mM EDETA buffer (PH7.6), 0.275 mM GSH, 0.11 mM NADPH and an excess (1 UNIT) of glutathione reductase. 0.05 ml of a solution containing 1 mg of cumene hydroperoxide per ml was added to start the reaction, which was then incubated with 0.1 ml of enzyme preparation for 1 min at 37 c. At 340 nm, spectrophotometer saw a drop in absorbance.

Statistical analysis
Data was displayed as mean ± SD. To compare available data, Analysis was done utilizing one-way ANOVA (Analysis of Variance) function in SPSS (version 16) program. When P value < 0.05, data were regarded as significant.

Results
The effect of resveratrol on renal function tests (creatinine and BUN)
The serum creatinine level in the group handled with d-galactose alone (in a dose of 120 mg/kg subcutaneously for 8 weeks) showed a significant increase while the resveratrol treated group (in a dose of 50 mg/kg/day for 8 weeks) revealed a non-significant decrease contrasted to control group. However, when Res group was contrasted to the D-gal group, it showed significant decrease. D-gal + Res group revealed a considerable decline contrasted to the values of D-gal group, and when contrasted to control group, showed insignificant change in the serum creatinine level. Table 1, figure 1.

Table 1: Serum creatinine level (mg/dl) in all studied groups

Groups No.	Control group (I)	D-gal group (II)	Res group (III)	D-gal + Res group (IV)
Mean± SD.	0.82±0.055	1.83±0.21	0.78±0.061	0.85±0.049
F	176.953			
P	0.001*			
group I versus	group I versus	group II versus	group II versus	group I versus
group II	group III	group III	group IV	group IV
< 0.001*	> 0.05	< 0.001*	< 0.001*	>0.05

The serum BUN level in the group handled with d-galactose alone indicated a significant rise contrasted to control group while Res group showed a non-significant decline. When Res group was contrasted to the D-gal group, it showed significant decrease. D-gal + Res group revealed a significant decline in BUN level contrasted to D-gal group, but when contrasted to normal control group, this group exhibited significant increase. Table2, figure 1.

Table 2: Serum BUN level (mg/dl) in all studied groups

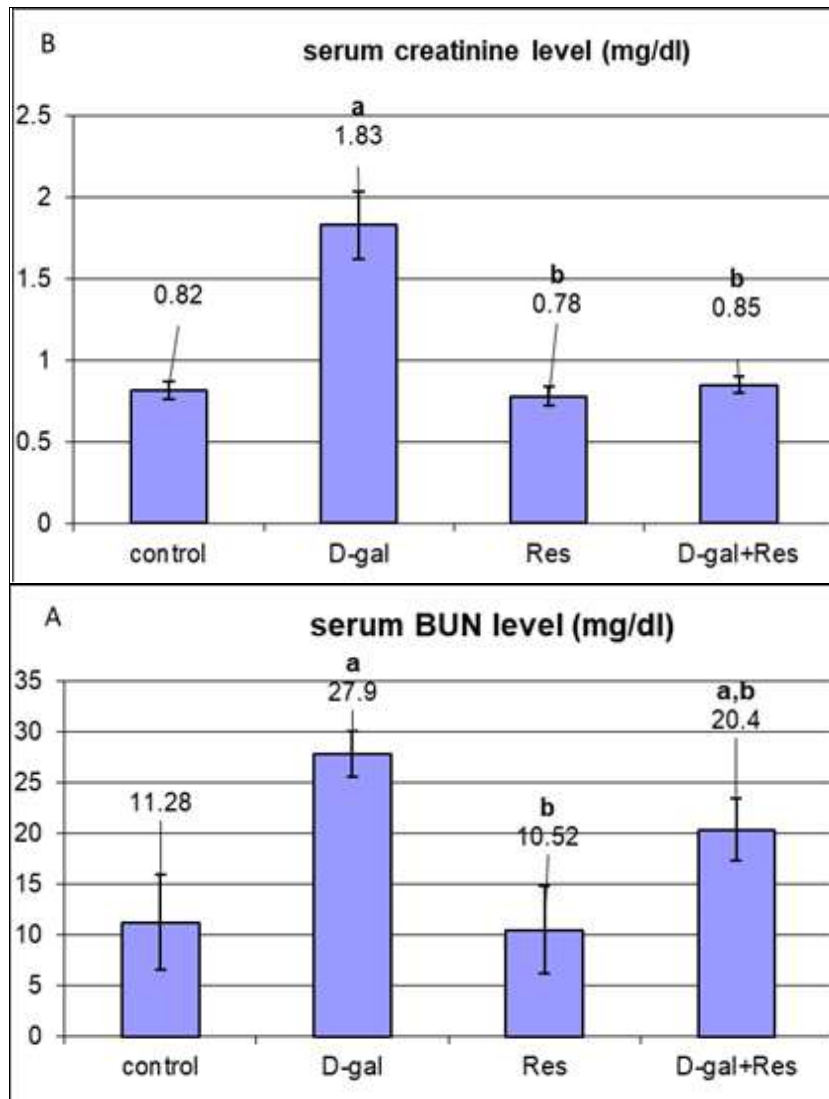| Groups No	Control group (I)	D-gal group (II)	Res group (III)	D-gal + Res group (IV)
Mean± SD	11.28±4.68	27.9±2.27	10.52±4.28	20.4±3.05
F	49.782			
P	0.001*			
group I versus	group I versus	group II versus	group II versus	group I versus
group II	group III	group III	group IV	group IV
< 0.001*	> 0.05	< 0.001*	< 0.001*	< 0.001*

Fig 1: Serum (A) BUN level (mg/dl) (B) creatinine level (mg/dl) in all studied groups a, b Denotes statistical significance at p ≤ 0.001. a Denotes statistical significance as compared with the control group. b Denotes statistical significance as compared with the D-gal group.

The effect of resveratrol on the inflammatory status (TNF alpha level)

The serum TNF alpha level in the group handled with d-galactose alone indicated a significant rise while Res group revealed insignificant changes in contrast to control group.

When Res group was contrasted to the D-gal group, it revealed significant decrease. D-gal + Res group showed a significant decrease in serum TNF alpha level ontrasted to D-gal group, but when contrasted to control group, D-gal + Res group showed significant increase. Table 3, figure 2

Table 3: Serum TNF alpha (TNF-α) level (pg/ml) in all studied groups

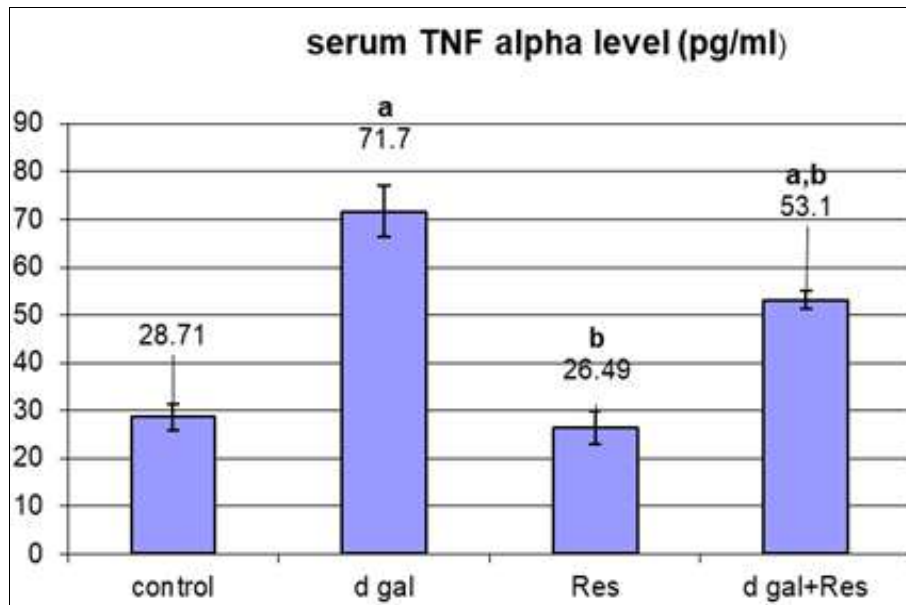| Groups No.	Control group (I)	D-gal group (II)	Res group (III)	D-gal +Res group (IV)
Mean± SD	28.71±2.78	71.7±5.27	26.49±3.52	53.1±1.9
F	358.332			
P	0.001*			
group I versus	group I versus	group II versus	group II versus	group I versus
group II	group III	group III	group IV	group IV
< 0.001*	> 0.05	< 0.001*	< 0.001*	< 0.001*

Fig 2: Serum TNF alpha level (pg/ml) in all studied groups. a, b Denotes statistical significance at p ≤ 0.001. a Denotes statistical significance as compared with the control group. b Denotes statistical significance as compared with the D-gal group.

The effect of resveratrol on the oxidative status (renal MDA and GPx levels)

The D-gal group indicated a noticeable rise in the level of renal MDA contrasted to control group while Res group revealed a significant decline in comparison with D-gal group. D-gal + Res group revealed a significant decrease regarding renal MDA level in contrast to values of D-gal group, and when contrasted to normal control group, this group showed insignificant change. Table 4, figure 3

Table 4: Renal MDA level (nmol/gm) in all studied groups

Groups NO.	Control group (I)	D-gal group (II)	Res group (III)	D-gal + Res group (IV)
Mean± SD.	0.66±0.053	0.87±0.052	0.56±0.048	0.68±0.056
F	61.967			
P	0.001*			
group I versus	group I versus	group II versus	group II versus	group I versus
group II	group III	group III	group IV	group IV
<0.001*	<0.001*	<0.001*	<0.001*	>0.05

The renal glutathione peroxidase (GPx) activity in D-gal group exhibited a significant decrease as contrasted to control group while Res group revealed a significant rise contrasted to D-gal group. D-gal + Res group indicated a significant rise in GPx activity contrasted to D-gal group, and when contrasted to normal control group, this group revealed insignificant change. Table 5, figure 3

Table 5: Renal glutathione peroxidase (GPx) activity level (umol/g tissue)

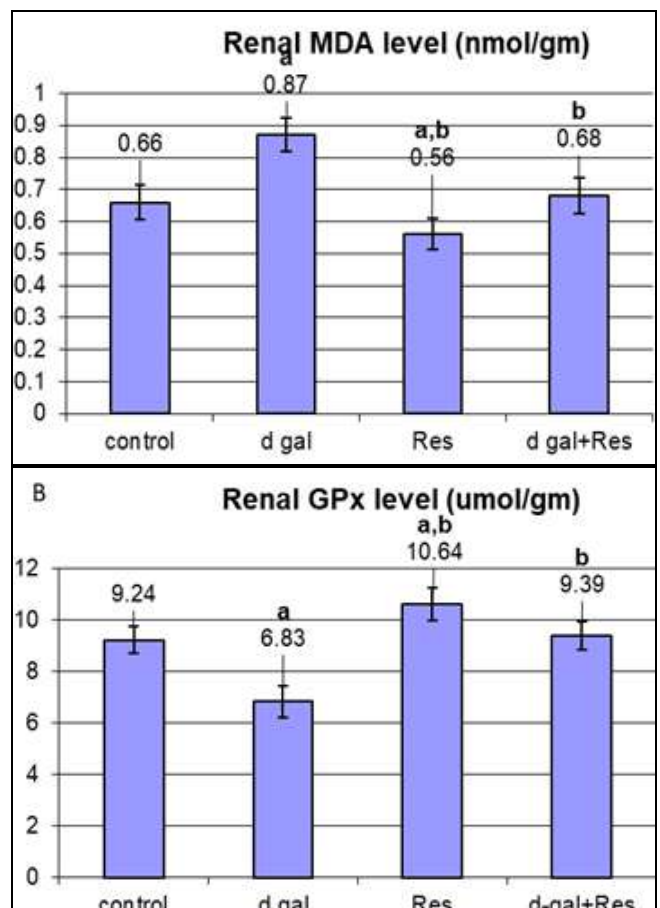| Groups No.	control Group (I)	D-gal Group (II)	Res Group(III)	D-gal + Res Group (IV)
Mean ± SD	9.24±0.51	6.83±0.62	10.64±0.64	9.39±0.54
F value	74.067			
P. value	0.001*			
group I versus	group I versus	group II versus	group III versus	group I versus
group II	group III	group III	group IV	group IV
p<0.001*	p<0.001*	p<0.001*	p<0.001	p>0.05

Fig 3: (A) Renal MDA level (nmol/gm) (B) Renal GPx level umol/g tissue) in all studied groups a, b Denotes statistical significance at p ≤ 0.001.a Denotes statistical significance as compared with the control group. b Denotes statistical significance as compared with the D-gal group.

Histopathological observations

Renal sections stained with H&E from animals of D-gal group showed severe structural damages including glomerular sclerosis and atrophy with a decrease in the number of normal glomeruli, extensive tubular destruction with areas of necrotic epithelial cells, congestion and edema

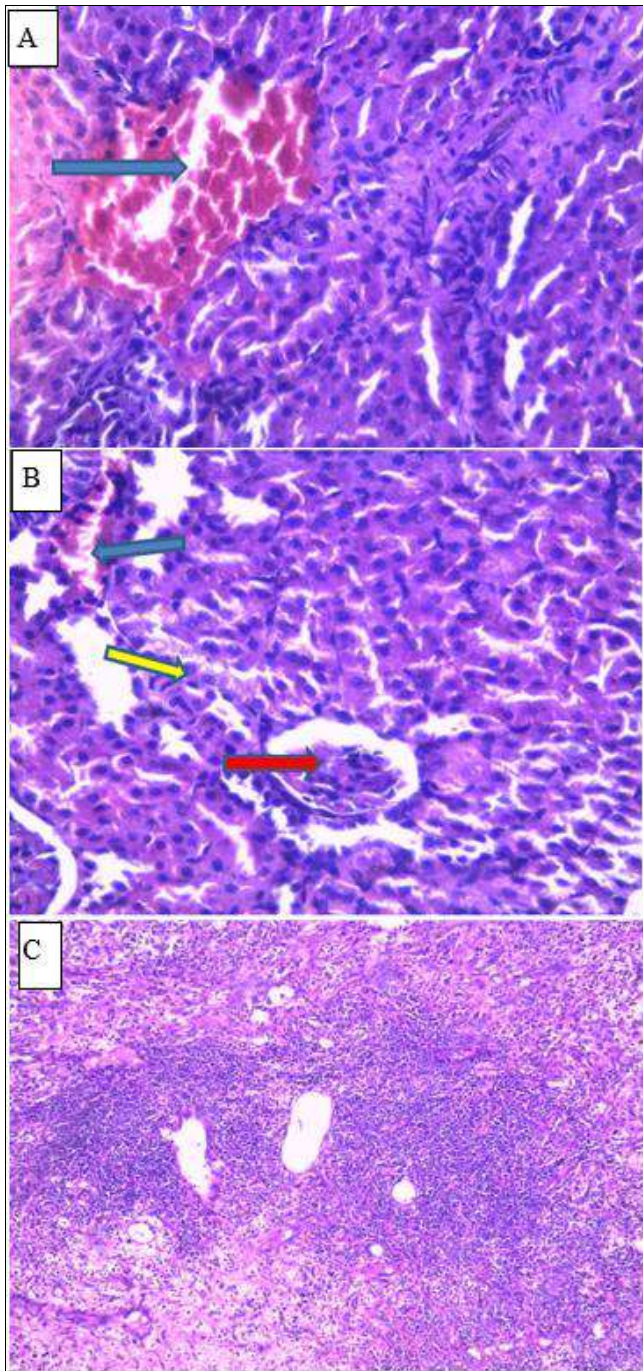with interstitial fibrosis along with heavy lymphocytic infiltration. Figure 4.

Fig 4: D-gal group showing (A) renal tissue with marked congestion and dilated vascular spaces (blue arrows),(B) glomerular atrophy (red arrows), tubular atrophy (yellow arrows) and congestion (blue arrows) (H&Ex400), (C) lymphocytic infiltration

While Res group revealed few histopathological changes when compared with normal control group as they showed normal structures of tubules and glomeruli. Figure 5 (A). D-gal + Res group: Kidney sections from this group showed marked improvement in renal structure as there was a reduction in glomerular and tubular damage compared to D-gal group. This suggests that resveratrol treatment could lessen kidney damage brought on by d-galactose. (H&Ex400). Figure 5 (B).
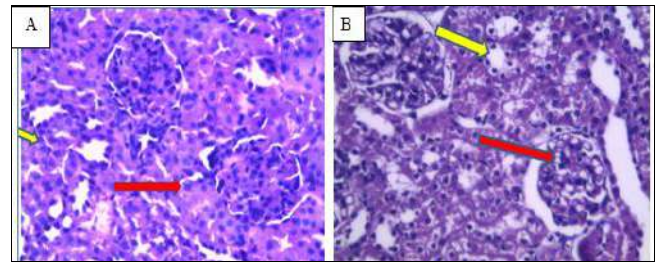

Fig 5: (A) Resveratrol group (Res group) showing regular morphology of normal renal glomeruli (Red arrow) and tubules (yellow arrow) (B) D-gal + Res group showing less damage of renal glomerular (Red arrow) and tubular (yellow arrow) structures with gradual improvement of renal tissues (H&Ex400)

Discussion
Resveratrol is a common polyphenol and phytoalexin that is found in edible materials and has many protective effects on different body organs [10]. To study the renoprotective effect of resveratrol, we damaged the kidneys in albino rats by d-galactose and studied ability of resveratrol to reverse this damage.

D-galactose (D-gal) administration in high concentrations produces a large amount of free radicals that exceeds scavenging ability of the oxidative defense system. This induces oxidative stress that leads to kidney changes similar to aging process ending in chronic kidney disease. The aging kidney is more sensitive to stress, has a lower potential for tissue regeneration, and is less able to recover from any acute injury [11].

In the current study, results revealed that resveratrol administration could successfully reverse the oxidative stress caused by d-galactose, showing a significant decrease in oxidative and renal markers, and at the same time an increase in the antioxidant markers. Serum levels of creatinine and BUN in different rat groups were determined, which are considered as sensitive indicators for assessing kidney function, revealing that chronic high dose d-galactose administration induced a significant increase in serum levels of both creatinine and BUN indicating that d-galactose can lead to renal damage probably owing to accelerated aging changes. The significant changes in these parameters are caused as a result of d-galactose on function and morphology of renal tissue as demonstrated by El-far *et al* [12]. Resveratrol could reverse all damaging effects of d-galactose on kidney function indicated by lowering the kidney function parameters including both creatinine and BUN in D-gal + Res group.

This was in agreement with the research done by Liu B. *et al*. [13] which showed the antioxidant effect of resveratrol. Additionally, the work done by Li L. *et al*. [14] who used multiple doses of resveratrol to reverse the toxic effects on kidney function.

In this study, increased serum level of tumor necrosis factor-alpha (TNF- α) in d-gal treated group may also be indicative of d-galactose-induced kidney injury. This was also observed by the study of El-far *et al*. [12] which showed that after d-galactose treatment, the expression of inflammatory factors like TNF α and interleukin-6 (IL-6) increased significantly. In the present study, resveratrol could significantly decrease the level of TNF-α, indicating that it has an anti-inflammatory effect on the aged rats, and this protected the kidney in D-gal + Res group. This was consistent with study of Cai *et al*. [15] which revealed that

resveratrol could ameliorate functional abnormalities, renal morphological changes, and renal inflammatory markers.

The study by El-far *et al.* [12] revealed a rise in level of ROS and apoptotic genes expression in the d-galactose treated group contrasted with control group.

In the present study, to assess the oxidative condition in renal tissue, the level of renal MDA and GPx levels was measured. Increased MDA levels impair the structure and performance of cellular proteins and antioxidant enzymes [16]. This makes MDA measurement a key indicator of aging process and organ damage including kidney aging and fibrosis [17].

In the present study, subcutaneous injections of d-galactose for 8 weeks significantly increased renal MDA levels compared to the normal control group while it significantly decreased the level of renal GPx indicating increased oxidative stress. This was in agreement with the study done by Yang *et al.* [18] which had similar results of significantly increased MDA leading to obvious kidney injury.

Li W. *et al.* [19] showed that d-galactose administration caused a significant decrease in superoxide dismutase (SOD) and catalase activities as well as an increase in MDA contents, leading to functional decline in the renal performance.

He *et al.* [20] showed that antioxidant enzymes including catalase, GPx and SOD were significantly decreased in the kidney tissues of the d-galactose treated group, which indicates that d-galactose could decrease the total antioxidant capacity.

This was all reversed by resveratrol co-administration with d-galactose as seen in the present study results, resveratrol could significantly decrease level of renal MDA and simultaneously Boost the level of renal GPx

Sadi *et al.* [21] reported that after resveratrol administration, there was a significant rise in level and activity of SOD and GPx in kidney together with decreasing the content of MDA.

In this study, histopathological findings showed marked morphological changes in tubular and glomerular areas after d-galactose administration that confirmed its renal damaging effect.

This was proved by other experimental studies such as the study done by Mo *et al.* [22] who showed extensive glomerular and tubular damages demonstrated by appearance of necrotic epithelial cells after d-galactose injection.

Taghipour *et al.* [23] reported in their study that vascular glomeruli were increased, and the glomerular capsular space dilated with leukocyte and inflammatory cell infiltration with continuous d-galactose administration at a dose of 500 mg/kg per day. The work done by Zhen *et al.* [24] also showed that there was necrosis and edema in the renal tissue of aged rats after d-galactose administration.

In the present study, resveratrol could significantly alleviate the histopathological injury caused by d-galactose in the kidney tissues. The histopathological study of the D-gal + Res group appeared closer to that of normal rats regarding renal capsules, renal tubules, and glomerular structures.

This was consistent with study by Li L. *et al.* [14] which showed that resveratrol treatment caused renal tubular epithelial cells' degree of swelling to decrease, and the aging kidney tissue gradually started to recover to normal.

Conclusions
In aging rats, resveratrol efficiently shields the kidney tissues from damage brought on by d-galactose. Its capacity to enhance the antioxidant and anti-inflammatory status may be linked to the mechanism behind its protective impact. According to this study, resveratrol may be used as a possible preventive measure against age-related kidney impairment.

Authors' Contributions
In terms of conception, execution, statistical analysis, and paper writing, all authors shared equal responsibility. The final paper work has been approved by all writers.

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