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Protective Effect of Gallic Acid on Cyclophosphamide-Induced Nephrotoxicity, Oxidative Stress, Genotoxicity, and Histopathological Alterations in Male Albino Rats

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ABSTRACT

Cyclophosphamide (CP) is a chemotherapeutic agent used to treat various types of cancer. Despite its nervous, hepatic, renal, and cytotoxic side effects, it is a highly effective agent whether used alone or in combination with other chemotherapeutics. This study was designed to examine the prophylactic effects of Gallic acid (GA) on CP-induced acute renal toxicity. Male Wistar albino rats were divided into six groups, 6 animals each: G1 was given normal saline and served as -ve control, while G2 and G3 were given i.p injections of 100 and 200 mg/kg GA, respectively, for 15 days. G4 was used as a +ve control and received a single i.p. injection of CP (150 mg/kg). G5 and G6 were treated with the two different doses of GA for 15 days before receiving a single i.p. injection of CP. Animals were euthanized 24 hrs after the last treatment, and their kidneys were carefully dissected out for histological, immunohistochemical investigation of Zona occluden-1(ZO-1), and biochemical examination, as well as the evaluation of P53, apoptotic markers and tumor necrosis factor-alpha (TNF-α) gene expressions. Blood samples were also taken to determine serum creatinine and urea levels. The intake of GA improved kidney function, as evidenced by lower levels of kidney toxicity markers (urea and creatinine). GA significantly reduced the percent of DNA fragmentation in renal tissue via modulating the levels of glutathione (GSH) and catalase (CAT) enzymes as well as malondialdehyde (MDA). Furthermore, GA reduced renal TNF-α and P53 gene expressions and improved the kidney's histological architecture as well as increasing of ZO-1 immuno-expression. In conclusion, the findings of this study indicate that GA protects against CP-induced renal toxicity via an anti-inflammatory and antioxidant mechanism. We believe that GA may have prophylactic effects against CP-induced nephrotoxicity.

INTRODUCTION

Cyclophosphamide (CP) is an active alkylating cytostatic and an immunosuppressive mediator that is applied in chemotherapy for Hodgkin's and non-

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Hodgkin's lymphoma, rheumatoid arthritis, leukemia, neuroblastoma, lupus erythematosus, Burkitt's lymphoma, multiple myeloma, multiple sclerosis, breast, lung cancer, and in organ transplantation (Hamsa & Kuttan, 2012 and Rehman *et al.,* 2012). Furthermore, CP is metabolized to several compounds in the liver by microsomal cytochrome P450 (Ayala *et al.,* 2014). The active metabolite 4-hydroxycyclophosphamide is stable in the presence of the cyclic tautomer aldophosphamide. These two compounds are circulated to tumor cells, where aldophosphamide cleaves to produce active phosphoramide mustard and acrolein. (Tripathi & Jena, 2009). Moreover, phosphoramide is responsible for anti-tumor effects as it binds to DNA forming an adduct that breaks cross-linking purine bases in DNA, inhibiting protein synthesis as well as the death of tumor dividing cells, while acrolein induces urotoxicity, nephrotoxicity, and hemorrhagic cystitis (Osawa *et al.,* 2011).

Unfortunately, the major problems in cancer treatment are the resistance of the cancer cells to chemotherapeutic drugs (Alfarouk *et al.,* 2015, Rueff & Rodrigues, 2016). Therefore, the alternative treatments allow the combination of cytotoxic agents with chemotherapeutic drugs to reduce the concentrations of the drugs, enhancing the treatment efficacy and decreasing their side effects (Hedigan, 2010). Herein, plants can utilize aromatic substances, such as phenolic acids and flavonoids that exhibit antioxidant properties due to their metal-chelating and hydrogen-donating capacities, thus allowing to decrease the occurrence of oxidative stress-related diseases (Rao *et al.,* 2010 & Engwa, 2018).In addition, phenolic agents can also link with iron and copper metal ions that can cause radical creation through the Fenton reaction and prevent this reaction (Kilic *et al.,* 2019). Among the phenolic compounds, Gallic acid (GA) (3, 4, 5-trihydroxy benzoic acid) is synthesized naturally from many plants such as red wine and grapes (Hornedo-Ortega *et al.,* 2020), green tea (Can Agca *et al.,* 2020), oak bark (Dróżdż & Pyrzynska, 2018), witch hazel (Wang *et al.,* 2003), sumac (Fereidoonfar *et al.,* 2019), nut, apple peels, gallnuts, tea leaves, bananas, areca, lemon, strawberries, pineapples, different berries, mango, and other fruits (Safaei *et al.,* 2018 and Ola Davies *et al.,* 2018). GA is composed of three hydroxyl groups attached to the aromatic ring in an ortho position (Fig.1) forming the strongest ROS scavenging activity of phenolic acid as well as antioxidant effect (Borde *et al.,* 2011 & Locatelli *et al.,* 2013).
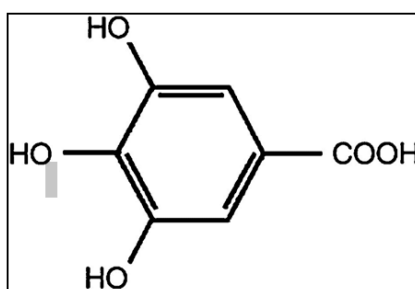

Fig. 1: Chemical structure of Gallic acid (3,4,5 - trihydroxybenzoic acid) (Reckziegel *et al.*, 2016).

Furthermore, other important characteristics of GA include anti-viral (Trujillo-Correa *et al.,* 2019), anti-bacterial (Rajamanickam *et al.,* 2019), anti-hyperglycemic (Variya *et al.,* 2020), anti-inflammatory (Asci *et al.,* 2017&Bustami *et al.,* 2018), and anti-cancer agent (Zhang *et al.,* 2019 & Khorsandi *et al.,* 2020).

Thus, due to the deleterious effects and nephrotoxic side effects of CP and the benefits of GA as a safe and cheap dietary compound that contains phytochemicals and has protective efficacy against CP-induced nephrotoxicity. The present study was

designated to help in finding a protective therapeutic approach to prevent the destructive effect of oxidative stress induced by CP and it may be beneficial for patients who have been using CP for a long time.

MATERIALS AND METHODS

Drugs and Chemicals:

Cyclophosphamide (Endoxan - Sigma-Aldrich Chemical Co. St. Louis, Missouri, USA) was dissolved in sterile water and freshly prepared immediately before each intraperitoneal injection.

Gallic acid (purity: 99.5%) extra pure was obtained from Sigma Aldrich Company (USA). All chemicals and reagents used during the experiment met the highest purity grade value according to international standards.

Animals:

Thirty-six adult male Wister albino rats, weighing (170- 200 g), aged 7-9 weeks old were used in this study. Animals were obtained from the Animal House Colony, National Research Centre, and Cairo, Egypt. The males were chosen according to an earlier study which found that the clastogenic effects of CP were more prominent in males than females (Krishna *et al.,* 1991). The animals were housed in stainless steel cages, 6 rats per cage, in a temperature-controlled environment (22 ± 2°C) with a 12-h light/dark cycle and humidity levels ranging from 50% to70%. Animals were acclimatized for 2 weeks before beginning the experiment. They were fed by standard rat pellet diet and tap water *Ad libitum* during the study. All animal studies are strictly conformed to the animal experiment guidelines of the Committee on Care and Use of Experimental Animal Resources, Medical Research Center, Ain Shams University.

Experimental Protocol:

Two weeks after acclimatization, rats were randomly divided into six experimental groups as follow: -

- **Group 1** (**negative control**): received 1ml/kg/day 0.9% NaCl intraperitoneally (i.p) for 15 days.
- **Group 2** and **Group 3**: received i.p. injections of 100&200 mg/kg GA respectively, for 15 days. GA doses were selected according to Ahmadvand *et al.* (2019) and Manshare *et al.* (2018).
- **Group 4**: (positive control) animals received single i.p. injection of 150 mg/kg CP to induce nephrotoxicity (150 ml /kg/i.p/single injection) (Abraham *et al.,* 2009).
- **Group 5**: animals were injected with the low dose of GA (100mg /kg) for 15 days before receiving a single dose of CP (150 mg /kg/i.p).
- **Group 6**: animals were treated with a high dose of GA (200 mg/kg) for 15 days then received a single i.p injection of CP (150 mg /kg/i.p).

All animals are monitored daily for health conditions during the treatment period. After the last treatment, rats were sacrificed under ether anesthesia and blood was collected directly by a cardiac puncture from the heart of each animal, allowed to clot for at least 30 min at room temperature. The blood was centrifuged at 3000×*g* for 10 minutes to obtain clear sera, which were then stored at –70°C to assess kidney functions. In addition, kidneys were rapidly dissected out, washed in ice-cold isotonic saline, blotted between two filter papers, and used in the investigations described below.

Biochemical Parameters:

Antioxidant Enzymes and Lipid Peroxidation Marker Assays: The left kidney from each animal was homogenized in chilled (10 mM) phosphate-buffered saline (PBS) with

optimal pH 7.4, centrifuged, and a part of the supernatant was used for evaluating the following:

- Malondialdehyde (MDA): the final product of lipid peroxidation using the thiobarbituric acid (TBA) test (Pedeson *et al.,* 1990).
- Catalase (CAT) Activity by assessing the decomposition of H_2O_2 at 240 nm as stated by Aebi (1983).
- Measurement of renal Glutathione levels (GSH): GSH content in the renal tissue was measured spectrophotometrically according to Ellman's method (Ellman, 1959).

Determination of Kidney Functions:

Urea and creatinine levels in serum were determined using commercially available kits (Todd *et al.,* 1974).

DNA Fragmentation (DNAF) Assay:

Genomic DNA was extracted from the renal tissue using the Wizard® Genomic DNA Purification kit (Promega Corporation, Madison, WI, USA) according to the manufacturer's instructions. To assess endonuclease-dependent ladder-like DNA fragmentation by gel electrophoresis, DNA was loaded onto agarose gel (15µg/lane). DNA laddering was determined by electrophoresis on a 2% agarose gel containing 0.5µg/ml ethidium bromide. DNA ladder (Jena Bioscience, USA) was used and served as a DNA base pair marker (Matassov, *et al.,* 2004). After electrophoresis, gels were visualized by the Gel Documentation system (Biometra, Germany). Furthermore, the percentages of fragmented DNA in renal tissue were assessed by the method described by Boraschi and Maurizi (1998).

Quantitative RT-PCR Analysis:

Total RNA was extracted from renal tissue homogenate using the RNA Purification Kit (Qiagen, Valencia, CA) according to the manufacturer's instruction. Then, cDNA was synthesized using the SuperScript cDNA synthesis Kit (Thermo Fisher, Scientific) according to the manufacturer's instruction, and quantitative real-time PCR (qPCR) was performed using SYBR Green Master Mix PCR Kit (Qiagen, Valencia, CA) on an ABI Prism 7500 Thermal Cycler (Applied Biosystems step one plus, Foster City, California, USA). The qPCR experiment was carried out after the manufacturer's instructions.PCR primers for candidate genes (Bax, Bcl$_2$, caspase-3, P53, and TNF-α) and house-keeping gene (β-actin) were purchased from Applied Biosystems (Table 1). The thermal cycling conditions and calculation of relative expression using $2^{-\Delta\Delta Ct}$ were done as previously described by (Livak and Schmittgen, 2001).

Table 1: Sequence of the primers used for real-time PCR.

BAX (Bcl-2 -associated X protein)	
Forward primer	5'-GTT TCA TCC AGG ATC GAG CAG-3'
Reverse primer	5'-CAT CTT CTT CCA GAT GGT GA-3'
Bcl-2(B-cell lymphoma protein 2)	
Forward primer	5'-CCT GTG GAT GAC TGA GTA CC-3'
Reverse primer	5'-GAG ACA GCC AGG AGA AAT CA-3'
Caspase-3	
Forward primer	5'- GGAAGCGAATCAATGGACTCTGG -3'
Reverse primer	5'- GCATCGACATCTGTACCAGACC -3'
P53	
Forward primer	5'- CCTCAGCATCTTATCCGAGTGG-3'
Reverse primer	5'- TGGATGGTGGTACAGTCAGAGC-3'
TNF-α	
Forward primer	5'- CTCTTCTGCCTGCTGCACTTTG-3'
Reverse primer	5'- ATGGGCTACAGGCTTGTCACTC-3'
Beta-actin (housekeeping gene)	
Forward primer	5'- TCT GGC ACC ACA CCT TCT ACA ATG -3'
Reverse primer	5'- AGC ACA GCC TGG ATA GCA ACG -3'

Histopathological Examination of the Kidney:

Renal tissue samples were flushed and fixed in 10% neutral buffered formalin for 72 hrs. Samples were trimmed and processed in serial grades of ethanols, cleared in xylene, infiltrated, and embedded into Paraplast tissue embedding media. 4μm thick tissue sections were cut by rotatory microtome, mounted on a glass slide, and stained by Hematoxylin and Eosin as a general morphological staining method according to Culling (2013).

Immunohistochemical Examination:

5 microns thick paraffin-embedded tissue sections were immunohistochemically prepared according to the manufacturer's protocol. Deparaffinized retrieved tissue sections were treated with 0.3% H_2O_2 for 20 minutes for blocking the endogenous peroxidase activity, incubated with Anti-ZO 1(1:1000 – Abcam - EPR19945-224) overnight at 4°C. Tissue sections were washed with PBS before incubating with the secondary antibody HRP Envision kit (DAKO) for 20 minutes. Then, slides were washed and incubated with diaminobenzidine (DAB) for 15 mins. Washed again by PBS, counterstained with haematoxylin, dehydrated, and cleared in xylene before being covered for microscopic examination. The specificity of the technique was assessed by negative controls.

Histological Analysis:

6 non-overlapping fields were randomly selected and scanned from each sample to determine the optical density of immuno-expression levels of ZO-1 in immuno-stained sections.

All microscopic examinations and data were obtained by using the Leica Application module for histological analysis attached to the Full HD microscopic imaging system (Leica Microsystems GmbH, Germany).

Statistical Analysis:

Results were presented as means ± standard error (S.E.M.). Statistical comparisons between different groups were done using one-way analysis of variance (ANOVA) followed by the Tukey HSD multiple comparison test. Significance was accepted at P≤ 0.05.

RESULTS

No mortality was detected in any of the experimental animal groups during the study period. The treatment with saline and GA for 2 weeks did not display statistical differences in any of the measured parameters when compared with the control group except for CAT enzymes, which increased significantly (P<0.01) with the GA treatment.

Biochemical Analysis:

Effect of GA on Oxidative Stress And Antioxidant Enzymes Levels In Rats'
Kidneys Treated With CP.:

MDA levels in the kidney of the CP-treated group were significantly elevated (P<0.01) when compared to the control group and GA protective groups. (Table 2). Meanwhile giving the animals two prophylactic doses of GA (100 mg /kg or 200mg /kg) before administrating CP-dose significantly (P<0.01) decreased MDA levels compared to those that received CP-alone. In contrast, both CAT and GSH enzymes in the kidney of CP-treated groups were significantly (P<0.01) reduced (70.27± 2.41 and 51.83±1.90 respectively) as compared with the control group (136.13± 1.62 and112.5±1.21 respectively) (Table 2). The reduction in the level of renal tissue contents of antioxidant enzymes (CAT and GSH) was elevated in GA protective groups.

Serum Levels of Renal Marker

The CP-treated group showed significantly (P<0.01) high serum levels of renal urea and creatinine when compared to the control group. GA administration for 15 days before CP injection significantly lowered serum levels of urea and creatinine in a dose-dependent manner when compared with the CP-treated group (Table 2).

Table 2: The effect of Gallic acid on lipid peroxidation (MDA), antioxidants enzymes, renal marker levels and DNAF in kidney tissues in adult male albino rats**.**

Parameter / Treatment	MDA level (nmol/gm)	CAT enzyme (u /gm)	GSH level (mmol/gm)	Urea level (mg/dl)	creatinine level (mg/dl)	DNAF
Control	13.05±0. 93	136.13±1.62	112. 5±1.21	19.93±0.18	1.30±0.11	±0.07##1.97
GA (100mg /kg)	13.05±0.43ns##	141.18±2.87**##	116.2±0.99 ns##	19.65±0.36 ns##	1.13±0.02 ns##	±0.09 ns##1.43
GA (200mg /kg)	13.93±0.27 ns##	138.08±3.09**##	115.86±1.17 ns##	19.16±0.45 ns##	1.31±0.09 ns##	±0.1 ns##1.54
CP-treated group	60.75±3.15**	70.27±2.41**	51.83±1.90**	49.7±1.45**	5.28±0.18**	±0.76**13.11
GA (100mg /kg) + CP	23.93±1.01**##	119.95±2.90**##	94.51±2.56**##	25.76±0.38**##	2.26±0.12**##	±0.09 ns##2.016
GA (200mg /kg) + CP	16.88±0.99ns##	126.58±2.78ns##	104.11±3.86ns##	22.99±0.21*##	1.88±0.13**##	±0.08 ns##1.41

All the values are presented as the mean ±SEM (n=6).   nsP>0.05 compared with the control group.
*P<0.05 compared with the control group.
##P<0.01 compared with the CP-treated group.   **P<0.01 compared with the control group.

Genotoxicity Studies:
DNA Fragmentation in Kidney Cells:

DNA injuries in kidney cells were evaluated as a DNA ladder representing a series of fragments using agarose gel electrophoresis assay (Fig. 2a) and colorimetrically as the percentages of fragmented DNA utilizing the diphenylamine (DPA) assay (Fig. 2b). The results indicate that the treatment with CP induced DNA damage in the kidney cells. The percentage of fragmented DNA in the kidney cells of rats treated with CP was found to be 6.6 times higher than in the control group. Meanwhile, treatment with the two doses of GA significantly (P<0.01) decreased the percentages of fragmented DNA in a dose-dependent manner as compared with the CP-treated group.
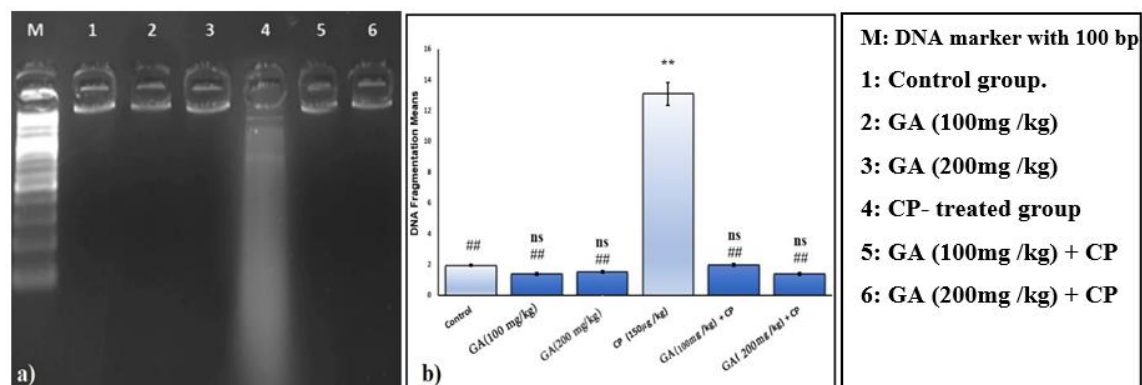

Fig. 2: The prophylactic effect of GA on the renal cellular DNA of rats treated with CP.
a) An agarose gel electrophoresis shows DNA fragmentation. Lane1, 2, 3, 5, 6: DNA without streaks or laddering. Lane 4: DNA with marked streaks and laddering.
b) mean percentages of fragmented DNA in different groups using DPA assay.
All the values are presented as the mean ±SEM (n=6).
nsP>0.05 compared with the control group. **P<0.01 compared with the control group. ##P<0.01 compared with the CP- treated group.

As shown in Fig. 3, the mRNA level of TNF-α, Caspase-3, P53, and Bax were significantly(P<0.01) increased in the CP-treated group when compared to the normal control group. The mRNA levels of the rear genes in the GA control groups were nearly identical to the control group. Similarly, GA administration before CP-intoxication

revealed a significant (P<0.01) improvement in these gene expressions. In contrast, Bcl-2 expression in the kidney of the CP-treated group was significantly (P<0.01) decreased as compared to the control group. While pre-treatment with GA significantly (P<0.01) increased the levels of Bcl-2 as compared to the CP-treated group (Fig. 3e).
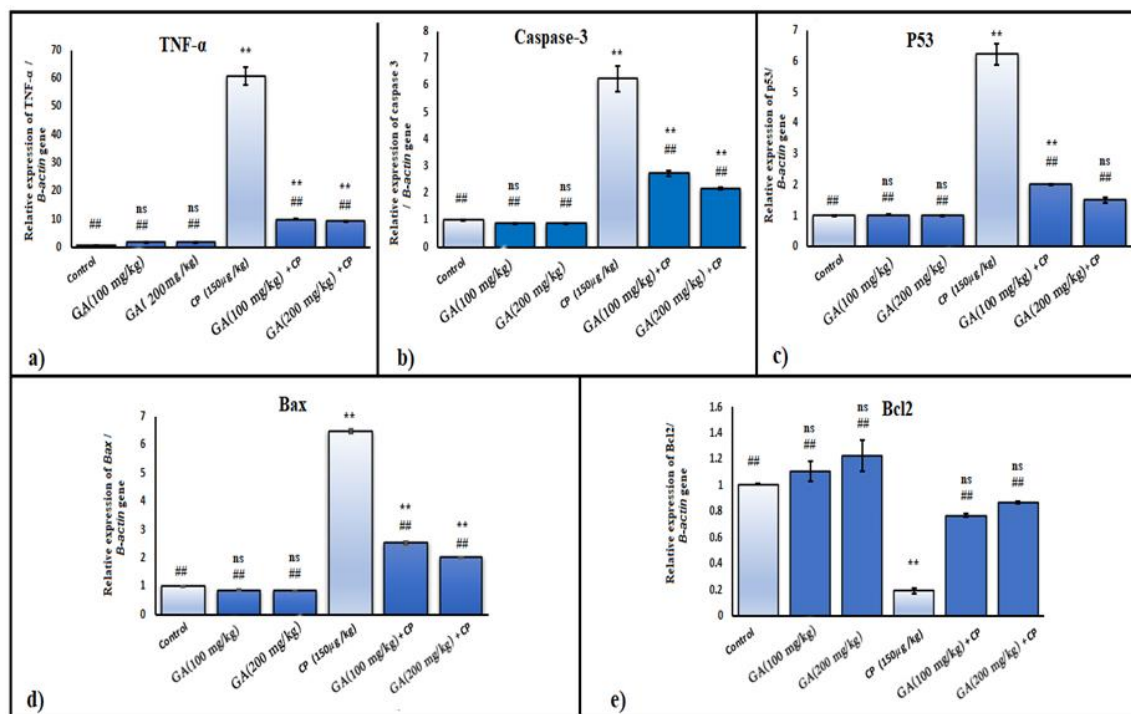

Fig. 3: Comparison between the TNF-α, Caspase-3, P53, Bax, and BCL-2 gene expressions in rat kidney tissue of the studied groups. Values represented as mean ± standard error mean.

nsP>0.05 compared with the control group.
∗∗P<0.01 compared with the control group.
##P<0.01 compared with the CP- treated group

Histological Investigation:

In the present investigation, the histological examinations mostly supported the results of serum enzymes and oxidative stress profiles. In H&E-stained sections, the control group and the two GA control groups (100&200mg/kg) showed normal and well-organized histological features of renal parenchyma with many apparent intact renal corpuscles (black star) and segments of nephron with intact tubular epithelium (arrow) with intact interstitial tissue as well as vasculatures as shown in Fig. 4 (A, B& C) respectively.

On the other hand, the rats injected with CP showed a moderate tubular dilatation of cortical tubular segments (red star) with moderate figures of tubular epithelium necrotic changes (dashed arrow), loss of luminal border integrity and periglomerular lymphocytic infiltrates (red arrow) as well as mild congested interstitial BVs. with a certain degree of tubular degeneration, and tubular necrosis, (Fig.4D).

However, kidney sections of treated rats with either of the two doses of GA pro tected the renal glomeruli and tubules from the deleterious effect of the CP induction in a dose-dependent manner. The kidney sections showed a significant improvement in the morphological features of renal parenchyma with minor sporadic records of tubular dilatation, focal necrotic and degenerative changes of the tubular epithelium **(red arrow)** as well as mild congested glomerular tufts **(star)** and minimal inflammatory cells

infiltrates compared to the rats treated with CP alone. Moreover, the high dose of GA restored the architecture of glomerulus tuft and renal tubules which appeared nearly similar to the control groups (Fig.4E &F).
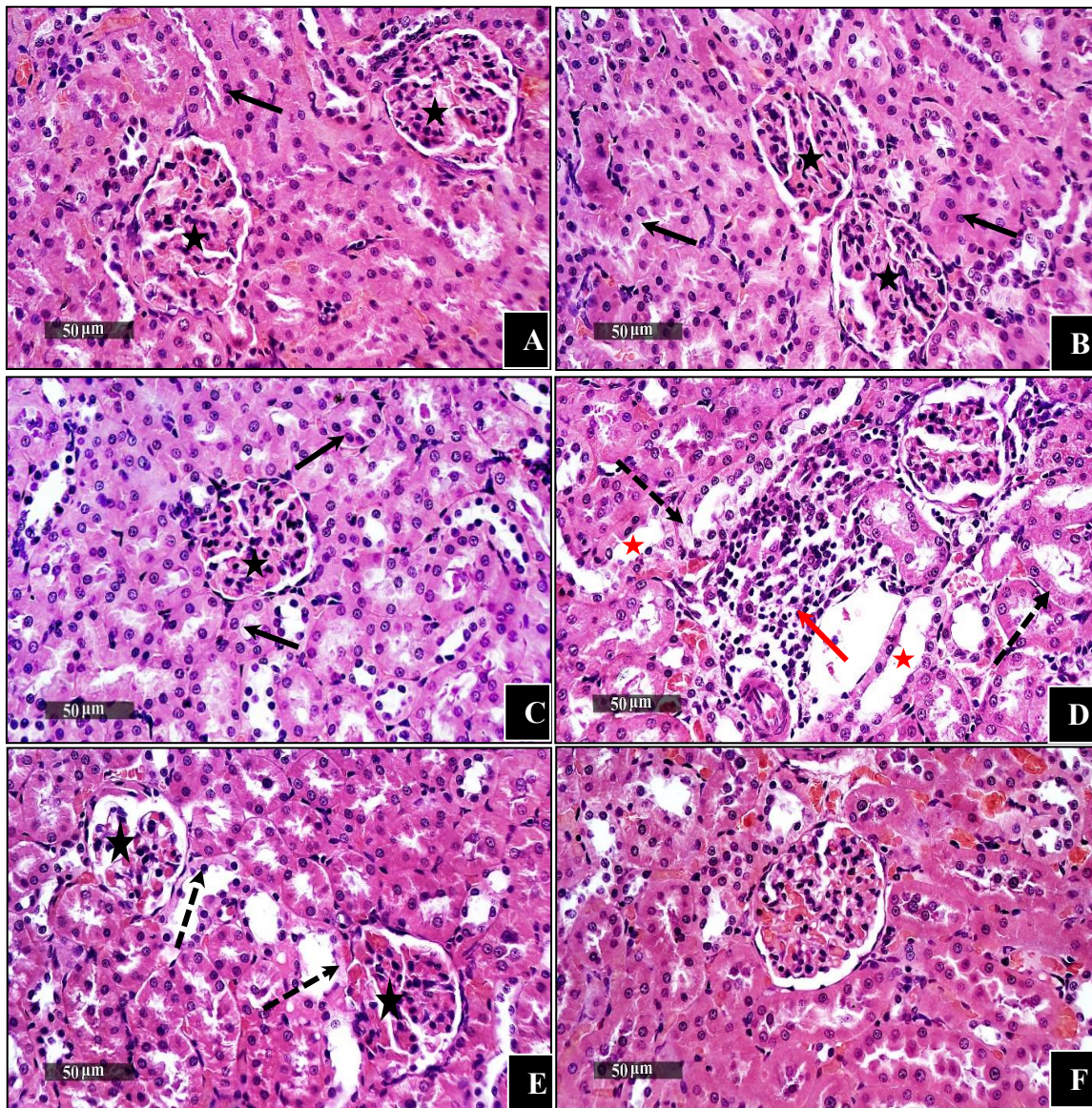

Fig (4): Photomicrographs of **Hematoxylin and Eosin-stained kidney sections** showing the protective effect of two doses of **GA** on CP-induced changes in the kidney of adult male rats. **(A):** Control group, kidney show normal architecture with normal glomeruli (**black star**) and intact tubular epithelium (**arrow**). **(B): GA** (**100mg /kg**), shows normal architecture with normal glomeruli (**black star**) and intact tubular epithelium (**arrow**). **(C): GA** (**200 mg /kg**). **(D):** CP-treated group, shows renal tubular dilatation **(red star),** moderate tubular epithelium necrotic changes **(dashed arrow),** and periglomerular lymphocytic infiltrates **(red arrow). (E): GA (100mg /kg) + CP,** shows nearly normal morphological features of renal parenchyma with minor tubular dilatation, focal necrotic and degenerative changes of tubular epithelium **(red arrow),** mild congested glomerular tufts **(star). (F): GA (200 mg /kg) + CP,** shows normal features of glomerulus tuft and renal tubules architecture. **(H& E, X 400).**

Immunohistochemical Investigation (IHC):

Along side the histological investigation, optical density expression of the tight-junction proteins, ZO-1was detected in the glomeruli histologically by IHC staining (Fig.5). The means and standard error are used to represent the values (Fig. 5G). There was no significant(P>0.05) correlation detected between the control group and either of the GA-treated groups (Fig. 5A, B & C). In contrast, there was a significant reduction of ZO-1protein expression after CP administration (P< 0.01) as compared to the control group(Fig. 5D). However, the treatment with the two doses of GA protected the tight junction between the cells from the CP effect by inducing a significant (P<0.01) elevation of ZO-1 expression in a dose-dependent manner when compared with the CP-treated group as shown in Fig.5 (E& F).
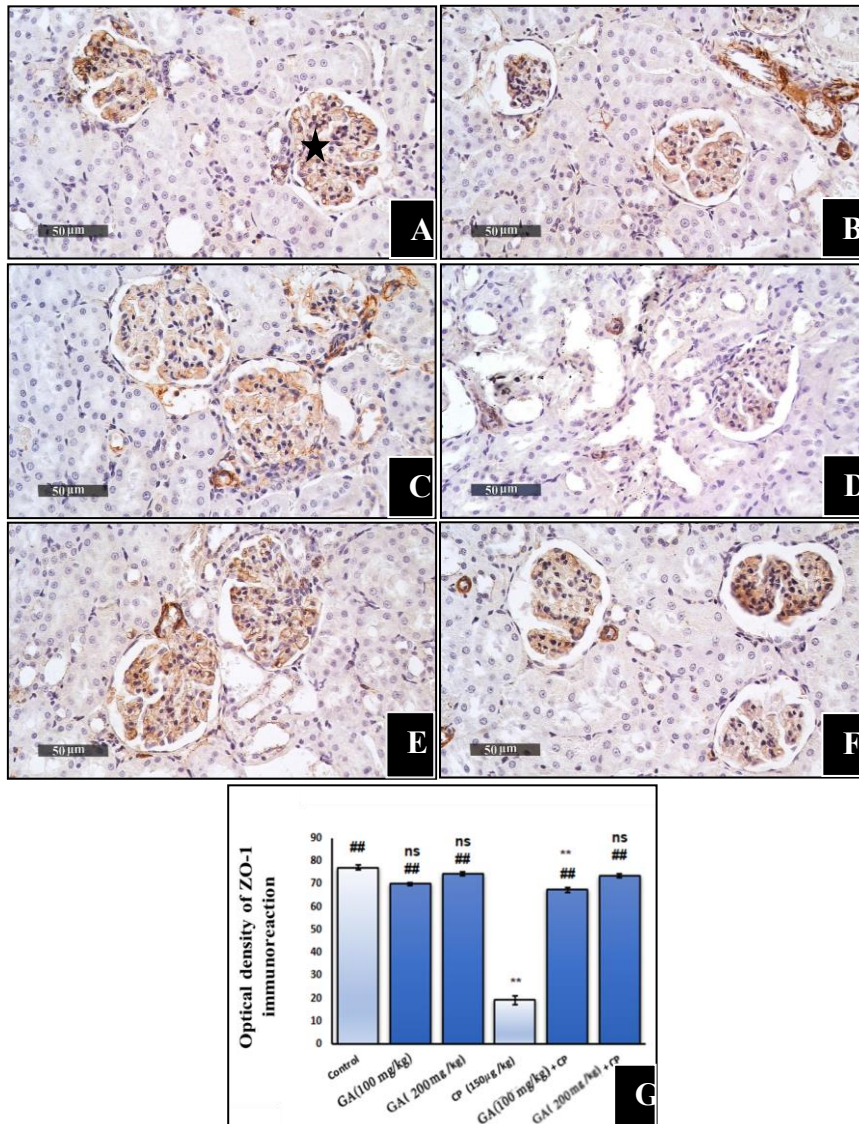

Fig. 5: Photomicrographs of ZO-1 Immunohistochemistry in kidney tissues. **(A):** Control group, show normal ZO-1 optical density immuno-reaction with intact brown colour **(star). (B):GA (100 mg /kg)** group, shows normal distribution of immuno-reactive cells. **(C): GA (200 mg /kg)** group**,** show normal distribution of immuno-reactive cells. **(D): CP- treated** group, show reduction of ZO-1 immuno-reactive cells. **(E): GA (100 mg /kg) + CP,** show increase of ZO-1 immuno-reactive cells. **(F): GA (200 mg /kg) + CP,** show marked increase of ZO-1 immuno-reactive cells. **(G):** Chart illustrates the optical density of ZO-1 immunoreaction in kidney tissues of all groups. The values are represented as mean ± S.E and significance (p < 0.05) compared to the control group; [ns]**P> 0.05** compared with the control group.[**]**P<0.01** compared with the control group. [##]**P<0.01** compared with the CP- treated group.

DISCUSSION

Cyclophosphamide is a highly effective chemotherapeutic agent used alone or in combination with other chemotherapeutics to treat various types of cancer, which causes tissue toxicity by its reactive metabolites. However, the results of the present study reported that a single dose of CP induced kidney damage as evidenced by increased renal function biomarkers, i.e., serum urea and creatinine. These results agreed with (Olayinka *et al*., 2015; Bokolo and Adikwu, 2018; Ayza *et al.,* 2020 and Aladaileh *et al*., 2021) who reported a significant increase in plasma levels of urea and creatinine after intraperitoneal injection of CP to rats, indicating marked damage to functioning nephrons.

Oxidative stress and free radical generation in renal tubular cells have been proposed to be the reason for CP-induced renal damage (Abraham *et al.,* 2007 &Al-Saeed *et al.,* 2017). In the present study, GA ameliorated CP-induced renal toxicity as indicated by the decrease of serum urea and creatinine levels by maintaining the renal cellular membrane integrity through improving the antioxidant status (Ahmadvand *et al.,* 2019). It seems that GA had a positive effect on renal function. Similar studies showed that administration of GA for seven or eight consecutive days in doses of 30, 200, or 400 mg/kg could improve kidney dysfunction in rats intoxicated with gentamicin, through its antioxidant activities and its capacity to keep the cellular membrane integrity (Ghaznavi *et al.,* 2018).

Also, previous studies indicated that GA succeed to protect the kidney in pathological conditions such as lindane-induced toxicity (Said, 2011&Manshare *et al.,* 2018) and Renal ischemia-reperfusion injury (RIR) (Ahmadvand *et al*., 2019) due to its anti-oxidative properties. The results offered by a previous study expressed that GA, 20 mg/kg for seven days isolated from *Peltiphyllum peltatum* attenuated the serum levels of urea, creatinine, and uric acid in rats treated with sodium fluoride (NaF) induced nephrotoxicity (Nabavi *et al.,* 2013).

In the current study, we observed that intraperitoneal administration of CP led to oxidative stress as evident from significant increases in MDA level and decreases in antioxidant enzymes such as GSH and CAT. MDA is one of the final products of lipid peroxidation. Lipid peroxidation causes the break of lipids with the formation of reactive compounds leading to DNA and protein oxidation, changes in the permeability of lipid bilayer membrane, and can dramatically alter cell integrity (Barrera, 2012). In the same manner, CP exhibited an increase in MDA level induce kidney injury as stated by Aladaileh *et al.* (2021).

Furthermore, increased serum levels of MDA can be the cause of many diseases associated with oxidative stress, such as diabetes (Kumari *et al.,* 2008). In the present study, animals pre-treated with GA displayed a significant decrease in MDA level especially with the highest dose of GA. Consistent with our findings, several reports highlighted that natural antioxidants such as Gallic and tannic Acids Akomolafe *et al*. (2014), eugenol, selenium, hydroxytyrosol, quercetin (Chashmi *et al*., 2017), erdosteine, α-tocopherol (Yurdakul *et al*., 2010) provoked the ameliorative effects on MDA levels. The restoration of oxidative stress by improving the antioxidant defense system might be ascribed to the free radical scavenging/antioxidant properties of the phytochemical constituents present in GA. These compounds can decrease lipid peroxidation and attenuate renal dysfunction and increase antioxidant enzyme activity. (Balagangadharan, 2012; Chilwant and Muglikar, 2012 and Aladaileh *et al.,* 2021).

Additionally, in the current study, the GSH and CAT activities were significantly decreased in CP-treated rats as reported earlier by Olayinka *et al.* (2015), this could be

due to the inactivation of cellular antioxidants by the ROS and oxidative stress with a cascade of effects on the functional and structural integrity of organelles and cell membranes (Nagaraj *et al.,* 2012).

Consistency with the results of this study, other researchers indicated that toxicity of CP is due to depletion of the GSH concentration and decreased antioxidant enzyme activity in renal tissue of rats (Said *et al*., 2015&Aladaileh *et al.*, 2021). The CP-induced depletion of GSH is primarily mediated by the interaction of its reactive metabolite; acrolein with cysteine which is one of the constituent amino acids of GSH (HamsaandKuttan, 2012). Therefore, some sulfhydryl (SH) compounds and cysteine itself have been observed to protect the animal from the toxic effect of CP (Rehman *et al.,* 2012).

According to the findings of the current study, GA improved antioxidant status by increasing GSH levels. Our findings are consistent with those of Olayinka *et al.* (2016), Ghaznavi *et al.* (2018), and Bahrami *et al.* (2021) who hypothesized that GA and other natural antioxidants could reduce lipid peroxidation and nitric oxide while increasing GSH content. Increased NO levels cause tissue damage primarily through the reaction with superoxide anion and the production of peroxynitrite, a powerful oxidant.

The increased CAT and GSH activities observed in GA-treated animals may be related to GA's ability to scavenge ROS and elevate levels of antioxidant enzymes' gene expression (Ghaznavi *et al*., 2018).

Moreover, different studies showed that GA could augment the activities of antioxidant enzymes SOD, CAT, and GSH in many pathological conditions such as myocardial infarction, CCl4-induced chronic liver injury, diabetes, and spinal cord injury (Oboh *et al*., 2016 & Kahkeshani *et al*., 2019). According to previous studies, GA has a huge antioxidant capacity that normalized the abnormal outcomes.

Also, the present study revealed that a single intraperitoneal administration of CP in rats induced a significant increase in both DNA strand breaks and a percent of DNA fragmentation in renal tissue that could be a result of excessive oxidative stress induced by CP injection (Rehman *et al*., 2012).

The current study found that pre-treatment with GA at both doses resulted in a significant reduction in the percent of DNA fragmentation. The current findings point to GA playing an important role in preventing CP-induced DNA damage and genotoxicity.

Moreover, increased ROS production by CP in renal tissues can damage several cell structures (Amien *et al*., 2015). Such oxidative stress can activate the p38 MAPK (mitogen-activated protein kinases). P38MAPK regulates numerous apoptotic and inflammatory pathways (Rashed *et al*., 2011).

Consistent with previous studies (Liu *et al*., 2016& Kang *et al*., 2019), the kidneys of CP-injected rats exhibited an increase in apoptosis, as evidenced by reducing Bcl-2 levels and elevating Bax, P53, and caspase-3 levels. CP-mediated apoptosis is supposed to be produced by increased ROS generation, which in turn sparks the DNA damage, and ultimately lead to the initiation of the mitochondrial apoptotic pathway by enhancing the expression of pro-apoptotic proteins and down-regulating of anti-apoptotic proteins (Liu *et al*., 2016; ALHaithloul *et al.,* 2019 & Kang *et al.,* 2019).

Numerous studies have suggested that using antioxidants may protect against CP-induced renal apoptosis (Liu *et al.,* 2016, Rehman *et al.,* 2012, El-Shabrawy *et al.*, 2020). Herein, the GA-treated group showed significant downregulate (p<0.05) in the expression levels of pro-apoptotic caspase-3, P53, and Bax genes along with significant up-regulate (p<0.05) in the expression level of anti-apoptotic Bcl-2 gene, suggesting that GA might possess anti-apoptotic effects. Similar results were reported by Jin *et al*. (2017).

These findings are consistent with those of Ahad *et al*. (2015), who discovered that GA significantly inhibited renal p38 MAPK and nuclear factor kappa B (Nf -κB) activation while significantly lowering renal transforming growth factor-beta (TGF- β) and fibronectin levels. The use of GA resulted in a significant decrease in serum levels of pro-inflammatory cytokines like interleukin 1 beta (IL-1 β), IL-6, and tumor necrosis factor-alpha (TNF- α).

In the current study, TNF- α gene expression levels increased significantly after CP injection. In the same manner, Said *et al*. (2015) stated that administration of CP significantly impaired oxidant/antioxidant balance and increased TNF-α level, with a significant impairment of kidney architecture and functions. TNF-α plays a key role in activating a large network of chemokines and cytokines, which mediates renal injury by inducing apoptosis (Yao *et al*., 2007; Sanz *et al.,* 2008 &Kotb *et al.,* 2021).

TNF-α can cause tubular cell death and tissue damage directly through TNF receptor type 1 (TNFR1) and indirectly through TNF receptor type 2 (TNFR2). TNF-α is known to coordinate the activation of a large network of pro-inflammatory cytokines, including interleukin-4, 1, 6 (IL-4β, IL-1, IL-6), monocyte chemotactic protein-1 (MCP-1) and TGF-β1 (Peres &da Cunha, 2013). TNF-α has been shown to elicit a series of different inflammatory molecules that causes tissue damage, such as prostaglandins, IL-8 (interleukin-8), and ROS (Giebelen *et al.,* 2007). The current study confirmed that GA reduced the elevated level of TNF-α after CP injection which indicates its protective effect against CP-induced nephrotoxicity. Similar results were stated before by Asci *et al.* (2017) and Bahrami *et al.* (2021).

The histopathological changes were consistent with the biochemical and molecular results that confirm the nephrotoxicity of CP. The renal injury in the present study was associated with changes in the histoarchitecture of the kidney, loss of glomerular epithelium, dilation, edema, vascular congestion, and inflammatory cell infiltration. Prior studies revealed that the renal injury induced by CP is linked with the inflammation as it generates ROS that triggers the inflammatory mediators and causes a fast production of pro-inflammatory cytokines such as TNF-α as mentioned above, thus inducing necrosis (Bhat *et al.,* 2018, ALHaithloul *et al*., 2019, Aladaileh *et al.,* 2021).

Moreover, the loss of glomerular epithelium could be linked with lipid peroxidation that causes the alteration of the membrane permeability as a result of inactivating membrane-bound proteins, this will lead to loss of membrane integrity and cell death (Nirmala and Lopus, 2020 &Aladaileh *et al.,* 2021).

On the other hand, our study revealed the anti-inflammatory and antioxidant efficacy of GA in a dose-dependent manner. It plays a nephroprotective role by reserving and stabilizing the cell membranes integrity and renal architecture via scavenging activities of free radical (Reckziegel *et al.,* 2016; Ghaznavi *et al*., 2018; Ahmadvand *et al.,* 2020 & Nouri *et al.,* 2021)

Also, this study is strongly supported by the immunohistochemical expression of one of the most studied tight junction proteins, ZO-1. These proteins play an important role in maintaining the tight junction complex structure (Ahn *et al*., 2016) and signaling transduction (Murphy *et al*., 2013). In addition, they found on the intracellular surface of the plasma membrane, which provides a link between the integral membrane proteins and the actin component of the cytoskeleton, thus connecting the skeleton of the adjacent cells (Anderson and Van Itallie, 2009). The disruption in tight junction integrity may increase cell permeability, leading to protein and fluid leakage, thus causing renal damage as stated by Wichapoon *et al*., 2014.

In the present study, CP exhibited a loss of tight junction protein by decreasing the ZO-1 expression. This decrease may be due to the increase of oxidative stress that

deactivates membrane-bound proteins, destroying the cell membrane (Smathers *et al.,* 2011, Aladaileh *et al.,* 2021). In line with this investigation, Yang *et al.* (2019) stated that ZO-1 was more sensitive to H_2O_2 and respond rapidly to oxidative stress. On the other hand, the administration of GA in a dose-dependent manner enhances the cellular integrity as demonstrated by increasing the expression of ZO-1 protein, this might be attributed to the antioxidative properties of GA That can scavenge ROS, strengthen the cellular antioxidant system, and restore the tight junction proteins. In the same manner, Cai *et al.* (2021) assumed that the pre-treatment with GA preserved the expression level of tight junction proteins ZO-1 in lipopolysaccharide (LPS-stimulated intestinal porcine enterocytes isolated from the jejunum (IPEC-J2) cells.

As a result, our findings highlight the protective roles of GA in mitigating tissue damage caused by oxidative stress via improving the levels of renal function markers, reducing the levels of urea, creatinine, and MDA, and increasing the levels of GSH and improving histopathological changes which may be associated with its antioxidant activities.

In conclusion, the current biochemical, molecular, histopathological, and immunohistochemical findings support Gallic acid's protective efficacy against CP-induced nephrotoxicity by enhancing the antioxidant defense mechanisms and by alleviating the inflammatory status. Due to the decrease of nephrotoxic side effects, GA administration may be beneficial for patients who have been using CP for a long time. Consequently, it could be combined with the pharmaceutical formulation of CP and may be used safely and effectively to treat cancer or autoimmune diseases. During CP therapy, cancer patients should consume more food sources that contain GA.

Authors' Contributions:
The authors participated equally in all parts of the research.

Conflict of Interest:
The authors declare that they have no conflict of interest.

Ethical Approval:
All applicable international, national, and institutional guidelines for the care and use of animals were followed. We respected the welfare of animals and excluded situations when animals were in pain.

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ARABIC SUMMARY

التأثير الوقائي لحمض الجاليك ضد السمية الكلوية ، والإجهاد التأكسدي ، والسمية الوراثية ، والتغيرات النسيجية المرضية التي يسببها السيكلوفوسفاميد في ذكور الجرذان البيضاء

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يعتبر السيكلوفوسفاميد علاج كيميائي يستخدم لعلاج أنواع مختلفة من السرطان. وعلى الرغم من آثاره الجانبية العصبية والكبدية والكلوية والسمية للخلايا ، إلا أنه عامل فعال للغاية سواء تم استخدامه بمفرده أو بالاشتراك مع علاجات كيميائية أخرى. وقد تم تصميم الدراسة الحالية لفحص التأثيرات الوقائية لحمض الجاليك (GA)على السمية الكلوية الحادة التي يسببها السيكلوفوسفاميد. حيث استخدمت ذكور الفئران البيضاء في هذه التجربة. وقسمت الحيوانات إلى 6 مجموعات ، احتوت كل مجموعه على 6 حيوانات وقد أعطيت المجموعة الأولى (المجموعة الضابطة) محلول ملحي ، بينما أعطيت كلا من المجموعة الثانية والثالثة حمض الجاليك بجرعات تعادل 100 و 200 مجم / كجم على التوالي لمدة 15 يومًا. تم حقن المجموعة الرابعة مرة واحدة بما يعادل 150 مجم / كجم من السيكلوفوسفاميد. بينما تم معالجة كلا من المجموعة الخامسة و السادسة بما يعادل100 و 200 مجم / كجم من حمض الجاليك على التوالى لمدة 15 يومًا قبل حقنهم بالسيكلوفوسفاميد وقد تمت كل المعالجات بواسطة الحقن داخل الصفاق وتم تشريح الحيوانات بعد 24 ساعة من آخر معالجة ، حيث تم استخراج الكليتين بعناية من أجل إجراء الدراسات النسيجية والكيميائية المناعية للـ ZO-1 والفحص الكيميائي الحيوي ، بالإضافة إلى تقدير التعبير الجيني لكلًا منP53 ، وعلامات موت الخلايا المبرمج ، ونخر الورم (TNF-α). كذلك تم تحديد مستويات كلًا من الكرياتينين واليوريا في الدم. وقد أثبتت النتائج أن تناول حمض الجاليك أدى إلى تحسين وظائف الكلى ، كما يتضح من انخفاض مستويات مؤشرات سمية الكلى (اليوريا والكرياتينين). كما أدى حمض الجاليك الى إحداث انخفاض ذو دلالة معنوية (p<0.05) فى نسبة تكسر الحمض النووي في أنسجة الكلى عن طريق تعديل مستويات الجلوتاثيون (GSH) وإنزيمات الكاتلاز (CAT) بالإضافة إلى مالونديالديهايد (MDA).

علاوة على ذلك ، قلل حمض الجاليك من التعبير الجيني الكلوي لكلًا من TNF-α و P53 كما حسّن البنية النسيجية للكلية بالإضافة إلى زيادة إفراز ZO-1. وختامًا قد أكدت نتائج الدراسة الحالية أن حمض الجاليك يحمي من التسمم الكلوي الناجم عن السيكلوفوسفاميد وذلك من خلال آلية مضادة للالتهابات ومضادة للأكسدة. كما نعتقد أن حمض الجاليك قد يكون له تأثير وقائي ضد السمية الكلوية التي يسببها السيكلوفوسفاميد.