Study on the alleviating effect of *Punica granatum* against toluene-induced renal and oxidative stress damage in rats

Fatma Zohra Arkoub[1,2]*, Rahma Boukarine[1,2], Leila Hamdi[1], Labiba Kahalerras[3], Kamel Khelili[1], and Cherif Abdennour[1]

[1]Laboratory of Animal Ecophysiology, Department of Biology, Faculty of Sciences, University Badji Mokhtar-Annaba, Annaba 23000, Algeria.

[2]Laboratory of Environmental Sciences and Agroecology, Department of biology, Faculty of Life and Natural Sciences, University Chadli Bendjedid- El Tarf, El Tarf 36000, Algeria.

[3]Department of Agronomy, Institute of Veterinary Sciences and Agronomic Sciences, University El Hadj Lakhdar-Batna-1, Batna 05000, Algeria.

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Abstract

Organic solvents exposure has been shown to affect the functional integrity of various organs. The current study was carried out to evaluate the renal dysfunction induced by toluene (Tol) toxicity, and the potential protective role of *Punica granatum* fresh peel aqueous extract (FPAE) in male rats. The orally treated rats were divided as follows: Control (C), positive controls (corn oil: 1.25 mL/kg BW; and FPAE: 400 mg/kg BW), Tol1: 275 mg/kg BW, Tol2: 550 mg/kg BW and a mixture each of FPAE-Tol1 and FPAE-Tol2. After 6-week study period, urea, creatinine, and uric acid were estimated, as well as renal histology and oxidative stress markers. Results showed that Tol group has significant increase of serum urea and creatinine levels, with a significant elevation in renal malondialdehyde (MDA) levels, and a significant decrease of glutathione content and glutathione peroxidase activity. FPAE co-administration partially retrieved the changes in almost all studied parameters compared with the Tol group. Tol induced histopathological kidney damage, which was minimized as a result of *P. granatum* treatment. In conclusion, this study provides evidence that FPAE attenuates renal oxidative injury induced by Tol, supporting the traditional claims of its beneficial effects, possibly due to its antioxidant potential.

Corresponding Author : *f.arkoub@univ-eltarf.dz*

1. INTRODUCTION

Nowadays, renal diseases represent a major global public health issue, significantly contributing to a high incidence of morbidity and mortality **[1].** Kidneys are vital organs composed of thousands of functional units called nephrons that continuously filter the blood to excrete waste matters **[2]**. They act like a chief player in eliminating toxic metabolites produced from catabolism, making them frequent targets of various xenobiotics as medications, industrial toxins, which are primarily excreted through those organs **[2]**.

Humans' constant exposure to hazardous pollutants is largely driven by lifestyle behaviors. Despite being less widely recognized than illicit drugs, inhalants abuse represents a common problem in the adolescents around the world**.** The ease of their availability and low cost, have made them easier for consumption **[3].** Toluene is an aromatic hydrocarbon commonly used for abuse in different ways**.** It is also present in gasoline and some cleaning products. As a lipophilic substance, it has a strong affinity for lipids and can easily diffuse into fatty tissues **[4].** Toluene abuse is associated with harmful effects on health, including euphoria followed by depression0. Prolonged or high-level

exposure can damage the central nervous system, heart as well as reproductive changes, hepatic and renal failure **[5,6].** These effects are primarily attributed to suppression of free radical scavenging function and the enhancement of ROS by toluene induced, leading to oxidative stress and lipid peroxidation **[7].** Toluene toxicity can be mitigated through antioxidant defense mechanisms, which help reduce ROS, neutralize free radicals, and enhance the elimination of toxic compounds. This toxicity can be mitigated through dietary supplementation with antioxidant-rich products, which reduce or eliminate reactive oxygen species and free radicals **[8]**.

Currently, natural antioxidants are used as potential health benefits that are abundant in various spices, herbs, vegetables, and fruits **[9,10].** The natural antioxidant defense system plays a crucial role in neutralizing many toxic effects in the body **[10,11].** The *Punica granatum,* commonly called pomegranate, is a seasonal crop of the Punicaceae family grown in the Mediterranean region, as well as Pakistan, India, and Iran**.** Pomegranate is a rich source of bioactive molecules such as hydrolyzable tannins (ellagitannin, punicalagin, punicalin and pedunculagin), flavonoids, anthocyanins etc…. These components place pomegranate in a higher grade compared to other fruits **[12].**

P. granatum have attracted a great deal of attention because of its potential health benefits, which include antioxidant properties and high potency in the elimination of free oxygen radicals, as well as anti-cancer, anti-inflammatory, anti-lipoperoxidation, and DNA repair activities; in which its consumption has been linked to a possible risk reduction of some cancers, heart diseases, diabetes, and obesity **[12]**, and to male fertility improvement **[13].**

Despite the studies carried out demonstrating its antioxidant properties and medicinal benefits of *Punica granatum*, however, none has dealt with the association between this fruit and toluene consumption. Therefore, the present study was conducted to shed some light on the likely attenuating potentials of *P. granatum* against toluene renal damage of Wistar rats.

2. MATERIAL AND METHODS

Chemicals

Toluene ($C_6H_5CH_3$, purity 99.5%), manufactured by Sigma-Aldrich Chemicals Co. (St. Louis, MO, USA), was purchased from a chemical shop in Annaba, Algeria. All other chemicals used in this experiment were analytical grade and supplied by Animal Ecophysiology Laboratory.

Fruit collection and extract preparation

Mature *Punica granatum* were obtained from a farmland (Besbes-El-Taref). Following collection, fresh fruits were cleaned under running tap water and the fresh peels were removed, crushed into fine pieces using a blender (Sinbo, Turkey). The pieces were extracted using distilled water (via cold maceration) for 72 h at room temperature with continuous shaking. The mixture was decanted and filtered using cotton gauze.

Animals and experimental design

Adult male Wistar rats weighing 230±30 g, were obtained from the Pasteur Institute of Algiers and housed at the breeding house of Badji Mokhtar Annaba University. They were kept in standard polypropylene cages with access to water and standard rat food purchased from the agro-food complex (El-Kseur, Bejaia). The animals were maintained at an ambient temperature of 22±2°C, humidity, and natural photoperiod.

After acclimatization period, rats were divided into seven groups (each containing ten rats) and were maintained for the experimental period of 45 days, as follows:

C: It is served as a normal control group. Animals of this group given distilled water.
CO: Animals of this group given 1.25 mL/kg BW of corn oil.
FPAE: Animals of this group received 400 mg/kg BW of *P. granatum* peel fresh aqueous extract.
Tol1: Rats received 275 mg/kg BW of toluene dissolved in corn oil as vehicle.
Tol2: Rats received 550 mg/kg BW of toluene dissolved in corn oil as vehicle.
FPAE-Tol1: Animals received Tol (275 mg/kg BW), followed after 2 hours by FPAE (400 mg/kg BW).
FPAE-Tol2: Rats received Tol (550 mg/kg BW), followed after 2 hours by FPAE (400 mg/kg BW).

Samples collection

Animals were sacrificed by decapitation and their kidneys were removed; one kidney was quickly frozen at -20°C for the assessment of oxidative stress parameters, while the second was fixed in formalin 10% for histological examination. Blood samples were collected in dry tubes, and serum was obtained by centrifugation at 3000× g for 10 min to measure biochemical parameters.

Determination of biochemical parameters

Serum urea, creatinine, and uric acid levels were determined using an automated chemistry analyzer (ERBA XL-600, ERBA Diagnostics Mannheim GmbH, Mannheim, Germany).

Determination of oxidative stress parameters

The reduced glutathione level (GSH) was measured spectrophotometrically at 412 nm, using the method of **Wekbeker and Cory [14].** Glutathione peroxidase (GSH-Px) activity was assessed according to the method of **Flohe and Gunzler [15].** Lipid peroxidation was measured as MDA determined by thiobarbituric acid (TBA) assay according to **Ohkawa et al. [16].** Total protein concentration was determined according to the method of **Bradford [17]** by using bovine serum albumin as a standard.

Histopathological examination

Formaldehyde-fixed tissues were dehydrated in gradual ethanol (70–100%) and were embedded in paraffin wax. Sections of 5 µm thicknesses were then prepared using a rotary microtome following staining by using hematoxylin and eosin (H&E) dye for microscopic observation under alight microscope (BioLab, Mumbai, India) **[18]**.

Statistical analysis

Statistical analysis of the data was performed using GraphPad Prism 7 (GraphPad Software, San Diego, CA, USA) and Statistical Package for the Social Sciences SPSS version 26.0 for Windows (IBM Corporation, Armonk, NY, USA). Results are expressed as mean ± SEM (Standard Error of the Mean). All measured parameters were analyzed using one-way ANOVA, and all statistical analyses were followed by a post hoc test (Tuckey's test), with significance set at $p<0.05$.

3. RESULTS

Kidney weight

The kidney absolute weight is shown in figure 1. Exposure to toluene (Tol2) significantly increased kidney weight compared to the control and positive control groups. In contrast, the combined group (FPAE-Tol2) showed an insignificant decrease in kidney weight compared to the Tol group.

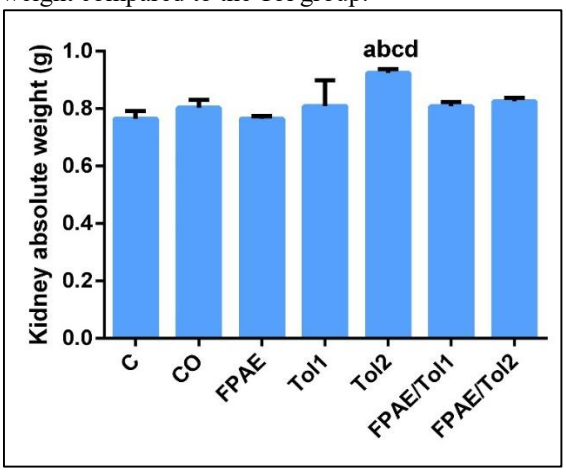

Figure 1. Kidney absolute weight of rats in different experimental groups (mean±SEM).
a: statistically different Vs control. **b**: statistically different Vs CO group. **c**: statistically different Vs PJ group. **d**: statistically different Vs PJ group.**e**: statistically different Vs Tol group.
C: Control, **CO**: Corn Oil, **FPAE**: Fresh Peel Aqueous Extract, **Tol1**: Toluene dose 1; **Tol2**: Toluene dose 2, **FPAE-Tol1**: Toluene dose 1 + Fresh Peel Aqueous Extract, **FPAE-Tol2**: Toluene + Fresh Peel Aqueous Extract).

Serum biochemical parameters

As shown in figures 2 and 3, oral administration of toluene resulted in a significant increase in serum urea and creatinine concentrations compared to the control and positive control groups. However, supplementation with pomegranate peel aqueous extract FPAE-Tol significantly reduced the concertation compared to the Tol2 group.
The serum uric acid of Tol2 group showed a significant decrease compared with the control and positive control groups (Figure 4). No significant differences were noted in the Tol1 group compared to the control animals.

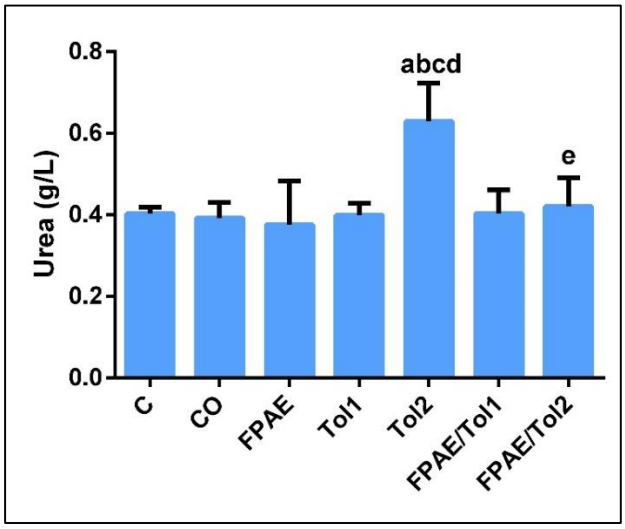

Figure 2. Urea levels in different experimental groups (mean±SEM).
a: statistically different Vs control. **b**: statistically different Vs CO group. **c**: statistically different Vs PJ group. **d**: statistically different Vs PJ group.**e**: statistically different Vs Tol group.
C: Control, **CO**: Corn Oil, **FPAE**: Fresh Peel Aqueous Extract, **Tol1**: Toluene dose 1; **Tol2**: Toluene dose 2, **FPAE-Tol1**: Toluene dose 1 + Fresh Peel Aqueous Extract, **FPAE-Tol2**: Toluene + Fresh Peel Aqueous Extract).

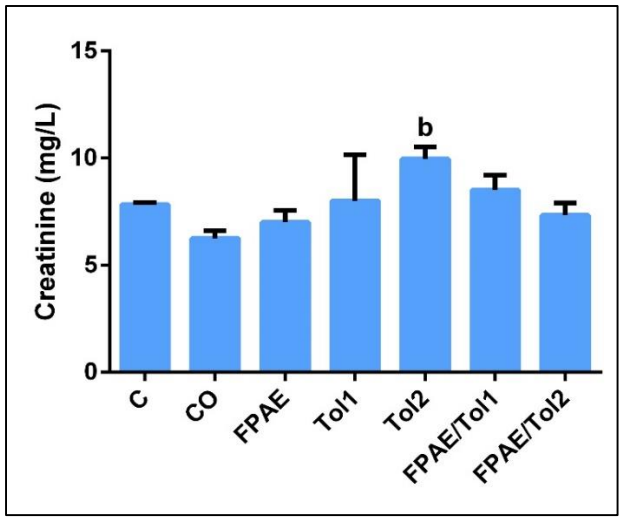

Figure 3. Creatinine levels in different experimental groups (mean±SEM).

a: statistically different Vs control. **b**: statistically different Vs CO group. **c**: statistically different Vs PJ group. **d**: statistically different Vs PJ group.**e**: statistically different Vs Tol group.
C: Control, **CO**: Corn Oil, **FPAE**: Fresh Peel Aqueous Extract, **Tol1**: Toluene dose 1; **Tol2**: Toluene dose 2, **FPAE-Tol1**: Toluene dose 1 + Fresh Peel Aqueous Extract, **FPAE-Tol2**: Toluene + Fresh Peel Aqueous Extract).

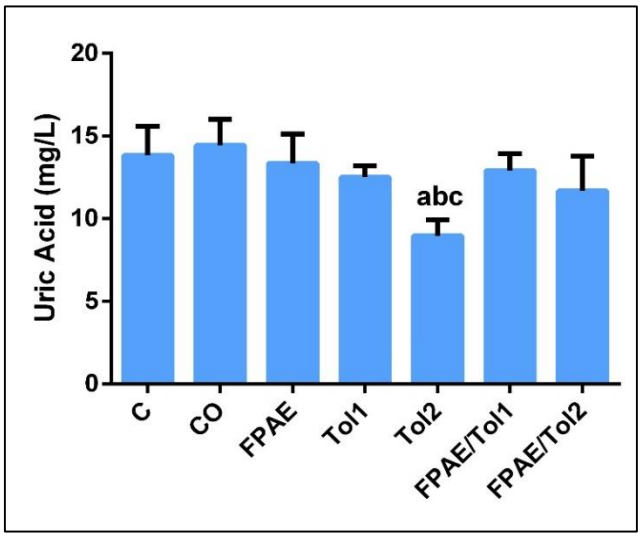

Figure 4. Uric Acid levels in different experimental groups (mean±SEM).

a: statistically different Vs control. **b**: statistically different Vs CO group. **c**: statistically different Vs PJ group. **d**: statistically different Vs PJ group.**e**: statistically different Vs Tol group.
C: Control, **CO**: Corn Oil, **FPAE**: Fresh Peel Aqueous Extract, **Tol1**: Toluene dose 1; **Tol2**: Toluene dose 2, **FPAE-Tol1**: Toluene dose 1 + Fresh Peel Aqueous Extract, **FPAE-Tol2**: Toluene + Fresh Peel Aqueous Extract).

Oxidative stress parameters

As indicated in table 1, oxidative stress results revealed a significant reduction in the renal GSH level and GPx activity, whereas the renal MDA concentration was significantly increased in rats of Tol2 group compared to the control and positive control groups (CO and FPAE). On the other hand, the group Tol1 did not show significant difference compared controls.

Conversely, the administration of *P. granatum* FPAE significantly increased the level of GSH and the activity of GPx compared to Tol2 group. Results also showed a significant decrease in MDA levels of FPAE supplemented rats compared to the group treated with Tol2.

Table 1. Oxidative stress parameters of rats in different experimental groups (mean±SEM).
C: Control, **CO**: Corn Oil, **FPAE**: Fresh Peel Aqueous Extract, **Tol1**: Toluene dose 1; **Tol2**: Toluene dose 2, **FPAE-Tol1**: Toluene dose 1 + Fresh Peel Aqueous Extract, **FPAE-Tol2**: Toluene + Fresh Peel Aqueous Extract).

Groups	GSH Kidney (nmoL/mg Prts)	GPx Kidney (nmoL GSH/mg Prts)	MDA Kidney (nmoL/mg Prts)
C	56.11±3.75	0.44±0.007	0.32±0.01
CO	54.83±2.71	0.43±0.02	0.34±0.01
FPAE	57.03±2.59	0.45±0.004	0.29±0.007
Tol1	54.81±3.13	0.42±0.02	0.36±0.008
Tol2	38.76±5.27^{abcd}	0.31± 0.02^{abcd}	0.53±0.02^{abcd}
FPAE-Tol1	53.88±0.78	0.42±0.009	0.34±0.02
FPAE-Tol2	51.27±0.81	0.40±0.01^{e}	0.43±0.01^{de}

Histological examination

As shown in Figure 5, Renal microscopic observation in the control rats (C, H, PJ, FPAE) revealed a normal renal parenchyma with normal architecture, consisting of the glomerulus (GL) surrounded by a narrow and clear space. Tol2 exposure (E) resulted in renal parenchyma changes and disorganization, characterized by extensive vacuolization, dilatation of Bowman's space, degeneration of the renal tubular epithelium, and glomerulus atrophy. Whereas renal tissues of rats in FPAE-Tol2 (G) groups were less affected compared with the Tol2 group.

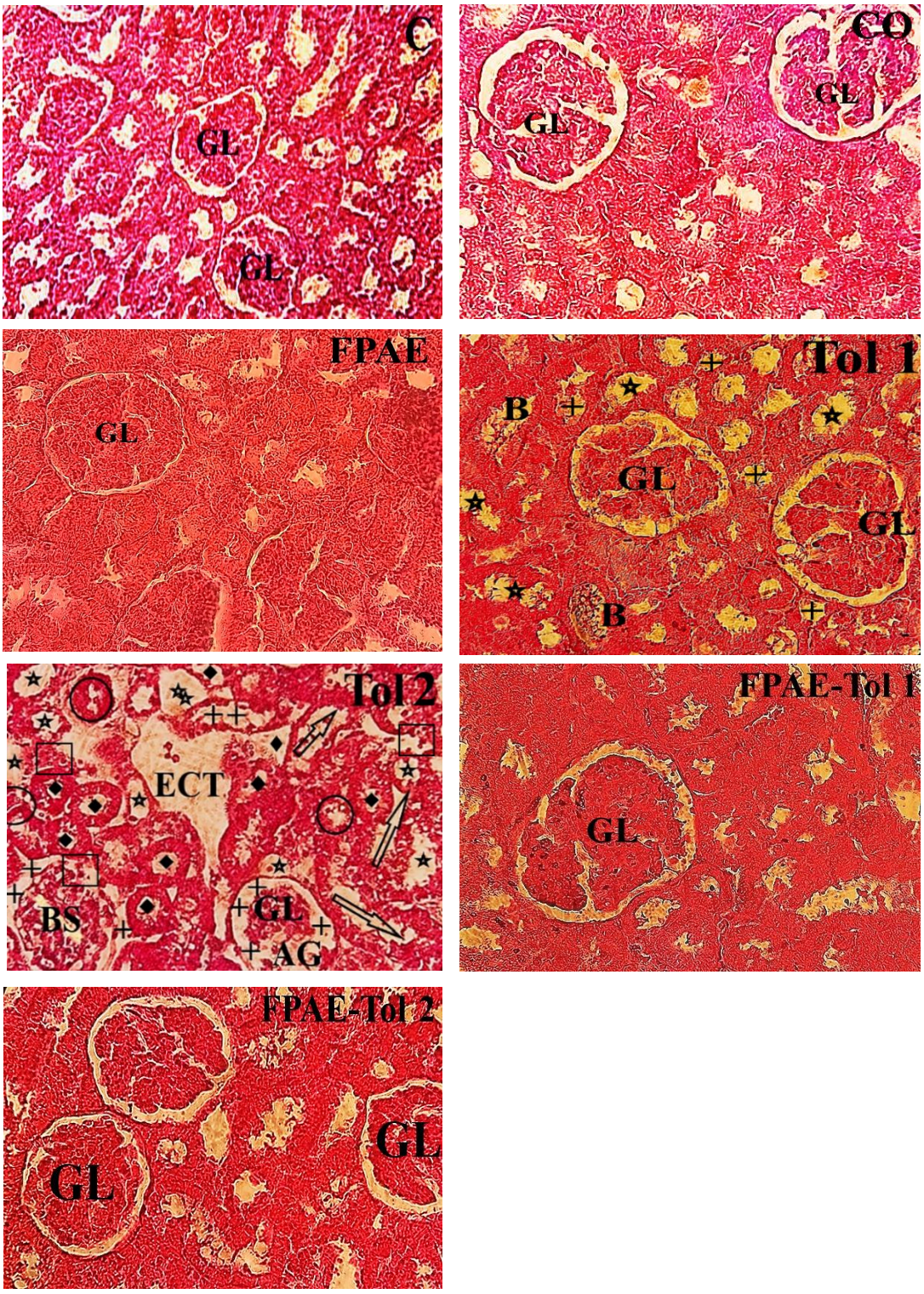

Figure 5. Light microphotographs of kidney tissues from different experimental groups (×40).

Control (A), CO (B), FPAE (C), and Tol1 (D) revealed a normal renal glomerulus (GL) and tubular epithelium (×10). Tol2 group (E) showed a deleterious morphological change versus those of the control groups, which was illustrated by glomerular atrophy (GA) with dilatation of Bowman's space (BS) and degeneration of the renal tubular epithelium (stars, circles, and arrows). Yet, FPAE-Tol1 (F) and FPAE-Tol2 (G) groups displayed a better-preserved epithelium and morphology with a normal architecture nearly like those of controls.

(GL: glomerulus, BS: Bowman's Space, AG: atrophy of the glomerulus with widening of Bowman's capsule, stars: dilatation of the convoluted tubule, (+): dilatation of Bowman's space, arrow: tubular dilatation and degeneration of the renal tubular, ECT: empty convoluted tubule). C: Control, CO: Corn Oil, PJ: Pomegranate Juice, PAE: Peel Aqueous Extract, Tol: Toluene; PJ-Tol: Toluene + Pomegranate Juice, PAE-Tol: Toluene + Peel Aqueous Extract).

4. DISCUSSION AND CONCLUSION

Humans are exposed to numerous environmental agents that can impair their physiologic systems capacity. Renal function is known to be highly sensitive to many chemical and physical agents generated by industrial and agricultural activities **[2,10,11]** The kidney is the target organ for many occupational and environmental chemicals and acts as chief player in their elimination **[19]**. As the primary excretory organ, it becomes a major target for various xenobiotics, such as toluene, which can be eliminated in the urine after 12 hours following exposure, mainly as metabolites.

Exposure to toluene showed a significant increase in rats' kidney absolute weight. This result agrees with that obtained when Wistar rats were exposed to this organic solvent. Indeed, following exposure to toluene at 4.5 ml/kg on male rats for 7 days, a drastic increase in absolute kidney weight was observed **[20]**, which could be attributed to an inflammation of renal tissues. It has been shown that exposure to toluene in provoked an elevation of pro-inflammatory cytokines levels (IL-4, IL-13, TNF-α, and IFN-γ) **[21]**. In people smelling toluene chronically from adhesives, accumulation of immune complexes in the kidneys has been observed **[22]**. This increase may also be the result of tubular cells swelling, their disorganization and congestion **[8].**

Toluene induced an increase in the blood urea and creatinine levels, which was concur with the results of previously reported work **[8, 20].** As markers, uric acid, urea and creatinine was stated to be essential elements to estimate renal function and glomerular filtration **[23].** Numerous mechanisms might explain the high blood levels of these metabolites, including dehydration **[24],** which has been observed in our study**.** According to the study of **Taros et al. [22]** exposure to toluene brings irreversible renal insufficiency due to distal tubular cell damage. Additionally, this increase may be attributed to the elevated proteins catabolism into amino acids to form urea and creatinine **[25]** Since toluene is mostly eliminated through the kidneys in the form of a hippuric acid, it is evident that it can damage renal glomerular cells; hence, the excretion of urea and creatinine could be disrupted **[6].** The significant decrease in serum uric acid may be interpreted by its strong free radical-scavenging molecule and its protective response against ROS production. Some major epidemiological studies have identified low uric acid levels as a translation to the greater oxidative stress generation **[26].**

The metabolic perturbation induced by toluene has been found to be related to its biotransformation, resulting in particular production of toluene epoxides, which are high reactivity species that can induce damage to various body systems **[7].** Therefore, it appears that it can disturb antioxidant defence system by decreasing GSH levels and GPx activity, along with a rise in MDA levels, which is also seen in our study. Our results seem to be conceivable with those obtained by other researchers **[27]**. It has been mentioned that NO production and its reaction with superoxide radicals generates highly cytotoxic ROS, which can significantly increase the progression of renal failure. This, in turn, resulting in a reduction of intracellular glutathione activity. Several scholars have highlighted the relationship between VOCs, including toluene, and elevated levels of H2O2, COX-2 and NO. These compounds affect enzymatic activity, DNA oxidation, thiol oxidation, nitrosylation, and lipid peroxidation **[7].** Lipid peroxidation can be assessed by measuring MDA, the end product of polyunsaturated fatty acids oxidation causing structural impairment and loss of function of cell membranes. Overproduction of ROS is a key pathogenic process contributing to the disruption of intracellular redox homeostasis or indirectly activate signal transduction pathways **[28]**, which is known to cause tissues pathological changes. Regarding the drop observed in GSH concentration the reason perhaps was associated not only to the excessive production of ROS but could also result from impaired regeneration by GSH reductase. Decreased regeneration of GSH may occur due to a deficiency in the reducing equivalent NADPH, which can result from the alterations in mitochondrial membrane permeability by oxidative stress **[29].**

In line with our previous findings in this study, several structural changes were observed renal in rats treated with Tol. Thus, the main renal histopathological features included abnormal nephrotic changes varied from degenerative to necrotic in some tubular epithelium besides to atrophy of glomerular part. renal tubular cells were swollen, had loss of staining capacity, and nuclei appeared to be dilated, presence of blood clot was noted in toluene-treated rats. Renal tubules swelling and distortion lining with congestion of interstitium was mentioned after exposure of rats to 900 mg/kg of toluene **[6]**. The critical reason of the cellular damage caused by toluene is a caspase dependent process, which plays an important role in the production of inflammatory mediators and apoptosis. It was suggested that Bax/Bcl2 ratio was directly associated with renal apoptosis and progressive diseases in other organs **[6].**

Pomegranate has a protective effect as it contains high content of phenolic compounds, phenolic acids, ellagic tannins (punicalin, punicalagin, gallagic, and ellagic acid), flavonoids (anthocyanins, catechins, rutin, epigallocatechin-3- gallate), and anthocyanins (delphinidin, cyaniding, and pelargonidin), which possess many prominent capacities **[9,30,31].** In terms of the investigation of *Punica granatum*'s role, our study revealed that treatment with PAE and PJ generally improved the impairment induced by toluene in kidney. Our results are in harmony with the findings other findings **[32],** who suggested that the administration of pomegranate peel extract significantly attenuated the damaging

impact of phenylhydrazin on the kidney in rat models. It has been affirmed that phytoconstituents such as gallic acid was proved to be very effective against oxidation by restoring damaged tissues in rats, which might be explained by their ability to donate a hydrogen atom from their phenolic hydroxyl groups **[30].** Other **a**uthors also established that flavonoids and ellagic acid can elevate the biosynthesis of glutathione and/or prevent its degradation by increasing the vital enzyme in GSH synthesis (C-glutamyl cysteine synthetase) and serum paraoxonase activity, potentially protecting against lipid peroxidation **[33].** Furthermore, punicalagin exhibits the capacity to inhibit inflammation and apoptosis by modulating the production of NO, COX, Bax and Bcl-2, therefore protecting cells from death **[12,13].**

In conclusion, the current study affirmed that the sub-chronic exposure to a moderate dose of toluene induces kidney injuries mediated via oxidative stress, confirmed by lipid peroxidation, and down-regulated antioxidant parameters. But further studies are needed to ascertain the precise mechanisms of its action on mitochondrial function. Supplementation of *Punica granatum* fresh peel aqueous extract has been proved to own excellent antioxidant activities that prevented the effects of this toxic solvent by the restoration of renal tissue structure and function.

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