# Saponin protects against cyclophosphamide-induced kidney and liver damage via antioxidant and anti-inflammatory actions

MOHAMMAD GHASEM GOLMOHAMMADI[1], SHOKOFEH BANAEI[2]* ,
MOJGAN TIMAR[1] and ALI ABEDI[2]

[1] Department of Anatomical Sciences, School of Medicine, Research Laboratory for Embryology and Stem Cells, Ardabil University of Medical Sciences, Ardabil, Iran

[2] Department of Physiology, School of Medicine, Ardabil University of Medical Sciences, Ardabil, Iran

## ABSTRACT

*Background:* The liver and kidney are organs affected by chemotherapy drugs such as cyclophosphamide (CP). This study examined the protective effects of treatment with saponin (SP) against CP-induced nephrotoxicity and hepatotoxicity. *Methods:* 24 adult male mice were divided into four groups ($N = 6$): Control group, CP (15 mg kg$^{-1}$), SP (2.5 mg kg$^{-1}$) and CP + SP. After treatment, blood samples were collected for the determination of biochemical parameters. Liver and kidney samples were taken for histological analysis and assessment of oxidative stress and inflammatory markers. *Results:* Cyclophosphamide decreased renal and liver functions and antioxidant enzymes, which significantly increased blood urea nitrogen and creatinine (BUN, Cr), liver enzyme levels, malondialdehyde, nuclear factor kappa β (NF-kB) and Interleukin 1 beta (IL-1B) concentrations. Moreover, histopathological findings of the CP group showed that there were acute tubular necrosis and glomerular atrophy in the renal tissues and lymphocyte infiltration in the liver samples. Treatment with saponin improved hepatic and renal functions, pathological changes and antioxidant capacity, and also decreased lipid peroxidation and inflammation. *Conclusion:* It seems that saponin could exert a hepato-nephroprotective effect against cyclophosphamide toxicity.

* Corresponding author. Tel.: +98 4533245526; fax: +984533522197. E-mail: s.banaei75@gmail.com

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## INTRODUCTION

The kidney and liver are systems affected by chemotherapy drugs like cyclophosphamide (CP). The liver is the organ in which chemical materials and drugs are metabolized, then excreted. Liver cells or hepatocytes are in contact with various chemical agents which may lead to hepatotoxicity, hepatic dysfunction and failure [1]. The kidney has several functions such as the regulation of water and electrolyte balance, arterial blood pressure and acid-base balance. Furthermore, the kidney excretes metabolic waste products and exogenous chemicals, and the active form of vitamin D is produced by mitochondria of the proximal tubules. There is a close relationship between the liver and kidney, and this functional interaction is pivotal to maintain internal homeostasis [2]. In the kidney, the liver and other organs, cytotoxicity effects of chemotherapeutic drugs which are widely prescribed in the various types of neoplasia and tumors are able to disrupt their physiologic functions [3].

These two systems (liver and kidney) metabolize and excrete chemical agents and are more involved in material detoxification than other organs. As a result, the toxicity of chemotherapeutic drugs can result in degenerative alterations and life-threatening kidney and liver injury [4]. One of the chemotherapeutic drugs prescribed for cancer patients, namely cyclophosphamide has side effects such as reno-cardiac toxicity, hepatotoxicity, genital disorders and testicular injury. The antineoplastic properties of CP are associated with histological and biochemical alterations in different organs. It has been identified that CP interrupts the balance of free radicals and antioxidant defenses, leading to the production of reactive oxygen species (ROS) responsible for DNA fragmentation, necrosis, apoptosis and cell death [5]. Oxidative injury of DNA plays an important role in the apoptosis of the germ cells and tissues. Although CP is widely applied in clinical practice, it has adverse influence on the liver that decomposes CP to active metabolites and on the kidney that excretes them. Previous studies have demonstrated that impaired humoral secretion, infertility, DNA damage, lipid peroxidation and morphological defects in the tissues are related to the treatment with CP [6].

Therefore, protecting organs such as the kidney, the liver, the cardiovascular system etc. against CP toxicity is inevitable. Herbal medicines have been confirmed to inhibit CP-induced tissue toxicity [7]. Saponins (SP) are natural compounds of Ginseng possessing various biological activities including anti-inflammatory, antioxidant, anti-microbial, anti-tumor and immune-modulating properties [8, 9]. Additionally, saponins scavenge ROS, reduce lipid peroxidation, improve antioxidant capacity and normalize AST (aspartate transaminase), ALT (alanine transaminase) and ALP (alkaline phosphatase) in the liver. Also, saponins decrease heat stress and suppress oxidative stress in tubular and glomerular epithelial cells in the kidney in a dose-dependent manner. It has been found that some saponins obtained from plants decrease tumor growth, have preventive effect on proteinuria, therapeutic effect on the liver and renal function as well as protective effect against cardiomyopathy [10, 11].

In recent decades, herbal remedies have been shown to prevent the side effects caused by chemical medicines. In the present study, we investigated the protective effects of saponin against CP-induced nephrotoxicity and hepatotoxicity using evaluation of antioxidant capacity and inflammatory mediators.

## MATERIALS AND METHODS

For the study, 24 adult male NMRI mice (6–8 weeks old, weighing 20–25 g) were divided into four groups and treated for 35 days [12]. The mice had free access to standard feed and water during the study period. The conditions of the animal house were controlled temperature about $21 \pm 2\,°C$ and 12/12 h light-dark cycle with humidity ($60 \pm 5\%$). Principles of laboratory animal ethics with regard to international standards and the ethics committee of Ardabil University of Medical Sciences were observed in all stages of research (ethics code: IR.ARUMS.AEC.1400.019).

## EXPERIMENTAL DESIGN

Male mice were randomly divided into 4 groups ($N = 6$) with a treatment period of 35 days. The groups were as follows:

1. Control + saline (0.2 mL/day, IP)
2. CP group (15 mg/kg/week, IP)
3. SP group (2.5 mg/kg/day, IP)
4. CP + SP group, received CP (15 mg/kg/week) with SP (2.5 mg/kg/day)

In darkness, CP (500 mg vial Sigma, USA) was dissolved in the saline and injected weekly intraperitoneally for 35 days ($15\,mg\,kg^{-1}$) [13]. Saponin powder was obtained from Sigma-Aldrich, USA, and dissolved in normal saline, away from light and under the hood, and was then administered at 2.5 mg/kg/day intraperitoneally [12].

## BIOCHEMICAL MEASUREMENT

At the end of the treatment period in each group, under anesthesia (xylazine ($10\,mg\,kg^{-1}$) and ketamine ($50\,mg\,kg^{-1}$)), blood samples (taken from the heart) and liver and kidney tissue samples were collected. The blood samples were centrifuged at 4,000 rpm for ten minutes. The blood urea nitrogen (BUN) and creatinine (Cr) levels in the serum were determined to assess kidney function, using the Auto-analyzer by original kits (Pars-Azmoon Company, Tehran, Iran). The activities of ALP, ALT and AST were assayed using Randox assay kits. These enzymes are responsible for the proper functioning of the liver.

## HISTOLOGICAL EVALUATION

Liver and kidney tissue samples were fixed in 10% buffered formalin solution, dehydrated in ascending grades of alcohol and embedded in paraffin. Sections of 5 μm were taken, stained with hematoxylin-eosin (H&E), and then examined in a blinded manner by a pathologist under light microscope. The histological score was semi-quantitatively assessed using a scale ranging from 0 to 3 (0: none, 1: mild, 2: moderate, and 3: severe damage) [14].

### Tissue malondialdehyde (MDA) and total antioxidant capacity (TAC) measurement

The liver and renal tissues were homogenized in 1.15% KCl. The tissue levels of MDA were evaluated by the ZellBio kit (ZellBio GmbH, Lonsee, Germany). The TAC levels were

determined using the Randox total antioxidant status kit (Randox Laboratories Ltd. Crumlin, United Kingdom) based on the manufacturer's method. The production of the radical cation $ABTS^+$, which has a blue color, is suppressed with antioxidant components in proportion to their concentration in the samples [15].

### Tissue glutathione peroxidase (GPx) and superoxide dismutase (SOD) assessment

To measure cytosolic enzyme activity, the liver and renal samples were homogenized in 1.15% KCl solution. SOD and GPx activity were measured according to the ZellBio kit and the manufacturer's protocol [16].

### Assessment of tissue levels of nuclear factor kappa β (NF-kB) and Interleukin 1 beta (IL-1B)

Liver and renal samples were collected after the treatment period and the tissue levels of NF-kB and IL-1B were measured using enzyme-linked immunosorbent assay (ELISA) kits in accordance with the manufacturer's protocol. The kits for assaying NF-kB and IL-1B levels were purchased from R&D Systems, Inc. (USA & Canada). A 100-mg renal and liver tissue pieces were placed into a 1.5 mL tube, 1 mL of 0.9% saline was added, and the piece was cut into smaller pieces. After grinding, the resulting suspension was centrifuged for 15 min (4 ºC, 1,500 rpm) and the supernatant was absorbed. The levels of NF-kB and IL-1B in liver and renal tissues were measured using ELISA kits [17]. The absorption was determined by the microplate absorbance reader at 450 nm. The lower detection limit was 10 pg mL$^{-1}$ for IL-1B and 0.1 ng mL$^{-1}$ for NF-kB.

## STATISTICAL ANALYSIS

All the data were expressed as mean ± standard deviation, using the one-way analysis of variance (ANOVA) with Graph Pad Prism version 8.4.3, and Tukey's post hoc test was done to determine significant differences between groups. Statistical significance was accepted at $P < 0.05$.

## RESULTS

In this study, the therapeutic and protective effects of saponin against CP-induced nephrotoxicity and hepatotoxicity were investigated. We hypothesized that with the intervention of appropriate therapeutics, it should be possible to manage the CP damage and provide beneficial hepato- and reno-protection. Biochemical analysis results are outlined in Fig. 1. The results of histological scores are shown in Figs 3 and 5.

### Protective effect of saponin on CP-induced alterations in serum biochemical parameters

Serum levels of AST, ALT and ALP enzymes in the CP group increased significantly compared to the control group ($P < 0.001$, Fig. 1). BUN-Cr levels also increased significantly in the CP
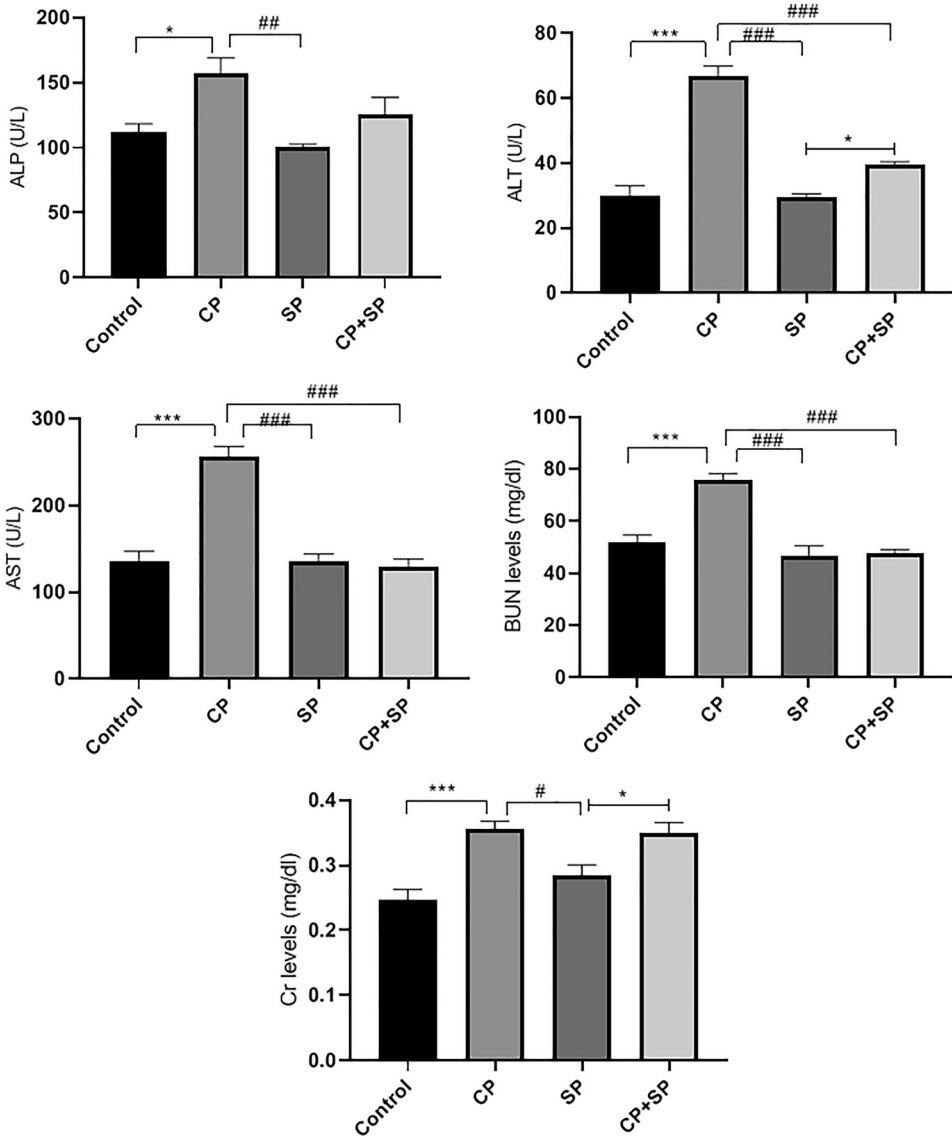
*Fig. 1.* The effect of cyclophosphamide (CP) and saponin (SP) on serum levels of ALP, ALT, AST, BUN, and Cr in treated mice. $^{*}P < 0.05$, $^{***}P < 0.001$ versus control group. $^{#}P < 0.05$, $^{##}P < 0.01$, $^{###}P < 0.001$ versus CP group. $^{*}P < 0.05$ versus SP group. Alkaline phosphatase (ALP), Alanine transaminase (ALT), Aspartate transaminase (AST), Blood urea nitrogen (BUN), Creatinine (Cr)

group ($P < 0.001$, Fig. 1). Saponin and co-administration of SP together with CP significantly reduced the levels of ALT, AST, BUN and Cr in comparison to the CP group.

## Protective effect of saponin on CP-induced renal and hepatic histopathological changes

Histological examination of the kidneys showed that no morphological changes were seen in the control group (Fig. 2A). In the SP group, healthy and normal appearance of glomerular and tubular cells was observed (Fig. 2C). In the CP group, acute tubular necrosis (ATN) congestion, pyknotic nuclei, glomerular atrophy and dilated bowman's space were very obvious (Fig. 2B). Saponin co-administration with CP resulted in noteworthy decline in histopathological alterations induced by cyclophosphamide (Fig. 2D). Correspondingly, the renal histological index was decreased in the saponin group in comparison to the CP group (Fig. 3).
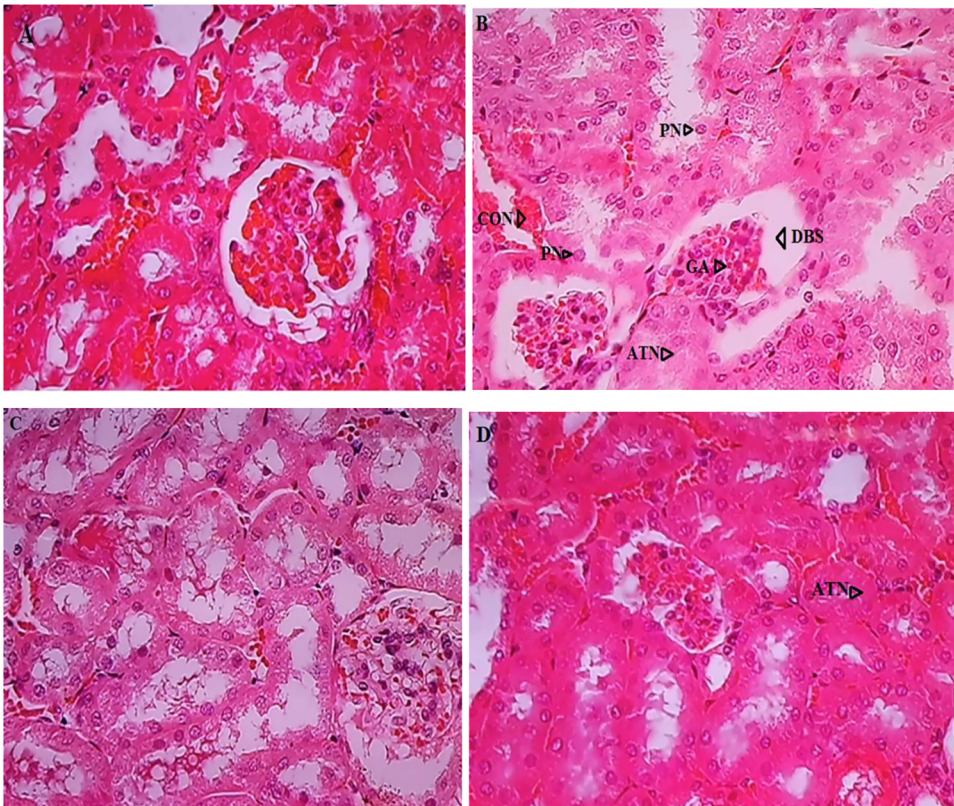


*Fig. 2.* (A and C) Photomicrographs of the kidney of mice in the normal control and SP groups show normal renal tissue structure, healthy appearance of glomerular and tubular cells. (B) CP-administered mice had congestion (CON), pyknotic nuclei (PN), glomerular atrophy (GA), dilated bowman's space (DBS) and acute tubular necrosis (ATN). (D) Acute tubular necrosis (ATN) in CP + SP group ($40 \times$ H&E)
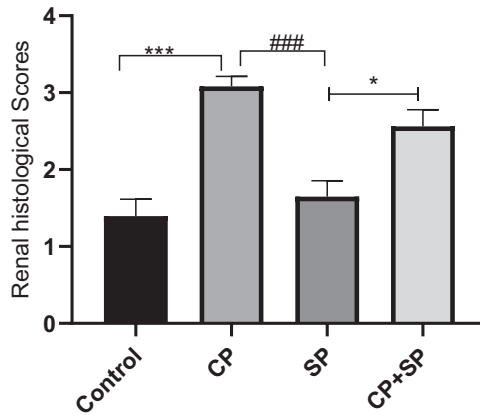
*Fig. 3.* Semi-quantitative analysis of the protective effect of saponin against CP-induced kidney damage. The histological score was semi-quantitatively evaluated using a scale ranging from 0 to 3 (0: none, 1: mild, 2: moderate, and 3: severe damage). The results were expressed as mean ± standard deviation. ***$P < 0.001$ versus control group, ###$P < 0.001$ versus CP group, *$P < 0.05$ versus SP group

Also, histological examination of the liver did not show any significant changes in the control group (Fig. 4A). Moreover, normal hepatic structure was seen in the SP-treated groups (Fig. 4C). In the CP group, the normal histological organization of the liver tissues was disrupted. Lymphocyte infiltration, acute triaditis, and neutrophil infiltration occurred after treatment with CP (Fig. 4B). CP Plus SP showed reduced degenerative changes in hepatocytes (Fig. 4D). In addition, the liver histological index was significantly decreased in the SP and CP + SP groups in comparison to the CP group ($P < 0.05$, Fig. 5).

## The effect of saponin and cyclophosphamide on the MDA and TAC levels

As shown in Table 1, in the CP group MDA levels were insignificantly increased as compared to the control group. Saponin administration more significantly reduced MDA concentration than the CP-treatment ($P < 0.01$). CP administration also decreased TAC levels in contrast to the control group. However, saponin treatment significantly elevated TAC levels compared to the CP group ($P < 0.05$).

## The effect of saponin and cyclophosphamide on SOD and GPx activity

There was an insignificant decline in the mean activities of GPx and SOD in the liver and kidney of the CP group in comparison to the control group. The saponin-treated groups exhibited a considerable increase in SOD and GPx levels as compared to the CP group (Table 1).

## The effect of saponin and cyclophosphamide on NF-kB and IL-1B production

We assessed whether saponin decreases the production of the pro-inflammatory cytokines including NF-kB and IL-1B, which are induced by CP. The toxic effects of CP significantly elevated the concentration of NF-kB and IL-1B in kidney and liver tissues compared to the
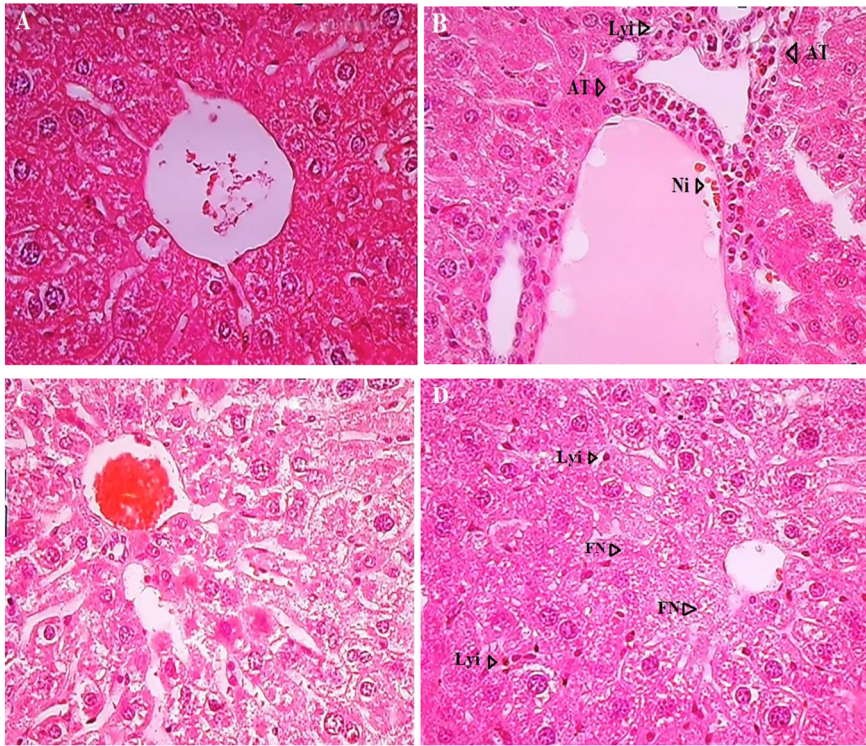
*Fig. 4.* (A and C) Photomicrographs of the liver of mice in the normal control and SP groups show normal hepatic tissue structure. (B) CP-administered mice had lymphocyte infiltration (Lyi), acute triaditis (AT) and neutrophil infiltration (Ni). (D) Lymphocyte infiltration (Lyi) and focal necrosis (FN) in the CP + SP group (40 × H&E)
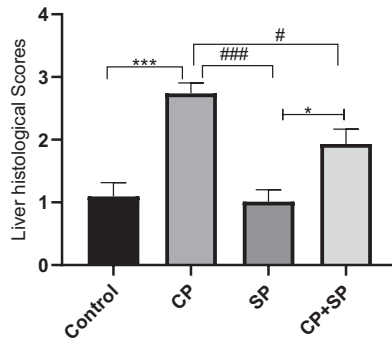


*Fig. 5.* Semi-quantitative analysis of the protective effect of saponin against CP-induced liver damage. The histological score was semi-quantitatively evaluated using a scale ranging from 0 to 3 (0: none, 1: mild, 2: moderate, and 3: severe damage). The results were expressed as mean $\pm$ standard deviation. [***]$P < 0.001$ versus control group, [#]$P < 0.05$, [###]$P < 0.001$ versus CP group, [*]$P < 0.05$ versus SP group

*Table 1.* Protective effect of saponin on cyclophosphamide-induced changes in renal and hepatic TAC, MDA and antioxidant enzyme levels

| Groups | Control | CP | SP | CP + SP |
|---|---|---|---|---|
| Kidney | | | | |
| MDA (nmol mg$^{-1}$ protein) | 1.61 ± 0.44 | 1.89 ± 0.22 | 1.15 ± 0.14[b] | 0.88 ± 0.10[c] |
| TAC (mmol L$^{-1}$) | 0.51 ± 0.07 | 0.43 ± 0.11 | 0.63 ± 0.03[b] | 0.62 ± 0.06[a] |
| SOD (U mg$^{-1}$ protein) | 8.87 ± 1.82 | 7.43 ± 1.02 | 8.53 ± 1.67 | 8.38 ± 1.02 |
| GPx (U mg$^{-1}$ protein) | 5.18 ± 1.33 | 4.39 ± 0.38 | 5.12 ± 0.83 | 4.70 ± 0.91 |
| Liver | | | | |
| MDA (nmol mg$^{-1}$ protein) | 0.67 ± 0.15 | 0.82 ± 0.21 | 0.68 ± 0.18 | 0.48 ± 0.06[a] |
| TAC (mmol L$^{-1}$) | 0.77 ± 0.07 | 0.70 ± 0.19 | 0.92 ± 0.16 | 1.01 ± 0.14[a] |
| SOD (U mg$^{-1}$ protein) | 9.47 ± 1.16 | 8.23 ± 0.82 | 10.11 ± 0.56 | 8.63 ± 1.39 |
| GPx (U mg$^{-1}$ protein) | 6.26 ± 0.57 | 4.93 ± 1.00 | 6.06 ± 0.48 | 5.70 ± 0.77 |

The results were expressed as mean ± standard deviation. a, b and c show the significance of the differences to CP group ($P < 0.05$, 0.01 and 0.001). SP; saponin, CP; cyclophosphamide, MDA; malondialdehyde, TAC; total antioxidant capacity, SOD; superoxide dismutase, GPx; glutathione peroxidase.
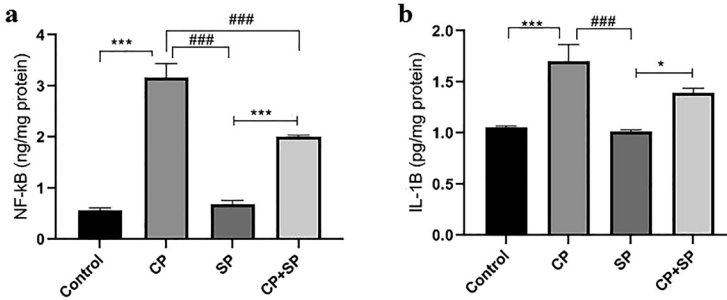


*Fig. 6.* The effect of cyclophosphamide (CP) and saponin (SP) on NF-kB and IL-B levels in the renal tissue of treated mice. ***$P < 0.001$ versus control group, ###$P < 0.001$ versus CP group, *$P < 0.05$ versus SP group
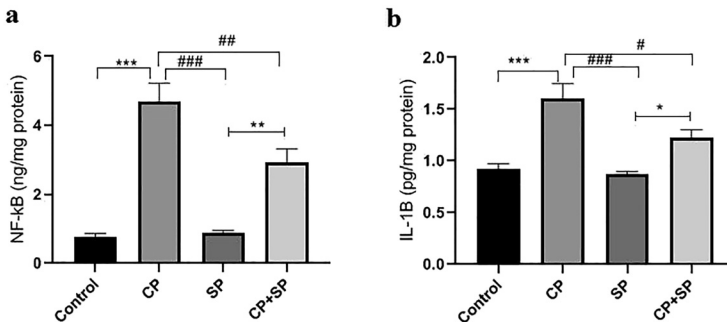


*Fig. 7.* NF-kB and IL-B levels in the liver tissue of treated mice. ***$P < 0.001$ versus control group, #$P < 0.05$, ##$P < 0.01$, ###$P < 0.001$ versus CP group, *$P < 0.05$, **$P < 0.01$ versus SP group

control group ($P < 0.001$). However, the tissue levels of these cytokines were significantly reduced in the saponin ($P < 0.001$) and CP plus SP ($P < 0.05$) groups in comparison to the CP group (Figs 6 and 7). The results reveal that saponin alone and co-administration of CP with SP lead to a decrease in the production of the NF-kB and IL-1B.

## DISCUSSION

Many investigations have been done about the antioxidant, anti-inflammatory and immuno-regulatory role of saponins. Saponins reduce the free radicals produced, regulate the expression of antioxidant enzymes and eliminate the toxic effects of the industrial chemicals, leading to resolution of inflammation and return to hemostasis [18, 19]. Therefore, in the present study, we hypothesized saponin with antioxidant and anti-inflammatory capacity would be able to inhibit the liver and kidney toxicity due to treatment with cyclophosphamide.

Our findings in the current study confirmed that exposure to CP significantly increased the serum levels of ALP, ALT, AST and BUN- Cr, suggesting hepatic and kidney dysfunction which was greatly decreased after saponin treatment. The kidney and liver as the organs of purification and detoxification in the body are impacted by xenobiotic toxicities due to their physiological actions and structure. CP, a clinical antineoplastic agent used in the treatment of different cancers elicits damages in healthy tissues and causes oxidative stress in liver, lung, kidney etc. Cytochrome P450-dependent transformation of CP uses molecular oxygen to oxidize organic solvents. Then, the oxidized solvents generate free radicals, especially hydroxyl radical. This radical can react with cellular components including nucleic acids, proteins and fatty acids. As a result, CP-induced tissue damage involves considerable cytoskeleton disruption, antioxidant depletion and lipid per-oxidation. Oftentimes, renal and hepatic functions are impaired by CP treatment, resulting in increased serum BUN- Cr, ALT, ALP, AST and MDA and decreased antioxidant capacity. In addition, CP-mediated hepatic and renal toxicity is characterized by cell apoptosis and damages elicited by inflammation. Overall, a noteworthy involvement of oxidative stress and inflammation mediated by the immune system in signaling pathways lead to hepato-nephrotoxicity [20].

Saponin and co-administration of SP together with CP significantly decreased these biochemical parameters. Probably, antioxidant properties of saponin could be effective in the improvement of hepatic and renal function. Additionally, the histological assessment showed that CP treatment led to glomerular and tubular changes, as shown by dilated bowman's space, glomerular atrophy, acute tubular necrosis and congestion. Saponin and CP plus SP attenuated the pathological changes in renal tissue caused by CP. Also, the renal histological index was reduced by saponin treatment. This cytoprotective effect of saponin may be due to its powerful antioxidant capacity [21].

In addition, cyclophosphamide caused pathological changes in the hepatocytes including acute triaditis and lymphocyte infiltration, whereas the administration of saponin and CP plus SP reduced the morphological changes of the liver tissues. Correspondingly, the liver histological index was significantly decreased by saponin treatment in the effective dose of 2.5 mg kg$^{-1}$. These protective effects of saponin may probably be due to its potent anti-inflammatory and antioxidant properties [22], which were demonstrated by the recovery and regeneration of pathological defects in the liver and renal structure.

Lipid peroxidation as a free radical generating system has been shown to be closely related to CP-induced cytotoxicity, and MDA is a main marker of the degree of lipid peroxidation [13].

In the present experiment, MDA levels were elevated and the antioxidant activities of GPx, TAC and SOD were reduced by CP administration. These data are consistent with previously published findings about the effects of CP on organ toxicity [23]. The CP therapy caused oxidative stress, lipid peroxidation in renal and hepatic tissues and reduced antioxidant defense system. Increased levels of free radicals play a major role in cytotoxicity induced by CP. Therefore, compounds that can inhibit the production of free radicals will be of particular importance. Saponin, as a free radical scavenger, has a considerable antioxidant effect. Our results confirmed that it reduced the amount of oxidative stress and subsequently lipid peroxidation, and improved antioxidant capacity, thus documenting the ability of saponin's dual function as both an enhancer of anti-oxidative enzyme defenses and a free radical scavenger.

Exposure to chemicals and drugs results in inflammation that is characterized by the protective response of the immune system against these agents. The level of pro-inflammatory cytokines including tumor necrosis factor alpha (TNF-α), IL-1B and IL-6 increase in the body. Transcription of these interleukins are induced by a protein playing an important role in the inflammatory process, NF-kB [24]. Oxidative stress has been reported to be related with NF-kB. Actually, a vicious cycle has been suggested between oxidative stress and the NF-kB signaling pathway. Oxidative stress is crucial for the activation of the NF-kB cascade. On the other hand, the expression of NF-kB leads to the aggravation of free radical generation [25].

Our results indicate that CP created a state of oxidative stress in kidney and liver tissues and then induced the production of pro-inflammatory cytokines such as NF-kB and IL-1B in these two organs, which is consistent with previous research in this regard [26]. Usually, inhibition of inflammation by anti-inflammatory medications is the most effective action in controlling the toxic effect of these agents. Inflammatory conditions have been treated with natural compounds. To avoid the unwanted side effects of anti-inflammatory medicines, such as nonsteroidal anti-inflammatory drugs (NSAIDs), it is necessary to find alternative anti-inflammatory agents with more efficacy than NSAIDs but with fewer side effects. Our findings demonstrated that saponin could significantly inhibit the production of inflammatory mediators induced by CP. Also, CP + SP significantly decreased the levels of NF-kB and IL-1B in kidney and liver tissues that had been increased by CP. Probably, the high anti-inflammatory and antioxidant activity of saponin is responsible for ROS detoxification and inflammatory reduction.

## CONCLUSION

In conclusion, liver and renal failure are the most common complications of chemotherapy drugs such as cyclophosphamide. Increased oxidative stress and inflammation are widely acknowledged components in the toxicity mediated by CP. Saponins have considerable antioxidant and anti-inflammatory effects and are well-known free radical scavengers. Our findings proved that the administration of saponin or CP plus saponin plays a protective role on CP-induced hepatic and nephric injury, as shown by the reduction of liver and renal dysfunction, pro-inflammatory mediator production and pathological changes, and by the improvement of antioxidant capacity.

*Author contributions:* Mohammad-Ghasem Golmohammadi: study design; Shokofeh Banaei: study design, histological evaluation, statistical analysis and manuscript writing. All authors approved the final manuscript.

*Conflict of interest:* The authors report no conflicts of interest.

*Data availability:* The data that support the findings of this study are available from the corresponding author upon reasonable request.

## ACKNOWLEDGMENTS

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