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Original Article

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In Vitro Evidences for Renal-Protective Properties of Phloretin in Nephrotoxicity Induced by Bisphenol-A *via* Mediation of *VCAM-1/ICAM-1* Genes Expression

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Abstract

Background: Oxidative stress is a major contributor to kidney dysfunction. Phloretin, a dietary flavonoid, exhibits antioxidant and anti-inflammatory activity. This study evaluated the protective effects of Phloretin against Bisphenol-A (BPA)-induced oxidative stress in human renal tubular epithelial (HK-2) cells.

Methods: HK-2 cells were exposed to BPA (1000 μM) for 24 *hr* with or without Phloretin (50, 100, 200 μM). Cell viability, oxidative stress markers Reactive Oxygen Species (ROS), Malondialdehyde (MDA), Total Antioxidant Capacity (TAC), cytokines (IL-6, IL-10), and *VCAM-1*/ICAM-1 expression were assessed.

Results: BPA markedly decreased cell viability and TAC while elevating ROS, MDA, IL-6, and *VCAM-1/ICAM-1* expression (all $p<0.001$). Phloretin significantly reversed these effects in a dose-dependent manner. At 200 μM, Phloretin increased cell viability by ~35–40% ($p<0.001$), reduced ROS and MDA by ~35–40% ($p<0.001$), and restored TAC by ~40–45% ($p<0.001$) compared to BPA. Inflammatory markers were also modulated: IL-6 was reduced by ~35–40% ($p<0.001$), while IL-10 was elevated by ~40–45% ($p<0.001$). Moreover, Phloretin suppressed *VCAM-1* and ICAM-1 expression by ~45–50% ($p<0.001$).

Conclusion: Phloretin significantly attenuates BPA-induced oxidative stress and inflammation in renal tubular cells, suggesting its potential as a natural nephroprotective agent.

Keywords: Inflammation, Kidney, Oxidative stress, Phloretin, Tubular epithelial cells

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Introduction

Oxidative stress is well-known as critical risk factors for kidney diseases which is induced by an imbalance between the production of free radicals and antioxidant barriers, and leads to damage to cell components including fats, proteins, and nucleic acids (1). Bisphenol-A (BPA) is a potentially endocrine-disrupting agent which is frequently found in plastic bottles, food and beverage containers. Bisphenol-A can leach from polymers and penetrates in food and water sources. Many studies documented the accumulation of bisphenol-A in the human body in recent years (2-4). The highest concentration of bisphenol-A (1-104 *ng/g* of tissue) was detected in the placenta and fetus. Exposure to bisphenol-A is associated with tissue oxidative stress and peroxidation, which leads to various diseases (5). Bisphenol-A as a xenoestrogen mimics the natural estrogen properties which leads to adverse health consequences. Moreover, the studies reports that bisphenol-A induces cytokine dysregulation and oxidative stress in the brain. The main mechanism of nephrotoxicity with bisphenol is the production of Reactive Oxygen Species (ROS) in kidney cells, which can lead to inflammation and cell apoptosis (6,7). The tubular expression of Vascular Cell Adhesion Molecule-1 *(VCAM-1)* has been linked to the presence of transferrin-receptor-positive interstitial cells in individuals suffering from primary glomerulonephritis. Evidence suggests that VLA-4 interactions with *VCAM-1* play a biologically significant role in interstitial renal disease, as demonstrated by the efficacy of anti-VLA-4 antibodies in preventing interstitial disease in rat models of mercuric chloride-induced nephritis. Additionally, the β2 integrins, specifically the CD11/CD18 complex, interact with intercellular adhesion molecule-1 (ICAM-1), which is typically found on interstitial cells and blood vessels. Increased expression of renal ICAM-1 has been observed across various kidney diseases in both human and animal studies (8).

Phenolics components are a vital agent which have tremendous antioxidant activity, as well as various health advantages. Phloretin as a potent flavonoid antioxidant which found abundantly in apples and strawberries. Previous studies have demonstrated the immune-modulating, anti-oxidant and anti-inflammatory properties of Phloretin (9,10). Although previous studies have demonstrated the antioxidant and anti-inflammatory properties of Phloretin in models of oxidative stress and systemic inflammation, its potential protective role against BPA-induced nephrotoxicity has not been well characterized. In particular, there is limited evidence on whether Phloretin can modulate oxidative stress markers, cytokine balance, and the expression of adhesion molecules (*VCAM-1* and *ICAM-1*) in human renal tubular epithelial cells exposed to BPA. Therefore, the present study was designed to address this gap by systematically evaluating the nephroprotective effects of Phloretin in an *in vitro* BPA-induced kidney injury model. The authors hypothesized that Phloretin would attenuate BPA-induced nephrotoxicity in HK-2 cells by reducing oxidative stress, modulating inflammatory cytokines, and suppressing *VCAM-1/ICAM-1* expression.

Materials and Methods

The human proximal tubular epithelial cells (HK-2) were obtained from the Pasteur Institute of Iran. Then, the cells were cultured in low-glucose (5.5 *mM*-glucose) DMEM medium with 10% fetal bovine serum, 100 *U/ml* penicillin, and 100 *mg/ml* streptomycin at 37°*C*. After the cell confluency reached 80-90%, the grouping was done as below:

1. Control: cells were incubated without treatment for 24 *hr*.
2. Cells incubated with bisphenol-A (1000 μM) for 24 *hr* (11).
3. Cells incubated with H_2O_2 as a well-known oxidant agent for 24 *hr* (12).
4. Cells incubated with bisphenol-A (1000 μM) and Phloretin (50 μM), 24 *hr*.
5. Cells incubated with bisphenol-A (1000 μM) and Phloretin (100 μM), 24 *hr*.
6. Cells incubated with bisphenol-A (1000 μM) and Phloretin (200 μM), 24 *hr* (13).
7. Cells incubated with Phloretin (200 μM) for 24 *hr*.

The concentration of BPA (1000 μM) was selected based on previous *in vitro* studies that used high micromolar ranges to reliably induce oxidative stress, inflammatory responses, and cytotoxicity in kidney and other mammalian cell models within a short exposure time (24 *hr*) (14,15).

Table 1. Primer sequences used for real-time PCR analysis of GAPDH, *VCAM-1*, and *ICAM-1* genes in HK-2 cells

Genes	Forward sequences	Reverse sequences
GAPDH	5′-GGC AAA TTC AAC GGC ACA GT-3′	5′-AGA TGG TGA TGG GCT TCC C-3′
VCAM-1	5′-TGT TGA GAT CTC CCC TGG AC-3′	5′-CGC TCA GAG GGC TGT CTA TC-3′
ICAM-1	5′-CTG CTA AAC TGT TCA TTG TAG-3′	5′-CTA TGG GTT TTA CCT GTG-3′

All experiments were performed in triplicate (n=3 biological replicates), and each measurement was conducted in technical duplicates.

Cell survival assessment via MTT analysis

In this order, the cells are treated with bisphenol-A, H_2O_2 and Phloretin for 24 *hr*. Then, at the end of incubation, the culture medium was discarded and culture medium containing MTT solution was added to all groups. MTT was regenerated by the succinate dehydrogenase system, which is an enzyme involved in the mitochondrial respiratory cycle. Finally, the spectrophotometry was done and the survival rate of each concentration was then plotted in the form of a graph, and based on the graph, interpretation and analysis of the results were performed.

ROS generation assessment via lowcytometry

A reactive probe (2′,7′-dichlorofluoresceindiacetate, DCFH-DA, 10 μM) was used to detect ROS. The cells were cultured in a 6-well plate at a density of 10 (6) cells/well and incubated with the medium for 24 *hr*, followed by incubation with DCFH-DA at 37°C for 30 *min* and washing with Phosphate-Buffered Saline (PBS). Finally, the level of free radical production was measured by flowcytometry method.

Measurement of Total Antioxidant Capacity (TAC) and Malondialdehyde (MDA) levels

For this purpose, the cell supernatant was removed from all groups and the concentration level of MDA as a biomarker of oxidative stress and TAC as a biomarker of antioxidant marker were measured using assay kits (ZellBio GmbH, Germany) and the spectrophotometry method.

Measurement of inflammatory cytokines

For this purpose, the cell supernatant was removed from all groups and the concentration level of IL-6 as a biomarker of pro-inflammatory cytokine and IL-10 as a biomarker of anti-inflammatory cytokine were measured using assay kits (Karmania Pars Gene, Iran) and the spectrophotometry method.

Real-time PCR gene expression

Total RNA underwent treatment with DNase I prior to the synthesis of the initial strand of cDNA. Real-time PCR was conducted following the methodology outlined in previous studies, utilizing SYBR Green Mastermix. The levels of mRNA are reported as fold changes, normalized against glyceraldehyde-3-phosphate dehydrogenase (GAPDH), in accordance with the procedures established by Schmittgen *et al*. The specific primers employed in the real-time PCR are listed in table 1.

Data analysis

Statistical analysis was performed using SPSS v26.0. Normality of the data was assessed with the Kolmogorov–Smirnov test. Comparisons between groups were conducted using one-way ANOVA followed by Tukey's HSD post hoc test. Results are expressed as mean±SEM from at least three independent experiments (n=3). Exact p-values are reported in the results and figure legends, with $p<0.05$ considered statistically significant.

Results

Phloretin improved cell viability in HK-2 cells exposed to bisphenol-A

Exposure to BPA (1000 μM) significantly reduced cell viability compared to control ($p<0.001$). The H_2O_2 group also showed markedly lower viability than control ($p<0.001$). Phloretin treatment restored cell viability in a concentration-dependent manner. At 50 and 100 μM, viability increased significantly compared to BPA alone ($p<0.05$ and $p<0.01$, respectively), while 200 μM Phloretin produced the

strongest effect, restoring viability by approximately 35-40% relative to BPA (p<0.001) (Figure 1).

Phloretin diminished free radicals (ROS) in HK-2 cells exposed to bisphenol-A

BPA exposure significantly elevated ROS levels compared to control (p<0.001). Treatment with Phloretin significantly reduced ROS in a dose-dependent manner. At 200 μM, ROS levels were reduced by ~35–40% compared to BPA (p<0.001), with smaller, but significant reductions at 50 and 100 μM (p<0.05 and p<0.01) (Figure 2).

Phloretin decreased lipid peroxidation in HK-2 cells exposed to bisphenol-A

MDA concentration was significantly higher in BPA-treated cells than in control (p<0.001). Phloretin reduced MDA levels in a concentration-dependent manner. At 200 μM, MDA was reduced by ~30–35% relative to BPA (p<0.001), while 50 and 100 μM produced moderate reductions (p<0.05 and p<0.01) (Figure 3).

Phloretin increased total antioxidant capacity in HK-2 cells exposed to bisphenol-A

BPA exposure markedly decreased TAC compared to control (p<0.001). Phloretin significantly increased TAC in a dose-dependent fashion, with 200 μM restoring TAC by ~40–45% compared to BPA (p<0.001). Lower doses of 50 and 100 μM produced smaller, but significant increases (p<0.05 and p<0.01) (Figure 4).

Phloretin decreased interleukin 6 (IL-6) levels in HK-2 cells exposed to bisphenol-A

IL-6 concentration was significantly elevated in the BPA group relative to control (p<0.001). Phloretin treatment reduced IL-6 levels in a concentration-dependent manner. At 200 μM, IL-6 was reduced by ~35–40% compared to BPA (p<0.001), while 50 and 100 μM treatments showed moderate but significant decreases (p<0.05 and p<0.01) (Figure 5).

Phloretin increased interleukin 10 (IL-10) levels in HK-2 cells exposed to bisphenol-A

BPA significantly lowered IL-10 concentration compared to control (p<0.001). Phloretin treatment increased IL-10 levels in a dose-dependent manner, with 200 μM producing the largest effect (~40–45% increase compared to BPA, p<0.001). The 50 and 100 μM doses also significantly elevated IL-10, albeit to a lesser extent (p<0.05 and p<0.01) (Figure 6).

Phloretin decreased VCAM/ICAM expression in HK-2 cells exposed to bisphenol-A

BPA markedly upregulated *VCAM-1* and *ICAM-1* gene expression compared to control (p<0.001). Phloretin treatment significantly reduced the expression of both adhesion molecules in a dose-dependent manner. At 200 μM, Phloretin suppressed *VCAM*-1 and *ICAM-1* expression by ~45–50% compared to BPA (p<0.001), while 50 and 100 μM produced smaller but significant reductions (p<0.05 and p<0.01). (Figure 7).

Discussion

The obtained results of the current study demonstrated that Phloretin has antioxidant and anti-inflammatory properties which leads to nephroprotective effect in kidney cells exposure to bisphenol-A.

Phloretin is a relatively strong antioxidant in inhibiting proximities leads to inhibiting lipid peroxidation (16). The strong antioxidant activity of Phloretin was found in other assays, such as hydroxyl radical scavenging and 1,1-diphenyl-2-picrylhydrazyl radical scavenging (17,18). Phloretin has immune-modulating properties and is widely used in organ dysfunction due to its antioxidant properties (19,20). In the current study, the effect of Phloretin on oxidative stress factors in nephrotoxicity caused by bisphenol-A in human kidney tubular epithelial cells was investigated and the results showed that there was a significant difference between cell viability in the studied groups. The highest average cell viability was observed in Phl200 concentration. Moreover, the protective effect of Phloretin on the production rate of free radicals was investigated, and the results showed that there was a significant difference between the production rates of free radicals in the investigated groups. The highest average production of free radicals was observed in the positive control group and BSA, whereas the lowest production of free radicals was observed in the negative control group and Phl200. Another aim of

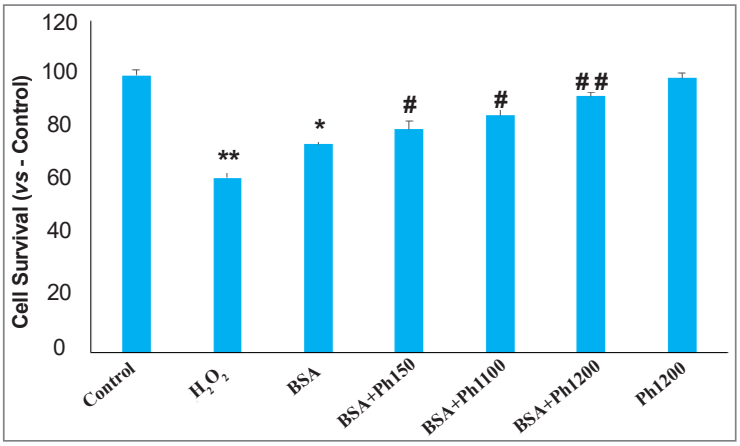

Figure 1. Effect of Phloretin on HK-2 cell viability following exposure to bisphenol-A (BPA, 1000 *μM*). Data are presented as mean±SEM (n=3 independent experiments). Statistical analysis was performed using one-way ANOVA followed by Tukey's HSD post hoc test. *p=0.021 *vs.* control; #p=0.008 *vs.* BPA. Abbreviations: BSA, bisphenol-A; Phl, Phloretin.

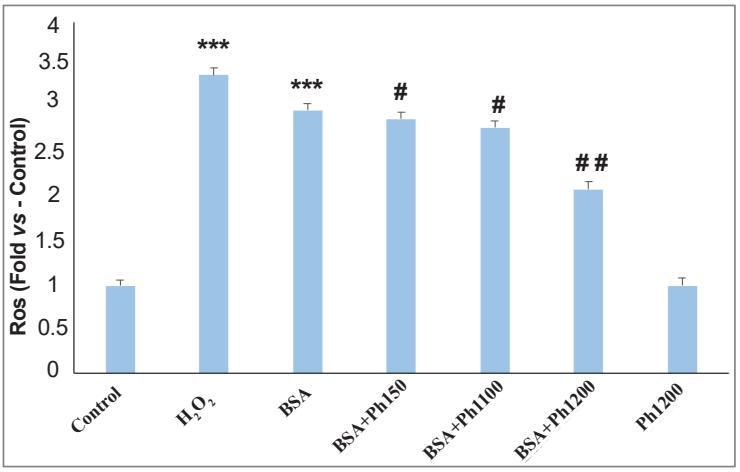

Figure 2. Effect of Phloretin on Reactive Oxygen Species (ROS) production in HK-2 cells after BPA exposure (1000 *μM*). Data are mean±SEM (n=3). One-way ANOVA with Tukey's post hoc test was applied. ***p<0.001 *vs.* control; #p<0.05 and p<0.01 *vs.* BPA.

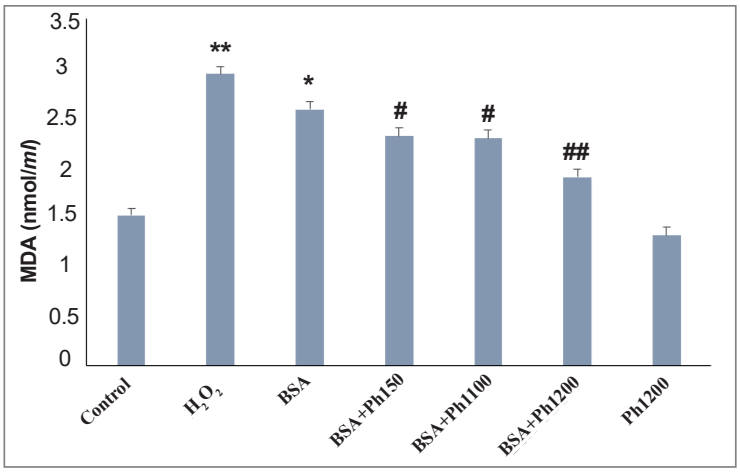

Figure 3. Effect of Phloretin on Malondialdehyde (MDA) concentration, a marker of lipid peroxidation, in HK-2 cells exposed to BPA (1000 *μM*). Data are mean± SEM (n=3). One-way ANOVA with Tukey's post hoc test. *p<0.05 and **p<0.01 *vs.* control; #p<0.05 and ##p<0.01 *vs.* BPA.

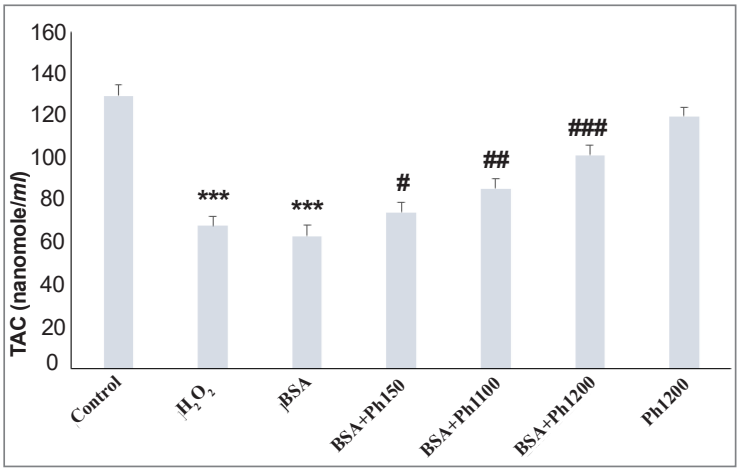

Figure 4. Effect of Phloretin on Total Antioxidant Capacity (TAC) in HK-2 cells after BPA exposure (1000 *μM*). Data are mean±SEM (n=3). One-way ANOVA with Tukey's post hoc test. ***p<0.001 *vs*. control; #p<0.05, ##p<0.01 and ###p<0.001 *vs*. BPA.

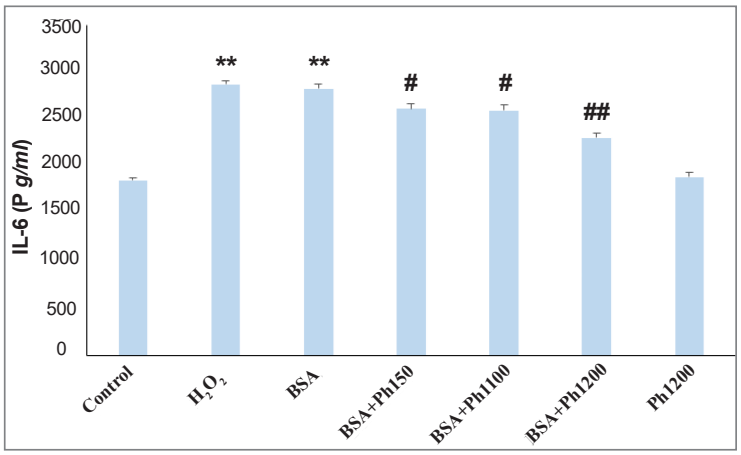

Figure 5. Effect of Phloretin on interleukin-6 (IL-6) levels in HK-2 cells exposed to BPA (1000 *μM*). Data are mean±SEM (n=3). One-way ANOVA with Tukey's post hoc test. **p<0.01 *vs*. control; #p<0.05 and ##p<0.01 *vs*. BPA.

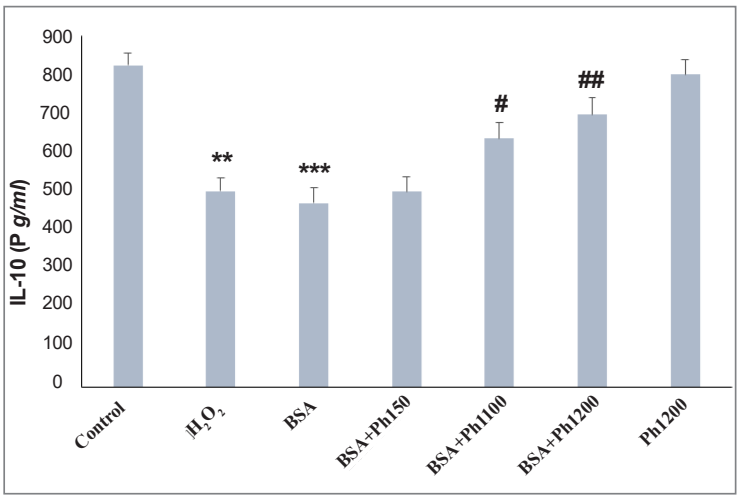

Figure 6. Effect of Phloretin on interleukin-10 (IL-10) levels in HK-2 cells exposed to BPA (1000 *μM*). Data are mean±SEM (n=3). One-way ANOVA with Tukey's post hoc test. **p<0.01 and ***p<0.001 *vs*. control; #p<0.05 and ##p<0.01 *vs*. BPA.

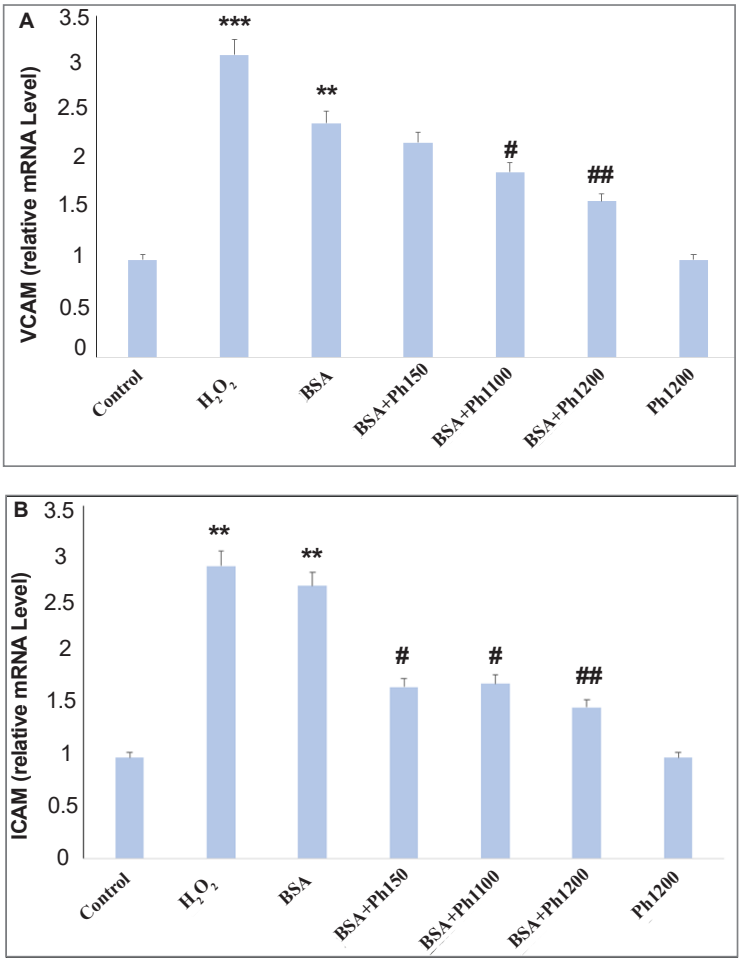

Figure 7. Effect of Phloretin on gene expression of (A) Vascular Cell Adhesion Molecule-1 *(VCAM-1)* and (B) Intercellular Adhesion Molecule-1 *(ICAM-1)* in HK-2 cells exposed to BPA (1000 *μM*). Data are mean±SEM (n=3). Gene expression was normalized to GAPDH and expressed as fold change relative to control. One-way ANOVA with Tukey's post hoc test. **p<0.01 and ***p<0.001 *vs.* control; #p<0.05 and ##p<0.01 *vs.* BPA.

the study was to investigate the protective effect of Phloretin against oxidative stress indicators, and the research findings showed that there was a significant difference between oxidative stress indicators, including MDA, in the studied groups. The highest average of oxidative stress indices including MDA, were observed in the positive control group and BSA, and the lowest amount of oxidative stress indices including MDA, were observed in the negative control group and Phl.

Another goal of this study was to investigate the protective effect of Phloretin on oxidative stress indices, including TAC, and the results of the study showed that there was a significant difference between the oxidative stress indices, including TAC, in the studied group. The highest average amount of oxidative stress indicators including TAC in the negative control group and Plr200, and the lowest average TAC were associated with the BSA group.

Moreover, the results of the study showed that there is a significant increase in the gene expression levels of *VCAM-1* and *ICAM-1* in the BSA group compare to the control cells; although, there was significant decrease in *VCAM/ICAM* gene expression levels in the cell exposure to Phloretin compared to the BSA groups. In individuals suffering from Chronic Kidney Disease (CKD), *VCAM-1* and *ICAM-1* may serve as potential indicators of endothelial dysfunction, a condition associated with the atherosclerotic process that leads to various complications concerning

renal health. Changes in the expression of integrins, along with *VCAM-1* and *ICAM-1* on parenchymal cells, could affect these cells' capacity to engage cytotoxically with invading inflammatory cells. Numerous studies have documented elevated levels of *ICAM-1* and *VCAM-1* in this context. The results of the present study documented that *VCAM-1* and *ICAM-1* might play a role in kidney cell toxicity induced by BSA. However, Phloretin prevented BSA-stimulated upregulation of *VCAM-1* and *ICAM-1* expression in a concentration-dependent manner which suggests that Phloretin may have beneficial effects in the onset and progression of kidney diseases. In previous study it has been shown that the flavonoid Phloretin suppresses stimulated expression of endothelial adhesion molecules and reduces activation of human platelets with prevented TNF-alpha-stimulated upregulation of *VCAM-1, ICAM-1*, and E-selectin expression (21).

Moreover, the results of the study showed that there is a significant difference between the levels of interleukin-6 in the studied groups. The highest amount of interleukin 6 was in the positive control group and BSA, and the lowest amount of interleukin 6 was in the negative control group and Phl200. Finally, the protective effect of Phloretin on the amount of interleukin 10 was investigated, and the results of the study showed that there was a significant difference between the amount of interleukin 10 in the studied groups. The highest level of interleukin 10 was observed in the negative control and BSA groups, and the lowest level of interleukin 6 was observed in the BSA control group.

The present study demonstrates that Phloretin protects renal tubular cells from BPA-induced oxidative stress and inflammation. Mechanistically, Phloretin appears to act through multiple pathways. The reduction in ROS and MDA suggests direct free radical scavenging and suppression of lipid peroxidation, consistent with prior reports of Phloretin inhibiting NADPH oxidase activity and enhancing endogenous antioxidant defenses *via* Nrf2/HO-1 activation (22). The observed increase in TAC further supports its role in strengthening antioxidant capacity. Phloretin also modulated cytokine balance, lowering IL-6 and elevating IL-10, which is in agreement with Zhao *et al*, study (23), who reported that Phloretin attenuated cisplatin-induced renal inflammation. These effects may be mediated through inhibition of NF-κB signaling, a pathway known to regulate pro-inflammatory cytokines. Furthermore, suppression of *VCAM-1* and *ICAM-1* gene expression suggests Phloretin can reduce adhesion molecule–mediated leukocyte recruitment, aligning with previous findings that flavonoids attenuate endothelial activation (24). The present results therefore complement existing literature by providing new evidence that Phloretin mitigates adhesion molecule upregulation in kidney tubular cells specifically exposed to BPA, a novel angle not extensively explored in earlier studies Mechanistically, the nephroprotective effects of Phloretin observed in the present study may be attributed to multiple actions. Phloretin is known to inhibit ROS generation by suppressing NADPH oxidase activity, thereby reducing oxidative stress-induced lipid peroxidation and DNA damage. In addition, it enhances cellular antioxidant defenses by modulating pathways such as Nrf2/HO-1, leading to increased TAC levels. Phloretin has also been reported to inhibit NF-κB activation, which may explain the reduced IL-6 secretion and enhanced IL-10 levels observed. The suppression of *VCAM-1* and *ICAM-1* gene expression further suggests that Phloretin may interfere with pro-inflammatory adhesion molecule signaling, limiting leukocyte recruitment and inflammatory injury in renal tubular cells.

In summary, the results of this study show a significant effect of the protective effect of Phloretin on cell viability indices, free radical production rate, oxidative stress indices including MDA, oxidative stress indices including TAC and interleukin 6 and 10 levels, and Phloretin significantly increases cell viability, interleukin 10 and the amount of TAC in cells exposed to bisphenol. Furthermore, Phloretin significantly reduced the number of free radicals and MDA in cells exposed to bisphenol and interleukin 6. Un *et al* 2021 study showed the effectiveness of Phloretin and phloridzin against cisplatin-induced nephrotoxicity in rats; treatment with Phloretin reduced the parameters of kidney damage and inflammation compared with the model group. In addition, Phloretin treatment increased the activity of SOD and GSH and increased and decreased MDA levels (25). The results showed significant

anti-oxidant, anti-inflammatory, and immune effects, which agree with the results of this study in terms of anti-oxidant properties. Muir *et al* reported in 2023 that the use of bisphenol A at a concentration of 100 *nM* in the cell culture medium causes podocyte inflammation by reducing the synthesis of nephrin and podocin, gap junction proteins involved in the mechanisms of proteinuria and podocyte survival (26). A study by Wahby *et al* in 2017 showed that the use of bisphenol A at a dose of 40 *mg/kg* in large laboratory rats causes kidney enlargement and hydronephrosis, which indicates the effect of this substance on the morphology of kidney tissue through the expansion of Bowman's capsule, hypercellular glomerulation, and destruction of epithelial cells of proximal tubules in kidney tissue (27). The results of this study are contradictory regarding the kidney function regards to use of Bisphenol A.

A study by Omrani *et al* in 2016 in Iran showed that the effect of Phloretin on the oxidative and inflammatory reaction in a sepsis model caused by cecal ligation in rats showed that the levels of Blood Urea Nitrogen (BUN) and Tumor Necrosis Factor alpha (TNF-α) in the CLP-induced sepsis group were significantly increased compared with the control group. In addition, the values of transcription factor tissue Glutathione (GSH) and liver nuclear factor κB (NF-κB p65) were higher in the CLP-induced sepsis group. This increase was significantly reduced in the CLP group treated with Phloretin (28). The results of the aforementioned studies also revealed the antioxidant properties of Phloretin and its effectiveness against kidney damage, which agrees with the results of this study.

The limitations of the current study should be acknowledged. First, this study used an *in vitro* model of acute BPA exposure at relatively high concentrations (1000 μM), which may not fully reflect chronic, low-dose environmental exposure in humans. Second, Phloretin concentrations up to 200 μM exceed typical dietary levels, raising questions about translational relevance. Nonetheless, these doses are commonly used in experimental studies to reveal mechanistic pathways. Finally, *in vitro* models cannot capture systemic metabolism and tissue interactions present *in vivo*. Future work should therefore investigate the protective effects of Phloretin in animal models and assess efficacy at physiologically relevant concentrations.

Conclusion

In summary, this study demonstrates that Phloretin protects renal tubular cells against BPA-induced oxidative stress and inflammation by improving antioxidant capacity, reducing ROS and lipid peroxidation, modulating cytokine balance, and suppressing adhesion molecule expression. These findings highlight Phloretin's potential as a natural nephroprotective agent, although further *in vivo* studies at physiologically relevant concentrations are required to confirm its translational value.

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Conflict of Interest

There was no conflict of interest in this manuscript.

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