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RESEARCH ARTICLE

Effects of Phloretin on Bisphenol-A Induced Liver and Kidney Toxicity in Prepubertal Female Rats

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ABSTRACT

Objective: The aim of this study was to investigate the protective effects of phloretin against bisphenol-A (BPA)-induced liver and kidney damage in rats using histopathological and biochemical parameters.

Materials and Methods: This study started on female rats on the postnatal 28th day via subcutaneous injection by dissolving the compounds in corn oil at 30-min intervals, starting with phloretin, and followed by BPA. The dose of BPA was 50 mg/kg bw/day, and the doses of phloretin were 0.5, 5, and 50 mg/kg bw/day. Treatments were administered every day for 15 days. Histopathological, morphometric, and biochemical parameters were analyzed.

Results: Histopathological evaluation revealed tubular degeneration, fibrous tissue formation, congestion, and edema in the kidney tissue and cellular degeneration and congestion in the liver tissue. BPA treatment resulted in a statistically significant increase in serum urea and alanine aminotransferase levels and a decrease in serum glucose and aspartate aminotransferase levels. Against these effects of BPA, a positive effect was detected only on serum urea levels in rats treated with 50 mg/kg bw/day phloretin. There was also no significant change in serum triglyceride, creatinine, and albumin levels in the BPA positive control group. The renal morphometric analysis revealed that treatment with 0.5 mg/kg bw/day phloretin reduced the BPA-induced glomerular damage.

Conclusion: Biochemical parameters and histopathological findings in the kidney and liver tissues revealed no clear evidence of a protective effect of phloretin against the damage caused by BPA. Hence, phloretin exhibits a low level of protection against liver and kidney damage.

Keywords: Bisphenol-A, phloretin, liver, kidney, female rats.

INTRODUCTION

Chemical compounds are indispensable components of our daily life, but many of these compounds, especially endocrine disruptors, can cause harmful effects on endocrine system structures and hormones.[1] However, studies also indicate that endocrine disruptor chemicals negatively affect liver and kidney functions.[2,3] Bisphenol-A (BPA) is a diphenylmethane derivative formed by two phenyl rings attached to two methyl groups. BPA ($C_{15}H_{16}O_2$) is one of the most produced chemicals worldwide.[4] The BPA values recommended by the U.S. Environmental Protection Agency are as follows: lowest-observed-adverse-effect level (LOAEL): 50 mg/kg bw/day, no-observed-adverse-effect level: 5 mg/kg bw/day, and acceptable daily intake: 50 µg/kg bw/day. The average daily exposure in adults is 0.5 µg/kg bw/day.[5] The effects of BPA on animals have been extensively investigated. The liver and kidneys are among the target organs identified in repeated-dose animal studies.[6] Several studies on BPA demonstrated that it affects biochemical parameters, exerting a negative effect on antioxidant enzymes and causing damage to liver and kidney tissues.[7,8] In the present study, BPA was used to induce liver and kidney damage.

Sheep that fed on red clover pastures were found to have fertility issues, and therefore the feeding area of the sheep was examined. It was observed that those pastures were denser in terms of phenolic compounds than other pastures. In this manner, phytoestrogens were identified. The possible estrogenic effect of plant-derived compounds was first discussed in the 1940s. Subsequently, interest in plant-derived estrogens increased with the advent of hormone replacement therapy.[9] Phloretin is one of the three chalcone derivatives (butein, marein, and phloretin) of the flavonoid group in the phytoestrogen classification.[10,11] It is a phytopolyphenol found in apples, strawberries, and other fruits and exhibits high antioxidant properties. It is also known to exhibit antitumor and anti-inflammatory properties and al-

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leviate liver damage. Moreover, it reduces the risk of serious chronic diseases.[12–14] In the present study, we investigated the protective effects of three doses of phloretin (0.5, 5, and 50 mg/kg bw/day) against BPA-induced liver and kidney damage.

There is no study examining the effects of phloretin against BPA-induced liver and kidney damage. In the present study, the effect of phloretin against BPA-induced liver and kidney damage was firstly examined.

MATERIALS AND METHODS

Chemicals

Phloretin (CAS No. 60-82-2) and BPA (CAS No. 80-05-7) were obtained from Sigma–Aldrich (USA). Creatinine (Cat. No. E-BC-K186), albumin (Cat. No. E-BC-K058), alanine aminotransferase (ALT) (Cat. No. E-BC-K235), aspartate aminotransferase (AST) (Cat. No. E-BC-K236), urea (Cat. No. E-BC-K183), and triglyceride (Cat. No. E-BC-K238) kits were obtained from Elabscience-Biotechnology (China). Glucose kit (Cat. No. E1623R) was obtained from Bioassay Technology Laboratory (China).

Animals and Housing

This study was conducted using 36 Wistar albino (*Rattus norvegicus*) female rats, aged 28 days, and weighing 130–150 g, which were obtained from Hacettepe University Experimental Animals Production Center with the approval number 2018/47-04. The rats were randomly grouped. During the 15-day experiment, the laboratory temperature was set at approximately 23°C ± 2°C, and the relative humidity was 48% ± 3%. The photoperiod was set as 12-h light and 12-h dark. Drinking water and normal pellet feed were provided *ad libitum* during the experiment.

Experimental Protocol

The rats were divided into five groups with six rats in each group, which was based on previous similar toxicological studies, and the smallest sample size was expected to be statistically significant. The five groups were as follows: (1) corn oil-control, (2) 50 mg/kg bw/day BPA positive control, (3) 50 mg/kg bw/day BPA+0.5 mg/kg bw/day phloretin dose, (4) 50 mg/kg bw/day BPA+5 mg/kg bw/day phloretin dose, and (5) 50 mg/kg bw/day BPA+50 mg/kg bw/day phloretin dose. As our aim was to evaluate the protective effect of different doses of phloretin against liver and kidney damage induced by BPA, we did not create a phloretin control group in which BPA was not applied. To ensure that BPA causes damage, we used the LOAEL value of 50 mg/kg bw/day.[5] Phloretin and BPA were dissolved in corn oil and administered to rats. Hence, a separate group was created for the corn oil group used as a vehicle and termed the "corn oil-control group." The purpose of creating this group was to eliminate the doubt that the corn oil exerts any effect on the results. Treatment was started on the rats on the postnatal 28th day. This age range was selected because the effect of chemicals is quite large during the prepubertal period (before puberty) of sexual differentiation (sensitive period) in rodents and humans. All rats were administered at the same age and randomly distributed to the groups, based on their weight. During the study, daily body weight, consumed feed, and water amount were recorded. BPA and phloretin were administered to the rats via subcutaneous injection at 30-min intervals, in the determined doses, starting with phloretin, and followed by BPA. The doses of phloretin were selected according to the phytoestrogen doses that people can take daily. Phloretin is found at rates of 80–420 mg/kg in apple peel, 3–223 mg/L in juice, and 2–5 mg/kg in fresh strawberries.[15] All treatment was administered every day for 15 days. Rats were sacrificed by cervical dislocation 24 h after the final dose under ketamine/xylazine anesthesia.

Liver and Kidney Organ Weights

After sacrificing the rats, the liver and kidney tissues were removed without damage and weighed, and these values were presented as absolute organ weights. To calculate relative organ weights, the organ weights were divided by terminal body weights, and the results were supported by dividing organ weights by brain weight.

Biochemical Analysis

Blood samples collected for biochemical analyses were centrifuged at 3600 rpm for 30 min at 4°C in the Eppendorf Centrifuge 5810R device (Germany), and serum was obtained. The levels of serum ALT, AST, urea, triglyceride, creatinine, albumin, and glucose were determined using kits. Analyses were performed using a BIOTEK uQuant (USA) spectrophotometer device.

Histopathological Analysis

At the end of the experiment, the liver and kidney tissues removed from the rats were fixed in 10% formaldehyde fixative for 24 h, after which they were washed in running water for 24 h. The tissues were blocked in paraffin, and sections of the paraffin blocks were cut at a thickness of 4 µm using a Leica (Germany) microtome. Slides were stained with hematoxylin and eosin. The preparations were examined under the Olympus BX51 system light microscope (Olympus Corporation, Japan) and photographed using Olympus cellSens Entry 4.1.1 program (Olympus Corporation, Japan).

Histomorphometric Measurement of Kidney Tissues

Glomeruli were histomorphometrically measured in all groups. For each group, 100 glomeruli were selected at random. The shortest and longest diameters of glomeruli were measured using the Olympus BX51 system light microscope (Olympus Corporation, Japan) and Olympus cellSens Entry 4.1.1 program (Olympus Corporation, Japan). The glomerular volume was determined using the formula $4\,\pi(d(G)/2)^3/3$, where d (G) is the arithmetic mean of the long and short diameters.[16]

Statistical Analysis

Data were analyzed using the statistical SPSS IBM-23 program (version 23, USA). Data homogeneity was evaluated using Levene statistics. ANOVA was used when the variances were homogeneous; otherwise, the Welch test was used. Tukey and Games–Howell tests were used as post hoc tests. Fisher's exact test was used to determine the statistical significance of histopathological data. All values were expressed as mean ± SD. $p<0.05$ was considered statistically significant.

Ethics Committee Approval

Permission required for the studies was obtained from Hacettepe University Experimental Animals Ethics Committee with the number 2018/47-04.

RESULTS

Liver and Kidney Organ Weights

Liver and kidney absolute organ weights, relative organ weights, and final body weights of female rats in the corn oil-control, BPA positive control, and phloretin treatment groups are presented in Table 1. Both initial and final body weights showed a statistically significant difference between the BPA and phloretin dose groups. To determine the accuracy of this difference, we investigated the % weight change but found no significant difference in the results. Overall, BPA positive control and phloretin dose groups showed no significant changes in body weights in this study. However, the absolute kidney weights statistically significantly decreased in the BPA positive control group compared with that in the corn oil-control group, but no difference was detected in the phloretin dose groups. The kidney weights calculated according to body weight significantly reduced in the 50 mg/kg bw/day phloretin dose group compared with that in the BPA positive control group, whereas the relative kidney weights calculated according to brain weight significantly increased compared with that in the 0.5 mg/kg bw/day BPA positive control group. Although no statistically significant change was observed in liver weights in the BPA positive control group, a significant increase was detected in the phloretin dose groups compared with that in the BPA group. The relative liver weights determined based on body weight showed no significant differences. In the 0.5 mg phloretin dose group, there was a significant reduction in the relative liver weights calculated according to brain weights compared with that in the BPA positive control group.

Biochemical Results

Table 2 shows the biochemical results of the control and experimental groups. Serum ALT levels significantly increased in the BPA positive control group compared with those in the corn oil-control group. Serum ALT values also significantly increased in all phloretin treatment groups compared with those in the corn oil-control group. However, there were no statistically significant differences between the BPA positive control and phloretin treatment dose groups. Regarding serum AST levels, a statistically significant decrease was detected in the BPA positive control group compared with those in the corn oil-control group. Similarly, serum AST values significantly decreased in all the phloretin treatment groups compared with those in the corn oil-control group. With the doses and methods used in this study, we detected no beneficial effect of phloretin on alterations in serum ALT and AST levels caused by BPA. Serum glucose levels statistically decreased in the BPA positive control group compared with those in the corn oil-control group. Similarly, serum glucose levels were significantly lower in the 0.5 and 50 mg/kg bw/day phloretin dose groups than in the corn oil-control group. The decrease in serum glucose levels in the 5 mg/kg bw/day phloretin dose group was not statistically significant compared with that in the corn oil-control group. There was no significant change between the BPA positive control and phloretin dose groups. Serum triglyceride levels also showed no statistically significant differences between the corn oil-control and BPA positive control groups, and the phloretin dose groups also showed no differences triglyceride levels compared with those in the corn oil-control or BPA positive control group. Nevertheless, the 0.5 mg/kg bw/day phloretin dose group showed statistically lower triglyceride levels than the 5 and 50 mg/kg bw/day phloretin dose groups. Regarding serum albumin levels, no statistically significant difference was detected between BPA positive control and corn oil-control groups. Similarly, serum albumin levels in the 0.5 mg/kg bw/day phloretin dose group showed no statistically significant differences compared with those in the corn oil-control and BPA positive control groups. Serum albumin levels in the 5 mg/kg bw/day phloretin dose group were significantly higher than those in the corn oil-control group. Moreover, serum albumin levels statistically significantly decreased in the 50 mg/kg bw/day phloretin dose group compared with those in the BPA positive control group and the 5 mg/kg bw/day phloretin dose group.

Regarding serum creatinine levels, no statistically significant differences were found among the corn oil-control, BPA positive control, and phloretin dose groups. Serum urea levels

Table 1. Absolute and relative organ weights of female rats in the corn oil-control, BPA-positive control and phloretin dose groups.

Measurements	Control Groups		Phloretin Groups			*P value*
	Corn Oil	**BPA (50 mg/kg bw/day)**	**Phloretin (0.5 mg/kg bw/day) + BPA (50 mg/kg bw/day)**	**Phloretin (5 mg/kg bw/day) + BPA (50 mg/kg bw/day)**	**Phloretin (50 mg/kg bw/day) + BPA (50 mg/kg bw/day)**	
Initial Body Weights (g)	102 ± 13	90 ± 15 [c, d, e]	117 ± 13 [b]	117 ± 11 [b]	119 ± 18 [b]	*0.014*
Terminal Body Weights (g)	140 ± 11	120 ± 19 [c, d, e]	157 ± 16 [b]	164 ± 12 [b]	153 ± 15 [b]	*0.001*
Weight Change (%)	40 ± 23	34 ± 3	35 ± 5	40 ± 5	30 ± 16	0.327
Brain Weights (g)	1.53 ± 0.12	1.5 ± 0.08	1.6 ± 0.07	1.52 ± 0.26	1.6 ± 0.14	0.696
Kidney Weights (g)	1.48 ± 0.15 [b]	1.2 ± 0.07 [a]	1.47 ± 0.18	1.41 ± 0.10	1.26 ± 0.16	*0.003*
Liver Weights (g)	6.92 ± 0.88	6.09 ± 0.73 [c, d, e]	7.79 ± 0.90 [b]	8.06 ± 0.73 [b]	7.9 ± 0.55 [b]	*0.001*
Relative Kidney Weights (g/body weight kg)	10.55 ± 0.53 [e]	10.11 ± 1.28	4.42 ± 0.39	9.07 ± 1.64	8.27 ± 0.99 [a]	*0.004*
Relative Liver Weights (g/body weight kg)	49.22 ± 3.39	51.15 ± 4.45	47.56 ± 2.90	51.32 ± 1.45	52.11 ± 5.64	0.157
Relative Brain Weights (g/body weight kg)	10.96 ± 0.76	12.66 ± 1.39 [c, d]	9.83 ± 0.56 [b]	9.8 ± 2.06 [b]	10.62 ± 1.69	*0.012*
Relative Kidney Weights (g/brain g)	0.97 ± 0.08 [b]	0.8 ± 0.05 [a, c]	0.96 ± 0.23 [b]	0.92 ± 0.15	0.79 ± 0.10	*0.012*
Relative Liver Weights (g/brain g)	49.22 ± 3.39	51.15 ± 4.45 [c]	47.56 ± 2.90 [b]	51.32 ± 1.45	52.11 ± 5.64	*0.029*

Values are given as mean ± SD. [a] Statistically different from the corn oil-control group, [b] statistically different from the BPA-positive control group, [c] statistically different from the 0.5 mg/kg bw/day phloretin dose group, [d] statistically different from the 5 mg/kg bw/day phloretin dose group, [e] statistically different from the 50 mg/kg bw/day phloretin dose group, (Significance level $p<0.05$). Bisphenol-A (BPA).

Table 2. Biochemical analysis of serum samples of female rats in the corn oil-control, BPA-positive control and phloretin dose groups.

Measurements	Control Groups		Phloretin Groups		
	Corn Oil	**BPA (50 mg/kg bw/day)**	**Phloretin (0.5 mg/kg bw/day) + BPA (50 mg/kg bw/day)**	**Phloretin (5 mg/kg bw/day) + BPA (50 mg/kg bw/day)**	**Phloretin (50 mg/kg bw/day) + BPA (50 mg/kg bw/day)**
ALT (IU/L)	5 ± 0.04 [b, c, d, e]	15 ± 0.02 [a]	16 ± 0.01 [a]	19 ± 0.01 [a]	20 ± 0.02 [a]
AST (IU/L)	164.74 ± 6.39 [b, c, d, e]	121.64 ± 12.33 [a]	126.15 ± 9.67 [a]	121.12 ± 9.09 [a]	123.79 ± 12.55 [a]
Albumin (g/L)	21.58 ± 0.30 [d]	21.62 ± 0.53 [e]	22.08 ± 0.84	22.92 ± 0.68 [a, e]	20.53 ± 0.68 [b, d]
Glucose (mg/dL)	84.73 ± 8.8 [b, c, e]	52.46 ± 4.17 [a]	55.26 ± 0.66 [a]	65.26 ± 12.57	53.1 ± 8.91 [a]
Creatinine (µmol/L)	26.00 ± 3.7	24.15 ± 3.70	24.76 ± 3.02	25.07 ± 6.31	24.15 ± 4.77
Triglyceride (mmol/L)	0.07 ± 0.02	0.08 ± 0.00	0.04 ± 0.01 [d, e]	0.12 ± 0.01 [c]	0.11 ± 0.06 [c]
Urea (mmol/L)	1.16 ± 0.26 [b, c, d]	1.82 ± 0.51 [a]	2.40 ± 0.44 [a, d]	2.05 ± 0.30 [a]	1.6 ± 0.17 [c]

Values are given as mean ± SD. [a]Statistically different from the corn oil-control group, [b]statistically different from the BPA- positive control group, [c]statistically different from the 0.5 mg/kg bw/day phloretin dose group, [d]statistically different from the 5 mg/kg bw/day phloretin dose group, [e]statistically different from the 50 mg/kg bw/day phloretin dose group, (Significance level $p < 0.05$). Bisphenol-A (BPA), Alanine Aminotransferase (ALT), Aspartate Aminotransferase (AST).

statistically significantly increased in the BPA positive control group compared with those in the corn oil-control group. Serum urea levels in all the phloretin dose groups were also higher than those in the corn oil-control group. This increase was sta-

tistically significantly different in the 0.5 and 5 mg/kg bw/day phloretin dose groups compared with that in the corn oil-control group. Moreover, the 0.5 and 50 mg/kg bw/day phloretin dose groups showed significant differences in serum urea levels.

Histopathological Results

The results of microscopic evaluation of the kidney tissue of the corn oil-control, BPA positive control, and phloretin dose groups are depicted in Figure 1. The incidence of the histopathological findings of the kidney tissues of rats is presented in Table 3. Kidney sections from the corn oil-control group demonstrated healthy kidney tissues. However, kidney sections from the BPA positive control and phloretin treatment groups demonstrated Bowman's capsule dilatation, tubular degeneration, degeneration in the renal parenchyma, congestion, glomerular atrophy, cell expulsion into the lumen, and fibrous tissue formation. No clear protective effect of phloretin against BPA-induced histopathological damage in the kidney tissue was detected. The results of microscopic evaluation of the liver tissue of the corn oil-control, BPA positive control, and phloretin groups are illustrated in Figure 1. The incidence of the histopathological findings of the liver tissues of rats is shown in Table 4. Liver sections from the corn oil-control group revealed healthy liver tissue. However, liver sections from the BPA positive control and phloretin treatment dose groups showed congestion, sinusoidal dilatation, edema, and degeneration in hepatic parenchyma, mononuclear cell infiltration, ballooning in hepatocytes, and steatosis. Ballooning in hepatocytes and congestion in the liver tissue were primarily detected in the BPA positive control group compared with those in the corn oil-control group, but these findings decreased significantly in all the phloretin dose groups compared with those in the BPA positive control group. Although phloretin protected against congestion and ballooning in hepatocytes, no strong protective effect was detected against other BPA-induced liver damage.

Histomorphometric Measurement of Kidney Tissues

The results of kidney morphometric analysis of the corn oil-control, BPA positive control, and phloretin treatment groups are shown in Table 5. A statistically significant decrease was observed in long diameter, short diameter, glomerular diameter, and glomerular volume in the BPA positive control group compared with those in the corn oil-control group. Similarly, the values in the 5 and 50 mg/kg bw/day phloretin dose groups were statistically different from those in the corn oil-control group in all measurements. The values in the 0.5 mg/kg bw/day phloretin dose group were also statistically significantly different from those in the BPA positive control group in all measurements. The 50 mg/kg bw/day phloretin dose group showed highly statistically significant differences in short diameter, glomerular diameter, and glomerular volume from those in the corn oil-control group.

DISCUSSION

Researchers have recently began focusing their attention on the physiological and pharmacological functions of bioactive substances found in plants, such as phloretin. Epidemiological and experimental studies on phloretin have demonstrated that this flavonoid exerts both positive and negative effects, as an exceptionally high-dose of phloretin exerts lethal effects on mice.[17,18] Nevertheless, several studies have demonstrated that phloretin treatment at specific doses exerts numerous protective effects, such as antidiabetic, anticancer, and anti-inflammatory.[17,19] According to the literature, the effects of phloretin differ depending on the type, age, and gender of the experimental animal, the phloretin dose, and the method of administration, and, if the effects against induced damage are being investigated, the substances that cause the damage. Although some of our study findings support the positive findings reported in the literature, our conclusion was that increasing the phloretin dose did not increase the protective effect and would not be safe.

Damage to the structural integrity of the cell membrane, especially in the liver cells, causes the release of ALT and AST in large amounts into the blood, increasing their serum levels.[20] A study in which D-galactosamine was used to cause hepatotoxicity showed an increase in serum ALT and AST levels. In this study, the effects of D-galactosamine on ALT and AST levels were reduced in parallel with increasing doses of phloretin used in the study (0.877 and 1.754 mmol/kg), and hepatic lesions were decreased.[21] Ren et al. investigated choline-induced hepatotoxicity. It was stated that blood ALT and AST levels increased in the choline model group compared to the normal control group. In the experimental groups where phloretin and choline were administered together, it was determined that ALT and AST levels decreased compared to the choline model group. This protective effect occurred in parallel with increasing doses of phloretin (100, 200 and 400 mg/kg/day).[22] In another study on mice, liver damage was induced by CCl_4 and the protective effect of phloretin at doses of 100, 200, and 500 mg/kg/day was evaluated. It was observed that increasing doses of phloretin reduced the excessive increase in serum ALT and AST levels induced by CCl_4.[23] The anticancer properties of phloretin were investigated by Alansari et al.[19] who also showed that the increase in serum ALT and AST levels caused by diethylnitrosamine-induced hepatocellular carcinoma was reduced after treatment with 25 mg/kg/day phloretin. In another study, mice fed on a western diet and high-fructose corn syrup showed increased serum ALT and AST levels, and treatment with 100 and 200 mg/kg/day phloretin significantly decreased the elevated serum ALT levels, whereas 50 mg/kg phloretin dose was ineffective, but the AST levels significantly decreased in all dose groups.[24] In these previous studies, the chemicals

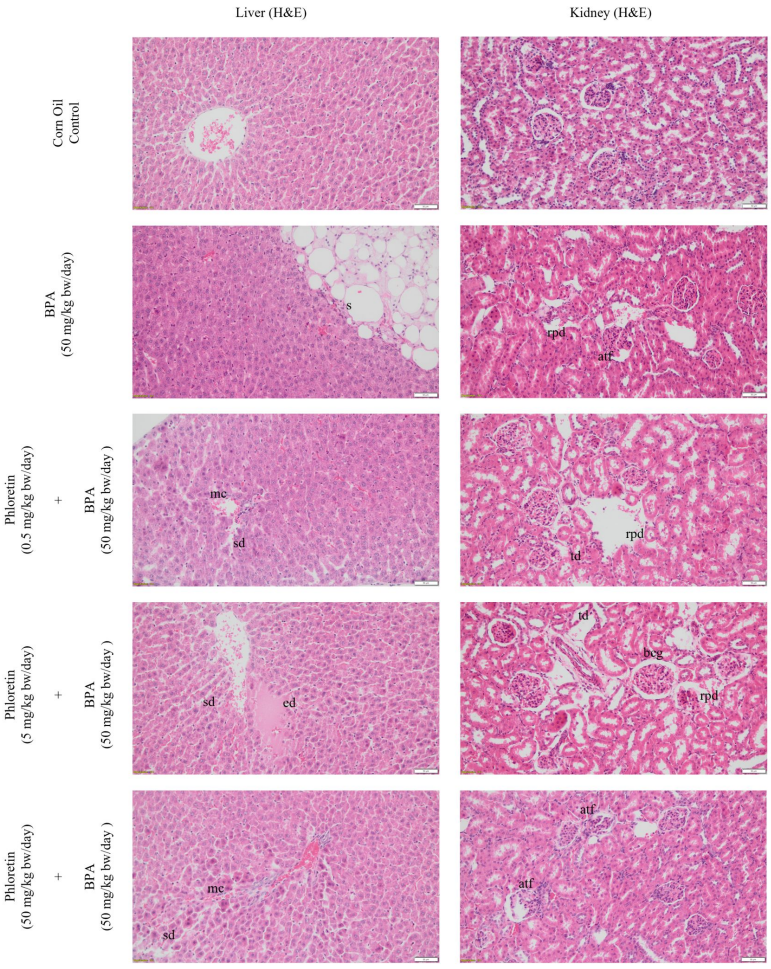

Figure 1. Representative photomicrographs of liver and kidney tissue of the corn oil- control, BPA-positive control and phloretin treatment groups. Normal histology in the kidney tissue of the corn oil-control group; degeneration in renal parenchyma (**rpd**) and glomerular atrophy (**atf**) are shown in BPA-positive control group; tubular degeneration (**td**) and degeneration in renal parenchyma (**rpd**) are shown in 0.5 mg/kg bw/day phloretin dose group; tubular degeneration (**td**), degeneration in renal parenchyma (**rpd**) and bowman capsule dilatation (**bcg**) are shown in 5 mg/kg bw/day phloretin dose group; glomerular atrophy (**atf**) is shown in 50 mg/kg bw/day phloretin dose group. Normal histology in the liver tissue of the corn oil-control group; steatosis (**s**) is shown in BPA-positive control group; minimal congestion (**mc**) and sinusoidal dilatation (**sd**) are shown in 0.5 mg/kg bw/day phloretin dose group; edema (**ed**) and sinusoidal dilatation (**sd**) are shown in 5 mg/kg bw/day phloretin dose group; minimal congestion (**mc**) and sinusoidal dilatation (**sd**) are shown in 50 mg/kg bw/day phloretin dose group; H&E stain, 200X.

used to generate animal models increased serum ALT and AST levels, whereas we used BPA in the present study that decreased serum AST and ALT levels. As we could not detect a significant decrease or increase in the phloretin treatment groups compared with the BPA group, we believe that the decrease in serum AST levels detected in the present study is due to BPA. In contrast, we detected an increase in serum ALT levels, which we again believe is due to BPA. Unlike the previous study, we could not obtain clear information regarding the effect of phloretin on serum ALT and AST levels.

The effects of phloretin on glucose metabolism have been extensively investigated.[25–27] For instance, a study investigating the effects of oral treatment of 5 and 10 mg/kg/day phloretin on body energy and glucose balance in diabetic C57BL BKS-DB mice reported that in parallel to phloretin dosage increases, blood glucose levels considerably lowered and glucose tolerance improved.[25] Shen et al. explored the hypoglycemic effect of phloretin in 8-week-old male rats with streptozotocin-induced diabetes and fed on high fat and high sugar.[26] To determine its protective and therapeutic effects, phloretin was administered to different groups before and after streptozotocin induction at 100 mg/kg daily for 4 weeks, and it was observed that phloretin was protective against diabetes and beneficial in the treatment of glucose and lipid metabolism.[26] Alsenea et al.

Table 3. Incidence of histopathological findings detected in kidney tissues of the corn oil-control, BPA-positive control and phloretin dose groups.

Histopatological Findings	Control Groups		Phloretin Groups		
	Corn Oil	BPA (50 mg/kg bw/day)	Phloretin (0.5 mg/kg bw/day) + BPA (50 mg/kg bw/day)	Phloretin (5 mg/kg bw/day) + BPA (50 mg/kg bw/day)	Phloretin (50 mg/kg bw/day) + BPA (50 mg/kg bw/day)
Bowman Capsule Dilatation	1 / 6 [c, e]	1 / 6 [c, d]	6 / 6 [a, b]	2 / 6	4 / 6 [a, b]
Tubular Degeneration	0 / 6 [b, d, e]	5 / 6 [a]	3 / 6	4 / 6 [a]	4 / 6 [a]
Degeneration in Renal Parenchyma	0 / 6	1 / 6	1 / 6	1 / 6	1 / 6
Minimal Congestion	0 / 6 [d]	3 / 6	3 / 6	4 / 6 [a]	3 / 6
Glomerular Atrophy	0 / 6 [b, d]	4 / 6 [a]	3 / 6	4 / 6 [a]	3 / 6
Cell Expulsion into the Lumen	0 / 6	3 / 6	1 / 6	1 / 6	2 / 6
Mononuclear Cell Infiltration	0 / 6	1 / 6	2 / 6	1 / 6	2 / 6
Fibrous Tissue Formation	0 / 6	1 / 6	1 / 6	1 / 6	2 / 6

Values are given as the number of rats with histopathological findings/number of rats examined in the group. [a]Statistically different from the corn oil-control group, [b]statistically different from the BPA-positive control group, [c]statistically different from the 0.5 mg/kg bw/day phloretin dose group, [d]statistically different from the 5 mg/kg bw/day phloretin dose group, [e]statistically different from the 50 mg/kg bw/day phloretin dose group, (Significance level $p<0.05$). Bisphenol-A (BPA)

Table 4. The incidences of histopathological findings detected in the liver tissue of the corn oil-control, BPA-positive control and phloretin dose groups.

Histopathological Findings	Control Groups		Phloretin Groups		
	Corn Oil	BPA (50 mg/kg bw/day)	Phloretin (0.5 mg/kg bw/day) + BPA (50 mg/kg bw/day)	Phloretin (5 mg/kg bw/day) + BPA (50 mg/kg bw/day)	Phloretin (50 mg/kg bw/day) + BPA (50 mg/kg bw/day)
Minimal Congestion	0 / 6 [b]	4 / 6 [a, c, d, e]	1 / 6 [b]	1 / 6 [b]	1 / 6 [b]
Sinusoidal Dilatation	0 / 6 [b, c, d]	4 / 6 [a]	5 / 6 [a]	5 / 6 [a]	3 / 6
Edema	0 / 6	0 / 6	0 / 6	1 / 6	1 / 6
Degeneration in Hepatic Parenchyma	0 / 6	0 / 6	0 / 6	1/ 6	1 / 6
Mononuclear Cell Infiltration	1 / 6	2 / 6	1 / 6	2 / 6	1 / 6
Ballooning in Hepatocytes	0 / 6 [b]	4 / 6 [a, c, d, e]	1 / 6 [b]	0 / 6 [b]	0 / 6 [b]
Steatosis	0 / 6	1 / 6	1 / 6	0 / 6	0 / 6

Values are given as the number of rats with histopathological findings / number of rats examined in the group. [a]Statistically different from the corn oil-control group, [b]statistically different from the BPA-positive control group, [c]statistically different from the 0.5 mg/kg bw/day phloretin dose group, [d]statistically different from the 5 mg/kg bw/day phloretin dose group, [e]statistically different from the 50 mg/kg bw/day phloretin dose group, (Significance level $p<0.05$). Bisphenol-A (BPA).

investigated the preventive and therapeutic effects of phloretin in 10-week-old male C57BL/6 mice with high-fat-diet-induced obesity.[27] Phloretin was administered intraperitoneally at 10 mg/kg twice weekly for 12 weeks, and the results showed that phloretin improved glucose homeostasis and insulin sensitivity and attenuated hepatic lipid accumulation.[27] Furthermore, Mao et al. examined the protective effect of 25 and 75 mg/kg phloretin doses against diabetes-induced endothelial damage through *in vitro* and *in vivo* studies.[28] They observed that both doses of phloretin were protective against endothelial damage in diabetic mice through AMP-activated protein kinase-dependent anti-EndMT (endothelial–mesenchymal transforma-

Table 5. Histomorphometric measurements of glomeruli in the corn oil-control, BPA-positive control and phloretin dose groups.

Measurments	Control Groups		Phloretin Groups			*P value*
	Corn Oil	BPA (50 mg/kg bw/day)	Phloretin (0.5 mg/kg bw/day) + BPA (50 mg/kg bw/day)	Phloretin (5 mg/kg bw/day) + BPA (50 mg/kg bw/day)	Phloretin (50 mg/kg bw/day) + BPA (50 mg/kg bw/day)	
Long Diameter (µm)	75.07 ± 15.22 [b, d, e]	62.21 ± 13.71 [a, c]	72.01 ± 12.59 [b]	68.08 ± 17.01 [a]	64.46 ± 12.38 [a]	***<0.0001***
Short Diameter (µm)	62.67 ± 12.19 [b, d, e]	48.95 ± 12.44 [a, c, d]	59.89 ± 13.30 [b]	56.3 ± 14.41 [a, b]	48.78 ± 8.67 [a]	***<0.0001***
Glomerular Diameter (µm)	68.87 ± 12.90 [b, d, e]	55.58 ± 12.30 [a, c, d]	65.96 ± 11.95 [b]	62.19 ± 14.93 [a, b]	56.62 ± 9.71 [a]	***<0.0001***
Glomerular Volume ($x10^6$ μm^3)	0.19 ± 0.10 [b, d, e]	0.1 ± 0.07 [a, c, d]	0.16 ± 0.08 [b]	0.15 ± 0.10 [a, b]	0.1 ± 0.05 [a]	***<0.0001***

Values are given as mean ± SD. [a]Statistically different from the corn oil-control group, [b]statistically different from the BPA-positive control group, [c]statistically different from the 0.5 mg/kg bw/day phloretin dose group, [d]statistically different from the 5 mg/kg bw/day phloretin dose group, [e]statistically different from the 50 mg/kg bw/day phloretin dose group, (Significance level $p<0.05$). Bisphenol-A (BPA).

tion) activation and reduced procalcification factors and vascular fibrosis.[28] Molecular studies on the effects of phloretin could explain the decrease in glucose levels. Sodium-dependent glucose cotransporter 1 (SGLT1) present in intestinal epithelial cells and glucose transporter protein type 2 (GLUT2) present in the intestinal membrane play a role in glucose absorption. Phloretin inhibits the function of GLUT2 and SGLT1, reducing the basolateral transfer of glucose from intestinal cells to the blood and reducing apical glucose uptake.[17,29,30] Phlorizin (phloretin glycoside) also inhibits the function of SGLT2, which is responsible for renal tubular reabsorption of glucose, increasing its urinary excretion.[17,31,32] Overall, the administration of phloretin reduces glucose levels by decreasing its absorption and increasing its urinary excretion.[17] It is evident that certain doses of phloretin exert curative effects on diabetes and glucose metabolism. In the present study, serum glucose levels decreased in all groups compared with those in the corn oil-control group. However, no significant difference was found between BPA-positive control and phloretin treatment groups. Therefore, phloretin does not appear to have an additional contribution to the decrease in serum glucose levels caused by BPA in all groups. The chemicals used to create a model in the abovementioned studies increased the serum glucose level. Furthermore, some studies have used diabetic animal models. BPA, which was used to induce damage in the present study, decreased the serum glucose level. Therefore, we believe that our results differ from those reported in the literature because BPA and phloretin were administered subcutaneously for 15 days. We believe that it would be valuable to explore the effects of phloretin on hyperglycemia and diabetes.

In mammals, the highest concentrations of creatinine are found in the skeletal muscle, where it plays a significant role in energy metabolism. Experimental data reveal a close relationship between disorders in creatinine metabolism and various muscle diseases. However, creatinine also plays a vital role in kidney metabolism[33] and is produced in the liver as well.[34] Serum creatinine is a biomarker for both the kidney and liver. Studies have demonstrated that treatment with phloretin causes a reduction in serum creatinine levels, which increases the damage caused by different chemicals.[35,36] In a study investigating the toxic effect of phloretin, no effect on serum creatinine level was found.[37] The effect of 50 mmol/kg intraperitoneal phloretin treatment on a sepsis model with cecal ligation and puncture in rats was investigated in another study, which showed that the levels of blood urea nitrogen, tumor necrosis factor-alpha, glutathione, and liver nuclear factor-κB p65 transcription factor increased in the CLP group and decreased in the phloretin treatment groups. However, no significant difference was observed in the levels of serum creatinine and creatinine phosphokinase.[38] Cui et al. investigated the effect of phloretin on kidney damage in mice with adenine/potassium oxonate-induced hyperuricemia.[39] They reported that treatment with 50 mg/kg phloretin significantly decreased the serum urea nitrogen level, which was elevated due to hyperuricemia. In contrast, the creatinine level decreased slightly, but not statistically. In the present study, the slight decrease observed in the BPA group was insignificant. However, no statistically significant difference was observed in serum creatinine levels in the phloretin treatment groups compared with those in the corn oil-control or BPA positive control groups. We concluded that phloretin doses exerted no significant effect no serum creatinine. Urea is a vital parameter to interpret kidney functions.[40,41] Studies have shown that increased serum urea levels due to damage caused by various chemicals are reduced by phloretin treatment.[35,38] In the present study, we detected a significant increase in urea

levels in the BPA group. We also detected a significant increase in urea levels in the phloretin treatment groups compared with those in the corn oil-control group. Nonetheless, the increase in urea levels in the phloretin treatment group was insignificant compared with that in the BPA group. This finding differs from other studies on phloretin. For instance, Un et al. investigated the effect of phloretin and phlorizin against cisplatin-induced damage in Balb/c female mice.[36] They administered 50 and 100 mg/kg phlorizin and phloretin to mice by oral gavage for 3 days and found significant improvement in cisplatin-induced elevated serum urea levels. They also administered phlorizin and phloretin to mice not treated with cisplatin and found no difference in serum urea levels between the phlorizin and phloretin treatment groups and the control group (no treatment).[36] Similarly, Pujari et al. reported that phloretin may not exert a direct increasing or decreasing effect on serum urea levels.[37] Hence, we believe that the phloretin doses we used affect in the present study exerted no effect on serum urea levels and that the statistically significant increase in the 0.5 and 5 mg/kg bw/day phloretin dose groups was caused by BPA.

Triglycerides are esters formed from glycerol and three fatty acids and were previously referred to as triacylglycerols. Triglycerides are essential in metabolism as an energy source and a carrier of dietary fat. Moreover, they are the primary component of low-density lipoprotein and are therefore clinically significant and routinely investigated in serum obtained from human and animal blood samples.[42–45] Studies on phloretin have shown that phloretin treatment individually does not affect serum triglyceride levels.[37] However, phloretin treatment can reduce the increases in serum triglyceride levels caused by chemicals.[24] Chhimwal et al. found that the increased triglyceride levels in rats fed on a western diet and high-fructose corn syrup were significantly reduced by the administration of phloretin at 100 and 200 mg/kg doses.[24] Furthermore, they evaluated the effects of phloretin by creating an *in vitro* model of nonalcoholic fatty liver disease (NAFLD) in Huh7 cells (human hepatoma cells). They found that 50, 100, and 150 μM phloretin doses reduced lipid accumulation by 12%, 31%, and 44% and intracellular triglyceride accumulation by 15%, 30%, and 56%, respectively.[24] However, our study showed no significant differences in serum triglyceride levels.

The most prevalent protein in mammalian plasma is albumin, which is typically considered a multipurpose transport protein.[46] It is believed that phloretin does not exert a toxic effect on serum albumin.[37] However, consistent with other hepatotoxicity and renal toxicity markers, increased serum albumin levels caused by chemical damage were found to decrease with phloretin treatment.[35] In the present study, we observed a significant difference in serum albumin levels in the 5 mg/kg bw/day phloretin treatment group compared with that in the corn oil-control group. We assumed that a statistically significant increase in the 5 mg/kg bw/day dose groups was caused by BPA. The liver is the target organ for endocrine-disrupting chemicals. The levels of hepatic enzymes may decrease or increase due to BPA, and pathological findings may appear in liver histology. Liver damage caused by BPA can also occur from the accumulation of BPA toxic metabolites and the production of reactive oxygen species in the liver.[20] Congestion, sinusoidal dilatation, edema, degeneration in hepatic parenchyma, mononuclear cell infiltration, ballooning in hepatocytes, and steatosis were detected in the positive control and phloretin treatment dose group. Several studies have indicated that treatment with phloretin decreases the histopathological findings of the liver.[19,24] Chhimwal et al. investigated the effect of phloretin on NAFLD in adult male C57BL/6J mice. They administered phloretin by oral gavage at doses of 50, 100, and 200 mg/kg for 16 weeks to rats that were fed on a western diet and high-fructose corn syrup.[24] Their study results suggested that phloretin effectively reduces the progression of NAFLD and inhibits hepatic inflammation and fibrosis by upregulating autophagy-mediated lipid degradation. Especially at high doses, phloretin has been found to decrease histological damage by reducing hepatic lipogenesis and facilitating fatty acid oxidation. However, our study revealed no evidence that phloretin improves BPA-induced liver injury at oral doses of 0.5, 5, and 50 mg/kg bw/day. A nephrotoxic impact may occur due to the accumulation of BPA and its hazardous metabolites and the kidney's inability to effectively remove these chemicals.[20] In the present study, BPA damage was mostly observed in the kidney. Bowman's capsule dilatation, tubular degeneration, degeneration in renal parenchyma, congestion, glomerular atrophy, cell expulsion into the lumen, and fibrous tissue formation were detected in the positive control and phloretin treatment groups. Several studies have shown that phloretin reduces histopathological findings.[36,39] For instance, treatment with 50 and 100 mg/kg of phloretin was found to significantly improve cisplatin-induced tubular injury, with no abnormalities due to high-dose phloretin administration being identified.[36] In another study, mice with hyperuricemia exhibited tubular atrophy, tubular dilatation, and tubulointerstitial damage with interstitial fibrosis, whereas phloretin administration improved renal morphological lesions and tubular necrosis in mice. Moreover, mice in the phloretin treatment group alone showed no renal histological lesions compared with mice in the control group.[39] Phloretin has also been suggested to exert protective effects against several types of chronic kidney disease.[47] Nevertheless, our study found no statistically significant difference in kidney histopathology. The effects of BPA and phloretin on the kidney in this study were supported by morphometric measurements. As we found in our previous study, BPA exposure led to a statistically significant decrease in all morphometric data.[3] The closest values to those in the corn oil-control group were determined in the 0.5 mg/kg bw/day phloretin dose group, and the data were statistically different from those in the BPA positive control group. In this context, a dose of 0.5 mg/kg bw/day phloretin may protect against BPA-induced kidney damage. However, we observed that with an increase in phloretin dose,

its protective effect against BPA-induced kidney damage decreased. Although some protection might be provided in the 5 mg/kg bw/day phloretin dose group, the results obtained in the 50 mg/kg bw/day dose group were highly similar to those in the BPA positive control group. Overall, no clear evidence was obtained concerning the protective effect of phloretin against BPA-induced kidney damage at oral doses of 5 mg/kg bw/day and especially 50 mg/kg bw/day.

Finally, based on our study results, treatment with 0.5 and 5 mg/kg bw/day phloretin administered as low doses may provide low protection against BPA-induced kidney and liver damage. However, the protective effect of 50 mg/kg bw/day phloretin used as a high-dose was low in this study. Geohagen et al. mentioned that a protective effect does not occur with an increase in phloretin dose; in contrast, it may exert adverse effects.[18] In this study, we did not administer phloretin without BPA. Therefore, we could not obtain information on the negative impact of increased phloretin doses. BPA may have caused the biochemical and histopathological adverse effects in this study. Nevertheless, our results support that increasing the phloretin dose does not increase the protective effect. In the study of Geohagen et al., phloretin was administered via intraperitoneal injection, and the results demonstrated that high-dose (2.40 mmol/kg) phloretin administration did not prevent lethality caused by acetaminophen, whereas low doses (0.2–0.4 mmol/kg) provided moderate hepatoprotection.[18] In another study, the authors concluded that long-term use of high-dose phloretin may cause liver damage.[48] However, there are also studies showing that there are positive effects that increase in parallel with the dose.[49] For instance, Zhao et al. reported protective effects exerted by 25 and 50 mg phloretin doses against cisplatin-induced kidney damage. They observed that phloretin doses increased the levels of serum creatinine, urea, and albumin in a dose-dependent manner, as well as oxidative stress markers in the control group.[35] In a previous study investigating the toxic effect of phloretin, 25 and 50 mg/kg/day doses of phloretin were administered to female and male mice for 28 days. The results of that study revealed no significant difference in the levels of ALT, AST, albumin, triglyceride, creatinine, and glucose compared with those in the control group. The authors of that study concluded that phloretin is safe to use.[37] We did not obtain evidence of a clear protective impact of phloretin in this investigation. Oral administration of phloretin is known to result in low absorption and bioavailability, as phloretin has exceptionally low water solubility.[17,49–51] We administered phloretin subcutaneously for 15 days in the present study; hence, a longer treatment period is recommended to elucidate the protective properties of phloretin. Regarding the data of the phloretin dose groups, the results are quite complex. However, our results revealed that the data of the 0.5 mg/kg bw/day phloretin group were closer to those of the corn oil-control group. To summarize, we could not detect an apparent protective effect of phloretin after 15 days of subcutaneous administration of 0.5, 5, and 50 mg/kg bw/day doses in female rats against BPA-induced liver and kidney damage.

CONCLUSION

Considering the studies conducted on phloretin, we believe that it would be valuable to investigate its positive effects. However, the results obtained to date are complicated. According to the results of this study and the literature, we can conclude that phloretin is not yet suitable for the development of pharmaceuticals or use as a nutritional supplement. It is necessary to gain a complete understanding of the beneficial or harmful effects of phloretin to take advantage of its benefits. Additional *in vivo* studies are required to confirm the safety of phloretin and clarify its molecular mechanisms.

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