

Potential hepatic and renal toxicity induced by the biflavonoids from *Ginkgo biloba*

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[ABSTRACT] Evidence continues to grow on potential health risks associated with *Ginkgo biloba* and its constituents. While biflavonoid is a subclass of the flavonoid family in *Ginkgo biloba* with a plenty of pharmacological properties, the potential toxicological effects of biflavonoids remains largely unknown. Thus, the aim of this study was to investigate the *in vitro* and *in vivo* toxicological effects of the biflavonoids from *Ginkgo biloba* (i.e., amentoflavone, sciadopitysin, ginkgetin, isoginkgetin, and bilobetin). In the *in vitro* cytotoxicity test, the five biflavonoids all reduced cell viability in a dose-dependent manner in human renal tubular epithelial cells (HK-2) and human normal hepatocytes (L-02), indicating they might have potential liver and kidney toxicity. In the *in vivo* experiments, after intragastrical administration of these biflavonoids at 20 mg·kg⁻¹·d⁻¹ for 7 days, serum biochemical analysis and histopathological examinations were performed. The activity of alkaline phosphatase was significantly increased after all the biflavonoid administrations and widespread hydropic degeneration of hepatocytes was observed in ginkgetin or bilobetin-treated mice. Moreover, the five biflavonoids all induced acute kidney injury in treated mice and the main pathological lesions were confirmed to the tubule, glomeruli, and interstitium injuries. As the *in vitro* and *in vivo* results suggested that these biflavonoids may be more toxic to the kidney than the liver, we further detected the mechanism of biflavonoids-induced nephrotoxicity. The increased TUNEL-positive cells were detected in kidney tissues of biflavonoids-treated mice, accompanied by elevated expression of proapoptotic protein BAX and unchanged levels of antiapoptotic protein BCL-2, indicating apoptosis was involved in biflavonoids-induced nephrotoxicity. Taken together, our results suggested that the five biflavonoids from *Ginkgo biloba* may have potential hepatic and renal toxicity and more attentions should be paid to ensure *Ginkgo biloba* preparations safety.

[KEY WORDS] Biflavonoids; *Ginkgo biloba*; Potential toxicity; Liver; Kidney; Apoptosis

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Introduction

Ginkgo biloba is one of the most extensively used botanical dietary supplements and phytopharmaceutical drugs worldwide. In the past few decades, it has proven to be beneficial

for the treatment of many chronic and acute diseases, such as Alzheimer's disease, ischemic heart disease, atherosclerosis, thrombosis, cancer, and diabetes [1-6]. Nowadays, ginkgo leaf extract preparations are available in the Asia, European and American markets in the forms of tablets, dropping pills, soft capsule, oral solutions, injectable solutions, and others. In 2012, total global sales of ginkgo products were \$1.26 billion, of which \$578 million were sold in China, \$152 million in Germany, and \$40 million to \$61 million in Australia, France, Brazil and South Korea [7]. The standardized extract preparation of *Ginkgo biloba* leaf commonly contains 24% flavonoids, 6% terpene trilactones (TTLs), and less than 5 ppm of ginkgolic acid [8,9], among which flavonoids and TTLs are the main active components in ginkgo leaf extract [10, 11].

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Biflavonoids are one kind of flavonoids in *Ginkgo biloba* and ginkgo leaf extract with the structure of flavonoid-flavonoid dimer. There are several biflavonoids existed in *Ginkgo biloba*, including amentoflavone, bilobetin, ginkgetin, isoginkgetin, sciadopitysin, tetrahydroamentoflavone, sequoiaflavone, and isocryptomerin [12]. Previous studies have been demonstrated that these biflavonoids possess extensively pharmacological properties, such as anti-inflammatory, antiviral, anti-tumoral, and anti-diabetic effects [13-17]. It was found that ginkgetin has dual inhibitory properties both on cyclooxygenase-2 and 5-lipoxygenase, which can enhance anti-inflammatory effects [18]. Amentoflavone could inhibit the production of prostaglandin E2 by down-regulating the expression of cyclooxygenase-2, which has certain anti-inflammatory activity [18]. Moreover, amentoflavone has a strong inhibitory effect on the respiratory syncytial virus (RSV) with IC_{50} of $5.5 \mu\text{g}\cdot\text{mL}^{-1}$ and also has a significant inhibitory effect on herpes simplex virus (HSV)-1 with IC_{50} of $16.5 \mu\text{g}\cdot\text{mL}^{-1}$ [19]. In addition, a patent proved that sciadopitysin has a good therapeutic effect on diabetes with no obvious adverse reactions and sciadopitysin is expected to be a new generation drug for prevention and treatment of diabetes and its complications [20].

In recent years, although herbal medicines exhibit a lot of effects, their potential toxicity has been frequently reported [21-25]. Similarly, despite the good pharmacological activity and potential therapeutic value of biflavonoids, the potential toxicity induced by biflavonoids has been sporadically declared [26-29]. A clinical report said that two cases of acute renal failure were observed after ingestion of *Taxus Celebica* for treatment of diabetes mellitus, and by compared with six cases of flavonoid-induced acute renal failure in the literature, the authors suggested that sciadopitysin may be the cause of acute nephropathy as *Taxus Celebica* contains sciadopitysin [27]. In another study, it was found that amentoflavone exhibited positive mutagenicity in *Salmonella typhimurium* assay [29]. The inhibitory effects of amentoflavone on human cytochrome P450 3A4 and 2C9 activities were also reported [28]. Additionally, recent studies demonstrated that several natural biflavonoids such as amentoflavone and sciadopitysin could inhibit the activity of human UDP-glucuronosyltransferase (UGT) which are the most important class of detoxification enzymes and highly expressed in metabolic organs including liver, intestine, and kidney [30-32]. Taken together, although evidences from the *in vitro* models and clinical case have suggested the health risks induced by individual biflavonoids, the effects of biflavonoids on health are still uncertain and require further investigation.

Hence, this study was aimed to investigate the potential toxicity of biflavonoids contained in *Ginkgo biloba* (namely amentoflavone, bilobetin, ginkgetin, isoginkgetin, and sciadopitysin) with *in vitro* and *in vivo* experiments. Based on previous studies, liver and kidney may be the target organs of these biflavonoids [27-28, 30-32], thus the effects of these compounds on human proximal tubular cell line (HK-2) and

human normal liver cell line (L-02) representing the two important organ systems were first tested *in vitro*. Then, an acute toxicity of orally administered these bioflavonoids was further investigated in BALB/c mice, along with the serum biochemical analysis and histopathological examinations of the main organs. Furthermore, the possible toxicity mechanisms were detected by immunohistochemistry analysis and TUNEL assay of the target organs.

Materials and Methods

Chemicals and reagents

Dulbecco's modified Eagle medium/Nutrient Mixture F-12 medium (DMEM/F-12), phosphate-buffered saline (PBS), Roswell Park Memorial Institute (RPMI) 1640 medium, 0.25% trypsin-EDTA, fetal bovine serum (FBS), and penicillin-streptomycin solution $100 \times (10\ 000 \text{ U}\cdot\text{mL}^{-1}$ penicillin and $10\ 000 \mu\text{g}\cdot\text{mL}^{-1}$ streptomycin) were purchased from Gibco (Grand Island, NY, USA). 3-(4, 5-dimethyl-2-yl)-2, 5-diphenyltetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), and trypan blue were purchased from Sigma Co. (St. Louis, MO, USA). Amentoflavone, sciadopitysin, ginkgetin, isoginkgetin, and bilobetin were obtained from Shanghai Yuan Ye Biotechnology Co., Ltd. (Shanghai, China). Sodium chloride, potassium chloride, potassium dihydrogen phosphate, sodium carboxymethyl cellulose, methanal, disodium hydrogen phosphate, and phenobarbital sodium were purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). BCL-2 antibody, BAX antibody, HRP-labeled Goat Anti-mouse IgG, and HRP-labeled Goat Anti-Rabbit IgG were purchased from Servicebio Biotechnology Co., Ltd. (Wuhan, China). All the other reagents were obtained from commercial sources and were of analytical grade.

Cell culture

The human proximal tubular cell line HK-2 cells were obtained from the Cell Bank of Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences (Shanghai, China), and cultured in DMEM/F12 supplemented with 10% heat-inactivated FBS, $100 \text{ U}\cdot\text{mL}^{-1}$ penicillin, and $100 \mu\text{g}\cdot\text{mL}^{-1}$ streptomycin in a humidified atmosphere incubator at $37 \text{ }^\circ\text{C}$ with 5% carbon dioxide (CO_2). Human normal liver cell line L-02 cells were obtained from Shanghai North Nuo Biotechnology Co., Ltd. (Shanghai, China) and cultured in RPMI-1640 supplemented with 10% heat-inactivated FBS, $100 \text{ U}\cdot\text{mL}^{-1}$ penicillin, and $100 \mu\text{g}\cdot\text{mL}^{-1}$ streptomycin in a humidified atmosphere of 5% CO_2 at $37 \text{ }^\circ\text{C}$. The cells were detached from the flasks by treatment with 0.25% trypsin-EDTA when the cells reached 90% confluency. The cell passage ratio of these two cell lines was both set at 1 : 4.

Cell viability assay

In order to assess the cytotoxicity of the biflavonoids from *Ginkgo biloba*, cell viability was determined by MTT assay. HK-2 or L-02 cells were plated in 96-well plates at a density of 1×10^4 cells per well. After 24 h incubation at $37 \text{ }^\circ\text{C}$ in an atmosphere of 5% CO_2 , cells were treated with

different concentrations of biflavonoids for 48 h. Then, the culture medium was removed and replaced by 100 μL fresh culture medium containing 0.5 $\text{mg}\cdot\text{mL}^{-1}$ MTT. After incubation at 37 °C for an additional 4 h, the MTT solution was removed and the formed formazan crystals were solubilized with 100 μL DMSO. The optical density was measured at 580 nm using Infinite M1000 Pro (TECAN, Germany). The cell viability of biflavonoid-treated group was quantified as a percentage compared to vehicle control.

Animal experiments and sample collection

BALB/c mice (female, body weight 18–20 g) were supplied by Shanghai SLAC laboratory Animal Co., Ltd. (Shanghai, China). Animals were housed at 25 ± 1 °C with a relative humidity of $50\% \pm 10\%$ under a 12 h light-dark cycle. Food and water were available *ad libitum*. After 3 days of an acclimation period, mice were randomly divided into 6 groups according to the body weight ($n = 7$), including vehicle control group (0.5% sodium carboxymethyl cellulose), amentoflavone group (20 $\text{mg}\cdot\text{kg}^{-1}$), sciadopitysin group (20 $\text{mg}\cdot\text{kg}^{-1}$), ginkgetin group (20 $\text{mg}\cdot\text{kg}^{-1}$), isoginkgetin group (20 $\text{mg}\cdot\text{kg}^{-1}$), and bilobetin group (20 $\text{mg}\cdot\text{kg}^{-1}$). The exposure dose and time of these biflavonoids were determined by the previously published studies in pharmacological activities [33–36] as well as our preliminary experiment. As intragastrical administration of these biflavonoids at 20 $\text{mg}\cdot\text{kg}^{-1}\cdot\text{d}^{-1}$ for 7 days induced obvious changes in serum biochemistry and histopathology in the preliminary experiment, thus the same exposure dose and time were used in this study. Each biflavonoid-treated group was intragastrically administered at the volume of 0.1 mL/10 g body weight per day for 7 days as well as the vehicle control. On the eighth day, mice were sacrificed under anesthesia by intraperitoneal injection of 1.5% pentobarbital sodium. Blood samples were collected from inferior vena cava and put into anticoagulant tubes. Serum was separated by centrifugation (5810R, Eppendorf, Germany) at 3000 $\text{r}\cdot\text{min}^{-1}$ for 10 min at 4 °C, and then stored at –80 °C until biochemical analysis. The major organs (heart, liver, spleen, lung, and kidney) were removed and fixed with 10% neutral-buffered formalin for histopathological examinations.

Biochemical analysis

Blood urea nitrogen (Bun), serum creatinine (CREA), alanine transaminase (ALT), aspartate transaminase (AST), alkaline phosphate (ALP), total bilirubin (TBIL), total cholesterol (TCHOL), and triglycerides (TG) of serum samples were analyzed using a Roche COBAS C8000 Automatic Analyzer with the appropriate kits.

Histopathological examinations

The tissues fixed in 10% neutral-buffered formalin were dehydrated by decreasing concentrations of ethanol, cleared in xylene, and embedded in paraffin. Then, the tissue was section with a microtome (Leica RM2016) into sequential slices of 4 μm and subjected to hematoxylin and eosin (HE) staining. HE-stained slices were employed to assess the de-

grees of organ injury using a light microscope (IX53, Olympus, Japan).

TUNEL assay

A Roche In Situ Apoptosis Detection Kit was used to detect cell apoptosis in kidney according to the protocol of manufacturer. Briefly, sections were incubated with proteinase K for 30 min at 37 °C. After washing 3 times with PBS, slides were incubated with TUNEL mixture for 2 h at 37 °C. Followed by staining with 4', 6-diamidino-2-phenylindole for 10 min, the FITC labeled TUNEL-positive cells was obtained using a fluorescence microscope (IX53, Olympus).

Immunohistochemistry analysis

Immunohistochemistry was used to assess the expression levels of apoptosis-related proteins, i.e., BCL-2 and BAX. Paraffin-embedded sections were cut at 5 μm and deparaffinized and rehydrated with a sequence of xylene and aqueous alcohol solutions. Then, the sections were incubated in 3% hydrogen peroxide solution for 20 min. After washing 3 time with PBS, sections were incubated overnight with primary antibodies (BCL-2 dilution ratio: 1 : 2000; BAX dilution ratio: 1 : 300) at 4 °C. Then, sections were incubated with HRP conjugated secondary antibody (1 : 200) for 50 min at room temperature after washing 3 times with PBS. Substrate was placed in sections for 50 min following 3, 3'-diaminobenzidine (DAB) staining and hematoxylin counterstaining. Finally, sections were dehydrated in ethanol and a mounting medium was used for cover-slipping. Staining intensity was analyzed under a light microscope (IX53, Olympus).

Statistical analysis

All the experimental data were presented as mean \pm standard deviation (mean \pm SD). Statistical significance was assessed by Microsoft Excel or Graph Pad Prism 6 software. Differences between each biflavonoid-treated group and vehicle control group in mouse body weight were determined by *T* test. Serum biochemical data were analyzed by one-way ANOVA, followed by Dunnett's multiple comparison test. A $P < 0.05$ was considered to be significant.

Results and Discussion

Biflavonoids induce cytotoxicity on HK-2 cells

HK-2 cell line is a widely-used human renal proximal tubular cell line derived from the healthy kidney of an adult male, which has similar biochemical properties and activities as human proximal tubule cells *in vivo* [37]. A plenty of previous studies have demonstrated that HK-2 is a suitable *in vitro* model for assessing drug-induced nephrotoxicity in humans [38–40]. In this study, in order to investigate the cytotoxicity of biflavonoids from *Ginkgo biloba* on renal cells, HK-2 cells were subjected to different concentrations of ginkgetin, isoginkgetin, amentoflavone, sciadopitysin, or bilobetin (i.e., 0.01, 0.1, 1, 10, and 100 $\mu\text{g}\cdot\text{mL}^{-1}$) for 48 h. Cell viability was measured by MTT assay and the results were shown in Fig. 1.

After incubation with different concentrations of biflavonoids, amentoflavone and sciadopitysin can significantly

reduce the cell viability of HK-2 at the concentrations of 1, 10, and 100 $\mu\text{g}\cdot\text{mL}^{-1}$ (Figs. 1A and 1B). Similarly, ginkgetin and isoginkgetin markedly inhibited the proliferation of HK-2 cells at same concentrations, and the effects of these two biflavonoids were comparable at the concentrations of 10 and

100 $\mu\text{g}\cdot\text{mL}^{-1}$ (Figs. 1C and 1D). Moreover, bilobetin can remarkably reduce the survival rate of HK-2 cells at 10 and 100 $\mu\text{g}\cdot\text{mL}^{-1}$ (Fig. 1E). Taken together, these results suggested that these five biflavonoids could induce cytotoxicity on HK-2 cells in a dose-dependent manner.

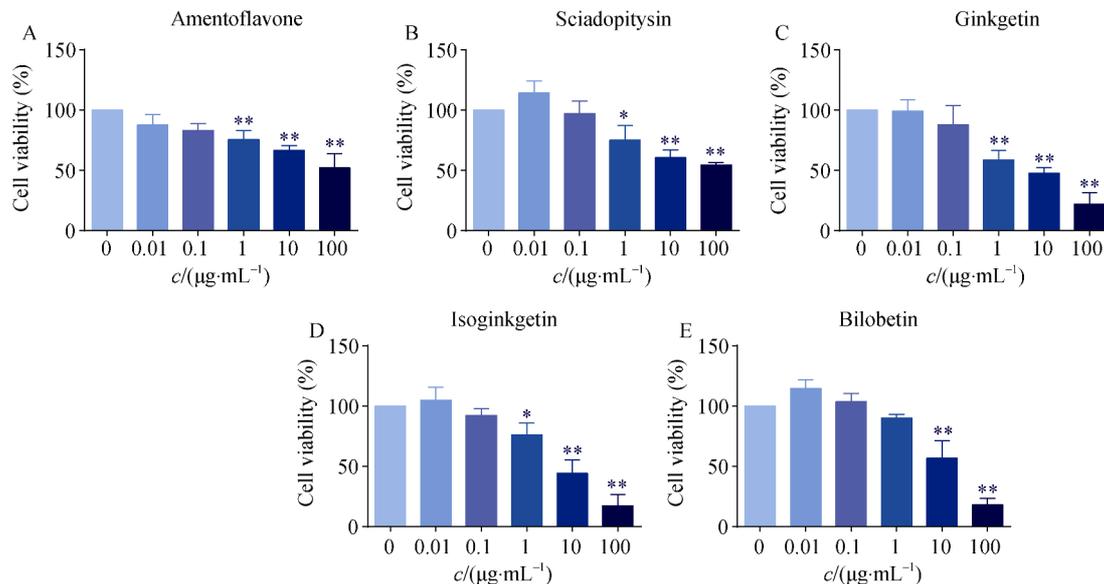


Fig. 1 The cytotoxicity of the five biflavonoids on HK-2 cells. (A) amentoflavone; (B) sciadopitysin; (C) ginkgetin; (D) isoginkgetin; (E) bilobetin. Data were obtained by MTT assay from three repeated experiments and expressed as mean \pm SD. * $P < 0.05$, ** $P < 0.01$ vs the control group

Biflavonoids induce cytotoxicity on L-02 cells

The human normal liver cell line L-02, expressing the main molecules of human hepatocytes, is a good *in vitro* model for investigating liver diseases as the cells have great proliferative capacity and maintain excellent hepatocyte func-

tions^[41-43]. In this study, we further investigated the cytotoxicity of the five biflavonoids on liver cells using L-02 cells. The concentration gradient setting of each biflavonoid was the same as HK-2 cell experiment. The cell viability was shown in Fig. 2.

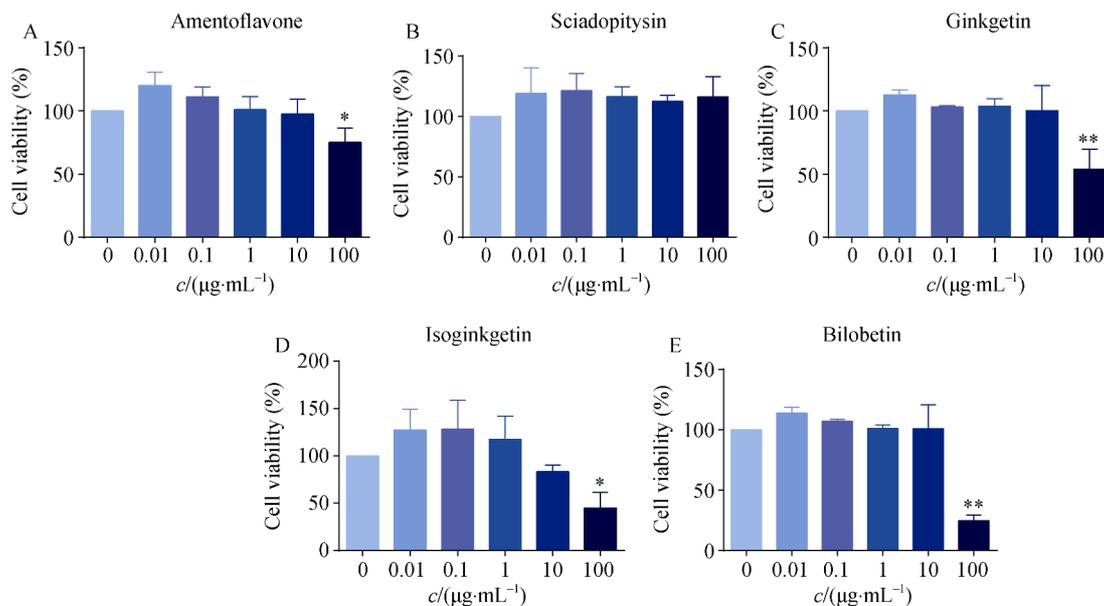


Fig. 2 The cytotoxicity of the five biflavonoids on L-02 cells. (A) amentoflavone; (B) sciadopitysin; (C) ginkgetin; (D) isoginkgetin; (E) bilobetin. Data were obtained by MTT assay from three repeated experiments and expressed as mean \pm SD. * $P < 0.05$, ** $P < 0.01$ vs the control group

Interestingly, only amentoflavone, ginkgetin, isoginkgetin, and bilobetin significantly inhibit the proliferation of L-02 cells at the higher concentration ($100 \mu\text{g}\cdot\text{mL}^{-1}$), whereas $0.01\text{--}100 \mu\text{g}\cdot\text{mL}^{-1}$ sciadopitysin treatments did not show any cytotoxicity compared with control group in this cell line, suggesting these biflavonoids may be more toxic to kidney than liver.

Biflavonoids decrease the body weight of mice

Based on the results of *in vitro* experiments, we further detected the potential health risk of these biflavonoids *in vivo*. As females are generally more sensitive to drug toxicity than males [44-45], female mice were selected to investigate the potential toxicity of these biflavonoids in this study. Ginkgetin, isoginkgetin, amentoflavone, sciadopitysin, or bilobetin was intragastrically administered to mice at a dose of $20 \text{mg}\cdot\text{kg}^{-1}$ per day for 7 days, then continuous observation and

recording were performed. The body weight of the mice was changed during the administration period, and the results were shown in Fig. 3.

Compared with the vehicle control group, the body weight of all the biflavonoids-treated mice decreased during the 7 consecutive days of administration, and the weight of mice in the group treated with amentoflavone or sciadopitysin decreased significantly from the third day. Moreover, the mouse body weight of ginkgetin and bilobetin groups showed a significant decrease from the fourth and fifth day of administration, respectively. Isoginkgetin-treated mice only obviously decreased body weight on the seventh day. At the same time, loss of appetite, reduction in activities, and bad hair condition were detected in the drug-administered mice. These results indicated that the biflavonoids from *Ginkgo biloba* indeed caused some health problems in mice.

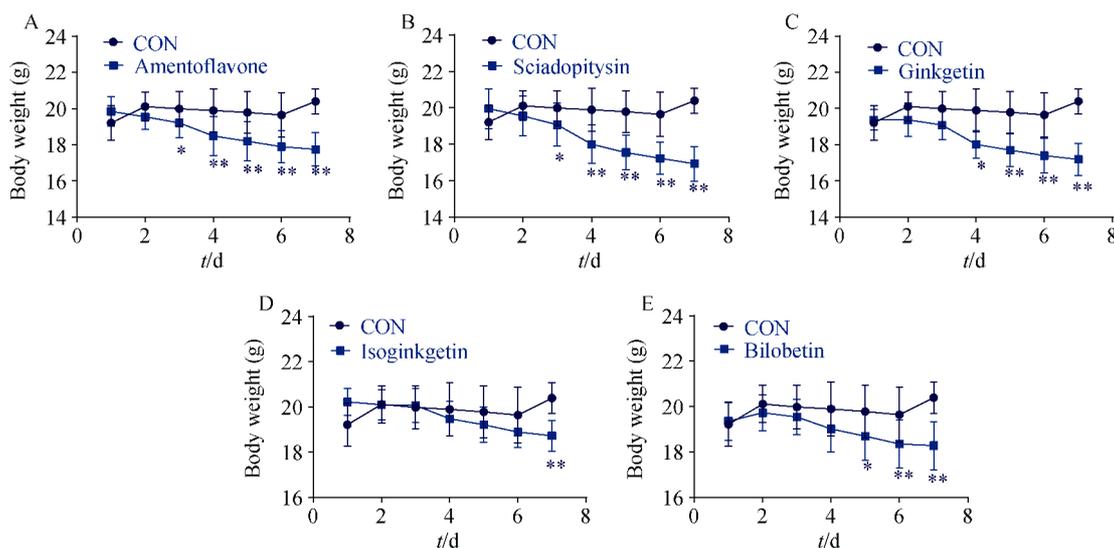


Fig. 3 The effect of biflavonoids from *Ginkgo biloba* on the body weight of mice. (A) amentoflavone; (B) sciadopitysin; (C) ginkgetin; (D) isoginkgetin; (E) bilobetin. CON means the vehicle control group. Data were expressed as mean \pm SD. $n = 7$. * $P < 0.05$, ** $P < 0.01$ vs the vehicle control group

Biflavonoids cause alterations in the levels of serum biochemical parameters in mice

As the *in vitro* results suggested that the five biflavonoids can cause cytotoxicity in kidney and liver cells, thus the serum biochemical parameters which are closely related to kidney and liver functions were analyzed in the *in vivo* experiment.

ALT, AST, and ALP activities with TBIL level are the most widely adopted biomarkers for drug-induced liver injury [46]. Among them, ALT is a gold standard for the detection of liver cell damage, whereas AST can also reflect the degree of liver injury [46, 47]. The elevation of serum ALP activity is a sensitive marker for cholestatic disease and chronic hepatitis/cirrhosis [48, 49] and increase in serum TBIL level indicates hepatobiliary diseases [50, 51]. These four serum parameters are complementarily used to identify severe liver injury [46]. In our study, it is worth noting that the serum ALP activity was markedly increased after all biflavonoid treatments compared

with the vehicle control group. In contrast, no significant increase was observed in ALT and AST activities as well as TBIL level. Meanwhile, the liver lipid metabolism related two parameters, TG and TCHOL, were also detected. An enhanced level of TG was only observed in amentoflavone- and bilobetin-treated mice along with no significant alterations identified in TCHOL. These findings suggested that these biflavonoids may have weak hepatotoxicity, which were in agreement with those from our *in vitro* experiments where these biflavonoids only significantly inhibited liver cell viability at the higher concentration or did not show any toxicity within the test dose range.

As serum CREA and Bun are the most useful and easily measurable biomarkers for diagnose of acute kidney injury in the clinic [52], the levels of these two parameters were further observed in this study. As the results, the values of serum CREA and Bun were significantly increased in the ginkgetin-treated

mice, while serum Bun level was also increased after sciadopitysin administration. It has been demonstrated that serum CREA and Bun levels increase only after a substantial decline of kidney functions [52-53]. Thus, our data indicated that ginkgetin and sciadopitysin may cause acute kidney injury.

Biflavonoids induce histopathological change in mouse liver and kidney

In order to confirm the liver and kidney injuries induced

by the biflavonoids from *Ginkgo biloba*, histopathological examinations with HE staining were performed to the main organs of the biflavonoids-treated mice, including heart, liver, spleen, lung, and kidney. Among them, there were no obvious lesions in the heart, spleen, and lung (data not shown), but histopathological changes were detected in the liver and kidney tissues (Figs. 5 and 6), which was consistent with the data from *in vitro* experiments and serum biochemistry.

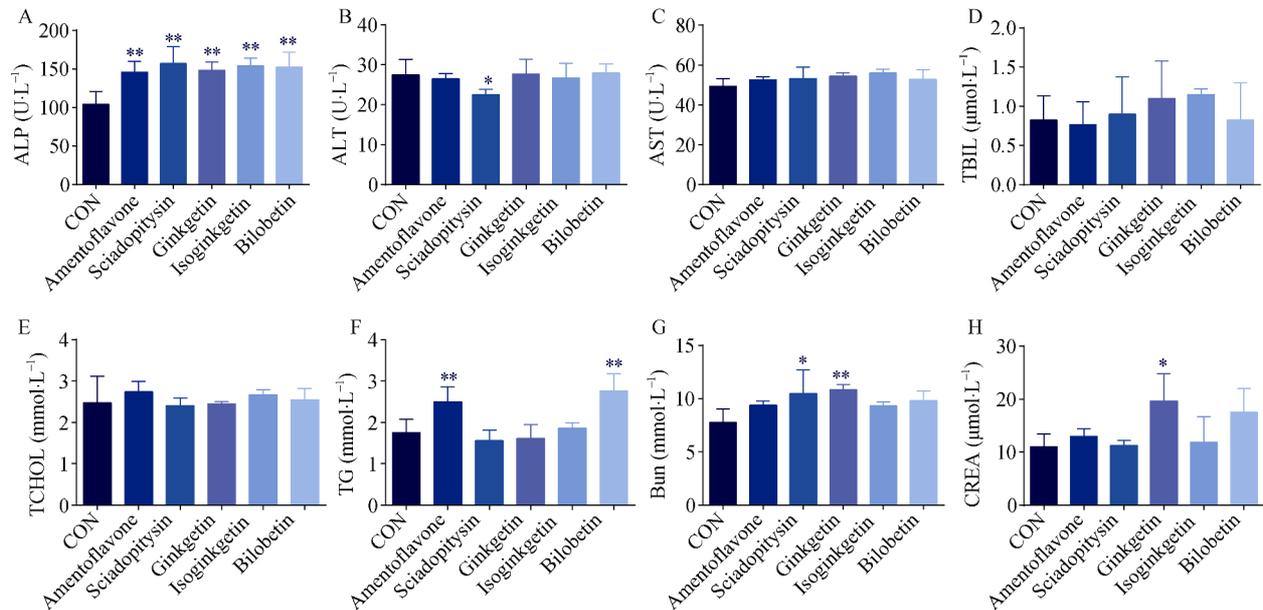


Fig. 4 Serum biochemistry analysis of the biflavonoids-treated mice. (A) ALP; (B) ALT; (C) AST; (D) TBIL; (E) TCHOL; (F) TG; (G) Bun; (H) CREA. Data were expressed as mean ± SD, **P* < 0.05 vs vehicle control group

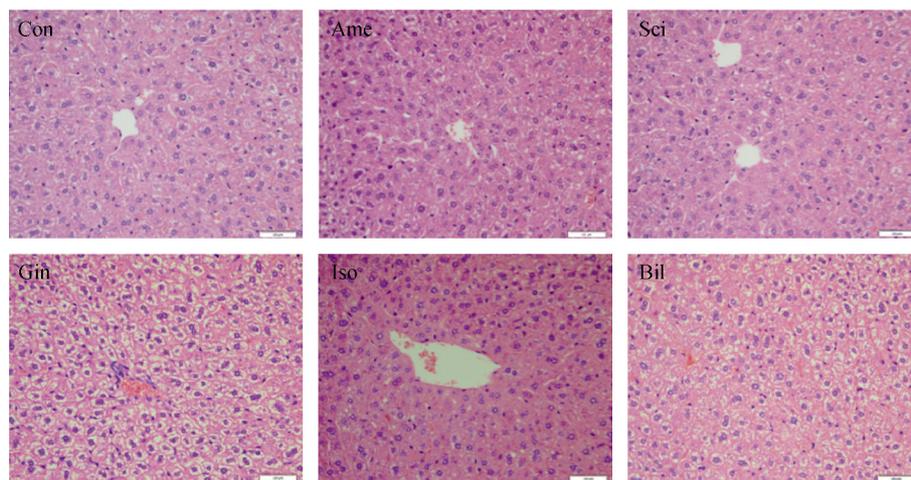


Fig. 5 HE staining of the liver tissues after biflavonoid treatments. Con: vehicle control group; Ame: amentoflavone; Sci: sciadopitysin; Gin: ginkgetin; Iso: isoginkgetin; Bil: bilobetin. Original magnification × 400

A comparison of liver sections obtained from the control and biflavonoids-treated mice indicated widespread hydropic degeneration of hepatocytes was detected in the ginkgetin and bilobetin groups, and no overt sign of toxicity was found in amentoflavone, isoginkgetin, and sciadopitysin groups.

Ginkgetin exposure also caused inflammatory cell infiltration in mouse liver. These data suggested that bilobetin and ginkgetin were more toxic to hepatocytes among the five biflavonoids, which were basically consistent with the *in vitro* results.

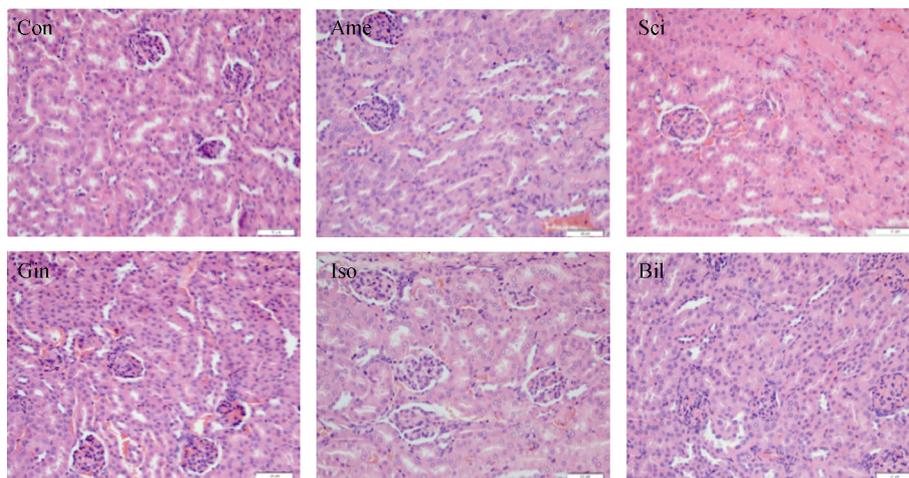


Fig. 6 HE staining of the kidney tissues after biflavonoid treatments. Con: vehicle control group; Ame: amentoflavone; Sci: sciadopitysin; Gin: ginkgetin; Iso: isoginkgetin; Bil: bilobetin. Original magnification $\times 400$

The HE staining of kidney tissues showed that control mice presented a normal kidney histoarchitecture with normal tubules and glomeruli. The main pathological lesions of biflavonoids-treated groups were confirmed to the tubule, glomeruli, and interstitium injuries. For instance, the mouse renal tubules of amentoflavone group had a small amount of protein casts, and the glomerular mesangial was slightly thickened, accompanied by renal interstitial hemorrhage. Bilobetin induced localized renal interstitial hemorrhage, glomerular mesangium thickening and glomerular telangiectasia. In sciadopitysin group, local renal interstitial small-scale hemorrhage occurred and glomerular capillaries were slightly hyperemia. Renal interstitial hemorrhage and glomerular capillary congestion were observed in ginkgetin group, whereas the renal tubules of isoginkgetin group were slightly edematous. Taken together, it was worth noting that these biflavonoids all can induce kidney injury and renal interstitial hemorrhage was the common pathological phenomenon, except the isoginkgetin treatment. Consistently, previous study showed that after ingestion of *Taxus Celebica* which contains sciadopitysin caused acute interstitial and tubular injuries with cola-colored urine in human and the possible mechanism may be related with uptake and accumulation the drug into tubular cells [27].

Biflavonoids induce apoptosis in mouse kidney

Apoptosis is a form of programmed cell death and plays a critical role in drug-induced nephrotoxicity [54-55]. Thus, as these biflavonoids were more toxic to kidney than liver, we further detected the possible mechanism of bioflavonoids-induced nephrotoxicity by identifying the apoptosis in kidney tissue with TUNEL assay. As shown in Fig. 7, there were few TUNEL-positive cells in kidney tissue of vehicle control mice. By contrast, the TUNEL-positive cells increased obviously in biflavonoids-treated mice, especially in ginkgetin and sciadopitysin groups, indicating the biflavonoids induced apoptosis in mouse kidney.

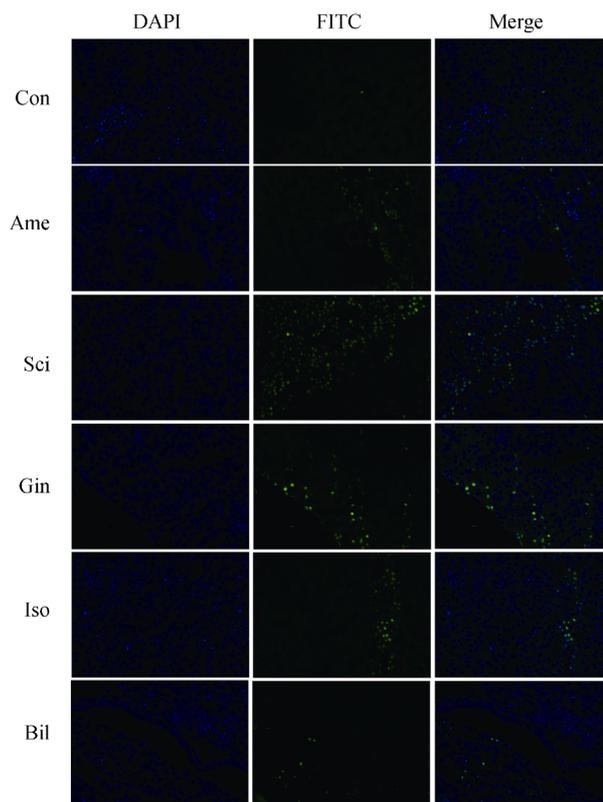


Fig. 7 Apoptosis was observed by TUNEL assay in the kidney tissues of biflavonoids-treated mice. The images of TUNEL-positive cells were captured by a fluorescence microscope (400 \times). Con: vehicle control group; Ame: amentoflavone; Sci: sciadopitysin; Gin: ginkgetin; Iso: isoginkgetin; Bil: bilobetin

In vertebrates, the interaction of Bcl-2 family proteins initiate the apoptosis and determine the cell fate. Among this family, BAX acts as a proapoptotic protein and BCL-2 is an antiapoptotic protein. BAX can convert into homooligomers that permeabilize the mitochondrial outer membrane, trigger-

ing the apoptosis, whereas BCL-2 is able to form a heterodimer with BAX and prevent the apoptosis, indicating BCL-2 and BAX are important factors in cell apoptosis^[56,57]. Thus, we further detected the expression levels of BAX and BCL-2 proteins in the mouse kidney using immunohistochemical analysis in this study. As shown in Fig. 8A, the expression of proapoptotic protein BAX was negligible in the kidney tissues of vehicle control group, whereas biflavonoid-treated groups revealed dark brown granules in their cytoplasm throughout a plenty of the cells in kidney. Moreover, the antiapoptotic protein BCL-2 was negatively expressed in the kidneys of biflavonoid-treated and vehicle control mice (Fig. 8B). These findings indicated that biflavonoids may cause nephrotoxicity by increasing apoptosis.

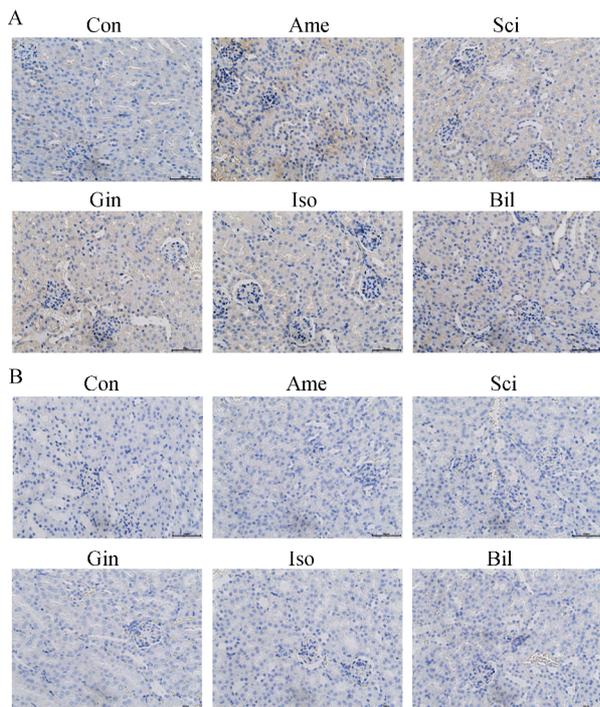


Fig. 8 Immunohistochemical analysis of BAX and BCL-2 in the kidney tissues of biflavonoids-treated mice. (A) BAX; (B) BCL-2. Original magnification $\times 400$. Con: vehicle control group; Ame: amentoflavone; Sci: sciadopitysin; Gin: ginkgetin; Iso: isoginkgetin; Bil: bilobetin

Conclusion

This is the first *in vitro* and *in vivo* study describing the potential toxicological effects of the biflavonoids contained in *Ginkgo biloba*. In light of the *in vitro* results, the biflavonoids, i.e., ginkgetin, isoginkgetin, amentoflavone, sciadopitysin, and bilobetin, all induced cytotoxicity in human proximal tubular cells and caused weaker toxicity in human normal liver cells. Consistently, oral administration of these biflavonoids to the mice for 7 days at $20 \text{ mg}\cdot\text{kg}^{-1}$ revealed acute kidney injury and mild hepatotoxicity. Furthermore, the activation of apoptosis was involved in biflavonoids-induced

nephrotoxicity. The data obtained from present investigation suggests that these biflavonoids may have potential hepatic and renal toxicity. As *Ginkgo biloba* extract occupy a large share in the market, the safety of long-term use of *Ginkgo biloba* preparations which contains these biflavonoids deserves further study. In addition, further clarifying the toxicological mechanism of these biflavonoids is needed, which will aid in rational, safe, and effective use of *Ginkgo biloba* preparations.

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