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Original Article

Bilobalide ameliorates carbon tetrachloride-induced oxidative damage in HepG2 cells by the induction of Nrf2-dependent HO-1 expression through PI3K/Akt and P38 pathways

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Abstract: Oxidative stress is implicated in the pathogenesis of hepatic damage induced by various factors. Bilobalide (BB) is a compound extracted from Ginkgo biloba leaf. It has been reported for its anti-oxidative activity and has been tested to treat brain damage. However, it has not been studied whether BB possesses hepatoprotective activity though regulating heme oxygenase-1 (HO-1) which is a key antioxidant defense against reactive oxygen species. In this study, HepG2 cells were stimulated with carbon tetrachloride (CCl4) to induce cellular oxidative damage, and were treated with different concentrations of BB. The viability of the cells and the leakage of ALT and AST were detected to evaluate cell damages. Levels of H_2O_2, MDA and 8-OHdG in HepG2 cell lysates were measured to assess the cellular oxidative stress. Besides, the expression of nuclear factor erythroid 2-related factor 2 (Nrf2) and HO-1 (mRNA and protein) were detected. The results showed that BB attenuated the cellular damage, inhibited the oxidative stress, and increased the expression of Nrf2 and HO-1 in the CCl4-stimulated HepG2 cells. Yet, the cytoprotective effects of BB were abolished by a HO-1 inhibitor zinc protoporphyrin (ZnPP). In addition, Nrf2 knockdown with siNrf2 blocked the induction of HO-1 expression by BB. Furthermore, LY294002 (a PI3K/Akt inhibitor) and SB203580 (a p38 inhibitor) significantly reversed the up-regulation of Nrf2/HO-1 expression and the cytoprotection by BB. Our findings indicated that the hepatoprotective effects of BB were associated with the Nrf2 mediated HO-1 expression. And PI3K/Akt and p38 pathways were involved in the hepatoprotective effects and the regulation of Nrf2/HO-1 signaling by BB. BB may be a useful agent for the treatment of oxidative stress-induced hepatic damage.

Keywords: Bilobalide, hepatoprotective effect, HO-1, Nrf2, PI3K/Akt, P38, in vitro

Introduction

The liver is one of the most important organs that play crucial roles in maintaining homeostasis. The clearance and transformation of many toxicants are performed in the liver, which makes the liver susceptible to toxicant-induced damage. In addition, a number of other factors also induce liver injury in the body. Every year, many people die of liver diseases. Yet, few drugs that offer sufficient protection to the liver against various damages are available in clinic [1]. Therefore, novel agents are in need to supplement or replace the currently used hepatoprotective drugs.

Among the complex hepatotoxic mechanisms, oxidative stress is one of the common mechanisms shared by toxicants, hepatic infection, non-alcoholic fatty liver disease and other pathogenic factors [2]. Anti-oxidative management has been shown to be effective in the management of hepatic damages in various models in vivo and in vitro [3]. One of the anti-oxidative treatment strategies is to increase the levels of antioxidant enzymes, which may rebalance the antioxidant and oxidant homeostasis in the body. As an antioxidant enzyme, HO is a key antioxidant defense against reactive oxygen species. Up-regulation of HO-1 expression has exhibited protective effects against oxida-

tive stress-induced damage in various disease models including hepatic injury [4]. Transcription factor Nrf2 is one of the main factors that regulate the expression of HO gene [5]. Protein kinase C, MAPK pathway, PI3K/Akt pathway and are recently regarded to be involved the activation of Nrf2-HO signaling [6, 7]. Studies showed that many agents increased HO-1 expression and exhibited cytoprotective effects though MAPK and PI3K/Akt pathways in a number of cellular and animal models [8, 9]. Therefore, induction of HO-1 expression is thought to be a potential therapeutic strategy for oxidative stress-induced damages including hepatic injury [10].

A number of chemicals can induce hepatotoxicity in animals and hepatic cellular systems. Several of them are frequently used to establish hepatotoxic models in vivo and in vitro. But some species like rat are relatively resistant to some of the chemicals which are used to induce hepatotoxicity, such as n-acetyl-p-aminophenol. Among the chemicals, CCl4 is one of the most widely used xenobiotics to induce hepatotoxicity and to investigate the hepatoprotective effects of novel agents [11]. The hepatotoxicity of CCl4 is associated with excess oxidative stress. Studies have proven that CCl4 can initiate protein oxidation, lipid peroxidation and DNA oxidative damage in cells [12]. In vitro studies, several hepatic cellular systems are used to evaluate the activity of hepatoprotective agents. HepG2 cell line has similar properties to normal human hepatocytes. It has been extensively used in assessing the hepatoprotective efficacy of agents in recent studies [13].

Medicinal plants are important sources of hepatoprotective drugs. Recently, some herbal products were tested to manage hepatic diseases [11, 14]. Ginkgo biloba leaf has a long history of medical use. Several compounds have recently been obtained from Ginkgo biloba leaf, such as ginkgolide A, B, C, J, G, K and bilobalide (BB). Some of the ginkgolides have exhibited hepatoprotective effects in several hepatic cellular and animal models through anti-inflammatory, anti-oxidative and other mechanisms [15]. A recent study reported that the freeze-dried methanolic leaf extract of Ginkgo biloba which contained several compounds (ginkgolide A, B, C, G, J and BB) attenuated the lantadenes-induced hepatic damage in guinea pig [16]. However, no study investigated whether BB itself has hepatoprotective effects. Some recent studies have demonstrated the anti-oxidative and cytoprotective activities of BB in human melanocytes [17], 3T3-L1 adipocytes [18] and PC12 cells [19]. Studies also showed BB exhibited neuroprotective effects in animals with brain ischemia [20], Aβ (25-35) induced learning and memory impairments [21], loss of dopaminergic neurons in substantia nigra [22] and some other disorders. But, to our knowledge, it is unclear whether BB can protect hepatic cells against oxidative stress-induced damage.

Therefore, this study aimed to investigate the possible hepatoprotective effect of BB on the CCl4-induced damage in HepG2 cells and to detect the possible underlying mechanisms.

Methods

Cell culture

HepG2 cell line (Xiangya Cell Bank, Changsha, China) was cultured in Dulbecco Modified Eagle's Medium (DMEM; GE Healthcare Life Sciences, Logan, UT, USA) supplemented with 10% fetal bovine serum (GrandIsland, NewYork, NY, USA), 100 U/ml penicillin and 100 μg/ml streptomycin in a humidified atmosphere of 5% CO_2 at 37°C.

Cell viability

3-(4,5-dimethylthiazole-2yl)-2,5-diphenyl tetrazolium bromide (MTT) assay was conducted to measure the cell viability. Briefly, after the incubation procedure described in the following experiments, cells were incubated with MTT (5 mg/ml) (Sigma-Aldrich, MO, USA) for 4 h. Then the medium was removed, and 100 μl of DMSO was added to dissolve the formazan crystals. The absorbance was read at the wavelength of 570 nm using a microplate reader (Bio-Rad, Hercules, CA, USA).

Detection of cytotoxicity of BB

To study the cytotoxicity, HepG2 cells were plated in 96 well plates (5×10^3 cells/well) and cultured for 24 h. Then the cells were respectively incubated with 0.1, 1, 5, 10, 20 and 30 μM BB for 0.5, 2, 4, 12, 24 and 48 h. Following the BB incubation, the viability of the HepG2 cells was determined using MTT assay.

Table 1. Viability of the HepG2 cells cultured with different concentrations of BB

	0.5 h	2 h	4 h	12 h	24 h	48 h
Control (BB 0 μM)	1.00±0.05	1.00±0.06	1.00±0.06	1.00±0.04	1.00±0.05	1.00±0.08
BB 0.1 μM	1.03±0.05	1.01±0.06	1.05±0.08	1.04±0.09	0.99±0.07	1.03±0.09
BB 1 μM	1.03±0.07	1.04±0.06	1.03±0.07	1.05±0.08	1.01±0.07	0.98±0.08
BB 5 μM	1.04±0.09	1.06±0.09	1.02±0.09	1.00±0.08	0.98±0.12	1.02±0.08
BB 10 μM	0.99±0.11	1.01±0.13	0.98±0.10	0.98±0.11	1.02±0.09	1.01±0.11
BB 20 μM	1.01±0.09	0.98±0.10	0.98±0.12	0.96±0.12	0.98±0.11	0.97±0.12
BB 30 μM	0.97±0.10	0.96±0.14	0.94±0.11	0.94±0.10	0.95±0.09	0.93±0.10

Data were expressed as mean ± SD. [a]$P<0.05$ if compared to the control group.

Table 2. Cytoprotective and anti-oxidative effects of BB in CCl4-simulated HepG2 cells

	H_2O_2 (mmol/L)	8-OHdG (ng/ml)	MDA (nmol/mgprot)	Viability	ALT (μIU/ml)	AST (mU/ml)
Control	1.27±0.09	1.09±0.15	0.18±0.03	1.00±0.07	28.69±2.12	25.90±4.63
CCl4	4.66±0.71[a]	3.07±0.40[a]	0.63±0.06[a]	0.48±0.05[a]	100.01±8.18[a]	60.77±4.82[a]
CCl4+BB 10 μM	3.15±0.40[b]	2.25±0.30[b]	0.44±0.05[b]	0.68±0.08[b]	76.61±8.50[b]	46.50±5.33[b]
CCl4+BB 20 μM	1.79±0.24[b,c]	1.66±0.18[b,c]	0.31±0.04[b,c]	0.90±0.10[b,c]	44.19±6.00[b,c]	36.11±2.84[b,c]

Data were expressed as mean ± SD. [a]$P<0.05$ if compared to the control group; [b]$P<0.05$ if compared to the CCl4 group; [c]$P<0.05$ if compared to the CCl4+BB 10 μM group.

Knockdown Nrf2 with siRNA in HepG2 cells

HepG2 cells were seeded in 6-well plates with a density of 2×10^5 cells/well and cultured for 24 h. then HepG2 cells were transfected with 50 nM Nrf2 siRNA or scrambled siRNA using Hi-Perfect transfection reagent (QIAGEN, Valencia, CA, USA). Western blot assay was carried out to verify the knockdown of Nrf2. After incubation with siRNA for 3 days, the cells were harvested for further experiments.

Cytoprotective effect of BB on the CCl4-simulated HepG2 cells

To detect the cytoprotective effect, HepG2 cells were seeded in the in 96 well plates at 5×10^3 cells/well. After grown in the wells for 24 h, cells were incubated with 10 or 20 μM BB with/without the presence of HO-1 inhibitor, PI3K/Akt inhibitor and MAPK pathway inhibitors (p38, JNK and JNK inhibitors) for 2 h (Sigma Chemical Co., St. Louis, MO, USA). Then CCl4 was added to the wells and the cells were further cultured with 0.4% CCl4 for 22 h. The cell culture medium was collected detect the leakage of ALT and AST using ELISA kits (Cusabio Company, Wuhan, China). Some wells of cells were used to analyze the cell viability using MTT assay. The other cells were lysed. The cell lysates were collected for the further detections.

Oxidative stress markers and HO-1 assessments

Levels of H_2O_2, MDA and 8-OHdG in the HepG2 cell lysates were detected using commercial detection kits (Nanjing Jiancheng Company, Nanjing, China; Xinqidi Company, Wuhan, China). Levels of HO-1 in cell lysates were detected by ELISA (Xinqidi Company, Wuhan, China).

Real-time PCR analysis

The expression of Nrf2 and HO-1 genes was quantified using quantitative real-time PCR (qRT-PCR). Briefly, the total RNA was extracted from cells with TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and reverse-transcribed into cDNA using iScript cDNA synthesis kit (Thermo Scientific, Shanghai, China). The qRT-PCR reactions were run on a Real Time System machine as follows: at 95°C for 30 s, then subjected to 40 cycles of 95°C for 10 s, and at 55°C for 45 s. The forward and reverse primer sequences were as follows: GAPDH, 5'-CTTTGTCAAGCTCATTTCCTGG-3' and 5'-TCTTCCTCTTGTGCTC TTGC-3'; Nrf2, 5'-CCGCTCGAGATGATGGACTTGG AGCTGCC-3' and 5'-GGGGTACCG TGTTTTTCTTAACATCTGGC-3'; HO-1, 5'-G CCAC-

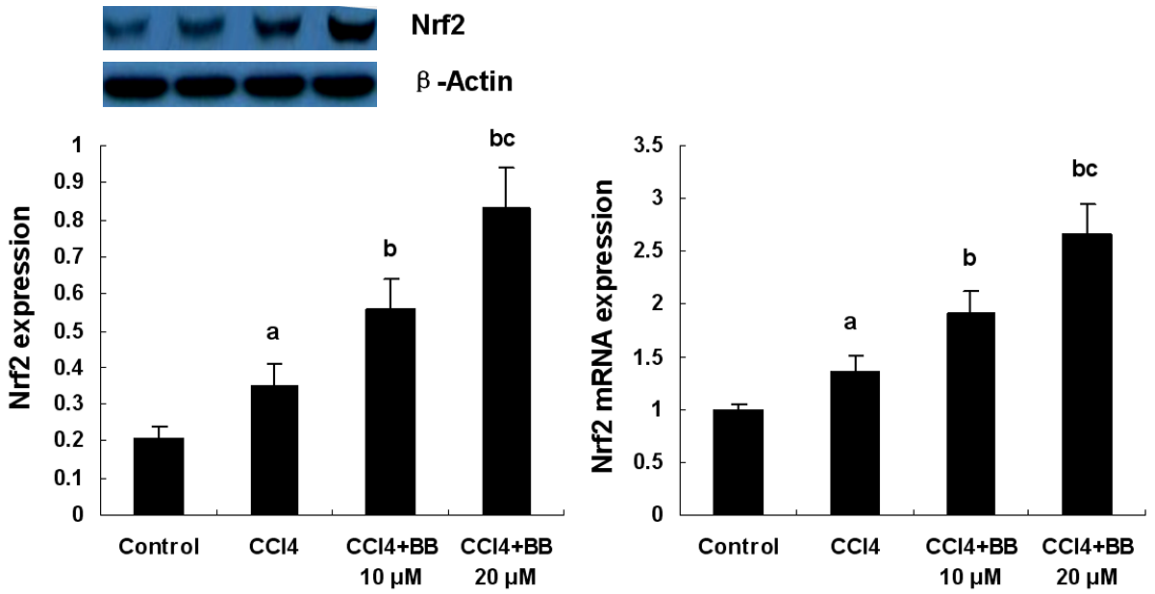

Figure 1. BB increased the expressions of Nrf2 protein and mRNA in the CCl4-simulated HepG2 cells. Data were expressed as mean ± SD. [a]*P*<0.05 if compared to the control group; [b]*P*<0.05 if compared to the CCl4 group; [c]*P*<0.05 if compared to the CCl4+BB 10 μM group.

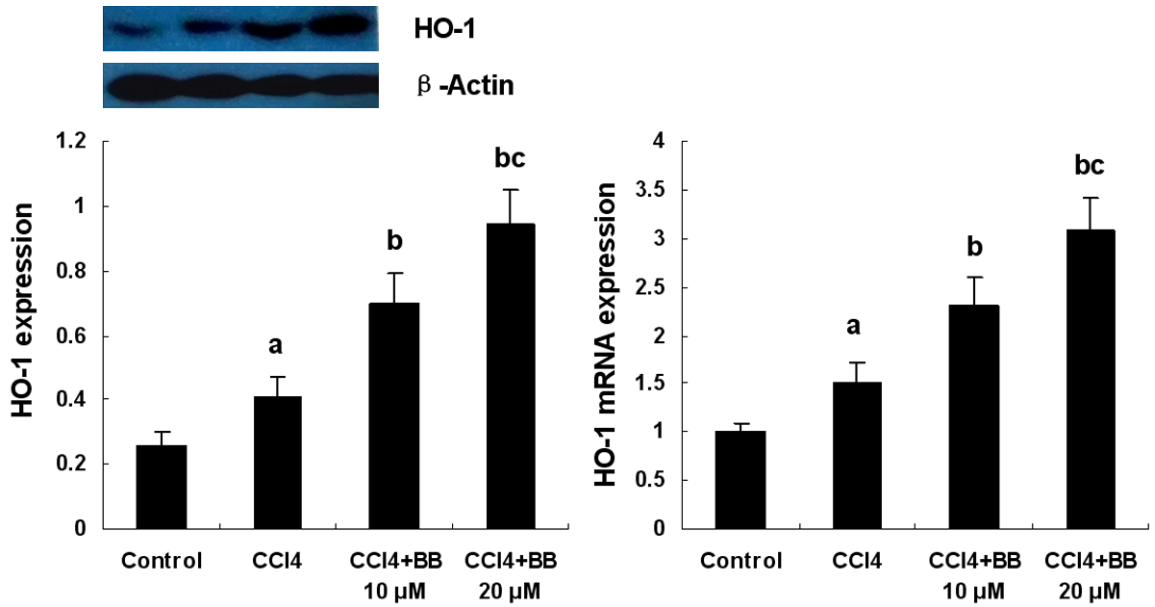

Figure 2. BB increased the expressions of HO-1 protein and mRNA in the CCl4-simulated HepG2 cells. Data were expressed as mean ± SD. [a]*P*<0.05 if compared to the control group; [b]*P*<0.05 if compared to the CCl4 group; [c]*P*<0.05 if compared to the CCl4+BB 10 μM group.

CAAGTTCAAGCAGCTCTA-3' and 5'-AGCAGCTCC-TGCAACTCCTCAA-3'.

Western blot

Cells were lysed using lysate containing RIPA buffer and protease inhibitor on ice. Total protein was extracted. Equal amounts of protein sample (40 μg) were separated by 12% SDS-PAGE gel and transferred to PVDF membranes. The membranes were blocked with 5% skim milk blocking buffer at room temperature for 1 h. The membranes were then incubated with rabbit anti-human Nrf2 (1:1000) or rabbit anti-human HO-1 (1:1000) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) overnight at

Table 3. HO-1 inhibitor diminished the effects of BB on the CCl4-simulated HepG2 cells

	H_2O_2 (mmol/L)	8-OHdG (ng/ml)	MDA (nmol/mgprot)	Viability	ALT (μIU/ml)	AST (mU/ml)
Control	1.35±0.07	1.13±0.14	0.20±0.04	1.00±0.06	30.31±2.36	26.65±4.04
CCl4	4.31±0.57[a]	3.41±0.55[a]	0.69±0.09[a]	0.49±0.07[a]	101.17±9.12[a]	61.58±5.70[a]
CCl4+BB 20 μM	1.60±0.23[b]	1.75±0.20[b]	0.33±0.05[b]	0.91±0.11[b]	48.09±6.14[b]	34.87±3.28[b]
CCl4+BB 20 μM+ZnPP	3.39±0.41[c]	2.89±0.35[c]	0.56±0.07[c]	0.59±0.08[c]	80.20±9.51[c]	48.91±4.10[c]

Data were expressed as mean ± SD. [a]*P*<0.05 if compared to the control group; [b]*P*<0.05 if compared to the CCl4 group; [c]*P*<0.05 if compared to CCl4+BB 20 μM group.

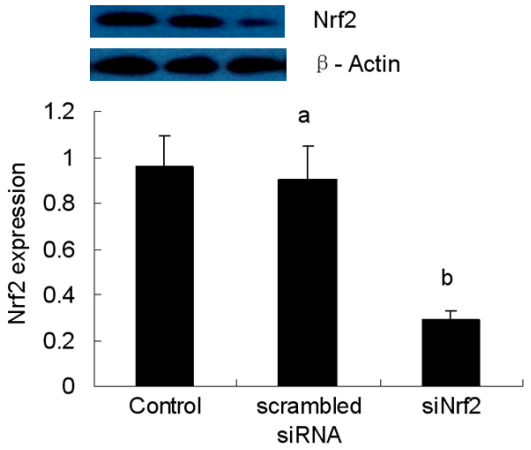

Figure 3. Nrf2 siRNA transfection reduced the expression of Nrf2 protein in HepG2 cells. Data were expressed as mean ± SD. [a]*P*<0.05 if compared to the control group; [b]*P*<0.05 if compared to the scrambled siRNA group.

4°C. After the rinsing step, the membranes were incubated with secondary antibody (1: 1000) (goat anti-rabbit IgG: Zhongshan Golden Bridge Biotechnology, Beijing, China) at room temperature for 2 h. β-Actin was used as a loading control. Protein bands were visualized by enhanced chemiluminescence and the intensity of the bands were quantified using image analyzer software.

Statistical analysis

The results were presented as the mean ± standard deviation. Statistical analysis was carried out using the SPSS 16.0. The significance of the data was analyzed with one-way ANOVA and Student-Newman-Keuls test. The difference was considered statistically significant when P<0.05.

Results

BB exhibited no obvious toxic effects

Toxicity study was carried out to ascertain that BB was safe for the treatment on HepG2 cells. The result of the MTT assay showed that there was no significant difference in cell viability among the normal control HepG2 cells and the HepG2 cells with BB (0.1, 1, 5, 10, 20 and 30 μM) incubation for 0.5, 2, 4, 12, 24 and 48 h (**Table 1**), suggesting that all the concentrations of BB used in the study had no marked cytotoxicity on HepG2 cells.

Protective effect of BB against oxidative stress in CCl4-stimulated HepG2 cells

As shown in **Table 2**, levels of H_2O_2, MDA and 8-OHdG in the lysates of the CCl4-stimulated HepG2 cells were much higher the normal control HepG2 cells, indicating CCl4 induced oxidative stress in the cells. However, BB (10 and 20 μM) treatment significantly diminished the increases of the oxidative stress markers in a dose-dependent manner.

BB increased Nrf2 and HO-1 expression in the CCl4-simulated HepG2 cells

As shown in **Figures 1** and **2**, CCl4-simulation increased the levels of transcription factor Nrf2 and HO-1 (mRNA and protein) if compared to the normal control cells. Treatment with BB (10 and 20 μM) further elevated the levels of Nrf2 and HO-1 (mRNA and protein) in the CCl4-simulated HepG2 cells, and the effects were dose-dependent.

Cytoprotective effect BB on the CCl4-simulated HepG2 cells

As shown in **Table 2**, the viability of the CCl4-stimulated HepG2 cells were much lower the normal control HepG2 cells; but the levels of ALT and AST in the culture medium markedly increased compared to the normal control HepG2 cells. However, BB (10 and 20 μM) treatment significantly diminished the changes of cell viability, ALT and AST induced by CCl4 in a dose-dependent manner.

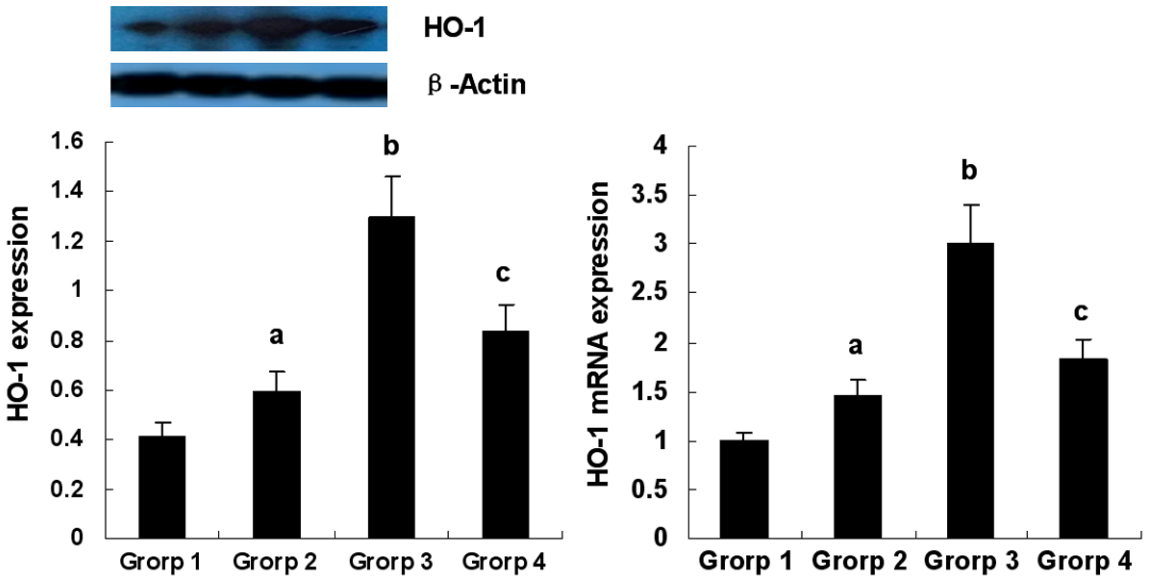

Figure 4. Nrf2 silence reversed the effects of BB on HO-1 expression in the CCl4- simulated HepG2 cells. Data were expressed as mean ± SD. [a]*P*<0.05 if compared to the group 1; [b]*P*<0.05 if compared to the group 2; [c]*P*<0.05 if compared to group 3. (Group 1: control group; Group 2: CCl4 group; Group 3: CCl4+BB 20 μM+ scrambled siRNA group; Group 4: CCl4+BB 10 μM+siNrf2).

Table 4. Nrf2 silence reversed the effect of BB on the viability of CCl4-simulated HepG2 cells

	Viability
Control	1.00±0.08
CCl4	0.51±0.06[a]
CCl4+BB 20 μM+ scrambled siRNA	0.90±0.11[b]
CCl4+BB 10 μM+siNrf2	0.60±0.07[c]

Data were expressed as mean ± SD. [a]*P*<0.05 if compared to the control group; [b]*P*<0.05 if compared to the CCl4 group; [c]*P*<0.05 if compared to the CCl4+BB 20 μM+ scrambled siRNA group.

HO-1 inhibitor diminished the effects BB in the CCl4-simulated HepG2 cells

As shown in **Table 3**, the anti-oxidative effects of BB in the CCl4-simulated HepG2 cells were markedly diminished by a HO-1 inhibitor ZnPP. Consistently, the cytoprotective effects of BB also were significantly abolished by ZnPP (**Table 3**). The results indicated that HO-1 played a crucial role in mediating the effects of BB.

Nrf2 silence reversed the effects of BB on HO-1 expression

We carried out Nrf2 siRNA transfection in HepG2 cells and verified the silencing effect by examining the expression of Nrf2 using western blot. We found that the expression of Nrf2 protein was dramatically reduced by Nrf2 siRNA transfection (**Figure 3**).

Furthermore, Nrf2 silence with siNrf2 significantly decreased the HO-1 expression (mRNA and protein) in BB-treated HepG2 cells (**Figure 4**). Consistently, siNrf2 significantly decreased the viability of the HepG2 cells (**Table 4**). The results suggested the involvement of Nrf2 in the induction of HO-1 expression by BB in CCl4-stimulated HepG2 cells.

Involvement of PI3K/Akt and p38 MAPK pathways in the effects of BB

In order to detect the upstream factors that mediated the effects of BB on Nrf2/HO-1 signaling, inhibitors of PI3K/Akt and MAPK (p38, JNK and JNK) pathways were used. We found LY294002 (a PI3K/Akt inhibitor) and SB203580 (a p38 inhibitor), but not SP600125 (a JNK inhibitor) or PD98059 (an ERK inhibitor), significantly abolished the elevations in cell viability (**Table 5**) and the expression of Nrf2 and HO-1 (mRNA and protein) (**Figures 5** and **6**) by BB in the CCl4-stimulated HepG2 cells. LY294002 and SB203580 also significantly reversed the reduction in ALT and AST leakage by 20 μM BB, but SP600125 and PD98059 had no effects on ALT and AST levels (**Table 5**).

Table 5. Involvement of PI3K/Akt and p38 MAPK pathways in the cytoprotective effects of BB (20 μM) on the CCl4-simulated HepG2 cells

	Viability	ALT (μIU/ml)	AST (mU/ml)
Control	1.00±0.08	30.80±2.71	26.61±4.10
CCl4	0.48±0.06[a]	101.44±8.90[a]	59.97±6.38[a]
CCl4+BB	0.90±0.11[b]	44.93±6.55[b]	35.72±4.05[b]
BB+CCl4+LY294002	0.68±0.08[c]	70.22±4.89[c]	46.60±3.63[c]
BB+CCl4+SB203580	0.65±0.10[c]	73.25±6.61[c]	48.15±3.04[c]
BB+CCl4+SP600125	0.85±0.13	48.51±42.95	38.55±4.91
BB+CCl4+PD98059	0.82±0.17	46.96±6.30	37.21±4.20

Data were expressed as mean ± SD. [a]$P<0.05$ if compared to the control group; [b]$P<0.05$ if compared to the CCl4 group; [c]$P<0.05$ if compared to the CCl4+BB group.

Discussion

In this study, we demonstrated that BB ameliorated the CCl4-induced cell damage, inhibited the production of H_2O_2, MDA and 8-OHdG, as well as increased the expression of Nrf2 and HO-1 in HepG2 cells. And the effects of BB were reversed by HO-1, p38 and PI3K/Akt inhibitors.

Herbal productions have been long used to treat liver disease. These agents exhibit hepatoprotective effects via various mechanisms such as inhibiting oxidative stress, inflammatory response, fibrogenesis and other pathogenic factors [1, 23]. Some extracts of Ginkgo bi loba leaf, such as ginkgolide A, have been tested to manage hepatic damage in recent studies [15]. Yet, little is known about the hepatoprotective effects of BB which is also a compound extracted form Ginkgo biloba leaf. Our toxic detection in this study showed that BB exhibited no marked cytotoxicity in HepG2 cells. In order to investigate its hepatoprotective effects, we stimulated the HepG2 cells with CCl4 which is a chemical extensively used to induce hepatic damage in vivo and in vitro. Our results showed CCl4 induced significant damage to the cells with the manifestations of decreased viability, as well as increased ALT and AST leakage. The cellular damages were similar to those observed in previous reports [24]. However, we found BB dose-dependently attenuated the CCl4 induced-damage to HepG2 cells. Although this is the investigation that demonstrated the hepatoprotective effects of BB in HepG2 cells, its cytoprotective activity has been proven in human melanocytes [17], 3T3-L1 adipocytes [18] and PC12 cells [19] in recent studies. Evidence from animal studies also showed BB had neuroprotective effects on brain damage [20-22].

Oxidative stress is involved in the CCl4-induce cell damage [25]. In order to detect the possible mechanisms by which BB protected HepG2 cells, levels of 8-OHdG (a marker of gene oxidative damage), H_2O_2 and MDA were measured. Results showed CCl4 significantly increased levels of these oxidative markers. It is regarded that HO-1 which is an important antioxidant defense is activated under the condition of excessive oxidative stress [26]. Consistently, we found the mRNA and protein expression of HO-1 in the CCl4-stimulated HepG2 cells increased in this study. However, our results showed that BB dose-dependently decreased the levels of H_2O_2, MDA and 8-OHdG, but further increased the expression of HO-1 mRNA and protein in the cells. A number of recent studies have well demonstrated that HO-1 is a key defense against cellular oxidative damage [26, 27]. Up-regulation of HO-1 expression has exhibited cytoprotective effects in various cellular systems [27, 28]. To confirm the role HO-1 in mediating the effects of BB in this study, a HO-1 inhibitor was used. Interestingly, HO-1 inhibitor markedly reversed the anti-oxidative and cytoprotective effects of BB in the cells. The data demonstrated that HO-1 played a crucial role in mediating the effects of BB.

As an antioxidant enzyme, HO-1 is activated by various stimuli. It has been recently proven that the expression of HO-1 gene can be regulated by Nrf2. Nrf2 is a critical transcription factor that regulates the genes encoding phase II detoxifying and antioxidant enzymes. The activation of the Nrf2-antioxidant response element (ARE) signaling pathway is regarded as a major mechanism of the cellular defense against oxidative stress. The induction of Nrf2 expression has been proven to eliminate reactive oxidants and ameliorate oxidative damage in many cellular and animal models [29, 30]. Therefore, Nrf2-ARE pathway has been recognized as an important therapeutic target for treatment of various diseases including hepatic

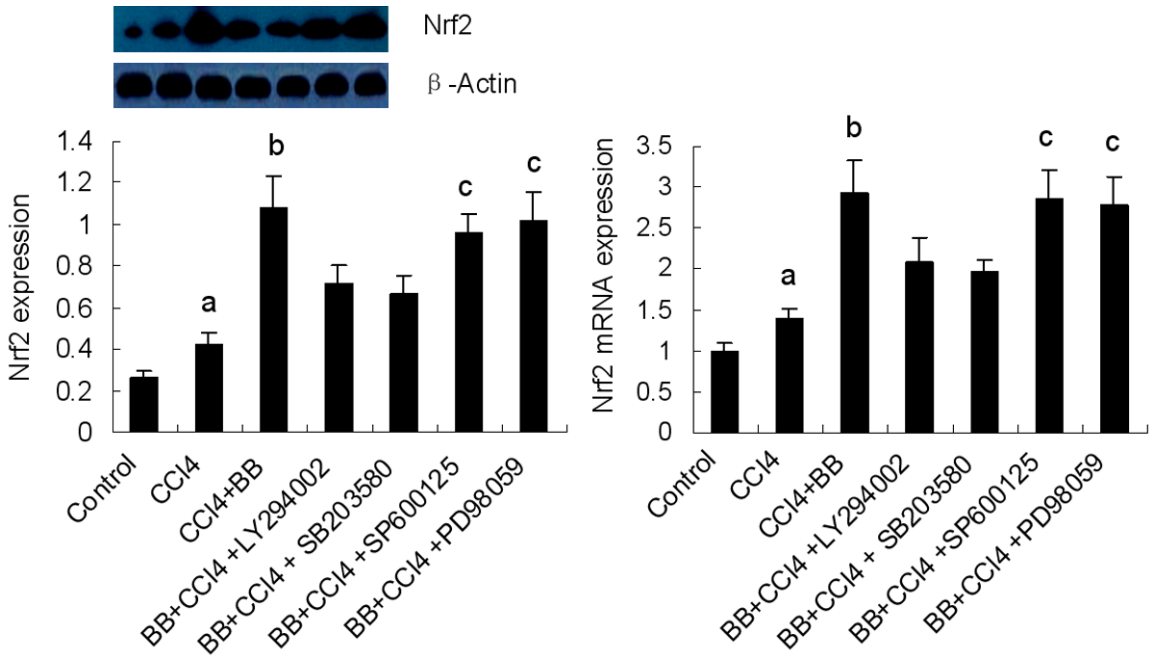

Figure 5. PI3K/Akt inhibitor LY294002 and p38 inhibitor SB203580 diminished the effects of BB (20 μM) on Nrf2 expression (protein and mRNA) in CCl4-simulated HepG2 cells. Data were expressed as mean ± SD. [a]*P*<0.05 if compared to the control group; [b]*P*<0.05 if compared to the CCl4 group; [c]*P*<0.05 if compared to the CCl4+BB group.

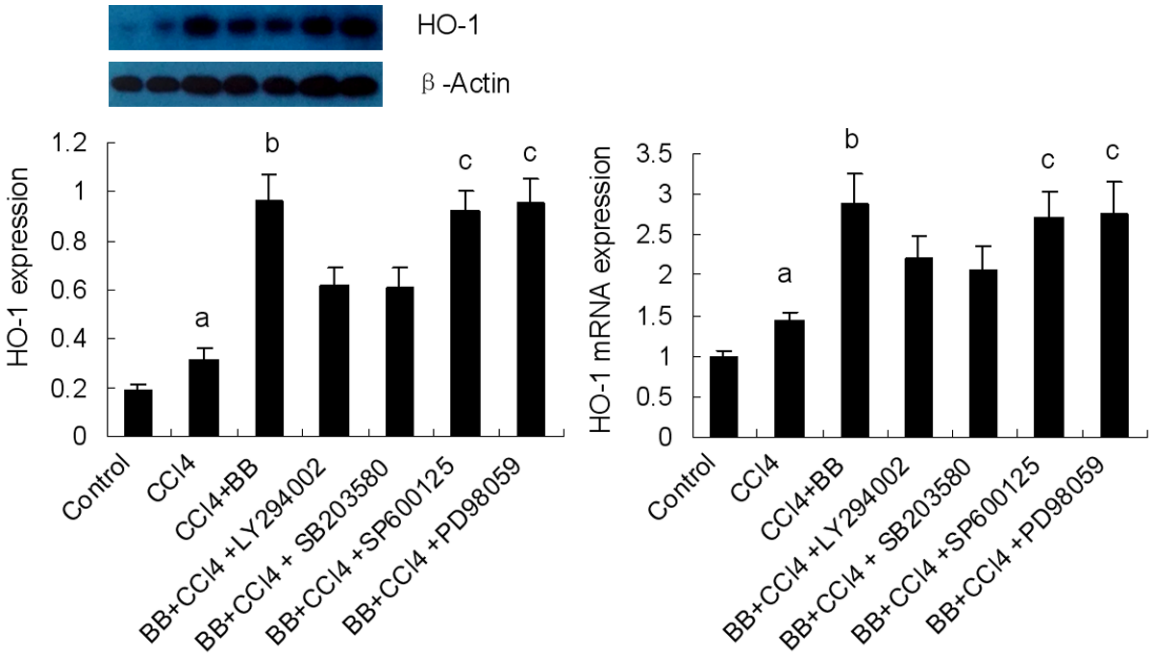

Figure 6. PI3K/Akt inhibitor LY294002 and p38 inhibitor SB203580 diminished the effects of BB (20 μM) on HO-1 expression (protein and mRNA) in CCl4-simulated HepG2 cells. Data were expressed as mean ± SD. [a]*P*<0.05 if compared to the control group; [b]*P*<0.05 if compared to the CCl4 group; [c]*P*<0.05 if compared to the CCl4+BB group.

damage [31, 32]. Nrf2-HO-1 signaling is regarded to have a crucial role in maintaining antioxidant and oxidant homeostasis. A number of recent studies have well demonstrated the cytoprotective effects resulting from the activation of Nrf2/HO-1 signaling [33, 34]. In this study, we found BB significantly increased the expression of Nrf2 mRNA which was in line with

the increase in HO-1 expression. Furthermore, we found the knockdown of Nrf2 by using siRNA markedly inhibited the effects of BB on the HO-1 expression and cell viability in CCl4-stimulated HepG2 cells. The findings were in agreement with a recent study showing that Nrf2 activation positively regulated HO-1 expression in HepG2 cells [35]. The data strongly suggested the involvement of Nrf2 in the mediating the effects of BB.

As an important regulator of cellular redox status, Nrf2 is modulated by several signaling. Some recent studies have proven that PI3K/Akt and MAPK pathways have a central role in Nrf2 activation associated HO-1 expression [36]. MAPKs are a family of protein serine/threonine kinases, including p38, ERK and JNK subgroups. In order to clarify the up-stream pathways that mediated the induction of Nrf2/HO-1 signaling by BB, inhibitors specific to PI3K/Akt and MAPKs pathways were used. We found that incubation of the HepG2 cells with LY294002 and SB203580 diminished the induction of Nrf2/HO-1 expression by BB in CCl4-stimulated HepG2 cells. SP600125 and PD98059, however, exhibited no significant influence on the effects of BB. Consistently, the cytoprotective effects of BB were also inhibited by LY294002 and SB203580. Our findings indicated that p38 and PI3K/Akt pathways were very important for the BB-induced Nrf2/HO-1 expression and cytoprotection. In line, PI3K/Akt and p38 pathways were reported to be involved in mediating the effects of some other natural compounds in previous studies [37]. In addition, similar to our findings, Shi C reported that BB prevented the apoptosis of SH-SY5Y cells through activation of the PI3K/Akt pathway [38].

Therefore, BB exhibited cytoprotective effect in HepG2 cells, which was associated with the induction of Nrf2-mediated HO-1 expression. PI3K/Akt and p38 pathways were involved in the hepatoprotective effects and the regulation of Nrf2/HO-1 signaling by BB. The findings suggested a therapeutic potential of BB for the treatment of oxidative-associated hepatic damage.

Disclosure of conflict of interest

None.

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References

[1] Madrigal-Santillán E, Madrigal-Bujaidar E, Álvarez-González I, Sumaya-Martínez MT, Gutiérrez-Salinas J, Bautista M, Morales-González Á, García-Luna y González-Rubio M, Aguilar-Faisal JL, Morales-González JA. Review of natural products with hepatoprotective effects. World J Gastroenterol 2014; 20: 14787-14804.

[2] Muriel P, Gordillo KR. Role of oxidative stress in liver health and disease. Oxid Med Cell Longev 2016; 2016: 9037051.

[3] de Andrade KQ, Moura FA, dos Santos JM, de Araújo OR, de Farias Santos JC, Goulart MO. Oxidative stress and inflammation in hepatic diseases: Therapeutic possibilities of N-Acetylcysteine. Int J Mol Sci 2015; 16: 30269-30308.

[4] Bakhautdin B, Das D, Mandal P, Roychowdhury S, Danner J, Bush K, Pollard K, Kaspar JW, Li W, Salomon RG, McMullen MR, Nagy LE. Protective role of HO-1 and carbon monoxide in ethanol-induced hepatocyte cell death and liver injury in mice. J Hepatol 2014; 61: 1029-1037.

[5] Heyninck K, Sabbe L, Chirumamilla CS, Szarc Vel Szic K, Vander Veken P, Lemmens KJ, Lahtela-Kakkonen M, Naulaerts S, Op de Beeck K, Laukens K, Van Camp G, Weseler AR, Bast A, Haenen GR, Haegeman G, Vanden Berghe W. Withaferin A induces heme oxygenase (HO-1) expression in endothelial cells via activation of the Keap1/Nrf2 pathway. Biochem Pharmacol 2016; 109: 48-61.

[6] Li L, Dong H, Song E, Xu X, Liu L, Song Y. Nrf2/ARE pathway activation, HO-1 and NQO1 induction by polychlorinated biphenyl quinone is associated with reactive oxygen species and PI3K/AKT signaling. Chem Biol Interact 2014; 209: 56-67.

[7] Zhao M, Guo H, Chen J, Fujino M, Ito H, Takahashi K, Abe F, Nakajima M, Tanaka T, Wang J, Huang H, Zheng S, Hei M, Li J, Huang S, Li J, Ma X, Chen Y, Zhao L, Zhuang J, Zhu P, Li XK. 5-aminolevulinic acid combined with sodium ferrous citrate ameliorates H2O2-induced cardiomyocyte hypertrophy via activation of the MAPK/Nrf2/HO-1 pathway. Am J Physiol Cell Physiol 2015; 308: C665-672.

[8] Chen XQ, Wu SH, Zhou Y, Tang YR. Lipoxin A4-induced heme oxygenase-1 protects cardiomyocytes against hypoxia/reoxygenation injury via p38 MAPK activation and Nrf2/ ARE complex. PLoS One 2013; 8: e67120.

[9] Nguyen CN, Kim HE, Lee SG. Caffeoylserotonin protects human keratinocyte HaCaT cells against H2O2-induced oxidative stress and apoptosis through upregulation of HO-1 expression via activation of the PI3K/Akt/Nrf2 pathway. Phytother Res 2013; 27: 1810-1818.
[10] Motterlini R, Foresti R. Heme oxygenase-1 as a target for drug discovery. Antioxid Redox Signal 2014; 20: 1810-1826.
[11] Chen S, Chen Y, Chen B, Cai YJ, Zou ZL, Wang JG, Lin Z, Wang XD, Fu LY, Hu YR, Chen YP, Chen DZ. Plumbagin ameliorates CCl4-induced hepatic fibrosis in rats via the epidermal growth factor receptor signaling pathway. Evid Based Complement Alternat Med 2015; 2015: 645727.
[12] Kadiiska MB, Gladen BC, Baird DD, Germolec D, Graham LB, Parker CE, Nyska A, Wachsman JT, Ames BN, Basu S, Brot N, Fitzgerald GA, Floyd RA, George M, Heinecke JW, Hatch GE, Hensley K, Lawson JA, Marnett LJ, Morrow JD, Murray DM, Plastaras J, Roberts LJ 2nd, Rokach J, Shigenaga MK, Sohal RS, Sun J, Tice RR, Van Thiel DH, Wellner D, Walter PB, Tomer KB, Mason RP, Barrett JC. Biomarkers of oxidative stress study II: are oxidation products of lipids, proteins, and DNA markers of CCl4 poisoning? Free Radic Biol Med 2005; 38: 698-710.
[13] Ma B, Wang J, Tong J, Zhou G, Chen Y, He J, Wang Y. Protective effects of Chaenomeles thibetica extract against carbon tetrachloride-induced damage via the MAPK/Nrf2 pathway. Food Funct 2016; 7: 1492-1500.
[14] Susutlertpanya W, Werawatganon D, Siriviriyakul P, Klaikeaw N. Genistein attenuates nonalcoholic steatohepatitis and increases hepatic PPARγ in a rat model. Evid Based Complement Alternat Med 2015; 2015: 509057.
[15] Ye N, Wang H, Hong J, Zhang T, Lin C, Meng C. PXR Mediated protection against liver inflammation by Ginkgolide A in tetrachloromethane treated mice. Biomol Ther (Seoul) 2016; 24: 40-48.
[16] Parimoo HA, Sharma R, Patil RD, Sharma OP, Kumar P, Kumar N. Hepatoprotective effect of Ginkgo biloba leaf extract on lantadenes-induced hepatotoxicity in guinea pigs. Toxicon 2014; 81: 1-12.
[17] Lu L, Wang S, Fu L, Liu D, Zhu Y, Xu A. Bilobalide protection of normal human melanocytes from hydrogen peroxide-induced oxidative damage via promotion of antioxidase expression and inhibition of endoplasmic reticulum stress. Clin Exp Dermatol 2016; 41: 64-73.
[18] Priyanka A, Nisha VM, Anusree SS, Raghu KG. Bilobalide attenuates hypoxia induced oxidative stress, inflammation, and mitochondrial dysfunctions in 3T3-L1 adipocytes via its antioxidant potential. Free Radic Res 2014; 48: 1206-1217.
[19] Zhou LJ, Zhu XZ. Reactive oxygen species-induced apoptosis in PC12 cells and protective effect of bilobalide. J Pharmacol Exp Ther 2000; 293: 982-988.
[20] Schwarzkopf TM, Koch KA, Klein J. Neurodegeneration after transient brain ischemia in aged mice: beneficial effects of bilobalide. Brain Res 2013; 1529: 178-187.
[21] Yin Y, Ren Y, Wu W, Wang Y, Cao M, Zhu Z, Wang M, Li W. Protective effects of bilobalide on Aβ (25-35) induced learning and memory impairments in male rats. Pharmacol Biochem Behav 2013; 106: 77-84.
[22] Li LY, Zhao XL, Fei XF, Gu ZL, Qin ZH, Liang ZQ. Bilobalide inhibits 6-OHDA-induced activation of NF-kappaB and loss of dopaminergic neurons in rat substantia nigra. Acta Pharmacol Sin 2008; 29: 539-547.
[23] Lam P, Cheung F, Tan HY, Wang N, Yuen MF, Feng Y. Hepatoprotective effects of Chinese medicinal herbs: A focus on anti-inflammatory and anti-oxidative activities. Int J Mol Sci 2016; 17: 465.
[24] Krithika R, Verma RJ, Shrivastav PS. Antioxidative and cytoprotective effects of andrographolide against CCl4-induced hepatotoxicity in HepG2 cells. Hum Exp Toxicol 2013; 32: 530-543.
[25] Ghaffari H, Venkataramana M, Nayaka SC, Ghassam BJ, Angaswamy N, Shekar S, Sampath Kumara KK, Prakash HS. Hepatoprotective action of Orthosiphon diffusus (Benth.) methanol active fraction through antioxidant mechanisms: an in vivo and in vitro evaluation. J Ethnopharmacol 2013; 149: 737-744.
[26] Zhao Z, Liao G, Zhou Q, Lv D, Holthfer H, Zou H. Sulforaphane attenuates contrast-induced nephropathy in rats via Nrf2/HO-1 pathway. Oxid Med Cell Longev 2016; 2016: 9825623.
[27] Liu B, Qian JM. Cytoprotective role of heme oxygenase-1 in liver ischemia reperfusion injury. Int J Clin Exp Med 2015; 8: 19867-19873.
[28] Wang N, Han Q, Wang G, Ma WP, Wang J, Wu WX, Guo Y, Liu L, Jiang XY, Xie XL, Jiang HQ. Resveratrol protects oxidative stress-induced intestinal epithelial barrier dysfunction by upregulating heme oxygenase-1 expression. Dig Dis Sci 2016; 61: 2522-2234.
[29] Jing X, Wei X, Ren M, Wang L, Zhang X, Lou H. Neuroprotective effects of tanshinone I against 6-OHDA-induced oxidative stress in cellular and mouse model of Parkinson's disease through upregulating Nrf2. Neurochem Res 2016; 41: 779-786.
[30] Wang X, Chen L, Wang T, Jiang X, Zhang H, Li P, Lv B, Gao X. Ginsenoside Rg3 antagonizes adriamycin-induced cardiotoxicity by improving endothelial dysfunction from oxidative stress

via upregulating the Nrf2-ARE pathway through the activation of akt. Phytomedicine 2015; 22: 875-884.
[31] Shin SM, Yang JH, Ki SH. Role of the Nrf2-ARE pathway in liver diseases. Oxid Med Cell Longev 2013; 2013: 763257.
[32] Joshi G, Johnson JA. The Nrf2-ARE pathway: a valuable therapeutic target for the treatment of neurodegenerative diseases. Recent Pat CNS Drug Discov 2012; 7: 218-229.
[33] Ye F, Li X, Li L, Yuan J, Chen J. t-BHQ provides protection against lead neurotoxicity via Nrf2/HO-1 pathway. Oxid Med Cell Longev 2016; 2016: 2075915.
[34] Cai Z, Lou Q, Wang F, Li E, Sun J, Fang H, Xi J, Ju L. N-acetylcysteine protects against liver injure induced by carbon tetrachloride via activation of the Nrf2/HO-1 pathway. Int J Clin Exp Pathol 2015; 8: 8655-8562.
[35] Lee MS, Lee B, Park KE, Utsuki T, Shin T, Oh CW, Kim HR. Dieckol enhances the expression of antioxidant and detoxifying enzymes by the activation of Nrf2-MAPK signalling pathway in-HepG2 cells. Food Chem 2015; 174: 538-546.
[36] Kang JS, Choi IW, Han MH, Kim GY, Hong SH, Park C, Hwang HJ, Kim CM, Kim BW, Choi YH. The cytoprotective effects of 7,8-dihydroxyflavone against oxidative stress are mediated by the upregulation of Nrf2-dependent HO-1 expression through the activation of the PI3K/Akt and ERK pathways in C2C12 myoblasts. Int J Mol Med 2015; 36: 501-510.
[37] Bae J, Lee D, Kim YK, Gil M, Lee JY, Lee KJ. Berberine protects 6-hydroxydopamine- induced human dopaminergic neuronal cell death through the induction of heme oxygenase-1. Mol Cells 2013; 35:151-157.
[38] Shi C, Wu F, Yew DT, Xu J, Zhu Y. Bilobalide prevents apoptosis through activation of the PI3K/Akt pathway in SH-SY5Y cells. Apoptosis 2010; 15: 715-727.