

# Cytoprotective effects of diosmetin against hydrogen peroxide-induced L02 cell oxidative damage via activation of the Nrf2-ARE signaling pathway

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**Abstract.** Oxidative stress is considered a crucial mediator in the pathogenesis of various liver diseases. The flavone diosmetin has been reported to exhibit antioxidant activities; however, the hepatoprotective effects of diosmetin against oxidative stress, and the underlying molecular mechanisms, remain unknown. The present study aimed to investigate the potential hepatoprotective effects of diosmetin on hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>)-induced oxidative damage in L02 cells and attempted to evaluate the role of the nuclear factor erythroid 2-related factor 2 (Nrf2)/antioxidant response element pathway in this process. L02 cells were divided into groups: Control (DMSO, diosmetin), H<sub>2</sub>O<sub>2</sub>, Trolox or tertiary butylhydroquinone and diosmetin (different doses). Protective effects in L02 cells were determined by CCK-8, cell apoptosis and lactate dehydrogenase leakage assays. Flow cytometry and inverted fluorescence microscope were used to measure the intracellular reactive oxygen species (ROS) and mitochondrial membrane potential (MMP). Protein expression levels of Nrf2, heme oxygenase-1 (HO-1) and NAD(P)H quinone oxidoreductase-1 (NQO1) were determined by western blotting and mRNA levels were determined by reverse transcription-quantitative polymerase chain reaction. The results revealed that H<sub>2</sub>O<sub>2</sub> induced notable injury to L02 cells, as demonstrated by decreased cell viability, increased lactate dehydrogenase release, apoptotic rate and intracellular ROS production, and by the loss of MMP. Conversely, diosmetin

(20-40  $\mu$ M) significantly reversed the damaging effects of H<sub>2</sub>O<sub>2</sub>, which indicated that diosmetin may exhibit potent hepatoprotective potential against H<sub>2</sub>O<sub>2</sub>-induced oxidative damage. Furthermore, pretreatment with diosmetin elevated mRNA and protein expression levels of Nrf2, HO-1 and NQO1. The present study is the first, to the best of our knowledge, to demonstrate that activation of the Nrf2/NQO1-HO-1 signaling pathway maybe involved in the cytoprotective effects of diosmetin against oxidative stress. Therefore, diosmetin may be considered a promising therapeutic agent for the treatment of various liver diseases associated with oxidative stress.

## Introduction

Oxidative stress occurs when redox homeostasis is disrupted, which is usually accompanied by damaging effects to cell survival. Additionally, oxidative stress has been implicated in various pathologies, including liver diseases, neurodegenerative diseases, cardiovascular diseases, cancer and diabetes (1-3). Overproduction of reactive oxygen species (ROS) is considered to serve a prominent role in oxidative stress; high concentrations of ROS may result in cell death and damage to cellular structures involving DNA, lipids and protein. Generally, the cellular antioxidant defense system counterbalances ROS production to maintain an appropriate balance between oxidants and antioxidants (4). Therefore, antioxidant therapy may be one strategy to prevent cells from excessive exposure to oxidative stress and correct cellular redox homeostasis (5).

Recent studies have demonstrated that the transcription factor nuclear factor erythroid 2-related factor 2 (Nrf2) tightly regulates the cellular antioxidant system (6-8). Nrf2 binds to and mediates the activation of antioxidant response element (ARE)-dependent antioxidant target genes, including heme oxygenase-1 (HO-1), NAD(P)H quinone oxidoreductase-1 (NQO1), superoxide dismutase (SOD1 and 2), catalase, glutathione peroxidase (GPx)1, GPx2, GPx4 and glutathione (6). The Nrf2-ARE signaling pathway is known to be one of the important ROS-induced physiological mechanisms in defense against oxidative damage (9). Therefore, the induction of Nrf2 and further upregulation of antioxidant genes is considered an important pathway to prevent diseases induced by oxidative

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stress, including liver diseases, such as hepatitis, alcoholic and non-alcoholic fatty liver diseases (10).

Previous studies have demonstrated that natural products, including flavonoids, may be used as regulators of the Nrf2-ARE signaling system in Nrf2 activation. Diosmetin (3',5,7-trihydroxy-4'-methoxyflavone) is a flavone initially found in the legume *Acacia farnesiana* Wild and *Olea europaea* L. leaves (11,12). Diosmetin occurs naturally in various sources, including citrus fruits, oregano and some specific medicinal herbs, including *Chrysanthemum morifolium*, *Origanum vulgare*, *Robiniapseudoacacia*, *Rosa agrestis* and *Lespedeza davurica* (13). Pharmacologically, diosmetin has been reported to exhibit antioxidant (14,15), antimicrobial (16), anti-inflammatory (17), anticancer (18) and estrogenic (19) activities, and is used in traditional Mongolian medicine to treat liver diseases (20). However, to date, very few studies have focused on the hepatoprotective effects of diosmetin against hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>)-induced liver cell damage, and the underlying molecular mechanism involved in the expression of antioxidant genes remains to be elucidated.

The present study aimed to demonstrate the protective effects of diosmetin against H<sub>2</sub>O<sub>2</sub>-induced oxidative stress in the normal human liver cell line L02 and to evaluate its role in activation of the Nrf2-ARE signaling pathway for cytoprotection.

## Materials and methods

**Cell culture and treatment.** Human normal hepatocytes (L02 cells) obtained from Nanjing Key Gen Biotech Co., Ltd. (Nanjing, China) were cultured in RPMI-1640 medium (HyClone; GE Healthcare, Chicago, IL, USA) supplemented with 10% fetal bovine serum (HyClone; GE Healthcare) and 100 U/ml penicillin-streptomycin at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub>. Diosmetin (Nanjing Zelang Medical Technological Co., Ltd., Nanjing, China) stock solution was prepared in dimethyl sulfoxide (DMSO) and diluted with RPMI-1640 medium (2.5, 5, 10, 20, 30 and 40 µM) prior to experimentation. Cells in the negative control group were treated with DMSO alone at a final concentration of <0.1% (v/v). The positive control was treated with Trolox (40 µM, Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) or t-BHQ (30 µM, Sigma-Aldrich; Merck KGaA). *In vitro* oxidative stress cell damage models were induced by 200 µM H<sub>2</sub>O<sub>2</sub> (Sigma-Aldrich; Merck KGaA).

**Cell viability, cell apoptosis and lactate dehydrogenase (LDH) leakage assays.** L02 cells were seeded in 96-well plates at a density of 5x10<sup>3</sup> cells/well and cultured overnight. Subsequently, cells were pretreated with various concentrations of diosmetin (0-40 µM), Trolox (40 µM) or t-BHQ (30 µM) for 24 h at 37°C prior to exposure to 200 µM H<sub>2</sub>O<sub>2</sub> for 6 h at 37°C. Cell viability was estimated using the Cell Counting kit-8 colorimetric assay (Dojindo Molecular Technologies, Inc., Kumamoto, Japan) according to the manufacturer's protocol. The release of LDH was evaluated using an LDH assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer's protocol. The proportions of apoptotic cells were evaluated using an Annexin V/fluorescein isothiocyanate staining kit (Beyotime Institute of Biotechnology, Haimen,

China) according to manufacturer's protocol. All cells were analyzed by flow cytometry (BD Accuri™ C6 1.0.264.21, BD Biosciences, San Jose, CA, USA).

**Measurement of intracellular ROS and mitochondrial membrane potential (MMP).** Intracellular ROS production was detected using an intracellular ROS assay kit (Beyotime Institute of Biotechnology) and MMP was measured using rhodamine 123 (Rh123; Sigma-Aldrich; Merck KGaA). Cells (5x10<sup>5</sup> cells/well) were pretreated with various concentrations of diosmetin and t-BHQ (30 µM) for 24 h at 37°C, and were then incubated with 200 µM H<sub>2</sub>O<sub>2</sub> for 6 h at 37°C. Following staining with 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA, in the ROS assay kit; 10 µM) for 20 min or Rh123 (1 µM) for 30 min at 37°C, cells were analyzed by flow cytometry (BD Accuri™ C6 1.0.264.21, BD Biosciences, San Jose, CA, USA), images of the stained cells were observed under an inverted fluorescence microscope (IX71; Olympus Corporation, Tokyo, Japan).

**Reverse transcription-quantitative polymerase chain reaction (RT-qPCR).** Total RNA was extracted from L02 cells using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) and was reverse transcribed into cDNA using a PrimeScript™ RT reagent kit (Takara Bio, Inc., Otsu, Japan) according to manufacturer's protocol. RT-qPCR was conducted using SYBR® Premix Ex Taq™ II kit (Takara Bio, Inc.) on an Applied Biosystems Quant Studio™ 6 Flex thermocycler (Thermo Fisher Scientific, Inc.). The RT-qPCR conditions were as follows: 95°C for 30 sec, 40 cycles of amplification (95°C for 5 sec, 60°C for 30 sec, and 72°C for 30 sec), and 95°C for 15 sec, 60°C for 1 min, 95°C for 15 sec. The PCR primers used were as follows: Nrf2 forward, 5'-GCGACGGAAAGAGTATGAGC-3', and reverse, 5'-ACCTGGGAGTAGTTGCGACA-3'; HO-1 forward, 5'-CTGACCCATGACACCAAGGAC-3', and reverse, 5'-AAAGCCCTACAGCAACTGTGCG-3'; NQO1 forward, 5'-GGCAGAAGAGCACTGATCGTA-3', and reverse, 5'-TGATGGGATTGAAGTTCATGGC-3'; and GAPDH forward, 5'-ACGGATTGTGTCGTATTGGG-3' and reverse, 5'-TGATTTTGGAGGGATCTCGC-3'. The 2<sup>-ΔΔC<sub>q</sub></sup> method was used for quantitative calculation (21).

**Western blot analysis.** Following treatments, cells were lysed using radioimmunoprecipitation assay lysis buffer (Beyotime Institute of Biotechnology). The lysates were centrifuged at 12,000 x g for 10 min at 4°C, and the supernatants were collected and stored at -80°C. Protein concentrations were determined using a Bicinchoninic Acid assay kit (Beyotime Institute of Biotechnology). Equivalent amounts of lysate protein (50 µg) were separated by 10% SDS-PAGE and were then transferred to polyvinylidene fluoride membranes (EMD Millipore, Billerica, MA, USA). The membranes were blocked with 5% skimmed milk powder diluted in TBS with Tween 20 at room temperature for 1 h. Then membranes were probed with monoclonal anti-Nrf2 (1:2,000; cat. no. ab62352), HO-1 (1:20,000; cat. no. ab68477), NQO1 (1:20,000; cat. no. ab80588; Abcam, Cambridge, UK) and β-actin primary antibodies (1:5,000; cat. no. T0022; Affinity Biosciences, Cincinnati, OH, USA) overnight at 4°C, and were then incubated with goat anti-rabbit (1:6,000; cat. no. 33101ES60)

or anti-mouse (1:6,000; cat. no. 33201ES60) horseradish peroxidase-conjugated secondary antibodies (YEASEN Biosciences, Shanghai, China; ww.yeasen.com) for 1 h at room temperature. Blots were visualized using an enhanced chemiluminescent method (EMD Millipore) and were analyzed using a gel image analysis system (Bio-Rad ChemiDoc XRS; Bio-Rad Laboratories, Inc., Hercules, CA, USA).

**Small interfering RNA (siRNA) assays.** Nrf2 siRNAs (cat. no. siB140820100848) and a negative control (cat. no. siP01001) were purchased from Guangzhou RiboBio, Co., Ltd. (Guangzhou, China). siRNAs (100 nM) were transfected into L02 cells for 24 h at 37°C using Lipofectamine® 2000 reagent (Invitrogen; Thermo Fisher Scientific, Inc.) prior to H<sub>2</sub>O<sub>2</sub>/diosmetin treatment. Subsequently, the expression levels of Nrf2, HO-1 and NQO1 were detected by western blotting;  $\beta$ -actin was used as an internal control.

**Statistical analysis.** Data are expressed as the mean  $\pm$  standard deviation, and the differences in mean values were analyzed by one-way analysis of variance followed by the least significant difference test.  $P < 0.05$  was considered to indicate a statistically significant difference. Data were analyzed using SPSS version 16.0 (SPSS, Inc., Chicago, IL, USA).

## Results

**Diosmetin attenuates H<sub>2</sub>O<sub>2</sub>-induced L02 cell cytotoxicity.** The viability of cells treated with diosmetin alone was similar to that of the control group. However, compared with in the control group, cells exposed to 200  $\mu$ M H<sub>2</sub>O<sub>2</sub> for 6 h revealed a significant decrease in cell viability ( $54.7 \pm 6.9\%$ ;  $P < 0.01$ ). Conversely, the viability of cells pretreated with various concentrations of diosmetin (2.5, 5, 10, 20, 30 and 40  $\mu$ M) was restored in a dose-dependent manner; with the exception of the 2.5  $\mu$ M-treated group, the cell viability of the other diosmetin-treated groups were significantly increased compared with in the H<sub>2</sub>O<sub>2</sub>-treated group ( $P < 0.05$  and  $P < 0.01$ ). The cytoprotective effects of 20, 30 and 40  $\mu$ M diosmetin were similar to those exerted by the positive control 40  $\mu$ M Trolox, and there were no significant differences in cell viability among these groups ( $P > 0.05$ ; Fig. 1A).

As presented in Fig. 1B, the cellular LDH release assay demonstrated that the LDH levels in the culture medium of H<sub>2</sub>O<sub>2</sub>-treated cells were significantly increased compared with in the control group ( $P < 0.01$ ). The LDH levels were not markedly different between the control group and the group treated with diosmetin alone ( $P > 0.05$ ). Pretreatment with the lowest concentration of diosmetin (2.5  $\mu$ M) for 24 h prior to H<sub>2</sub>O<sub>2</sub> exposure significantly reduced LDH release ( $P < 0.01$ ), and diosmetin reduced LDH release in a dose-dependent manner (2.5–40  $\mu$ M). The highest concentration of diosmetin (40  $\mu$ M) exerted a similar effect to the positive control (40  $\mu$ M Trolox). These findings indicated that diosmetin exerted protective effects against H<sub>2</sub>O<sub>2</sub>-induced cytotoxicity, as demonstrated by LDH release and cell viability assays.

There were also significant differences in the rates of cell apoptosis and death among the various groups. In cells treated with 200  $\mu$ M H<sub>2</sub>O<sub>2</sub>, the cell apoptotic rate ( $47.1 \pm 5.5\%$ ) was much greater than in the control group ( $0.7 \pm 0.2\%$ ,

$P < 0.0001$ ). However, pretreatment with increasing concentrations of diosmetin (5, 15 and 30  $\mu$ M) significantly reduced H<sub>2</sub>O<sub>2</sub>-induced cell apoptosis in a concentration-dependent manner (Fig. 1C and D).

**Diosmetin inhibits H<sub>2</sub>O<sub>2</sub>-induced intracellular ROS accumulation and MMP loss.** To directly determine the production of intracellular ROS, DCFH-DA-labeled cells were measured using an inverted fluorescence microscope (Fig. 2A). The results demonstrated that the control group of cells exhibited very weak green fluorescence; however, the fluorescence intensity of H<sub>2</sub>O<sub>2</sub>-exposed cells was markedly enhanced. Conversely, diosmetin pretreatment reduced the effects of H<sub>2</sub>O<sub>2</sub> on fluorescence intensity.

As illustrated in Fig. 2B and C, when cells were treated with 200  $\mu$ M H<sub>2</sub>O<sub>2</sub> alone, the intracellular ROS level was more than three times that of the control group. However, pretreatment with increasing concentrations of diosmetin (5, 15 and 30  $\mu$ M) significantly attenuated H<sub>2</sub>O<sub>2</sub>-induced ROS accumulation in a concentration-dependent manner ( $P < 0.01$ ). In addition, 30  $\mu$ M diosmetin inhibited ROS accumulation to a similar level as that in the positive control group, which was treated with tertiary butylhydroquinone (t-BHQ, 30  $\mu$ M).

As presented in Fig. 3A and B, the MMP of L02 cells treated with diosmetin alone was similar to that of the control group. However, in L02 cells treated with 200  $\mu$ M H<sub>2</sub>O<sub>2</sub>, MMP was significantly decreased ( $68.5 \pm 5.3\%$ ) compared with in the control group ( $P < 0.01$ ). Conversely, pretreatment with diosmetin significantly prevented the loss of MMP in a dose-dependent manner ( $P < 0.05$  or  $P < 0.01$ ). Furthermore, 30  $\mu$ M diosmetin exhibited a similar inhibitory effect to 30  $\mu$ M t-BHQ. These results further supported the conclusion reached by observations made under fluorescence microscopy (Fig. 3C).

**Diosmetin upregulates Nrf2, NQO1 and HO-1 expression in H<sub>2</sub>O<sub>2</sub>-stressed L02 cells.** The Nrf2-ARE signaling pathway is known to serve a pivotal role in cellular defense against oxidative stress. Since diosmetin may attenuate H<sub>2</sub>O<sub>2</sub>-induced oxidative stress in L02 cells, it was hypothesized that treatment with diosmetin may activate expression of the transcription factor Nrf2 and ARE-dependent antioxidant target genes, including NQO1 and HO-1. Therefore, activation of Nrf2, NQO1 and HO-1 were investigated in diosmetin-treated L02 cells using western blot analysis and RT-qPCR. As expected, pretreatment with diosmetin dose-dependently increased the protein expression levels of Nrf2, NQO1 and HO-1. Notably, 30  $\mu$ M diosmetin induced significant protein accumulation of Nrf2, NQO1 and HO-1 compared with in the H<sub>2</sub>O<sub>2</sub>-treated group ( $P < 0.01$ ; Fig. 4A and B).

To assess the functional role of diosmetin and Nrf2 in H<sub>2</sub>O<sub>2</sub>-induced oxidative stress and damage, the present study investigated whether diosmetin may rescue the expression of Nrf2 inhibited by siRNA. The results revealed that transient inhibition of Nrf2 by siRNA resulted in significant downregulation of HO-1 and NQO1 in three groups (control, H<sub>2</sub>O<sub>2</sub> and diosmetin; Fig. 4C and D). However, treatment with 30  $\mu$ M diosmetin rescued the inhibitory effects of Nrf2 siRNA to a certain extent compared with in the H<sub>2</sub>O<sub>2</sub>-induced group, and increased the expression of Nrf2, HO-1 and NQO1 ( $P < 0.01$  or



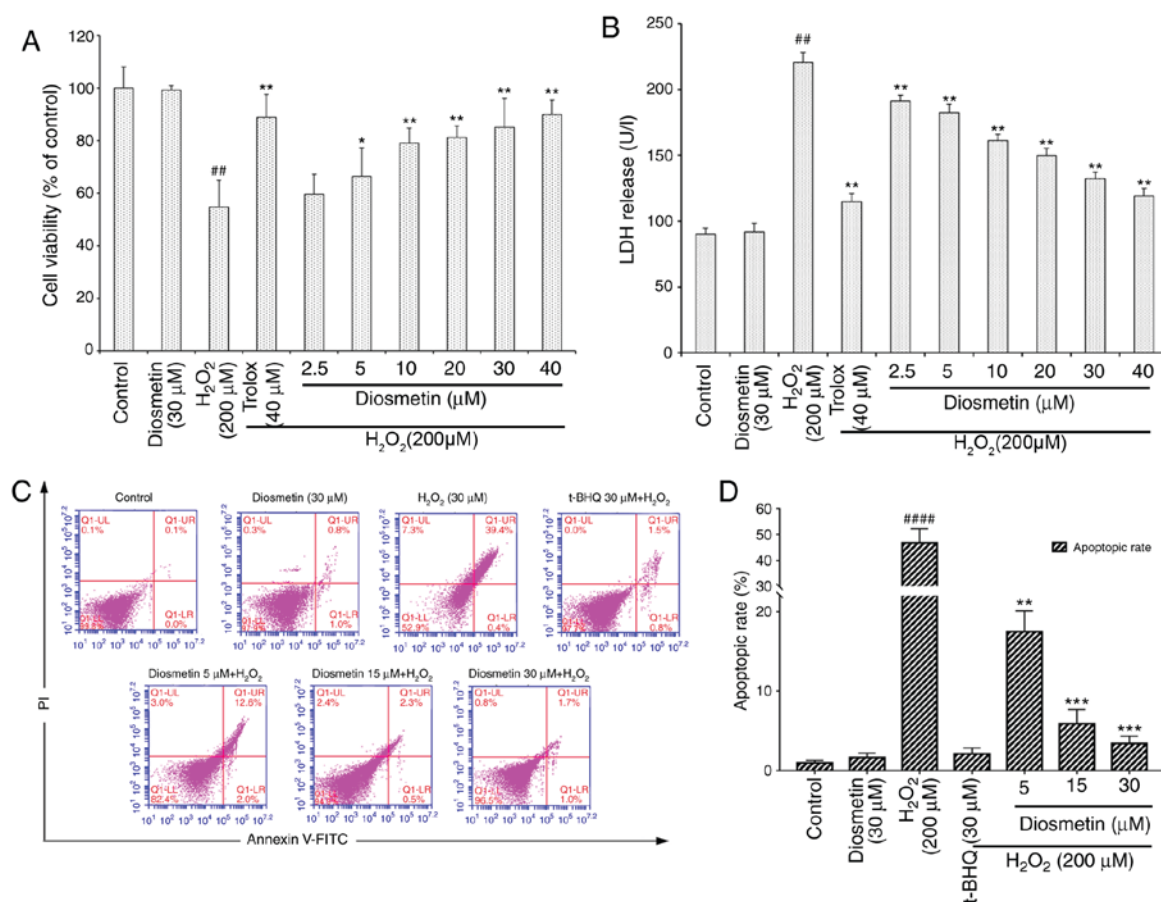


Figure 1. Protective effects of diosmetin on H<sub>2</sub>O<sub>2</sub>-induced L02 cell damage. (A) Cell viability was measured by Cell Counting kit-8 assay. (B) LDH levels in the culture medium were assessed using an LDH assay kit. (C) Apoptotic rates were detected using Annexin V-FITC/PI by flow cytometry. (D) Quantitative analysis of apoptotic rates. Data are expressed as the mean  $\pm$  standard deviation of four independent experiments. <sup>##</sup>P<0.01 and <sup>###</sup>P<0.0001 vs. the control group. <sup>\*</sup>P<0.05, <sup>\*\*</sup>P<0.01 and <sup>\*\*\*</sup>P<0.001 vs. the H<sub>2</sub>O<sub>2</sub> model group. FITC, fluorescein is thiocyanate; H<sub>2</sub>O<sub>2</sub>, hydrogen peroxide; LDH, lactate dehydrogenase; PI, propidium iodide; t-BHQ, tertiary butylhydroquinone.

P<0.05). Furthermore, pretreatment with diosmetin and t-BHQ also led to a significant increase in the mRNA expression levels of Nrf2, NQO1 and HO-1 in a dose-dependent manner (Fig. 4E).

Collectively, these data suggested that treatment with certain concentrations of diosmetin may activate the expression of Nrf2, which may regulate transcription of the antioxidant enzymes HO-1 and NQO1. Furthermore, increased expression of HO-1 and NQO1 may protect L02 cells from H<sub>2</sub>O<sub>2</sub>-induced oxidative stress and damage (Fig. 4F).

## Discussion

Oxidative stress has been reported to be involved in the pathogenesis of numerous human diseases, including hepatitis, alcoholic and non-alcoholic fatty liver diseases (2,22). It is widely believed that natural antioxidant products have broad protective effects against oxidative stress. Therefore, searching for natural antioxidant compounds with effective cytoprotective potential may provide novel therapeutic strategies for liver diseases. H<sub>2</sub>O<sub>2</sub>-induced cell injury is a broadly accepted cell model for evaluating the hepatoprotective effects of natural antioxidant compounds (23). The present study demonstrated that diosmetin may attenuate H<sub>2</sub>O<sub>2</sub>-induced L02 cell injury by increasing cell viability, decreasing LDH release and blocking

the loss of MMP. The protective effects of diosmetin against H<sub>2</sub>O<sub>2</sub>-induced L02 cell damage were associated with reduced ROS levels, activation of Nrf2 and upregulation of downstream phase II detoxifying enzymes, including HO-1 and NQO1. To the best of our knowledge, the present study is the first to demonstrate that diosmetin possessed potent hepatoprotective effects and suppressed numerous molecular events, which are implicated in oxidative stress, via activation of the ROS/Nrf2/NQO1-HO-1 signaling axis in human hepatocytes. Consequently, the findings of the present study indicated that diosmetin, as a natural antioxidant, may be used as a pharmacologically effective drug against oxidative liver disorders.

Two previous studies revealed that diosmetin exhibited antioxidant effects in other cell types. Ge *et al* (24) demonstrated that diosmetin may inhibit transforming growth factor- $\beta$ 1-induced intracellular ROS generation in human bronchial epithelial cells. Liao *et al* (14) reported that diosmetin may effectively attenuate 2,2-azobis(2-amidinopropane) dihydrochloride-induced erythrocyte hemolysis and CuCl<sub>2</sub>-induced plasma oxidation via the prevention of intracellular ROS generation. In addition, the antioxidant activity of diosmetin was revealed in a 1,1-diphenyl-2-picrylhydrazyl model system *in vitro* (15). However, to the best of our knowledge, there are no studies available to date regarding the effects of diosmetin on H<sub>2</sub>O<sub>2</sub>-induced oxidative stress in human

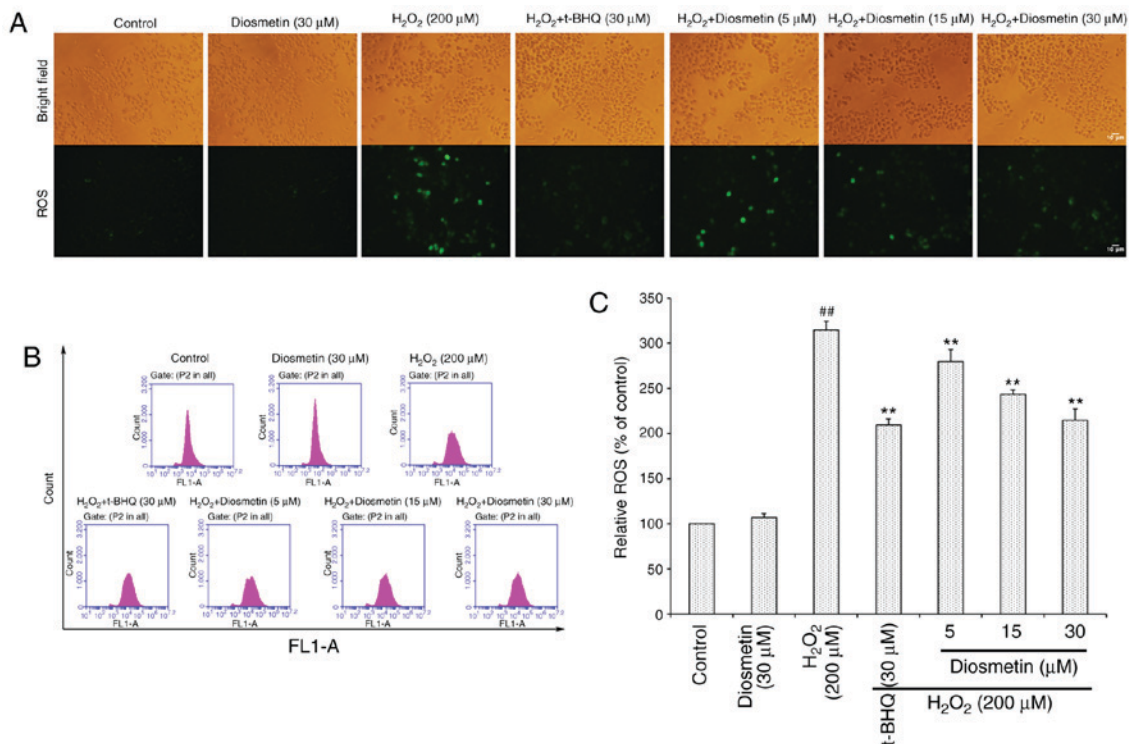


Figure 2. Detection of intracellular ROS. (A) Representative bright-field photomicrographs and photomicrographs of DCFH-DA fluorescence staining, as used to detect ROS production under an inverted fluorescence microscope. (B) Intracellular ROS levels were detected using DCFH-DA by flow cytometry. (C) Quantitative analysis of the mean DCF fluorescence intensity. Data are expressed as the means  $\pm$  standard deviation of three independent experiments. <sup>##</sup>P<0.01 vs. the control group. <sup>\*\*</sup>P<0.01 vs. the H<sub>2</sub>O<sub>2</sub> model group. DCFH-DA, 2',7'-dichlorodihydrofluorescein diacetate; H<sub>2</sub>O<sub>2</sub>, hydrogen peroxide; ROS, reactive oxygen species; t-BHQ, tertiary butylhydroquinone.

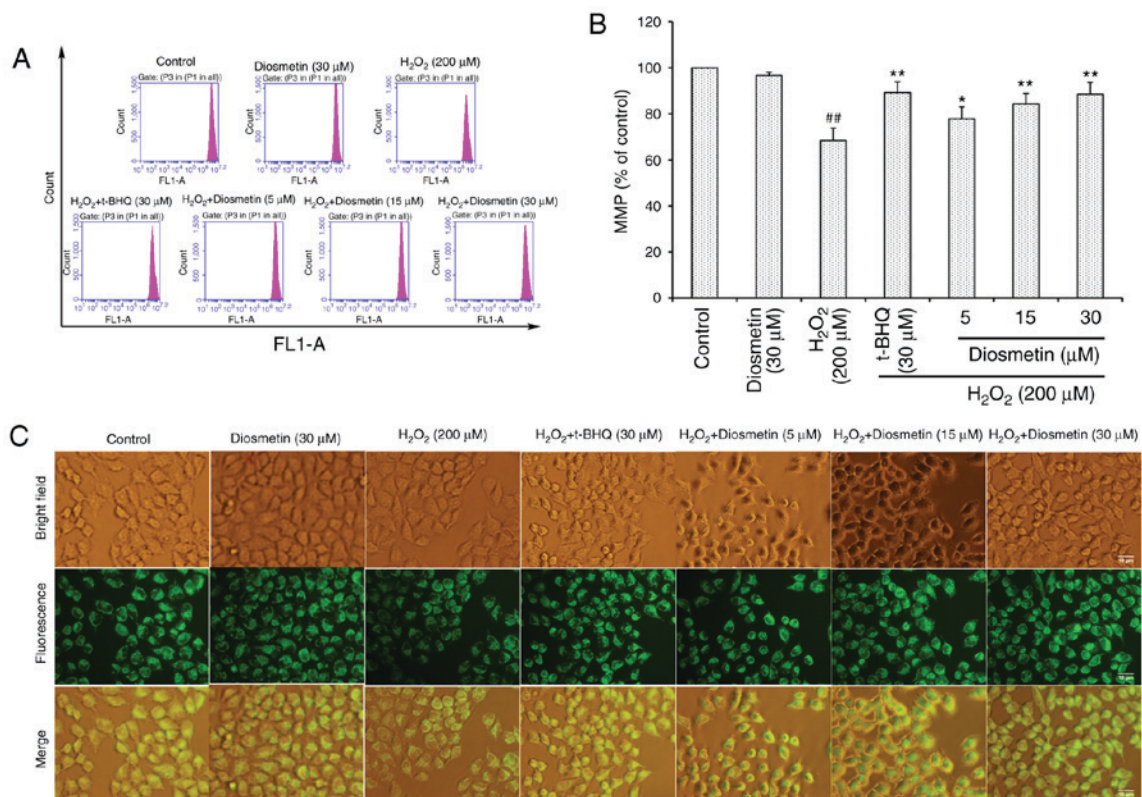


Figure 3. Effects of diosmetin on H<sub>2</sub>O<sub>2</sub>-induced MMP loss in L02 cells. (A) MMP was determined using rhodamine 123 by flow cytometry. (B) Quantitative analysis of mean rhodamine 123 fluorescence intensity. (C) Representative bright-field photomicrographs and photomicrographs of MMP fluorescence were observed under an inverted fluorescence microscope. Data are expressed as the mean  $\pm$  standard deviation of three independent experiments. <sup>##</sup>P<0.01 vs. the control group. <sup>\*</sup>P<0.05 and <sup>\*\*</sup>P<0.01 vs. the H<sub>2</sub>O<sub>2</sub> model group. H<sub>2</sub>O<sub>2</sub>, hydrogen peroxide; MMP, mitochondrial membrane potential; t-BHQ, tertiary butylhydroquinone.



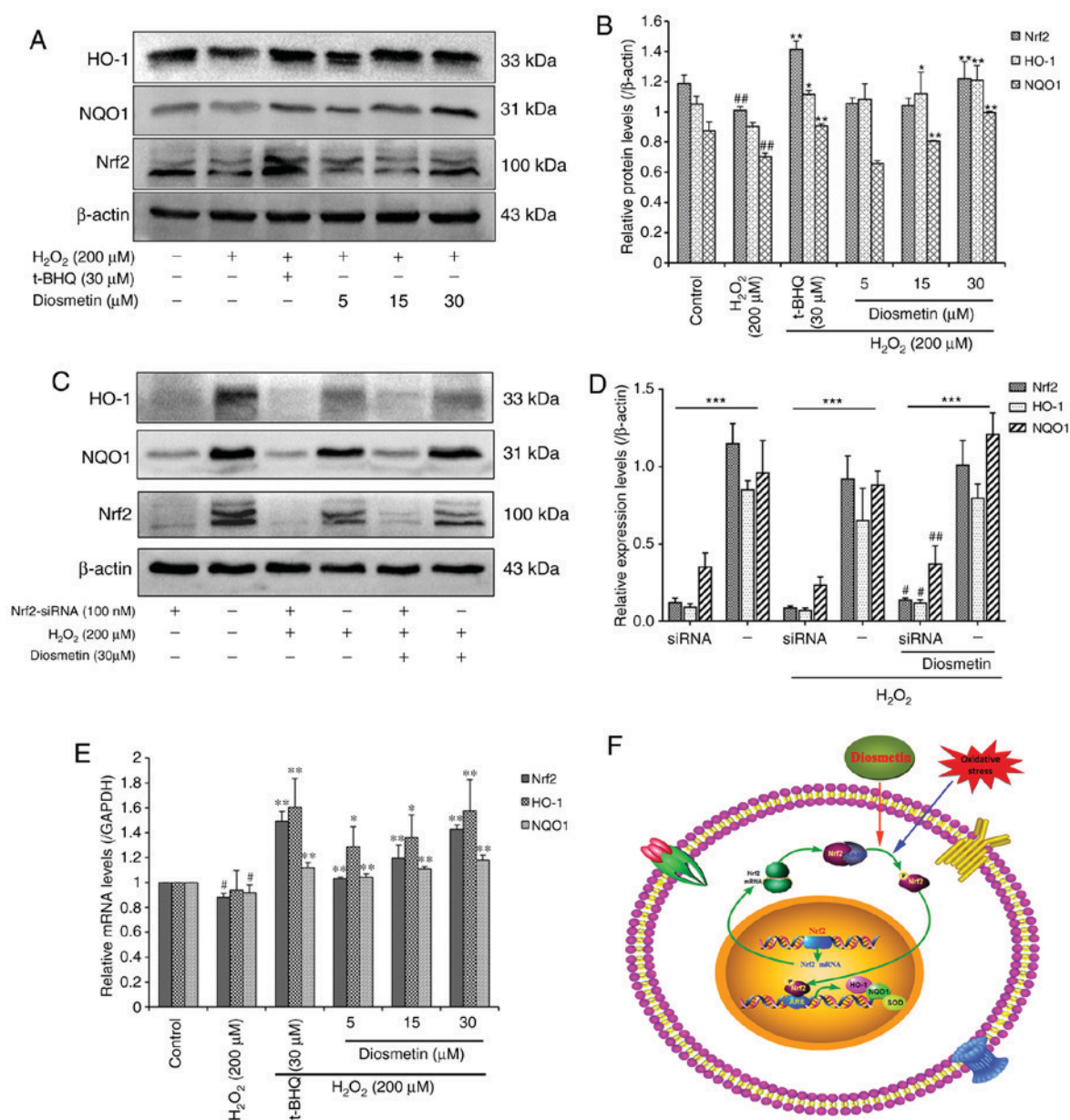


Figure 4. Effects of diosmetin on the expression levels of HO-1, NQO1 and Nrf2 in H<sub>2</sub>O<sub>2</sub>-induced L02 cells. (A) Relative protein expression levels of HO-1, NQO1 and Nrf2 were detected by western blotting. (B) Scanning densitometry was used for semi-quantitative analysis of western blotting. Data are presented as the mean  $\pm$  standard deviation of three independent experiments. <sup>#</sup>*P*<0.01 vs. the control group. <sup>\*</sup>*P*<0.05 and <sup>\*\*</sup>*P*<0.01 vs. the H<sub>2</sub>O<sub>2</sub> model group. (C) Expression levels of HO-1, NQO1 and Nrf2 following treatment with 100 nM Nrf2 siRNA and 30 μM diosmetin. (D) Scanning densitometry was used to semi-quantify the results of western blotting. Data are presented as the mean  $\pm$  standard deviation of three independent experiments. <sup>\*\*\*</sup>*P*<0.01 vs. Nrf2 siRNAs group with negative control. <sup>#</sup>*P*<0.05 and <sup>##</sup>*P*<0.01 vs. the H<sub>2</sub>O<sub>2</sub>+ Nrf2 siRNA group. (E) Relative mRNA expression levels of HO-1, NQO1 and Nrf2 were analyzed by reverse transcription-quantitative polymerase chain reaction. <sup>#</sup>*P*<0.05 vs. the control group. <sup>\*</sup>*P*<0.05 and <sup>\*\*</sup>*P*<0.01 vs. the H<sub>2</sub>O<sub>2</sub> model group. (F) Schematic representation of Nrf2-ARE signaling pathway activation by oxidative stress and diosmetin. In the cytoplasm, Keap1 inhibits the Nrf2 signaling pathway by promoting Nrf2 ubiquitination. When oxidative stress occurs in L02 cells, diosmetin facilitates the dissociation of Nrf2-Keap1, phosphorylation of Nrf2 and nuclear translocation. In the nucleus, Nrf2 promotes the expression of HO-1, NQO1 and SOD antioxidants by binding to the ARE regions. ARE, antioxidant response element; H<sub>2</sub>O<sub>2</sub>, hydrogen peroxide; HO-1, heme oxygenase-1; Keap1, Kelch-like ECH-associated protein 1; NQO1, NAD(P)H quinone oxidoreductase-1; Nrf2, nuclear factor erythroid 2-related factor 2; siRNA, small interfering RNA; SOD, superoxide dismutase; t-BHQ, tertiary butylhydroquinone.

liver cells. The results of the present study revealed that cells pretreated with diosmetin exhibited significantly increased cell viability and reduced LDH release compared with in cells exposed to H<sub>2</sub>O<sub>2</sub> alone, and the effects were similar to those of the positive control Trolox. Another property positive control t-BHQ was also used to demonstrate the protective effects of diosmetin. These results indicated that diosmetin exhibits an excellent antioxidant capacity to attenuate H<sub>2</sub>O<sub>2</sub>-induced oxidative stress in human hepatocytes.

Oxidative stress is considered to serve a marked role in the development of mitochondrial dysfunction, thus contributing to increased mitochondrial membrane permeability and resulting in depolarization of the MMP (25). Furthermore, a reduction in mitochondrial integrity may increase ROS production and decrease adenosine triphosphate production (26). In addition, high levels of ROS may in turn damage mitochondrial function, resulting in irreversible membrane damage and eventually cell death (27). The present study reported that

MMP was significantly decreased in H<sub>2</sub>O<sub>2</sub>-treated L02 cells, whereas diosmetin pretreatment reduced the loss of MMP in a dose-dependent manner, and the inhibitory effects of 30  $\mu$ M diosmetin were similar to those of the positive control (30  $\mu$ M t-BHQ). Therefore, the ability of diosmetin to maintain mitochondrial membrane integrity may be due to its ROS scavenging activity.

As a well-characterized oxidative stress inducer, H<sub>2</sub>O<sub>2</sub> may trigger intracellular ROS generation in various human cell lines (28). Additionally, H<sub>2</sub>O<sub>2</sub> is able to easily pass through the cell membrane via aquaporins or by simple diffusion, and evoke lipid peroxidation, and DNA and protein damage, which result in significant oxidative damage (29). The present study confirmed that cells exposed to H<sub>2</sub>O<sub>2</sub> generated a large amount of ROS in L02 cells; however, when L02 cells were pretreated with diosmetin, the H<sub>2</sub>O<sub>2</sub>-induced intracellular ROS accumulation was significantly attenuated. Therefore, the protective effects of diosmetin against H<sub>2</sub>O<sub>2</sub>-induced cytotoxicity may be mainly attributed to its ROS scavenging capacity.

It has previously been indicated that antioxidants may exhibit their antioxidant activity not by directly scavenging intracellular oxidants, but by inducing the endogenous antioxidant defense system (30). Activation of the antioxidant system is known to serve a significant role in cellular defense against oxidative impacts; detoxifying enzymes, including HO-1 and NQO1, which are regulated by Nrf2, are important parts of the system (4). Nrf2, which is a member of the cap 'n' collar family, is a basic leucine zipper transcription factor that serves as a critical regulator of antioxidants and detoxifying enzymes, in order to protect against oxidative stress-induced cell damage and apoptosis (31). When stimulated by inducers, Nrf2 is released from its cytosolic inhibitor, Kelch-like ECH-associated protein 1, after which translocates into the nucleus and binds to the ARE to promote the expression of numerous phase II enzymes, including NQO1 and HO-1 (8,32). Since the Nrf2/ARE signaling pathway has been reported to offer protection against oxidative damage, the induction of NQO1 and HO-1 regulated by the Nrf2/ARE signaling pathway may provide a therapeutic strategy for liver diseases in cases of oxidative stress (33). However, the regulatory mechanisms involved in mediating Nrf2 activation are not yet fully understood. The present study hypothesized that increased expression of NQO1 and HO-1 may be dependent upon activation of the Nrf2/ARE signaling pathway. As expected, the mRNA and protein expression levels of Nrf2 were increased in a dose-dependent manner in diosmetin-pretreated L02 cells, and the mRNA and protein expression levels of NQO1 and HO-1 were also dose-dependently increased. Collectively, the results of the present study indicated that diosmetin-mediated protection against H<sub>2</sub>O<sub>2</sub>-induced L02 cell injury may be attributed to upregulation of HO-1 and NQO1 via the Nrf2/ARE signaling pathway; to the best of our knowledge, the present study is the first to reveal activation of the Nrf2/ARE signaling pathway by diosmetin.

In conclusion, the present study demonstrated that diosmetin may exert hepatoprotective effects against H<sub>2</sub>O<sub>2</sub>-induced L02 cell damage by upregulating the expression of NQO1 and HO-1 via Nrf2 activation, which may contribute to the

suppression of ROS generation and increased MP. Therefore, the findings of the present study provided a scientific basis for the hepatoprotective effects of diosmetin and suggested that it may be used as a promising natural protective agent for the treatment of various liver diseases associated with oxidative stress.

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#### Availability of data and materials

The analyzed data sets generated during the study are available from the corresponding author on reasonable request.

#### Authors' contributions

CW, YL and CL drafted the paper and participated in the data analysis. SW and DW performed the RT-qPCR and western blot analysis. NW and QX performed the cell viability, cell apoptosis and LDH leakage assays. WJ measured the intracellular ROS. MQ measured the MMP. All authors read and approved the final manuscript.

#### Ethics approval and consent to participate

Not applicable.

#### Consent for publication

Not applicable.

#### Competing interests

The authors declare that they have no competing interests.

#### References

1. Zhang H, Davies KJA and Forman HJ: Oxidative stress response and Nrf2 signaling in aging. *Free Radic Biol Med* 88: 314-336, 2015.
2. Webb C and Twedt D: Oxidative stress and liver disease. *Vet Clin North Am Small Anim Pract* 38: 125-135, v, 2008.
3. Zhang B, Dong JL, Chen YL, Liu Y, Huang SS, Zhong XL, Cheng YH and Wang ZG: Nrf2 mediates the protective effects of homocysteine by increasing the levels of GSH content in HepG2 cells. *Mol Med Rep* 16: 597-602, 2017.
4. Espinosa-Diez C, Miguel V, Mennerich D, Kietzmann T, Sánchez-Pérez P, Cadenas S and Lamas S: Antioxidant responses and cellular adjustments to oxidative stress. *Redox Biol* 6: 183-197, 2015.
5. Hu Y, Wang S, Wang A, Lin L, Chen M and Wang Y: Antioxidant and hepatoprotective effect of *Penthorum chinense* Pursh extract against t-BHP-induced liver damage in L02 cells. *Molecules* 20: 6443-6453, 2015.

6. Leiser SF and Miller RA: Nrf2 signaling, a mechanism for cellular stress resistance in long-lived mice. *Mol Cell Biol* 30: 871-884, 2010.
7. Yan B, Ma Z, Shi S, Hu Y, Ma T, Rong G and Yang J: Sulforaphane prevents bleomycin-induced pulmonary fibrosis in mice by inhibiting oxidative stress via nuclear factor erythroid 2-related factor-2 activation. *Mol Med Rep* 15: 4005-4014, 2017.
8. Han MH, Park C, Lee DS, Hong SH, Choi IW, Kim GY, Choi SH, Shim JH, Chae JI, Yoo YH and Choi YH: Cytoprotective effects of esculetin against oxidative stress are associated with the upregulation of Nrf2-mediated NQO1 expression via the activation of the ERK pathway. *Int J Mol Med* 39: 380-386, 2017.
9. Nguyen T, Nioi P and Pickett CB: The Nrf2-antioxidant response element signaling pathway and its activation by oxidative stress. *J Biol Chem* 284: 13291-13295, 2009.
10. Tang W, Jiang YF, Ponnusamy M and Diallo M: Role of Nrf2 in chronic liver disease. *World J Gastroenterol* 20: 13079-13087, 2014.
11. Garavito G, Rincón J, Arteaga L, Hata Y, Bourdy G, Gimenez A, Pinzón R and Deharo E: Antimalarial activity of some Colombian medicinal plants. *J Ethnopharmacol* 107: 460-462, 2006.
12. Meirinhos J, Silva BM, Valentão P, Seabra RM, Pereira JA, Dias A, Andrade PB and Ferreres F: Analysis and quantification of flavonoid compounds from Portuguese olive (*Olea europaea* L.) leaf cultivars. *Nat Prod Res* 19: 189-195, 2005.
13. Patel K, Gadewar M, Tahilyani V and Patel DK: A review on pharmacological and analytical aspects of diosmetin: A concise report. *Chin J Integr Med* 19: 792-800, 2013.
14. Liao W, Ning Z, Chen L, Wei Q, Yuan E, Yang J and Ren J: Intracellular antioxidant detoxifying effects of diosmetin on 2,2-azobis(2-amidinopropane) dihydrochloride (AAPH)-induced oxidative stress through inhibition of reactive oxygen species generation. *J Agric Food Chem* 62: 8648-8654, 2014.
15. Bai N, Zhou Z, Zhu N, Zhang L, Quan Z, HE K, Zhang QY and Ho CH: Antioxidative flavonoids from the flower of *Inula Britannica*. *J Food Lipid* 12: 141-149, 2005.
16. Meng JC, Zhu QX and Tan RX: New antimicrobial mono- and sesquiterpenes from *Soroseris hookeriana* subsp. *erysimoides*. *Planta Med* 66: 541-544, 2000.
17. Domínguez M, Avila JG, Nieto A and Céspedes CL: Anti-inflammatory activity of *Penstemon gentianoides* and *Penstemon campanulatus*. *Pharm Biol* 49: 118-124, 2011.
18. Liu B, Shi Y, Peng W, Zhang Q, Liu J, Chen N and Zhu R: Diosmetin induces apoptosis by upregulating p53 via the TGF- $\beta$  signal pathway in HepG2 hepatoma cells. *Mol Med Rep* 14: 159-164, 2016.
19. Androutsopoulos V, Wilsher N, Arroo RR and Potter GA: Bioactivation of the phytoestrogen diosmetin by CYP1 cytochromes P450. *Cancer Lett* 274: 54-60, 2009.
20. Obmann A, Werner I, Presser A, Zehl M, Swoboda Z, Purevsuren S, Narantuya S, Kletter C and Glasl S: Flavonoid C- and O-glycosides from the Mongolian medicinal plant *Dianthus versicolor* Fisch. *Carbohydr Res* 346: 1868-1875, 2011.
21. Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. *Methods* 25: 402-408, 2001.
22. Marí M, Colell A, Morales A, von Montfort C, Garcia-Ruiz C and Fernández-Checa JC: Redox control of liver function in health and disease. *Antioxid Redox Signal* 12: 1295-1331, 2010.
23. Senthil Kumar KJ, Liao JW, Xiao JH, Gokila Vani M and Wang SY: Hepatoprotective effect of lucidone against alcohol-induced oxidative stress in human hepatic HepG2 cells through the up-regulation of HO-1/Nrf-2 antioxidant genes. *Toxicol In Vitro* 26: 700-708, 2012.
24. Ge A, Ma Y, Liu YN, Li YS, Guo H, Zhang JX, Wang QX, Zeng XN and Huang M: Diosmetin prevents TGF-beta1-induced epithelial-mesenchymal transition via ROS/MAPK signaling pathways. *Life Sci* 153: 1-8, 2016.
25. Beal MF: Mitochondria take center stage in aging and neurodegeneration. *Ann Neurol* 58: 495-505, 2005.
26. Somayajulu M, McCarthy S, Hung M, Sikorska M, Borowy-Borowski H and Pandey S: Role of mitochondria in neuronal cell death induced by oxidative stress; neuroprotection by Coenzyme Q10. *Neurobiol Dis* 18: 618-627, 2005.
27. Dumont M and Beal MF: Neuroprotective strategies involving ROS in Alzheimer disease. *Free Radic Biol Med* 51: 1014-1026, 2011.
28. Zorov DB, Filburn CR, Klotz LO, Zweier JL and Sollott SJ: Reactive oxygen species (ROS)-induced ROS release: A new phenomenon accompanying induction of the mitochondrial permeability transition in cardiac myocytes. *J Exp Med* 192: 1001-1014, 2000.
29. Sies H: Role of metabolic H<sub>2</sub>O<sub>2</sub> generation: Redox signaling and oxidative stress. *J Biol Chem* 289: 8735-8741, 2014.
30. Li T, Chen B, Du M, Song J, Cheng X, Wang X and Mao X: Casein glycomacropeptide hydrolysates exert cytoprotective effect against cellular oxidative stress by up-regulating HO-1 expression in HepG2 cells. *Nutrients* 9: pii: E31, 2017.
31. Huang Y, Li W, Su Z and Kong AN: The complexity of the Nrf2 pathway: Beyond the antioxidant response. *J Nutr Biochem* 26: 1401-1413, 2015.
32. Suzuki T and Yamamoto M: Molecular basis of the Keap1-Nrf2 system. *Free Radic Biol Med* 88: 93-100, 2015.
33. Ma Z, Li C, Qiao Y, Lu C, Li J, Song W, Sun J, Zhai X, Niu J, Ren Q and Wen A: Safflower yellow B suppresses HepG2 cell injury induced by oxidative stress through the AKT/Nrf2 pathway. *Int J Mol Med* 37: 603-612, 2016.