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Dihydroquercetin (DHQ) Induced HO-1 and NQO1 Expression against Oxidative Stress through the Nrf2-Dependent Antioxidant Pathway

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ABSTRACT: Dihydroquercetin (DHQ) is a well-known antioxidant agent. In the present investigation, we reported for the first time that DHQ stimulates the expression of phase II detoxifying enzymes through the Nrf2-dependent signaling pathway. The IC_{50} values of DHQ for reduction of 2,2-diphenyl-1-picrylhydrazol (DPPH), reducing power assay, lipid peroxidation assay, and xanthine oxidase inhibition were 5.96, 4.31, 2.03, and 13.24 μM, respectively. DHQ possessed considerable protective activity from oxidative DNA damage. A luciferase reporter assay also demonstrated that DHQ-activated signaling resulted in the increased transcriptional activity of Nrf2 through binding to the ARE (antioxidant response element) enhancer sequence. Furthermore, Western blotting and luciferase assay revealed DHQ activated ERK1/2, Akt, and JNK signaling pathways, subsequently leading to Nrf2 nuclear translocation. DHQ upregulated the Nrf2-related antioxidant genes heme oxygenase-1 (HO-1), NAD(P)H quinone oxidoreductase-1 (NQO1), and glutamate-cysteine ligase modifier subunits. Inhibition of Nrf2 by siRNA reduced DHQ-induced upregulation of these antioxidant genes.

KEYWORDS: *dihydroquercetin, antioxidant activity, Nrf2, antioxidant genes*

INTRODUCTION

Oxidative stress can be viewed as imbalance between prooxidants and antioxidants. Both excess production of reactive oxygen species (ROS) and deficiency in cellular antioxidant defenses (e.g., glutathione) result in endogenous oxidative stress. Increased oxidative stress is associated with cardiovascular risk factors such as hypercholesterolemia, hypertension, diabetes mellitus, and chronic renal failure.[1] Furthermore, reactive oxygen species are principal mediators of cardiomyocite dysfunction in various pathologic conditions including coronary heart disease and heart and renal failure.[2,3] Antioxidants are believed to be important in health maintenance by modulation of oxidative processes in the body.[4]

Flavonoids are widespread in many plant species and have a variety of beneficial biological effects,[5] including protection of cells against oxidative stress.[4] Dihydroquercetin (2-(3,4-dihydroxyphenyl)-2,3-dihydro-3,5,7-trihydroxy-4*H*-benzopyran-4-one) is a dihydroflavonol which eliminates free radicals in the body, improves the impermeability of capillary vessels, and recovers their elasticity effectively. Dihydroquercetin has a distinguished better antioxidant activity compared to other antioxidants.[6] In our preliminary study, we obtained an economically and environmentally friendly method for the extraction of dihydroquercetin from wood sawdust of *Larix gmelini* (Rupr.) Rupr.[7]

Many electrophilic compounds isolated from a plant-based diet activate the expression of phase II and antioxidant enzymes via the Nrf2−ARE signaling pathway.[8] The nuclear factor erythoid 2-related factor 2 (Nrf2) serves as a master regulator of cellular defense against oxidative stress. Under physiological conditions, Nrf2 is sequestered in the cytoplasm by Kelch-like ECH-associated protein 1 (Keap1) with Cullin 3-base E3 ligase, by which Nrf2 protein is ubiquitinylated and targeted for proteosome degradation.[9] Upon exposure to ROS, the sequestration complex breaks down and dissociated Nrf2 translocates into the nucleus, where it binds to cis-acting antioxidant-responsive elements (AREs) and promotes the transcription of a broad range of cytoprotective genes.[10] These genes encode enzymes that provide antioxidants, such as heme oxygenase-1,[11] glutamate cysteine ligase catalytic subunit (Gclc) and for glutathione (GSH) biosynthesis,[12] glutathione S-transferases (GSTs) [12] for detoxification of xenobiotics and electrophiles, NAD(P)H:quinione oxidoreductase 1 (NQO1), as well as multidrug transporters that efflux toxic metabolites.[13]

In the present study, we compared the antioxidant activity of dihydroquercetine (DHQ) and quercetin by reduction of 2,2-diphenyl-1-picrylhydrazol (DPPH), reducing power, and lipid peroxidation assays and xanthine oxidase inhibitory effect assays. Additionally, the protection from oxidative DNA damage was investigated. To the best of our knowledge, it has never been investigated whether DHQ exerts antioxidant

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effects by the Nrf2-dependent antioxidant pathway. HepG2 cells, a human hepatoma cell line, are considered as a good model to study in vitro xenobiotic metabolism and toxicity to the liver. HepG2 cells retain the activity of many phase I, phase II, and antioxidant enzymes; they can be constituted as a good tool to study cytoprotective, genotoxic, and antigenotoxic effects of active compounds,[14] HepG2 cells were selected for further studies in the present study. Our study provided evidence of a protective role for DHQ against oxidative injury via the Nrf2/ARE pathway. Moreover, the nuclear and cytoplasmic expression of Nrf2, expression of HO-1, NQO1, GCLC, and GCLM, as well as Akt, ERK, and JNK signaling pathways proteins were assayed. We also examined the involvement of GSH in the response of HepG2 cells upon DHQ treatment.

MATERIALS AND METHODS

Chemicals. Dihydroquercetin (purity ≥98%) (2*R*,3*R*)-2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy-2,3-dihydrochromen-4-one was isolated from wood sawdust of *Larix gmelini (Rupr.) Rupr.*, and the chemical structure was identified in our laboratory.[7] A 10 mg/mL stock solution of DHQ was prepared in dimethyl sulfoxide (DMSO) and stored at −80 °C.

Quercetin, allopurinol, dimethylsulfoximine (DMSO), xanthine oxidase, MTT (3-[4,5-dimehyl-2-thiazolyl]-2,5-diphenyl-2*H*-tetrazolium bromide), dimethylsulfoximine (DMSO), and propidium iodide (PI) were purchased from Sigma (St. Louis, MO). PD98059 (an MEK inhibitor), SP600125 (a JNK inhibitor), and LY294002 (a PI3K inhibitor) were purchased from Beijing Chemical Reagents Co. (Beijing, China). Other reagents and chemicals were of analytical grade and purchased from Beijing Chemical Reagents Co. (Beijing, China). Deionized water was produced by a Millipore Direct-Q purification system (Millipore Corp., Bedford, MA).

2,2-Diphenyl-1-picrylhydrazol (DPPH) Radical Scavenging Assay. The DPPH radical scavenging activities of the isolated flavonoids were tested. In brief, 10 μL of the sample (final concentrations were 0.5, 1, 5, 10, and 50 μM, respectively) was mixed with ethanol (90 μL) and then added to 0.004% DPPH (200 μL) in ethanol. The mixture was vigorously shaken and then immediately placed in a UV−vis spectrophotometer (UNICO) to monitor the decrease in absorbance at 517 nm. Monitoring was continued for 70 min until the reaction reached a plateau. Ascorbic acid (Sigma-Aldrich), a stable antioxidant, was used as a synthetic reference. Pure ethanol was used as a control sample. IC_{50} values denote the concentrations required to scavenge 50% of the free radicals generated by DPPH inhibition (%) = [(AB − AA)/AB] × 100, where AB and AA are the absorbance values of the blank sample and of the tested samples checked after 70 min, respectively.

Reducing Power Assay. Briefly, 0.5 mL of ethanol containing different concentrations of DHQ (final concentrations were 0.5, 1, 5, 10, 50, and 100 μM) was mixed with 0.5 mL of phosphate buffer (0.2 M, pH 6.6) and 0.5 mL of potassium ferricyanide (1%). The reaction mixture was incubated at 50 °C for 20 min. After 2.5 mL of 10% trichloroacetic acid was added, the mixture was centrifuged at 650 rpm for 10 min. From the upper layer, 0.5 mL of solution was mixed with 0.5 mL of distilled water and 0.1 mL of $FeCl_3$ (0.1%). DHQ providing 0.5 nm of absorbance (IC_{50}) was calculated from the graph of absorbance at 700 nm. Higher absorbance indicated higher reducing power.

Lipid Peroxidation Assay. Lipid peroxidation was induced by addition of 100 μL of 0.5 mM $FeSO_4$ in the reaction mixture containing PBS and chopped tissue and by incubating at 37 °C for 2 h. Liver slices were incubated with 100 μL of 0.5 mM $FeSO_4$ and DHQ concentrations of 0.5, 1, 5, 10, or 50 μM dissolved in DW. After 2 h, a homogenate of chopped liver was centrifuged at 800*g*, and the supernatant was used to measure lipid peroxidation. A control set was run in which all materials other than $FeSO_4$ or DHQ were added. Equivalent concentrations of quercetin (0.5, 1, 5, 10, and 50 μM) were used as a standard antioxidant for a comparison. The inhibition percentage of lipid peroxidation of the sample was calculated by the following equation: inhibition percentage (%) = [1 − (Ab532 sample/Ab532 control)] × 100, where Ab532 was the absorbance at 532 nm.

Determination of Xanthine Oxidase (XOD) Inhibitory Activity. In order to test the XOD inhibitory activity of DHQ, the XOD activity with xanthine as substrate was measured with a spectrophotometer at λ_{max} = 295 nm. First, 798 μL of 0.1 units XOD in buffer (200 mM sodium pyrophosphate/HCl, pH 7.5) and 2 μL (0.5, 1, 5, 10, and 50 μM) of DHQ in DMSO were mixed at 37 °C for 5 min. The control group did not contain test agent. The reaction was started by adding 200 μL of 0.6 mM xanthine in double distilled water to the mixture. The reaction mixture was incubated at ambient temperature. Finally, the absorption increments at 295 nm indicating the formation of uric acid were determined every minute up to 8 min. Quercetin was used as a positive control. Three replicates were made for each test sample. The percent inhibition ratio (%) was calculated according to the following equation: % inhibition = [(rate of control reaction − rate of sample reaction)/rate of control reaction] × 100.

DNA Damage Protection Activity. To perform the DNA nicking assay, a supercoiled pBR322 plasmid DNA was initially prepared. A mixture of 10 μL of DHQ (10, 20, and 30 μM) and plasmid DNA (0.5 μg) was incubated for 10 min at room temperature followed by addition of 10 μL of Fenton's reagent (30 mM H_2O_2, 50 μM ascorbic acid, and 80 μM $FeCl_3$). The final volume of the mixture was brought up to 20 mL. The mixture was incubated for 30 min at 37 °C. The DNA was analyzed on 1% agarose gel using ethidium bromide staining.

Cell Culture. The human hepatocarcinoma (HepG2) cell line was purchased from Harbin Medical University, China, and maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum and 100 U/mL penicillin and 100 μg/mL streptomycin (Beyotime Institute of Biotechnology, Beijing, China). The cells were kept at 37 °C in a humidified atmosphere containing 5% CO_2.

MTT Proliferation Assay. Inhibition of cell proliferation by cajanol was measured by the MTT assay. HepG2 cells were plated in 96-well plates at a density of 1×10^4 cells per well. After incubation with medium for 24 h, cells were incubated with various concentrations of DHQ for 48h . Then, MTT was added to cell cultures at a final concentration of 0.5 mg/mL. After 4 h of incubation, the adherent cells were solubilized with 100 μL of DMSO. Absorbance at 570 nm was measured using an ELISA reader.

Western Blotting. For isolation of total protein fractions, media were removed and cells were washed twice with ice-cold PBS and then lysed using cell lysis buffer [20 mM Tris pH 7.5, 150 mM NaCl, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM EDTA, 1% Na_3CO_4, 0.5 μg/mL leupeptin, 1 mM phenylmethanesulfonyl fluoride (PMSF)]. The lysates were collected by scraping from the plates and then centrifuged at 10 000 rpm at 4 °C for 5 min.

Total protein samples (20 μg) were loaded on 12% SDS polyacrylamide gels for electrophoresis, and then transferred onto PVDF transfer membranes (Millipore, Billerica, MA) at 0.8 mA/cm^2 for 2 h. Membranes were blocked at room temperature for 2 h with blocking solution (1% BSA in PBS plus 0.05% Tween-20). Membranes were then incubated overnight at 4 °C with primary antibodies (anti-Nrf2, anti-GCLC, anti-GCLM, anti-HO-1, anti-NQO1, anti-Akt, anti-JNK, anti-ERK 1/2, antiphospho-JNK (Thr183/Tyr185), antiphospho-Akt (Ser473), antiphospho-ERK 1/2 (Thr158 + Tyr187 + Thr202 + Tyr204), or anti-β-actin) at a dilution of 1:250 (Biosynthesis Biotechnology Company, Beijing, China) in blocking solution. After thrice washings in TBST (Tris buffered saline with Tween 20) for 5 s each, membranes were incubated for 1 h at room temperature with alkaline phosphatase peroxidase-conjugated antimouse secondary antibody (1:500 dilution) in blocking solution. Detection was performed by the BCIP/NBT Alkaline Phosphatase Color Development Kit (Beyotime Institute of Biotechnology, Beijing, China) according to the manufacturer's instructions. Bands were then recorded by a digital camera (Canon, EOS 350D, Tokyo, Japan).

Nuclear and Cytoplasmic Extractions. Nuclear and cytoplasmic extractions were performed using the Nuclear and Cytoplasmic

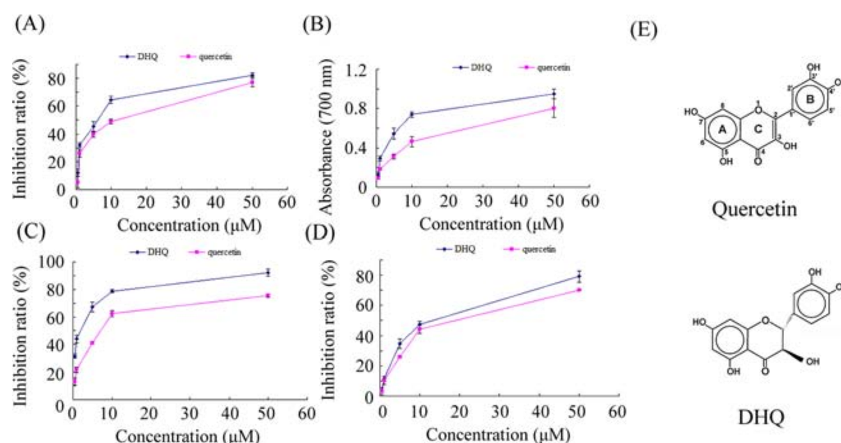

Figure 1. (A) Radical-scavenging activities were assessed by measuring the DPPH scavenging activities. (B) In the reducing power assay, the absorbance values at 700 nm of DHQ and quercetin were assessed. (C) The inhibitory effect of DHQ on lipid peroxidation of liposomes compared with quercetin. (D) XOD inhibitory activity of DHQ compared with quercetin. Values of each curve are mean values ± SD ($n = 3$). $p < 0.01$. (E) Structure of dihydroquercetine (DHQ) and quercetin.

Extraction kit (Biosynthesis Biotechnology Company, Beijing, China) following manufacturer's instructions. Then, nuclear and cytoplasmic proteins were assayed by Western blotting, as described above.

Transfection and Luciferase Reporter Assay. The vector was constructed as follows. Single-stranded oligonucleotides contained the GCLC ARE core sequence, (5′-CCCGTGACTCAGCGCTCCGTG-ACTCAGCGCTCCGTGACTCAGCGCT-3′; the ARE4 sequence is underlined[15]). A DNA fragment containing three copies of the ARE4 elements from GCLC gene was subcloned into a pGL3-promoter vector to construct pGL3-ARE4-Luc. HepG2 cells were transiently transfected with DNA mixture using the Effectane transfection reagent (QIAGEN) according to the manufacturer's published instructions. At 24 h following transfection, the cells were treated with 30 μM DHQ for 48 h or pretreated with 10 μM LY294002 (a PI3K inhibitor), PD98059 (an MEK inhibitor), and SP600125 (a JNK inhibitor) for 30 min and then treated with 30 μM DHQ for 48 h. Whole-cell lysates were prepared, and the luciferase activity was measured with a luciferase assay kit (Promega) and a luminometer.

Transfection of siRNAs. For silencing experiments, HepG2 cells were transfected with 60 nM Nrf2-specific siRNA (si-Nrf2) or control siRNA (si-control) using X-tremeGENE siRNA transfection reagent (Roche, Mannheim, Germany) according to the manufacturer's protocols. The Nrf2-specific siRNA and control siRNA sequences have the following sequences: human Nrf2, CAAACAGAAUGG-UCCUAAA; control, GAUCAUACGUGCGAUCAGA. After 24 h, the cells were treated with DHQ and then subjected to Western blot analysis.

GSH Assay. HepG2 cells were cultured in 6-well plates at a density of 5.0×10^4 cells per well. After incubation for 24 h, cells were exposed to DHQ (0, 10, 20, and 30 μM) for 48 h. After treatment of HepG2 cells, intracellular reduced GSH levels were enzymatically measured with glutathione reductase as previously described.[16] Cells were collected and lysed. Then, cell lysates were centrifuged and supernatants were transferred to sulfosalicyclic acid-containing tubes. GSH levels were spectrophotometrically measured by conversion of DTNB (5,5′-dithio-*bis*(2-nitrobenzoic acid) to its colored product upon reduction by GSH-dependent glutathione reductase.

Statistical Analysis. All results were expressed as mean values ± standard deviation ($n = 3$). Differences between groups were calculated by one-way ANOVA. An analysis of ANOVA variance with a Tukey post hoc test was used for multiple comparisons. All statistics were calculated using the STATISTICA program (StatSoft, Tulsa, OK). Correlations were calculated using the ReglinP function and inverted Student's t test. $p < 0.01$ was considered as statistically significant.

RESULTS

DHQ was found to effectively scavenge free radicals generated by DPPH. The concentration exhibiting 50% scavenging effect was 5.96 μM (Figure 1A). In the reducing power assay, the presence of antioxidant DHQ results in reduction of the Fe^{3+}/ferricyanide complex to its ferrous form. Figure 1B shows the extent of the reduction, in terms of absorbance values at 700 nm, for DHQ ranging from 0.5 to 50 μM, and the IC_{50} value was 4.31 μM. The inhibitory effect of DHQ on lipid peroxidation of liposomes is shown in Figure 1C. The IC_{50} value was 2.03 μM. The IC_{50} values of the positive control quercetin were 11.52, 14.33, and 7.11 μM for DPPH, reducing power, and lipid peroxidation assays, respectively. The results indicated that DHQ was more efficient as compared with quercetin.

The inhibition of XOD activity was spectrophotometrically measured on the basis of uric acid formation from xanthine.[17] DHQ showed a stronger inhibitory effect than quercetin, with IC_{50} values of 13.24 and 18.98 μM, respectively (Figure 1D).

Plasmid DNA was oxidized with Fenton's reagent treatment and checked on 1% agarose gel. Fenton's reagent caused DNA strand scission, bringing native circular DNA to nicked circular or linear DNA. As shown in Figure 2, DHQ was protected from

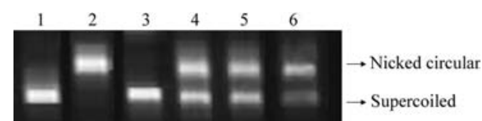

Figure 2. Effect of DHQ on oxidative DNA nicking caused by hydroxyl radicals. Lanes: 1, native pBR322 DNA; 2, DNA + Fenton's reagent; 3−5, DNA + Fenton's reagent + DHQ (30, 20, and 10 μM, respectively); 6, DNA + Fenton's reagent + quercetin (30 μM).

DNA damage in a concentration-dependent manner in a range of 10−30 μM. DHQ effectively reduced nicked DNA and increased formation of supercoiled DNA. At a concentration of 30 μM, protection was more effective than that of 30 μM quercetin.

The above results showed that DHQ was more effective than quercetin. Therefore, DHQ was used for the further experiments. The growth of the HepG2 cells in the presence of various concentrations of DHQ was examined by using the

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MTT assay. As shown in Figure 3, DHQ decreased the number of viable cells in a concentration-dependent manner after 48 h.

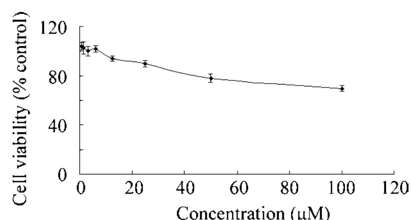

Figure 3. HepG2 cells were plated in 96-well plates at a density of 1×10^4 cells per well. HepG2 cells were treated with DHQ for 48 h, and viability was measured by MTT assay. The values for each DHQ concentration tested represent the average (mean ± S.D.) from eight replicate wells and are representative of three separate experiments.

The results indicated that the concentration (10, 20, and 30 μM) which we chose for the further experiments did not significantly contribute to cytotoxicity in HepG2 cells.

To further clarify the intracellular signaling pathways, the activation status of these signaling molecules was examined after DHQ treatment. DHQ treatment enhanced the phosphorylation-dependent activation of signaling components, such as AKT, ERK1/2, and JNK (Figure 4A and B). In ARE-luciferase-transfected HepG2 cells, we showed that DHQ increased the expression of a luciferase construct containing binding site for Nrf2. The activation of ARE binding by DHQ was inhibited by the ERK inhibitor PD98059, the PI3K inhibitor LY294002, and the JNK inhibitor SP600125 dramatically. The results showed that AKT, ERK, and JNK signaling pathways can indeed positively regulate Nrf2 activation (Figure 4C).

We analyzed nucleic and cytoplasmic Nrf2 expression upon treatment of HepG2 cells with DHQ. After treatment with DHQ (10−30 μM) for 48 h, Western blot analysis revealed that HepG2 cells treated with DHQ exhibited a dose-dependent increase in Nrf2 expression in the nucleus compared with control cells (Figure 5A). By contrast, Nrf2 expression in the cytoplasm decreased in a dose-dependent manner.

We performed Western blotting to further investigate the effect of DHQ on Nrf2-related antioxidant molecule expression. As seen in Figure 5B, 30 μM DHQ strongly induced the protein expression of HO-1 and NQO1 relative to controls in HepG2 cells. The protein expression of GCLC and GCLM also

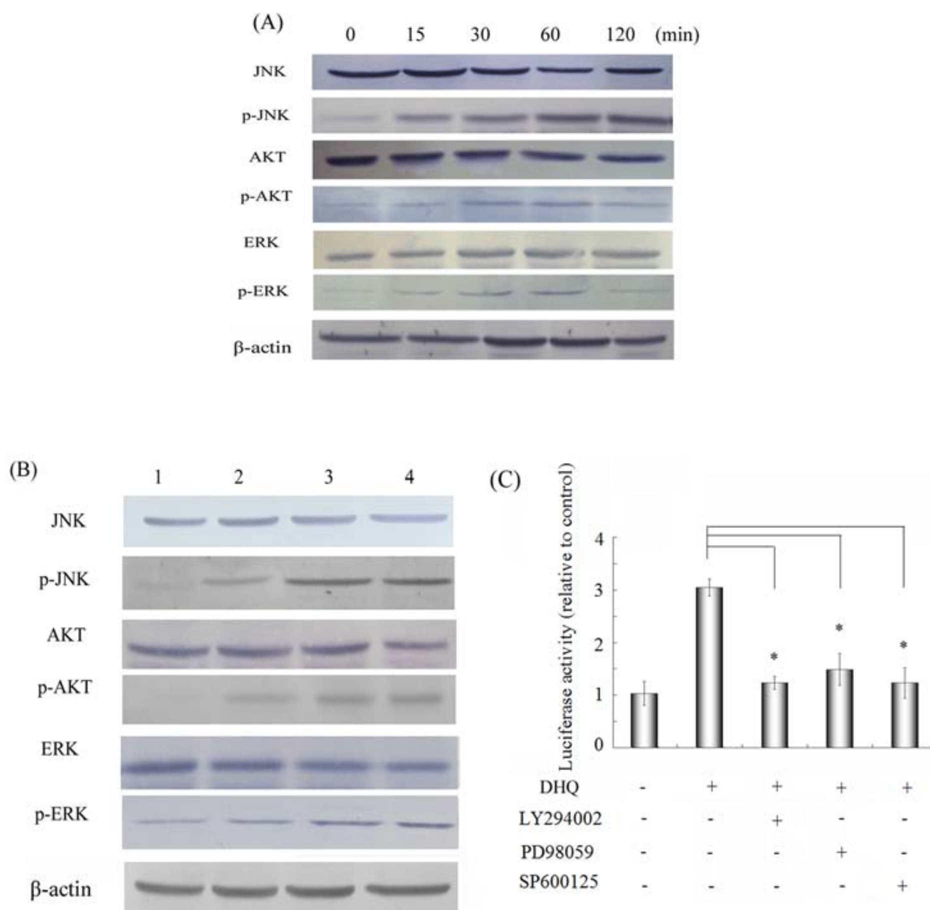

Figure 4. Effect of DHQ on AKT, ERK, and JNK signaling pathways. (A) Effect of DHQ on AKT, ERK, and JNK signaling pathways in a time-dependent manner. Cells were treated with 30 μM DHQ for 0−120 min. Total cell extracts were prepared and subjected to Western blot analysis in order to detect the active phosphorylated forms of AKT, ERK, and JNK. (B) Effect of DHQ on AKT, ERK, and JNK signaling pathways. HepG2 cells were treated with DHQ (1, control; 2−4, treated with 10, 20, and 30 μM) for 1 h, respectively. The test was repeated three times, and representative blots are shown. (C) The effect of kinase inhibitors on DHQ-mediated Nrf2/ARE activation in HepG2 cells. ARE-luciferase vector-transfected cells were treated with the named inhibitors, and the luciferase activity was measured using a luciferase assay kit (Promega) and a luminometer. $^*p < 0.01$; p value compared with only DHQ-treated cells.

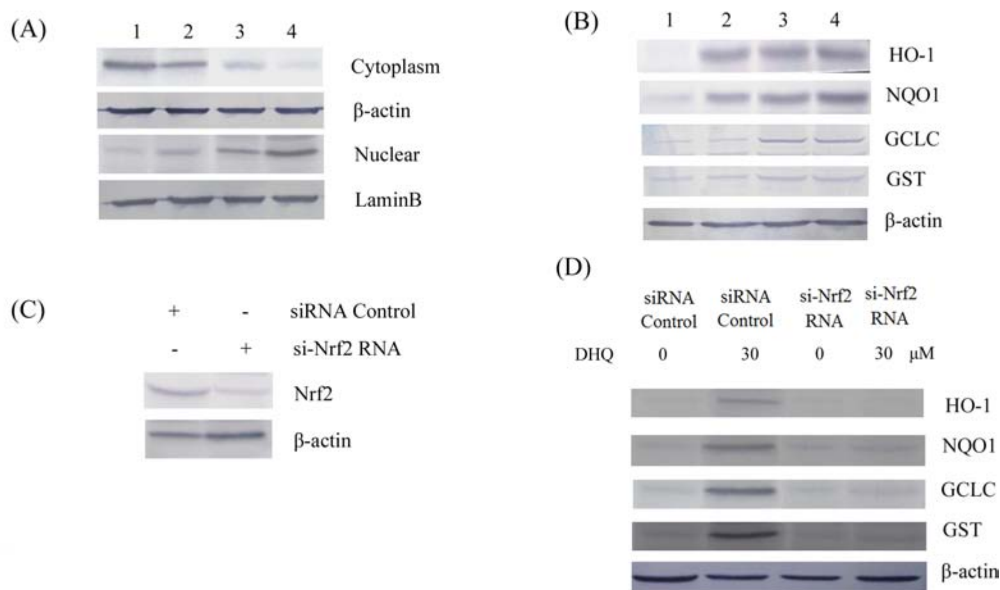

Figure 5. (A) DHQ stimulates Nrf2 nuclear translocation by Western blotting assay; HepG2 cells were treated with DHQ (1, control; 2−4, treated with 10, 20, and 30 μM) for 48 h, respectively. (B) GCLC, GST, NQO1, and HO-1 expression was analyzed by Western blotting in cultured HepG2 cells. Cells were treated with various concentrations of DHQ (10, 20, and 30 μM) for 48 h. The test was repeated three times, and representative blots are shown. (C) HepG2 cells were transfected with a specific si-Nrf2 RNA or a scramble siRNA control, and efficiency of silencing was evaluated by Western blot analysis. The results are a representative of three experiments (n = 3). (D) After transfection of si-Nrf2 for 24 h, the levels of GCLC, GST, NQO1, and HO-1 were measured by Western blot analysis. The results are a representative of three experiments (n = 3).

increased after DHQ treatment in HepG2 cells, which translated to the rate-limiting enzymes in GSH synthesis.

To confirm the importance of Nrf2 in DHQ-mediated antioxidant effects, we used siRNA for Nrf2 knockdown. As shown in Figure 5C, siRNA significantly inhibited Nrf2 expression. Knockdown of Nrf2 with siRNA markedly decreased DHQ-induced expression of several antioxidant genes: HO-1, NQO1, GCLC, and GCLM (Figure 5D).

GSH, a major component of the cellular antioxidant system, plays an important role in the detoxification of xenobiotic compounds, ROS, and free radicals.[18] Thus, we intended to investigate the effect of DHQ on intracellular glutathione synthesis. The intracellular glutathione content in DHQ-treated HepG2 cells was measured spectrophotometrically. Interestingly, DHQ enhanced intracellular GSH levels in a concentration-dependent manner (Figure 6A).

LY294002 (an inhibitor of the PI3K family), SP600125 (an inhibitor of JNK1/2), and PD98059 (an inhibitor of MEK1/2) were used in pretreatment of HepG2 cells for 30 min, and then, cells were cotreated with 30 μM DHQ. After treatment, cell extracts were prepared to determine the levels of GSH. As shown in Figure 6B, LY294002, SP600125, and PD98059 could effectively suppress the intracellular GSH levels.

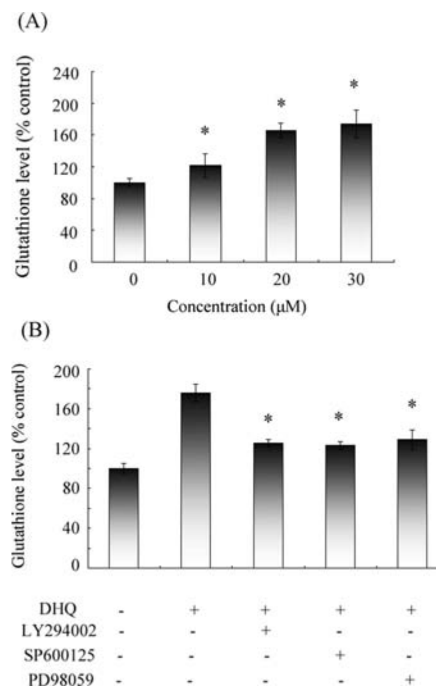

Figure 6. GSH level of intracellular HepG2 cells. (A) Different concentrations of DHQ (1, control; 2−4, treated with 10, 20, and 30 μM) were exposed to HepG2 cells for 48 h. Columns, mean of three experiments, data are presented as mean ± SD. $^{*}p < 0.01$; p value compared with the control group (0 μM). (B) Cells were treated with 10 μM LY294002, SP600125, or PD98059 and then challenged with 30 μM DHQ. ELISA analysis was applied to measure the contents of GSH. $^{*}p < 0.01$; p value compared with only DHQ-treated cells.

■ DISCUSSION

In the present investigation, we demonstrated that DHQ, prepared from wood sawdust of *Larix gmelini* (Rupr.) Rupr., possesses significant antioxidant activities. Different methods were used to evaluate the antioxidant properties, including DPPH reducing power and lipid peroxidation assays. DHQ was also found to inhibit XOD and reduce oxidative DNA damage. Additionally, DHQ can lead to Nrf2 nuclear translocation and eventually increasing phase II enzyme expression through activating ERK1/2, Akt, and JNK signaling pathways.

Because a single method cannot give a comprehensive prediction of the antioxidant efficacy of different compounds, a multimethod approach was used to evaluate the antioxidant

capacity.[19] The DPPH assay is based on the measurement of the scavenging ability of antioxidants toward the stable radical (DPPH). It is considered a valid and easy assay to evaluate the radical-scavenging activity (RSA) of antioxidants.[20] Other methods to measure the amount of peroxide, which is the primary oxidation product during the initial stages of oxidation, are the reducing power assay (ferricyanide method) and the lipid peroxidation assay.[21] Besides, the data on the inhibition ratio of lipid peroxidation obtained are dependent on the antioxidant properties of DHQ *in vivo*.[22] In the present work, all three assays were used to study the antioxidant activities of DHQ and quercetin as positive control.

The main function of XOD is to catalyze the oxidation of hypoxanthine to xanthine and of xanthine to uric acid.[17] The overaccumulation of uric acid can lead to hyperuricemia, which is linked to gout.[17] XOD is involved in purine metabolism. Previously, numerous natural products have been found to inhibit xanthine oxidase *in vitro* and *in vivo*.[23] Our results indicate that DHQ represents an efficient XOD inhibitor.

The Fenton reaction was used in both hydroxyl radical (•OH) scavenging activity and DNA nicking assay to generate hydroxyl radicals. Especially, the hydroxyl radical reactions of the iron-mediated system, such as deoxyribose and DNA nicking assays, have frequently been used to screen radical scavengers derived from a number of natural materials, including foods.[24] In this study, the results of the DNA nicking assays showed that DHQ scavenged hydroxyl radicals.

In HepG2 cells, we further investigated which signaling pathway is essential for the activation of Nrf2 by DHQ. We showed that Akt, ERK, and JNK signaling pathways resulted in activating Nrf2/ARE transcriptional activity. PI3K/Akt is activated by stress stimuli including oxidative stress and provides a pro-survival signal[25] facilitating the release of Nrf2 from KEAP1 and its subsequent translocation and regulating ROS-dependent Nrf2 activation.[26] On the other hand, ERK[27] can also mediate oxidative stress-independent Nrf2−ARE activation. We found that the level of active phosphorylated JNK was significantly higher in DHQ-treated than in untreated cells. Taken together, DHQ was able to activate JNK MAP kinase which is pivotal for activating Nrf2 in HepG2 cells. The effects of DHQ can be explained not only on the basis of nonenzymatic actions but also on the basis of their efficacy to enzymatic actions involved in Nrf2-dependent signaling pathways.

The cells' major strategy of coping with oxidative stress is to increase the antioxidant response by upregulating defense enzymes by activation of the nuclear factor-E2-related factor-2 (Nrf2)-antioxidant response element (ARE). Nrf2 is a transcription factor that is translocated into the nucleus upon oxidative signals. Here it binds to ARE which promotes expression of many phase II detoxifying and antioxidant genes.[28] Lee et al. have also shown that DHQ acts as a potential chemopreventive agent by regulating genes via an ARE-dependent mechanism.[29] It is interesting that DHQ activated the expression of Nrf2 and ARE-regulated antioxidant genes HO-1, NQO1, GCLC, and GCLM. The cytoprotective properties of HO-1 may be due to inhibiting NADPH oxidase activity by interrupting subunit assembly.[30] The induction of NQO1 represents a cytoprotective mechanism against oxidative damage. NQO1 is known to maintain both α-tocopherol and coenzyme Q10 in their reduced antioxidant state.[15]

The GSH redox system represents another important detoxification system involved in the protection of cells against oxidative damage.[18] GSH peroxidase and GSH reductase together with other phase II enzymes such as GSH synthetase and GSH-S-transferase, have a main role of detoxifying nucleophilic compounds, as well as maintaining optimal cellular levels of reduced GSH.[18] Our results suggested that DHQ induced enhanced formation of intracellular GSH. Therefore, we speculate that DHQ induced the expression of GSH peroxidase and GSH reductase, which improved the endogenous antioxidant capacity in HepG2 cells.

In previous studies, it was shown that DHQ can provide useful therapeutic potentials for the prevention or treatment of the neurodegenerative disorders caused by oxidative stress.[31,32] Furthermore, the present study shows that DHQ protected cells from oxidative stress by regulating Nrf2 activity and antioxidant enzyme NQO1 protein expression by the Akt, ERK, and JNK signaling pathways. Besides shedding light on the mechanisms underlying the antioxidant effects of DHQ, this investigation suggests that DHQ may be considered as an Nrf2 activator with medicinal potential in terms of chemoprevention and treatment of inflammation and atherosclerosis.

The antioxidant activities of the two flavonoids quercetin and DHQ are associated with two aromatic rings,[33] which effectively scavenge free radicals. DHQ oxidizes more actively, donates hydrogen atoms, and results in the oxidation product quercetin.[34] Adjacent hydroxyl groups in positions 3′ and 4′ are believed to be more effective for antioxidant activity in the flavonol class than groups in the 2′ and 4′ positions.[35] Quercetin and DHQ share these groups in the same positions. However, between C2 and C3, quercetin has a double bond, while DHQ has a single bond. Moreover, quercetin and DHQ possess planar and nonplanar structures, respectively. The differences in antioxidant activity may depend on the unique structures of these two flavonoids.[36] In addition, the difference of energy (ΔH_f) associated with the formation of various radicals to quercetin or DHQ is an important factor affecting their antioxidant activities;[37] it may testify the better antioxidant acitivity of DHQ. Because of structural features, DHQ possesses hydroxyl radical (•OH) scavenging activity in the nonenzymatic assays. At the same time, DHQ can induce antioxidant enzymes which also scavenge hydroxyl radical (•OH) and protect cells against oxidative stress. Therefore, we tested the enzymatic and nonenzymatic actions of DHQ together in the present study.

In conclusion, we showed that DHQ possessed both cytoprotective and antioxidant effects, which may be important for the prevention/treatment of cardiovascular diseases. Therefore, DHQ from wood sawdust of *Larix gmelini* (Rupr.) Rupr. extracts may be developed as a natural antioxidant for the food industry and may have a positive influence on various cardiovascular diseases.

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Notes

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