

Original Article

Four active monomers from Moutan Cortex exert inhibitory effects against oxidative stress by activating Nrf2/Keap1 signaling pathway

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ABSTRACT Paeonol, quercetin, β -sitosterol, and gallic acid extracted from Moutan Cortex had been reported to possess anti-oxidative, anti-inflammatory, and anti-tumor activities. This work aimed to illustrate the potential anti-oxidative mechanism of monomers in human liver hepatocellular carcinoma (HepG2) cells-induced by hydrogen peroxide (H_2O_2) and to evaluate whether the hepatoprotective effect of monomers was independence or synergy in mice stimulated by carbon tetrachloride (CCl_4). Monomers protected against oxidative stress in HepG2 cells in a dose-response manner by inhibiting the generation of reactive oxygen species, increasing total antioxidant capacity, catalase and superoxide dismutase (SOD) activities, and activating the antioxidative pathway of nuclear factor E2-related factor 2/Kelch-like ECH-associated protein 1 (Nrf2/Keap1) signaling pathway. We found that the *in vitro* antioxidant capacities of paeonol and quercetin were better than those of β -sitosterol and gallic acid. Furthermore, paeonol apparently diminished the levels of alanine transaminase and aspartate aminotransferase, augmented the contents of glutathione and SOD, promoted the expressions of Nrf2 and heme oxygenase-1 proteins in mice stimulated by CCl_4 . In HepG2 cells, paeonol, quercetin, β -sitosterol, and gallic acid play a defensive role against H_2O_2 -induced oxidative stress through activating Nrf2/Keap1 pathway, indicating that these monomers have anti-oxidative properties. Totally, paeonol and quercetin exerted anti-oxidative and hepatoprotective effects, which is independent rather than synergy.

INTRODUCTION

Oxidative stress breaking prooxidant-antioxidant balance in the body leads to oxidizing. The prooxidant/antioxidant dysfunction is due to excessive accumulation of reactive oxygen species (ROS). The burden of endogenous antioxidant defense is primarily provided by enzymes such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSH-Px), and nonenzymatic substances [1,2]. Additionally, oxidative stress affects cell viability, destroys cell structures, and ultimately leads to cell death [3]. Oxidative stress is closely associated with several age-related

chronic diseases, including cancer, Alzheimer's, Parkinson, and heart diseases [4]. Oxidative stress is an important cause of liver injury, CCl_4 has been widely used to study hepatotoxicity both *in vitro* and *in vivo*. CCl_4 is metabolized into trichloromethyl radical by CYP450 in the liver, and later trichloromethyl is converted to trichloromethyl peroxy radical in aerobic conditions. These peroxidation radicals induce lipid peroxidation, destroy the structure of cell membrane, release indicators of aspartate aminotransferase (AST) and alanine transaminase (ALT), and ultimately lead to liver injury of mice [5]. Thus, it is necessary to find some promising candidate drugs to resist oxidative damage.



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The current evidence demonstrates that a key transcription factor for the endogenous antioxidative enzyme, nuclear factor E2-related factor 2 (Nrf2), is a target for oxidant insults and apoptosis [6]. Under physiological conditions, Kelch-like ECH-associated protein 1 (Keap1) enables rapidly ubiquitination and degradation of Nrf2. However, in some circumstances, Nrf2 activators trigger the separation of Nrf2 from Keap1 and enter into the nucleus, resulting in the formation of a binding complex between Nrf2 and antioxidant-responsive element (ARE), and the promotion of the expressions of NAD(P)H quinone oxidoreductase 1 (NQO1) and heme oxygenase-1 (HO-1) [7]. This redox regulation in the oxidative stress response and signaling has been used to screen novel alternative agents for antioxidative effects.

Moutan Cortex (MC) exerts the pharmacological activities of antioxidation, anti-inflammation, removing blood stasis, dredging meridian, and expelling pus [8-10]. The main bioactive ingredients of MC consist of paeonol, gallic acid, quercetin, β -sitosterol, and paeonol [11,12]. Paeonol eliminating ligation-induced periodontitis in rat was through the regulation of the Nrf2/NF- κ B/NFATc1 signaling [13]. Gallic acid inhibiting tert-butyl hydroperoxide-induced hepatotoxicity was by activating the extracellular signal-regulated kinase-Nrf2-Keap1-mediated antioxidative response [14]. Quercetin was recognized as a potential hepatoprotective agent in the treatment of mice liver injury induced by lipopolysaccharide/D-galactosamine (LPS/D-GalN) [15]. However, a little information is available concerning the independent or synergistic effects of four active monomers on the hepatoprotective effects. Additionally, the inhibitory mechanisms of those compounds against oxidative damage and liver injury are unknown. Therefore, the aims of the present study are to evaluate the possible mechanisms of four monomers (paeonol, quercetin, β -sitosterol, and gallic acid) against hydrogen peroxide (H_2O_2)-induced oxidative damage in human liver hepatocellular carcinoma (HepG2) cells and against carbon tetrachloride (CCl_4)-induced liver injury in mice, and to further elucidate whether the effects of these monomers are independent or synergistic.

METHODS

Reagents and equipment

MC was purchased from the traditional herbal market in Dianjiang (Chongqing, China). Ethyl acetate, methanol, ethanol were obtained from Chongqing Taixin Chemical Reagent Co., Ltd. (Chongqing, China). H_2O_2 (30%) was purchased from Chongqing East Chemical (Group) Co., Ltd. (Chongqing, China). HepG2 cells were obtained from Beijing Beina Chuanglian Biotechnology Research Institute (Beijing, China). Antibodies against β -actin, Nrf2, HO-1, NQO1, Keap1, LaminB, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were obtained from Proteintech Group, Inc. (Wuhan, China). CCK-8 kits were purchased from

Selleck Chemicals (Shanghai, China). Total antioxidant capacity (T-AOC), CAT and SOD kits were acquired from Sino Best Biological Technology Co., Ltd. (Shanghai, China). ALT and AST and reduced GSH kits were purchased from Nanjing Jiancheng Institute of Biological Engineering (Nanjing, China). Electrophoretic mobility shift assay (EMSA) kits and the biotin-labeled probe ARE were obtained from Beyotime (Shanghai, China).

Extraction, isolation, identification of monomers from MC

Air-dried MC (250 g) was crushed and extracted with 85% ethanol for 24 h and ultrasound for 1 h. The combined extracts were concentrated *in vacuo* under reduced pressure and were then successively partitioned with different extract reagents, including petroleum ether, ethyl acetate, and n-butanol fraction. The fractions were performed on a silica gel column to separate and purified. Separation of the extract on a silica gel column obtained four monomers, and their structures were confirmed with spectral data from mass spectra (MS), ^{13}C NMR, and 1H NMR.

Cell culture

HepG2 cells were cultured in DMEM containing 15% (V/V) FBS, penicillin (100 U/ml), and streptomycin (100 U/ml) (Sijiqing, Zhejiang, China) in a humidified incubator at 37°C under 5% CO_2 . HepG2 cells were passaged at a ratio of 1:2 or 1:3 when the cells grew to more than 80% confluence.

Cell viability assay

HepG2 cells were transferred onto 96-well culture plates at a density of 5×10^3 cells per well. After a 48 h incubation, the cells were divided into four groups: control, H_2O_2 , chemicals, and positive control (vitamin E). The control group was not treated with chemicals or H_2O_2 . The H_2O_2 group was treated with different concentrations of H_2O_2 (3, 4, 5, 6, 7, 8, 9, 10, or 12 mM) for 24 h to screening molding concentration. The chemical and positive control groups were pretreated with different monomers (paeonol, quercetin, β -sitosterol, gallic acid, and vitamin E) with 10, 20, 40, and 80 μ M for 24 h to determine the dose concentration, respectively. Then, 1/10 volume of CCK-8 was directly added to the cell culture medium for 0.5 to 4 h. Finally, the absorbance was measured by microplate reader at 450 nm (BioTeK, Winooski, VT, USA).

Determination of ROS level of HepG2 cells induced by H_2O_2 after monomers pretreatment

As described above in 2.4, the cells were stimulated with 7 mM H_2O_2 for 5, 15, 30, 60, 120, or 300 min. In another 12-well plate, the cells were pretreated with monomers for 6 h, and then they

were incubated in the presence or absence of 7 mM H₂O₂ for 15 min. Finally, the cells were incubated with the DCFH-DA probe for 30 min, and the cellular fluorescence was detected by a fluorescence microscope (Nikon, Tokyo, Japan).

Determination of antioxidative markers after monomers pretreatment

HepG2 cells were transferred onto 12-well culture plates at a density of 2.5×10^5 cells per well. After a 48 h incubation, the cells were separately pretreated with four monomers at 20 μ M for 6 h and were then incubated in the presence or absence of 7 mM H₂O₂ for 24 h. The antioxidative markers were determined by SOD, CAT, T-AOC, ALT, AST, and GSH assays kits after pretreatment with monomers.

Preparation of relative proteins of HepG2 cells after monomers pretreatment

Cells were respectively pretreated with 20 μ M of four monomers for 6 h and then were stimulated with 7 mM H₂O₂ for 24 h. Total cellular proteins were extracted by the protein extract kits (KeyGEN Biotech, Jiangsu, China). Cells were separately pretreated with 20 μ M of monomers for 3 h, and later were stimulated with 7 mM H₂O₂ for 3 h, the cytosolic and nuclear proteins were extracted by the protein extract kits. The relative proteins were stored at -80°C for analysis.

Western blot analysis

The whole-cell lysates or nuclear/cytosolic proteins were separated by 10% or 12% SDS-PAGE and were transferred onto a polyvinylidene difluoride (PVDF) membrane. The membranes were blocked with 10% nonfat milk for 2 h and washed 5 times (5 min/wash) with the TBST solution. Next up, the PVDF membranes were incubated with different primary antibodies at 4°C overnight and washed. The membranes were incubated with the secondary antibody for 2 h and finally visualized using the ECL system (Beyotime Biotechnology, Shanghai, China).

EMSA assay

The EMSA assay was used to detect the binding of the protein to DNA. The ARE consensus oligo was as follows: 5'-ACT GAG GCT GAC TCA GCA AAA TC-3', 3'-TGA CTC CCA CTG AGT CGT TTT AG-5'. The nuclear Nrf2 protein combined with the ARE labeled probe. The free probe and bound macromolecular protein were segregated by EMSA and transferred to a magna nylon transfer membrane with positive electricity. The membrane was cross-linked by a ultraviolet (UV)-light cross-linker at 254 nm, and the strips were tested with the ECL system.

Effect of ML385 on the expression of Nrf2 protein of HepG2 cells

ML385 interacts with Nrf2 to inhibit the levels of Nrf2. HepG2 cells were cultured in 6-well plates at 2.5×10^5 cells/ml for 48 h. The cells were first treated with 2.5 μ M ML385 for 1 h and later with 20 μ M of the monomers in the chemical group for 6 h. Finally, 7 mM H₂O₂ was added to the cells for 24 h. The cellular proteins were separately extracted for analysis.

Animals, treatment and tissue preparation

Male Kunming mice (22 ± 2 g) were purchased by Chongqing Medical University. All animal experiments were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. The animal studies were performed after receiving approval of the Institutional Animal Care and Use Committee (IACUC) in Southwest University, P.R.China (IACUC approval No. 11-2018). The mice were randomly divided into six groups: control; 2% CCl₄; 2% CCl₄ + 100 mg/kg paeonol (paeonol); 2% CCl₄ + 100 mg/kg quercetin (quercetin); 2% CCl₄ + 50 mg/kg paeonol + 50 mg/kg quercetin (L-(paeonol + quercetin)); and 2% CCl₄ + 100 mg/kg paeonol + 100 mg/kg quercetin (H-(paeonol + quercetin)). The paeonol, quercetin, L-(paeonol + quercetin), and H-(paeonol + quercetin) groups were fed respectively with the monomers for a week. The control and 2% CCl₄ groups were given the same volume of 0.5% sodium carboxymethyl cellulose for a week. After that, all animals except the control mice were intraperitoneally injected with 2% CCl₄ (V/V in olive oil) for 24 h. Blood samples were taken from the retro-orbital venous plexus and were centrifuged at 12,000 \times g, 4°C for 10 min, and were stored at -80°C . The livers were harvested immediately and snap-frozen in liquid nitrogen for histopathology, immunohistochemistry, and western blot assays.

Histopathology and immunohistochemical staining

Liver tissue sections were fixed in 4% formalin and embedded in solid paraffin. The sections were mounted on silane-coated slides and stained with hematoxylin and eosin (H&E). The slides were prepared in terms of the instructions of the histostain TM-plus and diaminobenzidine (DAB) kits. These slides were incubated with Nrf2 antibodies at 4°C overnight and were with biotin-labeled goat antirabbit IgG for 1 h. Histopathological changes and the levels of protein expressions in livers were visualized by using a light microscope (magnification, 200 \times).

Statistical analysis

The comparison of the results was used the statistical package SPSS (ver. 20.0; IBM, Armonk, NY, USA). All data were presented as the mean \pm standard deviation. The data analysis was per-

formed by one-way analysis of variance (ANOVA). Differences between groups were determined by significant difference test. $p < 0.05$ was considered significant, and $p < 0.01$ was considered extremely significant difference.

RESULTS

Isolation and identification of four monomers from MC

The isolation and purification of monomers from MC were obtained by a silica gel column. Retention factors (R_f), melting point (Mp) and UV values were measured in Table 1. Their structures were confirmed by ^1H NMR, ^{13}C NMR (Supplementary Fig. 1) and MS (Supplementary Fig. 2) spectra. The four monomers (1-4)

were paeonol, quercetin, β -sitosterol, and gallic acid, respectively.

Compound 1 ^1H NMR (400 MHz, CDCl_3 , δ ppm): δ 2.56 (s, 3H, $-\text{COCH}_3$), 3.84 (s, 3H, $-\text{OCH}_3$), 6.42–7.64 (s, 3H, ArH), 12.75 (s, 1H, $-\text{OH}$); ^{13}C NMR (100 MHz, CDCl_3 , δ ppm): δ 202.54, 166.14, 165.30, 132.27, 113.94, 107.62, 100.87, 55.55, 26.12. In comparison to the reference data [16], the ^1H NMR, ^{13}C NMR and MS data confirmed the white crystal was paeonol.

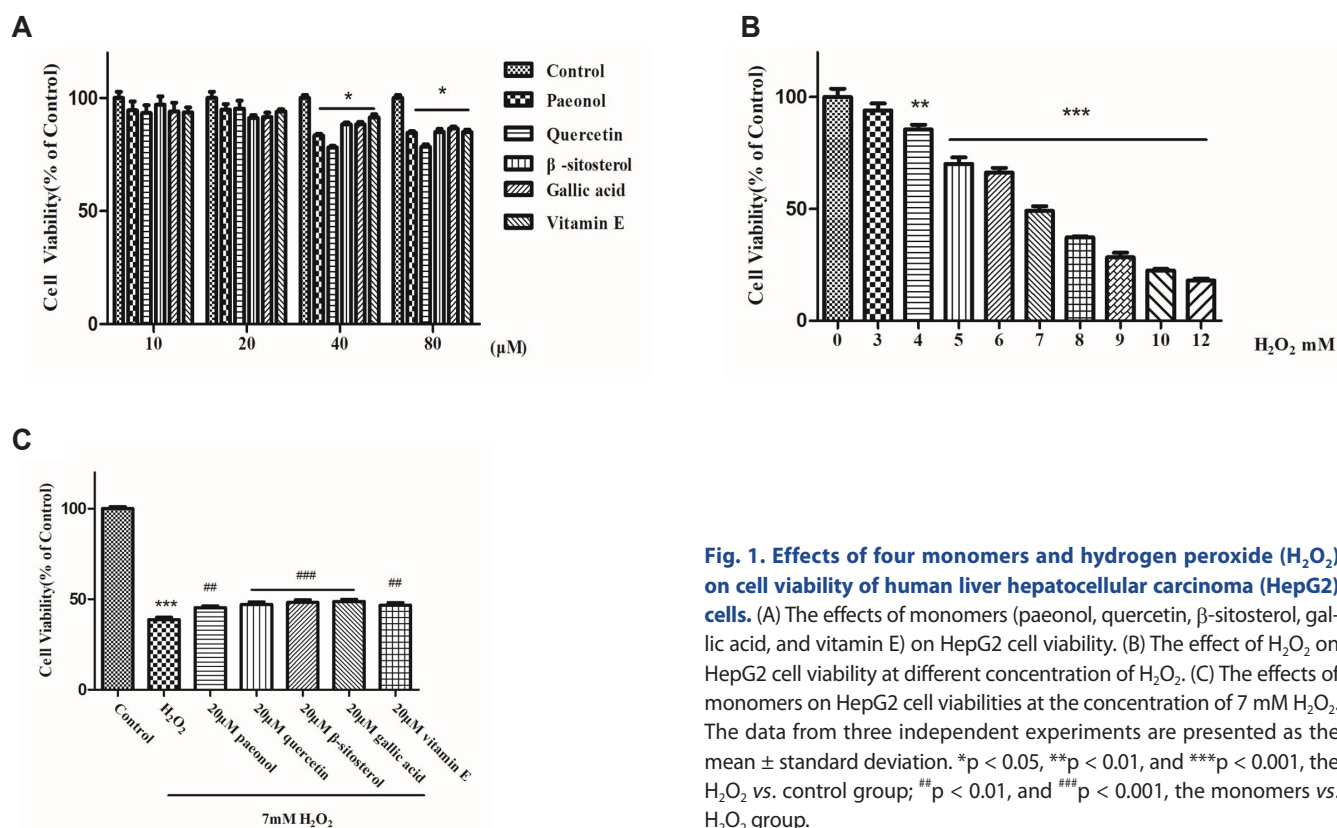
Compound 2 ^1H NMR (400 MHz, $\text{DMSO}-d_6$, δ ppm): δ 6.19–7.68 (m, 5H, ArH), 12.49 (s, 1H, 5-OH); ^{13}C NMR (100 MHz, $\text{DMSO}-d_6$, δ ppm): δ 176.30, 164.36, 161.19, 156.61, 148.17, 147.28, 145.52, 136.19, 122.43, 120.44, 116.07, 115.54, 103.48, 98.65, 93.81. In comparison to the reference data [17], the ^1H NMR, ^{13}C NMR and MS data confirmed the yellow powder was quercetin.

Compound 3 ^1H NMR (400 MHz, CDCl_3 , δ ppm): δ 0.68 (3H, s, H-18), 0.84 (3H, d, $J = 6.2$ Hz, H-26), 0.85 (3H, d, $J = 6.5$ Hz, H-27), 1.01 (3H, d, $J = 6.0$ Hz, H-29), 1.25 (3H, s, H-19), 3.52 (1H,

Table 1. Physical properties of active monomers from MC

Compound	Appearance	R_f (ethyl acetate:ethanol, V/V = 20:1)	Mp ($^\circ\text{C}$)	UV (nm) (EtOH)	$[\text{M} + \text{H}]^+$
Paeonol	White needle-like crystal	0.80	51.20–52.70	270.2	167
Quercetin	Yellow-green powder	0.76	313–314.20	258, 375	302.05
β -sitosterol	White powder	0.73	139–142	317.40	414.25
Gallic acid	White powder	0.48	235–240	216, 271, 239	173.05, 169.90

MC, Moutan Cortex; R_f , Retention factor; Mp, melting point; UV, ultraviolet; EtOH, ethanol; M, molecular weight; H, hydrogen.



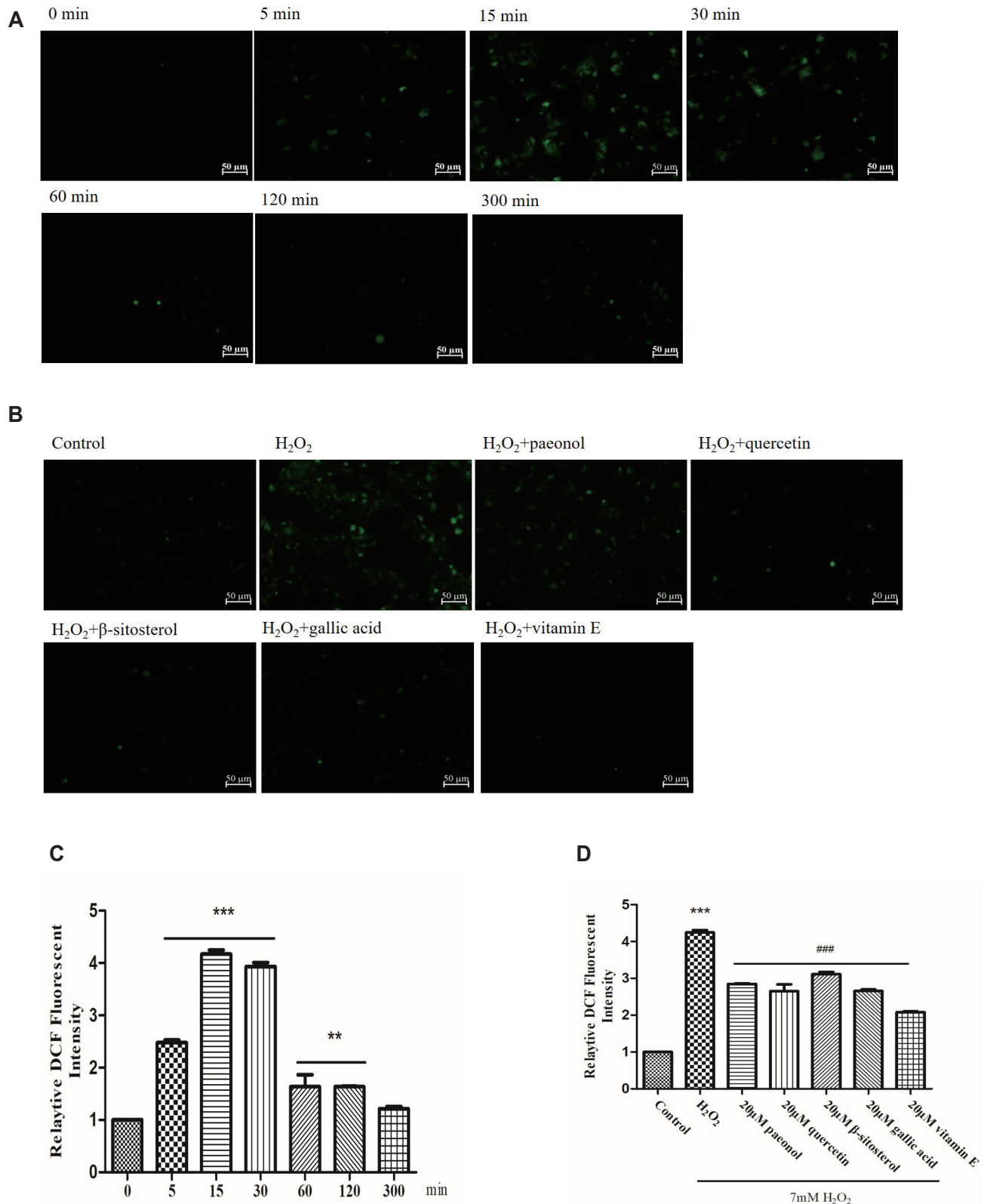


Fig. 2. Effects of four monomers on reactive oxygen species (ROS) generation of human liver hepatocellular carcinoma (HepG2) cells induced by hydrogen peroxide (H₂O₂). (A, C) ROS generation of HepG2 cells was measured at the different incubated time by fluorescence microscope assay. (B, D) ROS generation of HepG2 cells was inhibited by four monomers at 15 min incubated time through fluorescence microscope assay. The data from three independent experiments are presented as the mean ± standard deviation. **p < 0.01, and ***p < 0.001, the H₂O₂ vs. control group; ###p < 0.001, the monomers vs. H₂O₂ group.

br m, $J = 16.5$ Hz, H-3), 5.36 (d, $J = 5.5$ Hz, H-6); ^{13}C NMR (100 MHz, CDCl_3 , δ ppm): δ 140.78, 121.71, 71.82, 56.09, 55.99, 50.17, 45.88, 42.24, 42.33, 39.80, 37.33, 37.27, 37.27, 33.98, 32.42, 31.93, 31.69, 29.20, 28.90, 25.82, 24.37, 24.31, 23.10, 21.10, 19.81, 19.39, 18.98, 11.98, 11.86. In comparison to the reference data [18], the ^1H NMR, ^{13}C NMR and MS data confirmed the white powder was β -sitosterol.

Compound 4 was white powder: Mp 235°C – 240°C ; ^1H NMR (400 MHz, $\text{DMSO}-d_6$, δ ppm): δ 6.92 (2H, s, 2-H, 6-H), 8.82 (H, s, 4-OH), 9.17 (2H, s, -OH), 12.22 (1H, s, -COOH); ^{13}C NMR (100 MHz, $\text{DMSO}-d_6$, δ ppm): δ 167.90, 145.87, 138.45, 120.93, 109.20. In comparison to the reference data [19], the ^1H NMR, ^{13}C NMR confirmed the white powder was gallic acid.

Effects of monomers on cell viability of HepG2 induced by H_2O_2

Effect of paeonol, quercetin, β -sitosterol, gallic acid, and vitamin E and their doses on HepG2 cell viability were assessed through *in vitro* screening. As shown in Fig. 1A, compared to the control group, HepG2 cells viability began to decrease with the increase of monomers concentrations (as high as 40 and 80 μM) for 24 h. In other words, the monomers were not cytotoxicity at 10 and 20 μM on HepG2 cell. In order to compare the antioxidative effect of these monomers, subsequent experiments were uniformly administered at a concentration of 20 μM *in vitro*. As illustrated in Fig. 1B, HepG2 cell viability showed a dose-response effect from H_2O_2 exposure, and 50% cell viability was obtained with 7 mM H_2O_2 treatment for 24 h. As depicted in Fig. 1C, after pretreatment with the monomers at 20 μM for 6 h, and followed by stimulation with 7 mM H_2O_2 for 24 h, the cell viability was increased in the monomers groups when compared with that of the H_2O_2 group.

Inhibitory effects of monomers on ROS levels and antioxidant markers

The inhibitory effect of four monomers against H_2O_2 -induced oxidative stress was evaluated by the ROS levels and antioxidant

markers of HepG2 cells. From Fig. 2A and 2C, the fluorescence intensity of ROS was the strongest in HepG2 cells induced by 7 mM H_2O_2 for 15 min. As shown in Fig. 2B and 2D, the ROS intensity was reduced after the treatment of active monomers. In Table 2, the levels of T-AOC, SOD, and CAT were increased in the monomers groups and decreased in the H_2O_2 group. With respect to For the level of T-AOC, quercetin was much higher than the other monomers, as well as that of paeonol. As for the CAT level, paeonol had the best activities among monomers. This result revealed the orientation of the further animal experiment with the combined administration of paeonol and quercetin.

Effects of monomers on the levels of Nrf2, HO-1, and NQO1 expression of HepG2 cells

The levels of Nrf2, HO-1, and NQO1 expression of HepG2 cells were analyzed using western blot. As depicted in Fig. 3A, the levels of Nrf2, HO-1, and NQO1 were distinctly increased after pretreatment with paeonol, quercetin, β -sitosterol, and gallic acid. In comparison with the model group, the level of Keap1 was suppressed. Monomers triggered the separation of Nrf2 from Keap1, and Nrf2 translocated from the cytosol to the nucleus (Fig. 3B). After pretreatment with monomers, Nrf2 levels were increased in the nucleus and reduced in the cytosol. EMSA technology was employed to assess the binding of Nrf2 to ARE. From Fig. 3C, Nrf2-ARE complexes was increased in the four monomer-treated groups.

Activation of monomers on Nrf2/Keap1 signaling pathway in HepG2 cells

The inhibitory mechanism of four monomers against oxidative stress was investigated through the Nrf2/Keap1 pathway. ML385 as an inhibitory agent of Nrf2 was adopted to examine the expression of the downstream proteins related to this Nrf2/Keap1 pathway in HepG2 cells induced by H_2O_2 . As described in Fig. 4, when ML385 was added to HepG2 cells, the level of Nrf2 was inhibited, and those of its downstream NQO1 and HO-1 were as well markedly decreased. The inhibitory effect of the monomers

Table 2. Antioxidative effects of active monomers on different parameters in HepG2 cells

Parameter	T-AOC (U/ 10^6 cell)	SOD (U/mg)	CAT (U/ 10^6 cell)
Control	121.28 \pm 3.24	30.41 \pm 1.00	0.92 \pm 0.23
H_2O_2	81.75 \pm 0.01**	19.43 \pm 0.33**	0.23 \pm 0.01**
Paeonol	99.72 \pm 1.80 [#]	19.05 \pm 0.53	1.38 \pm 0.01 [#]
Quercetin	506.96 \pm 11.30 [#]	19.27 \pm 0.21	0.61 \pm 0.26 [#]
β -sitosterol	68.27 \pm 1.56 [#]	26.83 \pm 0.34 [#]	1.07 \pm 0.26 [#]
Gallic acid	78.75 \pm 2.89	28.62 \pm 0.52 [#]	0.84 \pm 0.13 [#]
Vitamin E	80.55 \pm 0.52	21.96 \pm 3.91	0.61 \pm 0.13 [#]

Values are expressed as the mean \pm standard deviation. HepG2, human liver hepatocellular carcinoma; T-AOC, total antioxidant capacity; SOD, superoxide dismutase; CAT, catalase; H_2O_2 , hydrogen peroxide. ** $p < 0.01$, the H_2O_2 vs. control group; [#] $p < 0.05$ and [#] $p < 0.01$, the chemicals vs. H_2O_2 group.

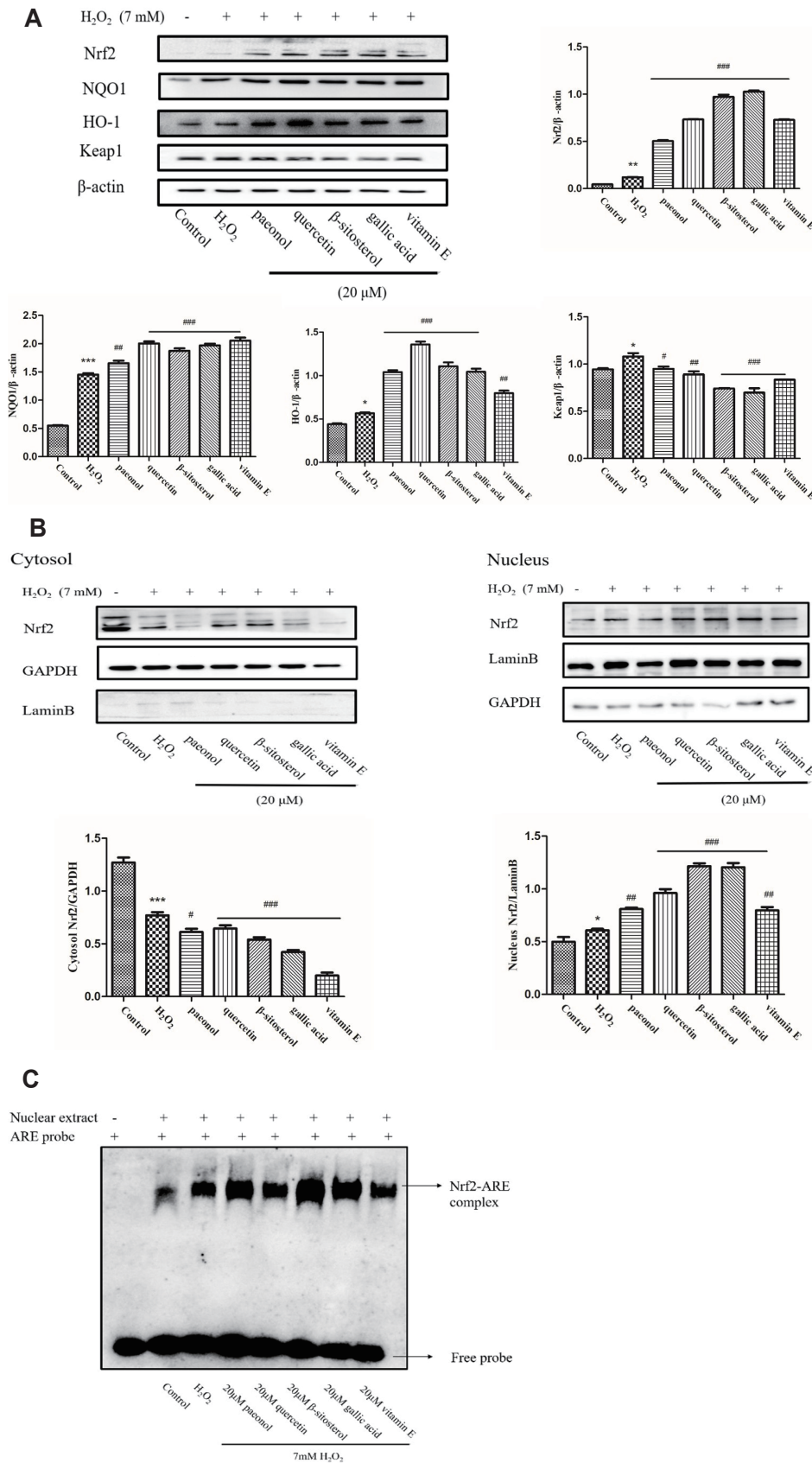


Fig. 3. Activation of four monomers on the Kelch-like ECH-associated protein 1 (Keap1)/nuclear factor E2-related factor 2 (Nrf2) signaling pathway in human liver hepatocellular carcinoma (HepG2) cells induced by hydrogen peroxide (H₂O₂). (A) The levels of Nrf2, Keap1, NQO1, and HO-1 expressions were measured by western blot assay. (B) Translocation of Nrf2 protein from the cytosol to the nucleus was also evaluated by western blot assay. (C) Effects of four monomers on the binding capacities of Nrf2-ARE complexes in HepG2 cells after H₂O₂ treatment. The data from three independent experiments are presented as the mean ± standard deviation. NQO1, quinone oxidoreductase 1; HO-1, heme oxygenase-1; ARE, antioxidant-responsive element. *p < 0.05, **p < 0.01, and ***p < 0.001, the H₂O₂ vs. control group; #p < 0.05, ##p < 0.01, and ###p < 0.001, the monomers vs. H₂O₂ group.

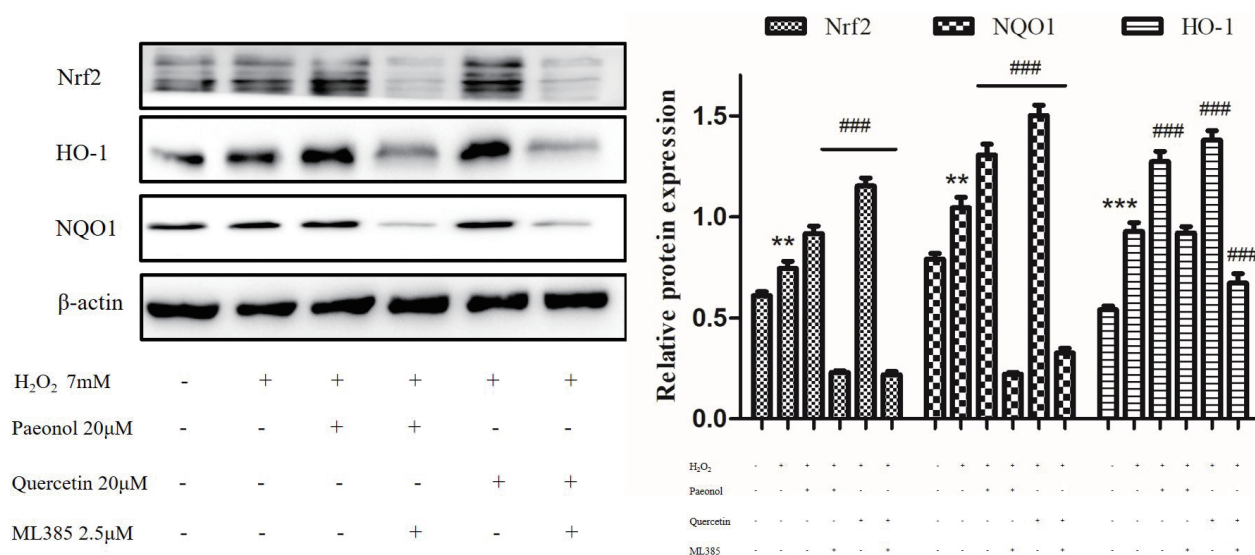


Fig. 4. The expressions of Nrf2, NQO1, and HO-1 proteins were determined in the presence or absence of paeonol, quercetin and ML385 in HepG2 cells induced by H₂O₂. The data from three independent experiments are presented as the mean ± standard deviation. Nrf2, nuclear factor E2-related factor 2; NQO1, quinone oxidoreductase 1; HO-1, heme oxygenase-1; HepG2, human liver hepatocellular carcinoma; H₂O₂, hydrogen peroxide. **p < 0.01, and ***p < 0.001, the H₂O₂ vs. control group; ###p < 0.001, the monomers vs. H₂O₂ group.

on oxidative stress was perhaps related to the Nrf2/ Keap1 pathway in HepG2 cells.

Effect of monomers on mice histopathological change and hepatic damage induced by CCl₄

Histopathological change and hepatic damage were evaluated by using H&E staining and commercial kits (Fig. 5). The H&E staining of mice liver tissue (Fig. 5A) showed that mice liver histology induced by the intraperitoneal injection of CCl₄ was significantly changed and accompanied with central venous hyperemia, inflammatory infiltration, and hepatocyte necrosis. By contrast, pretreatment with the monomers effectively prevented the development of histopathological changes. The activities of AST and ALT were measured to evaluate the hepatic damage (Fig. 5B, C). The levels of ALT, AST, and the AST/ALT ratio were facilitated in the liver injury group and were decreased in the monomers-treated groups. The levels of GSH and SOD were reduced in the CCl₄ group and were increased in the monomer groups (Fig. 5E, F). Paeonol distinctly ameliorated the liver injury, and quercetin slightly alleviated liver damage. Hence, the synergistic effects of paeonol and quercetin were not observed in mice stimulated by CCl₄.

Effect of monomers on levels of Nrf2 and HO-1 expression in mice stimulated by CCl₄

As described in Fig. 6A, compared to the model group, the Nrf2 level was increased in the paeonol group by immunohistochemical staining analysis. From Fig. 6B, the levels of Nrf2 and HO-1 of mice liver tissues were mildly increased in the CCl₄ group and

were apparently upregulated in paeonol, quercetin, L-(paeonol + quercetin) and H-(paeonol + quercetin) groups. However, the effect of paeonol was still better than that of quercetin, and the synergistic effect was not found in this study.

DISCUSSION

Paeonol, quercetin, β-sitosterol, and gallic acid extracted from MC had the antioxidative, anti-inflammatory, antifungal, anti-metastatic activities [20-25]. However, their inhibitory mechanisms against oxidative stress induced by H₂O₂ in HepG2 cells *in vitro*, and liver damage stimulated by CCl₄ in mice *in vivo*, and whether they exhibited their inhibitory effects were independent or synergy were still unknown. Our study suggested that four monomers had antioxidative capacities *in vitro* and *in vivo*. Furthermore, the inhibitory effects of the four monomers were independent and not synergistic in response to liver injury, and the potential mechanism mainly depended on the regulation Nrf2/Keap1 signaling pathway.

There was evidence proved that oxidative stress induced by H₂O₂ caused the self-generation of ROS [26]. Our study found that ROS was produced and ultimately led to cell death in the H₂O₂ group. The excessive ROS was evidently depleted with pretreatment with four monomers. Along with the continuous production of ROS, cellular innate antioxidant systems were inadequate to alleviate oxidative stress; thus, phase II defense enzymes and antioxidant proteins, such as enzymatic and antioxidants, were upregulated and played an essential role in reducing ROS [27,28]. SOD converted superoxide radicals into H₂O₂, while CAT degraded H₂O₂ into water and oxygen [29]. GSH was

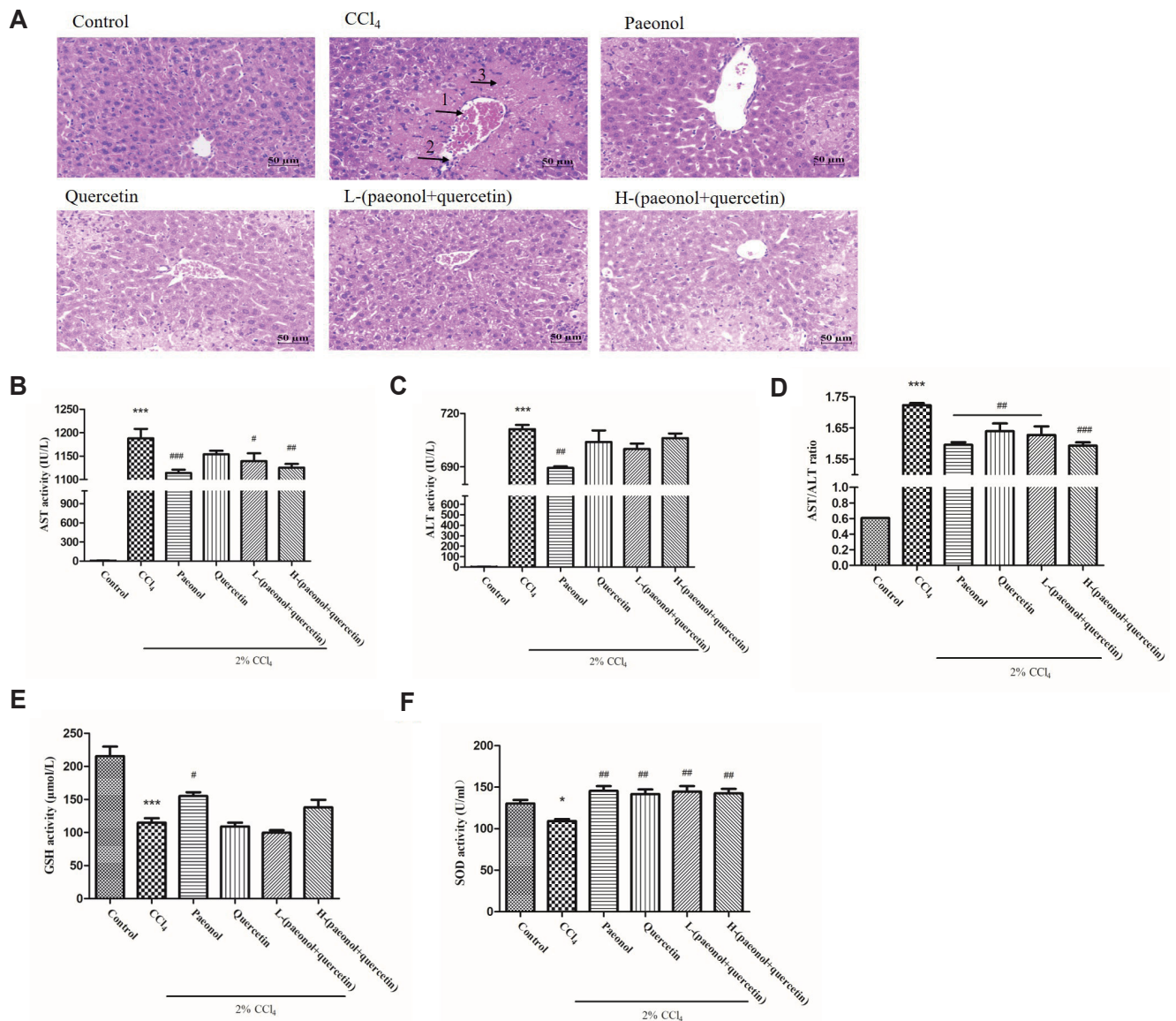


Fig. 5. Effects of separate or combined administration of paeonol and quercetin on liver tissues and serum markers of mice after carbon tetrachloride (CCl₄) intraperitoneal injection. (A) Representative histopathological changes in mice livers were observed with H&E staining. Magnification 200× (1, central venous hyperemia; 2, inflammatory infiltration; 3, hepatocyte necrosis). (B–F) Effects of active monomers on the levels of AST, ALT, AST/ALT ratio, GSH, and SOD in mice serums induced by CCl₄. Paeonol and quercetin groups were pretreated with 100 mg/kg paeonol and 100 mg/kg quercetin, respectively. L-(paeonol + quercetin) group was pretreated with 50 mg/kg paeonol and 50 mg/kg quercetin. H-(paeonol + quercetin) group was pretreated with 100 mg/kg paeonol and 100 mg/kg quercetin. The data from three independent experiments are presented as the mean ± standard deviation. AST, aspartate aminotransferase; ALT, alanine transaminase; GSH, glutathione; SOD, superoxide dismutase. **p* < 0.05, ****p* < 0.001, the CCl₄ vs. control group; #*p* < 0.05, ##*p* < 0.01, and ###*p* < 0.001, the monomers vs. CCl₄ group.

a low-molecular-weight free radical scavenger that had been implicated in oxidative damage [30]. T-AOC had been recognized as a critical index of oxidative defense [31]. Our results showed that antioxidant markers were reduced in the H₂O₂ group and were increased in the monomer groups. The hepatotoxicity induced by CCl₄ converted to trichloromethyl peroxy radical [7,32]. The reduction of ROS or inhibition of hepatotoxicity was a good strategy for the prevention of acute liver injury [33–35]. In our study, after the mice were treated with CCl₄, the phenomena of central venous hyperemia, inflammatory infiltration and hepatocyte ne-

crisis in mice liver would be occurred. The levels of ALT and AST and the ratio of AST/ALT were elevated, and the activities of SOD and GSH were decreased in the model group. Administration of paeonol, quercetin, L-(paeonol + quercetin), and H-(paeonol + quercetin) prevented liver injury induced by CCl₄. Furthermore, histopathological changes were effectively attenuated in the paeonol group but not in the combined paeonol and quercetin group. Obviously, scavenging excessive ROS, increasing the levels of antioxidant enzymes, inhibiting hepatotoxicity, and resisting cell death were beneficial for alleviating oxidative damage and liver

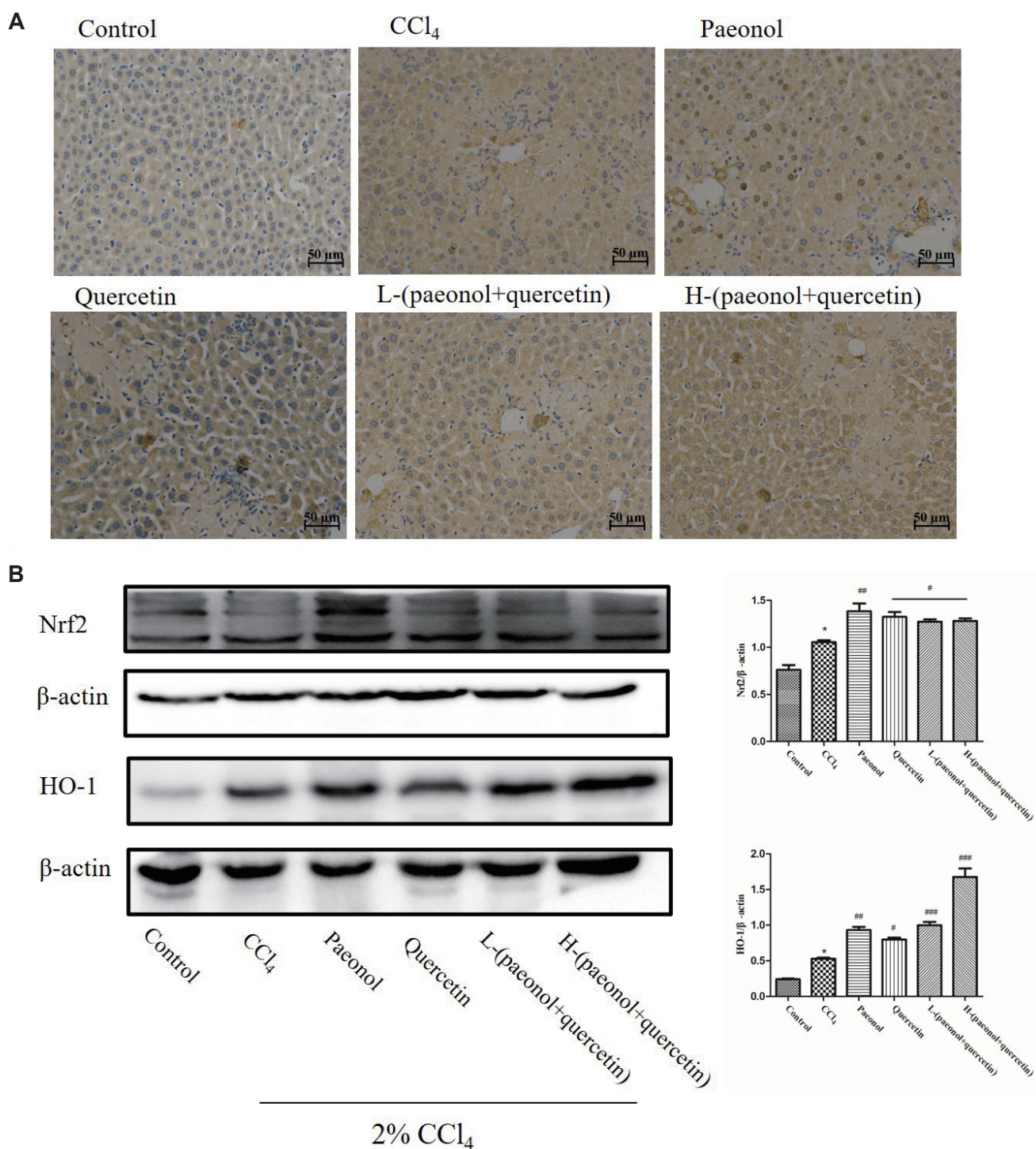


Fig. 6. Effects of separate or combined administration of paeonol and quercetin on the expressions of nuclear factor E2-related factor 2 (Nrf2) and heme oxygenase-1 (HO-1) proteins in mice livers induced by carbon tetrachloride (CCl₄). (A) Representative changes of Nrf2 in mice livers were obtained by immunohistochemical staining assay (magnification 200×). (B) The expressions of Nrf2 and HO-1 proteins were detected by western blot. The data from three independent experiments are presented as the mean ± standard deviation. **p* < 0.05, the CCl₄ vs. control group; #*p* < 0.05, ##*p* < 0.01, and ###*p* < 0.001, the monomers vs. CCl₄ group.

injury.

Nrf2 belonged to a small family of transcription factors that induced a set of antioxidant and detoxication enzymes [36]. In resting cells, Nrf2 was anchored in the cytoplasm by repressor

protein Keap1, which triggered the degradation of Nrf2 [37]. Under oxidative or electrophilic stress, the majority of cytoprotective proteins were regulated by Nrf2. The dissociation of Nrf2 from Keap1 migrated to the nucleus, which triggered the activation

of Nrf2-dependent transcription of ARE-responsive genes; this pathway seemed to be a strategy for augmenting the antioxidant defense [38,39]. Nrf2 was a highly unstable protein ($t_{1/2}$ -15 min). After the administration of the drug, Nrf2 levels first increased and later decreased in the nucleus; the highest level of Nrf2 was observed at 6 h [40]. Therefore, here Nrf2 level in the nucleus was measured after the administration of monomers for 6 h. Monomers pretreatment blocked Keap1, remarkably upregulated Nrf2 expression, promoted accumulation of Nrf2 in the nucleus, and increased the binding between Nrf2 and ARE in HepG2 cells treated with H₂O₂. Meanwhile, paeonol, quercetin, L-(paeonol + quercetin), and H-(paeonol + quercetin) group increased Nrf2 expression in CCl₄-induced mice. HO-1, which served as the primary antioxidative responder, was one of the key target genes of the Nrf2/Keap1 pathway [41,42]. NQO1 was a ubiquitous flavoprotein and a pivotal cytoprotective enzyme; it was responsible for converting reactive quinones (oxidants) to relatively stable hydroquinones [43]. Additionally, NQO1 was considered as a second antioxidative effector in the Nrf2/Keap1 pathway [44]. Our data demonstrated that pretreatment with active monomers sharply enhanced Nrf2 and HO-1 levels in the *in vitro* and *in vivo* experiments. ML385 was used to inhibit the transcription of the Nrf2 gene in HepG2 cells. After ML385 was adopted in this study, the HO-1 and NQO1 levels were inhibited as the Nrf2 level was reduced. The protective mechanism of these monomers against H₂O₂-induced oxidative stress and CCl₄-induced liver injury was mediated by the activation of the Nrf2/Keap1 signaling pathway.

In conclusion, pretreatment with paeonol, quercetin, β -sitosterol, and gallic acid attenuated oxidative stress in HepG2 cells, paeonol and quercetin exhibited a more significant alleviation on liver injury in mice. The underlying mechanism of the active monomers from MC against oxidative stress is the suppression of ROS generation, the attenuation of histological damage, up-regulation antioxidative enzymes and proteins, and the activation Nrf2/Keap1 signaling pathway. Meanwhile, our data proved that the inhibitory effects of paeonol and quercetin were independent, not synergistic in the liver injury of mice model.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

SUPPLEMENTARY MATERIALS

Supplementary data including two figures can be found with this article online at <http://pdf.medrang.co.kr/paper/pdf/Kjpp/Kjpp2020-24-05-01-s001.pdf>.

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