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Hepatoprotective effects and structure-activity relationship of five flavonoids against lipopolysaccharide/D-galactosamine induced acute liver failure in mice

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ABSTRACT

Acute liver failure (ALF) is a distinct clinical syndrome with high mortality and characterized by metabolic derangements, neurological complication, and multiple failures. Flavonoids exert great biological properties on anti-oxidation, anti-inflammation, and anti-apoptosis. After lipopolysaccharide (LPS)/D-galactosamine (D-GalN) administration, five flavonoids inhibited oxidative activities with reducing nitric oxide synthase (iNOS), malondialdehyde (MDA), and improving catalase (CAT), superoxide dismutase (SOD), total antioxidant capacity (T-AOC), nuclear factor erythroid-derived 2-related factor 2 (Nrf2) and heme oxygenase-1 (HO-1). They reduced the serum levels of alanine and aspartate aminotransferase (ALT, AST) and pro-inflammatory cytokines, prevented the phosphorylation of IKK, IκBα, and NF-κB/p65 in the NF-κB signaling pathway. Additionally five flavonoids inhibited hepatocyte apoptosis through increasing Bcl-2/Bax ratio and suppressing the Caspase family proteins. Chrysin, luteolin, apigenin, hesperetin and 3′, 4′-dimethoxy hesperetin have apparently hepato-protective effects against ALF induced by LPS/D-GalN. The study found, the C2−C3 double bond at A ring, and the hydroxyl group of C3′ or C4′ at B ring increased the protective activities, however, the effect of hydroxymethylation at C3′ and C4′ was reversed. In addition, apigenin has good hepatoprotective effects and potential as a promising therapeutic agent for ALF in clinical application.

1. Introduction

Acute liver failure (ALF) is a distinct clinical syndrome characterized by metabolic derangements, neurological complication, and multiple failures, associated with high mortality resulting from its unpredictable at the present stage and susceptibility factors are not well defined [1,2]. Common features of ALF are respiratory distress requiring mechanical ventilation and/or acute renal failure [3], cerebral edema and sepsis [4]. Liver transplantation is now the only effective treatment as patients experience life-threatening liver failure, unfortunately, its use limited since the shortage of donor livers and the expense of transplantation [5]. Therefore, continuously seeking a promising hepatoprotective agent is necessary.

Lipopolysaccharide (LPS) is a major component of the outer membrane of Gram-negative bacteria and can elicit strong immune responses [6]. Galactosamine has the ability to increase LPS-induced acute liver failure [7]. LPS and GalN-induced acute liver injury in mice is similar to clinical acute liver injury and the model has been widely

Abbreviations: ALF, acute liver failure; ALT, alanine transaminase; AP, apigenin; ARE, antioxidant response element; AST, aspartate aminotransferase; Bax, Bcl-2 associated X; Bcl-2, B-cell lymphoma 2; CAT, catalase; COX-2, cyclooxygenase-2; CR, chrysin; DMHP, 3′,4′-dimethoxy hesperetin; D-GalN, D-galactosamine; HO-1, heme oxygenase-1; HP, hesperetin; IκB, inhibitor of NF-κB alpha; IKK, inhibitor of nuclear factor kappa-B kinase; IE, leukocyte elastase; IL-1β, interleukin-1 beta; iNOS, inducible nitic oxide synthase; Keap1, Kelch-like ECH-associated protein 1; LU, luteolin; LPS, lipopoly-saccharides; MDA, malondialdehyde; NF-κB, nuclear factor kappa B; Nrf2, nuclear factor erythroid-derived 2-related factor 2; PGE2, prostaglandin E2; SI, silymarin; SOD, superoxide dismutase; T-AOC, total antioxidant capacity

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used in explore the mechanisms underlying the pathology of clinical hepatitis and discover novel liver-protective agents [8,9].

Flavonoids are common polyphenolic compounds that occur ubiquitously in life. Flavonoids exert great biological properties such as anti-oxidation [10], anti-inflammation and anti-thrombosis [11,12]. Polyphenol flavonoids including monomeric flavones, flavonols, flavanols and flavanones have a parent nucleus, diphenylpropane skeleton (C_6-C_3-C_6), which two benzene rings (ring A and B) linked by a three carbon chain that forms a closed pyran ring (C ring). Changes in the number and arrangement of hydroxyl groups as well as the nature and the extent of alkylation and/or glycosylation of these groups cause individual differences in flavonoids [13]. Sartor et al. reported that the presence of hydroxyl groups and C2−C3 double bonds inhibited leukocyte elastase (LE) activity and exerted anti-inflammatory effects [14]. Ueda et al. showed that the introduction of hydroxyl groups of flavonoids at A-5, A-7, and B-4′ resulted in a decrease of TNF-α activity, and the C-3 hydroxyl group presence or B-4′ hydroxyl group deletion led to the disappearance of TNF-α inhibitory activity [15]. García-Mediavilla et al. found that the presence of C2−C3 double bond enhances antioxidant activity [16]. In addition, Khoo et al. demonstrated that Chrysin (CR) inhibited Akt signaling and cell proliferation, and induced apoptosis [17]. Hougee et al. suggested that CR, luteolin (LU), and apigenin (AP) reduced the production of pro-inflammatory cytokines in LPS-stimulated peripheral blood mononuclear cell supernatants [18]. Zhou et al. investigated that AP prevented the D-GalN/LPS-induced liver injury in mice might be associated with the increments of Nrf-2-mediated antioxidative enzymes and modulation of PPARγ/NF-κB-mediated inflammation [19]. Hirata et al. studied that hesperetin (HP) exerted anti-inflammatory effects through reducing COX-2 gene expression in RAW 264.7 cells [20]. Kang et al. revealed that silymarin (SI) inhibited LPS-induced DNA binding activity of NF-κB/Rel in RAW 264.7 cells through the inhibition of the degradation of inhibitory factor-κB [21].

As above mentioned, slight changes in the structure of flavonoids made their function varied. In our previous study, three flavones (CR, LU and AP) and two flavanones (HP and DMHP) have been extracted and confirmed. Although numerous literatures have investigated the different mechanism of five flavonoids against inflammatory disease using the *in vitro* cell model, which through the inhibition of Akt or NF-κB signaling and the increment of Nrf-2-mediated anti-oxidative enzymes, limited references focused on revealing the *in vivo* anti-inflammatory mechanism on acute liver failure animal model induced by LPS/D-GalN, as well as comparison of anti-inflammatory capacities and structure-activity relationships. In order to elucidate the *in vivo* anti-inflammatory mechanism and structure-activity relationships, this study not only performed the hepatoprotective effects of five flavonoids against endotoxin-induced ALF in mice, which involved as Nrf2/Keap1 anti-oxidant pathway, NF-κB signaling pathway, production of inflammatory cytokines and expression of apoptosis-related molecules, but also carried out their structure-activity relationships for the further molecular design and synthesis of anti-inflammatory candidate.

2. Methods

2.1. Chemicals

CR, LU, AP, HP and DMHP (Fig. 1) were provided by the department of medicinal chemistry of Southwest University (purity 99% by HPLC, China). SI and LPS (*Escherichia coli* 055: B5) were purchased from Sigma-Aldrich Co. LLC. (Shanghai, China). D-GalN was obtained from Aladdin Reagent Database, Inc. (Shanghai, China). Diagnostic kits used for the determination of alanine transaminase (ALT), aspartate aminotransferase (AST), nitric oxide synthase (iNOS), malondialdehyde (MDA), catalase (CAT), superoxide dismutase (SOD) and total antioxidant capacity (T-AOC) activities were obtained from Nanjing Jiancheng Institute of Biotechnology (Nanjing, China). Rabbit inhibitor of nuclear factor kappa-B kinase (IKK), p-IKK, inhibitor of NF-κB alpha (IκB); p-IκB, nuclear factor kappa B (NF-κB) p65, cyclooxygenase-2 (COX-2), interleukin-1 beta (IL-1β), IL-6, nuclear factor erythroid-derived 2-related factor 2 (Nrf2), heme oxygenase-1 (HO-1), Bcl-2 associated X (Bax), B-cell lymphoma 2 (Bcl-2), Caspase 3, 9, β-actin, mouse Caspase 8 polyclonal primary antibodies, goat anti-mouse IgG-HRP-conjugated secondary antibody and goat antirabbit IgG-HRP-conjugated secondary antibody were purchased from Proteintech (Wuhan, China), and total protein extraction kits were from Sangon Biotech Co. Ltd. (Shanghai, China).

2.2. Mice

Male Kunming mice weighing (18–22 g) were obtained from Chongqing Academy of Chinese Materia Medica, China. Certificate of Conformity: SCXK (Jing) 2012-0011. Ten mice per group were chosen. Mice were selected from the pool eligible for inclusion in the study and were randomly divided into eight groups. All the animal experiments in this study were carried out in accordance with the Guide for the Care and Use of Laboratory Animals.

2.3. Animal welfare

Mice were housed in SPF-class animal laboratory with air-conditioning and 12 h dark-light cycle, ambient temperature at 23 °C(± 2 °C), and relative humidity of 50%. Mice were feed food and tap water. The mice were acclimatized for at least one week. The procedures used were as humane as possible. All studies involving animals are reported in accordance with the ARRIVE and US NIH guidelines for reporting experiments involving animals.

2.4. Experimental design

The control group received normal saline. The LPS/D-GalN model group was administered with saline once daily for 6 days. Flavonoid groups were divided into CR, LU, AP, HP, DMHP groups. SI was as a positive group. All flavonoids and SI respectively dissolved in 0.5% sodium carboxymethyl-cellulose and fed to mice by oral administration at a dose of 100 mg·kg^{-1} weight·day for 6 days in different groups. 1 h after final saline and flavonoids treatment, mice (except for control group) were injected with LPS/D-GalN (LPS, 10 μg·kg^{-1} body weight, D-GalN, 400 mg·kg^{-1} body weight, dissolved in saline) [22]. At 6 h post LPS/D-GalN treatment, all mice were sacrificed. Blood samples were collected from the eyeball of mice and centrifuged at 4 °C for 10 min at 1.4×10^4 rpm in glass tubes. The serum was stored at −80 °C in polystyrene tubes until use. Livers were harvested immediately and stored at −80 °C for histopathology and western blot assay.

2.5. Determination of mortality

The effects of five flavonoids pretreatment on LPS/D-GalN-induced mortality in mice were monitored over a 24 h period. The number of dead mice was counted every 2 h after LPS/D-GalN injection.

2.6. Histopathological changes

Several liver tissues were fixed in 4% paraformaldehyde and cut into 5 μm sections, and then embedded in paraffin blocks. After that sections were rehydrated and stained with hematoxylin and eosin (H&E) for histological assessment. The sections were observed using a fluorescence microscope system (TE2000, Nikon Japan) and representative images were presented.

2.7. The levels of hepatic damage and antioxidants

Blood was collected into different centrifuge tubes and centrifuged at 5000 rpm for 10 min, the serum stored at −20 °C until further use.

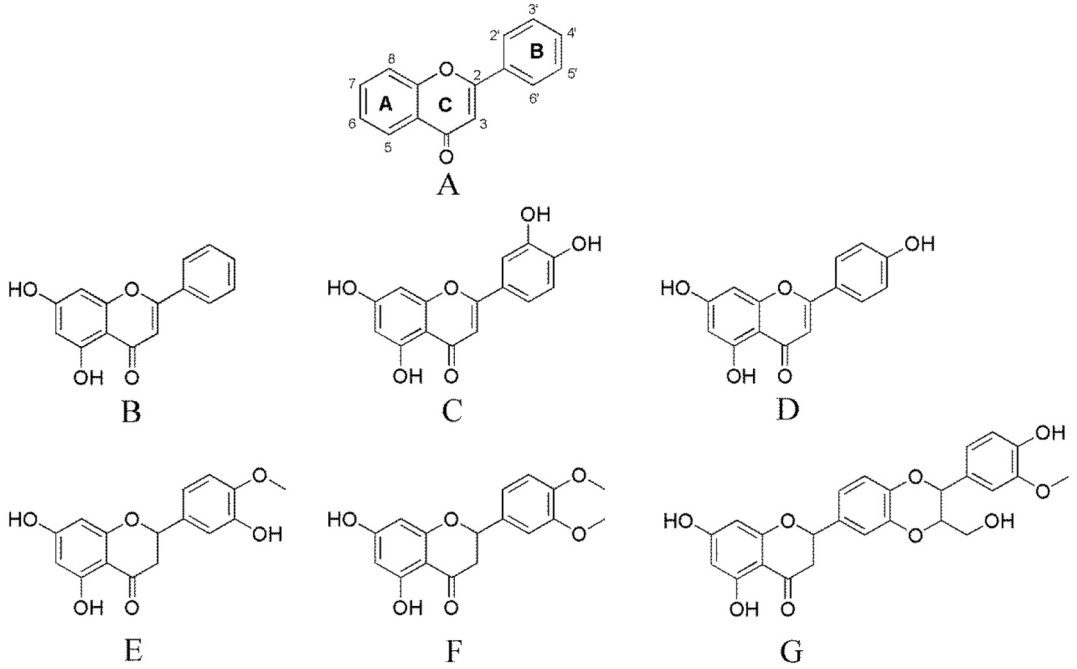

Fig. 1. Chemical structure of flavonoids. (A) Basic skeleton structure of flavonoid. (B) Chrysin. (C) Luteolin. (D) Apigenin. (E) Hesperetin. (F) 3′, 4′-Dimethoxy Hesperetin. (G) Silymarin.

The ALT and AST activities in serum were determined using manufacturer's instructions (Nanjing Jiancheng Biotechnology Institute, China). Livers were homogenized on ice and centrifuged at 5000 rpm for 10 min at 4 °C. Subsequently, supernatants were used to determine the concentrations of CAT, iNOS, MDA, SOD, and T-AOC. Biomarkers were measured using commercial assay kits (Nanjing Jiancheng Biotechnology Institute, China).

2.8. Western blot analysis

A western blot assay was performed to observe the protein expression. Proteins were extracted using commercially available kits, following the manufacturer's recommendation (Sangon Biotech Co. Ltd., Shanghai, China). The proteins are separated and transferred onto membranes. The membranes blocked in 5% skim milk at room temperature for 2 h. After that, the membranes were incubated with primary antibodies overnight at 4 °C and incubated with secondary antibodies at room temperature for 2 h. Then, target proteins were presented with representative images. The proteins were detected using Image Jet software and with β-actin as an internal standard.

2.9. Statistical analysis

All data were expressed as mean ± S.D. The statistical significance of the differences between groups was determined by one-way analysis of variance in SPSS 18.0. A p-value of ≤ 0.05 was used to determine statistical significance.

3. Results

3.1. Effects of flavonoids on LPS/D-GalN induced mortality and histopathological changes

The effects of different flavonoids pretreatment on LPS/D-GalN-induced mortality in mice are depicted in Fig. 2. Mice in the control group survived. Mice began to die after 8 hs of LPS/D-GalN injection and mortality reached 100% at 12 hs in the model group. However, compound groups remarkably decreased mice mortality induced by LPS/D-GalN. H&E staining assay was used to perform a histopathological assessment of liver injury. As shown in Fig. 3, the liver structure of the control group showed normal. The administration of LPS/D-GalN notably caused histological changes in the liver tissue as central vein venous congestion, inflammatory infiltration and the loss of hepatic architecture-vacuolation. However, flavonoids apparently attenuated liver changes and effectively blocked the development of liver histopathology induced by LPS/D-GalN.

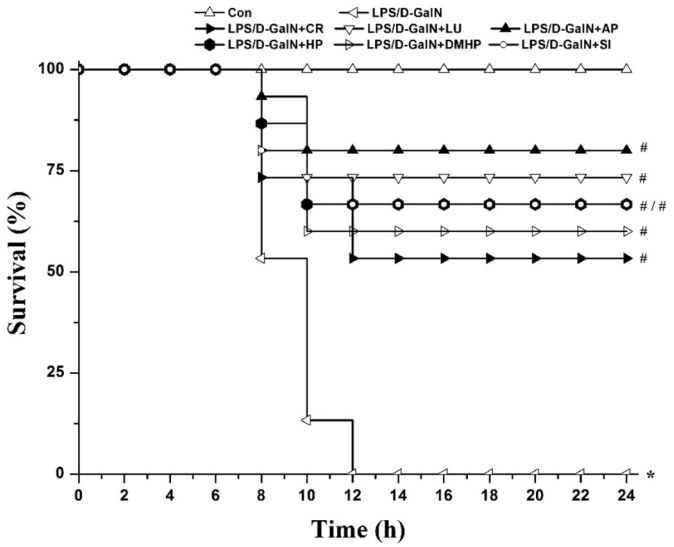

Fig. 2. Mortality in flavonoids-treated ALF mice induced by LPS/D-GalN endotoxin. The mortalities of the mice were measured every 2 h for 24 h. The administered dosages were as follows: LPS/D-GalN: LPS 10 $\mu g \cdot kg^{-1}$, D-GlaN 400 $mg \cdot kg^{-1}$; CR, LU, AP, HP, DMHP and SI (respectively treated with 100 $mg \cdot kg^{-1}$) + LPS/D-GalN; * Significantly different ($p < 0.05$) from the control group. # Significantly different ($p < 0.05$) from the model group.

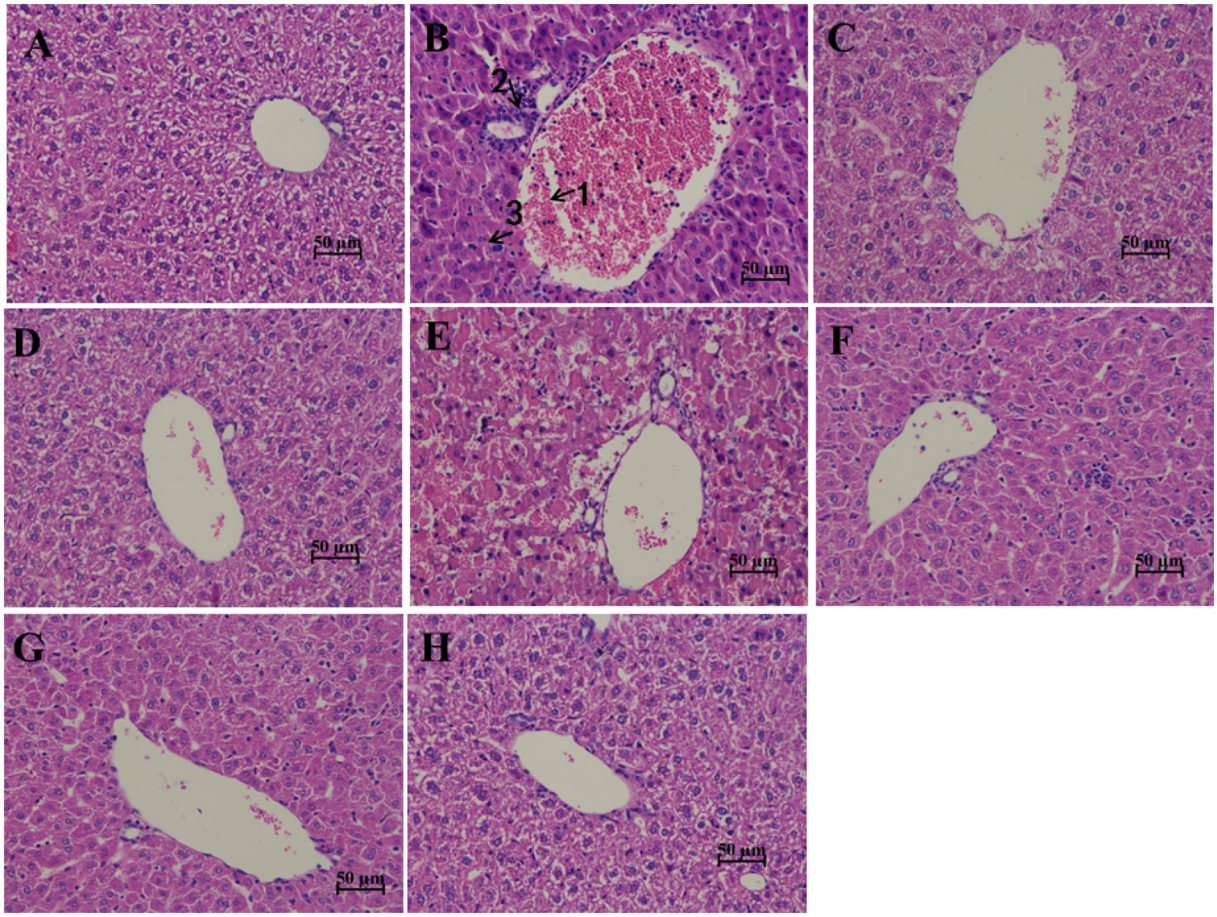

Fig. 3. Effect of flavonoids on histopathological changes of liver tissue in ALF mice after LPS/D-GalN administration. Histological assessments were performed by H&E staining. Typical images were chosen from each experimental group. Original magnifications were 200×. (A) Control. (B) Model (LPS/D-GalN: LPS 10 μg·kg^{-1}, D-GlaN 400 mg·kg^{-1}). (1. Central venous hyperemia; 2. Inflammatory infiltration; 3. The loss of hepatic architecture-vacuolation). (C) CR. (D) LU. (E) AP. (F) HP. (G) DMHP. (H) SI. (C–E) CR, LU, AP, HP, DMHP and SI (respectively treated with 100 mg·kg^{-1}) + LPS/D-GalN.

3.2. Effects of flavonoids on hepatic damage and oxidative markers

ALT and AST activities were measured to assess the liver injury. As illustrated in Fig. 4, compared with control group, the administration of LPS/D-GalN distinctly increased the activities of ALT and AST. However, pretreatment with flavonoid strikingly reduced AST and ALT activities. The quantitative analysis of iNOS, MDA, CAT, SOD, and T-AOC served as the evaluation of flavonoids on oxidative damage (Table 1). The enzyme activities of CAT, SOD, and T-AOC decreased in LPS/D-GalN group, whereas that increased after flavonoids treatment. Moreover, the levels of iNOS and MDA increased in LPS/D-GalN group and suppressed in flavonoid groups. In brief, flavonoids ameliorated hepatic damage and mitigated oxidative markers induced by LPS/D-GalN.

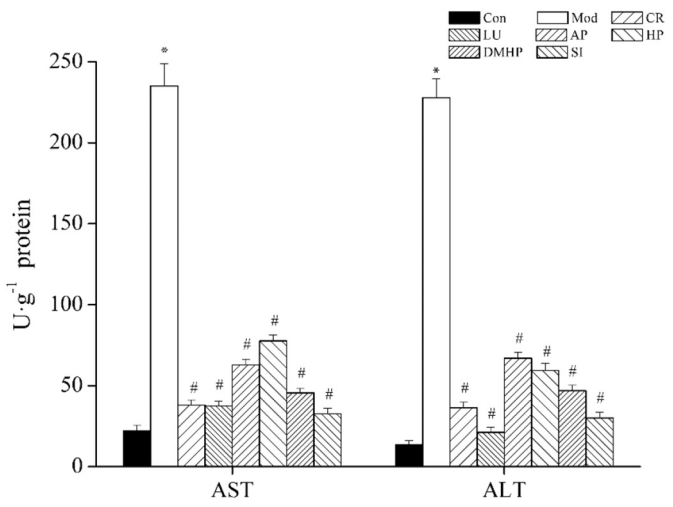

Fig. 4. Activities of ALT and AST in the serum of mice treated with flavonoids after LPS/D-GalN administration. These data represent the mean ± SD (n = 10). * Significantly different ($p < 0.05$) from the control group. # Significantly different ($p < 0.05$) from the model group.

3.3. Effects of flavonoids on stress response induced by LPS/D-GalN

Nrf2 is a transcription factor and important in various types of stress responses. To investigate the effect of flavonoids on LPS/D-GalN stimulated stress response, the amount of the relevant proteins (Nrf2 and HO-1) expression employed in western blot analysis (Fig. 5). After LPS/D-GalN administration, the expression of Nrf2 and HO-1 increased. Compared to model group, Nrf2 and HO-1 expression increased in five flavonoids treatment.

3.4. Effects of flavonoids on the NF-κB signaling pathway

Inflammatory cytokines play a critical role in the pathogenesis of ALF. The expressions of key proteins were detected by western blot. From Fig. 6, compared with the control group, the expression levels of the phosphorylation of IKK and IκB dramatically increased and NF-κB/p65 expression in the cytosol significantly reduced in the model group.

Table 1
The levels of flavonoids on the concentrations of MDA, iNOS, CAT, SOD and T-AOC in mice serum after LPS/D-GalN administration ($\bar{x} \pm s$, n = 10).

Group	MDA (nmol·mL^{-1})	iNOS (U·mg^{-1})	CAT (U·g^{-1})	SOD (U·mL^{-1})	T-AOC (U·mL^{-1})
Control	10.556 ± 1.746	10.709 ± 1.303	22.258 ± 2.262	349.087 ± 15.383	15.087 ± 0.931
Model	18.778 ± 1.819*	25.938 ± 3.484*	6.293 ± 0.785*	97.069 ± 10.718*	5.717 ± 0.570*
LPS/D-GalN + CR	14.444 ± 2.703	16.506 ± 1.532#	10.810 ± 1.193	207.403 ± 18.749#	8.510 ± 0.868
LPS/D-GalN + LU	10.444 ± 16.37#	15.229 ± 2.835#	15.071 ± 1.661#	234.534 ± 21.763#	11.840 ± 1.642#
LPS/D-GalN + AP	9.444 ± 0.778#	11.250 ± 2.071#	18.518 ± 1.271#	288.796 ± 19.850#	8.840 ± 1.213
LPS/D-GalN + HP	12.000 ± 1.262#	16.359 ± 1.361#	9.892 ± 1.843	208.005 ± 16.412#	5.797 ± 1.198
LPS/D-GalN + DMHP	12.333 ± 2.309#	21.664 ± 1.126	8.070 ± 0.838	133.244 ± 23.006	13.033 ± 0.967#
LPS/D-GalN + SI	8.333 ± 0.882#	13.313 ± 0.993#	17.781 ± 1.659#	294.825 ± 19.924#	17.781 ± 1.659#

* $p < 0.05$ *vs* control group.
# $p < 0.05$ *vs* model group.

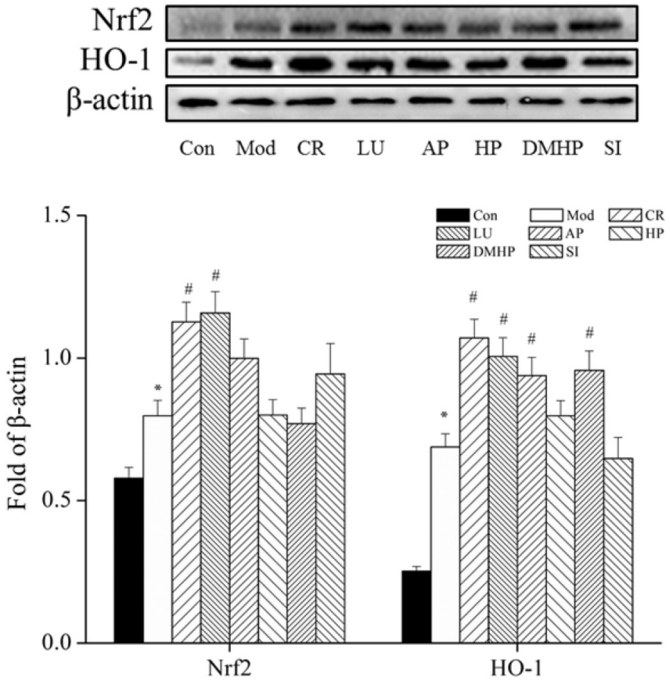

Fig. 5. Effects of flavonoids on the expressions of Nrf2 and HO-1 in mice livers induced by LPS/D-GalN. These data represent the mean ± SD (n = 10). * Significantly different ($p < 0.05$) from the control group. # Significantly different ($p < 0.05$) from the model group.

However, after flavonoids pretreatment, the expression of the phosphorylation of IKK and IκB inhibited, and NF-κB/p65 expressions in the nucleus reduced and that in the cytosol increased. As shown in Fig. 7, the amount of COX-2 enzyme and the expression levels of pro-inflammatory cytokines such as IL-1β and IL-6 evidently inhibited after flavonoids pretreatment, although LU and AP had no inhibitory effect on IL-6.

3.5. Effects of flavonoids on hepatocyte apoptosis induced by LPS/D-GalN

To explore the inhibitory mechanism of five flavonoids on hepatocellular apoptosis induced by LPS/D-GalN, the apoptotic-related signaling proteins (Bcl-2, Bax, cleaved Caspase 3, 8 and 9) were determined by western blot analysis (Fig. 8). After LPS/D-GalN administration, the expression amount of Bax increased and Bcl-2 decreased. The Bcl-2/Bax ratio in the model group, which indicated apoptosis, was markedly lower than that of the control group. The Bcl-2/Bax ratio of CR, LU, HP and DMHP increased compared to the model group. In addition, the amount of cleaved Caspases 3, 8 and 9 in Caspase family proteins reduced after flavonoids treatment. These results indicated that these flavonoids possibly inhibited LPS/D-GalN-induced hepatocellular apoptosis through affecting the expression of apoptosis-related factors.

4. Discussion and conclusions

Acute liver failure is characterized by hepatocyte death and activation of the innate immune response [23]. The combination of LPS and D-GalN was commonly used in experimental models of fulminant hepatic failure. Studies have shown that several dietary flavonoids have protective effects against some chronic diseases, including cognitive decline, diabetes, cardiovascular disease, and certain cancers [24–27], which are usually associated with apoptosis induction, an increase of free radical production, and the enhancement of oxidative stress. The aim of this study was to reveal the protective effects of flavonoids against LPS/D-GalN induced hepatotoxicity with the oxidation, inflammatory responses, and apoptotic signals.

When administration with LPS/D-GalN, the mice mortality rate was up to 100% at 12 h, and histopathological changes occurred such as central venous hemorrhage, inflammatory infiltration and the loss of hepatic architecture-vacuolation, it suggested that ALF model was successfully established by i.p. injection of LPS/D-GalN. While, five flavonoids markedly improved the mice survival exceeding 50% at 12 h and prevented histopathological changes as well as the inhibition of biochemical markers of AST and ALT activities in ALF [28].

Oxidative stress is a recognized phenomenon in liver injury induced by LPS/D-GalN [29,30]. MDA is a typical marker of oxidative stress. Overexpression of iNOS impairs liver morphology and increases nitrite production following acute endotoxemia [31]. SOD and CAT are major radical-scavenging antioxidant enzymes in the human body [32]. T-AOC serves as a preventive index of oxidant damage. Our results revealed that with the administration of LPS/D-GalN, the expression levels of MDA and iNOS increased, and enzyme activities of SOD and CAT decreased. On the contrary, this phenomenon changed after flavonoids pretreatment. The Nrf2/Keap1 signaling pathway regulates the expression of genes and functions which is related to oxidative stress and cell survival as antioxidant proteins, free radical metabolism, detoxification enzymes, inhibition of inflammation, proteasome function. The Nrf2 and HO-1 are critical for cellular stress responses. Under non-stressed conditions, Nrf2 is retained in the cytoplasm by its repressor protein Keap-1 in a 'silent' form. Oxidation and environmental stimulation modify the cysteine residues of Keap-1, making Nrf2 rapidly translocated into the nucleus and binding the upstream antioxidant response element (ARE) in the promoter regions of detoxification genes such as SOD, and HO-1 [33]. In this study, under the stimulation of LPS/D-GalN, Nrf2 dissociated from Keap1 and induced HO-1 expression. Landolfi et al. showed that the reduction of the C2—C3 double bond results in the loss of inhibitory activity of the PGE2-producing cyclooxygenase pathway [34]. It meant that the C2—C3 double bond enhanced the resistance to oxidative damage. Our study indicated that the expression levels of Nrf2 and HO-1 in the DMHP, HP, and SI groups were generally lower than those in the CR, LU, AP groups. Reduction of

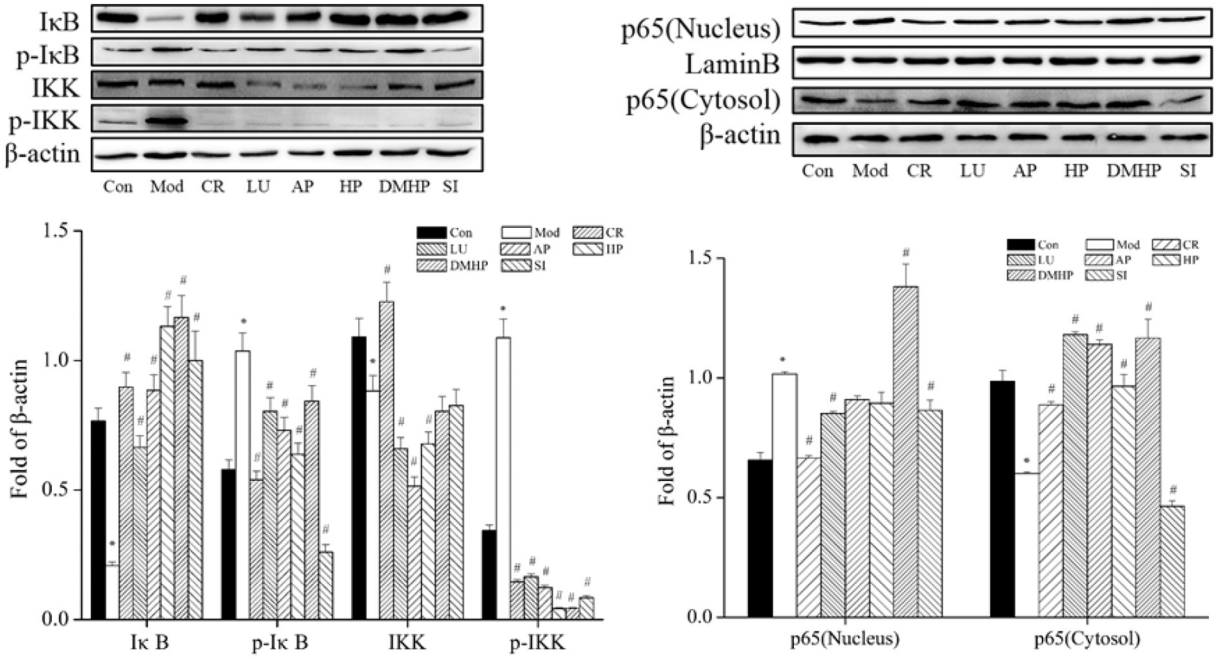

Fig. 6. Effects of flavonoids on NF-κB signaling pathway in mice livers after LPS/D-GalN administration. These data represent the mean ± SD (n = 10). * Significantly different ($p < 0.05$) from the control group. # Significantly different ($p < 0.05$) from the model group.

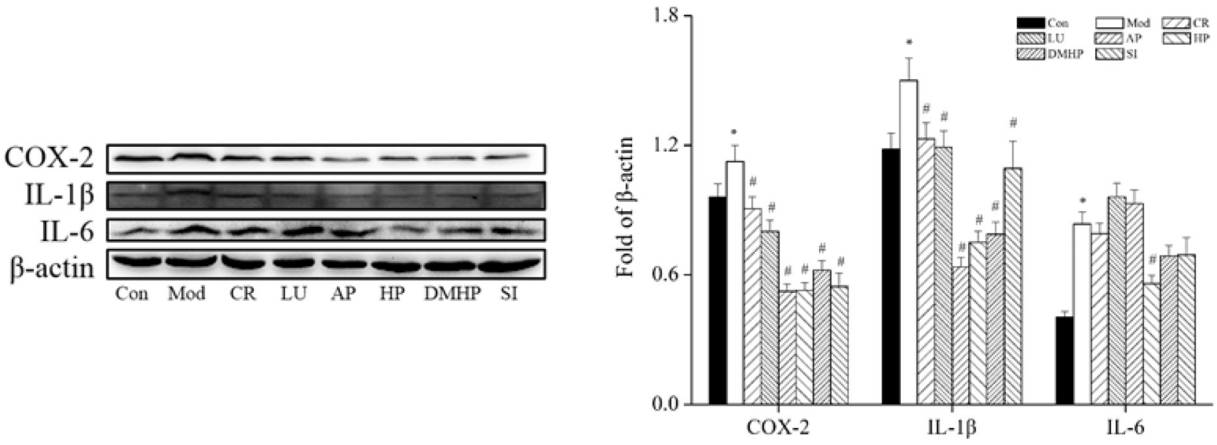

Fig. 7. Effects of flavonoids on the expressions of COX-2, IL-1β, and IL-6 in mice livers induced by LPS/D-GalN. These data represent the mean ± SD (n = 10). * Significantly different ($p < 0.05$) from the control group. # Significantly different ($p < 0.05$) from the model group.

the C2—C3 double bond diminishing the resistance to oxidative damage was agreed with the above reports.

The transcription factor NF-κB promotes immunity by controlling the expression of genes involved in inflammation. NF-κB activity is under the control of extracellular stimulation signals. IKK phosphorylation of IκB molecules promotes their degradation and releases NF-κB, which translocates to the nucleus to promote the transcription of target genes [35]. Our results showed that the phosphorylated levels of IκB and IKK, and the nuclear NF-κB/p65 expression in the model group were greatly higher than those in the control group. Pretreatment of flavonoids reduced these phosphorylated levels and inhibited the translocation of NF-κB/p65 from cytosol to nucleus. After the translocation of NF-κB/p65 into nucleus induced by LPS/D-GalN, the levels of several inflammatory cytokines such as IL-1β and IL-6 increased. Overexpression of inflammatory mediators is associated with the pathogenesis of inflammatory diseases, prevention of the NF-κB pathway can effectively block the expression of pro-inflammatory cytokines [36]. Ko et al. reported that the hydroxylation of flavanol at C4′ in B ring mediated the inhibition of COX-2 expression induced by phorbol ester [37]. Hou et al. showed that anthocyanins containing ortho-hydroxyl groups inhibited LPS-induced COX-2 expression in RAW 264.7 cells [38]. In our study, as for the inflammatory factors, LU and AP had stronger inhibitory effects than CR; moreover, the inhibition of AP and HP were more than LU and DMHP, respectively. The study also indicated that hydroxyl group at C3′ or C4′ position of B ring had better inhibition than that of non-hydroxy, two hydroxyl or methoxy groups. This result is confirmed by Shanmugam et al.'s report that the presence of the 4′-OH at B ring contributing to the anti-inflammatory effect, while the hydroxymethylation at C3′ and C4′ position diminishing the activity [39].

Apoptosis is a regulated and controlled process that is the primary mechanism for the removal of aged, damaged and unnecessary cells during an organism's lifecycle [40]. It is characterized by the activation of the cysteinyl aspartate-specific proteases (caspases) and the breakdown of the dead cell system into readily phagocytized apoptotic bodies [41]. In determining the susceptibility of cells to apoptosis, the Bcl-2/Bax ratio is more important than the individual Bax or Bcl-2 level. A high Bcl-2/Bax ratio always has a great anti-apoptotic activity [42]. In

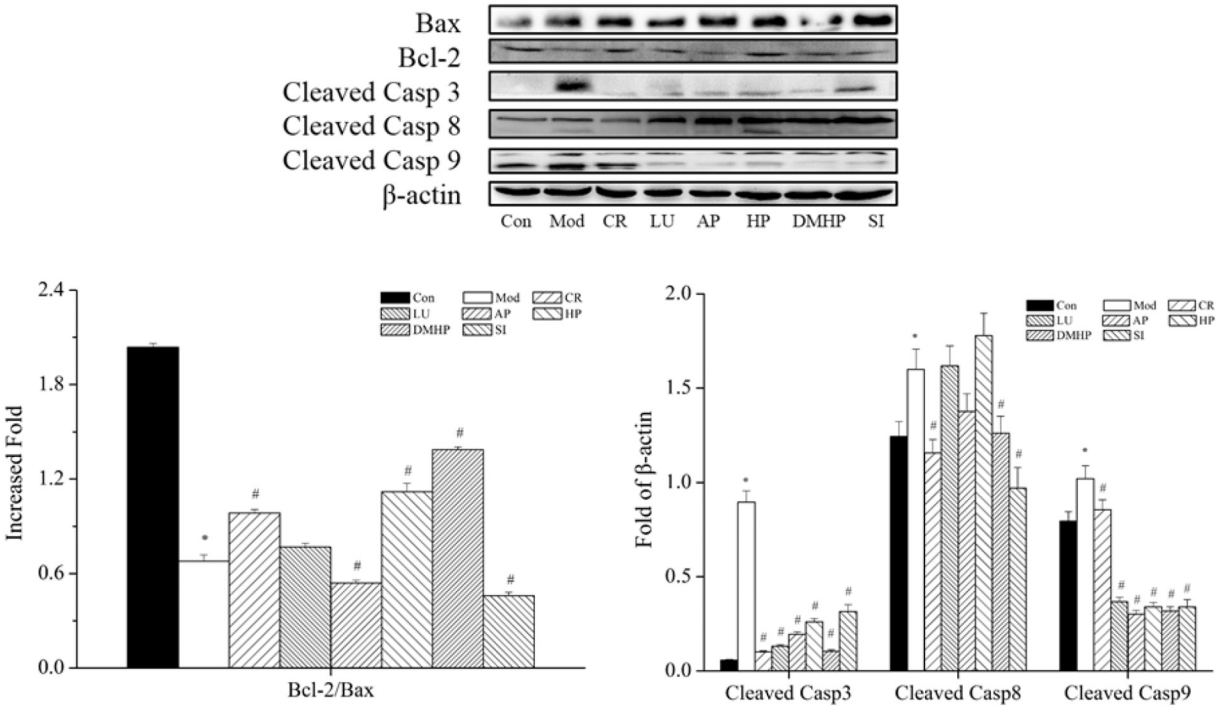

Fig. 8. Effects of flavonoids on the expression levels of Bax, Bcl-2, Caspase 3, 8, and 9 in mice livers induced by LPS/D-GalN. These data represent the mean ± SD (n = 10). * Significantly different ($p < 0.05$) from the control group. # Significantly different ($p < 0.05$) from the model group.

our study, when compared to the control group, the ratio of Bcl-2/Bax became lower at LPS/D-GalN administration, whereas that increased after CR, LU, HP and DMHP pretreatment. In contrast to the model group, the low ratios of Bcl-2/Bax in AP and SI groups were coherent with the reports of Banerjee and Katiyar. Banerjee et al. indicated that the molecular basis of the apoptotic potential of AP was ascribed to the disruption in pro-apoptotic and anti-apoptotic protein balance between Bcl-2 and Bax [43]. Katiyar et al. showed that SI treatment decreased the Bcl-2/Bax ratio in JB6 C141 cells [44]. Pro-apoptotic Bax and anti-apoptotic Bcl-2 are membrane-bound pore-forming proteins that interact through heterodimerization. They regulate the mitochondrial transmembrane channels of cytochrome *c* and activate the top Caspase proteins (*i.e.* Caspase 8 or 9) and the downstream effector of Caspase-3 to cause cell death [41,45]. In this study, the apoptotic-related signaling proteins (Bcl-2, Bax, cleaved Caspase 3, 8 and 9) were determined by western blot analysis (Fig. 8). These results indicated that five flavonoids inhibited LPS/D-GalN-induced hepatocellular apoptosis through affecting the expression of apoptosis-related factors. Cleaved Caspases 3, 8 and 9 proteins generally diminished by flavonoids, although LU and HP exhibited no significance on cleaved Caspase 8 protein.

In conclusion, we found that five flavonoids have hepatoprotective effects against LPS/D-GalN induced ALF. The underlying mechanism is implicated in inhibiting free radicals, promoting the expression of antioxidant proteins, suppressing inflammatory factors in NF-κB signal pathway, and attenuating hepatocyte apoptosis. As to the structure-activity relationship, the C2−C3 double bond at C ring, and the hydroxyl group of C3′ or C4′ at B ring increased the protective activities, however, the effect of hydroxymethylation at C3′ and C4′ was reversed. In addition, AP has good hepatoprotective effects and potential as a promising therapeutic agent for ALF in clinical application.

Author contributions

B. Z. designed the research, and D. H. provided suggestions for research. Y. H. and Z. X. performed most of the experiments, with the assistance of D. Y., J. W., L. J. analyzed the data. X. Y. and X. L. contributed analytic tools. Y. H. wrote the primary manuscript and B. Z. revised the manuscript.

Conflicts of interest

The authors state that they have no conflicts of interest.

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