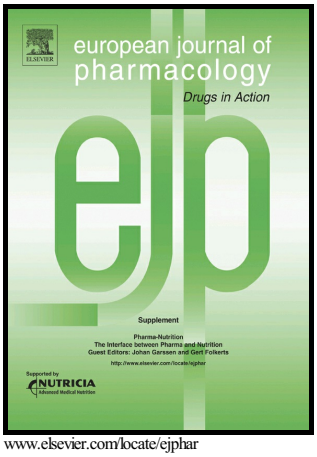


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PII: S0014-2999(18)30074-8
DOI: https://doi.org/10.1016/j.ejphar.2018.02.002
Reference: EJP71655

To appear in: *European Journal of Pharmacology*

Received date: 4 October 2017
Revised date: 15 January 2018
Accepted date: 6 February 2018

Cite this article as: Yulong Kong, Xiaoguang Gao, Changyuan Wang, Chenqing Ning, Kexin Liu, Zhihao Liu, Huijun Sun, Xiaodong Ma, Pengyuan Sun and Qiang Meng, Protective effects of yangonin from an edible botanical Kava against lithocholic acid-induced cholestasis and hepatotoxicity, *European Journal of Pharmacology,* https://doi.org/10.1016/j.ejphar.2018.02.002

# Protective effects of yangonin from an edible botanical Kava against lithocholic acid-induced cholestasis and hepatotoxicity

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**ABSTRACT**

Accumulation of toxic bile acids in liver could cause cholestasis and liver injury. The purpose of the current study is to evaluate the hepatoprotective effect of yangonin, a product isolated from an edible botanical Kava against lithocholic acid (LCA)-induced cholestasis, and further to elucidate the involvement of farnesoid X receptor (FXR) in the anticholestatic effect using *in vivo* and *in vitro* experiments. **The cholestatic liver injury model was established by intraperitoneal injections of LCA in C57BL/6 mice. Serum biomarkers and H&E staining were used to identify the amelioration of cholestasis after yangonin treatment. Mice hepatocytes culture, gene silencing experiment, real-time PCR and Western blot assay were used to elucidate the mechanisms underlying yangonin hepatoprotection.** The results indicated that yangonin promoted bile acid efflux and reduced hepatic uptake via an induction in FXR-target genes Bsep, Mrp2 expression and an inhibition in Ntcp, all of which are responsible for bile acid transport. Furthermore, yangonin reduced bile acid synthesis through repressing FXR-target genes Cyp7a1 and Cyp8b1, and increased bile acid metabolism through an induction in gene expression of Sult2a1, which are involved in bile acid synthesis and metabolism. In addition, yangonin suppressed liver inflammation through repressing inflammation-related gene NF-κB, TNF-α and IL-1β. *In vitro* evidences showed that the changes in transporters and enzymes induced by yangonin were abrogated when FXR was silenced. In conclusions, yangonin produces protective effect against LCA-induced hepatotoxity and cholestasis due to FXR-mediated regulation. Yangonin may be an effective approach for the prevention against cholestatic liver diseases.

**Keywords:** Cholestasis; FXR; Bile acids; Hepatic inflammation; Yangonin

## 1. Introduction

Cholestatic liver disease arises from toxic bile acid accumulation in liver when the formation or excretion of bile acids is interrupted (Sharanek et al., 2017). Bile acids are crucial for the absorption of intestinal fat and also implicated in the pathology of hepatic diseases (Trauner et al., 2008; Zhang et al., 2015). Lithocholic acid (LCA) is a hepatotoxic hydrophobic secondary bile acid formed by bacterial 7α-dehydroxylation of chenodeoxycholic acid and ursodeoxycholic acid in intestine (Zeng et al., 2016). However, excessive LCA can lead to a loss of gap-junction proteins, resulting in leaky junctions and a collapse of the bile osmotic gradient followed by decreased movement across membranes and cellular swelling (Hofmann, 2004; Owen et al., 2010; He et al., 2017). Cholestatic patients were found to have an elevated level of LCA in circulating blood (Chen et al., 2014). LCA administration to mice leads to hepatic parenchyma damage and disruption of bile flow which are similar to human with cholestatic liver disease (Fickert et al., 2006; Wang et al., 2012) and therefore has been widely applied to model intrahepatic cholestasis.

The therapeutic strategy against cholestasis is mainly focused on enhancement of hepatic bile acid efflux and detoxification. Nuclear receptors, transcription factors and their target genes have been demonstrated to play crucial roles in bile acid transport and detoxification (Zollner et al., 2010; Marschall et al., 2007). Farnesoid X receptor (FXR) is a member of ligand-activated transcription factors super family which acts as a master regulator of bile acid homeostasis due to its modulation of bile acid transport, synthesis and metabolism (Thompson et al., 2017; Xu et al., 2016; Halilbasic et al., 2016). Specifically, FXR can up-regulate bile salt export pump (Bsep) and multi-drug

resistance-associated protein 2 (Mrp2), while repress the expression of $Na^+$/taurocholate cotransporting polypeptide (Ntcp), cholesterol 7α-hydroxylase (Cyp7a1) and oxysterol 12α-hydroxylase (Cyp8b1) (Meng et al., 2015c; Song et al., 2014; Bechmann et al., 2013). In addition, chemokines and inflammatory cytokines are crucial in hepatic cell death, inflammation and subsequent regeneration and fibrosis in the process of cholestatic liver injury. NF-κB is a key regulator of inflammation since activated NF-κB is frequently detected in various inflammatory diseases and cholestatic liver injury (Wang et al., 2017). Recent report has demonstrated that FXR plays a novel role in the control of liver inflammation through inhibiting NF-κB signaling pathway (Wang et al., 2008).

Yangonin (Yan) is a product isolated from the root of an edible botanical Kava which is prepared as a kava-drinking used in the South Pacific Island Countries for centuries (Ligresti et al., 2012; Wang et al., 2015). Some pharmacological studies have revealed that Yan has pharmacological activities such as antioxidant properties and antiproliferative activity of cancer cell lines (Wruck et al., 2008; Zhongbo et al., 2017). However, whether Yan has hepatoprotective effect on cholestatic liver injury such as LCA-induced cholestasis is unknown.

The present study is to investigate the hepatoprotective effects of Yan against LCA-induced cholestasis in mice, and further to explore the potential mechanisms *in vivo* and *in vitro*.

## 2. Materials and methods

### *2.1 Materials*

Yangonin (purity > 98%) were purchased from Sigma-Aldrich (St. Louis, MO). LCA

(purity > 98%) was purchased from Aladdin Company (Shanghai, China). Obeticholic acid (OCA, purity > 98%) was purchased from AdipoGen International (San Diego, USA). Alanine aminotransferase (ALT) **(C009-1)**, aspartate aminotransferase (AST) **(C010-1)**, alkaline phosphatase (ALP) **(C059-1)** and total bilirubin kits **(C019-1)** were obtained from Nanjing Jiancheng Institute of Biotechnology (Nanjing, China). Total bile acids kit **(m1037200)** was purchased form Shanghai Enzyme-linked Biotechnology Co., Ltd. (Shanghai, China). A tissue protein extraction kit was obtained from Keygen Biotech. Co., Ltd. (Nanjing, China). A bicinchoninicacid (BCA) protein assay kit was purchased from the Beyotime Institute of Biotechnology (Jiangsu, China). All biochemical indicators kits and other chemicals were commercially available.

*2.2 Cell culture and cell toxicity assay*

The alpha mouse liver 12 (AML-12) cells were purchased from American type culture collection (Manassas, VA, USA) and cultured in Dulbecco's modified Eagle's medium and Ham's F12 medium (Gibco, Carlsbad, CA, USA). The cells were cultured in humidified environment containing 5% $CO_2$ at 37 °C. AML-12 cells were pretreated with different concentrations of yangonin (5, 10, 20, 40, 80, 160 and 320 μM) for 24 h. The toxicity of the compound was assayed using the MTT method.

*2.3 Cell proliferation assay*

The AML-12 cells were plated into 96-well plates at a density of $5 \times 10^4$ cells/ml for 24 h before treatment. **The cells were pretreated with various concentrations of yangonin (5, 10 and 20 μM) for 6, 12 and 24 h before challenged with LCA (30 μM)**

**for 24 h, and then measured using the MTT method.**

*2.4 LCA-induced cholestasis and liver injury in vivo*

Male C57BL/6 mice (8-10 weeks) were housed in laboratory animal facilities under a standard 12-hr light/dark cycle. All animals received human care and treatment protocols were in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the National Institutes of Health and were approved by the Institutional Animal Care and Use Committee at Dalian Medical University, Dalian, China. Yangonin (10, 20 or 40 mg/kg) was treated to mice by oral gavage once a day for 7 days. OCA (10 mg/kg) was administered to mice for 7 days. Since the fourth day, mice received intraperitoneal injections of LCA dissolving in corn oil (125 mg/kg) twice daily for 4 successive days. Mice were monitored twice a day throughout the whole experiment. No abnormal mice that lost more than 20% initial weight or that became moribund were found during the study. **On the 7th day after yangonin treatment, mice were sacrificed under anesthesia (pentobarbital sodium, 65 mg/kg, intraperitoneal injection).** Blood and liver were collected and snap-frozen on liquid nitrogen, then stored at -80 °C until use.



*2.5 Biochemical indicators determination*

The serum levels of ALT, AST, ALP, total bilirubin, total bile acids and hepatic total bile acids were detected using a commercial test kit based on the manufacturer's instructions.

*2.6 Histological assay*

Liver tissues were fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned at 5 μm, and stained with hematoxylin & eosin (H&E) for necrosis and other structural changes.

***2.7 Immunohistochemistry Analysis***

**Paraffin embedded tissue sections were rehydrated in xylene and graded ethanol solutions. The slides were exposed to 1% hydrogen peroxide/methanol for 10 min, washed in phosphate buffered saline (PBS), and then incubated with antigen retrieval buffer for 10 min at room temperature for antigen retrieval. After blocking with serum for 20 min, sections were then immunostained with α-smooth muscle actin (α-SMA) primary antibody (Proteintech Group, Chicago, USA) and incubated overnight at 4 °C. After washing with PBS, sections were incubated with biotinylated secondary antibody for 20 min at room temperature. Sections were then washed with PBS and incubated for 10 min with a streptavidin-biotin-peroxidase complex. After washing with PBS, diaminobenzidine was added as a visualizing agent. Nuclei were counterstained with hematoxylin. Positive staining for α-SMA was brown.**

*2.8 Quantitative real-time PCR*

Total RNA from mouse livers or cells was extracted using RNAiso Plus reagent. **The purity of RNA was assessed according to the ratio of absorbance at 260 nm and 280 nm. RNase-free DNase-treated total RNA (1.0 μg) was purified and randomly**

**reverse-transcribed to cDNA. Real-time RT-PCR was performed using PrimeScript RT reagent kit with gDNA eraser (TaKaRa Biotech, Kyoto, Japan). Quantitative real-time PCR was performed using SYBR Green PCR Master Mix and an ABI prim 7500 Sequence Detection System (Applied Biosystems, USA).** For each sample, the Ct values for the target gene and β-actin (as a calibrator) were determined based on standard curves. The calculated relative Ct value of each gene was divided by the relative value of β-actin. Then, the expression level of each gene in the control group was set to one-fold and used to determine the relative levels in the other samples (n-fold). **The sequence of the primers in mice is shown in Table 1.**

*2.9 Western blotting assay*

The protein samples from liver tissues and cells were extracted following standard protocols (Beyotime Biotechnology, Haimen, China), and the protein content was determined using a BCA protein assay kit. Proteins were subjected to SDS-PAGE and then transferred to a nitrocellulose membrane. **After blocking, membranes were incubated overnight with primary antibodies, including Bsep (H-180, 1/1000), Mrp2 (H-17, 1/1000), Ntcp (M-130, 1/1000), Cyp7a1 (sc-293193, 1/1000), Cyp8b1 (sc-101387, 1/1000) and FXR (H-130, 1/1000) (Santa Cruz Biotechnology, Santa Cruz, CA).** The blots were then incubated with horseradish peroxidise-conjugated antibodies for 2 h at room temperature. Protein expression was detected by the enhanced chemiluminescence (ECL) method and imaged with a Bio-Spectrum Gel Imaging System (UVP, USA). To eliminate variations due protein quantity and quality, the data were adjusted to β-actin

expression.

*2.10 RNA Silencing Experiment*

Mouse cells were seeded on to 12-well plate at a density of $6\times10^5$ cells per well. Twelve hours later, 200 nM negative control siRNA or siRNA targeting at mouse FXR (siGENOME SMARTpool, Dharmacon) was transiently transfected to mice primary cultured hepatocytes using lipofectamine 2000TM (Invitrogen, Carlsbad, USA) and incubated for 24 h. 20 μM yangonin was added to the culture medium for 24 h, and then 30 μM LCA was added to the culture medium for another 24 h. After that, the cells were harvested for cell viability assay or quantitative real-time PCR.

*2.11 Statistical analysis*

The data and statistical analysis comply with the recommendations on experimental design and analysis in pharmacology. Data were analyzed using GraphPad Prism 5 (GraphPad Software Inc., San Diego, CA). All data were expressed as means ± S.D. Unpaired student's t test or nonparametric Mann-Whitney U-test was used for statistical analysis between two groups. For multiple groups comparison, one-way ANOVA as well as nonparametric Kruskal-Wallis-Test was performed and followed by Dunnett's multiple comparison post hoc test. $P< 0.05$ was considered statistically significant.

**3. Results**

*3.1 Yangonin reduces the injury of the cells administered with LCA in vitro*

First *in vitro*, the effect of yangonin on the injury induced by LCA in AML-12 cells

was investigated. As shown in Fig. 1A, yangonin administered to cells at the concentrations of 5, 10, 20, 40, 80 μM for 24 h showed no statistically difference in cell viability, suggesting that yangonin in this concentration range had no damage to cells. Yangonin (5, 10 and 20 μM) pretreatment could dose-dependently increased cell viability for 6, 12 and 24 h, with a maximal increase for 24 h (Fig. 1B). Therefore, yangonin treatment for 24 h was selected for the following study. In addition, the significant decreases in ALT and AST by yangonin were observed in a dose-dependent manner (Fig. 1C and D). **The gene expression of FXR was decreased by LCA and was dose-dependently increased by yangonin (Fig. 1E). The expression level of small heterodimer parter (SHP), which is a FXR classical target gene, was also increased by yangonin (Fig. 1F).** Taken together, these data demonstrated the hepatoprotective effect of yangonin on LCA-induced hepatocyte injury *in vitro*.

*3.2 Yangonin protects against LCA-induced cholestatic liver injury in vivo*

To investigate whether yangonin has any effect on LCA-induced cholestasis and liver injury *in vivo*, male C57BL/6 mice were orally administered with yangonin (10, 20 and 40 mg/kg) for 7 days. Since the fourth day, mice received intraperitoneal injections of LCA (125 mg/kg) twice daily for 4 successive days. OCA which is a well-known FXR agonist was used as a positive control drug. As shown in Fig. 2A, the histopathological changes of the liver in model group included diffuse vacuolization, severe hepatic necrosis and infiltrating neutrophils. However, these changes were significantly ameliorated by yangonin. Besides, the increases in serum ALT and AST, the biochemical indicators of hepatocyte damage, were reduced by yangonin in a dose-dependent manner

(Fig. 2B). Similarly, serum ALP which is the biochemical indicator of biliary toxicity, was also significantly reduced in yangonin-treated mice (Fig. 2C). Yangonin further reversed the LCA-induced the increases in serum total bilirubin, total bile acids and hepatic total bile acids, all of which are the biomarkers of cholestasis (Fig. 2D). **In addition, α-SMA which is a marker of stellate cell activation, was increased by LCA and was reduced by yangonin treatment (Fig. 2E).** Specially, the mortality (including natural death and humanely sacrifice when losing > 15-20% of their weight) was increased to 60% by LCA at 4 days in mice, while yangonin treatment reversed the proportion to 20% (Fig. 2F). These results indicated that yangonin remarkablely protected against LCA-induced cholestasis and liver injury in a dose-dependent manner *in vivo*.

*3.3 Yangonin regulates FXR target genes involved in bile acid homeostasis*

To clarify the mechanism underlying hepatoprotective effect of yangonin, the expression of hepatic transporters involved in bile acid homeostasis was examined. LCA markedly decreased the expression of Ntcp, Mrp2 mRNA, while Bsep expression was slightly changed. Compared with model group, yangonin increased the Bsep mRNA and reversed LCA-induced decrease in Mrp2 mRNA, meanwhile yangonin further enhanced the decrease in Ntcp gene expression in a dose-dependent manner (Fig. 3A). Except for transporters, bile acid synthetic enzymes are also involved in bile acid homeostasis. LCA decreased Cyp7a1 and Cyp8b1 mRNA levels. Yangonin treatment further reduced Cyp7a1 and Cyp8b1 gene expression. Furthermore, yangonin increased the expression of Sult2a1, which is a phase II enzyme involved in bile acid metabolism (Fig. 3B). To confirm the above mRNA results, the respective protein levels were determined using

Western blot analysis. **Yangonin dose-dependently induced Bsep, Mrp2, and decreased Ntcp, Cyp7a1, Cyp8b1 protein level (Fig. 3C). Taken together, the above results suggested that hepatoprotection of yangonin against LCA may due to the regulation of genes involved in bile acid homeostasis, resulting in intrahepatic cholestasis attenuation.**

*3.4 Yangonin suppresses inflammation induced by LCA in mice*

To elucidate the mechanism underlying protection of yangonin against LCA-induced inflammation, hepatic inflammation-related protein was determined. LCA increased NF-κB gene expression, while yangonin treatment inhibited hepatic expression of FXR target gene NF-κB (Fig. 4A). In addition, the mRNA levels of TNF-α and IL-1β, two inflammatory genes which are mediated by NF-κB, were significantly increased by LCA, which were all restored by yangonin (Fig. 4B and C). These data suggested that the protective effect of yangonin against inflammation during LCA-induced liver injury may be due to its inhibition of hepatic FXR-regulated gene NF-κB.

*3.5 The protection of yangonin against LCA-induced hepatocyte injury is FXR-dependent*

The hepatoprotective effect of yangonin mediated by activating FXR had been measured in *in vivo* experiments. However, the level may not be enough to represent the effect of yangonin on FXR activation. Thus, the effect of yangonin on FXR activation was subsequently examined using FXR siRNA in mouse cells. As shown in Fig. 5A, the FXR expression was decreased after specific siRNA targeting FXR mRNA transfection, which was assured by Western blot analysis. *In vitro* evidences demonstrated that siFXR

remarkably abrogated the expression of Bsep, Mrp2 and Cyp7a1 induced by yangonin (Fig. 5B-D). Besides, the expression of NF-κB decreased by yangonin was abrogated by FXR gene silencing (Fig. 5E). These data further demonstrated the involvement of FXR activation in the hepatoprotective effect of yangonin.

## 4. Discussion

Accumulation of toxic bile acids in liver could cause cholestasis with the main features of diffuse vacuolization, severe hepatic necrosis and infiltrating neutrophils. Accumulating event suggests that bile acids play a mechanistic role in the pathogenesis of cholestatic liver injury (Chen et al., 2017; Fickert and Wagner, 2017; Zeng et al., 2017). Therefore, maintaining bile acid homeostasis is important for the prevention and treatment of liver injury.

Bile acid homeostasis is mediated to a large extent at the transcriptional level by nuclear receptors which play important roles in bile acid homeostasis. FXR has emerged as a promising therapeutic target in cholestatic disorders since FXR and its target genes may contribute to the bile acid homeostasis as well as the pathogenesis and progression of cholestasis (Guo et al., 2016). The protective effect of FXR in cholestasis is mediated by central regulation of bile acid transport, synthesis and detoxification (Meng et al., 2015b; Garzel et al., 2014; Meng et al., 2015a). In contrast, FXR-null mice are more susceptible to liver injury induced by ANIT and bile duct ligation (Cui et al., 2009). Increasing evidences indicate that FXR may be a novel therapeutic target to treat cholestasis and liver injury (Festa et al., 2017). Therefore, specific targeting FXR can serve as a novel therapeutic approach to treat cholestatic liver disease and FXR agonists

may exert effective protection against cholestasis.

In the present study, we have proposed a novel FXR ligand yangonin which mediated protective effect against LCA-induced cholestasis and hepatotoxicity. The evidences indicated that yangonin had at least three roles in protection against LCA-induced cholestatic liver injury. The first role is to decrease hepatic uptake and increase efflux of bile acids through an inhibition in hepatic uptake transporter Ntcp and an induction in efflux transporter Bsep, Mrp2 expression. The second role is to reduce bile acid synthesis in liver through repressing bile acid synthetic enzymes including Cyp7a1, Cyp8b1 and to increase bile acid metabolism through an induction in gene expression of Sult2a1. The third role is to suppress liver inflammation through repressing inflammation- related gene NF-κB, TNF-α and IL-1β. Further *in vitro* studies indicated that the hepatoprotective effect of yangonin against LCA-induced liver injury and intrahepatic cholestasis was due to FXR-mediated regulation. **To investigate whether yangonin has the ability to transactivate FXR, the effect of yangonin on FXR activation was subsequently examined using dual-luciferase reporter gene assay in HepG2 cells which were transiently cotransfected with FXR expression plasmid and FXR target gene Bsep promoter reporter vector. Yangonin dose-dependently increased the FXR reporter gene activity, suggesting that yangonin was a strong FXR activator (data not shown).**

The current study clearly demonstrated that yangonin exerted similar protection against LCA-induced liver cholestasis compared with OCA, as evidenced by the increased survival rate and ameliorative liver histology, as well as significant decrease in serum ALT, AST, ALP and total bilirubin. The mechanism of yangonin hepatoprotection against LCA-induced cholestasis was further investigated by targeted gene analysis. The

induction of FXR-target genes including Bsep, Mrp2 and Sult2a1 by yangonin revealed that this natural product accelerated transport and detoxification of bile acids *in vivo*. Particularly worth mentioning is that Ntcp, Cyp7a1 and Cyp8b were inhibited by LCA to defense against excessive bile acids entering hepatocytes and synthesis. Yangonin reduced the expression of FXR-target genes including Ntcp, Cyp7a1 and Cyp8b1, suggesting that this natural product decreased hepatic uptake and synthesis of bile acids.

Inflammatory response plays an important role in pathological conditions of the liver. Therefore, the precise control of inflammation is essential for the prevention of inflammatory disorders, as well as for inhibiting the exacerbation or progression of cholestatic liver injury. NF-κB has received considerable attention as a key regulator of inflammation because activated NF-κB is frequently detected in various inflammatory diseases and cholestatic liver injury. Recent report has demonstrated that FXR through inhibiting NF-κB signaling pathway play a novel role in the control of liver inflammation (Zhang et al., 2017; Verbeke et al., 2016). The present study showed that NF-κB gene was increased by LCA and yangonin reduced its expression, resulting in the decrease in inflammation during LCA-induced cholestatic liver injury.

In conclusion, Evidence is provided that yangonin dose-dependently protects against LCA-induced cholestatic liver injury and reverses the LCA-induced mortality. **The protective effect of yangonin on cholestasis may be due to its regulative effect on hepatic FXR-regulated genes including Ntcp, Bsep, Mrp2, Cyp7a1, Cyp8b1 and Sult2a1, which contribute to the increased transport and metabolism of toxic bile acids, as well as the decreased synthesis of bile acids,** and possibly due to the inhibition of hepatic inflammation which is also related to the FXR pathway. This study reveals

yangonin as a natural product which could possibly be developed to a promising anti-cholestatic drug in the future. Besides, these data support the notion that development of potent FXR agonists might be beneficial in the treatment of cholestatic liver diseases in clinical practice.

**Conflict of interest**

The authors declare that there are no conflicts of interest.



**Acknowledgments**

This present study was financially supported by a grant from the National Natural Science Foundation of China (Nos. 81302826 and 81473280) and the Program for Key Laboratory of Liaoning Province (LZ2015027).

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## Legend to figures

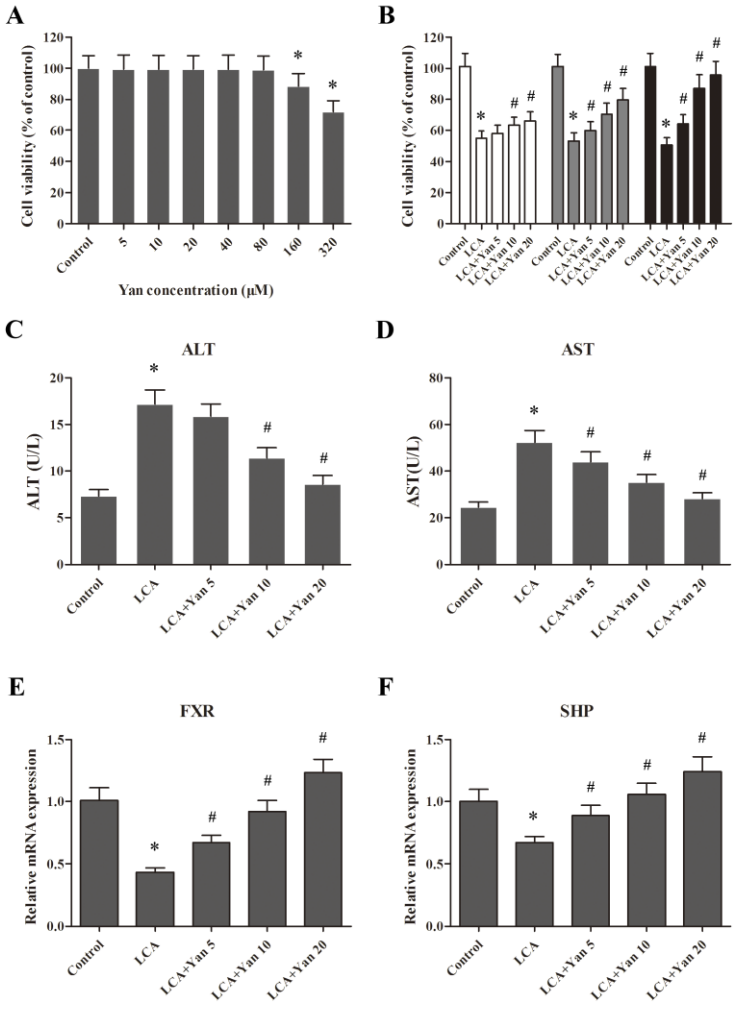
**Fig. 1** *In vitro* evidences that effects of yangonin on LCA-induced hepatotoxity in AML-12 cells. (A) Effects of yangonin (5, 10, 20, 40, 80, 160 and 320 μM) on the cell viability of cells for 24 h. Data are the mean ± S.D. (n=5). * $P< 0.05$ versus Control. (B) Effects of yangonin (5, 10 and 20 μM) on the viability of cells administered with LCA (30 μM) for 6, 12 and 24 h. Data are the mean ± S.D. (n=5). * $P< 0.05$ versus Control respectively; # $P< 0.05$ versus LCA respectively. Effects of yangonin (5, 10 and 20 μM) on (C) ALT and (D) AST in cells administered with LCA (30 μM). Data are the mean ± S.D. (n=5). * $P< 0.05$ versus Control; # $P< 0.05$ versus LCA.

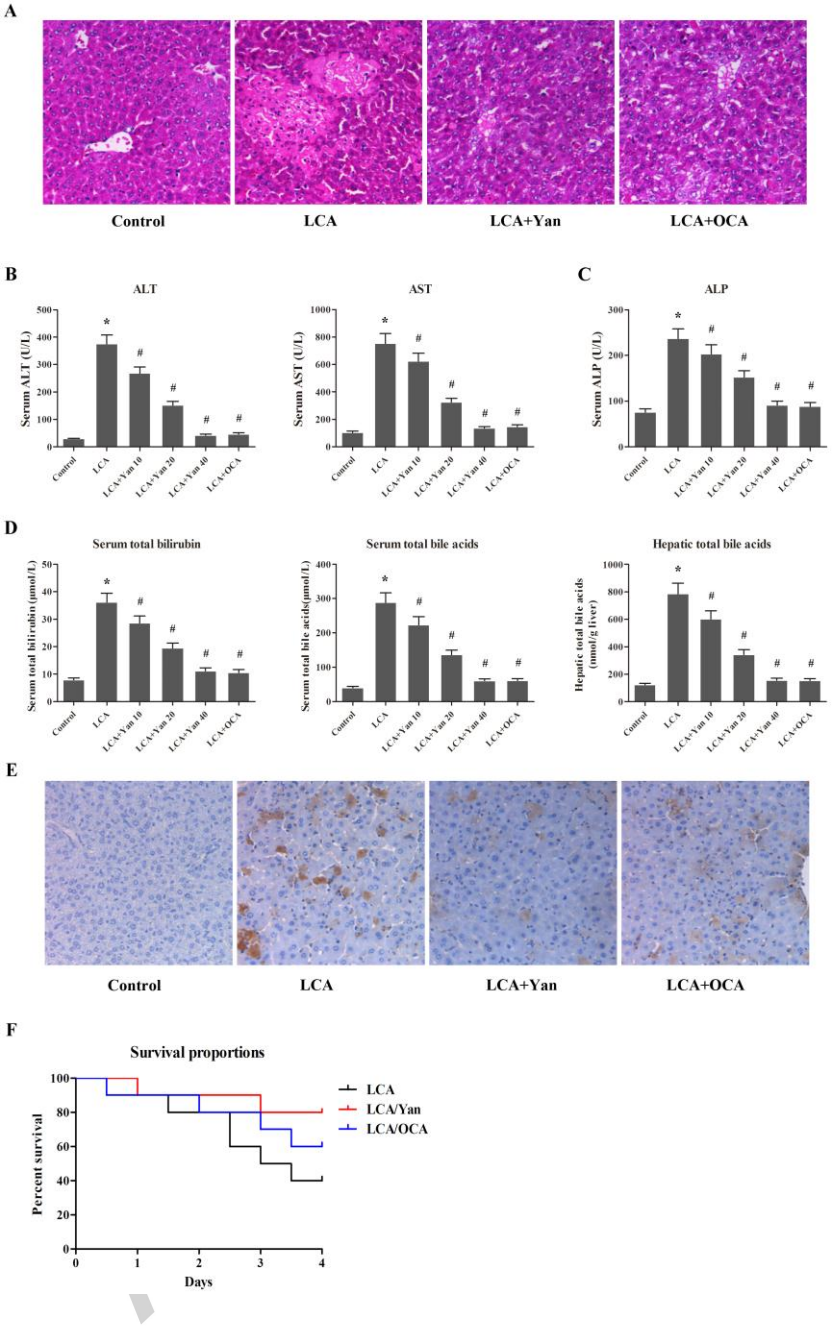
**Fig. 2** Hepatoprotection of yangonin against LCA-induced cholestatic liver injury *in vivo*. (A) The images of representative H&E stained liver sections (200 ×magnification) after LCA administration were shown. The data of serum ALT, AST (B) and ALP (C), as well as serum total bilirubin, total bile acids and hepatic total bile acids (D) indicated that LCA induced liver injury and yangonin dose-dependently attenuated the degree of liver injury in mice. Data are the mean ± S.D. (n=6). * $P< 0.05$ versus Control; # $P< 0.05$ versus LCA. **(E) The images of representative α-SMA positive cells in mice (200 ×magnification). α-SMA-positive cells were stained brown. (F)** Survival proportions over time in mice receiving LCA with yangonin (40 mg/kg) or OCA (10 mg/kg) were determined. Survival was recorded every 12 h after LCA first administration. The survival proportions were elevated in yangonin-treated and OCA-treated mice.

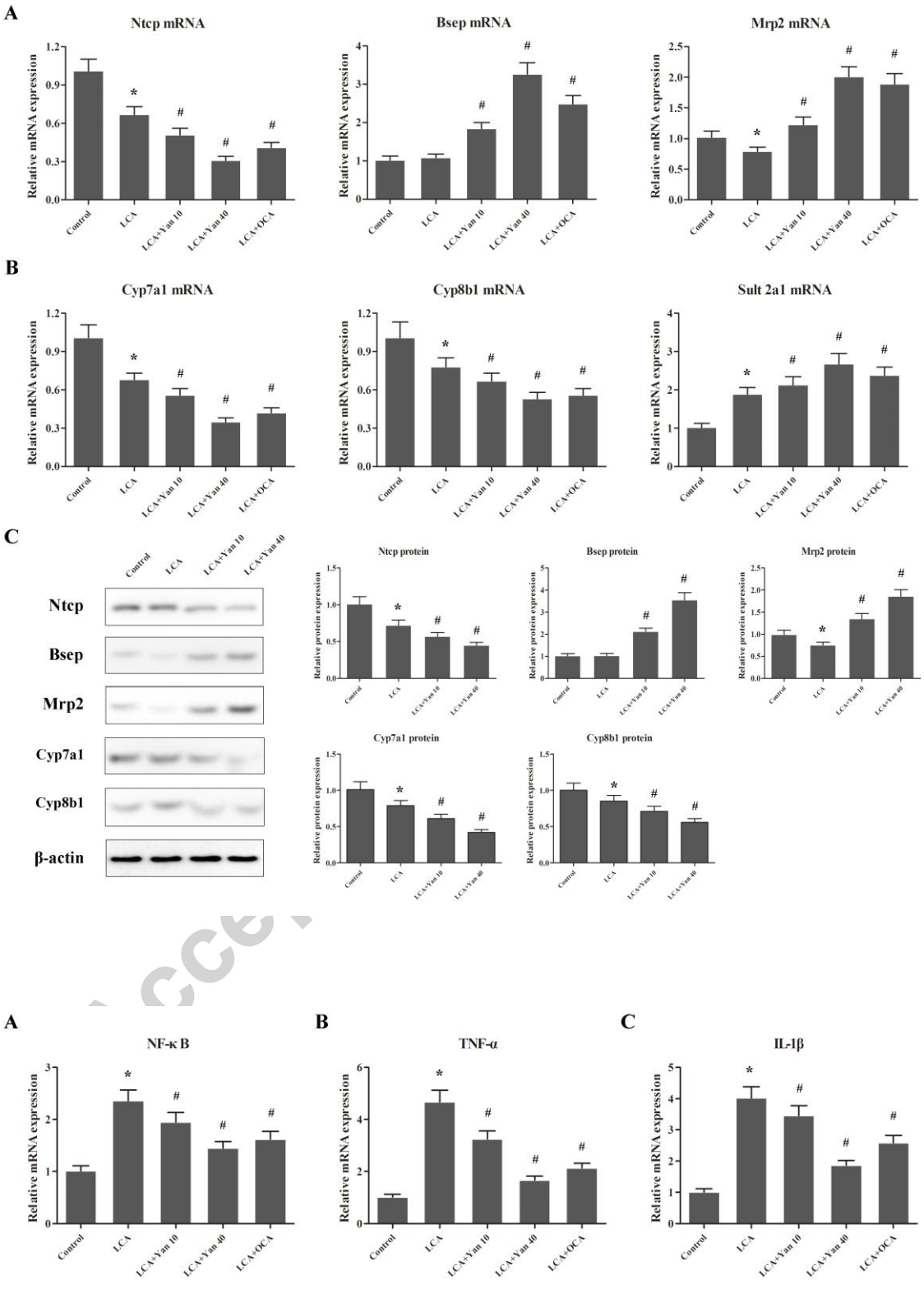
**Fig. 3** Yangonin alters gene and protein expression of hepatic transporters and metabolic enzymes involved in bile acid homeostasis in LCA-induced mice model of liver injury and cholestasis. (A) Quantitative real-time PCR analysis was performed to measure the gene expression of transporters including Ntcp, Bsep and Mrp2. (B) Western blot analysis was used to measure Ntcp, Bsep and Mrp2 protein expression. Specific band intensity was quantified, normalized to β-actin. (C) The mRNA levels of enzymes involved in bile acid homeostasis including Cyp7a1, Cyp8b and Sult2a1 were also determined. Data are the mean ± S.D. (n=6). * $P< 0.05$ versus Control; # $P< 0.05$ versus LCA.

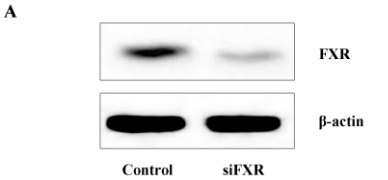
**Fig. 4** Yangonin suppresses inflammation induced by LCA in mice. Quantitative real-time PCR analysis was performed to measure the gene expression of (A) NF-κB, (B) TNF-α and (C) IL-1β. Data are the mean ± S.D. (n=6). * $P< 0.05$ versus Control; # $P< 0.05$ versus LCA.

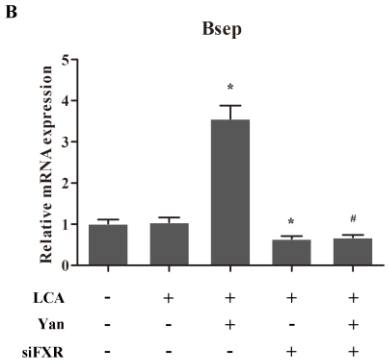
**Fig. 5** *In vitro* evidences on FXR activation by yangonin. (A) FXR silencing efficiency was measured by western blot. Effects of yangonin on the gene levels of (B) Bsep, (C) Mrp2, (D) Cyp7a1 and (E) NF-κB. Data are the mean ± S.D. (n=5). * $P< 0.05$ versus LCA alone; # $P< 0.05$ versus LCA+ yangonin.

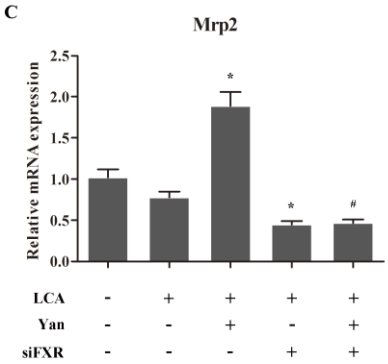


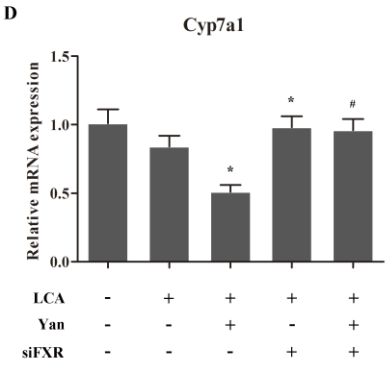






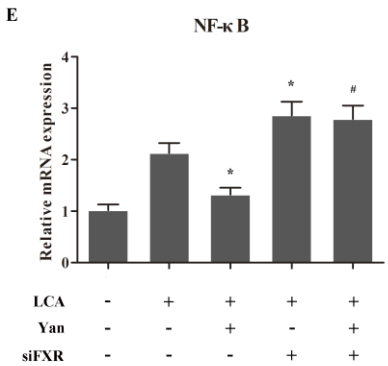



**Table 1 The primer sequences used for real-time PCR assay in mice**

| Gene | GenBank accession | Forward primer (5'-3') | Reverse primer (5'-3') |
|---|---|---|---|
| Ntcp | U95132.1 | GCATGATGCCACTCCTCTTATAC | TACATAGTGTGGCCTTTTGGACT |
| Bsep | NM_021022.3 | AGCAGGCTCAGCTGCATGAC | AATGGCCCGAGCAATAGCAA |
| Mrp2 | NM_013806.2 | AACTGCCTCTTCAGAATCTTA | GCCAGCCACGGAACCAGCTGCT |
| Cyp7a1 | NM_007824.2 | CAAGAACCTGTACATGAGGGAC | CACTTCTTCAGAGGCTGCTTTC |
| Cyp8b1 | NM_010012.3 | CCCCTATCTCTCAGTACACATGG | GACCATAAGGAGGACAAAGGTCT |
| Sult2a1 | NM_001111296.2 | GGAAGGACCACGACTCATAAC | GATTCTTCACAAGGTTTGTGTTACC |
| SHP | NM_011850.2 | GTCTTTCTGGAGCCTTGAGCTG | GTAGAGGCCATGAGGAGGATTC |
| FXR | AF293370.1 | CGCTGAGATGCTGATGTCTTG | CCATCACTGCACATCCCAGAT |
| β-actin | NM_007393.3 | TATTGGCAACGAGCGGTTC | ATGCCACAGGATTCCATACCC |