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Full Length Research Paper

Evaluation of anti-apoptotic, anti-injury and anti-hepatitis B virus effects of isochlorogenic acid C *in vitro*

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To study anti-hepatitis effect of isochlorogenic acid C from *Laggera alata*, anti-apoptotic and anti-injury properties of test compound were evaluated using the D-galactosamine (D-GalN)-induced apoptosis/injury model in human hepatocyte line HL-7702 cells. Hepatocyte apoptotic changes were demonstrated by DNA ladder analysis and caspase-3 activation. Hepatocyte injury was assessed by cell viability. Meanwhile, anti-hepatitis B virus (anti-HBV) effect of test compound was evaluated by the HBsAg, HBeAg, HBV DNA, HBV covalently closed circular DNA (HBV cccDNA) and heme oxygenase-1 (HO-1) expressions in HBV-transfected HepG2.2.15 cells. The results showed that test compound at concentrations of 10 to 100 µg/ml significantly reduced the caspase-3 and transformed growth factor β1 (TGFβ1) levels of the D-GalN-challenged hepatocytes. Also, test compound improved markedly cell viability of the D-GalN-injured hepatocytes and produced a maximum protection rate of 47.28% at a concentration of 100 µg/ml. Furthermore, test compound significantly inhibited productions of HBsAg and HBeAg. Its maximum inhibitory rates on the HBsAg and HBeAg expressions were 86.93 and 59.79%, respectively. In addition, test compound significantly induced the HO-1 expression of HepG2.2.15 cells. This study verifies that isochlorogenic acid C possesses potent hepatoprotective and anti-HBV effects. The anti-apoptotic and anti-injury effects of test compound could be achieved by its anti-oxidative properties and interfering the caspase-3 and TGF-β1 expressions. The anti-HBV target of test compound may mainly be at the downstream links of HBV replication process and is probably associated with blocking translation step. Furthermore, HO-1 induction may contribute to anti-hepatitis B effect of test compound.

Key words: *Laggera alata*, isochlorogenic acid C, D-Galactosamine, anti-hepatitis B, covalently closed circular DNA, heme oxygenase-1.

INTRODUCTION

Hepatitis is induced by viruses, alcohol, lipid peroxidative products, and various drugs. The continued inflammation of chronic hepatitis can slowly damages the liver, eventually resulting in cirrhosis and liver cancer. However, there is no suitable drug to treat patients with hepatitis, especially hepatitis B. In the context of liver disease, over activation of the apoptotic process may lead to hepatocellular damage, while inhibition of apoptosis may promote cell proliferation and transformation. Fulminant hepatic failure, induced by drugs, toxins or viral hepatitis, is characterized by a severe hepatocellular dysfunction with a massive death of hepatocytes, in which apoptosis may play a role in addition to necrosis (Prétet et al., 2003). The role of apoptosis in various liver diseases and the mechanisms by which apoptosis occurs in the liver may provide insight into these diseases and suggest possible treatments

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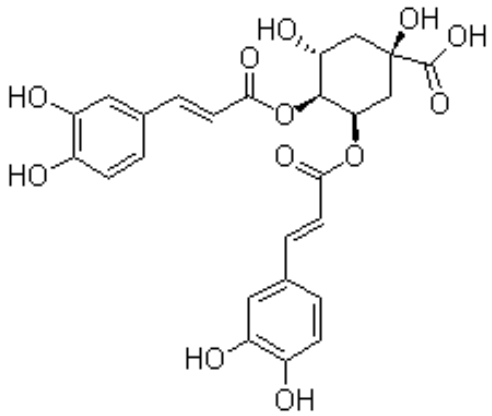

Figure 1. Structure of isochlorogenic acid C from *L. alata*.

(Neuman, 2001). Moreover, the current anti-hepatitis B drugs have some drawbacks such as rebound phenomenon of hepatitis B virus (HBV) replication after withdrawal of treatment, unpleasant side effects and drug resistance during the long-term use (Delaney et al., 2001). Therefore, there is a pressing need to develop more safe and effective anti-hepatitis agents. *Laggera alata* (Asteraceae) is distributed mainly in Africa and Southeast Asia. This plant has been used as the folk medicine for over three hundred years, especially for the treatment of some ailments associated with hepatitis (Jiangsu New Medical College, 1977). Most studies concerning *L. alata* focused on its folk use and phytochemical analyses (Bohlmann et al., 1985; Raharivelomanana et al., 1998; Zheng et al., 2003a, b, c). In previous investigations, we confirmed the potent anti-inflammatory and hepatoprotective activities of *L. alata* extract containing isochlorogenic acids (Wu et al., 2006; Wu et al., 2009; Wu et al., 2011). To further validate the beneficial effects of *L. alata* on liver disorders in humans, D-galactosamine (D-GalN)-induced apoptosis/injury model in human hepatocyte line (HL-7702 cells) was employed to evaluate anti-apoptotic and anti-injury effects of isochlorogenic acid C from *L. alata*. Anti-HBV effect of isochlorogenic acid C was evaluated by HBV-transfected HepG2.2.15 cells. The study is the first to find that isochlorogenic acid C possesses the antiapoptotic, anti-injury and anti-HBV effects. Hence, we report the details here.

MATERIALS AND METHODS

Reagents

D-Galactosamine (D-GalN), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), silybin and oxymatrine were purchased from Sigma Chemical Co. (St Louis, MO, USA). Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) diagnostic kits and malondialdehyde (MDA) detection kit were purchased from Nanjing Jiancheng Bioengineering Institute (China). Fetal bovine serum, 1640 medium and Dulbecco's modified Eagle's medium (DMEM) were purchased from Gibco-BRL (Grand Island, NY, USA). Caspase-3 activity kit, Bicinchoninic acid (BCA) protein assay kit and apoptosis DNA ladder detection kit were provided by Nanjing KeyGen Biotech. Co. Ltd, China. Human heme oxygenase-1 (HO-1) ELISA Kit was purchased from Beijing Yonghui Biological Technology Co., Ltd. Human TGFβ1 enzymeimmunoassay Kit was purchased from Boster Biological Technology, Ltd, China. HBV DNA PCR-fluorescence quantitation kit, EIA kits for the detection of HBsAg and HBeAg were provided from Shanghai Kehua Bio-engineering Co., Ltd. Lamivudine was provided from GlaxoSmithKline (China) Investment Co., Ltd. TA cloning Kit and G418 were provided from Invitrogen Corporation (USA). Plasmid safe ATP dependent DNase (PSAD) was provided from EPICENTRE Biotechnologies (USA). Plasmid Mini Preparation Kit was provided from Axygen Biosciences (USA). Conventional PCR Reagents were provided from Shanghai Sangon Biological Engineering Technology and Service Co., Ltd. All other reagents were of the highest commercial grade available.

Cells

HL-7702 hepatocytes were purchased from Cell Bank, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences and maintained in 1640-medium containing 2 mM glutamine and 10% (v/v) fetal bovine serum at 37°C (95% humidity, 5% CO_2). HepG2.2.15 cells were provided from the Second Military Medical University, China. The cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 2 mM glutamine, 10% (v/v) heat-inactivated fetal bovine serum and 380 µg/ml of G418 at 37°C (95% humidity, 5% CO_2).

Preparation of isochlorogenic acid C

L. alata (D. Don) Sch.-Bip ex Olivier was collected from Tengchong county, Yunnan Province, China. Plant sample was authentified and a voucher specimen (No. ZY982003LA) was deposited in the herbarium of College of Pharmaceutical Sciences, Zhejiang University, China. Isochlorogenic acid C (Figure 1) was isolated and authenticated as described in the method we reported previously (Wu et al., 2006). Isochlorogenic acid C (HPLC Purity ≥ 98%) was dissolved in the final concentration of 0.1% dimethyl sulfoxide (DMSO) solution in all tests.

Induction of apoptosis by d-GalN in HL-7702 hepatocytes

To induce hepatocyte apoptosis by D-GalN, DNA ladder detection was employed as the evaluation standard of hepatocyte apoptosis. Analysis of DNA fragmentation indicated that typical apoptosis ladder was observed at 6 h after HL-7702 hepatocytes were incubated with 60 mM D-GalN. Based on the pilot test, the hepatocyte apoptosis model was established as follows: HL-7702 hepatocytes were suspended in 1640-medium and then transferred to 6-well plates at a density of 1.0×10^5 cells/ml. After the cells were incubated for 6 h with 60 mM D-GalN, hepatocyte apoptosis was evaluated by analysis of DNA fragmentation and caspase-3 expression. For DNA ladder examination, the cells were collected by centrifugation and washed in cold PBS. The hepatocyte DNA was extracted using the apoptosis DNA ladder detection kit according to the manufacturer's instructions. The DNA fragmentation was assayed by electrophoresis on a 1.5% agarose gel containing 0.5 µg/ml ethidium bromide and its pattern was examined on the photographs taken under ultraviolet illumination.

Caspase-3 activity was evaluated using caspase-3 assay kit and

expressed in relative absorbance units and corrected for total protein content using BCA protein assay. In addition, hepatocyte injury was also assessed by measuring the amount of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) leakage as well as the MDA content with the AST, ALT and malondialdehyde (MDA) detection kits according to the protocol provided, respectively.

Anti-apoptotic effect of isochlorogenic acid C in HL-7702 hepatocytes

Cytotoxicity induced by isochlorogenic acid C treatment was measured using the MTT assay as follows: Hepatocytes were cultured in 1640-medium in the presence of 1 to 100 µg/ml isochlorogenic acid C for 48 h and then 10 µl of MTT (5 mg/ml) was added to cells in each well. After 4 h of culture, the medium was removed, and the blue formazan crystals that had formed were dissolved in DMSO. The absorbency of formazan generated from MTT was measured at 570 nm using a multi-well plate reader (MULTISKAN MK3, Thermo Fisher Scientific Inc., USA). Cytotoxicity was described according to the amount of formazan production relative to the untreated control cells. After HL-7702 hepatocytes were incubated for 48 h in culture medium containing 10 to 100 µg/ml isochlorogenic acid C, the cells were then cultured for 6 h in fresh culture medium containing 60 mM D-GalN. Oxymatrine at a concentration of 200 µg/ml was used as a reference drug. Hepatocyte apoptosis was assessed by measuring the Caspase-3 and TGFβ1 levels. Caspase-3 activity was examined as described earlier. The TGF-β1 level was detected by the TGF-β1 enzymeimmunoassay kit according to the manufacturer's procedure.

Anti-injury effect of isochlorogenic acid C in HL-7702 hepatocytes

After HL-7702 hepatocytes were incubated for 8 h with 80 mM D-GalN, the cells were then cultured for another 48 h in fresh culture medium containing 1 to 100 µg/ml isochlorogenic acid C. Silybin at a concentration of 100 µg/ml was used as the reference drug. Hepatocyte injury was detected using the MTT assay as described earlier. Anti-injury effect of test compound was assessed by cell viability and expressed as percentage protection.

Anti-HBV effect of isochlorogenic acid C in HepG2.2.15 cells

HepG2.2.15 cellular cytotoxicity induced by isochlorogenic acid C was analyzed with the MTT assay as follows: The cells were seeded in 96-well plate with a concentration of 1.0×10^5 cells/ml. Different concentrations of isochlorogenic acid C were applied to culture wells in triplicate. After incubation for 8 days, the MTT assay was carried out as described earlier. Cytotoxicity was described according to the amount of formazan production compared with the untreated control cultures.

For measurement of HBV antigens and HBV DNA, HepG2.2.15 cells were treated with various concentrations of test compound and the medium with compound was replaced every 4 days. On the forth day, the replaced medium was assayed for HBsAg and HBeAg. On the eighth day, the replaced medium was measured for HBsAg, HBeAg and HBV DNA. Lamivudine at a concentration of 100 µg/ml was used as the reference drug. HBsAg and HBeAg in replaced culture supernatants were respectively determined by enzyme-immunoassay kits according to the protocol provided with the kit. The results were read at 450 nm by a multi-well plate reader. HBV DNA level in replaced culture supernatants was detected with a HBV DNA PCR-fluorescence quantitation kit. In sum, HBV DNA was extracted and amplified with Bio-Rad iQ5 Real Time PCR system (Bio-Rad Laboratories Inc, Irvine, CA). The thermal programme comprised of an initial denaturation at 94°C for 2 min followed by 40 amplification cycles each of two steps: 95°C for 5 s and 60°C for 30 s. The forward primer is 5-CCG TCT GTG CCT TCT CAT CTG-3, the reverse primer is 5-AGT CCA AGA GTA CTC TTA TAG AAG ACC TT-3 and the Taqman probe is FAM-CCG TGT GCA CTT CGC TTC ACC TCT GC. A plasmid containing the full-length insert of the HBV genome was used to prepare the standard curve.

Effect of isochlorogenic acid C on HBV cccDNA and HO-1 expressions

After HepG2.2.15 cells were seeded in 6-well plates at a density of 1.0×10^5 cells/ml for 48 h, new DMEM medium containing test compound (50, 25 and 10 µg/ml) were added. The medium with compound was replaced every 3 days. On the sixth day, the cells of each well were harvested.

The cell pellet containing 1.0×10^6 cells were extracted with mini plasmid extraction kit. The extract product was further purified by plasmid safe ATP dependent DNase to remove the residual HBV relaxed circular DNA. HBV covalently closed circular DNA (HBV cccDNA) was detected by selective real-time fluorescent quantitative PCR with specific primers and Taqman MGB probe (Zhao et al. 2005). The forward primer is 5-TGA ATC CTG CGG ACG ACC-3; the reverse primer is 5-ACA GCT TGG AGG CTT GAA CAG-3 and the Taqman probe is 5-FAM-CCT AAT CAT CTC TTG TTC ATG TC-MGB-3. Two parallel controls were set, including positive controls of oxymatrine (50 µg/ml) and normal control with no antiviral drug.

For measurement of HO-1, HepG2.2.15 cells at a density of 1.0×10^5 cells/ml were treated with various concentrations of test compound in 6-well plates. The medium with compound was replaced every 3 days. On the sixth day, the cells were collected and their HO-1 levels were determined by human HO-1 ELISA kit according to the protocol provided. The absorbency was measured at 450 nm by a multi-well plate reader. The concentration of HO-1 in the samples is then determined by comparing the absorbency of the samples to the standard curve.

Statistical analysis

Experimental data were expressed as mean ± standard deviations of the mean (S.D.) and subjected to a one-way analysis of variance (ANOVA) and student's *t*-test. $P < 0.05$ was chosen as the criterion of statistical significance.

RESULTS

Induction of apoptosis in d-GalN-sensitized hepatocytes

The hepatocyte apoptosis model was established in HL-7702 hepatocytes. Typical apoptosis ladder was observed at 6 h after the hepatocytes were incubated with 60 mM D-GalN (Figure 2). As shown in Table 1, the caspase-3, aspartate aminotransferase (AST), alanine aminotransferase (ALT) and malondialdehyde (MDA) levels of the D-GalN-sensitized hepatocytes were significantly elevated.

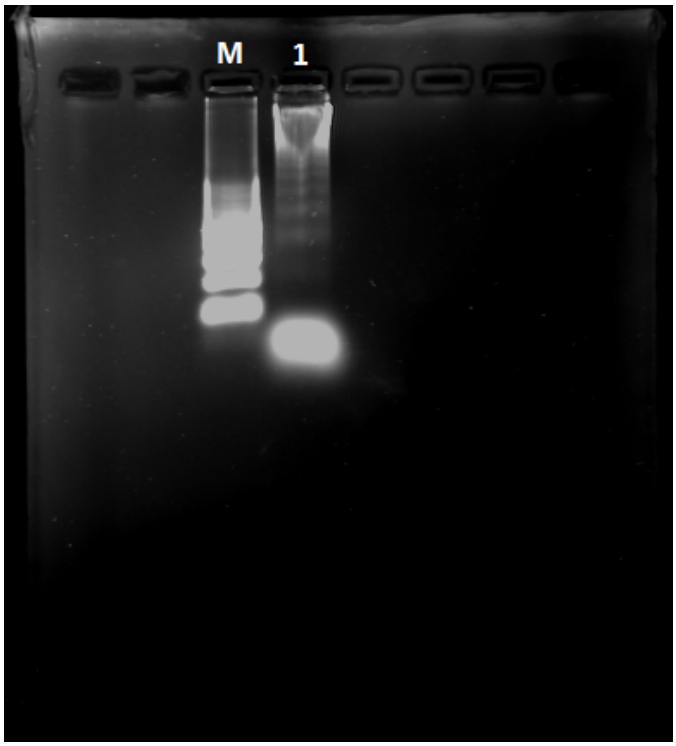

Figure 2. Analysis of DNA ladder from HL-7702 hepatocytes at 6 h after the D-GalN treatment. Lane M: the marker (molecular weight standard); Lane 1: the apoptotic hepatocytes.

Effect of isochlorogenic acid C on d-GalN-injured hepatocyte apoptosis

The cytotoxicity test indicated that isochlorogenic acid C was nontoxic to HL-7702 hepatocytes at concentrations of 10 to 100 µg/ml. Hepatocyte apoptosis was subsequently induced by exposure to 60 mM D-GalN after the cells were treated with isochlorogenic acid C. The results showed that isochlorogenic acid C at concentrations of 10 to 100 µg/ml significantly reduced the caspase-3 and transforming growth factor β1 (TGFβ1) levels of the D-GalN-challenged hepatocytes (Table 2).

Effect of isochlorogenic acid C on d-GalN-injured hepatocytes

Hepatocyte injury was induced by exposure to 80 mM D-GalN for 8 h and the cells was subsequently treated with isochlorogenic acid C. The results showed that isochlorogenic acid C improved significantly cell viability at concentrations of 10 to 100 µg/ml (Table 3). At a concentration of 100 µg/ml, it produced a maximum protection rate of 47.28%, while silybin was used as the standard drug indicated protection rate of 18.90% on hepatocyte injury.

Effect of isochlorogenic acid C on HBsAg, HBeAg and HBV DNA levels

Cellular toxicity test indicated that isochlorogenic acid C was harmless to HepG2.2.15 cells at concentrations of 10 to 100 µg/ml. Expressions of HBsAg and HBeAg in culture supernatants were assayed after the cells were incubated with test compound for 4 days (Table 4). The results indicated that isochlorogenic acid C at concentrations of 50 to 100 µg/ml had significant inhibitory effect on expression of HBsAg. It produced a maximum inhibition rate of 42.89% at a concentration of 100 µg/ml. The levels of HBsAg, HBeAg and HBV DNA in culture supernatants were measured after the cells were treated with test compound for 8 days (Table 5). At concentrations of 50 to 100 µg/ml, isochlorogenic acid C inhibited significantly expressions of HBsAg and HBeAg. Moreover, at a concentration of 100 µg/ml, it produced the maximum inhibition rates of 86.93 and 59.79% on expressions of HBsAg and HBeAg, respectively.

Effect of isochlorogenic acid C on HBV cccDNA pool and HO-1 expression

Effect of isochlorogenic acid C on the HBV cccDNA content was shown in Table 6. Isochlorogenic acid C exhibited no effect on HBV cccDNA pool of HepG2.2.15 cells. The reference drug oxymatrine at a concentration of 50 µg/ml markedly declined the HBV cccDNA level. In addition, the HO-1 level of HepG2.2.15 cells was determined after the cells were treated with test compound for 6 days (Table 6). At concentrations of 10 to 50 µg/ml, isochlorogenic acid C significantly enhanced the HO-1 expression. Oxymatrine, as the reference drug, showed the similar effect.

DISCUSSION

Physiologically, apoptosis is almost undetectable in the liver, with only 1 to 5 apoptotic cells/10,000 cells detected (Eichhorst, 2005). Liver disease is often associated with enhanced hepatocyte apoptosis, which is the case in viral and autoimmune hepatitis, cholestatic diseases, and metabolic disorders (Neuman, 2001). D-GalN is a toxin causing necrosis/apoptosis of the liver and is known as a suitable experimental model of hepatocyte damage (Keppler et al., 1968). Among the numerous models of experimental hepatitis, D-GalN induced liver damage is very similar to human viral hepatitis in its morphological and functional features (Lim et al., 2000). HepG2.2.15 cells are derived from human hepatoblastoma HepG2 cells that were transfected with a plasmid containing HBV DNA. The cells can stably secrete viral particles in culture medium (Sureau et al., 1986). In this study, the anti-apoptotic, anti-injury and anti-HBV effects of

Table 1. Changes of the related parameters of the D-GalN-induced hepatocyte apoptosis model.

Groups	Caspase-3 (nmol/g)	AST (IU/L)	ALT (IU/L)	MDA (nmol/ml)
Vehicle control	0.034 ± 0.001	2.33 ± 0.41	2.13 ± 1.04	2.21 ± 0.85
D-GalN-treated	0.330 ± 0.015[b]	4.49 ± 0.26[b]	5.63 ± 0.17[b]	8.65 ± 1.42[b]

Values are expressed as mean ± SD of four replicates. [a]P < 0.05 and [b]P < 0.01 represent the significance of the difference from the vehicle control.

Table 2. Effect of isochlorogenic acid C on D-GalN-induced HL-7702 hepatocyte apoptosis.

Groups	Concentration (μg/ml)	Caspase-3 (nmol/g protein)	TGFβ1 (ng/g protein)
Vehicle	-	0.090 ± 0.008	4.55 ± 0.71
D-GalN-Control	-	0.748 ± 0.037	23.53 ± 1.50
Oxymatrine	200	0.274 ± 0.009[b]	10.98 ± 0.41[b]
Isochlorogenic acid C	100	0.292 ± 0.004[b]	7.05 ± 1.00[b]
	50	0.538 ± 0.006[a]	15.95 ± 3.56[a]
	10	0.647 ± 0.001	23.20 ± 3.75

Values are expressed as mean ± SD of four replicates. [a]P < 0.05 and [b]P < 0.01 represent the significance of the difference from the D-GalN control.

Table 3. Effect of isochlorogenic acid C on survival of D-GalN-injured HL-7702 hepatocytes.

Groups	Concentration (μg/ml)	Absorbency (570 nm)	Protection rate (%)
Vehicle	-	1.289 ± 0.055[b]	-
D-GalN-Control	-	0.769 ± 0.053	-
Silybin	100	0.868 ± 0.036[a]	18.90
Isochlorogenic acid C	100	1.015 ± 0.072[b]	47.28
	50	1.012 ± 0.046[b]	46.66
	10	0.929 ± 0.032[b]	30.74

Absorbency values are expressed as mean ± SD of four replicates. Protection rate = [(A value in experimental group – A value in D-GalN-control group) / (A value in vehicle group – A value in D-GalN-control group)] × 100%. [a]P < 0.05 and [b]P < 0.01 represent the significance of the difference from the D-GalN control.

Table 4. Effect of treatment with isochlorogenic acid C for 4 days on HBsAg and HBeAg.

Compounds	Concentration (μg/ml)	HBsAg		HBeAg	
		P/N	Inhibition (%)	P/N	Inhibition (%)
Vehicle	-	19.08 ± 1.18	-	66.44 ± 8.24	-
Lamivudine	100	16.00 ± 0.58[a]	16.17	74.37 ± 3.25	-
Isochlorogenic acid C	100	10.90 ± 0.45[b]	42.89	56.65 ± 2.52	14.73
	50	14.73 ± 0.18[b]	22.83	64.48 ± 1.45	2.95
	10	15.83 ± 1.12[a]	17.07	72.33 ± 0.61	-

P/N (positive-to-negative) ratios were determined as the mean of absorbency values of test compound divided by that of the negative control. Inhibition (%) = [(P/N value in negative control group – P/N value in experimental group) / P/N value in negative control group] × 100%. Data are expressed as mean ± SD of three independent experiments. [a]P < 0.05 and [b]P < 0.01 compared with the vehicle group.

isochlorogenic acid C were studied using the D-GalN- induced hepatocyte apoptosis/injury model and

Table 5. Effect of treatment with isochlorogenic acid C for 8 days on HBsAg, HBeAg and HBV DNA.

Compounds	Concentration (μg/ml)	HBsAg		HBeAg		log (HBV DNA)
		P/N	Inhibition (%)	P/N	Inhibition (%)	
Vehicle	-	19.59 ± 1.13	-	81.65 ± 3.58	-	4.97 ± 0.13
lamivudine	100	16.71 ± 0.66[a]	14.70	85.20 ± 3.50	-	4.60 ± 0.09[a]
Isochlorogenic acid C	100	2.56 ± 0.07[b]	86.93	32.84 ± 3.04[b]	59.79	5.29 ± 0.03
	50	10.60 ± 0.48[b]	45.91	74.68 ± 2.03	8.55	5.20 ± 0.06
	10	16.86 ± 1.74	13.94	81.64 ± 0.74	0.01	4.95 ± 0.07

P/N (positive-to-negative) ratios were determined as the mean of absorbency values of test compound divided by that of the negative control. Inhibition (%) = [(P/N value in negative control group – P/N value in experimental group) / P/N value in negative control group] × 100%. Data are expressed as mean ± SD of three independent experiments. [a]$P < 0.05$ and [b]$P < 0.01$ compared with the vehicle group.

Table 6. Effect of isochlorogenic acid C on the HBV cccDNA and HO-1 expressions.

Group	Concentration (μg/ml)	log (HBV cccDNA)	HO-1 content (ng/g protein)
Normal	-	2.79 ± 0.03	36.00 ± 0.45
Oxymatrine	50	2.59 ± 0.12[a]	57.70 ± 3.21[b]
Isochlorogenic acid C	50	2.79 ± 0.10	59.36 ± 1.70[b]
	25	2.87 ± 0.04	50.42 ± 1.25[b]
	10	2.92 ± 0.04	47.53 ± 2.26[b]

Normal control with no antiviral drug was used as the negative controls. Oxymatrine was used as the positive controls. Data are expressed as mean ± SD of three independent experiments. [a]$P < 0.05$ and [b]$P < 0.01$ compared with normal group.

HepG2.2.15 cells, respectively. Based on the pilot test, we demonstrated the occurrence of hepatocyte apoptosis after HL-7702 cells were treated with D-GalN by the measurements of DNA ladder and Caspase-3. DNA fragmentation is one of the important features of cell apoptosis. Caspase-3 is a major executioner of liver apoptosis. In addition, some parameters of hepatocyte necrosis such as AST, ALT and MDA were also detected in this process. The results showed that the caspase-3 activity as well as the AST, ALT and MDA levels was significantly elevated and typical apoptosis ladder was observed, thus suggesting that the necrosis of a few hepatocytes occurred in the process of D-GalN-induced apoptosis. In this investigation, isochlorogenic acid C significantly reduced the caspase-3 and TGF-β1 levels, thus indicating pre-protective effect of test compound on D-GalN-indcued hepatocyte apoptosis. Additionally, D-GalN-induced hepatocyte injury is closely related to the formation of oxidative stress. Oxygen-derived free radicals released from activated hepatic macrophages are the primary cause of D-GalN-induced liver damage (Hu and Chen, 1992); and that isochlorogenic acids exhibit antioxidative, anti-inflammatory and antiviral activities etc (Maruta et al., 1995; Peluso et al., 1995; McDougall et al., 1998). Isochlorogenic acid C also improved significantly cell viability of the D-GalN-injured hepatocyte. Taken together, the antioxidative properties of isochlorogenic acid C may contribute to the reduction of hepatocyte apoptosis/injury.

Caspase-3 is usually thought of as the downstream effector protease most important for the classic nuclear changes associated with apoptosis (Enari et al., 1998). The cleavage of the anti-apoptotic protein Bcl-2 into a proapoptotic form is a necessary step in Fas-mediated apoptosis, and that caspase-3 mediates this processing (Cheng et al., 1997). Caspase-3 is also thought to be a key apoptotic “executioner” enzyme in mammalian cells because its activation triggers the cascade of enzymatic events that culminates in the death of the cell (Thornberry and Lazebnik, 1998). Fas-mediated hepatocytes death is implicated in much human liver pathology, and that caspase-3 is required for the initial events that occur in hepatocyte death following Fas ligation (Woo et al., 1999). The important role of TGF-β in orchestrating liver apoptosis is indicated by the hepatic fibrosis and apoptotic hepatocytes death of in transgenic mice that ectopically express TGF-β1 in the liver (Lee and Bae. 2002). TGF-β1-regulated apoptosis is cell type and context-dependent, indeed TGF-β1 provides signals for both cell survival and apoptosis (Perlman et al., 2001). The involvement of TGF-β1 in the production of oxidative stress required for the clearance of apoptotic bodies is further evidence of its integration into apoptotic pathways (Sánchez-Capelo, 2005). In this study, pre-treatment with

isochlorogenic acid C significantly reduced the caspase-3 and TGF-β1 levels, thus further suggesting that the anti-apoptotic potential of test compound is probably associated with interfering with the caspase-3 and TGF-β1 expressions.

Presence of HBsAg is the most common marker of HBV infection whereas HBeAg is used as an ancillary marker, primarily to indicate active HBV replication and associated progressive liver disease (Huang et al., 2001). HBV DNA polymerase is the site of action of the reverse transcriptase inhibitors such as lamivudine etc. Additionally, the template of HBV transcription, the cccDNA, plays a key role in the life cycle of the virus and permits the persistence of infection (Summers and Mason, 1982). In this study, isochlorogenic acid C significantly inhibited the HBsAg and HBeAg expressions of HepG2.2.15 cells. But it exhibited no inhibitory effect on HBV DNA and HBV cccDNA pool. It suggests that the site of anti-HBV effect of isochlorogenic acid C may mainly be at the downstream links of HBV replication process and is probably associated with blocking translation step.

HO-1 is the only inducible form among three known heme oxygenases (Otterbein and Choi, 2000). Over expression of HO-1 protects organs/tissues from immune-mediated organ injury, either through prevention of oxidative damage or via local immunomodulatory influence on inflammatory cells (Otterbein et al., 2003). Liver injury was significantly reduced after HO-1 induction in the acute hepatitis B model (Protzer et al., 2007). In addition, HO-1 showed a pronounced antiviral effect, which was confirmed in stably HBV-transfected hepatoma cells and in persistently HBV replicating transgenic mice (Protzer et al., 2007). Therefore, HO-1 might be a novel anti-HBV target. In this study, isochlorogenic acid C significantly induced the HO-1 expression in HepG2.2.15 cells, thus suggesting the hepatoprotective and anti-HBV effects of this compound could be achieved by HO-1 induction.

In conclusion, this investigation verifies that isochlorogenic acid C possesses the potent anti-apoptotic, anti-injury and anti-HBV effects. The anti-apoptotic and anti-injury effects of isochlorogenic acid C could be achieved by its anti-oxidative properties and interfering the caspase-3 and TGF-β1 expressions. The anti-HBV target of isochlorogenic acid C may mainly be at the downstream links of HBV replication process and is probably associated with blocking translation step. Induction of HO-1 may contribute to the hepatoprotective and anti-HBV effects of isochlorogenic acid C. Meanwhile, isochlorogenic acid C could be considered as a lead compound for the rational design of antiviral agents.

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