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Antiviral activity of methyl helicterate isolated from *Helicteres angustifolia* (Sterculiaceae) against hepatitis B virus

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ABSTRACT

The anti-HBV effect of methyl helicterate (MH), a triterpenoid isolated from the Chinese herb *Helicteres angustifolia*, was explored both *in vitro* and *in vivo*. In the HBV-transfected cell line HepG2.2.15, the secretion of HBsAg/HBeAg, the levels of HBV DNA and cccDNA, and the amount of viral RNA were significantly decreased after treatment with MH for 144 h. In addition, MH had no inhibitory effect on the mitochondrial DNA content. In DHBV-infected ducklings, MH significantly reduced the serum DHBV DNA, liver total viral DNA, and cccDNA levels. Furthermore, analysis of the liver pathological changes confirmed the hepatoprotective effect of MH. These results indicate that MH efficiently inhibits HBV replication both *in vitro* and *in vivo* and that MH may be a major bioactive ingredient in *H. angustifolia*.

1. Introduction

The hepatitis B virus (HBV) causes acute and chronic infections of the liver and is responsible for 1.2 million deaths annually (Harkisoen et al., 2012). Approximately 80% of HBV carriers suffer varying levels of hepatocyte destruction, which may develop into liver cirrhosis and hepatocellular carcinoma (HCC) (Aspinall et al., 2011; Park et al., 2006). The worldwide deaths from liver cancer caused by HBV infection most likely exceed 1 million per year (Gish, 2005).

Although the currently available anti-HBV drugs have improved the quality of life for HBV patients, the threat of drug resistance has prompted the search for novel anti-HBV agents. The identification of drugs from natural sources is especially important due to their novel structures and distinct action mechanisms. Many plants that have long been used as remedies are now being collected and examined in an attempt to identify potential antiviral drug sources (Micol et al., 2005). As an alternative to conventional chemical agents, a large number of phytochemicals have been recognized for their ability to control viral infections (Abad et al., 2000).

An example of a traditional herb that is often used in popular folk medicine for treating liver disease in China is *Helicteres angustifolia* (Sterculiaceae). Our pilot studies revealed that the aqueous extract from the root of this herb exhibits detectable inhibitory effects on HBV antigen expression and HBV DNA replication in both an *in vitro* model using the HepG2.2.15 cell line and in an *in vivo* duck model of HBV infection (Huang et al., 2011a,b). However, the components of the herb that are responsible for the anti-HBV activity are unknown. Thus, the identification of the active ingredients may provide clues of the underlying mechanism.

To further investigate the anti-HBV activity of this well-known Chinese medicine, we performed a bioactivity-guided isolation of the methyl helicterate (MH) from *H. angustifolia* and evaluated its anti-HBV activity both *in vitro* and *in vivo* relative to the positive control lamivudine (3TC). We evaluated the levels of HBV antigens, extracellular HBV DNA, intracellular neo-synthesized encapsidated viral DNA, HBV cccDNA, viral RNA, and mitochondrial DNA in the HepG2.2.15 cell line after *in vitro* MH treatment. We investigated the *in vivo* effects of MH by analyzing the serum and liver DHBV DNA levels, intrahepatic viral cccDNA level, and the hepatic pathological changes in DHBV-infected ducks. This is the first report of the anti-HBV effects of MH, which is an active substance derived from *H. angustifolia*.

2. Materials and methods

2.1. Chemicals

Methyl helicterate (MH) was isolated from *H. angustifolia* according to the protocol established in our previous study (Lin et al., 2012b). The MH molecular formula and molecular weight

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are $C_{40}H_{56}O_6$ and 632.89, respectively. In addition, the chemical structure of MH is shown in Fig. 1. MH is normally stored at 4 °C. This compound is dissolved in distilled water and diluted with physiologic saline for the animal tests.

Lamivudine (3TC), which has a molecular weight of 229.25, was purchased from GlaxoSmithKline, Inc., (USA); dimethyl sulfoxide (DMSO) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma (USA). Hepatitis B surface antigen (HBsAg) and hepatitis B e-antigen (HBeAg) kits were purchased from Rongsheng Biotechnology Co., Ltd., (Shanghai, China).

2.2. Cell culture

The HepG2.2.15 cell line, which supports the persistent replication of HBV and produces intact HBV particles (Sells et al., 1987), was provided by the Medical Experimental Center of Guangxi Medical University. The cells were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal calf serum, 100 units/mL penicillin, 100 mg/mL streptomycin, and 2 mmol/L L-glutamine (all from Invitrogen, USA) at 37 °C in a 5% CO_2 atmosphere with 100% humidity. The cells were harvested from the flask by treating the monolayer with 0.25% trypsin (Gibco-BRL, Grand Island, NY, USA) and 1 mmol/L EDTA, and the cells were then resuspended in culture medium for further use.

2.3. Cytotoxicity measurement

To evaluate the cytotoxicity, HepG2.2.15 cells were inoculated at a density of 1×10^5 cells/well (200 μL for each well) into 96-well culture plates (Costar, Corning Inc., NY, USA). After incubation for 72 h, the supernatants were replaced with fresh culture media containing serial 1:2 dilutions of MH (158.0–9.9 μM). The media in the wells was refreshed every other day. After treatment for 144 h, an MTT solution (10 mL/100 mL medium) was added to all of the wells, and the plates were incubated at 37 °C for 4 h. An acid-isopropanol combination was then added (100 mL of 0.04 N HCl in isopropanol) to the wells dissolve the dark blue crystals that developed from the MTT. The plates were then read directly with a microplate reader at a wavelength of 490 nm.

$$\text{Inhibition ratio}\,\% = [1 - (\text{OD}_{\text{sample}} - \text{OD}_{\text{empty control}})/(\text{OD}_{\text{cell control}} - \text{OD}_{\text{empty control}})] \times 100\%$$

2.4. Treatment of HepG2.2.15 cells with MH

HepG2.2.15 cells were inoculated in 42 flasks (75 cm^2, NUNC, Roskilde, Denmark) at a density of 1×10^5/mL (12 ml per flask). After incubation at 37 °C for 48 h, fresh culture media containing 1.0, 2.0, 4.0, 7.9, or 15.8 μM MH or 43.6 μM lamivudine (3TC) was added, and the media was replaced every 48 h. After 144 h of treatment, the supernatants from each group were collected independently to determine the secretion of albumin and the levels of HBV surface antigen (HBsAg), HBV e antigen (HBeAg), and HBV DNA. The cells were harvested by trypsin digestion, washed three times with phosphate buffered saline (PBS, pH 7.3), and used to determine the amount of cellular viral DNA and RNA.

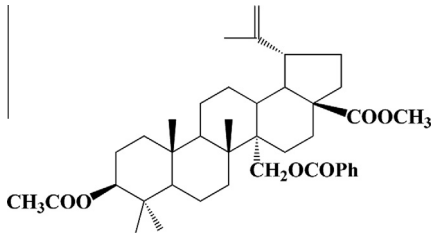

Fig. 1. Chemical structure of MH.

2.5. Detection of albumin in the supernatant

The albumin level in the supernatant was measured by radioimmunoassay with an albumin assay kit (Northward Biotechnical Institute, Beijing, China), according to the manufacturer's instructions.

2.6. Assays for HBsAg and HBeAg

The HBsAg and HBeAg levels were detected with ELISA kits (Rongsheng Biotechnology Co., Ltd., Shanghai, China) according to the manufacturer's instructions. The plates were read directly with a microplate reader at a wavelength of 490 nm.Inhibition ratio % = $(\text{OD}_{\text{control}} - \text{OD}_{\text{sample}})/\text{OD}_{\text{control}} \times 100\%$

2.7. Southern blot analysis of HBV DNA in the supernatant

The HBV DNA in the supernatant was detected through Southern blotting according to the method described by Kim et al. (2009). Briefly, the medium was centrifuged at 2000*g* for 15 min, and polyethylene glycol (M_r, 8000) was added to the supernatant to a final concentration of 10% (v/v). The virus pellets were collected by centrifugation at 10,000*g* for 15 min and resuspended in TNE buffer (10 mM Tris–HCl, pH 7.5, 100 mM NaCl, and 1 mM EDTA). The virus suspension was adjusted to 1% sodium dodecyl sulfate (SDS) and 0.5 mg/ml proteinase K and incubated at 55 °C for 3 h. The digest was extracted with 1:1 (v/v) phenol/chloroform, and the DNA was precipitated with ethanol. The DNA pellet was dissolved in TE buffer (10 mM Tris HCl, pH 8.0, and 1 mM EDTA), electrophoresed in a 0.8% agarose gel, and then blotted onto a Hybond-N^+ membrane (Amersham, Freiburg, Germany). The blot was hybridized with a ^{32}P-labeled HBV DNA probe in Church hybridization buffer (Church and Gilbert, 1984), washed with 2× standard saline citrate (SSC) and 0.2% SDS at room temperature for 1 h, and autoradiographed.

2.8. Measurement of intracellular neo-synthesized encapsidated viral DNA by Southern blotting

The effect of MH on the intracellular neo-synthesized encapsulated viral DNA was assessed as described previously (Zheng et al., 2011). The total DNA was extracted from 3×10^6 HepG2.2.15 cells using a Qiagen DNeasy Tissue Kit (Qiagen, Inc, Valencia, CA, USA). Ten micrograms of DNA were digested by Hind III at 37 °C and then precipitated in two volumes of ethanol. The DNA was dissolved in double-distilled water and separated on a 1.2% agarose gel, and the purified DNA samples were transferred onto nylon membranes (Amersham, Freiburg, Germany). After ultraviolet crosslinking and prehybridization, the membranes were hybridized with a ^{32}P-labeled DNA probe generated by a random-primed labeling kit (Amersham, Freiburg, Germany) using the full-length HBV DNA.

2.9. Northern blot analysis of viral RNA

The total RNAs were extracted from HepG2.2.15 cells using the Trizol method according to the manufacturer's instructions (Invitrogen). The RNAs were subjected to electrophoresis on a 1.2% formaldehyde–agarose gel and transferred onto a Hybond-N^+ membrane (Amersham). The HBV RNAs were detected with ^{32}P-labeled HBV DNA probes in Church hybridization buffer. The blots were then stripped and rehybridized with a ^{32}P-labeled GAPDH DNA probe for normalization (Feng et al., 2009).

2.10. Quantitative real-time PCR for HBV cccDNA

To observe the effect of MH on intracellular HBV cccDNA, HepG2.2.15 cells were incubated for 48 h in six-well plates at a density of 1.0×10^5 cells/mL, and new DMEM medium containing different concentrations of MH (1.0, 2.0, 4.0, 7.9, or 15.8 μM) was added. Two parallel controls were performed: a positive control with 43.6 μM 3TC and a negative control with 0.1% DMSO. The medium with MH was replaced every three days. On the sixth day, the cells from each well were harvested by trypsin digestion and washed three times with phosphate buffered saline (PBS, pH 7.3). The cell pellet containing 1.0×10^6 cells was extracted using a mini plasmid extraction kit (Qiagen). The extract product was further purified using plasmid-safe ATP-dependent DNase (Epicentre Technologies, WI, USA) to remove the residual single-stranded viral DNA, relaxed circular DNA, and/or cellular chromosomal DNA.

The intracellular cccDNA was quantified by selective real-time fluorescent quantitative PCR with specific primers and the Taqman MGB probe (Qi et al., 2012). The forward primer was 5-TTCTC ATCTGCCGGACCG-3, and the reverse primer was 5-CACAGCTT GGAGGCTTGAAC-3. The Taqman MGB probe was 5-FAM-CCTAATC ATCTCTTGTTCATGTC-MGB-3. The qPCR analysis was performed to detect the intracellular cccDNA using a RealQuant PCR kit (Invitrogen).

2.11. Examination of mitochondrial DNA

A detailed analysis of the effect of MH on the mitochondrial DNA synthesis was conducted. HepG2.2.15 cells were cultured for 24 h before the treatments with the various compounds were initiated. MH was added at a concentration of 1.0, 2.0, 4.0, 7.9, or 15.8 μM in DMEM. The cultures treated with 47.3 μM 2′3′-dideoxycytidine (ddC) were maintained in parallel as positive controls for the assessment of mitochondrial DNA damage. The compounds were repeatedly added every three days until day 9. The cellular DNA was then isolated according to standard protocols and digested with the restriction enzyme Bam HI. The hybridization and detection of the mitochondrial DNA were performed according to the Laboratory Manual of Molecular Cloning. The probe used was ^{32}pdCTP-labeled cytochrome oxidase III DNA.

2.12. Short-term toxic reaction of MH on ducklings

A short-term toxicity study was routinely conducted according to the method described by Li et al. (2008) with some modifications. Sixteen Pekin ducklings were randomly divided into the MH group (eight ducklings) and the normal control group (eight ducklings). The animals in the two groups were administered MH (100 mg/kg) or saline once a day for 14 days. The vital signs of the animals, including weight, feather color/luster, gait, food-taking, mental status, and response to stimuli, were observed throughout the experiment.

2.13. Inoculations of ducklings with DHBV virus and drug administration

The Pekin ducklings were maintained under normal daylight and fed standard commercial diet and water. The research was conducted according to protocols approved by the institutional ethical committee of Guangxi Medical University (Approval No.: 0120813361).

Normal one-day-old Pekin ducklings were divided into six groups of 10. The ducks received saline (groups I and II) or the corresponding drugs (groups III through VI) at a volume of 10 ml/kg once a day for a period of 14 days. Groups II through VI were intravenously infected with 5.7×10^6 viral DNA equivalents (VGE, 1VGE = 3.3×10^6 pg) of duck hepatitis B virus (DHBV). The treatments began seven days after infection. Group I served as the uninfected control group (normal control group). Group II served as the untreated control group (model control group). The ducks in the two groups were orally administered saline. Group III served as the positive control group, and the animals were intragastrically administered 100 mg/kg/day 3TC. Groups IV, V, and VI served as the MH-treated groups, and the ducks were orally administered 25, 50, and 100 mg/kg/day MH, respectively. These doses of MH were selected based on our previous studies (Huang et al., 2012).

Serum samples were collected via the crural vein of each duckling immediately before treatment (T0), seven (T7) and 14 (T14) days after the first treatment, and three days after withdrawal of the treatment (P3). The sera were stored at −70 °C until analysis.

At the end of the experiment, the ducks were sacrificed, and the liver tissues were dissected and washed immediately with ice-cold saline to remove the excessive blood. Subsequently, the liver samples were divided into two parts. One part was immediately stored at −80 °C for future analysis, and the other part was excised and fixed in a 10% formalin solution for histopathologic examination.

2.14. Measurement of duck serum DHBV DNA by real-time PCR

Real-time PCR was performed on the extracted DHBV DNA samples using a BioRad LightCycler (BioRad, Idaho, USA) and quantified using fluorescence probes. Each 25-μL reaction mixture contained 50 ng of DNA, 2 μL of the probes (Genecore, Shanghai, China), 2.5 μL of $MgCl_2$ (at a final concentration of 2.5 mmol/L), 0.25 μL of TaKaRa Ex *Taq* HS (5 U/μL; TaKaRa, Dalian, China), and 0.5 μL of the 10 μmol/L primers. The probe sequence (5′-CGGGCTC CCCTCTCCCACG-3′) was labeled with the 6-carboxy-fluorescein dye at the 5′-end and with the 6-carboxy-tetramethyl-rhodamine quencher dye at the 3′-end. The sense primer sequence was 5′-G AGCCCCTTCACCCCAAC-3′, and the antisense primer sequence was 5′-ATCTATGGTGGCTGCTCGAACT-3′. The primers and probe were synthesized by Shanghai Genecore (TaqMan minor groove binder, Shanghai, China). The PCR protocol consisted of an initial incubation step of 5 min at 95 °C and 40 cycles of 10 s at 95 °C, 20 s at 55 °C, and 30 s at 72 °C. The DHBV DNA level was quantified using a standard curve, which was described in our previous study (Lin et al., 2012a).

2.15. Southern-blot analysis of DHBV DNA in the duck liver

Four hundred milligrams of the duck liver tissues was ground in 4 mL of buffer (10 mM Tris–HCl, pH 7.6, 0.15 M NaCl, 1.27 mM EDTA, 20 mg/mL SDS, 5 μg/mL salmon sperm DNA, and 0.5 mg/mL proteinase K) at 50 °C for 3 h and then centrifuged at 13,000*g* for 10 min. The supernatant was extracted with phenol/chloroform and then precipitated in two volumes of ethanol containing acetic acid at 1/10. The DNA was then dissolved in 800 μL of TE buffer (10 mM Tris–HCl, pH 7.5, 2 mM EDTA, and 100 μg/mL RNase A). The DNA was separated on a 1.5% agarose gel and analyzed by Southern blotting using a DHBV DNA probe as described previously (Jilbert et al., 1992; Niu et al., 2003).

2.16. Determination of DHBV cccDNA in duckling liver

The intrahepatic viral cccDNA was extracted using a procedure that was described in detail by Jilbert et al. (1992). In brief, the liver samples were snap-frozen in liquid nitrogen, stored at −80 °C, and then analyzed for viral cccDNA. One hundred milligrams of liver was homogenized in 2 mL of 0.01 M Tris–HCl (pH 7.5) and 0.01 M EDTA, and the homogenate was diluted to 6 mL in 0.01 M Tris–HCl (pH 7.5) and 0.01 M EDTA and used to purify the non-protein-bound nucleic acids containing the viral cccDNA.

The non-protein bound nucleic acids were purified according to the method described by Wu et al. (1990) with the exception that the cccDNA-containing supernatant was extracted two times with an equal volume of phenol buffered with 0.05 M Tris–HCl (pH 8) and then extracted with an equal volume of phenol and chloroform (1:1) buffered with 0.5 M Tris–HCl (pH 8) before the ethanol precipitation at room temperature. Five micrograms of the cccDNA preparation was subjected to electrophoresis on 1.5% agarose gels. Southern blot analysis was conducted as described previously (Delmas et al., 2002), and the viral cccDNA was detected by hybridization with a ^{32}P-labelled probe representing the complete viral genome (AguesseGermon et al., 1998; Jilbert et al., 1992).

2.17. Histopathological examination of the duck livers

A portion of each harvested liver tissue was instantly fixed in 10% phosphate-buffered formalin. Subsequently, this portion was processed by routine histological procedures, such as embedding in paraffin, sectioning into 5-μM pieces, and mounting onto slides. The samples were stained with hematoxylin and eosin (H&E) for histopathological examination. The degrees of hepatocytic necrosis and degeneration, in addition to periportal tract and intralobular inflammation, were assessed according to the method described by Guo et al. (2007).

2.18. Statistical analysis

All of the statistical analyses were performed using the SPSS 11.5 software for Windows. One-way analysis of variance (ANOVA) was used to compare the means among different groups, and Tukey's test was used for the post hoc multiple comparisons. The data are presented as the mean ± S.E. Differences with a p-value less than 0.05 were considered statistically significant. The grade of the differences in the hepatic tissue pathology between groups was evaluated using the Mann–Whitney test.

3. Results

3.1. Cytotoxic effect of MH on HepG2.2.15 cells

The cytotoxicity of MH was determined using fresh culture medium containing serial 1:2 dilutions of MH. The inhibition ratio of the HepG2.2.15 cells exposed to different concentrations of MH is shown in Table 1. The 50% cytotoxic concentration (CC_{50}) was found to be 127.2 μM, and the maximum nontoxic concentration (MNCC) was 22.8 μM. These results were used to determine the dose range of MH for the subsequent experiments.

3.2. Effect of MH on the secretion of albumin

As shown in Fig. 2, there was no significant difference in the albumin level in the culture supernatant between the different groups.

Table 1
Evaluation of MH cytotoxicity to HepG2.2.15 using the MTT assay.

MH concentration (μM)	Inhibition ratio (%)
9.9	0.96
19.8	1.76
39.5	12.29
79.0	16.17
158.0	67.50

The 50% cytotoxic concentration (CC_{50}) was 127.2 μM, and the maximal non cytotoxic concentration (MNCC) was 22.8 μM.

3.3. Effect of MH on the secretion of HBsAg and HBeAg by HepG2.2.15 cells

As shown in Fig. 3A, MH exerted remarkable inhibitory effects on the secretion of HBsAg and HBeAg in HepG2.2.15 cells. The treatment of HepG2.2.15 cells for 144 h with various concentrations of MH resulted in a significant dose-dependent reduction in the HBsAg secretion. The 50% inhibiting concentration (IC_{50}) value was determined to be 8.9 μM. Similarly to the HBsAg secretion, the secretion of HBeAg was significantly reduced by MH in a dose-dependent manner with an IC_{50} value of 10.4 μM.

3.4. Effect of MH on the secretion of extracellular HBV DNA

To confirm that MH exhibits anti-HBV activity in HepG2.2.15 cells, the effect of MH on the HBV DNA level was evaluated. Consistent with the inhibitory effects that MH had on HBsAg and HBeAg secretion, treatment with 7.9–15.8 μM MH or 43.6 μM 3TC led to a significant reduction in the level of extracellular HBV DNA compared with the drug-free group (negative control) (Fig. 3B).

3.5. Effect of MH on intracellular viral DNA level

As shown in Fig. 3C, MH exhibited a dose-dependent inhibitory effect on the level of intracellular viral DNA. Compared with the negative control, treatment with 15.8 μM MH or 43.6 μM 3TC for 144 h resulted in a significant reduction in the level of intracellular viral DNA.

3.6. Effect of MH on the level of HBV RNA

As shown in Fig. 3D, the HBV RNA of the cells treated with MH was decreased markedly compared with that of the control, and this inhibitory effect increased with an increase in the MH concentration.

3.7. Effect of MH on the level of HBV cccDNA

The effect of MH on the level of HBV cccDNA is shown in Fig. 3E. The results indicated that MH at a concentration of 15.8 μM significantly reduced the HBV cccDNA content of HepG2.2.15 cells.

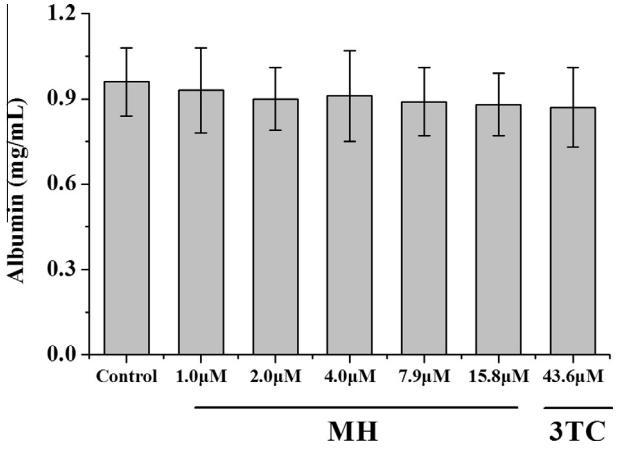

Fig. 2. Effects of MH on the secretion of albumin in the supernatant. The albumin level was determined by radioimmunoassay. The data are presented as the means ± S.E.

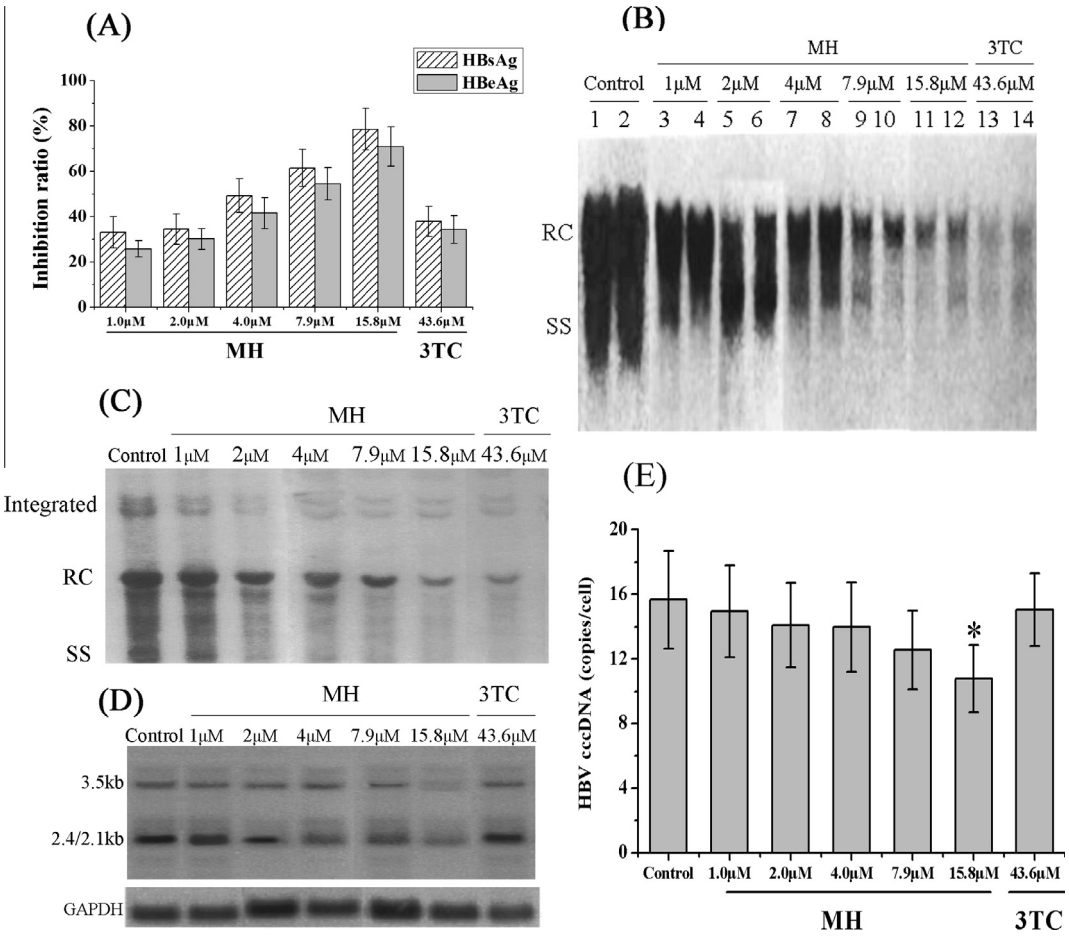

Fig. 3. Effects of MH on the secretion of HBsAg/HBeAg, the levels of viral DNA, and the expression of HBV RNA in the HepG2.2.15 cell line. The HBsAg and HBeAg levels in the supernatants were quantified using ELISA kits (A). The extracellular HBV DNA (B) and intracellular HBV DNA (C) levels were evaluated by Southern blotting with a ^{32}P-labeled HBV-specific DNA probe. The forms of HBV-DNA are indicated as integrated, relaxed circular (RC), and single-stranded (SS) HBV DNA. The viral RNAs were prepared from HepG2.2.15 cells and analyzed by Northern blotting with a ^{32}P-labeled HBV-specific DNA probe (D). In addition, the intracellular HBV cccDNA was measured by real-time PCR (E). The results are expressed as copy numbers per cell (mean ± S.E.) from three independent experiments. $^{*}P < 0.05$ vs. the negative control.

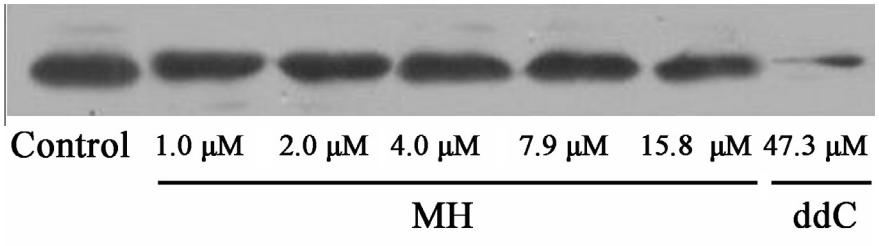

Fig. 4. Mitochondrial DNA levels in HepG2.2.15 cells repeatedly treated with MH at three-day intervals for a period of nine days.

3.8. Effect of MH on mitochondrial DNA

The hybridization of intracellular DNA to a cytochrome oxidase III probe was performed to evaluate the effect of MH on the mitochondrial DNA levels. The mitochondrial DNA levels in cells treated with MH were similar to those of the negative control but were significantly higher than those found in the cells treated with ddC (Fig. 4).

3.9. Short-term toxicity of MH on ducklings

Fourteen days after treatment, no significant differences in the body weight, feather color/luster, gait, food-taking, mental status, and response to stimuli were found between the MH group and the normal control group. This finding suggests that MH had no obvious short-term toxicity to ducklings.

3.10. Effect of MH on serum DHBV replication

The expression of DHBV could not be detected in the normal control group. As shown in Fig. 5A, the duck serum DHBV replication levels were markedly decreased during the treatment period in the groups that were treated with 3TC or a medium or high dose of MH compared with the model control group. Interestingly, the DHBV replication levels were increased after the three-day withdrawal period; however, the levels rebounded slightly in the medium- and high-dose MH-treatment groups compared with the 3TC group.

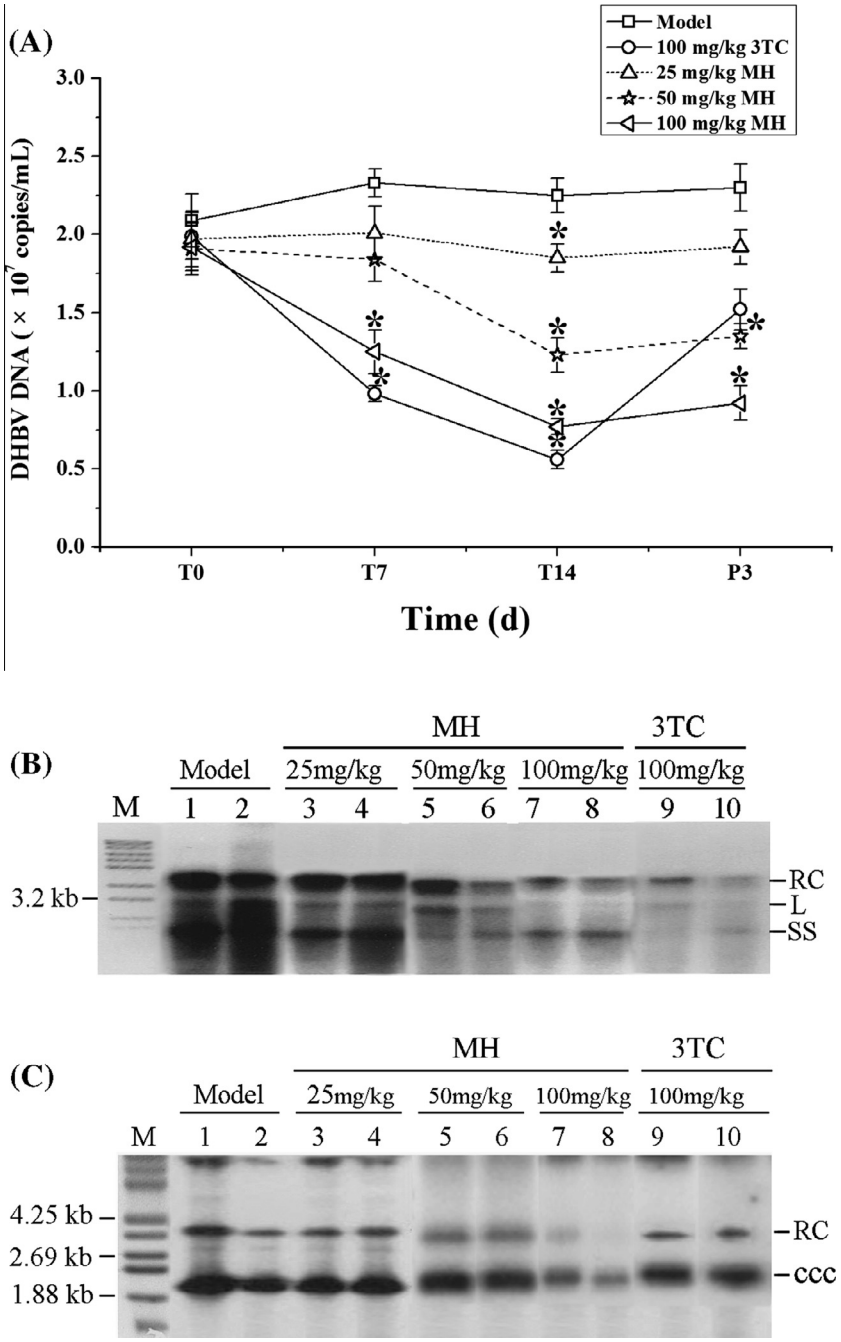

Fig. 5. Effect of MH on DHBV DNA levels. The serum DHBV DNA level (A) was assayed using real-time PCR. The data are presented as the means ± S.E. $^{*}P < 0.05$ vs. model control. The liver total DHBV DNA (B) and viral cccDNA (C) levels were analyzed by Southern hybridization. M, molecular standards; RC, relaxed circular DNA; L, linear DNA; SS, single-strand DNA; ccc, covalently closed circular DNA.

3.11. Effect of MH on DHBV DNA in the duckling liver

To further confirm the *in vivo* anti-HBV effect of MH in ducks, the DHBV DNA levels in the livers obtained on three days after the end of the treatment were examined through Southern blotting. Consistent with the inhibitory effect of MH on the serum DHBV DNA levels, the MH treatment dose-dependently reduced the relaxed circular (RC), linear (L), and single-stranded (SS) forms of DHBV DNA in the liver (Fig. 5B).

3.12. Effect of MH on DHBV cccDNA in liver tissue

The liver tissue was examined by Southern blot hybridization to determine the level of cccDNA. As shown in Fig. 5C, compared with the level of cccDNA in the infected model control ducks, the level of viral cccDNA in the ducks treated with 100 mg/kg MH was significantly reduced.

3.13. Histopathological examination of the duck livers

To evaluate the MH-induced pathological changes, liver sections were examined under a light microscope. The normal control liver presented a normal lobular architecture with central veins and radiating hepatic cords (Fig. 6A). The following typical pathological characteristics were obvious in the model group: marked fatty degeneration, portal inflammation, necrosis, and hepatocyte loosening (Fig. 6B). The groups that were treated with 100 mg/kg/day 3TC or 50 or 100 mg/kg/day MH appeared to exhibit less

pathological damage (Fig. 6C, E, and F). However, treatment with 25 mg/kg/day MH did not have a significant effect (Fig. 6D).

The results of the evaluation of all of the slides are summarized in Table 2. The liver sections were evaluated primarily on the basis of their hepatocytic degeneration, necrosis, and inflammatory cell infiltration. MH treatment resulted in dose-dependent improvements in each of the above mentioned three parameters, although superior improvements were observed in the degree of necrosis and cell infiltration compared with the degeneration. It is worth noting that treatment with 100 mg/kg/day MH elicited more significant improvements than treatment with 100 mg/kg/day 3TC.

4. Discussion

In this study, we examined the anti-HBV activity of MH both *in vitro* and *in vivo*. For the *in vitro* study, the HepG2.2.15 cell line is an appropriate model for identifying the molecular events in the intracellular viral replication cycle and the secretion of HBV particles, which contain multiple copies of the HBV genome that can stably integrate into the host cell genome (Han et al., 2011). As indicated by our results, cell growth retardation was not evident after the administration of low concentrations of MH (<22.8 μM); however, MH began to elicit cytotoxicity at high concentrations in a dose-dependent manner, with a 50% cytotoxic concentration (CC_{50}) of 127.2 μM. Thus, the nontoxic doses of MH (<22.8 μM) were used to determine the dose range of MH for the subsequent experiments. Furthermore, the assay of the level of albumin indicated that treatment with 1.0–15.8 μM MH had negligible effect on the secretion of albumin. These data further confirmed that MH has no cytotoxic effect on the cells.

The treatment of HBV-transfected HepG2.2.15 cells with MH for 144 h exhibited a potent inhibitory activity on the secretion of both HBsAg and HBeAg. It is worth noting that 15.8 μM MH was more potent than 43.6 μM 3TC at inhibiting the secretion of both HBsAg and HBeAg after 144 h of treatment. This finding provides strong evidence that MH elicits an inhibitory effect on HBV in HepG2.2.15 cells. The anti-HBV activity of MH was also confirmed by measuring its inhibitory effects on the levels of HBV DNA in HepG2.2.15 cells. After treatment with MH for 144 h, the levels of extracellular and intracellular HBV DNA were lower than the levels observed in the negative controls. Furthermore, the Northern blot analysis of the HBV RNA showed that MH can interfere with the transcription of HBV. As antigen expression, extracellular DNA, and intermediate DNA are all related to the transcription level of HBV RNA, a reduction in the HBV RNA might play a key role in the MH anti-HBV function.

In addition, the inhibitory effect of MH on the level of intracellular HBV cccDNA was also demonstrated in this study. HBV cccDNA is the first replication intermediate generated after HBV enters hepatocytes, and its presence indicates the initiation of intracellular HBV replication and the successful establishment of HBV infection (Seeger and Mason, 2000). The persistence of cccDNA in the nuclei of hepatocytes after the withdrawal of antiviral agents is believed to be primarily responsible for the recurrence of hepatitis B (Zoulim, 2005). Therefore, the nuclear cccDNA level is an indispensable index for the evaluation of the

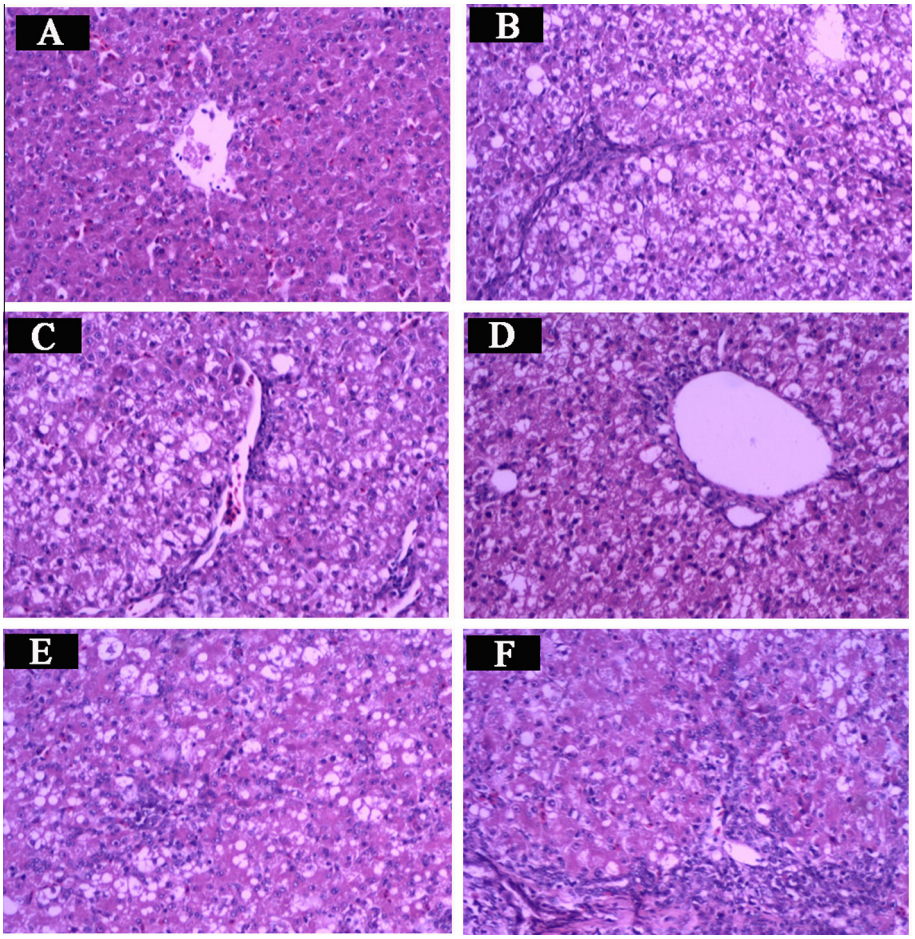

Fig. 6. Histopathological changes in duck livers. The liver sections were stained with hematoxylin and eosin (H&E), and the histopathological changes were examined under a light microscope (100×). (A) Normal control; (B) model control; (C) 100 mg/kg/day 3TC; (D) 25 mg/kg/day MH; (E) 50 mg/kg/day MH; (F) 100 mg/kg/day MH.

Table 2
Summary of the histopathological changes in the duck livers ($n = 10$).

Group	Dose (mg/kg/day)	Degeneration				Necrosis				Infiltration			
		–	+	2+	3+	–	+	2+	3+	–	+	2+	3+
Normal	–	10	0	0	0	10	0	0	0	10	0	0	0
Model*	–	0	1	6	3	0	3	4	3	0	2	6	2
3TC#	100	1	4	5	0	1	7	2	0	1	7	1	1
MH	25	0	2	7	1	0	5	4	1	0	4	5	1
MH#	50	2	6	2	0	3	5	1	1	2	5	3	0
MH#	100	5	3	2	0	7	2	1	0	5	4	1	0

Hepatic degeneration and necrosis and inflammatory cell infiltration were examined in the lobular and periportal tract regions. The degree of degeneration was determined as the percent of hepatocytic edema and fatty degeneration and was scored as follows: (–) none; (+) < 25%; (2+) < 50; (3+) ⩾ 50%. Necrosis was scored as follows: (–) none; (+) spotty necrosis; (2+) piecemeal necrosis; or (3+) bridging or widespread necrosis. Cell infiltration was scored as follows: (–) none; (+) mild infiltration; (2+) moderate infiltration; or (3+) massive infiltration or lymphoid nodules. Significance was determined by the Mann–Whitney test. $^{*}P < 0.05$ compared with the normal control group; $^{\#}P < 0.05$ compared with the model group.

effectiveness of an anti-HBV agent and is a predictor of a sustained antiviral response to therapy (Sung et al., 2005). In this study, a marked decrease in the intracellular cccDNA was observed after 144 h of treatment with 15.8 μM MH, which indicates that the anti-HBV effect of MH is most likely related to the inhibition of the formation of cccDNA.

The mitochondrial cytotoxicity of MH in HepG2.2.15 cells was also investigated to further assess the anti-HBV activity of MH and to evaluate its potential use for the treatment of HBV infection. The mitochondria play an important role in organ function, and it was hypothesized that decreases in the mitochondrial DNA level may cause delayed toxicity (He et al., 2008). The assay of the mitochondrial toxicity showed that MH had no inhibitory effect on the mitochondrial DNA content.

The *in vivo* anti-HBV activity of MH was also evaluated in DHBV-infected ducks, which is a standard model used to detect the *in vivo* activity and toxicity of anti-HBV agents (Zhou et al., 2007). In this study, treatment with 50 or 100 mg/kg MH for 14 days significantly reduced the serum DHBV DNA levels. Interestingly, the inhibitory effect of MH on the serum viral DNA was more effective than that of 3TC during the three-day period after termination of the drug treatments. In addition, MH also reduced the liver total viral DNA and cccDNA levels in a dose-dependent manner. These data suggest that MH can effectively suppress the replication of DHBV *in vivo*.

We previously found that MH was significantly beneficial for the prevention of CCl_4-induced liver injury, possibly by scavenging reactive free radicals, boosting the endogenous antioxidant system, inhibiting proinflammatory cytokines *via* the downregulation of NF-κB, inhibiting the Fas/FasL pathway, and blocking the CYP2El-mediated CCl_4 bioactivation (Lin et al., 2012b). In addition, MH was also found to be effective in the prevention of CCl_4-induced liver fibrosis in rats. The antifibrotic mechanism of MH was mainly attributed to its modulation of the TGFβ-Smad signaling pathway (Huang et al., 2012). These results strongly emphasize the preventive and therapeutic role of MH in liver injury and even fibrosis and provide a valuable reference for the investigation of the role of MH in HBV-induced liver injury. In this study, significant histological improvements were noted in the MH treatment groups three days after withdrawal of the treatment. Interestingly, the histopathological examination revealed that 100 mg/kg MH elicited a more significant improvement than 100 mg/kg 3TC.

In conclusion, our research study demonstrated that MH reduced the production of HBV-specific antigens and the levels of HBV DNA, cccDNA, and HBV RNA in a dose-dependent manner in HepG2.2.15 cells without impacting the cells. In addition, MH exerted no inhibitory effect on the mitochondrial DNA content. The *in vivo* experiments showed that MH significantly reduced the serum DHBV DNA, liver total viral DNA, and cccDNA levels. Furthermore, MH exhibited a protective effect on HBV-induced liver injury in DHBV-infected ducklings. Our study suggests that MH might play an important role in the treatment of hepatitis B and may contribute to the anti-HBV effects of *H. angustifolia*. This study also provides evidence that supports the further development of promising anti-HBV drugs.

Conflict of interest

The authors state that there are no conflicts of interest.

Acknowledgments

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