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Research Paper

In vitro and in vivo anti-hepatitis B virus activities of the lignan nirtetralin B isolated from *Phyllanthus niruri L.*

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ABSTRACT

Ethnopharmacological relevance: Nirtetralin B, a new lignan first reported by our team, is isolated from *Phyllanthus niruri L.* This plant has long been used in folk medicine for liver protection and antihepatitis B in many Asian countries. This study was designed to evaluate the anti-hepatitis B virus activity of nirtetralin B using HepG2.2.15 cells and duck hepatitis B virus (DHBV) infected ducks as in vitro and in vivo models.

Materials and methods: Nirtetralin B was isolated from *Phyllanthus niruri L.* (Euphorbiaceae) by extraction and chromatographic procedures and the anti-hepatitis B virus activity was evaluated both in vitro and in vivo. The human HBV-transfected liver cell line HepG2.2.15 was used in vitro assay. And the in vivo anti-hepatitis B virus activity was evaluated on the expression of HBV replication, HBsAg, HBeAg, ALT and AST on day 0, 7, 14, 17 after nirtetralin B was dosed intragastricly (i.g.) once a day for 14 days at the dosages of 25, 50 and 100 mg/kg/day in the duck hepatitis B virus (DHBV) infected ducks.

Results: In the human HBV-transfected liver cell line HepG2.2.15, nirtetralin B effectively suppressed the secretion of the HBV antigens in a dose-dependent manner with IC50 values for HBsAg of 17.4 μM, IC50 values for HBeAg of 63.9 μM. In DHBV-infected ducklings, nirtetralin B significantly reduced the serum DHBV DNA, HBsAg, HBeAg, ALT and AST. And analysis of the liver pathological changes confirmed the hepatoprotective effect of nirtetralin B.

Conclusion: The experimental data demonstrated that nirtetralin B exhibits anti-hepatitis B virus activity both in vitro and in vivo.

1. Introduction

Hepatitis B virus (HBV) causes both acute and chronic infections of the liver (Gitilin, 1997; Li et al., 2008). More than 400 million people worldwide are infected with hepatitis B virus (HBV), while approximately 80% of HBV carriers have different levels of hepatocyte destruction, which could develop into chronic hepatitis B, cirrhosis, or hepatocellular carcinoma (Pungpapong et al., 2007; Wu et al., 2012). HBV infection, responsible for over 1.2 million deaths annually, has produced a serious health problem, particularly in sub-Saharan Africa, China and South-East Asia (Parkin et al., 1999; Wong and Lok, 2006). Although several anti-viral drugs, including interferon-α and nucleoside analogs, have been approved for the treatment of hepatitis B, unresolved critical issues remain, including moderate to low efficacy, dose-dependent side effects, and the newly developed drug resistance (Perrillo, 2005; Yuen and Lai, 2011). Therefore, there exists a significant unmet medical need for safe and efficacious new anti-HBV drugs. On the other hand, natural products could provide a great opportunity for screening safer and more efficacious anti-HBV agents as a result of their structural diversity (Chattopadhyay et al., 2009).

Phyllanthus niruri L. (Euphorbiaceae) is a small herb widely distributed in tropical and subtropical regions in India, China and other countries. In many Asian countries, *Phyllanthus niruri.* has long been used in folk medicine for liver protection and antihepatitis B (Prakash et al., 1995; Cimanga et al., 2004). It has also been reported to exhibit anti-HBV effects and thoroughly investigated for its significant activities of anti-hepatitis B virus (Blumberg et al., 1990; Lee et al., 1996). Previous studies on this plant have led to the discovery of 21 lignans, among them niranthin and nirtetralin have been shown to possess anti-viral activity against human hepatitis B virus in vitro (Huang et al., 2003; Wei et al., 2012).

In previous studies, we have reported the discovery of a new lignan, nirtetralin B (Wei et al., 2012). In our recent work, the new

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lignan, nirtetralin B, was assayed with HepG2.2.15 cell line and DHBV-infected duckling model for antiviral activity against HBV. To our knowledge, this is the first report of the anti-HBV effects of nirtetralin B in vitro and in vivo. Results showed that nirtetralin B had significant anti-HBV activity both in vitro and in vivo. And our works provided a strong support for the development of nirtetralin B to a potential drug for the treatment of HBV infection.

2. Materials and methods

2.1. Chemicals

Nirtetralin B ($C_{24}H_{30}O_7$, 430.20, purity 98.2%) was isolated from *Phyllanthus niruri L.* according to the protocol established in our previous works (Wei et al., 2012). The chemical structure of nirtetralin B is shown in Fig. 1. Nirtetralin B was normally stored at 4 °C. This compound was dissolved in DMSO and diluted with culture medium for in vitro experiments. The final DMSO concentration did not exceed 0.1% for in vitro experiments. Nirtetralin B was dissolved in distilled water and diluted with physiologic saline for the animal tests. Lamivudine (3TC), obtained from GlaxoSmithKline (Fujian, P.R. China), served as the positive control. The molecular weight of 3TC is 229.25.

2.2. Cell culture

HepG2.2.15 (clonal cells derived fromhuman hepatoma cell line G2) cells were provided by the Chinese Academy of Medical Sciences (P.R. China) and maintained in modified eagle medium (MEM) supplemented with 10% fetal bovine serum and 380 μg/ml of G418, 50 U/ml of kanamycin, and 0.03% L-glutamine (all from Invitrogen, USA) at 37 °C in a 5% CO_2 atmosphere with 100% humidity.

2.3. Cell toxicity

The cytotoxic effect of nirtetralin B towards HepG2 2.2.15 was evaluated using the MTT assay (Ferrari et al., 1990; Korba et al., 1989; Han et al., 2008). Logarithmically growing cells were seeded in 96-well culture plates at a density of 1×10^5 cells/ml (200 μl/well). They were cultured for 24 h and then treated with various concentrations of nirtetralin B (34.9, 69.7, 139.5, 278.9, 550.9, 1115.8 μM). OD values were read at 450 nm after 144 h and the percent of cell death was calculated.

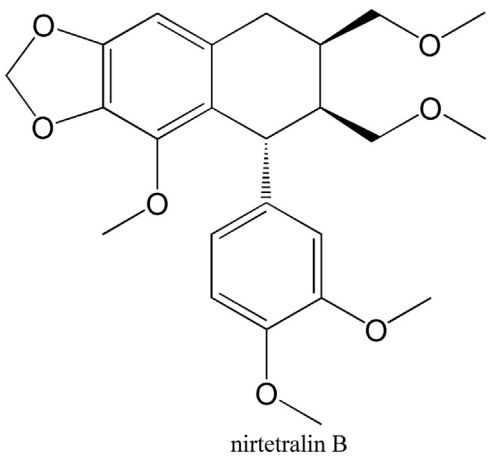

Fig. 1. Chemical structure of nirtetralin B. Molecular formula: $C_{24}H_{30}O_7$; molecular weight: 430.20.

2.4. Treatment of HepG2.2.15 cells with nirtetralin B

HepG2.2.15 cells were seeded at a density of 1×10^5 cells/ml (200 μl/well) in 96-well plates and maintained at 37 °C for 24 h, followed by treatment with various concentrations (8.1, 16.3, 32.3, 64.6, or 129.7 μM) of nirtetralin B or 43.6 μM lamivudine (3TC). 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.

2.5. Determination of HBsAg and HBeAg

HBsAg and HBeAg were simultaneously detected using 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 450 nm. The inhibition ratio (%) was calculated as follows: $(OD_{control} - OD_{sample})/OD_{control} \times 100\%$.

2.6. Detection of HBV DNA by FQ-PCR assay

To further evaluate the inhibitory effects of nirtetralin B on HBV replication, the extracellular and intracellular HBV DNA levels were determined by fluorescence quantitative PCR (FQ-PCR). The culture supernatants were used to measure extracellular HBV DNA and the cells were harvested for the analysis of intracellular HBV DNA. The viral DNA was extracted using Viral DNA Extraction Kit (TaKaRa, Dalian, China). Briefly, HBV DNA was extracted and amplified with a Step One Plus Real-Time PCR system using FastStart Universal SYBR Green Master (ROX). The forward primer was 5′-AAC CAT TGA AGC AAT CAC TAG AC-3′, and the reverse primer was 5′-ATC TAT GGT GGC TGC TCG AAC TA-3′ (Duflot et al., 1995). The PCR was carried out in a 25 μl reaction volume containing 12.5 μl 2 × SYBR Green Master (ROX), 1.0 μl 10 μM forward primer, 1.0 μl 10 μM reverse primer 8.5 μl ddH_2O and 2.0 μl HBV DNA. The thermal program comprised of an initial denaturation at 95 °C for 10 min followed by 40 amplification cycles with each of the two following steps: 95 °C for 15 s and 60 °C for 1 min. DHBV DNA was quantified using a standard curve. In this assay, the linear range was 1×10^5–1×10^{11} copies/ml. The inhibition ratio was calculated through the following formula: (negative control − treated test sample)/negative control × 100%.

2.7. Short-term toxic reaction of nirtetralin B on ducklings

A short-term toxicity study was routinely conducted according to the method described by Li et al. (2008) with some modifications. Twenty-four ducklings were divided randomly into six groups (4/group): normal control group (normal saline), 10, 30, 90, 148, 270 and 810 mg/kg nirtetralin B-treated groups. Drugs were administered orally for 14 days. Animal vital signs including weight, gait, food-taking, health status and response to stimuli were observed during the experiment.

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

Nirtetralin B concentration (μM)	Inhibition ratio (%)
34.9	0.26
69.7	1.15
139.5	8.30
278.9	24.80
550.9	31.35
1115.8	50.78

2.8. Animals and treatments

GuangxiSmall Partridge duck ducklings were provided by the experimental animal center of Guangxi Medical University. Both male and female ducks were used. All animals were treated according to the procedures outlined in the "Guide for the Care and Use of Laboratory Animals" prepared by the National Academy of Sciences and published by the National Institutes of Health. The research was conducted according to protocols approved by the institutional ethical committee of Guangxi Medical University (Approval no.: 2013051003). All experimental procedures were conducted in conformity with institutional guidelines for the care and use of laboratory animals in Guangxi Medical University, Nanning, China, and conformed to the National Institutes of Health Guide for Care and Use of Laboratory Animals.

Each one-day-old duck was injected into its tibial vein with 0.2 ml of serum from ducks with positive DHBV DNA serology (Chen et al., 2009). The drug treatment experiment was carried out 7 days after ducks were infected with DHBV, and then the positive ducks were randomly divided into five groups. The drug groups were treated with three dosages of nirtetralin B (25, 50, 100 mg/kg/day). The positive control group received 50 mg/kg/day 3TC and the model control group received normal saline. These drugs were administered orally once daily for 14 days continuously. Blood samples were taken at initiation of treatment (T0), the seventh day of treatment (T7), the fourteenth day of treatment (T14) and the third day (P3) of post-treatment follow-up. The serum was stored at −70 °C until analysis. At the end of the experiment, the ducks were sacrificed, and the liver tissues were removed and fixed in a 10% formalin solution for histopathologic examination.

2.9. Detection of serum DHBV DNA by FQ-PCR assay

DHBV DNA was detected at 0, 7, 14 days, and 3 days after cessation of treatment by FQ-PCR as described previously. The serum DNA was extracted using Viral DNA Extraction Kit (TaKaRa,

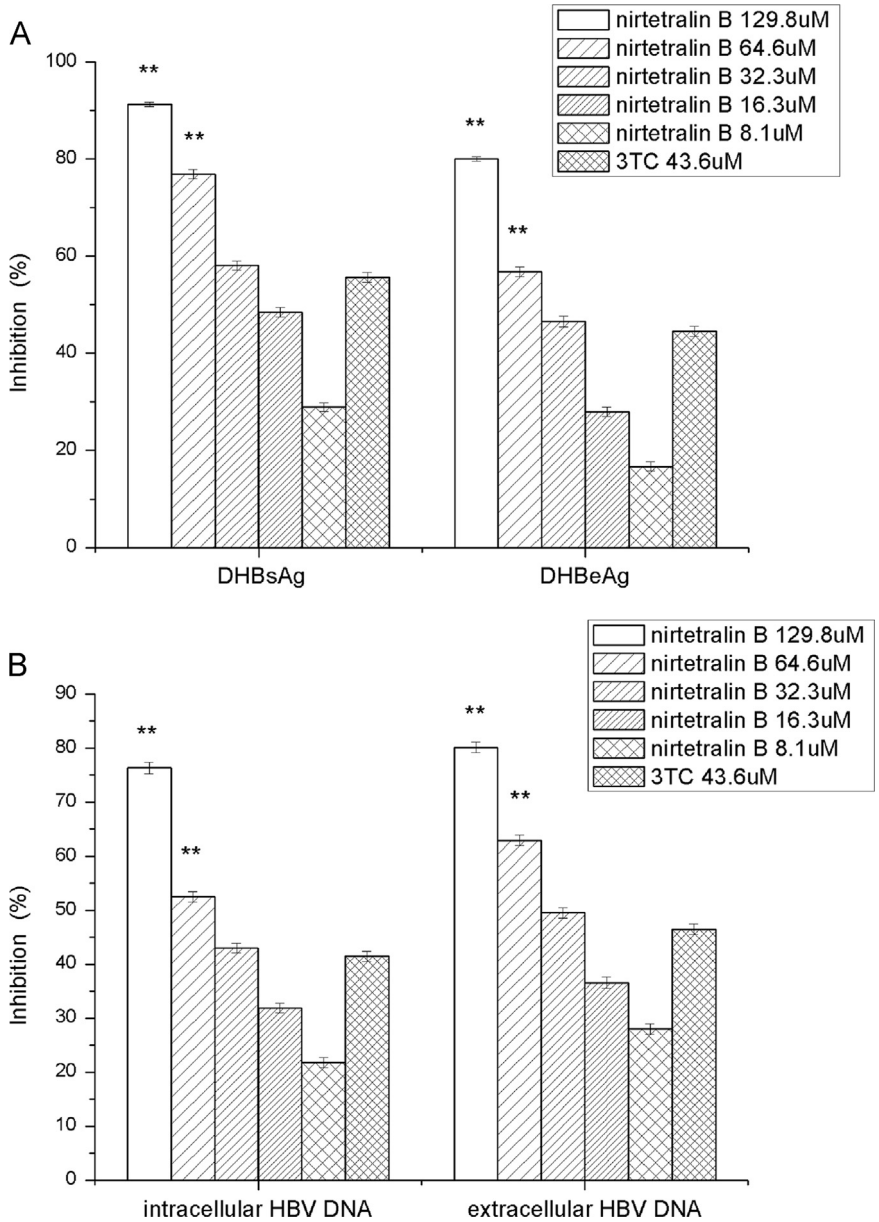

Fig. 2. Inhibitory effect of nirtetralin B on secretion of HBsAg/HBeAg (A) and HBV DNA (B) in the HepG2.2.15 cell line. HepG2.2.15 cells were cultured in the presence of nirtetralin B at various concentrations or of 3TC at 43.6 μM for 144 h, and then HBsAg and HBeAg in the supernatants were quantified using specific ELISA kits and HBV DNA was quantified by FQ-PCR. The data are presented as mean ± S.D. ($n=4$) of all experiments. $^{**}P<0.01$ compared to control.

Dalian, China). HBV DNA inhibition rate (%)=(copy number of the control − copy number of the study sample)/copy number of the control × 100%.

2.10. Determination of serum ALT and AST

Serum samples were collected from each group at days 0, 7, 14 of the treatment and day 3 post-treatment. The serum levels of alanine transaminase (ALT) and aspartate aminotransferase (AST) were measured by AST and ALT detection kits (Shanghai Zhicheng Bioengineering Institute, Shanghai, P.R. China).

2.11. Histopathological examination of duck liver

The DHBV-positive ducks were treated with nirtetralin B and 3 TC (i.g.) once daily for 14 days. On day 3 of post-treatment, the animals were sacrificed after operating by bleeding the tibial artery to die and then the liver tissues were removed. Every effort was made to minimize animal pain, suffering and distress according to the institutional ethical committee of Guangxi Medical University. A portion of each harvested liver tissue was instantly fixed in 10% phosphate-buffered formalin. Then, this portion was processed by embedding in paraffin, sectioning into 5-μm pieces. The samples were stained with hematoxylin and eosin (H & E), and examined using light microscopy for histopathological examination.

2.12. Statistical analysis

Statistical analysis was performed using SPSS 16.0 software for windows. One-way analysis of variance (ANOVA) was used to compare the means among different groups, and the Tukey test was used to conduct multiple post-hoc comparisons. Results are means ± S.D. Differences were considered statistically significant when P values were less than 0.05.

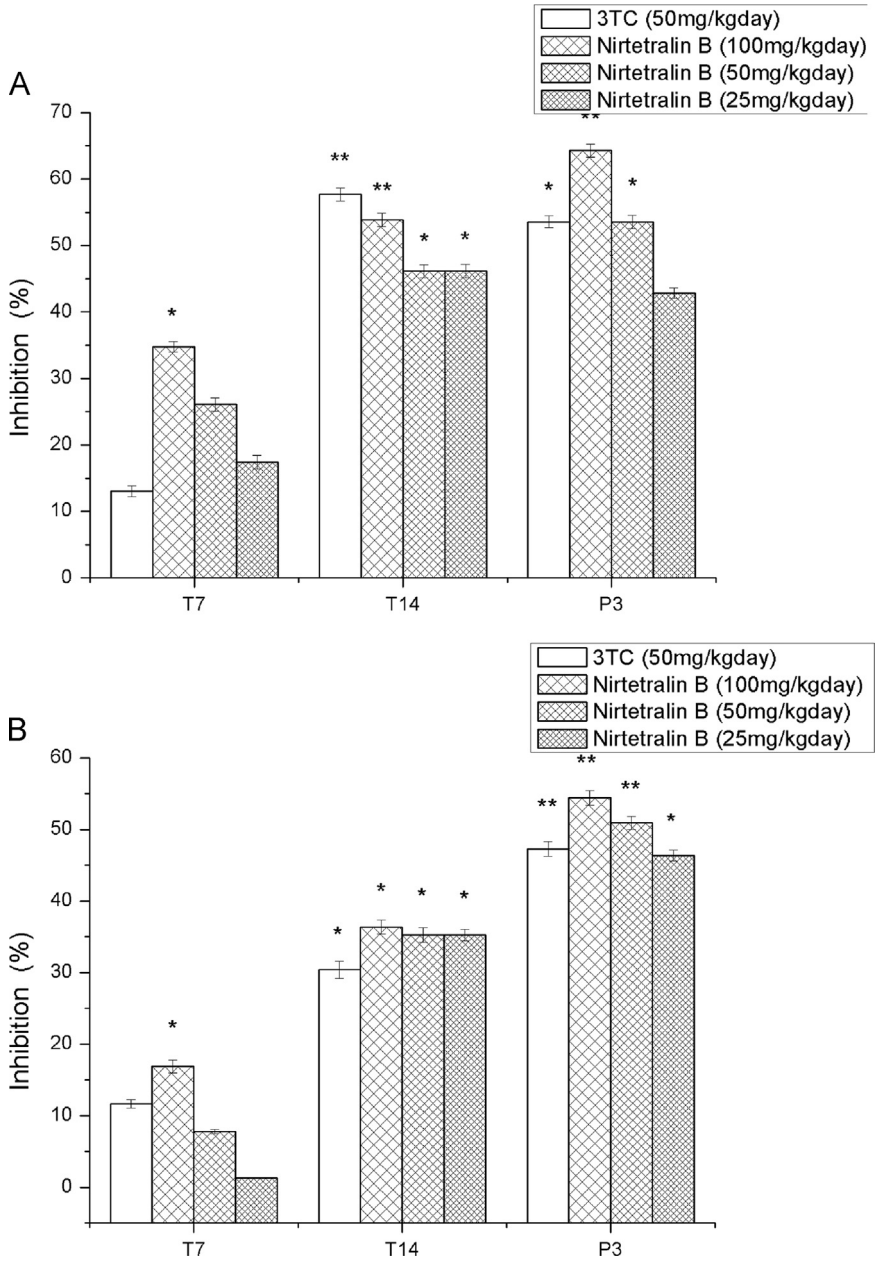

Fig. 3. Inhibitory effect of nirtetralin B on secretion of HBsAg (A) and HBeAg (B) in ducks plasma. The plasma were taken at days 0, 7, 14 of the treatment and day 3 post-treatment, and HBsAg and HBeAg were simultaneously detected by ELISA. Data were expressed as mean ± S.D. (n=6), and were statistically analyzed using the Tukey test. *P < 0.05, **P < 0.01 compared to control. *P < 0.05 and **P < 0.01 indicate significant difference between uninfected animals and HBV-infected groups.

3. Results

3.1. Cytotoxic effect of nirtetralin B on HepG2 2.2.15 cell viability

The cytotoxicity of nirtetralin B was determined using fresh culture medium containing serial 1:2 dilutions of nirtetralin B. The inhibition ratio of the HepG2.2.15 cells exposed to different concentrations of nirtetralin B is shown in Table 1. The 50% cytotoxic concentration (TC_{50}) was 1115.8 μM, and the maximum nontoxic concentration (TC_0) was 69.7 μM. These results were used to determine the dose range of nirtetralin B for the subsequent experiments.

3.2. Antiviral effects of nirtetralin B in HepG2 2.2.15 cells

Treatment of HepG2.2.15 cells with nirtetralin B at various concentrations for 144 h resulted in significant reduction of HBsAg and HBeAg secretion in a dose-dependent manner, with IC_{50} values of 17.4 μM and 63.9 μM, respectively (Fig. 2A). At the concentration of 8.1, 16.3, 32.3, 64.6, and 129.7 μM, nirtetralin B showed significant inhibitory effects on HBsAg secretion, with the highest inhibition at 93.1%. Similarly to the HBeAg secretion, the secretion of HBeAg was significantly reduced with the highest inhibition at 80.0%. In the same experiment, 3TC (43.6 μM) suppressed the secretion of HBsAg and HBeAg by 55.6% and 44.5%, respectively.

To confirm that nirtetralin B exhibits anti-HBV activity in HepG2.2.15 cells, the effect of nirtetralin B on the HBV DNA level was evaluated. Consistent with the inhibitory effects that nirtetralin B had on HBsAg and HBeAg secretion, treatment with various concentrations (8.1, 16.3, 32.3, 64.6, or 129.7 μM) of nirtetralin B or 43.6 μM 3TC led to a significant reduction in the level of HBV DNA in a dose-dependent manner (Fig. 2B). Nirtetralin B had similar activity to 3TC (43.6 μM) at the concentration of 32.3 μM and showed stronger inhibitory activity at the concentration of 64.6 or 129.7 μM.

3.3. In vivo anti-HBV activity of nirtetralin B in ducks

We fed the ducks with nirtetralin B at various concentrations once daily for 14 days, no toxicity was observed. To examine the in vivo anti-HBV activity of nirtetralin B, we first checked the HBsAg and HBeAg secretion in serum. As shown in Fig. 3, nirtetralin B significantly reduced plasma HBsAg and HBeAg secretion in a time-and dose-dependent manner with the highest inhibition at 64.29% and 54.55% respectively at the dose of 100 mg/kg/day, the effect being significant at the lowest dose tested (25 mg/kg/day) for the HBsAg. Even at day 3 of post-treatment, the plasma HBsAg and HBeAg secretion remained lower than control level. Moreover, the rebound of HBsAg and HBeAg secretion in

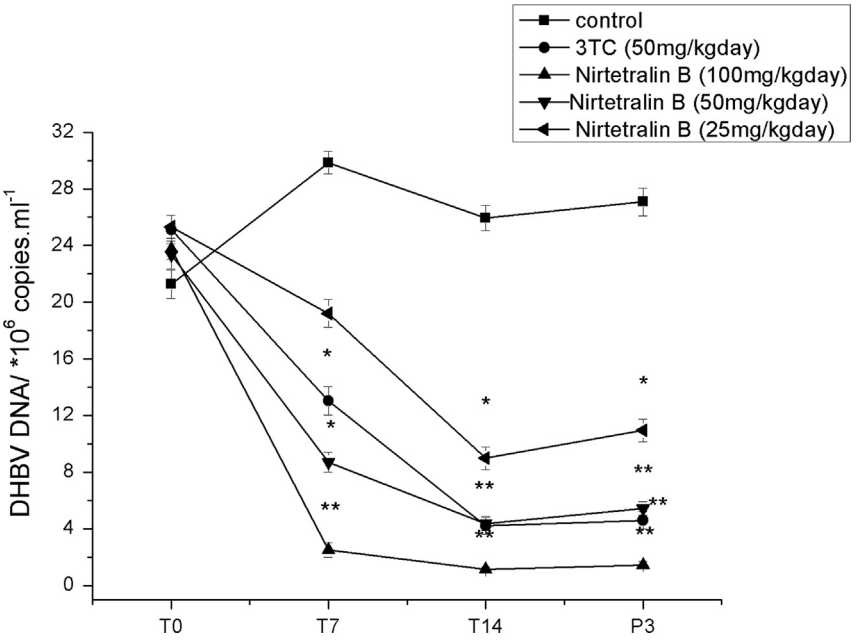

Fig. 4. Inhibition of DHBV DNA in ducks plasma in the nirtetralin B treatment study. The plasma DHBV DNA was quantified by FQ-PCR. Lamivudine was used as positive control. Data were expressed as mean ± S.D. ($n=6$), and were statistically analyzed using the Tukey test. *$P<0.05$, **$P<0.01$ compared to control.*$P<0.05$ and **$P<0.01$ indicate significant difference between uninfected animals and HBV-infected groups.

Table 2
Effect of nirtetralin B on serum ALT and AST.

Groups	ALT				AST			
	T0	T7	T14	P3	T0	T7	T14	P3
Control	40.50 ± 10.17	43.00 ± 9.96	44.83 ± 19.85	44.83 ± 19.85	42.50 ± 10.82	56.17 ± 8.80	60.50 ± 12.76	79.17 ± 18.76
3TC(50 mg/kg)	41.67 ± 8.38	40.17 ± 9.50	33.83 ± 8.08*	39.00 ± 11.83	43.17 ± 10.15	41.33 ± 10.17	39.67 ± 6.62*	48.83 ± 7.49
Nirtetralin B (100 mg/kg)	41.00 ± 10.86	36.50 ± 7.01*	31.83 ± 2.48**	35.00 ± 3.46*	43.00 ± 12.34	40.17 ± 11.20	33.83 ± 6.18**	42.83 ± 6.01*
Nirtetralin B (50 mg/kg)	41.67 ± 8.71	37.67 ± 14.62*	33.83 ± 7.68*	43.00 ± 8.92	42.16 ± 9.88	40.17 ± 7.19*	35.33 ± 8.09*	44.00 ± 5.22*
Nirtetralin B (25 mg/kg)	40.00 ± 12.18	45.33 ± 25.60	37.67 ± 8.43*	43.33 ± 12.46	41.83 ± 8.03	46.83 ± 11.77	38.17 ± 6.71*	45.50 ± 10.50

Data are expressed as mean expressed as mean ± S.E. in each group ($n=6$).

* Compared with the control group index: $P<0.05$.

** Compared with the control group index: $P<0.01$.

nirtetralin B-treated ducks was to a less degree as compared with 3TC-treated group.

To further confirm the anti-HBV activity of nirtetralin B in ducks, the plasma DHBV DNA levels of the infected ducks with and without treatment were evaluated by FQ-PCR. During this experimental period, no significant side effects were observed in animals receiving antiviral therapy, while one third of ducks in the control group died. After treatment with nirtetralin B and 3TC for 7 and 14 days, the level of DHBV DNA of each group decreased significantly ($P<0.05$ or $P<0.01$). As shown in Fig. 4, the treatment with nirtetralin B at the dose of 25, 50 and 100 mg/kg/day for 14 days exhibited a time and dose dependent inhibitory effect on DHBV DNA level. The rebound of the DHBV DNA levels in nirtetralin B treated ducks was to a less extent as compared with the 3TC treated group. No significant differences were observed in the DHBV DNA levels of any of the controls.

3.4. Analysis of ALT, AST levels in serum

DHBV infection resulted in a significant hepatic damage. The ALT, AST levels of model control group increased evidently compared to normal group. Oral administration of nirtetralin B at three different doses for 7 and 14 days resulted in lower levels of ALT and AST in serum (Table 2).

3.5. Histopathological examination of the duck livers

Typical photographs of liver sections by light microscopy are shown in Fig. 5. Hepatocyte structures of normal group were clear and regular, and single layer of cells arranged around the central vein in a radical pattern (Fig. 5A). The following typical pathological characteristics were obvious in the model group: revealing massive ballooning degeneration, hepatocyte, loosening necrosis and inflammatory cell infiltration (Fig. 5B). Samples treated with nirtetralin B, on the other hand, exhibited dose dependent improvement of the hepatocellular architecture (Fig. 5C–E). It is worth noting that nirtetralin B at 50 and 100 mg/kg/day resulted in a more significant improvement than 3TC at 50 mg/kg/day.

The results of the evaluation of all of the samples are summarized in Table 3. The liver sections were evaluated primarily on the basis of their hepatocytic degeneration, chronic inflammation and cell infiltration. Nirtetralin B treatment samples exhibited dose-dependent improvement of the hepatocellular architecture. Although treatment with 25 mg/kg/day nirtetralin B did not have a significant effect compared with 3TC treatment, nirtetralin B at 50 and 100 mg/kg/day resulted in a more significant improvement than 3TC at 50 mg/kg/day.

4. Discussion

Currently, nucleoside analogs play important roles in the antiviral therapy of hepatitis B. Although some agents (such as lamivudine and adefovir) are highly efficacious, they have been associated with serious side effects and resistant strains in a significant proportion of patients (Torresi and Locarnini, 2000;

Table 3
Summary of histopathological changes in duck livers.

Group	Severity											
	Degeneration				Chronic inflammation				Infiltration			
	−	+	2+	3+	−	+	2+	3+	−	+	2+	3+
Control	0	1	3	2	0	0	3	3	1	2	2	1
3TC	1	2	2	1	2	3	1	0	2	4	0	0
Nirtetralin B (100 mg/kg)	6	0	0	0	6	0	0	0	6	0	0	0
Nirtetralin B (50 mg/kg)	4	2	0	0	5	1	0	0	4	2	0	0
Nirtetralin B (25 mg/kg)	3	2	1	0	3	3	0	0	3	3	0	0

Hepatic degeneration, chronic inflammation, and inflammatory cell infiltration were examined in lobular and periportal tract regions. The degree of degeneration was determined as the percent of hepatocytic edema, ballooning degeneration and vacuoles degeneration, and scored as follows: (−) none; (+) <25%; (2+) <50; (3+) ≥50%. Chronic inflammation was scored as follows: (−) none; (+) mild chronic inflammation; (2+) moderate chronic inflammation; (3+) massive or widespread chronic inflammation. Cell infiltration was scored as follows: (−) none; (+) mild infiltration; (2+) moderate infiltration; (3+) massive infiltration or lymphoid nodules.

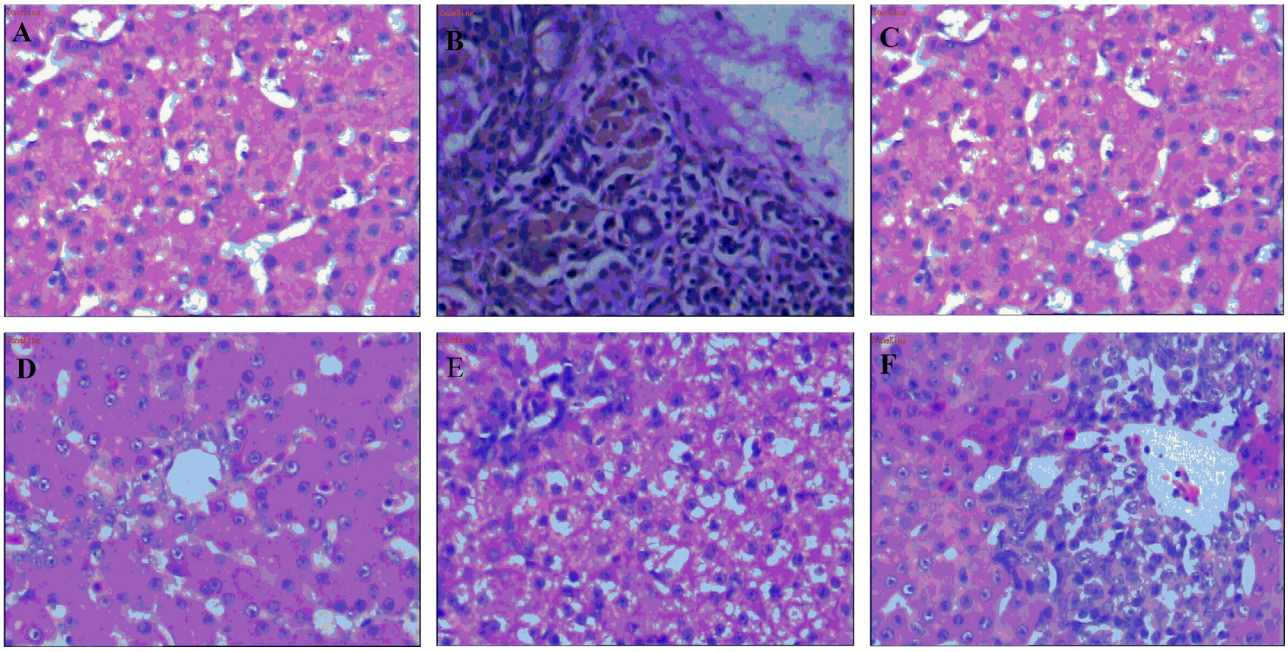

Fig. 5. Histopathological changes in duck livers. Normal control group (A) was DHBV uninfected duck liver tissue. Others were treated with nirtetralin B at 0 mg/kg/day (B), 100 mg/kg/day (C), 50 mg/kg/day (D), 25 mg/kg/day (E) or with 3TC at 50 mg/kg/day and (F) once a day for 14 days. Then, liver sections were stained with hematoxylin and eosin, and examined by light microscopy. Representative photographs were presented (magnification: 400 ×).

Sims and Woodland, 2006; Zheng et al., 2013). In addition, many patients have different extents of liver damage, and viral rebound with exacerbation of liver pathology after cessation of therapy. Compared with traditional nucleoside drugs, nirtetralin B, a new lignan first isolated by our team, has a novel chemical structure which possesses not only potent antiviral activity but also liver protective effect.

In the present study, the anti-HBV activity and hepatoprotective effect of nirtetralin B were investigated both in vitro and in vivo for the first time. For the in vitro study, we took advantage of the HepG2 2.2.15 cells, a widely used model for the evaluation of anti-HBV drugs. The results showed that cell growth retardation was not evident after the administration of low concentrations of nirtetralin B with TC_0 of 69.7 μM. And nirtetralin B inhibited the growth of HepG2.2.15 cells at high concentrations in a dose-dependent manner, with TC_{50} of 1115.8 μM. Thus, the nontoxic doses of nirtetralin B (< 69.7 μM) were used to determine the dose range of nirtetralin B for the subsequent experiments, indicating low cytotoxic effect of the compound on the cells. The treatment of HBV-transfected HepG2.2.15 cells with nirtetralin B for 144 h exhibited a dose-dependent inhibitory effect on the secretion of HBsAg and HBeAg antigens, with IC_{50} values of 17.4 μM and 63.9 μM respectively. The anti-HBV activity of nirtetralin B was also confirmed by measuring its inhibitory effects on the levels of HBV DNA in HepG2.2.15 cells. After treatment with nirtetralin B for 144 h, the levels of extracellular and intracellular HBV DNA were lower than the levels observed in the negative controls. Compared with 3TC (43.6 μM), nirtetralin B showed stronger inhibitory activity at the concentration of 64.6 or 129.7 μM.

To demonstrate the in vivo anti-HBV activity of nirtetralin B, we investigated the DHBV-infected ducks. The duck DHBV model represents a suitable and a widely used system for the study of in vivo activity of anti-HBV agents as well as their toxicity (Wang et al., 2002; Guha et al., 2004). In duck HBV infection model, nirtetralin B significantly reduced serum HBV DNA levels and the secretion of HBsAg and HBeAg in dose- and time-dependent manner. Compared with 3TC (50 mg/kg/day), nirtetralin B could inhibit the replication and rebound of DHBV DNA more effectively. Due to AST and ALT activities are associated with liver injury, they are both commonly considered as good indicators of hepatocyte integrity (Ozer et al., 2008). Oral administration of nirtetralin B (25, 50, 100 mg/kg/day) markedly reduced the elevated ALT and AST levels in serum. Furthermore, it is worth noting that the histopathological examination revealed a more significant improvement by nirtetralin B (50, 100 mg/kg/day) than 3TC (50 mg/kg/day).

In conclusion, evidences we provided firstly revealed that nirtetralin B, isolated from traditional Chinese herb *Phyllanthus niruri L.*, possessed significant activity against HBV replication and ameliorates hepatic pathology in vitro and in vivo. Results implied that further investigation would develop nirtetralin B to a promising anti-HBV drug.

Acknowledgments

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.jep.2014.09.019.

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