Antiviral Research 79 (2008) 114–120

Contents lists available at ScienceDirect

Antiviral Research

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In vitro and in vivo anti-hepatitis B virus activities of a plant extract from *Geranium carolinianum* L

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ARTICLE INFO

Article history:
Received 21 August 2007
Accepted 7 March 2008

Keywords:
Geranium carolinianum L.
Polyphenolic extract
Hepatitis B virus (HBV)
Duck hepatitis B virus (DHBV)

ABSTRACT

Natural products provide a large reservoir of potentially active agents with anti-hepatitis B virus (HBV) activity. We examined the effect of the polyphenolic extract from *Geranium carolinianum* L. (PPGC) on HBV replication both in vitro and in vivo. In the human HBV-transfected liver cell line $HepG_2$ 2.2.15, PPGC effectively suppressed the secretion of the HBV antigens in a dose-dependent manner with IC_{50} values of 46.85 μg/ml for HBsAg and 65.60 μg/ml for HBeAg at day 9. Consistent with the HBV antigen reduction, PPGC (100 μg/ml) also reduced HBV DNA level by 35.9%. In the duck hepatitis B virus (DHBV) infected ducks, after PPGC was dosed intragastricly (i.g.) once a day for 10 days, the plasma DHBV DNA level was reduced, with an ED_{50} value of 47.54 mg/kg. In addition, Southern blot analysis confirmed the in vivo anti-HBV effect of PPGC in ducks and PPGC also reduced the plasma and the liver DHBV DNA level in a dose-dependent manner. Furthermore, significant improvement of the liver was observed after PPGC treatment, as evaluated by the histopathological analysis.

1. Introduction

Hepatitis B virus (HBV), a member of the hepadnaviruses (hepatotropic DNA viruses) family, causes acute and chronic infection of the liver (Gitilin, 1997). Approximately 80% of HBV carriers have different levels of hepatocyte destruction, which could develop into liver cirrhosis and hepatocellular carcinoma (HCC) or liver cancer (Park et al., 2006). Worldwide, HBV is responsible for over 1.2 million deaths annually (Parkin et al., 1999). Several anti-viral drugs, including interferon-α and nucleoside analogues, have been approved for the treatment of Hepatitis B. However, unresolved critical issues remain with the current drugs (*e.g.* Lamivudine), such as the moderate to low efficacy, the dose-dependent side effects and the newly developed drug resistance (Perrillo, 2005). On the other hand, natural products could provide a great opportunity for screening safer and more efficacious anti-HBV agents.

Geranium carolinianum L. (Geraniaceae) is a widely used herb in China. Aqueous extract from the aerial parts of the plant is diuretic and hemostatic and it has been used for the treatment of diarrhea and rheumatic arthritis (National Committee of Pharmacopeia, 2005). Extract of *Geranium carolinianum* L. has also been shown to inhibit several kinds of virus (Wang and Lu, 1995; Zhang et al., 2001). An aqueous extract of this plant can improve the clinical symptoms of HBV infected patients (Zhu and Ren, 1995). Therefore it was supposed that *Geranium carolinianum* L. might possess the activity to inhibit the replication of HBV and the expression of viral antigens. However, its direct anti-viral activity against HBV has not been reported before.

In this study, the antiviral activity of the extract of *Geranium carolinianum* L. against HBV was investigated. To our knowledge, this is the first report of the anti-HBV effects of the polyphenolic extract from *G. carolinianum* L. Our observations suggest that PPGC have anti-HBV activity and the potential to be developed as an alternative or complementary anti-HBV agent.

2. Materials and methods

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.

2.1. Plant material

Aerial parts of *G. carolinianum* L. were collected from Xianfan District, Hubei Province, P.R. China. The collected samples were kindly identified by Prof. Jixian Guo in the Department of Pharmacognosy, School of Pharmacy, Fudan University, Shanghai, China. Voucher specimens were deposited in the School of Pharmacy, Fudan University.

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doi:10.1016/j.antiviral.2008.03.001

2.2. Extraction and isolation

The air-dried above ground parts of *G. Carolinianum* L. were soaked in 50% ethanol for PPGC extraction. After evaporation under vacuum, the residue was partitioned between $CHCl_3$ and H_2O (v/v 1:2). The aqueous phase was concentrated before loaded onto macroporous resin D101 (Tianjin Pesticides Co., Ltd., Resin Branch) and eluted with 50% ethanol. The eluent was evaporated to dryness and characterized as the polyphenolic extract through analysis by thin layer chromatography, absorption spectrometry and quantitative determination of tannins and flavonoids. Lamivudine (3TC), obtained from GlaxoSmithKline (Suzhou, China), was used as the reference compound.

2.3. Cell culture and experimental design

The $HepG_2$ 2.2.15 cell line was provided by the Chinese Academy of Medical Sciences. Cells were routinely cultured in Dulbecco's Modified Eagle Medium (DMEM, GIBCO, USA) supplemented with 10% (v/v) fetal bovine serum (GIBCO, USA) at 37 °C in a humidified incubator at 5% CO_2. Cells were seeded in 24-well tissue culture plates at approximately 5×10^5/well and maintained for 48 h prior to the PPGC treatment to allow the HBV DNA levels to stabilize. The cell medium and $HepG_2$ 2.2.15 cells were collected right before the first dose (day 0) and after 3, 6, 9, 12 days of treatment (in duplicate), and stored at −70 °C for analysis of Hepatitis B surface antigen (HBsAg), Hepatitis B e antigen (HBeAg) and HBV DNA level (Zhou et al., 2007).

2.4. Cell toxicity

The cytotoxic effect of PPGC towards $HepG_2$ 2.2.15 was evaluated using the MTT assay (Korba et al., 1989) to determine the drug concentrations that did not affect the cell viability. These drug concentrations were used in subsequent assays.

2.5. Determination of HBsAg and HBeAg

After incubation with various concentrations of PPGC at 37 °C in 5% CO_2 for 3, 6, 9, 12 days, the culture medium was collected. The concentration of HBsAg or HBeAg was detected by an enzyme-linked immunosorbent assay (ELISA) kit (KeHua Inc., China) following the manufacturer's protocol (Zhou et al., 2007; Shin et al., 2005).

$$\text{Inhibition ratio}\,\% = \left[\frac{\text{OD (control)} - \text{OD (sample)}}{\text{OD control} \times 100\%}\right].$$

2.6. Analysis of HBV DNA

For the intracellular HBV DNA analysis, cells in 24-well plates were lysed with 0.5 ml/well lysis buffer (4 M guanidine isothiocyanate, 7% 2-mercaptoethanol, 2% sarkocyl) and cells in 10 cm dishes were lysed in 6 ml lysis buffer. The cellular DNA was prepared as previously described (Korba et al., 1989). The DNAs (10 μg each) were then digested with EcoRI, electrophoresed on a 0.8% agarose gel and transferred to a nitrocellulose membrane. A purified 3.2 kb EcoRI HBV DNA fragment labeled with digoxigenin was used for the detection following the manufacturer's protocol (Roche Inc., USA) (Shin et al., 2005).

2.7. HBV DNA copy number determination and quantitative PCR

PCR reactions were performed in BIO-RAD iCycler (BIO-RAD, USA) following the manufacturer's manual. The oligonucleotide sequences of the primers were as follows: HBV upstream 5′-CCTCTTCATCCTGCTGCT-3′ downstream 5′-AACTGAAAGCCAAACA GTG-3′. The PCR was carried out in a 25 μl reaction volume containing 5.0 μl 5 × R-PCR Buffer, 0.3 μl 250 mM Mg^{2+}, 1.0 μl 10 mM dNTP, 1.0 μl 10 μM primers, 1.0 μl 25 × SYBR Green I, 1.0 μl 10^{-3} × Calibration, 0.25 μl 5 U/μl Taq-E (TaKaRa BIO, Japan), 13.7 μl ddH_2O and 2.0 μl HBV DNA. After initial incubation at 95 °C for 90 s to activate the Taq polymerase, 40 cycles of amplification were conducted using the following program: 95 °C for 5 s, 60 °C for 30 s and 72 °C for 20 s. The PCR products were finally held at 4 °C before analysis (Guo et al., 2007).

The GAPDH gene was used as the DNA standard. Primers for GAPDH were as follows: 5′-AAGGTCGGAGTCAACGGATT-3′ and 5′-CTGGAAGATGGTGATGATGGGATT-3′. The RT-PCR standard was generated using serial dilutions of a known DNA. The HBV DNA copy number was normalized by GAPDH and calculated by the iCycler iQ™ Real-Time System software (Version 3.0 for windows, BIO-RAD, USA) according to the standard curve. HBV DNA inhibition rate (%) = (copy number of the control − copy number of the study sample)/copy number of the control × 100%.

2.8. Inoculations of ducklings with DHBV virus

Ducklings at 1 day of age were intravenously infected with a 5.7×10^6 viral genome equivalent (VGE, 1 VGE = 3.3×10^6 pg) of DHBV (Jibert et al., 1992). Seven days later, PPGC, solubilized in isotonic saline solution, was administered (112, 56, 28 mg/kg) once daily orally in a liquid diet for 10 days. The isotonic saline liquid diet was also administered to the animals as negative control. Lamivudine (200 mg/kg) was used as the positive control. DHBV DNA levels were measured at 0, 5, 10 days, and 3 after cessation of treatment at 10 days (day p3) by dot blot analysis.

2.9. Measurement of duck liver DHBV DNA by Southern blot analysis

Four grams of duck liver tissues were ground in 4 ml of a buffer containing 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, followed by centrifugation at 13,000 × *g* for 10 min. The supernatant was extracted with phenol/chloroform before the DNA precipitation in two volumes of ethanol and 1/10 volume of acetic acid. The DNA was then dissolved in 800 μl of TE buffer. Finally, the purified DNA was separated on a 0.8% agarose gel and analyzed by Southern blot analysis using a DHBV DNA probe as described previously (Yao et al., 2001; Freman et al., 1988).

2.10. Histopathological examination of duck liver

The DHBV-positive ducks were treated with PPGC and 3 TC (i.g.) once daily for 10 days. The animals were sacrificed and the liver tissues were removed, fixed in formalin and embedded in paraffin. The tissue samples were then sliced at 5 μm of each section, stained with hematoxylin and eosin, and examined by light microscopy.

2.11. Statistical analysis

Results are means ± S.D. of the indicated number of independent experiments. Statistical significance was determined using Stat View Version 5.01 software (SAS Institute Inc., USA). The threshold of significance was set at $p = 0.05$.

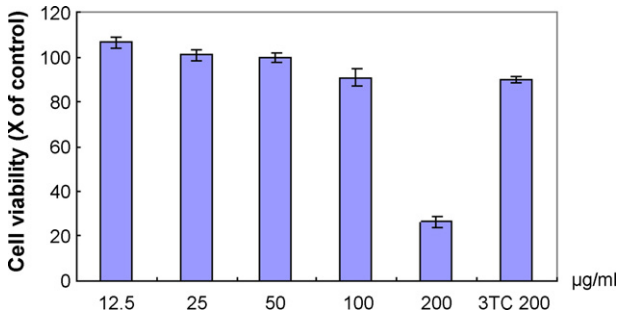

Fig. 1. The cytotoxicity of PPGC in $HepG_2$ 2.2.15 cells. The cell viability was measured by MTT method. PPGC could significantly inhibit the $HepG_2$ 2.2.15 cells above 100 μg/ml ($p < 0.05$). The data represent the mean ± S.D. ($n = 4$).

3. Results

3.1. Cytotoxic effect of PPGC on $HepG_2$ 2.2.15 cell viability

We first investigated the cytotoxic effects of the extract on the cell viability of $HepG_2$ 2.2.15 cells. The results from the MTT test showed that there was no significant difference of cell viability between PPGC-treated groups whose concentrations were below 100 μg/ml and the control group (Fig. 1). But at high concentration it had cytotoxicity. The 50% cytotoxic concentration was 160.79 μg/ml. The cytotoxicity of PA was measured to determine the treatment concentrations in the $HepG_2$ 2.2.15 cell culture.

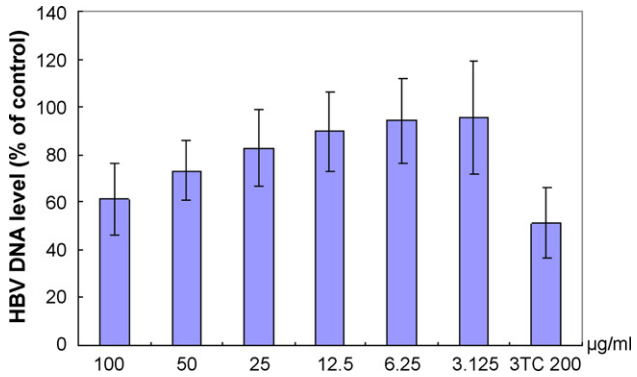

Fig. 3. Inhibitory effect of PPGC on HBV DNA level in $HepG_2$ 2.2.15 cells. $HepG_2$ 2.2.15 cells were cultured in the presence of PPGC at various concentrations (100, 50, 25, 12.5, 6.25, 3.125 μg/ml) or 3TC at 200 μg/ml for 9 days, and the HBV DNA levels were qualified by RT-PCR. The experiments were performed three times, and data are presented as mean ± S.D.

3.2. Characterization of PPGC

The results of spectrophotometric analyses of the PPGC revealed a total phenolic content of 567 ± 56 mg/g on a dry weight basis. It was positive in the $FeCl_3$ test and α-naphthol test. It was also positive in the Mg/HCl test. UV λ_{max} (MeOH) was 350 nm and 500 nm. Analysis of PPGC showed that it contained many biologically active compounds including tannins and flavonoids. In a separate study in our laboratory to identify the active ingredients,

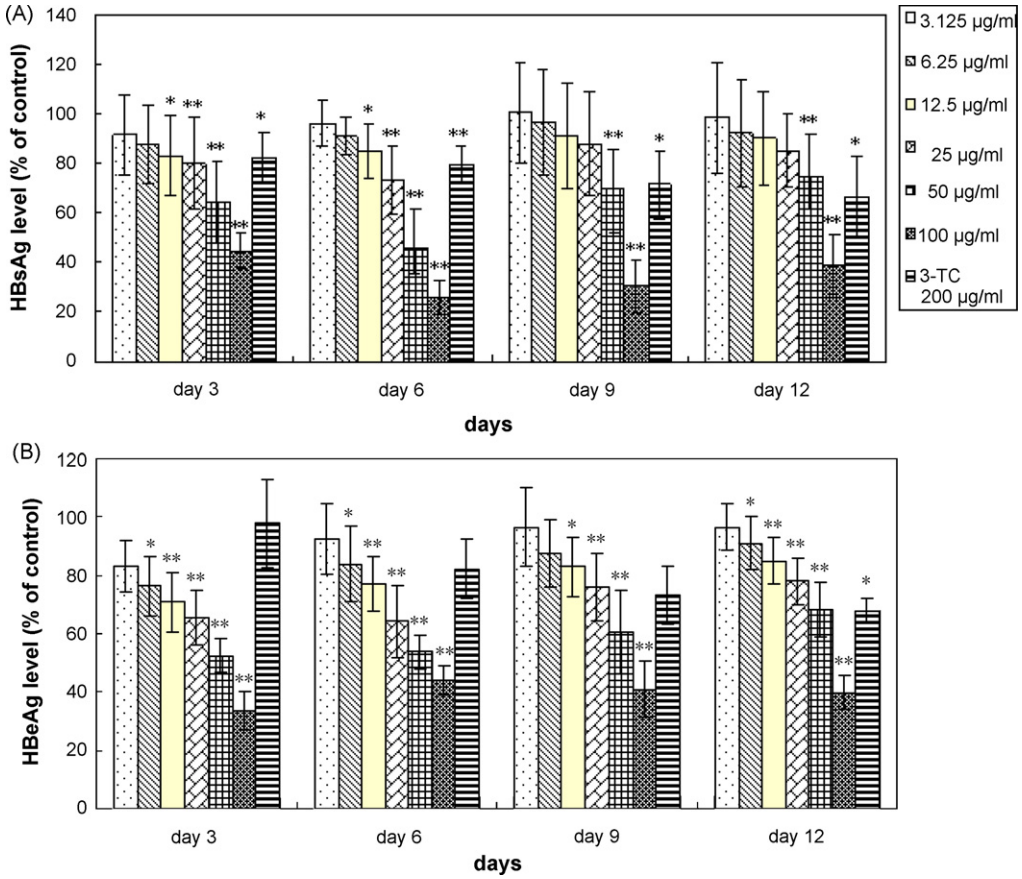

Fig. 2. PPGC reduces HBsAg and HBeAg secretion in a dose dependent manner in $HepG_2$ 2.2.15 cells. $HepG_2$ 2.2.15 cells were cultured in the presence of PPGC at various concentrations (100, 50, 25, 12.5, 6.25, 3.125 μg/ml) or 3TC at 200 μg/ml for 3, 6, 9, 12 days. The HBsAg (A) and HBeAg (B) in the supernatants were quantified using specific ELISA kits. Data are presented as mean ± S.D. of three experiments. $^{**}p < 0.01$, $^{*}p < 0.05$, as compared with the no drug control group.

PPGC were fractionated by extraction with solvents of increasing polarity and separated by chromatography. We identified gallic acid, hyperin (quercetin-3-β-D-galactopyranoside), ellagic acid, apigenin, kaempferol, quercetin, geraniin, hirsutine (quercetin-3-O-β-D- glucopyranoside), quercetin-3-O-β-D-(6′-galloyl) galactoside as the major ingredients present in PPGC. These results will be reported in another paper.

3.3. Antiviral effects of PPGC in HepG$_2$ 2.2.15 cells

Treatment of HepG$_2$ 2.2.15 cells with PPGC at various concentrations for 3 days resulted in a significant reduction of HBsAg and HBeAg secretion in a dose-dependent manner, with IC_{50} values of 83.55 μg/ml and 51.74 μg/ml respectively. After treatment for 6, 9, 12 days, PPGC still notably reduced HBsAg and HBeAg secretion for the 100 μg/ml PPGC group (Fig. 2). PPGC was more potent than 3TC for inhibiting both HBsAg and HBeAg secretion.

To further confirm the anti-HBV activity of PPGC in HepG$_2$ 2.2.15 cells, the HBV DNA levels were evaluated after PPGC treatment. Consistent with the inhibitory effects on HBsAg and HBeAg secretion, Treatment of HepG$_2$ 2.2.15 cells with PPGC at various concentrations for 9 days resulted in the reduction of the intracellular HBV DNA levels in a dose-dependent manner, as compared with the no drug control group (Fig. 3).

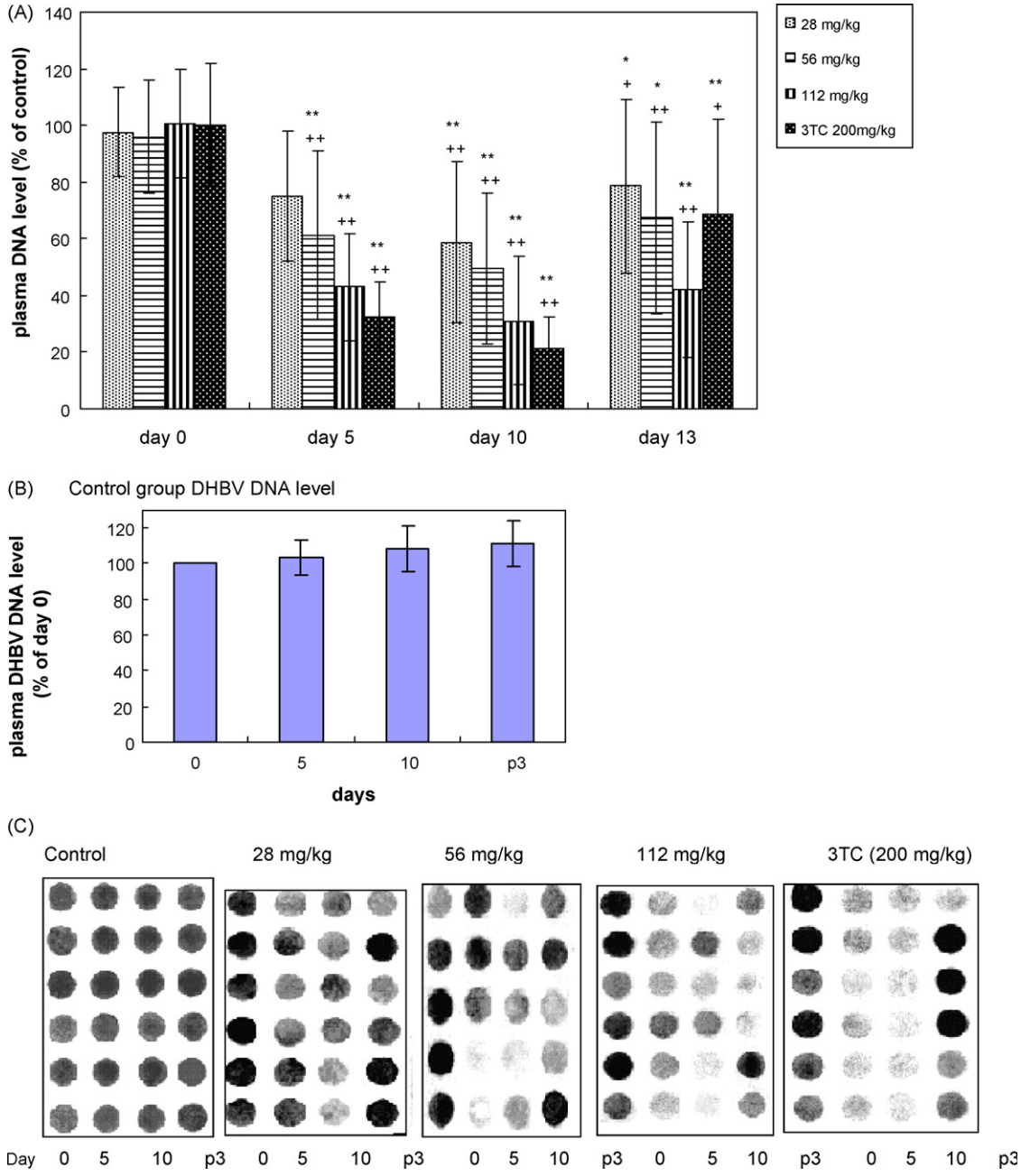

Fig. 4. Dot blots of DHBV DNA in ducks plasma in the PPGC treatment study. Plasma DHBV DNA was quantified by the dot blot hybridization method using cloned DHBV DNA as the control and analyzed with a Bio-mage analyzer. Lamivudine was used as positive control. Data were expressed as mean ± S.D. ($n = 6$), and were statistically analyzed using Dunnett's multiple comparison test. (A) PPGC treatment (50, 100 mg/kg) significantly inhibited DHBV DNA in ducks. The experiments were performed three times, and the data are presented as mean ± S.D. of all experiments ($n = 12$). $^{+}p < 0.05$; $^{++}p < 0.01$ vs. control of the same group; $^{\ddagger}p < 0.05$; $^{\ddagger\ddagger}p < 0.01$ vs. day 0. (B) There were no significant differences between the untreated controls on days 0, 5, 10, and 13 (p3).

3.4. In vivo anti-HBV activity of PPGC in ducks

We fed the ducks with PPGC at 6 g/kg (i.g.) once daily for 7 days, no toxicity was observed. To examine the in vivo anti-HBV activity of PPGC, we first checked the plasma DHBV DNA levels of the infected ducks with and without treatment. At dosage of 112 mg/kg, PPGC significantly inhibited DHBV DNA levels in the duck plasma (Fig. 4A). Densitometric quantitation of the dot blots revealed decrease in DHBV DNA levels (57.07%, 69.00% and 57.84% for days 5, 10 of the PPGC treatment and 3 days after the cessation of treatment respectively, $n = 6$) in ducks treated with 112 mg/kg PPGC, as compared with the untreated ducks. Moreover, the rebound of the DHBV DNA levels in PPGC treated ducks was to a less extent as compared with the 3TC treated group (Fig. 4C). No significant differences were observed in the DHBV DNA levels of any of the controls (Fig. 4B).

To further confirm the in vivo anti-HBV effect of PPGC in ducks, the DHBV DNA levels in the livers (obtained at days 5, 10 of PPGC treatment and 3 days after the treatment was stopped) were examined by Southern hybridization analysis. Consistent with the inhibitory effect on the plasma DHBV DNA level, PPGC treatment dose-dependently reduced the DHBV DNA levels in the liver (Fig. 5). Densitometric analysis of the autoradiographic signals indicated 26.96%, 45.37%, and 29.12% inhibition (days 5, 10 during the treatment and 3 days after the cessation of the treatment respectively) due to the PPGC treatment (112 mg/kg), whereas 3TC-treated (200 mg/kg) groups resulted in 43.98%, 59.74%, and 40.06% inhibition at these three data points.

3.5. Histopathological examination of the duck livers

To evaluate the pathological changes, liver sections from the above treatment groups were examined under the light microscope. In the control group, significant edemas could be observed in the endoplasmic reticulum, indicating the DHBV expression (Fig. 6). Samples treated with PPGC, on the other hand, exhibited dose-dependent improvement in the edema formation. It is worth noting that PPGC at 112 mg/kg resulted more significant improvement than 3TC at 200 mg/kg.

4. Discussion

In this paper, we examined the anti-HBV activities of PPGC both in vivo and in vitro. For the in vitro study, we took advantage of the $HepG_2$ 2.2.15 cells, a widely used model for the evaluation of anti-HBV drugs, and which contain multiple copies of the HBV genome that can stably integrate into the host cell genome (Sells et al., 1987). Our results indicated that PPGC inhibited both the HBV DNA level and the antigen for HBV in $HepG_2$ 2.2.15 cells. In the HBV-transfected $HepG_2$ 2.2.15 cells, PPGC exhibited a dose-dependent inhibitory effect on the secretion of HBsAg and HBeAg antigens after 6 days treatment, with an IC_{50} value of 46.85 and 65.60 μg/ml respectively. The anti-HBV activity of PPGC was further confirmed by its inhibitory effects on the levels of HBV DNA in $HepG_2$ 2.2.15 in a dose-dependent manner.

To demonstrate the in vivo anti-HBV activity of PPGC, 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; Mason et al., 1980). In DHBV-infected duck, PPGC reduced plasma (Fig. 4) and liver (Fig. 5) DHBV DNA levels in a dose-dependent manner. The in vivo anti-HBV activity was also confirmed by histopathological improvement (Fig. 6). It is worth noting that the histopathological examination revealed more significant improvement by PPGC at 112 mg/kg than 3TC at 200 mg/kg.

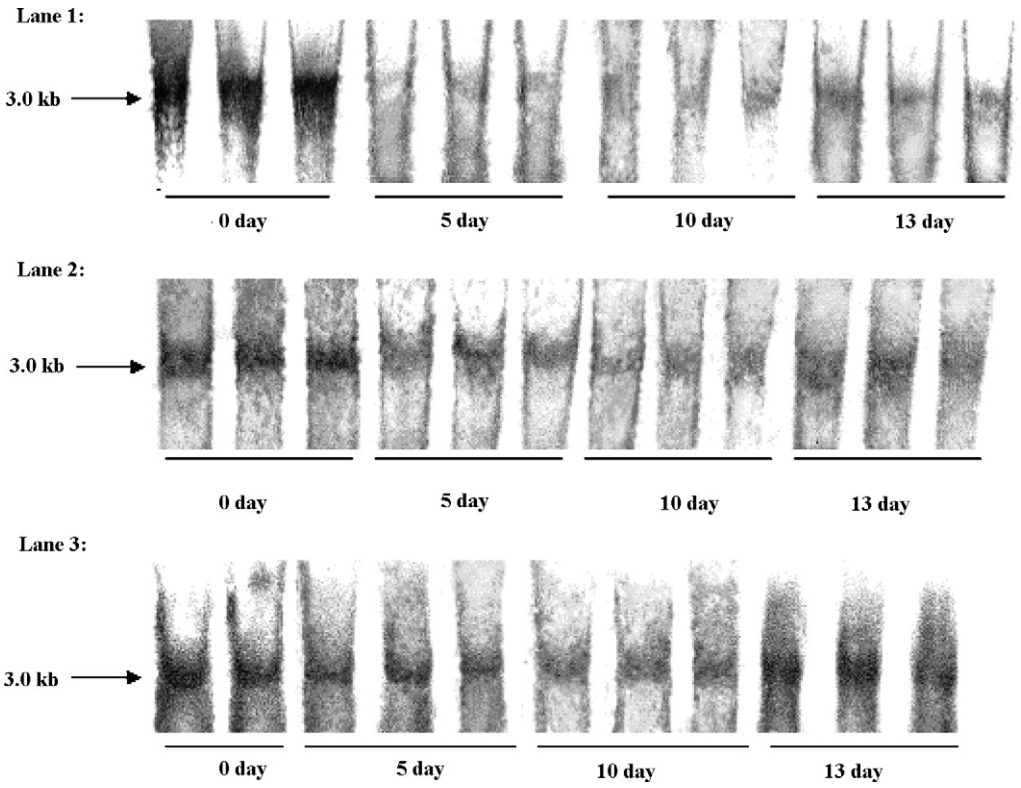

Fig. 5. PPGC inhibited the in vivo DHBV DNA levels in the infected ducks. After PPGC treatment (112 mg/kg for the second lane, and 56 mg/kg for the third lane), duck livers were removed and the DNAs were extracted at different days (days 5, 10 of the treatment, and 3 days after the cessation of the treatment or day 13). The DNA levels were then analyzed by Southern hybridization. 3TC (200 mg/kg, lane 1) was used as the control. The experiments were performed three times, and representative set of data are presented.

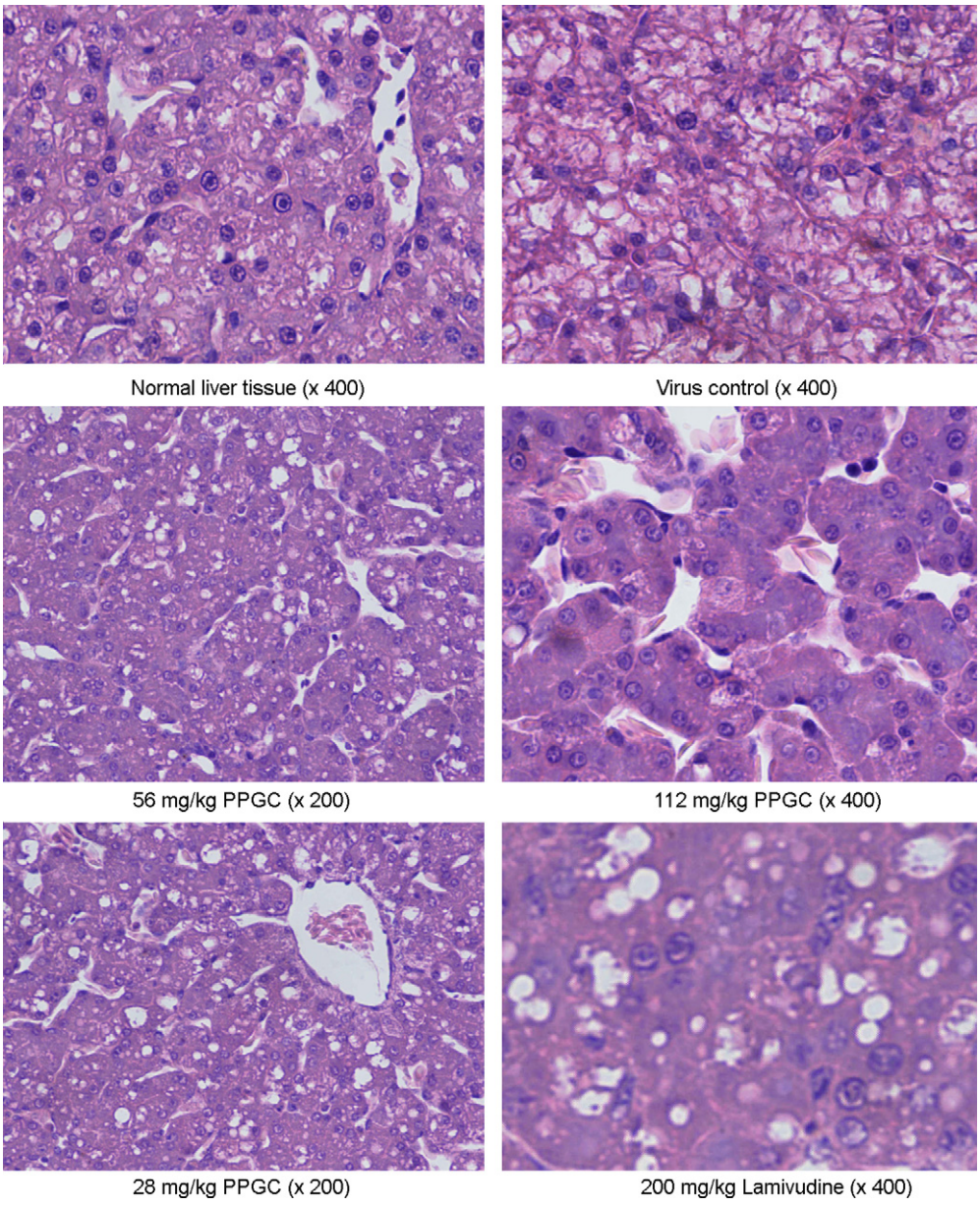

Fig. 6. Histopathological changes in duck livers. The infected ducks were treated with PPGC at 112, 56, 28 mg/kg or with 3TC at 200 mg/kg once a day for 10 days, and after the treatment, animals were maintained for three additional days. The liver sections were sainted with hematoxylin and eosin, and the histophthological changes were examined under a light microscope.

Interestingly, the in vivo anti-HBV effect of PPGC in ducks was more effective in inhibiting the rebound of plasma DHBV DNA as compared with the effect of 3TC, as indicated by the experimental groups 3 days after the termination of PPGC treatment. The relatively rapid rebound of plasma HBV DNA level in 3TC-treated ducks has been reported previously (Marion et al., 2002). This long duration of PPGC's activity may have significant clinical implications and supports the significance of developing PPGC as an anti-HBV drug.

Since the regulation of viral replication is quite different from that of the expression of HBV antigen, the fact that PPGC inhibits not only the HBV DNA replication but also the HBV antigen secretion indicates that there might be at least two targets of PPGC. The mechanism of the anti-HBV activity of PPGC is yet to be determined.

In conclusion, we provided the first evidence that PPGC can efficiently inhibit the HBV replication and the expression of HBsAg and HBeAg in $HepG_2$ 2.2.15 cells and inhibit the DHBV DNA replication in ducks in vivo. Data suggested that further investigation is warranted to develop PPGC as a potential alternative or complementary anti-HBV agent.

Acknowledgements

This work was supported by Science and Technology Commission of Shanghai Municipality. We thank Dr. Daofeng Chen (Department of Pharmacognosy, School of Pharmacy, Fudan University) for critical reading of this manuscript.

References

Freman, J.S., Jilbert, A.R., Dixon, R.J., 1988. Experimental duck hepatitis B virus infection, pathology and evolution of hepatic and extrahepatic infection. Hepatology 8, 507–513.

Gitilin, N., 1997. HepatitisB: diagnosis, prevention and treatment. Clin. Chem. 43, 1500–1506.
Guo, Q.L., Zhao, L., You, Q.D., Yang, Y., Gu, H.Y., Song, G.L., Lu, N., Xin, J., 2007. Anti-hepatitis B virus activity of wogonin in vitro and in vivo. Antiviral Res. 74, 16–24.
Jibert, A.R., Wu, T.T., England, J.M., 1992. Rapid resolution of duck hepatits B virus infections occurs after massive hepatocellular. J. Virol. 66, 1377–1388.
Korba, B.E., Wells, F.V., Baldwin, B., Cote, P.J., Tennant, B.C., Popper, H., Gerlin, J.L., 1989. Hepatocellular carcinoma in woodduck hepatitis virus-infected woodchucks: presence of viral DNA in tumor tissue from chronic carriers and animals serologically recovered from acute infections. Hepatology 9, 461–470.
Marion, P.L., Salazar, F.H., Winters, M.A., Colonno, R.J., 2002. Potent efficacy of entecavir (BMS-200475) in a duck model of hepatitis B virus replication. Antimicrob. Agents Chemother. 46, 80–88.
Mason, W.S., Seal, G., Summers, J., 1980. Virus of Pekin ducks with structural and biological relatedness to human hepatitis B virus. J. Virol. 36, 829–836.
National Committee of Pharmacopeia, 2005. Chinese Pharmacopeia. Chemical Industry Press, Beijing, vol. I, p. 80.
Park, N.H., Song, I.H., Chung, Y.H., 2006. Chronic hepatitis B in hepatocarcinogenesis. Postgrad. Med. 82, 507–515.
Parkin, D.M., Pisani, P., Ferlay, J., 1999. Estimates of the worldwide incidence of 25 major cancer in 1990. Int. J. Cancer 80, 827–841.
Perrillo, R.P., 2005. Current treatment of chronic hepatitis B: benefits and limitations. Semin. Liver Dis. 25 (Suppl. 1), 20–28.
Sells, M.A., Chen, M.L., Acs, G., 1987. Production of hepatitis B virus particles in $HepG_2$ cells transfected with cloned hepatitis B virus DNA. Proc. Natl. Acad. Sci. U.S.A. 84, 1005–1009.
Shin, M.S., Kang, E.H., Lee, Y.I.K., 2005. A flavonid from medicinal plants blocks hepatitis B virus-e antigen secretion in HBV-infected hepatocytes. Antiviral Res. 67, 163–168.
Zhang, H.N., He, S.T., Li, X.Q., 2001. A study of the treatment of experimental HSV keraticis with Laoguanbcao eye drops. J. Hunan College TCM 21 (3), 27–29.
Zhou, Z., Zhang, Y., Ding, X.R., Chen, S.H., Yang, J., Wang, X.J., 2007. Prodocutechnic aldehyde inhibits hepatitis B virus replication both in vitro and in vivo. Antiviral Res. 74, 59–64.
Zhu, M., Ren, J.J., 1995. Study on the anti-hepatitis B of aqueous extract from *Geranium carolinianum* L. Acta Acad. Med. Suzou. 15 (6), 1122.
Wang, C.Y., Giambrone, J.J., Smith, B.F., 2002. Detection of duck hepatitis B virus DNA on filter paper by PCR and SYBR green dye-based quantitative PCR. J. Clin. Microbiol. 40, 2544–2549.
Wang, Y.L., Lu, J.J., 1995. Study on the inhibitory effect of Chinese herb on herpes simplex virus. J. Tradit. Chin. Ophthalmol. 5, 78–82.
Yao, Y., Tang, N., Huang, A., 2001. Study on the replication of hepatitis B virus compared with that of duck hepatitis B virus in primary duck hepatocytes. Zhonghua Yi Xue Za Zhi 81, 1157–1161.