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Ethanol extract of *Polygonum cuspidatum* inhibits hepatitis B virus in a stable HBV-producing cell line

Jung-San Chang [a], Hong-Wen Liu [a], Kuo-Chih Wang [b], Mei-Chun Chen [a], Lien-Chai Chiang [a], Yi-Cheng Hua [b], Chun-Ching Lin [b,*]

[a] *Graduate Institute of Medicine, College of Medicine, Kaohsiung Medical University, 100 Shih-Chuan First Road, Kaohsiung 807, Taiwan*
[b] *Graduate Institute of Natural Products, College of Pharmacy, Kaohsiung Medical University, 100 Shih-Chuan First Road, Kaohsiung 807, Taiwan*

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Abstract

Chronic hepatitis B virus (HBV) infection is endemic in Asia and its consequences are among the major public health problems in the world. Unfortunately, the therapeutic efficacies of present strategies are still unsatisfactory with a major concern about viral mutation. In search of effective antiviral agent, we examined the efficacy of extracts of *Polygonum cuspidatum* Sieb. et Zucc. (*P. cuspidatum*) against HBV in $HepG_2$ 2.2.15 cells by quantitative real time polymerase chain reaction. The expressions of viral antigens, HBeAg and HBsAg, were also determined by enzyme linked immunosorbent assay. The ethanol extract of *P. cuspidatum* could inhibit dose-dependently the production of HBV ($p < 0.0001$) with an effective minimal dosage of 10 μg/ml. The water extract of *P. cuspidatum* might also inhibit the production of HBV at a higher dosage. The expression of HBsAg was significantly increased by both ethanol extract and water extract of *P. cuspidatum* dose-dependently ($p < 0.0001$) and time-dependently ($p < 0.0001$). Higher dose of water extract of *P. cuspidatum* (30 μg/ml) could inhibit the expression of HBeAg ($p < 0.05$). The extract of *P. cuspidatum* might contain compounds that would contribute to the control of HBV infection in the future. However, its promoting effect on the expression of HBsAg and its cytotoxicity should be monitored. Further purification of the active compounds, identification and modification of their structures to improve the efficacy and decrease the cytotoxicity are required.

Keywords: Antiviral assay; Chronic B hepatitis; HepG2; *Polygonum cuspidatum*; Polymerase chain reaction; TaqMan

1. Introduction

Chronic hepatitis B virus (HBV) infection is endemic in Asia. It is also one of the major public health problems in the world. It is estimated that approximately 300 million persons have chronic HBV infection worldwide (Ayoola et al., 1988). Chronic HBV infection could cause chronic hepatitis, cirrhosis, and hepatocellular carcinoma (HCC) (Beasley et al., 1981; Liaw et al., 1988; McMahon et al., 1990; Dienstag and Isselbacher, 2001a). These consequences of chronic HBV infection are also among the major public health problems in the world. Unfortunately, there are limited strategies to deal with this infection. Only IFN-α and nucleotide analogues are used to treat chronic hepatitis B. Nevertheless, their efficacies are still unsatisfactory (Doong et al., 1991; Ling et al., 1996; Tipples et al., 1996; Carreno et al., 1999; Manesis and Hadziyannis, 2001). Searching for effective antiviral agent is still required.

Abbreviations: α-SMA, α-smooth muscle actin; CC_{50}, cytotoxic concentration of 50%; DMSO, dimethyl sulfoxide; ELISA, enzyme linked immunosorbent assay; HBV, hepatitis B virus; HBeAg, e antigen of HBV; HBsAg, surface antigen of HBV; HCC, hepatocellular carcinoma; IC_{50}, concentration of 50% inhibition of viral replication; IFN, interferon; NF-κB, nuclear factor-κB; OD, optical density; PCE, the ethanol extract of *P. cuspidatum*; *P. cuspidatum*, *Polygonum cuspidatum* Sieb. et Zucc; PCW, the water extract of *P. cuspidatum*; SI, selectivity index; Th, helper T cell; TNF, tumor necrosis factor

* Corresponding author. Tel.: +886 7 312 1101x2122; fax: +886 7 313 5215.
E-mail address: aalin@ms24.hinet.net (C.-C. Lin).

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Polygonum cuspidatum Sieb. et Zucc. (*P. cuspidatum*) is frequently used as laxatives and anticancer drugs in Chinese traditional medicine. *P. cuspidatum* contains several anthraquinones. Anthraquinones and anthraquinone derivatives have been reported to possess antiviral and virucidal activities against viruses of several taxonomic groups (Andersen et al., 1991; Sydiskis et al., 1991; Barnard et al., 1992; Cohen et al., 1996). Extracts of *P. cuspidatum* has also been mentioned to inhibit several kinds of virus (Zheng et al., 1998). Moreover, it could inhibit the expression of surface antigen of HBV (HBsAg) (Zheng et al., 1998). Therefore, it was supposed that *P. cuspidatum* 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 mentioned before. We tested the hypothesis that extracts of *P. cuspidatum* possessed the antiviral activity against HBV.

2. Materials and methods

2.1. Preparation of plant extracts

The water extract (PCW) and the ethanol extract (PCE) of *P. cuspidatum* were prepared as reported before (Yen et al., 1991). Briefly, 100 g of *P. cuspidatum* were shade-dried and decocted for 1 h with 1 L of boiling reverse-osmotic water or ethanol three times. The decoctions were mixed, filtered, concentrated and lyophilized. The extracts of *P. cuspidatum* were dissolved in RPMI 1640 with 0.2% dimethyl sulfoxide (DMSO, Sigma) into the final concentrations (3, 10, 30 μg/ml for bioactivity and cytotoxicity test; 100, 300 μg/ml for cytotoxicity test only).

2.2. Cells and cell culture

The $HepG_2$ 2.2.15 human hepatoblastoma cell line (Sells et al., 1987) was used as the model system. The Hep G_2 2.2.15 cells were stably transfected with HBV clone. They were plated at a density of 2×10^5 cells/ml on 24-well cell culture plates and were routinely cultured with RPMI 1640 supplemented with 10% fetal calf serum, penicillin G 100 units/ml, streptomycin 100 μg/ml, amphotericin B 0.25 μg/ml, and G418 200 μg/ml, at 37 °C under 5% CO_2 (Innaimo et al., 1997). Different concentrations of the studied extracts were supplemented to the medium in triplicate 5 days after cells were plated. Control cultures received the carrier solvent (RPMI 1640 with 0.2% DMSO). Cells were grown in the presence of the studied extracts for 9 days with changing the medium daily. After incubation with studied extracts for 3, 6, and 9 days, the media and the cells were separated, collected and clarified by pelleting the cellular debris with a 5 min microcentrifuge spin at 6000 rpm. They were stored at −70 °C or were used for DNA extraction immediately.

2.3. DNA extraction

Viral DNA in the suspension and the β-actin DNA within the cells were extracted with QIAamp DNA Mini Kits (QIAGEN GmbH, Hildcr, Germany) and was re-suspended in 200 μl of elution buffer, according to the manufacturer's instructions. Briefly, DNA sample 200 μl was supplemented with QIAGEN Protease 20 μl, RNase 4 μl, and Buffer AL 200 μl. After vortexing for 15 s, the mixture was incubated at 56 °C for 10 min. Then, it was supplemented with absolute ethanol 200 μl and was carefully applied into QIAamp Spin Column within a 2 ml collection tube. It was supplemented with 500 μl of Buffer AW1 and AW2 sequentially after each centrifugation. Then, the mixture was supplemented with Buffer AE 200 μl (elution buffer) after centrifugation at full speed (20,000 × *g*; 14,000 rpm). It was incubated at room temperature for 5 min, and then was centrifuged at 6000 × *g* (8000 rpm) for 1 min.

2.4. Real-time polymerase chain reaction (PCR)

The quantity of HBV in the suspension of the studied extracts relative to that of the solvent control was estimated by real-time PCR (ABI PRISM 7700 Sequence Detector, PE Biosystems) based on the TaqMan technology. Sequences of primers and probes were designed against a highly conserved region among 25 published HBV genome sequences in GenBank and EMBL (Chen et al., 2001). The forward primer (5′-AGTGTGGATTCGCACTCCT-3′) and the reverse primer (5′-GAGTTCTTCTTCTAGGGGACCTG-3′) amplified a 120 bp fragment in the core region. The amplification was performed in 50 μl of reaction mixture containing DNA template 5 μl, 2 × TaqMan Universal Master Mix (PE Biosystems) 25 μl, forward primers 0.4 μM, reverse primer 0.4 μM, TaqMan probe (FAM-5′-CCAAATGCCCCTATCTTATCAACACTTCC-3′-TAMRA) 0.2 μM (PE Biosystems). The reaction mixture was first digested with uracil-*N*-glycosylase at 50 °C for 2 min to destroy the potential carry-over contamination. An incubation of 10 min at 95 °C allowed the activation of the *AmpliTaq* Gold DNA polymerase and the denaturation of the nucleic acids. Forty-five cycles of denaturation at 95 °C for 15 s and annealing-extension at 58 °C for 1 min were then carried out allowing the amplification-detection of HBV genomes. A serum sample quantified by b-DNA method was used as the standard sample to estimate the copy number of virus in each well and as the quality control of the PCR of HBV quantification. The internal control was estimated by commercialized β-actin kit (PE Biosystems) with reaction condition at 50 °C for 2 min first and 10 min at 95 °C. Then, 40 cycles of denaturation at 95 °C for 30 s and annealing-extension at 65 °C for 1 min were carried out. 2×10^5 cells of Hep G_2 2.2.15 were used as the standard sample to estimate the cell number in each well and as the quality control of the PCR of β-actin estimation. The analysis was accomplished within 2 min automatically at the end of the run. The concentration of 50% inhibition

of viral replication (IC_{50}) of extract of *P. cuspidatum* against HBV and the cytotoxic concentration of 50% (CC_{50}) against HepG2 2.2.15 cells were assayed and calculated by the above method. The selectivity index (SI) was determined by the ratio of the CC_{50} to the IC_{50}.

2.5. Cell number adjusted-HBsAg assay of the suspension

The suspension was collected as described above. The concentration of HBsAg was detected by SURASE B-96 (TMB) kit (General Biologicals CORP, Taiwan) according to the manufacturer's protocol. Briefly, 50 μl of the suspension was added into each well of reaction plate, including 3 negative controls, 2 HBsAg positive controls, and 2 blank controls. They were supplemented with anti-HBsAg peroxidase solution 50 μl, except 2 blank controls, and then were incubated at 37 ± 1 °C for 80 min. After incubation, the plate was washed. Hundred microlitres of reaction mixture containing equal volume of TMB Substrate Solution A and B was supplemented to each well. The plate was incubated at room temperature for 30 min. Then, the reaction was terminated with 2N H_2SO_4, 100 μl. The optical density (OD) was determined with the enzyme linked immunosorbent assay (ELISA) reader (Multiskan EX, Dynex Technologies, Denkendorf, Germany) at a test wavelength of 450 nm and a reference wavelength of 650 nm. The cell number adjusted OD was obtained from the OD divided by the cell number in each well.

2.6. Cell number adjusted-HBeAg assay of the suspension

The concentration of HBeAg was detected by EASE BN-96 (TMB) kit (General Biologicals CORP, Taiwan) according to the manufacturer's instructions. Briefly, 100 μl of the suspension were added into each well of reaction plate, including 3 negative controls, 2 HBeAg positive controls, and 2 blank controls. They were supplemented with anti-HBeAg peroxidase solution 100 μl, except 2 blank controls, and then were incubated at 37 ± 1 °C for 1 h. After incubation, the process was the same as the detection procedure of HBsAg above.

Data was calculated as percentage of control by the formula: (% of control) = $(OD_T)/(OD_C) \times 100\%$, where OD_T and OD_C indicated the cell number adjusted OD of the test compounds and the control, respectively.

2.7. Statistical analysis

The method of relative quantification was used for analysis of the result of PCR. The antiviral effect was assessed by the percentage of HBV DNA presented in the suspension relative to that of untreated controls (% of control). Statistical analysis was performed with ANOVA using JMP 5.0 software (SAS Institute Inc., USA). The Tukey honestly significantly different (HSD) test was used for the comparison of pairs of groups in ANOVA test. $p < 0.05$ was thought as statistically significant.

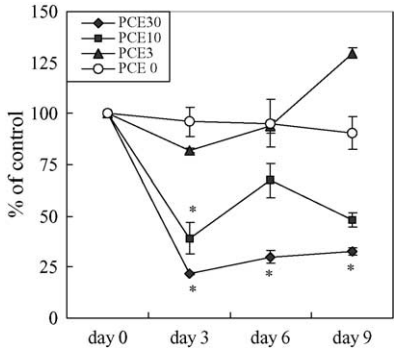

Fig. 1. Anti-HBV activity of ethanol extract of *P. cuspidatum* (PCE). The amount of HBV DNA in the suspension was quantified by real-time PCR for comparison. PCE could significantly inhibit the production of HBV dose-dependently ($p < 0.0001$). PCE 30 μg/ml was effective after 3 days of treatment, but PCE 3 μg/ml was not effective at all, ($^*p < 0.05$).

3. Results

3.1. Extract of P. cuspidatum decrease HBV DNA in the suspension

The ethanol extract of *P. cuspidatum* (PCE) could inhibit the production of HBV dose-dependently (0 μg/ml: 93.83 ± 4.84%; 3 μg/ml: 110.53 ± 4.834%; 10 μg/ml: 51.53 ± 4.84%; 30 μg/ml: 28.12 ± 4.84%; $p < 0.0001$; Fig. 1). PCE 30 μg/ml was effective after 3 days of treatment ($p < 0.05$). Concentration higher than 10 μg/ml might be required to exhibit this effect (Fig. 1). There were no significant difference of cell number by day and dose ($p = 0.3284$) between the PCE-treated group and solvent control group. However, at day 9, the group of PCE 30 μg/ml had a lower cell number than that of solvent control (Fig. 2). The

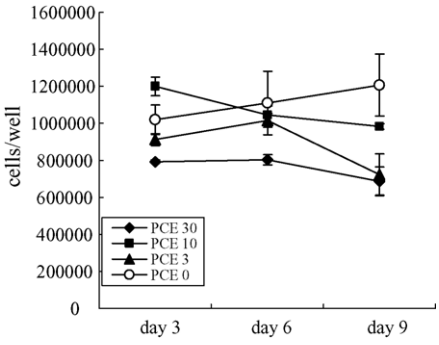

Fig. 2. Effect of ethanol extract of *P. cuspidatum* (PCE) on the cell number. The cell number per well estimated by the amount of β-actin DNA in the cell pellet was compared. There was no significant difference of cell number by day and dose ($p = 0.3284$) between the PCE-treated group and solvent control group. However, at day 9, the PCE 30 μg/ml group had a lower cell number than solvent control group.

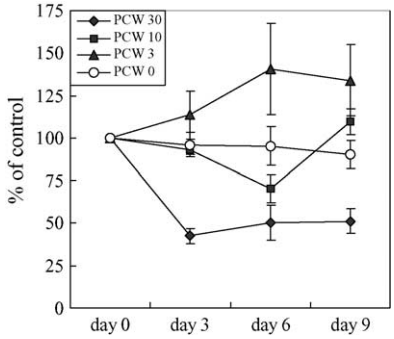

Fig. 3. Anti-HBV activity of water extract of *P. cuspidatum* (PCW). The relative HBV DNA amount of PCW to that of solvent control in the suspension was compared. PCW might inhibit the production of HBV at a dose of 30 μg/ml ($p < 0.05$).

calculated CC_{50} of PCE was 174.11 μg/ml and the IC_{50} was 20.4 μg/ml with a selectivity index of 8.53. The water extract of *P. cuspidatum* (PCW) might inhibit the production of HBV in a higher concentration (0 μg/ml: 93.83 ± 9.04%; 3 μg/ml: 129.35 ± 9.04%; 10 μg/ml: 99.30 ± 9.04%; 30 μg/ml: 48.12 ± 9.04%; Fig. 3). PCW 30 μg/ml ($p < 0.05$) might be effective against HBV. There were no significant difference of cell number between PCW-treated group and solvent control group (Fig. 4; $p = 0.6995$). The calculated CC_{50} of PCW was 132.23 μg/ml and the IC_{50} was 27.7 μg/ml with a selectivity index of 4.77.

3.2. The expression of cell number adjusted-HBV antigen in the suspension

Both ethanol (PCE) and water (PCW) extracts of *P. cuspidatum* might exhibit the anti-viral activity against HBV. Therefore, we were interested to know its effect on the expression of viral antigen. Unfortunately, the water extract, PCW, significantly promoted the expression of HBsAg dose-dependently ($p < 0.0001$) and time-dependently ($p < 0.0001$) (Fig. 5). PCW also increased the expression of HBeAg at a dose less than 10 μg/ml (Fig. 6). Nevertheless, PCW could inhibit the expression of HBeAg at a dose of 30 μg/ml (0 μg/ml: 100.92 ± 3.49%; 30 μg/ml: 61.19 ± 3.49%;

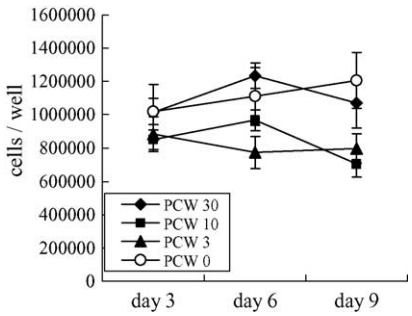

Fig. 4. Effect of water extract of *P. cuspidatum* (PCW) on the cell number. The cell number per well estimated by the amount of β-actin DNA in the cell pellet was compared. There were no significant difference of cell number between PCW treated group and solvent control group ($p = 0.6995$).

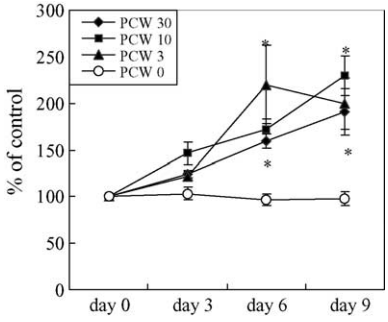

Fig. 5. Effect of water extract of *P. cuspidatum* (PCW) on the expression of cell number adjusted-HBsAg. The amount of HBsAg in the suspension was estimated by ELISA and corrected by the cell number estimated by PCR. PCW could significantly promote the expression of cell number adjusted-HBsAg dose-dependently ($p < 0.0001$) and time-dependently ($p < 0.0001$), ($^{*}p < 0.05$).

$p < 0.05$) and after day 6 of treatment (Fig. 6; $p < 0.05$). Besides, the ethanol extract, PCE, could also significantly increase the expression of HBsAg (0 μg/ml: 99.40 ± 7.72%; 3 μg/ml: 180.42 ± 7.72%; 10 μg/ml: 163.45 ± 7.72%; 30 μg/ml: 154.22 ± 7.72%; $p < 0.0001$). PCE might increase the expression of HBeAg at a dose of 3 μg/ml (0 μg/ml: 100.92 ± 4.35%; 3 μg/ml: 128.57 ± 4.35%, $p < 0.05$; 10 μg/ml: 98.32 ± 4.35%, $p > 0.05$; 30 μg/ml: 84.49 ± 4.35%, $p > 0.05$) as PCW did without the inhibition of HBeAg at higher dose.

4. Discussion

Our results clearly demonstrated the first time that the ethanol extract of *P. cuspidatum* (PCE) could inhibit the production of HBV. We also found the possible inhibitive effect of the water extract of *P. cuspidatum* (PCW) in higher dose. These findings might supplement with another therapeutic options, other than nucleotide analogues, to manage chronic

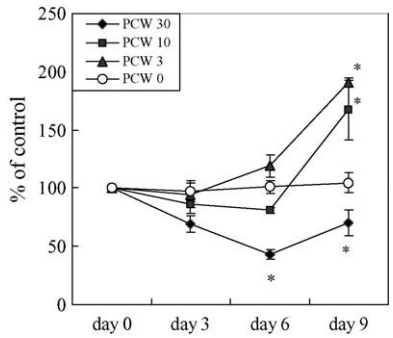

Fig. 6. Effect of water extract of *P. cuspidatum* (PCW) on the expression of cell number adjusted-HBeAg. The amount of HBeAg in the suspension was estimated by ELISA and corrected by the cell number estimated by PCR. PCW increased its expression at a dose of 3 μg/ml ($p < 0.05$) and at day 9 of treatment ($p < 0.05$). Nevertheless, PCW could inhibit the expression of cell number adjusted-HBeAg at a dose of 30 μg/ml ($p < 0.05$) and after day 6 of treatment ($p < 0.05$), ($^{*}p < 0.05$).

HBV infection in the future. HBV replicates within infected hepatocytes and expresses viral epitopes on them to induce T-cell mediated immune response to cause hepatitis (Chisari and Ferrari, 1995; Hollinger and Liang, 1996; Dienstag and Isselbacher, 2001b). Cytokines, such as IFN-α, TNF and Th1 cytokines, could directly inactivate intracellular HBV (Guidotti et al., 1999; Webster et al., 2000; Wieland et al., 2000) and/or could activate T cell to destroy the virus-infected hepatocytes (Mahoney, 1999; Rapicetta et al., 2002) to control the HBV infection. Obviously, there are two arms of therapy to manage chronic active hepatitis B, either by direct anti-viral therapy to inhibit replication of HBV or by indirect immunomodulatory therapy to enhance cellular immunity to destroy the virus-infected hepatocyte. However, immunomodulatory therapy with IFN-α had low efficiency with many limitations (Wong et al., 1993; Niederau et al., 1996; Hoofnagle and Di Bisceglie, 1997; Carreno et al., 1999; Manesis and Hadziyannis, 2001). Therefore, direct antiviral therapy might have increasing importance in managing chronic hepatitis B. Although direct antiviral therapy with lamivudine could efficiently control chronic active hepatitis B (Lai et al., 1998; Dienstag et al., 1999; Tassopoulos et al., 1999), drug resistant mutant could develop progressively after 6–9 months of initiation of therapy (Lai et al., 1998; Dienstag et al., 1999; Tassopoulos et al., 1999; Benhamou et al., 1999; Liaw et al., 2000; Leung et al., 2001). These unsatisfactory therapeutic results of IFN and nucleotide analogue strengthened the need of new anti-HBV agents. Both of the ethanol and water extracts of *P. cuspidatum* had not been evaluated before for their direct anti-viral activity against HBV. Our results implied that there might be at least two kinds of active compound of *P. cuspidatum*, water-soluble and ethanol-soluble, that possessed the anti-HBV activity. However, both of the extracts of *P. cuspidatum* showed some degree of cytotoxicity to the host cells.

Our results showed that the extracts of *P. cuspidatum* might increase the expression of viral antigens. One might supposed that significant cell lysis caused by studied extracts would release more of the viral proteins to bias the results. If this happened, one would find the cell number of experimental group to be decreased more significantly than that of control group as the viral proteins increased. However, we found that studied extracts increased HBsAg slightly at day 3 and significantly at day 6 without the significantly change of the cell number. Though PCE 30 μg/ml had a lower cell number at day 9, PCE 10 μg/ml did significantly increase the HBsAg at day 9 out of proportion to the change of cell number. Therefore, this increased expression could hardly be explained by the release of viral proteins simply due to cell lysis by the cytotoxicity of the extracts. Our findings were quite different from those of previous report (Zheng et al., 1998). It was reported that 20% of extract of *P. cuspidatum* could inhibit the expression of HBsAg without any effectiveness of the 10% extract (Zheng et al., 1998). On the contrary, in our experiment, both water and ethanol extract increased the expression of HBsAg. This conflicting data might be hard to explain. However, there was an obvious difference between the previous report and ours. We used a low concentration (<30 μg/ml; <3%) for test, while they had a high effective concentration (20%; 200 μg/ml). We found that high concentration (>100 μg/ml) of extract of *P. cuspidatum* would give too little number of viable cells at day 9 to be estimated by PCR in our preliminary experiments. Extract of *P. cuspidatum* and its active compounds were reported to have anti-neoplastic activity (Jayasuriya et al., 1992; Jayatilake et al., 1993; Zheng et al., 1998; Kimura and Okuda, 2001; Banerjee et al., 2002). We supposed that extracts of *P. cuspidatum* in high concentrations could induce apoptosis of the host cell of HBV, thus, decrease the cell number and decrease the production of the viral antigens, therefore, cause a false assumption on the reduction of the expression of HBsAg, if the results were ever not corrected by viable cell number. Further isolation and exclusion of the HBsAg promoting compounds from the extracts of *P. cuspidatum* is mandatory. Clinically, HBeAg indicates significant viral replication (Mahoney, 1999; Dienstag and Isselbacher, 2001a). The water extract of *P. cuspidatum* at higher concentration could inhibit the expression of HBeAg. This finding was compatible with the result that water extract at higher concentration could also inhibit the replication of HBV. However, the ethanol extract of *P. cuspidatum* could inhibit the expression of viral DNA, but could not inhibit the expression of HBeAg. This finding was in accordance with the concept that the regulation of viral replication is quite different from that of the expression of HBeAg. It might also imply that some compounds in the ethanol extract could inhibit the viral replication, while others promote the expression of HBeAg.

In conclusion, the ethanol extract of *P. cuspidatum* could inhibit HBV more potently than the water extract. Higher dose of water extract could inhibit the expression of HBeAg. It is worthwhile to isolate the active compounds and identify their structures to help us to find out new functional groups against HBV. Further structural modification might be needed to increase its efficacy and to decrease their cytotoxicity for further clinical application.

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