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ORIGINAL ARTICLE

Extract from *Phyllanthus urinaria* L. Inhibits Hepatitis B Virus Replication and Expression in Hepatitis B Virus Transfection Model *in Vitro**

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ABSTRACT **Objective**: To explore the effects of the extract from *Phyllanthus urinaria* L. on hepatitis B virus (HBV) replication and expression in HBV transient transfection model *in vitro*. **Methods**: The eukaryotic expression plasmid pHBV1.1, which contains 1.1-fold-overlength genome of HBV, was transfected into the human hepatoma cell line, HepG2, to establish and assess the HBV transient transfection model. The extract from *Phyllanthus urinaria* L. was prepared in different concentrations and methyl thiazolyl tetrazolium was used to detect the maximum nontoxic concentration of the drug. The extract from *Phyllanthus urinaria* L. were added into the transfected cell, at the concentrations of 0.8, 0.2 and 0.05 g/L, respectively. Four days after drug application, enzyme-linked immuno sorbent assay was used to detect the concentration of HBsAg in the supernatants, Southern blot was applied to analyze HBV DNA level, and Western blot was used to detect the expression of HBcAg in cells. **Results**: After the transfection of plasmid pHBV1.1 into HepG2 cells, the concentration of HBsAg in supernatants was increased obviously as compared with that of the normal cells ($P<0.05$), and all expected HBV replicative intermediates were confirmed by Southern blot analysis, which ensured the successful establishment of the HBV transient transfection model. After the application of drugs at the concentrations of 0.8 and 0.2 g/L, the level of HBsAg was obviously decreased in the supernatants, as compared with that of the virus group ($P<0.05$); Southern blot showed that the level of HBV rc DNA, ds DNA, ss DNA was obviously reduced compared with that of the virus group ($P<0.01$); Western blot revealed that the expression of HBcAg in the drug group was obviously inhibited, as compared with that of the virus group ($P<0.01$). **Conclusions**: The extract from *Phyllanthus urinaria* L. obviously inhibited replication and expression of HBV in HBV transfected cell lines *in vitro*, thus exerting distinctive anti-HBV effects.

KEYWORDS *Phyllanthus urinaria* L., hepatitis B virus, replication, expression, transient transfection model, Chinese medicine

Phyllanthus urinaria L. is the herb with full roots of Phyllanthus, which belongs to Euphorbiaceae plant.[1] It was firstly reported by Thyagarajan, et al[2] that Phyllanthus amarus had antiviral effect against hepatitis B virus (HBV), which enables 59% loss of HBsAg in HBV carriers. Meanwhile, it has been demonstrated that the water or alcohol extracts of full roots of *Phyllanthus* could obviously inhibit viral proteins in a dose-dependent manner in HepG2.2.15 cells *in vitro* and the duck hepatitis B virus model *in vivo*.[1,3]

However, previous studies often evaluated the anti-HBV effects of the drug in HepG2.2.15 cells, which derived from HepG2 cells with a fixed number of HBV genome integrated into the chromosomes. The defects of HepG2.2.15 cell line as *in vitro* model were obvious: (1) the viral replicating manner was different from the natural infection and the viral DNA could not be removed from chromosomes; (2) the viral replication level could not be artificially changed; (3) the level of viral replication is low.[4] The viral proteins and intracellular viral RNA could be expressed in HepG 2.2.15 cells, but all HBV replicative intermediates under natural infection state could not be detected.[5,6]

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Due to the restriction of HepG 2.2.15 cells, more and more studies have explored the antiviral effects and mechanisms of the drug, taking advantages of HBV transient transfection model, with HBV whole or part genome transferred into eukaryotic cells.

Therefore, our study observed the effects of extract from *Phyllanthus urinaria* L. on the replication and expression of HBV in HBV transient transfection model to ascertain whether *Phyllanthus urinaria* L. has direct antiviral effects on HBV replication.

METHODS

Plasmid

The recombinant eukaryotic expression plasmid, pHBV1.1, which contains 1.1-fold-overlength genome of HBV (genotype D), was kindly provided by the Key Laboratory of Molecular Biology on Infectious Diseases, Ministry of Education, Chongqing University of Medical Sciences, China.

Drugs and Drug Cytotoxicity

The Chinese herb *Phyllanthus urinaria* L., originated from Anhui province, China, was purchased from the out-patient department of Guo-Yi-Tang, Beijing University of Chinese Medicine, China. Adefovir (ADV) and entecavir (ETV), which served as positive control drugs, were provided by Gilead Sciences Inc. API, USA, and Yingxuan Pharmaceutical Co., Ltd., China, respectively.

The herb was washed, broken, and immersed in water. Then the mixture was bathed to 60 ℃ for 2 h, stirred for 4 to 5 times, and centrifugated. The supernatants were filtered by 0.45 μm membrane filter and the concentration of *Phyllanthus urinaria* L. was adjusted to 10 g/ L.[7] ADV was dissolved in ethanol with the final concentration as 100 μmol/L; ETV was dissolved in sterile ultrapure water with the final concentration as 0.2 μmol/L. The non-cytotoxic dose (TD0) of single drug on HepG2 cells were detected by using methyl thiazolyl tetrazolium (MTT, Amresco, USA) method.

Cell Culture and Transfection

HepG2, the human hepatoma cell line, derived from American Type Culture Collection, America (ATCC), USA, were grown at 37 ℃ in 5% CO_2 in modified Eagle medium (MEM) (GIBCO, USA) supplemented with 10% fetal bovine serum (GIBCO, USA), 100 mg/L streptomycin and 0.1 U/L ampicillin. Twenty-four hours prior to transfection, 7×10^5 HepG2 cells were seeded onto 6-well plates and incubated overnight at 37 ℃, 5% CO_2 for 16 h. Then, the cells were at 60% to 70% confluency, and the medium was removed. pHBV1.1 (4 μg) and 8 μL lipofection 2000 reagent (Invitrogen, USA) were diluted in 250 μL MEM without serum, respectively, and incubated for 5 min. After the media were mixed and incubated for another 20 min, the mixture was added into the well. The next day after transfection, the medium was removed and cells were given fresh culture medium and incubated for 48 h.

Enzyme-Linked Immunosorbant Assay

Twenty-four hours after transfection, the extract from *Phyllanthus urinaria* L. was added into the medium with the final concentration at 0.8, 0.2, and 0.05 g/L, respectively; ADV, with the final concentration at 0.5 μmol/L and ETV, with the final concentration at 0.001 μmol/L, served as positive control drugs. The cells in the virus control group were transfected with pHBV1.1 and the cells in the normal control group were normal cells without transfection or drug application. Four days after drug application, the concentration of HBsAg in supernatants was measured by using ELISA kit (Shanghai Kehua Company, China) according to the manufacturer's instruction. The sample was positive when OD value ⩾2.1, otherwise negative.

Southern Blot

Total DNA was extracted from transfected cells (6-well) at 4 days post-transfection. For the Southern blotting analysis, total viral DNA were isolated by proteinase K digestion and phenol:chloroform extraction method. DNAs were separated on 1% agarose gels and transferred to a nitrocellulose membrane (Amersham, USA). The presence of HBV viral DNA was probed with a digoxigenin (Dig)-labeled HBV DNA fragment at about full-length of HBV genome (nt1798-nt1821), which was generated with a Dig high prime DNA labeling and detection starter kit Ⅱ (Roche, Swiss).

Western Blot

Total cellular protein extracts were collected 4 days after drug application, separated on a 12% sodium dedecyl sulphate-polyacrylamide gel and blotted onto a nitrocellulose membrane. Membranes were then probed with the polyclonal rabbit anti-HBc antibody at the dilution of 1:50 (Becton, Dickinson

and Company, BD, USA). As secondary antibody, anti-rabbit IgG at the dilution of 1:2000 (Zhongshan Bio-Tech Co., Ltd, China) was used. Reblotting of the same membranes with an anti-β-actin antibody, diluted at 1:2000 (Zhongshan Bio-Tech Co., Ltd, China) was employed to demonstrate equal protein loading. The antigen-antibody complexes were visualized using the SuperSignal West Pico chemiluminescent substrate kit (Pierce, USA).

Statistical Analysis

All data were shown as the mean ± standard deviation ($\bar{x} \pm s$). Statistical comparisons between two groups were analyzed by Student's *t* test, and one-way ANOVA was used among more than two groups using SPSS Version 16.0 for Windows (SPSS Inc., Chicago, IL, USA). The *P* values less than 0.05 were considered statistically significant.

RESULTS

Identification of HBV Transient Transfection Model *in Vitro*

After transfection of 1, 2, 4 μg of pHBV1.1, respectively, into the HepG2 cells, the whole HBV genomic DNAs were extracted to analyze the HBV replicative viral intermediates by Southern blotting analysis upon the 4th day after transfection. All HBV expected replicative intermediates, including HBV relaxed circular (RC) DNA, double-stranded (DS) DNA, and single-stranded (SS) DNA, were detected in HepG2 cells. As the increase in the amount of pHBV1.1, the viral replication enhanced (Figure 1).

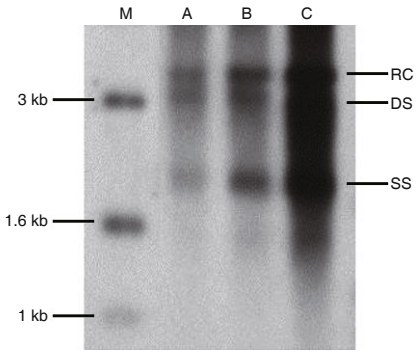

Figure 1. Expression of HBV DNA Level Detected by Southern Blotting Analysis

Notes: M: HBV DNA marker; A: transfection of 1 μg pHBV1.1; B: transfection of 2 μg pHBV1.1; C: transfection of 4 μg pHBV1.1

The level of HBsAg in medium was measured at day 2, 3, and 4 after transfection of 4 μg pHBV1.1 into HepG2 cells. The data showed that the samples were positive (the OD values ≥2.1) at day 3 and 4 after transfection, and the expression of HBsAg in medium was in a time-dependent manner The concentration of HBsAg in supernatants of pHBV1.1 group was increased obviously as compared with that of the normal cell group (*P*<0.05, Table 1). These results demonstrated that pHBV1.1 would initiate HBV viral replication and expression after transfection into HepG2 cells.

Table 1. HBsAg Level in Supernatants of HepG2 Transfected by pHBV1.1 Plasmid (OD value, $\bar{x} \pm s$)

Group	*n*	HBsAg level		
		Day 2	Day 3	Day 4
Normal cell	9	0.659 ± 0.010	0. 520 ± 0.005	0.359 ± 0.006
pHBV1.1	9	1.512 ± 0.013*	2.648 ± 0.042*	3.523 ± 0.194*
Positive control	9	3.378 ± 0.050	2.980 ± 0.050	3.725 ± 0.050
Negative control	9	0.061 ± 0.008	0.052 ± 0.005	0.066 ± 0.008

Note: *P<0.05, compared with the normal cell group

Drug Cytotoxicity of the Extract from *Phyllanthus urinaria* L. on HepG2 Cells

The drug cytotoxicity of the extract from *Phyllanthus urinaria* L. on HepG2 cells was determined by MTT. The data showed that the corresponding cell survival rate were 60.76%, 90.25%, 95.13%, 96.07%, 94.84% and 98.83%, respectively, when the drug concentration was 1.6, 0.8, 0.4, 0.2, 0.1 and 0.05 g/L. Thus, the TC0 of the Chinese medicine on HepG2 cells was 0.8 g/L.

Extract from *Phyllanthus urinaria* L. Reduced HBsAg Level

At day 4 after drug application, the extract from *Phyllanthus urinaria* L. at the concentration of 0.8 and 0.2 g/L, showed significant inhibition of HBsAg level (the OD value <2.1), compared with the virus group (*P*<0.05). While phyllanthus urinarial L. at the concentration of 0.05 g/L did not show the same inhibitory effect on HBsAg level (the OD value ≥2.1), as compared with that of the virus group (*P*>0.05). The positive control drug, ETV, at the concentration of 0.001 μmol/L, inhibited HBsAg expression significantly, compared with that of the virus group (*P*<0.05); ADV, at the concentration of 0.5 μmol/L, showed some inhibition on HBsAg level, compared with that of the virus group (*P*<0.05). However, the HBsAg expression in the ADV group showed positive (the OD value ≥2.1). The HBsAg level was higher in the ADV group than that of the ETV group (*P*<0.05, Table 2).

Extract from *Phyllanthus urinaria* L. Inhibited HBV DNA Replication

Four days after drug treatment, all viral replicative

Table 2. Effect of the Extract from *Phyllanthus urinaria* L. on HBsAg Expression Level ($\bar{x} \pm s$)

Group	*n*	Concentration	OD value
Normal cell	3		0.072 ± 0.060
Virus control	3		$3.042 \pm 0.156^{*}$
Phyllanthus urinaria L.	3	0.8 g/ L	$1.052 \pm 0.050^{\triangle\blacktriangle}$
Phyllanthus urinaria L.	3	0.2 g/ L	$1.911 \pm 0.048^{*\triangle\blacktriangle}$
Phyllanthus urinaria L.	3	0.05 g/ L	$3.139 \pm 0.131^{*\blacktriangle\circ}$
ADV	3	0.5 μmol/L	$2.521 \pm 0.067^{*\triangle}$
ETV	3	0.001 μmol/L	$1.637 \pm 0.066^{*\triangle\blacktriangle}$
Positive control	3		3.378 ± 0.050
Negative control	3		0.061 ± 0.008

Notes: $^{*}P<0.05$, compared with the normal cell group; $^{\triangle}P<0.05$, compared with the virus control group; $^{\blacktriangle}P<0.05$, compared with the ADV group; $^{\circ}P<0.05$, compared with the ETV group

intermediates were reduced significantly in *Phyllanthus urinaria* L. group at the concentration of 0.8 and 0.2 g/L, as compared with that of the virus group ($P<0.01$). And 0.05 g/L of *Phyllanthus urinaria* L. did not inhibit HBV DNA replication level, as compared with that of the virus group ($P>0.05$). Positive drugs ADV and ETV both inhibited HBV replication, as compared with that of the virus group ($P<0.01$), and there was no obvious differences between the two groups ($P>0.05$). Concentrations of 0.8 and 0.2 g/L of *Phyllanthus urinaria* L. exerted almost similar repression on HBV DNA level with ADV and ETV ($P>0.05$, Figure 2).

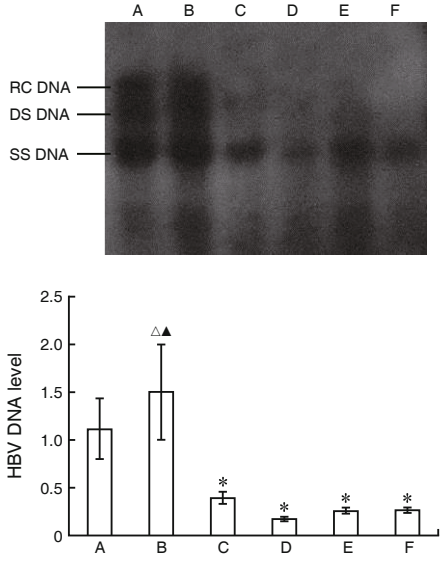

Figure 2. Inhibitory Effect of the Extract from *Phyllanthus Urinaria* L. on HBV DNA Replication

Notes: A: virus group; B: 0.05 g/L *Phyllanthus urinaria* L. group; C: 0.2 g/L *Phyllanthus urinaria* L. group; D: 0.8 g/L *Phyllanthus urinaria* L. group; E: 0.5 μmol/L ADV group; F: 0.001 μmol/L ETV group. $^{*}P<0.01$, compared with the virus group; $^{\triangle}P<0.01$, compared with the ADV group; $^{\blacktriangle}P<0.01$, compared with the ETV group

Extract from *Phyllanthus urinaria* L. Suppressed HBcAg Expression

HepG2 cells were harvested and the HBcAg level was examined 4 days after drug application by Western blotting analysis. The result showed that extracts from *Phyllanthus urinaria* L. inhibited intracellular expression of HBcAg in a dose-dependent manner. The HBcAg level in 0.8 and 0.2 g/L of *Phyllanthus urinaria* L. groups decreased obviously, compared with the virus group ($P<0.01$), and 0.05 g/L of *Phyllanthus urinaria* L. also showed inhibitory effect on HBcAg level, compared with the virus group ($P<0.05$). ETV and ADV showed obvious inhibition on HBcAg expression, compared with the virus group ($P<0.01$), and ADV exerted more robust inhibition on HBcAg expression than ETV ($P<0.01$). *Phyllanthus urinaria* L. at 0.8 g/L exerted almost similar repression on HBcAg level with ETV ($P>0.05$), while the HBcAg level in 0.2 and 0.05 g/L of *Phyllanthus urinaria* L. group were higher than that in the ETV group ($P<0.01$, Figure 3).

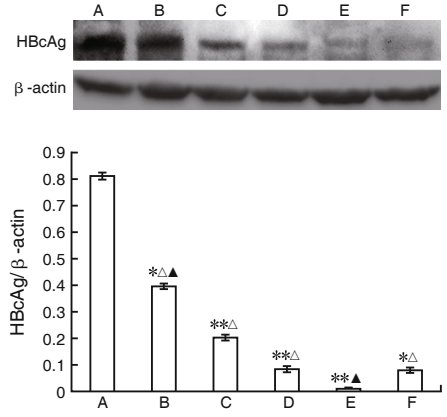

Figure 3. Extract from *Phyllanthus urinaria* L. Repressed Intracellular HBcAg Expression

Notes: A: virus group; B: 0.05 g/L *Phyllanthus urinaria* L. group; C: 0.2 g/L *Phyllanthus urinaria* L. group; D: 0.8 g/L *Phyllanthus urinaria* L. group; E: 0.5 μmol/L ADV group; F: 0.001 μmol/L ETV group. $^{*}P<0.05$, $^{**}P<0.01$, compared with the virus group; $^{\triangle}P<0.01$, compared with the ADV group; $^{\blacktriangle}P<0.01$, compared with the ETV group

DISCUSSION

HBV infection is a global health concern because infected individuals are at a high risk of developing liver cirrhosis, and eventually, hepatocellular carcinoma.[8] According to current estimates, a total of 350 million to 400 million people worldwide have chronic hepatitis B, resulting in 500,000 to 1,000,000 deaths per year. These are closely linked with the development of hepatocellular carcinoma, cirrhosis, and other complications. While an effective vaccine is available,

present regiments for hepatitis B treatments are costly and often have side effects. Only about one-third of patients treated with alpha interferon have shown a sustained response. Nucleoside analogues do not eliminate the virus completely and may cause resistant viral variants.[1-5] Thus, the development of new anti-viral treatment is urgent.

Chinese medicine has been widely used in treatment of HBV infection in China.[9,10] Most Chinese medicine used in the therapy of hepatitis B belong to "clearing heat and removing toxin" medicine, many of which exert therapeutic effects by adjusting the immune response and improving liver inflammatory injury, such as *Radix Sophorae Flavescentis*, compound *Radix et Rhizoma Rhei*, Bushen Formula (补肾方), Zhaoyang Pill (朝阳丸), etc.[11-13] It has been reported that *Phyllanthus urinaria* L. not only has anti-inflammation effects, but can also inhibit viral protein expression, and reduce virus titers.[14] Therefore, the application of *Phyllanthus urinaria* L. in the treatment of HBV infection has caught attention worldwide and has been widely studied.

However, till now, there is no definite evidence suggesting *Phyllanthus urinaria* L. could directly inhibit HBV DNA replication, for the restriction of HBV infection models. HepG2.2.15 cell line has been widely used for exploring HBV biological behavior *in vitro*. 2.2.15 cell, which constitutively released infectious HBV virions in the culture medium, was derived from HepG2 cells by stable transfection of a head to tail HBV dimer. This *in vitro* model can be used to observe the effects of drugs on viral protein expression. However, it is not suitable to study the drug effects on HBV DNA. On the other hand, although it has been reported that *Phyllanthus urinaria* L. could reduce virus titers in duck hepatitis B (DHBV) model, the direct effect of the Chinese medicine on HBV DNA replication is still doubtable because of the involvement of immune system *in vivo*. Therefore, in order to explore whether *Phyllanthus urinaria* L. has a direct inhibitory effect on HBV DNA level, we took advantage of HBV transient transfection model to evaluate the drug pression in this study.

HBV transfection model has often been used in the study of the pathogenesis of HBV and anti-viral drugs screening. 1.1-fold-overlength genome of HBV, containing 1.1 times of complete HBV genome, has been shown to initiate HBV replication efficiently in transfection cell line *in vitro* and in transgenic mice and has hepatocyte specificity.[15] In this study, the eukaryotic expression plasmid pHBV1.1, which contains 1.1-fold-overlength genome of HBV, was transfected into the human hepatoma cell line, HepG2, to establish and evaluate the HBV transient transfection model. The data suggested all viral replicative intermediates, including HBV RC DNA, DS DNA, and SS DNA, were detected in HepG2 cells after pHBV1.1 transfection. The HBV DNA level increased as the amount of pHBV1.1 increased. The HBsAg could be detected in the supernatants of HepG2 cells from day 3 after 4 μg of pHBV1.1 transfection, and the expression of the surface antigen was in a time-dependent manner. The results demonstrated that HBV could replicate and express in the pHBV1.1 transfection model and HBV transient transfection model was established successfully *in vitro*. The extract of *Phyllanthus urinaria* L. could reduce HBsAg level in supernatants, repress intracellular HBcAg expression and inhibit HBV DNA level in a dose-dependent manner.

Therefore, our results suggested that *Phyllanthus urinaria* L. could exert anti-viral effects by directly inhibitting HBV replication and expression. The positive controls, ADV and ETV, belong to the new generation of nucleoside analogues.[16] In clinical therapy, ADV and ETV have replaced lamivudine (LVD) in the treatment of chronic hepatitis B (CHB) because LVD may cause resistant viral variants.[17,18] In anti-HBV experiments, either ADV or ETV can serve as a positive control drug. However, we are not sure which one is more suitable for this experiment because neither ADV nor ETV has been used in HepG2-pHBV1.1 cell model. Thus, we aim to observe and compare the antiviral effects of the two drugs in HepG2-pHBV1.1 cell model. Therefore, the two drugs were used as positive controls in the study. We found that ADV and ETV shared almost similar repression on HBV DNA level, and there was no obvious difference between the two groups (P>0.05). ADV seemed to exert more robust inhibition on HBcAg expression than ETV (P<0.01).

The HBsAg expression showed positive in the ADV group (OD value>2.0) and negative in the ETV group (OD value<2.0), and there was obvious difference between the two groups (P<0.05). It had been reported that ETV is superior to ADV in inhibition of HBV DNA level in the treatment of chronic hepatitis B.[19] In this study, ETV had more significant

inhibitory effect on HBsAg than ADV, however, there was no obvious difference between ETV and ADV in repression of HBV DNA level. The inconsistence may be due to the different models used in the studies.

In summary, our experiments confirmed that the extract of *Phyllanthus urinaria* L. could inhibit HBV replication and expression in HBV transient transfection model *in vitro* and validated the drug's direct antiviral effects. Our study provided the experimental basis for the application of *Phyllanthus urinaria* L. in clinical treatment. Although various kinds of compounds have been isolated and identified from *Phyllanthus urinaria* L., there has been no reports about which chemical constituent exerts anti-HBV effect.[20] Our further study will focus on the molecular mechanisms of antiviral effects of *Phyllanthus urinaria* L. and the chemical basis responsible for its anti-HBV effect.

Conflicts of Interest

The authors do not have a financial conflict of interest and the corresponding author has signed permission from the other authors to act on their behalf.

Author Contribution

Wu Y contributed to study design, experimental studies, data analysis, manuscript preparation and revision. Lu Y contributed to Southern blot assay and statistical analysis. Li SY contributed to MTT, ELISA assays and statistical analysis. Song YH contributed to Western blot assay and statistical analysis. Hao Y and Wang Q contributed to manuscript revision.

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