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# Effects of 1,2,4,6-*tetra*-O-galloyl-β-D-glucose from *P. emblica* on HBsAg and HBeAg Secretion in HepG2.2.15 Cell Culture*

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**Abstract:** A polyphenolic compound, 1,2,4,6-*tetra*-O-galloyl-β-D-glucose (1246TGG), was isolated from the traditional Chinese medicine *Phyllanthus emblica* L. (Euphorbiaceae) and assayed for its potential as an anti-hepatitis B virus (HBV) agent. The cytotoxicity of 1246TGG on HepG2.2.15 as well as HepG2 cells was determined by observing cytopathic effects, and the effects of 1246TGG on secretion of HBsAg and HBeAg in HepG2.2.15 cells were assayed by enzyme immunoassay. Results indicates that treatment with 1246TGG (6.25 μg/mL, 3.13 μg/mL), reduced both HBsAg and HBeAg levels in culture supernatant, yet the inhibitory effects tend to decline with the assay time. This study provides a basis for further investigation of the anti-HBV activity and possible mechanism of action of 1246TGG.

**Key words:** 1,2,4,6-tetra-*O*-galloyl-β-D-glucose (1246TGG); *Phyllanthus emblica*; Euphorbiaceae; HBV; Antiviral agents

Hepatitis B virus (HBV), an important causative pathogen of cirrhosis-related liver failure and hepatocellular carcinoma (HCC), is a public health problem of worldwide concern, and is responsible for one million deaths each year worldwide [7]. China has the biggest HBsAg carrier population with more than one-third of the world's 350-400 million chronic HBV carriers[11]. Though prophylactic vaccines have been widely used since 1970s, eventual elimination of HBV infection remains an unfulfilled goal.

HBV belongs to the group of animal viruses known as the hepadnaviridae. Virus particles are present in large quantities in blood during HBV infection in humans, which consist of a membrane composed of envelope and nucleocapsid proteins containing circular DNA molecule. The envelope protein carries a hepatitis B surface antigen (HBsAg) while the capsid contains the hepatitis B core antigen (HBcAg) and hepatitis B e antigen (HBeAg)[12]. Currently, two therapies, conventional interferon alfa (IFNα) and

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lamivudine (LAM), are widely approved for treatment of chronic hepatitis B (CHB). Traditional Chinese medicines (TCMs) have a similar beneficial effect when compared with IFN or LAM for CHB on antiviral activity as evidenced by the loss of HBeAg and HBV DNA, which endows them with potential as alternative remedies for patients with CHB[17].

We have previously isolated a polyphonolic compound 1,2,4,6-tetra-O-galloyl-β-D-glucose (1246 TGG) from *Phyllanthus emblica* L. (Euphorbiaceae), which is a shrub or tree distributed in subtropical and tropical areas of the People's Republic of China, India, Indonesia, and the Malay Peninsula, and has been used in the Southwest of China for treating eczema, wart, diarrhea, and headache after a fever[13, 18]. Acyl glucoses have been shown to be potent antiviral agents against herpes simplex virus (HSV)[3, 9], human immunodeficiency virus (HIV)[2,10], severe acute respiratory syndrome coronavirus (SARS-CoV) [15] as well as other viruses. Here, we investigated the anti-HBV activity of 1246TGG by detecting the HBsAg and HBeAg secretion levels in HepG2.2.15 cell culture, a cell line derived by transfection of cloned HBV DNA into human hepatoblastoma cell line HepG2 and used to assay for anti-HBV agents[4].

## MATERIALS AND METHODS

### Compound

Compound 1246TGG was isolated and its structure was identified by the State Key Laboratory of Phytochemistry and Plant Resources in West China in the Kunming Institute of Botany, Chinese Academy of Sciences, using procedures as described in a previous paper [18]. Briefly, the ethanol extract of the fresh leaves and branches of *P. emblica* was suspended into water and then extracted with diethyl ether. The diethyl ether layer was partitioned between hexane and methanol, and the methanol layer was further chromatographed successively over Sephadex LH-20, silica gel, MCI-gel CHP 20P and Chromatorex ODS to obtain the desired compound (purity > 95%) in the form of a pale amorphous powder. Its structure was identified by comparison of the physical and spectral data with literature values and the 1H-1H COSY spectrum (Fig. 1). The isolated compound was then dissolved in dimethyl sulfoxide (DMSO) before use. The final concentration of DMSO was less than 0.2%.

### Cell culture

HepG2 and HepG2.2.15, both kindly supplied by Wuhan Institute of Virology, Chinese Academy of Sciences, were grown in growth media, RPMI-1640 (Gibco, USA) culture media supplemented with 10% heat-inactivated fetal bovine serum (FBS), 0.22% sodium bicarbonate (Sigma), and 50 μg/mL gentamycin (Gibco, USA). HepG2.2.15 cell culture was supplemented with an additional 380 μg/mL geneticin G418. The ingredients of the maintenance media were the same as the growth media except that

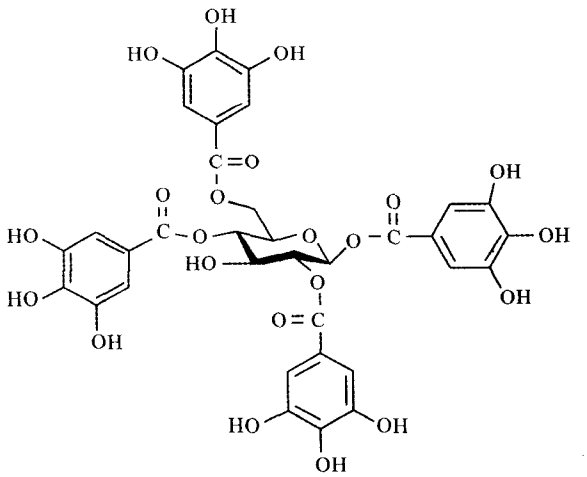

Fig.1. Structure of 1,2,4,6-tetra-*O*-galloyl-β-D-glucose (1246 TGG) from *Phyllanthus emblica* L. (Euphorbiaceae).

only 5% FBS was added. Cells were cultured at 37°C in a humid atmosphere with 5% $CO_2$.

**Cytotoxicity assay**

The cytotoxicity assay was performed by observing cytopathic effect (CPE). HepG2 or HepG2.2.15 cells were seeded onto 96-well tissue culture plates (Corning) $5\times10^3$ cells/well and incubated at 37°C in a humid atmosphere with 5% $CO_2$ for 24h before addition of 1246TGG. During the 10 d treatment period, varies concentrations of 1246TGG (ranging from 200μg/mL to 1.56μg/mL) diluted in maintenance media were added to the cultures every 3 d, namely, on the 1st, 4th and 7th day, and CPE of cell cultures under different compound concentrations were observed every 3 d, namely, on the 4th, 7th and 10th day. Cytopathic effects were classified into five levels as follows: >75%, between 75% and 50%, between 50% and 20%, <25% and no cytopathic effect. The assay was performed in four parallel wells. Concentrations without cytotoxicity were used for HBsAg and HBeAg inhibition assay.

**Determination of HBsAg and HbeAg**

For HBsAg and HBeAg secretion assay, HepG2.2.15 cells were seeded onto 24-well tissue culture plates (Corning) $3\times10^4$ cells/well and incubated at 37℃ in a humid atmosphere with 5% $CO_2$ for 24 h before the test. Similarly, 1246TGG at two concentrations (6.25 μg/mL and 3.13μg/mL) were diluted in maintenance media and added every 3 d during the 10 d treatment period, namely, on the 1st, 4th and 7th day. Before the second treatment of 1246TGG (on the 4th and 7th day) and at the end of the treatment period (on the 10th day), culture media of each compound concentration was collected and stored at -20℃. HBsAg and HBeAg levels in culture media were measured using an enzyme immunoassay kit (InTec) according to the manufacturer's instructions and absorbance at 450nm was measured using an ELISA reader (Bio-Rad). The assay was performed in four parallel wells.

**Statistical analysis**

Results were expressed as $\bar{x}$ or $\bar{x}\pm$S.D. of four parallel wells. Statistical calculations were carried out with the SPSS 13.0 for Windows software package (Statistica). One-Way ANOVA was used for statistical analyses; $P$ values $< 0.05$ were considered to be significant.

## RESULTS

**Cytotoxicity of 1246TGG**

The results of cytotoxicity assay are listed in Table 1. On the 10th day (after the third treatment), 1246 TGG at concentrations ranging from 200μg/mL to 12.5μg/mL all induced cytophathic effects to different extents. To confirm whether the cytotoxicity caused by 1246TGG was specific to HBV DNA transfected

Table.1 Cytotoxicity of 1246TGG on HepG2.2.15 cells ($\bar{x}$, n = 4)

| Time post treatment (d) | Concentration (μg/mL) | | | | | | |
|---|---|---|---|---|---|---|---|
| | 200.00 | 100.00 | 50.00 | 25.00 | 12.50 | 6.25 | 3.13 |
| 4 | +++ | +++ | ++ | + | - | - | - |
| 7 | ++++ | ++++ | ++++ | ++ | + | - | - |
| 10 | ++++ | ++++ | ++++ | +++ | ++ | - | - |

Cytotoxicity was determined by observing cytopathic effect (CPE), by classification into five levels as follows: ++++, >75%; +++, Between 75% and 50%; ++, Between 50% and 25%; +, <25%; -, No cytopathic effect.

cells, a parallel experiment was carried out on the HepG2 cell line and similar results were obtained (data not shown). These results indicate that 1246TGG possesses comparatively high cytotoxicity towards both HepG2.2.15 and HepG2 cells.

**Effects of 1246TGG on HBV antigen secretion**

To determine the inhibitory effects of 1246TGG on HBV antigen secretion, cells were treated with 1246TGG at concentrations of 6.25μg/mL and 3.13μg/mL every 3 d during the 10 d treatment period. As shown in Table 2, on the 4th day, HBsAg levels in the media supernatant was significantly reduced in the presence of 6.25μg/mL 1246TGG ($P<0.01$), while 3.13μg/mL of 1246TGG also inhibited HBsAg secretion but without a significant difference compared to the untreated group. On the 7th day, both 6.25μg/mL and 3.13μg/mL of 1246TGG led to a reduction of HBsAg level in media supernatant, however, no significant differences were found compared to the untreated group. At the end of the assay, no decrease of HBsAg levels was detected in the presence of either 6.25μg/mL or 3.13μg/mL of 1246TGG. Compared to the untreated group, the relative secretion levels of HBsAg in the presence of 6.25μg/mL 1246TGG were 62%, 84% and 100% on the 4th, 7th and 10th day respectively, and that of 3.13μg/mL 1246TGG were 84%, 87% and 94% respectively.

Similar results were obtained for HBeAg secretion assay. As shown in Table 3, treatment with 1246TGG at a concentration of 6.25μg/mL significantly inhibited HBeAg secretion both on the 4th ($P<0.05$) and 7th ($P<0.01$) day, while 3.13μg/mL of 1246TGG also reduced HBeAg levels but without significant differences compared to the untreated group. On the 10th day, no reduction of HBeAg levels in the presence of 1246TGG was found. Compared to the untreated group, the relative secretion levels of HBeAg treated with 6.25μg/mL 1246TGG were 67%, 60% and 103% on the 4th, 7th and 10th day respectively, while that of 3.13μg/mL 1246TGG were 82%, 92% and 100% respectively.

Table 2. Effects of 1246TGG on HBsAg secretion in HepG2.2.15 cell culture ($\bar{x}$±S.D, n=4)

| Group | Time post treatment (d) | | |
|---|---|---|---|
| | 4 | 7 | 10 |
| I | 0.69±0.08[a] | 1.31±0.07 | 1.47±0.13 |
| II | 0.96±0.13 | 1.37±0.18 | 1.38±0.18 |
| Untreated | 1.12±0.04 | 1.57±0.13 | 1.47±0.05 |

Group I, 6.25μg/mL; Group II, 3.13μg/mL. HBsAg levels in culture media were measured using an enzyme immunoassay kit. Data represent results of $OD_{450}$ measured by ELISA reader. [a] Untreated group $P<0.01$.

Table 3. Effects of 1246TGG on HBeAg secretion in HepG2.2.15 cell culture ($\bar{x}$±S.D, n=4)

| Group | Time post treatment (d) | | |
|---|---|---|---|
| | 4 | 7 | 10 |
| 6.25 (μg/mL) | 0.79±0.10[b] | 1.25±0.08[a] | 2.03±0.07 |
| 3.13 (μg/mL) | 0.96±0.07 | 1.91±0.06 | 1.98±0.10 |
| Untreated | 1.18±0.25 | 2.07±0.12 | 1.97±0.21 |

HBeAg levels in culture media were measured using an enzyme immunoassay kit. Data represent results of $OD_{450}$ measured by ELISA reader. [a] Untreated group $P<0.01$, [b] Untreated group $P<0.05$.

## DISCUSSION

Polyphenols, especially flavonoid, phenolic acids and other derivates might be potential antiviral agents [14]. Among these, galloyl glucoses, with various number of galloyl groups in the glucose core structure, have been reported to possess antiviral activity, mainly against HSV and HIV infection. Recently, an EtOAc extract fraction of the traditional Chinese medicine Galla Chinese, identified to be mixture of three galloyl glucoses 1,2,6-*tri*-O-galloyl-β-D-glucose, 1,2,3,6-*tetra*-O-galloyl-β-D-glucose and 1,2,3,4,6-*penta*-O-galloyl-β-D-glucose (PGG), was found to be efficient in inhibiting the NS3 protease of HCV [1]. PGG also decreased extracellular HBV in a dose-dependent manner in HepG2.2.15 cell culture [8]. In this study, 1246TGG was isolated from traditional Chinese medicine *P. emblica* and its activity in affecting HBV antigen secretion was reported for the first time.

1246TGG showed cytotoxicity towards HepG 2.2.15 cells and HepG2 cells at concentrations as low as 12.5μg/mL, which is significantly higher than what is observed with Vero cells, a normal cell line (unpublished data, YF Xiang, Y Pei, *et al*). The observed cytotoxicity differences of 1246TGG between HepG2.2.15/HepG2 cells and Vero cells suggests a possible anti-cancer activity in that PGG, a compound highly analogous to 1246TGG, has been shown to exhibit anti-cancer effects against prostate cancer, breast cancer, liver cancer, *et al* [16]. Since HepG2.2.15 and HepG2 are human hepatoblastoma cell lines, treatment with 1236TGG at high concentration might interfere with the growth or induce apoptosis of HepG2.2.15/HepG2 cells, which will subsequently lead to growth retardation and death of cells. However, such potential anti-cancer activity of 1246TGG still needs to be explored and confirmed.

Concentrations without cytotoxicity towards HepG 2.2.15/HepG2 cells were used to assay the effect of 1246TGG on HBV antigen secretion. As shown in the results, the activity of 1246TGG on suppressing antigen secretion was more obvious at early time periods (on the 4th and 7th day) during the assay. Specifically, 6.25μg/mL of 1246TGG could significantly reduce the HBeAg secretion both on the 4th and on the 7th day, and could significantly reduce the HBsAg secretion on the 4th day. Similarly, 3.13μg/mL of 1246TGG also decreased the HBeAg and HBsAg level on the 4th and 7th day but without significant differences ($P$>0.05). However, by the end of the assay, there is was almost no remaining inhibitory effect on antigen secretion. We noticed that HepG2.2.15/HepG2 cells grow rapidly, and during the whole 10 days of treatment, the number of cells in each well increased rapidly, leading to a corresponding increase of HBV DNA production and subsequently, the increase of both HBsAg and HBeAg secretion. It has already been reported that the hepatic uptake of resveratrol, another natural derived polyphenol, occurs by both passive diffusion and facilitated processes [5, 6], and similar processes probably occur in 1246TGG. Now that the quantity of cells maintained increase during the 10 day period of assay while the drug concentration remained the same, average uptake of 1246TGG into cells might decrease, resulting in the inhibition reduction of HBV antigen secretion. This might explain why the inhibitory effect of 1246TGG at the same concentration declined as time changes. However, the detailed metabolism of 1246TGG in hepatic cells remains to be explored to

determine whether the anti-HBV effect of 1246TGG might also be affected by the age of the cell culture.

To sum up, in this study, galloyl glucose 1246TGG was isolated and its potential as anti-HBV agent was investigated by measuring the HBsAg and HBeAg levels in HepG2.2.15 cell culture. Our results indicate that 1246TGG could inhibit the secretion of HBV antigen, especially during early period of treatment. However, due to the limitation of HepG2.2.15 cell line as evaluation system for anti-HBV activity of 1246TGG, activities at higher concentrations could not be determined. Besides, further studies are also needed to elucidate the detailed mechanism of anti-HBV activity of 1246TGG and to confirm its potential as a possible therapy for HBV infection in human.

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