Original Research

Anti-HBV Activities of Xanthones From *Swertia Punicea* Hemsl

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We studied the effects of two xanthones compounds isolated from *Swertia punicea* Hemsl (from Geutianaceae), swertianolin (I) and bellidifolin (II), on Hepatitis B surface antigen (HBsAg) and e antigen (HBeAg) in cultured human hepatocellular carcinoma cell line ($HepG_2$). The $HepG_2$ cells were first cultured for 24h, various concentrations of these two xanthones were then added to the culture medium. The culture medium containing the two xanthones was exchanged once every 4 days. After 8 days, the cytotoxic activities of these two xanthones were assessed by cytopathic effect. The $HepG_2$ cells were then treated with the two compounds at a concentration of swertianolin (1.6, 3.1, 6.2, 12.5, 25μg/ml) and bellidifolin (2.0, 3.9, 7.8, 25.5, 31.2μg/ml). Four or eight days later, the culture medium was collected and the expression of HBsAg and HBeAg were determined by radioimmunoassay. Our results show that swertianolin can suppress the expression of HBeAg with IC_{50} of 8.0μg/ml, while bellidifolin can inhibit the expression of HBsAg with IC_{50} of 13μg/ml at the eighth days. The Therapeutic Index for swertianolin and bellidifolin are 6.2 and 6.8, respectively. Our findings suggest that swertianolin and bellidifolin have anti-HBV activities in vitro.

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Key Words: *Swertia punicea Hemsl, swertianolin, bellidifolin, $HepG_2$, HBsAg, HBeAg*

INTRODUCTION

Hepatitis B is one of the most prevalent infectious diseases, especially in Asia. It has been reported that more than 350 million people worldwide are persistent carriers of HBsAg.[1,2] Infection with hepatitis B virus (HBV) results in severe liver diseases, including chronic hepatitis, cirrhosis and hepatocelluer carcinoma.[3] At present, interferon-α and lamivudine are the main licensed drugs for the treatment of chronic HBV infection. However, interferon-α is expensive and is associated with severe side effects. Long-term treatment with lamivudine may cause drug resistance.[4] Therefore, the development of more effective agents from crude extracts with anti-HBV activity remains of great importance. *Swertia punicea* Hemsl (from Geutianaceae) is a traditional medicinal plant mainly used for the treatment of hepatitis in some rural areas in China, and it has been approved for pharmacological and clinical trials in Hubei and Yunnan province in China. It has been reported that some of its active components, such as oleanolic acid, mangiferin, and swertiamarin, are useful for the treatment of liver diseases.[5-9] The $HepG_2$ cells has been developed as a model for screening novel agent with anti-HBV biological activities.[10,11] In this study, we reported that two active components isolated from the Chinese herb, *Swertia punicea* Hemsl, suppressed HBsAg or HBeAg the expression of the $HepG_2$ cells. The structures of these two components were identified as xanthones, namely, swertianolin (I) and bellidifolin (II).

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METHODS

Plant Collection and Identification

Swertia punicea Hemsl was collected at Hefeng county in Hubei province of China and identified by Professor Jiachun Chen, Tongji School of Pharmaceutical Sciences, Huazhong University of Science and Technology. A voucher specimen (No.040803) was stored in the herbarium of Hubei University of Chinese Medicine.

Preparation of Tested Compounds

The plant materials were air-dried and ground to a fine powder. Extraction was performed by soaking samples (500g dry weight) in 95% ethanol (5000ml) for 24h at 25℃. After filtration through filter paper, the residue was washed twice with 95% ethanol, followed by concentrating in vacuum at 40℃. The extract was further extracted with petroleum ether for 5 times to remove chlorophyll and subsequently partitioned in ether, EtOAc and water. The aqueous and EtOAc extractions were fractionated by chloroform and methanol gradient of sequential gel column chromatograph, respectively. The two compounds were obtained in chloroform and methanol (90:10 and 75:25, v:v) and further

purified by SephadexLH-20 column chromatography. The structures of the two compounds were identified respectively by comparing ^{1}H-NMR,^{13}C-NMR and MS data with literature. For bioassay, the two compounds were first dissolved in Dimethyl sulfoxide (DMSO), and then filtered through 0.45μm filter.

Reagents and Chemicals

HBsAg and HBeAg radioimmunoassay kits were purchased from the Chinese Isotope Co. (Beijing, China). Dulbecco's modified Eagle's medium (DMEM) and L-glutamine were obtained from Gibco Industries Inc. (Los Angeles, CA, USA). Fetal Bovine serum (FBS) was obtained from Hyclone (Logan, UT, USA). DMSO was obtained from Sigma (Dorset, UK). All chemical reagents for chromatography were of HPLC grade.

Cell Culture

$HepG_2$ cells were obtained from the Mount Sinai School of Medicine, USA, and were maintained in DMEM medium supplemented with 10% FBS, 50U/ml streptomycin and 3% L-glutamine. The cells were seeded into 96-well plates at a density of 2.0×10^4 /well, and incubated in 5% CO_2 at 37℃ for 24h. Various concentrations of the two xanthones were then added to the culture medium. The medium was removed every 4 days and fresh medium was added.

Cytotoxic Activity Assay

After 8 days, the viability of the cells was assesssed by cytopathic effect. The median toxic concentration (TC_{50}) values were calculated according to the method of Reed-Meuench.[12]

Determination of HBsAg and HBeAg

After the cytotoxic activity assay of these two xanthones, the $HepG_2$ cells were seeded into 24-well plates at a density of 1.0×10^5/well and allowed to attach overnight. The medium was changed to DMEM without serum, $HepG_2$ cells were treated with the two compounds at a concentration of swertianolin (1.6, 3.1, 6.2, 12.5, 25μg/ml) and bellidifolin (2.0, 3.9, 7.8, 25.5, 31.2μg/ml). The medium was removed every 4 days and fresh medium containing the two compounds was added until the eighth day. The culture medium of the fourth and eighth days was collected. The HBsAg and HBeAg in culture medium, which was secreted by $HepG_2$ cells, was measured by a radioimmunoassay kit according to the manufacture's instructions (Chinese Isotope Co.) and counted in a hemocytometer. The mean value (x) of cycles per minute (cpm) and standard deviation (s) of both experimental and control groups were calculated. The assays were performed in triplicate and the results were averaged. The antigen inhibition percentage (%) between the experimental group and the control group, the half maximal inhibitory concentration (IC_{50}), and therapeutic index (TI) were all calculated. The difference in cpm between the experimental and control groups were calculated using the Student's test.

$$antigen\ inhibition\ percentage\ (\%) = \frac{control\ group\ cpm - experimental\ group\ cpm}{experimental\ group\ cpm} \times 100\%$$

Figure 1. The equation.

RESULTS

Screening of Active Substances

In the course of our search for natural plant products as anti-HBV agents, the aqueous and EtOAc extracts of *Swertia punicea* Hemsl were found to show significant anti-HBV activity in vitro. Subsequent bioactivity fractionation resulted in the isolation of two pure compounds as the active compounds. The structures of these two active compounds were identified as swertianolin (I) and bellidifolin (II), respectively (**Figure 2**). [13-15]

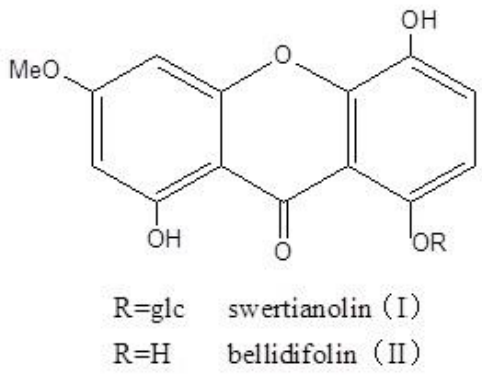

Figure 2. The structural formula of swertianolin and bellidifolin.

Cytotoxicity of the Two Compounds

We assessed the cytotoxicity of these two compounds by cytopathic effect, and found that TC_{50} of swertianolin and bellidifolin were 50μg/ml and 88μg/ml, respectively.

Suppression of HBsAg and HBeAg Production in HepG2

We assessed the effect of these two compounds on HBsAg and HBeAg production in HepG2 cells at the fourth and eighth days in culture. At the fourth day, IC_{50} of bellidifolin and swertianolin suppressing HBsAg or HBeAg expression of the $HepG_2$ cells could not calculated according to the experimental results. But the results from the eighth day showed that bellidifolin effectively suppressed HBsAg expression of the $HepG_2$ cells with IC_{50} of 13μg/ml and TI of 6.8. Swertianolin inhibited HBeAg expression with IC_{50} of 8.0μg/ml and TI of 6.2. The suppression on HBsAg and HBeAg expression of swertianolin and bellidifolin was not due to any cytotoxic activity of these two compounds, since the treated cells were still viable and continued to proliferate slowly during the incubation period of 8 days (**Table 1** and **Table 2**).

Table 1. Effect of swertianolin on HBsAg and HBeAg.

	HBsAg				HBeAg			
	4d		8 d		4 d		8 d	
concentration (μg/ml)	cpm (x ± s)	inhibition ratio (%)	cpm (x ± s)	inhibition ratio (%)	cpm (x ± s)	inhibition ratio (%)	cpm (x ± s)	inhibition ratio (%)
1.6	24723±541	-6.24	21348±2262	-7.79	6805±1835	-6.94	7061±1058	37.74
3.1	23312±2552	-0.18	22976±243	-16.01	6725±2443	-5.69	7418±1029	34.58
6.2	20820±1486	10.54	20821±2481	-5.13	6256±172	1.69	7322±867	35.43
12.5	20210±989	13.16	18895±1841	4.60	6176±800	2.95	8853±1168	21.94
25	20625±1196	11.37	18684±2380	5.66	6223±1490	2.20	9471±1748	16.48
control group	23271±1710		19805±2000		6363±1100		11340±3474	

Table 2. Effect of bellidifolin on HBsAg and HBeAg.

	HBsAg				HBeAg			
	4d		8 d		4 d		8 d	
concentration (μg/ml)	cpm (x ± s)	inhibition ratio (%)	cpm (x ± s)	inhibition ratio (%)	cpm (x ± s)	inhibition ratio (%)	cpm (x ± s)	inhibition ratio (%)
2.0	2785±317	-0.07	6928±302	27.89	3681±132	5.41	5134±431	19.70
3.9	2639±1047	5.15	5305±1008*	44.79	3647±27	6.28	5871±500	8.18
7.8	2769±279	0.48	3341±718**	65.22	3764±72	3.28	4844±229	24.23
15.6	2704±232	2.84	3548±675**	63.07	4361±139	-12.07	5253±1264	17.83
31.2	2112±374*	24.11	3272±214**	65.94	4237±413	-8.89	5983±1191	6.42
Control group	2783±102		9608±1650		3891±215		6393±1584	

**$p < 0.01$, *$p < 0.05$, compare with cell compare group,

DISCUSSION

Hepatitis B infection is a major health concern worldwide, especially in Asia. As a consequence, there is an increasing interest in the anti-HBV activities of natural products from Chinese herbs. *Swertia punicea* Hemsl is a traditional Chinese medicinal herb which has been used widely for many diseases including hepatitis for a long time. In this study, we isolated and identified two active xanthones from *Swertia punicea* Hemsl, which showed significant suppression effects on the expression of HBsAg or HBeAg in human hepatocellular carcinoma $HepG_2$ cells in culture. These two xanthones were identified as swertianolin and bellidifolin by analysis of the spectral data. Furthermore, we show for the first time that these two natural products from *Swertia punicea* Hemsl exhibit anti-HBV activities in vitro and this property may partly explain the reported effects of this medicinal plant in clinical application.[9] Therefore, our findings suggest that swertianolin and bellidifolin may possess potential in the development of effective anti-HBV drugs in the future.

CONFLICT OF INTEREST

None.

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