Study on the Chemical Constituents and anti-HBV Activity of *Hypericum japonica*

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Abstract. The most common cause of chronic viral hepatitis worldwide is from the Hepatitis B virus (HBV). Screening the safe and effective anti-HBV compounds in traditional Chinese medicine provides a large opportunity for the development of anti-HBV drugs. We found the ethanol extract of *H. japonica* had the best inhibitory effects on HBeAg secretion from 20 species of medicinal plants. In this study, silica gel Sephadex LH-20 and a thin layer of chromatography were used to screen and isolate active anti-hepatitis B virus compounds from *Hypericum japonica* that was extracted with 60% ethanol. Four compounds were separated from the ethyl acetate extract of *H. japonica* and their structures were identified by spectral chemical and structural analyses. In addition, using HepG2 2.2.15 cell line with the cytotoxicity and inhibitory effecting on the secretion of HBeAg and HBsAg of the 4 isolated compounds were examined by MTT and enzyme-linked immunosorbent assays. The four isolated compounds were determined and 1,3,6,7-tetrahydroxy-9H-xanthen-9-one (Compound 4) exhibited the highest activity in inhibiting secretion of HBeAg in HepG2 2.2.15 cells. The results indicated a 67.66% of inhibition rate, 40.25 μ g/mL of IC₅₀ value and TI > 2. We conducted a qualitatively evaluated research using duck hepatitis B virus model in vivo, the results indicated that ethanol extracts of *H. japonica* had anti-HBV effects and almost no sign of disease rebound after drug withdrawing. But the compound 4 has no direct inhibitory effect on HBV-DNA in duck experiment.

1 Introduction

The Hepatitis B virus (HBV) was discovered in 1966, but even after 50 years, it is still a major global public health problem [1], especially in Asian and African countries. Approximately 350-400 million people are affected with HBV in the world, and up to 1 million suffer from are HBV-related complications every year, including viral hepatitis and hepatocellular carcinoma (HCC) [2]. According to a national serological survey in 2006, China has one of the highest carrier rates of the Hepatitis B surface antigen (HBsAg), reaching 7.18% of the general population [2-7]. Currently, a great progress is being made in the treatment of the HBV with the development of effective and well-tolerated nucleotide analogues (NAs). However, the treatments of HBV, relying on some limited available antiviral drugs, are not successful in most of the cases. There are still some issues with the progress. First, there are still no highly effective anti-HBV drugs to remove the HBV-DNA from chronic infections completely. Second, the clinical anti-HBV drugs have some side effects.

However, anti-HBV drugs of constituents from traditional Chinese herbs have some unique advantages such as high efficiency, low toxicity, and multi-target

points. Therefore, screening safe and effective anti-HBV constituents in herbs is an important and necessary aspect within the research of anti-HBV drugs.

We screened out four herbs within this study: (*Hypericum japonica*, *Fagopyrum dibotrys* (D. Don) Hara, *Areca catechu* L and *Parmelia saxatilis* (L.) Ach) with anti-HBV activity from twenty kinds of herbs. The result indicated that *Hypericum japonica* has the potential to suppress the secretion of HBeAg effectively in a dose-dependent method. In addition, its inhibition rate was the highest out of the four herbs that were screened.

2 Materials and Methods

In this Duck-Model, Beijing ducks were infected with duck hepatitis B virus (DHBV) used to evaluate anti-HBV activity in this study. HepG2 2.2.15 cell line was used in this study to screen the ethanol extracts from these plants using in vitro assays. The cytotoxicity was examined by MTT assay, and the effects on the secretion of HBeAg and HBsAg were examined by enzyme-linked immunosorbent assay.

3 Results

3.1 Screening of the herbs with anti-HBV activity

Within this study, the “Records of Li Folk Medicine,” was used to identify 20 species of medicinal plants (table 1) and were selected to screen the herbs with anti-HBV activity in our study. HepG2 2.2.15 cell line was used to screen the ethanol extracts from these plants using in vitro assays[8-11]. The cytotoxicity was examined by MTT assay, and the effects on the secretion of HBeAg and HBsAg were examined by enzyme-linked immunosorbent assay (ELISA).

The data indicated that *H.japonica* could suppress the secretion of HBeAg effectively in the dose-dependent method and its inhibition rate was 67.66%, the IC50 value was 40.25 µg/mL.

Table 1. Inhibitory effects of 20 species of herbs on HBsAg and HBeAg secretion in highest non-toxic concentration in HepG2 2.2.15 cells ($\bar{x} \pm s$, n=3)

Ethanol extraction	Concentration n (µg/mL)	HBeAg		HBsAg	
		OD ₄₅₀	Inhibition rate (%)	OD ₄₅₀	Inhibition rate (%)
<i>Acanthus ilicifolius</i> L.	80	0.759 ±0.055***	34.90	2.746 ±0.136	NA
<i>Curcuma longa</i> L.	20	1.109 ±0.027	4.89	2.595 ±0.167	NA
<i>Zornia gibbosa</i> Spanog	40	0.784 ±0.051*	32.78	1.815 ±0.264	30.0
DMSO	0	1.166 ±0.046		2.597 ±0.153	
<i>Hypericum japonica</i> Thunb.ex Murray	40	0.538 ±0.018**	69.06	2.443 ±0.197	NA
<i>Clausena</i>	20	1.347 ±0.077**	22.55	2.461	1.0
<i>Lansium</i> (Lour.)Skeels		*		±0.096	
<i>Bidens pilosa</i> L.	160	1.438 ±0.073**	17.29	2.623 ±0.102	NA
DMSO	0	1.739 ±0.109		2.487 ±0.193	
<i>Clausena</i> <i>lansium</i> (Lour.)Skeels(see d)	ND	NT	NT	NT	NT
<i>Plumbago zeylanica</i> L.	ND	NT	NT	NT	NT
<i>Lygodium japonica</i> (Thunb.)Sw.	160	1.574 ±0.192	NA	2.009 ±0.146	22.93
DMSO	0	1.456 ±0.080		2.607 ±0.057	
<i>Fallopia multiflora</i> (Thunb.)Haral d.	160	1.234 ±0.060	17.80	2.070 ±0.101	10.5
<i>Uncaria macrophylla</i> Wall	40	0.973 ±0.106**	35.21	2.321 ±0.051	NA
<i>Geum aleppicum</i> Jacq.	10	1.574 ±0.0.192	NA	2.411 ±0.304	NA
DMSO	0	1.502 ±0.044		2.314 ±0.217	
<i>Phyllanthus urinaria</i> L.	40	0.835 ±0.053*	44.4	2.965 ±0.116	NA
<i>Mallotus apelta</i> (Lour.)Muell.-Arg.	40	0.949 ±0.076*	36.78	2.436 ±0.369	11.16
<i>Dichondra repens</i> Forst.	80	1.150 ±0.194*	23.4	2.196 ±0.041*	19.8

DMSO	0	1.502 ±0.044		2.742 ±0.170	NA
<i>Emilia sonchifolia</i> (L.)DC.	80	1.279 ±0.035*	20.96	2.769 ±0.119	NA
<i>Blumea balsamifera</i> (Linn.)DC.	20	1.198 ±0.023*	25.96	2.751 ±0.049	NA
<i>Clausena lansium</i> (Lour.)Skeels(lea ves)	10	1.368 ±0.037*	15.45	2.892 ±0.192	
DMSO	0	1.618 ±0.142		2.771 ±0.123	
<i>Atropa belladonna</i> L.	160	1.593 ±0.046	1.50	2.622 ±0.068	4.35
<i>Tylophora ovata</i> (Lindl.)Hook.ex Stend.	160	1.613 ±0.156	0.29	2.577 ±0.220	6.01
DMSO	0	1.618 ±0.142		2.838 ±0.220	
3TC(Positive control)	80	1.548 ±0.055**	30.16	1.234 ±0.04*	43.31
DMSO	0	2.217 ±0.122		2.174 ±0.10	

Note: NA, Inhibitory effects of 20 species of herbs on HBsAg and HBeAg secretion not tested. NT, toxic to cells and not tested. ND, the highest non-toxic concentration can not be determined. ***P<0.001; **P<0.01; *P<0.05, as compared with the control group (DMSO).

3.2 The inhibitory effects of Ethanol extract of *H. japonica* on HBsAg and HBeAg

In addition, the inhibitory effects of *H.japonica* on HBeAg and HBsAg had better results compared with 3-TC and Kushenin.. The results indicated that the ethanol extract of *H. japonica* did not have any inhibition on HBsAg; whereas, the ethanol extract of *H. japonica* had obvious inhibitory effects on HBeAg secretion, and the highest non-toxic concentration of inhibition rate was 69.06%, which was higher than that of Kushenin. The IC50 value was 14.27µg/mL and the therapeutic index (TI) > 2 (table 6); therefore, the ethanol extract of *H. japonica* had the further research value.

Table 2. The inhibitory effects of *H. japonica*, 3-TC and Kushenin on HBsAg and HBeAg Secretion in HepG2 2.2.15 cells ($\bar{x} \pm s$, n=3)

Drugs	Concentration (ug/mL)	HBeAg		HBsAg		Cell viability (%)
		OD450	Inhibition rate (%)	OD450	Inhibition rate (%)	
Ethanol extract of <i>H. japonica</i>	80	----	----	----	----	51.33 ±0.113
	40	0.538 ±0.018**	69.06	2.443 ±0.197	1.17	104.70 ±0.253
	20	0.847 ±0.123**	51.64	1.937 ±0.211	0.73	111.91 ±0.189
	10	0.917 ±0.004**	47.28	1.941 ±0.420	0.52	97.35 ±0.218
	5	1.166 ±0.241**	32.92	1.944 ±0.006	0.34	101.20 ±0.412
	2.5	1.421 ±0.081**	18.27	1.954 ±0.014	-0.13	95.05 ±0.319
	1.25	1.532 ±0.190**	11.89	1.959 ±0.332	-0.43	98.07 ±0.362
	0	1.739 ±0.109	0	1.951 ±0.042	0	100
3-TC	80	1.330 ±0.060	0.58	1.234 ±0.040**	43.33	103.47 ±0.114
	40	1.278 ±0.050	4.47	1.320 ±0.140**	39.30	106.43 ±0.067
	20	1.375 ±0.140	-2.82	1.638 ±0.090**	24.65	105.18 ±0.312
Kushenin	0	1.338 ±0.090	0	2.174 ±0.100	0	100
	3000	1.352 ±0.090**	37.40	0.883 ±0.110**	64.99	108.3 ±0.215
	1500	1.748 ±0.110**	19.06	1.321 ±0.330**	47.66	96.7 ±0.163
	7500	1.878 ±0.160**	13.04	1.888 ±0.180**	25.17	101.4 ±0.068
	0	2.159 ±0.070	0	2.523 ±0.070	0	100

Note: HepG2 2.2.15 cells were cultured in the presence of *Hypericum japonica*, 3-TC and Kushenin at various concentrations for 7 days. The HBsAg and HBeAg 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.

3.3 Inhibitory effects of different fractions from *H. japonica* on HBeAg

The *H.japonica* ethanol extracts were acquired by trichloromethane, ethyl acetate, n-butanol, and water, respectively, to obtain their different fractions. The four fractions from *H.japonica* ethanol extracts were tested for anti-HBV activity by MTT and ELISA methods, respectively. Results indicated it had the highest inhibition rate of ethyl acetate fraction on the secretion of HBeAg in HepG2 2.2.15 cell line (table 3). This fraction showed 63.46% of inhibition rate, 111.07 $\mu\text{g/mL}$ of IC₅₀ value and TI>2 (table 6). Consequently, it is necessary to isolate further the ethyl acetate fraction of *H. japonica*, which has the highest inhibition rate on the secretion of HBeAg in HepG2 2.2.15 cell line.

Table 3. The inhibitory effects of different fractions from *H. japonica* on HBeAg Secretion in HepG2 2.2.15 cells (n = 3, $\bar{x} \pm s$)

Fractions	Concentration ($\mu\text{g/mL}$)	OD ₄₅₀	Inhibition rate (HBeAg, %)	Cell viability (%)
Chloroform fraction	320	----	----	----
	160	----	----	----
	80	----	----	----
	40	----	----	55.30 $\pm$ 0.092
	20	0.726 $\pm$ 0.213**	40.27	95.00 $\pm$ 0.120
	10	0.768 $\pm$ 0.085**	39.66	98.00 $\pm$ 0.069
	5	0.810 $\pm$ 0.087**	36.36	103.90 $\pm$ 0.043
	2.5	0.949 $\pm$ 0.149**	25.47	103.81 $\pm$ 0.055
Ethylacetate fraction	0	1.216 $\pm$ 0.042	0	100
	320	0.444 $\pm$ 0.138**	63.46	109.60 $\pm$ 0.145
	160	0.559 $\pm$ 0.079**	54.03	116.5 $\pm$ 0.080
	80	0.562 $\pm$ 0.167**	53.78	123.60 $\pm$ 0.095
	40	0.828 $\pm$ 0.105**	31.88	122.30 $\pm$ 0.124
	20	0.915 $\pm$ 0.07**	24.72	107.60 $\pm$ 0.074
	10	0.978 $\pm$ 0.095**	19.60	123.30 $\pm$ 0.170
	0	1.216 $\pm$ 0.042	0	100
n-Butanol fraction	320	----	----	----
	160	----	----	----
	80	----	----	44.11 $\pm$ 0.332
	40	0.842 $\pm$ 0.068**	52.08	105.80 $\pm$ 0.145
	20	0.982 $\pm$ 0.011**	44.09	122.90 $\pm$ 0.202
	10	1.109 $\pm$ 0.051**	36.84	106.00 $\pm$ 0.220
	5	1.270 $\pm$ 0.034**	27.67	101.90 $\pm$ 0.103
	2.5	1.429 $\pm$ 0.096**	18.64	99.20 $\pm$ 0.299
Water fraction	1.25	1.436 $\pm$ 0.078**	18.24	98.60 $\pm$ 0.182
	0	1.948 $\pm$ 0.063	0	100
	320	0.892 $\pm$ 0.114	26.64	110.53 $\pm$ 0.091
	160	0.934 $\pm$ 0.153	22.33	109.21 $\pm$ 0.013
	80	0.993 $\pm$ 0.201	18.32	100.76 $\pm$ 0.021
	40	1.077 $\pm$ 0.116	11.41	101.53 $\pm$ 0.011
	20	1.146 $\pm$ 0.103	5.75	99.21 $\pm$ 0.073
	0	1.216 $\pm$ 0.042	0	100

Note: ----, toxic to cells and not tested, **P<0.01, *P<0.05, as compared with the no drug control group.

3.4 The inhibitory effects of different parts of ethyl acetate fraction on HBeAg

The next step was to isolate the ethyl acetate fraction, as well as obtain 3 parts (YZ1, YZ2 and YZ3) with the active anti-hepatitis B virus by the way of silica gel chromatography. Looking at Table 4, we see that, YZ2 is of the highest inhibition rate on the secretion of HBeAg in HepG2 2.2.15 cell line. This part indicated that it had 71.57% of inhibition rate, 85.21 $\mu\text{g/mL}$ of IC₅₀ value and TI>2 (table 6).

Table 4. The different inhibition parts of ethyl acetate fraction from *H. japonica* on HBeAg secretion in HepG2 2.2.15 cells (n = 3, $\bar{x} \pm s$)

Different parts of ethyl acetate fraction	Concentration ($\mu\text{g/mL}$)	OD ₄₅₀	Inhibition rate (HBeAg, %)	Cell viability (%)
	160	----	----	60.40 $\pm$ 0.151

YZ ₁	80	0.776 $\pm$ 0.028**	44.90	103.30 $\pm$ 0.208
	40	1.029 $\pm$ 0.015**	26.90	105.50 $\pm$ 0.012
	20	1.031 $\pm$ 0.039**	26.70	98.40 $\pm$ 0.398
	10	1.134 $\pm$ 0.065**	19.40	121.10 $\pm$ 0.359
	0	1.407 $\pm$ 0.009	0	100
YZ ₂	160	0.380 $\pm$ 0.080**	71.57	95.60 $\pm$ 0.148
	80	0.818 $\pm$ 0.017**	38.85	104.80 $\pm$ 0.262
	40	0.907 $\pm$ 0.045**	32.20	108.90 $\pm$ 0.319
	20	1.061 $\pm$ 0.058**	20.66	111.50 $\pm$ 0.217
	10	1.077 $\pm$ 0.069**	19.51	103.10 $\pm$ 0.570
YZ ₃	0	1.338 $\pm$ 0.085	0	100
	160	----	----	40.00 $\pm$ 0.071
	80	----	----	78.70 $\pm$ 0.197
	40	0.798 $\pm$ 0.033**	40.40	107.20 $\pm$ 0.117
	20	0.991 $\pm$ 0.042**	25.90	107.50 $\pm$ 0.352
	10	1.034 $\pm$ 0.018**	22.70	97.70 $\pm$ 0.059
	0	1.338 $\pm$ 0.085	0	100

Note: ----, toxic to cells and not tested, **P<0.01, *P<0.05, as compared with the no drug control group.

3.5 The isolation of the compounds with anti-HBV activities

Four compounds were separated from the ethyl acetate fraction of *H. japonica*. Their structures were identified as Vincetoxin side B, (2R,3R) double hydrogen quercetin-7-O- α -L-rhamnoside, quercitrin, and 1,3,6,7-tetrahydroxy-9H-xanthen-9-one (figure 1). Among the 4 compounds, 1, 3, 6, 7-tetrahydroxy-9H-xanthen-9-one exhibited the highest activity in inhibiting secretion of HBeAg in HepG2 2.2.15 cells (table 5). The results indicated a 67.66% of inhibition rate, 40.25 $\mu\text{g/mL}$ of IC₅₀ value and TI>2 (table 6).

Table 5. The inhibitory effect of the four compounds of the ethyl acetate fraction from *H. japonica* on HBeAg secretion in HepG2 2.2.15 cells (n = 3, $\bar{x} \pm s$)

Different parts of ethyl acetate fraction	Concentration ($\mu\text{g/mL}$)	OD ₄₅₀	Inhibition rate (HBeAg, %)	Cell viability (%)
Compound 1	40	----	----	28.10 $\pm$ 0.131
	20	----	----	35.80 $\pm$ 0.151
	10	1.103 $\pm$ 0.055	-0.094	100.10 $\pm$ 0.145
	5	1.055 $\pm$ 0.063	-0.046	109.30 $\pm$ 0.020
	2.5	1.027 $\pm$ 0.046	-0.018	101.90 $\pm$ 0.395
Compound 2	0	1.009 $\pm$ 0.034	0	100
	40	0.798 $\pm$ 0.042**	22.60	119.40 $\pm$ 0.076
	20	0.857 $\pm$ 0.070**	17.00	100.40 $\pm$ 0.111
	10	0.851 $\pm$ 0.025**	17.50	114.90 $\pm$ 0.242
	5	0.852 $\pm$ 0.065**	17.40	122.70 $\pm$ 0.019
Compound 3	2.5	0.971 $\pm$ 0.074**	5.80	108.60 $\pm$ 0.542
	0	1.032 $\pm$ 0.182	0	100
	80	0.803 $\pm$ 0.249**	22.10	95.80 $\pm$ 0.086
	40	0.900 $\pm$ 0.243**	12.80	103.40 $\pm$ 0.137
	20	0.974 $\pm$ 0.257**	5.60	101.50 $\pm$ 0.035
Compound 4	10	1.032 $\pm$ 0.132	-0.012	105.60 $\pm$ 0.149
	5	1.035 $\pm$ 0.223	-0.321	105.50 $\pm$ 0.157
	2.5	1.033 $\pm$ 0.246	-0.145	111.80 $\pm$ 0.140
	0	1.032 $\pm$ 0.182	0	100
	80	0.334 $\pm$ 0.057**	67.66	101.80 $\pm$ 0.577
Compound 4	40	0.571 $\pm$ 0.101**	44.65	117.30 $\pm$ 0.081
	20	0.682 $\pm$ 0.171**	33.81	103.10 $\pm$ 0.234
	10	0.853 $\pm$ 0.098**	17.31	112.50 $\pm$ 0.341
	5	0.960 $\pm$ 0.161**	6.94	105.40 $\pm$ 0.264
	2.5	1.019 $\pm$ 0.042**	1.23	99.10 $\pm$ 0.077
	0	1.032 $\pm$ 0.182	0	100

Note: ----, toxic to cells and not tested, **P<0.01, *P<0.05, as compared with the no drug control group.

Table 6. Evaluation of anti-HBV efficacy of the active site and isolated compound of *H. japonica*

Extract	IC ₅₀ (μg/mL)	TC ₅₀ (μg/mL)	TI
<i>H. japonica</i> ethanol extract	14.27	>40	>2
Ethylacetate extract	111.07	>320	>2
YZ ₂	85.21	>180	>2
Compound 4	40.25	>100	>2

Compound 1(vincetoxicoside B) Compound 2((2R,3R)-taxifolin-7-O-α-L-rhamnoside)

Compound 3 (quercitrin) Compound 4 (1,3,6,7-tetrahydroxy-9H-xanthen-9-one)

Fig.1. Chemical structures of the compounds. vincetoxicoside B

mp 174 ~ 180 °C, ESI-MS m/s:447[M-H]⁻, ¹H-NMR(400MHz,DMSO-d₆) δ:1.14 (3H,d,J=6.0Hz, rha-Me), 5.55(1H,J=1.7Hz, rhaH-1), 6.41(1H,m,H-6), 6.79(1H,d,J=2.1Hz, H-8),6.90(1H,d,J=8.1Hz, H-5), 7.57(1H,dd,J=8.6Hz, H-6'), 7.72(1H,d,J=2.1Hz, H-2'). ¹³C-NMR(100MHz, DMSO-d₆)δ:18.0(C-6''), 69.9(C-5''), 70.0(C-2''), 70.2(C-3''), 71.6(C-4''), 94.2(C-8), 98.4(C-6), 98.8(C-1''), 104.6(C-10), 115.2(C-6'), 115.6(C-5'), 120.1(C-2'), 121.8(C-1'), 136.1(C-3), 145.1(C-3'),147.5(C-4'), 147.9(C-2), 155.7(C-9), 160.4(C-7), 161.4(C-5), 176.0(C-4).

(2R,3R) -taxifolin-7-O-α-L-rhamnoside

mp 198~200°C. ¹H-NMR(400MHz,DMSO-d₆)δ:6.76 (1H, s, J=11.60Hz, H-2'), 6.75(1H, d, J=8.1Hz, H-5'), 6.75(1H, dd,J=1.0,8.0 Hz,H-6'), 6.17 (1H, d, J=1.9Hz, H-8),6.12(1H, d, J=1.8Hz, H-6), 5.45(1H, d, J=10.3Hz, H-2), 3.92(1H, d, J=11.6Hz, H-3),3.38(1H,dd,J=1.7,3.4 Hz, RhaH-2),3.36(1H, dd, J=3.1,9.4 Hz, RhaH-3),3.28(1H,m,RhaH-5),3.26(1H,t,J=10.0 Hz, RhaH-4),1.10(3H, d, J=6.0 Hz, Rha-Me); ¹³C-NMR(DMSO-d₆, 100MHz)δ:17.9(C-6''), 68.9(C-5''), 69.8(C-2''), 70.2(C-3''),71.5(C-4''), 75.5(C-3), 83.1(C-2), 95.4(C-8),96.5(C-6), 98.2(C-1''),102.0(C-10), 115.1(C-5'), 115.4(C-2'), 119.4(C-6'), 127.8(C-1'),145.0(C-3'), 145.8(C-4'),162.3(C-9),162.8(C-5), 164.1(C-7), 198.6(C-4).

Quercitrin

mp 180~181°C. ESI-MS m/z: 447[M-H]⁻. ¹H-NMR (400 MHz, DMSO-d₆)δ:7.30(1Hd, J=2.0HzH-2')7.29(1HddJ=8.42.0 HzH-6') 6.86 (1H, d, J=8.4 Hz, H-5'), 6.39(1H, d, J=2.0Hz, H-8), 6.20(1H, d, J=2.0Hz, H-6), 5.24(1H, d, J=1.6Hz, H-1'), 3.97(1H, brsH-2''), 3.51(1H, dd, J=9.53.2HzH-3''), 3.15(1H, t, J=10Hz, H-4''),

3.22(1H, dp, J=9.56.1HzH-5''), 0.88(3H, d, J=6.1Hz, H-6''); ¹³C-NMR (DMSO-d₆, 100MHz) δ:17.7 (C-6''), 70.0 (C-5''), 70.3(C-2''), 70.6(C-3''), 71.5(C-4''),101.1(C-1''), 120(C-6'), 115.5(C-5'), 148.4(C-4'),145.2(C-3'), 115.6(C-2'),1 21.1(C-1'), 104.1(C-10), 157.3(C-9), 93.6(C-8),164.2(C-7), 98.7(C-6),161.3(C-5),177.8(C-4), 134.2(C-3), 156.4(C-2).

1,3,6,7-tetrahydroxy-9H-xanthen-9-one

mp>300 °C, ESI-MS m/z: 260[M-H]⁻. ¹H-NMR (400MHz, DMSO-d₆) δ:7.36(1H, d, J=8Hz, H-7), 6.54(1H, d, J=8Hz, H-5), 6.32(1H,s, H-4), 6.14 (1H, s, H-2); ¹³C-NMR(DMSO-d₆, 100MHz) δ:93.6(C-4), 97.6(C-2), 101.5(C-9a), 102.5(C-5), 107.8(C-8), 111.5(C-8a), 143.9(C-7), 151.2(C-4b), 154.7(C-6), 157.3(C-4a), 162.5(C-1), 164.6(C-3), 178.8(C-9).

3.6 The inhibition effects on serum DHBV-DNA of compound 4

we conducted a qualitatively evaluated research using duck hepatitis B virus (DHBV) model in vivo [12-13]. The results showed that the compound 4 has no direct inhibitory effect on HBV-DNA in duck experiment. This indicated that compound 4 had the effect of anti-HBV activity through inhibiting the secretion of HBeAg.

4 Discussion

In this study, we conducted a qualitatively evaluated research using DHBV model in vivo, Ethanol extracts from *F. dibotrys*, *P. saxatile* and *H. japonica*, as well as the compound 4. Beijing ducks were infected with duck hepatitis B virus (DHBV) and they were used to evaluate anti-HBV activity. Treatments began on the 7th-day post infection. The ducks were infected with 26 mg/kg (high dose groups), 6.5 mg/kg (low dose groups) of ethanol extracts from *F. dibotrys*, *P. saxatile*, respectively. Drugs were given twice a day for ten consecutive days. The results indicated that the high dose groups, which were treated with ethanol extracts of *P. saxatile* or *H. japonica*, had anti-HBV effects and almost no sign of disease rebound after drug withdrawing. Treatment with ethanol extract from *F. dibotrys* also had certain levels of inhibitory effect on HBV-DNA, but the disease rebound after the withdrawing of treatment. The compound 4 has no direct inhibitory effect on HBV-DNA in duck experiment.

To our knowledge, this is the first report to show the 1, 3, 6, 7-tetrahydroxy-9H-xanthen-9-one has anti-HBV activity and low cytotoxicity in the in vitro assay, and our *P. saxatile* has anti-HBV activity in vivo.

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