

Full-length article

Effect of *Oenanthe javanica* flavone on human and duck hepatitis B virus infection¹Wei-na WANG², Xin-bo YANG^{3,6}, He-zhi LIU⁴, Zheng-ming HUANG^{5,6}, Guang-xia WU²

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Key words

Oenanthe javanica; flavones; hepatitis B virus; 2.2.15 cells; Duck hepatitis B virus; antiviral agents

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Abstract

Aim: To study the antiviral effect of *Oenanthe javanica* flavones (*OjF*) on human hepatoma HepG2.2.15 culture system and duck hepatitis B virus (DHBV) infection. **Methods:** (1) After incubation for 24 h, the 2.2.15 cells were treated with different concentrations of *OjF* for 12 d. The cell alteration was observed by microscope. The presence of HBsAg and HBeAg were measured using the enzyme immunoassay kit after 2.2.15 cells were treated with *OjF* for 9 d. (2) Ducklings infected with DHBV intravenously were divided into 5 groups and treated with *OjF*, acyclovir (ACV), and normal saline respectively for 10 d. All the ducklings were bled before, during, and after treatments at different times, and serum levels of DHBV-DNA were detected by a dot-blot hybridization assay. **Results:** (1) The 50% toxic concentration (TC₅₀) of *OjF* was 2.28 g/L. The maximum nontoxic concentration (TC₀) was 1.00 g/L. In nontoxic concentrations, *OjF* significantly inhibited HBsAg and HBeAg in 2.2.15 cells after 9 d of treatment ($P < 0.05$, $P < 0.01$). (2) The DHBV-DNA levels decreased significantly after the treatment with 0.50 and 1.00 g/kg of *OjF* ($P < 0.01$). The inhibition of the peak of viremia was maximum at a dose of 1.00 g/kg and reached 54.3% on d 5 and 64.5% on d 10, respectively. **Conclusion:** The results demonstrate that *OjF* is a strong inhibitor of HBsAg and HBeAg secretion in 2.2.15 cells and DHBV-DNA levels in the HBV-infected duck model.

Introduction

Oenanthe javanica (*Oj*), umbelliferate, has been widely used in traditional Chinese medicine for treatment of jaundice, hypertension, and polydipsia diseases for many years^[1]. Previous studies have shown that it has liver-protective^[2], hypotensive^[3], anti-arrhythmic^[4], anti-anaphylactic^[5], and antidiabetic^[6] effects. Recent studies also show that *Oj* was helpful in treatment of hepatitis B virus (HBV) infection in clinical trials^[7], and had an inhibitory effect on duck hepatitis B virus (DHBV)-induced hepatitis in Nestling ducks *in vivo*^[8] and *in vitro*^[9]. However, which is the active part and what are the active components of *Oj* in inhibiting HBV remain unclear. Using modern techniques, it has been revealed that *OjF* is one of the main active parts against HBV and it comprises approximately 2.2% of the whole plant content.

The present study aimed to investigate the anti-hepatitis activity of *OjF* in 2.2.15 cells *in vitro* and duck HBV infection in a duck model *in vivo*.

Materials and methods

Preparation of *OjF* *Oj* was collected from the Yanbian Autonomous Region in autumn and identification was performed by Prof Hui-zhong XIAO, Department of Phytochemistry, Yanbian Medical University. Dried whole plant was pulverized and extracted with 80% ethanol, and subsequently partitioned in ethyl acetate. The ethyl acetate soluble materials were fractionated by ethanol and distilled water gradient of column chromatography in polyamide. *OjF* was eluted and the content was determined according to aluminium nitrate reagent method. The content of hyperoside,

the major ingredient of *OjF*, was determined by reverse phase-high performance liquid chromatography (RP-HPLC)^[10,11]. The contents of *OjF* and hyperoside were 51.67% and 7% in whole extracts, respectively. The concentration used in the experiment was based on the dry weight of the extract.

Experimental animals Beijing ducklings within 1 d of hatching were obtained from an animal breeding farm, Chinese Academy of Medical Sciences [SCXK- (Beijing) 2002-001].

Reagents Minimum essential medium (MEM) was obtained from Gibco BRL (Gaithersburg, MD, USA). Fetal bovine serum (FBS) and G-418 were purchased from HyClone (Logan, Utah, USA). *L*-glutamine was obtained from Sigma (St Louis, MO, USA). HBsAg and HBeAg enzyme immunoassay (EIA) kits were purchased from China Isotope Co (Beijing, China).

Cell culture and treatment HepG2.2.15 cells (clonal cells derived from human hepatoma cell line G₂) were from the Institute of Medicinal Biotechnology, Chinese Academy of Medical Sciences. The 2.2.15 cells were cultured in complete MEM (containing 10% FBS, 100 kU/L benzylpenicillin, streptomycin, G-418, *L*-glutamine 0.03%, pH 7.0) in 75-cm² tissue culture flasks at 37 °C in a humidified 5% CO₂.

Cytotoxic studies The 2.2.15 cells were first seeded into 96-well plates (Corning Inc, Corning, NY, USA) at a density of 1.0×10⁵ cells per mL and cultured in 200 μL complete MEM containing 10% FBS. After 24 h of incubation, cells were washed three times with phosphate-buffered saline (pH 7.0) and treated with different concentrations (0.125, 0.25, 0.50, 1.00, 2.00, and 4.00 g/L) of *OjF* in serum-free medium for 12 d. The medium was replaced every 4 d in MEM supplemented with various concentrations of *OjF*. Untreated cells were used as control. The cell pathological changes (CPE) were observed by microscope. The degree of CPE was graded as: all positive cells (–), the number of negative cells <25% (+), 25%–49% (++), 50%–75% (+++), and >75% (++++).

Determination of HBsAg and HBeAg The 2.2.15 cells were incubated in 24-well plates at a density of 1.0×10⁵ cells per mL in 1 L MEM medium containing 10% FBS. After 24 h, the 2.2.15 cells were treated with different concentrations of *OjF* (0.125, 0.25, 0.50, and 1.00 g/L) in serum-free medium. Cells grew in the presence of drugs for 9 d with changes of medium every 3 d. After 6 and 9 d, supernatant was collected and performed at -20 °C. The HBsAg and HBeAg in culture medium were simultaneously measured by EIA kits on d 6 and d 9.

Experimented animals infection and drug treatment Beijing ducklings within 1 d of hatching were inoculated

intravenously with DHBV-DNA-positive serum from Shanghai ducks (0.2 mL/animal). Seven days after infection, ducklings were divided into five groups: the control group (normal saline); the positive drug group (ACV, 0.1 g·kg⁻¹·d⁻¹); and the *OjF* 0.25, 0.50, and 1.00 g·kg⁻¹·d⁻¹ groups. Drugs were administered orally, bid for 10 d. Serum samples were obtained before treatment (d 0), d 5, and d 10 during treatment, and d 3 (d 13) after the cessation of treatment. The serums were stored at -70 °C for future analysis.

Viremia analysis Viremia was assessed throughout the treatment and follow-up period by a semi-quantitative detection of DHBV-DNA in duck serum using a dot-blot hybridization. Fifty microliters of serum was spotted directly onto nitrocellulose filters. After denaturation and neutralization, the filters were hybridized with a full-length DHBV genomic DNA probe labeled with ³²P. The filters were autoradiographed and the spots were counted in a scintillation counter.

Histopathological examination of hepatocytes On d 13, each duckling was laparotomized to obtain the liver immediately after collecting blood from the leg vein. Fragments of the ducklings liver were fixed in 10% formalin solution, dehydrated with ethanol solution from 50% to 100%, embedded in paraffin and cut into 5 μm sections, and stained using haematoxylin-eosin dye for photomicroscopic observations.

Statistics The data were expressed as mean±SD, and analyzed by one-way repeated-measure ANOVA and *t*-test for comparisons between groups. *P*<0.05 was considered statistically significant.

Results

The cytotoxicity of *OjF* in 2.2.15 cells *OjF*-induced cytotoxicity was observed by microscope. After 12 d of incubation with 1.00 g/L *OjF*, no significant difference was found from that of the control. However, when *OjF* concentration increased, cell injury caused by *OjF* was observed (Table 1). The 50% toxic concentrations (TC₅₀) was 2.28±0.13 g/L and the maximum nontoxic concentrations (TC₀) was 1.00 g/L.

Inhibition of HBsAg and HBeAg production in 2.2.15 cells After 9 d of incubation, HBsAg and HBeAg production in the culture medium were determined (Table 2). The results showed that *OjF* suppressed HBsAg and HBeAg production in the 2.2.15 cells with median effective concentration (IC₅₀) of about 0.56 and 0.41 g/L respectively on d 6, 0.64 and 0.30 g/L respectively on d 9 (Table 3). *OjF*-induced suppression of HBsAg and HBeAg production in 2.2.15 cells was also reflected by the inhibition rate percentage (Figure 1, 2). The inhibitory effects appeared when cells were treated with 0.25 g/L *OjF*. A substantial increase of

Table 1. Cell toxicity of *OjF* on cultured 2.2.15 cells at d 12.

Parameter	Dose/g·L ⁻¹							TC ₅₀ /g·L ⁻¹
	4.00	2.00	1.00	0.50	0.25	0.125	0	
1 CPE	++++	+	-	-	-	-	-	2.41
	++++	+	-	-	-	-	-	
	++++	++	-	-	-	-	-	
	++++	+	-	-	-	-	-	
CPE %	100	31.25						
2 CPE	++++	+	-	-	-	-	-	2.29
	++++	++	-	-	-	-	-	
	++++	+	-	-	-	-	-	
	++++	++	-	-	-	-	-	
CPE %	100	37.50						
3 CPE	++++	++	-	-	-	-	-	2.15
	++++	++	-	-	-	-	-	
	++++	++	-	-	-	-	-	
	++++	+	-	-	-	-	-	
CPE %	100	43.75						
Mean								2.28

Positive cells (-); number of negative cells <25% (+), 25%–49% (++), 50%–75% (+++), and >75% (++++) in three experiments. CPE: cell pathological changes. TC₅₀ was the 50% toxic concentrations.

Table 2. HBsAg and HBeAg levels in 2.2.15 cells treated with *OjF*. n=9. Mean±SD. ^bP<0.05, ^cP<0.01 vs control.

Dose/g·L ⁻¹	10 ⁻³ ×Radioactivity/Bq			
	d 6		d 9	
	HBsAg	HBeAg	HBsAg	HBeAg
0	3.44±0.24	7.13±1.34	2.66±0.16	7.47±1.43
0.25	2.34±0.17 ^c	3.88±0.95 ^c	1.90±0.11 ^b	2.83±0.44 ^c
0.50	1.59±0.05 ^c	2.93±0.86 ^c	1.47±0.07 ^c	1.78±0.28 ^c
1.00	1.32±0.07 ^c	1.50±0.23 ^c	0.94±0.11 ^c	1.09±0.29 ^c

inhibitory effects was observed from 0.50 g/L *OjF*. When treated with 1.00 g/L *OjF*, the inhibition rate percentage on HBsAg and HBeAg in 2.2.15 cells were both more than 50%. The inhibition rate percentage on HBsAg was dose-dependent, and the inhibition rate percentage on HBeAg was both time- and dose-dependent.

Inhibitory effect of *OjF* on DHBV-DNA During the course of this study, no obvious side effects were observed in animals receiving antiviral therapy or in control animals.

The effects of *OjF* and ACV, which were used for comparison, on DHBV replication *in vivo* were determined

Table 3. Inhibitory effect on HBsAg and HBeAg in 2.2.15 cells treated with *OjF*.

	HBsAg				HBeAg			
	IC ₅₀ /g·L ⁻¹		TI ¹⁾		IC ₅₀ /g·L ⁻¹		TI	
	d 6	d 9	d 6	d 9	d 6	d 9	d 6	d 9
1	<0.25	0.62	9.64	3.91	0.56	0.25	4.33	>9.64
2	1.00	0.71	2.29	3.22	0.42	0.25	5.42	>9.16
3	0.43	0.59	5.00	3.67	0.25	0.41	>8.60	5.28
Mean±SD	0.56±0.39	0.64±0.06	5.64±3.72	3.60±0.35	0.41±0.15	0.30±0.09	6.12±2.22	8.03±2.39

¹ TI, therapeutic indices. The values were determined by IC₅₀ vs TC₅₀. The more the value of TI, the more suppression activity of *OjF*.

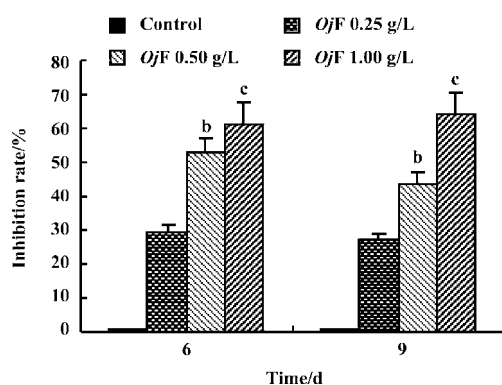


Figure 1. The inhibition rate percentage of *Oenanthe javanica* flavones (*OjF*) on HBsAg production in 2.2.15 cells. $n=9$. Mean \pm SD. ^b $P<0.05$, ^c $P<0.01$ vs control.

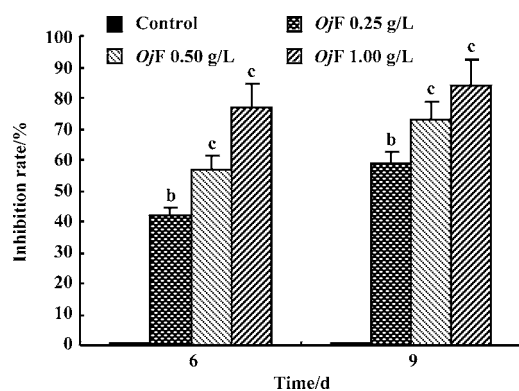


Figure 2. The inhibition rate percentage of *OjF* on HBeAg production in 2.2.15 cells. $n=9$. Mean \pm SD. ^b $P<0.05$, ^c $P<0.01$ vs control.

by quantification of DHBV-DNA by dot-blot hybridization. The levels of serum viral DNA were recorded in the 5 groups before the experiment. During treatment, serum levels of DHBV-DNA decreased in all 16 ducks treated with *OjF* 0.50 and 1.00 g·kg⁻¹·d⁻¹ (Table 4). The mean percentage inhibition of viral DNA levels with *OjF* 0.50 and 1.00 g·kg⁻¹·d⁻¹ was 54.3% and 64.5% respectively on the last day of treatment

Table 4. Mean changes of serum DHBV-DNA level in treated duck. $n=16$. Mean \pm SD. ^b $P<0.05$, ^c $P<0.01$ vs control. ^e $P<0.05$, ^f $P<0.01$ vs ACV.

Group	Dose/ g·kg ⁻¹ ·d ⁻¹	Absorbance			
		d 0	d 5	d 10	d 13
Control	-	0.88 \pm 0.10	0.92 \pm 0.10	0.79 \pm 0.04	0.85 \pm 0.08
ACV	0.1	0.87 \pm 0.06	0.27 \pm 0.16 ^c	0.32 \pm 0.09 ^c	0.74 \pm 0.14
<i>OjF</i>	0.25	0.83 \pm 0.17	0.79 \pm 0.21 ^{bf}	0.67 \pm 0.11 ^{cf}	0.78 \pm 0.12
	0.50	0.92 \pm 0.11	0.53 \pm 0.15 ^{cf}	0.48 \pm 0.10 ^{cf}	0.48 \pm 0.05 ^{cf}
	1.00	0.85 \pm 0.09	0.42 \pm 0.15 ^{ce}	0.28 \pm 0.05 ^c	0.33 \pm 0.03 ^{cf}

Table 5. The inhibition rate percentage of *OjF* on DHBV-DNA. $n=16$. Mean \pm SD. ^c $P<0.01$ vs control.

Dose/g·kg ⁻¹ ·d ⁻¹	Inhibition rate percentage/%		
	d 5	d 10	d 13
0	0	0	0
0.25	14.1 \pm 0.16	15.2 \pm 0.08	8.2 \pm 0.11
0.50	42.4 \pm 0.13 ^c	39.2 \pm 0.10 ^c	43.5 \pm 0.07 ^c
1.00	54.3 \pm 0.11 ^c	64.5 \pm 0.06 ^c	61.1 \pm 0.05 ^c

(Table 5). But 3 days after the cessation of treatment with ACV, the viral replication level returned to the pretreatment baseline. In ducks treated with *OjF*, the effect of DHBV-DNA inhibition lasted. No significant decrease of serum DHBV-DNA was observed during treatment with *OjF* 0.25 g·kg⁻¹·d⁻¹. In the control group, serum DHBV-DNA remained unaffected during the course of the study.

Histopathological features Histopathological profiles of the liver from model group ducklings revealed necrosis, steatosis, and often swelling of the hepatic cytoplasm. The protective effect of *OjF* was confirmed by histopathological examinations. Administration of *OjF* to the experimental animals (1.00 g·kg⁻¹·d⁻¹) showed a significant improvement of the hepatocellular architecture over the model group, as evident from a considerable reduction in necrosis and vacuolation (Figure 3).

Discussion

Hepatitis B virus causes acute and chronic hepatitis, which affects nearly 360 million people worldwide^[12]. Chronic infection with HBV has been associated with a high risk for the development of primary hepatocellular carcinoma^[13,14]. Effective antiviral therapy against HBV infection has not been fully developed. Studies have been hampered by the extremely narrow host range and limited access to experimental culture systems. Fortunately, techniques have been

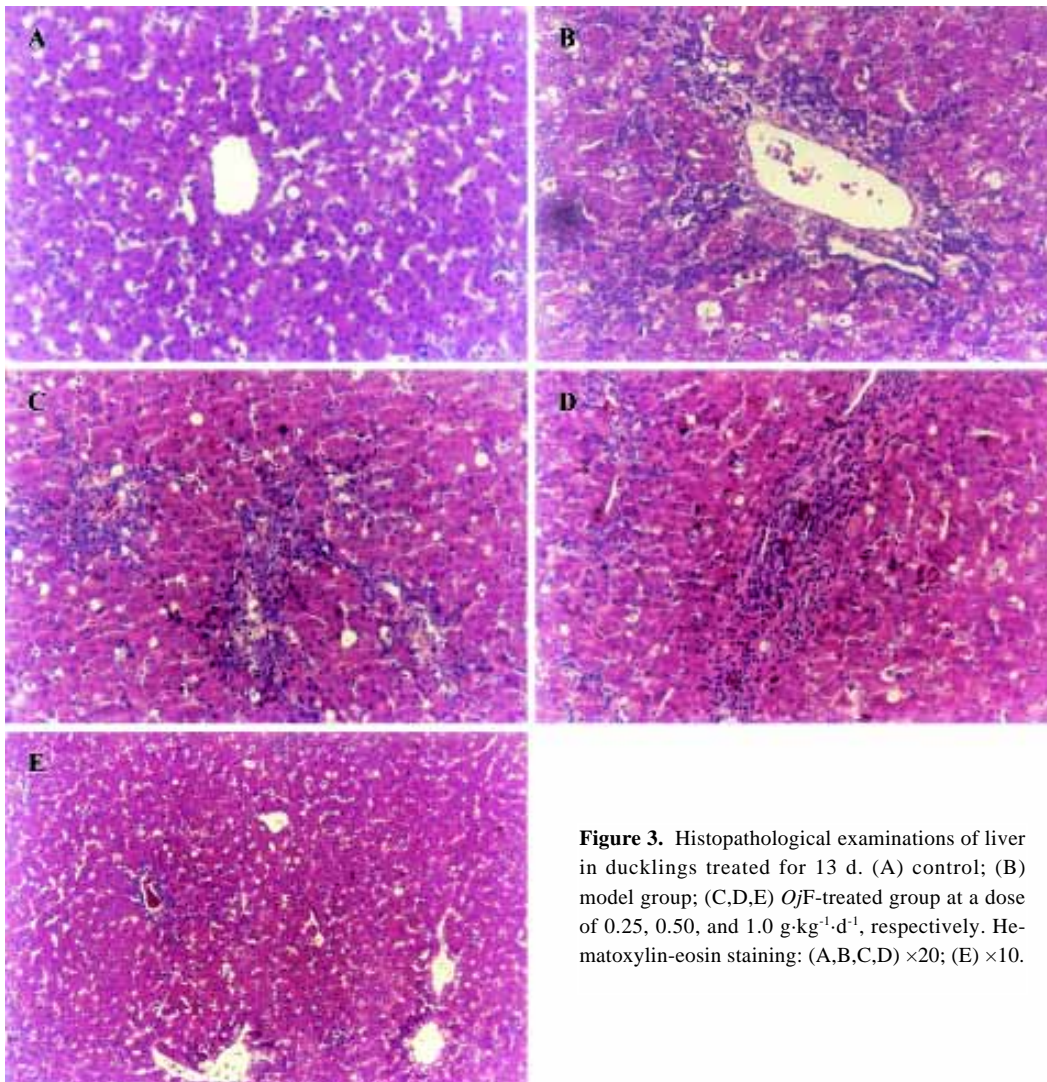


Figure 3. Histopathological examinations of liver in ducklings treated for 13 d. (A) control; (B) model group; (C,D,E) *OjF*-treated group at a dose of 0.25, 0.50, and 1.0 g·kg⁻¹·d⁻¹, respectively. Hematoxylin-eosin staining: (A,B,C,D) ×20; (E) ×10.

developed to propagate hepadnaviridae in tissue culture^[15,16] and animal systems^[17]. The 2.2.15 cells contain all HBV particles, and the Peking ducks allow multiplication of the HBV-like virus, which make it possible to study the various aspects of the viral life cycle and to examine the effectiveness of potential antiviral drugs.

In a previous study, we have shown that the acid-base extracts of *Oj* could protect hepatic cells, decrease the content of ALT, AST, and BiliT on a liver damage model caused by carbon tetrachloride (CCl₄) in rats^[18], and inhibit DHBV^[7,8]. Similar results of the inhibitory effect of *Oj* extracts on HBsAg and HBeAg production in cultured 2.2.15 cells was also observed^[19]. However, what the active part of *Oj* against HBV is is still unknown. *OjF* was obtained through extraction and separation, which made up more than 50% in the whole extracts. It might be an active part against HBV.

Through subsequent purification of *OjF*, hyperoside, persicarin, isorhamnetin, and quercetin were obtained. Among them, the assay of hyperoside was the highest (purity>96%), which was used as the criterion to control the quality of *OjF*.

This study demonstrated the inhibitory effect of *OjF* on HBsAg and HBeAg secretion by human hepatoma 2.2.15 cells and on serum DHBV-DNA levels of ducklings infected with hepatitis B virus. TC₅₀ of *OjF* was 2.28 g/L and TC₀ was 1.00 g/L in 2.2.15 cells, which suggested that the inhibitory action of *OjF* had no cytotoxicity. In nontoxic concentrations, *OjF* significantly inhibited the secretion of HBsAg and HBeAg. With *OjF* concentration increasing, a dose-dependent response was observed. At a TC₀ of 1.00 g/L, the inhibition rate percentage of *OjF* on HBsAg and HBeAg in 2.2.15 cells were both more than 50%, and the inhibition rate per-

centage on HBsAg exceeded that of *OjF* on HBeAg.

These results clearly illustrate an inhibitory effect of *OjF* on HBsAg and HBeAg production in 2.2.15 cells, which provide strong evidence to evaluate the effect of drugs against HBV in a cellular model, but it is still necessary to verify this in an animal model. Therefore, the inhibitory effect of *OjF* in the duck HBV model was investigated. Our experiments with *OjF* (0.50 and 1.00 g·kg⁻¹·d⁻¹) in ducklings pointed to a suppressive action on DHBV replication *in vivo*. With *OjF* 1.00 g·kg⁻¹·d⁻¹, the therapy caused a more pronounced decrease (64.5%) in viremia. It was well known that most antiviral medicines had the inevitable rebound effect after drug cessation. This shortcoming had limited the therapy to those diseases infected by viruses such as HB or AIDS. The similar phenomena appeared in the positive control drug ACV in the present study. *OjF* showed therapeutic effects as well as ACV, and no difference was observed after cessation of *OjF* therapy compared to *OjF*-treated animals. It suggested that *OjF* could maintain for a long time in treating viremia of HBV and the effect of DHBV-DNA inhibition showed a concentration-dependent response. Histopathological examination also confirmed the function of *OjF* protecting the liver in DHBV-infected ducklings. Xiong Q *et al*^[19] reported that *Apocynum ventum* extracts containing hyperoside and quercetin had hepatoprotective activity, and *OjF* contained these two compounds. These results demonstrated the antihepatitis B virus effect of *OjF*, which were consistent with antiviral activity of *OjF*^[7,8], making *OjF* a candidate for future evaluation in patients with HBV infection.

In order to elucidate the possible mechanism of *OjF* towards DHBV-DNA on DHBV-infected ducklings, the effect of *OjF* on DHBV-DNA was investigated (data not shown). Our results indicate that *OjF* might inhibit the DNA-dependent DNA polymerase reaction, which results in the termination of replication of DHBV-DNA. This hypothesis is currently under investigation in our laboratory.

In conclusion, *OjF* possessed the significant antiviral activity *in vitro* and *in vivo*, and it was one of the main active parts of *Oj* against HBV. Elucidation of the mechanism of its antiviral activity and identification of the active components in *OjF* will greatly enhance the understanding of viral gene expressions and provide new clues to assist in the development antiviral agents in the future.

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