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# *In vivo* and *in vitro* antiviral activity of hyperoside extracted from *Abelmoschus manihot* (L) medik[1]

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## Key words

hyperoside; hepatitis B virus; duck hepatitis B virus; antiviral agents

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## Abstract

**Aim:** To assess the anti-hepatitis B virus (HBV) effect of hyperoside extracted from *Abelmoschus manihot* (L) medik. **Methods:** The human hepatoma Hep G2.2.15 cell culture system and duck hepatitis B virus (DHBV) infection model were used as *in vivo* and *in vitro* models to evaluate the anti-HBV effects. **Results:** In the cell model, the 50% toxic concentration of hyperoside was 0.115 g/L; the maximum nontoxic concentration was 0.05 g/L. On the maximum nontoxic concentrations, the inhibition rates of hyperoside on HBeAg and HBsAg in the 2.2.15 cells were 86.41% and 82.27% on d 8, respectively. In the DHBV infection model, the DHBV-DNA levels decreased significantly in the treatment of 0.05 g·kg$^{-1}$·d$^{-1}$ and 0.10 g·kg$^{-1}$·d$^{-1}$ dosage groups of hyperoside ($P$<0.01). The inhibition of the peak of viremia was at the maximum at the dose of 0.10 g·kg$^{-1}$·d$^{-1}$ and reached 60.79% on d 10 and 69.78% on d 13, respectively. **Conclusion:** These results suggested that hyperoside 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

*Abelmoschus manihot* (L) medik is native to the Old World tropics and has been naturalized in some wild New World tropical areas. It is an edible hibiscus of the Malvaceae (Mallow) family, and is also used as a staple in folk medicine in Papua New Guinea, Vanuatu, Fiji, New Caledonia, or China for a variety of purposes, including: the control of fertility, to ease childbirth, to stimulate lactation, to help against menorrhagia, to induce abortion, and to prevent osteoporosis[1–3].

In recent studies, more researchers have been interested in the total flavonoids in the flowers of *A manihot*. Hyperoside, isoquercetin, and quercetin 3'-glucoside are important ingredients in total flavonoids. Among these, the content of hyperoside is the highest. Hyperoside (hyperin), quercetin-3-*O*-β-*D*-galactoside, is a flavonol glycoside which widely exists in many traditional medicines, such as *Semen cuscutae*[4], *St John's wort*[5,6], hawthorn[7], *Balbisia calycina*[8], and *Alchornea cordifolia*[9]. As an important bioactive compound, hyperoside has been documented to possess antiviral activity[10,11], antinociceptive[12–14], anti-inflammatory[15], cardioprotective[7,16,17], hepatoprotective[18–20], and gastric-mucosal-protective effects[21,22].

In previous studies, it has been shown that hyperoside demonstrates hepatoprotective properties in various chemically-induced hepatocyte injury models[18–20]. However, to our knowledge, the activity of hyperoside against viral hepatitis has never been tested. Therefore, in this study we aimed to evaluate the anti-hepatitis B virus (HBV) activity of hyperoside extracted from *A manihot*.

## Materials and methods

**Plant material** *A manihot* were collected from Xinghua, Jiangsu province and identified by Prof Xian-rong WANG at the Anhui Institute of Medical Science (Heifei, China).

**Extraction of hyperoside** Flower materials were extracted with 80% ethanol and subsequently partitioned in ethyl acetate. Ethyl acetate extracts were chromatographed over a

polyamide column using gradient mixtures of ethanol and distilled water. Ethanol extracts yielded total flavonoid following solvent removal under vacuum. The total flavonoid was dissolved in ethanol and crystallized to obtain pure hyperoside (about 97%). The structure of isolate was determined by reverse phase high performance liquid chromatography in comparison with authentic hyperoside (the National Institute for the control of Pharmaceutical and Biological Products, Beijing, China)

***In vitro* anti-HBV activity tests**

Cell culture The HBV-producing 2.2.15 cells were obtained from the Institute of Medicinal Biotechnology, Chinese Academy of Medical Sciences (Beijing, China). These cultures were derived from HepG2 cells that were transfected with a plasmid vector containing G418-resistance sequences and 2 head-to-tail dimmers of the HBV genome. The cells were found to produce elevated levels of HBeAg and HBsAg. 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 $cm^2$ tissue culture flasks at 37 ℃ in a humidified 5% $CO_2$.

Cell toxicity studies The 2.2.15 cells were first seeded into 96-well plates (Corning Inc, Corning, NY, USA) at a density of $1.0\times10^5$ cells per mL and cultured in 200 μL complete MEM containing 10% FBS. After 24 h of incubation, the cells were washed 3 times with phosphate-buffered saline (pH 7.0) and treated with different concentrations (0.20, 0.10, 0.05, 0.025, and 0.0125 g/L) of hyperoside in serum-free medium for 12 d. The medium was replaced every 4 d in MEM supplemented with various concentrations of hyperoside. Untreated cells were used as the control. Because the drug was fuscous, MTT assay could not be used to measure the toxicity of this drug. Therefore, as an index of toxicity, the cell pathological changes (CPE) were observed by a microscope. The degree of CPE was graded as: all positive cells (–), the number of negative cells <25% (+), 25%–49% (++), 50%–75%(+++), and >75% (++++). This test was done 3 times under the same conditions.

Determination of HBsAg and HBeAg The 2.2.15 cells were incubated in 24-well plates at a density of $1.0\times10^5$ cells per mL in 1000 μL MEM medium containing 10% FBS. After 24 h, the 2.2.15 cells were treated with different concentrations of hyperoside (0.05, 0.025, 0.0125, 0.00625, and 0.003125 g/L) in serum-free medium. The cells were grown in the presence of hyperoside for 9 d and the medium was replaced every 3 d. After d 6 and d 9, the supernatant was collected and performed at -20 °C. The HBsAg and HBeAg in the culture medium were simultaneously measured by EIA kits on d 6 and d 9. This test was done twice under the same conditions.

***In vivo* anti-HBV activity tests**

Animals and treatments Peking ducklings within 1 d of hatching were used as the in vivo model system. The animals were obtained from an animal breeding farm, Chinese Academy of Medical Sciences, [SCXK-(Peking)2002–001, Peking, China]. The animal quarters were maintained at 22± 2 °C and 50%±10% humidity with a 12 h light/12 h dark cycle. The ducklings were inoculated intravenously with duck hepatitis B virus (DHBV)-DNA-positive serum from the Shang-hai ducks (0.2 mL/animal). Seven days after the injection, the ducklings were divided into 5 groups: the control group (normal saline); the positive drug group (lamivudine or 3TC, 0.05 $g\cdot kg^{-1}\cdot d^{-1}$); and the hyperoside 0.02, 0.05, and 0.10 $g\cdot kg^{-1}\cdot d^{-1}$ groups. Drugs were administered orally twice daily for 10 d. Sera were obtained before treatment (d 0), on d 5 and d 10 during treatment, and d 3 (d 13) after the cessation of treatment. The serum levels of DHBV-DNA were detected by dot hybridization.

Detection of DHBV-DNA Fifty μL of serum was spotted directly onto the nitrocellulose filters. DNA hybridization was initiated by adding a recently prepared DHBV $^{32}$PDNA probe at $1.0\times10^6$ cpm/mL using the same prehybridization procedure over night. Filters were washed twice in 1×SSC (20×SSC: 3 mol/L NaCl, 0.3 mol/L sodium citrate, pH 7.0), 0.1% SDS at 65 °C for 2 h, and 1×SSC at room temperature for 30 min with gentle, constant agitation. The filter was dried and autoradiographed at -70 °C using X-ray film with an enhancer screen. After an autoradiographic image had been obtained, the filter was exposed in the phosphorimaging screen for 1–2 h, and the samples were quantitated by FujixBAS1000 (Fuji, Tokyo, Japan); the percentage density of the phosphorimaging units was calculated.

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 hematoxylin-eosin dye for photomicroscopic observations.

**Statistical analysis** All data were expressed as mean±SD and analyzed by one-way repeated-measure ANOVA and *t*-test for comparisons between groups. Values were considered significantly different at $P<0.05$.

## Results

***In vitro* anti-HBV activities**

Cytotoxicity of hyperoside in 2.2.15 cells Hyperoside-induced cytotoxicity was observed by microscope. After 12

d of incubation with 0.05 g/L hyperoside, no significant difference was found from that of the control. However, when the hyperoside concentration increased, cell injury caused by hyperoside was observed. According to the Reed-Meuench equation, the 50% toxic concentration ($TC_{50}$) was 0.115 g/L, and the maximum nontoxic concentration ($TC_0$) was 0.05 g/L (Table 1).

**Inhibitory effect of hyperoside on HBsAg and HBeAg expression in 2.2.15 cells** After 8 d of incubation, HBsAg and HBeAg produced in the culture medium were measured. The positive control drug was 3TC (0.05 g/L). The results showed that HBeAg and HBsAg of the cells incubated with hyperoside were less than that of the control cells, and the median effective concentrations ($IC_{50}$) were about 0.012 and 0.015 g/L, respectively, on d 4, 0.009 and 0.011 g/L, respectively, on d 8 (Tables 2, 3). The therapeutic indices, determined by $IC_{50}$ *vs* $TC_{50}$, were about 9.58, 12.78 of HBeAg, respectively, on d 4 and d 8 and 7.67 and 10.45 of HBsAg on d 4 and d 8, respectively (data not shown). The HBeAg inhibition rates of 3TC were 60.54% on d 4 and 54.68% on d 8, respectively (data not shown). The HBsAg inhibition rates of 3TC were 58.23% on d 4 and 41.48% on d 8 (data not shown). Table 2 shows that significant inhibition of HBeAg by hyperoside was observed at 0.0125 g/L ($P<0.01$), and a high inhibition was noted at the hyperoside concentration equal to 0.05 g/L on d 8. Furthermore, hyperoside also showed inhibitory activity on HBsAg excretion, about 82.27% at the concentration of 0.05 g/L on d 8. The inhibition rate percent-

**Table 1.** Cell toxicity of hyperoside on 2.2.15 cells.

| Parameter | Dose/g·L$^{-1}$ 0.20 | 0.10 | 0.05 | 0.025 | 0.0125 | 0 | $TC_{50}$/g·L$^{-1}$ | $TC_0$/g·L$^{-1}$ |
|---|---|---|---|---|---|---|---|---|
| CPE | ++++ | ++ | – | – | – | – | 0.115 | 0.05 |
| | ++++ | ++ | – | – | – | – | | |
| | ++++ | + | – | – | – | – | | |
| | ++++ | + | – | – | – | – | | |
| CPE% | 100% | 37.50% | | | | | | |
| CPE | ++++ | ++ | – | – | – | – | 0.121 | 0.05 |
| | ++++ | + | – | – | – | – | | |
| | ++++ | + | – | – | – | – | | |
| | ++++ | + | – | – | – | – | | |
| CPE% | 100% | 31.25% | | | | | | |
| CPE | ++++ | ++ | – | – | – | – | 0.108 | 0.05 |
| | ++++ | ++ | – | – | – | – | | |
| | ++++ | ++ | – | – | – | – | | |
| | ++++ | + | – | – | – | – | | |
| CPE% | 100% | 43.75% | | | | | | |
| Mean | | | | | | | 0.115 | 0.05 |

Positive cells(-); number of negative cells<25%, 25%–49%(++), 50%–75%(+++), and >75%(++++) in three experiments.

**Table 2.** Inhibitory effect of hyperoside on HBeAg expression in 2.2.15 cells. Each value is the mean±SD ($n$=9) of those cells treated with hyperoside. $^bP<0.05$, $^cP<0.01$ represent significant differences compared with the values seen in control groups.

| | 4 d | | | 8 d | | |
|---|---|---|---|---|---|---|
| Dose/g·L$^{-1}$ | cpm/Bq×10$^3$ | Inhibition/% | $IC_{50}$/g·L$^{-1}$ | cpm/Bq×10$^3$ | Inhibition/% | $IC_{50}$/g·L$^{-1}$ |
| 0.05 | 0.98±0.09$^c$ | 80.35±0.7 | | 0.89±0.08$^c$ | 86.41±1.5 | |
| 0.025 | 1.81±0.17$^c$ | 63.81±4.8 | | 1.23±0.17$^c$ | 77.87±5.0 | |
| 0.0125 | 2.47±0.15$^c$ | 50.78±1.8 | 0.012 | 2.45±0.25$^c$ | 60.16±7.4 | 0.009 |
| 0.00625 | 3.39±0.24$^b$ | 32.29±1.8 | | 3.86±0.16$^b$ | 37.48±6.8 | |
| 0.003125 | 4.17±0.18 | 16.72±1.7 | | 4.81±0.16$^c$ | 22.43±4.1 | |
| 0 | 5.01±0.17 | – | | 6.19±0.47 | – | |

**Table 3.** Inhibitory effect of hyperoside on HBsAg expression in 2.2.15 cells. Each value is the mean±SD ($n$=9) of those cells treated with hyperoside. $^bP<0.05$, $^cP<0.01$ represent significant differences compared with the values seen in control groups.

| | 4 d | | | 8 d | | |
|---|---|---|---|---|---|---|
| Dose/g·L$^{-1}$ | cpm/Bq×10$^3$ | Inhibition/% | $IC_{50}$/g·L$^{-1}$ | cpm/Bq×10$^3$ | Inhibition/% | $IC_{50}$/g·L$^{-1}$ |
| 0.05 | 1.76±0.12$^c$ | 67.83±0.7 | | 0.89±0.11$^c$ | 82.27±1.5 | |
| 0.025 | 2.04±0.17$^c$ | 62.44±2.3 | | 1.33±0.19$^c$ | 73.58±1.6 | |
| 0.0125 | 2.96±0.24$^c$ | 45.45±4.3 | 0.015 | 2.33±0.14$^c$ | 53.41±1.6 | 0.011 |
| 0.00625 | 3.99±0.10 | 26.58±3.7 | | 3.32±0.12$^b$ | 33.79±1.8 | |
| 0.003125 | 4.42±0.30 | 19.07±1.2 | | 4.07±0.07$^c$ | 18.86±3.4 | |
| 0 | 5.46±0.38 | – | | 5.02±0.16 | – | |

ages of both HBeAg and HBsAg were both time- and dose-dependent. It could also be observed that hyperoside had a relatively stronger inhibition on HBeAg than HBsAg.

***In vivo* anti-DHBV activities**

Inhibitory effect of hyperoside on DHBV-DNA Next, the anti-HBV activity of hyperoside was investigated *in vivo* using the DHBV-DNA-infected duckling model. During this experimental period, no significant side effects were observed in animals receiving antiviral therapy or in control animals. The levels of serum viral DNA were recorded in the 5 groups before the experiment (d 0). As shown in Table 4, with the exception of the control group, serum levels of DHBV-DNA of each group decreased with different extents after treatment with hyperoside and 3TC on d 5 and d 10, respectively. Among these, the hyperoside 0.10 g·kg$^{-1}$·d$^{-1}$ group, the 0.05 g·kg$^{-1}$·d$^{-1}$ group, and the 3TC group showed a significant decrease of DHBV-DNA ($P$<0.01). Three days after the cessation of treatment with 3TC, the viral replication level returned to the pretreatment baseline. In the ducks treated with hyperoside, the effect of DHBV-DNA inhibition lasted (Figure 1). The mean percentage inhibition of viral DNA levels with hyperoside 0.10 and 0.05 g·kg$^{-1}$·d$^{-1}$ was 60.94% and 56.24%, respectively, on the last treatment day (d 5).

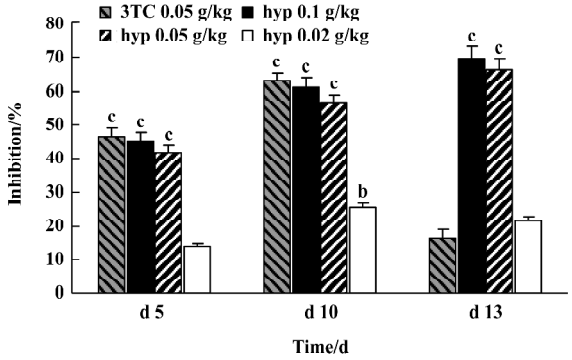


**Figure 1.** Inhibition rate of hyperoside on DHBV-DNA. Each value is the mean±SD ($n$=16) of ducks treated with hyperoside. $^bP<0.05$ and $^cP<0.01$ represent significant differences compared with the values seen in control groups.

**Histopathological features** Histopathological profiles of the liver from the model group ducklings revealed necrosis, steatosis, and often swelling of the hepatic cytoplasm. The protective effect of hyperoside was confirmed by histopatho-

**Table 4.** Mean changes of serum DHBV DNA level in treated duck. The serum DHBV DNA level was represented by optical density value (*OD*). Each value is the mean±SD ($n$=16) of ducks treated with hyperoside. $^bP<0.05$ and $^cP<0.01$ represent significant differences compared with the values before experiment.

| Group | Dose/g·kg$^{-1}$·d$^{-1}$ | *OD* | | | |
|---|---|---|---|---|---|
| | | d 0 | d 5 | d 10 | d 13 |
| Hyperoside | | | | | |
| | 0.10 | 1.357±0.20 | 0.726±0.19$^c$ | 0.532±0.19$^c$ | 0.410±0.16$^c$ |
| | 0.05 | 1.334±0.15 | 0.776±0.20$^c$ | 0.596±0.37$^c$ | 0.456±0.19$^c$ |
| | 0.02 | 1.305±0.10 | 1.145±0.21 | 1.015±0.20$^b$ | 1.062±0.24 |
| 3TC | 0.05 | 1.311±0.21 | 0.710±0.24$^c$ | 0.501±0.14$^c$ | 1.130±0.15 |
| Control | — | 1.321±0.12 | 1.329±0.11 | 1.362±0.06 | 1.354±0.06 |

logical examinations. Administration of hyperoside to the experimental animals (0.10 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 2).

## Discussion

Hepatitis B is a major epidemic disease in South-East Asia, China, and Africa, where approximately 10% of the population are chronic carriers[23]. HBV replicates within infected hepatocytes and expresses viral epitopes on them to induce T-cell mediated immune responses to cause hepatitis. Currently, there are 2 arms of therapy to manage chronic active hepatitis B: direct antiviral therapy to inhibit replication of HBV or indirect immunomodulatory therapy to enhance cellular immunity to destroy the virus-infected hepatocytes.

Due to the low efficiency and many limitations of immunomodulatory therapy with IFN-α, direct antiviral therapy could have increasing importance. However, although direct antiviral therapy with lamivudine could efficiently control chronic active hepatitis B, drug resistance could develop progressively after 6–9 months of the initiation of therapy[24,25]. These unsatisfactory therapeutic results strengthen the need for new anti-HBV agents. In this report, our results imply that hyperoside possesses anti-HBV activity.

In our experiment, the data shows that the $TC_{50}$ of hyperoside was 0.115 g/L and the $TC_0$ was 0.05 g/L in 2.2.15 cells, which suggests that the inhibitory activity of hyperoside had no cytotoxicity. In the nontoxic concentration, hyperoside inhibited HBeAg and HBsAg in a dose- and time-dependent manner. Nevertheless, hyperoside showed stronger inhibition on HBeAg with $IC_{50}$=0.012 g/L than HBsAg with $IC_{50}$=0.015 g/L on d 4. At 0.05 g/L, the inhibition rate percentage of hyperoside on HBeAg and HBsAg in 2.2.15 cells were 86.41% and 82.27%, respectively. Recent research showed that another flavonoid from *Phyllanthus urinaria* ellagic acid effectively blocked HBeAg secretion ($IC_{50}$=0.07 mg/mL). However, compared to hyperoside, it had no effect on HBsAg[26].

As an HBV-infected animal model, DHBV-infected Peking ducks were also used to evaluate the effects of hyperoside against HBV. At 0.05 and 0.10 g·kg$^{-1}$·d$^{-1}$, hyperoside significantly decreased DHBV replication with the inhibition rate percentage 65% and 70%, respectively, on d 10. On d 13, 3TC showed a rebound effect because of drug cessation like most antiviral drugs. However, in contrast with 3TC, the inhibition rate percentage of hyperoside on HBV-DNA showed no rebound after cessation on d 13. It indicated that hyperoside could maintain for a long time in treating viremia of HBV, and the effect of DHBV-DNA inhibition showed a concentration-dependent response. From histopathological examination, we could also confirm the protective effect of hyperoside on the livers of DHBV-infected ducklings. In summary, our results show that hyperoside possesses anti-HBV activity whether the tests were done *in*

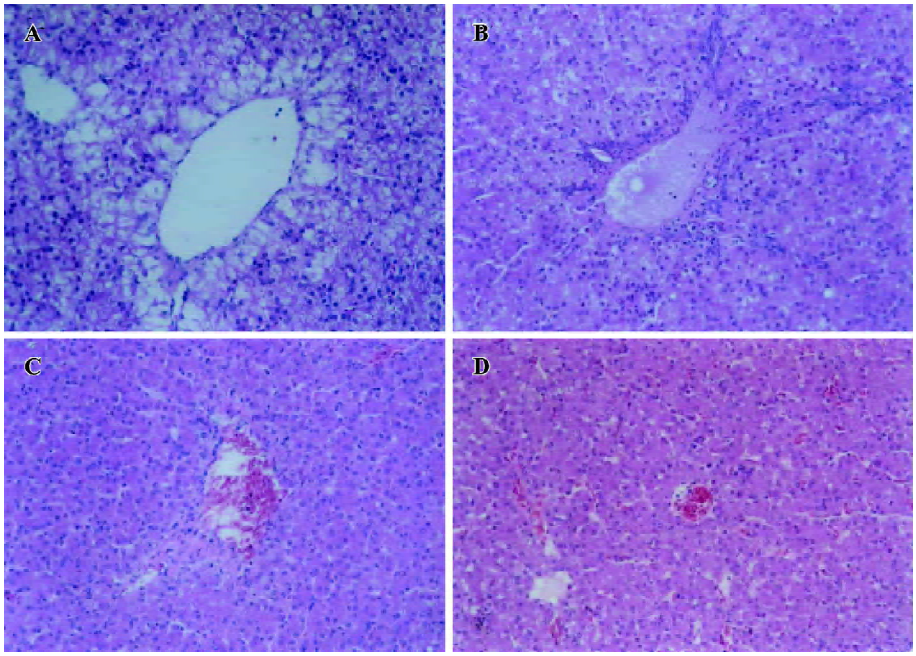


**Figure 2.** Histopathological examinations of liver in ducklings treated for 13 d. (A) model group; (B,C,D) hyperoside-treated group at a dose of 0.02, 0.05, and 0.10 g·kg$^{-1}$·d$^{-1}$, respectively. Hematoxylin-eosin staining: (A, B, C, D) ×20.

*vivo* or *in vitro*.

It is necessary to reveal the possible mechanisms of the anti-HBV activity of hyperoside. During the replication of hepadnaviruses DNA, DNA polymerase was a target enzyme of antihepatitis drugs. Therefore, further investigation of the DNA polymerase level in HBV replication is required. Xiong *et al*[18] used the *in vitro* D-GalN/TNF-α model to confirm that many flavonoids, including hyperoside, protect liver cells from TNF-α-induced hepatocyte apoptosis. TNF-α has been found to be a very important pathogenic mediator in patients with alcoholic liver disease and viral hepatitis, as well as in many animal liver injury models[27]. Recent research showed that the activation of the annexin A7 (Axn7) gene and the expression of the Axn7-GFP fusion protein could cause a decrease in HBsAg secretion[28]. However, the precise mechanism of the anti-HBV activity of hyperoside needs further investigation.

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