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Anti-hepatitis B virus activity of chlorogenic acid, quinic acid and caffeic acid in vivo and in vitro

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ABSTRACT

Chlorogenic acid and its related compounds are abundant plant polyphenols that have a diverse antiviral activity. In this study, HepG2.2.15 cells and duck hepatitis B virus infection model were used as in vitro and in vivo models to evaluate their anti-HBV activity. In the cell model, all the three compounds inhibited HBV-DNA replication as well as HBsAg production. Chlorogenic acid and caffeic acid also reduced serum DHBV level in DHBV-infected duckling model. Moreover, the anti-HBV activity of crude extracts of coffee beans, which have a high content of chlorogenic acid, was studied. Both the extracts of regular coffee and that of decaffeinated coffee showed inhibitory effect on HBV replication.

1. Introduction

Human hepatitis B virus (HBV) is the major epidemiological agent of acute and chronic hepatitis and HBV carriers have a high risk of development of cirrhosis and hepatocellular carcinoma. At present, there are two different therapeutic options for chronic HBV treatment, including immunomodulators and nucleoside analogs. However, their clinical utility is limited by the side effects of interferon α and the substantial resistance of the virus to early nucleosidic inhibitors (Buti and Esteban, 2005). Therefore, alternative strategies and drugs are being sought to combat this disease.

Several hundred of plant and herb species have been reported to have potential antiviral activity and a wide variety of active phytochemicals have been identified (Chattopadhyay and Naik, 2007; Jassim and Naji, 2003; Naithani et al., 2008). Chlorogenic acid is an important plant polyphenol that is widely distributed in the leaves and fruits of dicotyledonous plants such as coffee beans. It is the ester of caffeic acid with quinic acid (Fig. 1). Both chlorogenic acid and caffeic acid are powerful antioxidants and have been reported to have multi-antiviral activities against HIV (McDougall et al., 1998; Tamura et al., 2006), adenovirus (Chiang et al., 2002), HSV-1 and HSV-2 (Chiang et al., 2002; Khan et al., 2005). However, Helbig et al. reported that the polymeric oxidation products of chlorogenic acid showed antiviral activity against HSV-1, while the starting compound, chlorogenic acid, failed to inhibit viral replication (Helbig et al., 1997). The controversial results may be due to the different antiviral evaluating systems. Although the antiviral activities of chlorogenic acid and its hydrolysates have been well studied, little is mentioned about their activity against HBV, except that Namba and Matsuse (2002) have described that the suppression of hepatitis B virus surface antigen (HBsAg) was closely associated

Abbreviations: HBV, hepatitis B virus; HBsAg, hepatitis B virus surface antigen; HBeAg, hepatitis B e antigen; ELISA, enzyme-linked immuno ab sorbent assay; DHBV, duck hepatitis B virus; HIV, human immunodeficiency virus; HSV, herpes simplex virus; MEM, minimal essential medium; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide; RSV, respiratory syncytial virus; SARS, severe acute respiratory syndrome; SDS, sodium dodecyl sulfate; SRM, selected reaction monitoring; TPA, 12-O-tetradecanoylphorbol-13-acetate.

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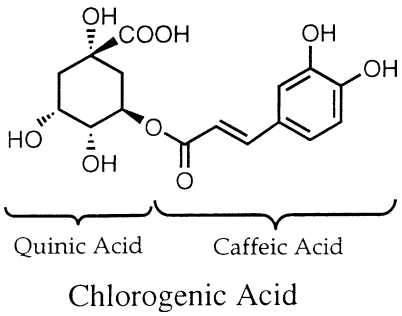

Chlorogenic Acid

Fig. 1. Chlorogenic acid is the ester of caffeic acid with quinic acid (McDougall et al., 1998).

to the presence of caffeic acid derivatives, especially chlorogenic acid (Namba and Matsuse, 2002).

To clarify whether chlorogenic acid, quinic acid and caffeic acid affect HBV replication, their antiviral activity was assessed in HepG2.2.15 cell line (Sells et al., 1987) and in the DHBV-infected duckling model. Results showed that chlorogenic acid, quinic acid and caffeic acid inhibited HBV replication in a dose-dependent manner in vitro and the inhibitory effect of chlorogenic acid and caffeic acid was also demonstrated in vivo. Considering that chlorogenic acids and its hydrolysates are the main components of the phenolic fraction of coffee beans, the antiviral activity of crude coffee extracts was also determined in HepG2.2.15 cell line. Data demonstrated that the coffee extract also inhibited HBV-DNA replication in vitro.

2. Materials and methods

2.1. Chemicals

Chlorogenic acid, caffeic acid and quinic acid (98–99% pure) were extracted from coffee beans. These pure compounds were also obtained from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China) for chlorogenic acid; Shanghai No. 1 Reagent Corporation (Shanghai, China) for caffeic acid and Alfa Aesar (Beijing, China) for quinic acid. Lamivudine was chemically synthesized in our Institute; α-^{32}P-dCTP was purchased from Beijing FuRui Biotechnology and Engineering Corporation (Beijing, China); the Nick translation kit was from Promega Corporation; Sephadex G-50, and Ficoll PVP were obtained from Pharmacia Corporation (Sweden), SDS was from Merck (Germany) and nitrocellulose filters were from Amersham Biosciences (England).

2.2. Cell culture and antiviral assays

Confluent cultures of HepG2.2.15 cells were treated with various doses of antiviral compounds in MEM supplemented with 10% fetal bovine serum (Hyclone). Fresh medium with the same concentration of compounds was replaced at day 4. The cells and culture medium were collected at day 8 for the intracellular and extracellular antiviral assays, respectively.

2.3. Cytotoxicity measurement

Cytotoxicity was assessed by the MTT assay as previously described (Wu et al., 2005). Briefly, HepG2.2.15 cells were cultured in triplicate for 8 days with compounds tested. The cells with media alone were used as untreated controls. MTT (5 g/l) reagent was added 4 h before the end of culture, and then cells were lysed with 10% SDS, 50% N,N-dimethyl formamide (pH 7.2), OD values were read at 570 nm after 6 h and the percent of cell death was calculated.

2.4. Measurement of secreted HBsAg and HBeAg

HBsAg and HBeAg in the culture medium were measured by using specific-ELISA kits (Sino-American biotechnology company, Henan, China) following the Manufacturer's recommendations. The supernatants collected after 8 days treatment were centrifuged at 4000 rpm for 5 min and used for ELISA.

2.5. Extraction of HBV-DNA in HBV core particles

HepG2.2.15 cells were treated with antiviral agents as described above. For the intracellular assay, the cells were collected for two purposes: half amount for quantifying HBV-DNA in core particles (Staprans et al., 1991), and the other half for quantifying cellular housekeeping gene glyseraldehyde-3-phosphate dehydrogenase (GAPDH) as quantitative reference. Briefly, for core particle HBV-DNA quantification, cells were lysed in 1 ml of 10 mM Tris (pH 7.5)/1 mM EDTA/50 mM NaCl/8% sucrose/0.25% Nonidet P-40. Nuclei and cell debris were removed by centrifugation at 13,000 rpm for 3 min at 4 °C, and the cytoplasmic extract was adjusted to 6 mM $MgCl_2$ and digested with DNase I (50 mg/ml) and RNase A (20 mg/ml) for 30 min at 37 °C. Cores were precipitated by adding 330 μl of 26% polyethylene glycol/1.4 M NaCl/25 mM EDTA, incubated at 4 °C for 30 min, and pelleted in an Eppendorf microcentrifuge for 4 min. Cores resuspended in 100 μl of 10 mM Tris (pH 7.5)/6 mM $MgCl_2$ were then redigested with DNase I for an additional 15 min at 37 °C, then HBV-DNA from the core was extracted and tested by real time fluorescent PCR. HepG2.2.15 genome (extracted by DNA fast200 of FASTAGEN Company, China) was used as template for GAPDH quantification.

2.6. Quantitative HBV PCR

HBV-DNA was quantified by using fluorescent PCR (Li et al., 2006). Briefly, 50 μl of the supernatants or core particle suspension was added into the extraction buffer, boiled for 10 min and centrifuged for 5 min, and then proper aliquots were used for the fluorescent PCR. Primers specific for HBV-DNA detection were: P1: 5′-ATCCTGCTGCTATGCCTCATCTT-3′, P2: 5′-CAGTGGG GAAAGCCCTACGAA-3′, and the probe was 5′-TGGCTAGTTTACTAGTGCCAT TTTG-3′. Primers specific for GAPDH: P3: 5′-GGTATCGTGGAAGGACTCATGAC-3′, P4: 5′-ATGCCAGTGAGCTTCCCGTTCAGC-3′.

2.7. Measurement of HBV-DNA by Southern blotting

HepG2.2.15 cells were inoculated at a density of 6×10^5 per 10 cm dish, and were treated with the tested compounds. Total DNA was extracted from 3×10^6 cells (Qiagen, Dneasy Tissue Kit). 10 μg DNA were digested by HindIII at 37 °C, and then precipitated in two volumes of ethanol. DNA was dissolved in double distilled water and separated on a 0.8% agarose gel, then analyzed by Southern blotting using a DIG-labeled full-length HBV-DNA probe as Sells et al. (1987) described with some modification.

2.8. Coffee powder and its crude extraction

Regular coffee powder (100% pure coffee, Rich Original from Maxwell House Coffee Company, NY) and decaffeinated coffee (Melitta, Decaf Classic, Medium Roast, 100% pure Arabica coffee, containing not more than 0.4% caffeine, the Melitta Group, Germany) were purchased in the supermarket (Shanghai, China). Forty grams coffee powder was brewed in a coffee pot with accessory

filter (Philips, HD7400, made in China) to obtain approximately 400 ml of fresh coffee, and lyophilized into crude extracts.

2.9. Determination of the contents of chlorogenic acid, quinic acid and caffeic acid in crude coffee extracts

The contents of the three compounds in coffee crude extracts were determined by the HPLC–ESI-MS/MS method. HPLC conditions were: flow rate: 0.3 ml/min; column: Zorbax XDB-C18 column (particle size 5 μm, 50 mm × 2.1 mm i.d.; Chadds Ford, PA) protected with an additional 0.2 μm filter (Upchurch Scientific, Oak Harbor, WA) and the injection volume: 10 μl. Mass spectrometer conditions: the spray voltage 3200 V, the sheath gas (N_2 gas) flow rate 25 Arb, the auxiliary gas (N_2 gas) flow rate 5 Arb, the capillary temperature 350 °C, source CID 10 V, the tube lens offset 180 V and the collision energy were 26, 19 and 28 V for chlorogenic acid, quinic acid and caffeic acid, respectively. The selected reaction monitoring (SRM) ion transitions used for quantification were m/z 353 → 191 for chlorogenic acid, 179 → 135 for caffeic acid, 191 → 85 for quinic acid. Peaks were only considered if the peak height was at least fivefold higher than the baseline noise.

2.10. Experimental inoculation of ducklings

Ducklings were maintained under normal daylight, fed with standard commercial diet and water, in accordance with the guidelines for animal care at the facilities of the Institute of Medicinal Biotechnology, Chinese Academy of Medical Sciences and Peking Union Medical College, under permit of the Ethical Committee for Animal Experiments of the Institute of Medicinal Biotechnology with notification number SYXK (Jing) 2007-0013. One-day-old Pekin ducklings were inoculated intravenously with DHBV-positive serum of Shanghai Sheldrake. Drugs were administered orally (bid) for 10 days and treatment was started at the seventh day after infection. The antiviral activity of the inhibitors was assessed by comparing the serum DHBV-DNA levels of inhibitor-treated and control ducklings at initiation of treatment (T0), the fifth day of treatment (T5), the tenth day of treatment (T10) and the third day (P3) of post-treatment follow-up. On day T0, T5, T10 and P3, blood samples were taken, and sera were prepared for analysis of DHBV-DNA levels.

2.11. Analysis of DHBV-DNA

DHBV-DNA from the serum of experimentally infected ducklings was detected by a specific dot blot assay at different time points as indicated. Samples of 50 μl of serum were spotted directly on nitrocellulose filters. After denaturation (0.5 M NaOH, 1.5 M NaCl), neutralization (0.5 M Tris–HCl [pH 8.0] with 1.5 M NaCl followed by 2XSSC), and fixation (80 °C for 2 h), filters were hybridized with a full-length, α-^{32}P-labeled DHBV genomic DNA probe. Filters were autoradiographed and quantitative measurement was done by examination of OD values (Han et al., 2008).

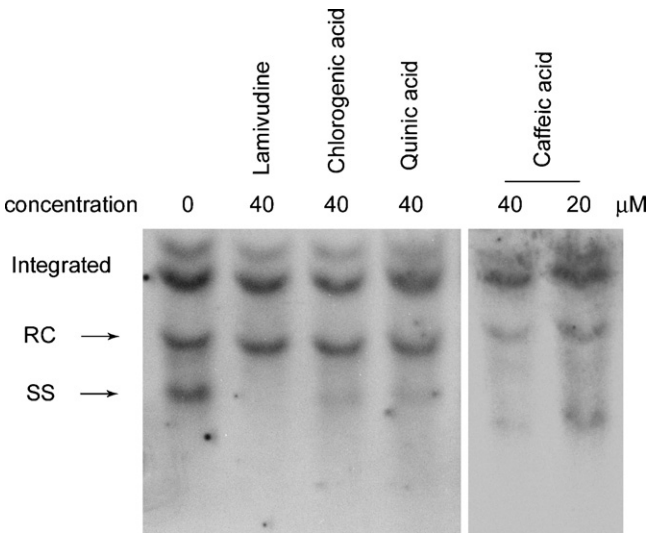

Fig. 2. Southern blotting analysis of HBV-DNA from HepG2.2.15 cells treated with chlorogenic acid, quinic acid or caffeic acid. Total DNA was extracted from HepG2.2.15 cells, digested with HindIII, separated by agarose gel electrophoresis and transferred to nylon membrane. HBV-DNA was detected by a DIG-labeled full-length HBV-DNA probe. Forms of HBV-DNA are indicated as integrated, relaxed circular (RC) and single-stranded (SS) HBV-DNA. Lamivudine was used as a positive control.

3. Results

3.1. Anti-HBV replication activity of chlorogenic acid, quinic acid and caffeic acid

HepG2.2.15 cells were cultured for 8 days in the presence or absence of tested compounds with the concentrations varying from 0.14 to 1000 μM. Lamivudine was used as a positive control. Data demonstrated that the three compounds reduced not only the virion DNA in the culture medium but also the core-associated DNA in the cytoplasma of HepG2.2.15 cells with little cytotoxicity (Table 1). The inhibitory effect of these compounds were also analyzed by the southern blotting assay (Fig. 2) The single-stranded form of HBV-DNA was almost undetectable at the indicated concentration. Altogether, these results suggested that chlorogenic acid and related compounds had inhibiting effects on HBV-DNA replication in vitro.

3.2. Anti-HBV antigens secretion activity of chlorogenic acid, quinic acid and caffeic acid

After 8 days with compound treatment, chlorogenic acid and quinic acid inhibited HBsAg secretion to some extent in HepG2.2.15

Table 1
Analysis of HBV replication and antigen secretion in HepG2.2.15 cells treated with chlorogenic acid, quinic acid and caffeic acid.

Compound	CC_{50}[a] (μM)	IC_{50}[b] (μM)			
		Extracellular			Intracellular
		HBV-DNA	HBsAg	HBeAg	HBV-DNA
Chlorogenic acid	>1000	1.2 ± 0.4	241.5 ± 198.2	>1000	1.3 ± 0.01
Quinic acid	>1000	10.1 ± 4.7	478.0 ± 179.6	>1000	1.6 ± 0.04
Caffeic acid	500 ± 200	3.9 ± 1.1	12.7 ± 9.9	109.3 ± 56.0	0.7 ± 0.2
Lamivudine	>1000	0.3 ± 0.04	294.9 ± 35.2	>1000	0.5 ± 0.1

The mean value of IC_{50} was from the results of three independent experiments (mean ± S.D).

[a] The cytotoxicity concentration of compound that reduced cell viability to 50% (CC_{50}).

[b] The concentrations of compound needed to inhibit HBV-DNA replication, HBsAg and HBeAg secretions to 50% (IC_{50}).

Table 2
Anti-HBV activity of crude water extracts of coffee beans.

Crude extracts of fresh coffee	CC_{50} (μg/ml)	IC_{50} (μg/ml)			
		Extracellular			Intracellular
		HBV-DNA	HBsAg	HBeAg	HBV-DNA
Regular	447 ± 110	82.4 ± 23.2	62.5 ± 6.5	133.6 ± 29.2	141.2 ± 37.5
Decaffeinated	403 ± 47	9.5 ± 8.5	41.7 ± 20.3	47.4 ± 13.2	46.1 ± 21.9

The mean value of IC_{50} was from the results of three independent experiments (mean ± S.D).

cell, but did not inhibit HBeAg secretion at a dose up to 1000 μM. However, caffeic acid showed a stronger inhibiting effect on both HBsAg and HBeAg secretion. Lamivudine, as a positive control, exhibited an inhibition of HBsAg secretion at an IC_{50} of 295 μM and showed little inhibition of HBeAg secretion at a dose up to 1000 μM (Table 1).

3.3. Anti-HBV activity of crude coffee extracts

To determine the anti-HBV activity of coffee beans, HepG2.2.15 cells were cultured for 8 days in the absence or presence of coffee extracts with the concentrations varying from 1.37 to 1000 μg/ml. As shown in Table 2, both the crude extract of regular coffee and that of decaffeinated coffee showed an inhibitory effect on HBV replication. The amount of virions in the culture medium and intracellular HBV-DNA were reduced by treatment with the crude extracts of regular coffee and decaffeinated coffee, and they also showed their efficacy on the egression of viral antigens. These results suggested that crude extracts of coffee could inhibit HBV-DNA replication and antigen secretion in HepG2.2.15 cells.

3.4. Contents of chlorogenic acid, quinic acid and caffeic acid in crude extracts of coffee beans

The amount of chlorogenic acid, quinic acid and caffeic acid contained in the crude water extracts of coffee beans has been studied. Their contents in regular coffee were about 43.3 ± 0.7, 41.6 ± 1.0 and 1.2 ± 0.03 mg/g, respectively, and those in decaffeinated coffee were about 63.4 ± 0.4, 28.7 ± 0.2 and 1.5 ± 0.00 mg/g, respectively. Altogether, the extract of regular coffee contained about 8.6% of the three compounds, and the content in the extract of decaffeinated coffee was about 9.36%.

3.5. Anti-DHBV replication in the experimentally infected duckling model after short-term administration of chlorogenic acid, quinic acid and caffeic acid

The antiviral activity of chlorogenic acid, quinic acid and caffeic acid was further evaluated in the DHBV-infected duckling model. Seven days after inoculation of virus, compounds were administrated orally at a dose of 100 mg/kg (six animals in each group) twice a day for 10 days. Lamivudine was given at a dose of 50 mg/kg and used as positive control. As shown in Table 3, chlorogenic acid reduced the DHBV viremia on T5, T10 and P3 with statistical significance. Its inhibitory efficacy was even higher than that of lamivudine. Especially on P3 DHBV-DNA rebounded in the lamivudine-treated group, but it showed sustain inhibition of DHBV-DNA in the chlorogenic acid-treated group. The DHBV viremia was also decreased by caffeic acid but only slightly changed by quinic acid. Hence, the above results indicated that chlorogenic acid and caffeic acid inhibited DHBV replication in the experimentally infected duckling model.

4. Discussion

Chlorogenic acid and related compounds are abundant natural polyphenol products and have been reported to demonstrate diverse biological actions such as antiviral (Khan et al., 2005; Chiang et al., 2002; McDougall et al., 1998; Tamura et al., 2006), antioxidant (Hung et al., 2006; Pavlica and Gebhardt, 2005), anti-carcinogenesis (Miura et al., 2007; Shimizu et al., 1999), hepatoprotective actions (An et al., 2008) and modulation of signal transduction pathway (Feng et al., 2005). In this study, we found that chlorogenic acid, quinic acid and caffeic acid possessed a substantial inhibitory activity against HBV-DNA replication in HepG2.2.15 cells. After 8 days treatment, both the virions in the culture medium and the core-associated DNA in the cytoplasma were reduced, and the level of HBsAg secreted in the culture medium was also down-regulated. However, the amount of HBeAg in the culture medium was decreased only by caffeic acid, suggesting that caffeic acid might perform its antiviral activity in a way different from the other two. Chlorogenic acid and caffeic acid also showed efficacy in the DHBV-infected duckling model.

Coffee is one of the most popular beverages all over the world. Chlorogenic acid, the ester of caffeic and quinic acid, is found widespread in plants and in high concentration in coffee: a single cup may contain 70–350 mg chlorogenic acid (Clifford, 1999). A growing body of evidence from epidemiological investigations suggests that coffee consumption is inversely associated with the risk of chronic liver disease, cancer and cirrhosis (Bravi et al., 2007; Corrao et al., 2001; Nkondjock, 2009; Ruhl and Everhart, 2005; Shimazu et al., 2005). Recently, Utsunomiya et al. reported that coffee extracts inhibited the multiplication of HSV-1 and poliovirus in vitro (Utsunomiya et al., 2008). However, little is known about the mechanism underlying these effects.

Table 3
Oral administration of chlorogenic acid, quinic acid and caffeic acid reduced DHBV-DNA level in DHBV-infected duckling serum.

Compounds	Dose	[a]Inhibition of DHBV-DNA in sera (%)		
	mg/kg, bid, p.o. for 10 days	Treatment for 5 days (T5)	Treatment for 10 days (T10)	Three days after withdrawal (P3)
Placebo control	–	−9.84	−9.63	−5.49
Chlorogenic acid	100	43.26**	31.18**	30.93**
Quinic acid	100	−14.73	5.11	8.39
Caffeic acid	100	21.03**	12.66	17.23*
Lamivudine	50	10.22*	22.29**	13.94

[a] Treatment with compounds started at 7 days post-inoculation. Inhibition% means [DHBV-DNA (T5, T10 or P3) − DHBV-DNA (T0)]/DHBV-DNA (T0) × 100%.
* $p < 0.05$, ** $p < 0.01$, compared to the placebo-treated group.

Comparing the world HBV distribution map (Center of Disease Control and Prevention, 2006) with the world coffee markets (United States Department of Agriculture, 2007), an interesting phenomenon attracted us that some countries with high consumption of coffee have relatively low incidence of HBV infection, such as Finland, Sweden and Norway, although other factors including race, environment, health care, and dietary habits should be taken into consideration. Since chlorogenic acid and its related compounds are abundant in coffee beans, we supposed that coffee extracts might have anti-HBV activity. Results turned out that the crude water extract of coffee beans, which had high content of chlorogenic acid, inhibited HBV-DNA replication as well as HBsAg and HBeAg secretion in vitro, and its polyphenol component possibly contributed to this effect. Caffeine seemed irrelevant to the antiviral activity, as the extracts of decaffeinated coffee showed even stronger effect than that of regular coffee.

Chlorogenic acid, as well as caffeic and quinic acid, is long known as antioxidant, and might therefore contribute to its anti-carcinogenic properties. Chlorogenic acid was shown to protect against TPA-induced carcinogenesis by up-regulation of cellular antioxidant enzymes and suppression of ROS-mediated NF-kappaB, AP-1, and MAPK activation (Feng et al., 2005). Additionally, Chiang et al. (2002) reported that caffeic acid inhibited HSV and adenovirus replication after infection without preventing virus adsorption, caffeoylquinic acid analogues are also active against HIV (Cos et al., 2004, 2008) and RSV (Li et al., 2005). Some caffeoylquinic acids showed activity against HIV-1 integrase, but caffeic acid and chlorogenic acid were not active. The mechanisms of action for the three compounds against HBV may be not similar. Chlorogenic acid and quinic acid reduced the amount of HBV-DNA more effectively than that of the viral antigens. However, the levels of both HBsAg and HBeAg secreted in culture medium were effectively reduced by caffeic acid with different IC_{50}s, suggesting it may interfere with the capsid envelopment and/or the viral egression. The precise mode(s) of action of chlorogenic acid and its hydrolysates need to be further explored thoroughly.

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