Kobe J. Med. Sci., Vol. 65, No. 2, pp. E71-E79, 2019

Antiviral Activity of *Cananga odorata* Against Hepatitis B Virus

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Received 10 June 2019 / Accepted 2 July 2019

Keywords: Antiviral, hepatitis B virus, Hep38.7-Tet cells, HepG2-NTCP cells, *Cananga odorata*.

Chronic hepatitis B virus (HBV) infection can lead to liver cirrhosis and hepatocellular carcinoma. Current therapeutic drugs for chronic hepatitis B using pegylated interferons and nucleos(t)ide analogs have limited efficacy. Therefore, the development of novel and safe antivirals is required. Natural products including medicinal plants produce complex and structurally diverse compounds, some of which offer suitable targets for antiviral screening studies. In the present study, we screened various crude extracts from Indonesian plants for anti-HBV activity by determining their effects on the production of extracellular HBV DNA in Hep38.7-Tet cells and HBV entry onto a HBV-susceptible cell line, HepG2-NTCP, with the following results: (1) In Hep38.7-Tet cells, *Cananga odorata* exhibited the highest anti-HBV activity with a 50% inhibitory concentration (IC_{50}) of 56.5 µg/ml and 50% cytotoxic concentration (CC_{50}) of 540.2 µg/ml (Selectivity Index: 9.6). (2) The treatment of HepG2-NTCP cells with *Cassia fistula*, *C. odorata,* and *Melastoma malabathricum* at concentrations of 100 µg/ml lowered the levels of HBsAg production to 51.2%, 58.0%, and 40.1%, respectively, compared to untreated controls, and IC_{50} and CC_{50} values of *C. odorata* were 142.9 µg/ml and >400 µg/ml. In conclusion, the *C. odorata* extract could be a good candidate for the development of anti-HBV drugs.

INTRODUCTION

Chronic hepatitis B virus (HBV) infection, a major health concern globally, increases the risk of developing liver cirrhosis and hepatocellular carcinoma. Despite the availability of preventive vaccines, there are approximately 250 million chronically infected people and in 2015, approximately 0.8 million deaths from hepatitis B-related diseases (1, 2). The prevalence of HBV infection is highly variable geographically with the largest number of infected individuals in Asia Pacific, Africa, and Western Pacific regions (1, 2). HBV has been classified into 10 genotypes (A-J) with more than 30 sub-genotypes (1, 3). HBV genotypes and certain subtypes play a role in infection manifestation, progression to complication, and response to antiviral treatment (1). Pegylated interferon-α (Peg-IFNα) and nucleos(t)ide analogs are currently approved for the treatment of chronic hepatitis B patients; however, those therapies have limited efficacy such as side effects and the possible emergence of HBV drug resistance (4). Therefore, the development of safe and more effective anti-HBV drugs is required.

HBV is an enveloped DNA virus that belongs to the family *Hepadnaviridae*, which has a partially double-stranded relaxed-circular DNA (rcDNA) and is approximately 3.2 kb in size. The HBV life cycle begins with virion binding to the surface of hepatocytes through a high affinity receptor, sodium taurocholate cotransporting polypeptide (NTCP) (5). After the uncoating of the HBV virion in the cytoplasm, the nucleocapsid carrying the rcDNA is released, followed by the transportation of rcDNA into the nucleus, where rcDNA is converted into covalently closed circular DNA (cccDNA). HBV cccDNA is transcribed into four major mRNAs encoding the viral proteins: HBV core (HBc), HBV e (HBe), HBV polymerase (Pol), HBV surface (HBs), and HBV X (HBx) proteins, including HBV-pregenomic RNA (pgRNA). The pgRNA is encapsidated within core particles of HBV

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and reverse transcribed by Pol to produce new rcDNA. Capsid-containing rcDNA is enveloped with HBs and released from the hepatocytes or recycled back into the nucleus to maintain the cccDNA pool. *In vitro* models for the study of the entire HBV life cycle, such as HepAD38 (6), HepG2.2.15 (7), and HepG2-NTCP cells (8), have recently been developed. The use of such culture systems has facilitated the development of direct-acting antivirals and host-targeting antivirals for the treatment of HBV.

Medicinal plants have been used for the treatment of numerous human diseases including infectious diseases since ancient times (9). Plants produce a wide variety of secondary metabolites, which possess unique chemical structures and bioactivities. A number of phytochemicals, including terpenoids, lignans, flavonoids, saponins, secoiridoids, lactones, and alkaloids, have been reported to suppress various viruses including HBV (9-11). Crude extracts of *Guiera senegalensis*, *Pulicaria crispa*, and *Fumaria parviflora* have been reported to possess anti-HBV activity (12). Ethanol extracts of *Artemisia capillaris* and its isolates, chlorogenic acid and chlorogenic acid analogs, inhibit both the secretion of HBs antigens (HBsAg) and HBeAg, and the replication of HBV DNA (11). Ethanol extracts of *Boehmeria nivea* leaves and roots have anti-HBV effects (13). Therefore, further studies exploring antiviral substances derived from medicinal plants is an attractive approach for the development of novel anti-HBV drug candidates.

Indonesia is one of the most biodiverse countries, with approximately 40,000 endemic plant species including 6,000 medicinal plants (14), which potentially offers an opportunity for the collection of numerous and diverse plant extracts for anti-HBV drug screening. Research Center for Science and Technology (PUSPIPTEK) botanical garden in Banten province has approximately 230,000 plants collected from different regions in Indonesia. In the present study, crude extracts of certain Indonesian medicinal plants collected from PUSPITEK botanical garden were examined for antiviral activity against HBV using two *in vitro* model systems: Hep38.7-Tet cells and HepG2-NTCP cells.

MATERIALS AND METHODS

Cell cultures and reagents

Human hepatocellular carcinoma HepG2 cells were cultured in Eagle's Minimum Essential Medium (Fujifilm Wako, Kyoto, Japan) containing 10% fetal bovine serum (FBS, Biowest, Nuaillé, France), 100 IU/ml penicillin/streptomycin (Nacalai Tesque, Kyoto, Japan), and nonessential amino acids (Nacalai Tesque). HepG2-NTCP cells (8) were cultured in Dulbecco's Modified Eagle Medium/Nutrient Mixture F12 with GlutaMAX (Thermo Fisher Scientific, Carlsbad, CA), supplemented with 10% FBS, 10 mM HEPES, 5 µg/ml insulin (Fujifilm Wako), 100 IU/ml penicillin/streptomycin, and 1 mg/ml G418 (Nacalai Tesque). Hep38.7-Tet cells (15) were cultured in basal culture medium with a composition similar to that used for the HepG2-NTCP cells, although with the addition of 400 µg/ml G418 and 400 ng/ml tetracycline (Fujifilm Wako). All cells were maintained at 37°C in a 5% CO_2 atmosphere in a humidified incubator.

Plant collection and preparation of crude extracts

Plant materials were collected from PUSPIPTEK botanical garden, in Serpong, Banten province, Indonesia, and identified by PUSPIPTEK botanists. Forty-six methanolic crude extracts from 43 Indonesian plants obtained from different plant parts (leaf, root, bark, fruit, and whole plant) were prepared as described previously (16). The crude extracts were dissolved in DMSO to obtain 100 mg/ml stock solutions and stored at –30°C. The complete list of samples is available upon request.

HBV preparation

HBV (genotype D) in this study was prepared from culture supernatant of Hep38.7-Tet cells as described previously (17, 18).

Hep38.7-Tet cells assay to assess antiviral effects on the post-viral entry stage

Hep38.7-Tet cells (1×10^5 cells/well) were seeded into a 24-well plate and cultured in a medium containing tetracycline until cell confluence. After tetracycline was removed, Hep38.7-Tet cells were treated with serial dilutions of plant extracts, culture medium as the untreated control, or 5 µM lamivudine (Fujifilm Wako) for 12 days. Sample-containing media were replaced every 3 days. The culture supernatants were harvested at day 12 post-treatment and the amounts of extracellular HBV DNA were determined using immunocapture real-time quantitative polymerase chain reaction (IC-RT-qPCR) assay.

HBV infection assay to assess antiviral effects on the viral entry and intracellular HBV RNA

HepG2-NTCP cells (2.3×10^5 cell/well) were seeded in a 24-well plate and incubated for 24 h. To examine the effect on the viral entry, HBV was pre-mixed with plant extracts at 100 µg/ml and the mixture was inoculated onto the HepG2-NTCP cells (6,000 genome equivalent (GEq)/cell) in the presence of 4% PEG8000 for 16 h at

37°C. After removing virus inoculum, the cells were rinsed with PBS three times and cultured further in fresh culture medium in the absence of plant extracts. Culture supernatants were collected at day 7–8 days post infection and used for the determination of relative HBsAg expression level by ELISA. To assess the effect of plant extracts on the intracellular HBV RNA, HepG2-NTCP cells were infected with HBV (6,000 GEq/cell) for 2 h at 37°C in the absence of plant extracts. Unbound virus was removed by washing and the cells were then re-fed with fresh medium containing the plant extracts for 6 days.

IC-RT-qPCR assay

IC-RT-qPCR for the efficient detection of HBV virion was performed as described previously with some modifications (19) (Fig. 1A). A 96-well Nunc MaxiSorp Immunoplate (Thermo Fisher Scientific) was coated with 4 µg/ml rabbit anti-HBs antibody (prepared by rabbit immunization with a mixture of two synthetic peptides GQNSQSPTSNHSP and RANTANPDWDFNP with an extra cysteine residue at the N-terminal of each peptide) in ELISA coating buffer (50 mM carbonate-bicarbonate buffer, pH 9.6) or normal rabbit IgG (MBL, Nagoya, Japan) as the control and incubated at 4°C overnight. After blocking with 2% bovine serum albumin in phosphate-buffered saline (PBS), 50 µl of 1:1 diluted culture supernatant was added into the anti-HBs antibody-coated wells and the immunoplate was incubated for 1 h at 37°C. After rinsing the wells with 0.05% Tween 20 in PBS (PBST) three times and PBS twice, 40 µl of 1:1 diluted SideStep Lysis and Stabilization Buffer (Agilent, La Jolla, CA) was added to each well to release HBV DNA. An aliquot of the DNA lysate was used as a template for HBV DNA quantification. RT-qPCR was performed with SYBR Premix Ex Taq II (Takara, Shiga, Japan) using a Bio-Rad CFX Manager as described previously (16). The primers used were Ayw-F (5′-CTCGTGGTGGACTTCTCTC-3′) and Ayw-R (5′-AAGATGAGGCATAGCCAGCA-3′). The plasmid HBV dilutions from 10^7 to 10^2 copies per reaction were used for generating a standard curve.

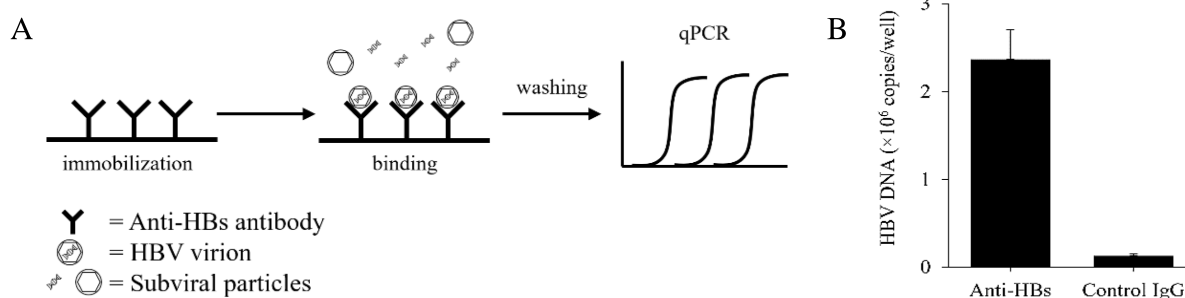

Figure 1. Quantification of extracellular HBV DNA in Hep38.7-Tet cell cultures using IC-RT-qPCR assay. (A) The layout of IC-RT-qPCR assay. (B) The immunoplate was coated with anti-HBs antibody or normal rabbit IgG. Culture supernatants from Hep38.7-Tet cells were added into the antibody-coated wells. HBV DNA amounts in the wells were quantified using qPCR. Data represent mean ± SD of triplicate wells.

Luciferase reporter assay

pLenti CMV Puro LUC (Addgene, Watertown, MA) vector was used in this study. Luciferase reporter assay was performed as described previously with minor modifications (20). Cultured HepG2 cells, at 1.3×10^6 cells in a 10-cm dish, were transfected with 0.5 µg of the vector using FuGENE6 (Promega, Madison, WI). At 8 h post-transfection, transfected cells were reseeded in a 96-well plate at 5×10^4 cells/well and the cells were treated or untreated with plant extracts for 48 h at 24 h post-transfection. The cells were harvested, followed by lysis and further preparation using the Steady-Glo Luciferase Assay System (Promega) according to manufacturer's instructions.

RNA extraction and RT-qPCR

Total RNA isolation, cDNA synthesis, and RT-qPCR were performed as described previously (16, 21). The primers used for RT-qPCR were HBV RNA-F (5′-GCTTTCACTTTCTCGCCAAC-3′) and HBV RNA-R (5′-GAGTTCCGCAGTATGGATCG-3′).

Anti-HBs ELISA

The levels of HBsAg expression in the culture supernatants were determined using HBsAg ELISA kit (DIASource Immunoassays S.A, Louvain-la-Neuve, Belgium) according to manufacturer's protocol. The HBsAg levels in plant extract-treated group were expressed as percentages of the untreated control group.

XTT assay

The cytotoxic effects of plant extracts on cell cultures were determined by XTT assay (16).

Statistical analysis

The data were analyzed by one-way ANOVA with Dunnett's post hoc test (control group vs. treated groups). A p-value <0.05 was considered significant.

RESULTS

Viral replication inhibition of Indonesian medicinal plant crude extracts in Hep38.7-Tet cells.

To examine the anti-HBV effects of crude extracts from Indonesian medicinal plants on the post-viral entry steps in HBV life cycle, a Hep38.7-Tet cells, a HepAD38 subclone (6), was used in this study. Since the Hep38.7-Tet cells can induce HBV replication under the control of cytomegalovirus (CMV) promoter and produce HBV virions, the cells are a useful tool for the screening of antivirals targeting the post-viral entry stage of the HBV life cycle, particularly the replication step. In our screening system, we evaluated the potential of crude extracts to reduce the production of HBV virions in the Hep38.7-Tet cells. It has been reported that extracellular vesicles secreted from HepAD38 cells contain virions and high amounts of incomplete particles, such as subviral particles, genome-empty virions, RNA virions, and naked virus (22). To detect HBV virion in the culture supernatants, we developed an IC-RT-qPCR assay. Using an antibody specific to the HBV surface protein, only HBV virion-derived DNA is amplified by RT-qPCR using primers with a specificity to detect HBV (Fig. 1A). We confirmed that IC-RT-qPCR assay detects HBV DNA in anti-HBs antibody-coated wells, but not in control IgG-coated wells (Fig. 1B).

Table I. Cytotoxic and anti-HBV effects of Indonesian medicinal plant crude extracts on Hep38.7-Tet cells.

Plant	Family	Part	Cell viability (%)[1]	HBV DNA (%)[1]
Platycerium bifurcatum	*Polypodiaceae*	Leaf	98.0 ± 3.0	75.5 ± 26.4
Asplenium scolopendrium	*Aspleniaceae*	Leaf	92.8 ± 3.8	87.2 ± 31.6
Drymoglossum piloselloides	*Polypodiaceae*	Whole plant	94.1 ± 1.0	75.8 ± 22.5
Anthurium plowmanii	*Araceae*	Leaf	115.0 ± 0.3	57.6 ± 1.9
Kigelia africana	*Bignoniaceae*	Bark	108.7 ± 1.4	86.0 ± 9.9
Cassia fistula	*Fabaceae*	Bark	98.8 ± 3.6	42.3 ± 6.8
Cananga odorata	*Annonaceae*	Leaf	94.5 ± 3.1	27.3 ± 3.2
Melastoma malabathricum	*Melastomataceae*	Leaf	110.9 ± 3.3	90.5 ± 44.1
Galphimia glauca	*Malpighiaceae*	Leaf	123.1 ± 4.8	82.2 ± 13.8
Lamivudine[2]	-	-	95.3 ± 4.7	0.7 ± 0.3

[1]Tested at 100 µg/ml. [2]Tested at 5 µM. Data represent mean ± SD of data of triplicate samples (cell viability) and duplicate samples (HBV DNA) from two independent experiments.

Using the IC-RT-qPCR assay, we tested the anti-HBV activities of the crude extracts from Indonesian medicinal plants in the Hep38.7-Tet cells. The cells were treated with crude extracts at single doses of 100 µg/ml for 12 days in tetracycline-free medium. Nine of the crude extracts showing greater than 80% cell viability were selected and subjected for the potential to reduce the production of extracellular HBV DNA (HBV virion) in the Hep38.7-Tet cells. Among them, *Cananga odorata* exhibited the highest inhibitory activity, with a 72.7% reduction in the production of extracellular HBV DNA in comparison to the untreated control (Table I). *Anthurium plowmanii* (42.4%) and *Cassia fistula* (57.7%) showed moderate inhibitory activity compared to the untreated control. The remaining six extracts had weak or no antiviral activities. Lamivudine, a positive control inhibitor, exhibited the highest anti-HBV activity among the samples tested (Table I).

Subsequently, we determined the dose-dependent effects of *C. odorata* on extracellular HBV DNA production in Hep38.7-Tet cells and their viability. *C. odorata* inhibited extracellular HBV DNA production in a dose-dependent manner with a 50% inhibitory concentration (IC_{50}) value of 56.5 µg/ml (Fig. 2A). No apparent cytotoxic effects were observed below 200 µg/ml (50% cytotoxic concentration (CC_{50}) = 540.2 µg/ml) and the selectivity index (SI = CC_{50}/IC_{50}) was 9.6 (Fig. 2B). To confirm whether *C. odorata* suppressed CMV promoter activity, HepG2 cells transfected with a CMV promoter plasmid were treated with 100 µg/ml *C. odorata* extracts and luciferase activity in the cells was measured. Treatment with *C. odorata* neither suppressed CMV promoter activity (Fig. 2C) nor reduced cell viability (Fig. 2D). Next, to clarify the mechanism of action of *C. odorata*, the effect of *C. odorata* on intracellular HBV RNA was examined using HepG2-NTCP cells infected with HBV. HBV-infected cells were treated with *C. odorata* extracts or lamivudine as a control for 6 days, followed by measuring the amounts of intracellular HBV RNA by RT-qPCR. The result showed that intracellular HBV RNA levels were not affected by either *C. odorata* or lamivudine (Fig. 2E).

Viral entry inhibition of Indonesian medicinal plant crude extracts on HepG2-NTCP cells.

NTCP was recently identified as a functional entry receptor for HBV (5) and NTCP-overexpressing cell line, HepG2-NTCP, has been used to screen for antivirals targeting virus entry (8, 23). We examined the anti-HBV effects of crude extracts of Indonesian medicinal plants during viral entry using HepG2-NTCP cells. At first, the

effects of plant extracts on cell viability at a concentration of 100 µg/ml were tested and 25 plant extracts exhibiting greater than 90% cell viability were examined for anti-HBV activity. HepG2-NTCP cells were exposed to HBV in the presence of the extracts for 16 h. After removing unbound virus, the cells were further incubated with fresh culture medium in the absence of the extracts for 7–8 days. HBV infection was evaluated by determining levels of HBsAg production by HepG2-NTCP cells. Among 25 extracts tested, *Moringa oleifera*, *C. fistula, Manihot esculenta*, *Tabebuia argentea*, *C. odorata*, *Plumbago auriculata*, *Citrus sp.* (Jeruk Sunkist), *Cissus sicyoides*, and *Melastoma malabathricum* lowered HBsAg production to 67.8%, 51.2%, 63.6%, 69.1%, 58%, 65.3%, 63.2%, 63.4%, and 40.1% respectively, at 100 µg/ml, compared to the untreated control (Table II). *C. odorata* suppressed the HBsAg production in a dose-dependent manner with an IC_{50} value of 142.9 µg/ml (Fig. 2F). The same treatment did not affect cell viability except at 400 µg/ml where cell viability reduced slightly (Fig. 2G).

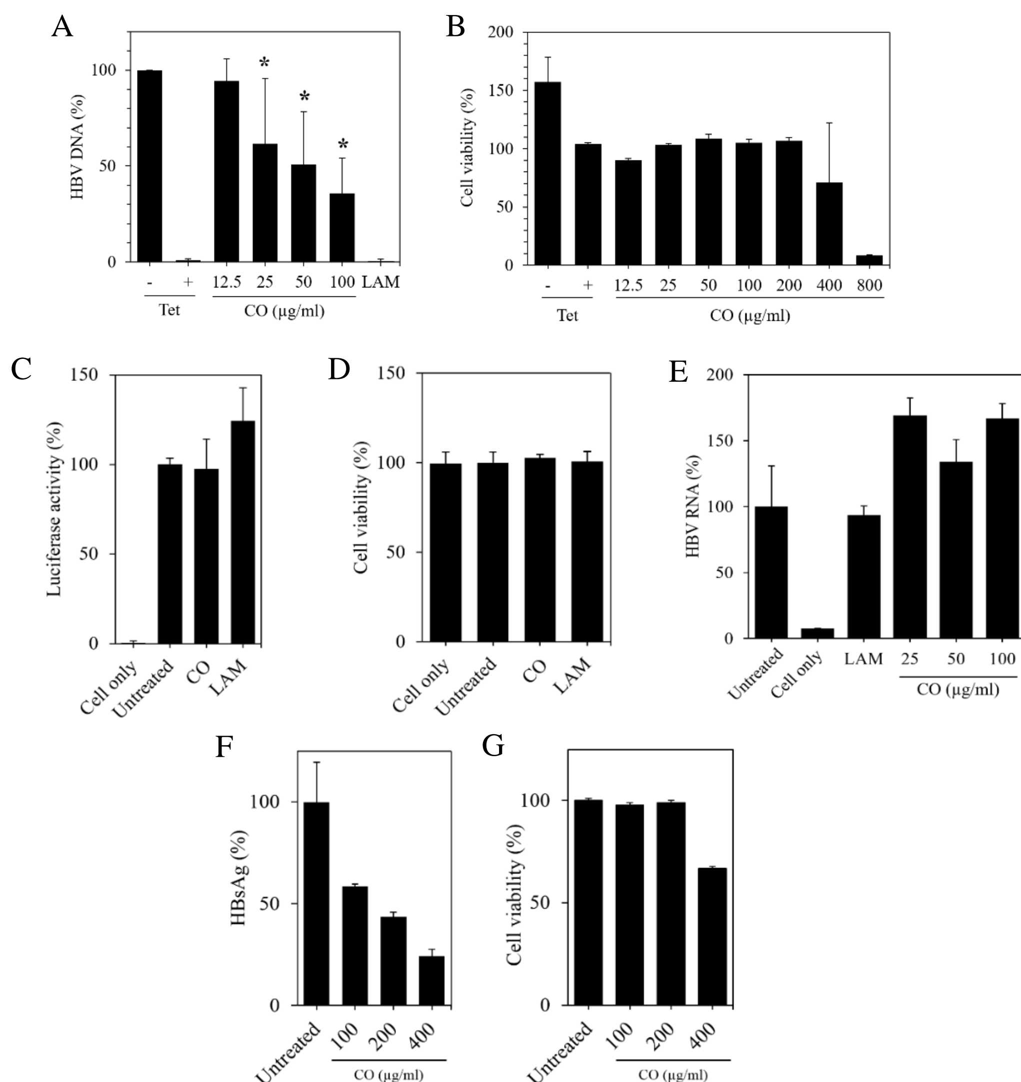

Figure 2. Anti-HBV activity of *C. odorata*. (A) Hep38.7-Tet cells were treated with serial dilutions of *C. odorata* extracts for 12 days. HBV DNA amounts in the culture supernatants were quantified using an IC-RT-qPCR assay and relative percentages of the HBV DNA amounts were compared with those of the untreated control. **p*<0.05 compared to Tet (-) control. Lamivudine (LAM) was used as the inhibitor control. Data represent mean ± SD for triplicate samples from two independent experiments. (B) Cell viability of Hep38.7-Tet cells treated with serial dilutions of *C. odorata* extracts. Percent cell viabilities compared to Tet (-) are shown. (C) HepG2 cells were transfected with CMV-driven luciferase plasmid vector, followed by treatment with *C. odorata* extracts at 100 µg/ml. Relative percentages of luciferase activity in the cells and cell viability (D) compared to the untreated control were measured. Cell only treatment represents mock-transfected cells as a negative control. (E) HepG2-NTCP cells infected with HBV were treated with *C. odorata* for 6 days. Intercellular HBV RNA was quantified by RT-qPCR. (F) HepG2-NTCP cells were exposed to HBV in the presence of *C.odorata* extracts at indicated concentrations for 16 h. After washing out unbound virus and *C. odorata* extracts, cells were further incubated for 8 days in the absence of *C.odorata* extracts. Levels of HBsAg in the culture supernatants were determined as described in materials and methods section. (G) Effect on viability of HepG2-NTCP cells was determined. Tet, tetracycline. CO, *Cananga odorata*. Data represent representative of mean ± SD of triplicate cultures (B, C, D, F, G) and quadruplicate cultures (E).

Table II. Cytotoxic and anti-HBV effects of Indonesian medicinal plant crude extracts on HepG2-NTCP cells.

Plant	Family	Cell viability (%)[1]	HBsAg level (%)[1]
Moringa oleifera	*Moringaceae*	106.6 ± 1.6	67.8 ± 2.6
Cosmos caudatus	*Asteraceae*	95.4 ± 2.8	81.4 ± 0.2
Portulaca oleracea	*Portulacaceae*	96.0 ± 1.1	92.7 ± 2.9
Platycerium bifurcatum	*Polypodiaceae*	98.0 ± 3.1	104.1 ± 20.2
Asplenium scolopendrium	*Aspleniaceae*	93.7 ± 2.0	106.6 ± 15.2
Drymoglossum piloselloides	*Polypodieceae*	94.9 ± 0.6	94.2 ± 4.7
Anthurium plowmanii	*Araceae*	99.2 ± 0.7	86.8 ± 1.8
Kigelia africana	*Bignoniaceae*	97.3 ± 0.5	78.9 ± 9.1
Cassia fistula	*Fabaceae*	91.9 ± 2.1	51.2 ± 2.6
Lagerstroemia indica	*Lythraceae*	98.2 ± 1.1	112.6 ± 5.3
Manihot esculenta	*Euphorbiaceae*	93.6 ± 1.2	63.6 ± 13.2
Tabebuia argentea	*Bignoniaceae*	97.6 ± 0.9	69.1 ± 6.1
Cananga odorata	*Annonaceae*	94.5 ± 0.1	58.0 ± 6.1
Graptophyllum pictum	*Acanthaceae*	99.4 ± 0.2	80.9 ± 15.0
Plumbago auriculata	*Plumbaginaceae*	104.9 ± 3.5	65.3 ± 10.7
Pluchea indica	*Asteraceae*	99.5 ± 2.5	87.4 ± 10.5
Tectona grandis	*Lamiaceae*	95.0 ± 1.1	93.3 ± 7.4
Citrus sp.(Jeruk Sunkist)[2]	*Rutaceae*	95.3 ± 2.0	63.2 ± 1.2
Cissus sicyoides	*Vitaceae*	100.7 ± 2.4	63.4 ± 13.4
Melastoma malabathricum	*Melastomaceae*	91.9 ± 2.4	40.1 ± 2.7
Citrus hystrix	*Rutaceae*	93.6 ± 2.1	70.6 ± 3.4
Galphimia glauca	*Malpighiaceae*	103.9 ± 3.6	95.6 ± 11.8
Phyllanthus emblica	*Pyllanthaceae*	93.6 ± 2.6	74.6 ± 6.7
Parkia javanica	*Fabaceae*	97.8 ± 2.6	73.0 ± 23.4
Persea americana	*Lauraceae*	101.4 ± 3.1	63.9 ± 29.7

[1]Tested at 100 µg/ml. Data represent mean ± SD of data of triplicate samples (cell viability) and duplicate samples (HBsAg level) from two independent experiments. [2]The scientific name has not been determined yet. Local name : Jeruk Sunkist.

DISCUSSION

We screened various crude extracts from Indonesian medicinal plants for anti-HBV activity using two *in vitro* model systems: Hep38.7-Tet cells and HepG2-NTCP cells. In the Hep38.7-Tet cells assay, the antiviral effects of plant extracts on the post-entry events of the HBV life cycle were evaluated. The Hep38.7-Tet cells were treated with plant extracts in the tetracycline-free medium for 12 days and the amounts of HBV DNA (HBV virion) in the culture supernatants were determined using an IC-RT-qPCR assay. Three plants, including *A. plowmanii*, *C. fistula*, and *C. odorata*, showed suppression of extracellular HBV DNA production in the Hep38.7-Tet cells. Among the plants, *C. odorata* exhibited the most considerable antiviral effects, with an IC_{50} value of 56.5 µg/ml and CC_{50} value of 540.2 µg/ml, without inhibiting CMV promotor activity. Treatment with *C. odorata* at the post-viral entry step did not affect intracellular HBV RNA levels in HBV-infected HepG2-NTCP cells, suggesting that *C. odorata* may inhibit other steps of the HBV life cycle except HBV RNA production. In the HBV infection system using HepG2-NTCP cells, anti-HBV effects of plant extracts during virus entry were examined. *C. fistula* and *M. malabathricum* had moderate anti-HBV effects on viral entry. Interestingly, *C. odorata* which suppressed extracellular HBV DNA production in Hep38.7-Tet cells (post-entry events) also exhibited anti-HBV effects on viral entry (IC_{50} = 142.9 µg/ml). Since the crude sample of *C. odorata* was prepared by extracting with methanol which can dissolve most of the polar and semi-polar compounds, several compounds from the crude extract of *C. odorata* may exert anti-HBV activity at both viral entry and post-entry events. Detailed mechanisms of how *C. odorata* showed the inhibitory effects need to be studied after the identification of compound(s) with anti-HBV activity in a future study. Additionally, we also observed that *C. fistula* and *A. plowmanii* inhibited both viral entry and post-entry events.

We did not yet identify the compound(s) responsible for the anti-HBV effect in this study. *C. odorata*, commonly called ylang-ylang, is a plant that belongs to the family *Annonaceae* and its essential oils is widely utilized in aromatherapy. This plant is known to possess various medicinal properties and used as traditional medicine for the treatment of malaria, asthma, gout, pneumonia, stomachache, headaches, eye inflammation, diabetes, and rheumatism (24, 25). In addition, extracts from the leaves and bark of *C. odorata* reportedly contain antimicrobial active compounds with antiplasmodial, antibacterial, and antifungal activity (24, 26). The methanolic extract of leaves of *C. odorata* contain megastigmane glycosides, monoterpene glucosides, and lignan diesters (27-29). To date, there have been no reports on the anti-HBV activities of *C. odorata* extracts.

C. fistula, belonging to the family *Fabaceae*, is widely grown in tropical and subtropical countries. Plants in genus *Cassia* including *C. fistula* exhibit various properties including antioxidant, anticancer, antifungal, and hepatoprotective activity (30, 31). Oxacyclododecan-2-one, imidazole, flavonoid, roseanone, eugenol, caryophyllene, β-copaene, azulene, dodecatetraenamide, and phenethylamine have been identified as bioactive compounds of *Cassia* genus (32, 33). Amentoflavone, a biflavonoid found in *C. fistula*, has been reported to exhibit antiviral activity against influenza virus, herpes simplex virus, human immunodeficiency virus, and hepatitis C virus (34-35). Some flavonoids also have been reported to possess anti-HBV activity at the virus entry and post-entry stage (37).

M. malabathricum is commonly found in Southeast Asia including Indonesia and the whole plant could be used as herbal medicine (38). Methanol extracts of the plant have been reported to have antioxidant and hepatoprotective properties in mice (39). 32-methyl-1-tritriacontanol, β-sitosterol, ursolic acid, flavonoids, and tannins were found in *M. malabathricum* dried leaves (38). *M. malabathricum* crude extracts exhibit moderate anti-HSV-1 activity, with the bioactivity potentially attributed to tannins (40). However, there have been no reports on the anti-HBV activity in *M. malabathricum*.

In conclusion, we demonstrated that *C. odorata, C. fistula*, and *M. malabathricum* possess notable anti-HBV activity. Among them, *C. odorata* exhibited the most promising anti-HBV activity. Further analyses of the crude extract of *C. odorata* is required to identify bioactive compound(s) that are promising candidates for the development of alternative antiviral drugs for HBV treatment.

ACKNOWLEDGMENTS

We thank Dr. Koichi Watashi and Dr. Takaji Wakita (National Institute of Infectious Diseases, Tokyo, Japan) for providing Hep38.7-Tet and HepG2-NTCP cells. This work was partly supported by 2018 JSPS bilateral joint research project, Inamori Foundation, KAKENHI Grants-in-Aid for Scientific Research (Grant Number JP16H05826), the Ministry of Health, Labour and Welfare of Japan, and the Research Program on Hepatitis from the Japan Agency for Medical Research and Development (AMED). The authors reported no conflicts of interest.

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