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Identification of an Oligostilbene, Vaticanol B, from *Dryobalanops aromatica* Leaves as an Antiviral Compound against the Hepatitis C Virus

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Chronic hepatitis C virus (HCV) infection can lead to liver cirrhosis and hepatocellular carcinoma. Although current medications using direct-acting antivirals (DAAs) are highly effective and well-tolerated for treating patients with chronic HCV, high prices and the existence of DAA-resistant variants hamper treatment. There is thus a need for easily accessible antivirals with different mechanisms of action. During the screening of Indonesian medicinal plants for anti-HCV activity, we found that a crude extract of *Dryobalanops aromatica* leaves possessed strong antiviral activity against HCV. Bioassay-guided purification identified an oligostilbene, vaticanol B, as an active compound responsible for the anti-HCV activity. Vaticanol B inhibited HCV infection in a dose-dependent manner with 50% effective and cytotoxic concentrations of 3.6 and 559.5 μg/mL, respectively (Selectivity Index: 155.4). A time-of-addition study revealed that the infectivity of HCV virions was largely lost upon vaticanol B pretreatment. Also, the addition of vaticanol B following viral entry slightly but significantly suppressed HCV replication and HCV protein expression in HCV-infected and a subgenomic HCV replicon cells. Thus, the results clearly demonstrated that vaticanol B acted mainly on the viral entry step, while acting weakly on the post-entry step as well. Furthermore, co-treatment of the HCV NS5A inhibitor daclatasvir with vaticanol B increased the anti-HCV effect. Collectively, the present study has identified a plant-derived oligostilbene, vaticanol B, as a novel anti-HCV compound.

Key words vaticanol B, hepatitis C virus, inhibition, antiviral

INTRODUCTION

Hepatitis C virus (HCV) belongs to the genus *Hepacivirus* of the *Flaviviridae* family. HCV infection causes acute and chronic hepatitis, which increases the risk of developing liver cirrhosis and hepatocellular carcinoma.[1] The WHO estimates that approximately 58 million people are chronically infected, and approximately 1.5 million new infections occur per year.[2]

The HCV genome is a positive-sense single-stranded RNA of approximately 9.6 kb in length. This genome consists of a 5′-untranslated region (UTR), a large open reading frame encoding a polyprotein precursor for three structural proteins (core, E1, and E2), and seven nonstructural proteins (p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B), and a 3′-UTR. The structural proteins are the main constituents of HCV virions. The p7 and NS2 proteins are involved in the process of virus assembly.[3] The other nonstructural proteins (NS3, NS4A, NS4B, NS5A, and NS5B) are essential for viral replication.[4] The NS3 protein contains a serine protease domain implicated in the cleavage and maturation of the viral polyprotein.[5] NS5B is an RNA-dependent RNA polymerase.[6] Among the viral proteins, NS3, NS5A, and NS5B are the primary targets of direct-acting antivirals (DAAs) currently used to treat chronic hepatitis C.[7]

Current therapy with DAAs is highly effective and well-tolerated, achieving a sustained virological response (SVR) rate exceeding 95% for patients with most HCV genotypes.[8,9] Although a low rate of failure has been observed with current DAA regimens, the emergence of resistance-associated substitutions (RAS) in the HCV genome is one of the major causes of DAA failure.[10,11] In addition, the high cost of medication restricts access to drugs for those who need them most, especially in low- to middle-income countries.[8]

Plants produce a wide variety of secondary metabolites possessing unique chemical structures and bioactivities, such as terpenoids, lignans, flavonoids, saponins, secoiridoids, lactones, and alkaloids. Most pharmacologically important drugs are derived from medicinal plants.[12] Indonesia is the second-largest biodiverse country globally, with approximately 40000 different plant species, including approximately 6000 medicinal plant species,[13] thus plants in Indonesia have been expected an excellent resource of bioactive secondary metabo-

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lites to discover new antiviral agents. In the previous research, we screened Indonesian medicinal plants for anti-HCV activity and reported some plant extracts displaying potential anti-HCV activity.[14–16] Moreover, we identified *N*-methylflidersine and O-methyloktadrenolon from *Melicope latifolia*, chalepin and pseudane IX from *Ruta angustifolia*, and quercetin and gallic acid from *Kalanchoe pinnata* as compounds responsible for the anti-HCV activity.[17–19]

The plant family Dipterocarpaceae is one of the most biologically diverse taxa of Indonesian plants and is widely distributed throughout the country. Dipterocarpaceae plants are known as a rich source of oligostilbenes, which are phenolic compounds consisting of resveratrol units from monomers to octamers.[20–22] Resveratrol and its dimers (*e.g.*, ε-viniferin and laevifonol), trimers (*e.g.*, ampelopsin E, α-viniferin, and Malaysianol), and tetramers (*e.g.*, vaticanol B and C, hopeaphenol, and vaticaphenol B) have been isolated from Dipterocarpaceae plants.[23] Oligostilbenes have attracted considerable attention in recent decades owing to their structural complexities and diverse biological activities, however, there has been little study investigating the antiviral potency of oligostilbenes thus far.[24,25]

In the present study, we identified an oligostilbene, vaticanol B from the crude extract of *Dryobalanops aromatica* leaves as a new anti-HCV compound. We also determined its antiviral mechanism of action.

MATERIALS AND METHODS

Plant Material *D. aromatica* leaves used in this study were obtained in 2013 from Pusat Penelitian Ilmu Pengetahuan dan Teknologi (PUSPITEK) Botanical Garden, in Serpong, Banten province, Indonesia, and verified by Mr. Suratman as an expert botanist of PUSPITEK. The herbarium specimen was deposited at the Research Center for Chemistry, National Research and Innovation Agency (BRIN).

Extraction of Crude Extracts and Isolation of Vaticanol B from *D. aromatica* Leaves Dried leaves of *D. aromatica* (1 kg) were ground into a powder and extracted using methanol under reflux conditions. The extracted solution was filtrated through Whatman No. 1 filter paper (Whatman International Ltd., Maidstone, U.K.) to remove insoluble materials and concentrated under a vacuum at 40 °C using rotavapor to yield the crude extract. The methanol crude extract was suspended in distilled water, then partitioned successively using *n*-hexane, ethyl acetate, and *n*-butanol, as described previously.[17,18] Ethyl acetate extract was subjected to silica gel column chromatography (0.063–0.200 mm; Merck, Darmstadt, Germany) and eluted with a combination of *n*-hexane, ethyl acetate, and ethyl acetate and methanol by gradient elution. Twelve major fractions (fractions 1 to 12) were obtained, of which fractions 7 (*n*-hexane: ethyl acetate 20 : 80 to 10 : 90) and 8 (ethyl acetate: methanol 100 : 0 to 90 : 10) showed strong antiviral activity. To isolate and identify the active compound, bulk-dried leaves of *D. aromatica* (5 kg) were collected, the maceration process was repeated for three days, and the crude extract (273 g) was obtained. The dried ethyl-acetate extract (104 g) was subjected to silica gel column chromatography and eluted with a stepwise gradient system consisting of *n*-hexane: ethyl acetate (100 : 0, 90 : 10, 80 : 20, 60 : 40, 40 : 60, 20 : 80, and 0 : 100) and ethyl acetate: methanol (80 : 20, 60 : 40, 40 : 60, 20 : 80, and 0 : 100) to obtain 110 fractions. The chromatogram profiles of the eluted fractions were analyzed by TLC using Silica gel 60 F254 (Merck) or RP-18 F254 plates (Merck). Bioassay-positive fraction No. 95 (eluted with 100% ethyl acetate) was further fractionated by silica gel column chromatography using a mobile phase gradient of chloroform and methanol, yielding 10 subfractions. Subfraction 7 (fr. 95.7), containing the main compound of fraction No. 95, was fractionated by recycling preparative HPLC (GS-320+GS-310, 21.5 mm × ID 1000 mm, flow rate: 8 mL/min, Japan Analytical Industry LC-980W) using 100% methanol, and three fractions were obtained. The third fraction 95.7.3 afforded an isolate (vaticanol B) as a pure solid (51.7 mg, purity >90%). Its chemical structure was determined by comparing NMR data with the published data.[26] NMR spectra were recorded using a JEOL ECA500 spectrometer (500 MHz). ^{1}H-NMR (acetone-d_6) δ: 7.19 (2H, d, J = 8.5 Hz, H-2a, 6a), 6.74 (2H, d, J = 8.5 Hz, H-3a, 5a), 5.73 (1H, d, J = 11.5 Hz, H-7a), 4.40 (1H, d, J = 11.5 Hz, H-8a), 6.24 (1H, d, J = 2.5 Hz, H-12a), 6.07 (1H,d, J = 2.5 Hz, H-14a), 7.12 (2H, d, J = 8.5 Hz, H-2b, 6b), 6.66 (2H, d, J = 8.5 Hz, H-3b, 5b), 5.17 (1H, d, J = 3.0 Hz, H-7b), 3.07 (1H, brd, J = 12.0 Hz, H-8b), 6.02 (1H,s, H-12b), 6.35 (2H, d, J = 8.5 Hz, H-2c, 6c), 6.47 (2H, d, J = 8.5 Hz, H-3c, 5c), 4.05 (1H, t, J = 11.5 Hz, H-7c), 4.51 (1H, d, J = 11.5 Hz, H-8c), 6.15 (1H, d, J = 2.0 Hz, H-12c), 6.43 (1H, d, J = 2.0 Hz, H-14c), 7.15 (2H, d, J = 8.5 Hz, H-2d, 6d), 6.72 (2H, d, J = 8.5 Hz, H-3d, 5d), 5.33 (1H, d, J = 5.5 Hz, H-7d), 4.64 (1H, d, J = 5.5 Hz, H-8d), 6.06 (2H, brs, H-10d, 14d), 6.26 (1H, t, J = 2.5 Hz, H-12d). ^{13}C-NMR (acetone-d_6) δ: 161.2 (C-11c), 159.4 (C-11d,13d), 159.9 (C-13c), 158.3 (C-11b), 158.1 (C-4a), 157.5 (C-4d), 156.3 (C-13a), 155.9 (C-4c), 155.4 (C-4b), 155.3 (C-11a), 154.4 (C-13b), 147.5 (C-9d), 142.7 (C-9b), 141.3 (C-9a), 141.2 (C-9c), 134.2 (C-1d), 133 (C-1b), 130.9 (C-1c), 130.3 (C-1a), 130.2 (C-2b, 6b), 129.7 (C-2a, 6a), 128.8 (C-2c, 6c), 127.8 (C-2d, 6d), 124 (C-10a), 122.9 (C-10c), 121.7 (C-14b), 115.54 (C-3a,5a), 115.49 (C-3d, 5d), 115.36 (C-3c, 5c), 115.25 (C-10b), 115 (C-3b, 5b), 107 (C-10d,14d), 106.5 (C-14c), 105.2 (C-14a), 101.7 (C-12d), 101.1 (C-12a), 95.9 (C-12b), 95.1 (C-12c), 94.2 (C-7d), 89.9 (C-7a), 57.1 (C-7c), 57.1 (C-8d), 52.7 (C-8b), 48.7 (C-8c), 48.4 (C-8a), 36.5 (C-7b). LC/MS was performed on API3000 (ABSciex) equipped with electrospray ionization (ESI) ion source and ESI-MS (positive mode) m/z: 907.5 $[M+H]^+$.

Cells and Viruses The human hepatoma-derived cell line Huh7it-1[14,27] was grown in Dulbecco's modified Eagle's medium (Gibco-Invitrogen, Carlsbad, CA, U.S.A.) supplemented with 10% fetal bovine serum (Biowest, Nuaillé, France), non-essential amino acids (Gibco-Invitrogen), and kanamycin (Sigma-Aldrich, St. Louis, MO, U.S.A.). Cultured cells were incubated at 37 °C in a 5% CO_2 humidified chamber. The cell culture-adapted HCV of the JFH1 strain (genotype 2a) was propagated and stored at −80 °C, as described previously.[14,28]

Viral Titration Viral titration was performed by focus-forming assay as described previously.[14,27]

Antiviral Activity Assay Extract samples from *D. aromatica* leaves and vaticanol B were dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich) and stored at −20 °C until further antiviral evaluation. An antiviral activity assay was carried out as described in our previous report.[14] Briefly, Huh7it-1 cells in a 48-well plate (5.5×10^4 cells/well) were inoculated with HCV at a multiplicity of infection (MOI) of 0.2 or 1 in culture medium containing extract samples or DMSO as the negative control for 2 h at 37 °C. After rinsing wells,

the cells were incubated for a further 22 to 46 h at 37 °C in a medium containing the same samples. Virus titers in the culture supernatant were determined by focus-forming assay. The 50% effective inhibition concentration (EC_{50}) was determined by non-linear regression analysis using GraphPad Prism 9 graphing software (GraphPad Software, La Jolla, CA, U.S.A.).

The antiviral efficacy in a combination with vaticanol B and the NS5A inhibitor daclatasvir (BMS-790052; Abcam, Cambridge, MA, U.S.A.) was assessed as described in our previous report.[14] In brief, Huh7it-1 cells were infected with HCV for 2 h at 37 °C. After washing, cells were incubated in a medium containing daclatasvir alone, vaticanol B alone, or a fixed dose of vaticanol B in the presence of variable concentrations of daclatasvir.

Virucidal Activity Assay The HCV suspension mixed with an equal volume of vaticanol B was incubated for 2 h at 37 °C.[14,29] Cells were inoculated with a dilution (35 times) of the pre-treated viral suspension and residual viral infectivity was determined by viral titration.

RT-Quantitative (q)PCR Total cellular RNA was extracted by using the ISOGEN-II reagent (Nippon Gene, Tokyo, Japan). cDNA was synthesized using ReverTra Ace® qPCR RT Master Mix (Toyobo Co., Ltd., Osaka, Japan) and subjected to RT-qPCR analysis using TB Green Premix Ex Taq (TaKaRa, Shiga, Japan). PCR amplification was carried out using a Bio-Rad CFX Manager, as described previously.[14] Comparative threshold cycle method was used to calculate the percentage of HCV RNA level normalized with glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

Replicon Inhibition Assay The transient reporter replicon assay was carried out as described in our previous report.[14] Briefly, Huh7it-1 cells (1×10^6 cells) were electroporated with 2 μg of SGR-GLuc/JFH1 RNAs using a NEPA electroporator (Nepagene) and immediately seeded into a 96-well plate at a density of 2×10^4 cells/well. Four hours later, vaticanol B, cyclosporin A (CyA, Sigma-Aldrich), or 0.1% DMSO was added to the medium, and the cells were incubated for 24 h at 37 °C. Gaussia luciferase activity in the culture medium was measured using a Pierce™ *Gaussia* luciferase Glow assay kit (ThermoFisher Scientific, Waltham, MA, U.S.A.).

Western Blot Analysis Immunoblotting was performed by a slightly modified version of a previously described procedure.[14] Cells were lysed with radio immunoprecipitation assay (RIPA) buffer (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) containing a protease inhibitor cocktail (Sigma-Aldrich) for 30 min on ice and the lysate was centrifuged at 15000 rpm for 30 min. Proteins were resolved by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and electrotransferred to polyvinylidene difluoride (PVDF) membranes. Membranes were incubated with antibodies specific for HCV NS3 (EMD Millipore, Temecula, CA, U.S.A.) and GAPDH (MBL, Nagoya, Japan) as primary antibodies. Horseradish peroxidase-conjugated goat anti-mouse immunoglobulin G (IgG; MBL) was used as the secondary antibody. Bands were detected with Immobilon Western (Millipore, Bedford, MA, U.S.A.), and the images were captured using an Image Quant LAS 4000 (GE Healthcare Life Sciences, Little Chalfont, U.K.).

Cell Viability Assay Cytotoxicity was evaluated using the 3-(4,5-dimethylthiazol-2-yl)-5(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium (MTT) assay, as described previously.[14] The 50% cytotoxic concentration (CC_{50}) was determined by non-linear regression analysis using GraphPad Prism 9 graphing software.

Statistical Analysis Data were analyzed with GraphPad Prism 9 (GraphPad Software). Differences between control and treated groups were evaluated by Student's *t*-test or one-way ANOVA. A *p*-value less than 0.05 was considered statistically significant.

RESULTS

Evaluating the Anti-HCV Activity of Crude Extract from *D. aromatica* Leaves and Isolating Its Bioactive Compound The crude extract from *D. aromatica* leaves was prepared by maceration with methanol and then screened for antiviral potency against HCV genotype 2a of the JFH-1 strain. The human hepatoma-derived cell line Huh7it-1 cells were inoculated with HCV in the presence or absence of the crude extract for 2 h at 37 °C. After removing the unbound virus and the extract, cells were further incubated for 46 h at 37 °C in the same extract. Virus titers in the culture supernatants were determined by focus-forming assay. The crude extract inhibited HCV infection in a dose-dependent manner with an EC_{50} value of 2.6 μg/mL (Fig. 1). The cytotoxicity of the crude extract against Huh7it-1 cells was examined by the MTT assay and the CC_{50} was >100 μg/mL (Fig. 1). The crude extract was next subjected to liquid–liquid partitioning to yield *n*-hexane, ethyl acetate, *n*-butanol, and water extracts, and the antiviral activity of each partition was evaluated. The ethyl acetate extract was found to be the most effective, with an EC_{50} value of 3.4 μg/mL (Table 1). The other extracts showed low to moderate antiviral activities ($EC_{50} > 21.7$ μg/mL). The ethyl acetate extract was then subjected to column chromatography, which was separated into 12 fractions (fractions 1–12). Two of these fractions (EA-Fr7 and EA-Fr8) showed strong activity with EC_{50} values of 1.6 and 0.9 μg/mL, respectively (Table 2). We considered EA-Fr8 as the most potent fraction for further purification.

To isolate the active compound from the most active fraction, the crude extract of *D. aromatica* leaves was partitioned

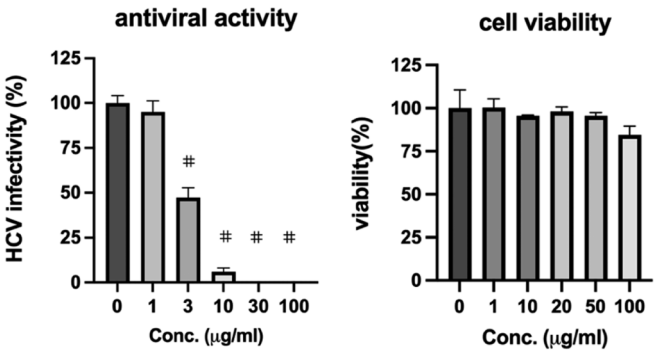


Fig. 1. Inhibitory Effect of the Crude Extract from *D. aromatica* Leaves against HCV

Cells were infected with HCV (MOI = 0.2) for 2 h at 37 °C in the presence of crude extracts or DMSO as an untreated control. After washing, cells were incubated with a medium containing the same samples. Viral titers in the culture supernatants at 48 h p.i. were determined by focus-forming assay. For determination of cytotoxicity, cells were incubated with various concentrations of crude extracts or DMSO for 48 h. Cell viability was determined by MTT assay. Data represent mean ± standard error of the mean (S.E.M.) from two independent experiments. One-way ANOVA analysis was performed to determine statistical significance. $^{\#}p < 0.01$.

Table 1. Anti-HCV Activity and Cytotoxicity of Solvent Partitions Obtained from *D. aromatica* Leaves

Sample	EC_{50} (μg/mL)	CC_{50} (μg/mL)
n-Hexane extract	27.9	>200
Ethyl acetate extract	3.4	125.5
n-Butanol extract	21.7	>200
Aqueous extract	97.8	>200

Data represent the mean from two or three independent experiments.

Table 2. Anti-HCV Activity and Cytotoxicity of Subfractions of Ethyl Acetate Extracts of *D. aromatica* Leaves

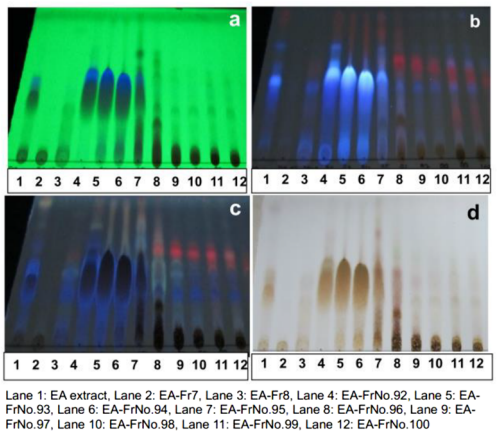
Fraction	HCV infectivity (%)[§]	EC_{50} (μg/mL)	CC_{50} (μg/mL)
EA-Fr1	79.8	na	na
EA-Fr2	176.6	na	na
EA-Fr3	87.6	na	na
EA-Fr4	0	5.4	>80
EA-Fr5	0.3	3.7	>80
EA-Fr6	0	3.5	>80
EA-Fr7	0	1.6	>80
EA-Fr8	0	0.9	>80
EA-Fr9	0	3.5	>80
EA-Fr10	0	3.9	>80
EA-Fr11	3.5	6.6	>80
EA-Fr12	138.0	na	na

[§]Tested at 20 μg/mL except for EA-Fr1 (10 μg/mL). Data represent the mean from two or three independent experiments. EA: ethyl acetate, na: not applicable due to the low antiviral potency.

to obtain the ethyl acetate extract. The ethyl acetate extract was then fractionated by open silica-gel column chromatography to yield 110 fractions. TLC analysis of the eluted fractions was conducted, and fractions No. 92 to No. 100 had similar TLC profiles with EA-Fr8 (Fig. 2A), while the other fractions did not (data not shown). As expected, fractions No. 92 to No. 100 exhibited anti-HCV activity, with an EC_{50} range of 4.7 to 18.6 μg/mL, along with CyA (1 μg/mL)[30] as a positive control inhibitor (Fig. 2B). Among them, the most active fraction No. 95 (EC_{50} = 4.7 μg/mL) was selected for further purification. Separation of fraction No. 95 by column chromatography yielded ten subfractions. Subfraction 7, which contained the main compound of fraction No. 95, was then subjected to recycling preparative HPLC, and three fractions were obtained. The third fraction 95.7.3 afforded an oligostilbene, vaticanol B (Fig. 2C). The chemical structure of vaticanol B is shown in Fig. 3A.

Vaticanol B Exhibits Potent Anti-HCV Activity at the Entry Step The purified compound, vaticanol B, showed dose-dependent inhibition against HCV with an EC_{50} of 3.6 μg/mL (3.97 μM) (Fig. 3B). Notable cytotoxic effects of vaticanol B against Huh7it-1 cells were not observed at concentrations up to 80 μg/mL and the CC_{50} value was 559.5 μg/mL (617.5 μM) (Fig. 3C). The selectivity index (SI, CC_{50}/EC_{50}) was 155.4. These findings demonstrate that vaticanol B is a compound responsible for the anti-HCV activity of *D. aromatica* leaves.

The HCV life cycle is divided into two stages: viral entry (adsorption and penetration) and post-viral entry (translation, replication, assembly, and release). We then conducted a time-of-addition experiment to determine the inhibitory step(s)



B

Fr. No.	EC_{50} (μg/ml)	CC_{50} (μg/ml)	Fr. No.	EC_{50} (μg/ml)	CC_{50} (μg/ml)
92	6.1	>20	97	10.2	>20
93	7.2	>20	98	6.6	>20
94	8.1	>20	99	18.6	>20
95	4.7	>20	100	11.1	>20
96	7.7	>20	CyA	64.7%[§]	118.1%[#]

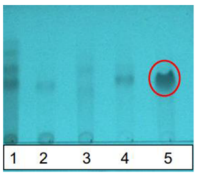


Fig. 2. Chromatogram Profiles, Anti-HCV Activity, and Cytotoxicity of the Ethyl Acetate Extracts of *D. aromatica* Leaves

(A) TLC profile of ethyl acetate (EA) extracts. Silica gel TLC was developed with *n*-hexane: EA (20 : 80; v/v) as a mobile phase and illuminated with UV 254 nm (a) or UV 365 nm (b), and sprayed with 10% sulfuric acid and heated at 105 °C, then observed under UV 365 nm (c) or white lamp (d). Samples from lanes 2 and 3 were identical to those from Table 2. Samples from lanes 1 and 4 to 12 were fractions separated from large-scale EA extracts by column chromatography. (B) Anti-HCV activity and cytotoxicity of EA-Fr No. 92 to EA-Fr No. 100. Cells were infected with HCV (MOI = 0.2) for 2 h at 37 °C in the presence of various concentrations of fraction samples or 0.1% DMSO as an untreated control. After washing, cells were incubated with a medium containing the same samples. Viral titers in the culture supernatants at 48 h p.i. were determined and the EC_{50} values were calculated. Data represent the mean from two independent experiments. [§]Inhibition ratio of HCV infection with cyclosporin A (CyA, 1 μg/mL) as a positive control. [#]Cell viability of CyA. (C) TLC profile of EA-fr. 95 subfractions. RP-18 TLC was developed with acetonitrile: methanol: water (1 : 2 : 3; v/v) as a mobile phase and illuminated with UV 254 nm. Lane 1, EA-Fr No. 95; Lane 2, fr. 95.7; Lane 3, fr. 95.7.1; Lane 4, fr. 95.7.2; and Lane 5, fr. 95.7.3. fr. 95.7 was subjected to recycling preparative HPLC, yielding three fractions: fr. 95.7.1, fr. 95.7.2, and fr. 95.7.3. Vaticanol B is marked in the red-color circle.

of vaticanol B on the HCV life cycle. Huh7it-1 cells were infected with HCV (MOI = 1), and a medium containing vaticanol B (20 μg/mL) or 0.1% DMSO (control) was added to the cells during (co-addition) and/or after virus inoculation (post-entry) (Fig. 4A). Viral titers in the culture supernatants at 24 h post-infection (p.i.) were determined. Addition of vaticanol B during viral inoculation reduced the infectivity of HCV to 63% of untreated control (Fig. 4B). Pretreatment of cells with vaticanol B for 2 h at 37 °C before viral inoculation did not affect HCV infectivity (Fig. 4C). Next, we performed a virucidal activity assay to examine the direct effect of vaticanol B on infectious HCV particles. HCV virions were premixed

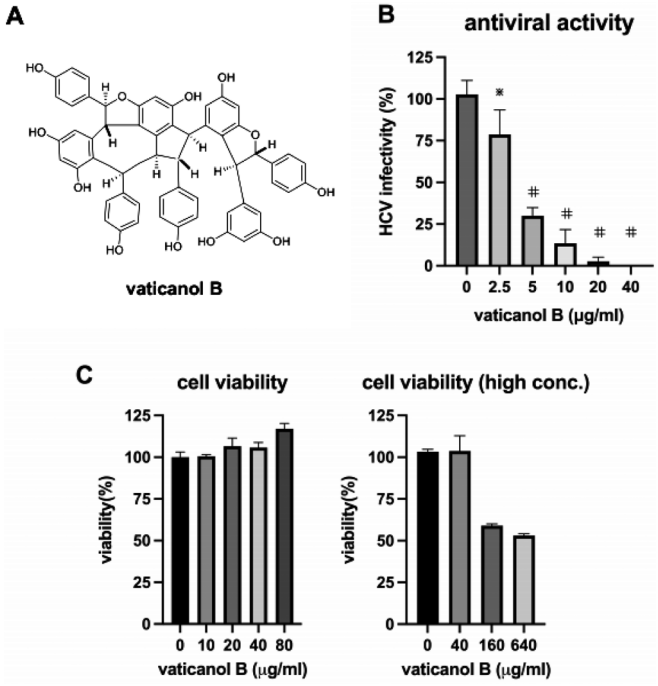


Fig. 3. Dose-Dependent Inhibition of HCV Infection by Vaticanol B

(A) Chemical structure of vaticanol B. (B) Cells were infected with HCV (MOI = 1) for 2 h at 37 °C in the presence or absence of vaticanol B. After washing, cells were incubated in the medium containing the same samples for 22 h. Viral titers in the culture supernatants were determined. Data represent mean ± S.E.M. from two independent experiments. One-way ANOVA analysis was performed to determine statistical significance. $*p<0.05$, $^{\#}p<0.01$. (C) Cells were incubated with vaticanol B or DMSO as an untreated control for 24 h. Cell viability was determined by MTT assay. Data represent mean ± standard deviation (S.D.) from triplicate wells.

with vaticanol B and incubated for 2 h at 37 °C. After diluting 35-fold before inoculating onto the cells, the residual virus titers were determined by focus-forming assay. Vaticanol B (40 μg/mL) significantly reduced the HCV infectious titers by 99.7% (Fig. 4D).

Vaticanol B Modestly Inhibits HCV Replication and HCV Protein Expression In order to examine the possible anti-HCV activity of vaticanol B at the post-entry step, we examined its effects on HCV RNA replication and HCV protein expression. Huh7it-1 cells inoculated with HCV for 2 h were treated with vaticanol B or 0.1% DMSO for 22 h. Total cellular RNA was extracted and levels of HCV RNA expression were determined by RT-qPCR analysis. Vaticanol B (40 μg/mL) treatment slightly reduced intracellular HCV RNA levels (32% reduction) (Fig. 5A). We also examined whether vaticanol B could suppress HCV replication in a subgenomic replicon system. Cells were electroporated with a subgenomic HCV replicon RNA encoding the *Gaussia*-luciferase gene and treated with vaticanol B, 0.1% DMSO, or CyA as a positive inhibitor control for 24 h. Treatment with vaticanol B (40 μg/mL) resulted in a 28% decrease in luciferase activity (Fig. 5B). Next, the effect on HCV NS3 protein expression was examined. After virus infection for 2 h at 37 °C, cells were treated with vaticanol B for 22 h and the expression of HCV NS3 protein in the cells was analyzed by Western blotting. The result showed that treatment with vaticanol B (20 and 40 μg/mL) resulted in modest suppression of HCV protein expression (Fig. 5C).

The Combination of Vaticanol B Plus NS5A Inhibitor Daclatasvir Increases the Antiviral Effect To evaluate whether vaticanol B can increase the inhibitory effect by using it in combination with clinically approved anti-HCV drugs, we examined the antiviral activity of the combination of vaticanol B and the NS5A inhibitor daclatasvir. After virus infection for 2 h at 37 °C, cells were incubated in a medium containing daclatasvir alone or a fixed dose of vaticanol B in the presence of variable concentrations of daclatasvir. As shown in Fig. 6, co-treatment with vaticanol B plus daclatasvir enhanced antiviral activity compared to that of daclatasvir monotherapy.

DISCUSSION

Although current DAAs markedly improved the cure rate of chronic HCV infection, high drug prices are a major barrier to access to DAA treatment. Also, DAA-resistant variants may impact their effectiveness during treatment. Therefore, continuous effort to improve the therapeutic option in the treatment of chronic HCV patients is required.

In the present study, we found that a crude extract from *D. aromatica* leaves exhibited potent antiviral activity against HCV (Fig. 1). Through a bioactive-guided purification, oligostilbene vaticanol B was identified as a compound of *D. aromatica* that is responsible for the anti-HCV activity (see Materials and Methods). Vaticanol B strongly inhibited HCV infection with an EC_{50} of 3.6 μg/mL (3.97 μM) (Fig. 3B). Vaticanol B showed extremely low cytotoxicity against Huh7it-1 cells, with the CC_{50} value of 559.5 μg/mL (Fig. 3C). Analysis of antiviral mechanisms demonstrated that vaticanol B inhibits HCV infection through the potent virucidal effect on the virions and the partial inhibition of viral replication (Figs. 4D, 5). To the best of our knowledge, this is the first report to demonstrate the antiviral activity of vaticanol B against HCV.

Structurally, oligostilbenes are quite different from other natural phenolics, such as flavonoids, phenolic acids, and phenolic alkaloids. Oligostibenes exist as dimers to octamers with various molecular frameworks resulting from different oxidative condensations of resveratrol monomers.[25,31] Resveratrol, a well-known stilbene, has been greatly investigated for its biological activity and shown to possess a broad spectrum of antiviral activities against different DNA and RNA viruses.[25,32] On the contrary, a certain number of natural oligostilbenes have been reported for antiviral activities. For example, α-viniferin, vaticanol E, pauciflorol B, and vaticahainol D as the resveratrol trimer, and vaticaffinol, pauciflorol C, davidol A, hopeaphenol A, neoisohopeaphenol A, and hemsleyanol D as the resveratrol tetramer was reported to exert antiviral activities against herpes simplex virus type 1 (HSV-1) and type 2 (HSV-2) by producing reactive oxygen species.[33] In another investigation, Ito *et al.* evaluated the antiviral potency of oligostilbenes isolated from the stem bark of *Shorea uliginosa* against HSV-1, HSV-2, and the influenza virus.[34] They found that hopeaphenol, shoreaketone, α-viniferin, and vaticanols B and G exerted anti-HSV activities, while hopeaphenol and shoreaketone inhibited influenza virus replication.[34] Dibalanocarpol and balanocarpol isolated from *Hopea malibato* Foxw. leaves exhibited modest HIV inhibitory activity.[35] Lee *et al.* reported two resveratrol dimers, ampelopsin A and ε-viniferin, and three resveratrol tetramers wilsonol C, vitisin A, and vitisin B as anti-HCV compounds from grapevine root extract.[36,37] The most potent oligostilbene, vitisin B, exhibited antiviral activity by disruption of the viral helicase NS3.[36]

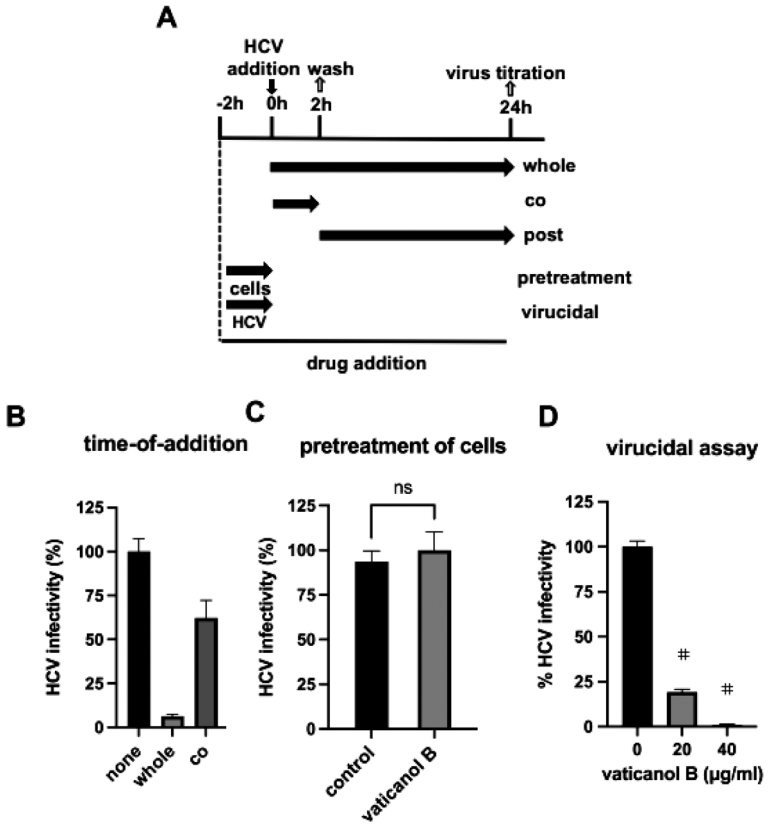


Fig. 4. Effect of Vaticanol B on Viral Entry

(A) Schematic experimental design of the time-of-addition assay. Cells were infected with HCV (MOI = 1) at 37 °C for 2 h in the presence or absence of vaticanol B. After washing out the free virus and compound, cells were incubated in the presence or absence of vaticanol B for 22 h. Viral titers in the culture supernatants were determined by focus-forming assay. Filled arrows indicate periods with vaticanol B treatment. (B) Cells were infected with HCV (MOI = 1) at 37 °C for 2 h. Vaticanol B (20 μg/mL) was added to the cells at the indicated time point, as shown in (A). Viral titers in the culture supernatants at 22 h p.i. were determined by focus-forming assay. Data represent mean ± S.D. from three independent experiments. (C) Cells were preincubated with 20 μg/mL vaticanol B for 2 h at 37 °C. After extensive washing, the cells were challenged with HCV infection. (D) HCV was premixed with vaticanol B for 2 h at 37 °C. After diluting 35-fold before inoculating onto the cells and the residual viral titers were determined. Data represent mean ± S.E.M. from two independent experiments. Student's two-tailed *t*-test (C) or one-way ANOVA analysis (D) was performed to determine statistical significance. $^{\#}p < 0.01$, ns: not significant.

D. aromatica, locally known as Kayu Kapur in Indonesia, is a tall tree belonging to the family Dipterocarpaceae. Of this family comprising 22 genera, seven genera (*Shorea*, *Hopea*, *Upuna*, *Cotylelobilum*, *Diterocarpus*, *Vatica*, and *Vateria*) are rich sources of oligostilbenes.[23] Vaticanol B, a tetramer of resveratrol, is isolated from Diterocarpus, Vatica, Shorea, Hopea, and Upuna plants.[22,26,38–40] Therapeutic potential of vaticanol B such as protection of endoplasmic reticulum stress-induced cell death, anti-inflammatory, antiproliferative, and antimicrobial activities have been reported.[39,41,42] In a more recent study, vaticanol B was reported as the inhibitor of entry and replication of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) and multiple SARS-CoV-2 variants.[43] Vaticanol B inhibited entry of SARS-CoV-2 by interfering with the binding of the receptor binding domain (the RGD motif) to the host angiotensin-converting enzyme 2 (ACE2) receptor.

Bioactivity-guided fractionation and TLC analysis of the ethyl acetate fractions led to the isolation of vaticanol B as an anti-HCV compound. However, the EC_{50} values of the plant crude extract (2.6 μg/mL, Fig. 1), ethyl acetate fraction (3.4 μg/mL, Table 1), ethyl acetate subfraction EA-Fr No. 95 (4.7 μg/mL, Fig. 2B), and vaticanol B (3.6 μg/mL, Fig. 3B) did not change significantly following the purification process. There are several possible explanations for this phenomenon. One explanation could be that the crude extract contains various plant constituents derived from the extraction procedures. The strong antiviral activity of the crude extract may demonstrate the comprehensive effect of other constituents with significant antiviral properties, in addition to the main antiviral compound, vaticanol B. These concomitant constituents have been removed during the purification of vaticanol B. Another factor to consider is that the *D. aromatic* leaves were collected twice in this study. Vaticanol B was successfully isolated and identified from the large-scale preparation of the ethyl acetate extract (Fig. 2). It has been reported that the yield of active constituents may vary during different times or seasons.[44] Therefore, the content of vaticanol B contained in the ethyl acetate fraction may differ slightly depending on when the leaves were collected.

A time-of-addition study revealed that vaticanol B acts mainly on the viral entry step, while acting modestly on the post-entry step as well. Indeed, antiviral activity of vaticanol B is mainly through a direct virucidal effect on the HCV virion and by a partial inhibitory effect(s) on HCV RNA replication and viral protein expression (Figs. 4D, 5). In this connection, vaticanol B has been reported to up-regulate p21 in a p53-independent manner while down-modulating cyclin D1 expression and mitogen-activated protein kinase-extracellular signal-regulated kinase (MAPK-ERK) activation.[39] Further studies are needed to determine whether up-regulation of p21 or down-modulating cyclin D1 expression and MAPK-ERK activation is involved in vaticanol B-mediated inhibition of HCV replication.[45–47]

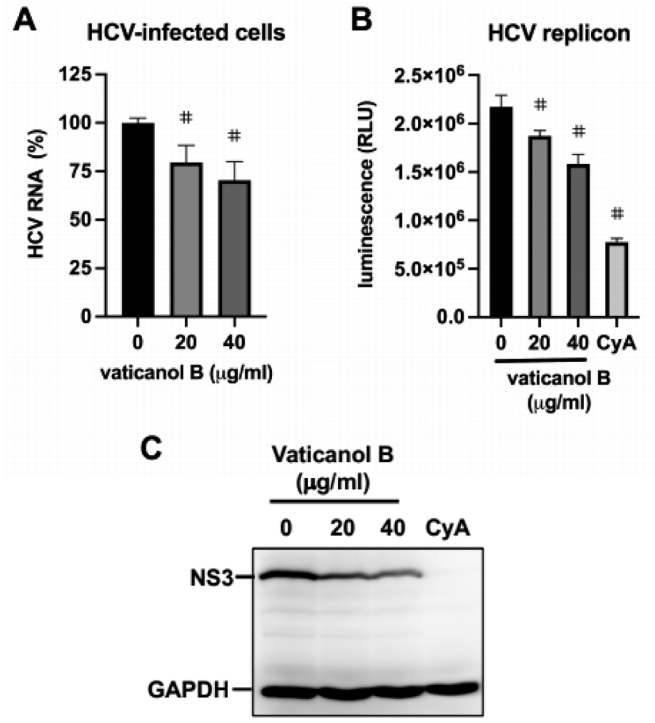


Fig. 5. Effect of Vaticanol B on HCV Replication and Viral Protein Expression

(A) Cells were infected with HCV (MOI = 1) for 2 h at 37 °C. After washing, infected cells were incubated in the medium containing vaticanol B or 0.1% DMSO for 22 h. Levels of HCV RNA expression in the cells were determined by RT-qPCR. HCV RNA levels were normalized to GAPDH mRNA expression levels. (B) HCV subgenomic replicon RNA was electroporated into Huh7it-1 cells and the cells were immediately plated into a 96-well plate (2×10^4 cells/well). At 4 h post-electroporation, cells were incubated in a compound-containing medium for 24 h at 37 °C and the culture supernatants were subjected to the luciferase assay. DMSO (0.1%) and CyA (2 mg/mL) were used as the negative and positive control, respectively. (C) Cells were infected with HCV (MOI = 1) for 2 h at 37 °C. After washing, infected cells were incubated in the medium containing vaticanol B, 0.1% DMSO, or 2 mg/mL CyA for 22 h. The total cell lysate was prepared and HCV NS3 expression was analyzed by Western blotting. GAPDH was detected as a loading control. (A, B) Data represent mean ± S.E.M. from two independent experiments. (C) Data represent a representative experiment result from three independent experiments. One-way ANOVA analysis was performed to determine statistical significance. $^{\#}p < 0.01$. CyA: cyclosporin A.

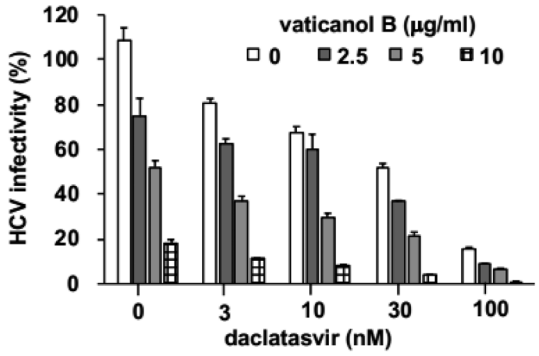


Fig. 6. Co-treatment Effect of Vaticanol B with Daclatasvir

Cells were infected with HCV (MOI = 1) for 2 h at 37 °C. After rinsing the wells, cells were co-treated with indicated concentrations of vaticanol B plus daclatasvir for 22 h. Virus titers from the culture supernatants were determined by focus-forming assay. The percentage of HCV infectivity compared to 0.1% DMSO as the untreated control is shown. Data represent mean ± S.E.M. from two independent experiments.

Current DAA therapies consist of a combination of inhibitors targeting viral proteins, such as NS3/4A protease inhibitors (*e.g.*, simeprevir and grazoprevir), NS5A replication-complex inhibitors (*e.g.*, daclatasvir, velpatasvir, and ledipasvir), and NS5B RNA polymerase inhibitors (*e.g.*, dasabuvir, beclabuvir, and sofosbuvir). Although a low rate of failure has been observed with current DAA regimens, the emergence of HCV mutants with RAS after DAA treatment is a major problem. Thus, a combination of DAAs with new antiviral agents targeting different parts of the HCV life cycle is important. We showed that vaticanol B exerts antiviral activity primarily through a direct virucidal effect against HCV virion, which is a different mode of action from those of current DAAs (Fig. 4D). Viral membrane-disrupting agents are less likely to induce drug resistance because the viral envelope is derived from the host cell membrane.[48,49] In addition, the data presented herein show that a combination of daclatasvir and vaticanol B enhanced the anti-HCV activity (Fig. 6). Thus, vaticanol B could offer a promising strategy to minimize the risk of emerging drug-resistant HCV mutants during DAA therapies.

In conclusion, we successfully identified vaticanol B as a compound of *D. aromatica* leaves that is responsible for the anti-HCV activity. Vaticanol B exerted the anti-HCV effect mainly through a direct virucidal effect. Although further studies are needed to assess its antiviral efficacy *in vivo*, vaticanol B is a potential compound for the prevention of HCV infection.

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Conflict of Interest The authors declare no conflict of interest.

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