

Antiviral effect of *Archidendron pauciflorum* leaves extract to hepatitis C virus: An *in vitro* study in JFH-1 strain

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ABSTRACT

Background: Hepatitis C virus (HCV) is a leading cause of chronic liver diseases. Drug resistance to the regimen is also increasing. Hence, there is a need for new anti-HCV agents that are less toxic and more efficacious. The aim of this study is to evaluate the possibility of *A. pauciflorum* extracts can be a antiviral drug.

Methods: Huh-7it cells were infected with the HCV genotype 2a strain JFH-I in the presence of methanol extracts of *Archidendron pauciflorum*. The methanol extract further partition used *n*-hexane, ethyl acetate, *n*-butanol, and water showed in which butanol extracts exerted the strongest IC₅₀ (6.3 g/ml). Further, the butanol fraction was fractionated and yielded into 13 fractions.

Results: The methanol extract of the leaves of *A. pauciflorum* exhibited concentration dependent inhibition against the JFH1 strain of HCV genotype 2a with an IC₅₀ is 72.5 µg/ml. The butanol fraction exhibited the highest anti-HCV activity with an IC₅₀ is 6.3 µg/ml. The butanol fraction was fractionated which yielded 13 fractions. Fractions 5 and 13 exhibited high anti-HCV activities with IC₅₀ is 5.0 µg/ml and 8.5 µg/ml and a time-of-addition study demonstrated that fraction 5 inhibited viral infection at the post-entry step, whereas fraction 13 primarily inhibited the viral entry step.

Conclusion: The extract *A. pauciflorum* can be used as a herbal-based antiviral drug.

Keywords: *Archidendron pauciflorum*, HCV, JFH-1

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Hepatitis C infection disease caused by the hepatitis C virus (HCV) primarily affects the function of liver. HCV is a major cause of chronic liver diseases and linked to the development of cirrhosis and hepatocellular carcinoma. Approximately 130–200 million people worldwide are infected with HCV,¹ which is spread primarily by blood-to-blood contact which associated with intravenous drug use, contaminated medical equipment, transfusion, and organ transplants. Boceprevir or telaprevir plus pegylated interferon alpha (IFN- α) and ribavirin are the standard therapies for patients with chronic HCV infections. This triple combination therapy increases the sustained viral, with the response rates up to 75% in patients with HCV genotype 1 infections, which respond relatively poor to IFN- α therapy.² However, it is still important to search for new and effective anti-HCV drugs to increase sustained viral responses as well as reduce the treatment side effects and costs.

Medicinal plants are attractive and important resources of bioactive compounds.³ Natural molecules derived from medicinal plants and natural products have been identified as anti-HCV agents in recent years.^{4–12} Among them, silymarin, ladanein, and (-)-epigallocatechin have been shown to be bioactive via oral administration in mice, healthy individuals, or HCV-infected patients,^{13–15} representing potential therapeutic use in clinical trials.

Djengkol is the common name of *Archidendron pauciflorum*. It is also called *Abrema jiringa* Kosterm, *Albizia jiringa* (Jack) Kurz, *Albizia lucida sensu auct.* *Archidendron pauciflorum* (Benth.) I. C. Nielsen, *Feuileea jiringa* Kuntz, *Pithecellobium jiringa* (Jack) Parin etc.¹⁶ *A. jiringa* is a leguminous tree plant belonging to the family of Fabaceae. This plant has been used as a traditional medicine for a range of ailments and consumed as a raw vegetable in Malaysia.¹⁷ Some chemical constituents is djenkolic acid (sulfur-containing amino acid), from leaves, have been reported containing five flavan-3-ol derivatives and new flavan-3-ol galates, galocatechin-3' and 4'-O-gallates, galocatechin7,3' and 7,4'-di-O-gallates. Procyanidinds-B3 and B-4, prodelphinidine B-1. Methanol extract leaves of *A. jiringa* active as antibacterial were mostly active for *Staphylococcus aureus*, *S. epidermidis*, and *Microsporum gypsum*.¹⁸ The shoot of *A.*

jiringa has been found to have high polyphenolic contents (>150 g gallic acid equivalents/mg dried plant) and antioxidant activities measured using ferric reducing antioxidant power (FRAP) method, also have high potent to 2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging activity.^{19,20} The beans of this plant are used as food sources in Indonesia and contain djenkolic acid and sulfur. Those elements can inhibit the pests as well as vitamin C and poly phenolic compounds, which have potential anti-oxidant activities.²¹ Plants of the Fabaceae family have been reported to exhibit various anti-viral activities. For example, glycyrrhizic acid from *Glycyrrhiza uralensis* Fisch was reported to exert anti-viral activities against coxsackievirus A16 and enterovirus 71, which are the most common causative agents of hand, foot, and mouth disease.²⁰ Extracts from *Acacia confusa*, were shown to exhibit anti-HCV activities.^{21–24} A sulfate prepared from galactomannan derived from *Mimosa scabrella* was reported to exert *in vitro* anti-herpetic and anti-rotaviral activities.²⁵ However, the possible anti-viral activities of *A. pauciflorum* extracts have not yet been reported.

In the course of our screening program of the anti-HCV activities of Indonesian medicinal plants, we observed the potential of the methanol crude extract and butanol fraction from the leaves of *A. pauciflorum* as anti-viral activities against HCV. Bioactivity-guided fractionation was performed and the mode of action of these fractions with anti-HCV activities was analyzed.

METHODS

Study Design

This was an *in vitro* study in human hepatocyte culture, Huh-7it cells using hepatitis C virus genotype-2a JFH-1 strain. Huh-7it cells were used to propagate HCV. All of the experiments were conducted in triplicate.

Collection of *A. pauciflorum*, extraction of crude extracts, and preparation of fractions

A. pauciflorum leaves used in this study were obtained from the Research Center for Chemistry, Indonesian Institute of Sciences (LIPI), Kawasan Puspiptek Serpong, Indonesia. The leaves samples were verified by botanical researchers at Herbarium, Research Center for Biology, LIPI, Cibinong, Indonesia. A herbarium

specimen was deposited in the Research Center for Chemistry-LIPI. *A. pauciflorum* leaves were dried, pulverized, and subsequently macerated in 100% methanol for 24 h. The extracts were filtered and the residue was resuspended in fresh 100% methanol. This maceration process was repeated over three days. The combined filtrates were evaporated at room temperature, not exceeding 40°C. The extract (80 g) was initially partitioned with *n*-hexane, ethyl acetate, *n*-butanol, and water to obtain a hexane fraction (18.2 g), ethyl acetate fraction (3.0 g), butanol fraction (30.0 g), and water fraction (25.3 g). Based on bioactivity results, the butanol fraction (2.5 g) was subjected to silica gel 60 column chromatography (0.063–0.200 mm; Merck, Darmstadt, Germany) with *n*-hexane: ethyl acetate and ethyl acetate: methanol by gradient elution, which yielded 13 subfractions (fraction number 1–13).

Sample preparation

The methanol crude extracts and subfractions were dissolved in DMSO to obtain stock solutions at concentrations of 100 and 50 mg/ml, respectively. The stock solutions were stored at –30°C until used.

Cell culture and virus preparation

Huh7it-1 is a well differentiated hepatocyte derived from cellular carcinoma cell line that was originally taken from a liver tumor in a 57 year-old Japanese male in 1982. The cell line was established by Nakabayashi and Sato J.²⁵

The human adapted variants of the HCV strain JFH-1 (JFH1a)²⁶ were inoculated into the Huh7it-1 cells. Culture supernatants were collected at 3–5 days post infection (p.i.), concentrated using Amicon 100K centrifugal filters, and stored at –80°C. Huh7it-1 cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (Biowest, Nuaille, France), 100 µg/ml kanamycin (Sigma-Aldrich, St. Louis, MO, USA), and non-essential amino acids (Invitrogen). Cells were grown at 37°C in an atmosphere of 5% CO₂.

Virus titration

Cells (2.4×10⁴ cells/well) were placed in a 96-well plate 1 day before the virus infection. Culture supernatants were serially diluted 10-fold with medium in a 96-well plate, and 50 µL of viruses

were adsorbed to the cells for 4 h at 37°C. Then, the cells were incubated with a medium containing 0.4% methylcellulose (Sigma-Aldrich) for 40 h. HCV titers were determined using a focus-forming assay as previously described.^{27,28} Antigen-positive cells were stained with anti-HCV anti-serum and horseradish peroxidase-conjugated goat anti-human IgG (MBL, Tokyo, Japan). Infectious foci were visualized using the Metal Enhanced DAB Substrate Kit (Pierce-Thermo Scientific). Foci in each well were counted and normalized to untreated controls to calculate the titre of virus.

Evaluation of anti-viral activities

Huh7it-1 cells (5.0×10⁴ cells/well) which cultured into a 48-well plate were infected with HCV at a multiplicity of infection of 0.1 in the presence of *A. pauciflorum* extracts or fractions (100, 50, 20, 10, 5, and 1 µg/ml) for 2 h at 37°C. After removing the residual viruses by rinsing the wells twice with serum-free medium, the cells were incubated for 46 h at the same sample concentrations. At 48 h p.i., the culture supernatants were collected and used for viral titration. In some experiments, the percent infectivity of the fractions at a concentration of 20 µg/ml was determined. Cells were treated with fractions 5 or 13 only during or only after viral inoculation for the remaining culture period until viral harvest to assess the mode of action of the plant extracts.²⁶

Cytotoxicity assay

Cytotoxicities of the crude extracts and the fractions against Huh7it-1 cells were evaluated using the MTT [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] assay (Sigma-Aldrich) following the manufacturer's instructions. Cells (2×10⁴ cells/well) were placed in a 96-well plate and treated with a medium containing serial dilutions of the plant extracts or fractions (1 to 300 µg/ml) for 48 h. Cyclosporine A were used as a comparator. The medium was replaced with 100 µl of MTT solution and incubated at 37°C for an additional 4 h. The medium was removed and 100 µl of dimethyl sulfoxide (DMSO, Sigma-Aldrich) was added to dissolve the generated precipitate. Absorbance at 550 nm was measured using a GloMax-Multi Microplate Multimode Reader (Promega). The percentage of viable cells versus the concentration of the test compound

was plotted. The concentration by which to mediate 50% inhibition (CC_{50}) was determined by non-linear regression analysis.

Data analysis

The data were presented in mean standard deviation. The data were analyzed using non-linear regression analysis. IC_{50} were calculated from the formula of non-linear regression.

RESULTS

Anti-HCV activities of extracts from *A. pauciflorum*

We examined crude extracts of 250 medicinal plants for anti-HCV effects using a cell-culture HCV system to investigate activities against HCV.²⁴ Huh7it-1 cells, which are highly permissive and obtained by curing replicon cells with IFN- α , were challenged with the HCV genotype 2a strain JFH1 in the presence of extracts. Virus titer in the culture supernatants at 48 h p.i. was determined using a focus-forming assay. Screening results revealed that a crude extract from *A. pauciflorum* dose-dependently suppressed production of infectious virus particles [50% inhibitory concentration (IC_{50}): 72.5 $\mu\text{g/ml}$] without cytotoxicity at the concentrations tested. Cytotoxicity of a crude extract to non-infected cells was determined using the MTT assay, which showed a CC_{50} of 190 $\mu\text{g/ml}$ and a selectivity index (SI, CC_{50}/IC_{50}) of 2.6 (Table 1).

The crude extract was partitioned into n-hexane, ethyl acetate, butanol, and water fractions and subsequently investigated for anti-HCV activities. The butanol fraction had the highest anti-HCV activity with an IC_{50} value of

Table 1. The effects of methanol crude extract from *A. pauciflorum* and its fractions on anti-HCV activities (IC_{50}) and cell viabilities (CC_{50})

Samples	IC_{50} ($\mu\text{g/ml}$)	CC_{50} ($\mu\text{g/ml}$)	SI
Crude extract	72.5	190	2.6
Hexane fraction	>60	415.2	>6.9
Ethyl acetate fraction	18.6	361.2	16.7
Butanol fraction	6.3	167.2	26.5
Water fraction	45.3	>600	>13.2

SI: Selectivity index, CC_{50} value was divided by the IC_{50} value

6.3 $\mu\text{g/ml}$ (Table 1). The ethyl acetate fraction exhibited moderate anti-HCV activity (IC_{50} , 18.6 $\mu\text{g/ml}$), whereas the hexane and water fractions exhibited weak or no anti-HCV activities. Cytotoxicities of these fractions to non-infected cells were determined and a CC_{50} value of the butanol fraction was 167.2 $\mu\text{g/ml}$, whereas those of other fractions were >300 $\mu\text{g/ml}$. We selected the butanol fraction for further fractionation due to the high SI value (26.5).

Anti-HCV activities of the isolated fractions

Furthermore, the butanol fraction was separated using silica-based open column chromatograph which yielded 13 fractions as described in figure 2. Cells were infected with HCV in the presence of each fraction (20 $\mu\text{g/ml}$) and the effects of each fraction on HCV infection were examined. All fraction exhibited varying levels of inhibition of HCV infection. However, we observed that fraction 5 and 13 exhibited a strong inhibition of 100% and 91%, respectively, of the untreated control (Table 2). The IC_{50} and CC_{50} values of fraction 5 were 5.0 and 33.2 $\mu\text{g/ml}$, respectively, whereas those of fraction 13 were 8.5 and 62 $\mu\text{g/ml}$ respectively.

Table 2. Effects of the butanol fractions from *A. Pauciflorum* on HCV JFH1 and cell viabilities

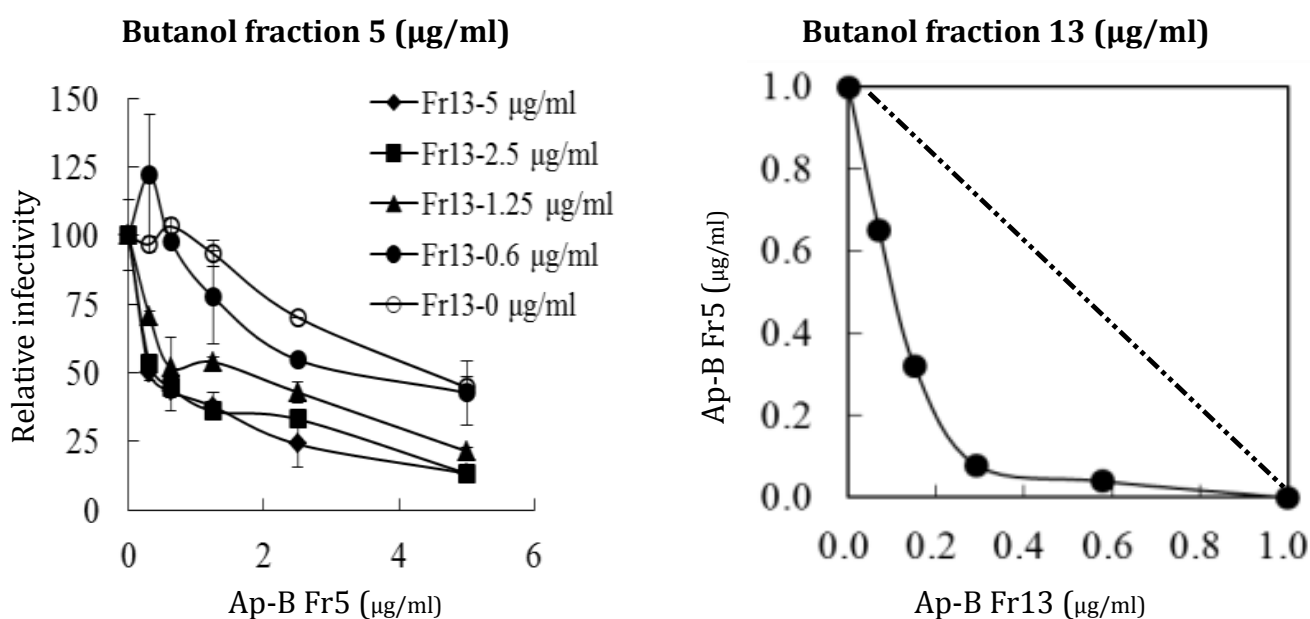
Samples	Inhibition (%)	Cell viability (%)	IC_{50} ($\mu\text{g/ml}$)	CC_{50} ($\mu\text{g/ml}$)
Fraction 1	-37 \pm 5.5	102 \pm 2.0	NT	NT
Fraction 2	-26 \pm 6.7	99 \pm 0.7	NT	NT
Fraction 3	7 \pm 3.1	97 \pm 0.1	NT	NT
Fraction 4	26 \pm 6.1	101 \pm 1.2	NT	NT
Fraction 5	100	108 \pm 0.2	5.0	33.2
Fraction 6	11 \pm 5.5	108 \pm 1.0	NT	NT
Fraction 7	59 \pm 5.5	84 \pm 4.9	NT	NT
Fraction 8	24 \pm 1.7	104 \pm 1.3	NT	NT
Fraction 9	28 \pm 2.5	112 \pm 3.2	NT	NT
Fraction 10	36 \pm 4.9	113 \pm 1.5	NT	NT
Fraction 11	44 \pm 6.1	114 \pm 0.4	NT	NT
Fraction 12	47 \pm 4.3	112 \pm 0.4	NT	NT
Fraction 13	91 \pm 0.6	92 \pm 1.4	8.5	62
Cyclosporin A	88 \pm 2.0	102 \pm 2.1	NT	NT

Inhibition and cell viability at the concentration of 20 $\mu\text{g/ml}$ were shown except for the concentration of cyclosporin A as an inhibitor control, which is 1 $\mu\text{g/ml}$. Values were shown as mean \pm SD values of two independent experiments; NT= not tested

Table 3. Mode of action studies of butanol fractions 5 and 13 from *A. pauciflorum*

	% Inhibition			Mode of action
	During + post-inoculation	During inoculation	Post-inoculation	
Fraction 5	98.9±0.5	63.4±4.1	95.4±1.2	Post-entry inhibition
Fraction 13	95.7±0.9	85.6±1.4	66.4±0.5	Entry inhibition

Fractions 5 and 13 at the concentration of 10 µg/ml and 20 µg/ml, respectively. Values presented the mean ± SD from three independent experiments

**Figure 1.** Synergetic effects of the butanol fraction 5 and 13

Mode-of-action of butanol fraction 5 and 13

To determine whether the anti-HCV effects of the two fraction were inhibited at the entry or the post-entry step, time-of-addition experiments were performed. As presented in Table 3, fraction 5 exhibited anti-HCV activity at the post-entry step, whereas fraction 13 exhibited anti-HCV activity primarily at the entry step. These findings suggested that each fraction contained different compounds with different mechanism of action.

The percent infectivity by treatment with fraction 5 was plotted for each fixed concentration of fraction 13 (Figure 1a). The curves shifted to the left with increasing

concentrations of fraction 13, demonstrating a synergistic effect of the two fraction against HCV infection. This synergistic effect was further evaluated by classic isobologram analysis and the dose-effect plots were drawn. The results demonstrated that the curve was below the line showing an additive effect, indicating that the effect of a combination of the two fractions on HCV infection was strongly synergistic (Figure 1b). Cytotoxicity was not observed at the tested concentrations via isobologram analysis (data not shown). These results collectively suggested that the strong anti-viral activities from the butanol fraction of *A. pauciflorum* leaves extracts have been contributed to several compounds with different anti-viral mechanisms.

DISCUSSION

Due to the high rate of pre-existing or emerging viral resistance, effective treatment of HCV-infected patients will likely require a combination of inhibitors targeting distinct viral or host functions. As described above, we demonstrated that both fractions 5 and 13 exerted inhibition activities with different modes of action. On the other hand, the anti-HCV effect by mono-treatment of each butanol fractions were not stronger than that of treatment with the butanol fraction alone. It was possible that fractions have synergistic mode in butanol fractions than each fraction alone. Therefore, we examined the anti-HCV effects of the two fractions in combination. Cells were infected with HCV and treated with combinations of fractions 5 and 13 at various concentrations.

A. pauciflorum (known as *A. jiringa*, *Pithecellobium jiringa* in Malaysia) is a typical plant in Southeast Asia including Indonesia. The bean of this plant contains jenkolic acid, sulfur, vitamin C and E, flavonoid, protein, and fatty acids.²¹⁻²⁴ The seed is used traditionally for the treatment of hypertension and diabetes, whereas the old leaves burnt to ashes were used against itching parts.²⁵ This plant was also found a high contain of polyphenolic compounds which has a potent antioxidant activity.²⁷ Although the compounds responsible for anti-HCV activities have not yet been isolated in this study, we observed that fraction 5 contained chlorophyll-related compounds, known to show antioxidant activity as major constituents (obtained from our preliminary study). We isolated a chlorophyll-related compound from another Indonesian plant by activity-guided purification and its compound exhibited anti-HCV activity (manuscript in preparation). Also, different groups previously reported that pheophytin and chlorin e6, which are chlorophyll degradation products, exhibited potential anti-HCV activities. The mechanism is through the inhibition of NS3 protease and probably disruption of the envelope protein, respectively.^{28,29} The activity of fraction 5 may have contributed to the presence of chlorophyll-related compounds. Further activity-guided purification and analytical studies are required to explore these anti-HCV substances. We have

not done the analysis of contents from butanol fraction 13.

In conclusion, crude extract from *A. pauciflorum* leaves, and the butanol fraction exhibited great potential as anti-HCV agents. The butanol fraction yielded a higher SI value compared to the crude extracts and other fractions. Among 13 butanol fractions, there were two fractions only fractions exhibited strong anti-viral activities. Synergetic effects were observed by a combination treatment of these two fractions. The results of the present investigation provide evidence of the potential of medicinal plants for herbal drug as anti-HCV. However, further study for the use of this fraction *in vivo* is still need to evaluate its efficacy and toxicity.

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Conflict of Interest

The authors affirm no conflict of interest in this study.

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