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AntiHepatitis C Virus Activity of *Alectryon serratus* Leaves Extract

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Abstract

Hepatitis C Virus (HCV) has infected approximately 2-3% (130-170 million) of the world's population. No vaccine is available to prevent HCV infection. Investigation of anti-HCV agent is thus deemed necessary. Various plants have been explored for their anti-HCV activity. *A. serratus* is a member of Sapindaceae family, which fruit and seed were traditionally used as insecticide. Anti-HCV activity tested on *A.serratus* leaves extract has been done. The result showed that leaves extract exhibited anti-HCV with IC_{50} value of 14.9 μg/ml and 9.8 μg/ml against HCV J6/JFH1 and JFH1a, respectively. The cytotoxicity assay results showed that *A.serratus* leaves extract was not toxic and has CC_{50} >100 μg/ml. Mode of action experiment results suggested that *A.serratus* extract inhibited HCV at the post-entry step. Further fractionation of leaves extract by open column chromatography resulted in 4 fractions. Only Fraction 1 (AP-5F.1) exhibited anti-HCV with IC_{50} value of 1.2 μg/ml against HCV JFH1a. Separation of AP-5F.1 by open column chromatography resulted in 15 fractions. Fraction number 13 (AP-5F.1.13) exhibited anti-HCV with IC_{50} value of 0.43 μg/ml against HCV JFH1a. Separation of AP-5F.1.13 by semi preparative-HPLC resulted in isolate identified by TLC and LC-MS method as chlorophyll derivate. There was a possibility that chlorophyll derivate has participated in performing the anti-HCV activity of fractions and extract besides the other compounds contained. In this study, we concluded that *A. serratus* leaves extract, AP-5F.1, and AP-5F.1.13 exhibited anti-HCV activity against JFH1a virus.

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Keywords: Alectryon serratus; leaves extract; antiHepatitis C Virus

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Nomenclature

HCV	Hepatitis C Virus	HPLC	High Pressure Liquid Chromatography
IC_{50}	Inhibitory Concentration 50	CC_{50}	Cytotoxicity Concentration 50

1. Introduction

Hepatitis C Virus (HCV) has infected approximately 2-3% (130-170 million) of the world's population. No vaccine is available to prevent HCV infection. Treatment for HCV has been developed and rapidly evolving. However, several obstacles remain in the current treatment of HCV[1]. Development of anti-HCV agent is thus deemed necessary. Various plants have been explored for their anti-HCV activity. Anti-HCV activity screening of 21 samples from 17 species of medicinal plants explored in the East Java Region, Indonesia revealed that extract of *Toona sureni* leaves (TSL), *Melicope latifolia* leaves (MLL), *Melanolepis multiglandulosa* stem (MMS), *Ficus fistulosa* leaves (FFL) showed antiviral activity with IC_{50} value at a range of 2.0 – 17.1 μg/ml. Time of addition experiments revealed that TSL and MLL inhibited both at the entry and post-entry steps, while MMS and FFL principally at the entry step[2]. Several numbers of isolated compounds were also reported to exhibit antiviral activity against HCV.

Glycycoumarin, glycyrin, glycyrol and liquiritigenin isolated from *Glycyrrhiza uralensis* were identified as anti-HCV compounds with IC_{50} value of 8.8, 7.2, 4.6 and 16.4 μg/ml, respectively. Those compounds acted on the post-entry step[3]. Quercetin and gallic acid isolated from *Kalanchoe pinnata* also inhibited HCV production in a dose-dependent manner, with IC_{50} value of 1.5 and 6.1 μg/ml, respectively, without exhibiting cytotoxicity. Quercetin acts at the post-entry step, whereas gallic acid at both the entry and post-entry steps[4]. In this study, the anti-HCV activity of *Alectryon serratus* has been determined. *A. serratus* is a member of Sapindaceae family, which fruit and seed were traditionally used as insecticide. It was reported that *A.serratus* leaves extract has antimalarial activity with IC_{50} value of 12.3 μg/ml and antioxidant activity with IC_{50} value of 1.96 μg/ml[5,6], but no reference was found regarding the antiviral activity of this plant. This study aims to determine the active substances of *A.serratus* extract which play a role in anti-HCV activity.

2. Methods

2.1. Plant material

Alectryon serratus leaves were collected from Alas Purwo National Park, Banyuwangi, East Java, Indonesia. Authentication and identification of plant were carried out at The Purwodadi Botanical Garden, East Java.

2.2. Extraction and fractionation

Leaves of *A.serratus* were dried at room temperature and pulverized. Dried leaves powder were extracted using ethanol 80% as solvent by ultrasonic assisted extraction for two minutes to three times replications. The ethanol extract was filtered and the obtained filtrates were concentrated using rotary evaporator to obtain ethanol extract of *A.serratus* (AP-5F). Further separation of AP-5F was done by open column chromatography using silica gel as stationary phase and gradient of chloroform-methanol system as development solvent. The separation resulted in 4 fractions (AP-5F.1–4) which were later continued for bioassay. A bioactivity-active fraction(s) was further fractionated under open column chromatography with sephadex LH-20 and 90% mobile phase methanol. The separation of AP-5F.1 resulted in 15 fractions (AP-5F.1.1-1.15). AP-5F.1.13 was further separated by semi preparative HPLC (solvent system: acetonitrile-methanol-water 75:2.5:22.5, column: Water XBridge 10x250 mm 5μm, flowrate: 2.5 ml/min, detection UV 365 nm).

2.3. Cells and viruses

Huh7it cells were cultivated in Dulbecco's modified Eagle's medium supplemented with fetal bovine serum, non essensial amino acids, penicillin and streptomycin. Cells were grown at 37°C in a 5% CO_2 incubator.

2.4. Analysis of anti-HCV activities

A.serratus extract and fractions were dissolved in dimethyl sulfoxide (DMSO) to obtain stock solutions at a concentration of 100 mg/ml. The stock solutions were stored at -20°C until it was used.

Analysis of anti-HCV method was described previously[7]. Huh7it cells were seeded in 24-well plates (5×10^4 cells/well). A fixed amount of JFH1a, with multiplication of infection (MOI) of 0.1 focus-forming units (ffu)/cell, was mixed with serial dilutions of the extracts (100, 50, 25, 12.5, 6.25 and 3.1 μg/ml) and inoculated to the cells. After 2 hours, the cells were washed with medium to remove the residual virus and further incubated in the medium containing the same concentrations of the test samples as those during virus inoculation.

Time of addition experiments were performed to assess the mode of action of the samples. Three sets of experiments were done in parallel: (i) to assess the antiviral effect at the entry step, the mixture of HCV and sample was inoculated to the cells. After virus adsorption for 2 hours, the residual virus and the sample were removed, and cells were refed with fresh medium without the sample for 46 hours; (ii) to assess the antiviral effect at the post-entry step, HCV was inoculated to the cells in the absence of the sample. After virus adsorption for 2 hours, the residual virus was removed and cells were refed with fresh medium containing the sample for 46 hours; (iii) as a positive control, HCV mixed with the sample was inoculated to the cells[8]. After virus adsorption for 2 hours, the residual virus and the sample were removed, and cells were refed with fresh medium containing the sample for 46 hours. Culture supernatants were obtained at 1 and 2 days post-infection (dpi) and titrated for virus infectivity. Virus and cells treated with medium containing 0.1% DMSO served as a control. The inhibition percentage of virus infectivity by the samples were calculated by comparing to the control using SPSS probit analysis, and IC_{50} values were determined.

2.5. Cytotoxicity assay

The cytotoxicity of the samples was assessed by MTT assay. Huh7it cells in 96 well plates were treated with serial dillution of the samples or control. The condition of the cells was observed after 46 hours incubation and the toxicity was checked under microscope. The medium was removed from 96 well plates and then MTT 10% 150 μl/well was put by multichannel pipette and incubated for 4 hours at 37°C. MTT solution was removed from 96 well plates and 100μl/well DMSO 100% was then put for dissolve. Absorbance was checked at 560 nm and 750 nm, shaker 0.5 min before reading absorbance.

3. Results and discussion

Anti-HCV activity screening of *A.serratus* leaves and stem extract revealed that leaves extract exhibited anti-HCV with IC_{50} value of 14.9 μg/ml and 9.8 μg/ml against HCV J6/JFH1 and JFH1a, respectively. The cytotoxicity assay results showed that *A.serratus* leaves extract has CC_{50} >100 μg/ml (Table 1). Mode of action experiment results suggested that *A.serratus* extract inhibited HCV at the post-entry step (Table 2).

Table 1. Anti-HCV activity of extract and fractions of *A.serratus*

Sample	IC_{50} (μg/ml)	CC_{50} (μg/ml)
AP-5F	14.9*	>100*
AP-5F	9.8	347.21
AP-5F.1	1.2	27.95
AP-5F.1	<10*	>100*
AP-5F.1.3	78.93	
AP-5F.1.9	NA	
AP-5F.1.13	0.43	39.73

All samples were tested against JFH1a, except for * was tested against J6/JFH1.

Table 2. Time of addition analysis of *A.serratus* extract (AP-5F)

Replicate	IC_{50} (µg/ml)			Mode of action
	During+post inoculation	During inoculation	Post inoculation	
1	6.25	11.3	6.25	Post-entry inhibition
2	5.5	20.1	7.9	
3	9.5	22.6	10.7	

Further fractionation of leaves extract by open column chromatography resulted in 4 fractions. Only Fraction 1 (AP-5F.1) exhibited anti-HCV with IC_{50} value of 1.2 µg/ml against HCV JFH1a. Separation of AP-5F.1 by open column chromatography using sephadex LH-20 as stationary phase and methanol 90% as mobile phase resulted in 15 fractions (AP-5F.1.1 – 1.15). Thin layer chromatography (TLC) profile of fractions is shown in Fig. 1.

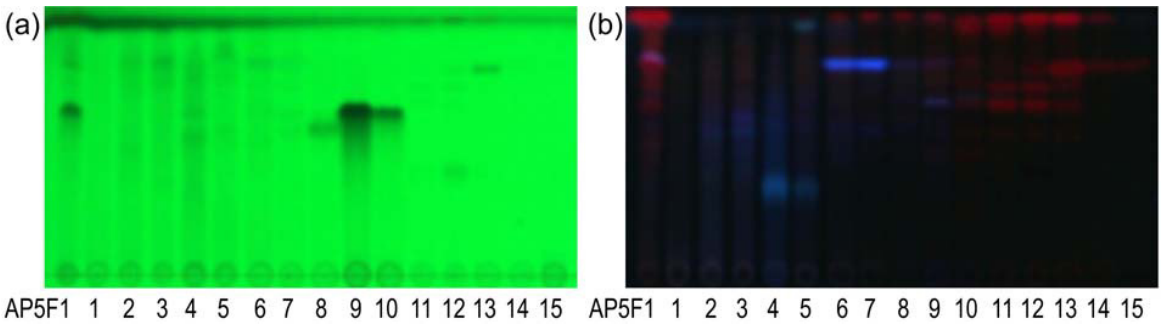

Fig. 1. TLC profile of fraction AP-5F.1.1 – 1.15 (15 fractions), detected under UV 254 nm (a), UV 365 nm (b)

Fraction number 13 (AP-5F.1.13) exhibited anti-HCV with IC_{50} value of 0.43 µg/ml against HCV JFH1a. On the TLC analysis (detection UV 365 nm) of AP-5F.1.13, a red fluorescent spot was detected. This results suggested that AP-5F.1.13 consists of chlorophyll compounds. Separation of AP-5F.1.13 by semi preparative-HPLC (solvent system: acetonitrile-methanol-water 75:2.5:22.5, column: Water XBridge 10x250 mm 5µm, flowrate: 2.5 ml/min, detection UV 365 nm) resulted in isolate identified by TLC and Liquid Chromatography-Mass Spectra (LC-MS) method as chlorophyll derivate. There was a possibility that chlorophyll derivate has participated in performing the anti-HCV activity of fractions and extract besides the other compounds contained. This suggestion was confirmed by previous study. It was reported that pheophorbide a and pyropheophorbide a, isolated from *Morinda citrifolia* showed potent anti-HCV activities with IC_{50} of 0.3 and 0.2 µg/ml, respectively[9]. Therefore, further purification is still required to isolate and identify the pure chlorophyll compounds that take a role in anti-HCV activity.

4. Conclusion

In this study, we concluded that *A. serratus* leaves extract (AP-5F), fraction AP-5F.1, and fraction AP-5F.1.13 exhibited anti-HCV activity against JFH1a virus.

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