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Research article

Anti-Hepatitis C Activity and Toxicity of *Scoparia Dulcis* Linn. Herb

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ABSTRACT

Hepatitis C Virus (HCV) infection is a serious public health problem since HCV is the ribonucleic acid (RNA) virus that easy to mutate. The HCV standard treatment has rapidly developed but the possibility of resistance and effectiveness of treatment needs to be considered. The medicinal plants are a source of various compounds that may potentially cure diseases including infectious diseases. Since a long years ago, medicinal plants were famous as an inherited treatment that believed to cure the disease. One of the medicinal plants is Scoparia dulcis (S. dulcis) that belongs to Scrophulariaceae family and traditionally used as remedies for digestive problems, hypertension, diabetes mellitus, bronchitis, and as an analgesic & antipyretic agent. The previous report showed that S. dulcis was known active as an antiviral against Herpes Simplex Virus (HSV) type 1 in vitro and in vivo. The aim of the study is to determine the biactivity potential of S. dulcis against HCV. Scoparia dulcis was extracted using 80% ethanol (EE) then further separated by liquid-liquid fractionation using dichloromethane (DCMF), ethyl acetate (EAF), butanol solvent (BF) and water (WF). The in vitro anti-HCV analysis was performed with Huh7it cells and HCV JFH1 (genotype 2a) by determining inhibition concentration 50 (IC_{50}). The toxicity (Cytotoxicity Concentration 50, CC_{50}) test was performed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay and mechanism of action were analyzed using time addition experiment. Phytochemical groups as the suspected active compounds of S. dulcis were identified by Thin Layer Chromatography (TLC) and observed under UV 254 nm, UV 365 nm, before and after sprayed using H_2SO_4 10% and heated at 105°C for 5 minutes. The IC_{50} test result of 80% EE and DCMF showed anti-HCV activity with a value of 12.7±4.8 µg/ml and 5.8±0.69 µg/ml, while EAF, BF, and AF respectively resulted in IC_{50} value of >100 µg/ml that suggested there was no inhibition effect on HCV JFH1. The DCMF was the most active fraction but toxic to the cell with CC_{50} value >23 µg/ml and selectivity index (SI) >3.9. According to the time addition experiment data, DCMF of S. dulcis inhibited post entry step HCV JFH1 infection that it means the possibility was to inhibit virus replication and or virion release. Scoparia dulcis contain chlorophyll, flavonoids and terpenoids as the suspected active compounds for inhibition of HCV JFH1 infecton. Futher study of post-entry inhibitions of HCV infection was needed.

Keywords: *Scoparia dulcis, anti-HCV, toxicity, Huh7it, HCV JFH1*

ABSTRAK

Infeksi Virus Hepatitis C (VHC) merupakan masalah kesehatan yang serius di dunia dikarenakan VHC adalah virus RNA yang mudah untuk bermutasi. Pengobatan VHC telah berkembang pesat namun kemungkinan adanya resitansi dan efektivitas pengobatan perlu untuk dipertimbangkan. Tanaman obat adalah sumber dari berbagai macam senyawa yang potensial untuk mengobati penyakit termasuk penyakit infeksi. Sejak bertahun-tahun sebelumnya tanaman obat dikenal untuk pengobatan turun temurun yang dipercaya dapat menyembuhkan penyakit. Salah satu dari tanaman obat adalah Scoparia dulcis (S. dulcis) yang berasal dari famili Scrophulariaceae dan secara tradisional digunakan untuk pengobatan masalah pencernaan, hipertensi, diabetes mellitus, bronkitis, dan sebagai agent analgesik dan antipiretik. Penelitian sebelumnya menunjukkan S. dulcis

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diketahui aktif sebagai antiviral terhadap Herpes Simplex Virus (HSV) secara in vitro dan in vivo. Tujuan dari penelitian ini adalah mengetahui potensi aktivitas dari S.dulcis terhadap HCV. Scoparia dulcis diekstraksi menggunakan etanol 80% (EE) dan dilanjutkan pemisahan menggunakan metode fraksinasi cair-cair dengan pelarut diklorometana (DCMF), etil asetat (EAF), butanol (BF), dan air (AF). Analisis antiHCV secara in vitro dilakukan dengan menggunakan sel Huh7it dan VHC JFH1 (genotip 2a) dengan menentukan inhibition concentration 50 (IC_{50}). Uji toksisitas (Cytotoxicity Concentration 50, CC_{50}) dilakukan dengan metode 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) dan analisis mekanisme aksi menggunakan uji time addition. Berbagai kelompok fitokimia yang diduga sebagai komponen aktif S. dulcis diidentifikasi dengan Thin Layer Chromatography (TLC) dan dilihat dibawah sinar UV 254 nm, UV 365 nm, sebelum dan sesudah disemprot dengan H_2SO_4 10% serta dipanaskan pada 105^oC selama 5 menit. Hasil uji IC_{50} menunjukkan 80% EE dan DCMF memiliki aktivitas anti-VHC dengan nilai IC_{50} 12,7±4,8 µg/ml dan 5,8±0,69 µg/ml, sedangkan EAF, BF, and AF berturut-turut menghasilkan nilai IC_{50} lebih dari 100 µg/ml yang menunjukkan tidak adanya hambatan terhadap VHC JFH1. Fraksi paling aktif adalah DCMF namun toksik terhadap sel dengan nilai CC_{50} >23 µg/ml dan selectivity index (SI) >3,9. Berdasarkan data pengujian time addition, DCMF S. dulcis menghambat infeksi VHC JFH1 pada post entry step yang berarti kemungkinan menghambat replikasi virus dan atau pelepasan virion. Scoparia dulcis terbukti mengandung klorofil, berbagai flavonoid dan terpenoid yang diduga sebagai komponen aktif penghambat infeksi HCV JFH1. Diperlukan penelitian lebih lanjut terhadap berbagai hambatan post entry pada infeksi VHC.

Kata kunci: *Scoparia dulcis, anti-VHC, toksisitas, Huh7it, VHC JFH1*

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INTRODUCTION

Hepatitis C Virus (HCV) is one of the causative agents of liver disease that potentially develop to liver cirrhosis and hepatocellular carcinoma (HCC).[1] More than 185 million people worldwide were infected by HCV, and 350.000 of them die every year.[2] In Indonesia, there had been estimated that 24 million people were infected with Hepatitis B (HBV) and HCV then 14 million of them had potentially become a chronic liver disease. Patients who had developed into chronic liver disease, around 1.4 million of them potentially develop into liver cancer.[2]

Until now there was no vaccine available for HCV infection. Various genotype and subtype of HCV probably caused difficult vaccine development. The current therapy of HCV infection is direct-acting antiviral agents (DAAs) combined with Interferon (IFN). The HCV infection therapy has improved Sustained Virological Response (SVR) >90%. Many therapies of HCV infection have been developed, but therapeutic efficacy still needs to be improved especially for high-risk populations with relatively low income. The important issues such as drug resistance and safety for long usage also need to be considered. Therefore, it is essential to develop effective, safe, inexpensive, and well-tolerated drugs for HCV infection.[3,4] Medicinal plants are a source of promising drug candidates for HCV infection.[5] Some plants were reported to have an antiviral activity of such as *Phyllantus amarus*, *Acacia nilotica*, *Boswellia carterii*, *Embelia schimperi*, *Piper cubeba*, *Quercus infectoria*, *Trachyspermum ammi*, and *Syzygium aromaticum.*[6]

Scoparia dulcis is a medicinal plant that belongs to Scrophulariaceae family. *Scoparia dulcis* traditionally used to treat some diseases such as digestive problems, hypertension, and diabetes. Another study reported that *S. dulcis* active as an antiviral against herpes simplex virus type 1 (HSV).[7] The phytochemical screening was showed that *S. dulcis* contained coumarin,[8] phenol,[9] saponins,[10] tannins,[11] flavonoids,[12] terpenoids,[13] and catecholamines.[14] In the previous publications, phytochemical groups terpenoids i.e Scopadulcic acid B was reported had antiviral activity against herpes simplex virus (HSV)[15] ; and the extract was reported to reduce virus titer of Coxsackie B1-B6 virus.[16] Some compounds of *S. dulcis*, Scopadulcic acid A was reported had antimalarial activity against *Plasmodium falciparum* in vitro and Scopadulcic

acid B exhibited inhibition of the tumor promoter 12-O-tetradecanoylphorbol-13-acetate (TPA).[7, 17] Furthermore, some compounds of *S.dulcis* were reported as an antitumor or anticancer agents such as Scopadulcic Acid B[18]; Scopadulcic acid C[19]; Benzoxazinoids[20]; and Betulinic acid.[21] Based on the above background, further study was conducted to determine the activity of anti-HCV and toxicity for extract and fractions from *S. dulcis* then analyzed their mechanism of action by time addition experiment, and to identify the presence of active compounds as antiviral of *S. dulcis*.

MATERIALS AND METHODS

Plant Material

Scoparia dulcis herb was obtained from Wain River Protection Forest Region of Balikpapan, East Kalimantan in September 2015 and determined at Lembaga Ilmu Pengetahuan Indonesia (LIPI) Purwodadi, Pasuruan, East Java.

Extraction and fractionation.

Simplicia of *S. dulcis* was extracted by the ultrasonic-assisted extraction method using 80% ethanol as a solvent. The extract was homogenized using ultrasonic then it was separated by filtration by three-time repetition. The Filtrate was collected then the solvent was evaporated by a rotary evaporator. The extract was dried in an oven at a temperature of 40°C and fractionated using dichloromethane 100%, ethyl acetate 100%, butanol 100%, and water successively.

Virus and cells.

Huh7it cells, a clone of human hepatocellular carcinoma-derived from Huh7 cell,[22] were cultured in Dulbecco's Modified Eagle Medium (Wako, Osaka, Japan) completed with 10% Fetal Bovine Serum (FBS, GIBCO), Non-Essential Amino Acids (NEAA, GIBCO), and 0.15 mg/ml kanamycin solution (SIGMA). A cell culture-adapted HCV variant (JFH1 strain of genotype 2a)[22] was propagated with Huh7it cells, suspended in 4ml medium containing JFH1 (1.8x107 ffu, Multiplication of Infection (MOI) 0.1), and incubated at 37 °C in 5% CO_2 for 4 hours with agitation every 30 minutes. Culture supernatant was harvested and removed cell debris by centrifugation on the third day. The supernatant was concentrated using Amicon-Ultra-15 centrifuge filter.[22]

Anti Hepatitis C Virus (Anti-HCV) activity.

Huh7it cells ($5.2x10^4$) were seeded for 24 hours before HCV infection. Hepatitis C virus with MOI of 0.1 was mixed with different concentrations of the plant extract/ fractions (100; 50; 25; 12.5; 6.2; 3.1µg/ml) and then inoculated into the Huh7it cells. After 2 hours of absorption, the cells were washed with medium and further incubated in the medium containing the same extracts for 46 hours.[23] Cultures supernatant were collected to assess the mode-of-action of the samples tested. The 50% inhibitory concentration (IC_{50}) effect was calculated and analyzed by SPSS probit. All experiments were conducted for three times replication to collect Standard Deviation (SD).

Cytotoxicity assay.

The cytotoxicity of the samples was assessed by 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) assay. Huh7it cells ($2.3x10^4$) in 96 well plates were treated with various concentrations of extract/ fractions for 48 hours. The medium was replaced with MTT 10% 150 µl/well containing medium and incubated for 4 hours. Insoluble precipitates were dissolved in Dimethyl Sulfoxide (DMSO) and measured the reaction color at 560 nm absorbance. Percentages of cell viability were compared to the control and calculated for 50% cytotoxic concentration (CC_{50}) values.[24] The ratio of CC_{50} and IC_{50} was calculated to obtain the Selectivity Index (SI) to determine the best candidate among the sample. The best one of *S. dulcis* extract or fraction according to the highest selectivity index was chosen for a time addition experiment.

Time addition experiment.

Time in addition experiments using much concentration of chosen extract/fraction were

performed for HCV JFH1 and Huh7it host cell culture by three set experiments: 1. The virus was inoculated to the cell after pretreatment cell with *S. dulcis* has chosen extract/fraction for 2 hours; 2. Virus was inoculated first (2hr incubation) then continued by adding *S. dulcis* chosen extract/fraction sample after virus fusion; 3. The chosen extract/fraction of *S. dulcis* was added before and after HCV JFH1 infection. All three set experiments were stained using 3,3'-Diaminobenzidine (DAB) staining (Thermo, UK) to visualize the cell infection.[5]

Identification of phytochemical groups in *S. dulcis*.

The identification of phytochemical groups contains in the *S. dulcis* extract and fraction was conducted by Thin Layer Chromatography (TLC). The profile was obtained using silica gel F254 as a stationary phase and chloroform: methanol (9:1 v/v) as a mobile phase. The plate was observed under UV 254 nm, UV 365 nm, and UV 365 after sprayed using H_2SO_4 10% and heated at 105^{o}C for 5 min.

RESULTS AND DISCUSSION

There were five samples resulted from *S. dulcis* separation i.e 80% Ethanol Extract (EE), Dichloromethane fraction (DCMF), Ethyl acetate fraction (EAF), Butanol fraction (BF), and Aqueous fraction (AF). The result of anti-HCV (IC_{50}), toxicity (CC_{50}), and Selectivity Index (SI) as a ratio of CC_{50} and IC_{50} of *S. dulcis* extract/fraction was presented in Table 1.

The result in Table 1 showed that 80% EE was active inhibited JFH1 with IC_{50} value of 12.7±4.8 µg/ml and less toxic with CC_{50} >100 µg/ml. Further analysis of fraction showed the most active fraction of 80% EE was DCMF with IC_{50} value of 5.8±0.69 µg/ml meanwhile the EAF, BF, and AF didn't show inhibition with IC_{50} value of >100 µg/ml.

Based on dose-dependent inhibition and cytotoxicity activity, it was showed that the anti HCV activity of DCMF from *S. dulcis* increased after concentration > 6.25 ug/ml but it was also followed by increased toxicity in cells (Figure 1). According to toxicity data, DCMF has the strongest toxicity among four fractions. The toxicity on DCMF may disturb HCV infection to the Huh7it or/and affected directly to virus inhibition.

To determine the anti-HCV mechanisms, a time of addition the experiment was performed in this study. *Scoparia dulcis* DCMF was analyzed for a mechanism of action at various dose extract during inoculation and post-inoculation. The results revealed that the mechanism of HCV JFH1 inhibition was dominantly in post-entry inhibition (post-inoculation) with IC_{50} value of 9.25 µg/ml (Table. 2) than entry inhibition (during inoculation).

The result in Table 2 was demonstrated the possible inhibition process in the assembly or/and release progeny virions. The inhibition of the virion replication and release can be affected by all virus life cycles and disturbed virus infection in the cells. Further analyzed on the specific inhibition on post-entry-step in host cells were needed.

The result of identification of phytochemical groups contained in the *S. dulcis* extract/fraction showed in Figure 2. Chlorophyll was identified as one of the phytochemical compounds contained in the EE and DCMF. It can be indicated by red bands at TLC profile when observed under UV 365 nm in figure 2B and 2D; and indicated by dark bands when observed under UV 254 nm in figure 2A (white arrows).[25,26]

Table 1. Anti-HCV activity (IC_{50}), CC_{50}, and SI of *S. dulcis* extract/fraction

***S. dulcis* Extract/ Fraction**	**IC_{50} (µg/ml) ± SD**	**CC_{50} (µg/ml)**	**SI (CC_{50}/ IC_{50})**
80% EE	12.7±4.8	>100	>7.87
DCMF	5.8±0.69	>23	>3.97
EAF	>100	>800	>8
BF	>100	>800	>8
AF	>100	>800	>8

IC_{50} : 50% Inhibition concentration of HCV JFH1 infection in Huh7it culture
CC_{50} : 50% Cytotoxicity concentration in Huh7it culture
SI : Selectivity index

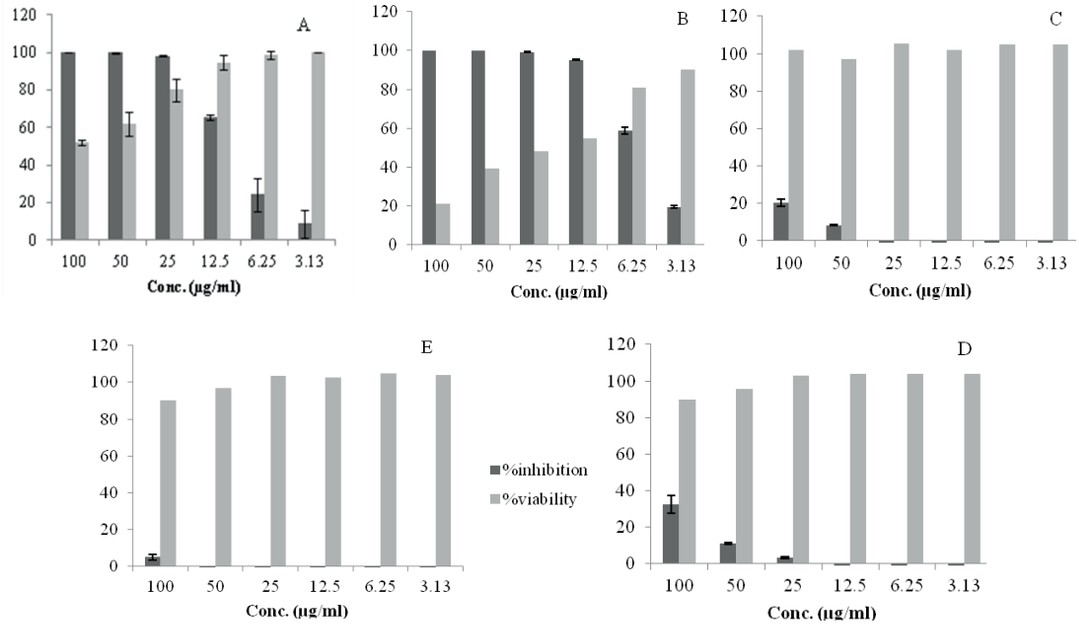

Figure 1. Dose-dependent Inhibition and Toxicity of *S. Dulcis;* A. 80% EE, B. DCMF, C. EAF, D. BF, E. AF.

Table 2. Mode of action of DCMF from *S. dulcis*

DCMF Concentration (ug/mL)	During and Post inoculation	During inoculation	Post inoculation
	%Inhibition	%Inhibition	%Inhibition
100	100	98.68	100
50	100	71.58	100
25	99.67	57.37	100
12.5	92.07	30.27	71.58
6.25	64.31	8.79	24.32
3.125	29.28	-3.11	20.03
IC_{50}	5.43 ug/mL	21.64 ug/mL	9.25 ug/mL

The identification of flavonoids and terpenoids compounds, after running TLC was taken using H_2SO_4 10% spray reagent which followed by heating at 105°C for 5 minutes. In Figure 2C and 2D, EE and DCMF were found to have a similar profile. Purple bands and yellow-brownish bands were identified in both samples. In figure 2C, the bands indicated flavonoids (yellow brownish band, white arrow) and terpenoids (purple bands, yellow arrow) compounds contained in EE and DCMF as well.[22, 25] Both samples were active and contain similar phytochemical compounds. Secondary metabolites such as flavonoids, alkaloids, coumarins, and terpenoids/polyphenol compounds have been reported to possess antiviral effects including anti-HCV activities.[3] The similarity of phytochemical compounds contained in both samples matched with the anti-HCV activities. Chlorophyll, terpenoids and flavonoids compounds in EE and DCMF were possible to have a role as anti-HCV active agents.

CONCLUSIONS

Scoparia dulcis EE and DCMF showed antiviral inhibition against HCV with the IC_{50} value of 12.7±4.8 and 5.8±0.69 µg/ml, respectively. Meanwhile, EAF, BF, and AF were not active as anti-HCV with IC_{50} value of >100 µg/ml. The DCMF was the most active fraction as anti-HCV but toxic to the host cells with CC_{50} value of >23

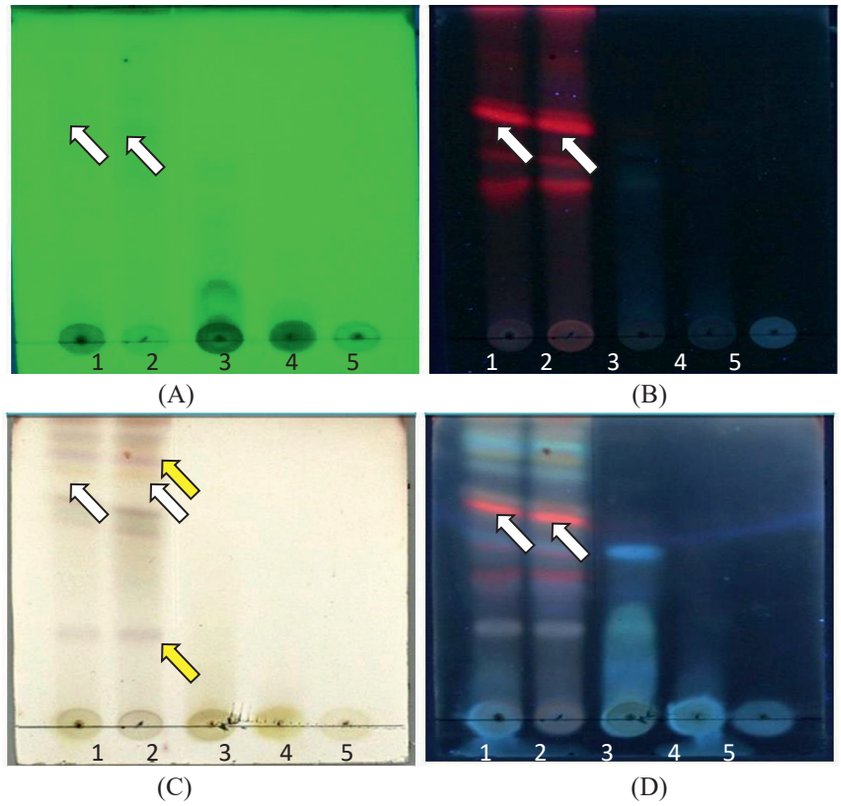

Figure 2. Thin Layer Chromatography profile of S. dulcis. Thin Layer Chromatography (TLC) profile of 1. 80% EE; 2. DCMF; 3. EAF; 4.BF; and 5. AF. The figures were observed in: A. Under UV 254 nm; B. Under UV 365 nm; C. Under visible lamp after sprayed using H2SO4 10% and heated at 105°C for 5 min; D. Under UV 365 nm after sprayed using H2SO4 10% and heated at 105°C for 5 min.

µg/ml and SI >3.97. The time addition experiment showed DCMF was inhibited on post-entry-step of HCV infection, it means the inhibition probably was on virus construction or/and virus release. Chlorophyll, terpenoids and flavonoids compounds in EE and DCMF were suspected to have a role as anti-HCV active agents.

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CONFLICT OF INTEREST

No conflict of interest of this paper.

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