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 (IC50). The toxicity (Cytotoxicity Concentration 50, CC50) 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 H2SO4 10% and heated at 105°C for 5 minutes. The IC50 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 IC50 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 CC50 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 infection. Further 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 resistansi 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 tujuan tanaman yang diperlukan dapat menyebabkan penyakit. Salah satu daripada 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

* Corresponding Author:
aty_ww@yahoo.com; aty-w@ff.unair.ac.id
Aty Widyawaruyanti, et al.: Anti-Hepatitis C Activity and Toxicity of Scoparia Dulcis Linn. Herb

INTRODUCTION

Hepatitis C Virus (HCV) is one of the causative agents of liver disease that potentially develop to liver cirrhosis and hepatocellular carcinoma (HCC). More than 185 million people worldwide were infected by HCV, and 350,000 of them die every year. 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.

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. Medicinal plants are a source of promising drug candidates for HCV infection. 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.

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). The phytochemical screening was showed that S. dulcis contained coumarin, phenol, saponins, tannins, flavonoids, terpenoids, and catecholamines. In the previous publications, phytochemical groups terpenoids i.e Scopadulcic acid B was reported had antiviral activity against herpes simplex virus (HSV) ; and the extract was reported to reduce virus titer of Coxsackie B1-B6 virus. 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).\textsuperscript{7, 17} Furthermore, some compounds of \textit{S. dulcis} were reported as an antitumor or anticancer agents such as Scopadulcic Acid B\textsuperscript{18}; Scopadulcic acid C\textsuperscript{19}; Benzoxazinoids\textsuperscript{20}, and Betulinic acid.\textsuperscript{21} Based on the above background, further study was conducted to determine the activity of anti-HCV and toxicity for extract and fractions from \textit{S. dulcis} then analyzed their mechanism of action by time addition experiment, and to identify the presence of active compounds as antiviral of \textit{S. dulcis}.

**MATERIALS AND METHODS**

**Plant Material**

\textit{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 \textit{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,\textsuperscript{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)\textsuperscript{22} was propagated with Huh7it cells, suspended in 4ml medium containing JFH1 (1.8x10^7 flu, Multiplication of Infection (MOI) 0.1), and incubated at 37 °C in 5% CO\textsubscript{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.\textsuperscript{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.\textsuperscript{23} Cultures supernatant were collected to assess the mode-of-action of the samples tested. The 50% inhibitory concentration (IC\textsubscript{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\textsubscript{50}) values.\textsuperscript{24} The ratio of CC\textsubscript{50} and IC\textsubscript{50} was calculated to obtain the Selectivity Index (SI) to determine the best candidate among the sample. The best one of \textit{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 H2SO4 10% and heated at 105°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 (IC50), toxicity (CC50), and Selectivity Index (SI) as a ratio of CC50 and IC50 of *S. dulcis* extract/fraction was presented in Table 1.

The result in Table 1 showed that 80% EE was active inhibited JFH1 with IC50 value of 12.7±4.8 μg/ml and less toxic with CC50 >100 μg/ml. Further analysis of fraction showed the most active fraction of 80% EE was DCMF with IC50 value of 5.8±0.69 μg/ml meanwhile the EAF, BF, and AF didn’t show inhibition with IC50 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 IC50 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 (IC50), CC50, and SI of *S. dulcis* extract/fraction |
|---------------------------------|------------------|------------------|------------------|
| *S. dulcis* Extract/Fraction    | IC50 (μg/ml ± SD) | CC50 (μg/ml)    | SI (CC50/IC50)   |
| 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               |

IC50 : 50% Inhibition concentration of HCV JFH1 infection in Huh7it culture

CC50 : 50% Cytotoxicity concentration in Huh7it culture

SI : Selectivity index

Copyright © 2020, IJTID, p-ISSN 2085-1103, e-ISSN 2356-0991
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 μg/ml.

The identification of flavonoids and terpenoids compounds, after running TLC was taken using H$_2$SO$_4$ 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. 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. 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.

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

| DCMF Concentration (μg/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 μg/mL                 | 21.64 μg/mL       | 9.25 μg/mL     |
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.

ACKNOWLEDGEMENT

We would like to say thank to Natural Product Medicine Research and Development (NPMRD) laboratory, Institute of Tropical Disease, Universitas Airlangga for financial support and research analysis.

CONFLICT OF INTEREST

No conflict of interest of this paper.

REFERENCES

1. WHO. Hepatitis C 2002 [Available from: http://www.who.int/csr/disease/hepatitis/whocdscsrlyo2003/en/index.html.
2. Guidelines for the Screenin, Care, and Treatment of Person with Hepatitis C Infection [Internet]. WHO Geneva. 2014. Available from: http://www.who.int/hepatitis/publications/hepatitis-c-guidelines-2016/en/.
3. Wahyuni TS, Aoki C, Hotta H. Promising Anti-Virus hepatitis C Compounds from Natural Resources. Natural product communications. 2016; 11(8): 1193–200.
4. Manns MP, Foster GR, Rockstroh JK, Zeuzem S, Zoulim F, Houghton M. The way forward in VHC treatment — finding the right path. Nat Rev Drug Discov. 2007; 6: 991–1000.
5. Wahyuni TS, Tumewu L, Permanasari AA, Apriani E, Adrianti M, Rahman A, et al. Antiviral activities of Indonesian medicinal plants in the East Java region against hepatitis C virus. Virology journal. 2013; 10: 259.
6. Lee J, Lim S, Kang SM, Min S, Son K, Lee HS, et al. Saponin inhibits hepatitis C virus propagation by up-regulating suppressor of cytokine signaling 2. PloS one. 2012; 7(6): e39366.
7. Murti K, Panchal M, Taya P, Singh R. Pharmacological Properties of Scoparia dulcis: A Review. Pharmacologia. 2012; 3(8): 3.
8. Ratnasooriya WD, Jayakody JR, Premakumara GA, Ediriweera ER. Antioxidant activity of water extract of Scoparia dulcis. Fitoterapia. 2005; 76(2): 220-2.
9. Le QU, Lay HL, Wu MC. Phenolic Composition, in Vitro Antioxidant and Anticancer Activities of Hypericin Japonicum Thumb and Scoparia Dulcis L. Herbal Medicine Journal. 2019; 4(1).
10. Jedage HD. Pharmacognostic, phytochemical investigation & pharmacological evaluation of scopolina dulcis linn. Plant extracts for nephro-protective activity. International Journal of Pharmaceutical Sciences and Research. 2014; 5(8): 3342–6.
11. Wankhar W, Sriviasan S, Rajan R, Rathinasamy S. Phytochemicals screening and antimicrobial efficacy of Scoparia dulcis Linn (Scrophulariaceae) against clinical isolates. Journal of Pharmacognosy and Phytochemistry. 2015; 3(6): 17–21.
12. Mishra MR, Behera RK, Jha S, Panda AK, Mishra A, Pradhan DK. A Brief Review on Phytoconstituents and Ethnopharmacology of Scoparia dulcis Linn. (Scrophulariaceae). International Journal of Phytomedicine 2011; 3: 422–38.
13. Krishnamurthy PT, Bajaj J, Sharma A, Manimaran S, Ravanappa PK, Pottekaal V. Hepatoprotective activity of terpenoids and terpenoid fraction of Scoparia dulcis. L. Oriental Pharmacy and Experimental Medicine. 2010; 10(4): 163–270.
14. Freire SM, Torres LM, Souccar C, Lapa AJ. Sympathomimetic effects of Scoparia dulcis L. and catecholamines isolated from plant extract. The Journal of pharmacy and pharmacology. 1996; 48(6): 624–8.
15. Hayashi K, Niwayama S, Hayashi T, Nago R, Ochial H, Morita N. In vitro and in vivo antiviral activity of scopadulanic acid B from Scoparia dulcis, Scrophulariaceae, against herpes simplex virus type 1. Antiviral research. 1988; 9(6): 345–54.
16. Kuriakose J, Ramamurthy N. Antiviral Effect of Scoparia dulcis against coxsackie B1-B6 virus. 23rd Swadeshi Science Congress 2013.
17. Riel MA, Kyle DE, Milhous WK. Efficacy of scopadulanic acid A against Plasmodium falciparum in vitro. Journal of natural products. 2002; 65(4): 614–5.
18. Nishino H, Hayashi T, Ariisawa M, Satomi Y, Iwashima A. Antitumor-promoting activity of scopadulanic acid B, isolated from the medicinal plant Scoparia dulcis L. Oncology. 1993; 50(2): 100–3.
19. Nkembo KM, Lee JB, Hayashi T. Selective enhancement of scopadulanic acid B production in the cultured tissues of Scoparia dulcis by methyl jasmonate. Chem Pharm Bull (Tokyo). 2005; 53(7): 780–2.
20. Wu WH, Chen TY, Lu RW, Chen ST, Chang CC. Benzoazinoids from Scoparia dulcis (sweet broomweed) with antiproliferative activity against the DU-145 human prostate cancer cell line. Phytochemistry. 2012; 83: 110–5.
21. Ahsan M, Islam SK, Gray AI, Stimson WH. Cytotoxic diterpenes from Scoparia dulcis. Journal of natural products. 2003; 66(7): 958–61.
22. Hafid AF, Permanasari AA, Tumewu L, Adianti M, Aoki C, Widyawaruyanti, et al. Activities of Ficus fistulososa Leave Extract and Fractions Against Hepatitis C Virus. Procedia Chemistry. 2016; 18: 179–84.
23. Apriyanto DR, Aoki C, Hartati S, Hanafi M, Kardon LB, Arisianti A, et al. Anti-Hepatitis C Virus Activity of a Crude Extract from Longan (Dimocarpus longan Lour.) Leaves. Japanese journal of infectious diseases. 2016; 69(3): 213–20.
24. Aoki C, Hartati, S., Santi, M.R., Lydwina, T., Firdaus, R., Hanafi, M., Kardon, L.B.S., Shimizu, Y., Sudarmono, P., Hotta, H. Isolation and identification of substances with anti-hepatitis C virus activities from Kalanchoe pinnata. Int J Pharm Pharm Sci. 2014; 6(2).
25. Daniel M, Bhattacharya SD, Arya A, Raole VM. Analytical methods for natural dyes, Natural Dyes: Scope and challenges: Scientific Publishers; 2019. 3 p.