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Activities of *Ficus fistulosa* Leave Extract and Fractions Against Hepatitis C Virus

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Abstract

Hepatitis C Virus (HCV) is a major global disease which often leads to chronicity and is potential to liver failure. There is no anti-HCV vaccine and the high diversity of viral genotypes will probably make it very difficult to develop a vaccine. Therefore, the development of new drugs for HCV treatment is highly required. It is commonly known that numerous important modern drugs have been developed from molecules originally isolated from natural sources. In this study, we tested the leave extract and fractions of *Ficus fistulosa* for their anti-HCV activities by cell culture method using Huh7it cells and HCV JFH1a. The result showed that ethanol extract of *Ficus fistulosa* (FFL) inhibited HCV JFH1a with IC_{50} value of 20.43±4.51 µg/ml. Toxicity test also indicated that FFL was not toxic with CC_{50} value of >200 µg/ml. The extract was further fractionated using chloroform (FFLC) and butanol (FFLB) successively. FFLC showed anti-HCV activity with IC_{50} value of 5.67±1.54 µg/ml and CC_{50} value of >100 µg/ml (Selectivity index >17.65). Further separation of FFLC by open column chromatography resulted in 12 subfractions (FFLC1-C12). Two subfractions, FFLC10, and FFLC11 showed high selectivity index (>100) with IC_{50} value of 0.60±0.30 µg/ml and 0.43±0.29 µg/ml, respectively. Therefore the leave extract (FFL) and fractions (FFL10, FFL11) of *Ficus fistulosa* would be a good candidate to develop antiviral against HCV.

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Keywords: Hepatitis C Virus; antiviral activity; *Ficus fistulosa*

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1. Introduction

Hepatitis C was first identified in 1989 as a disease that infects the liver, which is commonly caused by a virus. An estimated 130 to 170 million people, 2% to 3% of the world's population, are living with HCV infection and almost 350,000 people die of HCV each year. The prevalence of HCV in Indonesia is still more than 2 percent (based on serosurveys of voluntary blood donors). Three out of four people with Hepatitis C are potential to suffering from chronic hepatitis C and 10% up to 40% of people with untreated chronic hepatitis C will be likely to develop scarring of the liver (cirrhosis). Approximately 20% of people with cirrhosis will then develop liver failure, and 5% will develop liver cancer, both of which can be fatal[1-4].

HCV is a positive single stranded RNA virus of approximately 9,6 kB that encodes a long polyprotein precursor of ~3,000 amino acids, which is posttranslationally processed by host and virus proteases into mature proteins, including structural proteins (C, E1, E2, and p7) and nonstructural proteins (NS2, NS3, NS4A, NS4B, NS5A, and NS5B)[5,6]. The therapy using pegylated interferon alpha/ribavirin was only effective in approximately 80% and 40–50% of patients infected with HCV genotypes 2–3 and 1, respectively[7]. It has severe side effects such as headache, pyrexia, myalgia, and rigors and high cost[8-10]. Thus, the development of safe and inexpensive antiviral drugs is highly required.

More than 40% of all currently prescribed drugs is derived from chemicals that have been initially identified in plants. Many phytochemicals that have been identified display considerable inhibition of HCV at some stages of the life cycle[11,12]. *Ficus fistulosa* is locally known as *Beunying* and traditionally used to treat wounds. Ficus species are reported as anti Herpes Simplex Virus -1 and 2 (HSV-1, HSV-2), Varicella-Zoster Virus (VZV), Murine Sarcoma Virus (MuSV), anti Moloney Murine Leukemia Virus (MuLV)[13], antimicrobes[14], and antioxidant[15]. Our last publications also reported that *Ficus fistulosa* extract had Anti-HCV activities against 9 different genotypes (1a to 7a, 1b and 2b)[16]. The purpose of this study was to determine the active fractions of *Ficus fistulosa* againts HCV.

2. Methods

2.1. Cells and viruses

Huh7it cells were cultivated in DMEM-Dulbeco's Modiffied Eagle Medium (GIBCO Invitrogen) supplementing 10% Fetal Bovine Serum (FBS, GIBCO-Invitrogen), 1x Non-Essential Amino Acids (NEAA, GIBCO-Invitrogen), and 0.15 mg/ml Kanamycin solution (SIGMA) in 5% CO_2 at 37°C. The culture condition of Huh7it cells was observed under a microscope every day. JFH1a virus (50µl) was propagated by using Huh7it cells. It was suspended in 4 ml medium containing Huh7it ($1.8x10^7$ cells) and incubated at 37°C in 5% CO_2 for 4 hr with agitation for every 30 min. The infected cells were divided into eight T-75 flasks by supplying 10 ml culture medium per flask and incubated for 3 days. We harvested supernatant and removed cell debris by centrifugation at 1,500 rpm, 10 min, 4°C. The supernatant was concentrated by using Amicon-Ultra-15 centrifuge filter. Concentrated supernatant was aliquoted and stored at -80°C until use.

2.2. Extraction and fractionation of Ficus fistulosa

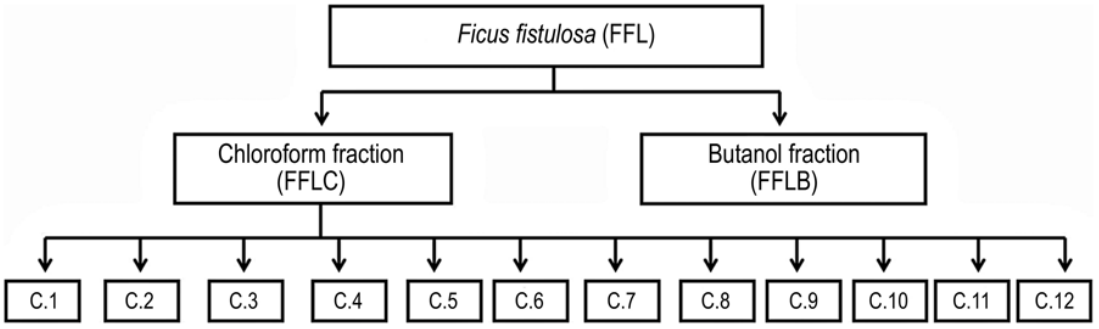

Fig. 1. Extraction and fractionation of *Ficus fistulosa*

Ficus fistulosa was extracted by ultrasonic assisted extraction method using ethanol 80% as a solvent. *F.fistulosa* extract (FFL) was then fractionated using chloroform and butanol successively to obtain chloroform fraction (FFLC) and butanol fraction (FFLB). FFLC was separated further by open column chromatography using sephadex LH-20 as a stationary phase and methanol-chloroform (95%:5%) as a mobile phase that resulted in 12 subfractions (FFLC1-C12).

2.3. Analysis of anti-HCV activities

Huh7it cells were seeded in 48-well plates (5×10^4 cells/well) and virus JFH1a with moi 0.1. A U-type 96 well plate was used to make dilution series of two-times higher than concentration used in an anti-HCV assay. JFH1a was mixed with the serial dilutions first and inoculated to the cells. After 2 hours, the cells were washed with a medium and incubated in the medium containing extracts for 46 hours to determine IC_{50}.

2.4. Virus titration and Immunostaining

The virus supernatant was dilluted serially 30-fold in a complete DMEM, inoculated onto Huh7it cells seeded on 96 well plate, incubated for 4 hrs, and replaced with MC-DMEM 0.4%. At 41 hr of post infection, cells were fixed by using 3.7% Formaldehyde for 15 min, permeabilized with 0.5% triton X-100 in PBS for 10 min, washed with PBS three times 200 µl/well, added with HCV-infected patient's serum for 60 min, followed by incubation with HRP-Goat anti human Ig antibody diluted with 2% BSA/PBS (x250) (50 µl/well) room temperature for 60 min, added with staining solution (DAB Thermo Scientific, USA), and incubated for 10-15 min at a room temperature until visualizing brown color. Consequently, the reaction was stopped by adding dH_2O.

2.5. Cytotoxicity assay

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

3. Results and discussion

Table 1. Antiviral activity (IC_{50}) against JFH1a, cytotoxicity (CC_{50}) and selectivity index (SI) of *Ficus fistulosa*.

Samples Ficus fistulosa	IC_{50} (µg/ml)	CC_{50} (µg/ml)	SI (Selectivity Index)
Ethanol extract (FFL)	20.43 ± 4.51	>200	>9.79
Buthanol fraction (FFLB)	74.10 ± 18.24	>1000	>13.50
Cloroform fraction (FFLC)	5.67 ± 1.54	>100	>17.65
Cloroform subfraction 1 (FFLC1)	78.70 ± 0.99	>500	>6.35
Cloroform subfraction 2 (FFLC2)	26.73 ± 5.61	>200	>7.48
Cloroform subfraction 3 (FFLC3)	34.78 ± 3.85	>200	>5.75
Cloroform subfraction 4 (FFLC4)	24.65 ± 5.31	>50	>2.03
Cloroform subfraction 5 (FFLC5)	34.43 ± 1.17	>100	>2.90
Cloroform subfraction 6 (FFLC6)	21.56 ± 8.69	>50	>2.32
Cloroform subfraction 7 (FFLC7)	11.32 ± 3.88	>50	>4.42
Cloroform subfraction 8 (FFLC8)	5.57 ± 2.80	>50	>8.98
Cloroform subfraction 9 (FFLC9)	1.50 ± 0.57	>50	>33.33
Cloroform subfraction 10 (FFLC10)	0.60 ± 0.30	>100	>166.67
Cloroform subfraction 11 (FFLC11)	0.43 ± 0.29	>50	>115.38
Cloroform subfraction 12 (FFLC12)	8.55 ± 6.58	>500	>58.48

In this study, we tested the extract of *Ficus fistulosa* (FFL) and the subfractions for their anti-HCV activities by cell culture method using Huh7it cells and HCV JFH1a. The result showed that FFL inhibited HCV JFH1a with IC_{50} value of 20.43±4.51 μg/ml. Toxicity test also indicated that FFL was not toxic with CC_{50} value of >200 μg/ml (Table 1). These results were similar with the previous report which suggested FFL as a good candidate for antiviral against HCV. The previous study reported that *F.fistulosa* leave extract was showed antiviral activity against HCV J6/JFH1-P47 strain and -P1 strains with IC_{50} value of 15.0 and 5.7 μg/ml, respectively. Time of addition experiment revealed that FFL inhibited at the entry step. Further analysis was needed to determine the possible anti-HCV compound contained in the extract[16].

Regarding to the data obtained from the study, the separation of FFL was performed. FFL was fractionated further by using chloroform and butanol successively to obtain chloroform fraction (FFLC) and butanol fraction (FFLB). Bioassay-guided fractionation was taken in this study. The results showed that FFLC possessed anti-HCV activity with IC_{50} value of 5.67±1.54 μg/ml and CC_{50} value of >100 μg/ml (SI >17.65). On the other hand, FFLB showed weaker inhibitory activity with IC_{50} value of 74.10±18.24 μg/ml and CC_{50} value of >1000 μg/ml. FFLC as an active fraction was separated further to get closer to the anti-HCV compound determination. The separation of FFLC was conducted by open column chromatography using sephadex LH-20 as a stationary phase and methanol-chloroform (95%-5%) as a mobile phase resulted in 12 subfractions (FFLC1-C12). Anti-HCV activity test was conducted to identify the inhibitory activity of subfractions. The results showed that five of twelve subfractions have IC_{50} value of 0.43-8.55 μg/ml. Meanwhile, the other subfractions have IC_{50} value of 11.32-78.70 μg/ml. The most potential inhibitory activity was showed by FFLC10 and FFLC11 with high selectivity index >166.67 and >115.38, respectively. Dose dependent inhibition showed that FFLC10 and FFLC11 exhibited JFH1a 100 % with concentration value < 2 μg/ml (Fig. 2).

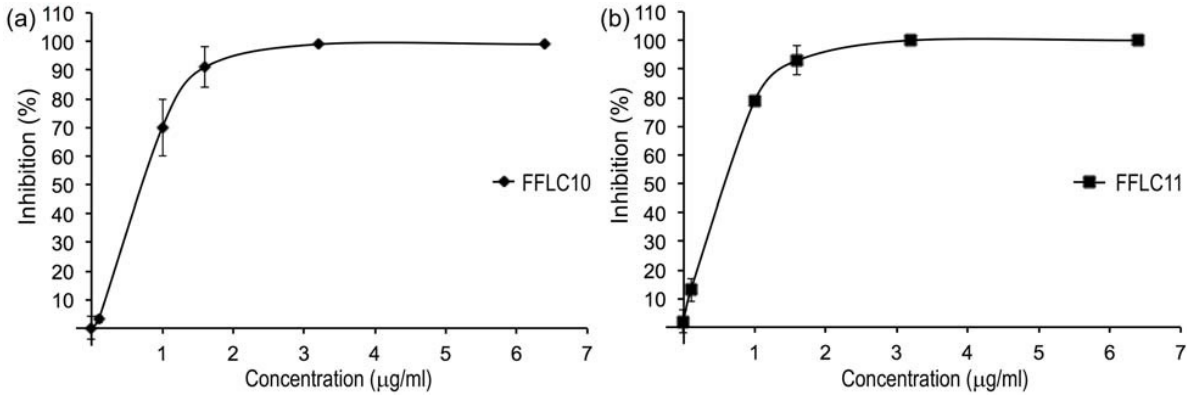

Fig. 2. Dose-dependent inhibition of JFH1a infection by (a) FFLC10 of *Ficus fistulosa*; (b) FFLC11 of *Ficus fistulosa.*

Identification of five active subfractions by Thin Layer Chromatography (TLC) revealed that subfractions contained flavonoids, terpenoids and chlorophyll compounds (Fig. 3). Chlorophyll compounds were shown by the presence of green spots after TLC plate was spayed using H_2SO_4 10% and heated 105^oC for 5 minutes (Fig. 3c) and by the red spots under uv 365 nm after sprayed and heated (Fig. 3d). FFLC8 until FFLC12 which contained green and red spots had IC_{50} less than 10 μg/ml. The presence of chlorophyll compounds was possible to increase the antiviral activity of subfractions. The previous study reported that chlorophyll catabolites, 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[17]. An alkylated porphyrin, chlorophyllide, also reported that directly and quantitatively disrupted Hepatitis B virus (HBV) virions, without detectably affecting cell viability or intracellular viral gene products[18]. These reports supported our suggestion about the chlorophyll compounds activity. Therefore, FFLC10 and FFLC11would become a good target for futher purification.

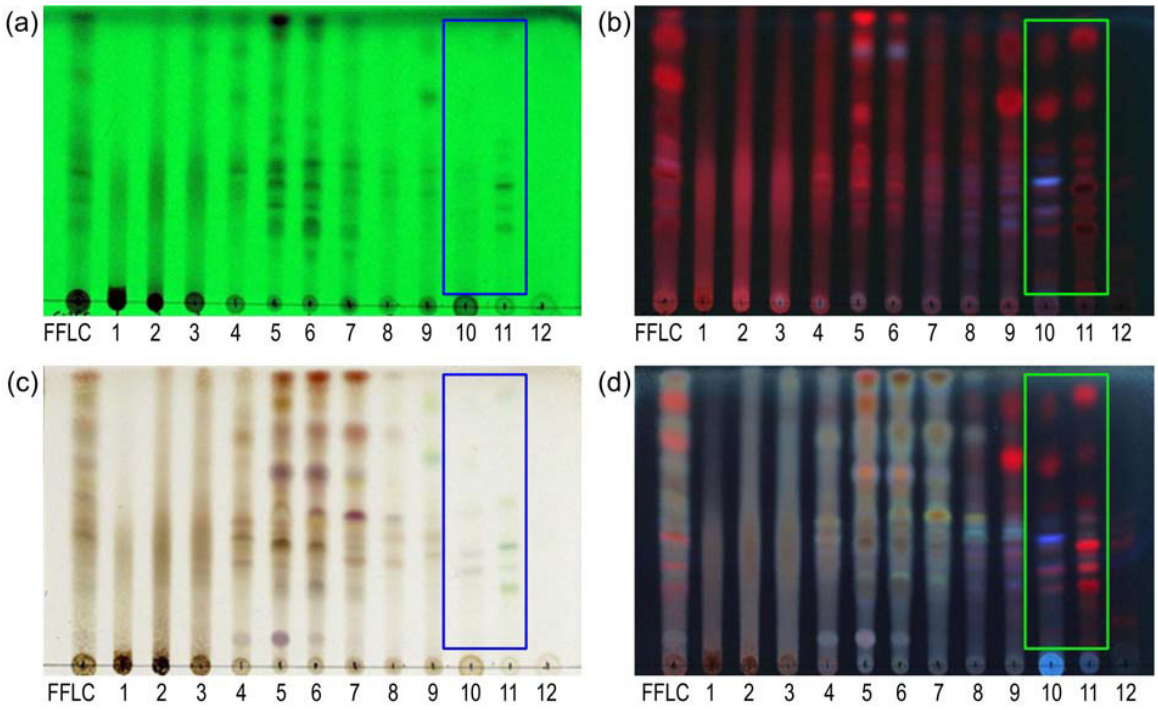

Fig 3. Thin Layer Chromatography (TLC) profile of sub fractions C12F C1-12 using silica gel F254 as stationary phase and chloroform:methanol 95:5 v/v as development solvent; observed under (a) uv 254 nm; (b) uv 365 nm; (c) using spray reagent H_2SO_4 10% and heated 105°C for 5 minutes; (d) observed under uv 365 nm after using spray reagent and heated.

4. Conclusions

The extract FFL, chloroform fraction FFLC, and subfractions FFLC10 and FFLC11 of *Ficus fistulosa* have a consistency of antiviral activities and would be a good candidate for the development of an antivirus against HCV.

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References

1. David L Thomas. Global control of hepatitis C: where challenge meets opportunity. *Nature Medicine 19, 850–858 (2013).*
2. Mohd Hanafiah K, Groeger J, Flaxman AD, Wiersma ST. Global epidemiology of hepatitis C virus infection: new estimates of age-specific antibody to HCV seroprevalence. *Hepatology. 2013 Apr;57(4):1333-42.*
3. Averhoff FM, Glass N, Holtzman D. Global burden of hepatitis C: considerations for healthcare providers in the United States. *Clin Infect Dis. 2012 Jul;55 Suppl 1:S10-5.*
4. Sulaiman HA, Julitasari, Sie A, Rustam M, Melani W, Corwin A, Jennings GB. Prevalence of hepatitis B and C viruses in healthy Indonesian blood donors. *Trans R Soc Trop Med Hyg. 1995 Mar-Apr;89(2):167-70.*
5. Penin, F., Dubuisson, J., Rey, F. A., Moradpour, D. and Pawlotsky, J.-M. Structural biology of hepatitis C virus. *Hepatology, 39: 5–19(2004).*
6. Darius Moradpour, François Penin & Charles M. Rice. Replication of hepatitis C virus. *Nature Reviews Microbiology 5, 453-463 (June 2007).*
7. Sarrazin C1, Hézode C, Zeuzem S, Pawlotsky JM. Antiviral strategies in hepatitis C virus infection. *J Hepatol. 2012;56 Suppl 1:S88-100.*
8. Manns MP, Wedemeyer H, Cornberg M. Treating viral hepatitis C: efficacy, side effects, and complications. *Gut. 2006 Sep;55(9):1350-9.*

9. Tariq Javed, Usman Ali Ashfaq, Sana Riaz, Sidra Rehman, Sheikh Riazuddin. In-vitro antiviral activity of Solanum nigrum against Hepatitis C Virus. *Virology Journal 2011, 8:26.*
10. Usman A Ashfaq, Tariq Javed, Sidra Rehman, Zafar Nawaz and Sheikh Riazuddin. An overview of HCV molecular biology, replication and immune responses. *Virology Journal 2011, 8:161.*
11. Dinesh Manvar, Mahesh Mishra, Suriender Kumar, and Virendra N. Pandey. Identification and evaluation of anti Hepatitis C Virus phytochemicals from Eclipta alba. *J Ethnopharmacol. 2012 Dec 18; 144(3): 545–554.*
12. Hu JF, Patel R, Li B, Garo E, Hough GW, Goering MG, Yoo HD, O'Neil-Johnson M, Eldridge GR. Anti-HCV bioactivity of pseudoguaianolides from Parthenium hispitum. *Journal of Natural Products. 2007;70:604–607.*
13. Yarmolinsky L, Huleihel M, Zaccai M, Ben-Shabat S. Potent antiviral flavone glycosides from Ficus benjamina leaves. *Fitoterapia. 2012 Mar;83(2):362-7.*
14. Mohamed Z. M. Salem, A. Z. M. Salem, L. M. Camacho and Hayssam M. Ali. Antimicrobial activities and phytochemical composition of extracts of Ficus spesies: An overview. *African Journal of Microbiology Research Vol. 7(33), pp. 4207-4219, 16 August, 2013.*
15. Muhammad Imran, Nasir Rasool, Komal Rizwan, Muhammad Zubair, Muhammad Riaz, Muhammad Zia-Ul-Haq, Usman Ali Rana, Ayman Nafady and Hawa ZE Jaafar. Chemical composition and Biological studies of Ficus benjamina. *Chemistry Central Journal 2014, 8:12.*
16. Wahyuni TS, Tumewu L, Permanasari AA, Apriani E, Adianti M, Rahman A, Widyawaruyanti A, Lusida MI, Fuad A, Soetjipto, Nasronudin, Fuchino H, Kawahara N, Shoji I, Deng L, Aoki C, Hotta H. Antiviral activities of Indonesian medicinal plants in the East Java region against hepatitis C virus. *Virology Journal 2013, 10:259.*
17. Ratnoglik SL, Aoki C, Sudarmono P, Komoto M, Deng L, Shoji I, Fuchino H, Kawahara N, Hotta H., Antiviral activity of extracts from *Morinda citrifolia* leaves and chlorophyll catabolites, pheophorbide a and pyropheophorbide a against hepatitis C virus. *Microbiol Immunol. 2014 Mar; 58(3):188-94.*
18. Haitao Guo, Xiaoben Pan, Richeng Mao, Xianchao Zhang, Lijuan Wang, Xuanyong Lu, Jinhong Chang, Ju-Tao Guo, Shendra Passic, Fred C. Krebs, Brian Wigdahl, Travis K. Warren, Cary J. Retterer, Sina Bavari, Xiaodong Xu, Andrea Cuconati and Timothy M. Block, Alkylated Porphyrins Have Broad Antiviral Activity against Hepadnaviruses, Flaviviruses, Filoviruses, and Arenaviruses, Antimicrob. Agents Chemother. *February 2011 vol. 55 no. 2 478-486.*