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Faloak (Sterculia quadrifida R.Br) Stem Bark Extract Inhibits Hepatitis C Virus JFH1

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ABSTRACT

The discovery of antiviral drugs is developing new alternative medication and economics prices. One of the natural resources for alternative medicine is the water extract of faloak stem bark which is a material used by Kupang Community as an antiviral drug. The HCV JFH1 lifecycle inhibition is expressed in IC_{50} (inhibitory concentration 50%) and the toxicity of water extract of faloak stem bark in hepatocyte cell line Huh7it is expressed in CC_{50} (cytotoxicity concentration 50%). Determining the results obtained using Microsoft Office Excel 2013. The water extract of faloak stem bark has an inhibition of HCV genotype 2a strain JFH1 with the IC_{50} 11.57 μg/mL and the toxicity water extract of faloak stem bark in the Huh7it cell line hepatocyte with the CC_{50} 1000 μg/mL. Mode of action activities from water extract of faloak stem bark can be inhibited all step HCV life cycle. The first step is entry step has the inhibition 93.97%, post-entry step has the inhibition 96.75%, and combination step (entry and post-entry step) has the inhibition was 100%. The active compound in the water extract of faloak stem bark was epicataechin 875 mg/kg. The water extract of faloak stem bark can be a candidate for antivirals against HCV JFH1.

Keywords: Faloak, Cytotoxicity concentration 50% R.Br, Hepatitis C Virus.

INTRODUCTION

Hepatitis C virus infection is a global problem and needed drugs can inhibit HCV infection. There is now a combination with pegylated interferon alfa (IFN-α) and ribavirin1. However until now the fulfillment of the drug needs has not been met well throughout the region of Indonesia, it takes an alternative treatment to help HCV infection. One medicinal plant that can be used as an alternative treatment for hepatitis is faloak. The part of the faloak plant commonly used as medicine is the stem bark6. Tests have been carried out on the faloak bark and flavonoid compounds have been found. Flavonoid
compounds can inhibit the hepatitis C virus in cell culture so that faloak plants can be tested to obtain active content and the effect of inhibiting the hepatitis C virus.

MATERIALS AND METHODS

Materials

The stem bark of faloak is obtained from Kupang City of East Nusa Tenggara Province. The only stemming by peeling is taken from the main stem. Weighed fresh samples, cut into small pieces and dried by air-dried until dried and then fertilized and weighed dry samples. Extraction is performed using a water solvent. The obtained viscous extract is dried using a freeze dryer until the extract is dry and weighed.

METHODS

Preparation and sample extraction

Determinants plants in UPT BKT Purwodadi Garden, East Java, Indonesia. Extraction was extracted for LC-MS/MS test to identify epicathechin compounds. A regular concentration series of (−)-epicatechin >98% (Sigma-Aldrich) was dissolved using 0.5 g of water extract from new bark to make a standard curve. A weigh of 0.5 g of homogeneous water extracts of bark dissolved in methanol and vortex until. Then the extract solution (0.1 mL) was added to 0.9 mL of methanol 5%, and then it was vortexed. The calculation of epicatechin level using dilution factor 1000, then the sample is injected into LC-MS/MS (AB-Sciex 4000) instrument. The result is a chromatogram and contains a peak epicatechin compound from the Analyst Instrument Control and Data Processing Software AB Sciex which is software in LC-MS/MS (AB-Sciex 4000) instrument.

Cells and Viruses

Analysis of anti-HCV activities

The anti-HCV test was carried out following the previous test. The samples was dissolved in dimethyl sulfoxide (DMSO) to obtain stock solutions at a concentration of 100 mg/mL, stored at -20°C until it was used. Huh7/t cells were seeded in 48-well plates (5x10⁴ cells/well). A fixed amount of infection (FFU)/cell, was mixed with serial dilutions of the extracts (100, 50, 25, 12.5, 6.25 and 3.125 μg/mL) and inoculated to the Huh7/t cells. After two h incubation, the cells were washed with Dulbecco’s modified Eagle’s medium to remove the residual virus and further incubated in the Dulbecco’s modified Eagle’s medium containing the same concentrations of test samples as those during virus inoculation.

Staining

Cell staining was carried out following the previous test. The Huh7/t cells were prepared medium in 96-well plate (2.3 x 10⁴ cell/mL) incubation 24 hours. The stored supernatant was thawed first and serially diluted in medium with 2% FBS, added into the Huh7/t cells, incubation 2 h at 37°C with 5% CO₂. Then, remove supernatant and overlaid with methylcellulose 0.5%, incubation at 37°C with 5% CO₂ for two days. After two-day infection cells, in each well were fixated using 200 μL 10% formaldehyde per well and incubated for 15 minutes. After the formaldehyde was removed, the cells were washed three times with 200 μL of Phosphate Buffered Saline (PBS) with a period of 5 min in between. After the PBS was removed, 100 μL of Triton-X 0.5% was added to each well. Afterward, incubation was done for 10 minutes. The Triton-X was then removed from all wells. Next, the wells were rinsed with PBS three times with 200μL of PBS with a period of 5 min in between. Then, 45 μL of 1/200 anti-HCV from human serum was added. Incubation for 1 h was then performed. After removing the antibody, each well was rinsed with PBS. After that, the second antibody, which was 45 μl of 1/300 human anti-hors eradish peroxidase (HRP) (Sigma-Aldrich), was added. It was incubated, removed, and rinsed again with the same method as before. Next, 100 μL of 3, 3-diaminobenzidine (DAB) (Thermo Scientific, USA) was added to each well as staining step. Then, it was incubated for 15 minutes. Finally, the virus focus was observed under light microscope and calculated with KatiKati program as cells counter.

Cytotoxic assay

The cytotoxic test followed the previous test. The cell viability of the samples was assessed by (3-(4,5-dimethyl thiazolyl-2)-2,5-diphenyltetrazolium bromide) (MTT) (Sigma-Aldrich) assay. Huh7/t cells (2.3x10⁴ cells/well) in 96 well plates were treated with serial dilution of the samples (1000 μg, 800 μg, 600 μg, 400 μg, 200 μg, 100 μg, 50 μg, 25 μg/12.5 μg). The condition of the cells was observed after 48 h incubation, and the toxicity was checked under a 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 100 µl/well DMSO 100% was then put for dissolve. Absorbance was checked at 560 nm and 750 nm, shaker 0.5 min before reading absorbance. The MTT reagent is absorbed by the cells and converted by reduction reaction to formazan by mitochondrial dehydrogenases. Percent cell viability compared to the control was calculated for each dilution of the samples and CC_{50} were determined.

Mode of action assay test is carried out by following the previous test^{2,3}. These experiments were performed to assess the way of action of the samples. It has two sets of tests; the first is entry step, the mixture of HCV and sample was inoculated to the cells. After 2 h the residual virus and the sample were removed, and cells added to the medium without samples for 46 hours. The second step is a post entry step, HCV was inoculated to the cells in the absence of the sample. After 2 h the residual virus was removed, and the cell was added to the medium containing the sample for 46 hours. Positive control used a medium containing 0.1% DMSO.

RESULTS

Taxonomically, faloka plants are included in the Genus Sterculia^{4,5}, so the initial assumption of faloka plants contains compounds that resemble plants in their genus. Plants that are one genus, namely Sterculia tragacantha, which has successfully isolated several flavonoid compounds, one of these compounds is epicatechin^{6}. On this basis, the search for epicatechin compounds was carried out qualitative and quantitative by using LC-MS/MS (AB-Sciex 4000)\(^{10}\). The test results using LC-MS/MS (AB-Sciex 4000) library software water extract faloka stem bark identified containing epicatechin at retention time (RT) and mass to charge ratio (m/z) shown in Figure 1.

![Fig. 1. Chromatogram results of the identification of epicatechin identification compounds in the water extract of water extract faloka stem bark to determine RT using LC-MS/MS (AB-Sciex 4000). (A) From the figure at RT 5.90 shows a compound based on software library from LC-MS/MS (AB-Sciex 4000) so that this pick graph is an epicatechin compound. (B) The epicatechin compounds in the water extract faloka stem bark is shown at m/z 289.2 based on the software library LC-MS/MS (AB-Sciex 4000) with the molecular formula C_{15}H_{15}O_6 for.](image)
After successfully identifying the epicatechin compounds in the water extract of falok stem bark, the next step is to determine the level of epicatechin compounds. Determination of epicatechin compounds using LC-MS/MS (AB-Sciex 4000) and standard compounds of standard compounds (S)-epicatechin >98% (Sigma-Aldrich). The results of the determination of the levels of epicatechin compounds in the water extract of falok stem bark shown in Figure 2.

Anti-HCV activities of Sterculia quadrifida R.Br stem bark

The water extract of falok stem bark at the concentration of 50 and 100 μg/mL showed 100% inhibition to HCV JFH1 replication. Lower doses such as 25 μg/mL, 12.5 μg/mL, 6.25μg/mL and 3.125 μg/mL showed the percentage of infectivity at the level of 95.39%, 67.50%, 29.775% and 8.31%, respectively. From the linear regression equation of the percent infectivity, IC₅₀ of water extract of falok stem bark 11.67μg/mL (Figure 3).

Cytotoxic Assay

The cytotoxic test using MTT assay aims to calculate the viability of cells and then compared with the viability after treated with DMSO. The results showed that the treatment using water extract of falok stem bark at the concentration of up to 100μg/mL was not toxic to the cells, while the cell viability decreased at treatment with 200 μg/mL. From the linear regression equation of the percent viability, we found that the CC₅₀ of water extract of falok stem bark was >1000 μg/mL (Figure 4).

![Fig. 3. Linear Regression curve of the inhibition of HCV JFH1 after treated with water extract falok stem bark. The data represent means ± SEM of data from two independent experiments](image)

![Fig. 2. Chromatogram results of the identification of epicatechin identification compounds in the water extract of falok stem bark to determine RT using LC-MS/MS (AB-Sciex 4000). (A) The epicatechin standard at RT 3.90, (B) The epicatechin compounds in the water extract of falok stem bark is shown at RT 3.90. The calculation of epicatechin concentration obtained by using Analyst Instrument Control and Data Processing Software AB Sciex obtained by linear regression equation y=104 x+2.88 and R-value =0.9993. Total epicatechin found in a high concentration of 875mg/kg. These results are in accordance with the initial assumptions that mention falok plants contain epicatechin compounds.](image)

![Fig. 4. Linear regression curve of the viability cell after treatment with water extract of falok stem bark. The data represent means ± SEM of data from two independent experiments](image)
After testing the effect of HCV JFH1 inhibition and Huh7t cell line cytotoxic test, it was continued by determining the selectivity index with the criteria >311. The selectivity index is determined by comparing CC_{50} and IC_{50}. The results of the comparison are obtained with > 8.57, then the next test which is the time of the additional test can be done.

**Mode of action anti-HCV**

Test to determine the inhibition step of water extract of falook stem bark the HCV JFH1 lifecycle using a concentration of 40 μg/mL and obtained the test results at the entry step was 93.97% inhibition. The post-entry step was 96.75% inhibition. Combination of entry and the post-entry step was 100% inhibition. The results can be seen in Figure 5.

![Figure 5. Linear regression curve of the viability cell after treatment with water extract of falook stem bark. The data represent means a SEM of data from three independent experiments. From the data, 100% inhibition was encountered during and post-inoculation step, the inhibition was 93.968% at the step during inoculation and the inhibition was 96.75% at the post inoculation step. Thus showing the water extract of falook bark can inhibit at three step in the HCV JFH1 life cycle.](image)

**DISCUSSION**

Water extract of falook stem bark has flavonoid content, namely epicatechin which is soluble in water. This result is similar to previous researchers, epicatechin compounds contained in the extract have the ability as anti-HCV and not toxic.

The results of the inhibitory test of water extract of falook stem bark were 11.67 μg/mL, these results met the criteria of inhibitory extracts in vitro testing that was equal to <25 μg/mL. The results of cytotoxic tests obtained results >1000 μg/mL, these results indicate cell viability at a concentration of 1000 μg/L cell viability was 73.97% and this was according to the criteria of >50%. It also obtained a selectivity index of >8.57. From these data water extract falook stem bark can be continued to the next test.

The test results of the step of inhibition in the HCV JFH1 life cycle obtained by the extract of falook bark water can inhibit at the entry step. In this entry step the water extracts of falook stem bark interacting with binding factors (GAGs and LDL-R), receptors (SR-BI, CD81, Ocludin and Claudin 1) and entry factors (EGFR, EphA2, TIR1 and NPC1L1) and VHC JFH1, which is expected to be able to inhibit the life cycle of JFH1 by inhibiting one of the above things by 93.968%. At the post-entry stage also water extract falook stem bark can inhibit the life cycle of JFH1 HCV. At this stage the water extract falook bark will interact internally, especially the non-structural parts that function in RNA replication such as NS3, NS3 / NS4A, NS4B, NSSA and NSSB20. From the data, it is found that there are obstacles around 96.78%, so that it has obstacles in one or several non-structural proteins. This research can be continued for testing specific proteins in the JFH1 VHC infection process in Huh7t cell line hepatocyte culture in vitro.

**CONCLUSION**

Falook plants for the first time identified and determined the content of epicatechin compounds. It can inhibit VHC JFH1 replication, is not toxic and can inhibit HCV JFH1 at the entry and post-entry step in the Huh7t hepatocyte cell line.

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**CONFLICT OF INTEREST**

Declared none

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