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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® Excell 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 epicatechin 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 [illegible]ded drugs can inhibit HCV infection. There is now a combination with pegylated interferon alfa (IFN-α) and ribavirin[1]. 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 bark[2]. 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[4] 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 epicatechin 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 50%, 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[5,6]. The sampels 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. Huh7it cells were seeded in 48-well plate (5×10^4 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 Huh7it 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.

Stainning

Cell staining was carried out following the previous test[5]. The Huh7it cells were prepared medium in 96-well plate (2.3 x 10^4 cell/mL) incubation 24 hours. The stored supernatant was thawed first and serially diluted in medium with 2% FBS, added into the Huh7it cells, incubation 2 h at 37°C with 5% CO_2. Then, remove supernatant and overlaid with methylcellulose 0.5%, incubation at 37°C with 5% CO_2 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 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-horseradish 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[5]. The cell viability of the samples was assessed by (3-(4,5-dimethyl thiazolyl-2)-2,5-diphenyltetrazolium bromide) (MTT) (Sigma-Aldrich) assay. Huh7it cells (2.3x10^4 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 µg12.5 µg). The condition of the cells was observed after 46 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 assaytest is carried out by following the previous test[5,7]. 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, faloak plants are included in the Genus Sterculia[8,9], so the initial assumption of faloak 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[10]. 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 faloak stem bark identified containing epicatechin at retention time (RT) and mass to charge ratio (m/z) shown in Figure 1.

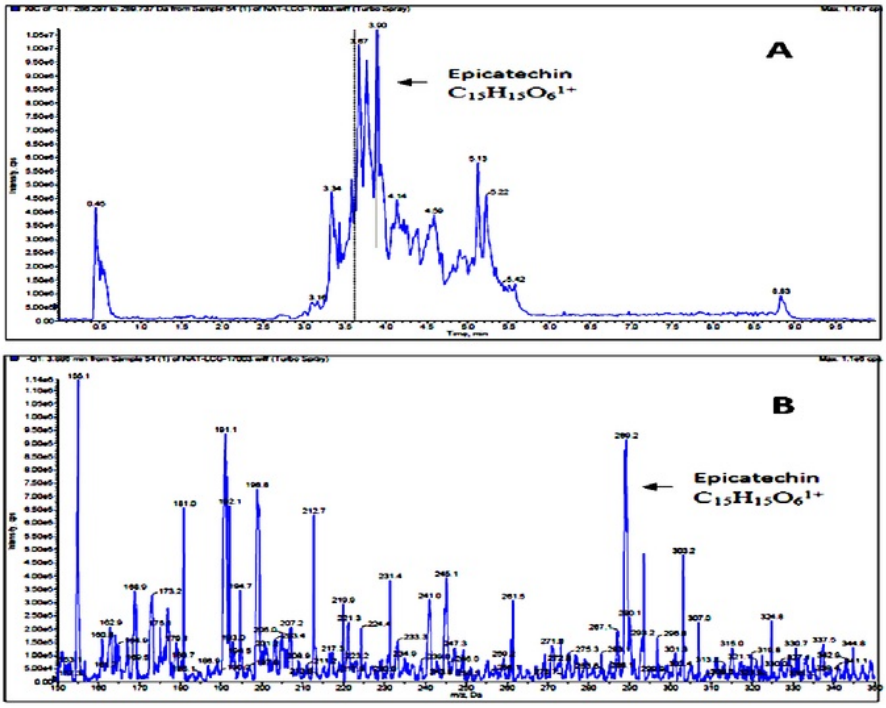

Fig. 1. Chromatogram results of the identification of epicatechin identification compounds in the water extract of water extract faloak stem bark to determine RT using LC-MS/MS (AB-Sciex 4000). (A) From the figure at RT 3.90 shows a compound based on software library from LC-MS/MS (AB-Sciex 4000) so that this pick graph is an epicatechin compound. (B) The epicathechin compounds in the water extract faloak 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$1+.

After successfully identifying the epicatechin compounds in the water extract of faloak 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 (-)-epicatechin >98% (Sigma-Aldrich). The results of the determination of the levels of epicatechin compounds in the water extract of faloak stem bark shown in Figure 2.

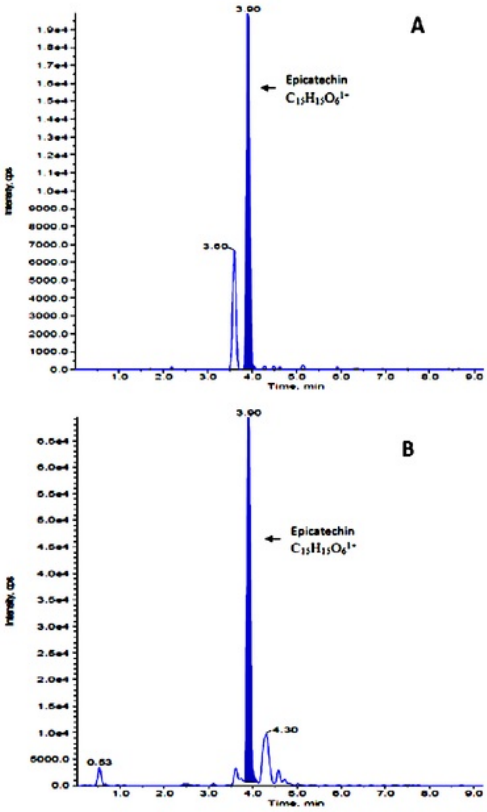

Fig. 2. Chromatogram results of the identification of epicatechin identification compounds in the water extract of faloak stem bark to determine RT using LC-MS/MS (AB-Sciex 4000). (A) The epicatechin standard at RT 3.90. (B) The epicathechin compounds in the water extract of faloak stem bark is shown 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 faloak plants contain epicatechin compounds.

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

The water extract of faloak 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, IC_{50} of water extract of faloak stem bark 11.67µg/mL (Figure 3).

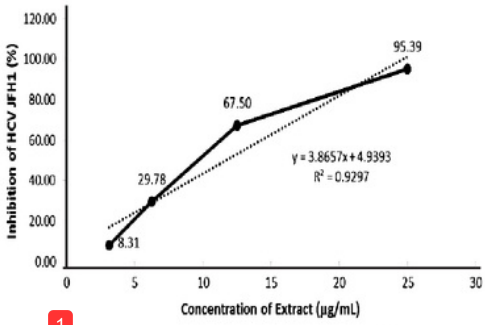

Fig. 3. Linear Regression curve of the inhibition of HCV JFH1 after treated with water extract faloak stem bark. The data represent means ± SEM of data from two independent experiments

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 faloak 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 CC_{50} of water extract of faloak stem bark was >1000 µg/mL (Figure 4).

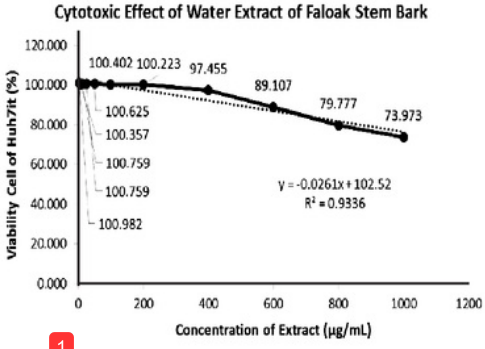

Fig. 4. Linear regression curve of the viability cell after treatment with water extract of faloak stem bark. The data represent means ± SEM of data from two independent experiments

After testing the effect of HCV JFH1 inhibition and Huh7it 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}[12]. 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 faloak 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.

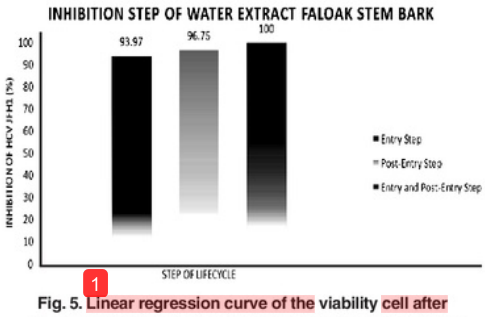

Fig. 5. Linear regression curve of the viability cell after treatment with water extract of faloak stem bark. The data represent means ± SEM of data from three independent experiments. From the data, 100% inhibition was encountered during and post-inoculation step, the inhibition was 93.968% at the step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

DISCUSSION

Water extract of faloak stem bark has flavonoid content, namely epicatechin which is soluble in water[13]. This result is similar to previous researchers, epicatechin compounds contained in the extract have the ability as anti-HCV[14,15] and not toxic[16].

The results of the inhibitory test of water extract of faloak 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[11]. 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%[11]. It also obtained a selectivity index of >8.57. From these data water extract faloak 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 faloak bark water can inhibit at the entry step. In this entry step the water extracts of faloak stem bark interacting with binding factors (GAGs and LDL-R), receptors (SR-BI, CD81, Occludin and Cloudin 1) and entry factors (EGFR, EphA2, TfR1 and NPC1L1[17,18,19] 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 faloak stem bark can inhibit the life cycle of JFH1 HCV. At this stage the water extract faloak bark will interact internally, especially the non-structural parts that function in RNA replication such as NS3, NS3 / NS4A, NS4B, NS5A and NS5B20. From the data, it is found that there are obstacles around 96.75%, 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 Huh7it cell line hepatocyte culture *in vitro*.

CONCLUSION

Faloak 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 Huh7it hepatocyte cell line.

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CONFLICT OF INTEREST

Declared none

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