Research Article

ISOLATION AND IDENTIFICATION OF SUBSTANCES WITH ANTI-HEPATITIS C VIRUS ACTIVITIES FROM *KALANCHOE PINNATA*

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ABSTRACT

Objective: The aim of this study was to examine extracts from Indonesian plants to identify a compound(s) responsible for antiviral activity against hepatitis C virus (HCV).

Methods: Huh7it-1 cells, a clone of human hepatocellular carcinoma-derived Huh7 cells, were infected with the HCV genotype 2a strain JFH1 in the presence of crude methanol extracts from the plants. The extracts were further fractionated and purified by anti-HCV bioactivity-guided analysis using a combination of various column chromatography techniques. The isolated compounds were examined for anti-HCV activity and cytotoxicity, and their structures determined by nuclear magnetic resonance spectroscopy and liquid chromatography–mass spectrometry.

Results: Screening of Indonesian plants revealed that a crude methanol extract from *Kalanchoe pinnata* exhibited anti-HCV activity with a 50%-inhibitory concentration (IC_{50}) of 17.2 µg/ml. An ethyl acetate fraction was found to possess strong anti-HCV activity, from which three compounds, i.e., quercetin, gallic acid and quercitrin, were isolated. Anti-HCV activity assay revealed that quercetin and gallic acid, but not quercitrin, inhibited HCV production in a dose-dependent manner, with IC_{50} values of 1.5 and 6.1 µg/ml, respectively, without exhibiting cytotoxicity. A time-of-addition study demonstrated that quercetin acted at the post-entry step whereas gallic acid at both the entry and post-entry steps.

Conclusion: An extract from *K. pinnata* and its constituents, quercetin and gallic acid, could be potentially used as a supplement for the treatment of HCV infection.

Keywords: Hepatitis C virus, Herbal plant, *Kalanchoe pinnata*, Inhibition, Antiviral.

INTRODUCTION

Hepatitis C virus (HCV) infection is a serious health problem that afflicts >180 million people worldwide [1]. HCV causes chronic liver disease in most cases, resulting in hepatitis, liver cirrhosis and, eventually, hepatocellular carcinoma [2,3]. Also, HCV causes extrahepatic manifestations, such as lipid and glucose metabolic disorders, and is closely associated with steatosis and type 2 diabetes [4, 5].

HCV is classified into seven genotypes, each of which is further divided into a number of subtypes, such as 1a, 1b and 1c [6]. The HCV genotype distributions vary geographically. Genotypes 1 to 3 are distributed worldwide [7] with genotypes 1b and 2a being most common in Asia, including Japan and Indonesia [8, 9]. Combination therapy of a pegylated interferon-α (PEG-IFNα) and ribavirin is used as the initial treatment of choice; however, this combination therapy is very expensive and effective in only approximately 50% of patients with HCV genotype 1 infections and 80% with genotype 2 and 3 infections. The protease inhibitors, telaprevir and boceprevir, have been approved for HCV therapy by the US Food and Drug Administration in 2011. A triple therapy with PEG-IFNα/ribavirin/telaprevir was shown to shorten the treatment duration and increase sustained virus response rates to 75% in patients with HCV genotype 1 infections, with the remaining patients left uncured. On the other hand, telaprevir and boceprevir increased the risk of adverse effects, such as anemia and skin rashes [10]. Thus, the development of safe and inexpensive antiviral drugs is still required for treatment of HCV infections. Traditional medicinal plants are promising sources for the discovery of novel biologically active natural products, which are useful for medical applications, including the treatment of HCV infections [11-13]. Indonesia is a country rich in biodiversity with forests that contain a wide variety of plants. We conducted a screening of about 250 Indonesian herbal plants to identify those possessing anti-HCV activities. Here we report that a crude extract from *Kalanchoe pinnata* leaves exhibited antiviral activities against HCV. To identify compound(s) responsible for the anti-HCV activities, we conducted bioactivity-guided purification and structure determination.

MATERIALS AND METHODS

Cell culture and virus preparation

A human hepatocellular carcinoma-derived cell line, Huh7, and its clone, Huh7it-1, which was generated by IFNα treatment of Huh7 cells harboring HCV JFH1 subgenomic RNA replicon, were cultured in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Biowest, Nuaille, France), 150 µg/ml of kanamycin (Sigma-Aldrich, St. Louis, MO, USA) and non-essential amino acids (Invitrogen).

The supernatants from Huh7it-1 cells infected with cell culture-adapted HCV (JFH1 strain of genotype 2a) [14, 15] were collected at 3 to 5 days post infection (p.i.), concentrated using 100K Amicon centrifugal filters and stored at −80°C.

Reagents

Quercetin and gallic acid were purchased from Sigma-Aldrich (St. Louis, MO, USA). Quercitrin was purchased from Extrasynthese (Genay, France) and Sigma-Aldrich.

Plant materials

K. pinnata leaves were obtained from the Research Center for Chemistry, Indonesian Institute of Sciences (LIPI), Serpong, Indonesia. The species were determined by botanists at the Botanical Research Center for Biology, LIPI, Chibinong, Indonesia. A herbalium specimen was deposited in the Research Center for Chemistry, LIPI.

Preparation and purification of *K. pinnata* extracts

Dried *K. pinnata* leaves (1 kg) were ground to powder and extracted using methanol under reflux conditions. The extract (80 g) was initially partitioned with the respective solvents to obtain an *n*-hexane (18.2 g), ethyl acetate (3.0 g), butanol (30.0 g) and water fractions (25.3 g). The ethyl acetate fraction (1.9 g) was subjected to silica gel 60 column chromatography (0.063–0.200 mm; Merck, Darmstadt, Germany) with *n*-hexane:ethyl acetate and ethyl acetate:methanol by gradient elution, which resulted in the isolation of quercitrin (120 mg) as fraction 8 (ethyl acetate:methanol 100:0 to 90:10). Eleven major fractions (fractions 1 to 11) were obtained using thin layer chromatography. Based on the bioactivity results, fractions 6 to 9 were subjected to further purification. Fractions 6 (*n*-hexane:ethyl acetate 30:70), 7 (*n*-hexane:ethyl acetate 20:80 to 10:90) and 9 (ethyl acetate:methanol 90:10 to 80:20) were combined and subjected to further chromatography using a Sephadex LH-20 column (Sigma-Aldrich) with a step-wise gradient of dichloromethane:methanol (1:1) to yield a flavonoid, quercetin (58.1 mg), and gallic acid (11.1 mg). The physical and spectral characteristics were determined using one- and two-dimensional nuclear magnetic resonance (NMR; 500 MHz; JEOL-JNM-ECA, Tokyo, Japan) and liquid chromatography–mass spectrometry (Mariner BiospectrometryWorkstation; Mckinley Scientific, Sparta, NJ, USA) [16]. The crude extract and its subfractions were dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich) at a concentration of 100 and 20 mg/ml, respectively, and stored at −30°C.

Virus titration

Huh7it-1 cells (2.4×10^4 cells/well) were placed in a 96-well plate 1 day before virus infection. Culture supernatants obtained from HCV-infected cells were serially diluted 10-fold in culture medium and inoculated to the cells. The virus was adsorbed to the cells for 4 h at 37°C and the cells were then incubated with a medium containing 0.4% methylcellulose (Sigma-Aldrich) for 40 h. HCV titers were determined using a focus formation assay as described previously [15, 17]. HCV antigen-positive cells were stained with HCV-infected patient's serum and horseradish peroxidase-conjugated goat anti-human IgG (MBL, Tokyo, Japan). The infectious foci were detected using the metal enhanced DAB substrate kit (Thermo Fisher Scientific Inc., Rockford, IL, USA). Foci were imaged and counted using the katikati counter software.

Anti-HCV activities assay

Huh7it-1 cells were seeded into 48-well plates at 5.0×10^4 cells/well 1 day before virus infection. Two-fold serial dilutions of crude extracts, subfractions or isolated compounds were prepared in culture medium in 96-well plates. The same volume of HCV (5.0×10^3 focus-forming units) was mixed with the samples that were prepared as described above and immediately inoculated into the Huh7it-1 cells. After incubation for 2 h at 37°C, the cells were rinsed twice with serum-free medium to remove residual virus, and incubated for an additional 46 h with the same medium. At 48 h p.i., the culture supernatants were collected and used for virus titration. In some experiments, cells were treated with the isolated compounds only during viral inoculation or only after viral inoculation for the remaining culture period until virus harvest to assess the mode-of-action of the test samples.

Cytotoxicity assay

Cytotoxicity of the crude extracts and pure compounds against Huh7it-1 cells was determined using MTT [3-(4,5-dimethylthiazol-2-yl)-5(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] assay (Sigma-Aldrich) following the manufacturer's instructions. Cells (2.0×10^4 cells/well) were seeded into 96-well plates and treated with various concentrations of extracts, subfractions or pure compounds for 48 h. The medium was replaced with 100 μl of MTT-containing medium and incubated for an additional 4 h. The medium was removed and 100 μl of DMSO was added to dissolve the generated precipitates. Absorbance at 550 nm was measured using a GloMax-Multi Microplate Multimode Reader (Promega). The percentage of viable cells was plotted versus the concentration of the test compound. The concentration by which to mediate 50% cytotoxicity (CC_{50}) was determined by non-linear regression analysis using GraphPad Prism graphing software.

RESULTS

Anti-HCV activities of extracts from *K. pinnata*

While screening Indonesian medicinal plants for the possible anti-HCV activities, we found that a crude methanol extract from *K. pinnata* showed anti-HCV activities with a 50%-inhibitory concentration (IC_{50}) of 17.2 μg/ml (Fig. 1a and Table 1). Cytotoxicity of the crude extract against Huh7it-1 cells was determined using MTT assay. Severe cytotoxicity was not observed at the concentrations tested and the CC_{50} value was 207.2 μg/ml. The selectivity index (SI; CC_{50}/IC_{50}) was 12.0.

To isolate an active substance(s) responsible for the anti-HCV activity, the crude extract was partitioned into *n*-hexane, ethyl acetate, butanol and water fractions and their anti-HCV activities were evaluated.

The result obtained showed that the ethyl acetate and butanol fractions had strong anti-HCV activities with IC_{50} values of 9.3 and 7.1 μg/ml, respectively (Fig. 1b and Table1) whereas the *n*-hexane and water fractions exhibited only 20% inhibition at the highest concentration tested (50 μg/ml). The cytotoxicity of these fractions against Huh7it-1 cells was examined and the result showed CC_{50} values of >100 μg/ml for all four fractions. We selected the ethyl acetate fraction for further purification.
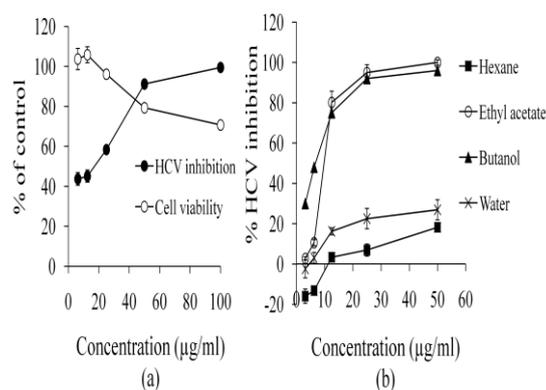

Fig. 1: Anti-HCV activities of a crude extract (a) and its fractions (b) from *K. pinnata*.

Data represent means ± SD of data from triplicate cultures.

Table 1: IC_{50} and CC_{50} values of a crude methanol extract and its fractions from *K. pinnata*

Sample	IC_{50} (μg/ml)	CC_{50} (μg/ml)	SI
Crude extract	17.2	207.2	12.0
n-Hexane fraction	119.0	>200	>1.7
Ethyl acetate fraction	9.3	149.4	16.1
Butanol fraction	7.1	148.4	20.9
Water fraction	65.2	166.8	2.6

SI: Selectivity Index (CC_{50}/IC_{50})

The ethylacetate fraction was separated using silica gel column chromatography, which yielded 11 subfractions (fractions 1 to 11). These fractions were evaluated for anti-HCV and cytotoxic activities, along with cyclosporin A (1 μg/ml) as a positive control. The result obtained revealed that fractions 6 to 9 at 10 μg/ml inhibited HCV infection by 60.4%, 98.6%, 96.2% and 91.7%, respectively, while cyclosporin A (1 μg/ml) by 82.2% (Table 2). The IC_{50} values of fractions 6 to 9 were 11.4, 0.8, 1.0 and 9.5 μg/ml, respectively, with their SI being 7.1, 33.3, 22.5 and 7.5, respectively.

Table 2: Anti-HCV activities of an ethyl acetate fraction and its subfractions from *K. pinnata*

Sample[a]	% HCV inhibition	IC$_{50}$ (µg/ml)	CC$_{50}$ (µg/ml)	SI
None (untreated)	0	NA	NA	NA
EA Fraction	51.9 ± 3.2	9.3	149.4	16.1
Fraction 1	27.8 ± 12.3	NT	NT	NA
Fraction 2	12.7 ± 4.0	NT	NT	NA
Fraction 3	24.2 ± 0.9	NT	NT	NA
Fraction 4	14.3 ± 3.4	NT	NT	NA
Fraction 5	1.5 ± 2.0	NT	NT	NA
Fraction 6	60.4 ± 6.3	11.4	81.4	7.1
Fraction 7	98.6 ± 0.9	0.8	26.6	33.3
Fraction 8	96.2 ± 1.4	1.0	22.5	22.5
Fraction 9	91.7 ± 0.9	9.5	71.3	7.5
Fraction 10	57.8 ± 6.0	NT	NT	NA
Fraction 11	24.6 ± 7.2	NT	NT	NA
Cyclosporin A	82.2 ± 2.2	NT	NT	NA

[a]Each sample was tested at 10 µg/ml, except cyclosporine Atested at 1 µg/ml.EA: ethyl-acetate, NT: not tested, NA: not applicable

Anti-HCV activities of the isolated compounds, quercetin, gallic acid and quercitrin

The fractions 6, 7, and 9 possessing antiviral activities were combined and subjected to further purification using a Sephadex LH-20, which yielded two pure compounds. Another pure compound was obtained byfurther chromatography of fraction 8. These three isolated compounds were identified as quercetin, gallic acid and quercitrin, respectively, by comparing their NMR spectrum data with those reported previously. The purity of the isolated compounds was estimated at approximately 90% based on ^{1}H NMR spectrometric data (data not shown). We then evaluated anti-HCV activities of those compounds.

The isolated quercetin and gallic acid exhibited dose-dependent HCV inhibition at non-toxic concentrations with IC$_{50}$ values of 1.5 and 6.1 µg/ml, respectively, whereas quercitirin showed no anti-HCV activity (Table 3).

We confirmed the anti-HCV activities of commercially available quercetin and gallic acid, with their IC$_{50}$ values being 4.5 and 13.9 µg/ml, respectively (Table 3 and Fig. 2). As for the cytotoxicity, CC$_{50}$ values of the isolated quercetin and gallic acid against Huh7it-1 cells were >40 and 65.3µg/ml, respectively, with their SI being >26.7and 10.7, respectively. Similar results were obtained with the commercially available quercetin and gallic acid.

Table 3: IC$_{50}$ values, CC$_{50}$ values, and SI of isolated compounds, quercetin, gallic acid, and quercitrin

Compound	IC$_{50}$(µg/ml)	CC$_{50}$ (µg/ml)	SI
Isolated quercetin	1.5	>40	>26.7
Quercetin[a]	4.5	>240	>53.3
Isolated gallic acid	6.1	65.3	10.7
Gallic acid[a]	13.9	88.7	6.4
Isolated quercitrin	>120	>120	>1
Quercitrin[a]	>120	>120	>1

[a]Purchased from Sigma-Aldrich or Extrasynthese.

Table 4: Time-of-addition analysis of quercetin and gallic acid

| Compounds | % Inhibition | | | Mode-of-action |
	During + post inoculation	During inoculation	Post inoculation	
Quercetin	79.9 ± 2.7	22.3 ± 3.1	75.5 ± 3.3	Post-entry inhibition
Gallic acid	95.4 ± 2.6	58.6 ± 2.3	82.7 ± 0.9	Entry and post-entry inhibition

Quercetin, 20 µg/ml; Gallic acid, 30 µg/ml. Data representmeans ± SD of data from triplicate cultures.
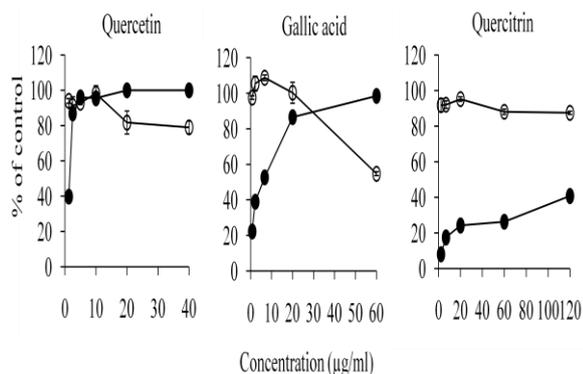

Fig. 2: Anti-HCV activities and cytotoxicity of quercetin, gallic acid and quercitrin

The percent HCV inhibition (●) and cell viability (○) are presented as the percentage of the mock-treated control. Data represent means ± SD of data from triplicate cultures.

Mode-of-action of quercetin and gallic acid

To determine the possible inhibition step(s) in the viral life cycle, cells were treated with the test compounds only during viral inoculation or only after viral inoculation for the remaining culture period until virus harvest. Quercetin exerted anti-HCV activity when it was added after viral adsorption, suggesting the inhibition at the post-entry step (Table 4). On the other hand, gallic acid inhibited HCV activities at both the entry and post-entry steps.

DISCUSSION

The present results demonstrated that extracts from *K. pinnata* leaves and two isolated compounds, quercetin and gallic acid, exhibited anti-HCV activities. *K. pinnata*, a member of the *Crassulaceae* family commonly found in tropical areas, has been used as a traditional medicinal herb for the treatment of inflammation and gastric ulcer owing to its antidiabetic, antibacterial, antiviral, antifungal, hepatoprotective and anticancer properties [18]. To date, there is no report regarding the anti-HCV activities of *K. pinnata* although previous studies reported that extracts from *K. pinnata* and plants of the *Kalanchoe* genus possessed antiviral activities against other viruses than HCV. For example, crude extracts of *K. pinnata*

exhibited antiviral activities against herpes simplex and Epstein-Barr viruses [19].Also, extracts of *K. farinacea* showed antiviral activities against influenza

A and herpes simplex type 1 viruses [20] and *K. gracilis* leaf extracts against entero virus 71 and coxsackievirus A16 [21]. These findings collectively suggest that extracts from plants of the *Kalanchoe* genus offer valuable sources of antiviral agents.

A variety of natural products have been described as anti-HCV agents [11-13]. The flavonoids quercetin and quercitrin were isolated as major compounds from the leaves of *K. pinnata*. Our present results have demnstrated that quercetin possesses potent anti-HCV activities (IC_{50}= 1.5 μg/ml) and considerably low cytotoxicity, with the SI of >26.7 (Table 3). On the other hand, quercitrin, a quercetin derivative conjugated with 3-*O*-alpha-L-rhamnopyranoside, did not exhibit an appreciable level of anti-HCV activity even at the highest concentration tested (120 μg/ml). The anti-HCV activities of quercetin have been previously reported by other groups and the antiviral mechanism(s) were thought to involve disruption of the interaction between heat shock protein (HSP) 70 and NS5A and the inhibition of NS3 protease activity [22-24]. Quercetin was also effective against a number of viruses, including hepatitis B virus, dengue virus, human cytomegalovirus, herpes simplex virus type 1, polio virus type 1, parainfluenza virus type 3, respiratory syncytial virus and adenovirus *in vitro*[25-31], suggesting that quercetin has potential use in the treatment of various viral infections. Gallic acid is also a common natural constituent of a variety of plants. In the present study, gallic acid was identified as one of the compounds responsible for the anti-HCV activity of *K. pinnata*.It exhibited moderate anti-HCV activities with an IC_{50} value of 6.1 μg/ml and SI value of 10.7 (Table 3). The time-of-addition analysis demonstrated that gallic acid acts at both the viral entry and post-entry steps.

The precise mechanism of HCV inhibition by gallic acid remains unclear; however, gallic acid is well known to possess antioxidant and hepatoprotective activities [32, 33] and antioxidant levels are reportedly significantly reduced in chronic hepatitis and cirrhosis [34]. It is conceivable, therefore, thatthe anti-HCV mechanism of gallic acid involves its antioxidant activities. Further studies are needed to elucidate the issue.

CONCLUSION

Extracts from *K. pinnata* leaves and two compounds, quercetin and gallic acid, possess anti-HCV activities. Quercetin and gallic acid could be good candidatesto design and develop novel antiviraldrugs for treatment of HCV infection.

ACKNOWLEDGMENT

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