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Original Article

Qualitative and Quantitative Analysis of 70% Ethanol Extract from *Ruta angustifolia* for Developing Anti-Hepatitis C Agents

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ABSTRACT

Background: Medicinal plants are potential sources for drug candidates. It possesses with various metabolites which have many pharmacology effects. *Ruta angustifolia* is one of medicinal plants that has been used traditionally for liver disease. Previous study it has been demonstrated to inhibit hepatitis C virus under in vitro cell culture. It decreased protein NS3 level and gave synergistic effect in combination with simeprevir and telaprevir. This plant provides a prospective candidate to develop as anti-HCV **Objective:** This study evaluates the phytochemistry screening for qualitative assay and determine the concentration of rutin as marker compound for developing *R. angustifolia* extract as anti-HCV agent. **Materials and Methods:** *R. angustifolia* leaves were extracted with 70% of ethanol. Extract and rutin were analysis their anti-HCV activity by in vitro culture cells of Huh7it. The concentration of rutin was determine by TLC densitometry. **Results:** The 70% ethanol extract of *R. angustifolia* dan rutin exhibit anti-HCV activities with IC_{50} value of 2.9 ± 0.8 µg/ml and 28.1 ± 5.6 µg/ml, respectively. Screening phytochemistry demonstrated to contain flavonoid, terpenoid, alkaloid and polyphenols. TLC densitometry analysis yield the concentration of rutin in extract 0.06 %. **Conclusion:** Extract of 70% ethanol of *R. angustifolia* has a potential anti-HCV activity. Extract of *R. angustifolia* may provide a good candidate for developing anti-HCV agents.

Key words: Hepatitis C Virus, *Ruta angustifolia*, Rutin, Medicinal plants, Medicine, Infectious disease.

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INTRODUCTION

Medicinal plants have thousands of species. From a total of around 40,000 species of medicinal plants that have been known in the world, 30,000 were allegedly located in Indonesia. This amount represents 90% of medicinal plants found in the Asian. From this amount, 25% of them or about 7,500 species are known to have herbal or medicinal properties. However, only 1,200 types of plants have been used as raw materials for herbal medicines or herbs.[1] With this potential, provide good prospects in developing herbal medicines that are important for health.

Our previous studies were reported medicinal plants which possess anti-HCV activities such as *Melicope latifolia*, *Glycyrrhiza uralensis*, and *Phyllanthus niruri*.[2-6] Various plants from several countries have also evaluated for their anti-HCV activities by inhibiting in the entry or post entry steps of HCV life cycle.[7-9] We also reported that 96% of extract *Ruta angustifolia* possess a potential anti-HCV activity.[3] *R. angustifolia* is belong to the genus of Rutaceae. Ruta genus plant has been used as traditional medicine, such as antiseptic, antihelmintic, anti-inflammatory, wound healing, pain relief drugs, to treat disorders of the digestive tract, respiratory tract, nervous system, skin, and musculoskeletal.[10] In Indonesia, *R. angustifolia* L. is commonly used as a traditional medicine for liver disease and jaundice, and in Chinese communities in Malaysia and Singapore it is used as a cancer treatment.[11] *R. angustifolia* contains alkaloids, coumarin and flavonoids. And in other studies isolation of compounds that have produced isolates of chalepin, scopoletin, γ-fagarin, arborinine, kokusaginine, and pseudane IX compounds. Chalepin and pseudane IX compounds provide strong inhibitory activity against hepatitis C virus.[3] *R. angustifolia* extract shows inhibitory activity at post-entry step and decreases the protein level of NS3 and NS5A hepatitis C virus. Combination of *R. angustifolia* extract and antiviral hepatitis C drugs have a synergistic effect.[12] In other studies, isolates of the chalepin and rutin can significantly inhibit the growth of cancer cells. Based on the previous studies, *R. angustifolia* is one of the plants that has the potential to be developed into Standardized Herbal Medicine and phytopharmaceutical. In its development, product raw materials must fulfil quality requirements in order to have pharmacological and safe effects Further analysis to ensure the quality was done by examine the quality of extract including the chemical compounds, metabolite and activity. Those elements related to the pharmaceutical quality paradigm, quality in terms of fulfil standard requirements (chemical, biological, pharmaceutical). Therefore, this research will determine the quality of extract of *R. angustifolia* as a raw material for the development of traditional medicines to guarantee the quality by determining the profile of metabolite including the concentration of standard compound. Therefore, this current study we analyzed the phytochemistry evaluation and determine the rutin substance in the 70% ethanol extract of *R. angustifolia* as raw material for developing anti-HCV agent.

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METHOD

Collection and extraction

R. angustifolia leaves were collected from area of Jombang, East Java and verified by expert botanist of Purwodadi Botanical Garden Indonesia Institute of Science, East Java, Indonesia. Leaves of the plants were dried at room temperature, pulverized, and extracted by 70% of ethanol. Extraction was performed by maceration process and filtrate was concentrated with a rotary evaporator in the temperature of 50°C and a rotary speed of 35-40 rpm, then poured on a porcelain cup and dried in an oven at 42°C until a constant weight.

Cell and viruses

A clone of human hepatocellular carcinoma-derived Huh7 cells, Huh7it-1, was cultivated in Dulbeco's Modified Eagle Medium (GIBCO Invitrogen, Carlsbad, CS, USA) supplemented with 10% Fetal Bovine Serum (Biowest, Nualle, France), 0.15 mg/mL Kanamycin (Sigma–Aldrich, St. Louis, MO, USA) and non-essential amino acids (GIBCO-Invitrogen) in 5% CO2 at 37°C. A cell culture-adapted HCV variant was propagated as described previously. In brief, Huh7it-1 cells (1.8×10^7 cells) were infected with JFH1 1.8×10^7 focus forming unit (ffu) for 4 h with agitation in every 30 min. The HCV-infected cells were incubated for 5 days. The supernatants a day 3 post infection were collected. Then, concentrated using an Amicon filter and stored at -80°C and evaluate the virus titration for antiviral experiments. *Dubelco's Phosphate Buffered Saline* (DPBS, GIBCO-Invitrogen), trypsin-EDTA (GIBCO-Invitrogen), 0.4% metil cellulose (Sigma–Aldrich), *Bovine Serum Albumin* (BSA, Roche), 10% formaldehyde solution (HCHO, Applicam), 0.5% triton X-100 (Promega), *3,3'-diaminobenzidine* (DAB) *thermo staining,* hepatitis C virus *human patient anti-serum* dan antibody HRP-*goat-antihuman Ig* (MBL), MTT*(3-(4,5-dimethylthiazol-2-yl)-5-3carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) reagen* (Sigma-Aldrich).[5,6]

Anti-viral activities of *Ruta angustifolia* extract and Rutin against Hepatitis C virus

R. angustifolia extracts and rutin (Sigma) were dissolved in dimethyl sulfoxide (DMSO) to obtain stock solutions at a concentration of 100 mg/ml. The stock solutions were stored at –20°C until used. Huh7it cells were seeded in 48-well plates (1.9×10^5 cells/well). A fixed amount of HCV virus, with a multiplication of infection (MOI) of 0.5 focus-forming units (ffu)/cell, was mixed with serial dilutions (100, 30, 10, 1 and 0.1 µg/ml) and inoculated to the cells. After 2 hours, the cells were washed with medium to remove the residual virus and further incubated in the medium containing the same concentrations of the test samples as those during virus inoculation. Culture supernatants were obtained at 2 days post-infection and titrated for virus infectivity.[13] Virus and cells treated with medium containing 0.1% DMSO served as a control. The percent inhibition of virus infectivity by the samples was calculated by comparing to the control using SPSS probit analysis, and IC_{50} values were determined.[5,6]

Virus titration and immunostaining

Huh7it-1 cells (2×10^4 cells/well) were seeded in a 96-well plate and incubated for 24 hours. Virus supernatants were diluted in the medium and inoculated onto the Huh7it culture cells and incubated for 4 hours. After virus absorption, the cells were cultured with medium containing 0.4% methylcellulose (Sigma–Aldrich) following 41 hours incubation. Infected cells were analyzed with immunostaining using anti-HCV patient anti-serum (250 time dilution on 2% BlockAce/1%BSA/PBS) and HRP-goat antihuman Ig antibody (300x on 2% lockAce/1%BSA/PBS). The HCV antigen positive cells were visualized with Metal Enhanced DAB substrate kits (Thermo Fisher Scientific, Rockford, USA). The infected cells were counted under microscopes and calculated the percentage inhibition.

Cytotoxicity analysis

The cytotoxicity analysis was conducted to determine whether the extract mediated any cytotoxicity effects. MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) assay was done by inoculating 100, 50, 10, 1 and 0.1 µg/mL of extract in 96 wells plate culture cells which have seeded for 24 hours. After 48 h incubation, the medium was replaced with MTT reagent containing medium and incubated for 4 h. Absorbance sample was evaluated under microplate reader at 450 and 600 nm, which is correlated with the amount of cell viability. The percentage of cell toxicity was calculated by comparing with untreated cells and further determine its 50% cytotoxic concentration (CC_{50}) values.[3,14,15]

Phytochemistry screening of *R. angustifolia* extract by Thin layer chromatography

Extract was conducting on TLC plate and examine its components for flavonoid, alkaloid, polifenol and terpenoid. The sample is bottled on a GF254 silica gel plate, then put into a chamber with a mobile phase of n-hexane: ethyl acetate by comparison through the optimization results. Identification is done by comparing the Rf value of the sample with the standard and TLC results are detected with a UV detector at a wavelength of 254 and 366 nm.[16]

Determine rutin concentration by TLC densitometry

A TLC chromatogram was performed prior to the analysis of rutin content in the extract. Various concentration of standart compounds, rutin, were prepared with concentration of 51, 102, 204 and 408 µg/ml. All concentration was applied in the TLC plate and eluated with mobile phase ethyl acetate: asam formiat and water (100:15:17 v/v). While extract was prepared by dissolved in ethanol and applied to the the TLC plate together with the standart. The stationary phase was silica gel GF254 and hexane-ethyl acetate (7:3) was used as mobile phase. The area of each sport was evaluated under Camag densitometer. The concentration of rutin in the extract was calculated.

RESULT

Screening phytochemistry of 70% ethanol extract of *R. angustifolia* demonstrated to possess alkaloid, flavonoid, terpenoid and polyphenol compounds (Table 1).

Anti-hepatitis C virus activity of 70% of extract ethanol *R. angustifolia* and rutin was showed to have potential inhibition with IC_{50} value of 2.9 ± 0.8 and 28.1 ± 5.6 µg/ml, respectively (Figure 1).

The content of rutin was evaluated by TLC densitometry. For the validation method, the linear regression data for respective calibration curves showed a good linearity for rutin analysis with r = 0.999. The relative standard deviation (RSD) of 0.34% (acceptable range < 10% RSD), with the regression equation y=433.4 +17.68x. The estimation of rutin concentration in the extract was 0.06%. ± 0.34.

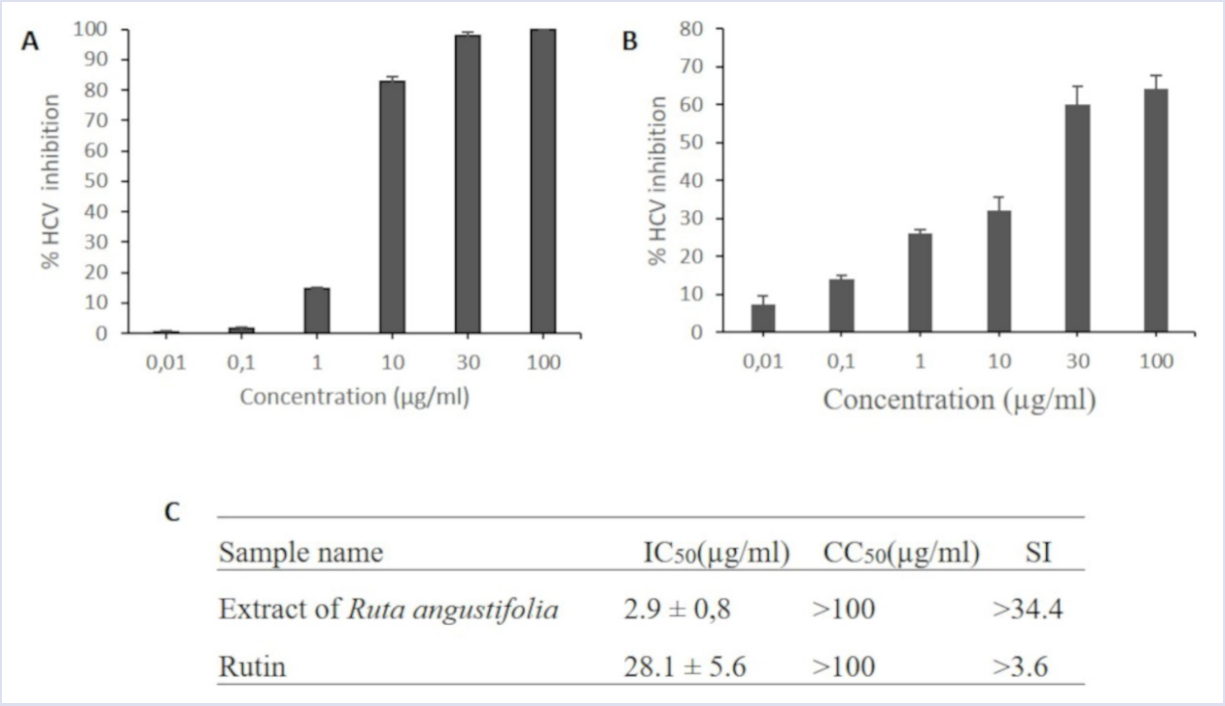
DISCUSSION

R. angustifolia has been reported to possess anti-HCV activity. Developing *R. angustifolia* as anti-HCV agent such as alternative or complementary product provide good opportunity. In conducting herbal drug product, assessment of raw material quality is needed. The evaluation of metabolite compounds is necessary to ensure the responsible compounds. Phytochemistry screening of extract showed to contain with alkaloid, flavonoid, terpenoid and polyphenol of *R.*

Table 1: Phytochemistry screening of 70% ethanol extract of *R. angustifolia*

	Detection reagent	Spot's color	Result
Flavonoid	Citrate borate	yellow	+
Terpenoid	Anisaldehyde	purple	+
Alkaloid	Dragendorff	orange	+
Polyphenol	$FeCl_3$	dark blue	+

Extract of *R. angustifolia* was detected by applying to the stationary phase of silica gel GF254 and mobile phase hexane: ethyl acetate (7:3).



C

Sample name	IC_{50}(µg/ml)	CC_{50}(µg/ml)	SI
Extract of *Ruta angustifolia*	2.9 ± 0,8	>100	>34.4
Rutin	28.1 ± 5.6	>100	>3.6

Figure 1: Dose-dependent anti-HCV activities of *R.angustifolia* extract (A) and Rutin (B). IC_{50} value, CC_{50} and selectivity index (SI) (C). Huh7it cells seeded in 48 well plates were infected with HCV and treated with *R. angustifolia extract* or rutin. Culture supernatant was collected for virus titration. The percentage HCV inhibition was calculated and compared with the control. Data represent means ± SEM of data from three independent experiments.

angustifolia extract. Several plants of the genus Ruta was reported to contain rutin. There are 14 species from the genus of Ruta such as *Ruta graveolen* and *Ruta chalepin*. Rutin was first isolated from leave of *R. graveolen*[17] and it was known as the main active flavonoid of *R. graveolen*.[18,19] It was demonstrated to have various activities such as anti-oxidant, anti-cancer, vaso-protective, neuroprotective and cardioprotective activity.[20] It was also possessed anti-viral activities against immunodeficiency virus, herpes simplex virus, enterovirus and dengue virus.[20,21] Our study found that rutin exhibit anti-hepatitis C virus activities against hepatitis C. The contain of rutin in the *R. angustifolia* extract could be a marker compound for anti-HCV drug development. Rutin is a flavonoid as glycoside compound with the chemical name of 3,3′,4′,5,7-pentahydroxyflavone-3-rhamnoglucoside or quercetin -3-rutinoside (Figure 2). Flavonoid compounds have been demonstrated to mediate antiviral activity such as Human immunodeficiency virus (HIV) by inhibit DNA polymerase of virus. Flavone *O*-glycoside inhibit HIV-1 reverse transcription. Kaempferol and luteolin possess synergistic effect in herpes simplex virus (HSV), while quercetin reveled inhibition in HSV and dengue virus. Many flavonoids, namely, dihydroquercetin, dihydrofisetin, leucocyanidin, pelargonidin chloride, and catechin, show activity against several types of virus including HSV, respiratory syncytial virus, polio virus and Sindbis virus. Inhibition of viral polymerase and binding of viral nucleic acid or viral capsid proteins have been proposed as antiviral mechanisms of action.[22] Rutin was also reported to inhibit HIV and HSV. Anti-viral activity of rutin was caused by the structure of compound that potential to interact with the receptor of virus or host in inhibiting the virus production. The polyhydroxylated substitutions of ring A and B, a 2,3-double bond, a free 3-hydroxyl substation, a 4-keto moiety, and C-3 position of glycosylated may contribute in the activities.[20,23]

CONCLUSION

Extract of 70% ethanol of *R. angustifolia* has a potential anti-HCV activity. It contains rutin compound which potential as a marker for anti-HCV drug development of *R. angustifolia*.

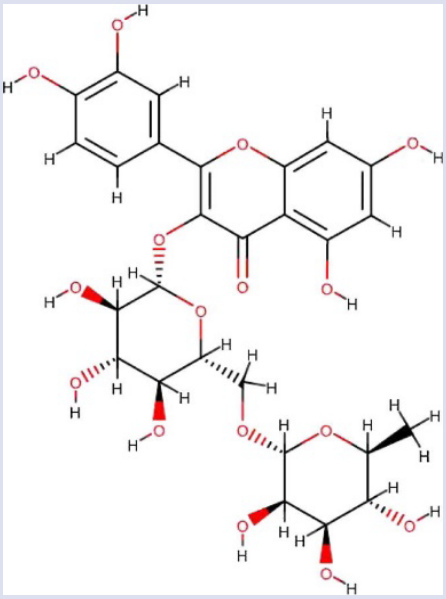


Figure 2: Chemical structure of rutin.

CONFLICTS OF INTEREST

None.

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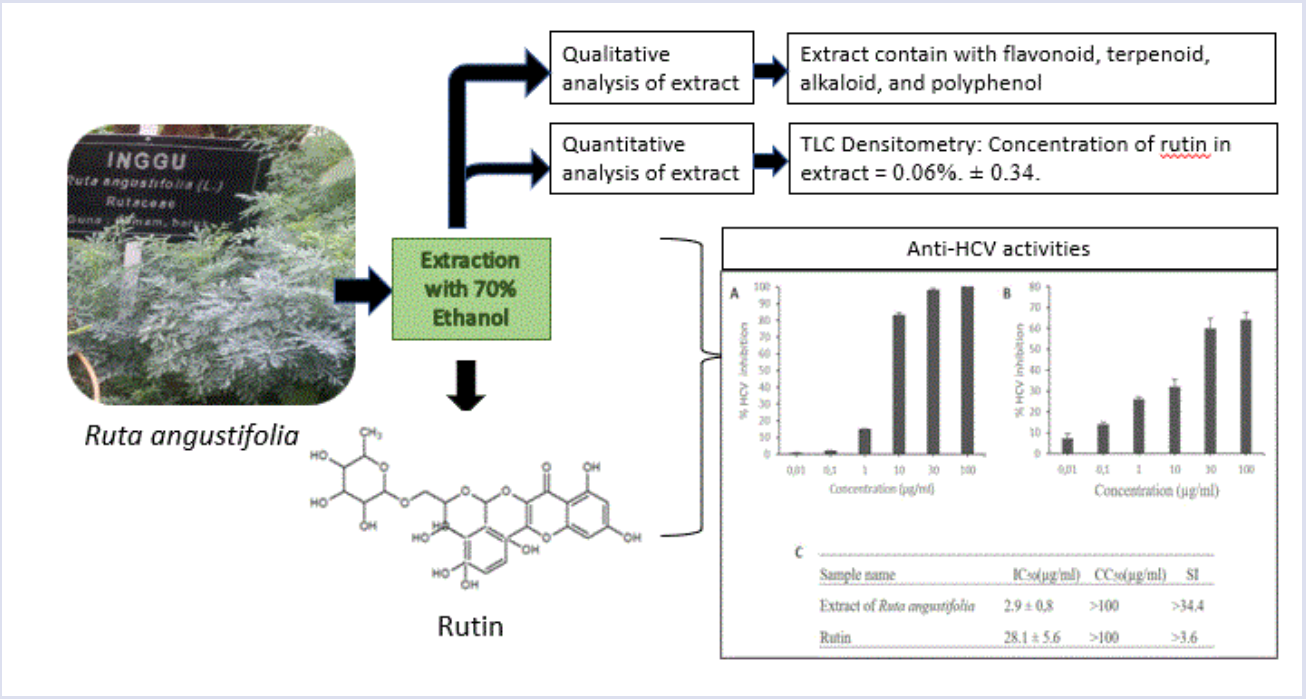
GRAPHICAL ABSTRACT



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