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Synergistic anti-hepatitis C virus activity of Ruta angustifolia extract with NS3 protein inhibitor

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Abstract:

Background: Medicinal plants are known to perform many pharmacological actions due to their chemical metabolites, which include antiviral effects. Previously, the extract of *Ruta angustifolia* was shown to have potential anti-hepatitis C virus (HCV) activity without any cytotoxicity, with a 50% inhibitory concentration of 3.0 μg/mL and a 50% cytotoxicity concentration of >100 μg/mL. Furthermore, the combination of medicinal plants and current anti-HCV agents, such as a direct-acting antiviral agent, was shown to potentiate their overall effectiveness. In the course of this study, the ethanolic extract of *R. angustifolia* was evaluated for its anti-HCV effects; specifically, the mechanism of action on HCV NS3 and NS5A protease was investigated.

Methods: Analysis of the use of this extract in conjunction with current NS3 inhibitor drugs, simeprevir (SMV) and telaprevir (TVR), was performed. Anti-HCV activity was performed by *in vitro* culture of hepatocyte cells. The cells were infected and treated with various concentrations of the sample. HCV inhibition was calculated and CompuSyn software analysis was used to determine the synergistic effect of the combination.

Results: Results demonstrated that *R. angustifolia* extract inhibited the post-entry step and decreased the protein levels of HCV NS3 and NS5A. The combination of extract and SMV and TVR mediated a synergistic effect.

Conclusions: These findings suggest that combining *R. angustifolia* extract with current anti-HCV drugs should be considered when developing alternative and complementary anti-HCV medicines.

Keywords: *Ruta angustifolia*, simeprevir, synergistic, telaprevir

DOI: 10.1515/jbcpp-2019-0348

Received: November 17, 2019; **Accepted:** November 18, 2019

Introduction

Medicinal plants are potential sources for finding new drugs. Metabolites of many plants have been reported to possess several biopharmacological effects, including antiviral activities; this includes inhibiting the activity of hepatitis C virus (HCV) [1]. *Ruta angustifolia* is a plant belonging to the Rutaceae family and has been used as traditional herbal remedy [2]. In Indonesia, it is known as a traditional herb for jaundice. In our previous study, a number of compounds exhibiting anti-HCV effects have been isolated from *R. angustifolia*, including chalepin, arborinine, γ-fagarine, kokusagenin, and pseudane IX [3]. Hence, strong evidence supports the combination of *R. angustifolia* extract and current anti-HCV drugs as warranted for the drug development of anti-HCV agents.

HCV infection is a global health problem that chronically infects more than 71 million people, putting them at risk for developing cirrhosis or liver cancer [4]. At this time, there is no vaccine available for preventing HCV infection. Although the most recent therapy being developed is a combination of direct-acting antivirals (DAAs), these include NS3 protease, NS5A protein, and NS5B RNA polymerase with a sustained virologic response (SVR) of >90%, especially in HCV genotype 1 patients. However, despite its efficacy, limits with this treatment remain problematic; the high cost is prohibitive to patient access. Furthermore, potential for drug resistance and side effects associated with long-term use have been observed [5]. Thus, the development of new anti-HCV agents and/or combination therapies is imperative to improve the overall efficacy of HCV treatment.

The goal of HCV treatment is curative and defined as achieving undetectable HCV RNA concentrations within 12 weeks (i.e. SVR12) or 24 weeks (i.e. SVR24) of treatment [4]. Anti-HCV agents can be divided into two classes: DAAs (discussed above) that directly target viral NS3 protease, NS5B polymerase, or NS5A protein and host-targeting antivirals, such as cyclophilin inhibitors [6]. Currently, the standard therapies for HCV infection include interferon (IFN)-α and other alternative IFN-free treatment regimens that use two or three types of DAAs in combination.

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The combination of anti-HCV compounds is often shown to provide a greater reduction in HCV RNA levels compared to the use of each agent singularly. For example, the inhibitory effect of IFN (concentration of 5 IU) on HCV is 55%. With the addition of glycyrrhizin (a plant extract of *Glycyrrhiza glabra* at a concentration of 10 µg/mL), the inhibitory antiviral effect of IFN increased to 95%. In addition, extracts from the *Acacia confusa* plant, when combined with IFN, telaprevir (TVR), and 2′-C-methylcytidine, showed a synergistic effect with a combination index (CI) of <1 [7], [8]. These data support the potential for increased efficacies when developing combination therapies between natural compounds and anti-HCV agents, including DAAs. This study determined the antiviral effect of *R. angustifolia* extract when combined with simeprevir (SMV) and TVR.

Materials and methods

Collection and extraction

The leaves of *R. angustifolia* used were from Lembang, West Java, Indonesia. The sample was identified by expert botanical researchers in Purwodadi, Indonesia. The leaf samples were then dried at room temperature, ground to powder, and extracted via maceration with 96% ethanol for a total of 3 days. The collected filtrate was then concentrated using a rotary evaporator until the desired thickness was obtained.

Cell and virus preparation

Hepatocyte cells (Huh7it) were cultivated in 10 cm dish with 10 mL Dulbecco's modified Eagle's medium (Wako Chemicals) and supplemented with fetal bovine serum (Biowest, Inc.), nonessential amino acids (Invitrogen), 100 IU/mL penicillin, and 100 µg/mL streptomycin (Invitrogen). Cells were then grown at 37 °C in a 5% CO_2 incubator. HCV (J6-JFH1 strain) was propagated as described previously [3], [9] and inoculated into Huh7it cells, which were then cultured for 2 days. After culture, the supernatants were collected, and the viral titers were determined.

Sample preparation for anti-HCV activity

Ruta angustifolia extract was dissolved in dimethylsulfoxide (DMSO) to make a stock solution at a concentration of 100 µg/mL. SMV and TVR, both made in stock at a concentration of 1000 nM, were the DAAs tested. All stock solutions were stored at −30 °C until used.

Analysis of anti-HCV activity

Huh7it cells were seeded in 48-well plates at a density of 5.4×10^4 cells per well. HCV was mixed with a serial dilution of the extract and inoculated into the cells at a multiplication of infection of 0.5 focus-forming units/cell. After 2 h, the cells were washed with medium to remove any residual virus and then further incubated in medium containing the antiviral compounds. The culture supernatants were collected 2 days after infection and then titrated to determine the virus infectivity [9]. Virus and cells treated with medium containing 0.1% DMSO served as the control. The percent inhibition of virus infectivity was calculated for each sample by comparing the infectivity of test samples to that of control using SPSS probit analysis to determine the 50% inhibitory concentration (IC_{50}) values.

Mode of action analysis

Mode of action analysis was evaluated by *in vitro* culture cells of HCV. *Ruta angustifolia* extract was analyzed by a three-series model in parallel. First, the extract was treated only during inoculation (2 h), the remaining virus was discarded, and the extract was refed into the medium until 46 h incubation. Second, the culture was treated with extract only after inoculation for 46 h. Third, the culture was treated with extract in both entry and post-entry steps.

Immunoblotting analysis

Treated Huh7.it cells were lysed with radioimmunoprecipitation assay buffer and the amount of protein was calculated. Equal amounts of protein were separated in sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels and transferred onto polyvinylidene difluoride membrane (Millipore, Bedford, MA, USA). First antibody HCV NS3-specific mouse monoclonal antibody (clone H23; Abcam, Cambridge, MA, USA) and glyceraldehyde-3-phosphate dehydrogenase antibody (MBL) were incubated for 1 h, and phosphate-buffered saline-0.05% Tween was used for membrane washing. Second antibody horseradish peroxidase-conjugated goat anti-mouse immunoglobulin (MBL) was incubated for 1 h and the respective protein was visualized using Clarity Western ECL substrate (Bio-Rad). Skim milk (5%) was added to block the nonspecific binding for 60 min incubation. Chemiluminescence was detected using ImageQuant LAS 4000 (GE Healthcare).

Combination treatment of *R. angustifolia* extract and anti-HCV drugs

The effects of combination drug treatment were tested by adding *R. angustifolia* extract to anti-HCV DAA drugs, SMV (Toronto Research Chemical) and TVR (Adooq Bioscience), an HCV NS3 protein inhibitor). Three series of analyses were conducted simultaneously: (1) *R. angustifolia* extract alone, (2) each of the anti-HCV drugs alone, and (3) a mixture of *R. angustifolia* extract and each anti-HCV drug (1:100 ratio for the combination with SMV and 1:200 for the combination with TVR). The percent inhibition of virus infectivity for each sample was then calculated as described in Section 2.4. CompuSyn software was used to calculate the CI to determine whether the drug combination exerted an additive, synergistic, or antagonistic antiviral effect [10], [11], [12].

Results

It is known that *R. angustifolia* extract possesses strong antiviral activity against HCV, with an IC_{50} value of 3.0 μg/mL and a 50% cytotoxicity concentration of >100 μg/mL. It was postulated that the potency of *R. angustifolia* extract may increase if combined with other anti-HCV drugs. Therefore, the primary purpose of this study was to further analyze the anti-HCV effects of *R. angustifolia* extract in combination with existing anti-HCV agents, such as SMV and TVR.

Mode of action analysis found that *R. angustifolia* extract inhibited HCV dominantly in the post-entry step (Figure 1B). Further analysis by immunoblotting demonstrated an inhibition effect of *R. angustifolia* extract on HCV NS3 and NS5A protein. The result showed that *R. angustifolia* extract suppressed HCV protein NS3 and NS5A in culture cells (Figure 1C and D).

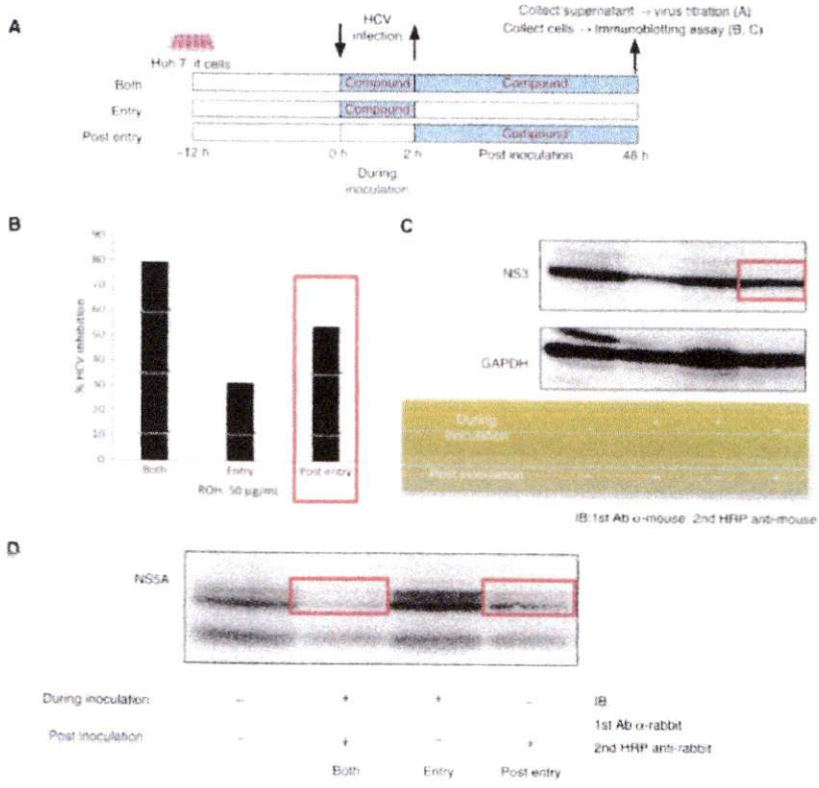


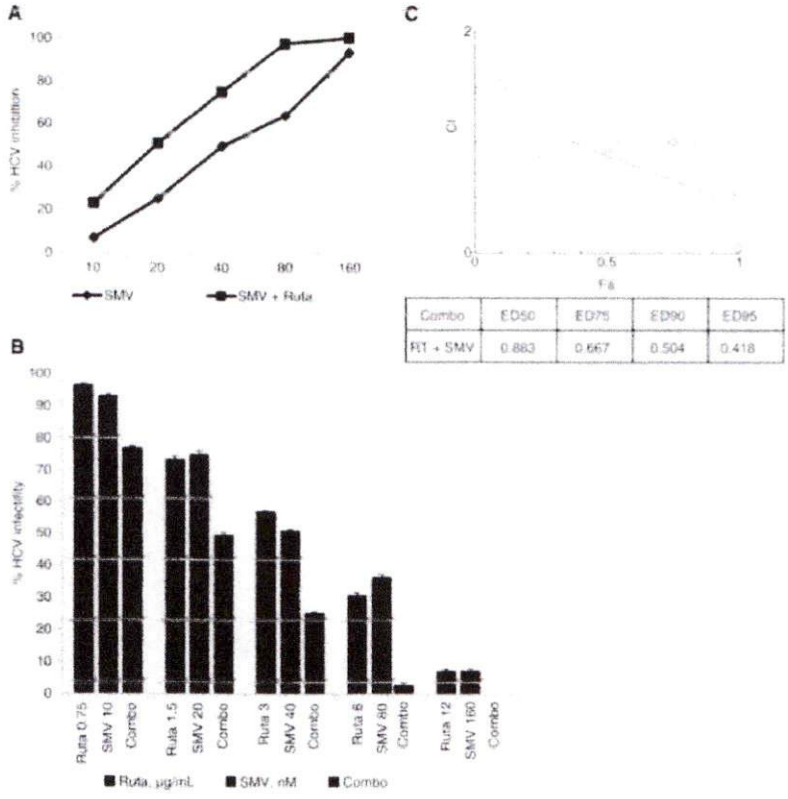
Figure 1: *Ruta angustifolia* extract possesses strong inhibition in the post-entry step against HCV. (A) Scheme of mode of action analysis. (B) Inhibition in the post-entry step is higher than that in the entry step. It decreases the NS3 (C) and NS5A (D) protein level.

The combination treatment of *R. angustifolia* extract and SMV revealed a higher inhibition of HCV compared to treatment with either of the drugs alone . The IC_{50} of SMV used singularly was 43.84 ± 0.96 nM, whereas the combination treatment improved the inhibitory effect of SMV to an IC_{50} value of 19.70 ± 0.28 nM. The results using CompuSyn software determined a [redacted] value of ED_{50} = 0.883 (<1); Table 1, Figure 2), thus showing that this combination treatment has a synergistic effect on the inhibition of HCV [10]. Combination was also performed with TVR and obtained the higher HCV inhibition compare to the TVR single drug alone (Figure 3).

Table 1: IC_{50} of single administration and combination of extract and SMV and TVR.

Samples	Anti-HCV activity (IC_{50}), nM
SMV	43.84 ± 0.96
TVR	10.48 ± 0.11
Combination extract and SMV	19.70 ± 0.28
Combination extract and TVR	3.64 ± 0.07

Data are mean ± SE from three independent experiments.



Combo	ED50	ED75	ED90	ED95
RT + SMV	0.883	0.667	0.504	0.418

Figure 2: *Ruta angustifolia* extract in enhanced anti-HCV activity of SMV.
Huh7it cells seeded in 48-well plates were infected with HCV and treated with *R. angustifolia* extract. Culture supernatant was collected for virus titration. The percentage HCV inhibition was calculated and compared to control. The combination treatment increased anti-HCV activities. Serial dilution of the concentration of extract and SMV was inoculated according to the method of Chou and Talalay. The addition of *R. angustifolia* extract increased HCV inhibition compare to the single treatment of SMV in doses dependent manner (A). *Ruta angustifolia* extract in combination with SMV decreased HCV infectivity more than the treatment of either drug alone (B). CompuSyn analysis of the drug combination treatment demonstrates a synergistic effect (CI < 1.0) (C). Data are mean ± standard error (SE) from three independent experiments.

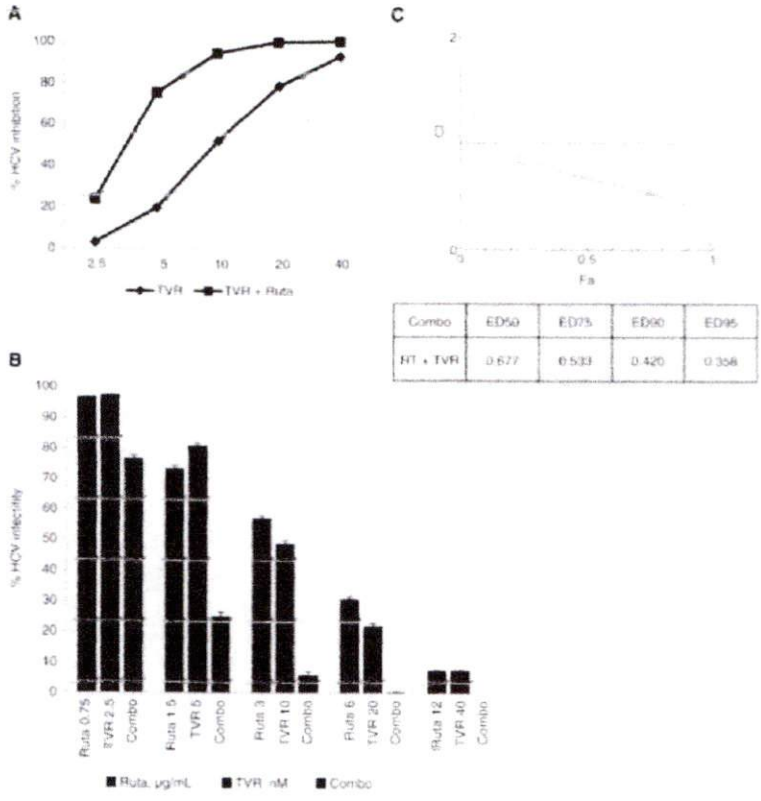


Figure 3: *Ruta angustifolia* extract showed to increase anti-HCV activity of TVR.
Huh7it cells seeded in 48-well plates were infected with HCV and treated with *R. angustifolia* extract. Culture supernatant was collected for virus titration. The percentage HCV inhibition was calculated and compared to control. The combination treatment increased anti-HCV activities. Serial dilution of the concentration of extract and TVR was inoculated according to the method of Chou and Talalay. The addition of *R. angustifolia* extract increased HCV inhibition compare to the single treatment of TVR in doses dependent manner (A). *Ruta angustifolia* extract in combination with TVR decreased HCV infectivity more than the treatment of either drug alone (B). CompuSyn analysis of the drug combination treatment demonstrates a synergistic effect (CI < 1.0) (C). Data are mean ± SE from three independent experiments.

Discussion

The chemical metabolites contained in medicinal plants are known to provide important pharmacological uses. Thus, the continued discovery of naturally produced compounds has been important in the development of new drugs. Moreover, societies that lack access or the financial means to purchase more modern, synthetic drugs use natural compounds as do the people who wish to minimize the potential side effects from synthetic drugs.

Natural compounds known to possess antiviral effects against HCV include the flavonoid compounds quercetin, naringenin, and catechin, which collectively inhibit HCV, thus demonstrating significant potential in reducing NS3 and NS5A protein levels in HCV-infected patients [13], [14]. Similarly, circumdatin G (an alkaloid compound) has been shown to protect patients from fungus-mediated anti-HCV activity [15]. Polyphenol compounds, such as ethyl gallate, catechin gallate, delphenidin, saikosaponin b2, and grosheimol, likewise have been shown to inhibit HCV in the initial stages of infection [16].

The purpose of HCV therapy is to eradicate the virus in a patient, the success of which is indicated by its SVR. Combination drugs are the current modality used to treat HCV; however, their efficacy is lacking. The drug treatment combination of IFN and RBV achieves only 50% SVR after 24 weeks. Furthermore, this treatment may cause serious side effects. The most currently used HCV treatment regimen involves using an IFN-free combination of two to three DAAs — NS3/4A protease, NS5A, and NS5B polymerase inhibitors. This treatment approach has better success compared to treatment using IFN alone (SVR > 90%) [4]. However, viral resistance and potentially undesirable side effects are still seen. Moreover, these antiviral synthetic drugs are

expensive, making them inaccessible to patients with limited income. The differential responses of various HCV genotypes to these treatments underscore the need to find new and less expensive anti-cofactor enzymes so that it will suppress the replication process of HCV [17].

In general, the HCV life cycle process consists of receptor binding, fusion, translation, replication, virion assembly, and released virion. Th entry step is defined as the stage consisting of receptor binding through translation, whereas t[29]tage of replication through virion release constitutes the post-entry stage. *Ruta angustifolia* extract has been shown to inhibit HCV in the post-entry step through its inhibition of NS3 and NS5A (see Figure 1).

SMV and TVR are DAAs; their direct mechanism of action as NS3/4A protease inhibitors is to disrupt the wo[1] of the protease and cofactor enzymes and thereby suppress the replication process of HCV [17].

NS3/4A inhibitors are conventionally grouped into two classes. The first class (first generation) are linear peptidomimetics that incorporate a reactive electrophilic ketoamide at the cleavage site; this then targets the catalytic Ser139 of the active site of the enzyme via a fully reversible mechanism. Examples of this class include TVR and boceprevir. The second class (second generation) includes competitive, reversible, macrocyclic, noncovalent inhibitors. Macrocycles are useful to improve affinity and selectivity for protein targets while preserving the sufficient bioavailability characteristics of small molecules. Belonging to this class is SMV [18], which has a macrocyclic str[1]ture; it is thought to have an advantage over first-generation protease inhibitors, and their linear structures, in terms of binding affinity and specificity for NS3 protease [19].

We demonstrated in this study that the combinatory addition of *R. angustifolia* extract increased the anti-HCV activities of SMV and TVR. Moreover, the extract exerted a synergistic effect with CI values of <1. The extract alone of *R. angustifolia* suppressed HCV production and reduced the HCV NS3 and NS5A protein level. These results suggest that combinations of SMV and TVR with *R. angustifolia* extract may good candidates to consider as combination.

Conclusions

The combination of *R. angustifolia* extract and the current anti-HCV drugs was shown to enhance the overall antiviral effectiveness by giving an additive synergistic effect. Therefore, the addition of *R. angustifolia* extract to existing drug combinations should be considered in the development of alternative and complementary anti-HCV treatment.

Acknowledgments

The authors are sincerely grateful to Prof. Hak Hotta and Dr. Chie Aoki Utsubo for providing HCV and hepatocyte cells.

Author contributions: All authors have accepted responsibility for the entire content of this manuscript and approved its submission.

Research funding: None declared.

Competing interests: The authors state no conflict of interest.

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