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Anti-Hepatitis C Activity of Combination of *Ruta angustifolia* Extract and Ribavirin

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Abstract

Background*: Hepatitis c virus infection is a global health problem which chronically infected 71 million people in the world. This infection has a risk of becoming liver cirrhosis and hepatocellular carcinoma. Since the current HCV therapy has been developed by direct-acting antivirals (DAA), however, most patients get limited access due to the high cost. Therefore, further development anti-HCV agent still greatly needed. Ruta angustifolia is a natural resource which was reported to possess anti-HCV activity. Ribavirin is an antiviral agent used to treat several virus infections, either DNA or RNA. Ribavirin was known to inhibit HCV infection by regulated immune system in host cells and interfering the replication of HCV by inhibit HCV RdRp.* ***Objective****: The current study evaluated the combination treatment of R. angustifolia extracts and ribavirin by in vitro culture cells of Huh 7it.* ***Method****: The study was conducted under an invitro cell culture of Huh 7it and infected with JFH1a. Result: The result demonstrated an enhancement effect of the extract by increasing the anti-HCV activity 3.5-fold higher compared to ribavirin alone. The 50% inhibitory concentration of ribavirin by single treatment was 10.43 ± 0.18 µg/mL, while in combination with Ruta angustifolia extract was 2.80 ± 0.03 µg/mL. Further analysis of the combination by CompuSyn software mediated a synergistic effect among the combination with a combination index value of 0.691.* ***Conclusion****: These results suggested that combination of Ribavirin and Ruta angustifolia should be considered in developing anti-hepatitis C virus agents.*

Keywords*: hepatitis c virus, Ruta angustifolia, ribavirin, medicine, infectious disease*

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INTRODUCTION

The medicinal plant is potential source for various bioactivities due to its metabolite properties, including their activities as antiviral and hepatitis (Ashfaq & Idrees, 2014; Dhama et al., 2018; Wahyuni et al., 2013a; Wang *et al*., 2018). The plant extract of *Ruta angustifolia L.* has been reported in a previous study to possess antiviral activity against hepatitis C virus with no toxic effect (Wahyuni et al., 2014). Mode of Action assay was demonstrated to inhibit dominantly in the post-entry step of HCV life cycle, and decreased the HCV NS3 level (Wahyuni et al., 2019). The isolated compounds of *R. angustifolia*, chalepin and pseudane IX exhibited to decrease viral RNA replication and reduced viral protein synthesis (Wahyuni et al., 2014). Those results demonstrated the potential of the ethanol extract of *R. angustifolia* L on HCV infection, making the extract a prospect as an anti-HCV agent. Medicinal plants are commonly used as complementary medicine to support the effectiveness of standard drugs. A combination of drugs may improve the effectiveness and decrease the resistance potency (Ulrich-Merzenich, 2014; Vickers & Zollman, 1999). A combination assay of ethanol extract of *R. angustifolia* have been done with Direct Acting Antiviral Agents (DAAs), simeprevir and telaprevir, which revealed a synergistic effect (Wahyuni et al., 2019). The combination aims to produce a synergistic or additive effect, where the impact of the combination of extracts and drugs will be greater for inhibition of the hepatitis C virus than the effect of drugs used individually (Connell et al., 2013). Conceptually, combination therapy of several hepatitis C antiviral agents with different mechanisms of action can increase antiviral effectiveness and avoid viral resistance (Chatterji et al., 2014). Ribavirin is an antiviral drug that acts on host cells (host targeting agents) by inhibiting ribonucleoprotein synthesis and interfering with the early stages of viral transcription (Rumi, 2009). Therefore, it is necessary to evaluate the combination therapy of ribavirin for anti-hepatitis C virus activity with natural ingredients, namely *Ruta angustifolia* to treat hepatitis C virus infection safely, have affordable treatment costs, have no toxicity and reduce drug resistance.

MATERIALS AND METHODS

Materials

The leaves of *R. angustifolia* was obtained from Jombang, East Java Indonesia and has been verified by a expert botanist researchers from Materia Medika Indonesia, Batu, East Java. Huh7it-1 hepatocyte cells and JHF1 hepatitis C virus (Cultivaed in Institute Tropical Disease, Airlangga University) were propagated as described previously (Wahyuni et al., 2013b; Wahyuni et al., 2014),). Dimethyl Sulfoxide (DMSO), medium of Dubelco's Modified Eagle Medium (DMEM, GIBCO-Invitrogen), 10% Fetal Bovin Serum (FBS, GIBCO-Invitrogen), Non-Essential Amino Acid (NEAA, GIBCO-Invitrogen), Dubelco's Phosphate Buffered Saline (DPBS, GIBCO-Invitrogen), Penicillin Streptomycin (GIBCO-Invitrogen), Trypsin-EDTA (GIBI BCO-Invitrogen), Bovine Serum Albumin (BSA, Roche), formaldehyde (HCHO, Applicant), TritonX-100 (Promega), Thermo staining 3,3'-diaminobenzidine (DAB), hepatitis patient antiserum C, HRP-Goat-anti-human *Ig* (MBL) were used for anti HCV assay. The material used in the toxicity test was MTT (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymetho xyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) reagents (Thermo fisher).

Method

Collection and extraction

R. angustafolia was collected from Jombang area, East Java, Indonesia. The leaves of the plant were dried and extracted with 96% ethanol three times using the maceration method. The filtrate was collected and concentrated with a rotary evaporator. The extract was stored at -20°C until it used.

Cell and virus preparation

The cells of hepatocyte (Huh7it) were cultivated in Dulbecco's modified eagle's medium (Wako Chemicals) and supplemented with fetal bovine serum (Biowest, Inc) and non-essential amino acids/NAA (Invitrogen). The mixture of 100 IU/mL penicillin and 100 µg/mL streptomycin (Invitrogen). Cells were incubated at the condition of 37°C in a 5% CO_2 incubator. While the virus of hepatitis C was propagated for 3, 4 and 7 days and following by titer assay for each day (Wahyuni et al., 2014).

Sample preparation for anti-HCV activity

Sample preparation was started by making a stock solution of extract in dimethylsulfoxide (DMSO) to obtain a concentration of 100 mg/mL. The ribavirin was provided in a sterile water solution with a concentration of 10.000 µg/mL. The stock solutions were kept at -20°C until used.

Anti-HCV activity assay

The initial treatment in conducting the sample activity test was seeding cells in 48 well plates with a cell density of $5.4x10^4$ and incubate for 24 hours, JFH1 virus was added with a multiplicity of infection (m.o.i) of 0.1. The ethanol extract of leaves of *R. angustifolia*

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with a concentration of 100, 30, 10, 1, 0.1, and 0.01 µg/mL were inoculated into the cells. Ribavirin was also evaluated for its activities at the concentration of 50, 30, 10, 1, 0.1, and 0.01 µg/mL. The test was carried out to determine the IC_{50} concentration of the ethanol extract of *R. angustifolia*. and ribavirin to be used as concentrations in the combination assay. Combination of *R. angustifolia* and ribavirin were determined at the concentration of 4 x IC_{50}; 2 x IC_{50}; 1 x IC_{50}; 1/2x IC_{50}; and 1/4x IC_{50} of both substances. The tested-concentration of ribavirin was 2.5, 5, 10, 20, and 40 µg/mL, while the tested-concentration of *R. agustifolia* extract was 0.75, 1.5, 3, 6, and 12 µg/mL. The evaluation was carried out either ribavirin alone or in combination with extract of *R. angustifolia*. The combination method is commonly referred to the Chou-Talalay method (Chou, 2010). In each well, 100 µL of the test material was inoculated with the mixture of virus and incubated for 2 hours, then cells were rinsed, and 200 µL of test material were added and incubated for approximately 46 hours. The supernatant containing hepatitis C virus was collected and further analyzed its virus titration. The virus titration was conducted in the Huh7it cells that had been seeded in 96 well plates with a density of $2.4x10^4$ for 24 hours. After inoculation of the virus supernatant for 2 days, cells were fixed with 3.7% formaldehyde 200 µL and cell permeabilization using 0.5% triton X-100 100 µL. Observation of infected cells was carried out using DAB thermo staining: substrate (1:9). Colonies of infected cells were evaluated under the microscope (Wahyuni et al., 2018; Wahyuni et al., 2013a).

Cytotoxicity test

A toxicity test was performed using the reagent of MTT reagent. Huh7it cells were exposed to the test materials, the ethanol extract of the leaves of *R. angustifolia L.*, ribavirin, and a combination of the extract and ribavirin. In this toxicity test, 60 µL of each extract concentration, ribavirin and the mixture of extract and ribavirin were inoculated into the cells with the concentration of 4 x IC_{50}; 2 x IC_{50}; 1 x IC50; ½ xIC_{50}; and ¼ x IC_{50} and incubated for 48 hours.

Then, the remaining medium was discarded, and 150 µL of medium containing MTT reagent was added and incubated for 4 hours. To dissolve the precipitate formed from the MTT reaction, DMSO was added. The absorbance was detected at 560 nm and 750 nm wavelength of the Microplate Multidetector Reader (Sigma). The absorbance of the sample was measured by comparing to the control (Wahyuni et al., 2019; Wahyuni et al., 2018).

Data analysis

Data of the number of infected cells with the virus will be calculated using the formula below and will get the percentage of infected cells.

$$\% \text{ Infected cells} = \left(\frac{Number\ of\ infected\ cells\ in\ sample}{number\ of\ infected\ cells\ in\ control}\right) x\ 100\%$$

To calculate % inhibition = 100% - % infected cells. The IC_{50} value was further determined using a probit log analysis with SPSS. While, the data in the form of sample absorbance in the toxicity test will be calculated using the formula below and obtained % cell viability data.

$$\% \text{ Cell viability} = \left(\frac{Sample\ absorbance}{Control\ absorbance}\right) x\ 100\%$$

Furthermore, to evaluate the synergistic or additive effect of the combination of the ethanol extract of the leaves of *R. angustifolia* and ribavirin, CompuSyn software was used, and further justified based on the combination index value (<1: synergistic, 1: additive, >1: antagonist). The data needed to be included in the CompuSyn software are the concentration and % inhibition of each extract and drug as well as the concentration and % inhibition of the combination (Chou, 2010).

RESULTS AND DISCUSSION

R. angustifolia has been analyzed its potency in anti-HCV activity in the previous work (Wahyuni et al., 2020; Wahyuni et al., 2019; Wahyuni et al., 2014). Further exploration its activities in the combination with Ribavirin was done in this study.

Three parallel assays of anti-HCV activities were carried out in vitro using Huh7it cells and JFH1a virus. Anti-hepatitis C activity test of the combination between the ethanol extract of the leaves of *R. angustifolia* and ribavirin on Huh7it cells and JFH1a virus was followed by the Chou-Talalay method. The results of the ed activity test of the ethanol extract of the leaves of R. angustifolia L. and ribavirin are shown in Figure 1. The combination can increase the activity, which is shown by the reduction of the IC_{50} value of ribavirin from 10.43 ± 0.18 µg/mL (single treatment) to 2.80 ± 0.03 µg/mL (in combination treatment). Thus, the combination of *R. angustifolia* extract with ribavirin could increase the inhibitory activity of ribavirin against the hepatitis C virus by 3.7 times higher compared to ribavirin alone. The combination index values was obtained through the CompuSyn software analysis. The combination index (CI) values were revealed at the concentration with 50, 75, 90 and 95 % inhibition activities. All of the CI values demonstrated less than 1, which indicates that the

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combination of *R. angustifolia* extract and ribavirin produces a synergistic effect, as shown in Table 1.

Ribavirin is one of the initial HCV infection treatments with an interferon combination. It modulates T helper-1 and -2 lymphocyte imbalance, interferes with cell guanosine triphosphate by inhibiting inosine monophosphate dehydrogenase, and the viral RNA-dependent RNA polymerase, impairment of translation by preventing the capping of messenger RNA and caused viral mutagenesis. Although the current treatments use Direct Acting Antiviral agents (DAAs), however ribavirin is still use in some areas (Mathur et al., 2018). While ethanol extract of *R. angustifolia*, and its isolated compounds, chalepin and pseudane IX, were shown to decrease NS3 protease which involved in the replication of virus. Moreover, it was reported that the combination of extract of *R. angustifolia* and NS3 inhibitor demonstrated a synergistic effect (Wahyuni et al., 2019; Wahyuni et al., 2014). Therefore, the combination of ribavirin and *R. angustifolia* extract may provide a different side of actions to inhibit HCV virus. The results of the toxicity test of the combination of the ethanol extract of the leaves of R. angustifolia and ribavirin showed that the concentration-tested was not toxic to hepatocyte cells. The percentage of cells viability at all concentration-tested was higher than 90%, as shown in the Figure 2.

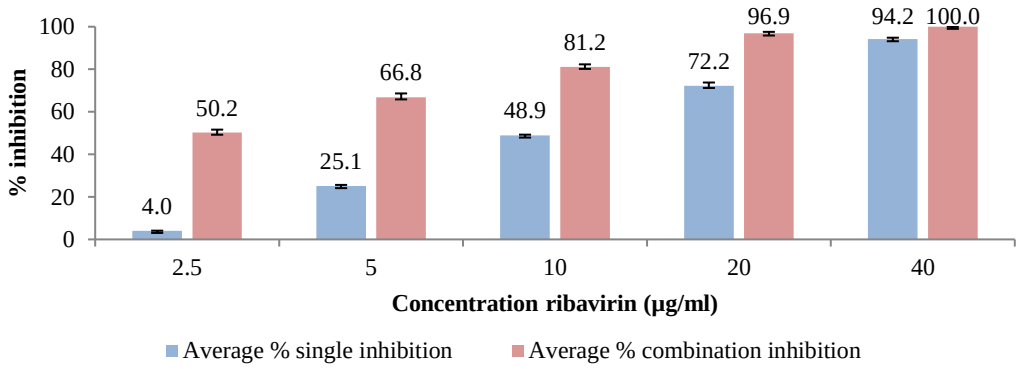

Figure 1. The percentage inhibition of single ribavirin and combination of ribavirin and ethanol extract of *R. angustifolia* according to the Chou-Talalay method. Data were represented 3 independent experiments

Table 1. The values of the combination index (CI) of the combination between the ethanol extract of the leaves of *R. angustifolia L.* and ribavirin

Combination	Combination index (CI) value			
	IC_{50}	IC_{75}	IC_{90}	IC_{95}
R. angustifolia L. Extract + Ribavirin	0.691	0.568	0.467	0.408

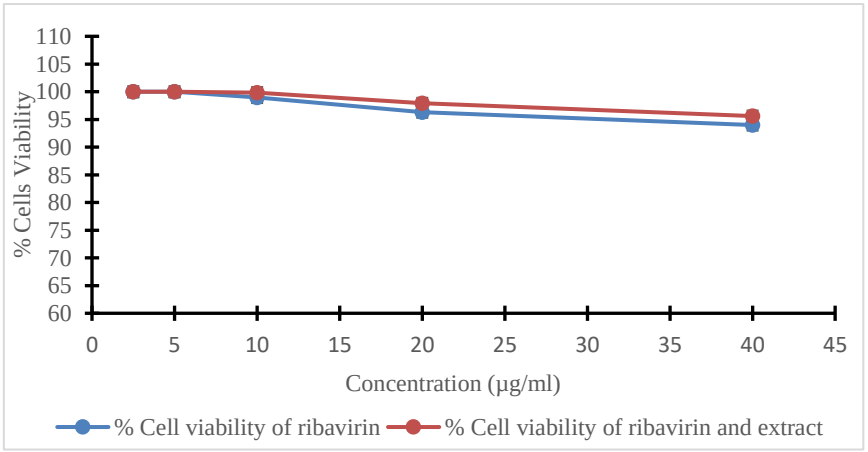

Figure 2. The percentage cells viability of single ribavirin and ribavirin combination with the ethanol extract of the leaves of *R. angustifolia L.* according to the Chou-Talalay method. Data were represented 3 independent experiments

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CONCLUSION

The combination of the ethanol extract of the leaves of *R. angustifolia* with ribavirin revealed a synergistic effect which increased the anti-HCV activity 3.7 times higher compare to the ribavirin alone without any toxic effect. These results suggested that a combination of ribavirin and *R. angustifolia* should be considered in developing anti-hepatitis C virus agents.

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AUTHOR CONTRIBUTIONS

Conceptualization, T.S.W, C.A.U., A.F.; Software, T.S.W., A.A.P.; Methodology, T.S.W., A.A.P.; Validation, T.S.W., A.W.; Writing - Original Draft, T.S.W., A.A.P.; Writing - Review & Editing, C.A.U., A.W.; Funding Acquisition, T.S.W.

CONFLICT OF INTEREST

The authors declared no conflict of interest.

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