

## The Phytochemistry Profile of *Piper Betle* Extract and Its Activity Against Hepatitis C Virus

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### ABSTRACT

Hepatitis C virus (HCV) is an RNA virus with a high mutation rate, making it prone to developing resistance. There is currently no vaccine for this disease, and the existing treatments are often costly. Therefore, this study aimed to provide alternative and complementary antiviral options from plant by evaluating the activity of *Piper betle* (*P. betle*) against HCV and its combination with existing antiviral drugs, Ribavirin and Simeprevir. The antiviral inhibition was identified by in vitro culture using Huh7it-1 cells and JFH1a HCV. The phytochemistry profile was also determined by Thin Layer Chromatography (TLC) and High-Performance Liquid Chromatography (HPLC). The result showed that the ethanol extract of *P. betle* had strong activity with an IC<sub>50</sub> value of 0.08 ± 0.028 µg/mL. The mechanism of action showed that the extract dominantly inhibited the post-entry steps. Furthermore, the combination of *P. betle* extract with simeprevir showed higher anti-HCV activity compared to the single use of the drug, but no effect was observed in the combination with ribavirin. The Western blotting analysis showed that the inhibition of NS3 protein levels was in a dose-dependent manner. Based on the phytochemistry evaluation, the extract was found to contain flavonoids, polyphenols, and alkaloids. These results suggested that the ethanolic extract of *P. betle* could be a good candidate for the development of alternative anti-HCV drugs.

**Keywords:** Hepatitis C Virus, *Piper betle*, Medicinal plant, Simeprevir, Ribavirin, Medicine.

### INTRODUCTION

Hepatitis C Virus (HCV) is the causal agent of liver infection, often leading to chronic conditions such as cirrhosis and hepatocellular carcinoma. This disease affects more than 70 million people worldwide (Kumar *et al.*, 2021; Chaudhari *et al.*, 2021). Current treatment primarily relies on direct-acting antiviral drugs (DAAs) which inhibit HCV replication. However, resistance, specifically in genotype 1b, and limited access to health care in low-income countries remain significant challenges.

Antiviral drugs are a class of medicine particularly used for the treatment of viral infections. Treatment strategies for anti-HCV drugs

are focused on two different role, first is targeting to the virus and the second is the target at host cell factors. (Kausar *et al.*, 2021). Current study was used Ribavirin and Simeprevir. Over the past two decades, ribavirin has been an integral component of infection treatment (Feld *et al.*, 2017). Ribavirin is reportedly strain-dependent and its efficacy is influenced by the specific sequence of multiple HCV nonstructural proteins (Mejer *et al.*, 2020). On the other hand, simeprevir is an NS3/4A protease inhibitor approved as a component of combination therapy (Ouwerkerk-Mahadevan *et al.*, 2016). It has been recently approved for the treatment of genotype 1 of chronic hepatitis C in combination with pegylated interferon and

ribavirin. This drug possesses significantly different characteristics from first-generation protease inhibitors. Previous studies showed that simeprevir is highly effective and safe, with few adverse events. Future combinations of two or more direct-acting antiviral agents are expected to be approved, that offering optimized sustained virologic responses for pan-genotypic HCV treatment (Izquierdo *et al.*, 2014).

Simeprevir, a second-generation HCV NS3/4A protease inhibitor has been approved for treating HCV genotype 1-infected patients in combination with peginterferon  $\alpha$ -2a or 2b and ribavirin, providing highly effective results with similar adverse events except for being milder and causing reversible jaundice (Kanda *et al.*, 2014). *Piper betle* L. is an economically and popular medicinal plants as an important cash crop belonging to the family *Piperaceae* and often referred to as the green gold. Plant leaves have been used as a traditional medicine to treat various health conditions in Asia. It is highly abundant and inexpensive, thereby promoting further investigations and industrialization development, including in the food and pharmaceutical industries. The phytochemicals of *Piper* species have shown strong antioxidant activity, as well as antibacterial, and antifungal activity against human pathogens (Nayaka *et al.*, 2021). Moreover, the species possesses therapeutic and preventive potential against several chronic disorders (Salehi *et al.*, 2019). Phytochemical compounds are reportedly responsible for various bioactivity. According to previous studies, *P. betle* contains diosgenin, eugenol, allylpyrocatechol, methyl eugenol, chavibetol, hydroxychavicol, triterpenes, and  $\beta$ -sitosterol which possess anti-platelet, anti-inflammatory, immunomodulatory, gastroprotective, and antidiabetic activity (Bhalerao *et al.*, 2013).

This study aimed to explore the potential synergistic effects of combining an antiviral drug with *P. betle* extract. This combination strategy was adopted to enhance antiviral efficacy and overcome potential drug resistance, offering a novel therapeutic method for combating viral infections, including HCV.

## MATERIALS AND METHODS

### Collection and extraction

Leaves of *P. betle* were obtained from the Jombang area, East Java, Indonesia. The samples were dried at room temperature, powdered, and then extracted by maceration method using 96%

ethanol as a solvent. The filtrate was evaporated in a vacuum and the concentrated extract was dried at a temperature of 40°C to a constant weight in the oven (Wahyuni *et al.*, 2021).

### The phytochemistry screening by TLC

The extract was screened for its phytochemical content by TLC including flavonoid, alkaloid, polyphenol, and terpenoid. Subsequently, the extract was applied on a GF<sub>254</sub> silica gel plate and eluted with a mobile phase of n-hexane: ethyl acetate (1:2 v/v). The eluted TLC plate was sprayed using a spray reagent and examined under UV at a wavelength of 254 and 366 nm. Further TLC was conducted with a nonpolar stationary phase (RP-18) and a mobile phase of acetonitrile: water (7:3 v/v) (Wahyuni *et al.*, 2021).

### HPLC analysis

The dried ethanolic extract of *P. betle* was dissolved in methanol (1 mg/mL), filtered, and subjected to a Shimadzu HPLC system. The analysis was conducted using Merck RP-18 column (4.6 x 250 mm, 5  $\mu$ m), an isocratic elution, deionized water, and acetonitrile (7:3 v/v), flow rate 0.5 mL/min, running time 30 min, and observed at wavelength 254 nm (Sabir *et al.*, 2021).

### Cells and virus

A clone of hepatocellular carcinoma, Huh7it-1 cells, was cultivated in DMEM-Dulbecco's Modified Eagle Medium (GIBCO Invitrogen). The medium was supplemented with 10% Fetal Bovine Serum (FBS, GIBCO-Invitrogen), 0.15 mg/ml kanamycin solution (SIGMA), and 1x Non-Essential Amino Acids (NEAA, GIBCO-Invitrogen). Following this, the cell was cultivated in 5% CO<sub>2</sub> at 37 °C. HCV JFH1a was propagated by mixing 4 ml Huh7it-1 cells with a concentration of 1.8x10<sup>7</sup> cell/well and 50  $\mu$ L of JFH1 suspension. The mixture was subsequently incubated at 37°C in 5% CO<sub>2</sub> for 4h. The suspension was divided into eight flasks and incubated to ensure the propagation of virus. HCV supernatant was harvested on the third, fifth, and seventh days. This was followed by concentration using an AMICON Ultracentrifugal filter and examination for viral titration then stored at -80 °C for further use (Wahyuni *et al.*, 2018; Widyawaruyanti *et al.*, 2021).

### Anti-HCV activity of *P. betle* extract

The *P. betle* extract was stocked at a concentration of 100 mg/mL by dissolving in dimethyl sulfoxide (DMSO) and then kept at -20 C until used. Huh7it cells were seeded in 48-well

plates ( $5.6 \times 10^4$  cells/well), 24 h before treatments. HCV JFH1a virus with a multiplication of infection (MOI) reaching 0.1 focus forming units (ffu)/cell was mixed with various concentrations of the sample (100, 30, 10, 1, 0.1, and 0.01  $\mu\text{g}/\text{ml}$ ) and inoculated to the cells. The cells and virus were incubated for 2 hours to facilitate complete infection. Following this, the cells were washed with the medium to remove the residual virus, and the same concentrations of the test samples used during inoculation were added. The culture infection was further incubated for 48 h. After 2 days post-infection, the supernatants were collected and titrated for virus infectivity. The percentage inhibition and  $\text{IC}_{50}$  values of virus growth were calculated by SPSS Probit analysis, and compared to the control (Bhalerao *et al.*, 2013; Wahyuni *et al.*, 2018; Widyawaruyanti *et al.*, 2021).

#### Virus titration and immunostaining

The titration was performed to determine the HCV virus production. The Huh7it-1 cells were seeded in 96 well plates in the concentration of  $2.6 \times 10^4$  cells/well and incubated for 24 hours. The supernatants collected during the HCV experiment were diluted in the medium and inoculated onto the Huh7it-1 cells, followed by incubation for 4 h. Afterward, the cells were washed with medium to remove the residual virus, and 200  $\mu\text{L}$  was added to each well. This was followed by incubation for 48 hours and fixing with Paraformaldehyde 4%. The infected cells were observed by immunostaining with the mixture solution of 250-time dilution anti-HCV patient anti-serum on 2% Block Ace/1%BSA/PBS and 300x HRP-goat antihuman Ig antibody on 2% Block Ace/1%BSA/PBS. 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 the percentage inhibition was calculated (Susiloningrum *et al.*, 2020; Wahyuni *et al.*, 2013; Widyawaruyanti *et al.*, 2021)

#### Western blot analysis

Cells were washed with PBS twice and lysed in 300  $\mu\text{L}$  RIPA buffer containing a cocktail protease inhibitor (Roche). The sample was shaken and incubated for 30 min on ice, followed by centrifugation at 12,000 rpm to collect the protein supernatant. Furthermore, equal amounts of total

extract protein (30  $\mu\text{g}$ ) were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The gel was transferred to polyvinylidene fluoride (PVDF) membranes. After blocking with 5% BSA for 1 h at room temperature, the membrane was incubated with mouse anti-NS3 monoclonal antibody (Abcam, Cambridge, USA) at 4°C overnight. On the second day, the membrane was washed in PBST and incubated with peroxidase-conjugated goat antimouse secondary antibody (MBL, Nagoya, Japan). To visualize the protein band, the membrane was treated with enhanced chemiluminescence assay (ECL) reagents (GE Healthcare, Pittsburgh, Pennsylvania, USA) (Permanasari *et al.*, 2021).

#### Cytotoxicity analysis

To analyze the viability of Huh7it-1 cells due to extract treatment, an MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) assay was conducted. Huh7it-1 cells with a density of  $2.3 \times 10^4$  were seeded on 96 well plates 24 hr before treatment. The following day, varying concentrations of *P. betle* extract including 100, 50, 10, 1, and 0.1  $\mu\text{g}/\text{mL}$  were inoculated into the cells. The cells were then incubated at 37°C with 5%  $\text{CO}_2$ . After 48 h incubation, the medium was replaced with an MTT reagent-containing medium and incubated for 4 h. The absorbance was assessed under wavelengths of 560 and 750 nm. The percentage of cell viability and 50% cytotoxic concentration ( $\text{CC}_{50}$ ) values were calculated by SPSS, and compared to the control (Susiloningrum *et al.*, 2020; Wahyuni *et al.*, 2018).

#### Combination treatment experiments

To analyze the effectiveness of the combination treatments, the  $\text{IC}_{50}$  values of commercial antiviral drugs, namely Simeprevir (SMV) (Toronto Research Chemical, Canada) and Ribavirin (RBV) (Sigma Aldrich, MO) were determined using SPSS. Combination treatment experiments were conducted using various concentrations of simeprevir (10, 20, 40, 80, and 160 nM) and ribavirin (2.5, 5, 10, 20, and 40  $\mu\text{g}/\text{mL}$ ) in combination with *P. betle* extract at concentrations of 0.025, 0.05, 0.1, 0.2 and 0.4  $\mu\text{g}/\text{mL}$ , respectively. Huh7it-1 cells were exposed to HCV in the presence of a combination of *P. betle* extract and commercial drugs (Wahyuni *et al.*, 2019; Wahyuni *et al.*, 2020; Permanasari *et al.*, 2021).

Table I. The phytochemistry screening of *P. betle* extract by TLC analysis

Compound	Spot detection	Result	Colors
Alkaloids	Dragendorff	Positive	orange-green brown
Flavonoid	Citrate borate	Positive	yellow
Terpenoids	Anisaldehyde-H <sub>2</sub> SO <sub>4</sub>	Positive	red-purple
Polyphenols	FeCl <sub>3</sub>	Positive	black

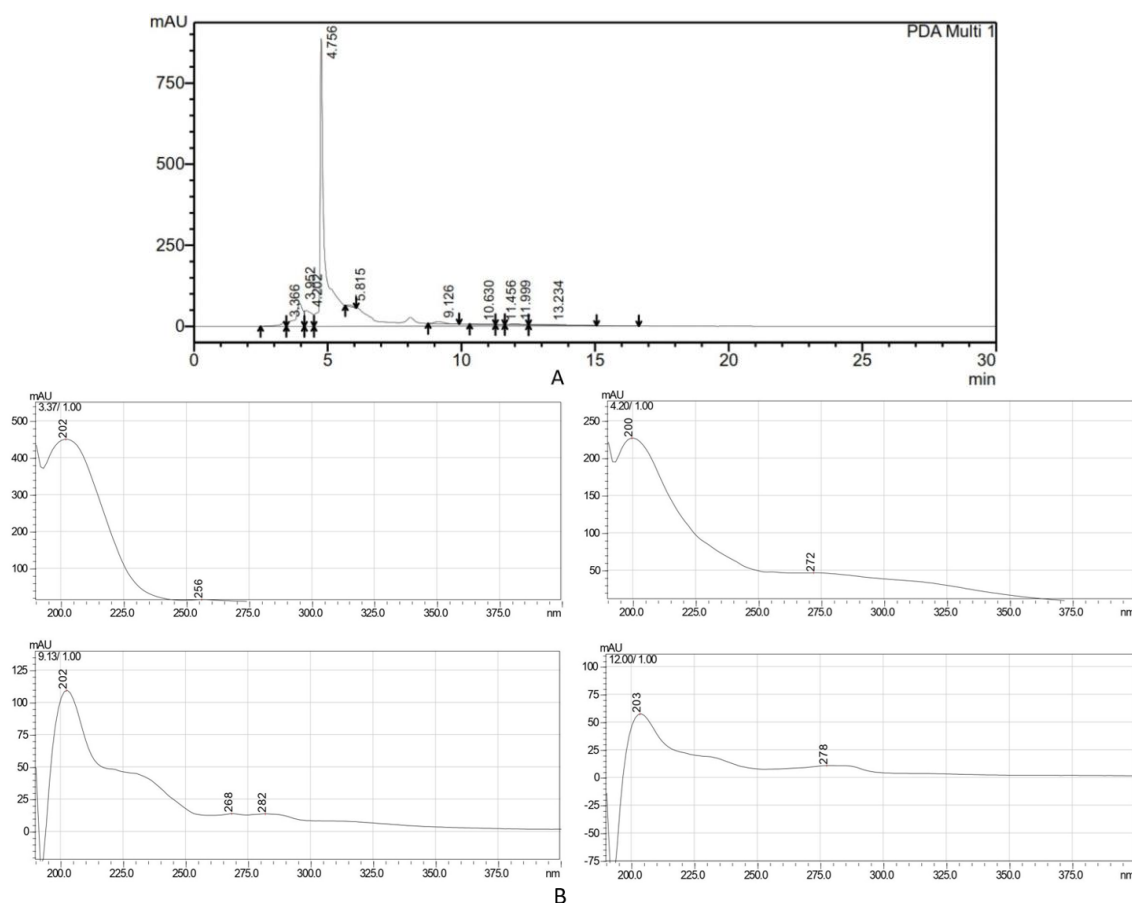


Figure 1. The HPLC profile of *P. betle* extract observed under UV 254 nm using RP-18 column (Merck, 4.6x250 mm, 5  $\mu$ m) and acetonitrile-water (70%-30%) as mobile phase with flowrate 0.5 mL/min (a), The UV spectra of several peaks at Retention Time 3.3 min, 4.2 min, 9.1 min and 12.0 min (b).

## RESULTS AND DISCUSSION

### The phytochemistry screening of *P. betle* extract by TLC

*P. betle* has been extensively studied for its various phytochemical constituents (Biswas *et al.*, 2022). Based on the results, the phytochemistry screening of 96% ethanolic extract showed the presence of alkaloids, flavonoids, terpenoids, and polyphenols (Table I). This result was in accordance with a previous study which found alkaloids, phenols, flavonoids, saponins, steroids, tannins, terpenoids, and glycosides in the

qualitative analysis of methanol extract of betel leaves (Syahidah *et al.*, 2017).

### HPLC profile of ethanolic extract of *P. betle*

The HPLC chromatogram profile of *P. betle* extract showed several peaks under 30 min observation (Figure 1a). Each peak was observed with its UV spectra to predict the group of compounds contained in the extract (Figure 1b). Meanwhile, the phytochemistry screening by TLC showed the presence of polyphenols, alkaloids, flavonoids, and terpenoids.

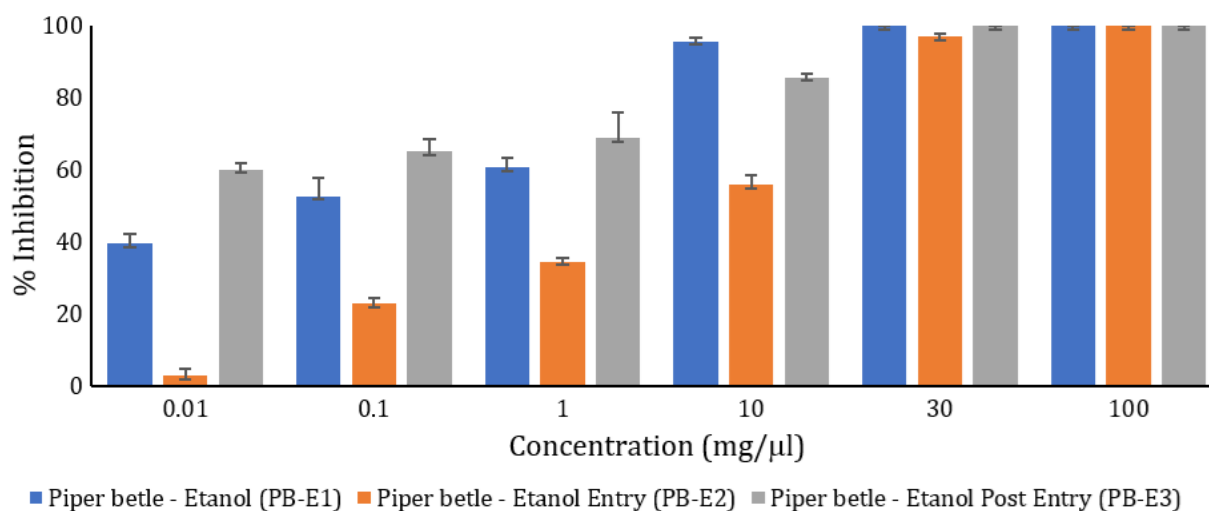


Figure 2. Graph of viral inhibition value (%) for the 96% ethanolic extract of *P. betle* leaves at the entry, post-entry, and both stages.

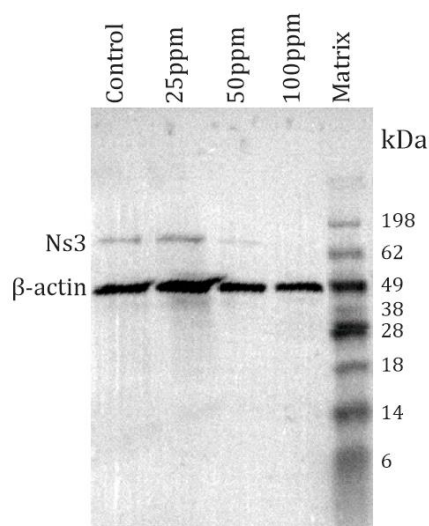


Figure 3. *P. betle* decreased HCV NS3 protein level. The culture of Huh7it-HCV infected cells (MOI = 0.5) was treated with various concentrations of compounds. After 48 h of incubation, cells were subjected to western blot analysis using monoclonal antibody against HCV NS3 protein.

These results were in accordance with the UV spectra of HPLC chromatogram peaks detected in the extract. The UV spectra showed  $\lambda_{max}$  values at 256, 268, 272, and 278 nm, corresponding to peaks detected at Rt 3.3 min, 4.2 min, 9.3 min, and 12.0 min. These UV spectra possibly represent alkaloids given their absorption maxima near 250 and 300 nm due to the dihydroindole chromophore. The polyphenols showed maximum absorbance at 265, 284, 289, and 315 nm (Halake & Lee, 2017) with their

prominent absorption band at 280 nm. The terpenoids showed a simple absorption at 235.60 nm. Furthermore, other UV spectra showed  $\lambda_{max}$  values at 200 and 272 nm for a peak at Rt 4.2 min; 202, 268, and 282 nm at Rt 9.1 min; as well as 203 and 278 nm at Rt 12.0 min. These UV spectra possibly represent flavonoids. The typical UV spectra of flavonoids include two absorbance bands. In flavanones and dihydroflavonols, band A lies at 300-330 nm and B at 277-295 nm.

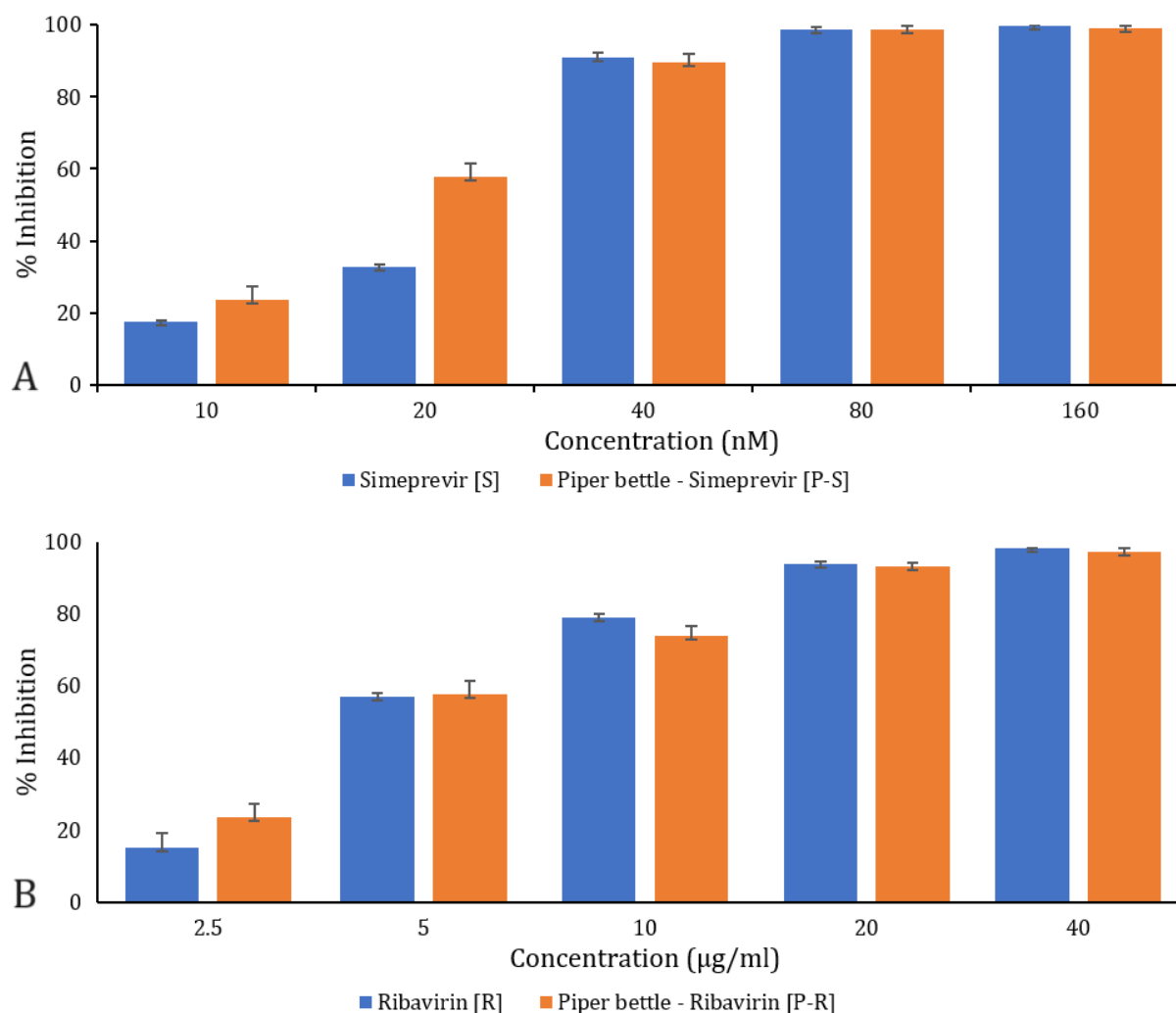


Figure 4. The average % inhibition of single simeprevir and combination with ethanolic extract of *P. betle* leaves (a), the average percentage inhibition of single ribavirin and combination with ethanolic extract of *P. betle* leaves (b).

\*The data were collected in three replications of the experiment, resulting in mean and standard deviation (SD) values.

#### Anti-HCV activity and mode of action (MOA)

The 96% ethanolic extract of *P. betle* was evaluated against HCV in cultured Huh7it-1 cells at several doses (Figure 2). Time addition experiments were performed to determine the inhibition step. The results showed that the ethanol extract had an  $IC_{50}$  value of  $0.08 \pm 0.028 \mu\text{g/mL}$  with inhibition at both entry and post-entry steps. However, based on the data, the most significant inhibition was at the post-entry step with an  $IC_{50}$  value of  $0.10 \pm 0.005 \mu\text{g/mL}$ , while that of the entry step was  $1.61 \pm 0.153 \mu\text{g/mL}$  (Figure 2).

#### Western blotting

The Western blot assay showed a decrease in the NS3 level of protein after treatment with the ethanol extract of *P. betle* compared to the control in the same concentration of cells (Figure 3). These results supported the MOA assay, which found a higher inhibition in the post-entry step. This step includes the process of translation, replication, assembly, and release. The post-entry inhibition was associated with the reduction of NS3 protein level as a crucial component of HCV replication machinery. The NS3 protein has a function to

support viral replication by cleaving the viral polyprotein (NS4A, NS4B, NS5A, and NS5B). The reduction in the NS3 level affected the subsequent processes including viral replication, construction, and release (Beran & Pyle, 2008; Wahyuni *et al.*, 2016).

### Combination treatment experiments

This combination led to an increase in the inhibition of HCV growth compared to the use of a single simeprevir sample. Simeprevir belongs to a class of drugs that block the NS3/4A protein in HCV resulting in the inhibition of polyprotein replication (Figure 4A). Consequently, combining the ethanolic extract of *P. betle* leaves with this drug increased the inhibitory activity against HCV, and reduced the IC<sub>50</sub> value compared to the single use.

Combination of ethanolic extract of *P. betle* leaves with ribavirin was found that extract only enhanced the effect of ribavirin at low concentrations (Figure 4B). In contrast, at higher concentrations, the use of extract did not produce additional effects.

Ribavirin works by inhibiting monophosphate dehydrogenase in HCV, causing mutagenesis in the polyprotein structure and ultimately inhibiting the replication process (Pradat *et al.*, 2014). Therefore, combining the ethanolic extract of *P. betle* leaves with ribavirin may not provide promising prospects at higher concentrations.

Table II. Combination treatment of simeprevir or ribavirin with *P. betle* extract

Sample	IC <sub>50</sub> *
<i>P. betle</i>	0.08 ± 0.03 µg/ml
Ribavirin	5.09 ± 0.29 µg/ml
<i>P. betle</i> + Ribavirin	4.79 ± 0.30 µg/ml
Simeprevir	21.02 ± 0.24 nM
<i>P. betle</i> + Simeprevir	16.9 ± 0.95 nM

\*Data was obtained from 3 independent experiments ± SD.

The results in (Table II) show that the combination treatment of simeprevir or ribavirin with *P. betle* extract did not show any potential effect. The addition of extract with ribavirin yielded similar IC<sub>50</sub> values, while the combination with simeprevir led to increased activity with the IC<sub>50</sub> values from 21.02 nM (alone) to 16.9 nM (combination). This combination provided better potency compared to the use of extract with ribavirin. The activity of *P. betle* observed in this

study was caused by its metabolites. Several compounds have been isolated from this plant, including phytol, acyclic diterpene alcohol, 4-chromanol, hydroxychavicol or allylpyrocatecol, eugenol, carvacrol, and chavibetol. Hydroxychavicol is recognized as an active antifungal and antibacterial. These compounds may also play an important role in anti-HCV activity.

### CONCLUSION

In conclusion, the ethanolic extract of *P. betle* leaves showed significant activity against HCV. Extract dominantly inhibited the post-entry step, decreased the NS3 protease level, and enhanced the anti-HCV effect of simeprevir. These results imply that extract may be a potential candidate for further examination in the development of complementary and alternative anti-HCV drugs.

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### CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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