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ANTI-VIRAL ACTIVITY OF *Phyllanthus niruri* AGAINST HEPATITIS C VIRUS

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ABSTRACT

Hepatitis C virus (HCV) infection is a global problem that causes liver disease and hepatocellular carcinoma. Although the current standard treatment provided a significant improvement on response rate with sustain virology response more than 90%, however, the high cost was remaining limited access to this therapy, resistance emergence and serious side effects which provide the necessities to find the new anti-HCV agents. The current study, we evaluated the ethanol extract of *Phyllanthus niruri* for its anti-HCV activities. Anti-HCV activity was determined by *in vitro* culture cells of Huh 7i. Anti-HCV activity of *P. niruri* extract revealed strong inhibition against HCV with IC_{50} values of 4.14 μ g/mL and yield stronger activity in the entry step of the HCV life cycle. Moreover, the *P. niruri* extract enhanced anti-HCV activity of simeprevir (NS3 protease inhibitor) with increase the activity up to 4-fold compared to a single treatment of simeprevir. Docking analysis was performed to predict the interaction phyllanthin and hypophyllantin, known compounds of *P. niruri* against HCV receptor. Both of phyllanthin and hypophyllantin were mediated a strong interaction with 4GAG, a protein that involved in entry step of HCV. These results suggested that the ethanol extract of *P. niruri* may be good candidates for the development of anti-HCV drugs.

Key words: Hepatitis C virus, antiviral activity, *Phyllanthus niruri*, entry step

INTRODUCTION

Hepatitis C virus (HCV) infection is a serious health problem and a potential cause of substantial morbidity and mortality. The infections could lead to chronic liver diseases, such as hepatitis, liver disorder and hepatocellular carcinoma. The prevalence of HCV infection is infected around 71 million people worldwide (The Polaris Observatory HCV Collaborators; European Union HCV Collaborators). There is no vaccine of HCV that have been

developed. The current treatment of HCV used of IFN-free regimen, includes a combination of direct-acting antiviral agents (DAAs), is capable of providing a sustained virological response (SVR) for more than 90% (Gonzalez-Grande *et al.*, 2016). However, the viral resistance issue, side effects, and economical view due to the high cost of current treatment agents remained the necessities to develop complementary and/or alternative drugs for the treatment of HCV.

Medicinal plants are rich with many chemical substances such as flavonoids, terpenoids, lignans, polyphenolics, coumarins, saponins, furyl com-

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pounds, alkaloids, polyphenols, proteins and peptides, that have been reported to possess various bioactivities including antiviral. Those compounds and their derivatives have been determined to possess anti-HCV activities that have promise for development into anti-HCV agents (Calland *et al.*, 2012; Jassim & Naji 2003; Khachatourian *et al.*, 2012; Wahyuni *et al.*, 2016). Alkaloid and coumarin compounds of *R. angustifolia*, pseudane IX and chalepin, respectively, possess a strong inhibition against HCV (Wahyuni *et al.*, 2014). Honokiol, a lignan compound from *Magnolia officinalis*, reported inhibiting HCV with an IC_{50} value of 4.5 μ M (Lan *et al.*, 2012), while a lignan compound from *Swietenia macrophylla*, 3-hydroxy caruilignan C, reduced HCV protein and RNA level with IC_{50} value of 10.5 μ M (Wu *et al.*, 2012).

Phyllanthus niruri belongs to the family of Euphorbiaceae and is widely distributed in tropical and subtropical regions. It has been traditionally used for many kinds of disease including to reduce fever, treat jaundice and liver diseases. The various species of *Phyllanthus* family such as the *P. amarus*, *P. niruri*, *P. urinaria* and *P. orbicularis* have been reported to demonstrate potential inhibitory effect against a broad spectrum of viruses. They showed to possess potent inhibition in human immunodeficiency virus (HIV), herpes simplex virus (HSV) and hepatitis B virus (HBV) (Forero *et al.*, 2008; Tan *et al.*, 2013). *Phyllanthus amarus* has been demonstrated to have potential inhibition on HCV replication (Ravikumar *et al.*, 2011). Nirtetrahn B, a lignan isomer compounds of *P. niruri* exhibited to suppress the secretion of the HBV antigens (Liu *et al.*, 2014). Other lignan compounds, phyllanthin and hypophyllanthin were reported to mediate anti-viral activities against hepatitis B virus and human immunodeficiency virus (HIV) (Bagalkotkar *et al.*, 2006; Venkateswaran *et al.*, 1987). This study was evaluated the anti-HCV activity of *P. niruri* *in vitro* culture cells and further analyzed the mechanism of action. To predict the mechanism of known compounds, docking analysis of phyllanthin and hypophyllanthin was done to evaluate the interaction with protein in Protein Data Base (www.rcsb.org) that involve in HCV activity.

MATERIALS AND METHODS

Extraction and sample preparation

The herb of *P. niruri* was harvested from Bandung, West Java, Indonesia. The plant was verified by a licensed botanist of Botanical garden, Purwodadi, Indonesia. The dried powder of the herb was pulverized and extracted by maceration method with 96% of ethanol. The obtained filtrates were evaporated to yield the ethanolic extracts of *P. niruri*. The stock solution

was prepared by dissolving the extract in dimethyl sulfoxide (DMSO) to obtain a stock concentration of 100 mg/mL. Serial dilutions of extracts were prepared to yield the concentrations of extracts 100, 50, 10, 1, 0.1 and 0.01 μ g/mL.

Cells and Hepatitis C virus preparation

Huh7it cells (Aoki *et al.*, 2014) were cultivated in Dulbecco's Modified Eagle Medium (GIBCO Invitrogen, Carlsbad, CA, USA) supplemented with 10% Fetal Bovine Serum (Gibco, Grand Island, NY, USA), 0.15 mg/mL Kanamycin (Sigma-Aldrich, St. Louis, MO, USA) and non-essential amino acids (GIBCO-Invitrogen) in 5% CO₂ at 37°C. The culture cells were cultivated and maintained by periodically re-feeding with a new medium. The adapted HCV variant was propagated in Huh7it (Apriyanto *et al.*, 2016). Culture supernatant from the infected cells was collected at day 2 and day 5 post-infection and concentrated using Amicon Ultra centrifugal filter unit. Virus titers were determined for antiviral assay (Hafid *et al.*, 2017).

Anti-Hepatitis C activity assay

Antiviral activity assay was conducted as described previously (Aoki *et al.*, 2014; Hafid *et al.*, 2017; Wahyuni *et al.*, 2013; Wahyuni *et al.*, 2014). Huh7it cells (5.4×10^4) were seeded for 24 hours. The HCV at the multiplication of infection (MOI) of 0.1 in the presence of different concentrations of the sample were inoculated to the culture cells. The cells were treated with a serial concentration of *P. niruri* extract. The mixture of extract and virus was incubated for 2 hours for virus adsorption and the cells were rinsed with the medium. Infected-cells were further incubated in the medium containing the same concentration of sample for 46 hours. Mode of action analysis was performed by time-of-addition experiments with three series of experiments. First, the culture was treated with the extract both in pre- and post- inoculation. Second, the culture was only treated with the extract at inoculation steps (2 hours). The third extract was added only after inoculation to examine the action of a substance in the post-entry steps of the HCV life cycle. Culture supernatants were collected for virus titration. The percentage inhibitory was determined and the 50% inhibitory effect (IC_{50}) was calculated by SPSS probit analysis (Wahyuni *et al.*, 2013; Wahyuni *et al.*, 2014). Combination treatment to evaluate the effect of *P. niruri* extract was done by added the extract to the serial concentration of simeprevir, the NS3 protein inhibitor.

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 on 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% BlockAce/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, 0.1 and 0.01 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 hours. Absorbance sample was evaluated under a 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 (CC₅₀) values (Apriyanto *et al.*, 2016).

Docking analysis of phyllanthin and hypophyllanthin

The ligand was prepared by making 2D and 3D structures of the phyllanthin and hypophyllanthin using ChemBioOffice program Ultra 11.0 and its energy was minimized using MMF94. The docking analysis continued by Molegro Virtual Docking ver 5.5 program Ver 5.5, resulted in rerank score describing the minimal energy by the ligand in interaction with the receptor

RESULTS

Anti-hepatitis C virus activity and cytotoxicity assay of *Phyllanthus niruri* extract

Ethanol extract of *P. niruri* was examined for antiviral activities against hepatitis C virus JFH1. The result showed that *P. niruri* possesses potential inhibition against hepatitis C virus without any cytotoxicity. Dose dependent on percentage inhibition and cell viability of *P. niruri* was shown in Table 1. The 50% inhibitory concentrations (IC₅₀) was determined by probit analysis and yield the IC₅₀ value 4.14 µg/mL without any cytotoxic effect with the 50% cytotoxic concentrations (CC₅₀) >100 µg/ml and selectivity indexes (SI: CC₅₀/IC₅₀) > 24.2

Mode of Action analysis of *Phyllanthus niruri* extract

To evaluate that actions of extract in the HCV life cycle, mode of action analysis of *P. niruri* extract was performed. The three parallel experiments were exerted, first the cells culture were treated with the plant extract in both pre- and post-inoculation, second cells were treated with extract only during inoculation to determine the action of extract in the entry step and third, cells were treated only post-inoculation to evaluate the action of extract in the post entry step. Pre-inoculation treatment obtained higher inhibition than post-inoculation treatment. It indicated that the extract of *P. niruri* mainly inhibits at the entry step. The percentage of HCV inhibition in the entry step was 70% while post-entry step was less than 50% (Figure 1) This result indicated that the mechanism of action of *P. niruri* was predicted in the attachment with some host receptor of entry to the host cell via endocytosis process.

Table 1. Dose dependent on percentage inhibition and cell viability of *Phyllanthus niruri*

Concentration (µg/mL)	% HCV inhibition	% Cell viability
0.01	13.3 ± 4.7*	99.8 ± 0.4
0.1	22.5 ± 4.1	99.0 ± 0.9
1	25.5 ± 5.8	98.8 ± 1.2
10	44.3 ± 3.3	98.7 ± 1.2
50	88.0 ± 5.1	98.7 ± 1.3
100	100.0 ± 0.0	98.3 ± 1.5

* Data represent means ± SEM of data from three independent experiments

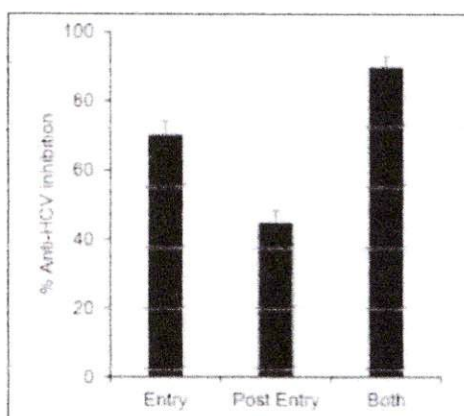


Fig. 1. Extract of *Phyllanthus niruri* (30 µg/ml) inhibits HCV mainly in the entry step. Three kinds of experiments were done in parallel. Entry: infected-culture cells were treated with extract only in pre-inoculation. Post entry: infected-culture cells were treated only post-inoculation, both: infected-culture cells were treated with extract in the pre-and post-inoculation. The percentage HCV inhibition, both: 88%, entry: 70%±4.3%. Data represent means ± SEM of data from three independent experiments.

Combination treatment of *Phyllanthus niruri* extract and simeprevir (NS3 protease inhibitor)

Simeprevir is an antiviral drug that acts to inhibit NS3 protein. Combination treatment by adding 25 $\mu\text{g/mL}$ of *P. niruri* extract was obtained increasing HCV inhibition (Figure 2). The potency of combination treatment was increased in 4-fold with the IC_{50} value $3.54 \text{ nM} \pm 0.05$, while the IC_{50} value of simeprevir single treatment was $11.23 \text{ nM} \pm 0.36$. *P. niruri* extract showed to reduce HCV NS3 protein level under western blot analysis (Figure 3).

Docking analysis of known compounds from *Phyllanthus niruri*

Phyllanthin and hypophyllanthin are known as isolated compounds from *P. niruri*. To predict the interaction of those compounds docking analysis was performed. Several proteins from Protein Data Base (www.rcsb.org) were evaluated to examine their interaction with Phyllanthin and hypophyllanthin. The result demonstrated a strong interaction between phyllanthin and hypophyllanthin with 4GAG. The rerank score value of phyllanthin and hypophyllanthin were -110.125 and -88.5645 kcal/mol, respectively, while the rerank score of standard ligands was -45.0229 kcal/mol. This result indicated the strong interaction between phyllanthin and hypophyllanthin to the 4GAG receptor. The receptor of 4GAG was reported to be a protein that involves in the entry step of HCV, neutralizing antibody AP33 in complex with E2 epitope (Colpitts and Baumert 2016; Zhu *et al.*, 2014).

The hydrogen bonding and steric van der Waals interaction were involved in the interaction between compounds to the receptor. Hydrogen bonding of phyllanthin with His 164 and Gln 42, and the steric van der Waals between phyllanthin with, Asp 167, Val163, His 164, Gln42, Lys39, Pro40 and Gly41 contributed the binding interaction of 4GAG and phyllanthin (Figure 4A). Hypophyllanthin revealed weaker interaction than phyllanthin with hydrogen bonding to His 164 and steric van der Waals-bond to Asp167, Val163, His164 and Thr165 (Figure 4B). While the standard ligand revealed hydrogen bonding to His 164 and Asp 167 and steric van der Waals to Asp 167. The interaction of phyllanthin and 4GAG was clearly described in the 3D profile (Figure 5).

DISCUSSION

Medicinal plants and many natural sources are rich with chemical substances that responsible in many bioactivities including antiviral. We previously summarized potential plants that have anti-HCV activity (Wahyuni *et al.*, 2016). Our current study reported anti-HCV activities of *P. niruri* against

HCV JFH1. Plant with the genus of *Phyllanthus* was reported to have many bioactivities such as antimicrobial, antioxidant, anticancer, anti-inflammatory, anti-plasmodial, hepatoprotective and antiviral (Calixto *et al.*, 1998; Kaur *et al.*, 2017). This study found that ethanolic extract of *P. niruri* inhibited HCV with IC_{50} value of $4.14 \mu\text{g/mL}$ without showing cytotoxicity. The extract of *P. niruri* may interfere with the inhibition effect in the several steps of the HCV life cycle. Further study to examine the effect of *P. niruri* extract in the entry or post-entry step of the HCV life cycle was evaluated by mode-of-action analysis. The result was described that *P. niruri* possesses a dominant inhibition in the entry steps of the HCV life cycle (Figure 1). The steps in HCV life cycle include entry into the host cells, uncoating and replication of viral genome, translation of virus proteins, and assembly and release of the virion (Lindenbach *et al.*, 2005; Ploss & Dubuisson 2012). In the attachment/entry step, the HCV lipoviral

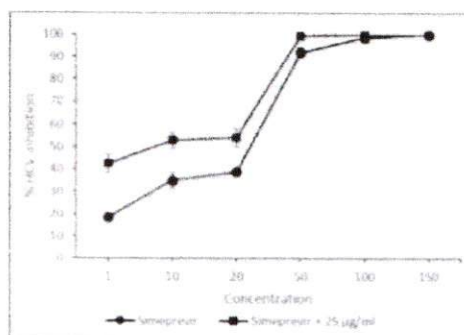


Fig. 2. Extract of *Phyllanthus niruri* (25 $\mu\text{g/mL}$) enhanced anti-hepatitis C activity of simeprevir. In parallel, culture cells were treated with a serial concentration of simeprevir only and the other was treated with a combination of simeprevir and extract. Data represent means \pm SEM of data from three independent experiments.

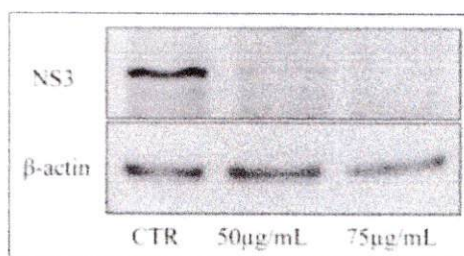


Fig. 3. Reduction of HCV NS3 protein level by *Phyllanthus niruri* extract. Huh 7.5it cells were infected with HCV and treated with ethanolic extract of *Phyllanthus niruri* (50 and 75 $\mu\text{g/mL}$) and the untreated control (CTR) were subjected to western blot analysis. β -actin served as an internal control to verify equal amounts of sample loading.

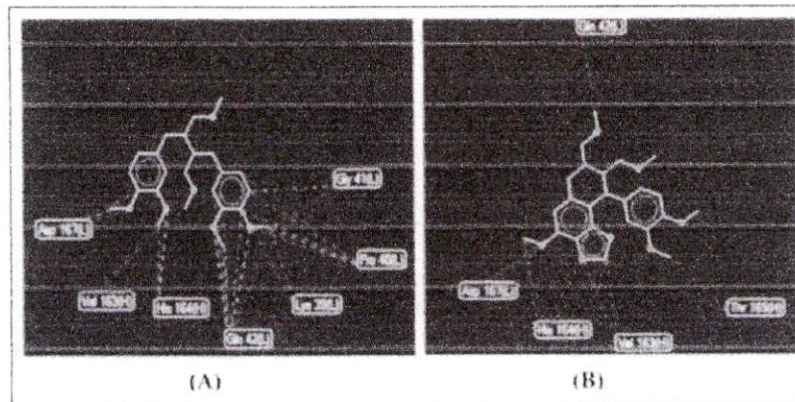


Fig. 4. Hydrogen bond interaction (dashed blue-line) and Steric-Van der Waals bond interaction (dashed red-line) between Standard Ligand and A) phyllanthin, B) hypophyllanthin, on the active site of HCV protein (4GAG.pdb).

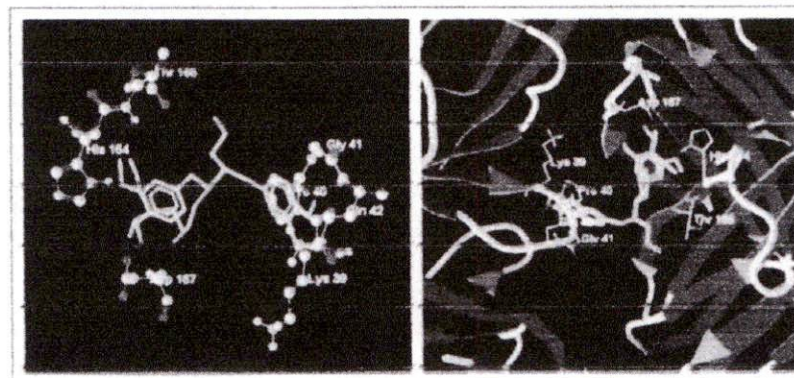


Fig. 5. The 3D profile of docking interaction of phyllanthin (green color) with 4GAG protein.

particles attach to the cell surface and interact with glycosaminoglycans (GAG), low-density lipoprotein receptor (LDLR), scavenger receptor class B type 1 (SCARB1) and CD81. Then, the viral interaction with CLDN1 results in internalization of the virus via clathrin-mediated endocytosis. Following acidification of the endosome and subsequent fusion of viral and endosomal membranes, the viral genome is released into the cytoplasm. On the other hand, the lateral movement of HCV-CD81 causes the transmission of the virus by cell-to-cell contact (Lindenbach & Rice, 2013; Zeisel *et al.*, 2015).

Combination treatment was done by adding the extract of *P. niruri* to the serial concentrations of simeprevir, an NS3 protein inhibitor which acts in the replication step of HCV. The addition of *P. niruri* extract increased anti-HCV activity of simeprevir in almost 4-fold with the IC_{50} value 3.54

$nM \pm 0.05$, while the IC_{50} value of simeprevir single treatment was $11.23 nM \pm 0.36$. These results indicated that *P. niruri* extract enhanced the anti-HCV activity of simeprevir. *P. niruri* was demonstrated to possess a reduction effect on HCV NS3 protease level at the dose of $50 \mu g/mL$ of extract (Figure 24) which indicated that *P. niruri* may also involve in the replication step of HCV life cycle.

The various metabolites in the plants do contribute to anti-HCV activities such as lignan, terpenoids, flavonoids, alkaloid, tannin and steroid (Geethangili & Ding 2018; Kaur *et al.*, 2017). Niranthin and hinokinin isolated compounds from *P. amarus* have strong anti-hepatitis B activities while coriandrin was reported to block HCV NS3 protease and NS5B RNA-dependent-RNA-polymerase (Huang *et al.*, 2003; Reddy *et al.*, 2018). *P. niruri* possessed various compounds, such as quercetin, niruriflavone, rutin, ellagic acid,

phyllanthin, hypophyllanthin, niranthin, lintetralin, phylltetralin, etc. (Bagalkotkar *et al.*, 2006). Phyllanthin and hypophyllanthin are the lignan compounds that were reported as major compounds of *P. niruri* (Calixto *et al.*, 1998). To predict the mechanism-of-action of phyllanthin and hypophyllanthin to the HCV receptor, docking analysis was done by Molegro Virtual Docking ver 5.5 program. Phyllanthin and hypophyllanthin possess smaller re-rank value to compare to the ligand, it indicated the stronger interaction to 4GAG receptor, a protein that involves in the entry step of HCV, neutralizing antibody AP33 in complex with E2 epitope (Colpitts & Baumert 2016). The interaction of phyllanthin and hypophyllanthin to the receptor 4GAG resulting in an entry inhibition of HCV to the hepatocyte cells. The interaction of phyllanthin more stable than hypophyllanthin. Thus, it predicted to have higher biological activity.

CONCLUSION

These results obtained strong activity of *P. niruri* extract against the hepatitis C virus that showed stronger inhibition in the entry step and enhances the activity of simeprevir. Phyllanthin and hypophyllanthin, known compounds of *P. niruri* predicted to possess strong interaction with 4GAG, a protein receptor that involves in the entry step of HCV. These results suggest that *P. niruri* would be a good candidate to develop anti-hepatitis C agents.

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