ENHANCEMENT OF ANTI-HEPATITIS C VIRUS ACTIVITY BY THE COMBINATION OF CHALEPIN FROM *RUTA ANGUSTIFOLIA* AND CURRENT ANTIVIRAL DRUGS

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Abstract. Hepatitis C virus (HCV) infection is a serious disease, which chronically infects 71 million people worldwide. Currently oral interferon (IFN)-free regimen usage involving a combination of direct-acting antiviral agents (DAAs) is capable of providing a sustained virologic response (SVR) of >90%. However, a number of DAA-resistant HCV strains have emerged and many patients do not have access to this therapy owing to its high cost. Combination drug therapy is one strategy for lowering cost and improving effectiveness of antiviral therapy. Chalepin from *Ruta angustifolia* is known to exhibit strong anti-hepatitis C activity. Anti-HCV efficacies of combinations of chalepin and current antiviral drugs, namely, cyclosporine A (CsA), daclatasvir (DCV). IFN-α, ribavirin (RBV), simeprevir (SMV), and telaprevir (TVR) were measured by treating HCV-infected cells *in vitro*. Chalepin enhanced anti-HCV activities of CsA, DCV, IFN-α, RBV, SMV, and TVR with a synergistic combination index of <1. The results suggest drug combinations that include chalepin should be considered when developing alternative and complementary medicine as anti-HCV agents.

Keywords: *Ruta angustifolia*, chalepin, combination drug treatment, direct-acting antiviral, Hepatitis C virus

INTRODUCTION

Hepatitis C virus (HCV) infection is a global public health concern. This viral infection has a potential to develop into a chronic stage having a high risk of causing liver damage and hepatocellular carcinoma subsequent to 25-30 years of infection (Zaltron *et al*, 2012). HCV infects some 185 million people worldwide, almost 3% of the global population, and approximately 71 million being chronically infected (Jardim *et al*, 2018).

There is currently no effective vaccine for prevention of HCV infection; however, drugs are available for treating infection

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(Abdelwahab and Ahmed Said, 2016). For instance, a combination of pegylated interferon-α (IFN-α) and ribavirin (RBV) provides a sustained virologic response (SVR) in 50% of patients (Alexopoulou and Karayiannis, 2015). The most currently used treatment employs direct-acting antivirals (DAAs) targeting HCV proteins, such as NS3 protease, NS5A protein and the NS5B RNA polymerase (Geddawy *et al*, 2017). DAAs dramatically boost SVRs, especially in patients infected with HCV genotype 1, however, high cost of the antivirals and potential of drug resistance make imperative development of new anti-HCV agents and therapy combinations to improve the efficacy of HCV treatment (EASL, 2018).

The goal of effective HCV treatment is to achieve undetectable blood HCV RNA within 12 weeks of treatment, *ie* SVR = 12 (SVR12) or within 24 weeks of treatment (SVR24) (EASL, 2018). Anti-HCV agents can be divided into two classes, namely, DAAs that directly target viral NS3 protease, NS5B polymerase or NS5A protein and host-targeting antivirals (HTAs), such as cyclophilin inhibitors (Dubuisson and Cosset, 2014; Gonzalez-Grande *et al*, 2016). The strategy of most HCV treatments is to apply a combination of several anti-HCV agents to maximize the drugs' effectiveness against HCV. The current standard therapy against HCV infection is IFN-α together with other alternative IFN-free treatment regimens that employ a combination of two or three types of DAAs; however, IFN-α plus RBV is still the most preferred treatment against HCV in some parts of the world (Gupta *et al*, 2017).

Medicinal plants possess a variety of secondary metabolites, which can increase bioactivity of certain useful drugs, including antivirals (Calland *et al*, 2012). A number of medicinal plants possess both crude extracts and isolated compounds that can inhibit HCV (Bose *et al*, 2017). Medicinal plants, *Ficus fistulla*, *Melanoleptis mutiglandulosa, Melicope latifolia,* and *Toona sureni,* all native to Indonesia, possess strong inhibitory effects against HCV infection (Wahyuni *et al*, 2013). Compounds isolated from these plants inhibit various stages of HCV life cycle (Wahyuni *et al*, 2013). For example, flavonoid compounds, such as catechin, naringenin and quercetin reduces NS3 and NS5A protein levels in HCV-infected patients (Ciesek *et al*, 2011, Khachatoorian *et al*, 2012). Circumdatin G (an alkaloid) from *Aspergillus ochraceus* was found active as antiHCV infection (Dai *et al*, 2001). Catechin gallate, curcumin, cynaropricin, delphinidin, ethyl gallate, cynaropricin, and saikosaponin b2 inhibit HCV in its initial stage of infection; embelin, epigenin, excoecariphenol, geraniin, mangostin, myrifabral A, oleonic acid, and rographolide block HCV in its replication phase; and glyzhirizin inhibits HCV during the assembly process (Calland *et al*, 2015, Wahyuni *et al*, 2016, Elsebai *et al*, 2015, Ciesek *et al*, 2011).

Chalepin, a coumarin extracted from *Ruta angustifolia* and a number of compounds isolated from other plants, (arborinine, chalepin, γ-fagarine, kokusagenin, and pseudane IX) exhibit anti-HCV activity. In particular, chalepin decreases levels of NS3 protease and HCV RNA in cell culture (Wahyuni *et al*, 2014).

Combination of several natural compounds have been tested for their efficacy together with existing antiviral drugs, and a combination of anti-HCV compounds often show a greater reduction in HCV RNA level than if each agent is used alone (Wahyuni *et al*, 2016, Lin *et al*, 2006). Extracts of *Phyllanthus amarus* leaves

used in combination with IFN-α exhibit synergistic effect against HCV in Rep2a cells (Ravikumar *et al*, 2011). Likewise, delphenidin, a polyphenol, improves the effectiveness of both boceprevir and IFN-α, in that a 5 μM supplement of delphenidin elicits a 5-fold decline in IC_{50} value of boceprevir and a 10-fold decline in IC_{50} value of IFN-α (Calland *et al*, 2015). Similarly, curcumin (the major compound in turmeric) enhances inhibitory effects of boceprevir (NS3 protease inhibitor), cyclosporine A CsA and Peg-IFN-α (Anggakusuma *et al*, 2014).

Hence, the anti-HCV effects of chalepin combined with commonly used DAAs daclatasvir (DCV), simeprevir (SMV) and telaprevir (TVR), cyclosporine A (CsA), IFN-α, and RBV. The findings should indicate the potential of developing a combination agents containing natural compounds and currently used anti-HCV agents.

MATERIALS AND METHODS

Cell and virus preparation

Human hepatocyte Huh7it cells (derived from Dr Chie Aoki, Kobe University, Japan) were cultivated in Dulbecco's modified Eagle's medium (DMEM) (Wako Chemicals, Japan) supplemented with 10 % fetal bovine serum (Biowest USA, MO), non-essential amino acids (Invitrogen, CA), 100 IU/ml penicillin, and 100 μg/ml streptomycin (Invitrogen) at 37°C under a humidified atmosphere containing 5% CO_2. HCV (JFH1 strain) (provided by Dr Chie Aoki, Kobe University, Japan) was propagated in hepatocyte cells as previously described (Wahyuni *et al*, 2013, Wahyuni *et al*, 2014), inoculated in Huh7it cells at 0.1 multiplication of infection (MOI) and cultured for two days as described above. Then supernatant was collected and viral titer determined (Wahyuni *et al*, 2013, Wahyuni *et al*, 2014).

Isolation of chalepin from *R. angustifolia* leaves

R. angustifolia leaves were obtained from Lembang, West Java, Indonesia and identified by an expert botanist at Purwodadi Botanical Garden, Indonesia and a dried specimen was deposited at the Faculty of Pharmacy, Airlangga University, Indonesia. *R. angustifolia* leaves (5.0 kg) was extracted with 10 L of absolute ethanol and extract dried by evaporation. Chalepin was isolated as previously described (Wahyuni *et al*, 2014). In brief, dried extract was pulverized and sequentially extracted with n-hexane, dichloromethane and methanol, followed by open column chromatography [100% methanol solvent system, a GS-320 and GS-310 (21.5 mm ID × 1000 mm) column, 5.0 ml/min flow rate, and detection at 210 nm], then high-performance liquid chromatography [Waters XBridge C18 column (250 mm in length)], a solvent system gradient of 0.1% trifluoroacetic acid and acetonitrile, a 2.5 ml/min flow rate, and 30°C column temperature. Chemical structure of chalepin (Fig 1) was determined using *nuclear magnetic resonance* spectroscopy (Bruker Ascend, IL) and liquid chromatography/mass spectrometry (Shimadzu, Japan). Chalepin [10 mg/ml dimethyl sulfoxide (DMSO)] was stored at -20 ºC until used (Wahyuni *et al*, 2014).

Anti-HCV activity assay

Huh7it cells were seeded in 24-well plate at a density of $1.9x10^5$ cells/well, inoculated with HCV (at 0.5 MOI) and incubated as described above for two hours. Supernatant was removed and HCV titer determined as described above.

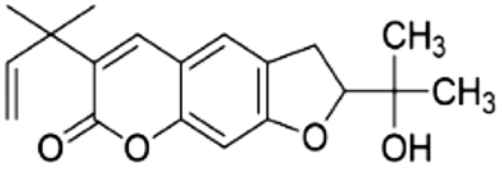

Fig 1-Molecular structure of chalepin.

Then inoculated cells were washed with medium and incubated for a further two hours in medium containing a serial dilution of test antiviral drug prior to determination of virus titer in supernatant. Negative control cells were treated with medium containing 0.1% DMSO. IC_{50} (50% inhibitory concentration) value of virus infectivity for each antiviral drug was calculated using SPSS probit analysis (Wahyuni *et al*, 2014). The antiviral drugs tested were CsA (WAKO Pure Chemical, Japan), DCV (Adooq Bioscience, Irvine, CA), IFN-α (Sigma Aldrich, MO), RBV (Sigma Aldrich, MO), SMV (Toronto Research Chemical, Canada), and TVR (Adooq Bioscience, Irvine, CA). Each experiment was performed in triplicate and IC value reported as mean ± SD.

In order to observe the effects of chalepin-antiviral combinations on HCV infectivity, two additional series of anti-HCV activity experiments were conducted employing chalepin alone and a mixture of chalepin and each anti-HCV drug (at various molar ratios of chalepin : drug. IC_{50} values were measured as described above. The effect of chalepin-anti-HCV drug combination compared to that of each compound alone on HCV infectivity is presented as a combination index (CI). The value of CI was calculated based on the median-effect that demonstrated the relationship between dose and the effect and determined by CompuSyn software (ComboSyn Inc) (Chou, 2006). A CI value of 1.0 was considered as an additive effect, CI <1.0 was considered as synergistic and CI >1.0 was considered to be antagonistic.

RESULTS

HCV-infected Huh7, it cells were treated with varying concentrations of known anti-HCV drugs (CsA, DAAs (DCV, SMV and TVR), IFN-α, and RBV) and chalepin isolated from *R. angustifolia* leaves, and IC_{50} values were determined using probit analysis (Table 1). Chalepin showed to possess a strong inhibition on HCV with IC_{50} value of 5.40 ± 0.5 μM which was stronger as compared to ribavirin.

In order to evaluate the effect of chalepin when combined with each of the six anti-HCV compounds on HCV infectivity. The combination treatment was done by treated of HCV-infected Huh7it with ¼x, ½x, 1x, and 2x of each IC_{50} of drugs. CI value was calculated by CompuSyn software analysis (Chou, 2006). Each combination exhibited synergism in anti-HCV activity as evidenced by CI values <1 (Fig 2). Combination of chalepin

Table 1
Fifty percent inhibition concentrations (IC_{50}) of known anti-hepatitis C virus drugs and chalepin isolated from *Ruta angustifolia* leaves.

Compound	IC_{50} (mean ± SD)
Chalepin	5.40 ± 0.5 μM
Cyclosporine A	0.30 ± 0.02 μM
Daclatasvir	0.35 ± 0.22 nM
Interferon-α (IU/ml)	1.10 ± 0.07 IU
Ribavarin	9.90 ± 0.19 μM
Simeprevir	37.5 ± 0.80 nM
Telaprevir	9.54 ± 0,24 nM

and interferon mediated a stronger synergistic effect as compared to the ribavirin and cyclosporin A. Combination with NS3 inhibitor (simeprevir and telaprevir) gave stronger effect than daclatasvir, an NS5A/B inhibitor.

DISCUSSION

Medicinal plants possess many chemical metabolites potentially providing important pharmacological properties and clinical applications. The discovery of natural products has been important in the development of new drugs and a number have reported to possess antiviral activity, including protection against HCV infection (Wahyuni, 2016).

The purpose of HCV therapy is to eradicate the virus in a patient, success of which can be indicated by its SVR. Combination drug treatment is one strategy used against HCV; however, combination drug treatment with IFN-α and RBV, standard drug regimen in treating most HCV cases, is only able to achieve a 50% SVR after 24 weeks and this treatment has a number of serious side effects (Witthoft *et al*, 2007). Currently, an IFN-free regimen consisting of combining two or three DAAs and inhibitors of NS3/4A protease, NS5A and NS5B polymerase is recommended as this treatment achieves better success (SVR >90%) than using IFN alone (Pawlotsky *et al*, 2015); however, viral resistance and potential undesirable side effects are still observed. In addition, synthetic drugs are expensive, making them inaccessible to patients with limited income. Differential responses to various HCV genotypes by these treatments underscores the need to

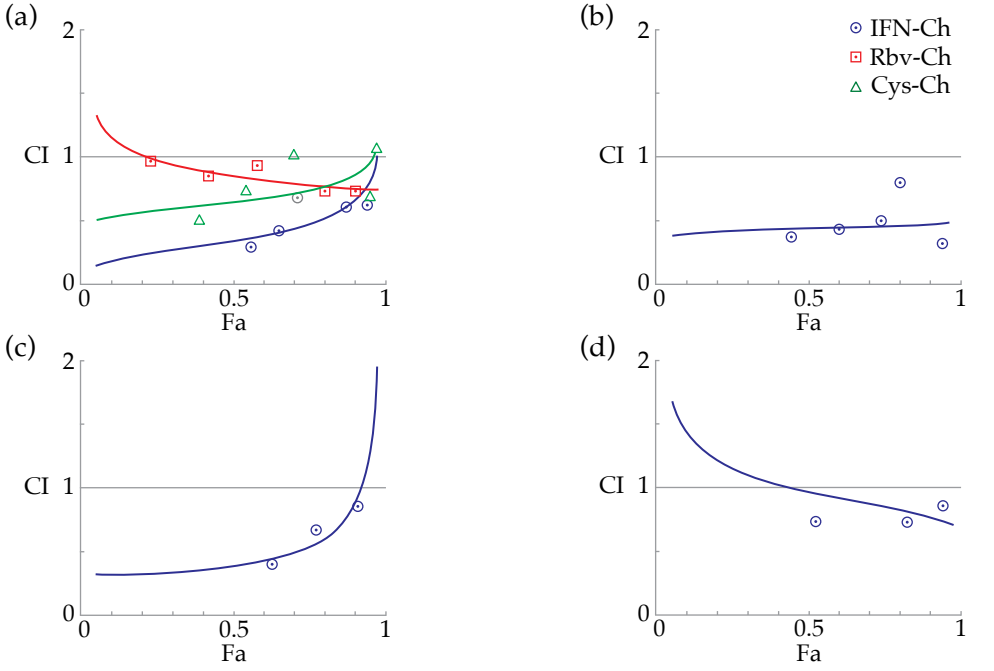

Fig 2-Combination index (CI) values of chalepin-anti-hepatitis C virus (HCV) drug mixture (a) IFN, interferon-α; CSA, cyclosporine A; RBV, ribavarin; (b) SMV, simeprevir; (c) TVR, telaprevir; (d) DCV, daclatasvir.

find new and less expensive alternatives for treatment of HCV (Gonzalez-Grande *et al*, 2016, Kish *et al*, 2017).

In our *in vitro* anti-HCV chalepin-drug combination tests, a synergistic effect was observed whether chalepin was combined with DDAs or non-DDAs. This is of interest given the different mechanisms by which these six anti-HCV drugs. IFNs bind a ubiquitous heterodimeric receptor, activating JAK-STAT signaling pathway and inducing expression of interferon stimulating gene, which limited the spread of infection due to the enhanced immune response (Huang *et al*, 2014). CsA inhibits HCV replication by enhancing expression of intracellular IFN-α (Liu *et al*, 2011), and also binds to cyclophilin, a protein that bind to CsA. Cyclophilins catalyzes *cis-trans* formation of peptide bonds which play an important role on interaction to the protein receptor (Nakagawa et al, 2005). Ribavirin, a guanosine analog, inhibits inosine monophosphate dehydrogenase resulting in an induction of mutagenesis and immunomodulation (Te *et al*, 2007). Simeprevir and teleprevir inhibit HCV NS3/4A proteases which are responsible for cleaving polyproteins allowing virus to release and generate a mature protein (Gonzalez-Grande *et al*, 2016, Verbinnen *et al*, 2015). On the other hand, daclatasvir is an inhibitor of NS5B, non-structural protein involved in HCV replication (Hessel *et al*, 2016).

Chalepin is a furanocoumarin and certain coumarin analogs were reported to induce IFNs levels and act against HCV NS5B protein (Hassan *et al*, 2016). Chalepin has been shown to reduce HCV RNA and NS3 protein expression (Wahyuni *et al*, 2014). These results reveal a potential prospective to develop chalepin as an alternative partner drug for anti-HCV combination therapy with the possibility to replace or reduce the number of the more expensive synthetic compounds used in current anti-HCV drug treatment.

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