

The Alkaloid Fraction from *Melicope latifolia* Leaves Inhibits Hepatitis C Virus

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ABSTRACT

Introduction: Hepatitis C Virus (HCV) is a major health problem, which infects approximately 170 million people among worldwide population. Moreover, there is no vaccine available to prevent HCV infection and the current anti-HCV drugs have not covered all the various genotypes and subtypes. Meanwhile, medicinal plants have been widely used to treat a variety of infectious disease. Our previous study reported that ethanol extract of *Melicope latifolia* has been shown to exert anti-HCV activity towards a number of different virus genotypes with mainly inhibition mechanism at the entry step. Further separation was needed to purify and identify the active anti-HCV constituent using bioactivity-guided isolation method. **Materials and Methods:** In vitro Anti-HCV assay was performed using hepatocyte cell line (Huh7it) and HCV genotype 2a (JFH1). The purification of *M. latifolia* ethanol extract (B1F) was done by liquid-liquid fractionation, vacuum liquid chromatography (VLC), and high-performance liquid chromatography (HPLC). The active fraction was further identified by thin layer chromatography (TLC) and the major constituent was determined by nuclear magnetic resonance (NMR) spectra data analysis. **Results:** The fractionation of *M. latifolia* leaves ethanol extract resulted an alkaloid fraction (B1F D2H.3) containing a major constituent *N*-methylflindersine. This alkaloid fraction was active to reduce HCV JFH1 with an inhibition concentration (IC₅₀) value of 6.21 µg/mL, a cytotoxicity concentration (CC₅₀) value of 82.64 µg/mL, and a selectivity index value of 13.31. **Conclusion:** An alkaloid fraction of *M. latifolia* (B1F D2H.3) was known to have major compound named *N*-methylflindersine. This alkaloid fraction exhibited strong anti-HCV against JFH1 in vitro. The results indicated that this alkaloid fraction may a good candidate for anti-HCV agent.

Key words: Anti-HCV, *Melicope latifolia*, *N*-methylflindersine.

INTRODUCTION

Hepatitis C Virus is one of the major health problems in the world that causes millions of people to die annually. According to WHO, it was estimated that the number of deaths from hepatitis C is 333,000 in 1990, 499,000 in 2010 and 704,000 in 2013. The increasing number of deaths illustrates the high incidence rates of hepatitis C virus over the decades.¹

Hepatitis C Virus is an RNA virus that has a diameter of 50 nm and a length of 9.6 kb. Hepatitis C virus is classified in the Hepacivirus genus and Flaviviridae family. This virus has varied genotypes (1-7) and more than 70 subtypes (1a, 1b, 2a, 2b etc).^{2,3} The viral genome encodes polyprotein precursor consisting of about 3000 amino acid residues, which is cleaved by the host and viral protease to generate 10 mature protein, structural proteins namely core E1, E2, a putative ion channel p7 and nonstructural proteins NS2, NS3, NS4, NS4B, NS5A, and NS5B.²

The recommended HCV therapy is direct-acting antiviral drugs (DAAs). Several inhibitors of viral nonstructural protein successfully improved sustained virology response (SVR) but the resistant factor, expensive price, and limited access to

the treatment need to be considered as important problems.^{4,5} In order to circumvent those problems, the development of new antiviral drugs is very much required.

Some plants have been reported to have hepatitis C antiviral activity. Plant extracts from *Toona sureni*,⁶ *Artocarpus heterophyllus*,⁷ *Ficus fistulosa*,⁸ *Alectron serratus*,⁹ and *Melicope latifolia*⁴ significantly inhibited HCV activity in vitro. A variety of active phytochemicals such as flavonoids, terpenoids, lignins, sulphides, polyphenolics, coumarins, saponins, furyl, alkaloids, polylines, thiophenes, protein, and peptides have been identified to inhibit various viruses.¹⁰

Melicope latifolia belongs to the Rutaceae family, commonly known in Indonesia as Ki Sampang and is traditionally used to cure fever and abdominal cramps. The tree typically has a height of about 20 m and is commonly found in primary and secondary open forests. *M. latifolia* plants are distributed in Malaysia, Philippines, Java-Indonesia, Kalimantan-Indonesia and Papua New Guinea.¹¹ The chemical compounds which were contained in the *M. latifolia* plant, are flavonoids, alkaloid, coumarins and terpenoids.^{10,12} In general, plant species in the Rutaceae family contain the chemical constituent of alkaloid group, including the *Melicope* genus.

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For instance, *M. ptelefolia* leaves were reported to contain the alkaloid compounds of *N*-methylflindersine and two new alkaloids named melicobisquinolinone A and B.¹³

Our previous study showed that 80% ethanol extract of *M. latifolia* leaves exhibited potent activity as anti-HCV with IC_{50} of 3.5 ± 1.4 μ g/mL against J6/JFH1 virus by inhibiting hepatitis C virus mainly at the entry step. Moreover, we also reported that *M. latifolia* inhibited various genotype of HCV with a percentage inhibition of higher than 87% against all genotypes i.e 1a,1b, 2a, 2b, 3a, 4a, 5a, 6a, 7a.¹⁰ However, further studies to identify the active fraction which are responsible for anti-HCV activities have not been conducted yet. Therefore, this study was conducted to identify active compound from *M. latifolia* and analyzed their anti-HCV activity and cytotoxicity.

MATERIALS AND METHODS

General

NMR spectra were recorded on a JEOL ECS-400, using $CdCl_2$ as the solvent. The HPLC system also includes two LC-10AD pumps and a SCL-10A controller. An Agilent RP-18 XDB column 4.6 x 250 mm was eluted with CH_3CN-H_2O (7:3 v/v) at 2 mL/minute of flow rate. Vacuum Liquid Chromatography (VLC) on silica gel GF_{254} (Merck, Cat No. 1.07730.0500) and Thin Layer Chromatography (TLC) was carried out on silica gel 60 F_{254} (Merck, Cat No. 1.05715.0001) and RP-18 silica gel plate (Merck, Cat No. 1.15389.0001). The identification of TLC profile was performed using TLC Visualizer (Camag).

Plants material

M. latifolia leaves were collected from Salak Mountain (900 m.a.s.l.), West Java, Indonesia. The species was determined and identified by Purwodadi National Botanical Garden-Indonesia Institute of Science, Malang, East Java (No. of determination: 0340/IPH.06/HM/III/2017).

Extraction, fractionation, and identification of major compound

M. latifolia leaves were dried at room temperature then extracted using 80% ethanol by ultrasonic assisted extraction for two minutes at three times of replications. The *M. latifolia* ethanol extract (B1F) was further fractionated by liquid-liquid fractionation using dichloromethane-water to obtain dichloromethane fraction (B1F D). Further separation of B1F D by vacuum liquid chromatography using a gradient solvent of chloroform-methanol (100%-97%) resulted in 9 subfractions (B1F D1-D9). The anti-HCV active subfraction (B1F D2) was then separated by semi preparative HPLC using acetonitrile : water (7-3 v/v), 2 mL/min of flow rate and resulted 5 subfractions (B1F D2H.1-B1F D2H.5). The active subfraction profile was analyzed using silica gel 60 F_{254} plate, scanned by TLC Visualizer on UV 254 nm and 366 nm, and sprayed with dragendorf, a specific reagent for alkaloid compound. The chemical structure of active subfraction was identified using NMR-JEOL ECS-400 and the spectra were then analyzed using MNova program.

Anti-HCV activity and cytotoxicity assay

Cell culture preparation

Huh7it cells were cultivated in DMEM (Dulbecco's Modified Eagle Medium) (GIBCO Invitrogen) and supplemented with 10% Fetal Bovine Serum (FBS), 1x Non-Essential Amino Acids (NEAA, GIBCO Invitrogen) 5 mL, and 0.15 mg/mL Kanamycin solution (SIGMA) in 5% CO_2 at 37 °C. The culture condition of Huh7it cells was observed under a microscope every day. The passage was performed while cells confluent >80%.⁷

HCV propagation

HCV genotype 2a (JFH1) propagation was performed on hepatocyte Huh7it cells (1.8×10^7 cells). Infected cells were incubated at 37 °C in 5% CO_2 for 4 hours with agitation every 30 minutes. HCV supernatants were harvested on day 3 and were concentrated through an Amicon Ultra-15 centrifugal filter (Millipore) by centrifugation at 3500 rpm, 15 min, 4 °C. DAB staining was used to visualize infected cells for virus titration.⁷

Cytotoxicity assay

The cytotoxicity analysis of the sample was assessed by MTT assay. Huh7it cells in 96 well plates were treated with serial dilution of the sample or control. The condition of the cells was observed after 48 hours incubation and the toxicity was checked under microscope. The medium was removed from 96 well plates and then MTT 10% 150 μ l/well was put by multichannel pipette and incubated for 4 hours at 37 °C. MTT solution was removed from 96 well plates and 1 DMSO 100% was added to dissolve formazan. The absorbance of sample was measured at 560 nm and 750 nm. The percentage of inhibition was calculated by comparing the absorbance sample with control. Probit analysis was conducted to calculate the CC_{50} .⁷

Analysis of anti-HCV activities

Extract and fraction of *M. Latifolia* were dissolved in dimethyl sulfoxide (DMSO) to obtain a stock solution at the concentration of 100 mg/mL. The stock solution was stored at -20°C until it was used. Huh7it cells were plated in 48-well plates (5×10^4 cells/well). A fixed amount of JFH1, with multiplication infection (MOI) of 0.1 was infected onto Huh7it cell then treated with the presence of extract and fraction of *M. latifolia*. The virus infected cells were stained with DAB thermo staining and calculated under microscope.⁷

Data analysis

The 50% growth inhibition (IC_{50}) and cytotoxicity (CC_{50}) was determined using an SPSS probit analysis by creating a curve relationship between the percentage of inhibition or cytotoxicity and the logs of doses.

RESULTS

Fractionation and identification of major compound

The fractionation of B1F D2 separated by semi preparative HPLC using acetonitrile: water (7-3 v/v), 2 mL/min of flow rate and resulted 5 subfractions (B1F D2H.1-B1F D2H.5). From those 5 subfractions, the most active subfraction was B1F D2H.3. Identification of B1F D2H.3 by HPLC was indicated that it was contain a major compound with a purity of 85.25% (Figure 1).

The chromatogram profile of B1F D2H.3 using stationary phase column 4.6x250 mm agilent RP-18 XDB, acetonitrile-water mobile phase 7:3 v/v, and flow rate 0.5 mL/minute. It showed the alkaloid fraction (B1F D2H.3) contain *N*-methylflindersine detected at retention time (Rt) 14.195 min and had 85.25% purity (Figure 2).

The results of ¹H-NMR and ¹³C-NMR of B1F D2H.3 showed the ¹H-NMR ($CDCl_3$, 400 MHz); δ H 5.53 (1H, d, $J=10.0$ Hz, H-3), 6.75 (1H, d, $J=10.0$ Hz, H-4), 7.32 (1H,d, $J=8.5$, H-7), 7.54 (1H, dt, $J=7.4$ Hz, H-8), 7.21 (1H, t, $J=7.9$ Hz, H-9), 7.96 (1H, dd, $J=7.9$ Hz, H-10), 1.52 (6H, s, 2-Me), 1.52 (6H,s, 2-Me), 3.69 (3H, s, N-Me) and ¹³C-NMR ($CDCl_3$, 400 MHz): δ 78.3 (C-2), 126.4 (C-3), 118.0 (C-4), 105.9 (C-4a), 160.5 (C-5), 139.4 (C-6a), 114.1 (C-7), 130.9 (C-8), 121.7 (C-9), 123.2 (C-10), 115.2 (C-10a), 155.2 (C-10b), 28.3 (2-Me), 29.2 (N-Me). The ¹H-NMR spectrum ($CDCl_3$, 400 MHz) showed four aromatic proton signals *ortho*, *meta* and *para* [δ H 7.96 (1H, dd, $J=7.9$ Hz, H-10), 7.54

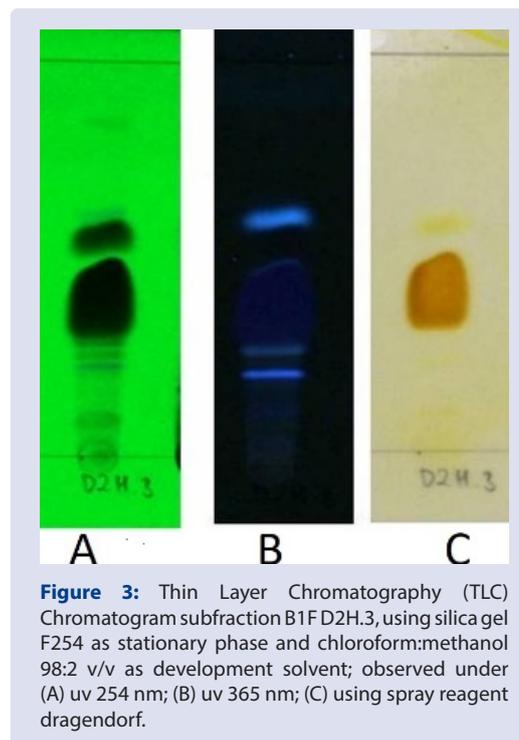
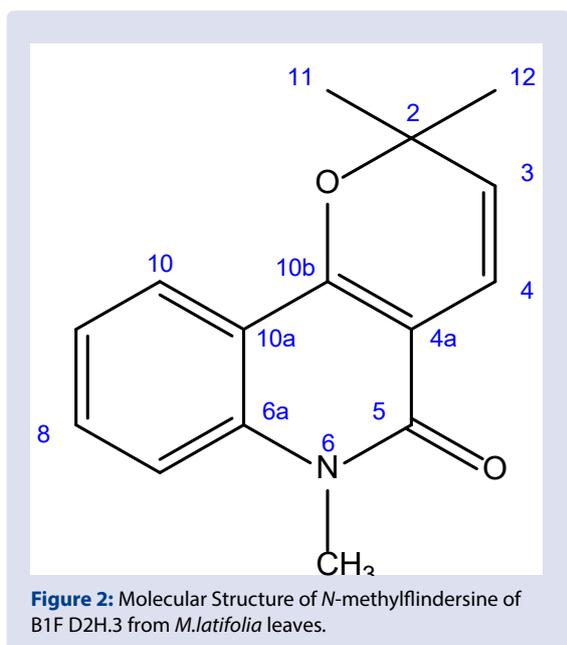
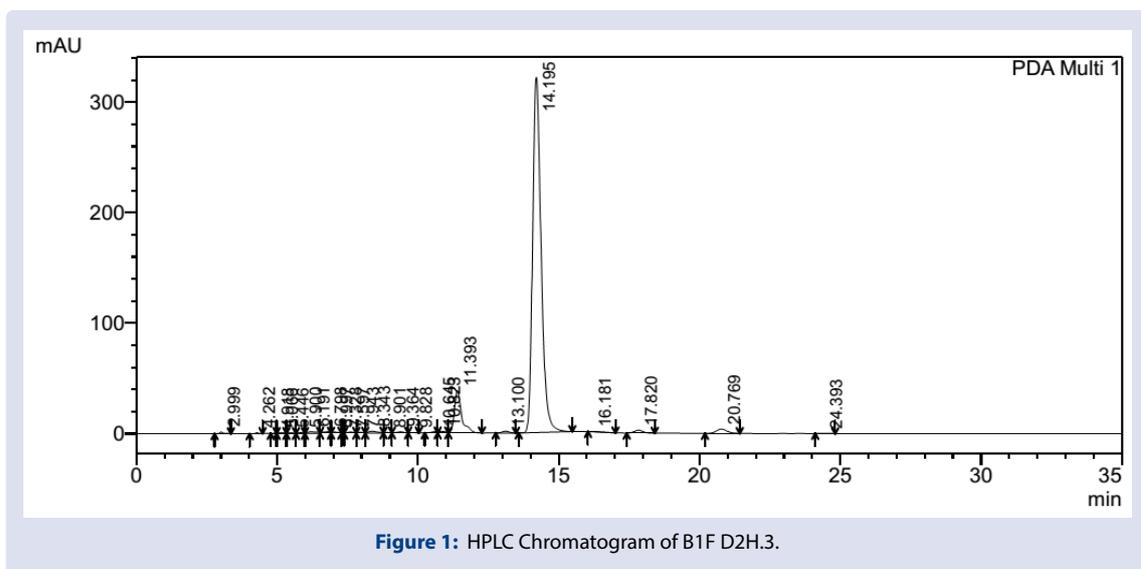
(1H, dt, J = 7.4 Hz, H-8), 7.32 (1H, d, J = 8.5Hz, H-7), 7.21 (1H, t, J = 7.9 Hz, H-9)], which is the characteristic of substituted 1,2 benzene.¹³ A pair of cis-vinylic doublet signals (J = 10.0 Hz) at δ H 6.75 (H-4) and 5.53 (H-3) and one methyl signal at δ H 1.53 (11-CH₃ and 12-CH₃) are proton signals from group 2, 2-dimethylpirano.¹⁴ The singlet signal at δ H 3.69 (N-CH₃) is a proton signal of N-methyl. The NMR spectral data were identical with those in references,¹³ and TLC analysis of subfraction B1F D2H.3 showed an orange spot with a dragendorf reagent (Figure 3). The result showed that B1F D2H.3 was similar as alkaloids *N*-methylflindersine.

Anti-HCV activity

The fraction and subfractions were subjected in vitro anti-HCV and cytotoxicity assay. First fraction (B1F D), and subfractions (B1F D1-B1F D9) were tested at a concentration of 30 μ g/mL. The result

showed that subfractions B1F D2, B1F D7, and B1F D9 exhibited strong activity against hepatitis C virus with inhibition percentages of 97.05%, 98.31%, and 98.30%, respectively. On the other hand, the same concentration of B1F D1, B1F D3-D6, and B1F D8 showed lower anti-HCV activity with percentage inhibition less than 28%. To determine the cytotoxicity effect, MTT test was performed in Huh7it. The results showed no toxic effect among all subfractions with mediated the cell viability higher than 85%, while the active subfractions B1F D2, B1F D7 and B1F D9 had viability levels of 99.77%, 93.63%, and 95.99%, respectively (Table 1).

To determine the IC₅₀ value, the active fraction and subfractions were evaluated for their inhibition percentages at various concentrations. The result showed that B1F D, B1F D2, B1F D7 and B1F D9 exhibited strong anti-HCV activity with an IC₅₀ value of 11.38 μ g/mL, 13.33 μ g/mL, 4.5 μ g/mL, and 2.9 μ g/mL, respectively. The cytotoxicity assay revealed that



all active fractions mediate CC_{50} value of $>100 \mu\text{g/mL}$. There was no cytotoxicity with exposure of sample up to $100 \mu\text{g/mL}$ for 48 h. The selectivity index (SI) that determined the effectiveness of a potential medicinal drug was analyzed by the ratio of CC_{50} and IC_{50} . It was shown that the highest SI value of >96.56 belongs to subfraction B1F D9 followed with B1F D7 and B1F D2 (Table 2). Due to a high amount of chlorophyll on B1F D7 and B1F D9, we focused to further separate B1F D2 using semi preparative HPLC to obtain the subfraction.

We obtained 5 subfractions (B1F D2H.1-B1F D2H.5) and analyzed their activity and toxicity as anti-HCV. The results determined that B1F D2H.3 had a strong active anti-HCV activity with an IC_{50} value of $6.29 \mu\text{g/mL}$, a CC_{50} value of $82.64 \mu\text{g/mL}$, and an SI >13.31 (Table 3). This result showed that subfraction D2H.3 may a potential target for separating the active anti-HCV constituent.

DISCUSSION

Developing anti-HCV agents from medicinal plants has become a currently significant issue. In recent years, many compounds were isolated from medicinal plants reported to be active anti-HCV agents. Chalepin and pseudane IX from *Ruta angustifolia* (leaves) revealed anti-HCV activities with IC_{50} value of 1.7 ± 0.5 and $1.4 \pm 0.2 \mu\text{g/mL}$ by inhibiting HCV replication and decreasing the NS3 protein level,¹⁵ Embelin and 5-O-Methyl embelin were isolated from *Embelia schimper* (Fruit) with IC_{50} of $21 \mu\text{M}$ and $46 \mu\text{M}$, by inhibiting HCV replication and decreasing the NS3 protein level,¹⁶ and Saikosaponin b2 from *Bupleurum kanoi* root with IC_{50} $16.1 \mu\text{g/mL}$ inhibited HCV by the neutralization of virus particle, attachment, and fusion.¹⁷

Anti-HCV activity and the mechanism of action from the ethanol extract of *M. latifolia* leaves had been reported in previous studies.¹⁰ In this study, we have fractionated and identified the active compound as anti-HCV agent from *M. latifolia*. The active fraction was contained major compound which similar with a known alkaloid compound called *N*-methylflindersine. This fraction has anti-HCV activity with IC_{50} value of $6.2 \mu\text{g/mL}$ and less cytotoxicity effect with CC_{50} value of $82.64 \mu\text{g/mL}$.

N-methylflindersine is the characteristic constituent of Rutaceae and Meliaceae families. It is known to possess insect growth inhibitor, antifeedant, and fungistatic activities.^{13,18} The antiviral activity of *N*-methylflindersine in *M. latifolia* has not been reported yet.

Melicope species have been investigated for their chemical compounds. *Melicope ptelefolia* leaves were reported to contain *N*-methylflindersine and two new alkaloids named melicobisquinolinone A and B. Another alkaloid were obtained from *Melicope semicarpifolia*, there were 2-acetylevolitrine (1), 2-acetylpteleine (2), and semecarpifoline (3). Several of these alkaloid exhibited significant antiplatelet aggregation activities in vitro.¹⁹ There was APS, an alkaloid from *Maytrenus ilicifolia* (root bark), which has known activity as anti-HCV with EC_{50} of $2.3 \mu\text{M}$ by decreasing HCV replication and NS5A level.²⁰ Although the mechanism of ethanol extract from *M. latifolia* was known to inhibit mainly at the entry step with $90.8 \pm 0.2\%$ and post entry step $60.6 \pm 4.9\%$, the mechanism of action of its alkaloid fraction, *N*-methylflindersine was still unknown. Further analysis of the mechanism of *N*-methylflindersine was necessary.

Table 1: Percentages of inhibition and cell viability in the fraction and subfractions from *M. latifolia* at the concentration of $30 \mu\text{g/mL}$.

Sample Code	Inhibition (%)*	Cell Viability (%)*
B1F D	78.25 ± 1.49	87.65 ± 5.32
B1F D1	0 ± 0	96.42 ± 2.39
B1F D2	97.05 ± 0.35	99.77 ± 0.88
B1F D3	3.91 ± 0.49	94.11 ± 3.54
B1F D4	27.14 ± 1.98	85.70 ± 0.89
B1F D5	40.68 ± 0.97	90.98 ± 3.24
B1F D6	0.13 ± 0.18	99.78 ± 1.28
B1F D7	98.31 ± 2.39	93.63 ± 3.68
B1F D8	24.89 ± 0	97.23 ± 3.80
B1F D9	98.30 ± 2.40	95.99 ± 0.88

*values are Mean \pm SD of duplicates experiments

Table 2: Antiviral activity (IC_{50}), cytotoxicity (CC_{50}), and selectivity index (SI) of fraction and subfraction of *M. latifolia* leaves.

Sample	IC_{50} ($\mu\text{g/mL}$)	CC_{50} ($\mu\text{g/mL}$)	SI (Selectivity Index)
B1F D	11.16	171.03	>15.33
B1F D2	13.38	142.85	>10.68
B1F D7	4.47	295.41	>66.09
B1F D9	2.89	280.03	>96.90

Table 3: Antiviral activity (IC_{50}), cytotoxicity (CC_{50}), and selectivity index (SI) of subfraction from *M. latifolia* leaves.

Sample	IC_{50} ($\mu\text{g/mL}$)	CC_{50} ($\mu\text{g/mL}$)	SI (Selectivity Index)
B1F D2H.1	79.34	349.91	>4.40
B1F D2H.2	252.51	>1000	>3.96
B1F D2H.3	6.21	82.64	>13.31
B1F D2H.4	27.18	171.07	>6.29
B1F D2H.5	71.20	699.86	>9.83

CONCLUSION

This study identified the potential of alkaloid fraction from *M. latifolia* as an anti-HCV agent. *N*-methylflindersine was identified as active anti-HCV fraction with a major constituent purity of 85.25%. These results suggest that *M. latifolia* and alkaloid fraction might be a good candidate to develop anti-HCV agents.

CONFLICTS OF INTEREST

The authors declare that they have no competing interest.

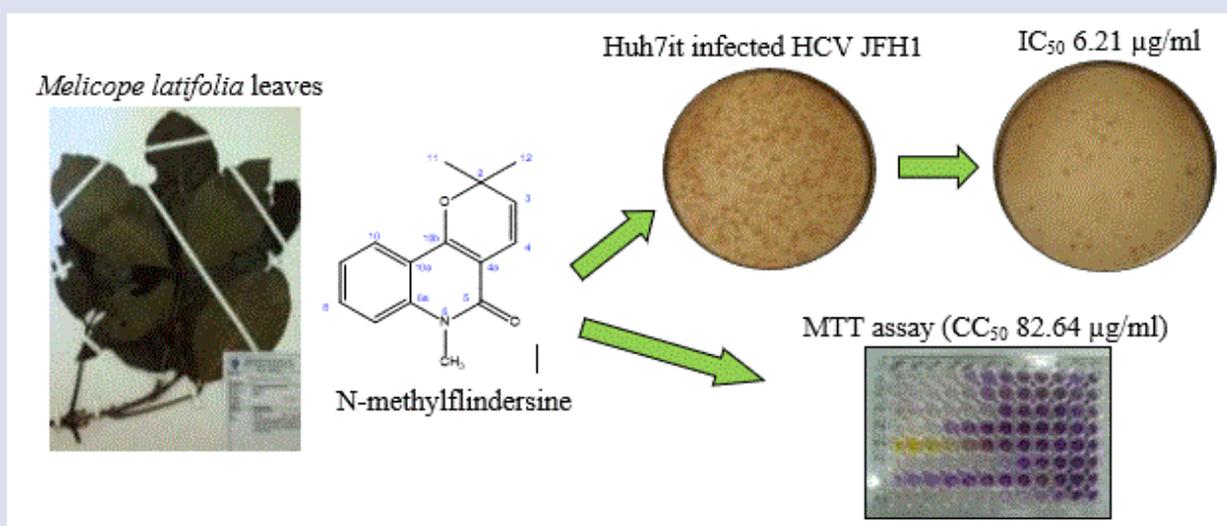
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GRAPHICAL ABSTRACT



ABOUT AUTHORS



Dwi Susiloningrum obtained her bachelor's degree in pharmacy at Faculty of Pharmacy, Muhammadiyah University of Surakarta in 2012. In 2013, she pursued her Pharmacists title and started to obtain her master's degree in Natural Product Chemistry, Pharmaceutical Science at Universitas Airlangga in the following year. Now she is focuses on study of the secondary metabolite as anti Hepatitis C Virus.



Adita Ayu Permasari is a researcher in Institute of Tropical Disease, Universitas Airlangga, Indonesia. She graduated in Master of Biology from Faculty of Science and Technology, Universitas Airlangga. She is currently work on antihepatitis virus cell culture and its mechanism of action. Some studies of hepatitis viruses have been published in some international scientific journals.



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