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

Dwi Susiloningrum¹, Adita Ayu Permanasari², Myrna Adianti²,³, Lidya Tumewu², Tutik Sri Wahyuni²,⁴, Mulyadi Tanjung²,⁵, Aty Widyawaryanti²,⁴, Achmad Fuad Hafid²,⁴,⁶*

ABSTRACT

**Introduction:** Hepatitis C Virus (HCV) is a major health problem, which infects approximately 170 million people across the world. 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 diseases. 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 (Huh7.5) 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 extract resulted in 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 a major compound named N-methylflindersine. This alkaloid fraction exhibited strong anti-HCV activity 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 affects millions of people each year. 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.

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). The viral genome encodes polypeptide 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, NS4A, NS4B, NS5A, and NS5B. 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. 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*, *Artocarpus heterophyllus*, *Ficus fistulosa*, *Electron serratus*, and *Melicope latifolia* significantly inhibited HCV activity in vitro. A variety of active phytochemicals such as flavonoids, terpenoids, lignins, saponins, coumarins, sappanols, saponin, alkaloids, polyamines, thiophenes, protein, and peptides have been identified to inhibit various viruses.

*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. The chemical compounds which were contained in the *M. latifolia* plant are flavonoids, alkaloids, coumarins and terpenoids. In general, plant species in the Rutaceae family contain the chemical constituent of alkaloid group, including the Melicope genus.
For instance, *M. ptelefolia* leaves were reported to contain the alkaloid compounds of N-methylindolines and two new alkaloids named melicobisquinolinone A and B. 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 μg/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. 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$_3$ 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$_3$CN-H$_2$O (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 TLC Visualizer on UV 254 nm and 366 nm, and sprayed with dragenord, 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 (Dulbeco’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%. HCV propagation

HCV genotype 2a (JFH1) propagation was performed on hepatocyte Huh7it cells (1.8x10$^5$ 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.

**Cytotoxicity assay**

The cytopathicity 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% 150μl 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 IC$_{50}$.

**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 (5x10$^4$cells/well). A fixed amount of JFH1, with multiplicative 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.

**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/minute of flow rate and resulted 5 subfractions (B1F D2H.1-B1F D2H.5). 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 alkaidal fraction (B1F D2H.3) contain N-methylindolines detected at retention time (Rt) 14.195 min and had 85.25% purity (Figure 2).

The results of ¹H-NMR and C-NMR of B1F D2H.3 showed the¹

H-NMR (CDCl$_3$, 400 MHz): δH 5.53 (1H, d, J=10.0 Hz, H-3), 6.73 (1H, d, J=10.0 Hz, H-4), 7.32 (1H, d, J=8.5, H-7), 7.54 (1H, dt, J=7.4Hz, H-8), 7.21 (1H, t, J= 7.9 Hz, H-9), 7.95 (1H, dd, J=7.9 Hz, H-10), 1.52 (6H, s, 2-Me), 1.52 (6Hs, 2-Me), 3.69 (3H, s, N-Me) and ¹C-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 [δH 7.96 (1H, d, J = 7.9 Hz, H-10), 7.54...
Susiloningrum, et al.: The Alkaloid Fraction from Melicope latifolia Leaves Inhibits Hepatitis C Virus

(1H, dt, J = 7.4 Hz, H-8), 7.32 (1H, d, J = 8.5 Hz, H-7), 7.21 (1H, t, J = 7.9 Hz, H-9)], which is the characteristic of substituted 1.2 benzene. A pair of cis-vinylic doublet signals (J = 10.0 Hz) at δH 6.75 (H-4) and 5.53 (H-3) and one methyl signal at δH 1.53 (11-CH 3 and 12-CH 3) are proton signals from group 2, 2-dimethylpirano. The singlet signal at δH 3.69 (N-CH 3) is a proton signal of N-methyl. The NMR spectral data were identical with those in references, 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 μg/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 IC 50 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 IC 50 value of 11.38 μg/mL, 13.33 μg/mL, 4.5 μg/mL, and 2.9 μg/mL, respectively. The cytotoxicity assay revealed that...
all active fractions mediate \( CC_{50} \) value of >100 \( \mu g/mL \). There was no cytotoxicity with exposure of sample up to 100 \( \mu g/mL \) 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 \( \mu g/mL \), a \( CC_{50} \) value of 82.64 \( \mu g/mL \), 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±0.2 \( \mu g/mL \) by inhibiting HCV replication and decreasing the NS3 protein level. Embelin and 5-O-Methyl embelin were isolated from *Embelia schimper* (Fruit) with \( IC_{50} \) of 21 \( \mu M \) and 46 \( \mu M \), by inhibiting HCV replication and decreasing the NS3 protein level. Several of these alkalds exhibited significant antiplatelet aggregation activities in vitro. There was APS, an alkaloid from *Maytrenus ilicifolia* (root bark), which has known activity as anti-HCV with \( EC_{50} \) of 2.3 \( \mu M \) by decreasing HCV replication and NS5A level. Although the mechanism of ethanol extract from *M.latifolia* was known to inhibit mainly at the entry step with 90.8 ± 0.2% and post entry step 60.6 ± 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 \( \mu g/mL \).

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

*values are Mean ± 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.

<table>
<thead>
<tr>
<th>Sample</th>
<th>( IC_{50} ) (( \mu g/mL ))</th>
<th>( CC_{50} ) (( \mu g/mL ))</th>
<th>SI (Selectivity Index)</th>
</tr>
</thead>
<tbody>
<tr>
<td>B1F D</td>
<td>11.16</td>
<td>171.03</td>
<td>&gt;15.33</td>
</tr>
<tr>
<td>B1F D2</td>
<td>13.38</td>
<td>142.85</td>
<td>&gt;10.68</td>
</tr>
<tr>
<td>B1F D7</td>
<td>4.47</td>
<td>295.41</td>
<td>&gt;66.09</td>
</tr>
<tr>
<td>B1F D9</td>
<td>2.89</td>
<td>280.03</td>
<td>&gt;96.90</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Sample</th>
<th>( IC_{50} ) (( \mu g/mL ))</th>
<th>( CC_{50} ) (( \mu g/mL ))</th>
<th>SI (Selectivity Index)</th>
</tr>
</thead>
<tbody>
<tr>
<td>B1F D2H.1</td>
<td>79.34</td>
<td>349.91</td>
<td>&gt;4.40</td>
</tr>
<tr>
<td>B1F D2H.2</td>
<td>252.51</td>
<td>&gt;1000</td>
<td>&gt;3.96</td>
</tr>
<tr>
<td>B1F D2H.3</td>
<td>6.21</td>
<td>82.64</td>
<td>&gt;13.31</td>
</tr>
<tr>
<td>B1F D2H.4</td>
<td>27.18</td>
<td>171.07</td>
<td>&gt;6.29</td>
</tr>
<tr>
<td>B1F D2H.5</td>
<td>71.20</td>
<td>699.86</td>
<td>&gt;9.83</td>
</tr>
</tbody>
</table>
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.

ACKNOWLEDGEMENTS
This research was supported by Mandat Research Grant funding year 2016 from Universitas Airlangga, and NPMRD (Natural Product Medicine Research and Development), Institute of Tropical Disease, Universitas Airlangga, Indonesia, funding year 2018.

REFERENCES
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 Permanasari 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.

Myrna Adianti obtained her bachelor's degree in biology at Faculty of Science and Technology, Universitas Airlangga. She was continued to obtain her master’s degree at Faculty of Medicine, Universitas Airlangga. She pursued her PhD at Kobe University, Japan. She is currently a lecturer at Faculty of Vocational Study, Universitas Airlangga and a researcher at Institute of Tropical Disease, Universitas Airlangga. Her major achievement are in the field of bioactivity including antiHepatitis C virus activity and antiamebic activity using cell culture.

Lidya Tumewu obtained Bachelor and master’s degree in pharmacy at Faculty of Pharmacy, Universitas Airlangga. She is a researcher at Institute of Tropical Disease, Universitas Airlangga. She is focuses on metabolite secondary of natural product and their bioactivity.

Tutik Sri Wahyuni was started her career in Faculty of Pharmacy, Department of Pharmacognosy and Phytochemistry. She took her PhD at Kobe University, Graduate School of Medicine in 2012 and studied on anti-hepatitis C virus activities of plants and its metabolites. Now, she is focuses on drug development of medicinal plants for hepatitis virus infection.

Mulyadi Tanjung is a lecturer at Faculty of Science and Technology, Universitas Airlangga. He was graduated as Bachelor of Chemistry from Andalas University. He was further obtain his Master and Doctoral degree at Institute of Technology Bandung. His major achievements are in the field of isolation and identification of compounds from endemic plants of Indonesia especially East area of Indonesia.

Aty Widyawaruayanti is a lecturer at Faculty of Pharmacy, Universitas Airlangga and researcher at Natural Product Medicine Research and Development (NPMRD), Institute of Tropical Disease, Universitas Airlangga. She obtained her bachelor’s degree in pharmacy at Faculty of Pharmacy, Padjajaran University. She was further pursued her Master and Doctoral degree at Faculty of Pharmacy, Universitas Airlangga. Her research is focuses on drug discovery from bioactive natural products especially drug discovery for antimalarial drugs.

Achmad Fuad Hafid is currently vice-director of Institute of Tropical Disease, Universitas Airlangga. He is a lecturer at Faculty of Pharmacy, Universitas Airlangga and a researcher as well. His acquired a Professor with expertise in the fields of phytochemistry and bioactive natural product discovery for therapeutic applications.