Identification of Bioactive Compound from Microalga BTM 11 as Hepatitis C Virus RNA Helicase Inhibitor

(Akron Zaenal Mustopa, Rifqiyyah Nur Umami, Prabawati Hyunita Putri, Dwi Susilaningsih, & Hilda Farida)

1Research Center for Biotechnology, LIPI, Jalan Raya Bogor Km 46, Cibinong, Bogor, 16911
2Department of Biochemistry, Bogor Agricultural University, Dramaga Campus, Bogor, 16680
Email: azmustopa@yahoo.com

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ABSTRACT
Hepatitis C virus (HCV) is the major causative agent of chronic liver disease. Recently, the inhibition of NS3 RNA helicase/ATPase activity is being explored as the specifically targeted antiviral therapy (STAT) against HCV infection. This study was aimed to elucidate potential candidates for anti-HCV therapy derived from Indonesian indigenous microalgae. The microalga designated as BTM 11 was isolated and cultured. Methanol extract of BTM 11 was screened as the opponent of purified HCV NS3 RNA helicase enzyme through colorimetric ATPase assay. Screening of chemical compound and fractionation by using gel filtration chromatography with eluent of methanol : chloroform (1:99) were conducted for identification and isolation of the bioactive compounds. The third fraction of fractionated sample showed a relatively strong ATPase inhibitory effect (81.23 ± 2.25 %) compared to the negative control. Further analysis of third fraction using thin layer chromatography (TLC) with eluent of chloroform : methanol (9:2) gave two spots with the Rf value of 0.8 and 0.37, respectively. In addition, high performance liquid chromatography (HPLC) analysis showed absorption peak with the highest abundance at the retention time of 12.483 and 16.617 minutes which absorbed at 266 and 230 nm wavelegnth, respectively. According to those analyses, this study suggests that bioactive compounds derived from BTM 11 were classified as the groups of flavonoids and feasible as potential candidates for anti-HCV therapy through the inhibitory effect of NS3 RNA helicase/ATPase activity.

Keywords: Hepatitis C Virus, NS3 RNA helicase, ATPase, Microalga, Flavonoids

INTRODUCTION
The hepatitis C virus (HCV) is the member of genus Hepacivirus, family Flaviviridae. HCV is a small enveloped virus with the genome of a single-strand of positive-sense RNA around 9.6 kb that consist of a 5’NTR, a long open reading frame (ORF) and a 3’NTR (Figure 1). The 5’NTR contains an internal ribosome entry site (IRES) mediating translation of a single polyprotein of approximately 3,000 amino acid residues. The polyprotein is cleaved by host and viral protease into at least 10 different products. The structural proteins (core, E1 and E2) are located in the amino terminus of the polyprotein followed by p7, a hydrofobic peptide with unknown function, and the non
HCV is a major causative agent in most cases of acute and chronic non-A non-B hepatitis and could lead to liver-related diseases. Since the discovery of HCV in 1989, an estimated of 170 million people are persistently infected with HCV worldwide and the case number continues to increase (Choo et al. 1989; Kato et al. 1990; Takamizawa et al. 1991; WHO 1999; Bartenschlager et al. 2000). Despite the fact that HCV infection commonly has sub-clinical or only associated with mild symptoms, persistent infection frequently progress to chronic hepatitis and may initiates steatosis, cirrosis, hepatocellular carcinoma and mortality in more than 70% of infected individual. Hepatocellular carcinoma is among the most lethal and prevalent cancers, and chronic HCV infection is one of the most prominent factors associated with this type of cancer (WHO 1999; Andrade et al. 2009).

The global prevalence of HCV infection has become a significant health problem, however, the current standard therapy which combine pegylated-alfa interferon (PEG-IFNα) with ribavirin (RBV) is inadequately effective, poorly tolerated and can triggers some of adverse drug reactions such as flu-like symptoms, fatigue, severe malaise, hemolytic anemia, anorexia, taste disorders and depression (Manns et al. 2006; Jang et al. 2011). Furthermore, no effective vaccine for preventing HCV infection is available so far. Serious efforts are being made to develop an IFNα-free therapy to reduce the numerous side effect caused by the systemic administration of this cytokine. Thus, a novel and more effective therapeutic strategy is urgently required (Walker et al. 2003).

The bioactive compounds isolated from marine organism often has potent biological activities. Indonesia is a country with mega biodiversity of marine organism, one of them is microalgae. Microalgae are primitive members of the plant kingdom with the approximate size of 3-20 μm. Some microalgae have been commercially produced as supplement food due to their high nutritional value such as, *Spirulina*, *Chlorella*, *Dunaliella salina* and *Porphyridium* (Chu 2011). Microalgae also had potential activity as antiviral agents, although they are not fully explored. The methanol extracts of microalgae had been showed an antiviral activity against herpes simplex virus (HSV) and Epstein-Barr virus (EBV) (Ohta et al. 1998; Kok et al. 2011), while another study showed the polysaccharides fraction of red microalgae were found to inhibit the production of retroviruses (Talyshinsky et al. 2002). In addition, the isolate of *Spirulina* plantesis had been found to have anti-enterovirus activity (Shih et al. 2003).

Recent biotechnological advances and molecular approaches have led to the development of new antiviral strategies against HCV infection targeting specific HCV protein required for HCV replication such as NS2 protease, NS3 helicase, NS3/4A protease, NS5A, and NS5B polymerase. NS3 helicase is a multifunctional protein with an N-terminal serine-type protease domain and a C-terminal RNA helicase/NTPase domain (Tai et al. 1996; Gallinari et al. 1998; Borowski et al. 2000; Soriano et al. 2009). In this study we devised a rational approach in the attempt to elucidate potential candidate of selective HCV NS3 RNA helicase inhibitors derived from Indonesian indigenous microalgae. This study may present an alternative way toward the development of therapeutic agent for chronic hepatitis C.

**MATERIAL AND METHODS**

Microalga *BTM 11* were inoculated and grown into Modified Bristol Medium – Sea
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Water (MBM-SW) medium by the measurement of OD$_{630}$ within 50 days of cultivation with two days of observation interval, and harvested at lag-phase. Pellets were collected from 500 mL culture by centrifugation at 8500 rpm for 10 minutes. Extraction was carried out using organic solvent according to Ohta et al. 1998 by mixing the pellet with 80% MeOH followed by four cycles of sonication on ice (1 minute working, 2 minutes free) for cell disruption and the homogenate was centrifuged. The soluble fraction was vacuum evaporated at 60°C.

The recombinant HCV NS3 RNA helicase protein was expressed and purified as described previously (Utama et al. 2000). E. coli BL21 (DE3)pLysS harboring expression plasmid (pET21b-466 amino acids of HCV NS3 RNA helicase gene) were cultured at 37°C in Luria-Bertani medium containing 100 μg/ml of ampicillin. The protein expression was induced by the addition of 0.3 mM IPTG when the OD$_{600}$ of the culture reached 0.3, for 3 hours. Following the induction, the cells were collected by centrifugation and three times of freezing and thawing, subsequently. Buffer B (10 mM Tris-HCl buffer (pH 8.5), 100 mM NaCl, 0.25% Tween 20) was used to resuspend the cells. Three cycles of sonication on ice (15 seconds working, 1 minute free) were carried out to disrupt the cells. The soluble fraction of the clarified cell lysate was applied on buffer B-calibrated TALON metal affinity resin (Clontech, Palo Alto, CA, USA) and the binding was carried out by gentle mixing for 3 hours at 4°C. The resin-bound protein was collected by brief centrifugation followed by two times washing with buffer B. Buffer B containing 400 mM imidazole was used to elute and purify the protein. The purified protein was confirmed by 8% SDS-PAGE and visualized by coomassie blue staining.

The inhibition of HCV NS3 RNA helicase/ATPase activity was measured by colorimetric ATP hydrolysis assay based on the measurement of free phosphate moiety released from ATP, as described previously (Utama et al. 2000). The 45 μl/well of reaction mixture containing 10 mM MOPS buffer (pH 6.5), 2 mM ATP, 1 mM MgCl$_2$, and purified HCV NS3 RNA helicase protein was incubated in the absence or presence of 5 μl methanol extract of microalgae in a 96 well microtiter plate at room temperature for 45 minutes. The reaction was stopped with the addition of 100 μl/well of dye solution (with the composition of water, 0.081% malachite green, 5.7% ammonium molybdate in 6N HCl, and 2.3% polyvinyl alcohol were 2:2:1:1, v/v). Following the addition of 25 μl/well of 30% sodium citrate, the absorbance at 620 nm was measured with the reference absorbance at 492 nm. The inhibition rate was calculated as the percentage of absorbance in the presence of methanol extract as the inhibitory substance compared to absorbance in the absence of methanol extract. All absorbance measurements were done in triplicate and the results were expressed as mean value ± standard deviation.

The chemical compounds present in the methanol extract of BTM 11 were identified by means of qualitative analysis according to the standard method (Harborne 1984). Alkaloids were identified by using Wagner’s, Mayer’s and Dragendorff’s test; tannins by treatment with 1% (b/v) FeCl$_3$; saponins by shaking with H$_2$O; flavonoids by 10% (b/v) NaOH and 2N H$_2$SO$_4$; triterpenoid and steroid by using Lieberman Buchard’s test.

Purification of HCV NS3 RNA helicase inhibitor was performed as described by Ohta et al. 1998. The MeOH extract obtained will be fractionated on a column of silica gel using a solvent gradient of MeOH:CHCl$_3$ (1:99). Then, the active fractions obtained were separated by TLC (Kieselgel 60, Merck) by using CHCl$_3$:MeOH (9:2) as developing solvent. Next, this semi-purified sample subjected to HPLC on Eurospher (C18) column with MeOH:water (0:100; 33:67; 50: 50; 67: 33; 100:0), flow rate 1 ml/1 minute, 254 nm.

RESULTS

Culture and extraction of microalgae

Samples of microalgae were collected and isolated from various aquatic regions in Indonesia including Pari, Batam and Ciatier. BTM 11, microalga isolated from Batam, was cultured and the growth was identified with the appearance of the green color filaments (Figure 2). The culture of BTM 11 was harvested at lag-
phase at 50 days of cultivation. Methanol was used as the polar solvent in the extraction method in order to obtain the chemical compound for the screening of NS3 RNA helicase/ATPase activity inhibitor.

Expression and purification of the recombinant HCV NS3 RNA helicase protein

The purified recombinant HCV NS3 RNA helicase protein were confirmed by SDS-PAGE 8% as shown in Figure 3. The confirmed size was 54 kDa. The enzymatic activities of the HCV NS3 RNA helicase purified protein were used for the colorimetric ATPase assay.

ATPase assay

The crude methanol extract of BTM 11 showed highly stable ATPase inhibitory effect compare to the other isolate (data not shown). The inhibition activities of the fractionated crude methanol extract of BTM 11 were represented as percentages in Table 1. The third fraction showed the highest inhibitory effect (81.23 ± 2.25 %).

Phytochemical studies

The screening result for phytochemical test of the methanol extract of BTM 11 were recorded in Table 2. The results indicated the presence of flavonoids group in polar solvent (methanol and distiller water).

Purification and identification of HCV NS3 RNA helicase inhibitor

The third fraction which showed high inhibition activity (81.23 ± 2.25 %) were pooled, and dialyzed. The purity of each preparation step was confirmed by TLC. The TLC assay showed that the third fraction have two spots with Rf 0.8 and 0.37 (Figure 4). Further chemical analysis was conducted using HPLC. Based on qualitative analysis using profiling chemical compound, the third fraction showed two peaks and had high abundance with retention time (Rt) at 12.483 minutes and 16.617 minutes (Figure 5). Two selected peaks were analyzed with PDA detector. The retention time 12.483 minutes was absorbed at 266 nm wavelength, while the retention time 16.617 minutes was absorbed at 230 nm wavelength (Figure 6 and Figure 7). According to Harbone (1984), the two absorbance results indicate that the bioactive compounds derived from the third fraction were classified as the group of flavonoids.

DISCUSSION

Based on the sequence motif analysis, the HCV NS3 protein was predicted as multifunctional protein containing serine protease, NTPase, and RNA helicase activities. The HCV NS3 RNA helicase/NTPase domain is classified into the DexH protein subfamily of the helicase superfamily II which is capable of unwinding RNA-RNA duplexes by disrupting the hydrogen bonds fueled by ATP hydrolysis during viral transcription and/or replication. (Tai et al. 1996; Gallinari et al. 1998; Borowski et al. 2000; Utama et al. 2000a; Utama et al. 2000b). Although the NS3 helicase represent an ideal candidate as the specifically targeted antiviral therapy, but the progress is lack behind other viral enzymes such as NS3/NS4 protease, NS5A protein and NS5B polymerase. In term of HCV therapy, two protease inhibitors (telaprevir and boceprevir) are expected to receive final approval for clinical use, while no helicase inhibitors have been completed the preclinical test (Belon & Frick, 2009; Soriano et al. 2011).

Screening of the chemical compounds followed by isolation of the bioactive compound might be one of the strategies for the development of helicase inhibitors. These compounds could proceed as lead molecules for analog synthesis,
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structure-activity studies, and possible identification of a novel drug design. However, measuring helicase-catalyzed RNA unwinding is difficult since the reaction products (single-stranded RNA) will rapidly re-anneal, thus, cannot be detected.

In this study, we perform a simple spectrophotometry method by monitoring the free phosphate moiety as the result of helicase-catalyzed ATP hydrolysis using colorimetric ATPase assay. Based on the color spectrum, higher inhibitory effect will be shown as lower color intensity. The results showed that the third fraction of fractionated sample derived from methanol extract of microalgae BTM 11 had the highest inhibitory effect compare to the negative control.

Microalgae frequently live in extreme environments of light, salinity, and temperature. In order to adapt to these extreme conditions, most of them produce a high variety of secondary metabolites that often have potent biological activities. In comparison with terrestrial plants, microalgae can be easily cultured in the laboratory scale with appropriate cultivation condition to provide a consistent source of bioactive compounds, however, the composition of bioactive compounds derived from microalgae might vary depends on the species (Ibañez et al. 2012).

The phytochemical analysis, TLC and HPLC result showed that the chemical inhibitor of HCV RNA helicase in this study belongs to the group of flavonoids. Flavonoid is one of the phenolic compound that had been extensively studied and known to have abundance structures. Another studies also showed the presence of flavonoid in the extract of microalgae by using new detection method of ultra high performance liquid chromatography tandem mass spectrometry (UHPLC-MS/MS) technology (Klejdus et al. 2010; Goiris et al. 2014).

The exploration of flavonoid regarding to its antiviral activity was mostly targeting HIV infection, but recently, flavonoid also known to have antiviral activity against herpes simplex virus, coxsackievirus B3 and also dengue virus (Tapas et al. 2008; Yin et al. 2014; Qamar et al. 2014; de Sousa et al. 2015). However, most of these studies are performed in vitro, and less information about in vivo studies of antiviral activity of flavonoid are known.

Flavonoid acts as inhibitor against HCV infection through several modes of action such as the inhibition of RNA binding of HCV RNA dependent-rRNA polymerase (Ahmed-Belkacem, et al. 2014) and decreasing HCV mature microRNA122 levels (Shibata et al., 2014). In this study, the specific mechanisms of action of the identified flavonoid are not precisely determined. However, the approach use in this study was based on the fact that the ATP hydrolysis provides the energy for the RNA unwinding reaction. The inhibition of the accessibility of the helicase-ATP binding site for the ATP may

Table 1. Inhibition of HCV NS3 RNA helicase/ATPase activity by the column chromatography fractionated samples.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>% Inhibition</th>
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<tbody>
<tr>
<td>1</td>
<td>64.85 ± 3.73</td>
</tr>
<tr>
<td>2</td>
<td>71.70 ± 5.66</td>
</tr>
<tr>
<td>3</td>
<td>81.23 ± 2.25</td>
</tr>
<tr>
<td>4</td>
<td>67.63 ± 1.41</td>
</tr>
<tr>
<td>5</td>
<td>62.21 ± 7.57</td>
</tr>
<tr>
<td>6</td>
<td>58.79 ± 8.96</td>
</tr>
<tr>
<td>7</td>
<td>27.70 ± 9.71</td>
</tr>
<tr>
<td>8</td>
<td>53.70 ± 6.32</td>
</tr>
<tr>
<td>9</td>
<td>56.95 ± 2.91</td>
</tr>
<tr>
<td>10</td>
<td>46.14 ± 4.13</td>
</tr>
</tbody>
</table>

Figure 3. SDS-PAGE analysis of Hepatitis C Virus NS3 RNA helicase with approximately 54 kDa in size.
lead to the reduction of ATPase activity and consequently declining the unwinding rate, which hampered the viral replication. Furthermore, according to Borowski et al. 2002, inhibitor of HCV RNA helicase/NTPase could act by several mechanisms such as, obstruction of NTP binding; inhibition through allosteric mechanism; inhibition of the coupling of NTP hydrolysis; competitive inhibition of RNA binding and also sterical blockade of the translocation of the helicase/NTPase along polynucleotide chain during unwinding stage.

It appears that the flavonoids derived from the methanol extract of microalga *BTM 11* have great potential as antiviral therapy, particularly

Table 2. Qualitative identification of chemical compound of *BTM 11* methanol extract.

<table>
<thead>
<tr>
<th>Chemical Compound</th>
<th>Identification</th>
</tr>
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<tbody>
<tr>
<td>Alkaloids</td>
<td>- Wagner - Meyer - Dragendorf</td>
</tr>
<tr>
<td>Tannin</td>
<td>-</td>
</tr>
<tr>
<td>Saponin</td>
<td>-</td>
</tr>
<tr>
<td>Flavonoid</td>
<td>+</td>
</tr>
<tr>
<td>Triterpenoid</td>
<td>-</td>
</tr>
<tr>
<td>Steroid</td>
<td>-</td>
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Figure 4. TLC of the third fraction. (a) spot detection with heat treatment, (b) spot detection with UV 254 nm.

Figure 5. Chromatogram of the third fraction using HPLC.

Figure 6. The retention time 12.483 minutes was absorbed at 266 nm wavelength.

Figure 7. The retention time 16.617 minutes was absorbed at 230 nm wavelength.
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for anti-HCV infection. Nevertheless, further isolation, purification, characterization, and modification as well as molecular study of the bioactive compounds need to be done in the attempt to obtain higher antiviral activity. Furthermore, the mechanism of antiviral effect and in vivo study remain to be elucidated.

CONCLUSION

As the concluding remarks, this study suggests that the extraction of bioactive compounds derived from microalgae BTM 11 which is classified as the groups of flavonoids, showed potential activity against HCV infection through the inhibitory effect of NS3 RNA helicase/ATPase activity.

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