Malays. Appl. Biol. (2018) 47(3): 121–127

POTENCY OF A MARINE SPONGE FROM THE GENUS *Stylissa* AS ANTI HEPATITIS C AGENT

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Accepted 6 May 2018, Published online 30 June 2018

ABSTRACT

Marine sponges have shown potential as a source of many bioactive metabolites. Our previous study on Indonesian marine sponges indicated that this organism possessed promising antiviral activity. The aim of this study was to screen antiviral activity of marine sponge extract from the genus *Stylissa* and its fractions against hepatitis C virus (HCV). Identification of metabolites in the active fractions was also carried out as well as investigation of the mode of action of the extract. The results showed that the extract inhibited the growth of HCV at IC_{50} of 4.4 µg/mL. The time of addition experiment revealed that the extract act at the post entry step. Bioassay-guided fractionations revealed two active fractions, namely fractions SF7 and SF8 which gave 99.3% and 86.4% inhibition, respectively, at a concentration of 50 µg/mL. ^{1}H NMR and GC-MS analyses indicated the presence of a mixture of saturated and unsaturated fatty acids in the active fractions.

Key words: Marine sponge, *Stylissa*, hepatitis C virus, fatty acids

INTRODUCTION

Hepatitis C is a major health problem, and according to the World Health Organization (2017), around 71 million people globally suffered from hepatitis C which distributed in eighty-seven countries (Gower *et al.*, 2014). In 2017 it was estimated that 399,000 people die each year from hepatitis C (World Health Organization, 2017). Chronic infection of hepatitis C virus can lead to the development of hepatic cirrhosis and hepatocellular carcinoma. The current standard treatment for hepatitis C is using combinations of direct-acting antivirals (DAAs) such as elbasvir-grazoprevir, without using interferon (IFN) since it causes severe side effects. Currently, there are four classes of DAAs, nonstructural proteins 3/4A(NS3/4A) protease inhibitors (PIs), NS5B nucleoside polymerase inhibitors (NPIs), NS5B non-nucleoside polymerase inhibitors (NNPIs), and NS5A inhibitors (Calland *et al.*, 2015). Although treatment of hepatitis C infection has been established, however, it is considered expensive, especially in developing countries. The cost of treatment as well as the presence of drug-resistence virus has urged research on finding an alternative treatment against hepatitis C virus. Recently, several anti-HCV agents from natural resources have been reported, such as a flavonoid delphinidin and the catechin EGCG (epigallocatechin-3-gallate); both compounds are plant origin (Calland *et al.*, 2015).

The oceans, which occupies most of the Earth's surface, are extraordinarily rich in species diversity, especially in tropical environments. While plants remains a major source of natural products, marine biodiversities have also gained popularity as the new pipeline of novel chemistry and biologically active metabolites (Kim, 2012). Amongst marine organisms, sponges have been the focus of study for many years. Marine sponges are simple, multicellular, and sessile invertebrates with approximately 15,000 species have been discovered worldwide. More than 5,300 metabolites have been reported from this organism (Ye *et al.*, 2015). Many

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of these metabolites showed pronounced bioactivity including antiviral. Several marine sponges have been reported to have anti-HCV activity, such as *Amphimedon* sp. (Fujimoto *et al.*, 2012) and an unidentified Demospongiae sponge (Furuta *et al.*, 2014); from this unidentified sponge anti-HCV compounds were isolated, namely halisulfate 3 and suvanine. Both compounds inhibited NS3 helicase-activity in a dose dependent manner with IC_{50} values of 4 and 3 μM, respectively (Furuta *et al.*, 2014). Several alkaloids such as discorhabdins A, C and dihydrodiscorhabdin C showed potency as anti-HCV agent with EC_{50} values < 10 μM were isolated from the sponge genus *Latrunculia* (Na *et al.*, 2010).

Our previous study has shown that marine sponges, *Homaxinella tanitai* and *Microxina subtilis* gave strong inhibition against HCV with IC_{50} of 27.1 and 40.5 μg/mL, respectively (Suciati *et al.*, 2017). In the current study, we investigated the anti-HCV activity of the ethyl acetate extract of a marine sponge genus *Stylissa* collected from the same location, Barrang Lompo Island, South Sulawesi, Indonesia. Flash column chromatography fractions of the sponge extract were also subjected to anti-HCV assay. Sponges from the genus *Stylissa* have been known to produce bromopyrolle alkaloids. Several of these compounds showed promising anti-viral effect against HIV-1 (Ebada *et al.*, 2014; O'Rourke *et al.*, 2016). In order to determine the mode of action of the extract time of addition experiment was conducted. Identification of metabolites in the active fractions was carried out by NMR spectroscopy and GC-MS analyses.

MATERIALS AND METHODS

Reagents

Virus JFH1a, cell Huh7it, *Dulbeco's Modified Eagle Medium* (DMEM, GIBCO-Invitrogen), *Fetal Bovine Serum* (FBS, GIBCO-Invitrogen), *Non-essential Amino Acids* (NEAA, GIBCO-Invitrogen), kanamycin (SIGMA), *Dulbesco's Phosphate Buffered Saline* (DPBS, GIBCO-Invitrogen), *Trypsin*-EDTA (GIBCO-Invitrogen), DAB *Thermo Staining*, DMSO, *Bovine Serum Albumin* (BSA, Roche), Formaldehyde (HCHO, Applicam), TritonX-100 (Promega), anti-serum HCV, HRP-*Goat-anti-human Ig* (MBL).

Biological Material

The sponge was collected by using SCUBA at a depth of 8-10 m from around Barrang Lompo Island, Makassar, South Sulawesi, Indonesia on May 17th, 2014. The sample was kept in a plastic pack in ice boxes immediately after collection. Sponge specimen was then stored in a freezer at -20°C until analysis. Sponge specimen was sent to Ecology Laboratory, Department of Biology, Faculty of Mathematics and Sciences, Institut Teknologi Sepuluh November, Surabaya, Indonesia for identification. Sponge sample was identified as genus *Stylissa*, however, identification to the species level was not possible. Voucher specimens were kept in ethanol 70% at the Faculty of Pharmacy, Universitas Airlangga under the accession number 17-5-14-11.

Extraction and fractionation

Fresh sponge (wet weight 128 gram) was diced into small pieces and extracted exhaustively in a combination of dichloromethane : methanol (1:1, 600 mL) by using ultrasonic vibration for 3 x 10 mins for each extraction. The solvent was removed by filtration, and the residue was re-extracted using the same procedure. The collected filtrate was evaporated under *vacuo* to give an aqueous residue, which was then partitioned with ethyl acetate (5 x 150 mL). The organic layer was removed, dried over anhydrous Na_2SO_4, and concentrated under reduced pressure to afford a crude oily extract (994 mg).

The sponge extract was subjected to Sepacore® flash column chromatography by using glass column filled with silica gel as a stationary phase. The sample was eluted with gradient elution of n-hexane, ethyl acetate and methanol in order of increasing polarity, flow rate 10 mL/min. The fractionation was monitored by using UV detector at 280 nm.

Anti-HCV Assay

The protocol as has been described in (Adianti *et al.*, 2014; Aoki *et al.*, 2014; Tumewu *et al.*, 2016; Suciati *et al.*, 2017). Huh7it-1 cell was obtained from treatment of Huh7 cells with INFα. The cells were seeded in 48-well plates (5×10^4 cells/well) a day before infection. Serial dillutions of the extract was prepared at concentrations of 100, 50, 25, 12.5, 6.25 and 3.13 μg/mL, and for flash column fractions single concentration was prepared at 50 μg/mL. The samples were then mixed with JFH1a virus, with a multiplication of infection (MOI) of 0.1, and inoculated to the Huh7it cells. The mixture was incubated at 37°C for 2 h, and the cells were rinsed twice with serum-free medium to remove the residual virus, followed by incubation for an additional 46 h with the same medium. The culture supernatants were collected at 48 h after infection, and used for virus titration.

Virus titration was conducted by placing Huh7it cells (2.4×10^4 cells/well) in a 96-well plate 1 day prior to virus infection. Culture supernatants obtained from HCV-infected cells were serially

diluted 25-fold in culture medium and inoculated to the cells. The virus was adsorbed to the cells at 37°C for 2 h, followed by incubation for 46 h with a medium containing 0.4% methylcellulose (Sigma-Aldrich). HCV titers were conducted using a focus formation assay. HCV antigen-positive cells were stained with HCV-infected patient's serum and horseradish peroxidase-conjugated goat anti-human IgG (MBL, Tokyo, Japan). A metal enhanced DAB substrate kit (Thermo Fisher Scientific Inc., Rockford, IL, USA) was used to detect the infectious foci, which were then imaged and counted using the Katikati counter software.

The mode of action of the extract was examined by conducting the time of addition experiments. Experiments were done in parallel: (i) to assess the antiviral effect at the entry step, a mixture of HCV and sample was inoculated into the cells. After virus adsorption for 2 hours, the residual virus and the sample were removed. The cells were then refed with fresh medium without the sample for 46 hours; (ii) to examine the antiviral effect at the post-entry phase, HCV was inoculated to the cells in the absence of the sample. After virus adsorption for 2 hours, medium containing samples were then added followed by incubation at 46 hours. The sample was mixed with the virus and inoculated to the cells, and was used as a positive control. After virus adsorption for 2 hours, the residual virus and the sample were removed, and cells were refed with fresh medium containing the sample for 46 hours. Culture supernatants were collected at 1 and 2 days post-infection (dpi) and titrated for virus infectivity. For control, the virus and cells were treated with medium containing 0.1% DMSO.

Cytotoxicity Assay

The cytotoxicity of the sample was determined by MTT method as has been described previously (Tumewu *et al.*, 2016; Suciati *et al.*, 2017). The method was based on the reaction between 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) with enzyme dehydrogenase inside living cells to form an insoluble formazan, which has purple color. This color corresponds to the number of viable cells. In this assay, Huh7it-1 cells were mixed with serial dilution of the samples or control in 96 well plates. After 48 hours incubation, the cells were observed under microscope. The medium was removed from 96 well plates and 150 µl/well of MTT 10% was added, and the mixture was further incubated for 4 hours at 37°C. The MTT solution was removed from 96 well plates and 100 µl/well of DMSO 100% was then added. Reaction mixtures were homogenized by shaking for 0.5 min before measurement of absorbance at 560 nm and 750 nm.

***Fatty acid methyl ester (FAME) and GC-MS Analysis* (Suciati, 2008)**

Fractions SF7 and SF8 (each 6 mg), were dissolved in HCl (1.5 M, 15 mL) in methanol. The solution was then refluxed at 60°C for 2 hours using a water bath. Toluene (1 mL) was added, the solvent was then evaporated in *vacuo*. The crude FAME fraction was purified over SiO_2 eluted with hexanes/EtOAc (1/1) to obtain FAME derivatives, which then subjected to GC-MS analysis. The GC-MS analysis was performed on GC-FID with Agilent Technologies 6890N and GC-MSD with the Agilent 6973 series equipped by Wiley 7n.1 database (2004) on HP-5 column (30 m x 0,250 mm x 0,25 µm). The temperature for GC-MS was started at 100°C, then increased to 250°C at a rate of 16°C/min, and held for 20 mins.

Data analysis

Determination of the anti-HCV and cytotoxicity assays were performed on 96 well plate microplate reader. The number of infected cells was counted and the percentage of inhibition of the samples against the virus was then calculated by comparing to the control. The 50% inhibitory concentration (IC_{50}) was determined by using GraphPad Prism 6 by plotting log concentrations as axis and % inhibition as ordinate.

The EI-MS data were compared to the Wiley 7n.1, NIST and SDBS databases. According to the method from the Commission of European Communities stated in the Commission Decision 2002/657/EC, the identity of a compound can be verified if its MS spectrum showed at least 4 identical fragments compared to standard MS data (European Commission, 2002). ^1H NMR spectra were measured on JEOL ECA 400 at 400 MHz using residual and deuterated chloroform peaks as a reference standard. The data obtained were then compared to literature.

RESULTS AND DISCUSSION

Anti-HCV activity

The ethyl acetate extract of *Stylissa* showed anti-HCV activity with 50% inhibitory concentration (IC_{50}) of 4.4 µg/mL (Table 1). Based on the results of MTT assay, severe cytotoxicity was not observed at the concentrations tested and the CC_{50} value was > 100 µg/mL. The selectivity index was evaluated (SI; CC_{50}/IC_{50}); the SI value was >22.7. The results (Table 2) showed that extract of *Stylissa* at low concentrations (3 and 6 µg/mL) gave higher inhibitions against Huh7it-1 cell lines at post entry step compared to entry step. This result suggested that *Stylissa* extract act at post entry step.

Table 1. Anti-HCV activity of the *Stylissa* extract

Concentration (µg/mL)	% inhibition	Note
100	99.8 ± 0.3	IC_{50}: 4.4 µg/mL
50	99.3 ± 0.9	CC_{50}: >100 µg/m
25	93.2 ± 1.6	SI: >22.7
12.5	73.2 ± 6.6	
6.25	56.6 ± 3.4	
3.13	36.4 ± 6.2	

Note: SI: Selectivity index (CC_{50}/IC_{50}). % inhibition represented as mean ± SD of three independent experiments.

Table 2. Time of addition experiment of the *Stylissa* extract

Concentration (µg/mL)	% inhibition		
	Entry phase	*Post entry phase*	Entry + post entry phase
100	100.0	100.0	100.0
50	95.6	100.0	100.0
25	73.5	73.5	91.2
12.5	69.1	77.9	82.4
6.25	2.9	82.4	69.1
3.13	0,0	73.5	42.7

Table 3. Anti-HCV activity of fractions

Fractions	% inhibition
SF1	< 10
SF2	< 10
SF3	< 10
SF4	< 10
SF5	< 10
SF6	69.5 ± 2.3
SF7	99.3 ± 1.0
SF8	86.4 ± 1.4
SF9	< 10

Note: Samples were tested at 50 µg/mL; data represented as mean ± SD of two independent experiment each performed in duplicate.

Tabel 4. ^{1}H NMR data of fractions SF 7 and SF 8

δ_H (ppm)	Predictions
0.8–0.9	$-CH_3$
1.2–1.3	$-CH_2-$
1.5–1.7	R_3C-H
2.0–2.1	$-C=C-CH_2-$
2.3–2.4	$-CH_2-COO-$
2.84	$-C=C-CH_2-C=C-$
3.75	-CH-OH
5.3–5.4	$-HC=CH-R$

Note: Spectra were recorded at 400 MHz in $CDCl_3$.

The ethyl acetate extract was subjected to flash column chromatography by using gradient elution of n-hexane, ethyl acetate and methanol in order of increasing polarity, and afforded 9 fractions, namely SF1–SF9. The anti-HCV assay was performed on each fraction at a concentration of 50 µg/mL. The results as can be seen in Table 3 showed that two fractions, namely fractions SF7 and SF8, gave > 85% inhibitions, and suggested that the anti-HCV metabolites present in semipolar fractions in which eluted in a combination of ethyl acetate and methanol from flash column chromatography.

Identification of metabolites in active fractions

^{1}H NMR spectra of fractions SF7 and SF8 (Table 4) indicated the presence of fatty acids from signals at δ_H 0.8–0.9 and 1.2–1.7 ppm assign as methyl and methylene groups, respectively (Knothe &Kenar, 2004). Signals for methine group was observed at δ_H 2.3–2.8 ppm, while an alkene signal was seen at δ_H 5.3–5.4 ppm which suggested unsaturated fatty acids. Samples were then derivatized to its methyl ester derivatives and subjected to GC-MS analysis. The Total Ion Chromatogram (TIC) of fraction SF7 exhibited a major peak at a retention time (Rt) 9.857 minutes and several minor peaks, while the TIC of fraction SF8 exhibited a major peak at 8.000 minutes and several minor peaks. The EI-MS of the major peak in SF7 showed molecular ion at $[M]^+$ at *m/z* 298, other peaks were observed at *m/z* 255 [M^+-43], 143 [M^+-155], 87 [M^+-212], 74 [M^+-224, McLafferty rearrangement ion], 55 [M^+-243], and 43 [M^+-255]. Wiley 7n.1 database predicted that the peak corresponded to methyl ester of octadecanoic acid with a similarity score of 99%. Criteria resembiance to the database can be considered equal if > 80% (Odchimar *et al.*, 2016). According to the European Commission Decision 2002/657/EC, the identity of a compound can be verified if its MS spectrum showed at least 4 identical fragments compared to standard MS (European Commission, 2002). Comparison of the EI-MS profile to other databases, such as NIST and SDBS revealed 7 identical peaks. All these data confirmed that fraction SF7 contain octadecanoic acid methyl ester. Other fatty acids present in fractions SF7 and SF8 were identified by this method.

Based on Tables 5 and 6 it can be seen that fractions SF7 and SF8 contain a mixture of saturated and unsaturated fatty acids. Saturated fatty acids present in both fractions are hexadecanoic acid (16:0) and octadecanoic acid (18:0). Fraction SF8 also contains other saturated fatty acids, tetradecanoic acid (14:0), heptadecanoic acid (17:0), and eicosanoic acid (20:0). Both fractions also contain unsaturated fatty acids, in fraction SF7 there are 11-hexadecenoic acid (16:1, Δ11), 10-

Table 5. Analysis of GC-MS Data of fraction SF7

No	Retention time (Mins)	Molecular weight and fragmens (*m/z*)	Prediction[a]	Similarity index (%)
1	8.542	41,43, 55, 69, 97, 83, 84, 87, 96, 97, 98, 152, 194, 236, 268	11-Hexadecenoic acid, methyl ester	99
2	8.667	43, 57, 74, 75, 83, 97, 129, 143, 171, 185, 199, 227, 241, 270	Hexadecanoic acid, methyl ester	99
3	9.719	55, 74, 97, 123, 153, 180. 207, 222, 264, 296	12-Octadecenoic acid, methyl ester	97
4	9.745	41, 55, 69, 74, 83, 87, 97, 180, 207, 222, 264, 296	10-Octadecenoic acid, methyl ester	95
5	9.857	43, 55, 74, 87, 97, 115, 143, 171, 199, 227, 255, 271, 298	Octadecanoic acid, methyl ester	99

Note: Prediction based on Wiley 7n1 database and further confirmed by NIST and SDBS databases.

Table 6. Analysis of GC-MS Data of fraction SF8

No	Retention time (Mins)	Molecular weight and fragmens (*m/z*)	Prediction[a]	Similarity index (%)
1	6.937	43, 57, 60, 73, 97, 129, 157, 185, 200, 228	Tetradecanoic acid	95
2	8.000	43, 74, 87, 97, 115, 143, 185, 227, 270	Hexadecanoic acid, methyl ester	99
3	8.229	43, 73, 97, 129, 171, 199, 227, 256, 270	Heptadecanoic acid	97
4	9.062	41, 51, 74, 96, 123, 152, 180, 199, 222, 247, 264, 296	6-Octadecenoic acid, methyl ester	97
5	9.199	43, 74, 97, 115, 143, 171, 199, 227, 255, 271, 298	Octadecanoic acid, methyl ester	98
6	10.437	15, 45, 74, 97, 116, 143, 171, 199, 227, 255, 283, 326	Eicosanoic acid, methyl ester	99

Note: Prediction based on Wiley 7n1 database and further confirmed by NIST and SDBS databases.

octadecenoic acid (18:1, Δ10), and 12-octadecenoic acid (18:1, Δ12), whereas at fraction SF8 there is 6-octadecenoic acid (18:1, Δ6).

According to Leu *et al.* (2004), it is known that unsaturated fatty acids such as arachidonic acid (AA), docosahexaenoic acid (DHA), and eicosapentaenoic acid (EPA) have antiviral activity by inhibiting RNA replication of viruses. Some unsaturated fatty acids such as α-linolenic acid, γ-linolenic, and linoleic acid only showed low antiviral activity. While saturated fatty acids such as oleic acid, myristic acid, palmitic acid, and stearic acid had no antiviral activity against HCV (Leu *et al.*, 2004). Thormar *et al.* reported that short-chain and long chains saturated fatty acids did not have or have low activity against the enveloped virus. While medium chain saturated and long chain unsaturated fatty acids are very active against the enveloped virus (Thomar *et al.*, 1987). HCV is an RNA enveloped virus (Kim & Chang, 2013). According to Beermann *et al.* (2003) category of fatty acids based on the number of carbon are short chain fatty acid (< 8 carbon atoms), medium chain fatty acid (8–16 carbon atoms) and long chain fatty acid (> 16 carbon atoms). Therefore, it is possible that the anti-HCV activity in fractions SF7 and SF8 due to the presence of a mixture of medium chain saturated fatty acids (tetradecanoic acid and hexadecanoic acid) and long chain unsaturated fatty acids (11-hexadecenoic acid (16:1, Δ11), 10-octadecenoic acid (18:1, Δ10), and 12-octadecenoic acid (18:1, Δ12) in fraction SF7 and 6-octadecenoic acid (18:1, Δ6)).

CONCLUSION

A marine sponge from the genus *Stylissa* has shown potency as an anti-HCV agent. Following bioassay-guided isolation, the semipolar fractions possessed higher activity than other fractions. ^{1}H NMR and GC-MS analyses indicated that metabolites present in the active fractions were a mixture of saturated and unsaturated fatty acids.

ACKNOWLEDGEMENTS

Authors acknowledge Faculty of Pharmacy Universitas Airlangga for research grant BOPTN 2017. Authors also thank Dr Takaji Wakita and Dr Hak Hotta through collaboration as part of the Science and Technology Research Partnership for Sustainable Development (SATREPS 2010-2014) for providing pJFH1 and Huh7 cell lines.

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