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Antiviral Activity of Marine Sponges *Homaxinella tanitai* and *Microxina Subtilis* against Hepatitis C Virus.

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ABSTRACT

The aim of the study was to screen antiviral activity of marine sponges extracts and fractions against hepatitis C virus, as well as to investigate the mode of action of the extract. The results showed that extracts of marine sponges *Homaxinella tanitai* dan *Microxina subtilis* inhibited the growth of HCV at IC_{50} of 27.1 and 40.5 µg/mL, respectively. Three fractions from *H. tanitai* and two fractions from *M. subtilis* gave more than 50% inhibiton againts HCV at concentration 100 µg/mL. The time of addition experiment revealed that *H. tanitai* and *M. subtilis* extracts both act at enty and post entry steps. These results suggest that *H. Tanitai* and *M. subtilis* extracts could serve as potential candidates for antiviral agents against hepatitis C virus.

Keywords: antiviral, hepatitis C virus, marine sponges, *Homaxinella tanitai*, *Microxina subtilis*

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INTRODUCTION

Hepatitis C virus (HCV) is a positive-stranded RNA virus in the genus Hepacivirus of the family Flaviviridae. HCV has been considered as causatif agent of both acute and chronic hepatitis leading to the development of hepatic cirrhosis and hepatocellular carcinoma (1,2). Approximately 130-150 million people globally infected by hepatitis C, and estimated 700.000 people die each year from hepatitis C (World Health Organization, 2016). Based on the heterogeneity of the viral genome, HCV is currently classified into seven genotypes (3–9) and more than 67 subtypes (1a,1b, 2a, 2b etc.) (3, 4). The viral genome, which is a singlestranded open reading encoding a polyprotein precursor consisting of 3000 amino acid residues that is processed by virus-encoded and host cellular proteases into structural and nonstructural proteins. The HCV proteins also play essential roles in the pathological processes associated with HCV infection, such as carcinogenesis as well as glucose and lipid metabolic disorders (4, 5). Current standar therapy for hepatitis C is using triple combination of pegylated interferon, ribavirin and specific NS3 serine protease inhibitor, such as telaprevir and boceprevir. The treatment is considered effective with viral clearance rate to >70% (10,11,12). However, it cause severe side effect, such as anemia and skin rashes from the use of telaprevir and boceprevir, and the presence of drug-resistence virus. Therefore the search for effective and safer therapy for hepatitis C by using natural resources, such as marine sponges is still needed.

The oceans, which occopies over 70% of the Earth's surface, have been the habitat of various living creatures, including algae, sponges, cnidarians, molluscs, bryozoans, ascidians and echinoderms as well as microorganisms. This species diversity makes the marine environment one of the most prolific sources of natural products. Many of these marine creatures produce unique and biologically active compounds which may not be found in the terrestrial ecosystem. These metabolites may be produced as a means of self-defence against predation, since many marine organisms have no spine or protective shell (13). Amongst marine resources, 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 5300 metabolites have been reported from this organism (14). Many of these metabolites showed pronounced bioactivity including antiviral.

In this study we investigated anti HCV activity of ethyl acetate extracts of *Homaxinella tanitai* and *Microxina subtilis* collected from Barrang Lompo Island, South Sulawesi, Indonesia. Flash column fractions of the sponge extracts were also subjected to anti HCV assay. In order to determine the mode of action of the extracts time of addition experiments were conducted.

MATERIAL AND METHODS

Biological Material

Sponges were collected by using SCUBA at a depth of 8-10 m from around Barrang Lompo Island, Makassar, South Sulawesi on May 17th 2014. Samples were kept in plastic pack in ice boxes immediately after collection. Sponge specimens were then freezed at -20°C until analysis. Identification of sponges were conducted by Ecology Laboratory, Department of Biology, Faculty of Mathematic and Sciences, Institut Teknologi Sepuluh November, Surabaya. Voucher specimens were kept in etanol 70% at the Faculty of Pharmacy, Universitas Airlangga under the accession number 17-5-14-3 and 17-5-14-4.

Extraction and fractionation

Fresh sponges (wet weight 100 – 200 gram) were diced into small pieces and filled in glass container. Samples were extracted in dichloromethane: methanol (1:1) by using ultrasonic vibration for 3 x 5 mins each extraction. The solvent was removed by filtration and the residue was then re-extracted using the same procedure twice. The collected filtrate was evaporated under reduced pressure to give an aqueous residue, which was then partitioned with EtOAc (3 x 50 mL). The organic layer was removed, dried over anhydrous Na_2SO_4, and concentrated under reduced pressure to afford brown viscous extracts.

Anti HCV Assay

The protocol as has been described in (15,16,17). Huh7it cells were seeded in 48-well plates (5×10^4 cells/well) a day before infection. The same volume of JFH1a, with multiplication of infection (MOI) of 0.1 focus-forming units (ffu)/cell,was mixed with serial dilutions of the extracts (100, 50, 25, 12.5, 6.25 and 3.1 µg/ml) and inoculated to the cells. Single concentration of 100 µg/ml samples were used for flash column fractions, as well as for time of addition experiments. The mixture was incubated at 37°C for 2 h, and the cells were rinsed twice with serum-free medium to remove residual virus, followed by incubation for an additional 46 h with the same medium. At 48 h p.i., the culture supernatants were collected and used for virus titration.

Virus titration was conducted by placing Huh7it-1 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 extracts were examined by conducting time of addition experiments. Two sets of 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 sample for 46 hours; (ii) to assess the antiviral effect at the post entry step, 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; (iii) as a positive control, HCV mixed with the sample was inoculated to the cells. 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 obtained at 1 and 2 days post-infection (dpi) and titrated for virus infectivity. Virus and cells treated with medium containing 0.1% DMSO served as a control. The percentage of inhibition of the samples against the virus were then calculated by comparing to the control.

Cytotoxicity Assay

The cytotoxicity of the samples was determined by MTT method as has been described previously (17). The method was based on colorimetric reaction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide with enzyme dehydogenase inside living cells to form a coloured formazan dye, which corresponded to the number of viable cells. In this assay Huh7it cells were mixed serial dillution of the samples or control in 96 well plates. After 46 hours incubation condition of the cells were observed under microscope. The medium was removed from 96 well plates and then 150 µl/well of MTT 10% was added, followed by incubation 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 homogenize by shaking for 0.5 min before measurement of absorbance at 560 nm and 750 nm.

RESULTS

Anti HCV activities of *Homaxinela tanitai* and *Microxina subtilis* extracts

The ethyl acetate extracts of *H.tanitai* and *M. subtilis* showed anti-HCV activities with 50%-inhibitory concentration (IC_{50}) of 27.1 µg/ml and 40.5 µg/ml, respectively (Table 1). Cytotoxicity of the crude extract against Huh7it-1 cells was determined using 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 values were >3.7 and >2.5 respectively.

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Table 1. IC_{50} and CC_{50} values of *H. tanitai* and *M. subtilis* extracts

Samples	IC_{50} (µg/ml)	CC_{50} (µg/ml)	SI[a]
H. tanitai	27.1	>100	>3.7
M. subtilis	40.5	>100	>2.5

[a] SI: Selectivity index (CC_{50}/IC_{50})

The ethyl acetate extracts of both marine sponges were subjected to flash column chromatography by using Sepacore® flash column chromatography, employing gradient elution of n-hexane, ethyl acetate and methanol in order of increasing polarity to obtain 7 fractions. Each fractions were then submitted to anti HCV assay using single concentration of 100 µg/ml. The percentage of inhibition of each fractions was calculated (Table 2).

Table 2. Anti HCV activities of *H. tanitai* and *M. subtilis* fractions

Samples	%Inhibition
H.tanitai Fraction 1	0
H.tanitai Fraction 2	0
H.tanitai Fraction 3	0
H.tanitai Fraction 4	88.8 ± 3.5
H.tanitai Fraction 5	96.6 ± 2.2
H.tanitai Fraction 6	69.2 ± 11.2
H.tanitai Fraction 7	49.4 ± 4.7
M. subtilis Fraction 1	0
M. subtilis Fraction 2	0
M. subtilis Fraction 3	0
M. subtilis Fraction 4	0
M. subtilis Fraction 5	36.8 ± 3.5
M. subtilis Fraction 6	88.9 ± 6.9
M. subtilis Fraction 7	80.8 ± 8.7

Samples were tested at 100 µg/ml; data represented as mean ± SD of two independent experiment each performed in duplicate.

Time of addition experiments were performed to determine the possible mode of action of the extracts. Single concentration of extracts were used in this experiment. Tested samples were added to the cell only during viral inoculation or only after viral inoculation for the remaining culture period until virus harvest. The results (Table 3) suggested that both extracts inhibited HCV activities at both entry and post-entry steps.

Table 3. Time of addition analysis of *H tanitai* and *M. subtilis* extracts

	%Inhibition			
Samples[a]	During inoculation	Post inoculation	During+Post inoculation	Mode-of-Action
H. tanitai	88.7	84.2	99.6	Entry and post-entry inhibition
M. subtilis	38.1	45.4	86.9	Entry and post-entry inhibition

[a]Samples tested at 100 µg/ml

DISCUSSION

Marine sponges have been the fruitful source of many bioactive metabolites, including antiviral. Several marine sponges have been reported to have anti HCV activity, such as *Amphimedon sp*.(18) and undescribed species of the genus *Latrunculia* (19). The ethyl acetate extract of *Amphimedon sp*. showed inhibition of both protease and helicase activities of hepatitis C virus NS3. Anti HCV metabolites have also been succesfully isolated from marine sponges, such as halisulfate 3 and suvanine both obtained from unidentified Demospongiae spong derived from Okinawa Japan. Both compounds inhibited NS3 helicase-activity in a dose dependent manner with IC_{50} values of 4 and 3 µM, respectively (12). Another sponge metabolite reported to have anti HCV activity is a series of alkaloid discorhabdins, i.e discorhabdins A, C and dihydrodiscorhabdin C with EC_{50} value < 10 µM (19).

In this study ethyl acetate extracts of *H. tanitai* and *M. subtilis* exhibited anti HCV activity with IC_{50} values of 27.1 and 40.5 µg/mL, respectively. In addition, both extract did not dispaly cytotoxicity at the highest concentration tested 100 µg/mL. Flash column chromatography on the extracts were carried out, and obtained seven fractions, which then subjected to anti-HCV assay. The results (Table 2) showed that three fractions from *H. tanitai* and two fractions from *M. subtilis* gave > 50% inhibitions against HCV at concentration of 100 µg/mL. In addition, these results also indicated that the possible anti HCV metabolites of *H. tanitai* and *M. subtilis* are semi polar or polar compounds, in which eluted in combination of ethyl acetate and methanol from flash column chromatography.

In order to determine the mode of action of extracts, whether it exerted the effect on the entry or post entry steps, time of addition experiments were carried out. To examine the HCV effect at the entry step, sample was only added at the first 2 h of experiment, followed by incubation of cells with fresh media without sample for 46 h. Meanwhile, to determine whether sample act the post entry step, virus was inoculated into the cell in the absence of sample, after virus adsorption for 2 h, sample was added and incubated for 46h. The results (Table 3) showed that extract of *H. tanitai* exhibited 88.7 and 84.2 % inhibition against HCV at 99.6 µg/mL during inoculation and post inoculation, respectively. However, better inhibition (100%) of extract was obtained when it was added through out experiment. This result suggested that *H. tanitai* extract act at both entry and post entry steps. At the same concentration extract of *M. subtilis* showed lower inhibition againts HCV compare to extract of *H. tanitai* at 38.1 and 45.4 % during inoculation and post inoculation, respectively. However, the extract gave higher inhibition at 86.9%, when it was added in both steps (during and after inoculation), which indicated that *M. subtilis* extract exerted the activity at entry and post entry steps.

CONCLUSION

The results of this study suggest that the ethyl acetate extracts and fractions of marine sponges *Homaxinella tanitai* and *Microxina subtilis* exhibit antiviral activity against hepatitis C virus. Both extracts exerted the effect at entry and post entry steps.

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