Advances in Biological Sciences Research, volume 22
7th International Conference on Biological Science (ICBS 2021)

Anti-Infective Properties of a Sea Cucumber Associated Actinobacteria *Kocuria* sp. HL 55

Joko Tri Wibowo[1,*]

[1] *Research center for Biotechnology National Institute of Research and Innovation Agency (BRIN) Komplek CSC-LIPI Jln. Raya Bogor Km 46, Cibinong 16911, Bogor, Jawa Barat, Indonesia*
[*] *Corresponding author. Email: joko.tri.wibowo@brin.go.id*

ABSTRACT
"Antibiotics golden era" can end when resistant strains spread immensely. To find new anti-infective agents, we examined sea cucumber associated bacteria isolated from the intestinal part of the echinoderm *Holothuria leucospilota*. Partial identification using 16S rRNA gene Sanger sequencing revealed *Kocuria Flava* (99.21% sequence similarity). The ethyl acetate extract from liquid bacterial cultures has a MIC of 4.2, 8.3, and 67 µg/mL against *Bacillus subtilis*, *Staphylococcus aureus,* and *Eschericia coli*, respectively. The extract also showed antiviral activity against Hepatitis C Virus (HCV) with an infectivity value of 77.25 ± 3.13%. The bioactive fraction from the extract showed two peaks from two putative compounds. One peak showed *m/z* 1140.219 $[M+H]^+$ were identified as putative kocurin. Another peak with *m/z* 1515.373 $[M+H]^+$ was unidentified. The results showed that *Kocuria* sp. HL 55, a bacterium associated with *H. leucospilota*, potentially produces new bioactive compounds. Further analysis for instance extensive 1D and 2D NMR is needed to identify and to characterize the unidentified compound.

Keywords: *Actinobacteria, Antibiotic, Antiviral, Sea cucumber.*

1. INTRODUCTION

The spreading of resistant microbes due to increased human movement causes higher morbidity and mortality, costlier treatment, more extended hospital stays, thus placing a more significant burden on the health systems [1]. Nonetheless, studies on the development of new antibiotics are needed despite complicated problems such as complex criteria (*i.e.,* high efficacy, broad-spectrum, low toxicity), high cost, and rediscovery of known antibiotics [2,3].

The number of studies on the isolation of invertebrate-associated bacteria to discover new bioactive compounds have been increased, with sponges, members of invertebrates received the most attention for this purpose. However, the information about this is still limited. *Holothuria leucospilota* has shown antioxidant and antitumor activities *in vitro* against HeLa, A549 (human lung carcinoma), and B16F10 (skin melanoma) cell lines [4,5]. Bioactive compounds namely echinoside B, leucospilotasides A-C, holothurins A, B, and B2 have been isolated from *H. leucospilota* [6]. Therefore, in this study, *H. leucospilota* associated bacteria are isolated and screened for their potential as novel anti-infective compound producers.

2. METHODOLOGY

2.1. Bacterial isolation and identification

Sea cucumber associated bacteria have been isolated from the intestine of *Holothuria leucospilota*. Then, a bioactive bacterium was partially identified using 16S rRNA Sanger Sequencing (forward primer: 27F 5'-AGAGTTTGATCCTGGCTCAG-3'; reverese primer: 1492R 5'-GGTTACCTTGTTACGACTT-3') [7].

2.2. Extract Preparation

A single bacterial colony from an agar plate was picked and transferred into an Erlenmeyer bottle containing 100 mL of medium marine broth (MB) and then incubated at room temperature (~ 23 °C) for 10 x 24 hours until the cultures reached a late stationary phase

(examined with OD_{600}). The ethyl acetate (EtOAc) extract was obtained by stirring liquid cultures with EtOAc 1:2 (v/v) at a stirring speed of 12,000 rpm for 30 seconds using an Ultra-Turrax T65. The EtOAc extract was concentrated using a rotary evaporator. Extracts were kept in a −20 °C room.

2.3. Antimicrobe assay

The assay was conducted using the microdilution technique against *Pseudomonas aeruginosa* (PA16) and *Escherichia coli* (DSM 1116) (both are Gram-negative bacteria); *Bacillus subtilis* (DSM 10), *Mycobacterium smegmatis* (ATCC 7000048), and *Staphylococcus aureus* (DSM 346) (Gram-positive bacteria); *Candida albicans* (DSM 1665) and *Rhodotorula glutinis* (DSM 10134) (yeasts); and a fungi *Mucor hiemalis* (DSM 2656). The detailed assay method can be found in [7]. Briefly, the test bacteria were incubated overnight in the medium Mueller-Hinton buillon (MHB) and fungi/yeast in the medium MYC pH 7.0 (contains 1.0% glucose, 1.0% phytone peptone, and 1.19% HEPES) at 30 °C, 160 rpm. The ethyl acetate extract was diluted from well A to H in 1:2 steps for each test organism in a microtiter plate. The minimum inhibitory concentration (MIC) was observed in the most diluted sample, which is still active.

2.3. Anti-Hepatitis C Virus (HCV) assay

The extract was also tested against Hepatitis C Virus (HCV) using Huh7.5 cells. EGCG (epigallocatechin gallate) serves as a positive control. Further information on the assay can be found in [7]. Briefly, Huh7.5 cells with *Firefly luciferase* gene expression, with or without the samples, were transfected with the *Renilla* reporter virus from Jc1. Antiviral activity was measured by calculating the percentage of *Renilla* compared to the negative control. *Firefly luciferase* was measured to observe the cytotoxic effect of the sample on the cells.

2.4. Chemical analysis

The EtOAc was fractionated using the HPLC condition as mentioned in [8]. Briefly, the extract was injected in a semi-preparative HPLC and collected every 30 s/150 µL in a microplate. The plate was then incubated with the test organism. Chemical analysis using HR-ESI-MS in positive mode was conducted to analyze the compounds in the bioactive fractions which correlate with the retention time.

3. RESULTS

The associated culturable associated bacteria from *H. leucospilota* were isolated and tested in a preliminary antibacterial assay. The active bacteria were partially identified using 16S rRNA gene Sanger sequencing. One of them (HL 55) matched *Kocuria flava* (accession number NR_044308.1) with a sequence similarity of 99.21% to the NCBI database. The 16S rRNA sequence of the bacterium can be accessed in the NCBI database with accession number MK696544 [7].

EtOAc extract of *K. Flava* HL 55 showed bioactivity contra Gram-positive bacteria *Bacillus subtilis* and *Staphylococcus aureus* with the MIC of 4.2 and 8.3 µg/mL. The extract also exhibited antibiotic activity against *Eschericia coli* with the MIC of 67 µg/mL (Table 1). However, there was no activity observed against the other test microorganisms.

The extract also showed antiviral activity against HCV with a percent infectivity of 77.25 ± 3.13%. This value was higher than EGCG (percent infectivity = 37.45 ± 3.38%) [7]. This result can be produced by a low concentration of active materials from the extract. Fractions and isolates with the same concentration may result in higher activity. Further study is needed to confirm the hypothesis.

The bioactive fraction from the extract showed two peaks which may represent two putative compounds. One putative compound with a mass per charge ratio (in positive mode) of 1140.219 $[M + H]^+$ was identified as putative kocurin (Fig. 1), while another compound with a mass per charge ratio (in positive mode) of 1515.373 $[M + H]^+$ was unidentified. These compounds need to be further isolated and analyzed to elucidate and/or confirm their structure.

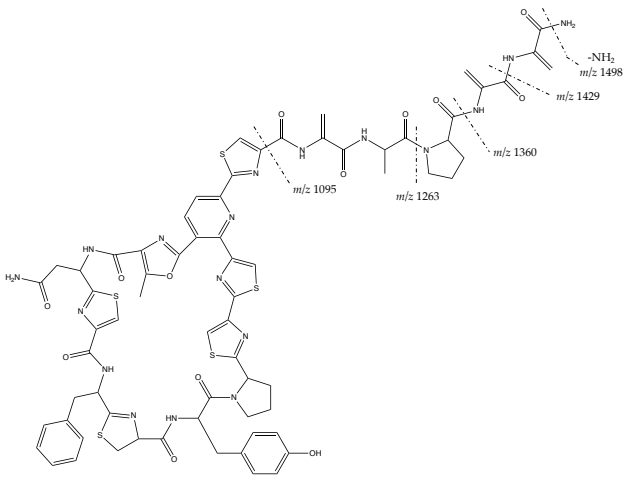

Figure 1. The structure of kocurin and the interpretation of the fragmentation based on MS^2 spectra [7].

Both putative compounds can be accountable for the bioactivity of *K. Flava* HL 55. Kocurin has shown antibiotic activity contra methicillin-resistant

Staphylococcus aureus (MRSA) [9], but no antiviral activity has been reported. Also, there is no bioactivity study for the putative compound with an *m/z* value of 1515.373 $[M + H]^+$. Therefore, it is interesting to further analyze the structure and bioactivities of that putative compound.

Sea cucumber associated actinobacterium *K. Flava* HL 55 can be the source of novel anti-infective compounds. However, further studies are required to isolate, identify, and characterize the active compounds from the bacterium.

ACKNOWLEDGMENTS

The author would like to express sincere gratitude to the collaboration program GINAICO (Germany-Indonesia for Anti-infective Cooperation) from the DAAD.

REFERENCES

[1] I. Frost, T.P. Van Boeckel, J. Pires, J. Craig, R. Laxminarayan, Global geographic trends in antimicrobial resistance: the role of international travel, Journal of travel medicine vol. 26, 2019.

[2] S. Bernardini, A. Tiezzi, V. Laghezza Masci, E. Ovidi, Natural products for human health: a historical overview of the drug discovery approaches, Natural product research vol. 32, 2018, pp. 1926-1950.

[3] K. Lewis, Platforms for antibiotic discovery. Nature reviews Drug discovery vol. 12, 2013, pp. 371-387.

[4] J. Baharara, E. Amini, N. Nikdel, F. Salek-Abdollahi, The cytotoxicity of dacarbazine potentiated by sea cucumber saponin in resistant B16F10 melanoma cells through apoptosis induction, Avicenna journal of medical biotechnology vol. 8, 2016, pp. 112.

[5] M. Soltani, K. Parivar, J. Baharara, M.A. Kerachian, J. Asili, Putative mechanism for apoptosis-inducing properties of crude saponin isolated from sea cucumber (*Holothuria leucospilota*) as an antioxidant compound, Iranian journal of basic medical sciences, vol. 18, 2015, pp. 180.

[6] R. Pangestuti, Z. Arifin, Medicinal and health benefit effects of functional sea cucumbers, J Tradit Complement Med., vol. 8, 2018, pp. 341-351, DOI:10.1016/j.jtcme.2017.06.007.

[7] J.T. Wibowo, M.Y. Kellermann, D. Versluis, M.Y. Putra, T. Murniasih, K.I. Mohr, J. Wink, M. Engelmann, D.F. Praditya, E. Steinmann, *et al.*, Biotechnological Potential of Bacteria Isolated from the Sea Cucumber *Holothuria leucospilota* and *Stichopus vastus* from Lampung, Indonesia. Mar. Drugs vol. 17, 2019. DOI:10.3390/md17110635.

[8] P.W. Okanya, K.I. Mohr, K. Gerth, R Jansen, R. Müller, Marinoquinolines A− F, Pyrroloquinolines from *Ohtaekwangia kribbensis* (Bacteroidetes). J. Nat. Prod., vol. 74, 2011, pp. 603-608.

[9] J. Martin, S.S.T. da, G. Crespo, S. Palomo, I. Gonzalez, J.R. Tormo, M. de la Cruz, M. Anderson, R.T. Hill, F. Vicente, *et al.*, Kocurin, the true structure of PM181104, an anti-methicillin-resistant *Staphylococcus aureus* (MRSA) thiazolyl peptide from the marine-derived bacterium *Kocuria palustris*, Mar Drugs vol. 11, 2013, pp. 387-398, DOI:10.3390/md11020387.