

RESEARCH ARTICLE

Cytotoxicity Evaluation of Bacteria Extract Isolated from Indonesia Sheep Manures and Fungal Fruiting Body

Leonny Yulita Hartiadi ^{1*}, Immaculata Titis Winiati ², Samirah ², Cristina Gómez ²

¹ Department of Pharmacy, School of Life Sciences, Indonesia International Institute for Life Sciences, Jakarta, Indonesia

² Department of Biomedicine, School of Life Sciences, Indonesia International Institute for Life Sciences, Jakarta, Indonesia

*corresponding author. Email: leonny.hartiadi@i3l.ac.id

ABSTRACT

Introduction: Cancer is one of the leading cause of death worldwide. Chemotherapy remains the most widely used approach to treat cancer. However, due to the lack of specificity, drug resistance, and undesirable side effects, the discovery of novel anticancer drugs is still a priority in cancer research. Bacteria are one of the major contributors for anticancer compounds as bacteria can produce secondary metabolites which may have cytotoxic activity. **Method:** A literature study was conducted to identify the isolates in i3L-USAID bacteria database that were potential candidates for novel anticancer drugs. The selected bacteria were cultured and their secondary metabolites were extracted. Cytotoxic activity of the extracts was assessed through morphological observation and MTT assay on HeLa and 3T3 fibroblast cell lines. **Results:** *Aneurinibacillus sp.* and *Ochrobactrum sp.* were selected. *Ochrobactrum sp.* extract significantly suppressed the growth of HeLa cells at 1000 µg/ml ($p < 0.05$) while it did not have any effect on 3T3 cells. Despite able to suppress the HeLa cell proliferation, *Aneurinibacillus sp.* extract significantly enhanced the growth of 3T3 fibroblast at the 1000 µg/ml ($p < 0.05$). The cytotoxicity activity was corroborated by cell death observed on HeLa cells treated with high concentrations of the extracts **Conclusion:** Secondary metabolites from *Aneurinibacillus sp.* and *Ochrobactrum sp.* could only exert cytotoxic effect at high concentration which is unfavourable for anticancer compounds. Although the extracts resulted in a reduction of HeLa cells proliferation, the extracts did not inhibit proliferation of 3T3, suggesting selectivity for non-tumorigenic over tumorigenic cells.

Keywords: cytotoxicity; anticancer; *Aneurinibacillus*; *Ochrobactrum*

INTRODUCTION

Cancer is a major health problem and one of the leading causes of mortality worldwide. The World Health Organization (WHO) reported in 2012, approximately 14 million new cancer cases occur worldwide and the number is predicted to increase by around 70% in the next two decades. Cancer occurs at substantial variation across and within countries due to complex interaction between host and cancer risk factors that are linked to aging population, genetic vulnerability, socio-economic status, as well as individual lifestyle (Bray *et al.*, 2018)

With the advance of research and development, there are many options provided for cancer treatment. A patient might be subjected to a single or combination therapy of the available treatment options such as surgery, radiation, chemotherapy, targeted therapy, immunotherapy, hormone therapy, stem cell transplant, and precision medicine (National Cancer Institute, 2017). Among these options, chemotherapy remains as the best option for standard protocol and widely distributed to combat various types of cancer, especially at the late stage (Miller *et al.*, 2016). Unfortunately, the development of drug resistance has reduced the effectivity of certain chemotherapy. This condition, along with undesirable side effects of chemotherapy, has increased the demand to discover a novel agent with greater therapeutic efficacy and fewer side effects (Demain & Vaishnav, 2011).

Many chemotherapies that serve as effective medicines for over 50 years in the market are isolated from natural resources, mainly plants and microbes (Demain & Vaishnav, 2011). Bacteria are type of microbes that demonstrate a growing number of studies for their role in cancer therapy. Actinomycin D, bleomycin, doxorubicin, and mitomycin are examples of anticancer drugs that have been successfully isolated from the

secondary metabolites of bacteria (Karpiński & Adamczak, 2018).

During the period 2014-2017, i3L conducted a research project funded by the USAID to identify novel microorganisms and microbial enzymes from Indonesian peatland and herbivores manures to improve bioconversion processes. More than 500 bacteria, yeast, and fungi were successfully isolated and identified until the genus level. Among the genus discovered, some of them have been described, or have members among the group, as antibiotic, antifungal and cytotoxic compounds producers. Therefore, these bacteria collection is a promising source for identification of new cytotoxic molecule to treat cancer.

This study aims to evaluate the previously isolated bacteria from USAID project for their potential cytotoxicity activity on cancerous and non-cancerous cell lines which could lead to the development of novel anticancer agents.

MATERIAL AND METHODS

Literature Study

Database containing all of the isolated microbes from previous USAID project was used and all of the bacteria identification codes (IDs) was recorded. Each ID was screened using PubMed, NCBI BLAST and Google Scholar to screen the relevant studies about the species. Selection of bacteria was determined based on several criteria, in which the selected genus must have: (i) limited or no publications about their potency to produce cytotoxic activity against cancer cell; (ii) an indication to possess antioxidant, antimicrobial, or to have bioactive components; (iii) shared phylogenetic similarities with another type of bacteria that have possessed the presence of potent anticancer compound.

Bacteria Culture

Selected bacteria were cultured from cryo-stock onto nutrient agar several times until a single colony grew. *Ochrobactrum sp.* isolates were pre-cultured in broth at $30 \pm 2^\circ\text{C}$ for 48 hours in peptic dextrose broth while *Aneurinibacillus sp.* isolates were pre-cultured at $50 \pm 2^\circ\text{C}$ for 24 hours in tryptic soy broth as previously described (Zu *et al.*, 2014; Lee *et al.*, 2014). The pre-cultured bacteria was further used for growth curve plotting.

Growth Curve Plotting

Growth curve plotting was carried out to determine the stationary growth phase of the bacteria in which during this phase the bacteria was expected to produce the optimal amount of secondary metabolites. 5 ml of pre-cultured bacteria was transferred to 100 mL of respective broth media and was incubated at the designated temperature. 200 μL of each bacteria culture was transferred to 96-well plate in triplicate. The bacteria-free broth was used as the blank. The apparent absorption (optical density [OD]) of the culture was measured using NanoQuant microplate reader at 600 nm every 12 hours. Then, the OD versus time points were plotted to show the growth rate.

Extraction of Bacteria's Secondary Metabolite

Bacteria culture obtained from the growth curve was used. Once the bacteria achieved the stationary growth phase, the culture was transferred to falcon tubes and centrifuged at 3000-4000 rpm for 15-20 minutes. The supernatant was collected and filtered using 0.22 μm membrane cellulose filter to remove bacterial cells. Cell-free supernatant was mixed with ethyl acetate in a ratio of 1:1 (v/v) and the ethyl acetate layer was collected and dried on water bath at 40°C . The dried crude extract was weighed and dissolved in 0.5% DMSO for cytotoxicity test.

Cell Culture

HeLa and NIH-3T3 fibroblast cell lines were obtained from Indonesia International Institute for Life Sciences. Both cells were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum and 100 $\mu\text{g}/\text{mL}$ penicillin and at 37°C under 95% humidified air and 5% CO_2 separately.

Cytotoxicity Test

Cells were seeded into 96-well plates at a concentration of 5×10^3 cells per well and starved in serum-free media for 24 hours. Cells were then treated with various concentrations (62.5, 125, 250, 500, and 1000 $\mu\text{g}/\text{mL}$) of crude extracts for another 24 hours. The cell viability was assessed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Briefly, 15 μL of dye solution was added into each well and incubated at 37°C in humidified atmosphere of 5% CO_2 for 4 hours. 100 μL of stop solution was added and the plate was incubated for 1 hour in room temperature under dark condition. Absorbance was then measured at 570 nm. Media only was used as a blank while cells grown in the media was used as a control. 10% DMSO was used as a negative control. The percentage of cell viability was calculated as followed:

$$\frac{[\text{sample absorbance} - \text{blank absorbance}]}{[\text{control absorbance} - \text{blank absorbance}]} \times 100\%$$

Morphological Observation

Morphological observation was carried out following 24 hours incubation period of the treatment, right before the MTT assay. Pictures were taken using ZEISS Axiocam microscope cameras via ZEN Imaging Software.

Data Analysis

The data obtained were statistically analyzed using two-way ANOVA compared to

control. The p value of 0.05 was used to determine the significance level. All data were taken from triplicate measurements and were expressed as mean \pm standard deviation.

RESULTS AND DISCUSSIONS

Bacteria Selection and Growth Curve

Based on our literature study, *Ochrobactrum sp.* and *Aneurinibacillus sp.* were selected as these 2 bacteria fulfilled the selection criteria. *Aneurinibacillus sp.* is reported to produce gramicidin S, a type of antibiotic while *Ochrobactrum sp.* belong to Proteobacteria phylum, known for the ability to produce secondary metabolites predominantly with antibacterial and anticancer compounds (Destriac *et al.*, 2013; Raimundo *et al.*, 2018; Gause *et al.*, 1944). Database showed that *Aneurinibacillus sp.* was isolated from barbary sheep manure in Taman Safari Indonesia, Cisarua, West Java and *Ochrobactrum sp.* was isolated from fungal fruiting body in Riau.

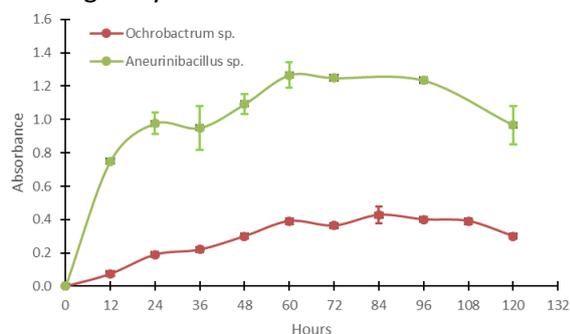


Figure 1. Growth curve of *Ochrobactrum sp.* and *Aneurinibacillus sp.*

As shown in figure 1, the stationary phase of *Ochrobactrum sp.* and *Aneurinibacillus sp.* was achieved during 84-96 and 60-96 hours after the incubation. During the stationary phase, the bacteria growth reached saturation as indicated by a plateau portion of the growth curve (Thirumurugan *et al.*, 2018). In this phase, bacteria release secondary metabolites that become important for the livelihood of the bacteria. Thus, the extraction was performed at 96 hours for both bacteria

to ensure maximum production of the secondary metabolites. The yield of dried secondary metabolites extract was 8.4 and 6.7 mg for *Ochrobactrum sp.* and *Aneurinibacillus sp.* respectively.

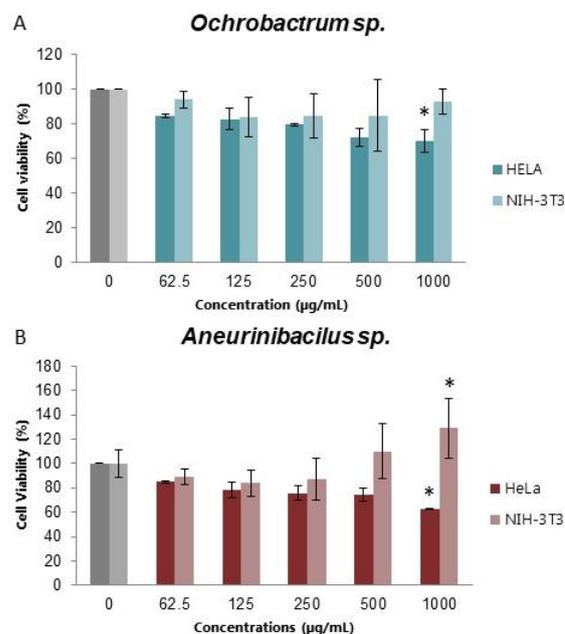


Figure 2. The effect of *Ochrobactrum sp.* and *Aneurinibacillus sp.* secondary metabolites extracts towards HeLa and NIH-3T3 cell lines

The secondary metabolites extracts were dissolved in 0.5% DMSO. 0.5% DMSO did not affect cell viability of HeLa and NIH-3T3 as both cells had a viability of 100% following 24 hours incubation with 0.5% DMSO (data not shown). Treatment with *Ochrobactrum sp.* and *Aneurinibacillus sp.* secondary metabolites extracts decreased cell viability of HeLa in a concentration-dependent manner where significance was observed at a concentration of 1000 $\mu\text{g/ml}$ (figure 2A and 2B, $p < 0.05$). In line with this, morphological observation on HeLa cells showed that *Ochrobactrum* and *Aneurinibacillus* extract resulted in less viable cells compared to control (figure 3). The cells morphology were also similar to death cells treated with 10% DMSO, showing loss of volume, circular morphology, and cell shrinkage (figure 3). A

significant amount of visible debris was observed, possibly be due to the presence of impurity from extract or cell death. The reduced number of viable cell indicates that at high concentrations, secondary metabolites of *Ochrobactrum sp.* and *Aneurinibacillus sp.* had cytotoxic activity. Nevertheless, the extracts were not potent enough to be developed as potential anticancer agents as the reduction of HeLa's cell viability only reached $78.9 \pm 6.5\%$ and $62.6 \pm 7.9\%$ for *Ochrobactrum* and *Aneurinibacillus* extracts respectively at a relatively high concentration. According to National Cancer Institute, an extract is considered to have a potent anticancer activity if the extract has an $IC_{50} < 30 \mu\text{g/ml}$ (Vijayarathna & Sasidharan, 2012).

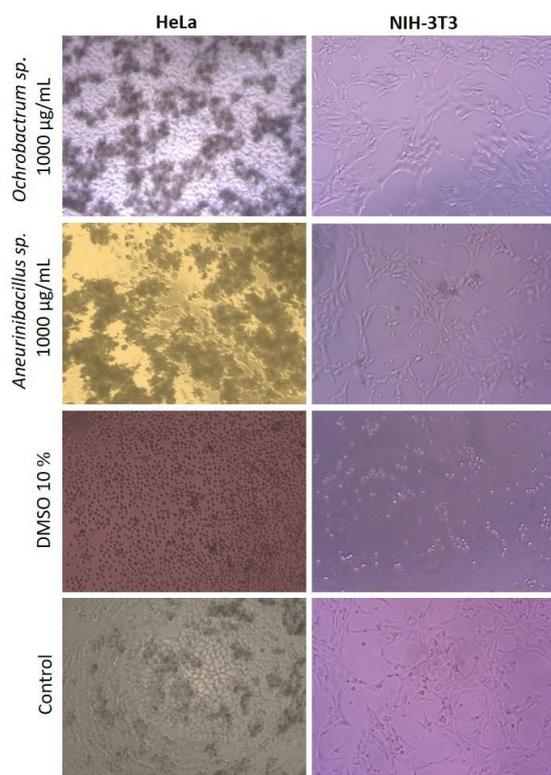


Figure 3. HeLa and NIH-3T3 morphology after treatment.

There was an overall slight decreased in the cell viability on 3T3 following *Ochrobactrum* extract treatments, however, this decrease was not statistically different (figure 2A). Interestingly, *Aneurinibacillus*

extract enhanced the growth of 3T3 significantly at a concentration of $1000 \mu\text{g/ml}$ with an increase in the cell viability up to 50% (figure 2B, $p < 0.05$). The morphology of 3T3-treated with *Ochrobactrum* and *Aneurinibacillus* extracts also showed healthy cells characteristics similar to the control (figure 3). Mariggio *et al.* (2009) reported that fibroblasts create the majority of extracellular matrix components in the formation of granulation tissue where hyaluronic acid becomes the most important component of this matrix. In a similar study, Favia *et al.* (2008) showed that the combination of hyaluronic acid and amino acids enhanced fibroblast proliferation. Therefore, *Aneurinibacillus sp.* might produce one of amino acids that contributes to the cell proliferation of 3T3-fibroblast. Moreover, no significant inhibition on 3T3 cell proliferation was observed from both extract treatments, suggesting that there is selectivity of the extracts towards non-tumorigenic compared to tumorigenic cells.

CONCLUSION

Literature study led to the selection of 2 bacteria, *Ochrobactrum sp.* and *Aneurinibacillus sp.*, that have not been tested previously for their cytotoxic effect. The present study demonstrates that secondary metabolites produced by *Ochrobactrum sp.* and *Aneurinibacillus sp.* have cytotoxic effect on HeLa cells only at very high concentrations, rendering them to be unsuitable candidate for anticancer development. Additionally, both of the extracts do not suppress cell proliferation of non-tumorigenic, 3T3 fibroblast cells, indicating some level of selectivity on healthy cells.

ACKNOWLEDGEMENT

The authors would like to thank Indonesia International Institute for Life Sciences (i3L)

for the equipment and financial support to conduct this study.

REFERENCES

- Bray, F., Ferlay, J., Soerjomataram, I., Siegel, R., Torre, L., Jemal, A. (2018). Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA: A Cancer Journal For Clinicians*, 68(6), 394-424. doi: 10.3322/caac.21492
- Demain, A. L., Vaishnav, P. (2010). Natural products for cancer chemotherapy. *Microbial Biotechnology*, 4(6), 687–699. doi:10.1111/j.1751-7915.2010.00221.x
- Desriac, F., Jégou, C., Balnois, E., Brillet, B., Le Chevalier, P., & Fleury, Y. (2013). Antimicrobial peptides from marine proteobacteria. *Marine drugs*, 11(10), 3632-60. doi:10.3390/md11103632
- Favia G, Mariggio MA, Maiorano E, Cassano A, Capodiferro S, Ribatti D. (2008) Accelerated wound healing of oral soft tissues and angiogenic effect induced by a pool of aminoacids combined to sodium hyaluronate (AMINOGAM). *J Biol Regul Homeost Agents*; 22:109-16.
- Gause, G. F., and M. G. Brazhnikova. 1944. Gramicidin S and its use in the treatment of infected wounds. *Nature* 154:703
- Karpiński, T., Adamczak, A. (2018). Anticancer Activity of Bacterial Proteins and Peptides. *Pharmaceutics*, 10(2), 54. doi: 10.3390/pharmaceutics10020054
- Lee, K. C., Kim, K. K., Eom, M. K., Kim, J. S., Kim, D. S., Ko, S. H., & Lee, J. S. (2014). *Aneurinibacillus soli* sp. nov., isolated from mountain soil. *International journal of systematic and evolutionary microbiology*, 64(11), 3792-3797. doi.org/10.1099/ij.s.0.066779-0
- Mariggio M, Cassano A, Vinella A, Vincenti A, Fumarulo R, et. al. (2009). Enhancement of Fibroblast Proliferation, Collagen Biosynthesis and Production of Growth Factors as a Result of combining Sodium Hyaluronate and aminoacids. *International Journal of Immunopathology and Pharmacology*. 2009 vol: 22 (2) pp: 485-492
- Miller, K. D., Siegel, R. L., Lin, C. C., Mariotto, A. B., Kramer, J. L., Rowland, J. H., ... Jemal, A. (2016). Cancer treatment and survivorship statistics. *CA: A Cancer Journal for Clinicians*, 66(4), 271–289. doi:10.3322/caac.21349
- National Cancer Institute. (2017). Types of Cancer Treatment.
- Raimundo, I., Silva, S. G., Costa, R., & Keller-Costa, T. (2018). Bioactive Secondary Metabolites from Octocoral-Associated Microbes-New Chances for Blue Growth. *Marine drugs*, 16(12), 485. doi:10.3390/md16120485
- Thirumurugan, D., Cholarajan, A., Raja, S. S. S., Vijayakumar, R. (2018). *An Introductory Chapter: Secondary Metabolites. Secondary Metabolites - Sources and Applications*. doi:10.5772/intechopen.79766
- Vijayarathna, S., & Sasidharan, S. (2012). Cytotoxicity of methanol extracts of *Elaeis guineensis* on MCF-7 and Vero cell lines. *Asian Pacific Journal of Tropical Biomedicine*, 2(10), 826-829. doi:10.1016/S2221-1691(12)60237-8

Zu, L., Xiong, J., Li, G., Fang, Y., & An, T. (2014).
Concurrent degradation of
tetrabromobisphenol A by
Ochrobactrum sp. T under aerobic
condition and estrogenic transition
during these processes. *Ecotoxicology
and environmental safety*, 104, 220-225.
doi:doi.org/10.1016/j.ecoenv.2014.03.01

5