Cytotoxicity of methanol extracts of Prochloron didemni originated from ascidians Lissoclinum patella and Didemnum molle collected from Manado Bay, North Sulawesi

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Abstract. Among coral reef ascidians inhabiting Manado Bay, North Sulawesi, Lissoclinum patella and Didemnum molle were found housing symbiotic microbe in their tunics. These tunicates benefit from the association by feeding directly on the microbial cells, and in turn the microbe were facilitated to produce functional compounds such as anticancer cyclic peptides. Preliminary screening for these compounds were conducted by cytotoxicity assays against human blood cells. The symbiotic microbe was molecularly identified as Prochloron didemni. Microbial samples were obtained by squeezing colonial tissues from the two newly collected host ascidians. Crude extracts were prepared by extraction in 80% methanol, and followed by in vitro cytotoxic assayed against human blood cells using May-grunwald-Giemsa method. The extract of the D. molle derived Prochloron showed more cytotoxic effects than the one of the L. patella. However, the cultivation of Prochloron cells originated from D. molle was not established compared to those of L. patella. Therefore for further production of anticancer potential compounds, cultivation of the L. patella derived Prochloron is being developed.

Key words: cytotoxicity, Prochloron, didemnid ascidians, anticancer potential

1. Introduction

Among over 3000 species of ascidian tunicates, invertebrate chordates, many can survive in relatively unclean waters, including Malalayang coast in Manado Bay where many seafood restaurant and malls surround. Ascidians, as the other invertebrates such as sponges and bryozoans, produce a rich variety of secondary metabolites presumably to avoid predation and as an anti-fouling mechanism. These compounds include cyclic peptides are not produced by the ascidian themselves but endosymbiotic microbes[1]. At least six species didemnid ascidian are found housing symbiotic microbes in their tunics in Manado Bay. Lissoclinum patella and Didemnum molle are among those ascidians that benefits from the association by feeding directly on the microbial cells, and in turn the microbes are facilitated to produce potential anticancer cyclic peptides. Prochloron didemni is a photosynthetic symbiotic microbe that is being investigated in regard with their molecular potentials[2][3] including anticancer potential of cyclic peptides[4].

The purpose of this study was to explore the anticancer potential of the methanol extracts of Prochloron didemnid isolated from L. patella and D. molle by cytotoxicity assays against human blood cells.
2. Materials and methods

Prochloron samples were collected by squeezing colonial tissues of the two ascidian hosts *L. patella* and *D. molle* soon after collection from Malalayang coastal area in Manado Bay by scuba diving at 10 m depth. The microbial cells were kept for a couple days in an aseptic standard culture medium for marine microalgae, consist of ammonium sulphate (NH$_2$SO$_4$), sodium phosphate (Na$_2$HPO$_4$.12H$_2$O) and the concentrated microelements so-called Clewat 32 with concentrations of 122.6, 23 and 15 ppm, respectively in seawater [5]. Each sample was homogenized in 80% methanol (1:2) and stirred for 24 hours. The solution was centrifuged with 3000 rpm for 15 min. The precipitate was re-extracted and re-centrifuged with the same method. The extracts were obtained from evaporating the supernatants with rotary vacuum evaporator.

The extract was assayed against human blood cells by using May-grunwald-Giemsa method. The human blood cells were taken from vena blood vessel with 5 cc disposable syringe from a donor. Some drops of blood cells with EDTA was put into a slide glass, and some other drops of blood cells with EDTA and the extract in another slide glass. Eustrek method was then applied on each slide glass, where each slide glass was fixed with 80% methanol for 5 minutes, and in Giemsa (Merck) stain for 30 minutes. The slide glasses were then rinsed in running water and air dried, prior to microscopic evaluation.

3. Results and Discussion

As shown in Figure 1, morphology of *Prochloron* cells from the two sources were similar in shapes and color, although the cells originated from *D. molle* were slight bigger (14-19 μm) than those from *L. patella* (11-19 μm). There was no nuclei appeared in cells, because they are prokaryotes, as described by [6], DNA located nearby cell wall, and the cell was mostly occupied with vacuoles [2]. This microbe has recently been molecularly identified as *Prochloron didemnid* using CAO gene.

![Figure 1. Prochloron cells originated from L. patella (a) and D. molle (b)](image)

Microscopic evaluation of the in vitro assays of toxicity effects of the two extracts are shown in Figures 2b and 2c. It can be seen that the extract of *Prochloron* derived from *L. patella* caused cell disorder, some cells lysed and appeared to be attached each other. Most of the cells had disorder in shape, due to cell wall damage. A few cells shrank, still in round shape, became smaller than the normal cells (Fig. 2b). As for the toxicity assays of the *D. molle*-derived *Prochloron* extract upon the blood cells shows significant different. As shown in Figure 2c, all cells were totally broken, no more cells appeared. The extract has lysed 100% cells. Methanol extract of *Prochloron* derived from *D. molle* shows more significant cytotoxic effects than the one from *L. patella*. Cytotoxic agent exhibits lysis of cell membrane or inhibition of macromolecular synthesis [7], affect DNA transcription, protein translation, drug efflux pumps, signaling pathways and the cytoskeleton [1]. As recently been reviewed by [8] in regard with cancer cell treatment, that cytotoxic effects of natural bioactive molecules attack macromolecules expressed by cancer cells in oncogenic signal transduction pathways. In vitro and in
vivo studies on the anti-cancer effects of marine natural products, and their activity in preventing tumor formation and the related compound-induced apoptosis and cytotoxicities are obviously required.

Figure 2. Normal blood cells (a), cells treated with the \textit{L. patella}-derived \textit{Prochloron} extract (b), and cells treated with the \textit{D. molle}-derived \textit{Prochloron} extract (c).

There has no report on the cytotoxicological study of the symbiotic microbes in ascidian \textit{L. patella} dan \textit{D. molle}, except a biochemical study on lipids content [3][1]. has recently reviewed the potential cytotoxic compounds produced by the symbiotic bacteria that ecologically useful for the ascidian hosts to defense and survive. Such compounds are potentially developed as therapeutic drugs for a wide variety of diseases. There are two ascidian natural products marketed for cancer treatment. Ecteinascidin (ET-743, trabectedin) from \textit{Ecteinascidia turbinate} is FDA approved and marketed under the trade name Yondelis®. Aplidin® (dehydrodidemnin B, plitidepsin)—first isolated from \textit{Aplidium albicans}—has attained orphan drug status [9]. Both are marketed by PharmaMar (Madrid, Spain)[1]. The clinical trial of anti-cancer compounds derived from the caribbean tunicate \textit{Ecteinascidia turbinate} were previously reported by [10][11]. The potential anti-cancer compounds of \textit{Prochloron didemnid} derived from \textit{Lissoclinum patella} have been also reported [4], [12],[13],[14], but still in vitro assays. No such a report is available in regard with \textit{D. molle}.

For further developing in vitro and in vivo cytotoxic assays as well, sufficient cell biomass is required. Therefore, mass cultivation of the symbiotic microbes away from its host is being investigated. However, the cultivation of \textit{Prochloron} cells originated from \textit{D. molle} was still difficult compared to those of \textit{L. patella}. More cellular treatments to separate cells from the tunics of host is being performed. The cyclic peptides that have already detected in P. didemnid associated with didemnid ascidians are being investigated for their potential in cancer by in vitro, in vivo dan in silico.

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References

[1] Watters DJ 2018 *Ascidian Toxins with Potential for Drug Development*. Mar. Drugs 2018, 16, 162; doi:10.3390/md16050162 www.mdpi.com/journal/marinedrugs

[2] Cox G 1993 *Prochlorophyceae*. In: Ultrastructure of Microalgae (T. Berner, Ed.). CRC Press, Inc. Boca Raton, USA. Pp. 53-70.

[3] Lewin R A dan L Cheng 1989 Prochloron A Microbial Enigma. Printed in the United States of America. 115 hal.

[4] Schmidt EW, Nelson JT, Rasko, DA, Sudek S, Eisen JA, Haygood MG, *Ravel J Patellamide A and C biosynthesis by a microcin-like pathway in Prochloron didemni, the cyanobacterial symbiont of Lissoclinum patella*. Proc. Natl. Acad. Sci. USA 2005, 102, 7315–7320. [CrossRef] [PubMed] Mar. Drugs 2019, 17, 491 25 of 31

[5] Hirata H, Andarias I & Yamasaki S 1981 *Effect of Salinity Temperature on the Growth of The Marine Phytoplankton Chlorella saccharophila*. Mem. Fac. Fish. Kagoshima Univ., 30 : 257-262.

[6] Bold H C dan M J Wynne 1985 *Introduction to The Algae; Stricture and Reproduction*. Prentice-hall, Englewood Cliffs, N. J. 720 hal.

[7] Shier WT 1988 *Hand Book of Natural Toxins in: Cytotoxic Effect Of Marine Toxins And Venoms* (Anthony T. Tu, Ed). Marcel Dekker Inc. New York. 486 hal.

[8] Khalifa S M, N Elias, MA Farag, L Chen, A Saeed, ME F Hegazy, MS Moustafa AAbd El-Wahed, S M. Al-Mousawi, S.G. Musharraf, F-R. Chang, A. Iwasaki, K. Suenaga, M. Alajlani, U. Göransson and H. R. El-Seedi. 2019. *Marine Natural Products: A Source of Novel Anticancer Drugs*. Mar. Drugs 17, 491; doi:10.3390/md17090491 www.mdpi.com/journal/marinedrugs

[9] Mayer CA *The Global Marine Pharmaceuticals Pipeline*. Available online: http://marinepharmacology.midwestern.edu/clínPipeline.htm (accessed on 18 September 2019).

[10] Da Rocha AB, Lopes RM, Schwartzmann, G. *Natural products in anticancer therapy*. Curr. Opin. Pharmacol. 2001, 1, 364–369. [CrossRef]

[11] Newman DJ, Cragg GM. *Advanced preclinical and clinical trials of natural products and related compounds from marine sources*. Curr. Med. Chem. 2004, 11, 1693–1713. [CrossRef]

[12] Donia MS, Hathaway BJ, Sudek S, Haygood MG, Rosovitz MJ, Ravel J, Schmidt EW. *Natural combinatorial peptide libraries in cyanobacterial symbionts of marine ascidians*. Nat. Chem. Biol. 2006, 2,729. [CrossRef]

[13] Donia MS, Ravel J, Schmidt EW *A global assembly line for cyanobactins*. Nat. Chem. Biol. 2008, 4, 341. [CrossRef] [PubMed]

[14] Williams D E, Moore RE, Paul VJ *The structure of ulithiacyclamide B. Antitumor evaluation of cyclic peptides and macrolides from Lissoclinum patella*. J. Nat. Prod. 1989, 52, 732–739. [CrossRef]