Available online at www.sciencedirect.com

ScienceDirect

Procedia Chemistry 16 (2015) 407 – 412

Procedia Chemistry

International Symposium on Applied Chemistry 2015 (ISAC 2015)

Aqueous-Methanol Extract of *Gracilaria verrucosa* Induces Cytochrome c Release From Mitochondria

Mona Fitria[a*], Fida Madayanti Warganegara[a]

[a]*Biochemistry Division, Chemistry Department, Faculty of Mathematics and Natural Sciences, Institut Teknologi Bandung Jl. Ganesha No. 10, Bandung 40132, Indonesia*

Abstract

Seaweeds is one of many sources of natural bioactive compounds that can be utilized in the fields of pharmaceuticals and health. However, research on utilization of the seaweed's bioactive compounds from Indonesia is still very limited. Therefore, the objective of this research is to study the ability to induce apoptosis of the extracts of *Gracilaria verrucosa* harvested from Sayang Heulang, a beach in Pameungpeuk, Garut, West Java. The study begins with bioactive compounds extraction using chloroform p.a, methanol p.a and 50 mM phosphate buffer pH 7.6 with a ratio 2:1:0.8, resulting crude aqueous-methanol and chloroform extracts. *G. verrucosa* produced 62.73% (w/w) crude aqueous-methanol extract and 1.07% (w/w) chloroform extract. Mitochondria for apoptosis test were isolated from rat liver. The treatment of 10% v/v crude aqueous-methanol extract from *G. verrucosa* for five hours at 37 ^{o}C toward mitochondrial suspension leads to formation of mitochondrial aggregates and study of the released of cytochrome c showed that those treatment released 8.7 µM cytochrome c which has equal to 11.4% of mitoxantrone 500 µM activity. In conclusion, our results indicate that aqueous-methanol extract of *G. verrucosa* able to induce apoptosis through mitochondrial pathway involving cytochrome c release from mitochondria.

Peer-review under responsibility of Research Center for Chemistry, Indonesian Institute of Sciences

Keywords: Seaweeds, apoptosis, cytochrome c, mitochondria

* Corresponding author. Tel.: +6281314771695
E-mail address: monafitria1985@yahoo.com

Peer-review under responsibility of Research Center for Chemistry, Indonesian Institute of Sciences
doi:10.1016/j.proche.2015.12.071

1. Introduction

Apoptosis or programmed cell death is essential for homeostasis and generally characterized by distinct morphological characteristics and energy-dependent biochemical mechanisms. There are two main apoptotic pathways, the extrinsic or death receptor pathway and the intrinsic or mitochondrial pathway. The stimuli that initiate the intrinsic pathway such as radiation, toxin, hypoxia, viral infection, and free radicals leads to mitochondrial damage and release of pro-apoptotic protein such as cytochrome c and Smac/DIABLO. This pathway results in DNA fragmentation and formation of apoptotic bodies[1]. Many human pathologies are associated with defects in mitochondria such as diabetes, neurodegenerative diseases or cancer[2].

Seaweeds is one of Indonesia's natural resources which has an enormous development opportunities in the future. Indonesian seaweed production reached 10.2 million tons in 2014[3], but its uses only as food source such as vegetables, salad, and main source of agar production[4]. It is one of many sources of natural bioactive compounds such as pigments, lipids, polysaccharides, proteins, phenolic compounds, flavonoids, terpenoids, steroids, glycosides and phytosterol[5,6,7] that can be utilized in the fields of pharmaceuticals and health. Saturated fatty acids and PUFA (Polyunsaturated Fatty Acids) from seaweeds have cytotoxic ability[8]. *Ulva fasciata*, a green seaweed from Jeju Island (Republic of Korea) was reported induces apoptosis of human colon cancer cells[9]. The same species from Gulf of Mannar, India showed similar anticancer activity in albino mice[10]. *Gracilaria tenuispitata*, a red seaweed from Kouhu Beach, Taiwan was found to have the ability to induces apoptosis of oral cancer cells[11]. *Gracilaria edulis* from Tamil Nadu, India also showed the ability to induce apoptosis of Ehrlich Ascites Tumor cells[12]. The above results indicate that the seaweed extract have an important role in apoptosis.

Research on utilization of the seaweed's bioactive compounds from Indonesia is still very limited. Therefore, this research aims to study the ability to induce apoptosis through mitochondrial pathway of the extracts of *Gracilaria verrucosa*, a red seaweed harvested from Sayang Heulang, a beach in Pameungpeuk, Garut District, West Java, Indonesia.

2. Methods

2.1 Sample collection

Gracilaria verrucosa were collected in November 2013 from Sayang Heulang, a beach in Pameungpeuk, Garut District, West Java. The samples was washed to remove sand, stones or pieces of coral, weighed and wrapped in plastic to be stored at -20 ° C.

2.2 Active compounds extraction

Active compounds were extracted according to the method described by Kumari et al. (2011) using chloroform p.a (Merck), methanol p.a (Merck) and 50 mM phosphate buffer pH 7.6 with a ratio 2:1:0.8, resulting crude aqueous-methanol and chloroform extracts. Both phases were separated and concentrated by rotary evaporator. Crude extract is calculated by gravimetric method and stored at -20 ° C[13].

2.3 Mitochondrial Isolation

Mitochondria were isolated from the rat liver according to the method described by Long et al. (2009) using SET buffer pH 7.4 (containing 0.25 M sucrose, 10 mmol/L Tris, and 0.5 mmol/L EDTA). The sample was homogenized using Potter Elvehjem homogenizer and centrifuged 1.000 *g* for 10 min at 4 °C. The supernatant was decanted, collected, and centrifuged 10.000 *g* for 10 minutes. The mitochondrial pellet was washed with isolation buffer, aliquoted, and stored at -80 ^{o}C[14].

2.4 Visualization of changes in mitochondrial morphology

Mitochondrial suspensions were treated with 10% (v/v) crude chloroform (in the mixture and isopropanol and 50 mM phosphate buffer pH 7.6 with a ratio of 3:10 (v/v) and aqueous-methanol extracts from *Gracilaria verrucosa*. Samples were incubated for five hours at 37 ^{o}C. Furthermore, mitochondrial morphology was evaluated under light microscopy at 1.000 times magnification and 2 times optical camera magnification.

2.5 Detection of cytochrome c released from mitochondria

The detection of cytochrome c released from mitochondria were conducted spectrometrically by observing absorption at 550 nm. Mitochondria suspensions were treated with 10% (v/v) aqueous-methanol extracts from *Gracilaria verrucosa*, incubated for five hours at 37 ^{o}C and centrifuged 10.000 g for 10 minutes. A very small crystal of potassium ferricyanide ($K_3Fe(CN)_6$) was added to the supernatant to be sure all of the heme iron of the cytochrome c was oxidized to Fe^{3+}. Cytochrome c-Fe^{3+} does not have absorbance peak at 550 nm. Cytochrome c-Fe^{3+} was reduced by adding sodium hydrosulfite ($Na_2S_2O_4$) and then observed at 550 nm[15].

3. Results and Discussions

3.1 Active compound extraction

Active compounds were extracted by cold extraction method that involve maceration with solvent and centrifugation. The addition of a phosphate buffer improved the efficiency of lipid extraction. It can prevent ionic adsorption effect that can be ionized phospholipids on samples containing large amounts of salts such as seaweeds[13]. Prior to extraction, the sample was dried with a freeze dryer to remove the water in the sample that may interfere the extraction process. Moisture content of *Gracilaria verrucosa* was 87.58% (g/g dry weight). Dried ground seaweeds were extracted with methanol p.a, chloroform p.a and 50 mM phosphate buffer pH 7.6 with a ratio of 2:1:0.8 (v/v) resulting 62.73% (g/g dry weight) of crude aqueous-methanol extract and 1.07% (g/g dry weight) of crude chloroform extract.

3.2 Mitochondria isolation

Mitochondria were isolated from the liver because the liver cells containing large amount of mitochondria, which is about 1.000-2.000 mitochondria or about one-fifth the volume of the cell. In this study, mitochondria were used to apoptosis induction test. Mitochondria were isolated from rat liver with stepwise centrifugation method using SET buffer pH 7.4 as buffer isolation[14]. Mitochondrial isolation begins with the liver tissues homogenization using Potter Evlehjem homogenizer. Isolation buffer containing Tris buffer to maintain pH, sucrose and chelating agent to maintain the stability of organelles[16]. Mitochondria were obtained by centrifugation at 10.000 *g*[14], whereas centrifugation at low speed (1.000 *g*) were precipitated cell debris, nuclei, cell wall fragments, and the cytoskeleton. All the isolation process must be performed at 4 ^{o}C to minimize the activation of damaging phospholipases and proteases[17]. Mitochondria are large enough to be seen in the light microscope[18] as shown in Fig. 1a. In this study, the mitochondria is visualized as small round shape, under light microscope with 1.000 times magnification and 2 times optical camera magnification.

3.3 Changes in mitochondrial morphology

Apoptosis can occur through extrinsic and intrinsic pathways. The intrinsic pathway occurs as a result of apoptotic stimuli such as cytotoxic compounds, oxidative stress, DNA damage, and hypoxia[19]. The intrinsic apoptotic pathway that involves mitochondria can be studied by observing changes in mitochondrial morphology using a light microscopy. In this study, crude aqueous-methanol and chloroform extract were treated toward mitochondrial suspension (10% v/v) and incubated for five hours at 37 °C. Mitoxantrone used as positive control while negative control used solvent, 50 mM phosphate buffer pH 7.6 for crude aqueous-methanol extract and a mixture of isopropanol with 50 mM phosphate buffer pH 7.6 (3:10 v/v) for crude chloroform extract. The results showed that the treatment of 10% v/v crude aqueous-methanol extract toward mitochondrial suspension leads to formation of mitochondrial aggregates (Fig.1), as well as mitoxantrone 500 μM, while those of chloroform extract showed negative results, as well as negative control. The formation of mitochondrial aggregates is one of the symptoms of apoptosis that precedes the release of cytochrome c from mitochondria[20]. Therefore, the results showed that crude aqueous-methanol extract from *G. verrucosa* can induce mitochondrial damaging and mitochondrial morphology changing which leads to apoptosis.

3.4 Cytochrome c released from mitochondria

Cytochrome c is a protein involved in the electron transport and has a heme unit with iron ion as the central atom[21]. Reduced cytochrome c (cyt c-Fe^{2+}) has absorption peak at 550 nm, while the oxidized cytochrome c (cyt c-Fe^{3+}) does not has absorption peak at these wavelengths. Detection of the release of cytochrome c is performed by adding 10% (v/v) of the active crude extract (aqueos-methanol extract) towards mitochondrial suspension and incubated for five hours at 37 °C. The concentration of cytochrome c released from mitochondria under various treatment can be calculated by Equation 1 below. The value of $\Delta\varepsilon_{550}$ (reduced-oxidized) was obtained by measuring absorbance of cytochrome c standard at various concentrations, both on the oxidized and reduced state. The relationship between concentration of cytochrome c standard and absorbance at 550 nm were plotted on graphs. The slope of each graphs is the molar extinction value for each state. The results showed that the value $\Delta\varepsilon_{550}$ (reduced-oxidized) was 11.35 $mM^{-1}cm^{-1}$.

$$[\text{cyt c}] = \frac{A_{550}\text{reduced} - A_{550}\text{oxidized}}{\Delta\varepsilon_{550}\ \text{reduced-oxidized}} \quad (1)$$

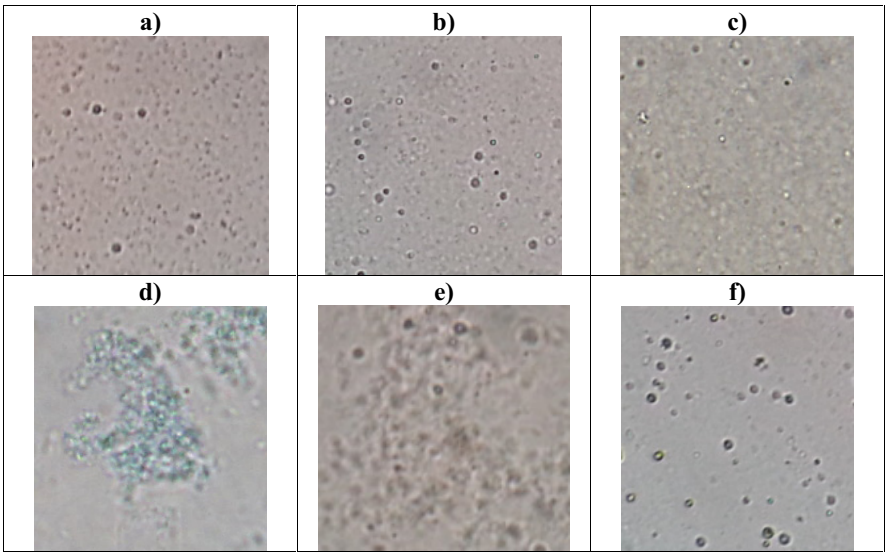

Fig 1. Mitochondrial visualization with various treatments (2.000 times magnification)

a) Mitochondria; b) Mitochondria + phosphate buffer; c) Mitochondria + phosphate buffer and isopropanol; d) Mitochondria + mitoxantrone 500 μM; e) Mitochondria + crude aqueous-methanol extract of G. verrucosa; and f) Mitochondria + crude chloroform extract of G. verrucosa

Cytochrome c released from mitochondria for each treatment was shown in Fig 2. The results showed that the the treatment of 10% (v/v) crude aqueous-methanol extract from *G. verrucosa* toward mitochondrial suspension released 8.7 ± 1.6 μM cytochrome c, the treatment of mitoxantrone 500 μM released 76.2 ± 2.5 μM of cytochrome c, while untreated mitochondria were not released cytochrome c. The activity of crude aqueous-methanol extract from *G. verrucosa* has equal to 11.4% of mitoxantrone 500 μM activity. The release of cytochrome c from mitochondria to the cytoplasm can activate caspase cascade leading to apoptosis.

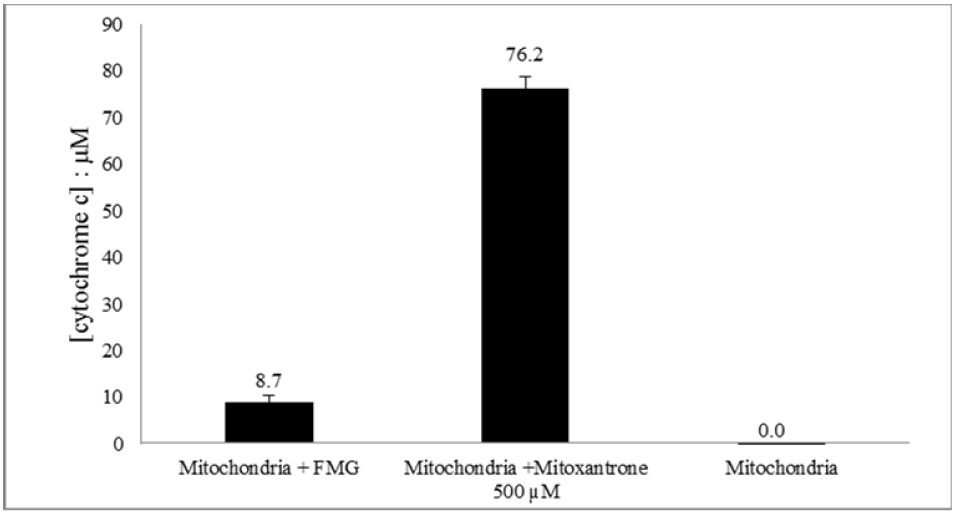

Fig 2. Cytochrome c released from mitochondria

4. Conclusions

The results indicate that aqueous-methanol extract of *G. verrucosa* can induces apoptosis through mitochondrial pathway that involve the release of cytochrome c from mitochondria. It also leads to formation of mitochondrial aggregates.

5. Acknowledgement

We are thankful to Politeknik Kesehatan Bandung and Pustanserdikjut Sumber Daya Manusia Kesehatan Badan PPSDM, Ministry of Health for the research grant.

References

1. Elmore S. Apoptosis : a review of programmed cell death. *Toxicologic Pathology* 2007; **35**:495-516.
2. Jourdain A, Martinou J. Mitochondrial outer-membrane permeabilization and remodelling in apoptosis. *The International Journal of Biochemistry* 2009; **41**:1884-1889.
3. Pregiawati LA. Komoditas rumput laut kian strategis, http://kkp.go.id/index.php/pers/komoditas-rumput-laut-kian strategis/?print= pdf, *Download* on 15 April 2015.
4. Kadi A. Potensi rumput laut di beberapa perairan pantai Indonesia. *Oseana* 2004; **29**(4):25-36.
5. Almeida CLFD, Falcao HDS, Lima GRDM, Montenegro CDA, Lira NS, Athayde-Filho PFD, Rodrigues LC, Souza MDFVD, Barbosa-Filho JM, Batista LM. Bioactivities from marine algae of the genus G*racilaria. International Journal of Molecular Sciences* 2011; **12**:4550-4573.
6. Silva M, Vieira L, Almeida P, Kijjoa A. The marine macroalgae of the genus *Ulva* : chemistry, biological activities and potential applications. *Oceanography* 2013; **1**(1):1-6
7. Holdt S, Kraan S. Bioactive compounds in seaweed : functional food applications and legislation. *Journal Applied Phycology* 2011; **23**:543-597.
8. Cetrullo S, Tantini B, Flamigni F, Pazzini C, Facchini A, Stefanelli C, Caldarera CM, Pignatti C. Effects antiapoptotic and antiautophagic of eicosapentanoic acid in cardiac myoblasts exposed to palmitic acid. *Nutrients* 2012; **4**:78-90.
9. Ryu MJ, Kim AD, Kang KA, Chung HS, Kim HS, Suh IS, Chang WY, Hyun JW. The green algae *Ulva fasciata* delile extract induces apoptotic cell death in human colon cancer cells. *In Vitro Cellular Developmental Bioogy-Animal* 2013; **49**:74-81.
10. Abirami RG, Kowsalya S. Anticancer activity of methanolic and aqueous extract of *Ulva fasciata* in albino mice. *International Journal of Pharmacy and Pharmaceutical Sciences* 2012; **4**(2):681-684.
11. Yeh C, Tseng C, Yang J, Huang H, Fang Y, Tang J, Chang F, Chang H. Antiproliferation and induction of apoptosis in ca9-22 oral cancer cells by ethanolic extract of *Gracilaria tenuistipitata*. *Molecules* 2012; **17**:10916-10927.
12. Patra S, Mathuraman MS. *Gracilaria edulis* extract induces apoptosis and inhibits tumor in ehrlich ascites tumor cells in vivo. *BMC Complementary and Alternative Medicine* 2013; **13**(331):1-12.
13. Kumari P, Reddy CRK, Jha B. Comparative evaluation and selection of a method for lipid and fatty acid extraction from macroalgae, *Analytical Biochemistry* 2011; **415**:134-144.
14. Long J, Ma J, Luo C, Mo X, Sun L, Zang W, Liu J. Comparison of two methods for assaying complex i activity in mitochondria isolated

from rat liver, brain, and heart, *Life Science* 2009; **85**:276-280.
15. Zabinski-Snopko RS, Czerlinski GH. Spectrophotometric titrations of ferricytochrome c with ferrohexacyanide in the pH range 4 to 7, *Journal of Biological Physics* 198; **9**:155-167.
16. Clayton DA, Shadel GS. Isolation of mitochondria from cells and tissues. *Cold Spring Harbor Protocol* 2015; 147-151.
17. Frezza C, Cipolat S, Scorrano L. Organelle isolation: functional mitochondria from mouse liver, muscle and cultured filroblasts. *Nature Protocols* 2007; **2**:287-295.
18. Alberts B, Johnson A, Lewis J, Raff M, Roberts K, and Walter P. *Molecular biology of the cell*. 5th ed. New York: Garland Science; 2007.
19. Iannolo G, Conticello C, Memeo L, Maria, RD. Apoptosis in normal and stem cells. *Critical Reviews in Oncology/Hematology* 2008; **66**:42-51.
20. Haga N, Fujita N, Tsuruo T. Mitochondrial aggregation precedes cytochrome c release from mitochondria during apoptosis. *Oncogene* 2003; **22**:5579-5585.
21. Nelson DL, Cox MM. *Lehninger principles of biochemistry*. 5th ed. New York: W.H. Freeman and Company; 2008.