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Original Research

LIPID EXTRACT OF *Gracilaria sp*. AS AN ANTI-APOPTOTIC CANDIDATE FOR CANCER THERAPY

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Abstract

It has been claimed that an Indonesian seaweed lipid extract possesses antibacterial properties. The mitochondrial pathway reported that a long fatty acid (more than 10 carbon atoms), which is known as the major component in seaweed lipid extract, acts as an apoptotic agent. It has also been claimed that unsaturated fatty acids can prevent apoptosis. on this study, we investigated the role of an Indonesian seaweed lipid extract on the mitochondrial pathway of apoptosis. Lipid isolation was the first step of the study, which was carried out using centrifugation and a chloroform-methanol-phosphate buffer 2:1:0,8. Rotary evaporators were used to separate the lipid extract from its solvent. Dry weight *Gracilaria sp*. yield extraction was 21,8%. By using several centrifugation techniques in SET buffer (0,25 M Sucrose, 0,5 mmol EDTA, and 10 mmol/L Tris base), pH 7,4, mitochondria were recovered from rat liver. By adding 10% (v/v) lipid extract to a suspension of mitochondria, toxicity testing from the lipid extract was undertaken. By using light microscopy, mitochondrial morphological damage was seen. *Gracilaria sp.* exhibits strong apoptotic activity.

Keywords: Apoptotic; Cancer; Gracilaria; Lipid extract.

INTRODUCTION

Macroalgae, or seaweed, is one of Indonesia's most plentiful natural resources. Seaweed is still only occasionally and imperfectly used in Indonesia. In Indonesia, only food (salad, agar) and agriculture (fertilizer, pet food, hydroponics, aquaponics) employ seaweed. Seaweed has a variety of ingredients that can be used not only in food production and agriculture but also in cosmetics and medicine.

In earlier investigations, seaweed was identified as one of the bioactive sources for drug development [1], [2]. It contains long-chain saturated and unsaturated fatty acids that have been

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shown to have cytotoxic properties [3]. Long-chain fatty acids with various double bonds are reportedly present in Indonesian seaweed sources [4]. In accordance with additional studies, sulfate-reducing and iron-oxidizing bacteria can cause carbon steel to corrode [5]. Seaweed lipid extracts from Indonesia were found to be able to prevent this from happening. Various illnesses, such as heart disease, stroke, AIDS, hypertension, and diabetes, typically follow the process of cell death in humans. For cancer/tumour treatment, cell death is also necessary [6]. Mitochondria play an important role in the body's metabolic processes such as amino acid biosynthesis, fatty acid metabolism, steroid metabolism, and apoptosis [7]. The cell death pathway in various diseases passes through the mitochondrial pathway [8]–[10]. This ability is a very potential opportunity to be developed in the medical field. By knowing the cytotoxic ability through the mitochondrial pathway, it is possible to find new drug candidates to treat diseases that cause the greatest death in the world. Therefore, this research studied the effect of seaweed extract from Indonesia on anti-cancer activity.

METHOD

Materials

The materials needed in this study include macroalgae *Gracilaria sp*. taken from Sayang Heulang Beach, Pameungpeuk Garut, West Java. Mitochondria were obtained from the liver of mice obtained from the Animal Biology Laboratory, SITH ITB. The materials used were Methanol p.a (Merck), Chloroform p.a (Merck), Phosphate buffer, Sucrose, EDTA and Tris.

Sample Preparation

Gracilaria sp. was collected from Sayang Heulang Beach, Pameungpeuk Garut, West Java Province. Prior to the extraction process, the seaweed was cleaned from foreign objects such as sand, stones, etc., drying using filter paper was carried out to remove moisture on the surface of the seaweed, water removal, and grinding [11]. After weighing, samples were immediately frozen at -20 C before further analysis.

Macroalgae Extraction

Oil extraction from seaweed was carried out using the method of Kumari with a slight modification [12]. The solvents used were chloroform p.a (Merck), methanol p.a (Merck) and 50 mM phosphate buffer (with the ratio between sample and solvent was 1:4). This process begins with drying seaweed using the freeze-drying method for 24 hours and then homogenized. The dried seaweed powder was extracted by chloroform-methanol-buffer phosphate (2:1:0.8) solvent mixture via vortex for 5 min and centrifugation 2057 x g for 15 min at room temperature. The seaweed powder was re-extracted using a chloroform: methanol solvent mixture at 2057 x g for 15 minutes. The supernatant obtained was collected and washed using Mili-Q water at 2057 x g for 5 minutes. The organic phase was separated and evaporated through a rotary evaporator. The lipids obtained were calculated gravimetrically and stored at -20 C for further analysis.

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Mitochondrial Isolation

Mitochondrial isolation was performed according to Long's modified method [13]. Rat livers were taken, weighed, ground, and homogenized in isolation buffer solution (Sucrose 0.25 M, EDTA 0.5 mmol and Tris 10 mmol/L) pH 7.4 and then centrifuged at 1000 x g for 10 min at 4°C. The resulting pellet was washed by 2 times the volume of isolation buffer. The supernatant obtained was collected and decanted and then centrifuged again at 10,000 x g for 10 minutes. The resulting pellet is the mitochondrial fraction. The pellet was washed by 2 times the volume of isolation buffer and suspended before being stored at -80 °C.

Mitochondrial Rupture Visualization Test

Mitochondria were added to macroalgae lipid extract at a concentration of 10% v/v and then incubated for 60 minutes. Negative control was done by adding water to the mitochondrial suspension, while positive control was done by adding phenol. Furthermore, incubation was carried out in an incubator at 37 °C. Mitochondria were then centrifuged at 10,000 x g for 10 minutes. The pellet obtained was collected and then taken 20 microliters and placed in a glass plate. The morphological appearance of mitochondria was observed through a light microscope with a magnification of 400x.

RESULT AND DISCUSSION

Macroalgae samples

Seaweed samples were taken from Sayang Heulang Beach, Pameungpeuk Garut representing the Rhodophyta or red algae class. Samples were taken in the morning at a depth of 20-50 cm from sea level with cloudy weather conditions, sea water temperature ± 26 °C and pH 7. Based on morphological analysis of the branching system and modification of the rope can be identified type (genus) of seaweed [14].

The taxonomy of seaweed is as follows.

Gracilaria sp.

Kingdom : Eukaryota
Phylum : Rhodophyta
Kelas : Rhodophyceae
Ordo : Gracilariales
Famili : Gracilariaceae
Genus : Gracilaria
Spesies :*Gracilaria sp.*

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Figure.1 *Gracilaria sp.* grows in Sayang Heulang Beach, Pameungpeuk, Garut

Extraction of Macroalgae Lipids

Macroalgae lipid extraction was carried out using chloroform-methanol solvent. The addition of phosphate buffer to the aqueous phase is intended to optimize extraction. Several previous studies have proven the role of fatty acids in the process of cell death. Polyunsaturated fatty acids have been reported to play a role in the prevention of heart disease, cardiovascular disease, and breast cancer [15], [16]. Prevention of cardiac cell death by omega-3 has also been reported [3]. Omega-3 can inhibit the production of molecules that promote cancer cell growth [17]. Omega-3 also causes cancer cell death [18]. Therefore, this study focused on the extraction of lipids contained in seaweed from Indonesia. The extracted oil from macroalgae is shown in Figure 2 and the extracted oil yield is shown in Table 1.

Table 1. Macroalgae Extraction Yield to Dry Weight % (w/w)

Seaweed	Water Content	Oil (% w/w) dw
Gracilaria sp.	86,16%	21,8%

Figure 2. Lipid of Gracillaria sp.

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Solvent-free oil obtained from macroalgae extraction through a rotary evaporator at 40ºC for 15 minutes showed a phase difference when tested for solubility in water, but the phase boundaries formed were not as clear as a mixture of cooking oil and water. The extraction yield (% w/w) per dry weight of macroalgae through the centrifugation process was 21.8% (Table 1).

Mitochondrial Isolation

Cells can be damaged in various ways, namely by changes in osmosis pressure, ultrasonic vibrations, or mechanical scraping. All these methods can break the cell membrane into small fragments which will then close again to form small compartments. However, these methods are not able to release some organelles such as the cell nucleus, mitochondria, Golgi bodies, lysosomes, and peroxisomes[19]. The cell suspension is reduced to small particles surrounded by a membrane, each of which has a different size, charge, and density. Some of these organelles can be separated from the cell suspension by centrifugation. Through this method, organelles are separated based on differences in size and density by rotation at a certain speed. The smaller the organelle size, the greater the speed used. Separation through centrifugation consists of several fractionation processes. To separate cell components such as the cell nucleus and cytoskeleton, low speeds (100 x g) are used. Both organelles are present in the cell pellet after centrifugation. The supernatant is then centrifuged again at medium speed (10,000 x g) to separate mitochondria, chloroplasts, lysosomes, and peroxisomes. If the centrifugation process is continued on the supernatant obtained at a higher speed, some small organelles such as plasma membranes, ribosomes and some large macromolecules will be obtained [20].

In this study, a medium-speed centrifugation method was used to isolate mitochondria from mice's livers [13]. The mitochondria obtained were then visualized through a light microscope at 1600x magnification (Figure 3).

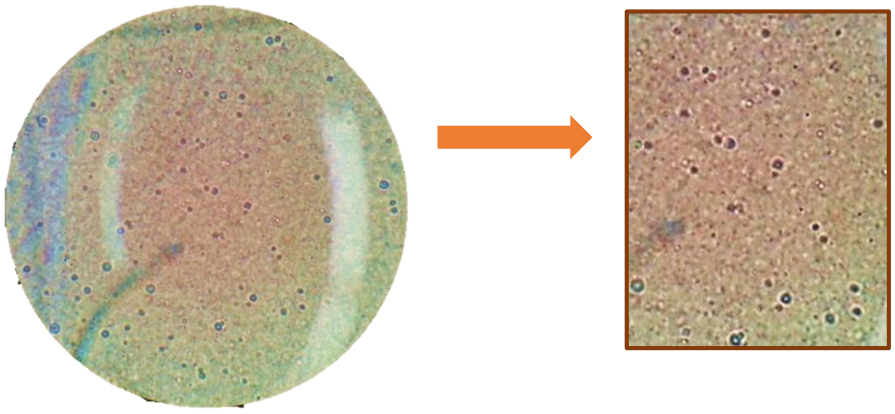

Figure 3. Isolation Results of Mitochondria from Mouse Liver

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Mitochondria are large enough to be seen through a light microscope. The description of mitochondria in general is a hard organelle, a long cylindrical shape with a diameter of 0.5-1 µm. Mitochondria consist of an inner membrane, outer membrane, and mitochondrial matrix. In Figure 3, the mitochondria obtained are round with two layers on the outer side. This is because they are dynamic and flexible plastic organelles that constantly change their shape and even fuse with each other and separate again. The two layers on the outer side are the inner membrane and outer membrane of the mitochondria. In its movement in the cytoplasm, mitochondria are often seen associated with microtubules. In one liver cell, there are about 1000-2000 mitochondria.

Cytotoxic Test and Visualization of Mitochondria by Light Microscopy

Mitochondria are organelles that have a very important role in cells. Mitochondria play a role in metabolic processes and are the site of amino acid oxidation reactions, fatty acid β-oxidation reactions, and the Krebs cycle. Mitochondria have an important role in the electron transfer process to produce energy. In addition to energy production, mitochondria also play a role in the process of programmed cell death (apoptosis) [21].

Cell death signals are characterized by the loss of mitochondrial membrane potential [21], accompanied by the release of cytochrome c from mitochondria [22]. Under normal circumstances, cytochrome c remains in the mitochondrial membrane because it has an important role in oxidative phosphorylation, namely producing ATP. Cytochrome c is guarded by the BCl2-Bax protein complex from leaving the mitochondria [23]. If this complex is disrupted, the Bax protein will separate and combine with other Bax proteins, forming a channel on the mitochondrial membrane. This canal will cause the release of cytochrome c from the mitochondria. Cytochrome c will activate caspases, and promote cell death [8].

In this study, the toxicity test of macroalgae oil extract on apoptotic activity in mitochondria was carried out with an extract concentration of 10% (v/v) to the mitochondrial suspension. As a positive control of apoptosis, mitochondria + phenol was used while as a negative control of apoptosis, mitochondria + water was used. Observation of mitochondrial morphology was observed through a light microscope with 1600x magnification (Figure 4).

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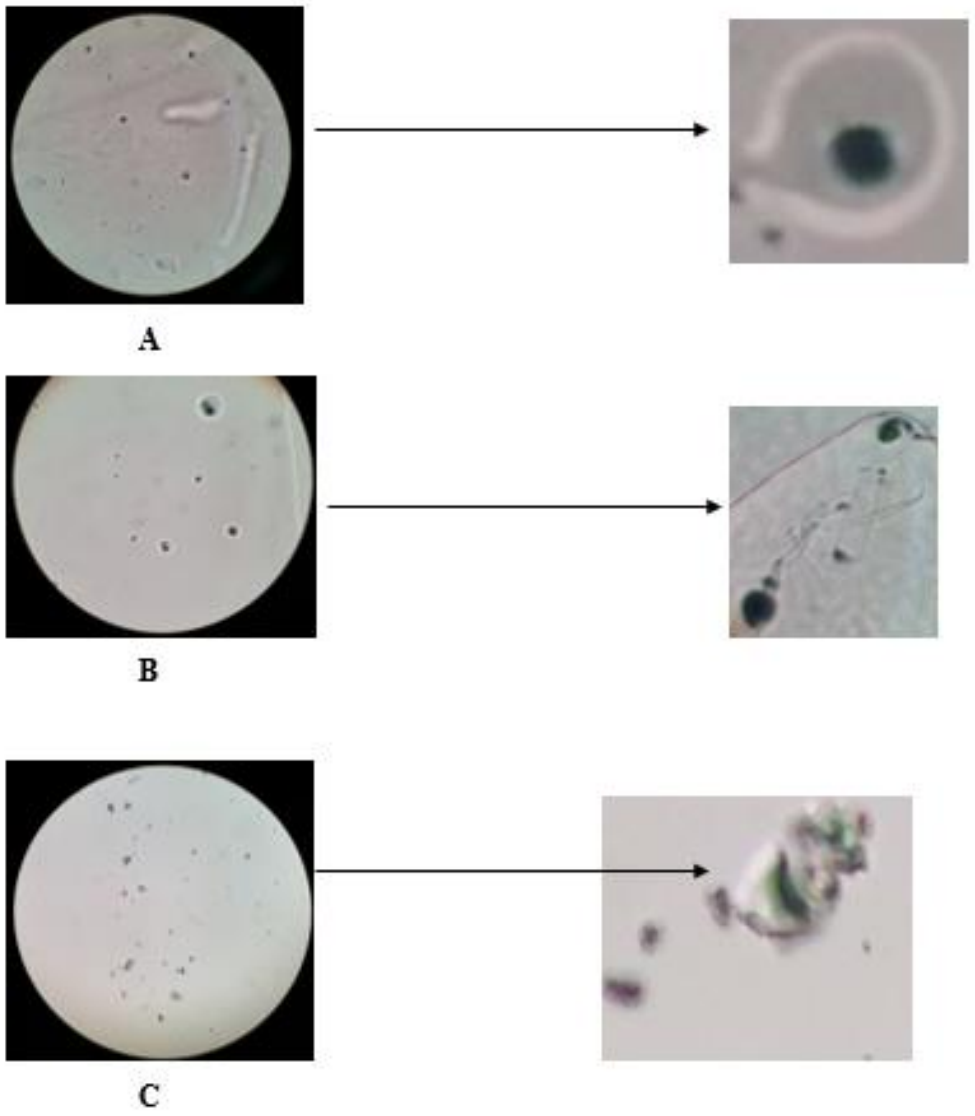

Figure 4. Cytotoxic Test of 10% (v/v) Macroalgae Extract on Mitochondria

(A. Mitochondria + water; B. Mitochondria + *Gracilaria sp*. Extract; C. Mitochondria + phenol)

Figure 4 shows that there is a damage to mitochondria treated with macroalgae extract and phenol (Figure B. mitochondria + *Gracilaria sp*. extract, C. mitochondria + phenol) and no damage to mitochondria treated with water (Figure 4. A). The damage that occurred in mitochondria treated with lipid extracts was not as great as the damage caused by the addition of phenol (positive control). This proves that lipid extracts have pro-apoptotic activity. The lipidic extract of *Gracilaria sp.* has activity as an apoptosis inductor that can cause several symptoms of apoptosis.

Induction of mitochondrial membrane permeability will promote apoptosis, whereas its inhibition can prevent cells from apoptosis. During apoptosis, the cell will undergo microscopic changes including cell shriveling, cytoplasm solidifying, nuclear membrane becoming discontinuous, and causing DNA to break into fragments that encourage the rupture of the cell nucleus and form nucleosome units. Furthermore, the cell splits to form apoptotic bodies and is eventually phagocytized by other cells [24]. The morphological features and biochemical changes that occur at this stage are generally the same, independent of the type of induction.

Conclusion

The addition of *Gracilaria sp.* lipid extract to the mitochondria causes disruption of the mitochondrial membrane so that cytochrome c is released outside the cell which is an early symptom of the apoptosis process.

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