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Inhibition of Apoptosis of Liver Cells of Mice Infected With *Plasmodium berghei* Through The Expression of Caspase-3 Using *Sargassum duplicatum* Extract

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Abstract

The process of apoptosis is an integrated process between external and internal factors involving several enzymes (Caspase-9, -8, -7, -6, -3) that act as major players in the process of apoptosis. This research aims to determine the potential of brown algae methanol extract Sargassum duplicatum against inhibition of apoptosis of liver cells of mice infected with Plasmodium berghei through the expression of caspase-3. Mice weighing 20–30 grams in Plasmodium berghei infection as much as 0.1 ml per head and left until the percent of parasitemia reaches 1-5%. Then mice (Mus musculus) were given methanol extract of Sargassum duplicatum seaweed at a dose of 1 gr / 100 ml, 10 gr / 100 ml, 100, gr / 100 ml, and 200 gr / 100 ml for 4 consecutive days and observed until day 6. After that, a histological preparation was made with immunohistochemistry staining to see the expression of caspase-3. The results of the observations will be analyzed descriptively. The results showed that Sargassum duplicatum methanol extract was able to inhibit liver cell apoptosis in mice infected with Plasmodium berghei. The decrease in Caspase-3 expression in this study is thought to be caused because the brown algae Sargassum duplicatum contains flavonoid compounds, tannins, and saponins which can reduce the pro-inflammatory cytokine caspase-3 through the role of NF-kB which is a transcription factor that plays a role in stimulating and coordinating innate and adaptive immune responses.

***Keywords**: Malaria, Caspase-3, Sargassum duplicatum, Plasmodium.*

Abstrak

Proses apoptosis merupakan suatu proses yang terintegrasi antara faktor eksternal dan internal yang melibatkan sejumlah enzim (Caspase-9, -8, -7, -6, -3) yang berperan sebagai pemain utama dalam proses apoptosis. Penelitian ini bertujuan untuk mengetahui potensi ekstrak metanol alga cokelat Sargassum duplicatum terhadap penghambatan apoptosis sel hati mencit terinfeksi Plasmodium berghei melalui ekspresi caspase-3. Mencit dengan berat badan 20 – 30 gram di infeksi Plasmodium berghei sebanyak 0,1 ml per ekor dan dibiarkan sampai persen parasitemia mencapai 1-5%. Kemudian mencit (Mus musculus) diberi ekstrak methanol rumput laut Sargassum duplicatum dengan dosis 1 gr/100 ml, 10 gr/100 ml, 100 gr/100 ml dan 200 gr/100 ml selama 4 hari berturut-turut dan diamati sampai hari ke 6. Setelah itu dibuat preparat histologi dengan pewarnaan immunohistokimia untuk melihat ekspresi caspase-3. Hasil pengamatan akan dianalisis secara deskriptif. Hasil penelitian menunjukkan bahwa ekstrak methanol Sargassum duplicatum mampu menghambat apoptosis sel hati pada mencit terinfeksi Plasmodium berghei. Terjadinya penurunan ekspresi Caspase-3 dalam penelitian ini diduga disebabkan karena alga cokelat Sargassum duplicatum mengandung senyawa flavonoid, tannin dan saponin yang dapat menurunkan sitokin proinflamasi caspase-3 melalui peranan NF-kB yang merupakan faktor transkripsi yang berperan dalam merangsang dan mengkoordinasi respon imun innate dan adaptif.

Kata kunci: Malaria, Caspase-3, Sargassum duplicatum, Plasmodium.

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INTRODUCTION

The existence of Plasmodium in the body will stimulate the immune system, especially the Reticulo Endothelial System (RES) by activating T lymphocyte cells, macrophages, and various cytokines such as Tumor Necrosis Factor-alpha (TNF-α) and Interleukin-1 (IL-1), causing induction of the release of Reactive Oxygen Intermediate (ROI) and Reactive Nitrogen Intermediate (RNI) oxygen metabolites that will react to form Nitric Oxide (NO) through the activation of inducible Nitric Oxide Synthase (iNOS / NOS / NOS type2) which plays a role in eliminating parasites, but due to its non-specific nature, it can cause pathological abnormalities[1].

The increase in free radicals due to malaria pathogenesis will cause various damages to body organs, including the liver, kidneys, and lungs. In liver cells, TNF-α will cause protein synthesis in the acute phase, and in muscle cells will cause catabolism and in some cells will cause apoptosis. Apoptosis is programmed cell death which is an important process in the normal regulation of homeostasis, this process generates a balance in the number of cells of a certain tissue through the elimination of damaged cells and physiological proliferation and thus maintains normal tissue function.[2,3]

The process of apoptosis is an integrated process between external and internal factors involving several enzymes (Caspase-9, -8, -7, -6, -3) that act as major players in the process of apoptosis where Caspase-3 is the caspase executor in cell apoptosis. This mechanism includes two pathways, namely: the extrinsic pathway, and the intrinsic pathway.[4]

The widely reported cases of resistance further enhance the progressiveness of studies to find new antimalarial drugs. Plants used as antimalarial drugs so far are mostly from land plants, while plants derived from the sea such as seaweed (algae) have not received much attention.[5,6] Currently, *Sargassum duplicatum* has not been optimally utilized,[7] even though *Sargassum duplicatum* is very useful, for example in the fields of health, microbiology, enzymology, and ecotoxicology.[8] Sargassum contains steroid compounds, alkaloids, phenols, flavonoids, saponins, and tannins.[9] The role of flavonoid compounds in inhibiting the growth of malaria parasites has been proven in some antimalarial medicinal plants.[10] The presence of antioxidants in the brown algae *Sargassum duplicatum* can increase the immune system in the form of cytokines, inhibiting the occurrence of cell apoptosis, namely by neutralizing free radicals so that macrophage activity in the body will be reduced.

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METHODOLOGY

Types of Research

The type of research used in this study is an experimental method (laboratory experiment).

Tools and Materials

The tools used are an Erlenmeyer measuring 1000 ml piece of Erlenmeyer measuring 500 ml, measuring cup, filter paper, a set of glassware, electronic scales, blender, rotavapor, mice confinement container, analytical balance, syringe, object glass container, scratch slide, electron microscope, tube centrifuge, hearing, sonde tool, volume pipette, mortar, spatula, test tube, vaporizer and digital camera, paraffin box, object glass, microtome, Micropipette.

While the ingredients used are brown algae, methanol, *Plasmodium berghei* culture, mice, CMC Na 0.5%, alceiver, aluminum foil, tissue, cotton, immersion oil and detergent, alcohol (30%, 50%, 70%, 80%, 90%, 100%), Albumin Glycerin, Caspase 3 antibody, secondary antibody labeled biotin, chloroform, DAB (3,3 diaminobenzidine), etellan, formalin 4%, FBS, Hematoksilin, H_2O_2 %, paraffin (I, II, III), PBS (Phosphate Buffer Saline), SA-HRP Peroxidase (Strep Avidin-Hesoradish Peroxidase), Sterile Aquades, and Xylol I, II, III.

Research Design

This research used a complete randomized design with 4 treatments and 3 repeats. The division of groups can be seen as follows:

1. Group I : Mice were given Plasmodium berghei infection but not treated.
2. Group II: Mice infected with Plasmodium berghei and given Sargassum duplicatum extract at a dose of 10 mg/kgBB.
3. Group III: Mice infected with Plasmodium berghei and given Sargassum duplicatum extract at a dose of 100 mg/kgBB.
4. Group IV: Mice infected with Plasmodium berghei and given Sargassum duplicatum extract at a dose of 200 mg/kgBB.

Work Procedure

Extraction

Sargassum duplicatum is taken and dried to air at room temperature. After drying, it is mashed with a blender (grinder) and the powder that has been smooth is weighed. Furthermore, extraction is carried out using the maceration method. A total of 100 grams of *Sargassum duplicatum* powder was put into Erlenmeyer with a size of 1000 ml, input methanol 250 ml and homogenized then left for 24 hours. After that, it is filtered and applied using a rotary evaporator so that a concentrated extract is obtained.

Testing the effectiveness of anti-malarial in vivo

Mice tried to be infected with as much as 200 µl of blood from donor mice and then observed the level of

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parasitemia. After the percentage of parasitemia (1-5%) is known, it is continually testing the effectiveness of malaria from the extract according to the predetermined dosage. The treatment was carried out for 4 consecutive days and observations were carried out until the 6th day (D0-D6). After that, hepatectomy is performed to take the liver organs of mice.

Making Liver Histology Preparations

Preparation of liver tissue is carried out by procedures according to Suntoro:[11]

1. Liver that has been fixed with Formalin 4 % washed with equates for 5 minutes, dehydrated in stratified alcohol ranging from 30 %, 50 %, 70 %, 80 %, 90 %, and 100% each for 5 minutes.
2. The remaining alcohol is cleaned by a clearing process, and the liver is soaked in Xylol I and Xylol II for 5 minutes each.
3. Infiltration process, liver organs are inserted in Paraffin I, Paraffin II, and Paraffin at III 60°C, each for 45 minutes.
4. In The process of embedding or planting, the liver is put in a Paraffin box for 15 minutes. Then sectioning or slicing is carried out through boiling in Paraffin blocks for a while then cutting with microtome with a thickness of 6 microns.
5. After taking the slicing results, the object glass is smeared with Albumin Glycerin so that the slicing results can stick to the object glass then placed on a hotplate with a temperature of 40 °C which aims to stretch the sliced results and melt paraffin on the object glass.

Observation of Caspase-3 Expression by Immunohistochemistry Method

Observation of Caspase-3 expression was carried out using immunohistochemistry painting methods according to Larasati:[12]

1. Hepatic histology preparations were washed with PBS (Phosphate Buffer Saline) pH 7.4 for 3 minutes, 3 times.
2. To remove endogenous perioxide, 3% H2O2 is used for 20 minutes. Next, the preparations are re-washed with PBS pH 7.4 for 3 minutes, 3 times.
3. The preparations were dripped with Caspase-3 primary antibodies, then incubated at 4°C for one night. After that the preparation is washed with PBS pH 7.4 for 3 minutes, 3 times.
4. Next, drip the preparation with a secondary antibody labeled AP (Alkaline Phosphatase) 1: 2500 anti-IgGgG AP Labelled) and then incubated at room temperature for one hour. After that, it is washed with PBS pH 7.4 for 3 minutes, 3 times.
5. Drops of preparations with Peroxidase Strep Avidin-Hesoradish Peroxidase (SA-HRP) and incubates at room temperature for 60 minutes. The preparations were then washed back with PBS pH 7.4 for 3 minutes, 3 times.

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6. Chromogen is administered by dripping with a solution of 3,3-diaminobenzidine (DAB), and incubated at room temperature for 20 minutes. Then washed with equates for 5 minutes, 3 times.
7. The next process is counterstain, using hamatoksilin incubated at room temperature for 20 minutes. Then dripped with tap water, and washed equates to 5 minutes, 3 times.
8. Dehydration is carried out with a stratified alcohol solution of 70%, 80%, 90%, and 100% and absolute alcohol I and II for 1 minute each.
9. Clearing is done with xylol I and xylol II for 3 minutes each, then the preparations are dried.
10. Mounting using an entellan, then covered with a glass object, labeled with the name of the preparation is then observed under a microscope.
11. If Caspase-3 is detected, it will be brown.

Data Analysis

Caspase-3 expressions will be analyzed descriptively.

RESULTS AND DISCUSSION

Photomicrograph of liver cells of mice (*Mus musculus*) with Immunohistochemistry staining can be seen in Figure 1.

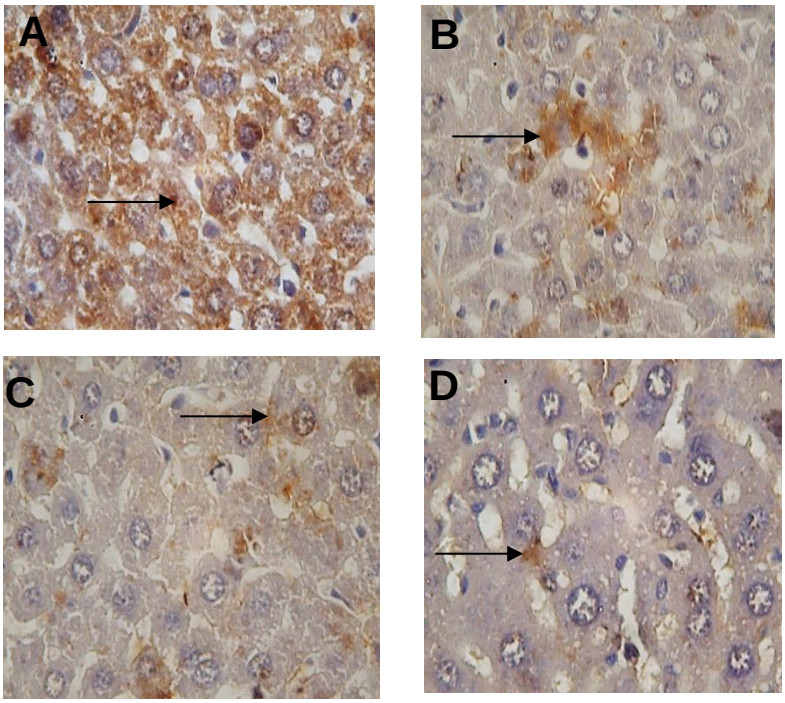

Figure 1. Photomicrograph of liver cells of mice by immunohistochemistry staining method. (A) group I, (B) group II, (C) group III, and (D) group IV.

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Based on the results that have been obtained, it can be explained that Plasmodium berghei infection in mice can cause apoptosis of liver cells. This is seen through the expression of Caspase-3 on liver cells when animals are infected with Plasmodium berghei (Figure 1A). Caspase-3 can be activated also through intrinsic pathways that can lead to the activation of caspase and the induction of apoptosis through the mitochondria. In mitochondria, apoptosis occurs through a potential reduction in the inner mitochondrial membrane and the release of a number of small molecules including apoptosis-inducing factor (AIF), endonuclease G, SMAC/DIABLO, HtrA2/Omi, and cytochrome c (Cyt c).[13] Under normal circumstances, cyt c is located in the space between the inner and outer mitochondrial membranes where it functions in oxidative phosphorylation. However, once released it will immediately join in the proteolytic activation of caspase-3 by caspase-9. The event led to the gathering of a multiprotein complex that activates caspase called 'apoptosome'. Apoptotic protease activating factor-1 (Apaf-1) forms the principal component of the apoptosome.[14] Atroph-1 is a protein that plays a role in the activation of caspase so that it is bound to cyt c, which is related to procaspase-9 through the breakdown of the caspase recruitment domain (CARD) and activates it. Caspase-9 can then reproduce procaspase-3, activating it through enzymatic breakdown to form caspase-3, which can then initiate a cascade of apoptosis involving caspase-2, -3, -6, and -7 effectors which ultimately results in DNA fragmentation.[3] This event is also followed by the translocation process of phosphatidylserine to the outer plasma membrane which will facilitate the introduction of apoptotic cells by macrophages, resulting in the clearance of dead cells.

Administration of *Sargassum duplicatum* extract can inhibit apoptosis of liver cells of mice infected with *Plasmodium berghei.* This can be seen by the large number of cells expressing caspase-3 in the negative control group (infected with *Plasmodium bergehi* but not given extract *Sargassum dulicatum*) when compared to the positive control group, the mice group infected with *Plasmodium bergehi* and given methanol brown algae Sargassum duplicatum dose 10 mg/kg BB (Figure 1B), 100 mg/kg BB (Figure 1C) and 200 mg/kg BB (Figure 1D).

The decrease in Caspase-3 expression in this research was caused because *Sargassum duplicatum* contains flavonoid compounds, tannins, and saponins which are thought to reduce the pro-inflammatory cytokine caspase-3 through the role of NF-kB which is a transcription factor that plays a role in stimulating and coordinating innate and adaptive immune responses. The mechanism of decreasing NF-kB activation includes

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inhibiting the translocation of NF-kB into the nucleus, inhibiting Ik-B phosphorylation, inhibiting the activation of genes that encode NF-kB transactivation and Ik-B degradation, or through the obstruction of the Ik-B degradation process by the proteasome. The decrease in NF-kB activation results in a decrease in NF-kB signaling in immune cells, thus regulating the decrease in TNF-α.[15]

Flavonoid compounds (prenylated stilbene) from Artocarpus integer have in vitro antimalarial activity in Plasmodium falciparum.[16] Flavonoid activity was also reported in studies conducted on plants. This plant is known as the basic ingredient of the malaria drug artemisinin with a lactone sesquiterpene content that is active against drugs resistant to Plasmodium falciparum. The results of research on several flavonoid compounds of this plant are known that flavonoids can increase the reaction of artemisinin to hemin and have antimalarial activity in vitro potential.[17]

CONCLUSION

Based on the results that have been obtained, it can be concluded that the methanol extract of brown algae *Sargassum duplicatum* can potentially inhibit liver cell apoptosis through the expression of Caspase-3.

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