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RESEARCH ARTICLE

Inability of Polysaccharides of Spirulina Platensis to Protect Hepatocyte Cells Line on Toxoplasma Gondii Infection *In Vitro*

Zhaza Afililla[1], Lucia Tri Suwanti[2,3*], Heni Puspitasari[3], Eko Agus Suyono[4], Arief Budiman[5], Mochamad Donny Koerniawan[6], Ulfah Juniarti Siregar[7]

1. *Magister Student of Faculty of Veterinary Medicine, Universitas Airlangga, Surabaya, 60115, Indonesia.*
2. *Department of Veterinary Parasitology, Faculty of Veterinary Medicine, Universitas Airlangga, Surabaya, 60115, Indonesia.*
3. *Institute of Tropical Disease , Universitas Airlangga, Surabaya, 60115, Indonesia.*
4. *Faculty of Biology, Universitas Gadjah Mada Yogyakarta, Indonesia.*
5. *Faculty of Technology, Universitas Gadjah Mada, Yogyakarta, Indonesia.*
6. *School of Architecture, Institut Teknologi Bandung, Bandung, Indonesia.*
7. *Faculty of Forestry, Institut Pertanian Bogor, Bogor, Indonesia.*

***Corresponding Author: Lucia Tri Suwanti**

Abstract

Toxoplasmosis is one zoonotic disease which caused by an obligate intracellular protrozoan parasite, *Toxoplasma gondii*. The parasite infects humans and warm-blooded animals (mammals and birds). The drug of choice against toxoplasmosis is combination between pyrimethamin with sulfadiazine, but both can induce side effects and toxic to host cell, so alternative therapeutic compounds are needed. Many reseachers have explored microalgae for food and medicine. One of the main components of *Spirulina platensis* that plays a role in enhanching immunity is polysaccharides. The present study investigated effect of Spirulina polysaccharides on *Toxoplasma gondii* infection in Vitro. Polysaccharides of *S. platensis* were extracted with hot water and alkhaline method. Extraction Rate (ER), sugar and protein levels were compared between the two methods. The Hepatocyte Cells Line (HCL) were infected with *T. gondii* and treated with polysaccharides of *S. platensis*. The parameters were Parasite burden and Viability Host Cell. The result shows that Extraction Rate, carbohydrate and protein levels were affected by extraction method. Carbohydrate level in polysaccharides with hot water extraction was higher than alkhaline extraction, on the contrary, ER and protein level were lower. Polysaccharides of *S. platensis* did not protect HCL infected with *T. gondii*. More than 5 mg/mL polysaccharides of *S. platensis* was toxic to host HCL.

Keywords: *Alkaline extraction, Hot water extraction, Polysaccharides, Spirulina platensis, Toxoplasma gondii.*

Introduction

Toxoplasmosis is one zoonotic disease which caused by an obligate intracellular protozoan parasite, *Toxoplasma gondii*. The parasite infects humans and warm-blooded animals (mammals and birds). Cats and another feline are definitive host that produce and spread the oocyst in environment. *Toxoplasma gondii* is estimated to infect one-third of population in the world [3].

Predominantly, transmission occurs through fecal–oral route by sporulated oocysts that contaminated food or water, or ingestion of tissue cysts from meat-infected or transplacental (congenital) [4]. Congenital infection causes clinical symptoms ranging from mild to severe symptoms including visual impairment, chorioretinitis, hydrocephalus or microcephaly, intracerebral

calcification, seizures, mental retardation and fetal death [2,13] and infection caused inflamation of variouse organs even death in individual with immune system disorder [19]. Acording to Mordue et al.[9] death of mice which infected with the virulent *T. gondii* due to severe liver damage. Although the prevalence of *T. gondii* infection in humans and animals are high, clinical treatments are limited. The drug of choice against toxoplasmosis is combination pyrimethamin with sulfadiazine [3] Because pyrimethamin and sulfadiazine induce side effects and toxic to host cell, alternative therapeutic compounds are needed [1].

In recent years, many reseachers have explored microalgae for food and medicine. This is related to component of the polysaccharide in cell wall, lipid, amino acid, pigment and micronutrent [18]. According to Sathasivam et al.,[12] *Spirulina, Chlorella, Haematococcus, Dunaliella, Botryococcus, Phaeodactylum, Porphyridium, Chaetoceros, Crypthecodinium, Isochrysis, Nannochloris, Nitzschia, Schizochytrium, Tetraselmis* and *Skeletonema* are the microalgae that have been identified for commercial use.

Commersial polysaccharides extracted from microalgae include alginat, agar and carrageenan [7]. In industry, *Spirulina* is one of the most important species as healthy food and nutritional supplement because it increase immune system activity, as anti tumor and growth promotor for livestock[8]. One of the main components that plays a role in enhanching immunity is polysaccharide [10].

In addition to the polysaccharides located on the cell wall of microalgae[17], microalgae also produce polysaccharides which are released into environment and called Exopolysaccharides (EPS) [8] and in their review mentioned that polysaccharides have activities as antibacterial, antioxidant, antivirus, anticancer and immunomodulator. According to Wang et al [21].

the alkaline extraction method has a higher efficiency to extracted polysacarides from Spirulina than the other methods. Polysaccharides of Spirulina utility as anti-toxoplasmosis is limited. We only found two published articles related to anti-toxoplasmosis activity of Spirulina [15,16]. They had done ethyl acetat, water and methanol extraction, but not polysaccharides extraction and reported that spirulina increase the humoral response of infected mice based on dose and type of extract. According to Xu et al. (2019) polysaccharides from fungus *Inonotus obliquus* protects mice from *Toxoplasma gondii*-induce liver injury. In this study we investigated the effect of polysaccharides from *Spirulina platensis* on *Toxoplasma gondii* infection in vitro**.**

Materials and Methods

Isolate of Parasite

Isolate of *T. gondii* used in this study were tachyzoites of the virulent RH strain, from Laboratory of Veterinary Parasitology, Faculty of Veterinary Medicine, Universitas Airlangga (Surabaya, Indonesia). The tachyzoites were maintained in peritoneal cavity of mice. After mice showed clinical sign, the tachyzoites were collected from intraperitoneal and centrifuged at 1500 rpm for 20 min. The tachyzoites in the pellet were released by forceful passage through a 27-gauge needle and centrifuged at 1500 rpm for 20 min and then the supernatant was discarded. The pellet was resuspended in PBS, and the number of tachyzoites was calculated by a haemocytometer.

Hepar Cells (Hepatocytes)

Hepatocyte Cells Line were kindly provided by the NPMRD Study Group, Institute of Tropical Disease, Universitas Airlangga. Parasite infections were performed in subconfluent cultures in 24-well cell culture plates.

Biomass *Spirulina Platensis*

Biomass *Spirulina platensis* was prepared by Faculty of Biology, Universitas Gadjah Mada Yogyakarta. Harvested algae was washed with fresh water to removed debris and epiphyte. The cleaned material was dried by oven at 80^{o}C overnight[7]. The dried samples were powdered by grinding and stored in -20^{O}C until used.

Alkaline Extraction

Alkanie extraction was done as previously described [21]. Fourty grams Spirulina powder were added with 1.6 kg distilled water and amount of 1 mol/L NaOH were

added until pH was adjusted to 10 as measured in pH meter. Mixture was incubated in waterbath 80°C for overnight with stired and followed by centrifugation at 4300 rpm for 20 minutes.

The pellets were resuspended with 1/5 initial volume and added five times volume with ethanol 95%, then incubated at freezer overnight and followed by centrifugation at 4300 rpm for 10 minutes. Then the precipitate was washed by aceton and evaporated by rotary evaporator. The final extraction products were dried and stored at -20° C for future use. And henceforth is called Polysaccharide Sprirulina (PSP).

Extraction Rate, Level of Protein and Carbohydrate

Extraction rate was calculated by formula: polysaccharide mass/Spirulina powder dry weight × 100%. Level of protein was counted by Bradford Method with spectrophotometer wavelength 595nm and by Phenol Sulfuric Acid Method ith spectrophotometer wavelength 490nm for level of carbohydrat.

Anti -Toxplasma in Vitro

Hepatocyte Cell Line were cultured with concentration 1×10^5 hepatocyes per well in 24-well cell culture plates one day before the assay. Plates were used for measurements of parasite burden and measurements of viability of host cells.

Parasite Burden

The cells were infected with 1×10^3 tachyzoites per well in DMEM for 6 h. Then the monolayer cell was washed twice with phosphate-buffered saline (PBS) to remove non-adhered parasites. Polysaccharide Sprirulina (PSP) were added at different concentrations respectively, 5, 10 and 20 mg/mL.

The positive control group was incubated in medium without PSP and drug. As a positive drug controls, 0.4µg/mL sulfadiazine were added separately under the same conditions [14]. After 24h of treatment, the cells were fixed with fresh in 10% buffered formalin and stained with Giemsa and observed under microscope. The number of infected cell were calculated to by observing the percentage of the number of infected cells in one field of view.

Viability Host Cells

The viability of the host cells were determined by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay. Well of microplate were divided into eight groups, each group got three times replication. Group 1: as negative control group, non infection and free from PSP. Group 2: as control positive group, infected and free from PSP. Group 3,4 and 5: infected and treated by PSP with concentration, 5, 10 and 20 mg/mL, respectively. Group 6,7 and 8: non infection cells and treated by PSP with different concentrations: 5, 10 and 20 mg/mL, respectively.

Exctract was solubilised in DMSO and diluted in DMEM. Infected dose 1×10^3 tachyzoites per well. Infection and treatment were done in the same time. After 48 h of treatment, the culture supernatant was removed, and 15µL of MTT solution (5mg/mL) in DMEM was added to each well for 4 h. The formazan crystals were subsequently solubilized by the addition of 100 µL of pure DMSO. The plate was read at 570 nm in a Promega Glomax MultiPlus Detection System (USA).

Result and Discussion

Extraction Rate, Level of Protein and Carbohydrate

Polysaccharide Spirulina (PSP) were extracted using two methods (alkaline and hot water) and both were analyzed and the result showed different levels of protein and carbohydrate. (Tabel 1) According to [11], *Spirulina platensis* has a soft cell wall that contain complex sugars and protein. Microalgae have higher quantities of proteins than carbohydrates [18]. Extraction Rate (ER) in this research was higher than Wang et al [21]. Protein level of alkaline extraction in this research, shows higher result than hot water extraction.

According to Kadam et. al.,[6], high level of protein in alkaline extraction is due to a condition where increasing on pH helped the insoluble hydrophobic protein from seaweed to be solubilized. In this researh, the number of protein level in alkaline extraction was two times higher than hot water extraction. Because the pH in alkaline extraction was 10.

Table 1: Extraction rate, protein and carbohydrate levels in hot water and alkaline extraction

No	Extraction Method	Extraction Rate (%)	Level of Protein (mg/mL)	Level of Carbohydrate (mg/mL)
1	Hot Water	60	15,73	221,5
2	Alkaline	64	35,20	156,0

The total protein content of the biomass depends on the microbial species [18]. The range of protein content is from 30% to 55% of the dry weight. Protein of *Spirulina platensis* contains amino acid: leucine, valine, isoleucine, phenylalanine, tyrosine, methionine, cysteine, and tyrosine. Microalgae carbohydrates are formed both inside the chloroplast and in the cytosol, and their presence are in the microalgal cell wall and intracellular vacuoles. The content and type of carbohydrates depend on the microalga mostly glucose, rhamnose, xylose, and mannose [18].

Parasite Burden

Tachyzoites of *T. gondii* were still alive although given PSP extract. These shown from field of view that more than 75% cells were not as good as one that were not given with PSP extract. While the cells that treated with Sulfadiazine 25% were still infected. Which means PSP were not able to protect the in vitro infection. This happened probably because polysaccharide roled in increasing immune system, while during in vitro there were no immunology cells, thus another research are needed for further finding in vitro infection. This also reinforced with MTT Assay result.

Viability Host Cells

Microalgae polysaccharides varied in their biological properties depending on their characteristics. For example, beta glucans are considered immune stimulators, which can lead to an immune response that promotes the activation of phagocytosis, radical oxygen species production, and a cellular immune response[18]. The immunomodulatory action of *S. platensis* has been suggested by some researchers to be mediated through the innate immune system and the researches were done in Vivo [11]. In mice, modulation immune system via increased proliferation of erythrocytes, granulocyte-monocyte, and fibroblast lineage cells derived from bone marrow cells of mice [11].

MTT Assay was done to find out about viability of host cells. Results of MTT Assay in this research showed that PSP with concentration 10 mg/mL and 20 mg/mL are toxic to hepatocyte cell, but non-toxic towards *T. gondii* infected hepatocyte.

The result also shows that hepatocyte with no infection and given 5 mg/mL PSP are still safe, because there are more than 50% of living cell after treatment and infection. In this research infected cells shows nearly same numbers of living cell, and even more above the control because *T. gondii* tachyzoites are living in the cell host and developing really fast. Bellow is the precentage of living cell after treatments and infection followed by MTT Assay (Table 2).

Table 2: MTT Assay result on precentage of living cell after undergo treatments and infection

Groups	Treatment	Precentage of living cell (%)
P1	Non infection without polisaccharide treatment	100,00 [a]
P2	Infection, without polisaccharides treatment	107,55 [a]
P3	Infection, with 5 mg/ml polisaccharides treatment	100,77 [a]
P4	Infection, with 10 mg/ml polisaccharides treatment	106,99 [a]
P5	Infection, with 20 mg/ml polisaccharides treatment	112,99 [a]
P6	Non infection, with 5 mg/ml polisaccharides treatment	53,45 [b]
P7	Non infection, with 10 mg/ml polisaccharides treatment	39,73 [c]
P8	Non infection, with 20 mg/ml polisaccharides treatment	38,79 [c]

Conclusion

Extraction method of polysaccharides involve significantly in ER result. Carbohydrate level in polysaccharides with hot water extraction was higher than alkaline extraction, on the contrary, ER and protein level were lower. Polysaccharides of *S. platensis* did not protect

HCL on *T. gondii* infection in vitro. More than 5 mg/mL polysaccharides of *S. platensis* was toxic to HCL, but not toxic toward infected HCL.

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