JIPK. Volume 14 No 2. November 2022
Sinta 1 (Decree No: 158/E/KPT/2021)
e-ISSN:2528-0759; p-ISSN:2085-5842
DOI=10.20473/jipk.v14i2.34766
Available online at https://e-journal.unair.ac.id/JIPK/index

JIPK
(JURNAL ILMIAH PERIKANAN DAN KELAUTAN)
Scientific Journal of Fisheries and Marine

Short Communication

The Activity of Mixed Microalgae Polysaccharides from Indonesia as Anti-Malaria in Vitro

Mahendra Pujiyanto[1], Zhaza Afililla[1], Lilik Maslachah[2], Thomas Valentinus Widiyatno[3], Mochamad Donny Koerniawan[4], Eko Agus Suyono[5], Arief Budiman[6], Ulfah Juniarti Siregar[7], and Lucia Tri Suwanti [8,9*]

[1]Faculty of Veterinary Medicine, Universitas Airlangga, Surabaya, East Java, 60115. Indonesia
[2]Department of Veterinary Basic Medicine, Faculty of Veterinary Medicine, Universitas Airlangga, Surabaya, East Java, 60115. Indonesia
[3]Department of Veterinary Pathology, Faculty of Veterinary Medicine, Universitas Airlangga, Surabaya, East Java, 60115. Indonesia
[4]Department of Architecture, School of Architecture, Planning, and Policy Development, Institut Teknologi Bandung. Bandung, West Java, 40132. Indonesia
[5]Department of Tropical Biology, Faculty of Biology, Universitas Gadjah Mada, Sleman, Yogyakarta, 55281. Indonesia
[6]Department of Chemical Engineering, Faculty of Engineering, Universitas Gadjah Mada, Sleman, Yogyakarta, 55281. Indonesia
[7]Department of Silviculture, Faculty of Forestry, Institut Pertanian Bogor, Bogor, West Java, 16680. Indonesia
[8]Department of Veterinary Parasitology, Faculty of Veterinary Medicine, Universitas Airlangga, Surabaya, East Java, 60115. Indonesia
[9]Department Institute of Tropical Disease, Universitas Airlangga, Surabaya, East Java, 60115. Indonesia

OPEN ACCESS
ARTICLE INFO

Received: April 05, 2022
Accepted: May 11, 2022
Published: May 21, 2022
Available online: August 30, 2022

*) Corresponding author:
E-mail: lucia-t-s@fkh.unair.ac.id

Keywords:
Anti-Malaria Drug
Infectious Disease
Malaria
Tropical Disease

Abstract

Malaria is an infectious disease caused by protozoan parasites of the genus *Plasmodium* that categorized as deadliest diseases in the world. Artemisinin and its derivatives are still recommended drugs for malaria therapy, however, there have been indications that *Plasmodium* parasites are resistant to this drug. Therefore, a study on polysaccharides from microalgae may be a potential as bioactive compound for anti-malaria. The aim of this study was to determine the effectiveness of the mixed microalgae polysaccharides as anti-malarial in vitro. Polysaccharides were extracted from three microalgae *Spirulina* sp., mixed microalgae Glagah and mixed microalgae East Java using the alkaline extraction method. The anti-malarial activity test refers to the concentration of polysaccharides used in calculating the IC_{50} value by probit analysis. The concentration of polysaccharides of the three microalgae used were 0; 0.01; 0.01, 1, 10 and 100 µg/mL. The results showed that the IC_{50} values of polysaccharides of Glagah, *Spirulina* sp. and East Java microalgae were 3.18 µg/mL, 5.43µg/mL, and 9.87 µg/mL, respectively. In Conclusion, polysaccharides of Indonesian mixed microalgae can be promoted as anti-malarial.

Cite this as: Pujiyanto, M., Afililla, Z., Maslachah, L., Widiyatno, T. V., Koerniawan, M. D., Suyono, E. A., Budiman, A., Siregar, U. J., & Suwanti, L. T. (2022). The Activity of Mixed Microalgae Polysaccharides from Indonesia as Anti-Malaria in Vitro. *Jurnal Ilmiah Perikanan dan Kelautan*, 14(2):396-403. http://doi.org/10.20473/jipk.v14i2.34766

1. Introduction

Malaria is an infectious disease caused by protozoan parasites of the genus *Plasmodium*. This disease is one of the deadliest diseases in the world (Talapko *et al*., 2019). Indonesia holds the second highest-ranking country (after India) in Southeast Asia for the highest number of malaria cases (WHO, 2020). Although there was a decline in 2010-2014, the trend of malaria cases in Indonesia tends to stagnate from 2014-2019. Plasmodium species that cause disease in humans in Indonesia are *Plasmodium falciparum*, *P. vivax*, *P. malariae* and *P. ovale,* which is *P. falciparum* appears to be the most common species (Elyazar *et al*., 2011). Malaria caused by *P falciparum* infection is the most severe disease because it can cause cerebral malaria, resulting in death.

Artemisinin and its derivatives are recommended drugs for malaria therapy due to their effectiveness. According to Simamora and Fitri (2007), artemisinin belongs to the group of sesquiterpene lactone compounds that have endoperoxide bridges. The structure of the endoperoxide bridge on the artemisinin molecule is broken by Fe2+ ions (heme compounds) to become highly reactive free radicals. Artemisinin radicals inhibit and modify various molecules in the parasite, causing the parasite to die. However, *Plasmodium* as the cause of malaria is already resistant to artemisinin (Afonso *et al*., 2006). Balikagala *et al*. (2021) reported that a total of 14 of 240 African patients between 2017 and 2019 who received intravenous artesunate therapy were shown to be infected with artemisinin-resistant *Plasmodium*. Among 14 patients, 13 were infected with *P. falciparum* which had mutations in the A675V or C469Y alleles in the *kelch13* gene. This is evidence that artemisinin resistance has occurred in Africa. Another malaria drug is chloroquine. Chloroquine acts at the stage of the intraerythrocytic cycle in which the parasite actively degrades hemoglobin (Parhizgar and Tahghighi, 2017). Cases of Plasmodium resistance to anti-malarial drugs are now a problem in endemic areas such as Indonesia (Khamis *et al*., 2018). Anti-malarial drug resistance has hampered progress in malaria control. Therefore, research is needed to find alternative drugs from natural ingredients.

Polysaccharide is one of components of natural ingredients and has been used as anti-malarials. Denou *et al*. (2019) revealed polysaccharides from the water extraction of *Argemone mexicana* (aerial parts), *Sarcocephalus latifolius* (root bark), and *Vitex doniana* (leaves) have potential as anti-malarials through an immunomodulating mechanism. Likewise, Zhu *et al*. (2012) proved that polysaccharides from the Chinese medicinal herb *Achyranthes bidentata* enhance anti-malarial immunity during Plasmodium infection. The anti-malarial activity of polysaccharides from various marine organisms (sea cucumber *Ludwigothurea grisea* and *Isostichopus badionotus*, red algae *Botryocladia occidentalis*, and marine sponge *Desmapsamma anchorata*) was analyzed on the growth of *P. falciparum* in vitro culture by Marques *et al*. (2016) and the results showed that polysaccharides inhibited the growth of parasites. Growth inhibition of *P. falciparum* due to polysaccharides act to inhibit the invasion of merozoites of *P. falciparum* into erythrocytes (Boyle *et al*., 2017).

Microalgae has been used as a potential biomass raw material in various fields ranging from health, cosmetics, aquaculture, and even for alternative fuels to replace fossil fuels (Chen *et al*., 2011). Microalgae contain polysaccharides/monosaccharides, carotenoids, phytosterols and various vitamins and function as antioxidants, anti-inflammatory, immunostimulant (Gügi *et al*., 2015). According to Wulandari *et al*. (2016), phycocyanin from the microalgae *Spirulina platensis* extracted using phosphate buffer has anti-malarial activity with an IC_{50} of 158.489 µg/mL. Other researchers have proven that the chloroform extract of *Skeletonema costatum* and *S. platensis* has anti-malarial activity based on the inhibition of the enzyme from P. falciparum (PfMQO), and the IC_{50} values of both are 0.043 µg/mL and 5.25 µg/mL, respectively (Setyowati *et al*., 2019). Mutanda *et al*. (2020) explained in their review that crude extracts from microalgae strains Chlorophyta, Heterokontophyta, and Rhodophyta had anti-plasmodial activity against *P. falciparum* (erythrocytic stage), as well as secondary metabolites of *Sargassum heterophyllum* (including sargaquinoic acid, sargahydroquinoic acid, sargaquinal, and fucoxanthin), has strong anti-plasmodial activity against chloroquine-sensitive *Plasmodium falciparum*.

Microalgae are eukaryotic photosynthetic microorganisms found in almost all habitats, both normal and extreme waters such as fresh water, lakes, rivers, oceans, estuaries, brackish, thermophilic, saline, and hyper-saline environments (Leliaert *et al*., 2012; Selvarajan *et al*., 2015), including in Indonesian marine waters. Indonesian researchers have found and succeeded in culturing the Glagah and East Java Mixed Microalgae (Suyono *et al*., 2016, 2018; Zakiyah *et al*., 2020). The mixed microalgae then were called consortium (Glagah consortium and East Java consortium). Mixed Microalgae from Glagah Yogyakarta contained 6 species of microalgae and 6 species of bacteria. The microalgae are *Cyclotella polymorpha*, *Cylindrospermopsis raciborskii*, *Golenkinia radiata*,

Corethron criophilum, *Chlamydomonas* sp., and *Syracosphaera turquoise*. Meanwhile, the bacteria are *Corynebacterium ulcerans*, *Corynebacterium bovis*, *Bacillus cereus*, *Bacillus megaterium*, *Pediococcus parvulus*, and *Staphylococcus vitulinus* (Suyono *et al*., 2018). Zakiyah *et al*. (2020) found microalgae from several beaches in East Java, namely microalgae divisions of Bacillariophyta, Chlorophyta, Cyanophyta, Dinoflagellates, Granuloreticulosa, and Ochrophyta. Study of mixed microalgae from East Java have only limited diversity based on identification at the genus level and distribution. Meanwhile research on mixed microalgae from Glagah beaches was still limited to the lipid profile as biodiesel material (Suyono *et al*., 2016). The use of the mixed microalgae, both from Glagah and East Java, has not been carried out in the food or health sector, including the polysaccharide content for anti-malarial.

This study aims to determine the effectiveness of polysaccharides of microalgae from Glagah Yogyakarta and East Java compared with polysaccharides of *Spirulina* sp. as anti-malarial based on parasitemia, growth inhibition of *Plasmodium falcifarum* in vitro.

2. Materials and Methods

2.1 Material

Spirulina sp. and Glagah mixed microalgae were obtained from Faculty of Biology, Universitas Gadjah Mada Yogyakarta, and East Java microalgae from Faculty of Forestry, Institut Pertanian Bogor. *Plasmodium falciparum* strain 3D7 sensitive to chloroquine was obtained from the Institute for Tropical Diseases Universitas Airlangga.

2.2 Methods

2.2.1 Microalgae polysaccharide extraction

Microalgal polysaccharides were extracted under alkaline conditions (pH 10) as was done according to Wang *et al*. (2018). Forty grams of powder microalgae were added to 1.6 L of water. The solution was prepared under pH 10 by adding 1 mol/L NaOH and incubated in water bath for 8 hours at 80°C. Then the solution was centrifuged at 4300 rpm for 20 min. The supernatant was concentrated to 1/5 of the initial volume and added five times the volume of 95% ethanol. The solution was then incubated in 4°C refrigerator overnight and followed by centrifugation at 4300 rpm for 10 minutes. The precipitate was washed with acetone absolute up to 2 times the volume, filtered, freeze-dried for 2 hours, and stored for further use.

2.2.2 In vitro anti-malarial activity test

The plasmodium used in this in vitro anti-malarial activity test is *Plasmodium falciparum* strain 3D7 which is sensitive to chloroquine and were obtained from the Institute for Tropical Diseases Universitas Airlangga. The parasites used in this study were synchronous parasites (Ring stage) with ±1% parasitemia (5% hematocrit). The concentration of polysaccharides of the three microalgae used were 0 (negative control); 0.01; 0.1, 1, 10 and 100 µg/mL. The concentration of polysaccharides was determined based on the research by Hafid *et al*. (2016) and Widyawaruyanti *et al*. (2014).

The polysaccharide solution, for each concentration, was taken as much as 2 µl and put into the well. Then 198 µl of parasite was added to each well (each concentration was made in duplicate) and incubated in mix gas (5% O_2, 5% CO_2 and 90% N_2) for 48 hours at 37°C. The cultures were harvested. Thin blood smears were made and stained with 20% Giemsa. Parasitemia was calculated by observing the number of infected cells in every 1000 erythrocytes under a microscope. Parasitemia data was used to calculate growth percentage and inhibition percentage, with the formula:

- Percentage of Parasitemia = (Number of parasites/ erythrocytes) x 100%
- Percentage of Growth = % Parasitemia after 48 hours - % parasitemia at 0
- Percentage of growth inhibition = 100% - Xu/Xk) x 100%)
- Xu = % growth in test solution
- Xk = % growth in negative control

2.2.3 Probit analysis

The percentage of growth inhibition data was used for probit analysis using SPSS version 20 program. Probit analysis was to determine the IC_{50} value, namely the concentration of microalgae polysaccharides that could inhibit parasite growth by 50%.

3. Results and Discussion

3.1 Results

Anti-malarial activity determined by calculating the IC_{50} value of microalgae polysaccharides on the growth of *P. falciparum* 3D7. There was an inverse correlation between polysaccharide concentration and *Plasmodium* growth (Figure 1). The higher the concentration of microalgae polysaccharides in the culture, the less the number of parasites that grew, indicated by the low percentage of parasitemia. Parasite growth was lowest in cultures with polysaccharide concentrations of 100 µg/mL and among the three microalgae, the lowest parasitemia was shown in cultures with Glagah microalga polysaccharides.

Table 1. Growth inhibition and IC_{50} value of microalgae polysaccharides *Spirulina* sp., Glagah, and East Java on growth of *P. falciparum* in vitro

Microalgae	Percentage of growth inhibition of *P. falciparum* in each concentration (µg/ml) of microalgae polysaccharides					IC50 (µg/ml)
	0.01	0.1	1	10	100	
Spirulina sp.	8.61	26.80	37.44	52.55	71.44	5.43
Glagah consortium	10.98	22.67	41.48	55.01	79.97	3.18
East Java consortium	1.67	15.55	30.84	54.31	66.69	9.87

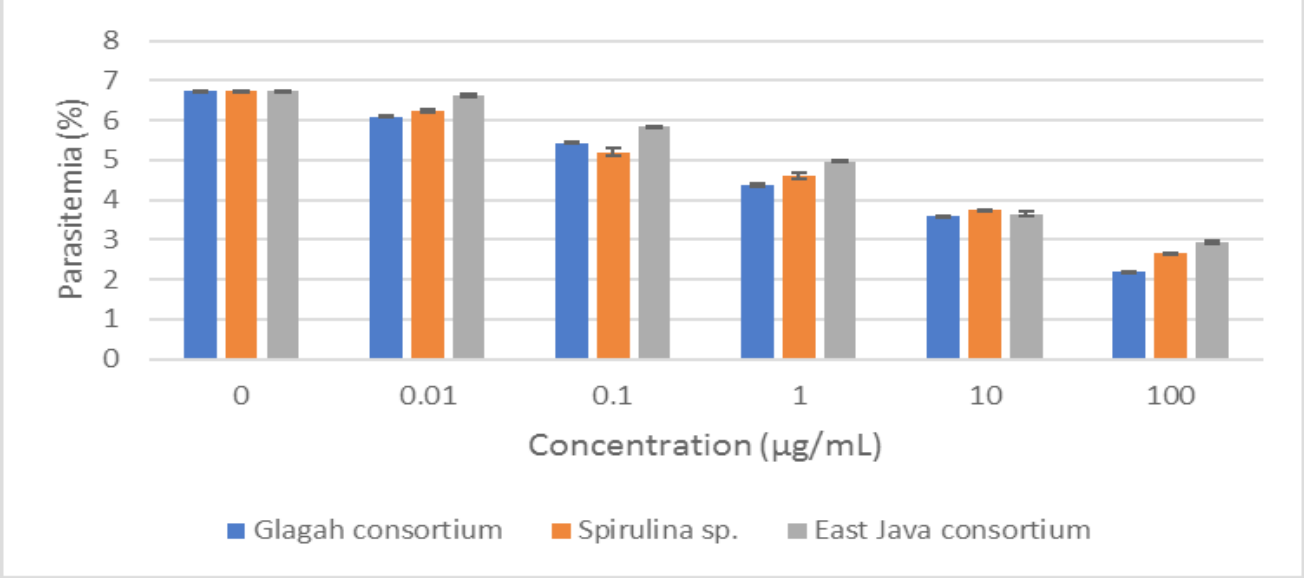

Figure 1. Parasitemia of *P. falciparum* 3D7 after microalgae polysaccharide exposure in vitro

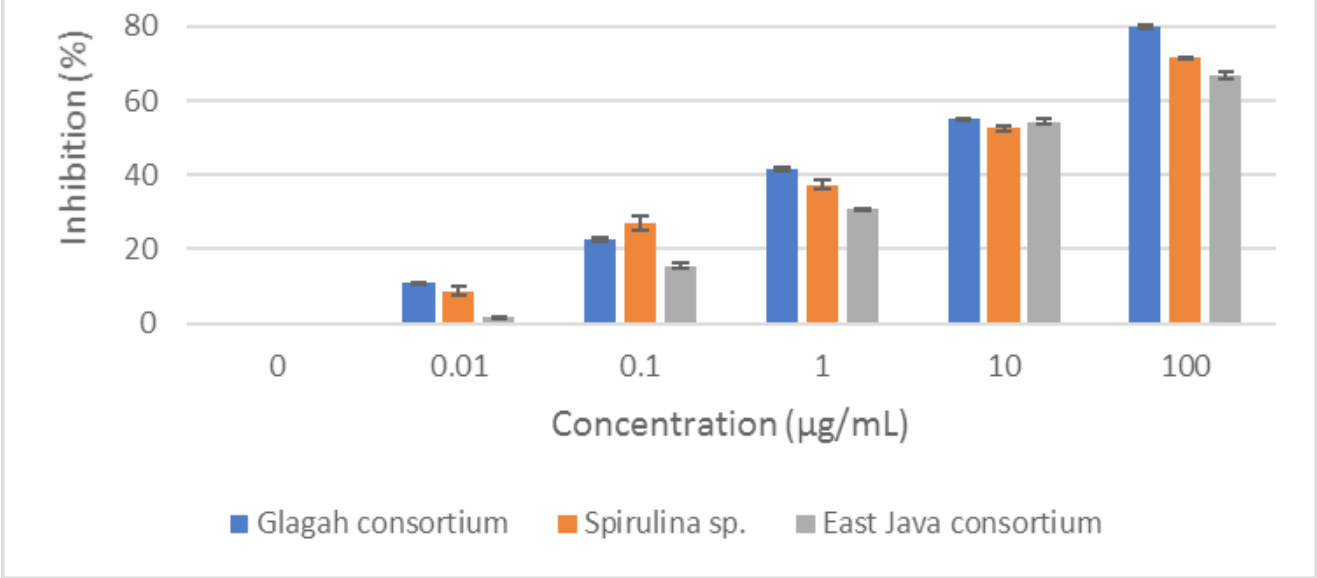

Figure 2. Growth inhibition of *Plasmodium falciparum* 3D7 after microalgae polysaccharide exposure in vitro

The growth of *P. falcifarum* in the medium with the addition of polysaccharides from the three microalgae (*Spirulina*, Glagah microalgae and East Java microalgae), at a concentration of 10 µg/mL there was growth inhibition of more than 50% (Table 1) (Figure 2). In the probit analysis, the IC_{50} values for the three microalgae polysaccharides were less than 10 µg/mL, with details of 3.18 µg/mL, 5.43 µg/mL, and 9.87 µg/mL respectively for Glagah, *Spirulina* and East Java microalgae (Table 1).

3.2 Discussion

In this study, the extraction of polysaccharides used the alkaline method. The determination of this method is based on the opinion of Wang *et al.* (2018) that the alkaline extraction method was a higher efficiency to extracted polysaccharides from *Spirulina* than the other methods. In addition to the alkaline (Lye) extraction method, in their research, Wang *et al*. (2018) extracted polysaccharides, using Hot-Water Extraction, Ultrasound-Assisted Extraction and Freeze-Thaw Method. This has also been proven by previous research where the extraction rate and protein level of alkaline extraction were higher than hot water extraction (Afililla *et al*., 2020). Based on Wang *et al.* (2018) and Afililla *et al.* (2020), the extraction of polysaccharides in this study only used one method, namely the alkaline extraction method.

Based on the order of IC_{50} values, the most active as anti-malarial activity of the three microalgae polysaccharides in this study was Glagah microalgae followed by *Spirulina* and East Java microalgae. The IC_{50} values were, respectively, 3.18 µg/ml, 5.43 µg/ml, and 9.87 µg/ml. This means that 3.18 µg/ml, 5.43 µg/ml, and 9.87 µg/ml of microalgal polysaccharides from Glagah, *Spirulina*, and East Java, respectively, can inhibit 50% of the growth of *Plasmodium falciparum* 3D7 parasites. This study indicates that the polysaccharides of all tested microalgae were active and have potential as anti-malarial. An extract was said to be very strong, strong, weak, and inactive as an anti-malarial if it has an IC_{50} value: < 5 µg/ml; 5 – 50 µg/ml; 50 µg/ml $< IC_{50} <$100 µg, and $>$ 100 µg/ml, respectively (Widyawaruyanti *et al*., 2014). Meanwhile, according to Setyowati *et al.* (2019), bioactive compounds are categorized as active and have potential as anti-malarial if the IC_{50} value of the compound is less than 10 µg/mL, and moderately active if the IC_{50} value ranges from 10 µg/mL to 50 µg/mL and has no activity when the IC_{50} value is more than 50 µg/mL.

The difference in the IC_{50} value of each microalgae studied was probably due to the origin of the microalgae, thus affecting the differences in the chemical composition and polysaccharide structure of each microalga. This hypothesis is based on the opinion of Wang *et al.* (2016) and Zeidan *et al.* (2017). According to Wang *et al.* (2016) that differences in cultivation, origin, and batch have a significant effect on the physicochemical properties and structure of polysaccharides. Mixed Microalgae Glagah originated from Glagah Beach Yogyakarta and cultured at the Faculty of Biology UGM Yogyakarta, while Mixed Microalgae East Java isolated from various beaches in East Java and cultured at IPB Bogor. *Spirulina* was also cultured at UGM Yogyakarta. Mixed Glagah microalgae showed the best results possibly because Glagah microalgae is a mixed microalgae consisting of 6 species of bacteria and 6 species of microalgae (Suyono *et al*., 2016). According to Zeidan *et al.* (2017), bacteria are able to produce various kinds of polysaccharides because bacteria can synthesize cytoplasmic polysaccharides (such as glycogen and bacterial starch) and polysaccharides in cell membranes such as peptidoglycan, lipopolysaccharide, lipooligosaccharide, teichoic acid, and lipoteichoic acid.

The use of several microalgae extracts for anti-plasmodium has also been carried out by several researchers (Pankaj *et al*., 2010; Setyowati *et al*., 2019; Wulandari *et al*., 2016). Pankaj *et al.* (2010) extracted phycocyanins from *Nostoc muscorum* (Cyanobacter) and reported that these phycocyanins could inhibit the growth of *Plasmodium* parasites with an IC_{50} value of 8.4 (10.27±2.79) µg/mL, while Wulandari *et al.* (2016) extracted phycocyanin from microalgae *Spirulina platensis* using phosphate buffer and reported the results that the anti-malarial activity, IC_{50} value, was 158.489 µg/mL. The mechanism of inhibition of plasmodium growth by phycocyanin may rely on the destruction of hemozoin polymerization by binding of phycocyanin to ferriprotoporphyrin-IX (Pankaj *et al*., 2010). Phycocyanin is a pigment protein belong to marine organisms, including microalgae, which can help carry out photosynthesis because it can capture light energy (Jiang *et al*., 2017). Setyowati *et al.* (2019) conducted a study of chloroform and ethanolic extracts of the microalgae *Spirulina platensis*, *Chlorella vulgaris*, *Skeletonema costatum*, *Chaetoceros calcitrans*, and *Nannochloropsis occulata* to inhibit the growth of *P. falciparum* by specific enzyme inhibiting mechanism of PfMQO. The results showed that only the chloroform extract of *S. costatum* and the ethanolic extract *of S. platensis* had anti-malarial activity with IC_{50} values of 0.043 µg/mL and 5.25 µg /mL, respectively.

In contrast to Pankaj *et al.* (2010) and

Setyowati *et al*. (2019) which explored phycocyanin (pigment) as an anti-malarial, while this study extracted polysaccharides as a compound of microalgae. According to the Review Falkenberg *et al*. (2019), bioactive compounds from macroalgae as natural products are fiber, minerals, antioxidants, vitamins, pigments, steroids, lectins, halogenated compounds, polyketides, polysaccharides, amino acids, proteins, polyunsaturated fatty acids, and other lipids. This study found that the polysaccharides *Spirulina*, Glagah and East Java microalgae are promising sources of anti-malarial for the development of new anti-malarial drugs. Further purification processes need to be carried out to obtain purified polysaccharides for explanation of their chemical structure and further study of their bioactivity.

Several researchers have carried out research on the use of marine polysaccharides as anti-malarial (Chen *et al*., 2009; Marques *et al*., 2016). Chen *et al*. (2009) reported that fucoidans from the Korean brown algae *Undaria pinnatifida* had an inhibitory effect on *Plasmodium* growth, both in vitro and in vivo, by inhibiting the invasion of *Plasmodium* merozoites into erythrocytes. Fucoidan is a type of sulfated water-soluble polysaccharide extracted from brown seaweeds. According to Marques *et al*. (2016), the inhibition of erythrocytes invasion by *Plasmodium*, possibly mediated by a coating of the parasite similar to that observed for heparin. This statement was a hypothesis from in vitro experiments on the anti-plasmodial activity of heparin-like sulfated polysaccharides from sea cucumbers *Ludwigothurea grisea* and *Isostichopus badionotus*, from red algae *Botryocladia occidentalis*, and from marine sponge *Desmapsamma anchorata* which showed that most compounds were significant inhibitor of *P. falciparum* growth at low anticoagulant concentrations (Marques *et al*., 2016).

4. Conclusion

Polysaccharides of Indonesian mixed microalgae can be promoted as anti-malarial. Polysaccharides from Glagah, *Spirulina* and East Java microalgae have anti-malarial activity in vitro with IC_{50} values, 3.18 µg/ml, 5.43 µg/ml, and 9.87 µg/ml, respectively.

Acknowledgment

The authors gratefully thank to Universitas Gadjah Mada and Institute Pertanian Bogor which have provided microalgae.

Authors' Contributions

All authors involved in the research and writing of the manuscript. The contributed of each author are as follows, LTS; as the lead researcher who had ideas, designed concepts, wrote, and revised the article. MP and ZA; collected data and compiled manuscripts. EAS, AB, and UJS; provided and clustered microalga as well as revised the article. LM and TVW; analyzed data and revised the article. MDK; proofread the article.

Conflict of Interest

All the authors declare that they have no competing interests.

Funding Information

This research was funded by Indonesian Directorate General of Higher Education for the research grant with contract number 415/UN3.15/PT/2021.

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