Int. j. adv. multidisc. res. stud. 2024; 4(5):875-879

Received: 27-08-2024
Accepted: 07-10-2024

International Journal of Advanced Multidisciplinary Research and Studies

ISSN: 2583-049X

Antibacterial Activity of Spirulina Pigment Fraction *Spirulina platensis*

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DOI: https://doi.org/10.62225/2583049X.2024.4.5.3337

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Abstract

Spirulina platensis is a microalgae that is rich in nutrients. These organisms have a high protein content, in addition to being rich in secondary metabolites. Pigment is the secondary metabolite that is most abundant in *Spirulina platensis*. The pigment of *Spirulina platensis* consists of chlorophyll, carotenoids, and phycocyanin. These three pigments have been proven to have bioactivity as antibacterials. The aim of this study was to test the antibacterial activity of the pigment fraction in *Spirulina platensis*. *Spirulina platensis* extraction using methanol, fractionation was carried out using diethyl ether solvent. Pigment identification is carried out using thin layer chromatography (TLC). Antibacterial activity was carried out using the well method using *S. aureus* and MDR E.coli bacteria. Pigment in *Spirulina platensis* can be extracted using methanol solvent with a yield of 13.25% and fractionated using diethyl ether solvent with a yield of 8.67%. The results of identification using TLC showed that the pigments interested in the fraction were chlorophyll and carotenoid. The antibacterial activity test showed that the pigment fraction could inhibit *S. aureus* and *E. coli* bacteria at concentrations of 0.2, 0.3, and 0.4%. The antibacterial activity against *S. aureus* bacteria for a concentration of 0.2, 0.3, and 0.4% has a clear zone of 1.008 ± 0.011 cm, 1.044 ± 0.013 cm, and 1.156 ± 0.013 cm. Meanwhile, the antibacterial activity against *E. coli* at a concentration of 0.2, 0.3, and 0.4% had 0.754 ± 0.014 cm, 0.821 ± 0.017 cm, and 0.899 ± 0.013 cm.

Keywords: Antibacterial Activity, Pigment, *S. aureus*, *E.coli*, *Spirulina platensis*

Introduction

Indonesia is one of the countries with the world's biodiversity center with various riches such as millions of species of animals, plants, microorganisms and ecosystems where all living things live their lives. Biodiversity is used as a source of life such as a source of food, clothing, shelter, medicine and other necessities of life (Sunarmi, 2014) [22].

This biodiversity causes high bioresources where a plant has its own bioactivity according to its chemical content, such as the microalgae *Spirulina platensis*. *Spirulina platensis* It is known to have various benefits because it contains various compounds, some of which are reported to have immunomodulatory, antioxidant, anticancer, antitumor, antiviral, antiinflammatory, cholesterol lowering, antirust, hepatitis prevention, metal chelating, blood formation stimulation activities. Apart from that, *Spirulina platensis* It is known as a source of protein and carbohydrates because of its high protein content, so it is often used as a food supplement and plays a role in the cosmetics sector (Karkos, *et al*., 2011; Ridlo, *et al*., 2016; Finamore *et al*., 2017) [11, 19, 6].

Spirulina platensis is a type of microscopic and filamentous cyanobacteria (blue-green algae) that can make its own food by photosynthesis. This microalgae does not have cellulose cell walls so it can be digested easily (Christwardana and Nur, 2013) [2]. *Spirulina platensis* used as a protein source with protein levels of up to 70%, apart from that Spirulina sp. contains vitamins, especially vitamin B12 and pro-vitamin A (β-carotene), as well as minerals, especially iron. These microalgae are also rich in tocopherol, phenolic acid, and γ-linolenic acid, all of which have various benefits for human life (Ridlo, *et al*., 2016) [19].

Spirulina platensis comes from the Oscillateriaceae family which naturally grows in warm media and alkaline pH so it is relatively easy to cultivate. This algae grows in fresh water and sea water spread across Asia, Europe, Africa, South and North America (Nayyef and Thalji, 2020) [15]. Many toxicological studies have proven the safety of Spirulina, and currently Spirulina is a substance or substances registered with the US Food and Drug Administration and it is recognized that this algae is safe

(Ridlo, *et al*., 2016) [19]. The abundance of chemical compounds in various species of Spirulina algae reported for their use is related to the secondary metabolites and bioactivity produced. One of the compounds from Spirulina that has bioactivity is pigment. The pigment of *Spirulina platensis* consists of chlorophyll, carotenoids, and phycocyanin. These three pigments have been proven to have bioactivity as antibacterials. Based on the background above, the aim of this research is to determine the antibacterial activity of the pigment fraction in *Spirulina platensis*.

Material and Method

Collection sample

Spirulina platensis) powder samples were obtained from the Denpasar, Bali, Indonesia. After the sample is taken, the powder put in a plastic container that contains sterile sea water and then temporarily stored in a coolbox (Nugraheni *et al*., 2010; Sabdaningsih *et., al* 2017) [16, 20].

Extraction

A total of 500 grams of *Spirulina platensis* powder is added with a little $CaCO_3$ and the antioxidant BHA (butylated hydroxyanisole) or BHT (butylated hydroxytoluene), then macerated quickly using methanol until the spirulina is white. The maceration process is carried out until the powder is no longer colored, and stir several times. The maceration process is carried out in a room at room temperature (25℃) and protected from light. The filtrate obtained was evaporated with a rotary evaporator at a temperature of 40℃ until a thick extract was obtained, then the percent yield was calculated. The thick extract obtained was dried with N_2 gas.

Fractionation

The thick extract was then partitioned in a separating funnel using diethyl ether. The ether fraction obtained was evaporated using a vacuum rotary evaporator. The remaining solvent contained in the thick extract is dried with the help of N_2 gas. From this stage, the diethyl ethyl fraction which contains pigment is obtained. The viscous fraction of the sample is then calculated and the % yield obtained is calculated.

Identification of chlorophyll *a* in fractions by TLC

Pigment identification in the fraction was identified using Thin Layer Chromatography (TLC) with a silica gel GF254 stationary phase and a mobile phase consisting of n-hexane: acetone: ether (6:3:2) (Wang, 1995) [24].

Phytochemical Screening

Pigment fraction was evaluated by phytocemical quantitative reactions for secondary metabolites. The screening was performed for phenol, terpenoid, alkaloid, steroid, flavanoids, saponins, and, tannins. The color intensity or the precipite formation was used as analytical responses to these tests (Khan *et al*. 2011; Yadav *et al*. 2011; Khayyat *et al*. 2017) [12, 25, 13].

Antibacterial activity

Pigment fraction were dissolved in dimethyl sulfoxide (DMSO) solvent to make solution with concentrations 0.2%, 0.3% and 0.4% (m/v) for antibacterial activity test. The samples solutions with various concentrations were tested against *S. aureus* and *E.coli* using the diffusion agar method with perforation techniques. Ciprofloxacin 0.005% b/v was used as a comparison control. Media containing bacterial suspension and pigment fraction were incubated at 37 ºC for 1 x 24 hours. The antimicrobial activity was determined by observing the inhibitory zones. The inhibitory zone around the paper disks indicated that pigment fraction were able to inhibit the growth of tested bacteria (Radjasa *et al*., 2007; Sibero *et al*., 2017) [18, 21].

Results and Discussion

Spirulina platensis powder was extracted with methanol solvent in a ratio of 1:10 until the powder was pale in color. Fig 1. The yield of the extract obtained was 13.25%.

Fig 1: Extraction with methanol

Extract that can be fractionated using diethyl ether solvent. Fractionation is a separation procedure between compounds based on their level of polarity. In principle, polar compounds are extracted with polar solvents while non-polar compounds are extracted with non-polar solvents (Uthia *et al*., 2017). The fraction yield obtained was 8.67% showed that diethyl ether solvent could attract pigments in *Spirulina platensis* extract.

Next, the extract that has been obtained is fractionated with diethyl ether solvent to extract the pigment. The fractionation results were then identified using TLC. TLC identification is shown in Fig 2.

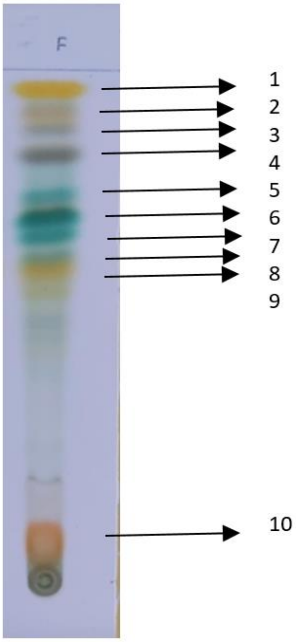

Fig 2: TLC analysis of the diethyl ether fraction (F) of spirulina a stationary phase of silica gel GF 254 and a mobile phase of hexane: ether: acetone (6:3:2)

Table 1

Spot	Spirulina *platensis*			
	Colour	**Rf**	**Pigment**	**Literature**
1	Yellow	0.97	β-caroten	0,6-1 (orange-karoten) (Britton, 2004) [1]
2	Yellow	0.90	Carotene	0,6-1 (orange-karoten) (Britton, 2004) [1]
3	Gray	0.82	Feofitin *a* epimer	0,74-0,82 (abu-abu feofitin) (Heriyanto dan Limantara, 2010) [9]
4	Gray	0.70	Feofitin *a*	0,74-0,82 (abu-abu feofitin) (Heriyanto dan Limantara, 2010) [9] 0,57-
5	Bluish green	0.65	Klorofil *a*	0,64 (hijau biru) (Heriyanto dan Limantara, 2010) [9]
6	Bluish green	0.60	Klorofil *a* epimer	0,57-0,64 (hijau biru) (Heriyanto dan Limantara, 2010) [9]
7	Bluish green	0.58	Klorofil *c*	0,57-0,64 (hijau kekuningan) (Heriyanto dan Limantara, 2010) [9]
8	Yellowwish green	0.55	Xantofil	0,10-0,30 (kuning muda) (Heriyanto dan Limantara, 2010) [9]
9	Yellow	0.55	Xantofil	0,10-0,30 (kuning muda) (Heriyanto dan Limantara, 2010) [9]
10	Orange	0.55	Xantofil	0,10-0,30 (kuning muda) (Heriyanto dan Limantara, 2010) [9]

The TLC results showed that the pigment fraction (diethyl ether) contained chlorophyll pigments and their derivatives as well as carotenoid pigments. In Britton's (2004) [1] research, the Rf β carotene value was 0.8 – 1.0. The Rf value is in accordance with the Rf in this study, namely 0.97. Based on the Rf value, stain 1 is identified as β carotene. Stains 3 and 4 are gray which shows the color of the pheophytin pigment. Pheophytin is a chlorophyll derivative compound that does not contain magnesium (Mg) metal (Gross, 1991; Kusmita *et al*., 2015) [7, 14]. The bluish green color formed in stains 5, 6, and 7 has an Rf of 0.65; 0.60 and 0.58 were identified as chlorophyll a. The results of research conducted by Wang *et al*., (1995) [24] show that chlorophyll a has an Rf value of 0.60. This value is not much different from the Rf chlorophyll a results of this study. These results are also supported by Wang *et al*., (1995) [24] who stated that chlorophyll a is bluish green. In this study there were also 3 bluish green stains. All three stains were the same compound, namely chlorophyll a. The difference was the epimer. Epimers are stereoisomer compounds that differ in configuration at only one stereogenic center. Meanwhile, stain number 8, which is yellow, has an Rf of 0.55, which is a xanthophyll pigment, the same as in the research of Strain *et al*., (1944). Xanthophyll is a carotenoid pigment which tends to be more polar than carotene (Britton, 2004) [1].To ensure the content of secondary metabolites contained in the extract phytochemical screening was performed. The results of phytochemical screening are shown in Table 2.

Table 2: Screening results of phytochemical pigment fraction

Test	Result	References	Conclusion
Flavonoids	A green solution	A yellow to red solution is formed overlaying amyl alcohol	(-) negative
Alkaloids	No precipitate	Mayer reagents form white deposits, and orange sediment drag	(-) negative
Saponin	Unstable foam	Formed a stable foam that lasts for 10 minutes	(-) negative
Terpenoids	A red solution is formed	A red solution is formed	(+) positive

Based on the phytochemical screening above, pigmen fraction positively contains terpenoids. Terpenoids are groups that have the basic structure of isoprene (Harbone, 1983). The most important terpenoid group is carotenoids with the molecular formula $C_{40}H_{64}$ formed by tetraterpenoids having 8 isoprene units (Wagner and Elmadfa, 2003 [23]; Merhan, 2017). Carotenoids have a straight chain structure formed by condensation of isoprene molecules (Damonkos *et al*, 2013) [5].

The next step is to test the antibacterial activity on the pigment fraction. The antibacterial activity test is carried out on *S.aureus* and *E.coli* bacteria. The results of antibacterial activity are shown in Fig 3 and Table 2.

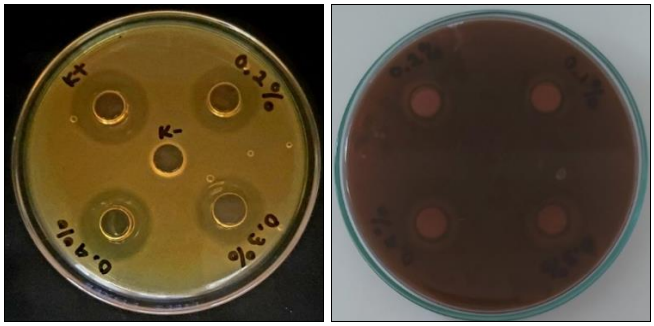

Fig 3: Antibacterial activity of pigment fraction against of *S.aureus* (a) and *E.coli* (b)

Table 2: Inhibition zone of chlorophyll c against of *S.aureus* and *E.coli*

Concentration (%)	Inhibition zone (cm)	
	S. aureus	***E.coli***
K+ 0.005 (ciprofloxacin)	1.231±0.012	1.012±0.015
K- DMSO	0	0
0.2	1.008±0.011	0.754±0.014
0.3	1.044±0.013	0.821±0.017
0.4	1.156±0.013	0.899±0.013

Based on this data, the antibacterial test against *S. aureus* bacteria for a concentration of 0.2% has a clear zone of 1.008 ± 0.011 cm, at a concentration of 0.3% has a clear zone of 1.044 ± 0.013 cm, and at a concentration of 0.4% has a clear zone of 1.156 ± 0.013 cm. Meanwhile, the antibacterial test against *E. coli* at a concentration of 0.2% had 0.754 ± 0.014 cm, for a concentration of 0.3% it had 0.821 ± 0.017 cm, and at a concentration of 0.4% it had 0.899 ± 0.013 cm. The higher the pigment fraction concentration, the resulting inhibition zone also increases. The higher the sample concentration, the greater the compound that diffuses into the planted bacteria, so it will further inhibit the growth of *S. aureus* and *E.coli* bacteria.

The ability of the Spirulina pigment fraction to inhibit the growth of *S. aureus* and E.coli bacteria is due to the presence of carotenoid pigments which are a group of terpenoids (Stephen *et al*., 2016). According to Radjasa (2009) [17], carotenoids are compounds that can be used as antibacterials. The mechanism by which carotenoids inhibit bacterial growth reacts with porins (trans-membrane proteins) on the outer membrane of the bacterial cell wall

and then forms strong polymer bonds, resulting in damage to the porins. Where these porins are a way for bacteria to get in and out of nutrients. So if the porins are damaged the supply of nutrients for bacterial growth will be absent and will cause the bacteria to not grow and die (Cowan, 1999) [3]. *E.coli* bacteria have a more complex cell wall structure than *S. aureus* bacteria. *E.coli* bacteria are gram-negative bacteria that are resistant to several antibacterials, this is due to the three layers of cell walls in this bacteria, so that some compounds are unable to damage the tissue of the cell walls of E.coli bacteria (Jawetz *et al*., 2007) [10]. The cell wall of gram-negative bacteria contains three polymers, namely the outer layer of lipoprotein, the middle layer of lipopolysaccharide, the inner layer of peptidoglycan, and the outer membrane in the form of a bilayer (has better resistance to compounds that leave or enter the cell and cause toxic effects). According to Helmiyati and Nurrahman (2010) [8], the cell walls that are easiest to denature are cell walls composed of polysaccharides compared to cell walls composed of phospholipids. *S. aureus* bacteria, which are gram-positive bacteria, have cell walls containing peptidoglycan, teichoic acid and teicuronic acid. According to Dewi (2010) [4], teichoic acid as a constituent of the cell walls of gram-positive bacteria is a water-soluble polymer that functions as a transport for positive ions in and out. This water-soluble nature shows that the cell walls of gram-positive bacteria are more polar, so that polar bioactive compounds easily enter the cell walls and damage the polar petidoglycan layer rather than the non-polar lipid layer.

Conclusion

The results of identification using TLC showed that the pigments interested in the fraction were chlorophyll and carotenoid. The pigment fraction of *Spirulina platensis* has antibacterial activity against *S. aureus* and *E. coli* bacteria. Antibacterial activity against *S. aureus* bacteria is greater than against *E.coli* bacteria.

Acknowledge

The authors would like the thank Yayasan Pharmasi Semarang for financial support to this research through HIBAH Yayasan 2024.

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