ANTIBACTERIAL ACTIVITY OF BIOPIGMENTS EXTRACTED FROM LABORATORY GROWN CULTURE OF *SPIRULINA PLATENSIS*

Phan Thi Thanh Nga[1] Pham Minh Diep[2]
Tran Mai Anh[3] Pham Thi Bich Dao[4]

[1, 2, 3, 4]Thanh Do University
Email: *thanhngato09@gmail.com[1]; mdiep12a4@gmail.com[2]; manhtrann298@gmail.com[3]; ptbdao@thanhdouni.edu.vn[4]*
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Abstract: *Spirulina platensis biopigments have been potential source of nutritional, pharmacological and cosmetical purposes due to the presence of bioactive pigments. In this study, Spirulina platensis was culture in the labotory to extract using a glucose/glycerol and water-based NADES. The chlorophyll a,b and carotenoid pigment concentration were 110.04 ± 9.58 and 89.25 ± 21.46 µg/mL, respectively. While the highest phycocyanin content was obtained as 2.25 ± 0.2 mg/mL. The biopigment components extracted from Spirulina platensis in natural deep eutectic solvent (Sp-NADES) were tested in vitro for their antibacterial activity for which one Gram positive bacterium (Staphylococcus aureus) and four Gram negative bacteria (Escherichia coli, Pseudomonas aeruginoas, Salmonella typhi, and Klebsiella pneumoniae) were used as test organisms. The Sp-NADES extract showed potent activity against Pseudomonas aeruginosa and Staphylococcus aureus. The Staphylococcus aureus, Pseudomonas aeruginosa inhibition zone of Sp-NADES extract were 8.75 ± 0.75 and 10.25 ± 1.42 mm in diameter. However, no inhibitory effect was found against Escherichia coli, Salmonella typhi and Klebsiella pneumoniae.*

Keywords: *Antibacterial activity; Biopigments; Spirulina platensis.*

1. Introduction

For the past 50 years, the discovery of pharmaceutical drugs has largely relied on the empirical screening of a vast number of pure chemical compounds to identify new potential leads. Throughout history, a specific group of bacteria known as actinomycetes has been recognized as the most significant producers of bioactive metabolites, which are chemical compounds with potential health benefits.

Cyanobacteria, commonly referred to as blue-green algae, are among the oldest organisms capable of photosynthesis. These organisms stand out because they can be cultivated without requiring organic materials for growth, offering a cost-effective advantage over other microorganisms. By optimizing the cultivation process in a controlled environment, it is possible to enhance the production of valuable compounds (Kreitlow et al., 1999).

In recent years, the search for cyanobacteria with antimicrobial properties has become increasingly important due to the global concern over the rapid rise in infections caused by microorganisms that are resistant to antibiotics. Research has demonstrated that biologically active compounds can be successfully extracted from cyanobacteria. Various strains of cyanobacteria are capable of producing both intracellular and extracellular metabolites that exhibit a wide range of biological activities. These include antibacterial (Kokou et al., 2012; Ozdemir et al., 2004; Santoyo et al., 2006; Sarada et al., 2011), antifungal (Santoyo et al., 2006), cytotoxic (Ebaid et al., 2017), immunosuppressive (Ebaid et al., 2017; Hu et al., 2018) and antiviral activities (Hayashi et al., 1996).

The purpose of study reported here was to investigate the antibacterial activity of natural deep eutectic solvent (NADES) extraction of laboratory grown culture of *Spirulina platensis.*

2. Research overview

Microalgae are highly regarded as valuable raw materials due to the wide variety of

compounds they contain, such as proteins, polysaccharides, long chain fatty acids, and pigments. These compounds have been utilized in various industries, including cosmetics, human nutrition, and energy production. Among these compounds, bioactive pigments are of particular interest because of their unique biological properties and vibrant colors, attracting significant attention from researchers. Considering the United Nations' sustainable development goals, which aim to decrease global dependence on synthetic materials, natural bioactive pigments are becoming increasingly significant. For example, phycocyanin, a blue pigment found in *Spirulina platensis*, is one of several pigments in microalgae, including chlorophylls, xanthophylls, and carotenoids like beta-carotene, zeaxanthin, and myxoxanthophyll.

To obtain bioactive pigments, various extraction methods are utilized (Martins et al., 2023). The choice of extraction method is crucial for producing new products, as it influences environmental impact. Commonly used aqueous-organic solvents for extracting bioactive compounds and pigments from natural sources include hexane, benzene, methanol, chloroform, petroleum ether, and acetone. However, these solvents pose several issues, including toxicity, volatility, and flammability, which can negatively affect environmental, operator, and consumer safety (Benvenutti et al., 2019). Consequently, researchers have explored innovative solvents such as ionic liquids (ILs), deep eutectic solvents (DES), and natural deep eutectic solvents (NADES) to enhance the sustainability of the extraction process. NADES are created through the interaction between a hydrogen bond acceptor and a hydrogen bond donor, making them eco-friendly, non-toxic solvents suitable for green extraction methods. They adhere to the principles of green chemistry and offer numerous benefits, including availability of components, biodegradability, tunable physicochemical properties, low toxicity, sustainability, and affordability, with most components derived from natural sources. This versatility addresses some limitations associated with ILs and DES (Benvenutti et al., 2019).

In recent years, *Spirulina* has gained growing recognition as a potential source of pharmaceutical compounds. Numerous studies have demonstrated its wide-ranging health benefits, including antioxidant, immunomodulatory, anti-inflammatory, anticancer, antiviral, and antibacterial activities. It also shows positive effects against conditions such as hyperlipidemia, malnutrition, obesity, diabetes, heavy metal-induced toxicity, and anemia (Bleakley & Hayes, 2017). Many algal extracellular products and extracts have shown antimicrobial effects (against viruses, bacteria, fungi, algae, and protozoa), though the precise structures and identities of the active components remain unclear (Borowitzka, 1995).

Among microalgae, *Spirulina* has increasingly been recognized as a natural antimicrobial agent. Research by Kokou et al. (2012) confirmed *Spirulina*'s antibacterial activity against six strains of *Vibrio*. Additionally, the antimicrobial properties of acrylic acid, which is present in relatively high amounts in *Spirulina*, were first identified in the late 1970s. Along with acrylic acid, other organic acids such as propionic, benzoic, and mandelic acids were also recognized as antimicrobial agents (Kokou et al., 2012).

Further studies evaluated different extracts of *Spirulina platensis* against bacteria and yeasts, identifying methanol extract as the most potent antimicrobial fraction (Ozdemir et al., 2004). Santoyo et al. (2006) confirmed the antimicrobial activity of *Spirulina platensis* extracts using ethanol, hexane, and petroleum ether against *Escherichia coli*, *Staphylococcus aureus*, *Aspergillus niger*, and *Candida albicans* (Santoyo et al., 2006). Moreover, purified C-phycocyanin from *Spirulina platensis* exhibited significant inhibition of various multidrug-resistant bacteria. For instance, it showed minimum inhibitory concentrations (MIC) of 125, 100, 75, and 50 mg/L against *Staphylococcus aureus*, *Escherichia coli*, *Klebsiella pneumoniae*, and *Pseudomonas aeruginosa*, respectively (Sarada et al., 2011).

3. Material and methods

3.1. Spirulina platensis production

Spirulina platensis was provided by Dalitra Technology Co., LTD. *Spirulina platensis* was cultured in Zarrouk medium under laboratory conditions at a temperature of 25 ± 3°C. The

culture was continuously aerated to provide agitation and a steady supply of CO_2. A light intensity of 9000 Lux was maintained, with a 12 hour light and 12 hour dark photoperiod. The biomass of *Spirulina platensis* was harvested using vacuum filtration. After collection, the biomass was freeze dried and stored in flasks for later use.

3.2. Spirulina platensis extraction

Freeze dried biomasses were extracted using ultrasound and and a NADES solvent composed of glucose, glycerol, and water in a 1:2:4 molar ratio, during 30 min with biomass/solvent ratio (1/20, *w*/*w*) (Wils et al., 2021). After the extraction, the mixture was centrifuged for 20 minutes at 8000 rpm (using 80-2 Electric Benchtop Centrifuge). The supernatant was collected, and the remaining solid residue was extracted two more times, resulting in a final biomass to solvent ratio of 1:60. Each extraction was performed three times.

3.3. Spectrophotometry analysis of the Spirulina platensis extracts

Spectrophotometric analysis was carried out using a UV1900 Advanced Double Beam UV Visible Spectrophotometer, Yoke, Shanghai, China. For this procedure the aliquots were filled with the extracts, and the maximum absorbance within a specific wavelength range was measured. The concentrations of bioactive pigments, including chlorophylls a and b, carotenoids, and phycocyanin, were then calculated using the appropriate equations below (Adeyemi, 2020; Yang et al., 1998).

$$C_{chlorophyll\ a+b}(\mu g\ mL^{-1}) = 17.76A_{646.6} - 7.34A_{663.6}$$

$$C_{carotenoids}(\mu g\ mL^{-1}) = 4.69A_{440} - 0.267\ C_{chlorophyll\ a+b}$$

$$C_{phycocyanin}(mg\ mL^{-1}) = (A_{620} - 0.474A_{652})/5.34$$

Where:

$C_{chlorophyll\ a+b}$ is the concentration of chlorophylls a and b in µg/mL; $C_{carotenoids}$ is the concentration of total carotenoids in µg/mL; $C_{phycocyanin}$ is the concentration of phycocyanin in mg/mL.

In addition, *A*646.6, *A*663.6, *A*440, *A*620, and *A*652 are the absorbance values at 646.6, 663.6, 440, 620, and 652 nm, respectively.

The yield of each pigment was determined following equation, also used by other authors (Hadiyanto et al., 2016) :

$$\text{Yield } (\frac{mg}{g}) = C_{pigment} \times \frac{V}{DB}$$

Where $C_{pigment}$ is the concentration of chlorophylls a and b, carotenoids, and phycocyanin expressed in mg/mL; *V* is the volume of solvent expressed in mL; and *DB* is the dried biomass used in the extraction, expressed in g.

3.4. Test microorganisms

The microorganisms used in antibacterial assays were supplied by Institute. The species employed include pathogenic Gram-positive bacteria (*Staphylococcus aureus* ATCC-6538) and Gram-negative bacteria (*Escherichia coli* ATCC-25922, *Pseudomonas aeruginoas* ATCC-9027, *Salmonella typhi* ATCC-13076, and *Klebsiella pneumoniae* ATCC-33495). The bacterial strains were inoculated on Plate Count Agar (PCA) and incubated for 24 h at 30°C then suspended in saline solution 0.9% NaCl and adjusted to yield approximately $1.0 \times 10^8 – 1.0 \times 10^9$ cfu/ml by using spectrophotometer (25% transmittance at 530 nm). Media component were purchased from Hi Media, Mumbai, India. All the chemicals used were of analytical grade.

3.5. Antibacterial testing

The antibacterial activity of *Spirulina platensis* extracts (Sp-NADES) was assessed *in vitro* using the agar well diffusion method (Perez et al., 1990). A volume of 100 µL of an adjusted bacterial culture (1.0×10^5 cfu/mL) was mixed with 100 mL of Muller Hinton Agar (MHA), mixed well and 25 mL of the mixture was poured into sterile 90 mm petri dishes.

After allowing the agar to solidify, the plates were labeled according to the inoculated organisms. Once solidified, wells with a 6 mm diameter were created using a sterile suction tube. Then, 100 µL of the Sp-NADES extracts were pipetted into each well. The plates were incubated overnight at 37°C, and the zones of inhibition were observed. The diameter of these zones was measured in millimeters. All tests were conducted under sterile conditions and repeated three times. The solvent control (NADES) showed no

antibacterial activity and positive control was Ampicillin (5 µg/mL).

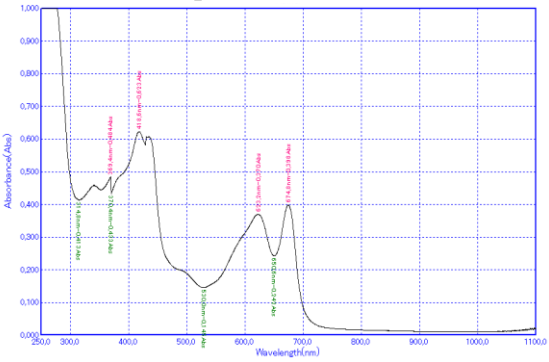
3.6. Statistical analysis

The data of all the parameters were statistically analyzed by Microsoft Excel 365 and expresses as mean ± Standard deviation.

4. Results

4.1. Determination of pigments in Spirulina platensis extracts

Figure 1. UV spectrum for the extracts obtained in the Sp-NADES extraction



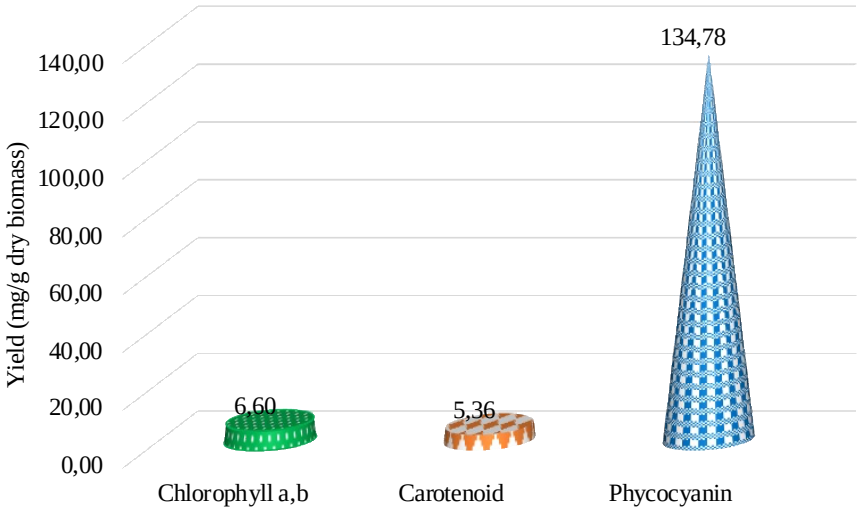
In this study, a glucose/glycerol/water based NADES was used. NADES was able to extract both water soluble and lipid soluble pigments at high yields. Given the significant antimicrobial and antioxidant properties of the bioactive pigments found in *Spirulina* extracts, quantifying these compounds is of great importance. Therefore, UV-VIS spectrophotometry was employed to measure the pigment concentration in the Sp-NADES extracts. Spectrums for Sp-NADES extracts is available for consultation in Figures 1.

The pigment concentration and yield of liquid extracts were given in Table 1. The highest chlorophyll a,b and carotenoid pigment concentration were 110.04 ± 9.58 and 89.25 ± 21.46 µg/mL, respectively. While the highest phycocyanin content was obtained as 2.25 ± 0.2 mg/mL. In this study, while chlorophyll a,b concentration is similar to other reports, phycocyanin concentration is almost half of values reported by other works. Sarada et.al (1999) have reported that dried *Spirulina* samples contain about 50% less phycocyanin than the fresh samples and lower phycocyanin content in this study it can be explained by loss during drying process (Sarada et al., 1999).

Table 1. The bioactive pigments content of Sp-NADES extract

	Chlorophyll a, b (µg/mL)	Carotenoid (µg/mL)	Phycocyanin (mg/mL)
Concentration	110.04 ± 9.58	89.25 ± 21.46	2.25 ± 0.2
	Chlorophyll a, b	Carotenoid	Phycocyanin
Yield (mg/g dry biomass)	6.6 ± 0.57	5.36 ± 1.29	134.78 ± 11.89

Figure 2. Yield of Sp-NADES extracts



The yields of bioactive pigments of Sp-NADES extracts are presented in Figure 2. From the results obtained, it can be drawn that total carotenoid content (5.36 ± 1.29 mg/g DB) was similar to the reported yield by Liestianty et al. (6 mg/g DB) and higher than in the study by Wils et al. (0.22 mg/g DB). Total chlorophyll content (6.6 ± 0.57 mg/g DB) was higher than the results obtained in the study (0.69 mg/g DB) (Wils et al., 2021). The phycocyanin yield (134.78 ± 11.89 mg/g DB) was also lower than what was reported by Liestianty et al. (180 mg/g DB), but also higher than in the study by Wils et al. (3.96 mg/g DB) (Liestianty et al., 2019; Wils et al., 2021).

On the other hand, carotenoid yield (5.36 ± 1.29 mg/g DB) was the lowest of the bioactive pigments obtained with ultrasound, while the total chlorophylls (6.6 ± 0.57 mg/g DB) and phycocyanin yield (134.78 ± 11.89 mg/g DB) was the highest. Thus, the ultrasound extraction using a glucose/glycerol based NADES proved to be more efficient than ultrasound extraction in the extraction of phycocyanin and chlorophylls.

Although the results were positive, further optimization of the extraction process may be necessary to increase the overall yield of bioactive pigments. This, in turn, could enhance the antimicrobial and antioxidant properties of the NADES extracts.

4.2. Antibacterial activity of Spirulina platensis extracts

The antibacterial activity of Sp-NADES extracts of *Spirulina platensis* are presented in Table 2, Figure 3. The Sp-NADES extracts tested exhibited different of antibacterial activity against tested microorganisms.

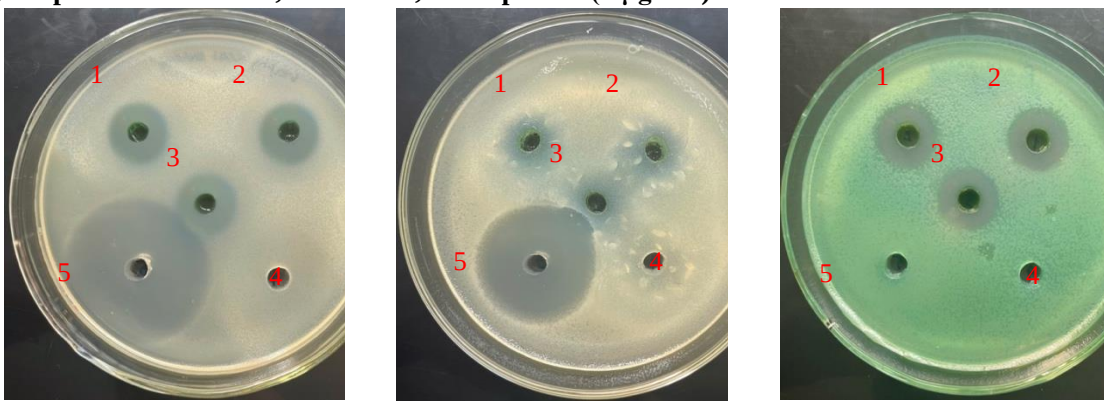
Antibacterial ability of Sp-NADES extract was determined based on its ability to inhibit bacterial growth, as indicated by the diameter of the inhibition zone on a petri dish. The inhibition zone of the Sp-NADES extract was clearly visible in the case of *Staphylococcus aureus* strains, with a diameter of 8.75 ± 0.75 mm. For *Pseudomonas aeruginosa*, the inhibition zone measured 10.25 ± 1.42 mm. However, no inhibitory effect was observed against *Escherichia coli, Salmonella typhi* and *Klebsiella pneumoniae*.

These results are consistent with the study by Martins et al., where pigment extracts in NADES solvents showed antibacterial activity against *Staphylococcus aureus* (Martins et al., 2023). In a previous study by Usharani et al., extracts of *Spirulina* in different solvents exhibited antibacterial activity, with hexane extracts showing activity against *Klebsiella pneumoniae* and *Staphylococcus aureus*, while extracts in ethanol, acetone, and methanol demonstrated activity against *Klebsiella pneumoniae*, *Escherichia coli*, and *Staphylococcus aureus*. It can be seen that the use of solvents in extraction processes poses potential toxicity risks (Usharani et al., 2015). Therefore, the results of the sp-NADES extract present a feasible solvent alternative for extracting bioactive pigments from *Spirulina platensis*.

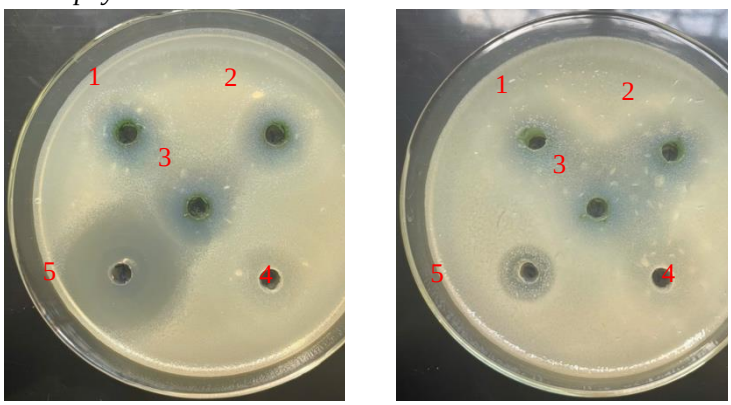
Table 2. Antibacterial activity of Sp-NADES extracts of *Spirulina platensis*

Microorganisms	Diameter of effective zone of inhibition (mm)		
	Sp-NADES extracts	Ampicillin	NADES
Staphylococcus aureus	8.75 ± 0.75	32.5 ± 3.84	(-)
Escherichia coli	(-)	29 ± 0.82	(-)
Pseudomonas aeruginosa	10.25 ± 1.42	(-)	(-)
Salmonella typhi	(-)	20 ± 0.82	(-)
Klebsiella pneumoniae	(-)	(-)	(-)
Results are the means of diameter values±standard deviation. (-) No activity; Ampicillin (5 μg/mL) Effective zone of inhibition (D, total zone of inhibition-diameter of well)			

Figure 3. Zone of inhibition exhibited by Sp-NADES extracts of *Spirulina platensis*
1, 2, 3. Sp-NADES extracts; 4. NADES; 5. Ampicillin (5 µg/mL)



Staphylococcus aureus *Escherichia coli* *Pseudomonas aeruginosa*



Salmonella typhi *Klebsiella pneumoniae*

5. Discussions

There is a growing interest in isolating antibacterial substances from cyanobacteria. In different studies, the antimicrobial effect of *Spiulina platensis, Fischerella sp., Oscillatoria anguistisima* and *Calothrix parietina, Anabaena, Oscillatoria, Pseudoanabaena, Synechocystis, Nostoc, Phormedium* and *Fischerella ambigua* extracts on some pathogenic microorganisms have been reported (Hu et al., 2018; Kokou et al., 2012; Kreitlow et al., 1999; Ozdemir et al., 2004). They have also reported that the extracts extracted in different solvents were effective against both Gram-positive and Gram-negative organisms. This is in agreement with our findings, since the *Spirulina platensis* extracted in NADES solvents had similar effects on both tow types of organisms used in this study.

Pigment components from *Spirulina platensis*, such as carotenoid, chlorophyll, and phycocyanin, have been studied for their antibacterial properties (Sarada et al., 1999; Yang et al., 1998). Purified phycocyanin extracted from *Spirulina platensis* exhibited activity against Gram-positive bacteria *Staphylococcus aureus* (with a MIC of 50–500 µg/mL) and other bacteria (Safari, 2020). On the other hand, fucoxanthin (a carotenoid derived from *Spirulina platensis)* showed similar inhibitory effects against *Staphylococcus aureus* with a minimum inhibitory concentration (MIC) of 125 µg/mL, and also against Gram-negative bacteria *Klebsiella pneumoniae* (MIC 250 µg/mL) (Karpiński & Adamczak, 2019). Further studies on the purification of these pigment components are necessary to better investigate their antibacterial activities.

6. Conclusions

Antibacterial activity test results showed that Sp-NADES extract inhibited two strains of bacteria *Staphylococcus aureus* and *Pseudomonas aeruginosa*. However, the antibacterial inhibitory ability of Sp-NADES extract was still modest, it is necessary to use extracts at higher concentrations. Nonetheless, this study also opens a novel and

greener strategy for both the extraction of bioactive compound.

The bioactivity of pigments from *Spirulina platensis* has been widely discussed, which presents opportunities for developing numerous products with bioactive pigments in various industrial sectors such as food, pharmaceutical, and nutraceutical. Further research is needed to determine the structural characterization of these compounds to discover their complete potential. Nevertheless, increasing their commercial prospects will require cost-effective production, extraction, and purification methods.

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HOẠT TÍNH KHÁNG KHUẨN CỦA SẮC TỐ SINH HỌC CHIẾT XUẤT TỪ TẢO XOẮN *SPIRULINA PLATENSIS* NUÔI CẤY TRONG PHÒNG THÍ NGHIỆM

Phan Thị Thanh Nga[1] Phạm Minh Diệp[2]
Trần Mai Anh[3] Phạm Thị Bích Đào[4]

[1, 2, 3, 4]Trường Đại học Thành Đô
Email: *thanhngato09@gmail.com[1]; mdiep12a4@gmail.com[2]; manhtrann298@gmail.com[3]; ptbdao@thanhdouni.edu.vn[4]*
Ngày nhận bài: 7/10/2024; Ngày phản biện: 28/10/2024; Ngày tác giả sửa: 04/11/2024;
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Tóm tắt: *Các thành phần sắc tố trong tảo xoắn Spirulina platensis là nguồn ứng dụng tiềm năng cho nhiều lĩnh vực như dinh dưỡng, dược phẩm và mỹ phẩm bởi sự hiện diện của các sắc tố có hoạt tính sinh học. Trong nghiên cứu này, Spirulina platensis được nuôi cấy trong phòng thí nghiệm và tách chiết sắc tố sinh học bằng dung môi NADES (glucose/glycerol/nước). Nồng độ sắc tố chlorophyll a, b và carotenoid lần lượt là 110,04 ±9,58 và 89,25 ±21,46 µg/mL. Trong khi hàm lượng phycocyanin thu được cao nhất là 2,25± 0,2 mg/mL. Các thành phần sắc tố sinh học chiết xuất từ tảo xoắn Spirulina platensis trong dung môi NADES (Sp-NADES) được thử nghiệm invitro về hoạt tính kháng khuẩn trên năm chủng vi khuẩn kiểm định bao gồm một vi khuẩn Gram dương (Staphylococcus aureus) và bốn vi khuẩn Gram âm (Escherichia coli, Pseudomonas aeruginoas, Salmonella typhi, and Klebsiella pneumoniae). Chiết xuất Sp-NADES có khả năng kháng vi khuẩn Pseudomonas aeruginosa và Staphylococcus aureus. Vùng ức chế Staphylococcus aureus ATCC-6538, Pseudomonas aeruginosa của chiết xuất Sp-NADES có đường kính là 8,75 ± 0,75 và 10,25 ± 1,42 mm. Tuy nhiên, không tìm thấy tác dụng ức chế nào đối với Escherichia coli, Salmonella typhi và Klebsiella pneumoniae.*

Từ khóa: *Hoạt tính kháng khuẩn; Sắc tố sinh học; Spirulina platensis.*