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EFFECT OF PIGMENT EXTRACTION SOLVENT FOR SPIRULINA PLATENSIS ON ITS ANTIOXIDANT AND ANTIOBESITY ACTIVITY IN VITRO

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ABSTRACT

Background: These guidelines Indonesia is a country with diverse biological resources, typically from marine resources, *Spirulina platensis*. The secondary metabolite compounds contained in *Spirulina platensis* are chlorophyll, phycocyanin, beta carotene, folic acid, phenolics, catechin hydrate and epicatechin, where these compounds have anti-obesity activity. One important factor in obtaining an extract with antioxidant and antiobesity activity is solvent optimization using an extraction method using fast maceration. This study aims to determine the effect of different extraction solvents to extract pigment compounds contained in *Spirulina platensis* on antioxidant and antiobesity activity with pancreatic lipase enzyme inhibitors in vitro. **Methods:** *Spirulina platensis* dry powder was extracted using the fast maceration method, using 4 solvents, namely 100% methanol, 100% acetone, methanol : acetone (70 : 30), acetone : methanol (70 : 30). Antioxidant activity test using DPPH and obesity activity with pancreatic lipase enzyme was employed using ELISA multimode reader. **Result:** The yields obtained from the maceration method from 100% methanol, 100% acetone, methanol : acetone (70:30), and methanol : acetone (30 : 70) were 13.10 ± 0.19%, 3.33 ± 0.46, 13.92 ± 0.78%, 9.26 ± 0.17 % respectively. The best antioxidant activity was found in the extract with methanol : acetone 30:70 solvent (79.84±0.62 µg/mL). The best lipase inhibitory activity was found in the extract with methanol : acetone 30:70 solvent (75.34±0.94 %).

KEYWORDS: antiobesity, antioxidant, solvent difference *Spirulina platensis.*

INTRODUCTION

According World Obesity Federation, it is estimated that 2.7 billion adults worldwide will be overweight by 2025 if the current poor dietary trends continue. An increase of 35% has occurred from 2.0 billion adults in 2014.[1] Data from World Health Organization in 2016 mentioned that there are 650 million adults, accounting for 13% of the adult population, and the prevalence of overweight is 39%. Obesity is very concerning because it is often associated with an increased rate of premature death and a high incidence of degenerative diseases such as type II diabetes, cardiovascular diseases, hypertension, hyperlipidemia, and several types of cancer.[2] Therefore, those suffering from obesity or being overweight are willing to make various efforts to lose weight, ranging from adjusting their diet, exercising, consuming various weight loss or slimming drugs, getting slimming injections, to undergoing plastic surgery. Most synthetic weight loss drugs or slimming agents available on the market, such as orlistat.

Orlistat is a lipase inhibitor that reduces fat absorption in the intestines. This drug is reported to be effective in reducing body weight. Orlistat is recognized as a long-term treatment that helps inhibit the absorption of dietary fats.[3] Orlistat works by inhibiting fat absorption, altering the body's fat metabolism by blocking the action of lipoprotein lipase enzymes that break down fats, so that fats are excreted from the body through feces. Fats can be absorbed when they have been converted by lipase into fatty acids from food that has not been hydrolyzed into free fatty acids and glycerol, so some fats are not absorbed by the intestines.[4] However, synthetic drugs, when consumed over a long period, can cause many undesirable side effects such as nausea, vomiting, dry mouth, anorexia, constipation, insomnia, and neurological symptoms.[5]

Alternative therapy for obesity can use herbs with the administration of spirulina algae extract. Spirulina is a species of cyanobacterium that is a blue-green algae plant and has organic nutrients. There are 3 species that are most commonly used in the medical field: *Spirulina platensis*, *Spirulina maxima*, and *Spirulina jusijormzs*,

which are also considered as nutrient source plants.[6] In the field of medicine and pharmacy, spirulina is one of the food ingredients that has also been widely used as a component in the production of health supplements. This is because spirulina has a fairly complete nutritional content. Chemically, spirulina consists of water (27.8%), protein (5.4%), carbohydrates (33.3%), fats, and fiber. Seaweed also contains enzymes, nucleic acids, amino acids, vitamins (A, B, C, D, E, and K), and macrominerals such as nitrogen, oxygen, calcium, and selenium, as well as micro-minerals that are 10-20 times higher than those found in terrestrial plants.[7] Spirulina also contains complex B vitamins, phycocyanin, gamma-linolenic acid protein, and *β*-carotene. Spirulina has phycocyanin and *β*-carotene content that can act as antioxidants.[8]

Based on the introduction above, research will be conducted on spirulina extract (*Arthrospira platensis*) samples using extraction with variations of methanol and acetone solvents. Previous research has been conducted on spirulina extraction using a methanol-acetone solvent with a 3:7 v/v ratio.[9] Additionally, research on the anti-obesity activity of spirulina (*Arthrospira platensis*) via in vitro lipase inhibitor using solvent variations in maceration, i.e., 100% methanol, methanol:acetone (70:30), 100% acetone, and acetone:methanol (70:30), has not been conducted in previous studies.

MATERIAL AND METHOD

a) Material

Identification and determination of spirulina (*Arthrospira platensis*) were conducted at the Indonesian Institute of Sciences Marine Bio-Industry Hall, Teluk Kodek Village, Pemenang Barat Village, Pemenang District, North Lombok Regency, Mataram, West Nusa Tenggara. The extraction solvents are 100% methanol, methanol:acetone (70:30), 100% acetone, acetone:methanol (70:30) (Merck, Germany). The solvents for TLC identification are n-hexane, ether, acetone (6:3:2). (Merck, Jerman). Placa de gel de sílice 60 F 20x20 cm (Merck, Germany). The materials for the antioxidant activity test are methanol and DPPH (Sigma Aldrich, USA). The materials for measuring antilipase activity are crude Porcine Pancreatic Lipase (PPL) (Sigma Aldrich, USA), para nitrophenyl butyrate (pNPB) (Sigma Aldrich, USA), phosphate buffer pH 7.4 (Sigma Aldrich, USA), DMSO (Merck, Germany), and orlistat standard. (Sigma Aldrich, USA).

b) Method

Extraction

Spirulina powder (*Arthrospira platensis*) was extracted using the quick maceration method with solvents of 100% methanol, methanol:acetone (70:30), 100% acetone, and acetone:methanol (70:30). The extraction process used the quick maceration method by soaking 5 grams of spirulina powder in each solvent (100% methanol, methanol:acetone (70:30), 100% acetone, acetone:methanol (70:30) in 20 ml amounts, five times, protected from light. The extraction results are filtered, and the filtrate is collected. The filtrate was then evaporated using a rotary evaporator at a temperature of 30°C, and subsequently evaporated with nitrogen gas to obtain a thick extract, which was then weighed.[10]

DPPH Radical Scavenging Assay

The samples were dissolved in methanol and a certain concentration series was made. The antioxidant activity was determined by placing 0.2 mL of solution of each sample series in a test tube, followed by adding 4.0 mL of 0.1 mM DPPH for each concentration. The mixture was then homogenized by vortex stirring for 1 minute and left for 30 minutes as the operating time. The absorbance of the solution was read at a maximum wavelength of 517 nm. The same steps were carried out in measuring the vitamin C standard series in an absorbance reader.[11], [12]

The antioxidant activity of the vitamin C standard used as a comparison. IC_{50} indicates the sample concentration required to capture 50% of the DPPH free radicals.[11], [13] The absorbance of fractions and isolates from *Curcuma aeruginosa* Roxb was calculated and expressed in the percentage (%) of antioxidant activity compared to the absorbance of the control using the formula:

$$\% \text{ antioxidant activity} = \frac{\text{Abs.control} - \text{Abs.Sample}}{\text{Abs.Control}} \times 100\%$$ ……(1)

Pancreatic Anti-lipase Activity

Thick extract of spirulina (*Arthrospira platensis*) from four different solvents, namely 100% methanol, methanol:acetone (70:30), 100% acetone, acetone:methanol (70:30), was then tested for pancreatic antilipase activity in vitro (% inhibition in 96 well plates on an ELISA reader). The enzyme stock concentration is set at approximately 0.1 mg/ml for every 1 mg of PPL powder dissolved in 1 ml of buffer solution (a). The extract is made at a concentration of 500 µg/ml (b), p-NPB is dissolved in 1% DMSO (c), and then diluted with 50 mM phosphate buffer pH 7.2 to a concentration of 2.5 mM in 100 µL (d). Solution (a) + (b) + (d) is mixed and incubated at 37˚C for 10 minutes. Each sample is replicated 3 times. Used as a standard, the positive control was orlistat and the negative control was without an inhibitor using 1% DMSO. One unit of activity is defined as the reaction rate that produces 1 µmol of p-nitrophenyl butyrate at 37˚C. The inhibition of lipase activity is expressed as the percentage decrease in activity when PPL is incubated with the test compound.[14]

$$\% \text{ Lipase Inhibition Activity} = 100 - \left[\frac{(B-b)}{(A-a)} \times 100\right]$$ ……..(2)

Explanation

A = absorbance of the negative control with the addition of enzyme and substrate

a = absorbance of the negative control without the addition of enzyme and substrate

B = absorbance of the inhibitor with the addition of enzyme and substrate
b = absorbance of the inhibitor without the addition of enzymes and substrates
inhibitor = extract sample and positive control

Identification of Pigments by TLC
Identification of pigments by TLC using silica gel GF 254 stationary phase and hexane:ether:acetone (6:3:2) mobile phase.[15]

Data Analysis
The data were expressed as the mean ± standard deviation (SD) of experiments in triplicate. This statistical analysis in this study was carried out using a Graph Pad Prism (version 9.1.2; Graph Pad Inc. software San Diego, CA, USA). IC_{50} value represented the concentration of the test sample causing 50% inhibition in which value <0.05 was considered significant.

RESULT
The extraction of spirulina was carried out using the rapid maceration method with four different types of solvents: methanol, acetone, methanol:acetone (70:30), and acetone:methanol (70:30). The purpose of the maceration method is to dissolve the pigments within the spirulina cells, causing them to be pushed out of the cells. The results of the maceration were filtered using Whatman filter paper, and the obtained filtrate was concentrated using an evaporator at a temperature of 30°C and a speed of 150 rpm. The % yield of the extract obtained is shown in **Figure 1**. The % yield of spirulina extract with methanol, acetone, methanol:acetone (70:30), and acetone:methanol (70:30) solvents were 13.10 ± 0.19%, 3.33 ± 0.46%, 13.92 ± 0.78%, and 9.26 ± 0.17%, respectively.

The optimal solvent for attracting carotenoid pigments is acetone.[17], [18] Based on the TLC results (**Figure 2**), it shows that the extract with the solvent acetone: methanol (70:30) can attract more pigments as indicated by the number of spots produced.

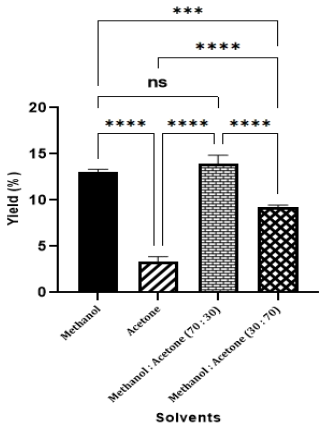

Figure 1: Results of Extraction Yield Test Using Four Solvent Extraction Methods, n=3, ns = not significant, * = < 0.001, **** = < 0.0001**

Figure 2: TLC Analysis of Spirulina Extract MA = Methanol: Acetone (70: 30), AM = Acetone: Methanol (70 : 30), A = Acetone, M = Methanol.

Antioxidant activity tests using the DPPH method were conducted on 4 extracts with different solvents. The activity test results showed that the methanol:acetone (30:70) extract had a free radical scavenging effect with increasing concentration. The antioxidant activity test results of the extract can be seen in **Figure 3**.

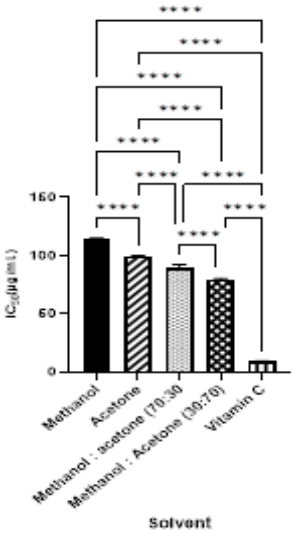

Figure 3: Results of Antioxidant Activity Test of the Extract, n=3, * = < 0.05, ** = <0.01, ** = < 0.0001.**

The obtained extract was subsequently tested for anti-obesity activity using pancreatic lipase enzyme inhibitors with an ELISA multimode reader instrument. The percentage inhibition results from the maceration extract with solvents methanol, acetone, methanol:acetone (70:30), and acetone:methanol (70:30) were obtained as 41.42 ± 1.42%, 54.67 ± 3.16%, 62.38 ± 3.25%, and 75.34 ± 0.91%, respectively (**Figure 4.**).

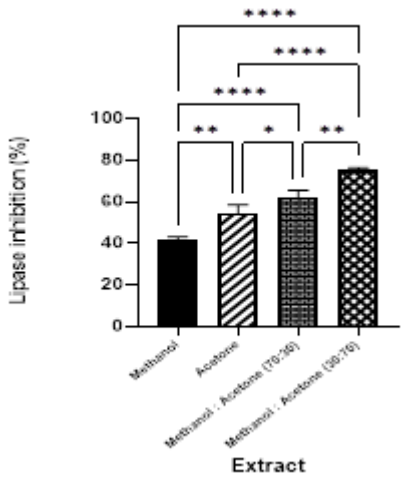

Figure 4: Results of Lipase Inhibition Test of Extract, n=3, * = < 0.05, ** = < 0.01, ** = < 0.0001.**

DISCUSSION

The yield from the rapid maceration method with the methanol:acetone (70:30) solvent was higher compared to the other solvents. The solvents used have the same polarity index: methanol 5.1 and acetone 5.1. Based on the results obtained, it shows that the extraction of compounds, especially pigments, does not depend on the polarity index. Many other factors can influence, including the structure of the solvent. Methanol with an OH group tends to attract chlorophyll more, while acetone with a ketone group tends to attract carotenoid pigments.[16]

Based on the TLC results shown in **Figure 2**, yellow color is observed at Rf 0.83 and Rf 0.96, gray color at Rf 0.48 and Rf 0.58, and bluish-green color at Rf 0.29 and Rf 0.39. Carotenoid pigments are yellow and chlorophyll is green.[16] The mobile phase used in this TLC is hexane: diethyl ether: acetone (6:3:2) and the stationary phase used is silica gel GF 60. The stationary phase is polar due to the presence of oxygen atoms on its surface. Mean while, the mobile phase is more non-polar, causing the movement of carotene to be faster compared to other pigments, which affects the resulting retardation factor (Rf) value. The Rf value of β-carotene is higher than that of xanthophyll and other pigments. The Rf value of *β*-carotene is 0.8 – 1.0. This Rf value is consistent with the Rf values in this study, which are 0.83 and 0.96. Based on the Rf value, the yellow-stained spot is identified as *β*-carotene.[17]

The gray stain indicates the color of the pigment pheophytin *a*. Pheophytin is a chlorophyll derivative compound that lacks the metal magnesium (Mg).[16], [19] The Rf value of feophytin *a* was 0.60.[19] The Rf values are close to the Rf in this study, which were 0.48 and 0.58. The bluish-green color with Rf values of 0.29 and 0.39 is identified as chlorophyll *a*. Chlorophyll *a* has Rf values of 0.57-0.64.[20] Chlorophyll *a* is bluish-green in color.[21]

Based on **Figure 3**, the DPPH antioxidant activity results show that the best IC_{50} is the extract with the methanol:acetone solvent (30:70), which is 79.84 ± 0.62 µg/mL. The activity of free radical scavenging is greatly influenced by phenolic hydroxyl compounds in their molecular structure. These compounds will react with free radicals, forming new radicals that are stabilized by the resonance of the aromatic nucleus.[22] The antioxidant activity of spirulina extract with a methanol:acetone (30:70) solvent using the DPPH method is the best compared to extracts with other solvents. This is due to phenolic compounds that act as antioxidants, whose activity depends on their chemical characteristics and mechanisms of action (for example, as metal chelators, hydrogen donors, or oxygen scavengers).[23] A compound is said to be a very strong antioxidant if the IC_{50} value is less than 50 ppm, strong for an IC_{50} value between 50 ppm – 100 ppm, moderate if the IC_{50} value is between 100 ppm – 150 ppm, and weak if the IC_{50} value is between 151 ppm – 200 ppm.[24]

Pancreatic lipase inhibition percentages < 41% fall into the weak category, 41%-80% into the moderate category, and > 80% into the strong category.[25] The spirulina extract with the acetone:methanol (70:30) solvent has an inhibition percentage of 75.34 ± 0.91, which falls into the moderate category.

CONCLUSION

The highest yield was in the extract using methanol:acetone 70:30 solvent (13.92±0.78%). The best antioxidant activity was found in the extract with methanol: acetone 30:70 solvent (79.84±0.62 µg/mL). The best lipase inhibitory activity was found in the extract with methanol: acetone 30:70 solvent (75.34± 0.94%).

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