Food Research 7 (Suppl. 3) : 59 - 66 (2023)
Journal homepage: https://www.myfoodresearch.com

FOOD RESEARCH

FULL PAPER

Effect of methanol solvent concentration on the extraction of bioactive compounds using ultrasonic-assisted extraction (UAE) from *Spirulina platensis*

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Article history:
Received: 11 November 2022
Received in revised form: 29 June 2023
Accepted: 27 September 2023
Available Online: 31 October 2023

Keywords:
Antioxidant activity,
Extraction,
Methanol,
Spirulina platensis

DOI:
https://doi.org/10.26656/fr.2017.7(S3).9

Abstract

Spirulina platensis is a blue-green algae that contains bioactive compounds as a source of antioxidants. Methanol is a polar solvent; it has similarities to the content of the bioactive compound *Spirulina platensis*, which is polar. This study aimed to determine the effect of different concentrations of methanol solvent (50%, 70%, 96%) using UAE on the content of bioactive antioxidant compounds from *Spirulina platensis*. The parameters observed were yield, antioxidant activity, phytochemical screening, and GC-MS. The results showed that the difference in the concentration of methanol solvent using UAE significantly affected the antioxidant activity of the *Spirulina platensis* extract. The highest antioxidant activity was found in *Spirulina platensis* extract with 50% methanol solvent concentration using UAE with an average inhibition value of 91.81%. The yield value from the *Spirulina platensis* extract with a 50% methanol concentration was 9.3%. The qualitative phytochemical screening test carried out on *Spirulina platensis* extract with 50% methanol solvent using UAE showed positive results for flavonoid compounds, alkaloids, tannins and phenols. GC-MS analysis on *Spirulina platensis* with 50% methanol as a solvent using UAE resulted in 5 types of the most dominant compounds, pentadecanoic acid, 9-Ooctadecenoic acid, hexadecanoic acid, hexadecanal, and 1-nonadecene.

1. Introduction

Antioxidants are natural or synthetic compounds that can play a role in maintaining and repairing damaged body cells, counteracting adverse effects, and slowing or preventing the oxidation process due to free radicals. Free radicals can come from our body's metabolism and outside the body, such as pollution, cigarette smoke, and radiation. In the industrial world, synthetic antioxidants are generally used because they are inexpensive and quite effective in preventing oxidation. Examples of synthetic antioxidants are Butyl Hydroxyanisol (BHA) and Butyl Hydroxy Toluene (BHT). However, synthetic antioxidants have drawbacks, as they are known to have a toxic effect and is carcinogenic.

Meanwhile, natural antioxidants are safer because they are obtained naturally from plant extracts. One of the natural antioxidants can be extracted from the microalgae *Spirulina platensis*. According to Ridlo *et al.* (2015), antioxidants are compounds that give or donate electrons that can inactivate oxidation reactions by preventing the formation of free radicals. One of the sources of natural antioxidants from aquatic biota is *Spirulina* sp.

The selected extraction method is very important because it will reflect the success rate of the extraction results. The conventional extraction method has drawbacks because it requires many solvents, a relatively long time, and the extract yields are less than optimal. Factors that affect the extraction results are the type and ratio of the solvent and the method used. Using both polar and non-polar solvents will affect the target compound to be extracted. According to Widyanto *et al.* (2020), the choice of solvent must be adjusted to the extracted compound. The solvent concentration will affect the antioxidant compounds that will be obtained.

Several methods can be used to extract the bioactive compounds in *Spirulina platensis*, including using methanol and water as a solvent. Both solvents are pretty effective in extracting bioactive compounds, especially phytochemical compounds. Methanol is a polar and universal solvent because, in addition to extracting polar components, it can also extract non-polar components. Extraction was done using different concentrations of

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eISSN: 2550-2166
Published by Rynnye Lyan Resources

methanol solvent to produce the best antioxidant. According to Moneim *et al.* (2022), using methanol as a solvent showed the more potent antioxidant activity of *Spirulina* extract than other solvents because of the phenolic content of the extract. The more polar the extract (methanol), the higher the total phenolic compound content.

This study aimed to determine the effect of different concentrations of methanol solvent on the content of bioactive compounds from *Spirulina platensis* and the best solvent concentration used for the extraction of *Spirulina platensis*.

2. Materials and methods

2.1 Materials and tools

The material used in this study was the microalgae *Spirulina platensis* obtained from PT Algaepark Indonesia Mandiri, Klaten, and methanol solvent. The tools used in this study were Sonicator (Powersonic 405), Rotary evaporator (IKA RV 10), and filter paper (Whattman no. 42).

2.2 Spirulina platensis extraction process

The extraction process begins by preparing a sample of 20 g of dry powder of *Spirulina platensis*, then dissolved in 100 mL of methanol solvent with a ratio between *Spirulina platensis* and methanol solvent of 1:5, using different concentrations (50%, 70% and 96%) of methanol solvent. The next step is the extraction process using a sonicator for 15 mins with a frequency of 40 kHz. The pure extract obtained was evaporated with a rotary evaporator until there is no more solvent dripping. The extraction results were obtained in the form of a thick extract.

2.3 Testing methods

2.3.1 Yield test

Yield is the percentage ratio of the weight of the resulting extract with the weight of the initial raw material. The calculation of the extract yield refers to the AOAC (2005) by calculating the weight of the extract obtained divided by the weight of the raw material sample multiplied by 100%. The following equation calculates the yield value:

$$\frac{(\text{Weight of extract obtained (g)})}{(\text{Weight of extracted sample (g)})} \times 100\%$$

2.3.2 Antioxidant activity test

Testing the antioxidant activity of the DPPH method refers to the research of Andayani *et al.* (2008) with slight modifications. The DPPH solution was prepared by weighing 2 mg of DPPH powder and dissolving it in 100 mL of methanol, then checking by seeing that the absorbance reaches a minimum of 550 A. The extract was dissolved by 0.2 mL DPPH solution and then mixed with 3.8 mL of methanol. The mixture was then vortexed, after which it was left in the dark for 30 mins. Absorbance measurements were carried out using a UV-Vis spectrophotometer at 515 nm. The blank solution used was methanol with added DPPH solution. Testing of antioxidant activity was carried out in triples. Free radical scavenging activity was calculated as the percentage of DPPH color reduction. The following equation calculated the antioxidant capacity (percent inhibition):

$$\text{Antioxidant activity (\%)} = \frac{\text{abs control} - \text{abs sample}}{\text{abs control}} \times 100\%$$

2.3.3 Phytochemical screening test

The phytochemical screening was carried out using the method described by Anthony *et al*. (2013).

2.3.3.1 Flavonoid test

Approximately 2 mL of sample extract was mixed with several fragments of magnesium ribbon, then drops of concentrated HCl were added until an orange/brick red color appears, indicating flavonoid compounds' presence.

2.3.3.2 Alkaloid test

Approximately 2 mL of sample extract was mixed with 1% HCl and then heated slowly. After that, Mayer's reagent or Dragendorf's is added to the mixture. The presence of alkaloids is indicated by the turbidity of the resulting precipitate which is yellow/orange in color.

2.3.3.3 Tannin test

Approximately 2 mL of sample extract was added with 5 drops of 10% NaOH then put into a test tube. After the addition of 2 mL of 2% $FeCl_3$ solution into the tube, dark brown, dark blue or dark green filtrate indicates a positive result.

2.3.3.4 Phenol test

Approximately 2 mL of the sample solution was poured into the drip plate followed by 2 mL of 2% $FeCl_3$ solution. Positive results are indicated by dark green, blue-black, or black-brown filtrate.

2.3.4 GC-MS test

The bioactive components were tested according to the method described by Riyadi *et al*. (2020), using Gas Chromatography Mass Spectrometry-GCMS, specifically with column Agilent 19091S-433 HP-5MS which has a length of 30 m and a diameter of 250 m to

https://doi.org/10.26656/fr.2017.7(S3).9

determine the chemical profile of *Spirulina platensis*. Approximately 1 L of *Spirulina platensis* extract was dissolved in methanol and injected into GC-MS. Helium gas acts as a carrier gas with a flow rate of 1 mL/min with the oven temperature at 325°C. The initial oven temperature was 150°C then held at 2°C/min. This process lasted for 10°C/min, which was increased to 240°C with a holding time of 11 mins. The total running time is 22 mins, ranging from 50-550 amu. The chemical structure profile screening was based on analysis of the mass spectrum fragmentation pattern and compared with the mass spectrum in the National Institute of Standards and Technology (NIST) and Wiley compound profile databases.

3. Results and discussion

3.1 Yield

The yield of the test results are shown in Table 1. Based on the results in Table 1, it can be seen that the best yield value was in the treatment which was an extract of *Spirulina platensis* using a 50% concentration of methanol as a solvent. Treatments A, B, and C, each with different concentrations of methanol (50%, 70% and 96%) had inversely proportional yield values. The higher the concentration, the lower the yield value. The yield value in treatment A was 9.3%, treatment B was 8.4%, while treatment C was 4% because 50% methanol has the most air content compared to 70% and 96% methanol. So that when the evaporation process is carried out at the same time the water has not evaporated completely, which causes higher yields and more water-soluble phenolic compounds. According to Sa'adah and Nurhasnawati (2017), air is a suitable solvent for ionic compounds. Water has a –OH group which is polar and provides both a dipole that needs to be solvated and an ion. Water solvents can dissolve bioactive compounds such as phenolics, flavonoids, alkaloids, tannins, and steroids. In addition, water is also able to extract components that are polar or semipolar. When evaporated the water evaporates longer so that the yield obtained is more.

Table 1. Results of calculation of yield of *Spirulina platensis*.

Treatment	Sample Weight (g)	Yield Value (%)
A	100	9.3
B	100	8.4
C	100	4.0

3.2 Antioxidant activity

The results of testing the antioxidant activity of DPPH on *Spirulina platensis* extract are shown in Figure 1. *Spirulina platensis* extract based on the results of the antioxidant activity test using the DPPH method had significantly different results between treatments. The average inhibition value of the 50% methanol solvent concentration treatment was 91.81%, the 70% methanol solvent concentration treatment had an average inhibition value of 78.94%, while the 96% methanol solvent concentration treatment had an average inhibition value of 58.61%. The water content dissolved the antioxidant-producing phenolic compounds in the *Spirulina platensis* extract. According to Novita *et al*. (2016), water is a very polar solvent compared to methanol, enabling it to extract a higher total phenol. Extraction using a higher water solvent causes the content of phenolic compounds such as alkaloids, tannins, saponins to be high because they are also extracted with water as a solvent. The level of polarity of the solvent can also be known from the dielectric constant, the greater the dielectric constant, the more polar the solvent. Water has a higher dielectric constant than methanol, so it is more polar than methanol. Methanol 70% is a solvent that is more polar than 96% methanol, but not more polar than methanol 50%, so phenolic compounds tend to dissolve more in 50% methanol because it has higher polarity than methanol 70%. According to Agustina (2017), the use of methanol and water solvents in extraction is because methanol solvent has a dielectric constant of 33, while water has a higher dielectric constant of 80. The polarity level of solvent can be known from the dielectric constant, the greater the dielectric constant of the solvent, the polarity of the solvent was also getting higher. Kothari and Seshadri (2010) reported that methanol and water extracts have high antioxidant activity because the extracted phenolic content is higher and can scavenge free radicals.

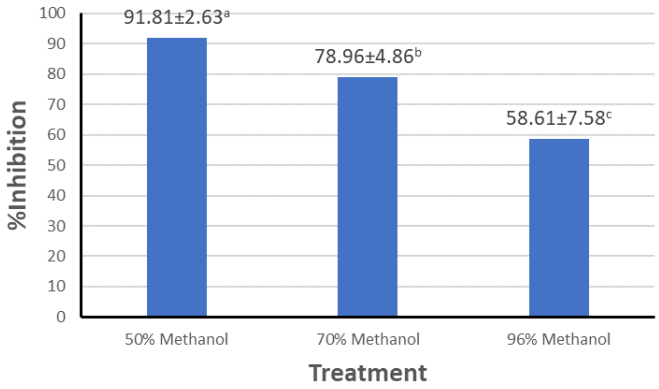

Figure 1. Antioxidant activity of *Spirulina platensis* extract. Values are presented as mean±SD. Values with different superscript are statistically significantly different ($p<0.05$).

3.3 Phytochemical screening

The results of phytochemical screening tests on *Spirulina platensis* extract at 50% methanol concentration are presented in Table 2. The results of the qualitative phytochemical screening test obtained on the extract of *Spirulina platensis* with a solvent concentration of 50% methanol were positive for

https://doi.org/10.26656/fr.2017.7(S3).9

flavonoids, alkaloids, tannins and phenols. The extract of *Spirulina platensis* has the potential as an antioxidant because it contains phytochemical compounds that produce antioxidants. Qualitative phytochemical screening tests refer to the research procedures described by Anthony *et al*. (2013). This method looks at the color test reaction using a color reagent. The purpose of the phytochemical screening test is to determine what groups of compounds are in the material under study. According to Pratama *et al*. (2020), the extract of *Spirulina platensis* which was tested for phytochemical screening were positive for flavonoid compounds, steroids, terpenoids, saponins, and phenolics. High antioxidant activity in the extract of *Spirulina platensis* can be shown by positive results on these secondary metabolites.

Table 2. Phytochemical screening of *Spirulina platensis* extract.

Compounds	Result
Flavonoids	(positive)
Alkaloids	(positive)
Tannins	(positive)
Phenols	(positive)

Spirulina platensis extract has a high content of flavonoid and phycocyanin compounds. The flavonoid compounds act as natural antioxidants, besides that *Spirulina platensis* also contains green pigments (chlorophyll) and carotenoids (Hayati *et al*., 2020). Alkaloids are organic compounds that belong to the group of secondary metabolites and are alkaline. This is because the content of nitrogen atoms (N) is owned by more than one alkaloid and can come from plants or animals (Susanto *et al*., 2019). According to Noer *et al*. (2018), tannins are polyphenolic compounds that can form complex compounds with proteins, and have enormous molecular weights reaching more than 1000 g/ mol. Tannins have a benzene ring compound structure (C6) that bonds with a hydroxyl group (-OH). The biological role of tannins is huge because they act as metal chelators and precipitate proteins. So that tannins can be used as biological antioxidants. Meanwhile Mekini'c *et al*. (2019) reported that phenol compounds are a group of secondary metabolites, having the form of an aromatic benzene ring consisting of one or more hydroxyl groups that are bonded directly to the aromatic ring. This compound in seaweed has a simple structure such as phenolic acid, until phlorotanin is the most complex.

3.4 Gas chromatography-mass spectrometry

The content of bioactive components by GC-MS in *Spirulina platensis* extract with a solvent concentration of 50% methanol produced various compounds. The activity potential of the *S. platensis* extract can be seen in Table 3.

The extract of *Spirulina platensis* contained 5 of the most dominant compounds, that is the pentadecanoic acid compounds which have an area of 9.34 and a retention time of 23.071, the compound 9-octadecenoic acid has an area of 6.27 and a retention time of 24.919, hexadecanoic acid compounds have an area of 5.17 and a retention time of 25.357. Hexadecanal compound has an area of 5.26 and has a retention time of 32.158, and 1-nonadecene compound has an area of 2.38 and has a retention time of 29.304. These compounds have different roles and functions.

Research conducted by Mellouk *et al*. (2017) showed that analysis of fatty acid profiles in red algae *Asparagopsis taxiformis* found pentadecanoic acid compounds which are the most common fatty acid compounds and have potential as antioxidants. Sharma *et al*. (2016), revealed that pentadecanoic acid showed the maximum area on GC-MS analysis which was identified as anticancer, antibacterial, anti-inflammatory. Meanwhile research conducted by Ihegboro *et al*. (2019), showed that 9-Octadecenoic acid extracted with methanol was found to have the most medicinal properties as antioxidants, anticancer, and anti-inflammatory.

According to Bharath *et al*. (2021), hexadecanoic acid compounds isolated from brown algae *Turbinaria ornata* showed potential antioxidant activity while exhibiting anticancer activity capable of inhibiting colon cancer cells through induced apoptosis and cell cycle arrest. Ihegboro *et al*. (2019) reported that other compounds found such as hexadecanoic acid compounds were also indicated as good anti-inflammatory agents. Meanwhile research conducted by Ragunathan *et al*. (2019) revealed that the hexadecanal found in the methanol extract of *Gracilaria corcita* functions as a source of antioxidants, besides that it also functions as an antibacterial that can restrain the growth of pathogenic microorganisms such as *Staphylococcus aureus*, *Bacillus subtilis* and *Pseudomonas aeruginosa*. According to Amudha *et al*. (2018), the bioactive compound 1-Nonadecene extracted from the aquatic plant *Enhalus acoroides* has biological activity as an antioxidant, anticancer, and antimicrobial.

4. Conclusion

The difference in the concentration of methanol solvent significantly affects the antioxidant activity of the *Spirulina platensis* extract. The best concentration used to extract *Spirulina platensis* was found at 50% methanol solvent concentration, producing antioxidant activity with an average inhibition value of 91.81%.

https://doi.org/10.26656/fr.2017.7(S3).9

Table 3. GC-MS result compounds and bioactive potency on *Spirulina platensis* extract.

S/N	Name	Formula	RT	Area (%)	Bioactive Potency
1.	Pentadecanoic acid	$C_{15}H_{30}O_2$	23.071	9.34	Antioxidant (Mellouk *et al*., 2017), Anticancer (Sharma *et al.,* 2016), Antibacterial (Sharma *et al.,* 2016), Anti-inflammatory (Sharma *et al.,* 2016), Antihypertensive (Dong *et al.,* 2022).
2.	Octadecanoic acid	$C_{18}H_{36}O_2$	23.343	0.41	Anticancer (Arora and Kumar 2018), Antimicrobial (Arora and Kumar 2018), Antibacterial (Sharma *et al.,* 2016), Anti-inflammatory (Othman *et al.,* 2015), Anti-fungal (Kalaivani and Amudha, 2021).
3.	9-Octadecenoic acid	$C_{18}H_{34}O_2$	24.919	6.27	Antioxidant (Ihegboro *et al.,* 2019), Anticancer (Ghavam *et al.,* 2021), Anti-inflammatory (Ghavam *et al.,* 2021), Antihypertensive (Ghavam *et al.,* 2021).
4.	Hexadecanoic acid	$C_{16}H_{32}O_2$	25.357	5.17	Antioxidant (Meechaona *et al*., 2007; Bharath *et al*., 2021), Anticancer (Bharath *et al.* 2021), Antibacterial (Sharma *et al.,* 2016), Anti-inflammatory (Aparna *et al.,* 2012; Ihegboro *et al.,* 2019), Antihypertensive (Okore *et al.,* 2021).
5.	Heptadecanoic acid	$C_{17}H_{34}O_2$	28.157	2.08	Antioxidant (Al-Douri and Shakya, 2019), Anticancer (Bharath *et al.,* 2021), Antibacterial (Syed *et al.,* 2021), Anti-inflammatory (Ali *et al.,* 2021), Antihypertensive (de Oliveira Otto *et al.,* 2018).
6.	1-Nonadecene	$C_{19}H_{38}$	29.304	2.38	Antioxidant (Amudha *et al.,* 2018), Anticancer (Amudha *et al.,* 2018), Antituberculosis (Amudha *et al.,* 2018), Anti-inflammatory (Skanda *et al.,* 2021).
7.	Oleic acid	$C_{18}H_{34}O_2$	29.931	1.25	Anti-tumor (Giulitti *et al*., 2021), Anticancer (Win, 2005), Anti-inflammatory (Santamarina *et al.,* 2021), Anti-cholesterol (Djamaludin and Chamidah, 2021).
8.	Cyclotetracosane	$C_{24}H_{48}$	30.124	1.45	Anti-diabetic (Tundis *et al*., 2012), Anticancer (Lee *et al.,* 2007), Antimicrobial (Aghatabay *et al.,* 2009).
9.	Cyclopentadecanone	$C_{15}H_{28}O$	30.489	1.46	
10.	Cyclohexane	C_6H_{12}	30.672	1.67	Antimicrobial (Shoaib *et al.,* 2019), Antibiotics (Shoaib *et al.,* 2019).
11.	Hexadecanal	$C_6H_{12}O$	32.158	5.26	Antioxidant (Ragunathan *et al.,* 2019), Antibacterial (Delnavazi *et al.,* 2014).
12.	Z,E-3, 13-Octadecadien-1-ol	$C_{18}H_{34}O$	32.357	1.91	Antioxidant (Jayachitra *et al.,* 2020), Anticancer (Jayachitra *et al.,* 2020), Antibacterial (Jayachitra *et al.,* 2020), Anti-inflammatory (Jayachitra *et al.,* 2020), Anti -obesity (Jayachitra *et al.,* 2020).
13.	Z-2-Dodecenol	$C_{12}H_{24}O$	32.573	1.71	
14.	1-Hexacosene	$C_{26}H_{52}$	32.787	2.17	
15.	1,2-dimethyl-3-pentyl-4-propyl-Cyclohexane	$C_{16}H_{32}$	33.331	2.19	
16.	Bis(2-ethylhexyl) phthalate	$C_{24}H_{38}O_4$	33.747	1.62	
17.	1,13-Tetradecadien	$C_{14}H_{26}$	33.847	2.20	

pirulina platensis extract compounds have bioactive potential such as antioxidants, anticancer, anti-inflammatory, anti-microbial, anti-cholesterol, antihypertention, antituberculosis, antibiotics, anti-obesity.

Acknowledgments

The author would like to thank (LPPM) Institute For Research and Community Services, Diponegoro University and Directorate of Research, Technology, and Community Service, Ministry of Education, Culture, Research and Technology for the National Competitive Basic Research Fund SP DIPA 023.17.1.690523/2022 02 revision dated 22 April 2022, Activity Implementation Agreement Number: 345-04/UN&.6.1/PP/2022.

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