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In Vitro Antioxidant and Anti-Obesity Activities of Ethanolic Extract from Microalgae Strain MRB-2

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Abstract. *Obesity has a 15-fold higher risk of coronary heart disease, stroke, and diabetes mellitus. Microalga is one of the natural resources that potentially treat obesity. The purpose of this study was to evaluate the total phenolic contents (TPC), antioxidant, and anti-obesity properties of ethanolic extract of microalgae strain MRB-2. The TPC was determined using the Follin-Ciocalteu method. The antioxidant activity was evaluated using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) method, and the anti-obesity was analyzed using an anti-lipase pancreatic assay. The morphology of microalga cells was also determined using Scanning Electron Microscopy (SEM). The results revealed that the TPC of ethanolic extract from the ultrasound extraction method was higher than the maceration method with the value of 2.75±0.26 mg GAE/g. While the scavenging activity toward DPPH radicals of ethanolic extract from the maceration method was higher than ultrasound, with a value of 38.92±1.94% at 0.8 mg/mL. The lipase inhibitory activity of extract from the maceration method was higher than ultrasound with a value of 20.81±2.24% at 0.38 mg/mL. Our results indicate that ethanolic extract of MRB-2 was potentially developed for anti-obesity foods and health-functional foods derived from new peatland microalgae.*

Keywords: *Anti-obesity, antioxidant, microalgae, total phenolic, peatland microalgae*

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INTRODUCTION

Obesity has been a worldwide concern as it has become a significant burden for the healthcare budget. Almost 25% of adults in developed countries fall into the obese category (Valenzuela et al., 2023). The number of obese populations kept increasing in developed and low- and middle-income countries (Popkin, 2014). Obesity has reduced quality of life and has been associated with health problems such as endocrine, metabolic, and cardiovascular disorders (Hruby et al., 2016). An individual with obesity has a 15-fold higher risk of coronary heart disease, coronary stroke, and diabetes mellitus (Valenzuela et al., 2023).

Individuals with obesity are initiated by the expansion of subcutaneous adipose tissues (SAT), where adipose cells increase up to three times, and some cells turn to hyperplasia. When the accumulation of SAT exceeds the threshold, fat is deposited in the visceral adipose tissue (VAT) in the abdominal part of the body, such as the pancreas, liver, and heart (Marseglia et al., 2015). Visceral adipose tissue lipid deposits are linked to an increased risk of cardiovascular diseases, including hypertension, ischemic heart disease, ischemic stroke, diabetes mellitus, and chronic kidney disease (Cesaro et al., 2023).

Interestingly, current studies showed that obesity is associated with chronic low-grade inflammation and continuous exposure to oxidative stress (Marseglia et al., 2015). The expansion of adipocyte cells is not supported by the formation of new blood vessels and sufficient blood flow. This condition induces cell hypoxia and oxidative stress (Trayhurn, 2013). Studies have shown that adipose tissue is the place where pro-inflammatory cytokines such as tumor necrosis factor-alpha (TNF-α), interleukin (IL)-1β, and IL-6 are produced (Marseglia et al., 2015). The more adipose tissue, the more pro-inflammatory cytokine is produced. Elevation of these pro-inflammatory cytokines promotes more production of reactive oxygen are generated. Continuous exposure to oxidative stress leads to cellular damage associated with metabolic syndrome, liver disease, hypertension, cancer prognosis, and neurological disorders (Hruby et al., 2016).

The common cause of obesity is an excess high-fat diet intake, less physical activity, and effortless access to abundant food. Thus, the anti-obesity industry is becoming more popular and promising for many (Lin & Li, 2021). One way to overcome obesity and its complications is to provide a natural product that can prevent the hydrolysis of triglycerides to fatty acid and glycerol while providing antioxidant activities to tackle the prolonged oxidative stress that is present in the person with obesity (Liu et al., 2020).

Currently, some research is focusing on preventing fatty acid absorption from the gastrointestinal tract while providing an antioxidant agent to combat oxidative stress during obesity. Among the many natural products, microalgae are a promising biological resource (Tun et al., 2020). Microalgae are photosynthetic microbes that are primarily found in aquatic environments. This organism has become a renewable resource for food, cosmetics, and medicine due to its ability to produce primary and secondary metabolites. Microalgae contain several compounds such as carotenoids, phenolics and polyunsaturated fatty acids (PUFAs) with antioxidant activity (Andriopoulos et al., 2022; Jerez-Martel et al., 2017; Coulombier et al.,2021). Microalgae *Chlorella sorokiniana* has been shown to inhibit pancreatic lipase in the gastrointestinal lumen, preventing fat absorption in the intestine (Banskota et al., 2016). Another research reported the potency of *Chlorella vulgaris* and *Chlorococcum amblystomatis* as anti-obesity, anti-steatosis, and anti-inflammatory properties (Regueiras et al., 2022). The extraction process of bioactive compounds is an important step in research, development and implementation. Several methods have been studied, such as microwave-assisted and supercritical fluid extraction. However, these methods have several drawbacks, such as high cost, high energy consumption, and

thermal effects (Lou et al., 2012). Furthermore, different extraction methods may result in differences in yields and the biological activity of the extract (Wigati et al., 2023). Thus, this study will use two kinds of extraction methods i.e. maceration and ultrasonication and study its effect on the biological activity of microalgae. Unfortunately, research on microalgae that live in peatlands is currently lacking, although their high organic content and low acidity may influence their metabolites and produce distinctive characteristics. Therefore, this study aimed to evaluate the total phenolic contents (TPC), antioxidant, and anti-lipase activities of microalgae strain-MRB2 from peatlands in Oxbow Hanjalutung Lake, Central Kalimantan, Indonesia, using different extraction methods. This study will be valuable to give new insight into the benefits of this microalga species.

MATERIALS AND METHODS

The research was conducted in the Research Center for Applied Microbiology, Research Organization of Life Science and Environment and Research Center for Food Technology and Processing, National Research and Innovation Agency, Indonesia at February-June 2023.

Preparation of Microalga Materials

The microalgae MRB-2 was isolated from Oxbow Hanjalutung Lake, a peatland in Central Kalimantan, Indonesia. The AF6 media was used to grow microalgae MRB-2 massively. The media was prepared according to NIES-collection with the following components (per 100 ml): $NaNO_3$ (14 mg), NH_4NO_3 (2.2 mg), $MgSO_4.7H_2O$ (3 mg), KH_2PO_4 (1 mg), K_2HPO_4 (0.5 mg), $CaCl_2.2H_2O$ (1 mg), Fe-citrate (0.2 mg), Citric acid (0.2 mg), Biotin (0.2 µg), Thiamin HCl (1 µg), Vitamin B6 (0.1 µg), Vitamin B12 (0.1 µg), and Trace Metals (0.5 mL) (Anam et al., 2020). A shaker was used to carry out the pre-cultivation with stable speed and continuous lighting. The MRB-2 was then cultivated for 14 days at 1000 lux light intensity and constant aeration in 5 L of AF-6 medium. The final product was ultimately obtained by freeze-drying the MRB-2 biomass after being harvested using centrifugation at 10,000 rpm.

Morphological Characteristic of Microalgae Cells MRB-2

The morphology of microalgae was observed by a microscope (Olympus BX53) using brightfield and differential interference contrast. The morphological features identified microalgae strain after being recorded photographically at 100x magnification and connected with a digital camera system.

Extraction of Samples

Extraction of MRB-2 was conducted by two methods: maceration and ultrasonication. Extraction of MRB-2 using maceration method following to (Ardiles et al., 2020) with modification. Briefly, 200 mg of microalga MRB-2 powder was put in 20 mL of ethanol as the solvent and sonicated on water bath sonication (Elmasonic S, Germany) for 15 min at room temperature, followed by maceration at room temperature (3x24 h). The filtrate was centrifuged at 4°C, 3500 rpm for 15 min. Furthermore, the crude extract was obtained by evaporating the solvent. According to (Huang et al., 2016), the second method was ultrasonication with modification. The instrument (Ultrasonic Cell Disrupter BSD-900 W, China) was set up with a power rate of 360 W for 10 min and used a probe of 6. The equal weight of samples and the same solvent were also used in this method. The following steps were centrifugation and evaporation, as above.

Scanning Electron Microscopic (SEM) Observation of Ruptured Microalgae Cells

The morphological surface of microalgae cells was analyzed on SEM (SU-3500 Hitachi), and the data was also compared between the samples before and after extraction. The sample powders were

mounted on stubs and coated with gold 30 mA and 60 s using a sputter coater. The acceleration voltage of work was operated at 3 kV with a magnification of 5000x.

Total Phenolic Content (TPC) Analysis

The total phenolic content (TPC) of the microalga strain MRB-2 extract was evaluated by the Folin–Ciocalteu method as modified by (Choochote et al., 2014). Briefly, 10 µL of methanolic extract (1 mg/mL) was mixed with 50 µL of Folin-Ciocalteu reagent in a microtube and then was incubated for 8 min. After that, 50 µL of 20% sodium carbonate and 790 µL of distilled water were added and mixed gently for 1 min. The prepared solution was ready to be injected into microplate 96 well and incubated for 2 h at room temperature in darkness. The absorbance of the reaction mixture was measured at 765 nm using a microplate spectrophotometer (Multiskan™, Thermo Scientific). Gallic acid was also prepared as a standard and diluted to be 0, 60, 80, 100, 120, and 150 µg/mL. As a result, the TPC of the extracts was expressed in milligram gallic acid equivalent (mg GAE/g extract).

Antioxidant Activity

The antioxidant activity of microalga MRB-2 extract was examined by the DPPH method referred to (Ayele et al., 2022) with slight modifications. Firstly, 80 µL of sample solution was added into 20 µL DPPH solution (1.01 mM). The mixture was left to stand for 30 min at room temperature in the darkness. The absorbance measurement was conducted at 517 nm with a microplate spectrophotometer (Multiskan™, Thermo Scientific). A methanol solution was used as blank, and quercetin was used as positive control.

The determination of percent scavenging activity following the formula (1):

$$\%\ \text{Inhibition} = \frac{(\text{Absorbance of control} - \text{Absorbance of sample})}{(\text{Absorbance of control})} \text{ X } 100\%$$

Anti-lipase Activity

The anti-obesity activity of extract was evaluated using anti-lipase assay. The pancreatic lipase inhibitory activity was evaluated according to (Banskota et al., 2016) with a slight modification. Either 10 µL of orlistat (final concentration 46 mg/mL) or extract (final concentration 0.38 mg/mL) was mixed with 10 µL of lipase enzyme (1 mg/mL in phosphate buffer saline, pH 6.8) and incubated for 5 min at 37°C. Then, 240 µL p-nitrophenyl butyrate (0.168 mM) was added, followed by measuring the absorbance of samples at 415 nm for 35 minutes (Multiskan GO, Thermo Scientific). Nitrophenol standard curves (1-20 µg/mL) were made to measure the rate of nitrophenol hydrolysis in the reaction. The inhibition of lipase activity (%) was calculated using the following formula::

$$Inhibition\ (\%) = \frac{A - B}{A} \times 100$$

Where A is the activity rate of untreated control, and B is the activity rate of samples or inhibitor standard.

RESULTS AND DISCUSSION

Morphology Structure of MRB-2 Microalga Cells

SEM imaging was used to visualize the surface morphology of microalgal residue before and after extraction. This method effectively compares structural changes such as aggregation of cells, collapse, and disruption of outer cell membranes (Zhang et al., 2018). The aggregated cells of MRB-2 were described as a single-cell (Figure 1). The cells were spherical/rounded, approximately 0.44-0.80 µm long and 0.72-0.82 µm high.

Before extraction, the condition of the cells was observed as intact with a smooth surface. The cell morphology has changed significantly after both maceration and ultrasound methods, suggesting a degradation (Figure 2; (b) and (c)). The SEM image showed that the maceration process initiated with ultrasonication for 15 minutes (Figure 2.b) had disrupted the microalgae cell completely compared to the ultrasonication for 10 minutes. This was in agreement with a previous study, which showed that a longer extraction time would have higher damage to the sample surface morphology (Lin et al., 2021). Other studies also indicated disruption of microalga cell walls post ultrasonic treatment (Huang et al., 2016; Khoo et al., 2020). Ultrasonic disruption is caused by physical/mechanical effects of intense shock waves, shear forces, high local temperature, microbubble resonance, and pressure (Liu Y. et al., 2022). The ruptured microalgae cells will allow the solvent to pass through the cells and extract the metabolites contained in the microalgae. However, both extraction methods in this study had their advantage, as it revealed that the total phenolic content was higher in the ultrasound method, while the maceration method had higher antioxidant and anti-obesity activities value.

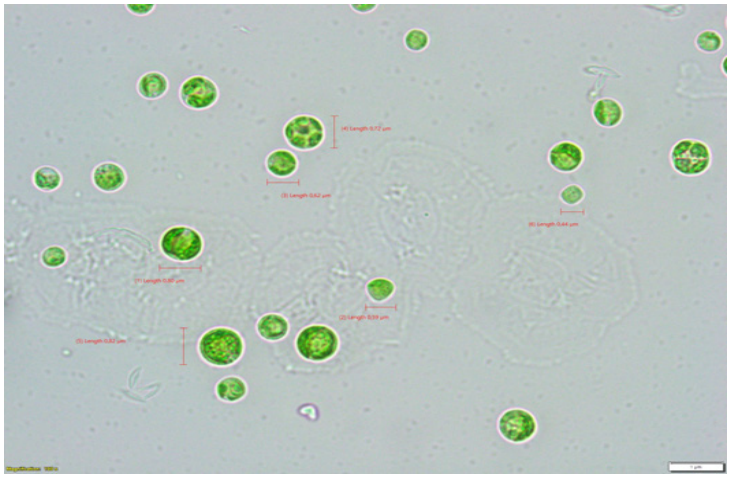
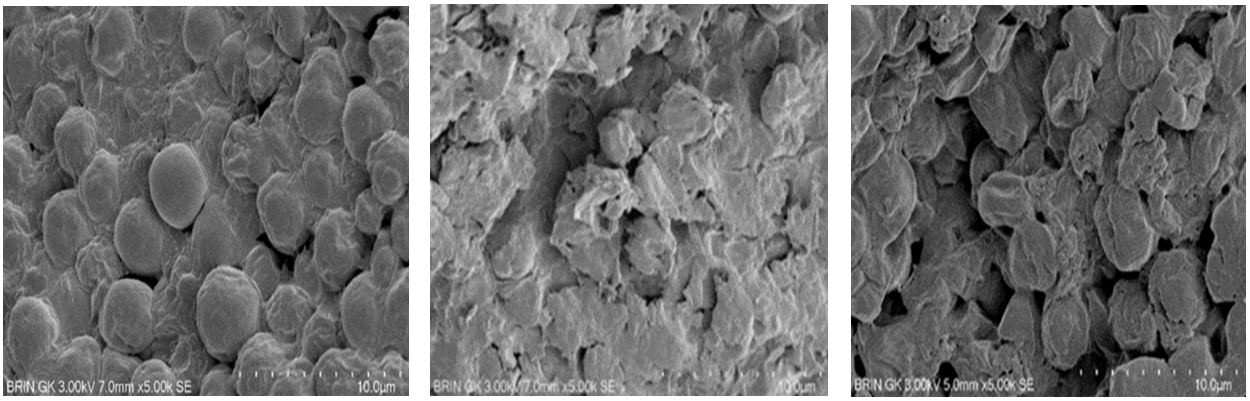


Figure 1. Microscopic overview of MRB-2



(a) (b) (c)

Figure 2. Scanning electron micrographs (SEM) of the microalgal cell surfaces. Cells before treatment (a); after treatments: maceration extraction (b); ultrasound extraction (c).

Total Phenolic Contents (TPC)

Microalgae have been reported as one of the most promising sources of phenolic compounds that are beneficial for food, cosmetics, and pharmaceutical sciences. Phenolic compounds benefit human health due to their several biological activities, including anti-inflammatory, regulating macronutrient digestion, and modulating oxidative stress (Del Mondo et al., 2021). Our study showed that the TPC obtained from the ultrasound method was higher than the maceration method, with the values of 2.75±0.26 mg GAE/g and 2.41±0.43 mg GAE/g, respectively. Somehow, this value is much lower compared to the previous research by (Choochote et al., 2014), who reported the TPC of the ethanolic extract from *Chlorella* sp.E53 was 35.5 mg GAE/g extract. In addition, the interaction of the solvent system and the matrix may be influenced by acoustic cavitation to alter the chemical properties of the produced extract (Pingret et al., 2013). This data is in line with the previous study, which suggested that the ultrasonication method is the most effective technique for obtaining TPC in Ulva rigida (Monteiro et al., 2020).

Antioxidant Activity

As the TPC value was promising, we continued examining the antioxidant activity. The extract processed by maceration exhibited higher DPPH radical scavenging activity (38.92±1.94% mg/mL) compared to ultrasonication, which is 8.31±0.42% at 0.8 mg/mL (Figure 4). A previous study by (Choochote et al., 2014) identified that the DPPH radical scavenging activity of ethanolic extract from *Chlorella* sp. E53 was 68.18% at 1.4 mg/mL (Manivannan et al., 2012) reported that phenolic compounds provide an electron or a hydrogen atom to form stable radical intermediates, acting in an important role as antioxidants. However, no correlation was found between TPC and antioxidant activity in our present study. There is a possibility that there were other metabolites in the ethanolic extract of microalga strain MRB-2 or that synergy between metabolites contributed to the antioxidant activity of the extract.

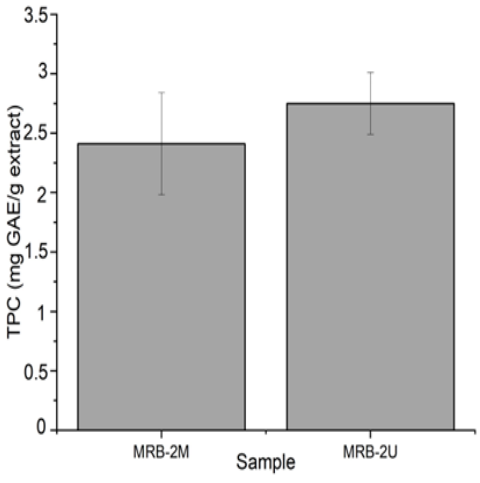


Figure 3. TPC of MRB-2 extract using different extraction methods, maceration (MRB-2M) and ultrasonication (MRB-2U)

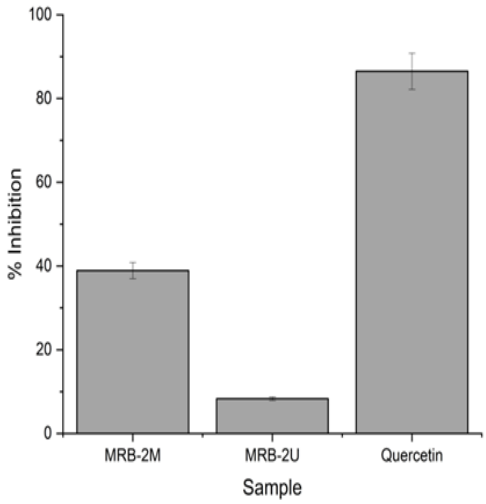


Figure 4. Antioxidant activity of MRB-2 extracts using different extraction method, maceration (MRB-2M) and ultrasonication (MRB-2U)

Anti-Lipase Activity

Lipase enzyme activity is one of the parameters for the obesity process. A compound that inhibits this enzyme activity can be developed as an anti-obesity agent (Liu T-T. et al., 2020). The lipase inhibitory activity of MRB-2 extract can be seen in Table 1. The result revealed that ethanolic extract of MRB-2 using maceration exhibited a higher lipase inhibitory activity compared to ultrasonic extraction with a value of 20.81±2.24% at 0.38 mg/ mL. The maceration and ultrasonication methods disrupt the microalgae cells and allow the ethanol solvent to enter the cell and extract the metabolites. The extraction method can affect the extraction of metabolites, which contributes to the biological activities of the extract (Zhao et al., 2023). According to the result, the different extraction methods showed differences in the lipase inhibitory activity of the samples. A previous study reported that microalgae *Chlorella sorokiniana* isolated from freshwater exhibited pancreatic lipase activity (Banskota et al., 2016).

Table 1. The lipase inhibitory activity of MRB-2

Sample	% Relative lipase inhibitory activity (0.38 mg/mL)
Maceration	20.81±2.24
Ultrasonication	8.25±2.38
Orlistat at 46 µg/mL	74.52±1.69

CONCLUSION

In conclusion, ethanolic extract of microalgae strain MRB-2 has the potential for antioxidant and anti-lipase activities. The TPC of the extract did not affect the antioxidant and anti-lipase activities of extract MRB-2. The extraction method affected the extraction of metabolites from microalgae.

AUTHOR CONTRIBUTION

The contribution of each author in this research is D.N investigation, writing-original; C.D. conceptualization, methodology, investigation, data analysis, writing–original & review; H.S. cultivation of microalgae, writing-editing; N.H. cultivation of microalgae, writing-editing; A.W.I. investigation, data analysis, review; S.H. writing-original, investigation, data analysis; R.M.H. writing-original and editing; M.D.L. writing-original and review, funding acquisition.

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CONFLICT OF INTEREST

There is no conflict of interest in this research team.

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