

Extraction Methods and the Effect of Solvents on Antioxidant and Anti-lipase Activities of *Nostoc* sp. MRB-1 from the Peatlands in Oxbow Hanjalutung Lake, Indonesia

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ABSTRACT

The increasing utilization of microalgae as a natural resource for bioactive compounds necessitates the discovery of efficient and cost-effective extraction methods for these compounds. Therefore, the present study aimed to evaluate two solvents and two extraction methods for obtaining extracts from microalgae *Nostoc* sp. MRB-1. The extracts were assessed for their phenolic content, antioxidant, and anti-lipase activities. The results indicated that the highest anti-lipase activity was obtained with maceration method using ethanol with value of $23.01 \pm 1.66\%$ at 0.38 mg/mL. While the maceration using hexane exhibited the highest antioxidant activity with a value of $39.68 \pm 0.07\%$ at 0.8 mg/mL. The results also showed that the phenolic content positively correlate to anti lipase activity. However, the content of phenolic compounds in the extract is not directly proportional to its antioxidant activity. This study revealed that extract of microalgae *Nostoc* sp. MRB-1 had high potential to be applied in food, nutraceutical and pharmaceutical industries.

Keywords: microalgae, antioxidant, anti-obesity, extraction method, *Nostoc* sp., solvent extraction

INTRODUCTION

Modern lifestyles are characterized by easier access to food, however, reduced physical activity has contributed to various health challenges for individuals. These phenomena have led to increased incidences of degenerative and cardiovascular diseases (Booth et al., 2012; Godheshwar et al., 2023). Simultaneously, advancements in health research and improved access to health facilities have enhanced life expectancy. As every individual wants to live longer and healthier, this shifting trend creates a bigger demand for nutraceuticals to promote general well-being (Wickramasinghe et al., 2020). Many individuals choose to regularly consume nutraceuticals to maintain their health status as the

cost of healthcare access continues to rise (Puri et al., 2022). These nutraceuticals were used for preventing and managing disease symptoms including cough and cold, high cholesterol, arthritis, sleep disorders, diabetes, hypertension, dyslipidemia, and pain relief. Consequently, it is expected that the market size of nutraceuticals will reach \$340 billion by the end of 2024 (Chopra et al., 2022). Several studies have reported that individuals with hyperlipidemia exhibit elevated levels of oxidative stress biomarkers (Martinez-Hervas et al., 2008; Miri et al., 2012). Therefore, it is advantageous to develop nutraceuticals that have dual action, such as products that demonstrate strong antioxidant activity normalize, while also normalizing lipid profiles. Such

nutraceuticals are anticipated to capture significant market share, particularly among consumers who are obese. There are 4 classes of obesity based on body mass index (BMI) elevation degree. A person is classified to be overweight if the BMI value of 30 kg/m². Meanwhile, BMI of obese class I, II, and III are 30-35 kg/m², 35-40 kg/m², and above 40 kg/m² respectively (Busebee et al., 2023). An individual with BMI more than 30 kg/m² has a higher risk for a wide range of non-communicable diseases, including diabetes, cardiovascular diseases, and cancer (Hruby et al., 2016; Lin & Li, 2021; Valenzuela et al., 2023).

Microalgae are autotrophic organism that can grow within short time frame and produce more biomass per unit area than any other organism (Dolganyuk et al., 2020). Their ability to generate various bioactive substances that exhibited antioxidant, antibacterial, antitumor, antiviral and immunostimulant effect led to the great interest of the food and pharmaceutical industry (Gürlek et al., 2019). Microalgae also accumulate essential minerals from the environment that benefit health. This includes potassium, zinc, iodine, selenium, iron, manganese, copper, phosphorus, sodium, nitrogen, magnesium, cobalt, molybdenum, sulfur, and calcium which could enrich the microalgae-based nutraceuticals (Costa et al., 2024). Some secondary metabolites produced by microalgae are carotenoids, essential amino acids, omega-3, vitamins, fiber, polyunsaturated fatty acids, polysaccharides, enzymes, peptides, and toxins. etc (Paniagua-Michel, 2015).

Certain microalgae such as *Chlamydomonas*, *Phaeodactylum tricornutum*, *Scenedesmus*, *Synechococcus*, *Dunaliella*, *Haematococcus*, *Chlorella*, and *Porphyridium*, have been investigated as potential nutraceuticals for obesity and dyslipidemia (Arif et al., 2021; Banskota et al., 2016, 2019; Gómez-Zorita et al., 2019; Kim et al., 2016). Exposure of human adipose tissue stem cells to 20% *Euglena* extract has been shown to reduce triglyceride content by up to 75%. This reduction in lipid content was associated with a 23% decrease in peroxisome proliferator-activated receptor γ (PPAR γ) protein expression, which subsequently affects its downstream signaling pathways, including fatty acid binding protein (Ap2) and lipoprotein lipase (Lpl). Another study conducted on an animal model of obesity demonstrated that administration of *Spirulina maxima* at doses of 150-450 mg/kg body weight led to reductions in triglyceride, total cholesterol, and LDL- cholesterol

levels (Gómez-Zorita, et al. 2019). Similar to other studies that supplementation of *Euglena* and *Spirulina* also resulted in decreased the expression of PPAR γ and Ap2 (Sugimoto, et al. 2018; Seo, et al. 2018). Clinical studies involving obese individuals who received 2 gr/day of *S. maxima* over three months exhibited reductions in body mass index (BMI), waist circumference, serum triglyceride, LDL-cholesterol, glucose and insulin levels, and total antioxidant state compared to placebo group receiving microcrystalline cellulose (Szulinska et al. 2017). This suggested that microalgae can exert both anti-lipid and antioxidant activities (Šantek et al., 2019). Numerous studies have reported the antioxidant activities of microalgae, which are correlated with their polyphenol and carotenoid content (Assunção et al., 2017; Choochote et al., 2014; Goiris et al., 2012; Monteiro et al., 2020a; Vieira et al., 2021). As a result, the choice of extraction method and solvent plays a crucial role in isolating the active ingredient for the design of microalgae-based nutraceuticals with antioxidant and anti-lipase properties.

Our group has isolated the microalgae *Nostoc sp. MRB-1* from the peatlands of Borneo Island, specifically in Hanjalutung Oxbow Lake. Information regarding the pharmacological activities of this strain in relation to hyperlipidemia and obesity has yet to be thoroughly addressed. However, to further examine these pharmacological activities, it is essential to establish an appropriate extraction method to obtain the active pharmacological compounds. Thus, this study aims to develop the most effective extraction method using both maceration and ultrasonication and two solvents i.e. ethanol and hexane for *Nostoc sp. MRB-1* microalgae to obtain extract with the best antioxidant and anti-lipase activity. The novelty of this study is providing most comprehensive study about extraction of *Nostoc sp. MRB-1* microalgae, and relationship between extraction method towards its biological activities such as antioxidant and anti-lipase activities.

MATERIALS AND METHODS

Cultivation of microalgae

The cultivation of microalgae *Nostoc sp. MRB-1* was conducted following the protocol described by Hidayati et al. (2020) with slight modification. The *Nostoc sp. MRB-1* was isolated from peatlands in Oxbow Hanjalutung Lake, Indonesia. The AF-6 was used as a growth medium containing NaNO₃, NH₄NO₃, MgSO₄.7H₂O, KH₂PO₄, K₂HPO₄, CaCl₂.2H₂O, CaCO₃, Fe-citrate, citric acid,

trace elements, and vitamins. Pre-cultivation was carried out in a 100 mL sterilized Erlenmeyer flask placed on a shaker at 150 rpm with constant lighting (2000 lux). The optical density of microalgae cells is quantified at a wavelength of 660 nm using the Varioskan Lux multimode microplates reader over a period of seven days, with measurements taken every two days. Furthermore, microalgae culture was scaled up gradually from 1 L to 5 L under constant aeration and lighting for 14 days. At the time of harvest, the culture was collected through the process of filtration and the biomass was then freeze-dried for further analysis.

Morphological characteristics of the cells

The morphology of microalgae was examined using a microscope equipped with brightfield and differential interference contrast, following the method described by Hossain et al. (2019) with slight modification. The microalgae MRB-1 was identified using a comparison study of distinctive morphological features among the members of *Nostoc* genera such as thallus, cell color, filament, vegetative cells, heterocyst, and akinets from the references. Furthermore, the differences between *Nostoc* and *Anabaena* genera, which share similar morphologies, were analyzed (Jiménez et al., 2023). The morphological features were documented through photography at a magnification of 100x using an Olympus BX53 microscope connected to a digital camera system.

Extraction of microalgae

The samples were extracted using two methods, namely maceration and ultrasound, referred to (Ma et al., 2014; Monteiro et al., 2020b) with modification. In total, there were four groups: maceration with ethanol p.a. (Merck Ltd, Darmstadt, Germany), maceration with hexane p.a. (Merck Ltd, Darmstadt, Germany), ultrasonication with ethanol p.a. (Merck Ltd, Darmstadt, Germany), and ultrasonication with hexane p.a. (Merck Ltd, Darmstadt, Germany). The maceration was conducted by extracting *Nostoc* sp. MRB-1 with ethanol and hexane in a 1:10 ratio. The microalgae were then sonicated at 30°C for 15 min, followed by maceration at room temperature for 3 x 24 h. The microalgae extracts were subsequently centrifuged at 3500 rpm and 4°C for 15 min. The supernatant was collected and evaporated. For the ultrasound extraction method, either ethanol or hexane was

used as a solvent. Two hundred milligrams of *Nostoc* sp. MRB-1 were mixed with 20 mL of ethanol and placed in an ultrasonic device (Biostellar, China) for extraction extraction at a power rating of 360 W for 10 min. The crude extract was obtained by collecting the supernatant and evaporating the solvent. A similar method was employed for the hexane ultrasound group.

Scanning electron microscopic (SEM) observation of ruptured microalgae cells

The morphology of microalgae cells was evaluated according to (Yap et al., 2014) with modifications. The morphological surfaces of microalgae cells before and after extraction were examined using a Scanning Electron Microscope (SEM) (SU-3500, Hitachi). The samples were then mounted on stubs and coated with gold at 30 mA for 60 s using a sputter coater. The SEM was operated at an acceleration voltage of 3 kV with a magnification of 5000.

Measurement of Fourier Transfer Infrared (FTIR) Spectroscopy

The vibration of functional groups on the extracts was measured using the Attenuated Total Reflectance (ATR) FTIR technique (Bruker Vertex 80) following (Arif et al., 2021). The extracts were placed on the ATR crystal and then measured in the infrared region (4000-300 cm⁻¹), with a resolution of 6 cm⁻¹, and the number of scans was 32. Molecule identification of the vibrations of the functional groups was analyzed using OMNIC software from Thermo Fisher Scientific.

Evaluation of total phenolic content

The total phenolic content (TPC) of the extract was determined using the Follin-Ciocalteu method (Darsih, et al., 2024). To a 96-well microplate containing the extract (10µL), 10 µL of the Follin-Ciocalteu reagent was added. Furthermore, the mixture was added with distilled water and 20% Na₂CO₃ (Merck Ltd, Darmstadt, Germany). The mixture was then incubated in a dark room for 2 h. The details of the method followed (Ayele et al., 2022) with slight modifications. A microplate reader (Multiskan™, Thermo Scientific) was used to determine the absorbance of the samples at 765 nm. The TPC was expressed as milligrams of gallic acid (Sigma Aldrich, Switzerland) equivalent per gram of extract (mg GAE/g).

Scavenging activity of DPPH radicals

The scavenging activity against (HiMedia, India) radicals was assessed following (Al-Rifai et al., 2017) with slight modification. Twenty microliters of 1.01 mM DPPH was added to the 96-well plates containing the sample. The mixture was incubated for 30 min in the dark. A microplate reader was then used to measure the absorbance of the mixture at 517 nm. The ascorbic acid p.a. (HiMedia, India) was used a positive control. The scavenging activity of the sample against DPPH radicals was calculated using equation (1).

$$\%inhibition = \frac{(A_{control} - A_{sample})}{A_{control}} \times 100 \dots\dots (1)$$

The lipase pancreatic inhibitory activity

The lipase pancreatic inhibitory activity of the extract was evaluated following (Wiyono et al., 2022) with modification. Around 10 µL of extract (0.19 µg/mL) was added with 10 µL of pancreatic enzyme (1 mg/mL in phosphate buffer saline, pH 6.8) and incubated for 5 min at 37°C. Furthermore, the solution was added with 240 µL of substrate p-nitrophenyl butyrate (0.165 mM in PBS). The absorbance was measured immediately using a microplate spectrophotometer at 415 nm at 0 min and after 35 min. Orlistat (Sigma Aldrich) was used as a positive control with a concentration of 46 µg/mL. Various concentrations of nitrophenol were assessed to create a standard curve.

Statistical Analysis

Total polyphenol contents (TPC), antioxidant and anti-lipase activities were conducted in triplicate (n=3). Statistical analysis was performed using SPSS for one-way analysis of variance (ANOVA) followed by post hoc Duncan's test. Differences were considered to be significant at p<0.05.

RESULTS AND DISCUSSION

The growth rate of microalgae MRB-1

The findings indicated significant increase in the growth of *Nostoc sp. MRB-1* microalgae cells from day 1 to day 7. The growth of the microalgae exhibited an exponential phase (Figure 1). The data indicate that algae growth reached exponential phase with the highest growth observed at OD (660 nm) = 0.24. The biomass was collected 14 days after cultivation, resulting in a yield of 1.76 grams per liter. This growth was influenced by the availability of nutrients and various environmental conditions. The growth of microalgae cells is influenced by various parameters, including temperature, light

intensity, pH level, nutri availability, salinity, and oxygen concentration (Chowdury et al., 2020; Darvehei et al., 2018; Josephine et al., 2022).

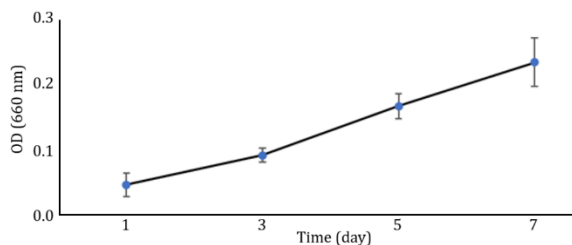


Figure 1. Growth rate of algae over a 7-day period under light intensity 2000 lux using AF-6 freshwater media. The graph illustrates the optical density (OD) of algae from triplo in double experiment, recorded at each two-day interval.

Morphology structure of microalgae MRB-1 cells

The microalgae MRB-1 cells are characterized by their spherical, oval, or barrel-shaped morphology, and are extremely small, measuring between 3.0 and 6.7 µm in size (Figure 2). The cells arranged linearly, forming elongated, unbranched filaments that closely resemble the filaments of *Anabaena*. However, *Nostoc* filaments are enveloped by unique, rigid, and often elastic mucilage, creating colonies that appear spherical, egg-shaped, or irregular. In addition to vegetative cells, the filaments contain heterocytes or heterocyst. Based on these observations, it is proposed that the microalgae MRB-1 be classified as *Nostoc Sp.*

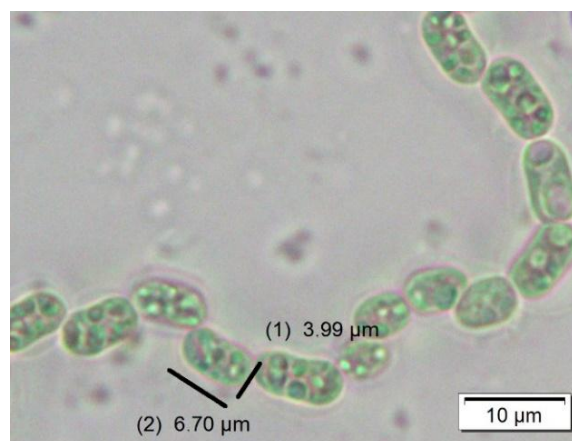


Figure 2. Microscopic overview of the microalgae *Nostoc sp. MRB-1* with 1000x magnification. The diameter of the cell is approximately 3- 6 µm. Scale bar: 10 µm

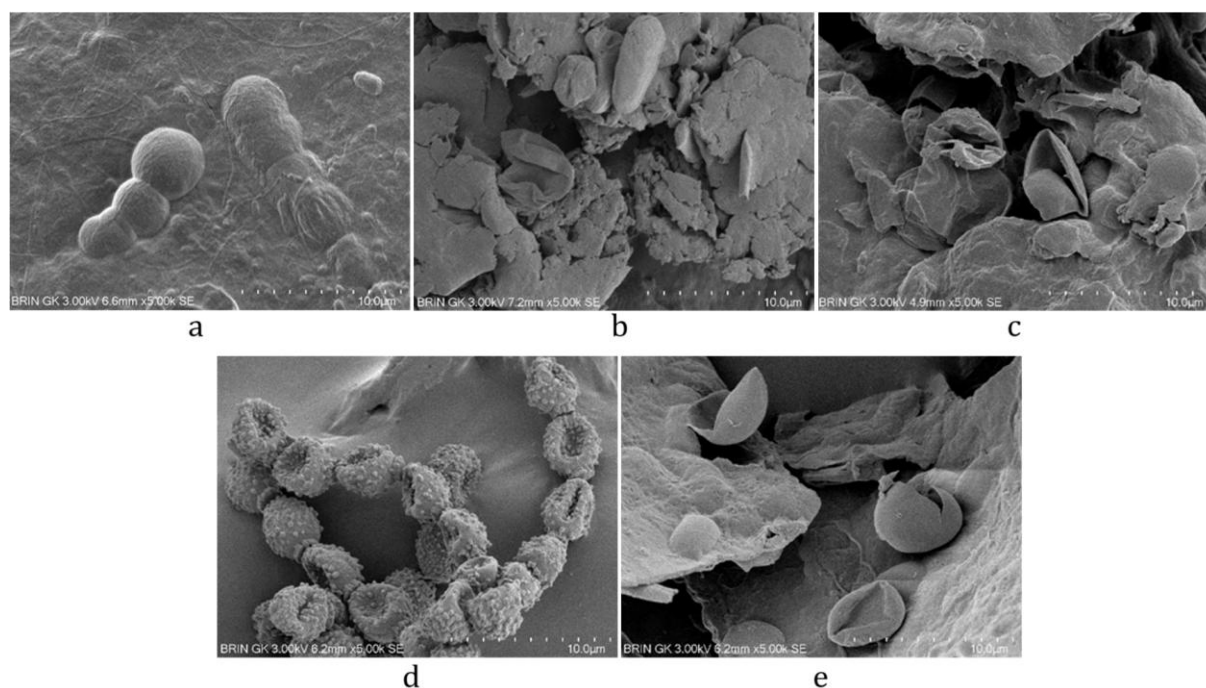


Figure 3. SEM of the microalga cell surfaces: (a) before extraction; (b) after maceration using EtOH; (c) after ultrasound using EtOH; (d) after maceration using hexane; (e) after ultrasound using hexane

Table I. The yield of extract from different extraction methods

Method	Solvent	Yield (%)
Maceration	Hexane	5.21
	EtOH	3.97
Ultrasound	Hexane	2.71
	EtOH	3.66

The cell morphology of the microalgae before and after treatments was examined using scanning electron microscopy (SEM) (Figure 3). The results indicated that the maceration and ultrasonication methods using different solvents alter the structure of microalgae MRB-1. Figure 3 illustrates the cell morphology after maceration and ultrasonication using hexane and ethanol solvents, showing the rupture of the cells due to external forces and solvents, which resulted in irregular shapes, compared to the oval microalgae cells before treatment. Maceration with hexane partially ruptured the cell walls of the microalgae, evidenced by some spots on the cell's surface. Maceration using EtOH, hexane, and ultrasound using hexane demonstrate shrinkage and significant creasing. These results align with previous studies (Eldalatony et al., 2016; Liu et al., 2022; Ma et al., 2014). The SEM confirms that

pretreatment using ultrasound and maceration with ethanol resulted in considerable cell destruction. Table 1 showed that extraction using the maceration method with hexane obtained the highest crude extract yield compared to other methods with value of 5.21%.

The physical and mechanical factors, such as strong shock waves, shear forces, localized high temperatures, microbubble resonance, and pressure, contribute to the ultrasonic disruption (Liu et al., 2022). The ruptured cells impact the extraction process of the metabolite compounds contained in the microalgae.

IR spectroscopy of *Nostoc* sp. MRB-1 cells powder after treatment

The FTIR analysis represents the composition and molecular functional groups of compounds present in the microalgae MRB-1. The

presence of organic compound groups in microalgae MRB-1 was reflected in the FTIR absorbance (Figure 4). According to the data (Figure 4), the FTIR spectrum displays a broad, strong absorption band at 3273 cm^{-1} , indicating stretching vibrations of the hydroxyl group (-OH stretching). This absorbance can be attributed to alcohol and acids (Dalal et al., 2021). The symmetric and asymmetric absorption of aliphatic (C-H) CH_2 , aromatic and or vinyl C-H absorption, and C-H anomer were identified at around $2900\text{--}2800\text{ cm}^{-1}$, suggesting the presence of hydrocarbons from lipids. Meanwhile the band observed between $1635\text{ to }1541\text{ cm}^{-1}$ was due to carbonyl (COO-) stretching vibrations from lipid's esters. These findings were consistent with previous studies (Dalal et al., 2021; Sudhakar & Premalatha, 2015). The absorbance at 1040 cm^{-1} indicates the presence of C-O-C polysaccharides from carbohydrates. The band at 526 cm^{-1} was the suggested number of hydrocarbons. This result was similar to Manjunatha & Girisha (2020). The presence of lipid groups, alcoholic groups, and carboxyl groups was identified using the FTIR analysis of microalgae MRB-1. Additionally, the results indicated a decrease in absorbance intensity at various wave numbers after treatment using different extraction methods and solvents. It can be concluded that extraction methods employing various solvents can effectively extract the compounds present in the microalgae cells.

Effect of extraction method and solvents on total phenolic contents (TPC)

One type of secondary metabolite that contributes to treating oxidative stress is phenolic compounds. The microalgae have been reported to contain these compounds and exhibit antioxidant activities (Minhas et al., 2016; Monteiro et al., 2020). The common analysis of TPC in the microalgae extract was the Follin-Ciocalteu assay (Monteiro et al., 2020). This method is straightforward and effective for quantitatively identifying these metabolites in the extract (Figure 5a).

The results demonstrated varying concentrations of TPC in different solvent and extraction methods, with values ranging from 2.28 ± 0.22 to 11.77 ± 1.47 mg GAE/g dry extract. The highest TPC was obtained when the microalgae MRB-1 was extracted using ethanol (EtOH) by the maceration method, resulting in a value of 11.77 ± 1.47 mg GAE /g dry extract. The ability of ethanol maceration to extract phenolic compounds

is 3 to 4 times greater than that of the other methods examined. Interestingly, studies have shown that even when using a similar solvent such as ethanol, ultrasound is less effective in extracting phenolic compounds. A similar trend regarding the inefficiency of ultrasound in microalgae extraction was observed with the hexane series, where ultrasound with hexane yielded a lower total phenolic content compared to the maceration method. Current studies confirm that hexane is not the optimal solvent for extracting phenolic compounds. This is because closely matched similar solvent solubility solvent solubility parameters are necessary to enhance the solubilizing capacity (Laksitorini et al., 2023). The maceration method, when preceded by water bath sonication, significantly ruptures the microalgae cell walls, allowing solvents to penetrate and extract the metabolite compounds, especially phenolic compounds. The rupture of cell walls is supported by the morphology of the cells observed in the SEM analysis (Figure 4). Ethanol, being a solvent with higher polarity is more effective at extracting the phenolic compounds present in microalgae. In contrast, hexane is a non-polar solvent that generally extracts non-polar compounds, such as lipids from microalgae. A previous study reported that biphasic solvents, such as hexane/water, selectively recovered non-polar triacylglycerides (Yap et al., 2014).

Effect of extraction method and solvents on the antioxidant activity

In this study, the antioxidant activity of the microalgae MRB-1 extract was evaluated using DPPH assays (Figure 5b). The results indicated variations in antioxidant activity among the extracts, with values ranging from 6.99 ± 1.66 to $39.68 \pm 0.07\%$ at a concentration of 0.8 mg/mL of dry extract. Among all the extracts, the highest DPPH scavenging activity was observed when the microalgae MRB-1 were extracted using the maceration method with hexane as the solvent, yielding an %inhibition value of 39.68 ± 0.07 at 0.8 mg/mL . In contrast, extracts obtained through both maceration and ultrasound methods using ethanol exhibited lower antioxidant activity. The results of this study demonstrated that the antioxidant activity of the extract did not correlate with the phenolic content, which with previous findings (Monteiro et al., 2020). Furthermore, it is suggested that other compounds, such as lipids, may contribute to the antioxidant activity of the *Nostoc sp. MRB-1* extract.

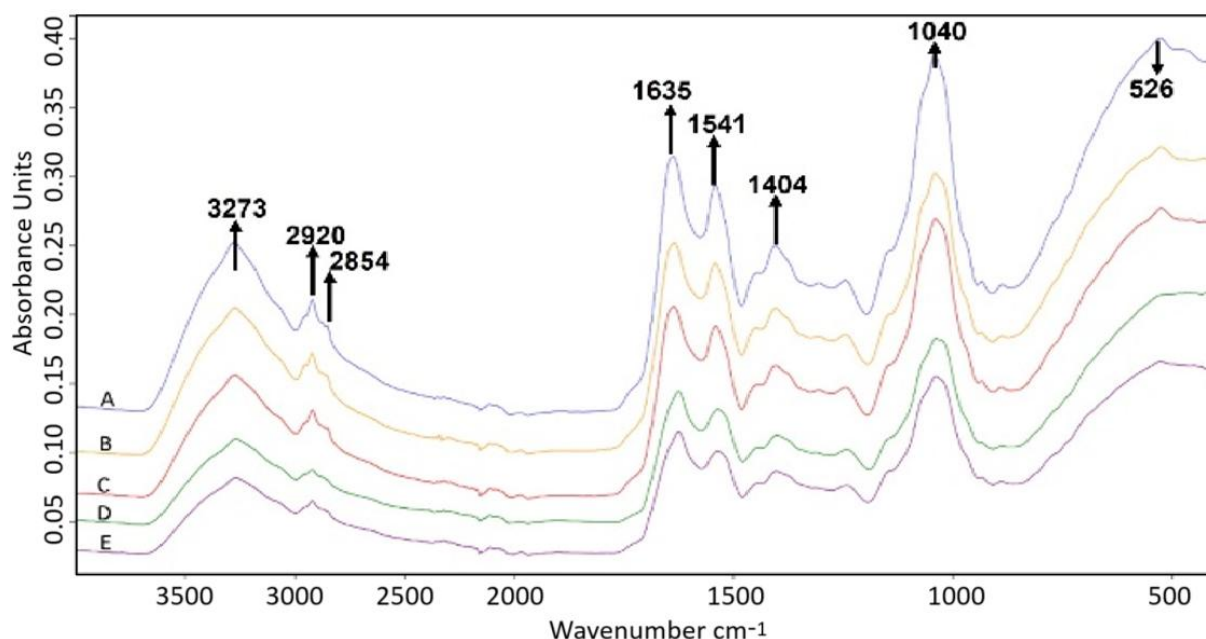


Figure 4. FTIR spectra of *Nostoc* sp. MRB-1 (A= before extraction; B = after maceration using EtOH; C= after ultrasound using EtOH; D= after maceration using hexane; E= after ultrasound using hexane)

Banskota et al. reported a correlation between lipid content and Oxygen Radical Absorbance Capacity (ORAC) values (Banskota et al., 2019).

Effect of extraction method and solvents on the lipase inhibitory activity

The effect of the extraction methods and solvents on lipase inhibitory activity revealed that the maceration method using ethanol (EtOH) exhibited the highest activity, with a value of $23.01\% \pm 1.66$ at a concentration of 0.38 mg/mL . In comparison, orlistat, a well-known lipase inhibitor used as a positive control, demonstrated a lipase inhibitory activity of $72.57\% \pm 3.57$ at $46 \text{ }\mu\text{g/mL}$ (Table I).

However, both the for both maceration and ultrasound methods using hexane as a solvent. revealed no detectable lipase inhibitory activity. It is important to note that the extraction method and type of solvent employed significantly influence the metabolites obtained. In this study, the observed lipase inhibition activity may be attributed to the presence of phenolic compounds. These phenolic compounds could inhibit the activity of pancreatic lipase enzymes by forming binding interactions with the enzyme's active sites through hydrogen bonds and π -stacking hydrophobic interactions (Martinez-Gonzalez et al., 2017). Previous studies have reported that *Nostoc commune* exhibited a

hypolipidemic effect (Ku et al., 2015; Tsai et al., 2022).

CONCLUSION

Evaluating different extraction methods with different solvents on *Nostoc* sp. MRB-1 revealed that maceration using hexane resulting in good antioxidant activity of the extract, while maceration using ethanol resulting extract with good anti-lipase activity. However, further study should be conducted on the phytochemical composition of *Nostoc* sp. MRB-1, the mechanism of active biological properties, and in vivo study.

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CONFLICT OF INTEREST

The authors declare that they have no known competing financial interests or personal relationships that could be perceived to influence the work reported in this paper.

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