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Identification of the Characteristics of Phytochemical Compounds in Microalgae Extracts

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A B S T R A C T

Microalgae are a source of bioactive compounds with potential as functional food ingredients. The effectiveness of bioactive compound extraction depends on the extraction method and solvent type. This study aimed to determine the effects of microalgal species (*Spirulina sp.*, *Chlorella sp.*, and *Nannochloropsis sp.*) and solvent type (ethanol, methanol, and acetone) on extract yield and phytochemical profiles using the Ultrasound-Microwave Assisted Extraction (UMAE) method. A factorial Completely Randomized Design (CRD) was employed with two factors, namely microalgal species and solvent type, with two replications. Phytochemical screening showed that *Spirulina sp.* extracts contained alkaloids, phenols, flavonoids, saponins, tannins, and terpenoids in all solvents, but steroids were not detected. *Chlorella sp.* showed a similar profile, except that tannins were not detected in the acetone extract. *Nannochloropsis sp.* showed variations in its phytochemical profile, with flavonoids and tannins detected only in the methanol extract. These results indicate that microalgal species and solvent type influence the yield and phytochemical profiles of extracts obtained using the UMAE method.

Contribution to Sustainable Development Goals (SDGs):

SDG 8: Decent Work and Economic Growth

SDG 9: Industry, Innovation and Infrastructure

SDG 12: Responsible Consumption and Production

SDG 14: Life Below Water

1. INTRODUCTION

1.1. Research Background

Microalgae are microscopic organisms commonly found in both freshwater and marine environments. These organisms lack structures such as roots, stems, or leaves but can photosynthesize to produce the nutrients they need independently [19]. Research on microalgae has not yet advanced to the same extent as that on seaweed. The advantages of microalgae—such as higher growth rates, greater photosynthetic efficiency, and the ability to be cultivated indoors—could create significant opportunities to explore their applications in the food and health industries [20]. Furthermore, Indonesia's territory is predominantly composed of

marine waters, which account for approximately 75% of the total area, while land accounts for only about 25%. However, this vast marine potential has not yet been fully managed or utilized.

Cyanobacteria (blue-green algae) are prokaryotic organisms that generally grow in lakes and high-salinity environments; one example is *Spirulina sp.* [18]. Meanwhile, eukaryotic microalgae are dominated by *Chlorophyta* (green algae), with most species being unicellular or filamentous and living in freshwater. Two types of green microalgae widely used in commercial applications are *Chlorella sp.* and *Nannochloropsis sp.* [20].

Extraction is the initial step for separating the desired chemical components from the sample matrix [23]. The extraction process occurs when a solvent penetrates the microalgal biomass to dissolve the desired compounds, which are then transported out of the cells via diffusion; thus, success



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depends on the selection of an appropriate solvent and an efficient extraction method [12]. A solvent's ability to extract bioactive compounds from natural materials is influenced by differences in the polarity of the solvent used. Each solvent type tends to dissolve specific compounds, resulting in different extract compositions. Variations in the bioactive compounds extracted lead to diverse characteristics and functional potentials of the extracts.

Non-conventional methods such as Microwave-Assisted Extraction (MAE) and Ultrasound-Assisted Extraction (UAE) operate more quickly because they use wave energy to accelerate diffusion and disrupt cellular structures without generating excessive heat [17]. One of the modern extraction techniques widely used today is Ultrasound-Microwave-Assisted Extraction (UMAE), which can accelerate the extraction process and achieve high yields using only a small amount of solvent. Ultrasound-Microwave-Assisted Extraction (UMAE) is a technique that combines ultrasonic and microwave extraction.

This study focused on qualitative phytochemical screening as an initial approach to identify the presence or absence of secondary metabolites in microalgal extracts. Qualitative screening provides preliminary information regarding the phytochemical profile of each extract, allowing comparison of different microalgal species and solvent types before conducting more detailed quantitative analyses. Therefore, quantitative determination of individual compounds was beyond the scope of the present study.

1.2. Literature Review

Microalgae are photosynthetic, single-celled organisms that exhibit high diversity and are found in various aquatic habitats. Although microalgae are small in size, they possess remarkable biosynthetic capacity, enabling them to produce a variety of compounds, such as proteins, polyunsaturated fatty acids, pigments, vitamins, and polysaccharides [12]. Microalgae are a diverse group of microorganisms, with an estimated 200,000 species [21]. Based on pigment color, phytoplankton algae are classified into several types, namely *Chlorophyta* (green algae), *Chrysophyta* (golden algae), *Pyrrhophyta* (fire algae), *Euglenophyta*, and *Cyanophyta/Cyanobacteria* (blue-green algae) [17].

Spirulina sp. is a blue-green autotrophic microalga with columnar cells arranged in twisted, spiral-like filaments, and is therefore classified as a filamentous blue-green alga (*cyanobacteria*). *Spirulina sp.* contains a very high amount of protein, reaching about 60–70% [1]. *Chlorella sp.* is a unicellular green microalga that cannot move and can live either solitary or in colonies. *Chlorella sp.* is a unicellular green microalga rich in vitamins, minerals, and protein, and is frequently used as a natural feed for various marine organisms [6]. *Nannochloropsis sp.* is a non-motile unicellular microalga commonly found in both marine and freshwater environments [11].

Ultrasound-Assisted Extraction (UAE) is an extraction technique that utilizes high-frequency sound waves (20–100 MHz) to aid in the extraction of compounds [17]. This principle works by producing spontaneous bubbles (cavitation) in the liquid phase at temperatures below its boiling point. This cavitation then causes structural damage to the material's cell walls, allowing the solvent to penetrate and dissolve the chemical components within [8]. Microwave-Assisted Extraction (MAE) is a non-contact heating method capable of minimizing temperature

differences and accelerating energy transfer via electromagnetic waves; heat from microwave radiation causes water trapped within the sample cells to evaporate, resulting in a drastic increase in pressure inside the cell walls. This internal pressure forces the cell walls to stretch, swell, and eventually rupture. This physical disruption of plant cells facilitates and accelerates the release of target compounds from the matrix into the solvent [7]. Ultrasound-Microwave Assisted Extraction (UMAE) is an extraction method that combines ultrasonic and microwave technologies to separate natural compounds from plants. This method offers numerous advantages, such as reducing extraction time, lowering costs, and improving process efficiency.

Extraction using ethanol is considered effective because of its properties as a neutral solvent with a wide solubility range, enabling it to dissolve a variety of compounds, including both hydrophilic compounds that are soluble in air and lipophilic compounds such as fats. Methanol has a higher degree of polarity than ethanol, enabling it to form stronger hydrogen bonds—both as a hydrogen donor and acceptor—in molecular interactions. Meanwhile, acetone is more effective at dissolving lipophilic components than polar compounds, making it highly suitable for extracting compounds such as vegetable oils and carotenoids from plant materials [10].

1.3. Research Objective

This study aims to determine the effects of various microalgal species (*Spirulina sp.*, *Chlorella sp.*, and *Nannochloropsis sp.*) and solvents (ethanol, methanol, and acetone) on the phytochemical characteristics of extracts obtained by Ultrasound Microwave-Assisted Extraction (UMAE).

2. MATERIALS AND METHODS

2.1. Materials

The materials used in this study were *Spirulina sp.*, *Chlorella sp.*, and *Nannochloropsis sp.* in powder form, obtained from BPBAP Situbondo and BPBAP Jepara. The solvents used for extraction were 70% ethanol (Merck), 70% methanol (Merck), and 70% acetone (Merck). The analytical reagents were ethanol (Merck), methanol (Merck), distilled water, sodium carbonate (Merck), aluminum chloride (Merck), amyl alcohol, potassium acetate (Merck), sulfuric acid (Merck), hydrochloric acid (Merck), ammonia, HCl (Merck), magnesium powder (Merck), and the alkaloid reagents Dragendorff (Merck), Meyer (Merck), and Wagner (Merck).

2.2. Research Procedures

This study used a completely randomized design (CRD) with two treatment factors: microalgae species (*Spirulina sp.*, *Chlorella sp.*, and *Nannochloropsis sp.*) and solvent types (ethanol, methanol, and acetone). Each treatment was replicated twice. The variables observed included the qualitative identification of alkaloids, flavonoids, phenols, tannins, saponins, terpenoids, and steroids in the microalgal extracts. The results of the phytochemical screening were analyzed descriptively based on the presence (+) or absence (–) of secondary metabolites.

2.3. Microalgae Extraction

The microalgae powder was first sieved using an 80-mesh sieve. Five grams of the microalgae powder were weighed on an

analytical balance and placed in a 500-mL Erlenmeyer flask. Solvent was added at a solid-to-solvent ratio of 1:10 (w/v); thus, 50 mL of solvent was used for the 5-gram sample. The Erlenmeyer flask was then sealed with aluminum foil. The extraction process was performed using the Ultrasound Microwave-Assisted Extraction (UMAE) method. The first stage involved extraction with a sonicator (GT Sonic) at 40 kHz for 15 minutes at a controlled temperature of 40°C. Afterward, the sample was extracted using a microwave (Samsung MS23K3515AS/SE) at 300 watts for 5 minutes. The extract was then filtered to separate the filtrate. The extract was separated from the solvent and evaporated using a rotary evaporator at 40°C and 100 mbar to obtain the microalgal extract. The resulting microalgal extract was weighed and subsequently analyzed according to the specified analytical parameters.

2.4. Phytochemical Qualitative Tests

2.4.1. Qualitative Test for Alkaloids

50 mg sample was dissolved in two drops of 2 N sulfuric acid (H₂SO₄) solution. The solution was then applied to a test plate and reacted separately with Dragendorff's, Mayer's, and Wagner's reagents to detect the presence of alkaloids. The test results are considered positive if a red-to-orange precipitate forms with the Dragendorff reagent, a yellowish-white precipitate with the Mayer reagent, and a brown precipitate with the Wagner reagent [6].

2.4.2. Qualitative Test for Flavonoids

A 50 mg sample was mixed with 0.05 mg of magnesium powder, then 0.2 mL of amyl alcohol (37% HCl in 95% ethanol, 1:1, v/v) and 4 mL of 70% ethanol were added. The solution was then examined on the amyl alcohol layer. The test result was considered positive if a red, yellow, or orange color formed on that layer [5].

2.4.3. Qualitative Test for Phenols

A 50-mg sample was dissolved in 0.25 mL of 70% ethanol, and then two drops of a 5% FeCl₃ solution were added. The presence of phenolic compounds is indicated by the formation of a green to bluish-green color in the solution [5].

2.4.4. Qualitative Test for Tannins

A 50-mg sample was mixed with 20 mL of hot water, then filtered to obtain the filtrate. Two drops of a 1% FeCl₃ solution were added to the filtrate. The test result was considered positive if the solution turned blue or blackish-green, indicating the presence of tannins [5].

2.4.5. Qualitative Test for Saponins

A 50-mg sample was placed in a test tube, then 20 mL of hot water was added, and the mixture was shaken until foam formed. The test result was considered positive if the foam remained stable for 30 minutes and did not disappear after the addition of one drop of 2 N HCl solution [5].

2.4.6. Qualitative Test for Steroids and Triterpenoids

A 50 mg sample was mixed with 2 mL of chloroform and placed in a test tube. Next, five drops of acetic anhydride and three drops of concentrated sulfuric acid (H₂SO₄) were added. The test result is considered positive if the solution first turns brownish-red and

then turns blue or green. A blue color change indicates the presence of steroid compounds, while a brownish-red color indicates the presence of triterpenoids [5].

3. RESULT AND DISCUSSION

3.1.1. Phytochemical Compounds in Microalgae Extracts

Microalgae are microscopic photosynthetic organisms that serve as the "biological factories" of the future, thanks to their ability to produce a wide range of bioactive compounds with complex structures and high functionality. As a first step toward identifying the phytochemical compounds present in the extract, this study conducted a phytochemical screening of the microalgae extract. Based on the observational data, the profile of active compounds contained in the microalgae extract is presented in detail in Table 1.

Table 1. Results of Phytochemical Screening of Microalgae Extracts

Microalgae	Solvent	Alkaloid	Phenol	Flavonoid	Saponin	Tannin	Steroid	Terpenoid
<i>Spirulina sp.</i>	Ethanol	+	+	+	+	+	-	+
	Methanol	+	+	+	+	+	-	+
	Acetone	+	+	+	+	+	-	+
<i>Chlorella sp.</i>	Ethanol	+	+	+	+	+	-	+
	Methanol	+	+	+	+	+	-	+
	Acetone	+	+	+	+	-	-	+
<i>Nannochloropsis sp.</i>	Ethanol	+	+	-	-	-	+	-
	Methanol	+	+	+	-	+	+	-
	Acetone	+	+	-	-	-	+	-

Description: (-) : does not contain the tested compound
(+) : contains the tested compound

The differences in phytochemical screening results among the three microalgal species indicate that each species possesses distinct phytochemical characteristics. These variations are primarily influenced by differences in phylogeny, secondary metabolite biosynthetic pathways, and each species' ability to produce and accumulate bioactive compounds. This is consistent with [15], which states that secondary metabolite production in microalgae is highly dependent on species and on various environmental factors that regulate their biosynthetic pathways.

In this study, phytochemical screening was conducted on microalgal extracts obtained using the Ultrasound-Microwave Assisted Extraction (UMAE) method. Ultrasound-Microwave Assisted Extraction (UMAE) is an extraction method that combines ultrasonic and microwave technologies to isolate natural compounds from plants. This method offers numerous advantages, such as shortening extraction time and improving process efficiency. Furthermore, the synergy between ultrasonic cavitation and microwave heating enables more effective disruption of cell walls, thereby optimizing the release of target compounds.

In this study, *Spirulina sp.* was extracted using three solvents: ethanol, methanol, and acetone. The results for all extract types obtained using different solvents showed the same phytochemical screening profile: they were positive for alkaloids, phenols, flavonoids, saponins, tannins, and terpenoids, but negative for steroids. These findings are consistent with the results of a study [3] that conducted a phytochemical screening of methanolic extracts of *Spirulina sp.* and detected the presence of saponins, tannins, and alkaloids, while also quantitatively confirming the presence of flavonoids through total flavonoid testing. However, there was a discrepancy regarding the presence of terpenoids,

which were not detected in that study. This discrepancy is most likely due to variations in the *Spirulina* sp. strains used, differences in cultivation conditions (including pH, salinity, temperature, and light intensity), and the extraction techniques employed. Additionally, the extremely low concentration of terpenoids in the samples may have been below the screening method's detection limit, rendering them undetectable in the qualitative analysis.

The presence of alkaloids, phenols, flavonoids, saponins, tannins, and terpenoids in *Spirulina* sp. is closely related to the microalgae's physiological adaptation mechanisms to the environment. *Spirulina* sp. naturally synthesises secondary metabolites as defence compounds against oxidative stress caused by high light exposure, ultraviolet radiation, changes in temperature and salinity, and nutrient limitations. Phenolic compounds and flavonoids function as antioxidants capable of scavenging free radicals, thereby protecting cells from oxidative damage, while terpenoids act as cell membrane-protective molecules and antioxidants. Alkaloids serve as defence compounds against microorganisms and predators, whereas saponins and tannins possess antimicrobial activity and help protect cells from environmental stress. According to [9], *Spirulina* is a microalga rich in phytonutrients and secondary metabolites, owing to a metabolic system that produces various bioactive compounds as a means of adaptation to extreme environments.

The results of the phytochemical screening of *Chlorella* sp. extracts showed that the use of ethanol and methanol as solvents yielded similar phytochemical profiles, namely positive for alkaloids, phenols, flavonoids, saponins, tannins, and terpenoids, while steroids were not detected. Meanwhile, results obtained using acetone as a solvent were nearly identical, but tannins were not detected. This indicates that the type of solvent influences the extraction efficiency of certain classes of secondary metabolites, particularly tannins. Furthermore, [2] explains that a solvent's effectiveness is influenced by its polarity; thus, although all three solvents can extract most secondary metabolites from *Chlorella* sp., the amount extracted may vary. Other factors, such as microalgal strain, culture medium, harvest age, light intensity, solvent, and extraction method, also play a role.

The difference in results for the acetone treatment, namely the absence of detectable tannins, is believed to be due to differences in the solvents' ability to dissolve these compounds. Tannins are polyphenolic compounds with high polarity, making them more soluble in polar solvents such as methanol and ethanol than in acetone. The results of the tannin test on the *Chlorella* sp. microalgae extract were indicated by a color change to blackish green. This color change whether to blackish green or blackish blue occurs due to a reaction between tannins and Fe^{3+} ions, which form a complex after the addition of FeCl_3 solution [14]. However, in the case of *Chlorella* treated with acetone, no blackish green or blackish blue color change occurred; instead, the solution turned dark green.

Phytochemical screening of *Nannochloropsis* sp. revealed differences in secondary metabolite profiles depending on the type of solvent used. In ethanol and acetone, the extracts contained only alkaloids, phenols, and steroids, while flavonoids, saponins, tannins, and terpenoids were not detected. In contrast, methanol not only extracted alkaloids and steroids but also flavonoids and tannins. These results indicate that methanol has better extraction capacity for polar compounds than ethanol or

acetone in *Nannochloropsis* sp. According to [16], *Nannochloropsis* sp. is indeed a rich source of phenolic compounds, including phenolic acids and flavonoids, such as gallic acid and ferulic acid—as well as other secondary metabolites; however, their composition and quantities are significantly influenced by the species, cultivation conditions, and the solvent used. The presence of steroid compounds in *Nannochloropsis* sp. is thought to be related to the composition of its membrane lipids, which naturally contain various types of phytosterols.

The presence of flavonoids and tannins only in the methanol extract indicates that methanol is more effective at dissolving polar compounds. This is consistent with the principle of extraction: a solvent's effectiveness depends on its polarity. Furthermore, the failure to detect flavonoids and tannins in both the ethanol and acetone extracts is likely due to their relatively low concentrations, which were below the detection limit of the phytochemical screening method.

Compounds not detected in qualitative phytochemical screening do not necessarily indicate complete absence from the sample; they may be present at concentrations below the method's detection limit. High-performance chromatography techniques, such as High-Performance Liquid Chromatography (HPLC) and Liquid Chromatography–Mass Spectrometry (LC-MS), are more sensitive methods for identifying and quantifying secondary metabolites [4]. Furthermore, the predominance of extracted pigments is suspected to cause a masking effect on color changes which serve as positive indicators thereby obscuring the presence of certain compounds and potentially introducing bias in the interpretation of screening results. Therefore, further research is recommended using these methods to confirm the presence of compounds that were not detected in this study.

Based on the results of the phytochemical screening of microalgal extracts using the Ultrasound Microwave-Assisted Extraction (UMAE) method, it can be concluded that the type of microalgae and the extraction solvent influence the profile of detected secondary metabolites. *Spirulina* sp. exhibited the most consistent diversity of bioactive compounds across all types of solvents, whereas *Chlorella* sp. and *Nannochloropsis* sp. showed results that varied more significantly depending on the polarity characteristics of the solvents. The ability of UMAE to enhance cell wall disruption by combining ultrasonic cavitation and microwave heating is believed to increase the release of bioactive compounds from the microalgal cell matrix. The differences in the secondary metabolite profiles observed indicate that extraction efficiency is determined not only by the extraction method but also by the characteristics of the microalgal species and the solvent's suitability for extracting the target compounds.

4. CONCLUSION

The type of microalgae and the type of solvent influence the yield and phytochemical profile of the extracts produced using the Ultrasound-Microwave Assisted Extraction (UMAE) method. Phytochemical screening results showed that *Spirulina* sp. extracts consistently contained alkaloids, phenols, flavonoids, saponins, tannins, and terpenoids in all solvents, but did not contain steroids. *Chlorella* sp. extracts exhibited a similar phytochemical profile, except that tannins were not detected in the acetone extract. Meanwhile, *Nannochloropsis* sp. extracts

showed variations in secondary metabolite content, with flavonoids and tannins detected only in the methanol extract.

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