ANTIOXIDANT ACTIVITY OF SEQUENTIALLY EXTRACTED *Haematococcus pluvialis* USING MULTIPLE IN VITRO ASSAYS

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Abstract

Oxidative stress causes cell damage and various chronic conditions; therefore, natural antioxidants, especially from microalgae, are needed. This study evaluated the antioxidant properties of *Haematococcus pluvialis* extracts obtained by sequential solvent extraction (n-hexane, ethyl acetate, and methanol) using ultrasonic-assisted maceration. The extracts were characterized for yield, total phenolic content, total flavonoid content, and carotenoid content, and their antioxidant activities were assessed using four in vitro assays (DPPH, ABTS, FRAP, and H_2O_2 scavenging). Sequential extraction yielded cumulative recoveries of 56.13% (30.13% n-hexane, 14.93% ethyl acetate, and 11.07% methanol), exceeding single-solvent methanol extraction (47.13%). The ethyl acetate fraction exhibited the highest phenolic (27.87±0.92 mg GAE/g) and flavonoid (5.96±0.25 mg QE/g) content and contained the highest pigment concentrations (astaxanthin 4,124.25±60.94 µg/mL; total carotenoids 4,686.65±69.25 µg/mL). Consistently, the ethyl acetate extract exhibited the strongest antioxi-dant activity across all assays (DPPH IC_{50}=46.71 µg/mL; ABTS IC_{50}=35.85 µg/mL; FRAP IC_{50}=23.94 µg/mL; H_2O_2 IC_{50}=54.64 µg/mL), outperforming the other sequential fractions and the single-solvent methanol extract. These results indicate that semi-polar extraction with ethyl acetate effectively concentrates phenolic, flavonoid, and pigment antioxidants from *H. pluvialis*. Sequential extraction enhances recovery and facilitates the identification of the most bioactive fraction, supporting the potential use of ethyl acetate extracts as natural antioxidants for nutraceutical, cosmetic, and pharmaceutical applications.

Keywords: astaxanthin, carotenoids, ethyl acetate, flavonoids, phenolic

Aktivitas Antioksidan *Haematococcus pluvialis* yang Diekstrak secara Berurutan menggunakan Beberapa Uji In Vitro

Abstrak

Stres oksidatif mengakibatkan kerusakan sel dan berbagai kondisi kronis, sehingga dibutuhkan antioksidan alami terutama dari mikroalga. Penelitian ini mengevaluasi sifat antioksidan ekstrak *Haematococcus pluvialis* yang diperoleh melalui ekstraksi bertingkat dengan pelarut berurutan (n-heksana, etil asetat, metanol) menggunakan maserasi berbantu ultrasonik. Ekstrak dikarakterisasi berdasarkan hasil rendemen, total fenolik, total flavonoid, dan karotenoid. Aktivitas antioksidannya dinilai menggunakan empat uji in vitro (DPPH, ABTS, FRAP, dan penangkapan H_2O_2). Ekstraksi bertingkat menghasilkan rendemen kumulatif 56,13% (30,13% n-heksana; 14,93% etil asetat; 11,07% metanol), melebihi ekstraksi

pelarut tunggal metanol (47,13%). Fraksi etil asetat menunjukkan fenolik tertinggi (27,87±0,92 mg GAE/g) dan flavonoid tertinggi (5,96±0,25 mg QE/g) serta kandungan pigmen terbesar (astaxanthin 4.124,25±60,94 µg/mL; karotenoid total 4.686,65±69,25 µg/mL). Secara konsisten, ekstrak etil asetat memperlihatkan aktivitas antioksidan terkuat pada semua uji (DPPH IC_{50}=46,71 µg/mL; ABTS IC_{50}=35,85 µg/mL; FRAP IC_{50}=23,94 µg/mL; H_2O_2 IC_{50}=54,64 µg/mL), mengungguli fraksi bertingkat lainnya dan ekstrak metanol tunggal. Hasil ini menunjukkan bahwa ekstraksi semi-polar dengan etil asetat efektif dalam mengkonsentrasikan senyawa fenolik, flavonoid, dan pigmen antioksidan dari *H. pluvialis*. Ekstraksi bertingkat mampu meningkatkan perolehan dan memudahkan identifikasi fraksi dengan aktivitas biologis tertinggi. Hasil ini mendukung potensi penggunaan ekstrak etil asetat sebagai sumber antioksidan alami untuk aplikasi nutraseutikal, kosmetik, dan farmasi.

Kata kunci: astaxanthin, etil asetat, flavonoid, fenolik, karotenoid

INTRODUCTION

Free radicals are highly reactive atoms or molecules characterized by the presence of one or more unpaired electrons in their outer orbitals. These unstable species include Reactive Oxygen Species (ROS), Reactive Nitrogen Species (RNS), and transition metal complexes. Free radicals are generated endogenously through cellular metabolic processes and exogenously from environmental factors such as air pollutants, cigarette smoke, and chemical exposure. Excessive accumulation of ROS disrupts cellular homeostasis and induces oxidative stress, leading to molecular damage, such as lipid peroxidation, protein denaturation, DNA hydroxylation, and ultimately apoptosis (Rani *et al*., 2021; Engwa *et al.,* 2022). Antioxidants serve as the primary defense mechanism against oxidative stress by neutralizing free radicals and stabilizing the reactive intermediates. They play a crucial role in protecting the body against oxidative stress-related disorders, including cancer and inflammatory diseases such as eczema (Abeyrathne *et al*., 2022; Martemucci *et al*., 2022). The growing demand for safer and more sustainable antioxidant agents has highlighted the strong potential of natural sources as effective alternatives for antioxidant production.

Natural antioxidants derived from biological sources have gained increasing attention, particularly those obtained from microorganisms with high metabolic versatility. Microalgae are unicellular eukaryotic organisms that represent a promising natural source of antioxidants for application in the food, cosmetic, and nutraceutical industries (Ferreira de Oliveira & Bragotto, 2022). They synthesize a broad spectrum of bioactive metabolites, including phenolic compounds (such as gallic acid and quercetin), natural pigments (β-carotene, phycocyanin, astaxanthin, and lutein), polyunsaturated fatty acids (PUFAs), polysaccharides, vitamins, and sterols (Gauthier *et al.,* 2020; Coulombier *et al*., 2021; Chamidah *et al*., 2025; Sunandar *et al*., 2025). Among these species, *Haematococcus pluvialis* stands out as the richest natural source of astaxanthin, a xanthophyll carotenoid with well-documented radical-scavenging and anti-inflammatory properties (Ren *et al.,* 2022; Prayogo *et al.,* 2025). *H. pluvialis*, a green microalgae species, is widely recognized as the most abundant natural source of astaxanthin. During the cyst or resting stage, algae accumulate substantial quantities of this pigment (Yin *et al*., 2023). Its unique biochemical composition, particularly its high carotenoid content, makes *H. pluvialis* an exceptional candidate for the development of natural antioxidant-based formulations and biotechnological applications (Oslan *et al*., 2021).

The effective recovery of antioxidant compounds from *H. pluvialis* is strongly influenced by the extraction strategy employed, as its metabolites exhibit considerable diversity in terms of polarity, solubility, and chemical structure. The chemical diversity of *H. pluvialis* poses challenges for extraction because its antioxidant metabolites vary widely in polarity and solubility. Extraction methods significantly influence the bioactive compound content of the extract, as they determine the efficiency of phytochemical release from the cellular matrices. The selective interaction between solvents and

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phytochemicals occurs according to their polarity; polar compounds dissolve in polar solvents, while nonpolar compounds dissolve in nonpolar solvents (Putri *et al*., 2022). The successive (graded) maceration method enables the separation of compounds based on polarity: *n*-hexane extracts nonpolar compounds, ethyl acetate extracts semipolar compounds, and methanol extracts polar compounds (Rahayu *et al*., 2022). In contrast, non-fractionated extraction produces a total extract containing all compounds that are soluble in the chosen solvent. The advantage of graded extraction lies in its ability to yield a larger extract recovery with diverse bioactive compounds of varying polarities (Arel & Ningsih, 2022; Riasari *et al*., 2022).

Accurate evaluation of antioxidant potential requires appropriate biological assays that reflect the different mechanisms of action. The antioxidant activity of natural extracts cannot be fully characterized using a single assay because different assays measure different underlying mechanisms. DPPH and ABTS reflect free radical scavenging ability, FRAP measures reducing power, and H_2O_2 scavenging evaluates the neutralization of specific ROS species (Hartung & Daston, 2009; Munteanu & Apetrei, 2021). Previous studies on *H. pluvialis* have reported variability in antioxidant activity depending on extraction method and assay type example, Zhao *et al.* (2016) observed high DPPH and H_2O_2 scavenging activity in extracts obtained via magnetic field-assisted extraction, while Tan *et al*. (2021) reported strong ABTS activity in extracts obtained using acid-assisted extraction. However, most studies have used only one or two assays, resulting in incomplete characterization of antioxidant mechanisms.

Although *H. pluvialis* is known for its antioxidant potential, research using sequential extraction with multiple antioxidant assays is still limited. Consequently, the effect of solvent polarity on the distribution of phenolics, flavonoids, and carotenoids and its impact on antioxidant activity has not been fully elucidated. This study aimed to determine the effect of solvent polarity on the extracted compounds, identify the bioactive components in each fraction, evaluate their antioxidant activity using DPPH, ABTS, FRAP, and H_2O_2 assays, and identify the fraction with the strongest antioxidant potential.

MATERIALS AND METHODS

Sample Extraction

Dried red-pigmented *H. pluvialis* powder was obtained from PT Evergen Resources (Kendal, Central Java, Indonesia). The extraction process was carried out based on the modified method of Sijabat *et al.* (2023), using a multistage maceration technique combined with ultrasonic-assisted extraction (UAE) to obtain fractions with different polarities. Sequential extraction was performed using solvents of increasing polarity: n-hexane (non-polar), ethyl acetate (semi-polar), and methanol (polar), all of which were purchased from PT Alfa Kimia Indonesia (ALKEMI). Approximately 5 g of dried biomass was extracted using 50 mL of solvent at a 1:10 (w/v) ratio. Each extraction stage involved ultrasonication using a sonicator (Emerson Branson) operating at 60 Hz for 15 min to enhance solvent penetration, followed by 24 h of maceration at room temperature with gentle agitation. The mixture was filtered through Whatman filter paper, and the residue was re-extracted using the subsequent solvent under identical conditions. All filtrates obtained from the three extraction stages were concentrated using a rotary evaporator (SCILOGEX) at 40 °C to obtain solvent-specific extracts. The extraction yield (%) was calculated using the following equation:

$$\text{Yield (\%)} = \frac{\text{Sample after extraction (g)}}{\text{Sample before extraction(g)}} \times 100$$

Carotenoids

Carotenoid analysis of *H. pluvialis* extract was performed based on the modified method described by Koopmann *et al.* (2022). The extract was prepared at a concentration of 100 ppm by dissolving the sample in acetone Aseton p.a. (Merck). The carotenoid solution was analyzed using a UV–Vis spectrophotometer (Cary 60, Agilent Technologies) at 663.2, 646.8, 470, 479, 645, and 663 nm. The absorbance values obtained at each wavelength were used to calculate

the carotenoid concentrations according to the respective equations for chlorophyll a, chlorophyll b, total chlorophyll, and total carotenoids.

Chlorophyll a (μg/mL) = (12.2 × A663.2) – (2,79 × A646.8)

Chlorophyll b (μg/mL) = (21.5 × A646,8) – (5.1 × A663,2)

Carotenoids(μg/mL) = ((1000 × A470) – (1.82 × Chlorophyll a) – (85.02 × Chlorophyll b))/(198)

Astaxanthin (μg/mL) = 0.8 × Carotenoids

β-Carotene (μg/mL) = 0.854 × A479 – 0.312 × A645 + 0.039 × A663 – 0.005 (Nagata, 2009)

Total Flavonoid Content

The total flavonoid content of the *H. pluvialis* extract was determined using a modified method described by Al-Maharik *et al.* (2023). The aluminum chloride (Merck) reagent was prepared by dissolving 1 g of $AlCl_3$ in 10 mL of distilled water (WaterOne, Onemed) (WaterOne, Onemed), and the potassium acetate (Merck) reagent was prepared by dissolving 0.98 g of potassium acetate in 10 mL of distilled water (WaterOne, Onemed). An aliquot (0.5 mL) of the extract (100 μg/mL) was mixed with 1.5 mL of methanol p.a. (Merck), 0.1 mL of aluminum chloride solution, 0.1 mL of potassium acetate solution, and 2.8 mL of distilled water (WaterOne, Onemed) (WaterOne, Onemed). The mixture was incubated in the dark at room temperature for 30 min to prevent the photodegradation of DCF. After incubation, the absorbance was measured at 415 nm using a UV–Vis spectrophotometer (Cary 60; Agilent Technologies). A quercetin (Sigma-Aldrich) standard curve was prepared at concentrations ranging from 20 to 100 μg/mL, following the same procedure. The TFC was calculated using a linear regression equation (y = ax + b) and expressed as milligrams of quercetin equivalent per gram of extract (mg QE/g extract).

Total Phenolic Content

The total phenolic content of the *H. pluvialis* extract was determined based on the modified method of Li *et al.* (2024) using the Folin–Ciocalteu colorimetric assay. The Folin–Ciocalteu (Merck) reagent was prepared by diluting 4 mL of stock solution with 45 mL of distilled water (WaterOne, Onemed), and the sodium carbonate (Merck) solution was prepared by dissolving 3 g of sodium carbonate in 47 mL of distilled water. An aliquot (0.5 mL) of the extract (100 μg/mL) was mixed with 2.5 mL of Folin–Ciocalteu reagent and 2 mL of sodium carbonate solution. The mixture was homogenized and incubated in the dark for 60 min at room temperature to prevent light-induced degradation of the pigments. After incubation, the absorbance was measured at 765 nm using a UV–Vis spectrophotometer (Cary 60, Agilent Technologies). A gallic acid (Merck) standard curve was prepared at concentrations of 20–100 μg/mL under the same conditions. The TPC was calculated using a linear regression equation (y = ax + b) and expressed as milligrams of gallic acid equivalent per gram of extract (mg GAE/g extract).

DPPH Radical Scavenging Activity

The antioxidant activity of *H. pluvialis* extract was evaluated using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging method, as described by Luís *et al.* (2018), with modifications. The extract was prepared using methanol p.a. (Merck) at concentrations of 100, 50, 25, 12.5, and 6.25 mg/mL were mixed with the 0.1 mM DPPH (Merck) methanol solution at a 1:10 ratio (v/v) and incubated for 60 minutes at 37°C in the dark. Absorbance was measured at 734 nm using a UV–Vis spectrophotometer (Cary 60, Agilent Technologies), with methanol serving as the blank. The inhibition percentage was calculated, and the IC_{50} value, representing the extract concentration required to scavenge 50% of the DPPH radicals, was obtained from the regression curve.

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

A= control absorbance
B= sample absorbance

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ABTS Radical Cation Scavenging Activity

The ABTS radical scavenging activity was determined as described by Wołosiak *et al.* (2021), with modifications. The ABTS radical solution was prepared by mixing 7 mM ABTS (Merck) with 2.45 mM potassium persulfate (Merck) and incubating the mixture in the dark for 16 h. The solution was diluted with ethanol p.a.grade. (Merck) to an absorbance of 0.700 ± 0.02 at 734 nm, before use. Extract solutions at concentrations of 100, 50, 25, 12.5, and 6.25 mg/mL were mixed with the ABTS working solution at a 1:10 ratio (v/v) and incubated for 60 min at 37°C in the dark. Absorbance was measured at 734 nm using a UV–Vis spectrophotometer (Cary 60, Agilent Technologies), with ethanol serving as the blank. The percentage inhibition was calculated, and the IC_{50} value, representing the extract concentration required to scavenge 50% of the ABTS radicals, was obtained from the regression curve.

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

A= control absorbance
B= sample absorbance

Ferric Reducing Antioxidant Power (FRAP)

The ferric reducing antioxidant power (FRAP) of *H. pluvialis* extract was analyzed as described by Misfadhila *et al.* (2022), with modifications. The FRAP reagent was freshly prepared by mixing 10 mM TPTZ (Sigma-Aldrich) in 40 mM HCl p.a. (Merck), 20 mM $FeCl_3$ (Merck), and 300 mM acetate buffer (Merck) at pH 3.6 in a 1:1:10 (v/v/v) ratio. The extracts (100, 50, 25, 12.5, and 6.25 mg/mL) were mixed with the FRAP reagent at a 1:10 ratio (v/v) and incubated for 60 min at 37°C in the dark. Absorbance was recorded at 593 nm using a UV–Vis spectrophotometer (Cary 60, Agilent Technologies) with methanol p.a. (Merck) was used as a blank. The reducing activity was calculated as percentage inhibition using the same equation, and the IC_{50} value was determined from the linear regression of extract concentration versus inhibition percentage, representing the concentration required to reduce 50% of ferric ions.

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

A= control absorbance
B= sample absorbance

Hydrogen Peroxide (H_2O_2) Scavenging Activity

The hydrogen peroxide scavenging activity of *H. pluvialis* extract was determined according to Kathiravan & Kalava (2021) with modifications. The H_2O_2 reagent was prepared by combining $FeSO_4$ (Merck), sodium salicylate (Merck), and hydrogen peroxide (Merck) to initiate radical formation. Extract solutions (100, 50, 25, 12.5, and 6.5 mg/mL) were mixed with the H_2O_2 reagent at a 1:10 ratio (v/v) and incubated for 60 min at 37°C in the dark. Absorbance was measured at 510 nm using a UV–Vis spectrophotometer (Cary 60, Agilent Technologies) and methanol p.a. (Merck) was used as a blank. The scavenging percentage was calculated using the inhibition formula, and the IC_{50} value was obtained from the regression curve, indicating the concentration required to scavenge 50% of the hydrogen peroxide radicals.

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

A= control absorbance
B= sample absorbance

Data Analysis

The experiment was designed using a Completely Randomized Design (CRD) with a single-factor extraction method, comparing sequential and single-solvent extractions. Data were analyzed using one-way analysis of variance (ANOVA) to assess the effects of extraction method and solvent type on the measured parameters. Significant differences ($p < 0.05$) were further analyzed using the Honest Significant Difference (HSD) post-hoc test. Nonparametric data were evaluated using the Kruskal–Wallis test. All statistical analyses were performed using the SPSS software (version 24.0; IBM Corp., USA).

RESULTS AND DISCUSSION

Yields

Extraction is a fundamental separation technique that involves the transfer of a

solute from its original matrix to a suitable solvent. This process relies on differences in the solubility of the components within a mixture, allowing the selective isolation of target compounds (Tambun *et al*., 2016). In this study, three solvents, namely N-hexane (non-polar), ethyl acetate (semi-polar), and methanol (polar), were used to extract *H. pluvialis*, thereby facilitating the isolation of diverse bioactive compounds with varying polarities and structural characteristics (Sijabat *et al*., 2023). The yields obtained using each solvent and extraction method are presented in Table 1.

Based on the data presented in Table 1, both the solvent type and extraction method significantly affected the yield of *H. pluvialis* extract. Sequential extraction using *n*-hexane, ethyl acetate, and methanol produced yields of 30.13%, 14.93%, and 11.07%, respectively, with *n*-hexane yielding the highest extract yield. The cumulative yield from sequential extraction reached 56,13%, which exceeded that of single-solvent extraction with methanol (47,13%). This higher overall yield demonstrates that sequential extraction enhances both extraction efficiency and selectivity compared to single-step extraction, making it an effective strategy for maximizing the recovery and characterization of diverse bioactive compounds from natural products. The variation in the extract yield is primarily attributed to differences in solvent polarity, as higher polarity increases the solubility of phytochemical constituents (Naima *et al*., 2015). Similarly, Supriatno and Lelifajri (2018) reported that not all compounds can be effectively extracted using a single-solvent system; thus, applying solvents with different polarities helps to overcome this limitation. The choice of solvent strongly influences extraction efficacy and the resulting phytochemical profile, as polar and non-polar solvents dissolve distinct classes of compounds (Santy *et al*., 2025). Sequential extraction is considered more efficient because each solvent selectively dissolves a specific group of compounds according to its polarity (Nawaz *et al*., 2019). Therefore, sequential solvent extraction can be regarded as an effective strategy for maximizing the recovery of both nonpolar and polar bioactive compounds from *H. pluvialis*.

Total Phenolic Content

The total phenolic content (TPC) is a quantitative parameter used to determine the concentration of phenolic compounds in a sample. Phenolic compounds are well-recognized bioactive molecules that protect cells against oxidative stress and reduce the risk of chronic diseases (Priyanthi & Sivakanesan, 2021). Structurally, these compounds contain one or more hydroxyl groups attached to an aromatic ring and can be broadly classified as flavonoids or non-flavonoids (Way *et al.,* 2020). Their structural diversity encompasses subclasses such as flavonoids, phenolic acids, and tannins (Yumita *et al*., 2023). The TPC values of *H. pluvialis* extracts obtained using different solvents and extraction methods are listed in Table 1.

The results revealed considerable variation in the phenolic content of *H. pluvialis* extracts, depending on the solvent used. The ethyl acetate fraction exhibited the highest phenolic concentration (27.87±0.918 mg GAE/g), whereas the n-hexane fraction

Table 1 Yield, total phenolic content, and total flavonoid content of *H. pluvialis* extracts

H. pluvialis extracts	Yield (%)	Total phenolic content (mg GAE/g)	Total flavonoid content (mg QE/g)
N-Hexane SE	30.13±0.02^{b}	17.13±0.26^{a}	3.24±0.17^{c}
Ethyl acetate SE	14.93±0.01^{a}	27.87±0.91^{d}	5.96±0.25^{d}
Methanol SE	11.07±0.01^{a}	20.92±0.92^{b}	1.64±0.09^{b}
Methanol SSE	47.13±0.03^{c}	24.60±0.93^{c}	4.76±0.22^{a}

Different superscipt on the same column showed a significance differences,
SE = sequential extraction; SSE = single-solvent extraction

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exhibited the lowest (17.13±0,262 mg GAE/g). The cumulative phenolic yield from sequential extraction was 65.92 mg GAE/g, which was substantially higher than that obtained from single-solvent extraction with methanol (24.60 mg GAE/g). These results indicate that semi-polar solvents such as ethyl acetate are more effective for phenolic extraction than non-polar or highly polar solvents. This observation further confirms that solvent polarity plays a crucial role in determining the extraction efficiency and compound selectivity. According to Kaczorová *et al.* (2021), phenolic compounds containing multiple hydroxyl groups are more soluble in polar solvents, such as water, ethanol, and methanol, whereas those with methoxy substituents exhibit a higher affinity for semi-polar or less polar solvents, such as ethyl acetate, acetone, and chloroform. These findings are consistent with those reported by Rao *et al.* (2010), who found that *H. pluvialis* extracts obtained using hexane, methanol, and ethyl acetate contained 47.52±1.25 µg/mg, 85.23±0.18 µg/mg, and 95.02±0,89 µg/mg of phenolic compounds, respectively. Furthermore, Castillo *et al.* (2022) identified several specific phenolic constituents in *H. pluvialis* red-stage extracts, including phloroglucinol, p-coumaric acid, gallic acid, and catechin, using both targeted and untargeted LC–MS analyses.The presence of these compounds supports the view that *H. pluvialis* contains a diverse range of phenolic molecules contributing to its strong antioxidant potential. Phenolic compounds serve as primary antioxidants owing to their redox properties, allowing them to donate hydrogen atoms or electrons to neutralize free radicals and terminate oxidative chain reactions that damage biomolecules. Numerous studies have reported a strong positive correlation between TPC and antioxidant activity in both plant and algal extracts, where samples with higher phenolic content tend to exhibit significantly greater radical-scavenging activity (Wali *et al.*, 2020).

Total Flavonoid Content

Flavonoids are one of the most important groups of plant secondary metabolites in the polyphenol family. These compounds play a significant role in human health and are widely utilized in the pharmaceutical, cosmetic, nutritional, and nutraceutical industries. Structurally, flavonoids are benzopyrone derivatives that are classified as polyphenols because of the presence of multiple hydroxyl (-OH) groups attached to specific positions on their aromatic rings (Nicolescu *et al.*, 2025). The total flavonoid content (TFC) of *H. pluvialis* extracts obtained using different solvents and extraction methods is presented in Table 1.

The analysis revealed that the ethyl acetate extract of H. pluvialis exhibited the highest total flavonoid content (5.96 ± 0.254 mg QE/g). The cumulative flavonoid yield from the sequential extraction was 10.84 mg QE/g, which exceeded that of the single methanol extraction (4.76 mg QE/g). The superior performance of ethyl acetate can be attributed to its semi-polar nature, which enables it to efficiently dissolve both polar and non-polar bioactive compounds. In single-solvent extraction, all flavonoids are solubilized directly in methanol, whereas in sequential extraction, a substantial portion of flavonoids is first extracted into the ethyl acetate phase, leaving a smaller fraction in the methanol extract (ME). The methanol extract also showed a relatively high flavonoid content (4.76±0.22 mg QE/g), which can be explained by the strong polarity and hydrogen bonding capacity of methanol. These properties facilitate the solubilization of flavonoid glycosides, which are polar derivatives commonly found in microalgal cell matrices. According to Riasari *et al.* (2022), each solvent dissolves compounds of similar polarity following the principle of "like dissolves like." Semi-polar solvents, such as ethyl acetate, are effective for extracting alkaloids and aglycone flavonoids, whereas polar solvents, such as methanol, are more suitable for isolating flavonoid glycosides and tannins.

Previous studies have confirmed that H. pluvialis contains a diverse range of flavonoids, including quercetin, dihydroquercetin, kaempferol, dihydrokaempferol, naringenin, apigenin, luteolin, genistein, and daidzein (Goiris *et al.*, 2014). According to Palavicini *et al.* (2022), less polar flavonoids, such as

isoflavones, flavanones, methylated flavones, and flavonols, are preferentially extracted using organic solvents such as chloroform, dichloromethane, or ethyl acetate, whereas more polar flavonoid glycosides and aglycones are best extracted using alcohols or alcohol–water mixtures. These solvent-dependent differences are consistent with the polarity and structural variability of flavonoids, particularly their variations in the degree of hydroxylation and glycosylation. Consistent with these results, Ferdous and Yusof (2021) reported that polar solvents such as methanol, ethanol, and ethyl acetate extract greater amounts of phenolic and flavonoid compounds than non-polar solvents such as hexane and dichloromethane. Similarly, Akbar et al. (2021) found that the ethyl acetate extract of Padina sp. contained the highest total flavonoid content, followed by the methanol and n-hexane extracts. The high total flavonoid content observed in the ethyl acetate extract of H. pluvialis strongly correlated with its superior antioxidant activity. Flavonoids are known to act as potent free radical scavengers because of their hydroxyl groups, which enable them to donate hydrogen atoms or electrons to neutralize reactive oxygen species (ROS). Therefore, the elevated flavonoid levels in the extract contribute significantly to its ability to inhibit oxidative chain reactions and protect biomolecules from oxidative damage (Muflihah *et al*., 2021).

Carotenoids

Microalgal pigments are organic molecules containing chromophoric groups with long conjugated double bonds or ring structures that can absorb light within specific regions of the visible spectrum. These pigments are generally classified into four major groups: chlorophylls, carotenoids, phycobiliproteins, and, more recently, polyphenolic pigments that have also been identified (Aizpuru & González-Sánchez, 2024). Carotenoids are naturally occurring pigments found in plants, microorganisms, and certain animals, and they are responsible for the diverse range of colors observed in nature. Their strong antioxidant properties have led to their wide application as functional ingredients in the food, pharmaceutical, feed, and cosmetic industries (Martínez-Cámara *et al*., 2021). The carotenoid pigment contents of H. pluvialis extracts obtained using different solvents and extraction methods are listed in Table 2.

Based on the results presented in Table 2, the highest pigment concentrations were obtained from the *H. pluvialis* extract using ethyl acetate as the solvent, with astaxanthin (4,124.25 µg/mL), β-carotene (3.33 µg/mL), and total carotenoids (4,686.65 µg/mL) content. The lowest pigment content was recorded in the sequential methanol extract, yielding 871.38 µg/mL astaxanthin, 0.79 µg/mL β-carotene, and 990.21 µg/mL total carotenoids. These results clearly demonstrate that ethyl acetate is the most effective solvent for carotenoid extraction, whereas sequential methanol extraction produces a comparatively lower pigment recovery. The superior performance of ethyl acetate can be attributed to its semi-polar nature, which enables the extraction of both polar and non-polar compounds. Moreover, its low toxicity and high volatility make it an ideal solvent for carotenoid recovery, including β-carotene and astaxanthin (Irfan *et al.,* 2024). Similar

Table 2 Carotenoid pigments of *H. pluvialis* extracts

H. pluvialis extracts	Astaxanthin (µg/mL)	β-Carotene (µg/mL)	Total carotenoids (µg/mL)
N-Hexane SE	3,255.68±96.34^{b}	3.12±0.084^{b}	3,699.64±109.47^{b}
Ethyl acetate SE	4,124.25±60.94^{c}	3.33±0.053^{c}	4,686.65±69.25^{c}
Methanol SE	871.38±64.05^{a}	0.79±0.066^{a}	990.21±72.79^{a}
Methanol SSE	3,243.13±142.70^{b}	2.92±0.26^{b}	3,685.38±162.15^{b}

Different superscipt on the same column showed a significance differences,
SE = sequential extraction; SSE = single-solvent extraction

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observations were reported by Pan-utai *et al*. (2021), who found that the ultrasound-assisted extraction of *H. pluvialis* using ethyl acetate for 25 min produced the highest total carotenoid content (272.17 μg/L). Similarly, Desai *et al*. (2016) demonstrated that ethyl acetate achieved over 70% astaxanthin recovery from *H. pluvialis*, further confirming its efficiency for carotenoid isolation.

Carotenoids are broadly classified into carotenes, which are nonpolar hydrocarbons (e.g., β-carotene), and xanthophylls, which are polar oxygenated derivatives possessing oxygen-containing groups at the ends of the carbon chain while preserving a conjugated double-bond system (Popova, 2017). This structural arrangement contributes to their distinctive color and photoprotective properties. Furthermore, El-Baz and Ali (2023) highlighted that natural carotenoids are among the most potent antioxidants, capable of quenching singlet oxygen and scavenging free radicals. Their strong oxidative stress–mitigating capacity is associated with the prevention of several chronic diseases, including cardiovascular disorders, cancer, diabetes, liver fibrosis, and osteoporosis.

Antioxidant Activity

Antioxidant activity is a key parameter for evaluating the biological potential of microalgal extracts, as it plays an essential role in mitigating oxidative stress and preventing related disorders. In this study, antioxidant capacity was expressed as the half-maximal inhibitory concentration (IC_{50}), which represents the concentration of extract required to scavenge 50% of free radicals. The IC_{50} value serves as a more precise indicator of antioxidant potential than the Radical Scavenging Activity (RSA) percentage. While RSA reflects radical inhibition at a single concentration, IC_{50} provides a quantitative measure of the concentration required to achieve 50% inhibition. A lower IC_{50} value indicates a stronger antioxidant capacity, as less extract is required to produce the same level of radical scavenging. Therefore, the IC_{50} offers a standardized and comparable metric for evaluating antioxidant potency across different samples and studies (Rozirwan *et al*., 2023). The antioxidant activities of *H. pluvialis* extracts, evaluated using DPPH, ABTS, FRAP, and H_2O_2 assays, are presented in Table 3.

DPPH Radical Scavenging Activity

The DPPH assay results presented in Table 3 indicate that the ethyl acetate SE exhibited the strongest antioxidant activity (IC_{50} = 46.71 μg/mL), whereas the methanol SE exhibited the weakest activity (IC_{50} = 83.03 μg/mL). The positive control, vitamin E, exhibited the lowest IC_{50} value (3.04 μg/mL), indicating its superior antioxidant strength. The lower antioxidant activity of the methanol SE can be attributed to the preferential extraction of major antioxidant compounds into the ethyl acetate fraction during sequential extraction, leaving fewer phenolic and flavonoid compounds in the methanol extract. This finding is consistent with that of Istiqomah *et al*. (2021), who reported that ethyl acetate fractions of *Schleichera oleosa* bark exhibited stronger DPPH scavenging activity (IC_{50} = 57.3 μg/mL) compared to ethanol (87.52 μg/mL) and hexane (136.03 μg/mL), confirming

Table 3 Antioxidant activity of *H. pluvialis* extracts

H. pluvialis extracts	DPPH IC_{50} (μg/mL)	ABTS IC_{50} (μg/mL)	FRAP IC_{50} (μg/mL)	H_2O_2 IC_{50} (μg/mL)
N-Hexane SE	79.46±2.05^{d}	56.60±1.89^{d}	41.22±1.50^{d}	79.96±1.76^{d}
Ethyl acetate SE	46.71±2.06^{b}	35.85±1.68^{b}	23.94±1.75^{b}	54.64±3.16^{b}
Methanol SE	83.03±2.19^{d}	77.59±1.45^{e}	48.41±2.19^{e}	92.79±1.83^{e}
Methanol SSE	65.53±1.27^{c}	50.54±0.60^{c}	35.05±2.84^{c}	72.87±1.81^{c}
Vitamin E	3.04±0.17^{a}	4.53±0.27^{a}	3.14±0.23^{a}	4.53±0.27^{a}

Different superscipt on the same column showed a significance differences,
SE = sequential extraction; SSE = single-solvent extraction

the higher extraction efficiency of semi-polar solvents for antioxidant compounds. Furthermore, Tan *et al. (*2021) reported that *H. pluvialis* extracts obtained using hydrochloric acid (HCl)-assisted extraction exhibited the highest DPPH radical scavenging activity, with an IC_{50} value of 20.32 µg/mL, which was significantly lower than the IC_{50} values obtained using other extraction methods, indicating stronger antioxidant activity. The differences in IC_{50} values among studies are likely influenced by variations in extraction techniques, solvent systems, and the resulting phytochemical composition of the extracts.

The strong DPPH radical scavenging activity observed in the ethyl acetate extract can be attributed to its higher phenolic and flavonoid content, which are known to act as effective hydrogen or electron donating antioxidants. Phenolic and flavonoids act as antioxidants primarily via hydrogen atom and electron transfer mechanisms, while flavonoids further enhance antioxidant protection by chelating metal ions and suppressing radical formation (Sudirman *et al*., 2022). Antioxidant activity can be categorized based on IC_{50} values, where compounds with IC_{50} values ranging from 10 to 50 µg/mL are considered to exhibit strong antioxidant activity, those with IC_{50} values between 50 and 100 µg/mL show intermediate antioxidant activity, and compounds with IC_{50} values greater than 100 µg/mL are classified as having weak antioxidant activity (Phongpaichit *et al*., 2007). Based on this classification, the ethyl acetate extract of *H. pluvialis* was classified as having strong antioxidant activity, whereas the methanol extract was classified as intermediate.

ABTS Radical Cation Scavenging Activity

The ABTS assay displayed a similar pattern, as shown in Table 3, where the ethyl acetate SE exhibited the strongest activity (IC_{50} = 35.85 µg/mL), while the methanol SE exhibited the weakest activity (77.59 µg/mL). Vitamin E served as the most potent standard (4.53 µg/mL). In sequential extraction, antioxidant compounds with high reactivity, particularly phenolic and flavonoid aglycones that exhibit strong activity toward the ABTS radical, are preferentially partitioned into the ethyl acetate fraction. Consequently, the subsequent methanol fraction predominantly contains more polar residual compounds, which generally possess lower radical-scavenging capacity. These results align with those of Ferdous *et al.* (2025), who reported that ethyl acetate extracts of microalgae (*Tetraselmis* sp., *Thalassiosira* sp., and *Nannochloropsis* sp.) exhibited the highest ABTS scavenging activity (41.57–30.92 mg TEAC/g extract). The consistency across species highlights that semi-polar solvents effectively extract the phenolic and flavonoid compounds responsible for radical scavenging. Tan *et al.* (2021) reported that *H. pluvialis* extracts obtained using hydrochloric acid (HCl)-assisted extraction exhibited the highest ABTS radical scavenging activity, with an IC_{50} value of 20.32 µg/mL, which was significantly lower than the IC_{50} values obtained using other extraction methods, indicating stronger antioxidant activity. Based on IC_{50} values, the ethyl acetate extract exhibited the strongest antioxidant activity compared to the hexane and methanol extracts. This higher activity is closely associated with the higher presence of phenolic and flavonoid compounds in the ethyl acetate fraction, which are well-known as major bioactive antioxidants. Lower IC_{50} values indicate stronger antioxidant capacity, whereas higher IC_{50} values reflect weaker activity (Edison *et al*., 2020).

Ferric Reducing Antioxidant Power (FRAP)

In the FRAP assay, which measures the ability of antioxidants to reduce Fe^{3+} to Fe^{2+}, the ethyl acetate SE again showed the highest reducing capacity (IC_{50} = 23.94 µg/mL), while the methanol SE showed the weakest activity (48.41 µg/mL) (Table 3). Vitamin E exhibited the strongest reducing ability (3.14 µg/mL). The relatively low FRAP activity observed in the methanol sequential extract can be attributed to the sequential extraction process, in which most redox-active antioxidant compounds were already recovered during the preceding n-hexane and ethyl acetate extraction steps, leaving only residual polar constituents in the methanol

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fraction. Consistent with the ABTS results, Ferdous *et al*. (2025) also reported that ethyl acetate extracts of microalgae species showed superior FRAP values, indicating the presence of redox-active compounds capable of electron donation and ferric reduction. According to Hacke *et al*. (2025), the methanol extract of *H. pluvialis* exhibited strong ferric-reducing activity, with an IC_{50} value of 36.3 ± 1.9 µg/mL, indicating high antioxidant potency. The high FRAP activity was attributed to the abundance of polar phenolic and flavonoid compounds in the methanol fraction, which act as effective electron donors. High phenolic and flavonoid contents are indicative of strong antioxidant activity, as evidenced by enhanced DPPH and FRAP activities (Ibrahimi & Hajdari, 2020).

Hydrogen Peroxide (H_2O_2) Scavenging Activity

Table 3 shows that the H_2O_2 scavenging assay further confirmed the superior performance of the ethyl acetate SE (IC_{50} = 54.64 µg/mL), while the methanol SE showed the weakest activity (92.79 µg/mL). Vitamin E demonstrated the strongest scavenging effect (4.53 µg/mL). Hydrogen peroxide scavenging involves electron donation by antioxidants, reducing peroxides to water, and preventing hydroxyl radical formation through Fenton-type reactions (Yu *et al*., 2024). The high activity of the ethyl acetate extract suggests the presence of potent electron-donating compounds capable of efficient peroxide neutralization. A previous study by Zhang *et al*. (2022) reported that astaxanthin crystals isolated from *H. pluvialis* exhibited a very low IC_{50} value (49.46 µmol L^{-1}) against hydroxyl radicals, indicating strong antioxidant activity. Antioxidant activity based on IC_{50} values can be classified into four categories: weak antioxidants with IC_{50} values ranging from 151–200 ppm, moderate antioxidants with IC_{50} values of 100–150 ppm, strong antioxidants with IC_{50} values of 50–100 ppm, and very strong antioxidants when with IC_{50} value below 50 ppm (Sudirman *et al*., 2022). The antioxidant mechanism of phenolic compounds against peroxyl radicals (ROO•) involves the transfer of a hydrogen atom from the phenolic hydroxyl group to the radical, resulting in the formation of a stabilized phenoxyl radical with a single unpaired electron. Enolic phenolic compounds, such as chlorogenic acid, exhibit strong free radical-scavenging activity through this mechanism. Consequently, the total phenolic content is positively correlated with the antioxidant activity of a sample (Mahardani & Yuanita, 2021). The total phenolic content of the extracts showed a strong correlation with antioxidant activity, as phenolic compounds may act individually or synergistically. Their radical-scavenging ability is primarily attributed to the presence of hydroxyl (–OH) groups, which function as hydrogen and electron donors. Through electron transfer mechanisms, these hydroxyl groups neutralize free radicals and reduce the levels of reactive oxygen species responsible for oxidative stress (Ghaisani *et al*., 2025).

Overall Antioxidant Performance

Across all antioxidant assays, the ethyl acetate extract consistently demonstrated the strongest activity, followed by the methanol extract, while the n-hexane and sequential methanol extracts showed comparatively weaker results. This trend corresponds with the total phenolic and flavonoid contents, further confirming that these compounds are the primary contributors to the antioxidant activity of *H. pluvialis*. The semi-polar nature of ethyl acetate enables the extraction of both hydrophilic and lipophilic phenolic and flavonoid molecules, which contain hydroxyl (–OH) groups capable of donating hydrogen or electrons to neutralize free radicals and reduce ferric ions, respectively. Flavonoids act through multiple antioxidant mechanisms, including free radical scavenging, metal chelation, and inhibition of reactive oxygen species (ROS) formation. According to Santy *et al*. (2025), flavonoids can neutralize oxygen and nitrogen radicals, inhibit radical-generating enzymes and chelate transition metals. Rauf *et al*. (2024) noted that polyphenols and flavonoids evolved in plants as defense mechanisms against oxidative stress and provide similar protective benefits when consumed by humans. Collectively, these results indicate that ethyl acetate, particularly

within a sequential extraction process, is the most effective solvent for isolating antioxidant compounds from *H. pluvialis*, underscoring its potential applications in nutraceuticals, pharmaceuticals, and functional foods.

The higher antioxidant activities observed in the ABTS and FRAP assays compared to those in the DPPH and H_2O_2 assays are primarily attributed to differences in their reaction mechanisms. Both ABTS and FRAP exhibit faster reaction kinetics and greater sensitivity toward electron-donating compounds than DPPH. The ABTS assay is particularly suitable for assessing hydrophilic antioxidants because of its water-soluble radical cation and rapid reaction rate, which often results in higher activity values under comparable conditions (Jia *et al.*, 2024). Similarly, the FRAP assay measures the reducing power of antioxidants by quantifying their ability to convert Fe^{3+} to Fe^{2+}, and shows high responsiveness to phenolic and flavonoid compounds. Consequently, these methods typically yield higher antioxidant readings than DPPH or H_2O_2 assays, particularly for extracts rich in redox-active molecules.

CONCLUSION

This study demonstrates that sequential solvent extraction enhances the recovery and separation of bioactive compounds from *Haematococcus pluvialis* compared to single-solvent extraction. Solvent polarity strongly influenced the extract composition, with the semi-polar ethyl acetate fraction containing the highest levels of phenolics, flavonoids, and carotenoid pigments. This fraction consistently exhibited the strongest antioxidant activity across all in vitro assays, confirming the major contribution of semi-polar metabolites to the antioxidant potential of *H. pluvialis*. Overall, ethyl acetate-based sequential extraction represents an efficient and targeted strategy for obtaining antioxidant-rich fractions from *H. pluvialis*. Therefore, the ethyl acetate extract is considered the most promising candidate for further development as a natural antioxidant source for nutraceutical, cosmetic, and pharmaceutical applications.

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