J Appl Biotechnol Rep. 2025 March;12(1):1554-1569

Journal of
Applied Biotechnology Reports

doi 10.30491/jabr.2024.467256.1755

Original Article

The *In vitro* Antioxidant, Anti-obesity, and Anti-inflammatory Properties of Microalgae Extracts of *Tetraselmis suecica* Cultivated under Sodium Acetate and Sodium Nitrate Supplementations

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Received July 10, 2024; **Accepted** October 22, 2024; **Online Published** March 20, 2025

Abstract

Introduction: Microalgae are versatile microorganisms known for producing bioactive compounds with potential health benefits. This study focused on the biological properties of *Tetraselmis suecica*, cultivated under supplementation of sodium acetate and sodium nitrate, to identify their potential as functional ingredients.

Materials and Methods: *Tetraselmis suecica* was first cultivated in media supplemented with sodium acetate and sodium nitrate. Biomass was quantified, and crude extracts were prepared for the measurement of total phenolic content. Extracts with the highest biomass and phenolic content were further evaluated for biological activities, including antioxidant and anti-inflammatory effects. The chemical composition of the extracts was analyzed using Fourier-Transform Infrared Spectroscopy (FTIR) and Liquid Chromatography-Mass Spectrometry (LC-MS).

Results: The combined supplementation of sodium acetate and sodium nitrate produced the highest dry cell weight and phenolic content in both ethanol and ethyl acetate extracts. The ethanol extract demonstrated significant antioxidant activity and inhibitory effects against pancreatic lipase activity. The ethyl acetate extract exhibited notable radical scavenging activity and anti-inflammatory effects by inhibiting proteinase denaturation and attenuating TNF-α production in LPS-activated RAW264.7 macrophages. The FTIR analysis revealed the functional groups, including amines, esters, and halocarbons in the extracts, while LC-MS identified bioactive compounds such as alpha carotene, cantaxanthin, rutin, quercitrin, and apigenin-O-rutinoside.

Conclusions: The study demonstrates that *Tetraselmis suecica* extracts, cultivated under sodium acetate and sodium nitrate supplementation, possess significant antioxidant and anti-inflammatory properties, providing potential for use in nutraceutical and pharmaceutical applications.

Keywords: *Tetraselmis suecica,* Bioactivities, Nutrient Supplementation, Secondary Metabolites

Citation: Nor Shahril NS, Abu Bakar MH, Kassim MA, Karunakaran T, Shariff KA. The *In vitro* Antioxidant, Anti-obesity, and Anti-inflammatory Properties of Microalgae Extracts of *Tetraselmis suecica* Cultivated under Sodium Acetate and Sodium Nitrate Supplementations. J Appl Biotechnol Rep. 2025;12(1):1554-1569. doi:10.30491/jabr.2024.467256.1755

Introduction

The versatility of microalgae in producing valuable compounds in various environmental conditions has garnered more attention. Microalgae are adaptive microorganisms capable of producing various biologically active metabolites that can be optimized in controlled conditions.[1] It has been reported that over 15,000 novel compounds were identified from diverse species of algae.[2,3] These compounds can be used to develop many bioproducts such as nutraceuticals, pharma ceuticals, cosmetics, and health supplements.[4] Among the valuable compounds derived from microalgae are pigments, lipids, vitamins, proteins, polysaccharides, and chlorophylls.[5] These bioactive compounds such as phenolics and carotenoids from microalgae often possess several biological properties that can be beneficial to human health, including antioxidant, antimicrobial, anti-diabetic, anti-inflammatory, and anti-obesity properties.[6]

Several growth parameters including nutrient supplemen tation, salinity level, light intensity, temperature, and radiation have been studied and optimized to modulate the accumulation of secondary metabolites in microalgae.[7] Nutrient supplemen tation can significantly influence the production of specific metabolites in microalgae.[8] It has been shown that nutrient supplementation in microalgae cultivation plays a crucial role in influencing their productivity and biological activities.[9] The excessive and limited use of different nutrients supplements in the cultivation media can trigger the

synthesis of various bioactive compounds. Previous studies have shown that nitrogen limitation may promote the production of a certain class of lipids including 16-carbon fatty acids and fewer polyunsaturated fatty acids, which are valuable for biofuel production.[10] Conversely, nitrogen-rich conditions may enhance the synthesis of proteins.[11] Moreover, supplementation of several carbon sources may enhance lipid accumulation within algae cells in an open pond cultivation.[12] Importantly, nutrient manipulation in microalgae cultivation media can be employed to enhance the production of bioactive compounds with therapeutic or commercial value. It has been shown that increasing the availability of certain nutrients can boost the production of antioxidants or pharmaceutical precursors.[13]

The supplementation of carbon and nitrogen sources has been proven to enhance microalgae growth during cultivation. It has been found that carbon sources can improve microalgae metabolism, uptake of other nutrients such as phosphate and nitrogen, and biochemical composition.[14,15] Among all carbon sources, sodium acetate is one of the most effective for cultivating microalgae and has been widely used. Sodium acetate helps maximize microalgae growth and the production of lipids in *Dunaliella salina*.[16] The capability of sodium acetate in enhancing microalgae growth and lipid production was also proven in the cultivation of *Micractinium reisseri* FM1.[17] The same study also found that pigment production in the microalgae increased when sodium acetate was supplied during cultivation. The supplementation of external sodium acetate also enhanced the biochemical compositions in terms of DHA and fatty acids production in *Crypthecodinium* sp. Cultivation.[18] The supplemented cultivation of the rising star in the phycology industry, *Haematococcus pluvialis*, was also conducted under sodium acetate supplementation, and the study also discovered that sodium acetate enhanced biomass and pigment production.[19] The production of biomass and pigments in other microalgae such as *Scenedesmus obliquus* and *Chlorella vulgaris* was also improved when microalgae were cultivated under the supplementation of sodium acetate compared to cultivation without the supplementation.[20,21]

Other than carbon, nitrogen sources are also one of the main components in microalgae cultivation as they can improve biomass production and growth rate in microalgae.[22] Sodium nitrate is the most common nitrogen source in microalgae cultivation since it is the most stable and preferred form of nitrate for microalgae assimilation during their growth.[23,24] Previous studies have also discovered the advantages of sodium nitrate supplementation in microalgae cultivation such as in *Nannochloropsis oceanica* where the microalgae showed gradual growth and improved protein production compared to cultivation without nitrate supplementation.[25] Moreover, the growth and cell density of *Isochrysis galbana* were enhanced under the supplementation of sodium nitrate.[26] The growth of other microalgae, such as *Tetraselmis* sp., *Scenedesmus bijµgatus*, *Tetradesmus obliquus*, and *Coelastrella sp.* was also improved during cultivation with sodium nitrate supplementation, proveding the capability of sodium nitrate in maximizing the growth of microalgae.[23,27,28]

Marine microalgae are renowned for their production of bioactive compounds, driving exploration for nutraceutical and pharmaceutical applications. A green marine microalga, *Tetraselmis suecica* belonging to the class Chlorophyceae, is rich in beneficial compounds such as carotenoids, vitamins, and lipids.[29] *Tetraselmis suecica* is widely utilized in aquaculture as feedstocks for molluscs and fishes.[30] Previous reports have discovered that *Tetraselmis suecica* extracts possess several biological activities including antimicrobial, anti-inflammatory, and antioxidant activities on human cancer cell lines.[31,32] However, most studies concerning the biological properties of secondary metabolites from *Tetraselmis suecica* have only been conducted using normal cultivation media without any modifications to nutritional supplementation. Therefore, the aim of the present study was to utilize *Tetraselmis suecica* to evaluate the effects of combined supplementation of carbon sources (molasses, glycerol, sodium acetate) and nitrogen sources (urea, ammonium sulfate, and sodium nitrate) toward secondary metabolite accumulation for biological evaluations. To this aim, the extracted microalgae biomass was first quantified, characterized, and evaluated for antioxidant, anti-inflammatory, and anti-obesity properties. In view of the initial evidence, this study provides a new dimension of microalgae cultivation combining carbon and nitrogen supplementations to enhance biomass and bioactive metabolite accumulation for biological significance.

Materials and Methods

Materials

The culture of a green marine microalga, *Tetraselmis suecica*, was obtained from the School of Industrial Technology, Universiti Sains Malaysia (USM). RAW 264.7 macrophage (CL0190) was purchased from Elabscience. (USA). Gallic acid, quercetin, porcine pancreatic lipase, 4-nitrophenyl butyrate, and astaxanthin were purchased from Sigma Aldrich (USA). Orlistat, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid (ABTS), butylated hydroxytoluene (BHT), and lipopolysaccharide (LPS) were purchased from Macklin (China). 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Merck (Germany), 2,4,6-Tris(2-pyridyl)-s-triazine (TPTZ) from TCI (Japan), aspirin and Dulbecco's Modified Eagle Medium (DMEM) from Gibco (New York), Griess reagent from Shanghai Yuanye (China), and Biolegend LEGEND MAX™ Mouse TNF-α ELISA Kit (#430907) from Biolegend (Canada). All other chemicals and solvents used in this study were analytical or HPLC grade.

Normal Batch Cultivation of Microalgae

Tetraselmis suecica was first cultivated using a batch cultivation mode with or without added supplementation of carbon and nitrogen sources in the F/2 Guillard's medium (Table 1). Meanwhile, the batch cultivation mode without nutrient supplementation serves as a control. All cultures were cultivated under standardized parameters, which are at 30 °C, with a light intensity of 1500 lux, aeration of 1 liter per minute oxygen, pH 8.0 and under a photoperiod of 12:12 light-dark cycle for 14 days. The absorbance reading of the cultures was measured daily at 680 nm, and the standard curve was plotted.

Table 1. The Comparison of Normal and Supplemented f/2 Guillard's Medium Composition for the Cultivation of *Tetraselmis suecica*

Composition	Normal	Supplemented with sodium acetate	Supplemented with sodium nitrate	Supplemented with combination of sodium nitrate and sodium acetate
Sodium phosphate monobasic dihydrate	5 g/L	5 g/L	5 g/L	5 g/L
Trace elements solution (EDTA Disodium Salt, Iron (III) Chloride Hexahydrate, Copper Sulphate Pentahydrate, Zinc Sulphate Heptahydrate, Cobalt Chloride Hexahydrate, Manganese (II) Chloride Tetrahydrate, Sodium Molybdate Dihydrate)	1 ml	1 ml	1 ml	1 ml
Vitamins solution (Thiamine-HCl (Vitamin B1), Biotin (Vitamin H), Cyanocobalamin (Vitamin B12))	1 ml	1 ml	1 ml	1 ml
Sodium nitrate	-	-	0.075 or 0.750 g/L	0.075 or 0.750 g/L
Sodium acetate	-	0.5 or 2.0 g/L	-	0.5 or 2.0 g/L

Added Supplementation of Sodium Acetate and Sodium Nitrate

The purpose of cultivating microalgae throμgh a batch cultivation mode with added nutrient supplementation was to increase the biomass concentration and enhance the accumulation of potential secondary metabolites from microalgae. The selection of added sodium acetate and sodium nitrate supplementation was mainly based on previous studies on the use of organic and inorganic supplementation to enhance cell growth and lipid production during microalgae cultivation.[33–38] These carbon and nitrogen sources were added individually at the beginning of the microalgae cultivation. The amount of these carbon and nitrogen supplementations was set at high and low concentrations in accordance with the literature; urea (0.025 and 0.200 g/L), sodium nitrate (0.075 and 0.750 g/L), ammonium sulfate (0.010 and 1.000 g/L), glycerol (0.250 and 0.750 ml/L), sodium acetate (0.500 and 2.000 g/L), and molasses (0.05% and 0.45% v/v).[33–38] The dry cell weight for each cultivated with and without added carbon and nitrogen supplementations was measured based on the standard curve of *Tetraselmis suecica* cultures against absorbance reading at 680 nm. The supplemented group from each carbon and nitrogen source with the highest microalgae dry cell weight was selected for combined supplementations.

Sequential Solvent Extraction

Sequential solvent extraction method was conducted to obtain crude extracts from microalgae using different types of solvents with increasing polarity (hexane < ethyl acetate < ethanol). Briefly, mixtures of microalgae biomass and solvent were sonicated for 30 minutes at 30 °C. The samples were then centrifμged at 4000 rpm for 4 minutes. The supernatant was filtered, collected, and the pellet was mixed again with solvent (30 ml). The mixture was centrifμged at 4000 rpm for 4 minutes, and these steps were repeated two times. All the supernatant was collected and dried under a fume hood overnight at room temperature. The temperature was kept below 30 °C to avoid degradation of secondary metabolites. The samples were then stored at -20 °C for further analysis.

Total Phenolic Content

Total phenolic content was determined using a method described with slight modifications.[39] Firstly, Folin-Ciocalteu's reagent was prepared by diluting 10% of the reagent with deionized water. The crude extract with a concentration of 0.2 mg/ml (0.5 ml) was added to the prepared Folin-Ciocalteu's reagent (2.5 ml). The mixture was incubated at room temperature for 5 minutes in a dark condition. Then, 20% sodium carbonate (2 ml) was added for 30 minutes of incubation at room temperature in a dark condition. After the incubation, the absorbance reading was measured at 765 nm. All tests were done in triplicates. The standard curve of gallic acid was used to determine the total content of phenolics in the samples, which can be expressed as μg of gallic acid equivalent per mg of extract (μg GAE/mg extract). The following formula was used to calculate the total phenolic content:

$$\text{Total phenolic content } (\mu\text{g GAE/mg extract}) = c \times \frac{v}{m}$$

Where *c* represents concentration obtained from standard curve, v is volume of extract used (ml) and m is mass of extract used (mg).

Ferric Reducing Antioxidant Power (FRAP) Assay

The antioxidant activity of microalgae extracts was evaluated using the ferric reducing antioxidant power (FRAP) assay, following our previous method with some modifications.[39] Firstly, the FRAP reagent was prepared with a 10:1:1 ratio of 300 mM acetate buffer (pH 3.6), 10 mM 2,4,6-tris(2-pyridyl)-s-triazine (TPTZ) (in 40 mM HCl), and 20 mM ferric chloride, respectively. These solutions were placed in a 37 °C water bath before mixing. To prepare the 300 mM acetate buffer, 1.55 g of sodium acetate and 8 ml of glacial acetic acid were added to 250 ml of distilled water. The pH was adjusted to 3.6, and the remaining distilled water was added. For the 10 mM TPTZ solution, 0.047 g of TPTZ powder was added to 15 ml of 40 mM hydrochloric acid. Additionally, 0.08 g of ferric chloride hexahydrate was added to 15 ml of distilled water to prepare a 20 mM ferric chloride solution. In the FRAP assay, 100 µl of microalgae extract (1 mg/ml) was added to 3 ml of the FRAP reagent. The mixture was incubated at 37 °C for 30 minutes in the dark, and the absorbance was measured at 593 nm. Ascorbic acid served as the positive control, while acetate buffer was the negative control. A standard curve of ferrous sulfate was plotted in a concentration range of 0.001 to 1000 µg/ml. The antioxidant activity of the microalgae samples was expressed as ferrous equivalent (FE) in µg/ml based on the ferrous sulfate standard curve. All tests were conducted in triplicate.

ABTS Radical Scavenging Activity

The 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical scavenging activity was conducted following the method from reference.[40] The ABTS reagent consisted of a mixture of 7 mM ABTS and 2.45 mM potassium persulfate solutions in a 1:1 ratio. To prepare the 7 mM ABTS solution, 0.054 g of ABTS powder was added to 14 ml of methanol, while the 2.45 mM potassium persulfate solution was prepared by mixing 0.027 g of potassium persulfate with 14 ml of methanol. The mixture was then stored in the dark at room temperature for 12-16 hours. Subsequently, the mixture was diluted with methanol until the absorbance reading reached 0.700 ± 0.05 at 734 nm. For the assay, 20 µl of microalgae samples (1 mg/ml) were added to 200 µl of the ABTS reagent and incubated at room temperature for 10 minutes in the dark. The absorbance reading was then measured at 734 nm using a microplate reader. Butylated hydroxytoluene (BHT) was used as the positive control, while methanol served as the negative control. All tests were performed in triplicate. The ABTS radical scavenging activity (%) was calculated using the following formula:

$$\text{ABTS scavenging activity (\%)} = \left(\frac{\text{Absorbance of control} - \text{Absorbance of sample}}{\text{Absorbance of control}}\right) \times 100$$

DPPH Radical Scavenging Assay

The 2,2-diphenyl-1-picryl-hydrazyl-hydrate (DPPH) radical scavenging assay was conducted following a previous method with slight modifications[41]. Firstly, the DPPH reagent was prepared by dissolving 2 mg of DPPH powder in 50 ml of methanol. All microalgae crude extracts were weighed to 1 mg per tube and diluted in 1% DMSO. Six concentrations of each sample (1000, 100, 10, 1, 0.1, and 0.01 µg/ml) were prepared accordingly. 150 µl of extracts were mixed with 150 µl of DPPH reagent and incubated for 30 minutes at room temperature in a dark condition. The absorbance reading was measured at 517 nm using a microplate reader. All tests were done in triplicates. The DPPH scavenging activity of the extracts was determined using the following formula:

$$\text{DPPH Scavenging activity (\%)} = \left(\frac{\text{Absorbance of control} - \text{Absorbance of sample}}{\text{Absorbance of control}}\right) \times 100$$

Pancreatic Lipase Inhibition Assay

The *in vitro* anti-obesity activity of microalgae extracts was carried out using a pancreatic lipase inhibition assay.[42] Tris-HCl buffer (0.0025 M, pH 7.4, 0.125 mM NaCl) was first prepared in deionized water. The porcine pancreatic lipase (PPL) was dissolved in Tris-HCl buffer (1 mg/ml). The substrate, p-nitrophenyl butyrate (PNPB) solution, was prepared in Tris-HCl buffer (0.00125 M). All microalgae crude extracts were weighed to 1 mg per tube and diluted in 1% DMSO. Six concentrations of each microalgae extract (1000 µg/ml, 100 µg/ml, 10 µg/ml, 1 µg/ml, 0.1 µg/ml, and 0.01 µg/ml) were prepared accordingly. Then, 100 µl of microalgae extract was added to 50 µl of PPL solution and incubated for 15 minutes at 37 °C. 100 µl of PNPB solution and 50 µl of Tris-HCl buffer were added to the mixture prior to a 1-hour incubation at 37 °C. The absorbance reading was measured at 405 nm. All tests were done in triplicates. The percentage inhibition of pancreatic lipase was then calculated using the formula:

$$\text{Inhibition percentage (\%)} = \left(\frac{\text{Absorbance of control} - \text{Absorbance of sample}}{\text{Absorbance of control}}\right) \times 100$$

Proteinase Inhibitory Activity

The inhibitory activity of proteinase was carried out in accordance with a method described by[43] with slight modifications. Briefly, 2 ml of trypsin (0.03 mg/ml), 1 ml of Tris-HCl buffer (20 mM, pH 7.4), 0.98 ml of methanol and 0.02 ml of microalgae samples (1 mg/ml) were dissolved and incubated at 37 °C for 5 minutes. To initiate the reaction, 1 ml of 0.8% casein was added and the mixture was incubated at 37 °C for 20 minutes. Lastly, 2 ml of 70% perchloric acid was added and the absorbance reading of the supernatant was measured at 210 nm. Aspirin was used as a positive control and buffer was used as a negative control. All tests were done in triplicates. The proteinase inhibitory activity was determined using the formula:

$$\text{Proteinase inhibitory activity (\%)} = \left(\frac{\text{Absorbance of control} - \text{Absorbance of sample}}{\text{Absorbance of control}}\right) \times 100$$

Nitric Oxide Production Assay

The anti-inflammatory activity of the microalgae extracts was assessed by measuring the production of nitric oxide by LPS-activated RAW 264.7 cells.[44] RAW 264.7 cells were seeded in a T75 flask and cultured in complete Dulbecco's Modified Eagle Medium (DMEM) consisting of incomplete DMEM, fetal bovine serum (FBS), and penicillin/ streptomycin (60:30:10) at 37 °C inside a CO2 incubator for 48 hours. Then, the cells were sub-cultured into a 96-well plate (200 µl per well) and incubated again at 37 °C for 24 hours at a density of 2×10^5 cells/well. After 24 hours, 100 µl of LPS solution (0.5 mg/ml) and 100 µl of microalgal extracts (0.001-0.1 µg/ml) were co-incubated for 24 hours at 37 °C. Then, 100 µl of the supernatant was collected and transferred into a new 96-well plate. 100 µl of Griess reagent (0.01 g/ml) was added to the supernatant and incubated for 10 minutes. The absorbance reading was measured at 540 nm. A standard curve of sodium nitrite was plotted ranging from 0.001 to 100 µg/ml. The amount of nitric oxide produced by the cells after sample treatment was calculated from the sodium nitrite standard curve, and the morphology of the cells was observed under an inverted microscope.

Quantification of TNF-α Using Enzyme-linked Immunosorbent Assay (ELISA)

The subsequent anti-inflammatory properties of the microalgae extracts were studied by quantifying the production of a pro-inflammatory cytokine, tumor necrosis factor-alpha (TNF-α), from RAW 264.7 cells. The concentration of TNF-α was determined using an ELISA kit, Biolegend LEGEND MAX™ Mouse TNF-α ELISA Kit (#430907), following the manufacturer's instructions. In brief, RAW 264.7 cells were seeded in a T75 flask and cultured in complete Dulbecco's Modified Eagle Medium (DMEM) at 37 °C inside a carbon dioxide incubator for 48 hours. Then, the cells were sub-cultured into a 96-well plate (200 µl per well) and incubated again at 37 °C for 24 hours at a density of 2×10^5 cells/well. After 24 hours, 100 µl of LPS solution (0.5 mg/ml) and 100 µl of microalgal extracts (0.001-0.1 µg/ml) were co-incubated for 24 hours at 37 °C. 50 µl of supernatant from the cells was collected and added to the ELISA pre-coated plate kit along with 50 µl of the assay buffer. The plate was sealed and incubated at 25 °C in an incubator shaker at 200 rpm for 2 hours. All solutions were discarded, and the plate was washed four times with washing buffer before adding the biotinylated antibody reagent. The plate was sealed and incubated in the incubator shaker at 25 °C for 1 hour. Then, the contents were discarded, and the plate was washed four times with the washing buffer. The avidin-HRP solution (100 µl) was added to the wells, and the plate was incubated again for 30 minutes at 25 °C. The contents were then discarded, and the plate was washed five times. 100 µl of substrate, di-(2-ethylhexyl)-2,4,5-trimethoxy benzalmalonate (TMB), was added to each well, and the plate was incubated for 15 minutes at room temperature in the dark. The blue color was observed in the wells containing TNF-alpha cytokines. Lastly, 100 µl of stop solution was added to the wells. The absorbance reading was measured at 450 nm within 30 minutes. The standard curve of the lyophilized Mouse TNF-α standard provided by the kit was plotted, and the production of TNF-alpha cytokines was determined from the standard curve. All tests were done in duplicates.

Fourier-Transform Infrared Spectroscopy (FTIR)

The FTIR analysis was performed using a Shimadzu IR-Prestige-21 Fourier Transform Infrared Spectrophotometer to elucidate the functional groups and structural changes of the compounds within the samples. There were two types of microalgae samples used for FTIR analysis: biomass powder and crude extract. The biomass powder was heated at 60 °C overnight, and the crude extracts were dissolved in solvents (hexane, ethyl acetate and ethanol) prior to the analysis. The biomass powder (1 mg) was ground with potassium bromide (900 mg). The mixture was then pressed until it formed a circular disk before the measurement. The FTIR spectra were recorded in the spectral range from 40-400 cm^{-1}.

Liquid Chromatography with Mass Spectrometry (LC-MS)

The metabolic profiling of secondary metabolites via liquid chromatography with tandem mass spectrometry (LC-MS) was conducted in accordance with the previous method.[45] The analysis was carried out using LC (Waters Acquity UPLC) with MS (Waters XEVO-TQS Micro), containing a C18 column (Waters, 2.10×100 mm) equipped with an autosampler (Waters Acquity FTN) and a column manager (Waters Acquity CM). MassLynx software was used for data analysis. The capillary voltage was 1.50 kV, and the cone voltage was 40 V. The source temperature was 150 °C, desolvation temperature was 500 °C, and desolvation gas flow was 1000 liters per hour. The target column temperature was 40 °C, and the target sample temperature was 20 °C. The concentration of the samples used was 1 mg/ml, and the samples were dissolved in methanol prior to the analysis. All samples were filtered with a nylon filter with a pore size of 0.22 µm before the analysis. The analysis was conducted using acetonitrile as eluent A, 0.1% formic acid as eluent B, water as eluent C, and methanol as eluent D. The flow rate was set to 0.3 ml per minute, and the volume of samples used was 1.0 µl per injection. The elution gradient program was set as follows: 0-10 min, 85% B, 15% D; 10-15 min, 60% B, 40% D; 15-18 min, 85% B, 15% D. The total elution time was 18 minutes. The scanning range was set from 300 to 900 m/z in positive ionization mode.

Statistical Analysis

All values were expressed as Mean ± Standard Deviation.

The statistical differences of the values for all tests were evaluated by performing Analysis of Variance (ANOVA) and post-hoc test, Tukey's Test. The value of $p<0.05$ was used to determine the significant difference of all values.

Results

The Combined Supplementation of Carbon and Nitrogen Sources Enhanced Dry Cell Weight of Microalgae

As shown in Figure 1a, the addition of 2.0 g/L of sodium acetate increased the dry cell weight to 0.974 ± 0.04 g/L after 14 days of microalgae cultivation, compared to normal cultivation. Figure 1b demonstrates that the supplementation of 0.750 g/L of sodium nitrate resulted in the highest dry cell weight of 0.344 ± 0.03 g/L, surpassing normal cultivation (0.261 ± 0.06 g/L). High concentrations of sodium nitrate and sodium acetate were then combined for supplementation. Figure 1c illustrates that the combined supplementation significantly enhanced the cell dry weight to 1.095 ± 0.03 g/L compared to single supplementation with sodium acetate or sodium nitrate.

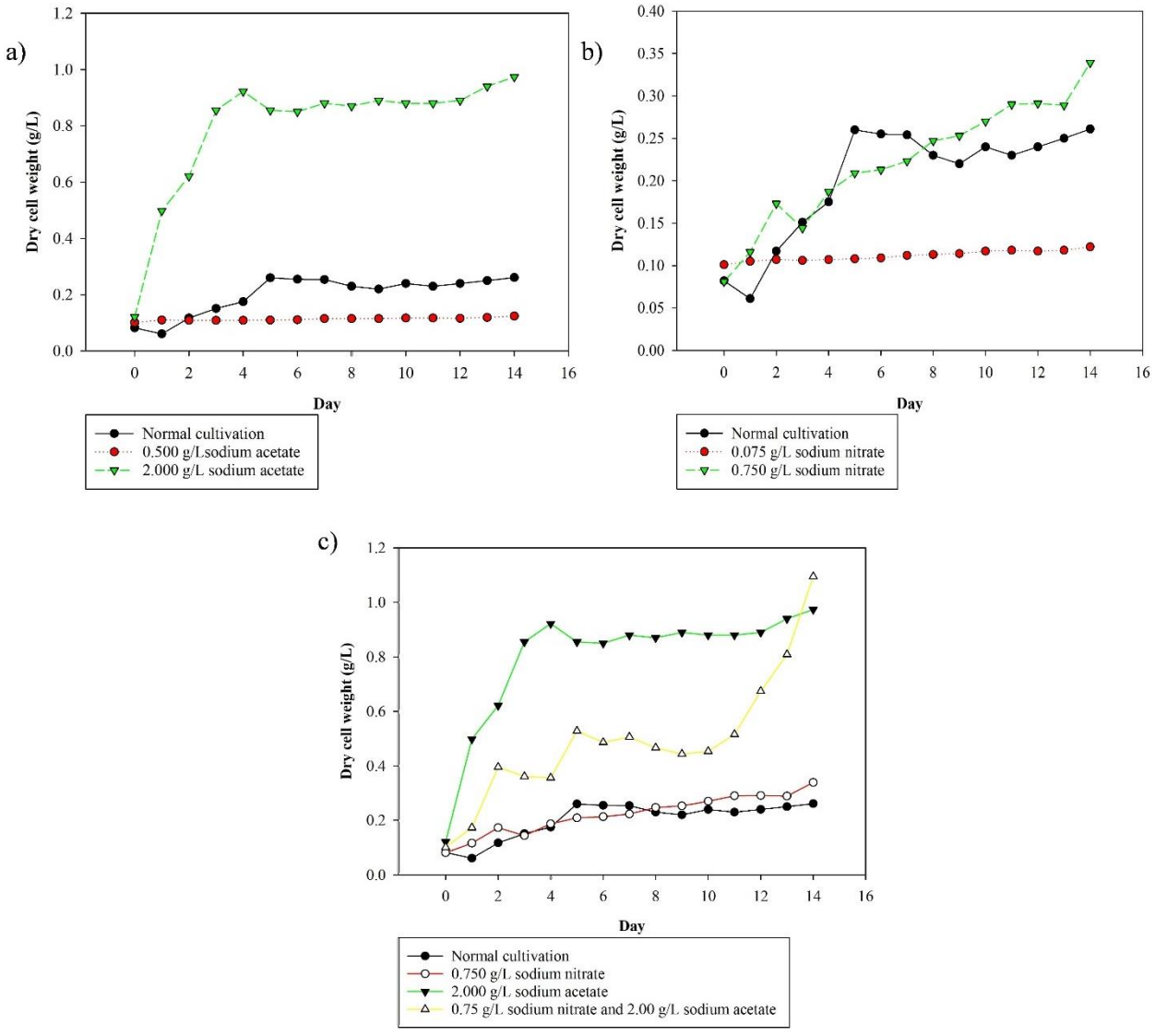

Figure 1. The Growth Profile of *Tetraselmis suecica* under the Single and Combined Supplementation of Sodium Acetate. (**a:** sodium acetate; **b:** sodium nitrate; **c:** combination of sodium acetate and sodium nitrate). All results are expressed as Mean ± Standard deviation.

Microalgae Extract of Combined Supplementations Enhanced Total Phenolic Contents and Antioxidant Properties

Measuring the total phenolic content (TPC) in microalgae can provide insights into the antioxidant potential and health-promoting properties of the microalgae biomass. The crude extract from microalgae biomass cultivated under combined supplementation of sodium acetate and sodium nitrate was obtained using a sequential solvent extraction (hexane, ethyl acetate, and ethanol). According to Figure 2a, the ethanol extract from combined supplementations demonstrated a higher total phenolic content (591.80 ± 0.92) compared to the ethanol extract of normal cultivation

(382.20 ± 0.69). Furthermore, the ethanol extract from the supplemented group exhibited the highest FRAP reducing ability (152.03 ± 5.84 FE µg/ml) compared to the same extract from the control group (78.70 ± 1.90 FE µg/ml) and other extracts as shown in Figure 2b.

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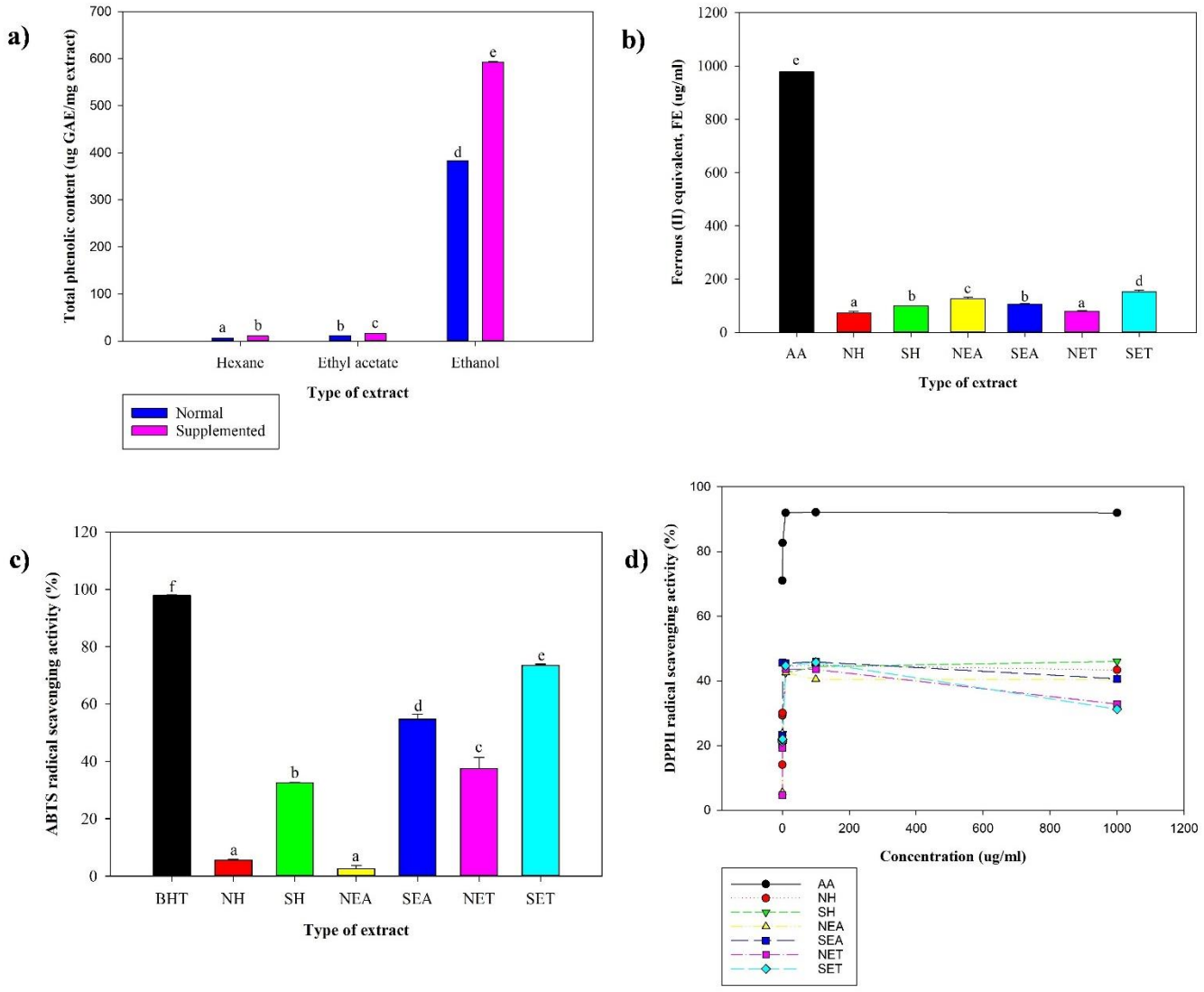

Figure 2. The Total Phenolic Content and Antioxidant Activities in *Tetraselmis suecica* Extracts Derived from Normal and Supplemented Cultivation. (**a:** total phenolic content; **b:** FRAP assay; **c:** ABTS assay; **d:** DPPH assay. AA: Ascorbic acid, BHT: Butylated hydroxytoluene, NH: normal hexane, NEA: normal ethyl acetate, NET: normal ethanol, SH: supplemented hexane, SEA: supplemented ethyl acetate, SET: supplemented ethanol). All results are expressed as Mean ± Standard deviation. Values in each column which have different letters are significantly different (p<0.05).

Ethanol Extract of Combined Supplementation Enhanced Inhibitory Effect against Pancreatic Lipase Activity

Pancreatic lipase enzyme is one of the digestive enzymes in our body that plays an important role in hydrolyzing almost 50-70% of total dietary fats[47]. In the present study, we measured the inhibitory effect of microalgae extracts derived from *Tetraselmis suecica* cultivated under combined supplementations of sodium acetate and sodium nitrate. As shown in Figure 3, the ethyl acetate extract from the supplemented group exhibited the highest inhibitory activity against pancreatic lipase (76.93 ±

0.14%) compared to other extracts. This sμggests that combined supplementation of sodium acetate as a carbon source and sodium nitrate as a nitrogen source during *Tetraselmis suecica* cultivation improved the anti-obesity activity of the microalgae extract. Table 3 shows the IC_{50} values based on pancreatic lipase inhibition of *Tetraselmis suecica* extracts under normal and supplementation cultivation conditions. Among all extracts, the ethanol extract from the supplemented group showed the lowest IC_{50} value at 23.83 μg/ml compared to other extracts from both normal and combined supplemented cultivation conditions. Thus, the present study discovered a new insight into the anti-obesity properties of *Tetraselmis suecica* extract derived from a batch cultivation mode under combined carbon and nitrogen supplementations.

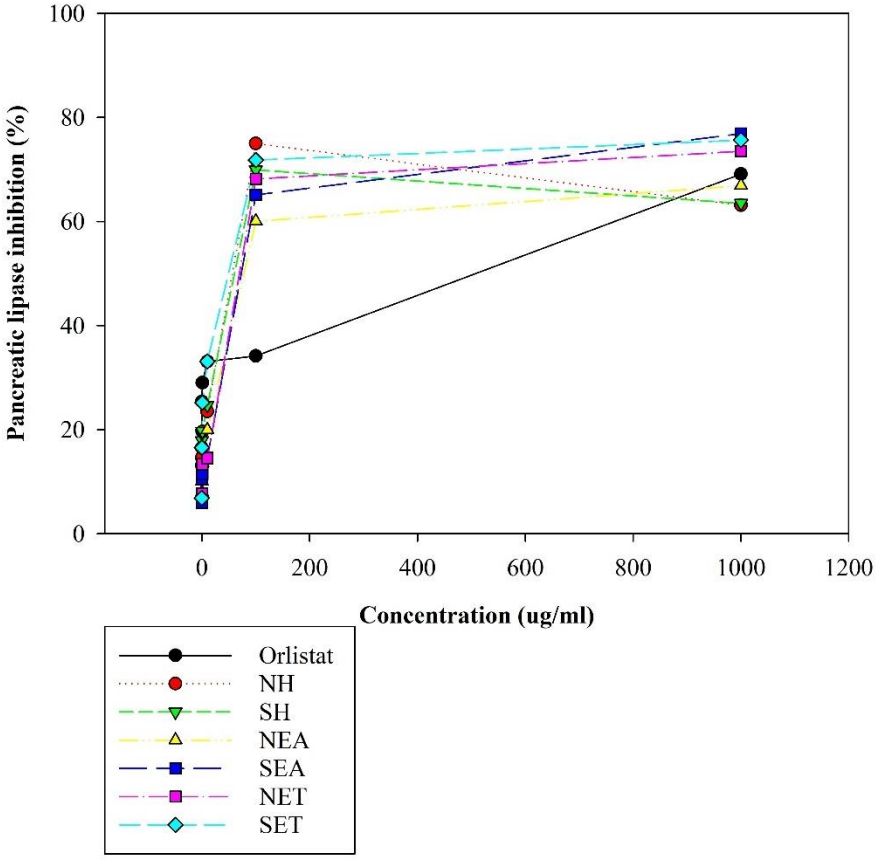

Figure 3: The Inhibition of *Tetraselmis suecica* Extracts against Pancreatic Lipase (NH: normal hexane, NEA: normal ethyl acetate, NET: normal ethanol, SH: supplemented hexane, SEA: supplemented ethyl acetate, SET: supplemented ethanol). All results are expressed as Mean ± Standard deviation.

Ethyl Acetate Extract of Combined Supplementation Enhanced the Anti-inflammatory Properties

We next evaluated the anti-inflammatory properties of microalgae extracts using proteinase inhibitory activity, nitric oxide production assay, and measurement of TNF-α levels. As depicted in Figure 4a, the ethyl acetate extract (1 mg/ml) from the supplemented group exhibited the highest inhibitory activity against proteinase denaturation (45.27 ± 0.02%) compared to other extracts. All extracts from both the control and supplemented groups exerted anti-inflammatory activity by lowering the production of nitric oxide in LPS-activated cells (Figure 4b). TNF-α is a pro-inflammatory cytokine produced by macrophages in response to various stimuli, including microbial pathogens and inflammatory mediators. The present study demonstrated that the ethyl acetate extract from the supplemented group decreased the production of the pro-inflammatory cytokine TNF-α in LPS-activated cells (Figure 4c).

This finding was in line with our morphological observation, in which the inflamed RAW 264.7 macrophages often changed from circular cells into dendritic-like and irregularly shaped cells upon LPS stimulation. However, when the cells were treated with microalgae extracts, the number of dendritic-like and irregularly shaped cells was reduced, indicating that the treatment of microalgae extracts on the macrophages reduced the inflammatory effects of the cells (Figure 5).

Chemical Characterization of the Tetraselmis suecica Extracts

The FTIR analysis is widely used to characterize the chemical compounds present in the extracts based on the

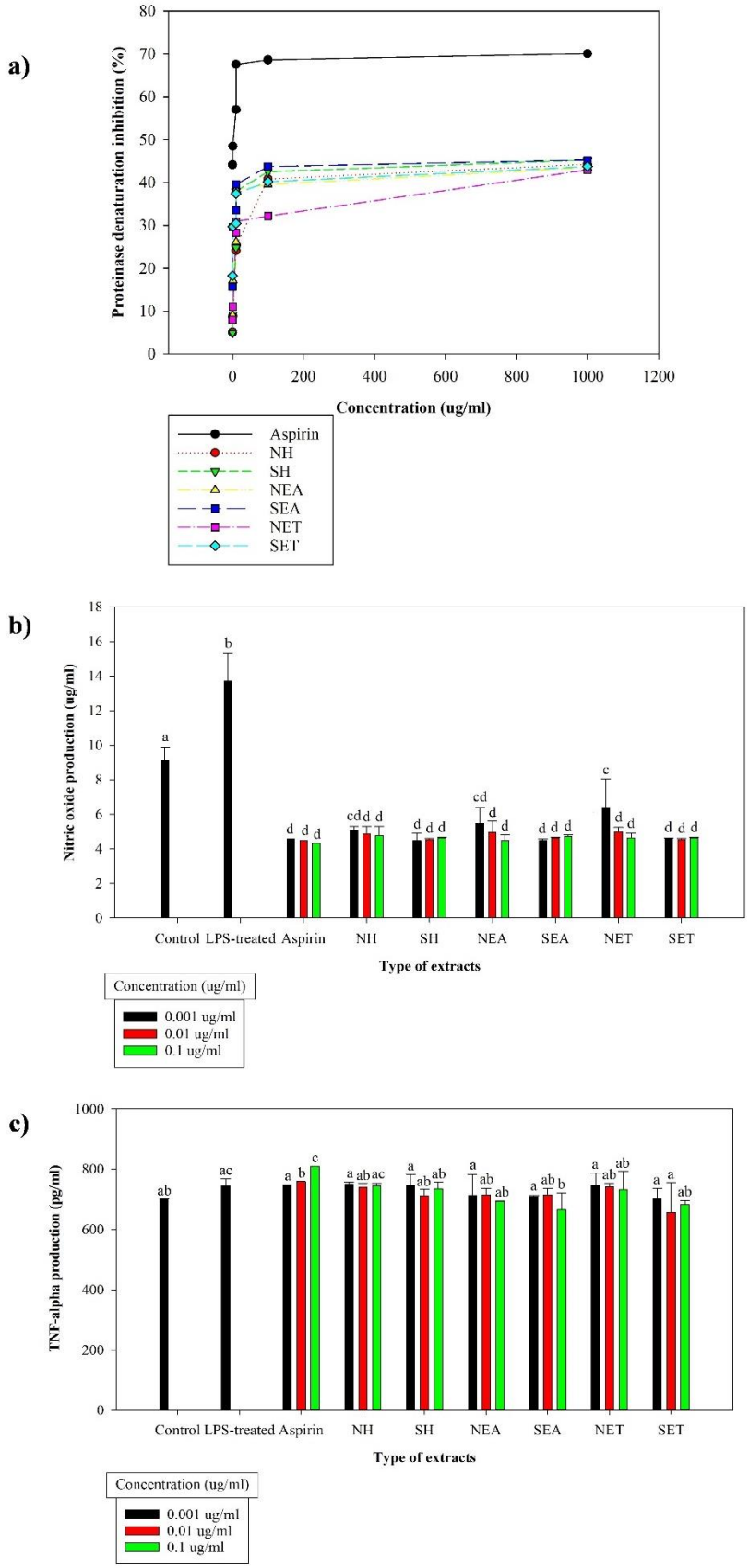

Figure 4. The Anti-inflammatory Activities of *Tetraselmis suecica* Extracts. (**a:** proteinase denaturation inhibition; **b:** nitric oxide production; **c:** TNF-alpha production. NH: normal hexane, NEA: normal ethyl acetate, NET: normal ethanol, SH: supplemented hexane, SEA: supplemented ethyl acetate, SET: supplemented ethanol). All results are expressed as Mean ± Standard deviation. Values in each column which have different letters are significantly different (p<0.05).

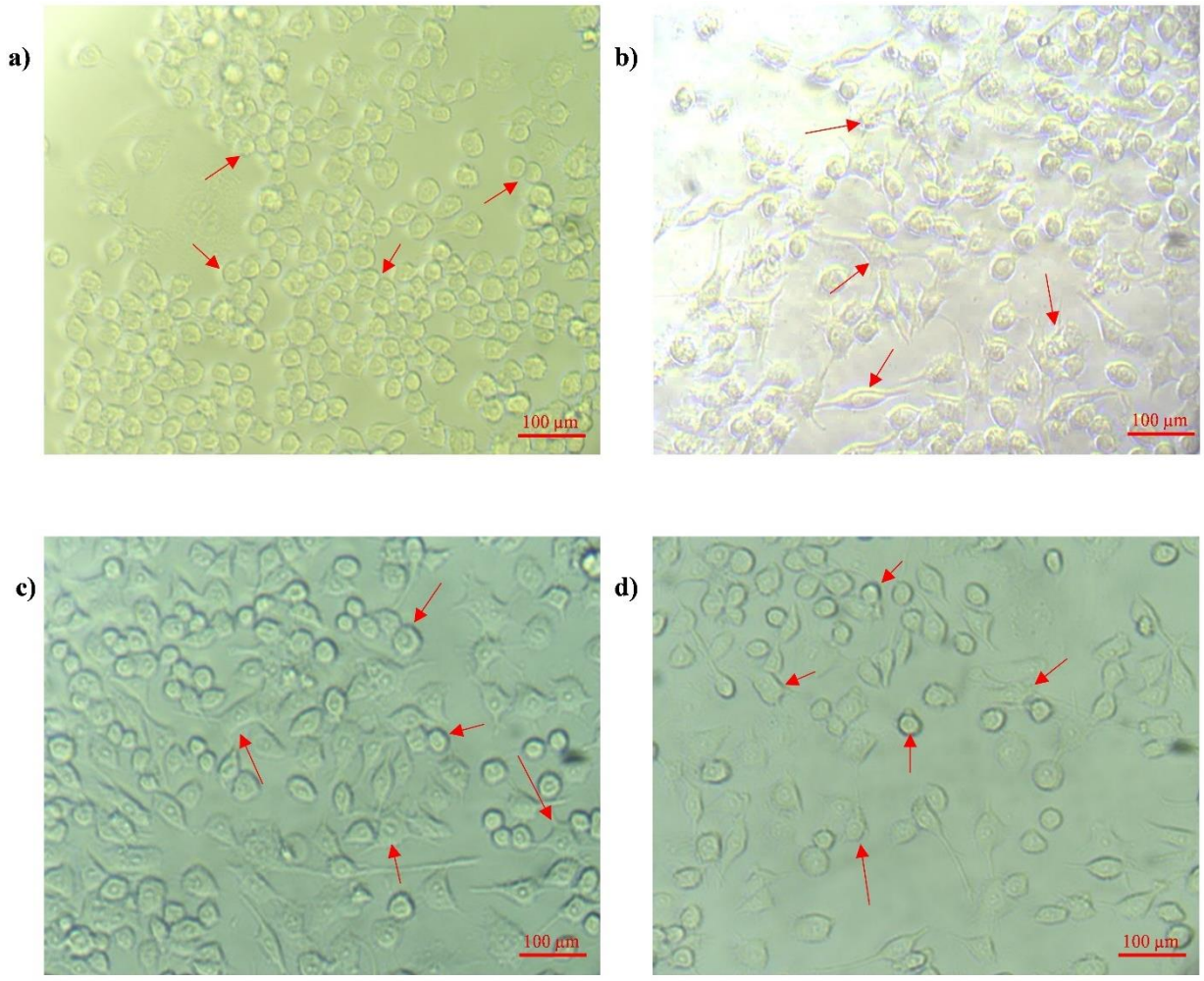

Figure 5. Cell Morphology after 24 hours of Coincubation of LPS and *Tetraselmis suecica* Extracts. (**a:** Control group: cells were circular and regular in shape; **b:** LPS-treated group: cells were irregular in shape and turned into dendritic-like cells; **c:** normal cultivation extract-treated group: the mixture of circular and dendritic -like cells; **d:** supplemented cultivation extract-treated group: the mixture of circular and dendritic-like cells) (magnification 20×0.40).

Table 2. The IC_{50} Values based on DPPH Radical Scavenging Activity of Microalgae Extracts of *Tetraselmis suecica* Cultivated under Normal and Supplemented Cultivations

Sample	IC_{50} (ug/ml)
AA	0.0002
NH	162.10
NEA	351.10
NET	207.70
SH	290.20
SEA	123.00
SET	164.10

AA: ascorbic acid, NH: normal hexane, NEA: normal ethyl acetate, NET: normal ethanol, SH: supplemented hexane, SEA: supplemented ethyl acetate, SET: supplemented ethanol.

Table 3. The IC_{50} values based on pancreatic lipase inhibition activity of *Tetraselmis suecica* microalgae extracts cultivated under normal and supplemented cultivations

Sample	IC_{50} (ug/ml)
Orlistat	183.20
NH	50.02
NEA	98.80
NET	64.48
SH	58.53
SEA	66.95
SET	23.83

AA: ascorbic acid, NH: normal hexane, NEA: normal ethyl acetate, NET: normal ethanol, SH: supplemented hexane, SEA: supplemented ethyl acetate, SET: supplemented ethanol.

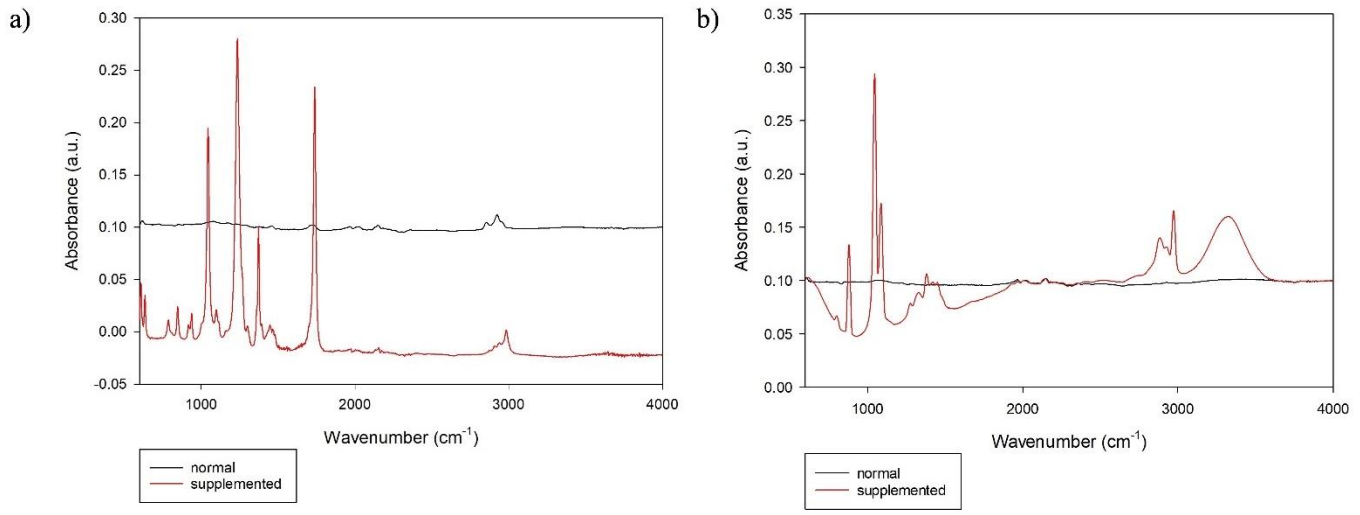

Figure 6. FTIR Analysis of *Tetraselmis suecica* Extracts from Normal and Supplemented Cultivation. (**a:** ethyl acetate extract; **b:** ethanol extract).

presence of functional groups[48]. The FTIR spectra showed differences between the functional groups present in the ethyl acetate extracts of the normal and supplemented groups (Figure 6). It was shown that various peaks mostly appeared in the supplemented group compared to the normal group. The FTIR spectra of crude extracts revealed the presence of C-Br, C-Cl, C=C, CO-O-CO, C-OH, C-N, S=O, C=O, and C-H functional groups in the supplemented ethyl acetate extract, while the presence of C-Cl, CO-O-CO, C-O, C-N, C=C=C, C-H, and N-H functional groups in the ethanol extract. Table 4 shows that the ethyl acetate extract from the supplemented group contains 8 functional groups consisting of halocarbons, alkenes, anhydrides, secondary alcohols, alkanes, amines, sulfonate, and aldehydes. Besides, the ethanol extracts from the supplemented group revealed the presence of seven functional groups, which are halocarbons, aromatic esters, aromatic amines, allenes, amine salts, alkanes, and alkynes.

Table 4. The Characterization of Functional Groups in Ethyl Acetate and Ethanol Extracts by FTIR Analysis

Frequency range	Absorption	Functional groups	Compound class	NEA	SEA	NET	SET
500-750	623.65	Strong C-Br	Halocarbon	-	+	-	-
600-800	786.96	Strong C-Cl	Halocarbon	-	+	-	-
	617.22	Strong C-Cl	Halocarbon	-	-	-	+
960-980	985.48	Strong C=C bending	Alkene	-	+	-	-
1040-1050	1045.49	Strong CO-O-CO stretching	Anhydride	-	+	-	-
	1045.42	Strong CO-O-CO stretching	Anhydride	-	-	-	+
1087-1124	1099.43	Strong C-OH stretch	Secondary alcohol	-	+	-	-
1020-1250	1234.44	Medium C-N stretching	Amine	-	+	-	-
1250-1310	1276.88	Strong C-O stretch	Aromatic ester	-	-	-	+
1266-1342	1328.95	Strong C-N stretching	Aromatic amine	-	-	-	+
1335-1372	1371.39	Strong S=O stretching	Sulfonate	-	+	-	-
1735-1750	1737.56	Strong C=O stretching	Aldehyde	-	+	-	-
1900-2000	1932.67	Medium C=C=C stretching	Allenes	-	-	-	+
2800-3000	2972.31	Strong N-H stretching	Amine salt	-	-	-	+
2840-3000	2883.58	Medium C-H stretching	Alkane	-	-	-	+
	2910.58	Medium C-H stretching	Alkane	-	+	-	-
2840-3000	2939.52	Medium C-H stretching	Alkane	-	+	-	-
2840-3000	2983.88	Medium C-H stretching	Alkane	-	+	-	-
3267-3333	3325.28	Strong, sharp C-H stretching	Alkyne	-	-	-	+

NEA: normal ethyl acetate, SEA: supplemented ethyl acetate, NET: normal ethanol, SET: supplemented ethanol, -: absent, +: present.

Table 5. Tentative Identification of Secondary Metabolites in Ethyl Acetate and Ethanol Extracts by LC-MS Analysis

No.	Compound name	Theoretical molecular mass $[M+H]^+$	Experimental molecular mass $[M+H]^+$	Retention time (min)	Molecular formula	NEA	SEA	SET	Ref
1	Alpha-carotene	537.45	537.88	8.57	$C_{40}H_{56}$	-	+	+	72
2	Cantaxanthin	565.40	565.85	10.38	$C_{40}H_{52}O_2$	-	+	+	72
3	Rutin	611.16	611.53	10.45	$C_{27}H_{30}O_{16}$	-	+	+	72
4	Quercitrin	449.11	449.39	10.88	$C_{21}H_{20}O_{11}$	-	+	+	72
5	Apigenin-*O*-rutinoside	579.17	579.34	11.08	$C_{27}H_{30}O_{14}$	-	+	+	72
6	Caffeoylglucoside	359.10	359.31	14.15	$C_{15}H_{18}O_{10}$	-	+	+	72

NEA: normal ethyl acetate, SEA: supplemented ethyl acetate, SET: supplemented ethanol, -: absent, +: present.

Tentative chemical profiling was carried out via LC-MS analysis to characterize possible chemical constituents present in ethyl acetate (NEA; SEA) and ethanol (SET) extracts for normal and supplemented groups, respectively. As tabulated in Table 5, the LC-MS analysis revealed the presence of six chemical constituents that were annotated in supplemented ethyl acetate and supplemented ethanol extracts, suggesting the presence of bioactive components in the extracts of *Tetraselmis suecica* cultivated under varying carbon and nitrogen supplementations.

Discussion

Tetraselmis suecica is a marine green microalga that has been widely utilized in the aquaculture industry as feedstock for molluscs and fishes due to its high content of good quality protein.[30,49] The present study aimed to explore the potential antioxidant, anti-obesity, and anti-inflammatory effects of microalgae extracts derived from *Tetraselmis suecica*, which were cultivated under varying combinations of carbon and nitrogen sources. In essence, the microalgae were grown in culture media supplemented with sodium acetate as a carbon source and sodium nitrate as a nitrogen source. The resulting biomass was quantified, and crude extracts were then obtained for further analysis, focusing on the measurement of total phenolic content. We demonstrated that sodium acetate at a concentration of 2 g/L exhibited the highest cell dry weight. A previous report showed that sodium acetate is highly beneficial as a carbon source in improving the biomass and lipid productivity of the microalgae *Chlorella pyrenoidosa* via mixotrophic cultivation mode.[50] Another study demonstrated that acetate can be used as a carbon source for the cultivation of *Scenedesmus abundans*, where the highest biomass was recorded. This is because the acetate from sodium acetate supplementation was utilized to form acetyl Co-A, which then acts as a precursor to improve carbon metabolic flux, leading to improved biomass production.[51] Considering this, sodium acetate was selected as the preferred carbon source as it can enhance biomass production and is also biodegradable, providing less and safer toxicity to the environment compared to other selected carbon sources.[52] Due to its favorable biomass production and safer characteristics, sodium acetate can be considered a viable supplementation in *Tetraselmis suecica* cultivation.

Nitrate is widely used in microalgae cultivation to improve microalgae growth and biochemical composition.[23,24] The supplementation of nitrate into *Isochrysis* sp. cultivation increased biomass production and cell density.[53] Nitrogen sufficiency in the cultivation of *Chlorella zonfingiensis* also improved growth and chlorophyll production.[54] Furthermore, an increasing supplementation of nitrate into *Isochrysis galbana* significantly increased cell density.[26] This was supported by a study where the increasing amount of sodium nitrate supplementation promoted notable growth in *Scenedesmus obliquus* compared to ammonium chloride supplementation in terms of cell density.[55] The cultivation of microalgae supplemented with nitrate also enhanced cell growth in Tetradesmus obliquus IS2 and *Coelastrella* sp. IS3 compared to ammonium supplementation.[28] In this context, we further selected sodium nitrate at a concentration of 0.750 g/L for the cultivation of *Tetraselmis suecica* considering its enhancement properties on dry cell weight of microalgae biomass.

After a single supplementation of carbon and nitrogen sources, high concentrations of sodium nitrate and sodium acetate were selected for a 14-day cultivation of microalgae. It was shown that the combination of these two added supplements improved the cell dry weight of microalgae biomass compared to single supplementation. The combination of total organic carbon and total nitrogen sources gradually improved the biomass yield, nutrient uptake, and lipid production in 6 out of 13 strains of *Chlorella* sp. Microalgae.[56] In another study, the combined supplementation of carbon (sucrose) and nitrogen (nitrate) also resulted in the maximum biomass and lipid production in *Chlorococcum* sp. cultivation compared to the single supplementation of the aforementioned carbon and nitrogen sources.[57] Moreover, the combined supplementation of sodium acetate (0.7 g/L) and sodium nitrate (9.5 ml/L) improved the final biomass production and total carotenoids content.[58] In this regard, the present study provides a strong new approach in combining the selected carbon and nitrogen sources into one time batch cultivation of microalgae for the improvement of biomass production at the end of cultivation.

Subsequently, extracts derived from cultures supplemented with combinations of sodium acetate and sodium nitrate that exhibited the highest biomass yield and phenolic content were selected for the biological evaluations. Crude extracts were obtained through solvent extraction, with the ethanol extract from supplemented cultures showing significantly higher total phenolic content compared to normal cultivation. Ethanol was found to be effective in extracting phenolic compounds due to its polar properties. A previous report on *Tetraselmis suecica* extracts has highlighted their antioxidant activity, particularly through DPPH and ABTS radical scavenging assays.[59] However, most studies previously focused on normal cultivation conditions, leaving a gap in understanding antioxidant activities under nutrient supplemented conditions.

To further validate antioxidant properties, ABTS radical scavenging activity was employed. Ethanol extracts from supplemented cultures exhibited significantly higher ABTS scavenging activity compared to those from normal cultivation, indicating superior antioxidant properties. A previous study showed that the microalgae extract of *Tetraselmis suecica* with highest ferric reducing antioxidant power (FRAP) value and ABTS radical scavenging activity

was due to the lutein content in the extract.[29] Similarly, ethanol extracts from supplemented cultures showed the highest DPPH scavenging activity, further confirming their potent antioxidant capacity compared to other extracts. The study demonstrated that combined supplementation of sodium acetate and sodium nitrate positively influenced the antioxidant activity of *Tetraselmis suecica* extracts. The supplementation of nutrients specifically nitrogen and carbon sources exerted beneficial effects on microalgae's antioxidant properties. For example, the addition of nitrate in Walne culture medium for the marine microalga *Isochrysis* sp. reduced oxidative stress in the cells. Additionally, the supplementation of nitrate into the same microalgae cultivation also increased the accumulation of metabolites such as eicosapentaenoic acid.[53] The supplementation of nitrate specifically sodium nitrate in the cultivation of *Nephroselmis sp.* N3C46 considerably enhanced antioxidant capacity due to a higher amount of the total carotenoid contents.[60] Overall, the current study provides valuable insights into the antioxidant potential of *Tetraselmis suecica* extracts under combined nutrient supplementation, emphasizing the importance of cultivation conditions in optimizing secondary metabolite production and antioxidant activity.

Subsequently, these extracts were assessed for their potential antioxidant, anti-obesity, and anti-inflammatory properties through in vitro experiments. A pancreatic lipase inhibition assay was utilized to assess the anti-obesity activity of the extracts. Results demonstrated that the ethyl acetate extract from the supplemented group exhibited the highest inhibitory activity against pancreatic lipase, indicating improved anti-obesity activity compared to normal cultivation extracts. This finding aligns with a previous report,[61] which shown the anti-obesity potential of microalgae extracts through pancreatic lipase inhibition. Another report revealed that the fractions of *Tetraselmis suecica* extracts derived from normal cultivation exhibited an excellent effect on inhibiting adipocyte differentiation and promoting glucose absorption into the cells, reflecting the potential anti-obesity activity of *Tetraselmis suecica.*[62] Notably, the study introduced a novel insight into the use of combined nutrient supplementation for enhancing the anti-obesity properties of *Tetraselmis suecica* extracts. Furthermore, the study explored the anti-inflammatory potential of the extracts through proteinase denaturation inhibition assay, nitric oxide production, and TNF-α cytokine production in LPS-activated cells. A previous report indicated that ethyl acetate fractions from *Tetraselmis suecica* under normal cultivation resulted in lower production of TNF-alpha cytokines in RAW264.7 cells compared to hexane and methanol fractions.[63] The current study showed that the ethyl acetate extract from the supplemented group exhibited the highest inhibitory activity against proteinase denaturation, indicating its potential anti-inflammatory effects. Additionally, all extracts from both control and supplemented groups demonstrated anti-inflammatory activity by reducing nitric oxide production in LPS-activated cells. The ethyl acetate extract from the supplemented group also decreased the production of TNF-α cytokine, further supporting its anti-inflammatory properties. Moreover, treatment with microalgae extracts reduced the inflammatory effects of macrophages, as evidenced by the reduction in dendritic-like and irregular-shaped cells upon LPS stimulation. Overall, the present study provides valuable insights into the potential anti-obesity and anti-inflammatory properties of *Tetraselmis suecica* extracts obtained under combined sodium acetate and sodium nitrate supplementation. The findings highlight the importance of cultivation conditions in enhancing the bioactivity of microalgae extracts, particularly in the context of anti-obesity and anti-inflammatory effects.

To gain insight into the chemical composition of the selected extracts, FTIR and LC-MS techniques were employed for chemical profiling. FTIR spectra illustrated distinct functional group differences between ethyl acetate and ethanol extracts from both normal and supplemented groups. The supplemented ethyl acetate extract exhibited additional functional groups compared to the normal group, including halocarbons, alkenes, anhydrides, secondary alcohols, alkanes, amines, sulfonate, and aldehydes. Similarly, the supplemented ethanol extract showed the presence of halocarbons, aromatic esters, aromatic amines, allenes, amine salts, alkanes, and alkynes. Previous studies have also demonstrated similar functional group patterns in *Tetraselmis* sp. extracts supplemented with sodium nitrate. Another study using extract from *Tetraselmis* sp. also revealed the presence of esters from the peaks absorbed at 1250-1310 cm^{-1} due to the C-O stretch,[64] highlighting the influence of nutrient supplementation on compound composition.[65] Furthermore. the LC-MS analysis revealed a higher number of chemical compounds in the supplemented ethyl acetate and ethanol extracts compared to the normal group. These compounds were classified into major groups, including carotenoids, phenolics, and flavonoids. Notably, the supplementation of combined sodium acetate and sodium nitrate induced the production of new carotenoids such as alpha carotene and canthaxanthin. Previous studies discovered that green microalgae especially *Tetraselmis* sp. is one of the rich sources of carotenoids such as violaxanthin, zeaxanthin and fucoxanthin.[66,67] Furthermore, phenolic compounds and flavonoids were identified in supplemented groups, including rutin, quercitrin, apigenin-O-rutinoside, and caffeoyl glucoside. These compounds have demonstrated antioxidant,[68] anti-inflammatory, and anticancer activities in previous studies.[69–71] This suggests that *Tetraselmis suecica* has beneficial medicinal active chemicals that could be useful for the development of new health-benefit supplements in the pharmaceutical and medical industries.

Conclusion

In conclusion, the combined action of supplementing sodium acetate and sodium nitrate significantly improved biomass production and increased the total carotenoid content in the ethanol extract. The ethanol extract from the supplemented group exhibited significant antioxidant activity. The ethyl acetate extract from the supplemented group showed potential anti-obesity and anti-inflammatory activities, with most compounds in both ethyl acetate and ethanol extracts from the combined supplemented group being phenolics and carotenoids. It can be concluded that the addition of sodium acetate and sodium nitrate supplementation can be considered one of the key strategies in *Tetraselmis suecica* cultivation to promote the accumulation of secondary metabolites with diverse biological activities for industrial applications in pharmaceuticals, nutraceuticals, and food.

Authors' Contributions

NSNS: Writing - original draft, Methodology, Investigation, Formal analysis and Data curation. MHAB: Supervision, Conceptualization, Methodology, Writing - review & editing, Validation and Funding acquisition. MAK: Conceptualization, Methodology and Validation. TK: Supervision, Conceptual ization, Methodology, Investigation and Formal analysis. KAS: Conceptualization, Formal analysis, Funding acquisition and Resources. All authors read and approved the final manuscript.

Funding

This work was financially supported by the Research University (Individual) Grant Universiti Sains Malaysia (Ref No.: 1001/PTEKIND/8011116) – as a main funder. TWAS-COMSTECH Joint Research Grant-18-420 RG/PHA/AS_C is the co-main funder of this work for equipment purchased.

Conflict of Interest Disclosures

The authors declare that they have no conflicts of interest.

Availability of Data and Materials

Samples of the compounds and research data are available from the authors.

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