# Biochemical Composition and Bioactive Properties of *Chlorella minutissima* (Chm1) as a Potential Source of Chemical Compounds for Nutritional Feed Supplement and Disease Control in Aquaculture

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Received: 12 April 2020, Revised: 9 June 2020, Accepted: 7 August 2020

## Abstract

Microalgae contain high levels of proteins, lipids, carbohydrates and other bioactive metabolites with direct relevance to aquaculture. This investigation was done to assess the bioactive properties and describe the neutraceutical and pharmacological benefits of a green microalga, *Chlorella minutissima* (Chm1). The alga has a total phenolic content of 30.94±0.06 mg GAE $g^{-1}$. Relative antioxidant efficiency showed that *C. minutissima* exerted potent ABTS scavenging activity and high ability of reducing copper ions in a concentration-dependent manner with $IC_{50}$ values of 48.13 μg GAE $ml^{-1}$ and 13.90 μg GAE $ml^{-1}$, respectively. Evaluation of antibacterial activities using microtiter plate dilution assay revealed that *C. minutissima* showed strong activity against *Aeromonas hydrophila* with a minimum inhibitory concentration (MIC) of 125 μg $ml^{-1}$. The algal extract is also effective against *Vibrio cholerae*, *Vibrio parahaemolyticus* and *Staphylococcus aureus* in each case with MIC value of 250 μg $ml^{-1}$. Also, *C. minutissima* extract was able to inhibit the growth of *Pseudomonas fluorescens* with MIC value of 500 μg $ml^{-1}$. Proximate composition of the dried microalga showed that *C. minutissima* consists of high protein, ash and crude fat content with values of 42.61±0.11%, 17.79 ± 0.04% and 11.70 ± 0.01%, respectively. The results show that *C. minutissima* is an excellent candidate organism as a potential source of chemical compounds important for feed formulation and disease control in aquaculture.

**Keywords:** Antibacterial activity; antioxidant activity; bioactive compounds; bacterial fish pathogen; *Chlorella*; microalgae; proximate analysis
DOI 10.14456/cast.2021.9

## 1. Introduction

Bacterial fish pathogens are regarded as principal causes of diseases in aquaculture occurring in nurseries and rear ponds that can cause massive fish mortalities leading to significant economic losses [1]. These bacterial fish infections are caused by several species of *Aeromonas* sp., *Vibrio* sp. and *Pseudomonas* sp. causing hemorrhages, lesions and ulceration in several fish species [2]. Antibiotics are being used in the aquaculture industry to prevent these infections. However, there is an increasing occurrence of antibiotic resistance in most aquaculture and thus antibiotics are becoming less effective. Also, the detection of drug

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residues in aquatic animal tissues and the possibility of infection of drug-resistant bacteria to consumers (humans) have become major considerations for public health that result from the application of antibiotics in aquaculture [1, 3]. Thus, discovery of different mitigation strategies to solve such unforeseeable and unceasing occurrence of fish disease is considered an urgent need. The use of other existing natural resources such as microalgae is considered as one of the strategic solutions to address such issues. Microalgae are a natural source of food for animals (such as fish fry and larval shrimp) important in aquaculture, and some species (*Chlorella*, *Isochrysis*, *Tetraselmis*, *Chaetoceros*, *Nannochloropsis* and *Pavlova*) are being used commercially as dried algal feed or live prey [1, 2]. These organisms are known to have diverse bioactive metabolites with antioxidant, anticoagulant, antibacterial, antifungal, anticancer, and antiviral activities [1]. These organisms use inorganic nutrients and light to produce biomolecules with high nutritional value like lipids, carbohydrates, pigments, and proteins. Also, other chemical compounds such as polysaccharides, sterols, polyphenols, carotenoids, vitamins and polyunsaturated fatty acids are reported to have potential use in the prevention and reduction of fish disease [2, 4, 5].

The genus *Chlorella* is a group of unicellular, green-pigmented microalgae capable of synthesizing a wide array of bioactive chemical compounds using various pathways with good therapeutic effects. These microalgae are capable of exhibiting high growth rate, high adaptability to various environmental conditions with minimal media requirements. Also, some species such as *Chlorella vulgaris* are considered as Generally Regarded as Safe (GRAS) for consumption by animals and humans as alternative source of single cell proteins. Thus, the feasibility of these organisms as alternative source of beneficial biomolecules and chemical compounds for large-scale production are acknowledged. *Chlorella minutissima* is a coccoid green microalga that is characterized by having chlorophyll and parietal chloroplasts. This species is known to produce secondary metabolites when subjected to environmental stress like high temperature, high intensity of light, and nitrogen starvation. Substances derived from this microalga such as polyphenols, sterols, phycobilins, polysaccharides, carotenoids and bioactive peptides are reported to have antioxidant, antibacterial and anticholinesterase activities [6, 7].

The Philippine freshwater ecosystem is known to have a number of *Chlorella* species with functional and bioactive properties yet to be explored. However, only a few studies have been documented about the nutritional properties, antibacterial and antioxidant activities of these microalgae. Previously, a protein-rich strain of *Chlorella sorokiniana* was reported to contain bioactive peptides including phosphoglycerate kinase, Fe-superoxide dismutase and chloroplast Rubisco activase. These bioactive peptides were able to exhibit ACE inhibitory, anti-amnestic, antioxidant and antithrombotic activities that suggest the potential of this species as alternative source of high value natural product [8]. The present study is the first report in the Philippines exploring the bioactive properties of a green microalga, *Chlorella minutissima* Fott & Nováková with potential to be use as novel antibiotics for disease control in aquaculture and as an alternative source of protein rich biomass for feed formulation. The investigation was done to analyze the proximate composition, total phenolic content, antibacterial, and antioxidant (using ABTS radical scavenging and copper reduction antioxidant capacity (CUPRAC) assay) activities of *C. minutissima*. In addition, correlation analysis on the phenolic content of the microalgal extract and its antioxidant (ABTS radical scavenging and copper reducing capacity) activity was established.

## 2. Materials and Methods

### 2.1 Algal culture

The microalga, *Chlorella minutissima* was procured from the Philippine National Collection of Microorganisms, National Institute of Molecular Biology and Biotechnology (BIOTECH), University of the Philippine Los Baños. Culture of *C. minutissima* (100 ml) was inoculated into three sterile flasks containing one liter of BG 11 medium. The mass production set up was

cultivated for 24 days with mean temperature of 23 ± 2 °C. Three fluorescent white lamps (light intensity of 120 μmol photons $m^{-2}$ $s^{-1}$) were utilized to provide illumination on a 16:8 light to dark cycle. The algal biomass was centrifuged at 10,000 rpm for 10 min and was rinsed with sterile distilled water. The rinsed biomass was centrifuged again and the biomass was freeze-dried using a Virtis Freeze mobile 25 SL lyophilizer [1, 5]. This freeze-dried biomass was used for determining the total phenolic content as well as antioxidant and antibacterial assays. In addition, algal biomass of *C. minutissima* obtained after centrifugation was oven dried at 60ºC for 24 h and used for determining the proximate composition [5].

## 2.2 Proximate composition analysis

The ash content was obtained by complete burning of the dried algal biomass in an oven at a temperature of 550°C for 4 h until an ash (grayish powder) was produced. Crude fiber content was analyzed using the Weende method [9]. Briefly, 0.3 g of the dried algal biomass was digested with 1.25% HCI followed by 1.25% NaOH. The residue obtained after the digestion process was dried at 105°C for 3 h and weighed. The protein content of *C. minutissima* was analyzed by Kjeldahl method [9]. The total amount of nitrogen in crude protein was determined using 6.25 as the conversion factor. The total crude fat concentration of the microalga was estimated using the Bligh and Dyer method. Ten grams of the dried algal biomass was mixed with the extraction solvent (1:2 chloroform/methanol) and kept overnight after addition of $CaCl_2$. The chloroform layer was separated from the algal sample and then evaporated to dryness (in an evaporator) and finally placed in an oven set at 105°C for 30 min. The total crude lipid concentration was obtained by calculating the weight differences between flasks [9]. The moisture content of *C. minutissima* was determined by subjecting the algal biomass (2 g) to dryness at 105°C until a constant weight was obtained [9]. The total carbohydrate concentration was estimated using the equation below:

$$\%\, Total\ Carohydrate\ = 100 - (\%\, Protein + \%\, Crude\ Fiber\ + \%\, Crude\ Fat + \%Ash)$$

## 2.3 Microalgal extract preparation

The lyophilized algal biomass was soaked overnight in methanol (1g biomass: 20 ml methanol) with stirring. The extract was filtered using a filter paper (Whatmann No. 1) and subjected to a rotary evaporator (at 40°C) under reduced pressure to concentrate the algal extract [1, 5].

## 2.4 Determination of total phenolic content

Analysis of the total phenolic content of *C. minutissina* was done using a Folin-Ciocalteau's reagent [10]. Briefly, a portion (0.5 ml) of the diluted algal extract (using distilled water as diluent) was added with equal volume of 10% sodium carbonate solution and Folin-Ciocalteau's reagent and set aside for 5 min. The solution was then added with 5 ml sterile distilled water. The optical density (OD readings) of the reaction mixture was measured at 720 nm using a Shimadzu UV-1601 spectrophotometer. The blank consisted of distilled water with other reagents used in the analysis. The standard gallic acid was used for the construction of the calibration curve. The total phenolic concentration of the extract was reported as mg gallic acid equivalents (GAE) $g^{-1}$ of the algal sample [10].

## 2.5 ABTS radical scavenging assay

ABTS free radical scavenging activity of *C. minutissima* extract was done using a method that is based on the decolorization of 2,2-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt [11]. In this method, ABTS chromophore is transformed to its radical cation upon addition of sodium persulfate. This radical cation (bluish in color) is converted to its neutral form (colorless) in the presence of antioxidants (such as phenolic compounds in the

extract) in the reaction mixture. Briefly, 40 µl of algal extract at different phenolic concentrations (diluted using distilled water) and 40 µl of 90% methanol for the control were mixed with 3 ml of ABTS radical mixture with a starting absorbance of 0.72±0.05 at 734 nm. The solutions were stirred and set aside for 5 min at ambient temperature. Then, optical density reading was read at 734 nm (water was used to zero the spectrophotometer) [11]. The blank consisted of distilled water with other reagents used in the assay. The percent ABTS inhibition was analyzed using the formula below:

$$\text{ABTS Inhibition (\%)} = \frac{\text{Abs}_{734}\,(\text{control}) - \text{Abs}_{734}\,(\text{sample})}{\text{Abs}_{734}\,(\text{control})} \times 100$$

## 2.6 Copper reduction antioxidant capacity (CUPRAC) assay

The cupric ion reducing capacity of the algal extract was analyzed by colorimetric method [12]. For this, 0.5 ml of the prepared algal extract concentrations (5, 10, 15, 20, and 25 µg GAE $ml^{-1}$) diluted using distilled water was mixed with 1 ml each of 0.0075 M neocuproine, ammonium acetate ($C_2H_3O_2NH_4$) buffer (1M, pH 7.0) solution, as well as 0.01 M $CuCl_2$ into a sterile test tube. The solutions were kept for 30 min at ambient temperature. The blank consisted of distilled water with other reagents used in the assay. The optical density (absorbance readings) of each reaction solution was noted at 450 nm and compared against a blank [12].

## 2.7 Test bacterial pathogens

The Philippine National Collection of Microorganisms, National Institute of Molecular Biology and Biotechnology (BIOTECH), University of the Philippines Los Baños (UPLB) provided the test organisms used in this study. Pathogenic strains of Gram-negative bacteria (*Pseudomonas aeruginosa* BIOTECH 1824, *Pseudomonas fluorescens* BIOTECH 1123, *Pseudomonas putida* BIOTECH 1506**,** *Vibrio cholerae* BIOTECH 1967, *Aeromonas hydrophila* BIOTECH 10089, and *Vibrio parahaemolyticus* BIOTECH 10210**)** and Gram-positive bacteria (*Listeria monocytogenes* BIOTECH 1958, *Staphylococcus epidermidis* BIOTECH 10098, and *Staphylococcus aureus* BIOTECH 1823) were pre-cultured using Luria Bertani (LB) broth or Marine broth (for *Vibrio* species) with shaking overnight at 35°C. The purity of each bacterial culture was maintained by continuously checking the biochemical tests and morphological characteristics of each pathogen [5, 13].

## 2.8 Micro-dilution antibacterial assay

The antagonistic activity of *C. minutissima* extract against the Gram-negative and Gram-positive pathogenic bacteria was analyzed using microtiter plate dilution assay [13]. Initially, bacterial stocks of each pathogenic bacterium with cell density of $1 \times 10^5$ cells $ml^{-1}$ were prepared. Using a 96-well microtiter plate, 100 µl of the bacterial stock cultures were added with 100 µl of *C. minutissima* extract at different dilutions (1000 µg $ml^{-1}$-7.81 µg $ml^{-1}$). Methanol was also used in the assay as the negative control. The microtiter plate was incubated at 35°C for 12 h, after which minimum inhibitory concentration (MIC) for each bacterium was recorded. MIC of the *C. minutissima* extract is the minimum concentration capable of inhibiting bacterial growth after 12 h incubation period. To determine the minimum bactericidal activity (MBC) of the algal extract, a loopful of the sample from each MIC wells (that exhibited growth inhibition of bacteria) was streaked into freshly prepared culture (tryptic soy agar and marine agar) medium. The plates were kept at 35°C for 24 h and were evaluated for visible colony growth for each dilution subculturing. No visible bacterial growth meant that *C. minutissima* was bactericidal at that particular dilution [5, 13].

### 2.9 Statistical Analyses

The experimental assay in this study was done with three replicates and data were expressed as means ± standard deviations (mean ± SD). The statistical tests for the linear correlation between antioxidant activity and phenolic concentration of the algal extract using Pearson's correlation coefficient (R) were analyzed using MS Office Excel 2007.

## 3. Results and Discussion

### 3.1 Proximate composition analysis

The growing concern on the limitation of the use of arable land areas and the decrease in fish harvested from freshwater and open ocean highlights the need for sustainable animal feed source from aquaculture [14]. Microalgae are regarded as promising substitutes (to traditional plant-based feeds) for nutrient sources of protein, lipids and carbohydrates needed for partial replacement in animal feed formulation. These organisms are rich in protein (40-60% of dry biomass) as well as carbohydrates and lipids and their compositions vary depending on the species and growth condition [15-17]. Thus, evaluation of the chemical composition and assessing the possibility of several strains of microalgae for mass production will give baseline information needed for the development of new feed formulation essential in aquaculture.

The proximate composition analysis of the dried biomass of *C. minutissima* exhibited high nutritional value (Table 1). The algal strain possesses high concentration of protein with an estimated amount of 42.61 ± 0.11% of the total dried biomass. This result is comparable to other microalgae being used in feed formulation in aquaculture such as *Chlorella vulgaris* and *Spirulina platensis* which have protein contents of 55.70 ± 2.10% and 61.74 ± 1.06%, respectively [18]. Also, this microalga has a higher protein content than other traditional plant-based protein sources used in animal feed formulation such as *Azolla pinatta* (28.01 ± 1.15%) and soybean (37.69 ± 0.00%) [18, 19]. The study revealed that *C. minutissima* showed protein content that is higher than 40% of its total dry biomass proving its potential as alternative protein source in feed formulation for aquaculture [5]. Table 1 showed other dominant biochemical components like ash (17.79 ± 0.04%), carbohydrate (14.06 ± 0.03%) and crude fat (11.70 ± 0.01%). The ash content shows the concentration of inorganic substances found in the algal biomass. On the other hand, a low concentration of crude fiber (9.13 ± 0.15%) was observed in *C. minutissima* biomass. This value is good in feed formulation since animals do not a need high concentration of fiber in their diets [5, 14].

**Table 1.** Proximate composition of *Chlorella minutissima*

| Proximate composition | Percent composition (%) |
|---|---|
| Moisture Content | 4.71 ± 0.10 |
| Ash Content | 17.79 ± 0.04 |
| Crude Protein | 42.61 ± 0.11 |
| Crude Fat | 11.70 ± 0.01 |
| Crude Fiber | 9.13 ± 0.15 |
| Carbohydrate | 14.06 ± 0.03 |

## 3.2 Total phenolic content (TPC)

The amount and kind of phenolic compounds extracted from a sample are dependent on the polarity of the solvent and the binding (complex formation) of phenolic substance with the corresponding solvent (used for extraction) for it to become more soluble [13, 20]. Phenolic compounds have high solubility in polar solvents (such as methanol) giving a high concentration of these target compounds in the sample extract. The TPC of *C. minutissima* was analyzed using the Folin-Ciocalteu reagent [10] and is expressed in gallic acid equivalent (calibration curve equation: $y = 0.0682x - 0.0214$, $r^2 = 0.997$). The results showed that the total phenolic concentration in *C. minutissima* extract is 30.94 ± 0.06 mg GAE $g^{-1}$. The phenolic concentration obtained from methanol extract of *C. minutissima* is greater than that observed from other species of microalgae found in the Nile River in Egypt such as *Oscillatoria agardhii* and *Anabaena sphaerica* with 20.91 ± 0.21 mg GAE $g^{-1}$ and 14.81 ± 0.02 mg GAE $g^{-1}$, respectively [21]. Likewise, lower TPC was also observed from previous studies using methanolic extract of *Chlorella marina* (0.647 ± 0.052 mg GAE $g^{-1}$) [22], *Scenedesmus quadricauda* (606.91 ± 0.028 μg GAE $g^{-1}$) [5] and *Desmodesmus* sp. (652.66±0.042 μg GAE $g^{-1}$) [3] in comparison with the current study.

## 3.3 Antioxidant activities

Microalgae have polyphenols with a wide range of antioxidant activities. Thus, utilization of these natural polyphenols for possible use in drug synthesis should be studied. The antioxidant activities of *C. minutissima* extract were evaluated using ABTS scavenging and CUPRAC assay. Two antioxidant assays were utilized in the study to better understand the antioxidant capacities of the microalgal extract.

### 3.3.1 ABTS radical scavenging activity

The ABTS radical scavenging assay is a spectrophotometric technique that uses the reduction of stable colored radicals to evaluate the antioxidant capability of a sample extract. In this assay, ABTS chromophore is generated through the reaction of sodium persulfate and ABTS, which changes ABTS into its radical cation. The radical cation is bluish in color and is capable of absorbing light at 734 nm [11]. The methanolic extract of *C. minutissima* exhibited a concentration-dependent inhibition of ABTS radical production (Table 2). The highest antioxidant activity (60.61%) for the algal extract was observed at 62.5 μg GAE $ml^{-1}$ of the prepared concentration of the extract, whereas the standard, that is, ascorbic acid, exhibited the highest activity of 64.53% at 187.5 μg $ml^{-1}$ concentration. This means that the antioxidant activity of the microalgal extract is thrice more effective than ascorbic acid (standard antioxidant) in scavenging ABTS free radical. *Chlorella minutissima* extract exerted a dose dependent ABTS free radical inhibition and scavenging activity (Table 2 and Figure 1). This activity can be ascribed to phenolic substances like flavonoids, vitamin C and carotenoids present in *C. minutissima* extract that serve as potent free radical scavengers which can be used as nutritional feed supplement in aquaculture for the control and prevention of fish diseases caused by bacteria and other external oxidative stressors. The activity of *C. minutissima* extract exhibited potent antioxidant activity when compared to ascorbic acid (positive control) with $IC_{50}$ of 48.13 μg GAE $ml^{-1}$ and $IC_{50}$ = 147.9 μg $ml^{-1}$, respectively. Also, the effective concentration ($IC_{50}$) of *C. minutissima* extract showed greater antioxidant activity than other strains of microalgae from KwaZulu-Natal in South Africa such as *Chlorella sorokiniana* and *Chlorella minutissima* with $IC_{50}$ values of 85.03 μg GAE $ml^{-1}$ and 86.05 μg GAE $ml^{-1}$, respectively [7].

**Table 2.** ABTS radical scavenging activity and $IC_{50}$ value of phenolics from *C. minutissima* and ascorbic acid

| Sample | Phenolic concentration (μg GAE $ml^{-1}$) | | | | | $IC_{50}$* |
|---|---|---|---|---|---|---|
| | 12.5 | 25.0 | 37.5 | 50.0 | 62.5 | |
| | ABTS Inhibition (%) | | | | | |
| *Chlorella minutissima* | 16.38 ± 1.53 | 29.87 ± 0.00 | 40.62 ± 0.51 | 51.66 ± 0.82 | 60.61 ± 1.63 | 48.13 μg $ml^{-1}$ |
| | Concentration (μg $ml^{-1}$) | | | | | |
| | 37.5 | 75.0 | 112.5 | 150.0 | 187.5 | |
| | ABTS Inhibition (%) | | | | | |
| Ascorbic acid | 11.70 ± 1.54 | 24.56 ± 0.62 | 36.70 ± 0.51 | 50.87 ± 0.82 | 64.53 ± 1.64 | 147.9 μg $ml^{-1}$ |

*$IC_{50}$ is the effective concentration that inhibits the activity of ABTS (2,2-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt) cation radical by 50%.

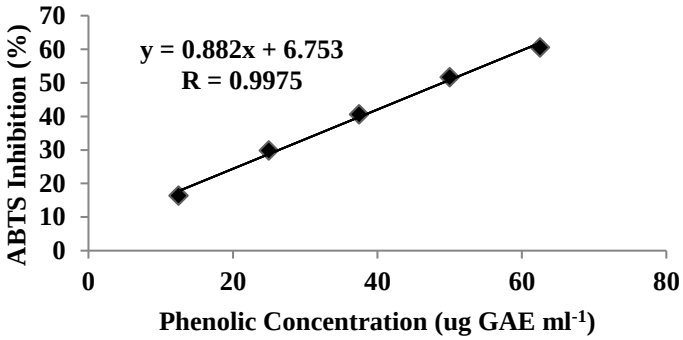


**Figure 1.** Correlation between the total phenolic content and antioxidant activity (ABTS radical scavenging assay) of *C. minutissima*

The coefficient of correlation (R) between phenolic concentration and antioxidant activity of *C. minutissima* using ABTS scavenging assay is presented in Figure 1. Based on the correlation analysis, a positive correlation (R=0.9975) exists between total phenolic concentration and ABTS antioxidant activity of the methanol extract of *C. minutissima*. This shows that the phenolic compound present in the algal extract enhances the antioxidant property of *C. minutissima*. Previous studies regarding antioxidant activity of green microalgae, *Scenedesmus quadricauda*, *Trentepohlia umbrina*, and *Desmodesmus* sp. showed positive correlation between antioxidant activities and phenolic contents of the algal extracts, a result that is in agreement in the current investigation [3, 5, 23].

### 3.3.2 Copper reduction antioxidant capacity (CUPRAC)

CUPRAC assay is a technique used in assessing the antioxidant capability of an extract by evaluating the capability of the algal extract to reduce (Cu (II)-Neocurpine) to colored Cu (I)-Neocurpine chelate, which exhibits a maximum absorbance at 450 nm. This method has unique advantages over other antioxidant assays such as the range of working pH near to physiological pH (as compared to the FRAP and Folin assay, which work at acidic and alkaline conditions, respectively) and efficient redox potential. Moreover, this assay is applicable in the detection of sulfhydryl (-SH) containing antioxidants (unlike FRAP method) as well as lipophilic and hydrophilic antioxidants (unlike other antioxidant assays such as

DPPH and Folin) in the sample extracts [24]. The presence of hydrophilic, lipophilic and sulfhydryl (-SH) containing antioxidants in the algal extract can exhibit promising biological activities that can be used for aquaculture [12]. *Chlorella minutissima* extract showed dose-dependent copper ion reduction ability. Table 3 shows the maximum absorbance of 0.860 of methanolic extract at 25 μg GAE $ml^{-1}$ concentration, whereas the standard, that is, ascorbic acid, shows an absorbance of 0.542 at 50 μg GAE $ml^{-1}$ concentration. In this method, a higher absorbance reading means greater antioxidant activity. The observed trend in this antioxidant assay is similar to that obtained from the ABTS assay in which 62.5 μg GAE $ml^{-1}$ concentration showed the highest antioxidant activity. The study reveals that *C. minutissima* extract contains phenolic compounds having antioxidant properties that can be ascribed to its excellent copper reducing ability. Previous studies show that microalgal compounds such as tocopherol, carotenoids, polyphenolic compounds and pigments were observed to have reduction potential like antioxidant properties [3, 5]. Lipophilic antioxidants like tocopherols and carotenoids are highly effective in inhibiting nucleic acid formation and cytoplasmic leakage in bacteria. On the other hand, hydrophilic antioxidants such as flavonoids are capable of causing damage in bacterial cell wall by dissolving extracellular proteins [6, 23]. In this study, the presence of these compounds in the algal extract contributed to a stronger antioxidant property than that of the standard antioxidant (such as ascorbic acids) used in the food industry.

**Table 3.** Copper reduction antioxidant capacity (CUPRAC) and $IC_{50}$ value of phenolics from *C. minutissima* and ascorbic acid

| Sample | Phenolic concentration (μg GAE $ml^{-1}$) | | | | | $IC_{50}$* |
|---|---|---|---|---|---|---|
| | 5.0 | 10.0 | 15.0 | 20.0 | 25.0 | |
| | CUPRAC (Absorbance at 450 nm) | | | | | |
| *Chlorella minutissima* | 0.182 ± 0.001 | 0.389 ± 0.001 | 0.535 ± 0.004 | 0.635 ± 0.016 | 0.860 ± 0.002 | 13.90 μg $ml^{-1}$ |
| | Concentration (μg $ml^{-1}$) | | | | | |
| | 10.0 | 20.0 | 30.0 | 40.0 | 50.0 | |
| | CUPRAC (Absorbance at 450 nm) | | | | | |
| Ascorbic acid | 0.112 ± 0.002 | 0.213 ± 0.007 | 0.328 ± 0.004 | 0.429 ± 0.012 | 0.542 ± 0.011 | 46.30 μg $ml^{-1}$ |

*$IC_{50}$ is the effective concentration that gives CUPRAC value of 0.5 absorbance reading at 450 nm. Computed by interpolation.

The strong positive correlation (R) between the antioxidant activity of *C. minutissima* and the phenolic concentration using CUPRAC assay (R= 0.9927) shows that phenolic compounds present in the algal extract contributed significantly to the antioxidant activity of the microalga (Figure 2). In addition, *C. minutissima* extract exhibited potent antioxidant activity as compared to ascorbic acid (positive control) with $IC_{50}$ of 13.90 μg GAE $ml^{-1}$ and $IC_{50}$ = 46.30 μg GAE mg $ml^{-1}$, respectively. The result of this analysis proves that the antioxidant activity of *C. minutissima* extract is stronger than ascorbic acid, which proves its promising characteristic as natural alternative source of bioactive compounds useful in drug discovery.

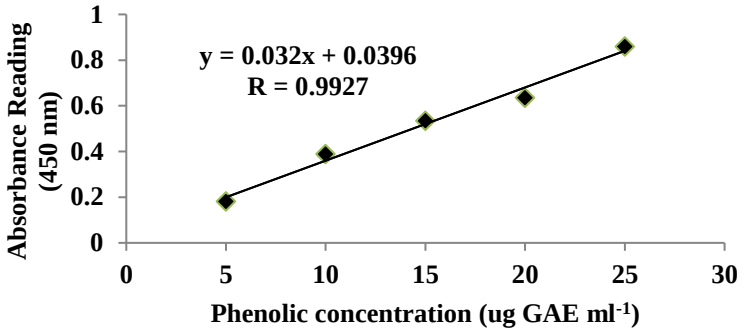


**Figure 2.** Correlation between the total phenolic content and antioxidant activity (copper reduction antioxidant capacity (CUPRAC) assay) of *C. minutissima*

## 3.4 Antibacterial activity

*Chlorella* is a common genera of microalgae widely used as feed supplement for aquaculture. These microalgae are being utilized as cultured microalgae in the rearing of the larvae of freshwater and marine fish, bivalves and other crustaceans. In addition, these cultured microalgae in ponds and culture tanks are reported to have a positive impact on the overall bacterial load of the aquaculture rearing system. These microalgae are capable of suppressing the growth and proliferation of opportunistic bacterial pathogens such as *Aeromonas* sp., *Pseudomonas* sp., and *Vibrio* sp., thus improving the survival rate of fish larvae [6, 25]. The enhanced effect of adding cultured microalgae in rearing tanks can be attributed to factors such as oxygen radicals produced during photosynthesis as well as other bioactive compounds produced by the microalgae [6].

The antibacterial activity of the *C. minutissima* extract against selected bacterial pathogen is shown in Table 4. The algal extract exhibited potent antibacterial effect against *A. hydrophila* with MIC and MBC values of 125 μg $ml^{-1}$ and 250 μg $ml^{-1}$, respectively. The extract is also effective against *V. cholerae*, *V. parahaemolyticus* and *S. aureus*, each with MIC values of 250 μg $ml^{-1}$. Also, *C. minutissima* extract was able to prevent the growth of *P. fluorescens* with MIC value of 500 μg $ml^{-1}$. The minimum bactericidal concentration (MBC) value of the algal extract for *V. cholerae*, *V. parahaemolyticus* and *S. aureus* (each with MBC of 500 μg $ml^{1}$) was greater than that obtained for *P. fluorescens* (MBC of 1000 μg $ml^{-1}$). The antibacterial activity of the extract against *V. parahaemolyticus* is similar to an earlier study showing that axenic culture of *C. minutissima* when co-cultivated with other *Vibrio* species for 24-96 h exhibited antagonistic activity against the pathogens [6]. Bioactive substances such as polyphenols and bioactive peptides were detected in several *Chlorella* species that are capable of inactivating adhesins and other protein envelope of bacterial pathogens [5]. Antioxidants derived from microalgae such as flavones, astaxanthin, vitamin E, peptides, vitamin C, polyphenols, carotenoids, and amino acids are beneficial to aquatic animals by providing enhanced immune responses against pathogens via free radical neutralization and repair of membrane system and biomolecules due to oxidative damage. The use of these antioxidants has shown a great potential as nutritional and immunostimulating feed supplement by exhibiting antimicrobial activities against important disease causing bacterial pathogens in aquaculture. *Arthrospira platensis* fed to commercially important shrimp and fish, exhibited increased phagocytic activity and resistance against *Escherichia coli*, *Vibrio harveyii*, *Bacillus subtilis*, and *Salmonella typhimurium*. In addition, *Porphyridium* sp. supplemented in fish feed exhibited antiviral, antitumor, antioxidant, and anti-inflammatory activities, proving the important effect of microalgae on the survival and development of cultivated aquatic organisms [26, 27]. The amount and type of free fatty acids present in the algal extract can cause disruption in the efficiency of the oxidative phosphorylation and electron transport chain of bacterial cells, leading to cell lysis and death [28, 29].

**Table 4.** Antibacterial activities of *C. minutissima* extract

| Bacterial Pathogen | Minimum inhibitory concentration (μg ml$^{-1}$) | Minimum bactericidal concentration (μg ml$^{-1}$) |
|---|---|---|
| Gram-positive bacteria | | |
| *Staphylococcus aureus* BIOTECH 1823 | 250.00 | 500.00 |
| *Listeria monocytogenes* BIOTECH 1958 | >1000.00 | ND |
| *Staphylococcus epidermidis* BIOTECH 10098 | >1000.00 | ND |
| Gram-negative bacteria | | |
| *Aeromonas hydrophila* BIOTECH 10089 | 125.00 | 250.00 |
| *Vibrio cholerae* BIOTECH 1967 | 250.00 | 500.00 |
| *Vibrio parahaemolyticus* BIOTECH 10210 | 250.00 | 500.00 |
| *Pseudomonas fluorescens* BIOTECH 1123 | 500.00 | 1000.00 |
| *Pseudomonas aeruginosa* BIOTECH 1824 | >1000.00 | ND |
| *Pseudomonas putida* BIOTECH 1506 | >1000.00 | ND |

*ND = Not detected

Several species of green microalgae, such as *Trentepohlia umbrina, Chlorococcum humicola, Chlorella vulgaris, Scenedesmus quadricauda, Desmococcus olivaceous* and *Desmodesmus sp.* were screened for antibacterial activities in search of new bioactive compounds effective in controlling bacterial pathogens [3, 5, 23, 30, 31], but in this investigation, for the first time in the Philippines, the antibacterial activities of *C. minutissima* methanolic extract against *A. hydrophila, V. cholerae, V. parahaemolyticus* and *P. fluorescens* were reported. Comparing the results obtained in this study with other microalgae, it seems that the methanolic extract of *Chlorella minutissima* showed relatively strong antibacterial activity against bacterial fish pathogen. Thus, *C. minutissima* is considered as a candidate freshwater microalgae with excellent biological activities that can be used for the development of new antibiotics. Further experiment should be conducted on the isolation, purification, chemical structure elucidation, and identification of these bioactive substances to better understand the mechanisms of antibacterial activity and its potential for large-scale production.

## 4. Conclusions

*Chlorella minutissima* (Chm1) possess bioactive metabolites with potential neutraceutical and pharmacological benefits. It is capable of producing chemical compounds in high concentration such as proteins, lipids and phenolic compounds with direct relevance to aquaculture application. The green microalga exhibits potent antioxidant and antibacterial activities as well as high protein content suitable for large-scale production. Further experiments focusing on the identification and characterization of the biologically active compounds using GC-MS analysis should be conducted to understand the mechanisms involving in the antibacterial and antioxidant properties of the algal extract. Also, studies on

the use of *C. minutissima* biomass as nutritional partial feed replacement in aquaculture feed formulation and feeding experiment should be conducted.

## 5. Acknowledgements

The author acknowledges the support of the Philippine National Collection of Microorganisms and Food Laboratory, National Institute of Molecular Biology and Biotechnology (BIOTECH), University of the Philippines Los Baños. The technical assistance of Mrs. Arsenia B. Sapin in the analysis of the algal sample and the constructive comments of the blind reviewer are acknowledged with gratitude.

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