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ORIGINAL ARTICLE / ÖZGÜN MAKALE

TOTAL PHENOLIC CONTENT AND *IN VITRO* ANALYSIS OF ANTIOXIDANT, ANTIBACTERIAL, AND ALPHA-GLUCOSIDASE INHIBITION PROPERTIES OF *CHROOCOCCUS MINUTUS* (KÜTZING) NÄGELI (CHROOCOCCALES, CYANOBACTERIA)

CHROOCOCCUS MINUTUS (KÜTZİNG) NÄGELİ'NİN (CHROOCOCCALES, CYANOBACTERIA) ANTİOKSİDAN, ANTİBAKTERİYEL VE ALFA-GLUKOZİDAZ İNHİBİSYON ÖZELLİKLERİNİN TOPLAM FENOLİK İÇERİĞİ VE İN VİTRO ANALİZİ

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ABSTRACT

Objective: *Cyanobacteria are novel sources of bioactive substances with important biological activities. In this study, the total phenolic content (TPC) and bioactive (antioxidant, antidiabetic, and antibacterial) properties of a freshwater cyanobacteria, Chroococcus minutus were investigated.*

Material and Method: *Methanol extract of C. minutus were prepared and subjected to different biological assays to determine the TPC, antioxidant, and alpha-glucosidase inhibition properties. The antibacterial activity was done using microtiter plate dilution assay against various medically important bacterial pathogens.*

Result and Discussion: *C. minutus has a TPC of 11.27 ± 0.14 mg GAE/g. Antioxidant efficiency of C. minutus are characterized by having potent DPPH scavenging activity with IC_{50} value of 255 µg/ml. Assessment of alpha-glucosidase inhibition property showed that C. minutus extract have potent inhibition activity with IC_{50} of 5.50 µg/ml as compared to acarbose (standard antidiabetic drug). In addition, C. minutus extract exhibited potent antibacterial activities against Bacillus cereus, Listeria monocytogenes, and Methicillin-resistant Staphylococcus aureus. The current investigation shows the potential of C. minutus as source of active metabolites with important use in pharmaceutical applications.*

Keywords: *Biological activity, cyanobacteria, freshwater, Philippines*

ÖZ

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Amaç: *Siyanobakteriler, önemli biyolojik aktivitelere sahip yeni biyoaktif madde kaynaklarıdır. Bu çalışmada, bir tatlı su siyanobakterisi olan Chroococcus minutus'un toplam fenolik içeriği (TPC) ve biyoaktif (antioksidan, antidiyabetik ve antibakteriyel) özellikleri incelenmiştir.*

Gereç ve Yöntem: *C. minutus'un metanol özütü hazırlanarak, TPC, antioksidan ve alfa-glukozidaz inhibisyon özelliklerini belirlemek için farklı biyolojik deneylere tabi tutulmuştur. Antibakteriyel aktivite, tıbbi açıdan önemli çeşitli bakteriyel patojenlere karşı mikrotitre plaka seyreltme deneyi kullanılarak yapılmıştır.*

Sonuç ve Tartışma: *C. minutus'un TPC'si 11.27 ± 0.14 mg GAE/g'dir. C. minutus'un antioksidan etkinliği, 255 µg/ml IC_{50} değeri ile güçlü DPPH temizleme aktivitesine sahip olmasıyla karakterize edilir. Alfa-glukozidaz inhibisyon özelliğinin değerlendirilmesi, C. minutus özütünün, akarboza (standart antidiyabetik ilaç) kıyasla 5.50 µg/ml IC_{50} ile güçlü inhibisyon aktivitesine sahip olduğunu gösterdi. Ek olarak, C. minutus özütü, Bacillus cereus'a, Listeria monocytogenes'e ve Metisiline dirençli Staphylococcus aureus'a karşı güçlü antibakteriyel aktiviteler sergilemiştir. Mevcut araştırma, farmasötik uygulamalarda önemli kullanımı olan aktif metabolitlerin kaynağı olarak C. minutus'un potansiyelini göstermektedir.*

Anahtar kelimeler: *Biyolojik aktivite, siyanobakteriler, tatlı su, Filipinler*

INTRODUCTION

Cyanobacteria have notable attractiveness as novel sources of bioactive substances with diverse biological activities (antioxidant, antimicrobial, antidiabetic, anti-inflammatory, and antiviral effects) [1,2]. Several strains of cyanobacteria (*Oscillatoria limnosa, Anabaena,* and *Synechocystis aquatilis*) were reported to show antibacterial activities against *Bacillus subtilis, B. thuringensis, B. megaterium, Escherichia coli, Pseudomonas aeruginosa,* and *Candida tropicalis* [1,3]. These reported activities are associated with various intracellular and extracellular metabolites that are produced by several strains of cyanobacteria with known diverse biological activities [3]. Even though the chemical composition of the bioactive metabolites in cyanobacterial extract vary, the mechanism of action of these metabolites are usually similar, namely via photosystem II mediated electron flow inactivation of other organisms such as algae and bacteria. These allelopathic metabolites may serve an important role in species dominance and succession in nature [4]. One popular example of this substance is cyanobacterin which was isolated in *Scytonema hofmanni* and are reported to be toxic to cyanobacteria and a variety of eukaryotic algae.

Several species of cyanobacteria produce diverse kinds of secondary metabolites with antioxidants and antidiabetic properties. Previous studies show the isolation of several bioactive compounds from various cyanobacterial species such as flavonoids, protocatechuic acid, phycobiliproteins, gallic acids, chlorogenic acids, and catechin. These compounds are considered to have potent antioxidant and antidiabetic properties that can be tapped as novel alternative source of active substances for drug synthesis [1,5,6]. Cyanobacteria are considered reliable source of novel natural bioactive metabolites, since these organisms can be mass propagated in small and big bioreactors for large scale production. In addition, the growth characteristic of the cyanobacterial cells can be manipulated and controlled, so that no toxic substances will be included in the harvested algal biomass [1,6].

Cyanobacteria with high growth rate and novel bioactive metabolites have yet to be studied and exploited for pharmaceutical purposes, and isolation and characterization of algal strains with potential for pharmaceutical application remain the focus of continuing research [1,3,5]. Given these promising benefits which can be obtained from cyanobacteria, it is necessary to explore the potential of local species to produce bioactive compounds. Thus, the current study was conducted to determine total phenolic content and evaluate the antioxidant, antibacterial, and antidiabetic (using α-glucosidase inhibition assay) properties of a freshwater cyanobacteria, *Chroococcus minutus*.

MATERIAL AND METHOD

Chemicals

The chemicals used were of analytical grade and obtained from Sigma-Aldrich (Singapore City, Singapore). On the other hand, the solvents used were of analytical and HPLC grade and were obtained from Sigma-Aldrich (Steinheim, Germany, and Singapore City, Singapore) or Merck (Darmstadt, Germany).

Cyanobacterial Culture and Cultivation

The cyanobacteria, *C. minutus* were obtained from PNCM-BIOTECH of the University of the Philippines Los Baños. Initially, *C. minutus* culture (100 ml) was inoculated into three one liter sterile flasks containing BG 11 medium [2]. The mass cultivation set up run for 14 days under light condition (fluorescent white lamps with light intensity of 120 μmol photons m^{-2} s^{-1}) and incubated at a mean temperature of 23 ± 2 °C. The cyanobacterial biomass was harvested via centrifugation set at 10,000 rpm for 10 min. The algal pellet was rinsed several times with sterile water and freeze-dried using a lyophilizer (Virtis Freeze mobile 25 SL) [2].

Preparation of Cyanobacterial Extract

Dried and pulverized biomass of *C. minutus* (1 gram) was subjected to extraction using 20 ml methanol in an ultrasonic bath for 30 minutes with continuous stirring for 1 hr. The sample mixture was concentrated (via centrifugation) at 12,000 rpm for 20 min at a temperature of 20°C. The algal extract was further concentrated using a rotary evaporator set at 40 °C under reduced pressure. The concentrated algal extract was kept under refrigerated condition (4°C) to preserve its biological activity for use to different biological assays needed in the study [2,6].

Determination of total phenolic content (TPC)

The TPC of *C. minutus* was determined following the method proposed by Nuñez-Selles et al. [7]. Initially, about 0.5 ml of *C. minutus* crude extract was mixed with 0.5 ml 10% sodium carbonate solution and 0.5 ml of Folin-Ciocalteau's reagent for 1 minute. The sample mixture was allowed to stand for 5 minutes at ambient temperature. Furthermore, the volume of the sample mixture was adjusted

using 5 ml sterile distilled water. Absorbance reading of all the sample mixture was measured at 720 nm using an Ultraviolet-Visible spectrophotometer (Shimadzu, Kyoto, Japan). The TPC is presented as microgram of gallic acid equivalent (GAE) per gram of the algal extract (calibration curve equation: y = 0.0682x – 0.0214, r^2 = 0.997) [2,7].

2,2-Diphenyl-1-picrylhydrazyl (DPPH) Radical Scavenging Assay

The radical scavenging effect against DPPH of the *C. minutus* extract was analyzed using the methods done by Ribeiro et al. [8]. Briefly, 5.0 ml of 0.1 mM DPPH solution was mixed with 100 µl of the *C. minutus* extract. The reaction mixture was mixed (via vortex mixer) and was kept at ambient temperature for 20 minutes. The absorbance readings of the different *C. minutus* extract concentrations and the controls were taken at 517 nm wavelength using a UV-VIS spectrophotometer. The scavenging inhibition activity (%) was computed using the equation:

$$\boldsymbol{Inhibition}\ (\%) = \left(\frac{\boldsymbol{A_{control} - A_{sample}}}{\boldsymbol{A_{control}}}\right) \times \mathbf{100}$$

where: $A_{control}$ = absorbance reading of the control (DPPH solution without algal sample) and A_{sample} = absorbance reading of the algal extract sample (DPPH solution with test sample). Ascorbic acid was used as the control in this assay. The effective concentrations (IC_{50}) of the control antioxidant and *C. minutus* extract needed to nuetratlize 50% of DPPH radicals were determined using the standard curve generated from the scavenging activities of different concentrations of the algal extract [8,2].

Antibacterial Activity

Three Gram-negative bacteria (*Aeromonas hydrophila* BIOTECH 10089, *Pseudomonas aeruginosa* BIOTECH 1824, and *Escherichia coli* BIOTECH 1634) and three Gram-positive bacteria (*Bacillus cereus* BIOTECH 1509, *Listeria monocytogenes* BIOTECH 1958, and Methicillin-Resistant *Staphylococcus aureus* BIOTECH 10378) were tested against *C. minutus* crude extract using microtiter plate dilution assay. These reference pathogens were obtained from PNCM, BIOTECH-UPLB. The bacterial strains used in this study are known bacterial pathogens reported in the Philippines causing diseases such as listeriosis, gastroentiritis, food poisoning, cellulitis, sepsis, and urinary tract infections (Table 1) [9]. Thus, the antibacterial assay was done to assess the effectivity of the algal extract against these medically-important bacterial pathogens. Initially, bacterial pathogens were pre-cultivated using Luria Bertani broth medium and incubated at with shaking for 24 hours at 37 ºC. Purity and viability of each test organisms were regularly monitored by conducting regular biochemical tests and morphological characterization [2,9]. Microtiter plate dillution (two-fold serial dilution technique) assay was used to determine the antibacterial activities of *C. minutus* extract [2,9]. Briefly, 100 µl of broth cultures of the bacterial pathogens (cell density of 1x 10^6 cells/ml) were gently mixed with 100 µl of *C.*

minutus extract set at different dilutions (1, $\frac{1}{2}$, $\frac{1}{4}$, $\frac{1}{8}$, $\frac{1}{16}$, $\frac{1}{32}$, $\frac{1}{64}$, and $\frac{1}{128}$ of algal crude extract concentrations). The antibacterial assay was done in triplicates and were incubated for 12 hours in an incubator set at 35°C. After incubation, antibacterial activity of *C. minutus* extract against different bacterial pathogens were taken [2,9].

Table 1. Bacterial pathogens used in the assay and their respective pathogenicity

Bacterial Pathogen	**Pathogenicity (Diseases)**
Gram-positive bacteria	
Bacillus cereus **BIOTECH 1509**	Food poisoning and gastrointestinal illness: emetic (vomiting) syndrome and the diarrhoeal syndrome
Listeria monocytogenes **BIOTECH 1958**	Causes listeriosis
Methicillin-Resistant ***Staphylococcus aureus*** **BIOTECH 10378**	Food poisoning and infection in hospitals
Gram-negative bacteria	
Pseudomonas aeruginosa **BIOTECH 1824**	Generalized inflammation and sepsis
Aeromonas hydrophila **BIOTECH 10089**	Gastroentiritis in humans; ulcers, tail rot, fin rot and hemorrhagic septicemia in fish
Escherichia coli **BIOTECH 1825**	Gastrointestinal and urinary tract infection

α-Glucosidase Inhibition Assay

The ability of *C. minutus* extract to inhibit α – glucosidase was assessed by spectrophotometric assay using *p*-nitrophenyl-α-glucopyranoside (*p*NPG) following the methods of Nair et al. [10]. The presence of α-glucosidases in algal extract converts *p*NPG (substrate) to *p*-nitrophenol (*p*NP) and is measured spectrophotometrically at 410 nm wavelength. Initially, a mixture containing 75 µl of α – glucosidase (2.5 U/ml), 100 µl of algal extract or 100 µl of 0.1 M phosphate buffer pH 6.8 (for the case of the control) were mixed in sterile test tubes. The total volume of the mixture was adjusted to 500 µl by adding 30 µl of 10mM p-nitrophenyl-α-D-glucopyranoside (Sigma N1337) and 295µl buffer before incubation. The mixtures were then incubated at 37 ^{0}C for 12 minutes after which 3 mL of 50 mM NaOH were added in the mixture [6,10]. Absorbance reading of each reaction mixtures (samples) was taken at 410 nm. The percent α – glucosidase inhibition was determine using the equation below:

$$\boldsymbol{\alpha - Glucosidase\ Inhibition}\ (\%) = \left(\frac{\boldsymbol{A_{control} - A_{sample}}}{\boldsymbol{A_{control}}}\right) \times \mathbf{100}$$

Statistical Analyses

The data obtained from the experiments are given as means ± standard deviations (mean ± SD) of three replicates [2]. In addition, correlation analysis was done using MS Office Excel 2017.

RESULT AND DISCUSSION

Total Phenolic Content and Antioxidant Activity

Bioactive compounds from cyanobacteria such as carotenoids, polyunsaturated fatty acids, mycosporine-like amino acids (MAAs), phenolic compounds, and polysaccharides are known potent antioxidants [11]. However, limited reports are available on the antioxidant properties, identification, and quantification of these compounds and its important role in the algal defense against exposure to high concentration of reactive oxygen species (ROS) [12]. Polyphenols are secondary metabolites found in several cyanobacterial species which can be tapped nowadays for pharmaceutical application because of the reported potent antioxidant, antibacterial, and antidiabetic properties [11,12,13]. The TPC of *C. minutus* is 11.27 ± 0.14 mg GAE/g extract. This result was greater than those observed from other cyanobacteria such as *Lyngbya* sp., *Oscillatoria* sp., *Spirulina* sp., and *Microcystis* sp. with TPC of 5.02 ± 0.20, 2.96 ± 0.14, 1.78 ± 0.07, and 2.65 ± 0.14 mg GAE/g, respectively [13]. However, Singh et al. [14] reported that cell-free extract of *Anabaena dolium, Calothrix geitonos, Oscillatoria ocuta,* and *Nostoc ellipsosporum* have higher TPC than *C. minutus*, which are 47.77 ± 3.4, 22.80 ± 2.3, 290.23 ± 2.2, and 39.03 ± 1.8 mg GAE/g, respectively. In general, variations in the amount of phenolic compounds in the cyanobacterial extract is dependent on the extraction protocol, type and polarity of solvent used in the extraction as well as solubility of algal polyphenols in the extraction solvents [11,12].

Table 2. DPPH radical scavenging activity and IC_{50} value of *Chroococcus minutus* extract and ascorbic acid

Chroococcus minutus		**Ascorbic Acid**	
Phenolic concentration (μg GAE/ml)	$DPPH^+$ Inhibition (%)	Concentration (μg/ml)	$DPPH^+$ Inhibition (%)
100	30.28 ± 0.21	100	18.86 ± 0.21
200	43.97 ± 0.84	200	38.12 ± 0.37
300	55.02 ± 0.58	300	57.16 ± 0.42
400	62.69 ± 1.10	400	76.43 ± 0.89
500	71.32 ± 0.05	500	91.35 ± 0.37
IC_{50}*	**255 μg/ml**	**IC_{50}***	**262 μg/ml**

*IC_{50} is the effective concentration that inhibits the activity of DPPH radical by 50%. Computed by interpolation.

Phenolic compounds derived from cyanobacteria are potent free radical scavengers. These compounds are capable of scavenging DPPH by donation of electron or hydrogen atom provision. Phenolic compounds transform DPPH into a colorless product (2, 2-diphenyl-1- hydrazine), causing a decrease in optical density of the sample extract [15]. The antioxidant activity of *C. minutus* extract was evaluated using the DPPH radical scavenging assay. Results showed that the activity against DPPH free radicals increased when the concentration of *C. minutus* extract (100 - 500 μg/ml) increases (Table 2). The IC_{50} value of the cyanobacterial extract (255 μg/ml) showed a more potent antioxidant activity than

that obtained from the control antioxidant, ascorbic acid with IC_{50} of 262 μg/ml. The antioxidant activity of *C. minutus* is considered more effective than those obtained from other species of cyanobacteria such as *Calothrix brevissima, Westiellopsis prolifica, Scytonema simplex,* and *Anabaena constricta* with IC_{50} value of 2.24 ± 0.18, 3.52 ± 0.26, 1.42 ± 0.19, and 0.91 ± 0.04 mg/ml, respectively [14]. Antioxidant activity of cyanobacteria may be associated to several polyphenols (such as gallic acids, vanillic acids, quercetin, ferulic acids, kaempferol, and rutin) present in *C. minutus* extract. These active substances are reported to have potent free radical scavenging ability [14]. In addition, factors such as strains of the cyanobacteria and culture growth conditions may also show an important effect on the amount and profile of phenolic compounds (in the extract) and thus affecting the antioxidant activity of the algal extract [2,14].

Antibacterial Activity

Cyanobacteria are good sources of active metabolites with potent antimicrobial activities. These reported antimicrobial activities are associated with various intracellular and extracellular metabolites that are produced by several strains of this group of cyanobacteria with known diverse biological activities [16,17]. *Chroococcus minutus* exhibited antibacterial activities against known pathogenic bacteria (Methicillin-resistant *Staphylococcus aureus* (MRSA), *Bacillus cereus* and *Listeria monocytogenes*) (Table 3). The antibacterial property of *C. minutus* extract against *B. cereus* and *L. monocytogenes* is comparable to those observed from extracts of *Moorea producens* which exhibited minimum inhibitory concentration (MIC) value of 250 μg/ml for both bacterial pathogens [18]. In addition, *C. minutus* extract exhibited effective antibacterial activity against MRSA like that of *Nostoc* sp. with reported MIC value of 0.8 μg/ml [19]. The antibacterial activity exhibited by *C. minutus* in this study may be associated to known secondary metabolites in cyanobacteria such as gallic acids, quercetin, ferulic acids, and kaempferol which are toxic to several microorganisms such as bacteria [16,19]. The study also reported that *C. minutus* extract did not exhibit antibacterial activities against Gram-negative bacteria (Table 3). In general, *C. minutus* extract is more effective in Gram-positive than Gram-negative bacteria. These differences in the antibacterial activities may be associated with a multilayered structure of cell wall in Gram-negative bacteria that serve as additional barrier that protects the bacterial cells from bioactive compounds present in the algal extract [20].

α-Glucosidase Inhibition Activity

Diabetes is a metabolic disease characterized by having high concentration of glucose in blood [21]. The management of this metabolic disorder is done by lowering the postprandial increase in blood glucose levels via inhibition of important carbohydrate-degrading enzyme (α-glucosidase), responsible for the breakdown of complex carbohydrates into glucose [22]. In this study, evaluation of the potential antidiabetic activity of *C. minutus* was done *in vitro* via α-glucosidase inhibition assay. The inhibitory activity of *C. minutus* extract on the target enzyme is presented in Table 4. The algal extract exhibited a

concentration-dependent reduction in α-glucosidase inhibition. *C. minutus* extract exhibited the highest inhibition of α-glucosidase (91.29 ± 0.05%) at phenolic extract concentration of 8 µg GAE/ml. *C. minutus* extract has potent α-glucosidase inhibition property (IC_{50} of 5.5 µg/ml) – more effective than acarbose (IC_{50} of 6771 µg/ml) which is a known standard antidiabetic drug. The IC_{50} value of *C. minutus* extract against α-glucosidase is comparable to that observed for methanolic extract of *Arthrospira platensis* and *Cylindrospermum* sp. with IC_{50} values of 145 µg/ml and 84 ±6.8 nM, respectively [23,24]. The antidiabetic properties of cyanobacteria can be attributed to bioactive substances such as phenolic compounds, pigments, and polysaccharides that are reported to possess α-glucosidase inhibitory activity [25,26,27]. These compounds are known inhibitors of hydrogen ion (via hydrogen scavenging mechanism) that is being discharged from the active site of the target enzyme (α-glucosidase) [6,26,27]. In conclusion, *Chroococcus minutus* extract exhibited promising bioactivities such as antioxidant, antibacterial, and alpha-glucosidase inhibition properties that can be used for pharmaceutical application. It is recommended that additional studies should be done to identify the bioactive compounds present in the cyanobacterial extract. Also, *in vivo* experimental trials should be conducted to confirm the effectivity and assess the safety of *C. minutus* extract for future medical application.

Table 3. Antibacterial activities of *Chroococcus minutus* extract

Bacterial Pathogen	**Antibacterial Activity**
Gram-positive bacteria	
Bacillus cereus **BIOTECH 1509**	++++
Listeria monocytogenes **BIOTECH 1958**	++++
Methicillin-Resistant *Staphylococcus aureus* **BIOTECH 10378**	+++
Gram-negative bacteria	
Pseudomonas aeruginosa **BIOTECH 1824**	ND
Aeromonas hydrophila **BIOTECH 10089**	ND
Escherichia coli **BIOTECH 1825**	ND

*+ = full crude extract concentration; + + = $\frac{1}{2}$ of crude extract concentration; + + + = $\frac{1}{4}$ of crude extract concentration; + + + + = $\frac{1}{8}$ of crude extract concentration; ND = None Detected.

Table 4. Alpha-glucosidase inhibition activity and IC_{50} value of *Chroococcus minutus* extract and acarbose

Chroococcus minutus		**Acarbose****	
Phenolic concentration (µg GAE/ml)	Alpha-glucosidase Inhibition (%)	Concentration (µg/ml)	Alpha-glucosidase Inhibition (%)
4	13.73 ± 0.72	2000	17.96 ± 1.36
5	34.10 ± 1.34	4000	31.69 ± 1.22
6	67.90 ± 0.91	6000	45.32 ± 1.90
7	83.08 ± 2.25	8000	57.26 ± 0.49
8	91.29 ± 0.05	10000	62.35 ± 0.49
IC_{50}*	**5.5 µg/ml**	**IC_{50}***	**6771 µg/ml**

*Inhibitory concentration that inhibits alpha-glucosidase activity by 50%.
**Reference alpha-glucosidase inhibitor and anti-diabetic drug.

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AUTHOR CONTRIBUTIONS

Concept: *E.D.L.R.A*; Design: *E.D.L.R.A*; Control: *E.D.L.R.A*; Sources: *E.D.L.R.A*; Materials: *E.D.L.R.A*; Data Collection and/or processing: *E.D.L.R.A*; Analysis and/or interpretation: *E.D.L.R.A*; Literature review: *E.D.L.R.A*; Manuscript writing: *E.D.L.R.A*; Critical review: *E.D.L.R.A*; Other: *E.D.L.R.A.*

CONFLICT OF INTEREST

The author declares no conflict of interest.

ETHICS COMMITTEE APPROVAL

The author declares that the ethics committee approval is not required for this study.

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