Antimicrobial activity of ethanol fraction from cyanobacteria

*Chroococcus turgidus*

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**Abstract.** *Chroococcus turgidus* belongs to cyanobacteria, a photosynthetic prokaryote, which produces many valuable bioactive compounds and shows interesting biological activities. In this study, we investigated the antimicrobial activity of the ethanol fraction from *C. turgidus* and identified compounds that responsible for the activity. *C. turgidus* biomass was extracted using the reflux method and then fractionated by column chromatography. The antimicrobial activity was evaluated against *Staphylococcus aureus*, *Escherichia coli*, *Bacillus subtilis*, and *Candida albicans* using agar disc diffusion method. The result showed significant activity against all tested microbes. The largest zone of inhibition was 18.1 ±0.14 mm, achieved by the fraction against *S. aureus*. Characterization using Gas Chromatography-Mass Spectrometry revealed the volatile organic compounds with 1,2-Benzenedicarboxylic acid, bis(2-ethylhexyl)ester as the dominant compound. This study revealed that the bioactive compounds produced by *C. turgidus* could be a potential source of natural antimicrobials.

**1. Introduction**

Problems related to microbial resistance are still growing and efforts to find safer antimicrobial agents instead of synthetic antibiotics are still being encouraged. Natural compounds are considered biodegradable, harmless or safer than synthetic ones because of their limited side effects [1], and microalgae have become an interesting source of those materials to study. The first investigation on the antibiotic activity of algae was carried out by Pratt et al. [2]. Since then many studies have been conducted to prove the antimicrobial effect of algal metabolites, especially from cyanobacteria [3][4].

Cyanobacteria is an ancient group of prokaryotic photosynthetic microorganism that has a wide distribution, can be found in almost all ecological habitats such as freshwater, marine, tree bark, thermal springs, and Antarctic region [5].

Cyanobacteria are rich of metabolites and showing interesting biological activities ranging from antibiotics, immunosuppressant, anticancer, antiviral, anti-inflammatory, proteinase-inhibiting agents [6], antifungal [7] to antimalarial [8]. It is reported that antibacterial activities related to the content of fatty acids [9][10]. Fatty acids also have the ability to inhibit fungi without giving a negative effect to the host [11]. One of the promising members of cyanobacteria is *Chroococcus turgidus*. 
C. turgidus contain many bioactive compounds like lipopeptide 40%, amino acid 5.6%, fatty acid 4.2%, amida 9%, polysaccharide, biosurfactant, vitamin, chlorophyll, and phycobiliprotein [12]. A study from Abou El-Kheir et al. [13] reported that methanolic extract of C. turgidus inhibited the growth of Salmonella typhi and Acinetobacter baumannii. Other study reported antibacterial and antifungal activity of those extract [14]. It was also reported that methanolic extract of C. turgidus exhibited positive inhibition against E. coli [15]. Most studies use methanol as a polar solvent for extraction, only a few use ethanol. Therefore in this study, extraction was carried out using ethanol solvents, which harmless compare to methanol. The main goal of this study was to investigate the antimicrobial activity of the ethanol fraction from C. turgidus against Gram-positive bacteria, Gram-negative bacteria, and fungi and later identified compounds that responsible for the activity with GC-MS analysis.

2. Materials & Methods
2.1. Cultivation of Microalgae C. turgidus.
Microalgae was cultivated in Johnson medium consisting of: MgSO₄ (0.5 g/L), CaCl₂ (0.2 g/L), MgCl₂ (1.5 g/L), NaHCO₃ (0.045 g/L), KH₂PO₄ (0.035 g/L), KNO₃ (0.5 g/L), NaCl (27 g/L), and micronutrient solution (1 ml/L) under 2500 lux light intensity and continuous aeration. The culture growth was evaluated daily based on its cell density using Spectrophotometer UV-VIS (Hitachi U-3900H) at 680 nm [16][17]. Microalgae was cultured for 14 days and harvested at stationary phase by centrifugation (Kubota 6200, Japan) at 6000 rpm for 5 minutes. Biomass was oven-dried at 50°C.

2.2. Preparation of Algal Extract
Dried biomass was ground to a fine powder and subjected to extraction using reflux method. 20 g of grounded algal biomass was extracted with 96% ethanol (1:10 w/v) for 2 hours. The obtained extract was filtered and evaporated using rotary evaporator (Janke & kunkel RV 05-ST) to get a concentrated extract. The extract was subjected for further analysis.

2.3. Phytochemical Analysis
Phytochemicals content such us alkaloid, flavonoid, saponin, tannin, triterpenoid, and steroid were determined by the method described by Harborne [18].

2.4. Isolation of Compounds
The compounds content of the extract were separated by chromatographic techniques. Thin-layer chromatography (TLC) was carried out as a first step to determine the best solvent for column chromatography. This process was developed using hexane, ethyl acetate, and ethanol in various ratio (2:7:1, 3:6:1, 5:4:1, 6:3:1, 7:2:1, 8:1:1, 8:1, 5:5, 8:2) as mobile phase. TLC sheets silica gel 60 F₂₅₄ (Merck) was used as a stationary phase. The spots on the TLC plate were marked. The Rf value of each spot was determined as the comparison between compound distance from the origin (midpoint) and solvent front distance from the origin [19]. Column chromatography was carried out according to the method described by Salituro & Dufresne [20]. Fractions were collected then combined based on the similarity of Rf and tested its antimicrobial activity.

2.5. Antimicrobial Assay
The antimicrobial activity of extract and fractions were evaluated against Staphylococcus aureus, Escherichia coli, Bacillus subtilis, and Candida albicans using agar disc diffusion method [21]. The density of microbes used was 10⁶ cell/ml. Sterile paper discs were placed at the surface of double-layer agar. 15 μl solution of each concentration 20,000 ppm and 30,000 ppm was dropped on the paper disc, allowed to dry and incubated at 30°C for 18-24 hours. Antbiotic chloramphenicol and
antifungal metronidazole (concentration of 20 ppm) were used as positive control while ethanol 96% as a negative control. The zone of inhibition was observed and measured as a millimeter in diameter.

2.6. GC-MS Analysis
Analysis of active fraction was performed using GC-MS 6973 N (Agilent Technologies) with parameters: program temperature 70°C-290°C, rate of temperature 15°C/min, used DB 5 capillary column (60 m in length and 0,25 μm in diameter), and used helium as a carrier gas. The active fraction was diluted in 1 ml ethanol then 2 μl was injected with the syringe to the GC-MS. The mass spectra of the component were compared with the Wiley10 Library and were obtained the name, molecular weight, and structure of compounds.

3. Results and Discussion
3.1. The Growth of Microalgae C.turgidus.
The result showed an increase of growth curve significantly (day 1-6), which was an indication of exponential (logarithmic) phase (Figure 1). This phase occurs when there are abundant nutrients and spaces for the cell to grow. After the exponential phase, microalgae entered stationary phase (day 6-9). In this phase, cell density reaches its saturated point due to the utilized most of the nutrients and the limited spaces for growth. Competition of nutrients, light, and spaces for growth eventually caused the cell to enter the death phase that indicated by the decrease of the growth (day 10-14) [22].

![Figure 1. The growth curve of microalgae C.turgidus.](image)

Biomassa was harvested at stationary phase by centrifugation. In this phase, secondary metabolites optimally produced, among them is a fatty acid, which is potential as antibacterial [23]. Some factors that play a role in the production of antimicrobial agents are temperature, pH, incubation period, medium constituents, and light intensity [24]. The result of biomass extraction showed the yield of ethanol extract as much as 66,95% (13,39 g).

3.2. Antimicrobial Activity of Ethanol Extract
The antimicrobial analysis indicated that all tested microbial strains showed moderate sensitivity to the ethanol extract of C. turgidus with the highest activity against C. albicans (14,5±2,12 mm inhibition zone). The diameter of the inhibition zone is more extensive with a higher concentration of extract. An antibacterial agent usually less effective against Gram-negative bacteria because of their complex and multilayered cell wall, which makes it more difficult for the active compound to
penetrate [25]. In contrast to that statement, our result showed that there was no significant difference between the inhibition of Gram-positive and Gram-negative bacteria.

Table 1. Antimicrobial activity of ethanol extract

| Microbial strains | Diameter of inhibition zone (mm) |
|-------------------|----------------------------------|
|                   | Cons 20,000 ppm                  | Cons 30,000 ppm                  |
| B. subtilis       | 12 ±14.0                         | 13 ± 14.0                        |
| S. aureus         | 12.5±0.70                        | 13.5±0.70                        |
| E. coli           | 13.5±2.12                        | 13 ±1.41                         |
| C. albicans       | 12 ± 0                           | 14.5 ±2.12                       |

The previous study reported antimicrobial activity of some different extract. A study from Bharanidharan et al. [26] reported that hexane extract of C. turgidus inhibited 7 bacterial strains, i.e S. aureus, S. epidermidis, E.coli, V. parahaemolyticus, Enterococcus faecalis, Enterobacter aerogenes, and Eubacterium lentum with the largest zone of inhibition was 13.3 ± 1.84 against V. parahaemolyticus. Other study reported antibacterial and antifungal activity of methanolic extract from C. turgidus against S. aureus (13 mm), Salmonella typhi (8 mm), Pseudomonas aeruginosa (8 mm), Aspergillus flavus (5.5 mm), Penicillium notatum (10 mm), and Candida albicans (7 mm) [14]. A study from Abou El-Kheir et al. [13] reported that methanolic extract of C. turgidus inhibited the growth of Salmonella typhi and Acinetobacter baumannii with diameter of inhibition zones were 27 mm and 17 mm, respectively. It was also reported that methanolic extract of C. turgidus exhibited positive inhibition (92.6%) against E. coli [15].

3.3. Phytochemical Analysis

The result showed that the majority gave a negative reaction during phytochemical tests. The positive reaction only revealed in the flavonoid test (Table 2). According to Harborne [18], flavonoids are the most abundant group of phenolic compounds. Flavonoids are known as compound that have antibacterial activity. Flavonoids are active against several bacteria such as Streptococcus [27], E. coli and S. aureus [28].

Table 2. Phytochemical tests of ethanol extract

| Phytochemical tests | Reaction |
|---------------------|----------|
| Alkaloid            | Mayer    |
|                     | Drageadorff |
|                     | Wagner   |
| Flavonoid           | +        |
| Saponin             | -        |
| Tannin              | -        |
| Steroid and Triterpenoid | -     |

3.4. Isolation of Compounds

TLC analysis in various eluent (2:7:1, 3:6:1, 5:4:1, 6:3:1, 7:2:1, 8:1:1, 8:1, 5:5) did not show a good separation of compounds because the spots were concentrated at the bottom of plate. Good results shown in the eluent 8:2 with the formation of several spots with Rf value 0.9, 0.7, 0.5, and 0.1 (Figure 2). Based on this result, the composition eluent (8:2) was used as the eluent in column chromatography.
Figure 2. TLC chromatogram of ethanol extract that was developed using hexane and ethyl acetate (8:2)

There were 40 fractions produced from the separation using column chromatography and based on fraction simplification 12 combined fractions were obtained (Table 3). These fractions were tested its antimicrobial activity.

Table 3. Combined fractions of column chromatography

| Combined fraction | Rf Value | Fraction number |
|-------------------|----------|----------------|
| 1                 | 0.6 ; 0.52 ; 0.49 | Fraction 1     |
| 2                 | 0.56 ; 0.49 ; 0.47 ; 0.43 ; 0.36 | Fraction 2     |
| 3                 | 0.54 ; 0.49 ; 0.45 ; 0.34 | Fraction 3     |
| 4                 | 0.47 ; 0.43 ; 0.34 | Fraction 4     |
| 5                 | 0.52 ; 0.47 ; 0.45 ; 0.36 ; 0.27 | Fraction 5     |
| 6                 | 0.49 ; 0.43 ; 0.36 ; 0.27 | Fraction 6     |
| 7                 | 0.47 ; 0.36 ; 0.30 | Fraction 7     |
| 8                 | 0.45 ; 0.36 ; 0.30 ; 0.27 | Fraction 8     |
| 9                 | 0.47 ; 0.4 ; 0.32 ; 0.27 | Fraction 9     |
| 10                | 0.41 ; 0.34 ; 0.29 ; 0.23 | Fraction 10    |
| 11                | 0.43 ; 0.32 ; 0.29 | Fraction 11    |
| 12                | 0.25 | Fraction 12-40 |

3.5. Antimicrobial Assay

The results showed that almost all fractions had antimicrobial activity against all tested microbes (Table 4). The majority of inhibitory zones formed in Gram-positive bacteria are greater than Gram-negative. This is because Gram-positive bacteria (S. aureus) have a simpler cell wall structure that tends to be more sensitive to antibacterial components while the cell wall structure of Gram-negative bacteria is more complex and consists of 3 layers, namely the outer layer in the form of lipoprotein, middle layer in the form of peptidoglycan, and the inner layer is lipopolysaccharide [29][30]. This is confirmed by the opinion that the Gram-positive bacterial cell membrane layer is composed by a more permeable peptidoglycan layer [31] while the outer membrane on Gram-negative bacteria contains lipopolysaccharide which becomes an impermeable protective layer [32]. This is what often causes Gram-negative bacteria to be more resistant to external treatment. Fractions were also showed good antifungal activity against C. albicans that indicated by the inhibition zone were formed, ranging from the least 6.6 ± 0.21 mm to the highest zone at 12.9 ± 4.80. From the result, it was showed that fraction
Table 4. Diameter of inhibition zone of fractions against tested microbial (K+: chloramphenicol for bacteria and metronidazole for fungi; K - : ethanol)

| Fraction | B. subtilis | S. aureus | E. coli | C. albicans |
|----------|------------|-----------|---------|-------------|
| F1       | -          | 9.4 ± 0.21| -       | 11.6 ± 0.35 |
| F2       | 7.1 ± 0.28 | -         | 5.3 ± 0.35 | 7.8 ± 2.19 |
| F3       | 11.4 ± 0.35| 9.1 ± 0.42| 6.1 ± 0.28| 11.3 ± 2.26 |
| F4       | 9.9 ± 0.21 | 10.2 ± 0.14| 7.2 ± 0.35| 10.8 ± 0.07 |
| K (+)    | 14.2       | 12.4      | 9.5     | 12.0        |
| K (-)    | -          | -         | -       | -           |
| F5       | 5.7 ± 0.56 | 5.3 ± 0.42| 9.4 ± 0.14| 9.8 ± 1.83 |
| F6       | 5.4 ± 0.21 | 6.0 ± 0.14| 9.0 ± 0.14| -           |
| F7       | 8.6 ± 0.21 | 8.8 ± 0.14| -       | 11.2 ± 0.07 |
| F8       | 6.2 ± 0.14 | 6.7 ± 0.63| 7.2 ± 0.21| 7.2 ± 2.12 |
| K (+)    | 10.3       | 11.4      | 11.2    | 12.1        |
| K (-)    | -          | -         | -       | -           |
| F9       | 6.0 ± 0.42 | 10.8 ± 0.14| 10.4 ± 0.63| 10.1 ± 0.98 |
| F10      | 5.4 ± 0.70 | 5.5 ± 0.49| 8.9 ± 0.007| 7.2 ± 0.77 |
| F11      | 12.1 ± 0.84| 11.4 ± 0.28| 10.2 ± 0.07| 6.6 ± 0.21 |
| F12      | 17.1 ± 0.21| 18.1 ± 0.14| 14.6 ± 0.07| 12.9 ± 4.80 |
| K (+)    | 11.7       | 12.5      | 10.7    | 11.4        |
| K (-)    | -          | -         | -       | -           |

Notes: samples concentration were 20,000 ppm; chloramphenicol and metronidazole concentration was 20 ppm

3.6. GC-MS Analysis

The mass spectrum of the unknown component was compared with the spectrum of the known components stored in the WILEY10N.14 library. The name, molecular weight, and structure of the components were obtained. The results showed that 15 major compounds (SI > 90%) were identified at retention time ranging from 6.73 to 24.72 as shown in Table 5. The GC-MS analysis of fraction 12 showed that the most dominant compound was 1,2-Benzenedicarboxylic acid,bis(2-ethylhexyl)ester with a percent area of 84.84% and a retention time of 24.72 minutes. The structure of this compound shown in Figure 3. A few studies have revealed the potential of this compound as antibacterial, but other studies reveal their potential as anticancer. 1, 2- Benzenedicarboxylic acid, bis(2-ethylhexyl) ester was successfully isolated from the *Thevetia peruviana* twigs extract. This compound was also known to be present in 13 other medicinal plants, including Indian Ginseng (leaves of *Panax pseudoginseng* subsp himalaicus). Bioactivity experiments revealed positive anti-cancer activity of this compound on PC3, MCF, HCT-116, A549, and MIAPACA cell lines, and this compound was proved to be a strong immunomodulatory B-cell stimulant. Therefore, this compound potentially used as biomarker [33]. Phenolic compounds such us Phenol, 2,4-bis (1,1-dimethylethyl) and Phenol, 4,4’-(1-methylethylidene) bis, and E-15 heptadecenal were also detected. A study from Seow et al. [34] revealed the antimicrobial activity of these compounds (Phenol, 2,4-bis (1,1-dimethylethyl) and E-15 heptadecenal) that were detected in ethyl acetate fraction of *Gynura segetum*. Phenol also showed bioactivity as antiseptic and disinfectant [35]. Phenolic compounds include extensive organic substances, namely aromatic compounds with hydroxy groups and some of them have antibiotic material. Polyphenols and flavonoids are the largest and most found in plants [18]. Some fatty acids were detected such us palmitic acid and stearic acid. Kabara [36] and Desbois et al. [37] reported that
fatty acids such as oleic, palmitic, stearic, myristic, linoleic, and linolenic acids showed antibacterial activity against *Staphylococcus* sp. The exact mechanism of fatty acids in causing bactericidal effects is still uncertain, but it is suspected because these molecules initiate the peroxidation process and inhibit fatty acid synthesis from bacteria [38]. In addition, fatty acids can reduce the absorption of nutrients and inhibit cellular respiration [39]. Fatty acids also have the ability to inhibit fungal growth [11].

![Figure 3. The structure of 1,2-Benzenedicarboxylic acid, bis(2-ethylhexyl)ester](image)

Table 5. Major compounds of active fraction (fraction 12) of *C. turgidus* using GC-MS analysis

| No. | Compounds                                                                 | Retention time | Area (%) | Molecular formula | SI (%) |
|-----|---------------------------------------------------------------------------|----------------|----------|-------------------|--------|
| 1   | Benzene, 1,3,5-trimethyl /(Mesitylene)                                     | 6.73           | 0.83     | C_{9}H_{12}        | 94     |
| 2   | Methyl salicylate                                                          | 13.93          | 0.69     | C_{8}H_{8}O_{3}    | 95     |
| 3   | 1,2-benzene-dicarboxylic acid, dimethyl ester                              | 17.24          | 1.73     | C_{10}H_{10}O_{4}  | 91     |
| 4   | 2,5-Cyclohexadiene-1,4-dione, 2,6-bis (1,1-dimethyl) ether                 | 17.37          | 0.28     | C_{14}H_{20}O_{2}  | 99     |
| 5   | Phenol, 2,4-bis (1,1-dimethyl) ether                                        | 17.74          | 1.16     | C_{14}H_{22}O      | 93     |
| 6   | 1-Hexadecene                                                              | 18.38          | 0.21     | C_{16}H_{32}       | 98     |
| 7   | Benzoic acid,2-ethylhexyl ester                                            | 19.40          | 0.82     | C_{15}H_{22}O_{2}  | 95     |
| 8   | 1-Octadecene                                                              | 19.92          | 0.20     | C_{18}H_{38}       | 99     |
| 9   | 7,9-Di-tert-butyl-1-oxaspiro (4,5) decu-6,9-diene-2,8-dione                | 20.93          | 0.35     | C_{17}H_{34}O_{3}  | 99     |
| 10  | Palmitic acid                                                             | 21.10          | 0.77     | C_{16}H_{32}O_{2}  | 99     |
| 11  | E-15-Heptadecenal                                                         | 21.30          | 0.14     | C_{17}H_{32}O      | 99     |
| 12  | Cycloecosane                                                              | 21.30          | 0.14     | C_{20}H_{40}       | 96     |
| 13  | Stearic acid                                                              | 22.37          | 0.32     | C_{16}H_{36}O_{2}  | 97     |
| 14  | Phenol, 4,4’-(1-methylene)bis(2-ethylhexylidene)                          | 22.65          | 1.15     | C_{15}H_{16}O_{2}  | 98     |
| 15  | 1,2-Benzenedicarboxylic acid,bis(2-ethylhexyl)ester                       | 24.72          | 84.84    | C_{24}H_{38}O_{4}  | 91     |

4. Conclusions
Based on this study, *C. turgidus* showed significant activity against all tested microbes. The antimicrobial activity may be due to the presence of 1,2-Benzenedicarboxylic acid, bis(2-ethylhexyl)ester as the dominant compound, phenolic compound, fatty acids, and other constituents. This study revealed that the bioactive compounds produced by *C. turgidus* could be a potential source
of natural antimicrobials. Further work is needed to purify and identify the compound that precisely responsible for the antimicrobial activity.

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6. References

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