Exploration of *Chlorella sp.* as Antibacterial to *Aggregatibacter Actinomycetemcomitans* Biofilm

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Abstract. *Aggregatibacter actinomycetemcomitans* is a facultative anaerobic gram-negative, which is major pathogenic bacteria cause of aggressive periodontitis that has the ability to form biofilm. The therapy of aggressive periodontitis has been done with tetracycline antibiotics, meanwhile it may cause resistance problem. *Chlorella* sp. has antimicrobial activity against the anaerobic gram-negative bacteria as an adjunctive therapy of aggressive periodontitis. **Purpose:** This study aimed to determine the antibacterial potency of various concentrations of *Chlorella sp.* to *A. actinomycetemcomitans* biofilm. **Method:** The antibacterial potency of *Chlorella* sp. to *A. actinomycetemcomitans* were examined by biofilm test, divided into 5 groups, each group consisted of 4 samples. The control groups were: K (aquadest), K+ (tetracycline), and treatment groups were given *Chlorella* sp. in various concentrations: P1 (0.625%), P2 (1.25%), and P3 (2.5%). The inhibition of biofilm formation were examined on microtiter plate by measure its Optical Density (OD) value on ELISA Reader. Data were analyzed using One Way ANOVA followed by LSD test. **Result:** Treatment with *Chlorella* sp. in all treatment groups decreased the OD value, as well with tetracycline (p<0.05). The decrease in OD values indicated that more biofilms were inhibited due to its antibacterial potency. Treatment with 2.5% of *Chlorella* sp. showed the greatest biofilm inhibition among the treatment groups (p<0.05). **Conclusion:** *Chlorella* sp. extract has antibacterial potency against *A. actinomycetemcomitans* biofilm

**Keywords:** *Aggregatibacter actinomycetemcomitans*, biofilm, *Chlorella* sp., antibacterial

1. **Introduction**

Periodontal disease is a dental problem that has a high prevalence. In Indonesia, prevalence of the periodontal disease reached up to 96.58% for all age groups [1]. Aggressive periodontitis is a complex periodontal disease and affecting children before puberty and young adults under 30 years old [2]. Physical activity, socio-economic, education, employment, smoking, hypertension, and stress are consider as risk factors of periodontal disease [3].

In periodontal disease, anaerobic gram-negative bacteria are dominant compared to aerobic gram-positive bacteria [4]. Some pathogenic bacteria spesies that cause periodontal tissue distruption are *Capnocytophaga gingivalis*, *Peptostreptococcus micros*, *Eubacterium nodatum*, *Aggregatibacter Actinomycetemcomitans*, *Porphyromonas gingivalis*, *Prevotella intermedia*, *Tannerella forsythia*, *Treponema denticola*, dan *Fusobacterium nucleatum* [5].

Biofilm is colonization of the bacteria as defense mechanism for its survival [6]. Microorganism that colonized and embedded in a biofilm matrix will be more resistant to antimicrobials compared to planktonic cells [7]. Biofilm formation is commonly consider to occur in four main stages: (1) formation of pellicles on the surface of the teeth continue with the onset of bacterial attachment, (2) microcolony formation, (3) biofilm maturation and (4) detachment or biofilm cell dispersion which may then colonize at new areas [8].
Aggregatibacter Actinomyccetemcomitans is the primary etiologic agent of aggressive periodontitis [8]. Aggregatibacter Actinomyccetemcomitans has several virulence factors, which are lipopolysaccharide (LPS), cytolethal distending toxin (CDT), leukotoxins, and part of the RTX (repeat of toxins) that has different mechanism. LPS would trigger an inflammatory response, CDT works as immunosuppressive, while leukotoxins and RTX cause imbalance of the immune system. Therefore, aggressive periodontitis describes as a type of periodontal disease which often associated with immune system disorder [9].

Patients diagnosed with aggressive periodontitis often does not respond predictably on scaling and root planing treatment regarding to its complex multi-factorial etiology [8]. Hence, antibiotic treatment frequently as adjunctive therapy is reported to be an optimal therapy and supports a favorable clinical outcome in treating aggressive periodontitis [9].

Tetracycline antibiotics are one of the antibiotics that works as a broad spectrum bacteriostatic that inhibits protein synthesis and active against both gram-positive and gram-negative bacteria [10]. Tetracycline is used for treating aggressive periodontitis and effective in inhibiting Aggregatibacter actinomyccetemcomitans bacteria because it could penetrate periodontal tissue and tetracycline has anti collagenase effects which can inhibit tissue damage and help bone regeneration [11].

Chlorella sp. is a one of the microalgae that has an ability as an alternative antimicrobial [12,13]. Previous research conducted by Christian showed that Chlorella sp. able to inhibit the formation of anaerobic gram-negative bacterial biofilms, which is Porphyromonas gingivalis in chronic periodontitis, while Aggregatibacter actinomyccetemcomitans bacteria are also anaerobic gram-negative bacteria but causing different periodontal disease [14].

Based on previous studies on the cytotoxicity test of Nannochloropsis oculata which has similar morphological and taxonomic characteristics with Chlorella sp. states that Nannochloropsis oculata is toxic at concentrations > 2.5% and thus assumed to be not toxic for therapeutic used at concentration of <2.5% [15]. Based on these data, researchers want to develop Chlorella sp. antibacterial potency against Aggregatibacter actinomyccetemcomitans, which is the main bacterium in periodontal disease, especially aggressive periodontitis, at concentrations of 0.625%, 1.25%, and 2.5%.

2. Experimental Method
This study was true experimental laboratory research with posttest only control group design. The antibacterial potency of Chlorella sp. to A. actinomyccetemcomitans were examined by biofilm test method, divided into 5 groups, each group consisted of 4 samples. The control groups were given aquadest (K- group), and tetracycline (group K+) while treatment groups were given Chlorella sp solution in various concentrations of 0.625% (P1 group), 1.25% (P2 group), and 2.5% (P3 group).

2.1. Chlorella sp preparation
Chlorella sp. were obtained from Balai Budidaya Air Payau Situbondo, dried as powder and dissolved into aquadest, prepared in three concentration, each of 0.625%, 1.25% and 2.5%. Identification of secondary metabolite compound of Chlorella sp were performed by AlCl3 Spectrophotometry (λ=415 mm) for flavonoid content and Colorimetric method using Bromocresol reagent Green (λ=470 mm) for the content of total alkaloid.

2.2. Bacterial suspension of A. actinomyccetemcomitans
A. actinomyccetemcomitans were innoculated into Congo Red Agar and incubated for 48 hours at 37°C in anaerobic condition. The bacteria was confirm to produce biofilm when it resulted in black colony formation, however, if the colony was red, the strain did not produced biofilm.

2.3. Biofilm test method
The next step was the biofilm test on microtiter plate assay. A. actinomyccetemcomitans were innoculated into BHI broth (Oxoid) then adjusted with 0.5 Mc Farland and diluted to 1: 100. The bacterial suspension were put into 96-well round bottomed microtitter plate, each of 0.1 mL and incubated overnight at 37°C. According to the groups, 0.1 mL of aquadest (K- group), 0.0001% tetracycline, Chlorella sp with
Concentrations of 0.625%, 1.25%, and 2.5% were each applied into 96-well round microtiter plate then incubated overnight at 37°C. All contained wells were washed 3 times with 0.2 mL phosphate buffered saline, then added 0.2 mL of crystal violet as biofilm staining, rinsed by distilled water and dried, added 0.2 mL Tween 80 2%. Biofilm formation were examined by measured the optical density (OD) on ELISA reader with a wavelength of 570nm. Data were analyzed using One Way ANOVA followed by LSD test.

3. Result and Discussion
Qualitative test for secondary metabolites of *Chlorella* sp. powder showed positive result on the compound flavonoid, alkaloid, terpenoid, tannins, saponins, steroid. Quantitative test result revealed the contain of flavonoid in *Chlorella* sp powder was 0.014 % w/w and alkaloid was 0.05 % w/w.

Inoculation of *Aggregatibacter actinomycetemcomitans* in Congo red agar resulted in black colony as shown in figure 1, showed that the bacteria could produce biofilm.

![Figure 1: Colony of A. actinomycetemcomitans Congo red agar](image)

The antibacterial potency of various concentration of *Chlorella* sp. to *Aggregatibacter actinomycetemcomitans* biofilm was examined on the OD value. The table below shows the average of OD value of *Aggregatibacter actinomycetemcomitans* biofilms.

| Groups | Average±SD |
|--------|------------|
| K(-)   | 0.24 ± 0.05|
| K(+)   | 0.17 ± 0.03|
| P1     | 0.52 ± 0.08|
| P2     | 0.29 ± 0.17|
| P3     | 0.20 ± 0.07|

Decreasing OD value indicated the inhibition of biofilm formation which refered to antibacterial potency. Table 1 and figure 2 showed that tetracycline (K+ group) had the lowest OD value as it was on the control groups. In the treatment groups, the higher concentration of *Chlorella* sp resulted to the lower average of OD value (p<0.05). Treatment with 2.5% *Chlorella* sp (P3 group) showed the lowest OD value (p<0.05).
Aggressive periodontitis is an infection that occurs in the periodontal tissues and alveolar bone supporting the teeth because of certain microorganisms and increases the depth of the periodontal pocket in a fast period of time [16]. The periodontal tissue damage occurs very quickly with a minimal amount of plaque and calculus. Aggressive periodontitis is commonly occurring among adolescents at the onset of puberty or in individuals at the age of the second or third decade [8].

Biofilm is bacteria that colonized and perfectly arranged to protect the bacteria from external influences [17]. Biofilm consist of 10-25% bacteria cells and 79-90% extracellular polymeric substances (EPS), which is primary matrix materials of biofilm [18, 19]. EPS provides the properties of antimicrobial resistance in biofilms by inhibiting antibiotic transport via biofilm [18].

*Aggregatibacter actinomycetemcomitans*, is an exogenous bacteria that cause infection in periodontitis [9]. *Aggregatibacter actinomycetemcomitans* is a gram-negative facultative anaerobic bacterium that most commonly cause periodontitis which resulting destruction in periodontal tissue. It also can avoid and deactivate immune system of the body [20].

In this study, the concentration of *Chlorella* sp. 0.625%, 1.25%, and 2.5% referred to the results of previous studies conducted by Revianti and Parisihni [15] concerning the cytotoxicity of *Nannochloropsis oculata*, which is one of microalgae that has similar characteristics to *Chlorella* sp., stated that *Nannochloropsis oculata* was toxic at concentrations more than 2.5% and nontoxic at concentrations of ≤ 2.5%.

The result of the statistical test, it can be concluded that tetracycline antibiotics and *Chlorella* sp. at all concentrations have antibacterial potency in inhibiting formation of *Aggregatibacter actinomycetemcomitans* biofilms. In the treatment groups that given *Chlorella* sp. microalgae solution with a concentration of 2.5% has the greatest antibacterial potency in inhibiting the biofilm formation compared to other concentrations. The positive control group, which is tetracycline has the greatest antibacterial effect in inhibiting the bacterial biofilm *Aggregatibacter actinomycetemcomitans* which was not statistically different from *Chlorella* sp. 2.5% concentration (p <0.05).

In the positive control group tetracycline has a high antibacterial potency against bacterial biofilms *Aggregatibacter actinomycetemcomitans* due to tetracycline containing antibacterial compounds. Tetracycline has an ability to enter into microorganism’s cell through passive diffusion or active transport depends on the energy. Tetracycline will be attracted to the binding site in the ribosomal 30S subunit in the bacteria and prevent the binding of aminoacyl-tRNA to the acceptor site in the mRNA-ribosomal complex. This prevents the addition of amino acids to the peptides that are being formed which inhibit the protein synthesis of the bacteria [10].

![Figure 2. Graphic of average OD value of Aggregatibacter actinomycetemcomitans biofilm. Notes: K(-) (Aquadest), K(+) (Tetracycline 0.0001%), P1 (Chlorella sp. 0.625%), P2 (Chlorella sp. 1.25%), P3 (Chlorella sp. 2.5%).](image)
In the treatment group there the value of OD decreasing as the concentration of *Chlorella* sp. increased which showed that there was an increase in antibacterial antibacterial potency in inhibiting *Aggregatibacter actinomyctemcomitans* bacterial biofilms. This antibacterial action assumed to be related to the active compound of *Chlorella* sp. that act as antibacterial by interfering with the components of the bacterial cell wall [21].

*Chlorella* sp. is one of the microalgae which have bioactivity as an antibacterial and capacity to inhibit the formation of bacterial biofilms. *Chlorella* sp. contains important compounds such as flavonoids, alkaloids, terpenoids, tannins, glycosides, saponins, and steroid as an active compound [21], [22]. Based on the qualitative test result of secondary metabolites compound of *Chlorella* sp., flavonoid and alkaloid might have the role on the antibacterial potency.

Qualitative test for secondary metabolites of *Chlorella* sp. powder showed positive result on the compound of flavonoid with concentration of 0.014 w/w and alkaloid was 0.05 w/w. Flavonoids have ability as antibacterial by changing the permability of the bacterial cell wall and inhibit bacterial motility. Flavonoids are bacteriostatic agent, but at high concentrations can be bactericides in gram-negative and gram-positive bacteria [23]. Alkaloid compounds can interfere the components of peptidoglycan in bacterial cells so that the cell wall layer is not well-formed and causes cell death. Alkaloids could also change the genetic balance in the DNA chain so that it promotes lysis of the bacteria cell [24, 25].

Besides flavonoid and alkaloid, there are more secondary metabolites, such as terpenoid, tannins, saponins, and steroid that might play role as antibacterial against the *Aggregatibacter actinomyctemcomitans* but it is still not readily available for qualitative test.

Terpenoid works by inhibiting synthesis of protein. Terpenoid will accumulate and cause changes of the component in bacterial cells, which damage the lipid bilayer of the cell membrane due to its hydrophilic group [26]. Tannins affects the bacterial growth by inhibiting the transcriptase enzyme. It would also disturbing the bacterial peptidoglycan layer, therefore the bacterial cells will not be form [27, 28]. Saponin compounds could interfere the permeability of bacterial cell membrane and cause the release of various important components in bacterial cells, such as proteins, nucleic acids, and nucleotides which triggered lysis of the bacteria cell [23]. Steroid has an ability as an antibacterial, works by associating with lipid membranes and sensitivity of the bacterial cell to steroid components cause leakage in bacterial liposomes. Steroids can interact with cell phospholipid membranes that are permeable to lipophilic compounds, causing membrane integrity to decrease so that the morphology of the cell membrane changes and increasing in fragility of the cells which will cause lysis of the bacteria cell [29].

Tetracycline and *Chlorella* sp. 2.5% have antibacterial potency to inhibit *Aggregatibacter actinomyctemcomitans* biofilm growth. Both antibacterial have different mechanisms. The mechanism of action of tetracycline antibiotics is by inhibiting bacterial protein synthesis in the ribosome so that it has greater antibacterial potency, while the active compounds in *Chlorella* sp. interfere with components of the bacterial cell wall [13, 30]. Therefore, it shows different result.

In this study, the particles of *Chlorella* sp. were still coarse, causing the difficulty of the solution to penetrate the bacterial cell wall. Hence, the inhibition of *Aggregatibacter actinomyctemcomitans* bacterial biofilm does not work optimally [18]. *Chlorella* sp. solution in a variety of relatively small concentrations of 0.625%, 1.25%, and 2.5% causes the bioactive content as antibacterial, such as flavonoids and alkaloids that was produced were smaller. This make the result shows smaller amount in bacterial biofilm antibacterial potency.

*Chlorella* sp. potentially developed as an antimicrobial alternative material for pathogenic bacteria that cause aggressive periodontitis. However, further research is needed regarding the use of *Chlorella* sp. solution whose particles are smaller is expected to provide a greater inhibitory effect on *Aggregatibacter actinomyctemcomitans* biofilm. Moreover, still need to be explored about the specific components in *Chlorella* sp. which act as antibacterial, how to obtain or manage them, doses of drug that can be used as therapy for aggressive periodontitis, drug formulations and dosage forms, and in-vivo test as a biocompatibility test including the clinical trials.
4. Conclusion

Chlorella sp solution has antibacterial potency against bacterial biofilms Aggregatibacter actinomycetemcomitans with the most effective concentration is 2.5% that has antibacterial potency which is almost the same as tetracycline antibiotics.

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