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Research Article

Antibiofilm Effect of *Clitoria ternatea* Flower Juice on *Porphyromonas gingivalis* *in vitro*

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KEYWORDS

biofilm;
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ABSTRACT

Introduction: *Clitoria ternatea* flower contains flavonoid such as anthocyanin that gives the blue color to its flower and has antimicrobial activity. **Objectives:** The aim of this study was to examine the effect of flower juice of *Clitoria ternatea* against *Porphyromonas gingivalis* biofilm viability *in vitro*. **Methods:** This study was experimental laboratory research using biofilm assay method. *P. gingivalis* was cultured in BHI broth in 37°C for 24h under anaerobic condition. Fresh flowers of *Clitoria ternatea* were extracted using mortar and pestle and diluted into 6 different concentrations: 100%, 50%, 25%, 12.5%, 6.25%, and 3.125% with phosphate buffer saline (PBS). Chlorhexidine (0.2%) was used as positive control and biofilm without treatment as negative control. The flower juice was distributed into 96 well-plates that contained biofilm of *P. gingivalis* and incubated for 1h, 3h, 6h, and 24h in 37°C, anaerobic atmosphere. Biofilm was measured using crystal violet dye with microplate reader (490 nm). Data were statistically analysed using one-way ANOVA test and Post Hoc test with $p < 0.05$ was set as significant different. **Result:** Result showed that *Clitoria ternatea* flower juice significantly reduced the *Porphyromonas gingivalis* biofilm viability in all concentration and all incubation time. The most effective concentration to inhibit *Porphyromonas gingivalis* biofilm was 100% in 1h incubation time which biofilm was diminished (Optical Density=0.00). One way ANOVA test and Post Hoc test showed a significant biofilm reduction in all concentration and all incubation time after treatment with the flower juice compared to control ($p < 0.05$). **Conclusion:** *Clitoria ternatea* flower juice has antibiofilm effect against *Porphyromonas gingivalis*. This result showed this flower juice may be useful for combating periodontal pathogens. However, further studies using other bacteria are still needed to confirm this result.

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INTRODUCTION

The most common dental and oral diseases in the Indonesian population are dental caries and periodontal disease.¹ Periodontitis is a chronic disease caused by an inflammatory response of the dental supporting tissue to microbial biofilms (dental plaque).² This inflammatory response can damage dental attachment apparatus (i.e., gingiva, cementum, periodontal ligaments, and alveolar bone). If left untreated, periodontitis can cause tooth loss.³ A recent study pointed to a possible association of periodontitis with systemic diseases, such as cardiovascular disease, diabetes, renal disease, respiratory dysfunction, rheumatoid arthritis, osteoporosis, and premature/low weight newborns.⁴

Periodontal disease is one of a number of oral infections initiated by biofilm formation.⁵ These biofilms consists of bacteria that adhere to polysaccharide matrices and other organic and inorganic materials.⁶ In the early stages of biofilm formation, the bacterial colony is dominated by aerobic bacteria (*Streptococcus mutans* and *Streptococcus sanguis*). As the biofilm grows, it is dominated by anaerobic bacteria (*Aggregatibacter actinomycetemcomitans* and *Porphyromonas gingivalis*).⁷ The biofilm is resistant to other microorganisms, the host's defenses, and toxic substances, such as chemicals and antibiotics.⁶

Anaerobic bacteria, such as *Aggregatibacter actinomycetemcomitans*, *Porphyromonas gingivalis*, *Bacteroides forsythus*, and *Treponema denticola*, play an important role in periodontitis.⁸ The aim of periodontitis treatment is to improve periodontal health, including reducing anaerobic bacteria colonies in dental plaque.² Although such treatment usually involves only plaque and calculus removal, local or systemic antibiotics can help treat periodontal disease, especially among patients with recurrent periodontitis.⁹

Natural ingredients can be used as an alternative treatment for periodontal disease. The popularity of natural ingredients has increased due to their ready availability, low cost, and minimal side effects.¹⁰ They are also environmentally friendly. Many studies have investigated the ability of herbal or fruit juice to inhibit oral pathogens and biofilm formation.¹¹⁻¹³ In terms of natural ingredients, components of *Clitoria ternatea* or the telang flower have been used. This plant belongs to the vine family and is often used as decoration or medicine. *C. ternatea* is found on Ternate island, Indonesia as well as in many countries in southeast Asia, such as India, Bangladesh, and Malaysia. In Indonesia, especially in West Java, the flower parts of the plant are often soaked in hot water to make a drink, which has many uses, including stomatitis treatment. The flower parts are also used to prepare an eye drop treatment for

red eye and conjunctivitis.¹⁴

C. ternatea has been known for its property as anti-inflammatory, antipyretic, and analgesic.¹⁵ The root of the plant contains a flavonol glycoside, which has a strong antibacterial effect.¹⁶ The flower and seed parts of *C. ternatea* contain an active peptide compound, clotide, which functions as a strong antimicrobial agent.¹⁷ A previous study showed that *C. ternatea* exerted a strong antimicrobial effect against various pathogenic bacteria, such as *Escherichia coli*, *Vibrio cholera*, and *Staphylococcus aureus*, thereby making it an effective treatment for many infectious diseases.¹⁰ Flowers from *C. ternatea* contain methanol, chloroform, petroleum ether, hexane, and aqueous.¹⁵ Despite the beneficial properties of *C. ternatea*, very few studies have examined its potential against pathogenic bacteria in the oral cavity. Therefore, the aim of this study was to investigate the effect of *C. ternatea* flower juice against biofilms of *P. gingivalis*.

MATERIAL AND METHODS

P. gingivalis Culture

P. gingivalis ATCC 33277 was cultured using brain heart infusion (BHI) broth at 37°C for 24h under anaerobic conditions in a GasPak jar system.¹⁸

C. ternatea Extract

The flowers of *C. ternatea* were used in this research. The flowers were separated from the pod until only the petals were left. The petals were then washed until clean under running water. The moistened petals were then crushed using a mortar and pestle until the juice extract of *C. ternatea* was obtained. Subsequently, the juice extract was diluted into five different concentration (100%, 50%, 25%, 12.5%, 6.25%, and 3.125%) using phosphate buffer saline (PBS).

Biofilm Assay

The effect of the flower juice extract on *P. gingivalis* ATCC 33277 was analyzed using a biofilm assay with crystal violet dye. For the bioassay, 200 µL of *P. gingivalis* (1.5×10^8 CFU/mL) were inoculated into 96 well-plates and incubated for 2×24h at 37°C under anaerobic conditions to encourage biofilm growth. After 48h, the well plates were rinsed with phosphate buffer saline (PBS). The growing biofilms were treated with different concentrations (100%, 50%, 25%, 12.5%, 6.25%, and 3.125%) of *C. ternatea* flower juice extract. As a positive control, a culture was treated with chlorhexidine (0.2%). Untreated culture in BHI broth was used as a negative control. The inhibitory effect of the juice was observed after 1h, 3h, 6h, and 24h incubation.

After each incubation period, the *C. ternatea* juice was removed, and the well plates were rinsed twice with PBS. Crystal violet dye (0.05%) was then added to the well plates and incubated for 15 min. Subsequently, 200 μ L of 90% ethanol were added, and a microplate reader was used to determine the optical density (OD) at a wavelength of 490 nm.

Additionally, the velocity of biofilm formation of non-treated groups was observed 6, 24, 48, and 72h using the formula below:

$$BF=AB-CW ; BF=AB/CW ; SBF=(AB-CW)/G$$

where BF denotes biofilm formation, AB denotes the optical density at 600 nm ($OD_{600\text{ nm}}$) of the stained bacteria, CW denotes the $OD_{600\text{ nm}}$ of the bacterial medium, SBF is the specific biofilm formation, and G signifies the bacterial growth in culture at $OD_{600\text{ nm}}$.²¹

Statistical Analysis

The data was statistically analyzed using a one-way ANOVA test and a Post Hoc LSD test in which $p < 0.05$ was set as the significant difference. The Kolmogorov-Smirnov test was used previously as data normality test.

RESULTS

The *C. ternatea* flower juice extract significantly reduced *P. gingivalis* biofilm mass in all incubation period ($p < 0.05$). The *C. ternatea* extract at a concentration of 100% had the highest inhibitory effect on *P. gingivalis* 1h post incubation period, with OD value of 0.004 ± 0.031 . There were significant differences compared to the negative control, with an OD value of 0.379 ± 0.038 ($p < 0.05$) as shown in Fig.1. At 3h post incubation period, the most effective concentration was 50%, with an OD value of 0.398 ± 0.115 (Fig.2).

The results showed that the *C. ternatea* extract at a 50% concentration had the highest inhibitory effect on *P. gingivalis* biofilm formation after 6h of incubation, with an OD value 0.472 ± 0.145 as compared with that of the negative control (OD value: 1.27 ± 0.219) (Fig. 3). As shown in Fig.4, at 24h post incubation, the *C. ternatea* extract inhibited *P. gingivalis* biofilm formation, with the smallest OD value (0.117 ± 0.079) at a concentration of 100%.

Table 1 showed the results of the velocity measurements of biofilm formation. The OD values 6, 24, 48, and 72h post incubation were 1.089 ± 4.685 , 0.913 ± 5.340 , 2.089 ± 7.026 , and 1.672 ± 5.429 , respectively. The OD values for SBF were 2.563, 2.148, 4.797, and 3.943 after 6, 24, 48, and 72h of incubation, respectively.

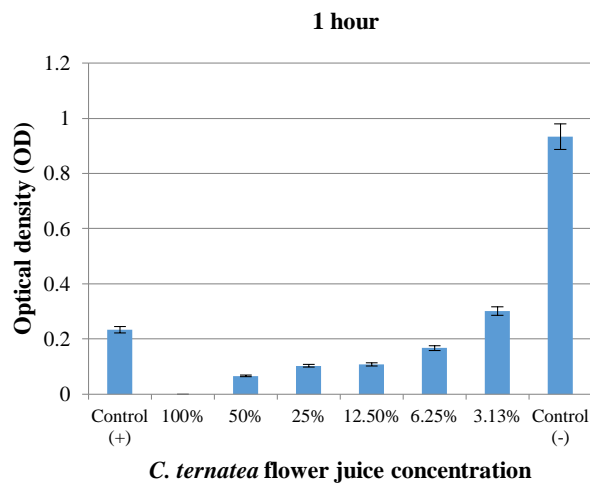


Figure 1. Graphic of the inhibitory effect of *C. ternatea* flower juice extract on *P. gingivalis* biofilm formation after 1 hour post-incubation.

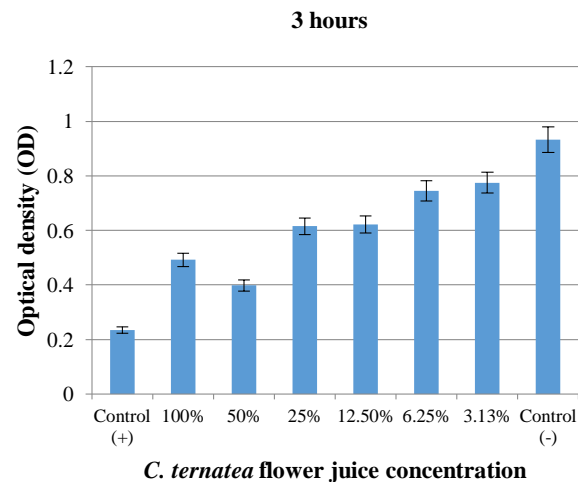


Figure 2. Graphic of the inhibitory effect of *C. ternatea* flower juice extract on *P. gingivalis* biofilm formation after 3 hours post-incubation.

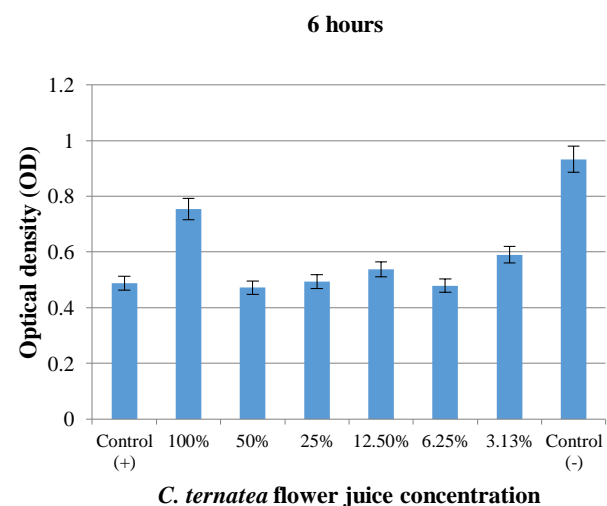


Figure 3. Graphic of the inhibitory effect of *C. ternatea* flower juice extract on *P. gingivalis* biofilm formation after 6 hours post-incubation.

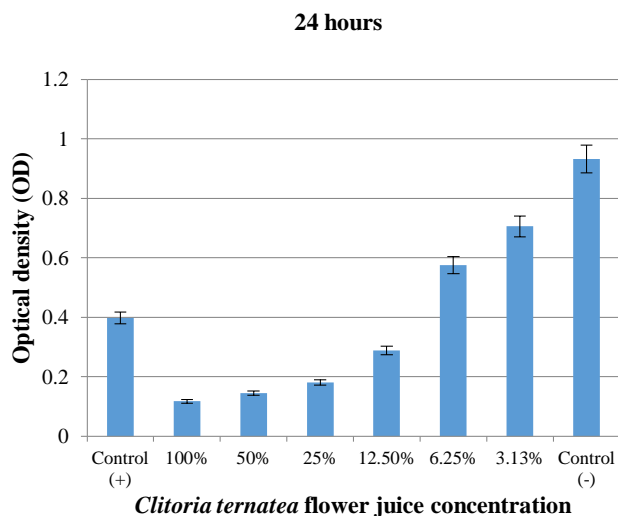


Figure 4. Graphic of the inhibitory effect of *C. ternatea* flower juice extract on *P. gingivalis* biofilm formation after 24 hours post-incubation.

Table 1. The velocity of biofilm formation

Variables	6h	24h	48h	72h
BF = AB-CW	1.089	0.913	2.039	1.672
BF = AB/CW	4.685	5.340	7.026	5.429
SBF = (AB-CW)/G	2.563	2.148	4.797	3.934

BF: biofilm formation; AB: OD_{600 nm} of the stained bacteria; CW: OD_{600 nm} of the bacterial medium; SBF: specific biofilm formation; G: bacterial growth in culture at OD_{600 nm}.

DISCUSSION

The *C. ternatea* plant is used as a traditional herbal medicine in Indonesia. Although all parts of the plant can be used, the flower parts are most commonly used due to their antimicrobial, anti-inflammatory, and antioxidant properties.²² As the potential benefits of parts of the *C. ternatea* plant in oral health are not well known, the present study examined the effect of *C. ternatea* flower juice extract on biofilm formation of the pathogenic bacterium *P. gingivalis*. *P. gingivalis* can adhere to hard surfaces or intraoral mucosa, and it plays an important role in chronic and aggressive periodontitis.²³

Previous research showed that the *C. ternatea* flower inhibited the growth of pathogenic bacteria, such as *S. mutans*, *Lactobacillus casei*, and *S. aureus*, in the oral cavity.¹⁰ *C. ternatea* also inhibited the growth of Gram-negative bacteria, such as *Escherichia coli* and *Candida*

albicans.²⁴ The flower petals of *C. ternatea* contain anthocyanins, which are responsible for the color of the petals. Anthocyanins are polar particles that dissolve in water, ethanol, methanol, and other polar solvents²⁵ and have antioxidant, antimicrobial, and anti-inflammatory properties.^{26, 27}

As shown by the formula used to calculate the speed of biofilm formation, it was rapid at all the post incubation times measured. According to the formula, *P. gingivalis* biofilm formation at all the incubation times with BF=AB-CW, the category was strong (OD>0.30) for this bacterium. With BF=AB/CW, after 6, 24, and 72h, the category was moderate (4.00<OD<5.99). After 48h of incubation, the category was strong (OD≥6.00). With SBF=(AB-CW)/G, at all the incubation times, the category was strong (OD≥1.10).

The inhibition of biofilm formation was greatest at *C. ternatea* flower juice extract concentrations of 100% and 50% due to the presence of increased amounts of anthocyanins, which are flavonoids with antibiofilm activity. The effective incubation time period in inhibit biofilm in this study was 24h which biofilm begin the maturation phase. A previous study showed that anthocyanins affected the growth of Gram-negative bacteria, such as *E. coli*, by inhibiting the production of fimbriae, which are required by bacteria to form biofilms.²⁸ Research also demonstrated that anthocyanins inhibit *P. gingivalis* and the activity of gingipains, which are *P. gingivalis* virulence factors.²⁹ Although anthocyanin shows activity against many microbes, Gram-positive bacteria are more sensitive to anthocyanin than Gram-negative bacteria. Gram-negative bacteria have an outer membrane, which functions as a permeability barrier and impedes the absorption of compounds.²⁷

Another study showed that *C. ternatea* extracts contain tannin, phlobatannin, flavonoid, anthraquinone, alkaloid, saponin, cardiac glycosides, volatile oils, steroids and terpenoids.³⁰ Flavonoid, inhibited the membrane function and penetrated the lipid bilayer of bacteria, thereby destroying the barrier function of the outer membrane.³¹ Furthermore, this compound caused membrane fusion, which resulted in cell leakage. However, the compound showed less success in terms of penetrating the lipopolysaccharide membrane of Gram-negative bacteria.³² Therefore, its antibacterial mechanism is limited. In another study, the flavonoid inhibited bacterial energy metabolism required for the synthesis of macromolecules (DNA, RNA, and protein).³³

A previous study showed antimicrobial activities of the methanol extracts of the leaf, stems, flower, seed, and

roots of *C. ternatea* against 12 bacterial species, 2 yeast species, and 3 filamentous fungal by the agar diffusion and broth dilution methods.³⁰ These results confirmed the effectiveness of *C. ternatea* in the inhibition of the growth of pathogenic microorganism. In a study in which *C. ternatea* extract was obtained using the maceration method with water, methanol, and chloroform, the extract showed antimicrobial activity against *Escherichia coli*, *Salmonella typhimurium*, *Klebsiella pneumonia*, and *Pseudomonas aureginosa*.¹⁷

CONCLUSION

C. ternatea flower juice extract exerted an antibiofilm effect on *P. gingivalis*. The results suggested that the extract may be useful in combating periodontal pathogens. Further studies examining the effect of the extract against other bacteria are needed to confirm these results.

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CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest.

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