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Research Report

# The effect of butterfly pea flower (*Clitoria ternatea L.*) kombucha against *Streptococcus viridans*

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## *ABSTRACT*

***Background:*** *Dental caries is a multifactorial disease driven by the formation of bacterial biofilms, particularly Streptococcus viridans, which contribute to root canal infections if not appropriately managed. Sodium hypochlorite (NaOCl) is commonly used as an irrigant in root canal therapy, but its application is limited due to its toxicity and corrosiveness. Consequently, there is a pressing need for safer and more effective natural alternatives. Kombucha derived from butterfly pea flower (Clitoria ternatea L.) has been identified as a promising candidate with antibacterial and antibiofilm properties due to its bioactive secondary metabolites. The fermentation process involving a symbiotic culture of bacteria and yeast (SCOBY) may further enhance the efficacy of these bioactive compounds.* ***Purpose:*** *This study conducted to analyze the effect of kombucha from butterfly pea flower (Clitoria ternatea L.) on the biofilm of Streptococcus viridans in vitro, utilizing a spectrophotometric method to assess the impact across various concentrations.* ***Methods:*** *An experimental laboratory study was conducted in vitro employing a post-test-only control group design. Kombucha prepared from butterfly pea flower was fermented for periods ranging from 12 to 154 days, followed by dilution into several concentrations (100%, 50%, 25%, 12.5%, 6.25%, 3.12%, and 1.56%) using the dilution method. Direct contact between the kombucha and Streptococcus viridans was established, and the resulting biofilm inhibition was assessed by measuring the Optical Density (OD) using a spectrophotometer at a wavelength of 650 nm.* ***Results:*** *The inhibitory percentage of butterfly pea flower kombucha against Streptococcus viridans biofilm decreased progressively with concentrations of 100%, 50%, 25%, 12.5%, 6.25%, 3.125%, and 1.56%. The highest inhibitory percentage was observed at a concentration of 100%.* ***Conclusion:*** *Kombucha of butterfly pea flower (Clitoria ternatea L.) demonstrated potential in inhibiting Streptococcus viridans biofilm formation, with a concentration of 25% determined as the MBIC50. However, an MBEC90 value could not be established, as no biofilm inhibition percentage exceeding 90% was observed in the test results.*

***Keywords:*** *kombucha; Clitoria ternatea; antibiofilm; Streptococcus viridans; butterfly pea flower*

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## INTRODUCTION

Caries is a multifactorial disease involving bacterial biofilm, fermentable carbohydrates, tooth hard tissues, and time.[1] The oral cavity has a unique ecology for the growth and development of microorganisms to form biofilms. The main etiology of caries is that bacteria that are cariogenic in nature ferment carbohydrates and produce acids which in turn result in a demineralization process on the tooth surface.[2] Without treatment, caries can lead to root canal infections caused by anaerobic bacteria, such as *Streptococcus viridans*, found in 63% of root canal infections.[3,4] Biofilms, composed of microorganisms and exopolysaccharides, enhance bacterial resistance to antimicrobial agents.[5] Irrigation materials have lately been employed as a way for removing bacteria in the root canal system. It is expected that the application of irrigation materials will remove bacteria present in root canals, particularly those that are inaccessible with mechanical instruments.[6] Chemical cavity cleaners containing 5% NaOCl are used to try to halt the growth of these bacteria, but they have drawbacks such as toxicity, bad odor, and irritation to periradicular tissue. These drawbacks created the opportunity for natural products to be used as alternatives.[7,8]

Natural alternatives, such as butterfly pea kombucha (*Clitoria ternatea L.*), have shown potential as antibacterial and antibiofilm agents.[9,10] Kombucha, a product of symbiotic fermentation by bacteria and yeast (SCOBY), produces bioactive compounds. In contrast, butterfly pea contains alkaloids, tannins, anthocyanins, and flavonoids effective


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against gram-positive and gram-negative bacteria.[11] Fermentation enhances the bioactivity of natural compounds by breaking down complex molecules into simpler ones.[12]

This study aims to evaluate the effect of butterfly pea kombucha on *Streptococcus viridans* biofilm in vitro using a spectrophotometer to measure absorbance values. A concentration range (100%, 50%, 25%, 12.5%, 6.25%, 3.125%, 1.56%) was applied to determine the minimum effective concentration for biofilm inhibition.[13]

## MATERIALS AND METHODS

This study was an in vitro laboratory experimental research with a post-test only group design. Samples of *Streptococcus viridans*, obtained from bacterial stock at the Research Center of the Faculty of Dental Medicine, Airlangga University, were divided into 9 groups: positive control (NaOCl), negative control (no antibiofilm agent), and 7 treatment groups with butterfly pea kombucha concentrations (100%, 50%, 25%, 12.5%, 6.25%, 3.125%, 1.56%). Butterfly pea kombucha was fermented using a bacterial and yeast consortium (SCOBY) and diluted to specific concentrations. *Streptococcus viridans* cultures were standardized to McFarland 0.5 ($1.5 \times 10^8$ CFU/ml), grown on BHIB media, and incubated at 37°C for 24 hours to form. biofilms. The biofilms were treated according to their respective groups, washed with PBS, stained with crystal violet, and OD was measured using a spectrophotometer at a wavelength of 540 nm. The data were examined utilizing the Shapiro-Wilk test for normality, Levene's test for homogeneity, One-Way ANOVA for group significance, and the Tukey HSD test for post hoc analysis.

## RESULTS

This study used butterfly pea flower kombucha (*Clitoria ternatea L.*) to determine the activity of *Streptococcus viridans* biofilm formation. This study used butterfly pea flower kombucha with concentrations of 100%, 50%, 25%, 12.5%, 6.25%, 3.125%, 1.56% to compare the inhibitory power between butterfly pea flower kombucha and positive control, namely NaOCl on the formation of *Streptococcus viridans* biofilm and the treatment was repeated eight times. Microplate results after treatment in Figure 1.

The inhibitory test for biofilm development of butterfly pea blossom kombucha and NaOCl was measured using a spectrophotometer at a wavelength of 540 nm, represented in Optical Density (OD) units. The outcome of the optical density reading from the investigation is presented in Table 1.

Based on the results of the OD reading of *Streptococcus viridans* biofilm using a spectrophotometer, it was found that the butterfly pea flower kombucha treatment group with concentrations of 100%, 50%, 25%, 12.5%, 6.25%, 3.125%, and 1.56% and the positive control, namely NaOCl, had a lower biofilm OD value than the OD value of the negative

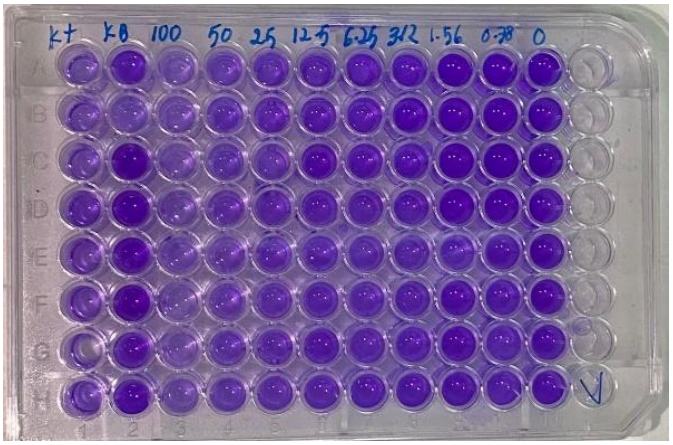


**Figure 1.** Microplate results after treatment.

**Table 1.** *Streptococcus viridans* bacterial biofilm formation

| Sample | K(+) | K(-) | 100% | 50% | 25% | 12.5% | 6.25% | 3.125% | 1.56% |
|---|---|---|---|---|---|---|---|---|---|
| 1 | 0.037 | 0.337 | 0.096 | 0.0124 | 0.152 | 0.217 | 0.344 | 0.313 | 0.344 |
| 2 | 0.06 | 0.309 | 0.087 | 0.087 | 0.143 | 0.247 | 0.356 | 0.351 | 0.341 |
| 3 | 0.047 | 0.274 | 0.071 | 0.011 | 0.198 | 0.294 | 0.295 | 0.263 | 0.32 |
| 4 | 0.051 | 0.373 | 0.059 | 0.091 | 0.177 | 0.258 | 0.272 | 0.289 | 0.36 |
| 5 | 0.021 | 0.384 | 0.075 | 0.085 | 0.138 | 0.269 | 0.301 | 0.343 | 0.298 |
| 6 | 0.049 | 0.382 | 0.067 | 0.114 | 0.14 | 0.255 | 0.296 | 0.256 | 0.345 |
| 7 | 0.03 | 0.351 | 0.094 | 0.136 | 0.098 | 0.239 | 0.331 | 0.358 | 0.343 |
| 8 | 0.046 | 0.361 | 0.053 | 0.105 | 0.149 | 0.292 | 0.212 | 0.315 | 0.353 |


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**Table 2.** *Streptococcus viridans* bacterial biofilm development means concentration value and standard deviation

| Group | N | Means Concentration ± Standard Deviation |
|---|---|---|
| Control (-) | 8 | 0.346 ± 0.038 |
| Control (+) | 8 | 0.042 ±0.013 |
| 100% Concentrated Butterfly Pea Flower Kombucha | 8 | 0.075 ± 0.016 |
| 50% Concentrated Butterfly Pea Flower Kombucha | 8 | 0.106 ± 0.018 |
| 25% Concentrated Butterfly Pea Flower Kombucha | 8 | 0.149 ± 0.029 |
| 12.5% Concentrated Butterfly Pea Flower Kombucha | 8 | 0.259 ± 0.026 |
| 6.25% Concentrated Butterfly Pea Flower Kombucha | 8 | 0.301 ± 0.046 |
| 3.125% Concentrated Butterfly Pea Flower Kombucha | 8 | 0.311 ± 0.039 |
| 1.56% Concentrated Butterfly Pea Flower Kombucha | 8 | 0.338 ± 0.02 |

**Table 3.** Percentage of reduction in *Streptococcus viridans* biofilm formation

| Group | Percentage of Reduction |
|---|---|
| Control (-) | 0% |
| Control (+) | 87.69% |
| 100% Concentrated Butterfly Pea Flower Kombucha | 78.27% |
| 50% Concentrated Butterfly Pea Flower Kombucha | 69.25% |
| 25% Concentrated Butterfly Pea Flower Kombucha | 56.87% |
| 12.5% Concentrated Butterfly Pea Flower Kombucha | 25.26% |
| 6.25% Concentrated Butterfly Pea Flower Kombucha | 13.14% |
| 3.125% Concentrated Butterfly Pea Flower Kombucha | 10.21% |
| 1.56% Concentrated Butterfly Pea Flower Kombucha | 2.42% |

control, namely *Streptococcus viridans* biofilm without treatment.

Based on the biofilm OD readings presented in the table, calculations can be performed. Additionally, the average OD value of *Streptococcus viridans* biofilm for each group can be determined (Table 2).

As shown in Table 3, the calculation results of the inhibitory ability of butterfly pea flower kombucha and NaOCl against *Streptococcus viridans* biofilm, the inhibitory ability of NaOCl has the highest ability with a percentage of 87.69%. Meanwhile, in butterfly pea flower kombucha, the highest inhibitory ability is at a concentration of 100% with a percentage of 78.27%.

The result of the Shapiro-Wilk Test showed that the research data were normally distributed. The result of the Levene Test showed that the research data was homogeneous. The butterfly pea flower and kombucha treatment groups had a substantial impact on the development of *Streptococcus viridans* biofilm, according to the results of the One Way ANOVA. When compared to other groups, the Tukey HSD Post Host-hoc Test result indicated that each group differed significantly in its ability to suppress the production of biofilms.

## DISCUSSION

In both primary and permanent teeth, caries is a complicated multifactorial illness marked by the demineralization of hard tooth tissues (cementum, dentin, and enamel).[1] Without appropriate treatment, microorganisms in caries can cause pulp infection through dentin, leading to pulp exposure and pulpitis.[14] *Streptococcus viridans,* a facultative anaerobic bacterium commonly found in the oral cavity, plays a key role in caries development by synthesizing sucrose-derived polysaccharides such as dextran and levan.[15]

*Clitoria ternatea L.*, commonly known as butterfly pea, is used as an ornamental plant and a traditional food dye in Indonesia. It exhibits antioxidant, antibacterial, antifungal, anti-inflammatory, anticancer, and antidiabetic activities.[11] Previous studies have shown that its extracts can act as antibiofilm agents against *Streptococcus mutans* and *Staphylococcus aureus*.[13] This study investigated the antibiofilm effects of butterfly pea kombucha against *Streptococcus viridans* biofilm using a post-test-only group design and optical density (OD) measurements. Lower OD values indicate greater biofilm inhibition, which was further calculated into percentage inhibition using inhibition efficacy formulas.

The results showed that butterfly pea kombucha at 100%, 50%, and 25% concentrations significantly inhibited biofilm formation compared to the negative control (untreated bacteria) and produced inhibition percentages closer to the positive control (2.5% NaOCl). In contrast, concentrations of 12.5%, 6.25%, 3.12%, and 1.56% showed negligible biofilm inhibition, with OD values approaching the negative control. The minimum biofilm inhibition concentration (MBIC50), defined as the concentration achieving at least 50% inhibition, was identified at 25%, with an inhibition percentage of 56.87%. However, no concentrations achieved a minimum biofilm eradication concentration (MBEC90), as none showed over 90% inhibition.

The antibiofilm activity of butterfly pea kombucha is attributed to secondary metabolites such as alkaloids, flavonoids, acetic acid, saponins, terpenoids, and tannins. These compounds disrupt biofilm formation through various mechanisms, such as reducing quorum-sensing genes, damaging bacterial membranes, inhibiting extracellular

polymeric substances (EPS), and interfering with biofilm adhesion and structure.[10,16] Acetic acid in kombucha plays a pivotal role by degrading biofilm matrices, lowering intracellular pH, and inducing bacterial cell death.[17]

Higher concentrations of butterfly pea kombucha correlated with lower pH values, enhancing its inhibitory effects. At 100% concentration, the kombucha showed the lowest pH (2.12) and the highest inhibition (78.27%), which closely approached the positive control's efficacy. NaOCl, the gold standard root canal irrigant, demonstrated the highest inhibition due to its robust oxidative and protein precipitation mechanisms.[18,19]

The study also highlights that the Tukey HSD post-hoc analysis confirmed significant differences in inhibition between treatment groups, emphasizing that higher concentrations of kombucha had stronger antibiofilm effects. While butterfly pea kombucha showed promising antibiofilm activity, particularly at 100% concentration, it remained less effective than NaOCl in eradicating biofilms.

This research underscores the potential of butterfly pea kombucha as an alternative biofilm inhibitor. By leveraging its natural components and acetic acid production through fermentation, future studies should explore enhancing its efficacy to approach or surpass the performance of conventional antibiofilm agents.

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