ORIGINAL ARTICLE

# Antibacterial potential of Biduri leaf extract (*Calotropis gigantea*) against the growth of *Streptococcus mutans* ATCC 35668 colonies: an experimental laboratory

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## ABSTRACT

**Introduction**: Caries is an oral cavity infection that attacks the hard tissue of the teeth. Caries are caused by cariogenic bacteria such as *Streptococcus mutans (S.mutans)*. *S.mutans* plays a role in the process of biofilm and plaque build up. One alternative ingredient that has antibacterial properties is Biduri leaves (*Calotropis gigantea*). The research aimed to analyze the antibacterial potential of Biduri leaf extract on the growth of *S.mutans* colonies. **Methods**: This type of research is a laboratory experimental research in vitro using the post-test-only control group design method. The antibacterial test used the colony counting method. The extraction process used the maceration method for 1 day with a shaker. The concentrations used were concentrations of 20, 40, and 60%. Control (+) used 0.2% chlorhexidine and control (-) used distilled water. Incubation process was for 24 hours. Counting the growth of *S.mutans* colonies using a colony counter was carried out by three observers. Data analysis used the *Kruskal-Wallis* test followed by the *Mann-Whitney* test. **Results**: The results showed that there was a significant difference in the growth of *S.mutans* ($p<0.05$) after being exposed to Biduri leaf extract at a concentration of 20, 40, and 60%. **Conclusion**: The Biduri leaf extract has antibacterial potential against S.mutans at concentrations of 20, 40, and 60%, and the best concentration in reducing the growth of *S.mutans* colonies is the concentration of 60%.

## KEYWORDS

Antibacterial, *Calotropis gigantea*, 0.2% chlorhexidine, colony counting, *Streptococcus mutans*

## INTRODUCTION

Dental caries is a multifactorial disease caused by a combination and interrelationship between host, diet, cariogenic bacteria and time.[1] According to The Global Burden Diseases in 2016, dental caries is a disease that affects almost half of the world's human population, around 3.35 billion people.[2] Oral bacteria have the most important role in initiating caries because they can change the pH of the oral cavity to become acidic so that the enamel layer will easily demineralize, which is the beginning of the caries process.[3]

*S.mutans* is the main bacterium that causes dental caries by sticking to the glycoprotein layer on the surface of the tooth, metabolizes sucrose to produce acid, and converts sucrose into extracellular polymer glucans, thus encouraging the buildup of biofilms. Apart from that, this bacterium is the most commonly found in plaque.[3] *S.mutans* is classified as a Gram-positive facultative anaerobic bacterium. *S.mutans* can grow rapidly at $37^0$C and the doubling time occurs at 160 minutes

from incubation and reaches maximum growth after 24 hours in the form of regular and smooth or irregular and sticky.[4]

*S.mutans* is able to produce extracellular polysaccharide, and has to form a biofilm layer. The *S.mutans* cell membrane is composed of proteins, lipids, and a small number of carbohydrates. *S.mutans* has a glucosyltransferase (GTF) gene whose function is to control the synthesis of glucose and fructose which are components of extracellular polysaccharides or bacterial capsules.[5] The GTFB and GTFC genes in *S.mutans* function as adhesions of bacterial cells to the tooth surface. Apart from that, *S.mutans* also has Glucan Binding Protein (GBP) which contributes to optimal plaque development and minimizes stress on the bacterial population.[6]

One method used to prevent the formation of biofilm and plaque is a mouthwash made from 0.2% chlorhexidine. CHX 0.2% is effective in reducing biofilm and preventing plaque build up, and in in vitro research, CHX 0.2% succeeded in reducing the growth of *S.mutans* colonies.[7] However, long-term use of 0.2% chlorhexidine will cause new problems such as staining on teeth, dry mouth (*xerostomia*), and changes in taste sensation.[8] The use of CHX allows Type IV and Type I hypersensitivity reactions to occur accompanied by anaphylaxis, leading to death due to a severe anaphylactic response.[9] Therefore, it is necessary to conduct research on alternative ingredients that have antibacterial properties with minimal side effects.

Recently, a lot of research has been done regarding the pharmacological effects of the Biduri plant. Biduri plants often grow in long dry areas and sandy beaches. Biduri plants grow wild along the coast in Watu Ulo, Jember. The Biduri leaf has been researched more than the other parts of the plant as the leaf is easier to obtain.The leaf is the part of the plant that is always available continuously so that the leaves can be taken at any time when needed.

Besides that, leaves contain more active compounds.[10] Alibasyah (2020) found that Biduri leaf extract positively contains antibacterial compounds such as flavonoids, phenols, tannins, saponins, terpenoids, and alkaloids which are secondary metabolite compounds.[11,12] Thomas *et al* (2017) explained that the alkaloid, flavonoid and tannin content in Biduri leaves can produce antibacterial power.[13] According to Waseem *et al* (2020) the total flavonoid content in Biduri leaves is +11.2 mg/mL.[14] and the total flavonoid content of Biduri leaf extract according to Pudji *et al*[15] is 9,630 mgQE/g extract, higher than Biduri flowers.[15]

Alibasyah`s (2020) research on Biduri leaf extract (*Calotropis gigantea*) using 70% ethanol solution with variable concentrations of 5, 10, 15, 20, 25 and 30% showed the ability of Biduri leaf extract to inhibit the growth of *Aggregatibacter actinomycetemcomitans* in various concentrations and showed an increase along with increasing extract concentration.[11] Hidayah`s (2020) research regarding the inhibitory power of Biduri leaf extract in concentrations of 10, 20 and 30% against *Staphylococcus aureus* showed that a concentration of 20% has a medium category of inhibitory power.[16] Dewi's research (2018) explained that Biduri leaf extract in concentrations of 40, 60, 80, and 100% had antibacterial power against *Staphylococcus aureus* and the antibacterial power increased as the concentration increased.[17]

Based on the description above, the aim of this study was to determine the antibacterial power of Biduri leaf extract (*Calotropis gigantea*) on the growth of *S.mutans* colonies using extract concentrations of 20, 40, and 60%. The research method used was the colony counting method using a colony counter. The research aimed to analyze the antibacterial potential of Biduri leaf extract on the growth of *S.mutans* colonies.

## METHODS

The research method used was laboratory experimental research (True Experimental Design) in vitro using the post-test-only control group design method. The research samples were *S.mutans* ATCC 35668 and Biduri leaf extract with concentrations of 20, 40, and 60%. The positive control used was 0.2% chlorhexidine and the negative control used was sterile distilled water.

Identification and production of Biduri leaf extract was carried out at the Plant Laboratory, Jember State Polytechnic. Biduri leaves were extracted using a maceration technique with 70% ethanol solvent in a ratio of 1: 5. 2 kg of Biduri leaves were obtained from the coast of Watu Ulo Beach. The leaves were washed thoroughly in running water to remove dirt then air-dried for a week. Biduri leaves were placed in the oven at $40^0$C for 2 x 24 hours to remove the water content in the leaves and prevent rotting.

The dried Biduri leaves were ground using a blender and sieved using a 30 mesh sieve. The extract was made by weighing 100 grams of simplicia then adding 70% ethanol solvent and stirring using a shaker for 1 day. The pure extract was condensed using a rotary evaporator with a temperature of $50^0$C and a rotation of 90 rpm. The thick extract was diluted using sterile distilled water solution until extracts with concentrations of 20%, 40% and 60% were obtained.[11,12,15]

The Mueller Hinton Agar (MHA) and Mueller Hinton Broth (MHB) were prepared as bacterial culture media. Both MHA and MHB were made by mixing powder with distilled water according to the composition on the packaging. The media solution was put into an Erlenmeyer flask and covered with aluminum foil. The agar medium in the Erlenmeyer flask was sterilized by autoclaving at $121^0$C for 20 minutes.[18]

*S.mutans* suspension was prepared by preparing 1 dose of bacteria and placed in a test tube containing 2 ml MHB. The test tube was covered with cotton and incubated for 24 hours at $37^0$C. The bacterial suspension was formed then adjusted for turbidity to the McFarland 0.5 standard, 1.5x108 CFU/ml. The bacterial suspension was then diluted to minimize and reduce the number of bacteria suspended in the liquid.

The sample containing the bacterial suspension was put into the first dilution tube of 1 ml ($10^{-1}$) with a ratio of 1:9. 1 ml was taken from tube ($10^{-1}$) then was transferred to tube ($10^{-2}$). 1 ml was taken from tube ($10^{-2}$) then was transferred to tube ($10^{-3}$). Each dilution was carried out aseptically and then homogenized using a vortex. The samples used in the research were samples at the 4th dilution ($10^{-4}$).

Antibacterial test method used the colony counting/plate counting method. The diluted bacterial suspension was then inoculated into each Petri dish using the pour plate technique. 15 ml of liquid MHA at a temperature of 40 - $45^0$C was put into a Petri dish then 1 ml of bacterial suspension was added and then homogenized by rotating it to form the number 8 or rotating it back and forth in the opposite direction quickly for 10 - 20 seconds. After being homogenized, the suspension in agar was exposed to 1 ml of Biduri leaf extract and then homogenized again. The Petri dish was left to solidify. The sample was incubated in the incubator for 24 hours at $37^0$C. After incubation, bacterial colonies that grew were counted using the colony counter.

The experiment was repeated 5 times in the same way for each sample group. There were several things needed to be considered when calculating the number of bacterial colonies: bacterial colony counts had to meet the Standard Plate Count requirements containing 30-300 CFU/mL (If the number of colonies per sample was more than 300 CFU/mL, it was categorized as too numerous to count (TNTC); counting as one individual colony for separate colonies of the same appearance and overlapping colonies still counted as different individuals. The number of bacterial

colonies in CFU/ml was quantitatively counted using a formula, number of colonies per petri dish times dilution factor divided by volume of inoculated suspension.

## RESULTS

The results of research on the antibacterial potential of Biduri leaf extract (*Calotropis gigantea*) on the growth of *S.mutans* colonies showed that there were differences in the growth of *S.mutans* colonies in several groups of samples after incubation for 24 hours (Figure 1).

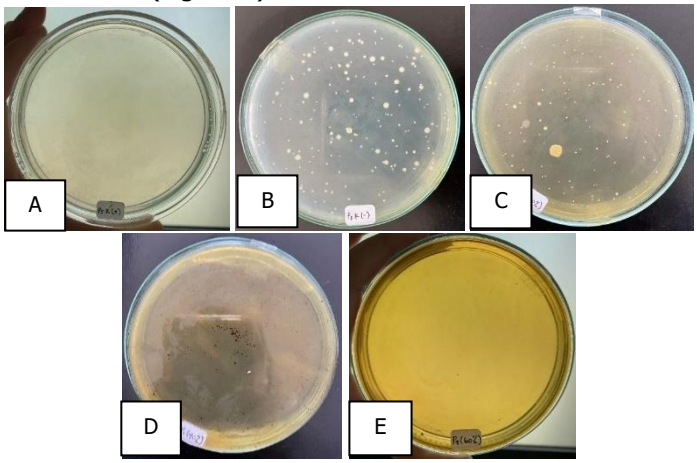


**Figure 1.** Bacterial colonies grow on the media after incubation for 24 hours in each sample group. (A) The positive control of 0.2% chlorhexidine showed no growth of bacterial colonies. (B) Negative control of sterile distilled water. (C) Concentration of 20%, the growth of colonies formed was less than the negative control. (D) Concentration of 40%, there was colony growth of 1 colony. (E) Concentration of 60%, no bacterial colony growth was found.

Figure 1. shows that at K (+) and a concentration of 60% there was no growth of *S.mutans* colonies. At a concentration of 40%, one colony of bacteria was still found in one repetition. At K (-) and a concentration of 20% there was a lot of growth of *S.mutans* colonies. The average results of bacterial colony growth for each group are presented in Table 1 and Figure 2.

**Table 1.** Average number of *S.mutans* colonies ($\underline{X}$) and standard deviation of colony growth in each sample group.

| Sample group | N | $\underline{X}$ ($10^{-4}$) | Standar deviation |
|---|---|---|---|
| K(+) | 5 | 0 | 0 |
| K (-) | 5 | 185 | 1.84 |
| 20% | 5 | 80 | 28.38 |
| 40% | 5 | 0.2 | 0.45 |
| 60% | 5 | 0 | 0 |

Description: number of sample (N), average number of S.mutans colonies ($\underline{X}$)

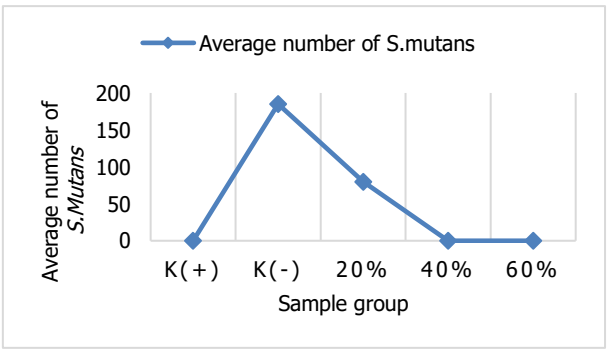


**Figure 2.** Average number graph of *S.mutans* colonies.

In Figure 2, the 60% concentration showed no growth of *S.mutans* colonies, which means that the 60% concentration had the ability to eliminate bacteria as well as the control (+) using 0.2% CHX.

Data analysis used non-parametric tests because the data were not normal and not homogeneous. The results of the *Kruskal Wallis* test showed that the significant value was 0.000 (p<0.05), meaning that there were significant differences in the growth of *S.mutans* ATCC 35668 colonies in each sample groups. Once it was known that there were significant differences in colony growth between sample groups, the *Mann Whitney* test was then carried out to determine the differences between the two sample groups. The results of the *Mann Whitney* test can be seen in table 3.

**Table 3** *Mann Whitney* test result

| Sample group | K(+) | K(-) | 20% | 40% | 60% |
|---|---|---|---|---|---|
| K(+) | - | 0,005* | 0,005* | 0,317 | 1,000 |
| K(-) | - | - | 0,009* | 0,007* | 0,005* |
| 20% | - | - | - | 0,007* | 0,005* |
| 40% | - | - | - | - | 0,317 |
| 60% | - | - | - | - | - |

*: There are significant differences (p<0,05)

Table 3 shows that there were no significant differences in growth of *S.mutans* colonies in control (+) with a concentration of 40%, control (+) with a concentration of 60%, and concentration of 40% with a concentration of 60%.

## DISCUSSION

In Figure 1 there were the differences in the color of the bacterial culture media. Bacterial media with a 60% label looked darker, while control (+) control (-) media was clearer. This is in accordance with Alibasyah's research which showed that media with a higher concentration had a darker color. This is because the higher the extract concentration, the more concentrated the media solution will be because the amount of extract is greater than the amount of solvent, resulting in a darker and brownish yellow color of the media.[11]

In Figure 2 The average number of colonies was obtained from the results of counting colonies on the colony counter by 3 observers and then averaging them to avoid counting errors. Table 1 and Figure 1 show the average growth of *S.mutans* colonies after exposure to the extract. In both table and figure, it was known that the average growth of *S.mutans* colonies decreased at all extract concentrations, starting from concentrations of 20, 40 to 60%.

This shows that Biduri leaf extract has antibacterial power against the growth of *S.mutans* colonies. This is in line with research by Alibasyah (2020) which stated that the antibacterial power of biduri leaf extract will increase along with increasing extract concentration because the number of active compounds contained in high extract concentrations is greater.[11] In addition, if the extract concentration increased, the growth of *S.mutans* colonies decreased. This is due to differences in the number of active compounds at each extract concentration.[11,12]

The research results of the 20% concentration had a significant difference to the control (+), meaning that this concentration did not have the same antibacterial power as the control (+). This is due to the fact that the number of antibacterial compounds at a concentration of 20% is less and less stable, so that at this concentration bacterial growth is still found. In the research by Hidayah (2020) which examined the inhibitory power of Biduri leaf extract with a concentration of 20% against *Staphylococcus aureus*, it was only able to inhibit the growth of *Staphylococcus aureus* in the moderate category.This may be due to the fact that

the active compound content in the extract was small so it was not able to produce maximum inhibitory power.[16]

The results of the first repetition of the 40% concentration (P1(40%)) still found the growth of one colony of *S.mutans* (Figure 1). A concentration of 40% when compared with the control (+) using 0.2% CHX was not able to kill the whole *S.mutans* colonies. The same result if a concentration of 40% was compared with a concentration of 60%.

In accordance with research by Dewi (2018) regarding the inhibitory power of Biduri leaves against *Staphylococcus aureus*, the inhibitory power produced by a 40% concentration compared to a 60% concentration is not much different, but the 40% concentration does not have the same inhibitory power as a 60% concentration.[17] This shows that the antibacterial power of the 40% concentration is still not stable and is not able to eliminate all *S.mutans* colonies as can be done by the 60% concentration and the control (+) using 0.2% CHX.

Apart from that, research by Alibasyah explained that the highest concentration of extract will also produce greater antibacterial power because it contains more antibacterial compounds.[11,12] In accordance with the research, the best concentration for reducing the growth of *S.mutans* colonies is a concentration of 60% because at a concentration of 60% no bacterial growth was found. A concentration of 60% compared with K (+) shows that there is no significant difference in the results.

In other words, a concentration of 60% has the ability to eliminate *S.mutans* as well as CHX 0.2% as a control (+). However, if this Biduri leaf extract is used in humans as a mouthwash, Biduri leaf extract with a concentration of 60% is not yet equivalent in its ability as an antibacterial when compared to 0.2% chlorhexidine. Apart from the very large difference in concentration, the content of the 0.2% chlorhexidine mouthwash has been clinically tested and is biocompatible for use in the oral cavity.[9] This is in accordance with the results of the study which showed that in the control (+) there was no growth of *S.mutans* colonies.

The phytochemical screening results of Biduri leaf extract contain active compounds such as alkaloids, flavonoids, phenols, saponins, sapocenin, calcium oxalate, terpenoids, kalotropine, amirin, uskarin, gigantin, tannin, and kalotoxin. This is in line with Alibasyah (2020) who identified phytochemical compounds in Biduri leaves which are thought to act as antibacterial compounds, including alkaloids, flavonoids, steroids, phenols, tannins.[11,12]

Differences in the amount of active compounds extracted can be influenced by the solvent used during maceration. The solvent that is suitable for extracting natural materials is 70% ethanol solvent. 70% ethanol solvent can extract more active compounds than 96% ethanol solvent. The 70% ethanol solvent consists of 70% ethanol which is effective for extracting active compounds, and 30% consists of water which minimizes the toxicity of the ethanol solvent and is effective for extracting non-polar compounds. This is the reason why 70% ethanol solvent is called a semi-polar solvent, because it can extract both polar compounds and non-polar compounds in plant extracts.[19]

The most phytochemical compounds in Biduri leaves are alkaloids 4.05%. Alkaloids are spread throughout all parts of the plant because alkaloids act as a defense system for plants. Alkaloids are toxic to predators or pests but help plants during dry conditions and extreme temperatures. Alkaloids as antibacterials are bactericidal. The mechanism action of alkaloids as antibacterials is by inhibiting the peptidoglycan component of bacterial cells so that the cell wall layer cannot form completely and will cause cell death.[20] The alkaloid that shows antibacterial ability is the sanguinarine type alkaloid. Sanguinarine alkaloids have been proven to inhibit the attachment of bacteria to the tooth surface and provide anti-plaque effects.[21] Therefore, Biduri leaf extract which contains alkaloids is also effective against *S.mutans* which is a bacteria that initiates plaque.

Flavonoids are the second most abundant content in Biduri leaf extract 4.01%. Flavonoids have been proven to have antibacterial, anti-inflammatory, analgesic,

anti-allergic, cytostatic and antioxidant properties. Flavonoids are lipophilic compounds that are bacteriostatic by damaging bacterial cell membranes. The mechanism of action of flavonoids as antibacterials is by damaging the bacterial cell membrane in the phospholipid section so that the permeability of the cell membrane is reduced and results in damage to the bacterial structure.[19]

Flavonoid content will reduce the hydrophobicity of plaque bacterium, *S.mutans*. Increasing the concentration of flavonoids will reduce the hydrophobicity of plaque bacteria so that the ability of the bacteria to attach to the tooth surface will decrease or even disappear.[22] Apart from that, apigenin type flavonoids have also been proven to be able to fight *S.mutans* by increasing membrane permeability and inhibiting acid production by *S.mutans* in biofilms.[23]

Another secondary metabolite compound that has antibacterial properties is saponin. Saponin compounds can control the growth of *Streptococcus* in the formation of biofilms in the oral cavity.[24] The mechanism of saponin as an antibacterial is by denaturing proteins so that cell membrane permeability is disturbed, cell membranes are damaged, cell leakage occurs and bacteria will lyse.[20] Saponin can damage the structure of the *S.mutans* cell membrane and will move across the cytoplasmic membrane so that the cell's cytoplasmic fluid comes out and causes the *S.mutans* cell membrane to become unstable, causing lysis.[25]

Terpenoid chemical compounds are also known to be contained in Biduri leaf extract. Research on administering terpenoids to *S.mutans* shows that terpenoids of the diterpenoid, monoterpenoid and sesquiterpenoid types have antibacterial effects and are able to destroy the biofilm that forms.[26] Terpenoid bioactive compounds are known to be bacteriostatic and bactericidal. However, bacteriostatic properties are more dominant than bactericidal properties. Due to their lipophilic nature, terpenoids work by strongly binding to porin proteins in bacterial cell membranes until the bacterial cell membrane is damaged and the permeability of the bacterial cell membrane is reduced so that the bacteria will slowly die.[27]

Tannin is another chemical compound that acts as an antibacterial against *S.mutans.* The toxicity of tannin compounds can damage bacterial cell membranes. The mechanism of tannin as antibacterial is that it binds to proteins in the bacterial cell membrane and damages the permeability of the cell membrane and inactivates essential enzymes needed by bacteria. Tanin inactivates the glucosyltransferase enzyme in bacterial cells so that bacterial cell structures cannot form.[20] Kováč's research (2023) concluded that tanins and flavonoids were proven to have antibacterial abilities against bacteria that cause caries, bacteria that cause periodontal disease and bacteria that cause other oral diseases, one of these bacteria is *S.mutans*.[28]

The limitation of this research was that there were only 3 variations of concentration, 30, 40, and 60%, in which this concentration range was too small, so it was not known which concentration of extract worked most effectively in reducing *S.mutans* in the oral cavity. Apart from that, this research was not accompanied by an extract toxicity test, so it was not yet known whether the research samples were toxic to the human oral cavity or not.

## CONCLUSION

Biduri leaf extract (*Calotropis gigantea*) concentration 20, 40, and 60% has antibacterial potential against *S.mutans* colonies. It is also known that the best concentration of Biduri leaf extract (*Calotropis gigantea*) in reducing the growth of *S.mutans* colonies is at a concentration of 60%. The implication of this findings is Biduri leaf extract (*Calotropis gigantea*) can be used as the ingredients of herbal mouthwash.

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**Conflicts of Interest:** The authors declare no conflict of interest.

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