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ANTIBACTERIAL EFFECTIVENESS OF BEETROOT AGAINST *STREPTOCOCCUS MUTANS*

Ulfa Yasmin*, Ibnu Adjiedarmo*, Yulia Christianti*, Sulistiawati, Mellani Cindera Negara****

*Department of Pediatric Dentistry, Faculty of Medicine Sriwijaya University
**Department of Periodontic Dentistry, Faculty of Medicine Sriwijaya University
Email: liachrsty1@gmail.com

KEYWORDS

beetroot, antibacterial effectiveness, MBC, MIC, Streptococcus mutans, inhibition zone

ABSTRACT

Introduction: *Antibacterial effectiveness is the level of an antibacterial agent's ability in controlling bacteria through growth-inhibiting or killing activities. A natural antibacterial agent that has the potential to be used as an alternative is beetroot (Beta vulgaris L.) because it contains phenolic compounds, flavonoids, saponins, tannins, and betalains. Antibacterial agents have been widely used as preventive for dental caries in children with the main causative bacteria being Streptococcus mutans. This study aims to evaluate the antibacterial effectiveness of beetroot extract against Streptococcus mutans.* ***Methods:*** *This study was in vitro experimental laboratory study with a post-test-only control group design. The maceration method used to obtain beetroot extract which will diluted into various concentrations, 12,500 µg/ml, 25,000 µg/ml, 50,000 µg/ml, and 100,000 µg/ml. Antibacterial effectiveness was tested by measuring the inhibition zone using disc diffusion, determining the Minimum Inhibitory Concentration (MIC) using broth dilution, and the Minimum Bactericidal Concentration (MBC) using agar dilution with 0,2% chlorhexidine gluconate as the positive control. Analysis of inhibition zone data was performed statistically using one-way ANOVA and Tukey Post Hoc. MIC and MBC tests were analyzed descriptively.* ***Results:*** *The results show all concentrations of beetroot extract were able to form an inhibition zone with the largest mean was 0.40 ± 0.05 mm, but still lower than the positive control. MIC and MBC of extract were 25,000 µg/ml and 50,000 µg/ml respectively.* ***Conclusion:*** *It can be concluded that beetroot extract has antibacterial effectiveness against Streptococcus mutans, therefore it can be used as a natural alternative for dental caries prevention.*

INTRODUCTION

Substances that limit the growth of bacteria in order to prevent or treat an infection are known as antibacterial.[1,2] Bacterial susceptibility needs to be investigated to find out how effective these substances are. Antibacterial effectiveness is the level of ability of antibacterial materials to control bacteria through their activity to inhibit the growth or kill the target bacteria.[3] Harti and Harmita in their study explained that the effectiveness of a substance can be measured in vitro by measuring the zone of inhibition, determining the Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC).[3,4]

Natural antibacterial materials have now been widely developed because they are considered efficacious, and safer, with fewer side effects and relatively low costs compared to synthetic antibacterials.[1,5] One of the natural ingredients from plants that has potential as an antibacterial is beetroot (*Beta vulgaris* L.).[6]

Beetroot is a red to dark purple tuber vegetable which is generally used as a natural dye or additive in various food products.[7–9] Previous studies have shown that beetroot can improve health through various therapeutic nutrients including antioxidant, anti-inflammatory, antitumor, hepatoprotective, cardioprotective, and antibacterial.[6,7,10]

Previous studies have proven the antibacterial activity of beetroot against several Gram-positive and negative bacteria, including *Staphylococcus aureus*, *Bacillus subtilis*, *Enterococcus faecalis*, and *Escherichia coli*.[7,10–14] Omogbai et al. and Saani et al. concluded that the bacteria that were more susceptible to beetroot extract were Gram-positive than Gram-negative.[11,13]

The antibacterial activity of beetroot has the potential to be utilized in dentistry, especially as an alternative caries prevention therapy from synthetic antibacterial ingredients, such as chlorhexidine mouthwash, which has been shown to cause various side effects.[15,16] The process of dental caries involves various factors, one of which is cariogenic bacteria in plaque.[17] Setyorini et al. in their research proved the number of colonies of *Streptococcus sp.* on dental plaque decreased after gargling with beetroot juice.[15] Types of bacteria in *Streptococcus sp.* which is closely related to the early formation of caries is *Streptococcus mutans*.[18]

Streptococcus mutans is a Gram-positive facultative anaerobe that has several characteristics that play a major role in the occurrence of caries.[19] These characteristics are being able to synthesize glucan extracellular polymers from the sucrose in large quantities for colonization of dental hard tissues, metabolize various carbohydrates into organic acids (acidogenic), and thrive under conditions of environmental stress, such as low pH (aciduric).[20]

Previous studies on the antibacterial properties of beetroot extract against several Gram-positive pathogenic bacteria have been carried out, but research on the Gram-positive bacteria that causes early caries lesions in children, namely *Streptococcus mutans*, is still limited.[11–14] Based on this, research on the antibacterial effectiveness of beetroot extract against *Streptococcus mutans* needs to be done with chlorhexidine as a positive control.

METHODS

This experimental laboratory research was conducted in vitro with a post-test-only control group design. The fresh beets used to come from plantations in the District of Berastagi, Karo Regency, North Sumatra. Extraction and dilution of beetroot extract

were carried out at the Biochemistry Laboratory, Faculty of Medicine, Sriwijaya University. The antibacterial effectiveness test was carried out at the Microbiology Laboratory of the Palembang Health Laboratory Center (BBLK). This research has received ethical approval from the Medical and Health Research Ethics Committee (KEPKK) Faculty of Medicine, Sriwijaya University as stated in the letter with protocol number 026-2021. This research was conducted from February to April 2021.

This research uses tools including stationery, oven, autoclave, knife, blender, filter paper, Erlenmeyer flask, Laminar Air Flow (LAF), rack and test tube, petri dish, loop needle, cotton, cotton swab, measuring cup, micropipette, disc paper, caliper, digital scale, maceration container.

The materials used in this study were beetroot (*Beta vulgaris* L.), 95% ethanol solvent, distilled water, *Streptococcus mutans* ATCC 25175, nutrient broth media, blood agar media, and 0.2% chlorhexidine gluconate as the positive control, 70% alcohol, physiological solution (0.9% NaCl). Every tool made of glass and metal is sterilized by oven at 100ºC or autoclave at 1 atm pressure, at 121ºC for ± 15 minutes. Tools made of plastic are sterilized using 70% alcohol by wiping, then washed and dried.[21]

Production of Extracts and Variations in Concentration of Beetroot Extract

The extract of *Beta vulgaris* L. was made by the maceration method. A total of 4 kg of beets are cleaned and washed in running water, then sliced thinly. Drying using an oven at 40ºC for 24 hours to dry. The dried beets were finely ground using a blender to obtain a dry powder.[10,11]

250 g of powder was dissolved in 750 ml of 95% ethanol solvent in a maceration container for 48 hours at room temperature. The soaking water was filtered using filter paper, then evaporated using an oven at 45ºC to obtain a thick extract of 11 g. The extract was then stored at 4ºC until used for analysis.[10,11]

The thick extract of beetroot was diluted with distilled water and calculated by the solution dilution equation to obtain variations in the concentration of the extract. The concentrations used in this study were 12,500 µg/ml, 25,000 µg/ml, 50,000 µg/ml, and 100,000 µg/ml.

Preparation of Media

The nutrient broth medium was taken at 0.26 grams and the distilled water was measured to 20ml, then both were put into an Erlenmeyer flask. Heating of the media is done while stirring until homogeneous and boiling. The media was sterilized using an autoclave at 121°C, at a pressure of 1 atm for 15 minutes. A total of 1 ml of nutrient broth liquid media was taken with a pipette, put in

a tube, closed, and ready to be used as a bacterial culture medium.[22]

Blood agar was prepared by weighing 8 gr of powder and dissolving it with 200 ml of distilled water in an Erlenmeyer flask. The medium was stirred and heated to boiling, then 10 ml of blood was added. The media was sterilized by autoclaving at 121°C, at a pressure of 1 atm for 15 minutes. Sterile blood agar is cooled and transferred to a petri dish.[23]

Preparation of Bacterial Suspension

Streptococcus mutans ATCC 25175 was obtained from the Microbiology Laboratory of BBLK Palembang. The bacterial suspension was made by inserting a physiological solution and adding 1 ose (inoculating loop) of culture into a test tube. The mouth of the test tube was closed with cotton. The bacterial suspension was adjusted to the McFarland turbidity standard (5x108 CFU/ml).[24]

Antibacterial Effectiveness Test Inhibition Zone Diameter Measurement by Disc Diffusion

The suspension of *Streptococcus mutans* bacteria was smeared on blood agar in a petri dish using a sterile cotton swab. Each disc paper was soaked first at the concentration of extract and positive control, then positioned on the media according to the marks that had been made, then incubated for 24 hours at 37ºC.[11,25] The diameter of the clear zone around the disc was measured using a caliper in mm and then calculated by the following equation.[26]

$$nw_{halo} = \frac{\frac{d_{iz} - d}{2}}{d}$$

Description:

nw_{halo} = Normalization calculation of the inhibition zone width
d_i = Diameter of the zone of inhibition
d = Diameter of the disc

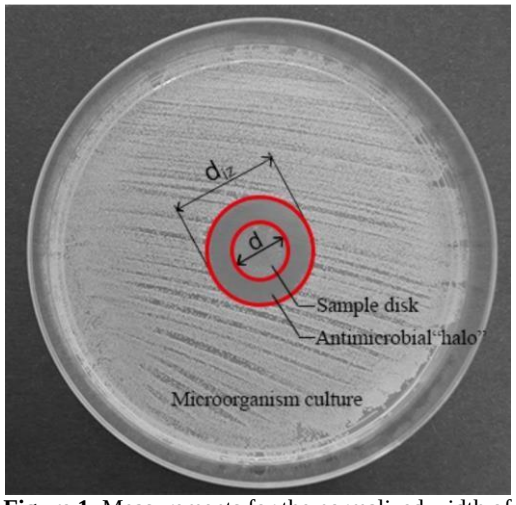

Figure 1. Measurements for the normalized width of the antibacterial zone[26]

Determination of MIC Value by Liquid Dilution

A total of five test tubes were added to each 2ml of nutrient broth using a micropipette. 0.2% chlorhexidine gluconate and beetroot extract with concentrations of 12,500 µg/ml, 25,000 µg/ml, 50,000 µg/ml, and 100,000 µg/ml were added in 5ml into the test tube. 0.1 ml of *Streptococcus mutans* suspension was also added to each tube. The inoculated tubes were incubated at 37 ± 1°C for 24 h. The tube was removed and visually observed for the presence or absence of turbidity in the solution after incubation. The extract with the lowest concentration that was able to

inhibit the growth of *Streptococcus mutans* without showing turbidity was recorded as MIC.[11]

Determination of MBC Value with Solid Dilution

Petri dishes containing blood agar were inoculated with samples from each test tube from the MIC test, then incubated at 37°C for 24 hours. The lowest concentration of the extract that did not show growth on the medium was taken as MBC.[11]

The inhibition zone measurement data were analyzed using One Way ANOVA and Post Hoc Tukey tests with a significance value of 0.05 on the SPSS application. The results of the MIC and MBC tests were analyzed descriptively.

RESULTS

Each antibacterial effectiveness test was repeated five times. The results of the measurement of the diameter of the inhibition zone and the calculation using the formula are shown in table 1. The concentration of 100,000 µg/ml is the concentration of beetroot extract which produces an inhibition zone against Streptococcus mutans bacteria with the largest average measuring 0.40 ± 0.05 mm. The positive control, 0.2% chlorhexidine gluconate, was the sample group that had the largest mean zone of inhibition, namely 1.02 ± 0.21 mm.

Table 1. Results of Inhibition Zone Measurements After Incubation For 24 hours

The Normalized Width of Antibacterial Zone (mm)					
Replication	Beetroot Extract Concentration (µg/ml)				Positive Control (0.2% CHX)
	12,500	25,000	50,000	100,000	
1	0,09	0,18	0,33	0,38	0,89
2	0,09	0,23	0,30	0,41	1,10
3	0,15	0,19	0,27	0,35	0,90
4	0,13	0,21	0,32	0,35	0,85
5	0,10	0,21	0,28	0,49	1,36
Average	0,11 ± 0,26	0,20 ± 0,01	0,30 ± 0,02	0,40 ± 0,05	1,02 ± 0,21

One Way ANOVA statistical analysis showed a significance value of <0.05, so it can be stated that there was a significant difference in the mean diameter of the inhibition zone between the sample groups against *Streptococcus mutans*. The analysis was continued with the Post Hoc Tukey test and the results obtained were that there was a significant difference in the mean value between the beet extract and beet extract groups as well as between the beet extract groups and the positive control group, indicated by a p-value <0.05.

Observations on the MIC test showed that the beetroot extract tube with a concentration of 25,000 µg/ml, 50,000 µg/ml, 100,000 µg/ml, and positive control, 0.2% chlorhexidine gluconate, showed clarity, while the concentration of the extract was 12,500 µg/ml. still showed the growth of *Streptococcus mutans* bacteria was not inhibited. This was evidenced by the presence of turbidity in the form of white lumps in the solution so that the MIC of beetroot extract was set at a concentration of

25,000 µg/ml. The results of the MIC are not sufficiently judged by direct observation because the color of the extract gets darker with increasing concentration, especially at concentrations of 50,000 µg/ml and 100,000 µg/ml.

The results of observations and calculations of *Streptococcus mutans* colonies on the results of the MBC test are shown in Table 2 and Figure 2. Subcultures of beetroot extract with concentrations of 12,500 µg/ml and 25,000 µg/ml showed the growth of *Streptococcus mutans* bacteria still occurred on agar media with an average number of colonies is >300 CFU/ml. Samples of beetroot extract with a concentration of 50,000 µg/ml, 100,000 µg/ml, and 0.2% chlorhexidine gluconate were able to kill *Streptococcus mutans* bacteria, which was indicated by the absence of bacterial colony growth, so the concentration of 50,000 µg/ml was determined as MBC.

Table 2. The Calculation of *Streptococcus mutans* Colonies After 24 Hours Incubation

The Calculation of Colonies (CFU/ml)					
Replication	Beetroot Extract Concentration (µg/ml)				Positive Control (0.2% CHX)
	12,500	25,000	50,000	100,000	
1	>300	>300	0	0	0
2	>300	67	0	0	0
3	141	30	0	0	0
4	120	>300	0	0	0
5	>300	>300	0	0	0
Average	>300	>300	0	0	0

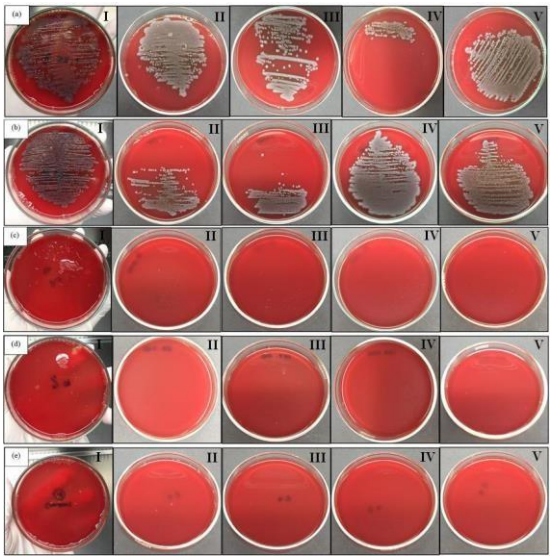

Figure 2. The results of the MBC test on *blood agar* of beetroot extract with concentrations of (a) 12,500 µg/ml, (b) 25,000 µg/ml, (c) 50,000 µg/ml, (d) 100,000 µg/ml, and (e) positive control 0.2% *chlorhexidine gluconate*

DISCUSSION

The results of the bacterial sensitivity test through the measurement of the inhibition zone proved that the entire concentration of beetroot extract was able to produce an inhibition zone around the paper disc. Table 1 shows that the smaller concentration decreased the mean value of the inhibition zone. This result is in accordance with the concentration-dependent principle; increasing the concentration will increase the antibacterial power.[27] Saani et al. in his research using beetroot ethanol extract against Gram-positive bacteria showed similar results where a large increase in the inhibition zone value occurred when the concentration of the extract was increased.[13] The concentration of 25,000 µg/ml was determined as the lowest concentration of beetroot extract which was bacteriostatic against *Streptococcus mutans*. The MIC test

using the visual observation method in this study was able to determine the MIC, but the weakness of this method is the increasing color density of the solution due to the increased concentration of the extract, so the assessment is less accurate and subjective.[28] Concentrations of beetroot extract of 50,000 µg/ml and 100,000 µg/ml have a dark brown-black solution which makes it difficult to interpret the clarity of the solution. Measurement of solution density with Enzyme-Linked Immunosorbent Assay (ELISA) reader and High-Pressure Liquid Chromatography (HPLC) test is a method that can be used as a further alternative test to assess quantitatively through the absorbance of the solution to obtain more precise and accurate results.[27,29]

The results of the MBC test (Table 2) showed that the concentration of beetroot extract which was able to kill bacteria was greater than MIC, namely 50.000 µg/ml. The MIC of antibacterial compounds can be equal to or greater than the MIC value. This proved that the increased antibacterial concentration was able to increase the antibacterial activity from inhibiting to killing bacteria.[30] This decrease in the number of colonies was supported by the research of Setyorini et al. which proves that the number of *Streptococcus sp.* on the dental plaque can decrease significantly after gargling with beetroot juice.[15]

The weak antibacterial ability of *Streptococcus mutans* can be influenced by several factors, one of which is the extract concentration is too low. Concentrations that are too low result in a small amount of the active compound being accumulated. Suryadi et al. in their research stated that the concentration of antibacterial material used was one of the determining factors for how much antibacterial activity the material had.[31] Antibacterial activity in the form of inhibiting and killing *Streptococcus mutans* is caused by the role of bioactive compounds contained in beetroot extracts such as phenols, flavonoids, betalains, saponins, and tannins.[10,11] Phenolic compounds and flavonoids are secondary metabolites that are mostly contained in beetroot extract.[10] Phenolic compounds act as antibacterials by disrupting and damaging the outer structure of bacteria, especially Gram-positive bacteria, which causes cytoplasmic leakage.[11,14] The mechanism of action of flavonoids, derived from phenolic compounds, against bacteria is that they can inhibit nucleic acid synthesis, cytoplasmic membrane function, and virulence factors such as enzymes in bacteria.[11,32] Bacterial cells eventually lyse, resulting in reduced important components of bacteria so that bacterial activity is disrupted, growth is inhibited and bacterial death.[32]

The saponins contained in the beetroot extract inhibit the growth of *Streptococcus mutans* bacteria by reducing the permeability of the bacterial membrane by lowering the surface tension of the cell wall.[15,33]

Antibacterial substances will enter the cell easily when the membrane permeability changes, the metabolism of bacteria is disturbed and causes the bacteria to die.[33]

Tannin compounds can inhibit bacterial growth through their ability to disrupt cell membrane permeability, inhibit enzyme activity that plays a role in the genetic function of bacteria, as well as remove substrates, ions, and minerals in bacteria.[15,34]

Betalain compounds can bind Fe2+, Ca2+, dan Mg2+ ions which play an important role in the basic needs of bacteria and the penetration of bacterial cell membranes. The mechanism of betalain as an antibacterial has not been discussed in detail and depth in previous studies.[35]

The results of this study indicate the antibacterial effectiveness of beetroot extract against *Streptococcus mutans*, a Gram-positive bacteria. Beetroot extract is known to be more susceptible to Gram-positive than Gram-negative bacteria.[11,13] This is because the cell wall structure of Gram-positive bacteria only consists of a simple, stiff, and thick layer of peptidoglycan, while the structure of Gram-negative bacteria is more complex even though the peptidoglycan layer is thin.[7,11,13,19] The outer membrane layer of Gram-negative bacteria consists of a double-layered, highly hydrophilic lipopoly-saccharide molecule and a periplasmic space.[7,19]

The positive control group had a higher mean zone of inhibition than the beetroot extract group. This can be influenced by different antibacterial mechanism factors between 0.2% chlorhexidine gluconate and beetroot extract in inhibiting and killing bacteria.[15] The chlorhexidine molecule can be adsorbed quickly on cells and has a positive charge that will bind to the negative charge on the bacterial cell wall electrostatically so that it disrupts the cell wall and osmotic pressure of the bacterial cell, while beetroot extract is known to not work with this mechanism.[19,31] Chlorhexidine is also able to freeze or harden the cytoplasm of bacteria with the formation of phosphate complexes that result in cell death.36,37 The use of 0.2% chlorhexidine gluconate has been reported to cause side effects, including discoloration of teeth and tongue, impaired sense of taste, and irritation of the oral mucosa.[16]

Several previous studies suggest that the ethanol extract of beetroot has minimal toxicity effects. Research by Gamal et al. reported that a dose of 500 mg/kg BW of beetroot ethanol extract was not toxic to Wistar rats.[38] Kim et al. in their in vivo research showed that the ethanol extract of beetroot was able to provide preventive and therapeutic effects on healing liver damage due to its high phenol and flavonoid content.[39]

Beetroot has been shown to be beneficial for health, but improper consumption can have undesirable effects on the body. Pluta RM in his study explained that beetroot can cause methemoglobinemia if consumed in excess,

especially in patients treated with sodium nitrate.[40] Johri N et al. stated that the high oxalate content in beetroot can also increase the risk of kidney stone formation.[41]

The ethanol extract of beetroot with the right amount of use has many health benefits and low toxicity effects, so it needs to be developed to the stage of in vivo and clinical research to find out more about the antibacterial effectiveness of beetroot extract as an alternative antibacterial.

CONCLUSION

The conclusion from the results of this study is beetroot extract has antibacterial effectiveness against *Streptococcus mutans* with an average value of inhibition zone starting from a concentration of 12,500 µg/ml which is 0.11 ± 0.26 mm with the MIC and MBC values were 25,000 µg/ml and 50,000 µg/ml respectively.

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