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Antibacterial Activity of Robusta Instant Coffee Powder Against Acidogenic and Aciduric Species of *Streptococcus mutans* Bacteria

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ABSTRACT
Streptococcus mutans is a major species that causes dental caries due to its acidogenic and aciduric properties. Coffee beans contain several compounds, such as caffeine, phenols, tannins, trigonelline, chlorogenic acid, and caffeic acid, which are known for their antibacterial properties. Robusta instant coffee powder is a stable product that is not easily contaminated with bacteria, fungi, and yeast. It is a zero-waste and soluble material. This study aimed to analyze the antibacterial activity of robusta instant coffee powder (RICP) against *S. mutans*. The antibacterial activity of RICP against *S. mutans* was tested using three antimicrobial susceptibility tests: 1) disk diffusion method, 2) minimum inhibitory concentrations (MIC), and 3) minimum bactericidal concentration (MBC). The results of the disk diffusion method test showed that the RICP concentration of 0.2 g/mL to 0.8 g/mL against *S. mutans* had an inhibition zone of 31.14 mm to 42.01 mm. MIC and MBC values of RICP against *S. mutans* were both 0.0313 g/mL. In conclusion, robusta instant coffee powder (RICP) demonstrates antibacterial activity against the acidogenic and aciduric *S. mutans* bacteria.

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1. INTRODUCTION

Dental plaque is a community of bacteria found on the tooth surface in a biofilm embedded in the host polymer matrix (Marsh, 2004). Plaque forms in an organized manner and has a diverse microbial composition that is relatively stable over time under healthy conditions (microbial homeostasis). However, significant changes can disrupt this balance, leading to the growth of certain bacteria. This disturbance can result in oral diseases (Marsh, 2006).

Studies have shown that dental caries are associated with an increased proportion of acid-producing and acid-resistant bacteria, particularly *Streptococcus mutans*, which can demineralize enamel (Marsh and Nyvad, 2008). These bacteria can quickly convert sugars into acids, leading to a low pH environment that is favorable for their growth but detrimental to other healthy enamel-associated species (Marsh, 1989).

Various studies have explored herbal remedies with minimal side effects. Coffee, a widely consumed tropical plant, is often consumed as a daily beverage in the form of roasted coffee bean powder. The Robusta instant coffee powder (RICP) produced by freeze-dried technology is a stable, mold-resistant, and soluble material. Freeze-drying technology maintains the quality of the product, including stability and rehydration power (Hariyadi, 2013). Roasted coffee beans contain phenols, tannins, trigonelline, chlorogenic, and caffeic acids, which are reported to have antibacterial activity (Patay et al., 2016; Wulandari et al., 2021; Setyati et al., 2023). The study used Robusta coffee beans (*Coffea canephora*), a type of coffee widely grown in Indonesia. In the Jember region alone, the Robusta coffee area covers about 18,000 hectares and produces over 11,000 tons per year (Ulum, 2021).

Previous research has demonstrated that *C. canephora* seed extract has antibacterial effects on *Streptococcus sanguinis* (Sulistiawati et al., 2019), *S. mutans*, and *S. sobrinus* (Antonio et al., 2011). Additionally, both green coffee bean extract and roasted coffee beans have shown antibacterial activity against *Porphyromonas gingivalis*, *Prevotella intermedia*, *Fusobacterium nucleatum*, and *Aggregatibacter*

actinomycetemcomitans (Tasew et al., 2020). Therefore, researchers aimed to investigate the antibacterial activity of robusta instant coffee powder (RICP) against acidogenic and aciduric species of *S. mutans* bacteria with a focus on proving and analyzing the inhibition, MIC, and MBC of Robusta instant coffee powder against these bacteria.

2. RESEARCH METHOD

Preparation of Robusta Instant Coffee Powder

The process of producing Robusta instant coffee powder involves brewing pure Robusta coffee powder obtained from the market and then using freeze-drying technology. Freeze-drying technology starts with freezing the material and then removing most of the water from the material through the sublimation mechanism. The Robusta instant coffee powder in this study was produced at the Center for Development of Advanced Science and Technology (CDAST) at Universitas Jember. Robusta coffee powder was brewed in hot water at 90°C in a ratio of 1 gram: 4 mL (500 grams of coffee powder in 2000 mL of hot water). In percentage terms, it becomes 25%. If the concentration is increased or decreased, it is likely to affect the outcome. The brewed coffee was filtered through a 100 mesh sieve and then placed in a freeze-drying machine at a temperature of -40°C for 2 x 24 hours to convert it into a powder. The resulting Robusta instant coffee powder is then stored in a sterile bottle.

Bacterial Isolate

The acidogenic and aciduric bacteria used in this study were *S. mutans* ATCC 25175.

Antimicrobial Susceptibility Test of RICP

The antimicrobial susceptibility test of RICP was conducted using the Kirby Bauer disk diffusion method on Mueller Hinton Agar (MHA) plates. The test organism (*S. mutans*) was cultured in Mueller Hinton broth (MHB), and then incubated overnight at 37°C. The culture was adjusted for turbidity using the McFarland 0.5 standard, resulting in an inoculum of 1.5×10^8 CFU/mL. The bacterial inoculum was spread onto the MHA media using a sterile cotton swab in a zigzag pattern from edge to edge. This was repeated three times, rotating the plate 60° each time (Hudzick, 2009). Four paper discs containing RICP at different concentrations (0.2 g/mL, 0.4 g/mL, 0.6 g/mL, and 0.8 g/mL), and one paper disc as a negative control (sterile aquadest) were placed on the surface of the MHA media inoculated with test bacteria at a minimum distance of 20 mm using sterile tweezers. The plates were then incubated for 24 hours at 37^0C in a facultative anaerobic incubator in an inverted position. The assay was repeated three times. After incubation, the plate was observed for the formation of the inhibition zone around the paper disk, indicating the antimicrobial activity of the compounds in the RICP. The zone of inhibition was measured in mm using a digital calliper.

Determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of RICP

The MIC of RICP was determined using the broth microdilution method, as described by Vu et al. (2016). Two-fold serial dilutions of Robusta Instant Coffee Powder (RICP) were added to wells of sterile 96-well plates containing MHA medium inoculated with bacterial cells (at a concentration of 10^5 colony-forming units per milliliter). The RICP concentrations used were 0.0156 g/mL, 0.0313 g/mL, 0.0625 g/mL, 0.125 g/mL. Sterile water was used as a negative control and did not affect bacterial growth. After 24 hours of incubation at 37°C, the MIC was determined as the lowest concentration that completely inhibited bacterial growth. The test was repeated three times against the test bacteria at all test concentrations. Iodonitrotetrazolium chloride at a concentration of 20 µl and 0.2 mg/mL was added to the test wells at the end of the incubation period and then incubated at 37°C for 3 hours. The presence of live bacteria was determined by the color change of the dye from yellow to pink.

MBC was determined, as described by Vu et al. (2016), by taking 20 µl of the bacterial broth suspension that showed no color change and then spreading it on MHA plates, which were then incubated at 37°C for 24-48 hours. The lowest concentration of RICP determined without bacterial growth observed was considered as MBC.

Data Analysis

Inhibition zone data were analyzed using a one-way analysis of variance (ANOVA) test to determine whether there were statistically significant differences between the groups, followed by Fisher's Least Significant Difference (LSD) test to identify where those differences lay.

3. RESULT AND DISCUSSION

Antimicrobial Susceptibility Test of RICP Using the Disk Diffusion Method

The disk diffusion method is the standard approach used in many microbiology laboratories for routine antimicrobial sensitivity testing. This testing method has several advantages, including its simplicity, cost-effectiveness, the ability to test a wide range of microorganisms and antimicrobial agents, and easy interpretation of results. As a result, this technique is commonly used for screening antimicrobial materials (Das and Shrivastava, 2010). The antimicrobial susceptibility test results obtained using the disc diffusion method in this study showed that Robusta Instant Coffee Powder (RICP) from all treatment groups tested against *S. mutans* exhibited varying sizes of inhibition zones (refer to Table 1). The highest inhibitory effect was observed at a concentration of 0.8 g/mL, followed by concentrations of 0.6 g/mL, 0.4 g/mL, and 0.2 g/mL, respectively. The higher the concentration of RICP, the greater the inhibition of *S. mutans*, likely due to the higher content of antimicrobial compounds in RICP.

Table 1. Mean inhibition zone values for *S. mutans* exposed to RICP

Research groups	N	Mean Zone of Inhibition (mm)	SD
Negative control (Aqudest)	3	0.00	± 0.00
RICP 0.2 g/mL	3	31.14	± 2.32
RICP 0.4 g/mL	3	37.60	± 4.47
RICP 0.6 g/mL	3	40.20	± 4.57
RICP 0.8 g/mL	3	42.01	± 2.90

Description:
RICP: Robusta Instant Coffee Powder
N: number of repetitions
SD: Standard Deviation

The mean values of inhibition zones from the five research groups were compared using a one-way ANOVA test, which analyzes both within-group and between-group variation. This analysis used the inhibition zone data as the dependent variable and the different concentrations of RICP data as the independent variable. The results of the one-way ANOVA test showed a significance value of 0.00 ($p < 0.05$), indicating a significant difference in the variation of inhibition zone values among all research groups. To further determine the differences between the research groups, the LSD test was conducted. The results showed that all treatment groups (K1 (RICP 0.2 g/mL), K2 (RICP 0.4 g/mL), K3 (RICP 0.6 g/mL), and K3 (RICP 0.8 g/mL)) were significantly different from the negative control group (K0 (sterile Aquadest)) with a significance value of 0.00 ($p < 0.05$). The results of these analyses showed that RICP has antibacterial activity against *S. mutans*.

The RICP demonstrated antibacterial activity against *S. mutans*. According to Monentea et al. (2015), brewed robusta coffee and coffee grounds contain chlorogenic acid, caffeine, and melanoidin, which have antibacterial properties. *S. mutans* is a gram-positive bacterium. Gram-positive bacteria are more susceptible to phenolic compounds such as chlorogenic acid, which is hydrophobic. Hydrophobic compounds are easily absorbed by gram-positive bacteria that do not have an outer membrane composed of phospholipids (Duangjai et al., 2016). Chlorogenic acid kills pathogenic bacterial strains by inducing irreversible permeability changes in the cell membrane, leading to a loss of the ability to maintain membrane potential and cytoplasmic macromolecules, including nucleotides (Lou et al., 2011). Studies have also shown that caffeine and melanoidin have antibacterial activity (Almeida et al. 2006; Wang et al., 2011; Almeida et al. 2012). Caffeine can inhibit DNA repair mechanisms, possibly due to its ability to bind specifically to single-stranded DNA (Grigg et al., 1972; Selby and Sancar,1990). Additionally, some authors have proposed that melanoidin inhibits bacterial growth through a metal-chelating mechanism (Rufian-Henares and de la Cueva, 2009). Therefore, RICPs are effective as antibacterial agents due to their various active compounds.

Minimum Inhibitory Concentration (MIC) of RICP

The MIC test is used in addition to the disk diffusion method. While the disk diffusion method is effective for testing bacterial growth inhibition, it cannot determine the minimum inhibitory concentration (MIC) because it does not enable the measurement of the antimicrobial agent that diffuses into the agar medium (Balouiri et al., 2016). Therefore, the dilution method is the most suitable for determining MIC values as it allows for a quantitative estimation of the concentration of the tested antimicrobial agent.

In this study, we used the microdilution method to determine the Minimum Inhibitory Concentration (MIC) of RICP against *S. mutans*. The microdilution method offers many advantages, including good reproducibility, the use of economical reagents, and minimum space requirements. We used INT (iodonitrotetrazolium or 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyl-2H-tetrazolium) dye reagent to determine the MIC endpoints. The results were obtained using the broth microdilution method to test RICP at

two-fold dilutions (0.0156 g/mL, 0.0313 g/mL, 0.0625 g/mL, 0.125 g/mL) in disposable sterile 96-well plates containing Mueller-Hinton broth media inoculated with S. mutans bacterial cells. We introduced the INT color reagent to the wells to visualize the presence of live bacteria. A change in color to pink after adding the color reagent indicated the presence of live bacteria. The results of the MIC test of RICP at concentrations of 0.0313 g/mL, 0.0625 g/mL, and 0.125 g/mL showed no bacterial growth, as indicated by the absence of pink color in the test media. However, at the concentration of 0.0156 g/mL, there was growth of *S. mutans* bacteria, indicated by the presence of a pink color on the test media. Therefore, we determined the MIC value of RICP against *S. mutans* to be 0.0313 g/mL.

The INT color reagent solution is used to to detect live bacteria in each well visually. This is indicated by the presence of a red color. This color reaction is based on the transfer of electrons from NAD+ to NADH, which is catalyzed by TDH (Threonine dehydrogenase) from bacteria. During the active period of bacterial growth, electrons are transferred from NADH to INT, leading to a reduction process that forms red formazan crystals (Aristyawan et al., 2017). As a result, if bacterial growth occurs in this microdilution test, a red color will appear in the microplate wells.

Table 2. Results of MIC test using the broth microdilution method on *S. mutans* exposed to RICP.

Research groups	**The presence of bacterial growth is indicated by the observation of a pink coloration in the broth microplate test media.**
Negative control (Aqudest)	Yes
RICP 0.0156 g/mL	Yes
RICP 0.0313 g/mL [a]	No
RICP 0.0625 g/mL	No
RICP 0.1250 g/mL	No

Description
RICP: Robusta Instant Coffee Powder
[a]: MIC value

Minimum Bactericidal Concentration (MBC) of RICP

The Minimum Bactericidal Concentration (MBC) is widely used to measure the bactericidal activity of an antimicrobial agent. It is defined as the lowest concentration of the agent that is needed to kill 99.9% of the initial inoculum after 24 hours of incubation under specific conditions. The MBC can be determined after broth microdilution by subculturing samples from wells where no microbial growth is observed onto agar plates to the surviving cells after 24 hours of incubation (CLSI, 1998). In a test with *S. mutans* exposed to RICP, it was found that a concentration of 0.0156 g/mL allowed bacterial growth, while concentrations of 0.0313 g/mL, 0.0625 g/mL, and 0.125 g/mL showed no bacterial growth (Tabel 3). Therefore, the MBC value of RICP against *S. mutans* is 0.0313 g/mL. The bacterial growth is characterized by the formation of cream-colored bacterial colonies on the agar plate test media.

Table 3. Results of MBC test on *S. mutans* exposed to RICP

Research groups	**The presence of bacterial growth is indicated by the formation of cream-colored colonies of bacteria on the agar plate test media.**
Negative control (Aqudest)	Yes
RICP 0.0156 g/mL	Yes
RICP 0.0313 g/mL [b]	No
RICP 0.0625 g/mL	No
RICP 0.125 g/mL	No

Description:
RICP: Robusta Instant Coffee Powder
[b]: MBC value

4. CONCLUSION

The study concluded that Robusta instant coffee powder (RICP) has antibacterial activity against acidogenic and aciduric species of *S. mutans* bacteria with MIC and MBC values of 0.0313 g/mL.

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