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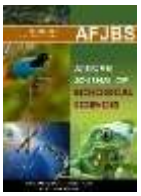

Combination of *Pseudomonas azotoformans* and *Ruta angustifolia* Extracts as Anti-*Streptococcus mutans*

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Abstract

Streptococcus mutans bacterial infection can cause tooth damage such as caries or cavities which can disturb comfort. The activity of *S. mutans* bacteria can be controlled with ingredients that contain antibacterial substances. The aim of this research is to determine the inhibitory power of a combination of compounds contained in the endophytic bacteria *Pseudomonas azotoformans* UICC B-91 and *Ruta angustifolia* leaves as an antibacterial for *S. mutans*. This research using disc diffusion inhibition method with 7 types of test formulations, namely a combination of *P. azotoformans* UICC B-91 and *R. angustifolia extracts* (1:1, 1:2, 2:1), *P. azotoformans* UICC B- 91 extract, *R. angustifolia* extract, positive and negative control. The result shows that all formulations are proven to produce an inhibitory zone against *S. mutans* bacteria. The combined formulation with a ratio of 1:1 produces an average inhibition zone of 7.5 mm, the 1:2 combination has an average inhibition zone of 7.75 mm and the 2:1 combination has an average inhibition zone of 10.09 mm. The best results in inhibiting the growth of *S. mutans* bacteria were in the combined formulation of *P. azotoformans* and *R. angustifolia* 2:1, which produced an average inhibition zone of 10.09 mm in the "strong" category.

Keywords: Bioactive; *Pseudomonas azotoformans*; *Ruta angustifolia*;antimicrobial;*Streptococcus mutans*

Introduction

Streptococcus mutans bacteria are normal flora in the mouth which if there are excessive amounts can cause infection or disease in the oral cavity. Dental and oral diseases caused by the activity of *Streptococcus mutans* are caries or cavities. Based on data from Riskesdas in 2018, the proportion of dental and oral problems in Indonesia has increased drastically from 23.2% in 2007 to 57.6% in 2018 with the largest share being caries (45.3%). The bacteria that cause caries are very cariogenic because they are able to make acid from carbohydrates which can be harmed, as a result the bacteria are helped to stick to teeth and stick to each other. Hasrul's research (2016) stated that *Streptococcus mutans* has resistance to the antibiotics amoxicillin and cefritriazone [1]. Liao *et al* (2017) also showed the resistance of *Streptococcus mutans* to fluoride, which for the last five decades has been believed to be an anti-dental caries agent [2]. The existence of bacterial resistance or resistance to antibiotics that are believed to be effective for treatment can trigger serious problems in the health sector. The most worrying thing is the rapid spread of multi-resistant bacteria that can cause common infections and resist treatment with existing antimicrobial drugs [3].

Efforts to prevent *Streptococcus mutans bacterial infections* are urgently needed to overcome the high incidence of dental caries [4]. Dental caries can cause pain, infection, tooth loss and death in serious cases, unless good treatment is carried out, this can be prevented [5]. Therefore, the use of antibacterial agents is very necessary to prevent the growth of *Streptococcus mutans bacteria* . Apart from several groups of chemical compounds, antibacterial agents can be obtained from various sources, one of which is natural resources. In research *by Sari et al.* (2022), the addition of the metal antibacterial agents TiO_2 and ZnO in making composite films was proven to have an inhibitory effect on *Staphylococcus aureus* and *Escherichia coli bacteria* [6]. Many antibacterial compounds are also produced by several microorganisms, especially bacteria. One source of new compounds that can be used as antibacterial compounds is endophytic bacteria from the yacon plant, because they contain sesquiterpene lactones in the leaves which can act as antibacterials [7]. Another natural antibacterial that has been used since time immemorial is using herbal plants. Plants that contain bioactive compounds can be used as antibacterials [8]. Plants used as antimicrobials usually contain bioactive compounds in the form of flavonoids, tannins, glycosides, saponins, alkaloids, steroids and essential oils which are antioxidant compounds with strong antibacterial activity.

One of the studies conducted by Pratiwi *et al.* (2023) and Putri *et al.* (2024) showed that the endophytic microorganism *Pseudomonas azotoformans* UICC B-91 was proven to be able to inhibit *C. albicans* ATCC 10231 and *T. mentagrophytes* [9, 10]. Several antimicrobial compounds that have been identified from *Pseudomonas azotoformans* are rhamnolipid which is capable of inhibiting various *Bacillus species, 1-acetyl-beta-carboline* which is capable of inhibiting MRSA, *2-aminophenol, 5-methyl-2,4-imidazolidinedione, 1,4-benzenediamine, N-(1-methylethyl)-N-phenyl, pyrrole[1,2-a] pyrazine-1,4-dione, pyrrole[1,2-a] pyrazine-1,4-dione,hexahydro-3-(phenylmethyl),* and al which is able to inhibit *Neisseria meningitidis* and *S. aureus* [11]. *Pseudomonas azotoformans* UICC B-91 is also known to contain alkaloid bioactive compounds in the CHON structure with a molecular weight of around 100-450 Da [12]. Alkaloids have antibacterial power and an inhibitory mechanism by blocking the formation of peptidoglycan components in bacterial cells [13]. Alkaloids exhibit broad bioactivity, have the potential to become new broad-spectrum natural antimicrobial compounds, and a low propensity to generate resistance [14].

One natural ingredient that is believed to be able to treat various diseases is the *Ruta angustifolia plant.* The leaves of *Ruta angustifolia* have been proven to contain bioactive compounds that have been qualitatively studied, including steroid compounds, flavonoids, tannins and quinones [15]. Several studies have shown that the flavonid content contained in plants has shown resistance activity against *Streptococcus membranes* by increasing proton permeability and also inhibiting acid production by *Streptococcus mutans* in biofilms. *Ruta angustifolia* extract in concentrations of 40%, 80% and 100% has also been proven to inhibit the growth of *Fusobacterium nucleatum* [16].

The combined formulation of *Pseudomonas azotoformans* and *Ruta angustifolia extracts* has been proven to inhibit the growth of *Candida albicans* with moderate criteria [17]. Based on this, this research aims to determine the inhibitory power of the combined formulation of *Pseudomonas azotoformans* and *Ruta angustifolia extract compounds* as an anti-bacterial agent for *Streptococcus mutans* which causes dental and oral diseases, especially dental caries or cavities.

Materials and Methods

Materials

Ethanol 96%, distilled water, *Nutrient Broth media* (Himedia, 30 gr/l)), Bacteriological Agar (Oxoid, 13 gr/l), *Ruta angustifolia extract* , pure extract of *Pseudomonas azotoformans UICC B-91*, *Streptococcus mutans* isolate ATCC 35668, amoxicilin .

Methods

Extract preparation

Pseudomonas azotoformans UICC B-91 extract was obtained from the Rina Hidayati Pratiwi collection [12] and *Ruta angustifolia extract* was obtained from the Shafa Noer collection [16].

Formulation preparation

The extract formulation of *Pseudomonas azotoformans* UICC B-91 and *Ruta angustifolia* was obtained by mixing 0.05 g of extract in 100 ml of sterile distilled water each which was then stirred until homogeneous. The positive control formulation uses the antibiotic amoxicillin 32 µg/ml, and the negative control uses sterile distilled water.

Preparation of test bacteria

Streptococcus mutans bacteria were rejuvenated by streaking the bacteria using a wire mesh from pure isolates on nutrient agar slant media and incubating for 24 hours. Bacteria that have grown on slanted agar are then inoculated in liquid *nutrient broth media* using a wire loop which is then incubated for 24 hours until the liquid media looks cloudy.

Antibacterial activity test preparation

The antibacterial activity test was carried out using the disc diffusion method. The NA media that had solidified in a petri dish was then spread by a suspension of *Streptococcus mutans bacteria* in 100 µl of *nutrient broth media using a cotton swab* . After that, 5 6mm paper discs were placed on which 100 µl of the test solution had been dripped. Then incubated for 24 hours to see the growth of microbes and the clear zone that forms around the disc.

Results and Discussion

Results

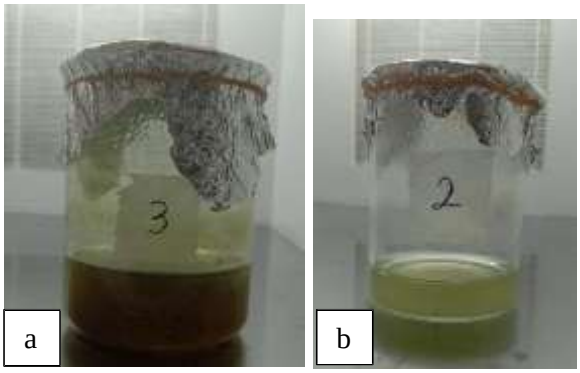

Fig 1. Extraction result: (a) *Ruta angustifolia* extract, (b) *Pseudomonas azotoformans* extract

The results showed that around the discs treated with the test solution there was a clear zone as a response to inhibiting bacteria (Figure 2). This shows that the test solution contains substances that can inhibit the growth of *Streptococcus mutans bacteria.*

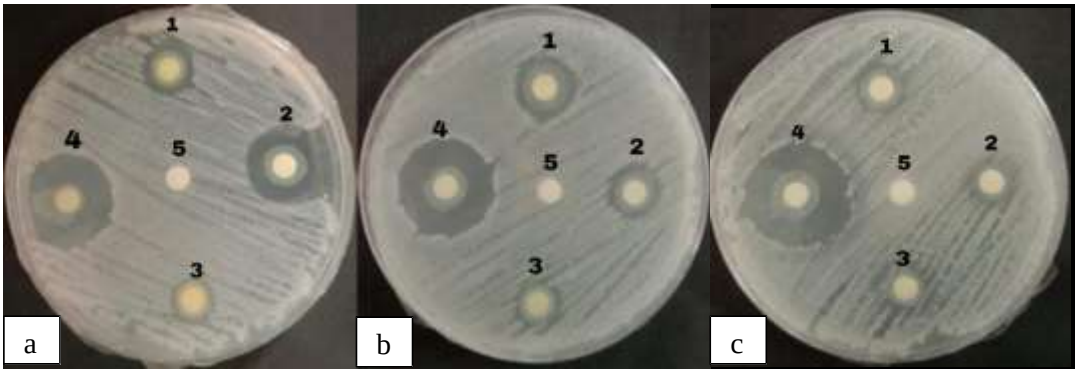

Fig 2. Disc diffusion inhibition zone test results: (a) Combination 1:1, (b) Combination 1:2, (c) Combination 2:1. Note: 1) Combination 2) P. azotoformans extract 3) R. angustifolia extract 4) Control +, and 5) Control –

Table 1. Results of Batch 1 Inhibitory Zone Diameter Measurements

Treatment	Diameter	Average diameter	Category
Control +	1. 19.5 2. 18.5 3. 19	19	Strong
Control -	1. 0 2. 0 3.0	0	-
Pseudomonas azotoformans	1. 8.5 2. 9 3. 8	8.5	Medium
Ruta angustifolia	1. 6 2. 6 3. 7.5	6.67	Medium
Combined (1:1)	1. 8.5 2. 8.5 3. 6.5	7.83	Medium
Combined (1:2)	1. 4 2. 7 3. 9	6.67	Medium
Combined (2:1)	1. 9 2.10 3.9.5	9.5	Medium

Table 2. Results of Batch 2 Inhibition Zone Diameter Measurements

Treatment	Diameter	Average diameter	Category
Control +	1. 18 2. 20 3. 19	19	Strong
Control -	1. 0 2. 0	0	-

	3.0		
Pseudomonas azotoformans	1. 9.5		
	2. 9	9.17	Medium
	3. 9		
Ruta angustifolia	1. 7		
	2. 7	7.33	Medium
	3. 8		
Combined (1:1)	1. 7		
	2. 7.5	7.17	Medium
	3. 7		
Combined (1:2)	1. 6		
	2. 9.5	8.83	Medium
	3. 11		
Combined (2:1)	1. 9.5		
	2.11	10.67	Strong
	3. 11.5		

Antibacterial activity is carried out by measuring the diameter of the inhibition zone formed around the disc using a ruler in millimeters using the following formula:

$$\frac{(v-c)+(h-c)}{2}$$

Information:

Dv : Vertical diameter

Dc: Disc diameter

Dh: Horizontal diameter

Discussion

This study aims to test the inhibitory power of the bacterial extract *Pseudomonas azotoformans* UICC B-91 and *Ruta angustifolia leaves* against *Streptococcus mutans bacteria*. The indicator of success in this research can be seen from the clear zone that forms around the paper disc as a sign of the sensitivity of the bacteria to the antibacterial ingredients contained in the test solution. Observations and measurements on the media were carried out after 24 hours of incubation.

Streptococcus mutans bacteria are included in the Gram-positive bacteria which are normal flora in the mouth. This bacteria can grow at temperatures ranging from 18-40 degrees Celsius. *Streptococcus mutans* can metabolize various types of carbohydrates , creating an acidic atmosphere in the mouth as a result of this process. These bacteria can thrive in an acidic atmosphere and can stick to the surface of the teeth and bond with each other [18]. This *Streptococcus mutans* bacteria is the main cause of oral cavity infection problems, namely dental caries or cavities, which is the largest part of dental and oral diseases in Indonesia in 2018 [19]. Caries arises as a result of several aspects that are interconnected with each other, namely teeth and saliva (host), microorganisms, substrate and time [20].

There are a number of factors that can influence the action of antimicrobial agents, including the age of the bacteria, the concentration of the antimicrobial agent, temperature, the content of the antimicrobial agent, and so on. The speed of death of a microbial population is greatly influenced by the age of the microbe. Usually younger microbes have lower resistance compared to older bacteria (stationary phase). The ability of a material to inhibit or form microbes depends on the high or low concentration of the antimicrobial agent. Generally, microbes die more quickly when given high concentrations of antimicrobial substances [21].

The results showed that *Pseudomonas azotoformans extract* UICC B-91 and *Ruta angustifolia* has been proven to inhibit the growth of *Streptococcus mutans bacteria* as seen from the formation of a clear zone around the disc. The inhibitory power occurs due to the antimicrobial content in the extract of *Pseudomonas azotoformans* UICC B-91 in the form of alkaloid compounds [22]. Alkaloid compounds are one of the secondary metabolites that can be found in nature in abundance and have physiological activity. Alkaloids work as antibacterials by disrupting the peptidoglycan components in bacterial cells, so that the cell wall layer does not form completely and causes the cell to die [23].

The clear zone is also formed due to the presence of antimicrobial compounds in *Ruta angustifolia* in the form of flavonoids, steroids, tannins and quinones [15]. Flavonoids as antibacterial compounds work by 3 mechanisms, namely by inhibiting nucleic acid synthesis, inhibiting cell membrane function and inhibiting energy metabolism [24]. The mechanism of action of steroids as antibacterials in inhibiting bacterial growth is related to the lipid membrane and sensitivity to steroid components which cause leakage in bacterial liposomes [25, 26]. Tannin works as an antibacterial by inactivating bacterial enzymes and disrupting the flow of proteins in the inner layers of cells [27]. Meanwhile, quinone compounds undergo

a change process, namely electron reduction, thereby changing the quinone into half hydroquinone (semiquinone) and producing free radicals which will later disrupt the development of bacterial cells through genetic pathways and the proteins produced [28].

Based on the results of data analysis, it can be seen that the best antimicrobial activity in the working group is found in the combined formulation of *Pseudomonas azotoformans* UICC B-91 and *Ruta angustifolia* 2 : 1 which has the "medium" category with an average of 9.5 mm in batch 1 and the "strong" category with an average of 10.67 mm in batch 2. Meanwhile, the smallest inhibitory zone activity is found in *Ruta angustifolia* extract with an average inhibitory zone diameter of 6.67 mm in batch 1 and 7.33 in batch 2, both of which are included in the "medium" category. If seen from these results, antibacterial activity increases with an increase in the concentration of one of the extracts, which results in a higher zone of inhibition. This proves that the higher the extract concentration, the greater the resulting inhibitory power [29].

The results show that the concentration of *Pseudomonas azotoformans* UICC B-91 plays a strong role in inhibiting the growth of *Streptococcus mutans* bacteria compared to *Ruta angustifolia* extract. This can be caused by the action of alkaloid compounds found in *Pseudomonas azotoformans* UICC B-91 by directly disrupting the formation of the peptidoglycan structure in the bacterial cell wall so that the bacteria lyse. Destruction of the cell wall in bacteria causes disruption of the synthesis of the components that make up the cell wall, this causes the cell wall to be very weak and undergo lysis [30].

Conclusion

Based on the results of the research that has been carried out, the following conclusions can be drawn:

1. *Pseudomonas azotoformans* Extract UICC B-91 and *Ruta angustifolia* has been proven to inhibit the growth of *Streptococcus mutans bacteria* ranging from "medium" to "strong" inhibitory strength.
2. The combined extract of *Pseudomonas azotoformans* UICC B-91 and *Ruta angustifolia* 2:1 produces the best inhibitory power in inhibiting the growth of *Streptococcus mutans bacteria* based on the inhibition zone produced with an average of 10.09mm in the "strong" category.
3. The lowest inhibition zone results were produced by *Ruta agustifolia extract* with an average inhibition zone of 7mm with "medium" strength.

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