DENTINO
JURNAL KEDOKTERAN GIGI
Vol X. No 2. SEPTEMBER 2025

ANTIBACTERIAL EFFECTIVENESS OF RAMANIA LEAVES EXTRACT GEL (BOUEA MACROPHYLLA GRIFFITH) AGAINST THE GROWTH PSEUDOMONAS AERUGINOSA

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ABSTRACT
Background: *Pseudomonas aeruginosa is a gram-negative anaerobic bacteria and frequently associated with necrotic pulp infections. Necrotic pulp can be treated with root canal treatment (RCT). Sterilization of the root canal is one of the stages in RCT. The most used intracanal medicament in the sterilization stage of root canals is calcium hydroxide. This material may cause pulp irritation and inflammation in the surrounding tissues. These drawbacks are the reason for the necessity of alternative intracanal medicaments from natural materials such as ramania leaves (Bouea macrophylla Griffith). Flavonoids, saponins, tannins, steroids, and phenols are secondary metabolites derived from Ramania leaves that have been shown to have antibacterial properties.* ***Purpose:*** *To analyze the antibacterial effectiveness of ramania leaves extract gel (Bouea macrophylla Griffith) against Pseudomonas aeruginosa.* ***Methods:*** *Experimental study with a post-test only with control group design consisting of 5 treatment groups, namely ramania leaves extract gel with a concentration of 6,25%, 12,5%, 25%, 50%, and control (calcium hydroxide mixed with gel base) were repeated 5 times. The method used in the antibacterial test is using the agar well diffusion assay.* ***Results****: The Post-Hoc Bonferroni test confirmed statistically that there is significant differences among the treatment groups.* ***Conclusion:*** *The ramania leaves extract gel (Bouea macrophylla Griffith) at concentrations of 6.25%, 12.5%, 25%, and 50% has antibacterial potential against the growth of Pseudomonas aeruginosa.*

Keywords: *antibacterial, bouea macrophylla griffith, intracanal medicament, necrotic pulp, pseudomonas aeruginosa, root canal treatment.*

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INTRODUCTION

Based on the Indonesian Health Survey in 2023, the percentage of oral and dental problems in Indonesia is 56.9% and in South Kalimantan is 57.7%.[1] Decayed tooth cases are still a dental and oral health problem that often occurs in the South Kalimantan community.[2,3] The Indonesian Health Survey in 2023 also showed that of all oral health problems in South Kalimantan, the percentage of damaged, decayed, or diseased teeth was 43.9%.[1] If a decayed tooth is left untreated, it can cause pulp necrosis.[4]

A tooth that has experienced pulp necrosis can be treated with Root Canal Treatment (RCT).[5] The sterilization of root canals is one of the important steps in the RCT procedure which consists of irrigation and intracanal medicament. Intracanal medicament is considered an important step in eliminating root canal bacteria that cannot be removed mechanically.[6] The gold standard for intracanal medicament is calcium hydroxide ($Ca(OH)_2$) with the most commonly used product in a paste-like form.[7] $Ca(OH)_2$ powder that is mixed with a viscous vehicles such as a gel base shows a more stable release effect of Ca^+ ions and OH^- ions than liquid vehicles, so that the pH rise is more controlled and the antibacterial effect can last longer.[8]

Calcium hydroxide ($Ca(OH)_2$) has a low compressive strength, which can lead to the dissolution of intracanal medicament materials.[9] $Ca(OH)_2$ is also difficult to thoroughly remove from the root canal and if not completely removed, the residue may interfere with the sealer's ability to seal the root canal.[10,11] One of the gram-

negative anaerobic bacteria in the dental root canal is Pseudomonas aeruginosa. According to Jaiswal, et al (2020), Pseudomonas aeruginosa was found in 8.7% of cases of root canal treatment failure.[12] This bacteria was also found in 6.8% of teeth after endodontic treatment in cases of apical periodontitis.[13]

Calcium hydroxide ($Ca(OH)_2$) has been proved to have the ability to inhibit bacteria in the root canal, but its ability to inhibit persistent bacteria such as Pseudomonas aeruginosa is not so significant after being treated for 7 days.[14] Based on this matter and the drawbacks of $Ca(OH)_2$, the interest in alternative intracanal medicaments from natural materials has increased. Ramania leaves (Bouea macrophylla Griffith) is one of the natural ingredients from South Kalimantan that has potential as an alternative medicine because it has been tested to have antibacterial, antioxidant, antifungal, and anticancer properties because it contains secondary metabolites, such as flavonoids, saponins, steroids, tannins, and phenols.[15,16] According to Hayati, et al (2023), ramania leaves extract concentrations of 6.25%, 12.5%, 25%, 50%, and 100% showed the ability to inhibit the growth of Actinomyces spp.[15] Based on this background and the absence of research on the antibacterial effectiveness of ramania leaves extract gel against the growth of Pseudomonas aeruginosa bacteria, the authors want to conduct research on this matter.

MATERIALS AND METHODS

Material

This research has been approved and authorized by the Ethics Committee of the Faculty of Dentistry, Lambung Mangkurat University No. 029/KEPKG-FKGULM/EC/X/2024. The tools that are used for the research are mask, handscoon, oven (Memmert UL30), petri dish, autoclave (Runeyes®), anaerobic incubator (BIOBASE), UV-Vis spectrophotometer (PG Instruments Ltd.), analytical scales (Duratron), digital caliper, test tubes (Pyrex®), erlenmeyer tubes (Iwaki), measuring cups (Pyrex®), beaker glass (Iwaki), rotatory evaporator (Eyela), waterbath (Wincom HH-4), pipette, ose, filter paper no. 40 (Whatman™), blender (Whatman™), blender (Mitochiba), magnetic stirrer, mortar, pestle, sterile tip (OneMed), sterile cotton stick (OneMed), bunsen burner, glass slab, and stainless steel spatula.[15,17,18] The materials that are used for the research are ramania leaves from Banjarbaru City, South Kalimantan, Pseudomonas aeruginosa bacterial culture (ATCC 15442), calcium hydroxide, McFarland's solution 0.5, Brain Heart Infusion Broth (BHIB), Nutrient Agar (NA), potassium dichromate ($K_2Cr_2O_7$), ethanol 96%, distilled water, aluminum foil, propylene glycol, glycerin, natrium carboxymethylcellulose (Na-CMC), and nipagin..[15,17]

Methods

This research is a pure experimental study with a posttest only design with control group design using 5 groups, namely ramania leaves extract gel with a concentration of 6.25%; 12.5%; 25%; 50%; and calcium hydroxide mixed with gel base as a control. There were 5 repetitions of each treatment group, resulting in a total of 25 samples. The evaluation of antibacterial activity was conducted using the agar well diffusion assay at the Biomedical Laboratory, Faculty of Dentistry, Lambung Mangkurat University.

Steps of Procedure

Ramania leaves extract (Bouea macrophylla Griffith) was obtained by maceration method. The dark green ramania leaves that were chosen had measurements ranging from 11–45 cm in length and 4–13 cm in width, starting with the fourth leaf from the top to the fifth leaf before the base of the twig.[15] The ramania leaves extract gel was obtained based on the formulation as in Table 1.

Table 1. Ramania Leaves Extract Gel Formulation

Ingredients	Ramania Leaves Extract Gel			
	6.25%	12.5%	25%	50%
Ramania Leaves Extract	6.25 g	12.5 g	25 g	50 g
Na-CMC	5 g	5 g	5 g	5 g
Propylene Glycol	5 g	5 g	5 g	5 g
Glycerin	10 g	10 g	10 g	10 g
Nipagin	0.1%	0.1%	0.1%	0.1%
Distilled Water	Ad 100 g	Ad 100 g	Ad 100 g	Ad 100 g

The antibacterial test was done by well diffusion method. Nutrient agar media that has been inoculated with bacteria is prepared with 2-3 wells, then the gel of ramania leaves extract and calcium hydroxide mixed with gel base using a 1:1 ratio as a control is inserted into each well. After that, the incubation was done for 24 hours at 37°C. The results of the observation are obtained from the inhibition zone which is a clear zone formed around the wells after incubation, then the inhibition zone is measured by using digital calipers. The diameter of the inhibition zone can be categorized based on the classification by Davis and Stout.[16,19]

Table 2. Classification of Zone of Inhibition against Bacteria According to Davis and Stout.

Category	Average Diameter of Inhibition Zone (mm)
Weak	<5
Moderate	5-10
Strong	10-20
Very Strong	>20

To identify which groups had significant differences, the research data were statistically analyzed using the One Way ANOVA test followed by the Bonferroni Post Hoc test.

RESULTS

The evaluation of antibacterial activity was conducted using the agar well diffusion assay. The diameter of the clear zone surrounding the well can be measured with a digital caliper to observe the inhibitory effect. The mean diameters of the inhibition zones and the standard deviations, resulting from the antibacterial activity of the ramania leaves extract (Bouea macrophylla Griffith) gel against Pseudomonas aeruginosa, are presented in Table 3.

Table 3. Mean and Standard Deviation of Inhibition Zone Diameter of Ramania Leaves Extract Gel (Bouea macrophylla Griffith) against the Growth of Pseudomonas aeruginosa.

Treatment Groups	N	Mean (mm) ± SD	Inhibition Zone Category
$Ca(OH)_2$ + Gel Base (Control)	5	5,68±0,58	Moderate
RLEG 6,25%	5	7,10±0,31	Moderate
RLEG 12,5%	5	8,87±0,05	Moderate
RLEG 25%	5	11,36±0,32	Strong
RLEG 50%	5	15,38±0,40	Strong

$Ca(OH)_2$: Calcium hydroxide
RLEG : Ramania leaves extract gel

Based on Table 3, the control group has the smallest inhibition zone diameter, while the ramania leaves extract gel at a concentration of 50% has the largest inhibition zone diameter. An increase in the concentration of ramania leaves extract gel corresponds to a larger inhibition zone diameter. In comparison to the control group, all ramania leaves extract gel treatment groups have larger inhibition zones.

For statistical analysis, IBM SPSS Statistics 27.0 for Windows was used. The One Way ANOVA test is used for the parametric test, and the results show a significance value (p) = 0.000 (<0.05), indicating a significant difference in the inhibition zone's diameter. Furthermore, to determine which treatment groups had significant differences, the Bonferroni Post Hoc test was used. The significance of values can be seen in Table 4.

Table 4. The Results of Post Hoc Bonferroni Test of Ramania Leaves Extract Gel (Bouea macrophylla Griffith) on the Growth of Pseudomonas aeruginosa.

Treatment Groups	$Ca(OH)_2$ + Gel Base (Control)	RLEG 6,25%	RLEG 12,5%	RLEG 25%	RLEG 50%
$Ca(OH)_2$ + Gel Base (Control)		0,001*	0,001*	0,001*	0,001*
RLEG 6,25%			0,001*	0,001*	0,001*
RLEG12,5%				0,001*	0,001*
RLEG 25%					0,001*
RLEG 50%					

* : There is a significant difference (<0,05)
$Ca(OH)_2$: Calcium hydroxide
RLEG : Ramania leaves extract gel

DISCUSSION

Ramania leaves extract gel (Bouea macrophylla Griffith) was tested for its antibacterial efficacy using the agar well diffusion assay against the growth of Pseudomonas aeruginosa at concentrations of 6.25%, 12.5%, 25%, and 50%. The results showed that the ramania leaves extract gel was effective in inhibiting the growth of Pseudomonas aeruginosa. According to this research findings, the ramania leaves extract gel at 50% concentration

has the strongest antibacterial properties in inhibiting the growth of Pseudomonas aeruginosa with an average diameter of the inhibition zone formed of 15.38 mm, which exceeds the average diameter of the inhibition zone in the ramania leaves extract gel at 6.25% concentration at 7.10 mm, 12.5% concentration at 8.87 mm, 25% concentration at 11.36 mm, 50% concentration at 15.38 mm, and calcium hydroxide ($Ca(OH)_2$) mixed with gel base as a control at 5.68 mm.

Stronger inhibition of Pseudomonas aeruginosa growth occurs with higher concentrations of ramania leaves extract gel. This happens because the increase in the concentration of antibacterial compounds causes an increase in the amount of secondary metabolites contained, so that the ability of antibacterial compounds to inhibit the bacteria will increase.[20] These findings are in line with the research conducted by Hayati, et al (2023).[15]

Various factors can affect antibacterial effectiveness, such as the concentration and content of the extract being used, the type of the bacteria, the temperature, and the pH of the environment.[21] Secondary metabolites such as flavonoids, tannins, steroids, phenols, and saponins are present in the extract of ramania leaves. These compounds have been tested for antibacterial properties.[16]

Flavonoids act as antibacterial agents by binding to bacterial DNA, thereby disrupting cell membrane permeability. This disruption leads to cellular leakage and ultimately results in bacterial lysis.[22] The mechanism of saponins as antibacterial agents is to interact with the cell wall to cause the formation of complex compounds through hydrogen bonds so as to reduce cell wall tension and cells experience leakage. The mechanism of tannins as antibacterial agents is to bind to bacterial enzymes and form complex compounds so that the bacteria's enzymes can be deactivated and damage the protein-binding structure of the bacteria cells.[15,22] Steroids act as antibacterial agents by inducing leakage in liposomes and interacting with the phospholipid components of bacterial cell membranes, which are permeable to lipophilic substances. This interaction compromises membrane integrity, alters membrane structure, and leads to cell fragility and eventual lysis. Phenol act as antibacterial agents by disrupting bacterial cell membranes, activating enzymes, and denaturing proteins. These actions reduce membrane permeability, inhibit the transport of essential organic ions into the cell, impair bacterial growth, and ultimately lead to cell death. Triterpenoids act as antibacterial agents by interacting with porins in the bacterial outer membrane, thereby altering cell wall permeability and ultimately causing bacterial lysis. The mechanism of alkaloids as antibacterial agents is by inhibiting the constituent components of peptidoglycan, thus preventing the formation of intact cell walls and eventually causing cell death.[15]

In addition to secondary metabolites known from qualitative phytochemical tests, in a research done by Nguyen, et al (2020), further phytochemical tests were also done on ramania leaves extracts, namely by using GC-MS (Gas Chromatography-Mass Spectrometry) analysis techniques, so that other active compounds that have potential as antibacterials can be identified specifically, such as caryophyllene, humulene, phytol, trans-geranylgeraniol, and diisooctyl phthalate.[23] Other than secondary metabolites, the species of the bacteria used in the research can also affect the results of antibacterial tests. This study used Pseudomonas aeruginosa as the bacteria tested.[21] This study used Pseudomonas aeruginosa as the bacteria tested. Gram-negative bacteria, such as Pseudomonas aeruginosa, are typically more resistant than gram-positive bacteria because of their complex membrane structure.[12,24]

According to the research findings, all groups of ramania leaves extract gel have a larger inhibition zone diameter compared to the $Ca(OH)_2$ mixed with the gel base group as a control. This can happen because the ramania leaves extract gel contains several secondary metabolite compounds that act as antibacterial agents and work together synergistically in inhibiting the growth of the bacteria, while in $Ca(OH)_2$ mixed with gel base as a control only OH^- ions alone act as antibacterial agents.[8,15] Other than that, another factor that may influence the inhibition zone's diameter is the tested preparation's viscosity because the higher the viscosity, the slower the diffusion rate in the agar media of the tested antibacterial compounds.[25] In this research, $Ca(OH)_2$ mixed with gel base as a control had the highest viscosity among all concentrations of ramania leaves extract gel because the control group used $Ca(OH)_2$ powder mixed with the gel base, while the ramania leaves extract gel was made by mixing the thick extract of ramania leaves with gel base.

The findings of this research showed the potential of ramania leaves extract gel as an alternative for intracanal medicament in root canal treatment to inhibit the growth of Pseudomonas aeruginosa bacteria, but further research needs to be done to qualify as an alternative material for intracanal medicaments in root canal treatment, such as being biocompatible, non-toxic, and non-irritating to the tissue.

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