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Research Article

DIFFERENCE BETWEEN ANTIMICROBIAL EFFECTS OF PAPACARIE® AND PAPAIN ON *STREPTOCOCCUS MUTANS IN VITRO*

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ABSTRACT

Objective: The aim is to compare the antimicrobial effects of papain and Papacarie with dilution and diffusion tests.

Methods: There were two treatment groups and one Group control. The treatment group received papain and Papacarie, and the control group received chlorhexidine, in five liquids with different concentrations of 0.5%, 0.25%, 0.125%, 0.0625%, and 0.03%. The dilution and diffusion tests were used to determine the minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC), and zone of inhibition for each treatment material.

Results: MICs of papain and Papacarie were 12.5%, indicating that at a concentration of 12.5%, the material can inhibit the growth of *Streptococcus mutans*. Papain does not have an MBC value but the Papacarie has an MBC at 25%, which indicating that at a concentration of 25%, Papacarie has bactericidal effects on *S. mutans*. The zone of inhibition of papain was lower than Papacarie.

Conclusion: Based on chemomechanical caries removal materials, the antimicrobial effects of Papacarie were better than those of papain.

Keywords: Chlorhexidine, Minimum bactericidal concentration, Minimum inhibitory concentration, Papacarie®, Papain, Zone of inhibition.

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INTRODUCTION

Dental caries is caused by oral bacteria. *Streptococcus mutans* is the pathogen that is most commonly related to the development of caries. The main habitat of *S. mutans* is the tooth surface. The bacteria are usually localized on certain parts of the tooth surfaces, such as pits, fissures, occlusal surfaces, proximal areas, tooth surface adjacent to gingiva, or on other caries lesions [1]. Since *S. mutans* is known to cause caries, studies have focused on inhibiting the growth of these bacteria. One of the methods known to prevent caries is the use of antimicrobial agents that eliminate *S. mutans* [2].

To ensure minimally invasive treatment, chemomechanical materials have been developed to eliminate minimally health tissues during cavity preparation. In 1998, in Sweden, a chemomechanical material, Carisolv was developed. Its drawbacks including the need for special instruments, high cost, bad taste, and short-lived action. In 2003, in Brazil, a chemomechanical material called Papacarie® was developed, with improvement in the drawbacks of Carisolv. Papacarie, whose main components are papain, blue toluidine, and chloramine, is a chemomechanical material that is relatively easy to use, does not require the use of special instruments, and is less expensive than Carisolv. Moreover, the active ingredient papain is a natural antimicrobial agent. A chemomechanical agent should have antimicrobial effects given that bacteria can colonize on caries lesions [3,4].

Papain is a proteolytic enzyme produced by papaya latex. In addition to its effects on infected dentinal collagen, papain works as antibacterial by affecting extracellular polysaccharide synthesis [3,4]. In Indonesia, papaya is abundant and can be easily found. Papain enzyme can be obtained by drying papaya latex through heating in the sun, heating by instruments, and spray drying. Spray drying seems to be the best way to produce the papain enzyme because spray drying is obtained from the fine extract that is easily dissolved in water; thus, this form of the liquid has high proteolytic activity [5-7]. This is important because *S. mutans* colonizes on caries lesions, which degrades dentinal collagen, and this ingredient may thus prevent such degradation. The relatively easy process of spray drying can help to circumvent the difficulties in obtaining Papacarie. In a previous study by Waluyatrie and Kuswandari, in 2014, the difference between papain 0.1% and 0.2% in suppressing *S. mutans* population in the oral environment was examined [8]; however, no further research has been conducted to determine the difference between antimicrobial characteristics of Papacarie and papain on *S. mutans*. Therefore, we investigated the antimicrobial effects of Papacarie and papain on *S. mutans in vitro.*

Recently, the antimicrobial drug chlorhexidine has been used in the oral environment in the form of gel as a topical application and the form of liquid as a mouthwash. Chlorhexidine is the most recommended antimicrobial agent because it inhibits the formation of plaque and due to its bacteriostatic and bactericidal properties; it has anticaries effects on *S. mutans*. Chlorhexidine is the "gold standard" of antimicrobial agents used against *S. mutans*; hence, in this study, chlorhexidine was used as the control [7].

Previous studies have proved that papain 0.1% and 0.2% liquids can suppress *S. mutans* population [8]. However, to the best of our knowledge, the antimicrobial effects of Papacarie and papain on *S. mutans* have not been compared.

METHODS

Three treatment groups of samples were used, one each for papain, Papacarie, and chlorhexidine (control group), in five liquids at concentrations of 0.5%, 0.25%, 0.125%, 0.0625%, and 0.03%. The findings in each sample were analyzed with the dilution and diffusion tests to determine the minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC), and zone of inhibition.

Determining MIC

To prepare the *S. mutans* growth medium, brain heart infusion (BHI) was mixed with 2% sheep blood. *S. mutans* was grown in this medium and then placed in an incubator at 37°C in anaerobic conditions for 48 h. After that, it is diluted in saline, its turbidity standardized with Sastroasmoro [9].

MIC is the lowest standard concentration of drugs or compounds in materials that can inhibit the growth of plants and bacteria. To determine MIC for *S. mutans,* Papacarie, papain, and chlorhexidine were individually added in small amounts to the media in which bacteria were growing. The levels of concentrations are established first, and then the tubes of growth medium were incubated anaerobically, at 35°C–37°C for 24–72 h. After the incubation, the tubes were assessed; if one exhibited no bacterial growth (stayed clear), the concentration of treatment drug was considered successful in inhibiting the growth of *S. mutans*. The lowest concentration of each of the three ingredients that inhibited organism growth was the MIC.

Determining MBC

MBC is the lowest concentration of drugs or compounds in materials that can kill the growth of bacteria. To determine the MBC, the bacteria were regrown in MIC tubes; the tubes that contained the highest concentration of Papacarie, papain, and chlorhexidine, or the tubes that stayed clear, on agar growth medium, were incubated anaerobically for 24–72 h at 37°C. After the incubation, the growth of bacterial colonies was assessed. In tubes that were clear with the lowest concentrations of Papacarie, papain, or chlorhexidine, the concentration was considered the MBC [9].

RESULTS

The MIC test was performed by visual assessment for turbidity in the reaction tubes. Turbidity indicated bacterial growth; tubes with turbidity were marked "+," and the ones that showed no bacterial growth were marked "–." Of the tubes with Papacarie and those with papain, Tube I (50% concentration), Tube II (25% concentration), and Tube III (12.5% concentration) were clear. Therefore, for Papacarie and papain, MIC was considered to be 12.5%, indicating that the minimum concentration of Papacarie and papain that could inhibit *S. mutans* growth was 12.5%. Meanwhile, all the tubes with chlorhexidine seemed clear, and thus MIC for chlorhexidine was 3.12%. The MIC results of each sample are presented in Table 1, and the visual turbidity assessment is shown in Fig. 1.

Table 1: Minimum inhibitory concentrations of papain, Papacarie, and chlorhexidine (control)

No.	Tested ingredients and concentrations (%)	Tube I	Tube II	Tube III
1	Papain 50	–	–	–
2	Papain 25	–	–	–
3	Papain 12.5	–	–	–
4	Papain 6.25	–	+	+
5	Papain 3.12	+	+	+
6	Papacarie 50	–	–	–
7	Papacarie 25	–	–	–
8	Papacarie 12.5	–	–	–
9	Papacarie 6.25	–	–	+
10	Papacarie 3.12	+	+	+
11	Chlorhexidine 50	–	–	–
12	Chlorhexidine 25	–	–	–
13	Chlorhexidine 12.5	–	–	–
14	Chlorhexidine 6.25	–	–	–
15	Chlorhexidine 3.12	–	–	–

For papain and Papacarie against *Streptococcus mutans*, minimum inhibitory concentrations (MICs)=12.5; for chlorhexidine against *S. mutans*, MIC=3.12%. Minus signs (–) indicate no *S. mutans* growth (tube was clear); plus signs (+) indicate *S. mutans* growth (turbidity in the tube)

The dilution test to determine MIC can be seen in Fig. 1. Of the tubes containing papain, Tubes I (50%), II (25%), and III (12.5%) are clear; thus, MIC of papain against *S. mutans* is 12.5%. Of the tubes containing Papacarie, Tubes I (50%), II (25%), and III (12.5%) appear clear; thus, MIC of Papacarie against *S. mutans* is 12.5%. Of the tubes containing chlorhexidine, Tubes I (50%), II (25%), III (12.5%), IV (6.25%), and V (3.12%) appear clear; thus, MIC of chlorhexidine on *S. mutans* is 3.12%.

Then, the samples are scraped onto blood agar plates, incubated at 37°C for 2×24 h in anaerobic conditions, and assessed for any growth on the blood agar plate.

On the agar plate after scraping, any bacterial growth is indicated by white spots. Agar plates with papain 50%, 25%, and 12.5% have white spots, which indicated *S. mutans* growth. This means that in this test, papain did not have any bactericidal effect. Agar plates with Papacarie 12.5% have white spots, which indicate *S. mutans* growth, whereas those with Papacarie 50% and 25% have no white spots. This means that Papacarie has bactericidal effects at a minimal concentration of 25%. Agar plates with chlorhexidine have no white spots for any concentrations, indicating that chlorhexidine has a bactericidal effect at a minimal concentration of 3.12%.

In antimicrobial tests with the diffusion test, a zone of inhibition would be apparent. ANOVAs are used in this study because there are >2 data groups. With the normality test, $p>0.05$, which indicates that the data distribution of these groups is not normal. After that, the data are transformed. However, because the data transformation process to normalize the distribution did not work, the Kruskal–Wallis nonparametric test was conducted. The fact that $p>0.05$ indicated that there were different zones of inhibition in the tested group samples. Zones of inhibition formed between tested samples and *S. mutans* are described in Table 3.

Table 3 shows that the higher the concentration of samples, the larger the zone of inhibition around *S. mutans.* The largest zone diameter, 2.899 mm, was formed by chlorhexidine 12.5%. Meanwhile, the average zone diameter with papain was 0.694 mm and t12,5% Papacarie was 2,444 mm.

To determine which groups had significantly different effects, *post hoc* analyses were performed with the Mann–Whitney U-test. The level of significance for each group is shown in Table 4.

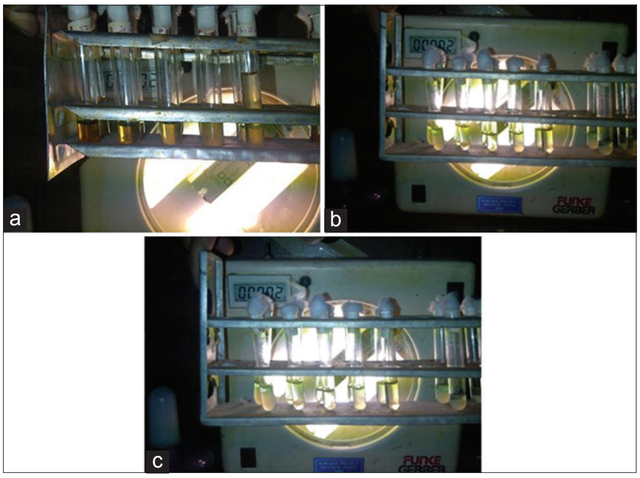

Fig. 1: Minimum inhibitory concentrations are determined using the dilution test. The clear tubes appear different from the turbid ones. The concentrations of these sample are 50%, 25%, 12.5%, 6.25%, and 3.063%. (a) Tubes containing papain: Tubes I, II, and III are clear. (b) Tubes containing Papacarie: Tubes I, II, and III are clear. (c) Tubes containing chlorhexidine: Tubes I, II, III, IV, and V are clear

The *post hoc* test results in Table 4 show that p≥ 0.05 for only the comparison between Papacarie 12.5% and chlorhexidine 12.5%, so there was no statistically significant difference between these agents.

Zones of inhibition formed around the blank disk on the agar plate, which can be seen in Fig. 3. The blank disk, which is given only droplets of 12.5% papain, exhibits a zone of inhibition in translucent form around the turbid area that indicates inhibited bacterial growth. This is also true of the blank disks that have been given droplets of 6.25% and 3.12% Papacarie. The blank disk that had been given droplets of chlorhexidine shows the biggest zone of inhibition.

DISCUSSION

This study was a comparison of the antimicrobial effects of Papacarie and papain on *S. mutans* with those of chlorhexidine as the control. The active ingredients of Papacarie include papain, which has been proven to have bactericidal and bacteriostatic effects at certain concentrations.

Papacarie has been examined for its chemomechanical characteristics; treatment using this is considered minimally invasive because it minimizes the elimination of healthy tissues. One of the ingredients of Papacarie, papain, is a natural substance that is found in papaya latex.

The papain used by scientists can be obtained alone from refined papaya latex. The methods of extracting papain from its solvent include cooling and heating methods. A solvent is a liquid substance that can dissolve other substances without chemical alterations. A good solvent for such an investigation must be inexpensive and easily available, must be physically and chemically stable, must reacted neutrally, does not evaporate easily, is not flammable, is selective in bonding with other substances, and does not affect other substances' properties. In this study, the solvent used was acetonide [9,10].

Chlorhexidine was used as the control because it is an efficacious antimicrobial used in the oral environment. It has been proven to be effective on Gram-positive and Gram-negative bacteria. Chlorhexidine

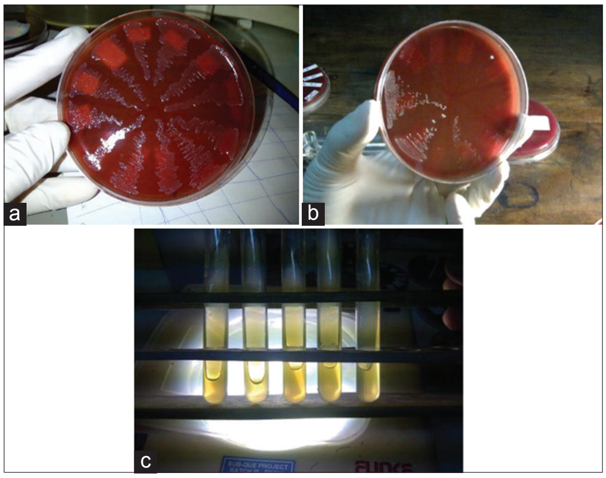

Fig. 2: Bacterial growth on an agar plate to determine the minimum bactericidal concentration. (a) The agar plates for papain show bacterial growth at all concentrations. (b) The plates for Papacarie show bacterial growth at 12.5% concentration. (c) The plates for chlorhexidine show no bacterial growth at any concentration

Table 2: Determination of minimum bactericidal concentration of papain and Papacarie by scraping sample on a blood agar plate

No.	Tested ingredients group (%)	Plate 1	Plate 2	Plate 3
1	Papain 50	+	+	+
2	Papain 25	+	+	+
3	Papain 12.5	+	+	+
4	Papacarie 50	–	–	–
5	Papacarie 25	–	–	–
6	Papacarie 12.5	+	+	+
7	Chlorhexidine 50	–	–	–
8	Chlorhexidine 25	–	–	–
9	Chlorhexidine 12.5	–	–	–
10	Chlorhexidine 6.25	–	–	–
11	Chlorhexidine 3.12	–	–	–

There is no minimum bactericidal concentration (MBC) of papain against *Streptococcus mutans*; MBC for Papacarie against *S. mutans*, MBC=25%; for chlorhexidine against *S. mutans*, MBC=3.12%. Minus signs (–) indicate no *S. mutans* growth (no white spots in the tube); plus signs (+) indicate *S. mutans* growth (white spots in the tube)

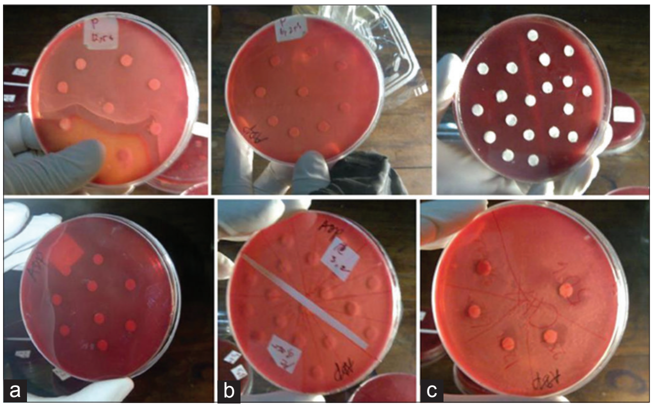

Fig. 3: Zones of inhibition formed after test sample penetration on blank disks. (a) Blood agar plate with a blank disk into which papain is added. (b) Blood agar plate with a blank disk into which Papacarie has been added. (c) Blood agar plate with blank disk into which chlorhexidine has been added

Table 3: Distribution of zones of inhibition against *Streptococcus mutans* formed by papain, Papacarie, and chlorhexidine

Tested samples with the concentrations (%)	Number of samples	Mean±standard deviation (mm)	95% Confidence interval for mean		p
			Lower	Upper	
Papain 12.5	9	0.6944±0.20833	0.5343	0.8546	0.00
Papain 6.25	9	0.6667±0.17678	0.5308	0.8025	
Papacarie 12.5	9	2.444±0.80795	1.8234	3.0655	
Papacarie 6.25	9	1.7778±0.26352	1.5752	1.9803	
Papacarie 3.062	9	1.2778±0.36324	0.9986	1.5550	
Chlorhexidine 12.5	9	2.8889±0.3333	2.6327	3.1451	
Chlorhexidine 6.25	9	2.6667±0.39528	2.3628	2.9705	
Chlorhexidine 3.062	9	1.5833±0.39528	1.2795	1.8872	

Results of Kruskal–Wallis test, p<0.05

Table 4: Significance value of each sample group in different concentrations

No.	Sample group in different concentrations	Number of samples	p
1	Papain 12.5% versus Papacarie 12.5%	9	0.000
2	Papain 12.5% versus chlorhexidine 12.5%	9	0.00
3	Papacarie 12.5% versus chlorhexidine 12.5%	9	0.222
4	Papain 6.25% versus Papacarie 6.25%	9	0.000
5	Papain 6.25% versus chlorhexidine 6.25%	9	0.000
6	Papacarie 6.25% versus chlorhexidine 6.25%	9	0.001
7	Papain 3.12% versus Papacarie 3.12%	9	0.000
8	Papain 3.12% versus chlorhexidine 3.12%	9	0.000
9	Papacarie 3.12% vs. chlorhexidine 3.12%	9	0.001

The significance test used was the Mann–Whitney U-test, with significance level $p \leq 0.05$

has the potency to fight against *S. mutans* by affecting its metabolism activity. In low concentrations, it has a bacteriostatic action against *S. mutans*, due to its hydrophobic-hydrophilic characteristics that damage the transportation of cellular membranes and intracellular constituents. In high concentrations, chlorhexidine shows bactericidal properties, which quickly and irreversibly precipitates cytoplasmic deterioration [11].

S. mutans used in this study was serotype C, which is a dominant strain in the oral environment. BHI, added with 2% blood sheep and blood agar plate, was used as the medium because it fosters quick growth of *S. mutans.* After enough bacterial colonies were grown for this research, the next step was to analyze the samples with *S. mutans* [11].

Since the diffusion test is a method aimed only at one type of microorganism, it can be performed more quickly and economically. In the diffusion test, the bacteria colonies on the agar plates cannot be too densely packed or too numerous so that the zone of inhibition can be assessed. In this study, the only type of bacteria tested was *S. mutans*. The examination was initiated with a concentration of 12.5% for each antimicrobial because trial and error revealed that Papacarie 50% and 25% were too viscous for action; hence, vehicle thickening occurred, and the bacteria could not penetrate the agar medium. Therefore, the lowest concentrations of the antimicrobials used were 12.50%, 6.25%, and 3.12%. The inhibitory potency was affected by the antimicrobial substance's concentration, amount of the microbes, temperature, types of microbes, pH, and dissolved organic substances.

In this study, the antimicrobial effects on *S. mutans* were highest for chlorhexidine, followed by Papacarie and papain. In addition to the visual examination, the results of the dilution test were supported with the findings regarding the zone of inhibition in the diffusion test.

The dilution test demonstrated that a concentration of 12.5% had antimicrobial effects on papain and Papacarie on *S. mutans* and antimicrobial effect on chlorhexidine was at 3.12%. This shows that at a low concentration, chlorhexidine still shows better antimicrobial effect than the other two antimicrobials.

In the research of bactericidal effect, the bacteria were grown in clear tubes on blood agar plates. After the incubation, the bacterial growth occurred in every concentration of papain and Papacarie 12.5%. This indicated that papain has no bactericidal effect, and the minimum bactericidal effect of Papacarie was 25%. The possible reason why Papacarie had better antimicrobial effects than papain is its composition that includes other antimicrobial ingredients; one of them chloramine, which has bactericidal and antiseptic effect. Chloramine T disinfectant, a new combination of active chloramine that can inactivate Gram-positive and Gram-negative bacteria *in vivo*, is also bactericidal *in vivo* when applied to contaminated wounds.

The diffusion test is used to measure the zones of inhibition formed around *S. mutans.* Papain can inhibit bacterial growth because it can metabolize the microorganism protein by breaking down peptide bonds in protein to simple compounds such as dipeptides and amino acids. Papain enzyme is included in sulfhydryl protease enzymes, indicating that it has sulfhydryl residue in its active location that works on the bacteria cell wall and cytoplasmic membrane [7,8]. Table 3 shows that the zone of inhibition with Papacarie is larger than papain, but the largest inhibitory is found on chlorhexidine. This happened because Papacarie contains papain that has an enzyme activity of 4.482 mmol/mg/h; hence, Papacarie has better bacteriostatic effects than papain. Papain used by researchers is crude papain, which is papaya latex that has been refined once. The papain enzyme activity can be improved by further refinements until the desired enzyme activity is achieved.

Table 4 shows that Papacarie 12.5% and chlorhexidine 12.5% have similar effects. This may be because Papacarie has chloramine whose antimicrobial effect is similar to that of chlorhexidine at the same concentration.

The zone of inhibition with papain was smaller than that with the other two antimicrobials because papain did not contain any other antimicrobial substances and its enzyme activity is lower than that of Papacarie. The zone of inhibition by papain was the largest at a concentration of 12.5%, and the differences among all antimicrobials were significant at the same concentration.

This study has its drawbacks. In the assessment of the zone of inhibition, papain was tested only at low concentrations (12.5%, 6.25%, and 3.12%). This was because Papacarie 50% and 25% could not penetrate into the agar medium. Therefore, the diffusion test was performed, starting with 12.5% concentrations of all antimicrobials.

The enzymatic activity of papain was far lower than that of Papacarie and so the zones of inhibition around *S. mutans* were also significantly different. The enzymatic activity of papain can probably be improved through further refinement but at a greater cost and with the need for higher technology.

Although papain has a poorer antimicrobial effect than Papacarie, it is still considered a chemomechanical material in the elimination of various tissues because it has bacteriostatic effects.

CONCLUSION

At the same concentration, papain and Papacarie have the same bacteriostatic effects. At the same concentration of 25%, however, Papacarie has better bactericidal effects. In addition, papain at the same concentration produced a smaller zone of inhibition than Papacarie.

CONFLICTS OF INTEREST

There are no conflicts of interest to declare.

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