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Original Article

Nigella sativa 3% Inhibition Test of Natural Toothpaste Compared *Cetylpyridinum chloride* (CPC) Toothpaste 0.01-0.1% on *Aggregatibacter actinomycetemcomitans*

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ABSTRACT

Periodontitis generally begins with gingivitis progresses to alveolar bone increasing the risk of systemic disease. The primary etiological factor in the etiology of periodontal disease is *Aggregatibacter actinomycetemcomitans* bacteria. *Cetylpyridinium chloride (CPC) has a bactericidal effect by disrupting the function of bacterial membranes in the cytoplasm. CPC can also absorb negative charges from bacteria, increase bacterial cell wall permeability, decrease cell metabolism, and reduce bacterial attachment to teeth. Use of antimicrobial toothpaste, such as Cetylpyridinium chloride (CPC), is one strategy to prevent periodontal disease, but CPC is hazardous in some quantities. As a result, it should be compared to a natural toothpaste, specifically Nigella sativa toothpaste. The objective of this study is to compare the antibacterial activity of natural toothpaste containing Nigella sativa 3% with toothpaste containing CPC 0.01% - 0.1% on Aggregatibacter actinomycetemcomitans. Experimental studies are used in this kind of research. The* colony count method was used to assess the natural toothpastes *Nigella sativa* 3% and toothpaste containing CPC 0.01- 0.1% for their capacity in inhibiting the *Aggregatibacter actinomycetemcomitans.* Natural toothpaste containing *Nigella sativa* 3% was completely inhibiting *Aggregatibacter actinomycetemcomitans,* compared to 0.01-0.1% CPC toothpaste. The significance level for the statistical test results was 0.000 ($p<0.05$). The conclution of this research are the natural toothpaste containing *Nigella sativa* 3% and toothpaste containing CPC 0.06%–0.1% can effectively suppress the growth of the microorganisms *Aggregatibacter actinomycetemcomitans.*

Keywords: : Periodontitis, *Aggregatibacter actinomycetemcomitans, Nigella sativa toothpaste 3%, Cetylpyridinium chloride* toothpaste 0.01-0.1%, and natural toothpastes.

Highlights: This research are expected to provide information about tooth paste contain *Nigella sativa* 3% and *Cetylpyridinium* chloride 0.01-0.1% which can inhibit the growth of *Aggregatibacter actinomycetemcomitans*.

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INTRODUCTION

Periodontitis, an inflammatory and pathological that impacts the connective tissue connection of the teeth, affects the majority of the adult population of the world. The Gram-negative microorganisms typically form a biofilm and cause it to be characterized by an increased host reaction against them, which ultimately results in tooth loss.[1] Pathogenic bacteria in the sulcus gingiva are the main cause of periodontitis, which has a complex etiology. *Porphyromonas gingivalis* and *Aggregatibacter actinomycetemcomitans* are two bacteria that are recognized as being significant periodontal pathogens and responsible for the devastating periodontal disease.[2]

Periodontitis can be prevented and treated at a low cost by maintaining oral hygiene with practices like cleaning your teeth.[3,4] According to Maria et al., periodontal pathogens in dental plaque were reduced when teeth were brushed with antibacterial toothpaste.[5] Periodontal therapy aims to restore lost shape, function, and aesthetics of all supporting structures and tissues (gingiva, periodontal ligament [PDL], cementum and alveolar bone) of the periodontal tissues and prevent periodontitis. The goal of standard periodontal therapy is to reduce the overall bacterial burden and change environmental factors affecting the microbial habitat so as to maintain homeostasis in the periodontal microbiota.[2] Abrasives are found in toothpaste and are used to polish teeth. A surface-active element in toothpaste known as a foaming agent is detergents.[5,6] Moreover, herbal extract is employed because it has beneficial benefits, such as anti-inflammatory, that prevent gingivitis and maintain oral health.[7,8]

The medical herb *Nigella sativa*'s antibacterial and anti-inflammatory properties have been studied.[9–11] Among the evidence-based herbal remedies, *Nigella sativa* is hailed as a "miracle herb".[10,12,13] The perennial plant *Nigella sativa*, commonly known as black cumin, black seed, habbatul barakah, black caraway, kalojeera, kalonji, or kalanji, is native to several Middle Eastern and Mediterranean countries as well as southern Asia. It is a member of the *Ranunculaceae* family.[12–15] The herb *Nigella sativa* contains tannins, thymoquinone, flavonoids, and thymol. The anti-inflammation and anti-oxidant properties of *Nigella sativa*, mint, cloves, aniseed, and olive leaf extracts increase tooth paste performance.[3,7,13] *Nigella sativa* may therefore be utilized as an adjuvant in periodontal therapy.[7,16] According to a preliminary investigation, supragingival plaque bacteria could not grow when used with toothpaste containing 2% SLS and *Nigella sativa* extract (Dentomaxxima). *Nigella sativa* extract also prevented bacterial plaque.[3,7]

Currently available toothpaste contains *Cetylpyridinium chloride* (CPC) as one of its primary active ingredients, an antibacterial agent.[17] The quaternary ammonium compound CPC possesses a range of antimicrobial properties. Water, ethanol, chloroform, benzene, and water are all solvents for CPC.[18] After two weeks of consistent use, the state of the oral cavity will improve. CPC is an anti-bacterial, anti-plaque, and gingivitis treatment.[19] In a prior investigation into the efficiency of CPC-containing mouthwashes through a clinical trial, Rawlinson et al. shown that two different types of CPC-containing mouthwashes with concentrations of 0.05% and 0.1% may clinically suppress plaque growth.[20] CPC also has a bactericidal effect by interfering with the function of the bacterial cytoplasmic membrane and disturbing bacterial metabolism, resulting in reduction of cell development and, eventually, cell death. Plaque index can be decreased by reducing the number of bacteria present in the plaque.[18,19,21]

Cetylpyridinium chloride (CPC) works by infiltrating the bacterial cell

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membrane, producing leaking inside the cell, and ultimately killing the bacteria.[18] CPC has the power to absorb bacterial negative charges, improve bacterial cell wall permeability, slow down cell metabolism, lessen bacterial adhesion to tooth surfaces, and prevent bacterial cell growth. The drawback of these artificial substances is that they leave dark stains on the teeth.[22]
According to literacy about CPC as an antibacterial agent, few have undertaken research on the inhibition of growth the *Aggregatibacter actinomycetemcomitans* bacteria. Therefore, the purpose of this study was to learn more about the inhibition of growth the *Aggregatibacter actinomycetemcomitans* by toothpaste containing CPC and *Nigella sativa*, as well as to compare the differences in CPC's inhibition of *Aggregatibacter actinomycetemcomitans* at concentrations of 0.01% - 0.1%.

MATERIALS AND METHODS

This research was performed at the Airlangga University Faculty of Dentistry Research Center in Surabaya, East Java. The research design in this study was a post-test-only laboratory experiment with a control group. The sample used in this investigation was the bacteria *Aggregatibacter actinomycetemcomitans*, which is available at the Faculty of Dental Medicine Airlangga University Research Center. Federer's formula **(n-1) (t-1)≥15**, where n=sample size for each intervention and t=number of interventions, for determining the number of samples was used to determine the number of samples used in the study.[22] The total number of group in this study was 12 made up of 11 treatment groups (*Nigella sativa* toothpaste 3% 1 group, and CPC toothpaste 10 groups), and 1 control groups (control media). Each concentration was repeated 3 times. Therefore, three samples are required at a least for each treatment to be repeated.

The independent variable in this study is toothpaste consisting of CPC 0.01-0.1% and *Nigella sativa* 3%. In this study, 3% extract was used because, in the previous study, formulation stable with the concentration of the active ingredient viscous extract of black cumin seeds (*Nigella sativa*) by 3%. The dependent variable in this study was the growth of the *Aggregatibacter actinomycetemcomitans* bacteria. The controlled variables in this study were the incubation time and temperature of *Aggregatibacter actinomycetemcomitans* bacteria, the sterility of the materials, and the skills of the operators during the study.

Research Procedure

This research is based on the development of previous research by Setiawati et al.,[23] and the procedure is based on research conducted by Toar et al.[22]

1. Tool Sterilization
   To prevent microorganism contamination, all tools and materials used throughout the investigation must be sterile and clean. Metal and glass instruments are sterilized by carefully washing, drying, and wrapping them in aluminum foil. Metal tools were sterilized for 60 minutes at 121°C in the autoclave, and glass tools were sterilized at 110°C for 15 minutes. Plastic tool sterilization is possible with 70% ethanol.

2. Preparation of *Aggregatibacter actinomycetemcomitans* Bacterial Culture Media

3. *Aggregatibacter actinomycetemcomitans* was used to create bacterial culture medium for the study. Brain Heart Infusion – Broth (BHI-B) media was then used to inoculate the bacteria, resulting in the suspension of the *Aggregatibacter actinomycetemcomitans* bacteria.

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4. Preparation of *Aggregatibacter actinomycetemcomitans* Bacterial Suspension.

   The following procedure is to make an *Aggregatibacter actinomycetemcomitan* bacterium suspension. A sterile ose needle was used to collect bacterial colonies that had been grown on agar media and placed inside a test tube's BHI-B medium. After then, the test tube was kept at 37°C for 24 hours. *Aggregatibacter actinomycetemcomitan* bacteria must be suspended in a solution that meets the McFarland turbidity requirement of 0.5. Two ingredients make up McFarland's 0.5 standard solution: 1% Barium chloride ($BaCl_2$) and 1% Sulfuric acid (H_2SO_4). The two solutions are combined in the necessary amount, shaken, and then combined. The concentration of $1.5x10^8$ CFU/ml in McFarland's standard solution is equivalent to a bacterial cell suspension. If the BHI-B media appears more turbid than the 0.5 McFarland solution, the solution can be introduced into the BHI-B media a little slowly to bring it up to the 0.5 McFarland turbidity standard.

5. Inhibitory test of the growth of *Aggregatibacter actinomycetemcomitans* bacteria.

6. The next step was to prepare test tubes that were labeled for testing the inhibition of Brain Heart Infusion – Broth (BHI-B) and CPC media at concentrations of 0.01%, 0.02%, 0.03%, 0.04%, 0.05%, 0.06%, 0.07%, 0.08%, 0.09%, and 0.1% on the growth of the bacteria *Aggregatibacter actinomycetemcomitans*. The tube also contained *Nigella sativa* 3%. The tubes were all then filled with 0.1 mL of bacterial suspension, and they were all cultured at 37°C for two consecutive days. Take 0.1 mL of the suspension from each tube, spread it on the Mueller Hinton Agar (MHA) media, and incubate the MHA media for two cycles of 24 hours at 37°C. The next procedure was to calculate the number of bacterial colonies on MHA media and perform data analysis.

7. The Shapiro-Wilk normality test, the homogeneity test utilizing Levene's test, and the One-Way Anova parametric test were employed in this study's data analysis, which comprised more than two sample variables. To evaluate if the data are regularly distributed or not, the Shapiro-Wilk normality test is utilized. If it is, the parametric test can be performed. If (p-Value) > 0.05, then homogeneous data are obtained from two or more data groups, according to the homogeneity test with Levene's test, which tries to determine the homogeneity of the data. Parametric one-way Anova test. This test is run when the data is discovered to be regularly distributed. One-Way Anova seeks to determine whether there is a significant difference between the means of two or more sets of data. Then proceed with the post hoc test with the Games-Howell test if the data is not homogeneous, test it aims to find out which groups are different significant.

RESULTS

After being incubated for two consecutive 24-hour periods at 37°C, *Aggregatibacter actinomycetemcomitans* bacteria underwent a colony count test on MHA media. The results revealed that there were changes in the number of colonies that grew. Table 1 shows that the number of *Aggregatibacter actinomycetemcomitans* colonies decreases with increasing CPC concentration. *Aggregatibacter actinomycetemcomitans* bacteria did not grow in MHA media with CPC

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concentrations of 0.06% to 0.1%. The following graph illustrates in Figure 1 the percentage of inhibition on *Aggregatibacter actinomycetemcomitans* bacteria growth.

The results of the inhibition test data were reviewed to see if the data were normally distributed using the Shapiro-Wilk test. If the study's data are normally distributed (p>0.05), the statistical test is conducted using the one-way Anova parametric procedure. The SPSS 27 program and statistical tests were used to examine the study's findings. The following table displays the results of the Shapiro-Wilk normality test.

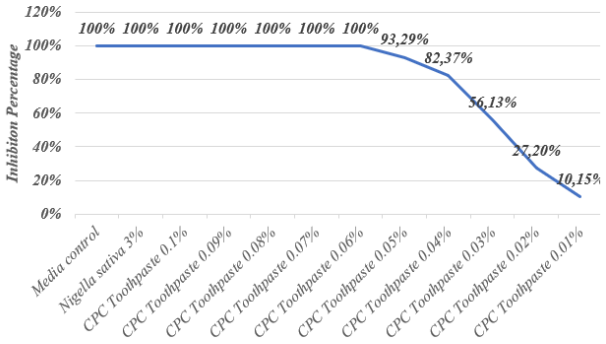

Figure 1. Graph of Percentage of Bacterial Growth Inhibition *Aggregatibacter actinomycetemcomitans*

Table 1. Shapiro-Wilk Normality Test

Sample Group	*Sig. (p)*	*Description*
Media Control	0	Non-normal Distribution
Nigella sativa toothpaste *3%*	0	Non-normal Distribution
CPC toothpaste 0.1%	0	Non-normal Distribution
CPC toothpaste 0.09%	0	Non-normal Distribution
CPC toothpaste 0.08%	0	Non-normal Distribution
CPC toothpaste 0.07%	0	Non-normal Distribution
CPC toothpaste 0.06%	0	Non-normal Distribution
CPC toothpaste 0.05%	0.637	Normal Distribution
CPC toothpaste 0.04%	0.298	Normal Distribution
CPC toothpaste 0.03%	0.688	Normal Distribution
CPC toothpaste 0.02%	0.380	Normal Distribution
CPC toothpaste 0.01%	0.567	Normal Distribution

Note: t>0,05 (Normal Distribution)

The normality test results in Table 1 reveal that the data are normally distributed, and the significance level is more than 0.05 (t>0.05) in several groups, especially in the CPC toothpaste group whose values are 0.05%, 0.04%, 0.03%, 0.02%, and 0.01% respectively. In contrast, the data was not normally distributed and there was no significant change in the 0.06% to 0.1% group because there was no bacterial colony formation. Levene's test is then used to do a homogeneity test on the normally distributed data.

Table 2. Levene's Test Homogeneity Test Results

Levene's Test				
Variable	Levene Statistic	df1	df2	Sig.
Number of Colonies	8.522	12	29	0.000

In Table 2, the data on the total number of colonies in the sample group was not homogenous, according to the results of the Levene homogeneity test, because the p-value was less than 0.05 (p = 0.000). If the data is normally distributed but not homogeneous, data analysis is done using a parametric test technique using the One-Way Anova test with a follow-up test using Games Howell.

Table 3. One-Way ANOVA Test Results

ANOVA			
Variable	df	F	Sig
Number of Colonies	12	654.973	0.000

Note: p < 0.05 (Significantly Different)

Based on the ANOVA test on Table 3 findings in Table 3, a significance value (p) of 0.000 (p>0.05) was found, indicating a significant difference in the growth of the

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bacterial colonies of *Aggregatibacter actinomycetemcomitans* in each sample group.

DISCUSSION

Cetylpyridinium chloride (CPC) and *Nigella sativa* toothpaste at concentrations of 0.01%, 0.02%, 0.03%, 0.04%, 0.05%, 0.06%, 0.07%, 0.08%, 0.09%, and 0.1% were the subjects of this laboratory experiment to test their ability to inhibit the growth of *Aggregatibacter actinomycetemcomitans* bacteria. The colony counting technique counts the number of colonies that have developed on MHA media in order to show the antibacterial activity of CPC and *Nigella sativa*. Based on the percentage of CPC inhibition at each concentration, the Minimum Inhibitory Concentration (MIC) and Minimal Bactericidal Concentration (MBC) of *Aggregatibacter actinomycetemcomitans* were determined.

A genuine infection can be contracted via exposure to the exogenous bacterium *Aggregatibacter actinomycetemcomitans.* It is capable of generating virulence factors.[24] *Aggregatibacter actinomycetemcomitans* tissue-destructive virulence factors include released proteins such as cell stress protein and lipopolysaccharide (LPS), which is found on the bacterial cell wall. In vitro and in vivo studies have shown that LPS increases bone resorption.[25] However, it is thought to be a less potent cytokine inducer than the released protein. Because it acts as an osteoclast "growth factor" and encourages bone resorption, the cell stress protein chaperonin 60 is thought to be a powerful bone-degrading agent.[26]

Aggregatibacter actinomycetemcomitans bacterial colonies are growing in CPC at concentrations of 0.01%, 0.02%, 0.03%, 0.04%, and 0.05%, according to the results of the colony count. When compared to the CPC concentration, which is 0.06%, 0.07%, 0.08%, 0.09%, and 0.1%, no colony growth was observed. This demonstrates that the number of *Aggregatibacter actinomycetemcomitans* bacterial colonies grown on MHA media decreased with increasing CPC content. In order to make it simple to determine the values of MIC and MBC, this study also determined the percentage of CPC inhibition. The Minimal Inhibitory Concentration (MIC) for CPC is in the 0.05% concentration range. CPC, a quaternary ammonium molecule has a broad-spectrum antibacterial impact on both Gram-positive and Gram-negative bacteria. Because CPC has a cationic group, it is simpler to bind to the bacterial cell membrane's surface negative charge, which will alter permeability and damage the cell membrane, leading to the leaking of cell components and cell death.[19]

Nigella sativa 3% did not exhibit colony growth *Aggregatibacter actinomycetemcomitans* bacteria, according to the results of the colony. Setiawatie et al. research on which showed that *Prevotella intermedia* and *Porphyromonas gingivalis*, the two bacteria that cause periodontitis, were vulnerable to *Nigella sativa*'s antibacterial effect, confirmed this result.[7] Recently, it was discovered that toothpaste containing *Nigella sativa*, 2% Sodium lauryl sulfate, and non-Sodium lauryl sulfate had no effect on fibroblasts. According to these findings, *Nigella sativa* may be the main component of dental paste used as an adjuvant therapy for periodontitis.[7]

In a study conducted by Kapil et al., the antibacterial activity of 0.2% thymoquinone gel was evaluated *in vitro*.[27] The results showed that *Porphyromonas gingivalis, Aggregatibacter actinomycetemcomitans*, and *Prevotella intermedia* were all very sensitive to 0.2% thymoquinone gel up to dilution levels of 10-9 and 10-8, respectively. This demonstrated that each of the three biological strangles was advised that the drug's effectiveness be assessed against more periodontal infections due to the intricacy of periodontal diseases in this area.[1,27] Thymoquinone was proposed as

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a potential natural product source by Kouidhi et al. to its activity in altering resistance and its selective antibacterial efficacy against oral bacteria.[1]

Thymoquinone and *Nigella sativa* have been addressed as anti-inflammatory and antioxidant mediators with therapeutic benefits in a number of studies.[13,28,29] Thymoquinone causes an antioxidant effect by scavenging a variety of free radicals, and it is as effective at scavenging superoxide anions as superoxide dismutase is at doing so.[30] Thymoquinone has been shown to have substantial anti-inflammatory benefits in clinical studies as well. Pro-inflammatory cytokines such as interleukin-1β (IL-1β), interleukin-6 (IL-6), and *tumor necrosis factor*-α (TNF-α), and prostaglandin E2 (PGE2) are reduced whereas anti-inflammatory cytokine IL-10 is elevated as a result of decreased macrophage production and lower Nitric oxide (NO) levels.[13,29–31] Thymoquinone's potential method of action for its anti-inflammatory and anti-oxidant activity may be related to its ability to suppress eicosanoid synthesis. Through the inhibition of COX and LOX molecular pathways, thymoquinone and *Nigella sativa* extracts have been shown in experiments to significantly limit lipid peroxidation and the production of *eicosanoids,* particularly thromboxane B and leukotrienes B4.[15,29,32] According to the current review, thymoquinone may be crucial in avoiding the beginning and progression of periodontal disease due to its potential anti-inflammatory and anti-oxidant properties.[13,14]

The active component, thymoquinone, accounts for 30% to 48% of the seeds of *Nigella sativa*.[3] *Nigella sativa* may be utilized as a treatment or adjuvant in bacterial illnesses because of its strong anti-bacterial action.[3,12,14] The substances thymoquinone, thymol, and tannins found in black cumin have been shown to be helpful in preventing the growth of the subgingival and supragingival plaque bacteria that are the main cause of periodontitis. Both Gram-positive and Gram-negative bacteria are known to be resistant to the growth of these substances.[33,34] The quinon derivative thymoquinone contains the element thymol. Quinon has strong antimicrobial qualities. The adhesin surface, polypeptide cell walls, and membrane-bound enzymes are among the targets in bacterial cells. Thymoquinone is known to combine with nucleophilic amino acids in proteins to generate irreversible compounds that can inactivate proteins and cause malfunctions. The periodontitis-causing supragingival and subgingival plaque bacteria are effectively stopped by the antibacterial THQ in black cumin extract.[33,35]

Nigella sativa, a traditional treatment, is frequently employed to treat a range of diseases. The *Nigella sativa* extract showed significant antibacterial effectiveness against the germs that cause pulpitis and periodontitis.[4,10,36] According to Setiawatie et al., *black cumin* extract demonstrates free radical scavenging activity at a concentration of 3%.[3] According to the results of the cytotoxic test performed on cell cultures of fibroblasts and osteoblasts, 3% of the *Nigella sativa* extract had viability levels above 90%.[7,16] The active ingredients in *Nigella sativa* have been connected to the plant's positive effects on health. Alkaloids, saponins, and 28–36% protein, as well as 0.4–2.5% essential oil, are all present in the seeds. Although *Nigella sativa* has many pharmacologically active chemicals, thymoquinone, dithymoquinone, thymol, and thymohydroquinone are the most commonly reported active components.[11,34]

The active component thymoquinone may be responsible for *Nigella sativa*'s anti-inflammatory and anti-destructive properties. Thymoquinone has been utilized to illustrate the primary pharmacological characteristics of *Nigella sativa*, such as its anti-inflammatory, antioxidative, anti-bacteriostatic, analgesic, hypoglycemic, and anti-carcinogenic properties.[3,13,34] It has also been demonstrated that thymoquinone inhibits pro-inflammatory cytokines such as

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ILs, TNF-α, and MMP8.[1,3,14,29] By stopping the development of biofilms in *Porphyromonas gingivalis* and *Prevotella intermedia*, thymoquinone additionally demonstrated its antibacterial activity.[7,36] These results suggest that *Nigella sativa* has antibacterial, anti-inflammatory, and anti-destructive characteristics, especially when applied to tissues with periodontitis.[3,7,36] The findings of this study can be used to develop an alternative antibacterial agent in the form of toothpaste since natural toothpaste has less negative effects on tooth and mucosal discoloration and because *Nigella sativa* toothpaste can be created in alcohol-free preparations.[3,8,33,37] In the area of periodontal disease, toothpaste containing *Nigella sativa* can stop supragingival plaque bacteria from growing. A reduced periodontal index and a significantly lower quantity of sub-gingival bacteria were observed in Wistar rats administered with *Nigella sativa* extract in drinking water in animal tests as compared to the control group. Other studies looked into the *Nigella sativa* extract's potential to stop periodontal inflammation in its tracks. Because it slows down alveolar bone resorption, *Nigella sativa* taken orally aids in the prevention of periodontal disease. In comparison to the chitosan group, the administration of periodontal chips containing *Nigella sativa* significantly improved the clinical condition of patients with chronic periodontitis.

Nigella sativa extract is ideal for application in herbal medicine in the field of dentistry as toothpaste, mouthwash, root canal irrigation material, pulp capping material, and dental implant coating due to its considerable antioxidant, antibacterial, anti-inflammatory, and cytoprotective qualities. By researching the development of *Nigella sativa* formulations in nanobiotechnology, future dental materials can be created.

STRENGTH AND LIMITATION

Only a few have researched the inhibition of *Aggregatibacter actinomycetemcomitans* bacteria. So, in this study, the researchers wanted to find out more about the inhibition ability of toothpaste containing *Nigella sativa* 3% and CPC 0.01-0.1% against *Aggregatibacter actinomycetemcomitans* bacteria. In addition, future studies are needed to determine the effectiveness of toothpaste containing *Nigella sativa* 3% and *CPC* 0.01-0.1% in inhibiting other bacteria.

CONCLUSIONS

According to the study's findings and analysis, *Aggregatibacter actinomycetemcomitans* bacteria can be inhibited from growing when natural toothpaste containing *Nigella sativa* 3%, which is comparable to CPC 0.06%–0.1%, is used.

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ETHICAL CLEARANCE

The research protocol was approved by Faculty of Medicine Research Ethics Commission Udayana University, ETHICAL CLEARANCE No: 1078/UN 14.2.2.Vll.14/LT/2022.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest for this research.

AUTHOR CONTRIBUTION

EMS : Conceptualization, Data curation, Funding acquisition, Investigation, Methodology, Resources, Supervision, Writing – review & editing. PW: Conceptualization, Formal analysis, Software, Validation, Visualization, Roles/Writing – original draft. RPR : Conceptualization, Formal analysis, Investigation, Methodology. AE: Conceptualization, Formal analysis, Software, Validation, Visualization, Writing. DS : Resources, Supervision, Validation. IJS : Investigation, Project administration, Validation, Writing. LB : Investigation, Project administration, Validation, Writing. RSM : Investigation, Project administration, Validation, Writing.

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