

Inhibitory effect of calcium hydroxide combined with *Nigella sativa* against *Enterococcus faecalis*

Myrna Nurlatifah Zakaria¹, Yusfien Shabrina Putri¹, Asih Rahaju¹, Sri Fatmawati² and Arief Cahyanto³

¹Department of Endodontology and Operative Dentistry, Faculty of Dentistry, Universitas Jenderal Achmad Yani, Cimahi, Indonesia

²Department of Chemistry, Faculty of Sciences, Institut Teknologi Sepuluh Nopember, Surabaya, Indonesia

³Department of Dental Materials Science and Technology, Faculty of Dentistry, Universitas Padjadjaran, Bandung, Indonesia

ABSTRACT

Background: Calcium hydroxide is the gold standard medicament for root canal treatment. *Enterococcus faecalis*, the primary cause of intraradicular persistent endodontic infection, is often identified even after endodontic treatment. Thymoquinone, an active ingredient of *Nigella sativa*, has an antimicrobial effect on both gram-negative and positive bacteria, including *E. faecalis*. **Purpose:** This study aimed to evaluate the inhibitory effect of calcium hydroxide combined with *Nigella sativa* extract and determine the best ratio for the combined material. **Methods:** This is an experimental study comprised of six groups ($n = 4$ per group) based on the material and its ratio, namely; (1) calcium hydroxide; (2) *Nigella sativa* extract; and groups of the combination of calcium hydroxide and *Nigella sativa* extract with a ratio (3) 70:30, (4) 50:50, (5) 30:70, (6) 10:90. The inhibitory effect against *E. faecalis* was evaluated by the agar well diffusion method in Muller–Hinton agar. Observation of the inhibitory zone was performed on the first, third, and seventh days. The collected data were analysed by a one-way ANOVA and LSD post hoc test. **Results:** Calcium hydroxide has the highest inhibitory effect, and the combination of *Nigella sativa* extract with calcium hydroxide ratio 50:50 was second. The inhibitory zone of these two groups was significantly higher than in any other group ($p < 0.05$). **Conclusion:** *Nigella sativa* extract combine with calcium hydroxide did not enhance calcium hydroxide's antimicrobial property against *E. faecalis*. An equal amount of *Nigella sativa* and calcium hydroxide is the best combination ratio, with a stable effect for up to seven days.

Keywords: Calcium hydroxide; endodontic infection; *Enterococcus faecalis*; intracanal medicament; *Nigella sativa*

Correspondence: Myrna Nurlatifah Zakaria, Department of Endodontology and Operative Dentistry, Faculty of Dentistry, Universitas Jenderal Achmad Yani. Jl. Terusan Jenderal Sudirman, Cimahi 40531, Indonesia Email: myrna.nurlatifah@lecture.unjani.ac.id

INTRODUCTION

Deep caries or traumatic injury often become a pathway for bacteria to enter the pulp and resume infection to the periapical tissue, known as endodontic infection. The endodontic infection may form as primary, secondary, or persistent infection.¹ One bacterium commonly found in persistent infections is *Enterococcus faecalis* (*E. faecalis*). Virulence factors of *E. faecalis* increase the bacteria's survival in harsh environments as it adheres to and penetrates the dentinal tubules. Bacterial virulence is one of the primary factors of root canal treatment failure.² The persistent infection commonly results in failed tissue regeneration seen as an unhealed radiolucency in the apical area or as unrelieved symptoms such as pain or ongoing sinus tract problems.³

In multiple visit endodontic treatments, the root canal system needs to be filled by an intracanal medicament to suppress the amount and inhibit the growth of bacteria, reduce pain, and provide long-term disinfection. Calcium hydroxide has been the most commonly used material in endodontics since 1920, with various indications from pulp capping, apexification, apexogenesis, and root canal disinfection.⁴ The main mechanism of action of calcium hydroxide is due to the release of calcium and hydroxyl ions in the presence of water. The hydroxyl ion elevates the environment pH to an alkaline condition, which has a detrimental effect on bacteria. It reduces bacterial virulence and eventually reduces symptoms, assisting the healing process and tissue regeneration.⁵ However, several studies mentioned the limitations of calcium hydroxide in eliminating *E. faecalis* because of the calcium hydroxide

paste's viscosity, penetration ability, and bacterial virulence, which can survive alkaline and starvation conditions.^{6,7}

Nigella sativa is a herb that is widely used by people, especially in the Middle East. *Nigella sativa* seeds and oils have been used as an analgetic, antibacterial, anti-allergy, anti-cancer, and anti-inflammatory agent. Thymoquinone is an active ingredient in *Nigella sativa*, which can inhibit the growth of bacteria and function as an antimicrobial agent. Thymoquinone can damage the bacteria cell's integrity, resulting in the death of bacteria cells through necrosis and apoptosis.^{8–10} *Nigella sativa* extract effectively eliminates both gram-negative and positive bacteria, including *E. faecalis*. A previous study reported that *Nigella sativa* is dose-dependent, in which 100% extract had better antibacterial action against *Enterococcus* than 25%, 50%, and 75%.¹¹ Other studies mentioned that *Nigella sativa* extract with low viscosity could penetrate and provide a good antibacterial effect, unlike the high viscosity extract.^{12,13} The *Nigella sativa*'s ability to eliminate *E. faecalis* and the low viscosity makes them of interest as an intracanal disinfection agent. It was hoped future research of the compound could synergistically improve the antimicrobial efficacy of calcium hydroxide.

Based on previous studies on the high potency of *Nigella sativa*, particularly as an antimicrobial agent, we proposed using this agent as supplemental to the commonly used endodontic intracanal medicament, calcium hydroxide. As far as we know, this has not been previously studied. Therefore, the proper ratio in combining the materials has yet to be determined. Whether this combination can work synergistically also needs to be evaluated; one of the ways is by evaluating the inhibitory effect of both materials in different combinations. Considering calcium hydroxide is a time-dependent medicament, the evaluation was done on three specific days (days one, three and seven) to evaluate the stability of the material.¹⁴ Therefore, we investigated the efficacy of the *Nigella sativa* extract and its combination with calcium hydroxide in several ratios to evaluate the

inhibitory effect against *E. faecalis*, and determined the best ratio for the combining material.

MATERIALS AND METHODS

This is an experimental study with a posttest-only control design, consisted of six groups ($n = 4$ per group), paired numeric analytic with 95% confidence interval) according to the sample components and ratio: (1) calcium hydroxide; (2) *Nigella sativa* extract; a combination of calcium hydroxide with *Nigella sativa* (3) ratio 70:30; (4) ratio 50:50; (5) ratio 30:70; (6) ratio 10:90. To ensure the bacteria itself was not affected by the distilled water used as a solvent for the calcium hydroxide a group by using only distilled water was used as negative control. The extraction of *Nigella sativa* was carried out by maceration using methanol as a solvent at the Department of Chemistry, Faculty of Sciences, Institut Teknologi Sepuluh Nopember, Surabaya, Indonesia.¹⁵ Calcium hydroxide powder (Merck, Darmstadt, Germany) was mixed with distilled water with the w/p ratio of 0.8 to provide the best consistency as intracanal medicament paste to have acceptable flowability. The total crude extract (100% concentration) and calcium hydroxide were weighed on an analytical scale to obtain the exact weight for each group according to its ratio (Table 1).

Table 1. Tested groups and weight ratio

Group	Ratio	<i>Nigella sativa</i> (milligram)	Calcium Hydroxide (milligram)
1	0:100	-	260
2	100:0	260	-
3	70:30	182	78
4	50:50	130	130
5	30:70	78	182
6	10:90	26	234

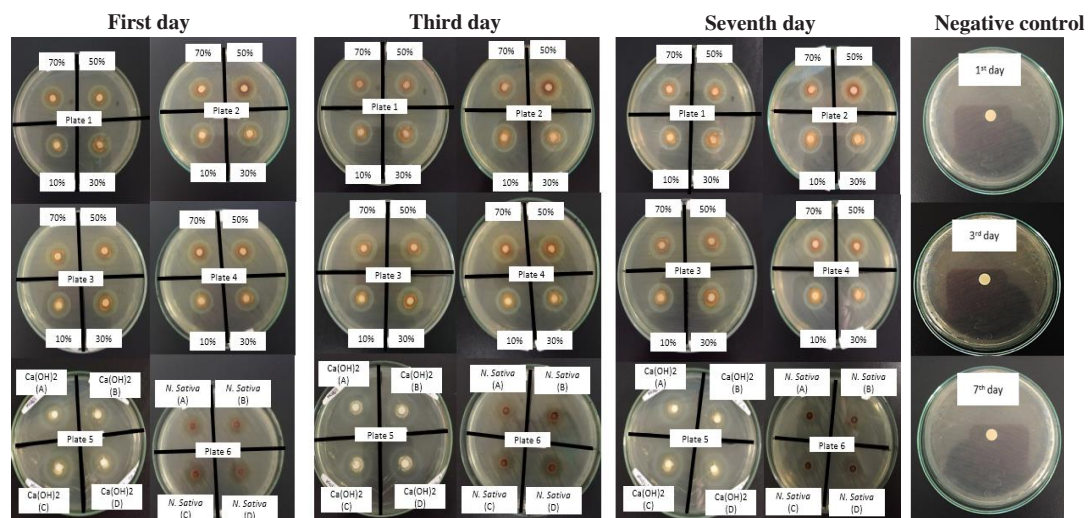


Figure 1. Antibacterial inhibitory zone of *Nigella sativa* extract, calcium hydroxide and its combination, to *Enterococcus faecalis*.

The *E. faecalis* ATCC 2912 was reidentified and cultured to achieve a pure suspension of *E. faecalis*. The concentration of the bacteria was standardised by spectrophotometry based on the McFarland standard at 0.5. The antibacterial test was carried out by preparing a bacterial suspension followed by the well diffusion test.¹⁵ Mueller–Hinton agar plates were prepared, and the bacteria suspension was plated in each agar plate. Each agar plate was divided into four, and then a well was made with a sterilised perforator. Afterwards, each sample group was placed into the well. The agar plates with the tested material were then incubated at 37° C for the evaluation interval. All work was conducted on a workbench and in a sterile protocol.

Observation of the inhibitory zone was performed on the first, third, and seventh days. Digital callipers were used to measure the diameter of the inhibition zone around the well. The measurement was repeated three times for each inhibition zone, and the average value was taken. Collected data were analysed using a one-way analysis of variance (ANOVA) test (p<0.05) to compare the inhibitory zones between groups, and a post hoc least significant difference (LSD) test was performed to determine which specific groups were statistically significant from others (p<0.05).

RESULTS

The inhibition zone of *Nigella sativa* extract, calcium hydroxide, and its combinations are depicted in Figure 1. Descriptively, all samples showed an inhibitory area surrounding the sample-containing well, except for the negative control group.

The mean value of each group and the one-way ANOVA test are presented in Table 2. In all three intervals evaluated,

calcium hydroxide has higher antibacterial efficacy than the others. The combination of *Nigella sativa* extract with a calcium hydroxide ratio 50:50 was second after calcium hydroxide. On all observation days, the mean value of an inhibitory zone for *Nigella sativa* extract was the lowest of all medicament groups. The inhibitory zone for all groups did not show notable differences until the seventh day of observation. Comparative analysis of the study groups by the post hoc LSD test (p<0.05) showed significant differences between tested groups.

Table 2. Antibacterial inhibitory zone of tested groups to *E. faecalis*

Group	Mean (mg/dl) ± SD	Day	P
Calcium hydroxide	21.63 ± 0.13	First	*0.001
<i>Nigella sativa</i>	16.8 ± 1.28		
Ratio 70:30	17.58 ± 0.88		
Ratio 50:50	19.2 ± 0.98		
Ratio 30:70	18. ± 0.51		
Ratio 10:90	16.98 ± 0.67		
Calcium hydroxide	21.63 ± 0.13	Third	*0.001
<i>Nigella sativa</i>	16.8 ± 0.91		
Ratio 70:30	17.83 ± 0.61		
Ratio 50:50	18.93 ± 0.93		
Ratio 30:70	17.65 ± 0.75		
Ratio 10:90	16.93 ± 0.77		
Calcium hydroxide	21.53 ± 0.15	Seventh	*0.006
<i>Nigella sativa</i>	16.73 ± 0.93		
Ratio 70:30	17.7 ± 0.52		
Ratio 50:50	18.78 ± 0.93		
Ratio 30:70	17.65 ± 0.69		
Ratio 10:90	16.83 ± 0.71		

*one-way ANOVA p<0.05 (significant difference)

Table 3. Intergroup comparisons of the antibacterial inhibitory zone of the groups against *E. faecalis*

Group	Day	Calcium hydroxide	<i>Nigella sativa</i>	Ratio 70:30	Ratio 50:50	Ratio 30:70	Ratio 10:90	Negative control
Calcium hydroxide	First							
<i>Nigella sativa</i>		0.000*						
Ratio 70:30		0.000*	0.200					
Ratio 50:50		0.001*	0.001*	0.012*				
Ratio 30:70		0.000*	0.050*	0.450	0.059			
Ratio 10:90	0.000*	0.767	0.317	0.001*	0.088			
Calcium hydroxide	Third							
<i>Nigella sativa</i>		0.000*						
Ratio 70:30		0.000*	0.064					
Ratio 50:50		0.000*	0.001*	0.048				
Ratio 30:70		0.000*	0.119	0.740	0.025*			
Ratio 10:90	0.000*	0.813	0.100	0.001*	0.180			
Calcium hydroxide	Seventh							
<i>Nigella sativa</i>		0.001*						
Ratio 70:30		0.040*	0.220					
Ratio 50:50		0.271	0.029*	0.230				
Ratio 30:70		0.021*	0.329	0.802	0.230			
Ratio 10:90	0.001*	1.000	0.220	0.029*	0.329			

*post hoc LSD, significant difference (p<0.05 level)

Intergroup comparisons between groups on each day by the post hoc analysis using the LSD test are described in Table 3. On the first day and third day of observation, the inhibitory zone of calcium hydroxide was significantly higher compared to all other tested groups. However, on the third, the inhibitory zone did not differ significantly between the calcium hydroxide group and the combination group of *Nigella sativa* and calcium hydroxide. In all three evaluations, a consistent significant difference between different combination ratios of *Nigella sativa* and calcium hydroxide extract groups compared to *Nigella sativa* alone was only observed in the 50:50 ratio group. The 50:50 ratio had a higher inhibitory zone than *Nigella sativa* alone, but less than the calcium hydroxide group. The addition of *Nigella sativa* extract to calcium hydroxide did not increase the inhibitory zone of calcium hydroxide.

DISCUSSION

Our study revealed that the calcium hydroxide group has the highest inhibition zone. However, others reported that calcium hydroxide is ineffective against *E. faecalis*, a prevalent bacterium in persistent infection.^{6,7} Controversial effectiveness of calcium hydroxide against *E. faecalis* is influenced mainly by the different methods used in the studies. Almost all studies that allow direct contact of calcium hydroxide to the bacteria resulted in high antimicrobial efficiency. It was found to be less effective in studies using root canal models or in vivo studies where the complexity of root canal morphology contributes significantly to the limitation of material to be in intimate contact with the bacteria.^{16–18} This is mainly caused by the high viscosity of calcium hydroxide, limiting its ability to reach small, narrow areas and it has difficulty penetrating the dentine tubules. In this study, the calcium hydroxide was in close contact with the bacteria and could diffuse through the agar plate, resulting in an enhanced inhibitory effect.

Based on our study, the inhibition zones of all medicament groups were formed from day one and were still stable until day seven, showing that the samples were still active during all evaluations. In contrast, a study on the antibacterial activity of *Nigella sativa* in various germination phases against clinical bacterial strains found that the effectiveness of *Nigella sativa* extracts was highest on the fifth and eleventh days. However, between the fifth and eleventh days, the antibacterial inhibition of *Nigella sativa* extract decreased.¹⁵ Another study also reported that the components of *Nigella sativa* extract such as tannins, saponins, phenols, and terpenoids concentrations decreased from the third until the seventh day of the observation.¹⁷ However, *Nigella sativa* as a single component without the addition of calcium hydroxide had a lower inhibitory zone than those with the addition of calcium hydroxide. Thymoquinone is the bioactive constituent of *Nigella sativa*, which is reported to have an antimicrobial effect.^{8–10} Other constituents with the same effect include thymol,

monoterpene, and tannin. The mechanism of action of *Nigella sativa* depends on its ability to penetrate through the bacterial membrane and damage the integrity of the bacteria cells. Gram-negative bacteria have good permeability defences due to their outer membrane structure. Thus, the *Nigella sativa* extract is more effective against gram-positive bacteria.^{19,20}

Calcium hydroxide can release stable hydroxyl ions for up to 14–21 days, maintaining the environment's alkalinity. Therefore, this intracanal medicament can be used for long-term interappointment dressings, which is in line with the results of this study. However, we did not observe any enhancement of calcium hydroxide's inhibitory effect when adding *Nigella sativa* extract. The combination did not seem to have a synergic effect from the inhibitory zone evaluation in all ratios evaluated because all combinations had a lower inhibitory effect. Also, the 50:50 ratio did not differ significantly compared to calcium hydroxide.

Looking at the inhibitory effect between the different combination ratios, calcium hydroxide combined with *Nigella sativa* extract in 50:50 ratio indicates a higher inhibitory effect than any other combination ratios or *Nigella sativa* extract alone. Compared to different ratios, this combination was the only combined material with a significantly higher inhibitory zone than *Nigella sativa* extract alone. However, compared to calcium hydroxide, this combination had a significantly lower inhibitory zone, confirming that the addition of *Nigella sativa* extract did not improve the inhibitory property of calcium hydroxide. The addition of the extract may impede the liberation of hydroxyl ions. The effectiveness of calcium hydroxide is strongly related to the dissociation of calcium hydroxide to hydroxyl and calcium ions.^{21,22} In our present study, the addition of *Nigella sativa* extract decreased the inhibitory effect of calcium hydroxide. One possibility is that the addition of extract inhibits the hydroxyl ion release and contributes to a lower pH generated by the calcium hydroxide. This can be further investigated by evaluating the hydroxyl ion release and pH of the samples.

The method used in this study could affect penetration ability and contribute to the active component's success to diffuse through the agar plate. When preparing the samples, we noticed that the combination with a ratio of 50:50 formed a homogenous paste consistency and had better flowability than other ratios, affecting the agar plate's penetration ability. However, the conditions between in vitro and oral cavity will be different. Calcium hydroxide can maintain its pH level for sustained periods in the media compared to infected teeth. It will not be influenced by the buffering effect of dentinal fluid and hydroxyapatite content. This causes the antibacterial efficacy of calcium hydroxide to survive for a long time on the agar media.²⁰ Another limitation of this study is the simplified in vitro condition on agar media compared to the complex root canal system in vivo, which will affect the antimicrobial property. Therefore, further study will be needed before using the results clinically.

The evaluation methods or concentration could also be contributing to the results. An in vitro study reported a moderate ability of thymoquinone to reduce biofilm formation to *Staphylococcus aureus* and *Staphylococcus epidermidis*. In contrast, the thymoquinone concentration needs to be doubled for the same settings to have the same effect on *E. faecalis*. This showed that the effectiveness of *Nigella sativa* extract as an antibacterial agent depends on its concentration.¹² According to a study, the minimum concentration of thymoquinone to inhibit *E. faecalis* is 256 µg/ml.¹³ In our present study, we use a total crude extract without evaluating different concentrations. This was based on a previous study reporting that 100% extract had better antibacterial action against *Enterococcus* than 25%, 50% and 75%.¹¹ However, this concentration was used for *Nigella sativa* without combining the extract with calcium hydroxide. This conclusion could be improved by a further study using different concentrations of *Nigella sativa* extract, such as by serial dilution of the extract. Dilution may provide a more aqueous vehicle for the dissociation of calcium hydroxide ions. Active component evaluation of the extract should also be conducted to confirm the concentration of thymoquinone on the extract, which could also be a contributing factor.

Nigella sativa extract and its combination with calcium hydroxide can provide antibacterial effects to *Enterococcus faecalis* as a common bacterium in persistent infections. The inhibitory effect of *Nigella sativa* extract and its combination with calcium hydroxide is not more significant than calcium hydroxide itself. In conclusion, *Nigella sativa* extract combined with calcium hydroxide did not improve calcium hydroxide antimicrobial properties against *E. faecalis*. A combination of 50:50 ratio provides a better inhibitory zone compared to other combinations.

ACKNOWLEDGEMENTS

The authors are grateful to the Faculty of Dentistry, Universitas Jenderal Achmad Yani (UNJANI), Indonesia, in collaboration with the Faculty of Dentistry, Universitas Padjadjaran, Indonesia, for supporting this study and also to the LPPM UNJANI.

REFERENCES

- Mohammadi Z, Dummer PMH. Properties and applications of calcium hydroxide in endodontics and dental traumatology. *Int Endod J*. 2011; 44(8): 697–730.
- Zakaria MN, Takeshita T, Shibata Y, Maeda H, Wada N, Akamine A, Yamashita Y. Microbial community in persistent apical periodontitis: A 16S rRNA gene clone library analysis. *Int Endod J*. 2015; 48(8): 717–28.
- Zargar N, Marashi MA, Ashraf H, Hakopian R, Beigi P. Identification of microorganisms in persistent/secondary endodontic infections with respect to clinical and radiographic findings: Bacterial culture and molecular detection. *Iran J Microbiol*. 2019; 11(2): 120–8.
- Ba-Hattab R, Al-Jamie M, Aldreib H, Alessa L, Alonazi M. Calcium hydroxide in endodontics: an overview. *Open J Stomatol*. 2016; 06(12): 274–89.
- Zakaria MN. Save the pulp is the essential issues on pulp capping treatment. *J Dentomaxillofacial Sci*. 2016; 1(2): 73–6.
- Vasudeva A, Sinha DJ, Tyagi SP, Singh NN, Garg P, Upadhyay D. Disinfection of dentinal tubules with 2% Chlorhexidine gel, calcium hydroxide and herbal intracanal medicaments against *Enterococcus faecalis*: An in-vitro study. *Singapore Dent J*. 2017; 38: 39–44.
- Lima RKP, Guerreiro-Tanomaru JM, Faria-Júnior NB, Tanomaru-Filho M. Effectiveness of calcium hydroxide-based intracanal medicaments against *Enterococcus faecalis*. *Int Endod J*. 2012; 45(4): 311–6.
- Ahmad A, Husain A, Mujeeb M, Khan SA, Najmi AK, Siddique NA, Damanhouri ZA, Anwar F. A review on therapeutic potential of *Nigella sativa*: A miracle herb. *Asian Pac J Trop Biomed*. 2013; 3(5): 337–52.
- Mahmoudvand H, Sepahvand A, Jahanbakhsh S, Ezatpour B, Ayatollahi Mousavi SA. Evaluation of antifungal activities of the essential oil and various extracts of *Nigella sativa* and its main component, thymoquinone against pathogenic dermatophyte strains. *J Mycol Med*. 2014; 24(4): e155–61.
- Shokri H. A review on the inhibitory potential of *Nigella sativa* against pathogenic and toxigenic fungi. *Avicenna J phytomedicine*. 2016; 6(1): 21–33.
- Nader MI, Al-Thwaini AN, Abdul-Hassan IA, Ali AW. Effect of *Nigella sativa* (Black seed), *Salvadora persica* (Siwak) and Aluminum potassium sulphate (Alum) Aqueous extracts on isolated bacteria from teeth root canal. *Iraqi J Biotech*. 2010; 9(1): 99–104.
- Chaieb K, Kouidhi B, Jrah H, Mahdouani K, Bakhrouf A. Antibacterial activity of Thymoquinone, an active principle of *Nigella sativa* and its potency to prevent bacterial biofilm formation. *BMC Complement Altern Med*. 2011; 11(1): 29.
- Kouidhi B, Zmantar T, Jrah H, Souiden Y, Chaieb K, Mahdouani K, Bakhrouf A. Antibacterial and resistance-modifying activities of thymoquinone against oral pathogens. *Ann Clin Microbiol Antimicrob*. 2011; 10: 29.
- Abbaszadegan A, Sahebi S, Gholami A, Delroba A, Kiani A, Iraj A, Abbott PV. Time-dependent antibacterial effects of Aloe vera and *Zataria multiflora* plant essential oils compared to calcium hydroxide in teeth infected with *Enterococcus faecalis*. *J Investig Clin Dent*. 2016; 7(1): 93–101.
- Islam MH, Ahmad IZ, Salman MT. Antibacterial activity of *Nigella sativa* seed in various germination phases on clinical bacterial strains isolated from human patients. *E3 J Biotechnol Pharm Res*. 2012; 4(1): 8–13.
- Attia DA, Farag AM, Afifi IK, Darrag AM. Antimicrobial effect of different intracanal medications on various microorganisms. *Tanta Dent J*. 2015; 12(1): 41–7.
- Dianat O, Saedi S, Kazem M, Alam M. Antimicrobial activity of nanoparticle calcium hydroxide against *enterococcus faecalis*: An in vitro study. *Iran Endod J*. 2015; 10(1): 39–43.
- Sangalli J, Jardim Júnior EG, Bueno CRE, Jacinto RC, Sivieri-Araújo G, Filho JEG, Cintra LTÂ, Dezan Junior E. Antimicrobial activity of psidium cattleianum associated with calcium hydroxide against *enterococcus faecalis* and *candida albicans*: An in vitro study. *Clin Oral Investig*. 2018; 22(6): 2273–9.
- Farkhondeh T, Samarghandian S, Shahri AMP, Samini F. The neuroprotective effects of thymoquinone: A review. *Dose-Response*. 2018; 16(2): 1–11.
- Ugur AR, Dagi HT, Ozturk B, Tekin G, Findik D. Assessment of in vitro antibacterial activity and cytotoxicity effect of *Nigella sativa* oil. *Pharmacogn Mag*. 2016; 12(47): S471–4.
- Zakaria MN, Siti Halimah IR, Sidiqa AN, Artilia I, Cahyanto A. Antimicrobial activity of calcium hydroxide synthesized from Indonesian limestone against *Enterococcus faecalis*. *Mater Sci Forum*. 2021; 1044: 171–7.
- Guerreiro-Tanomaru JM, Chula DG, Lima RK de P, Berbert FLVC, Tanomaru-Filho M. Release and diffusion of hydroxyl ion from calcium hydroxide-based medicaments. *Dent Traumatol*. 2012; 28(4): 320–3.