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(Research Article)

# Epigallocatechin-3-gallate combination on the growth of *Enterococcus faecalis* and cytotoxicity on fibroblast cells

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## Abstract

**Background:** Calcium hydroxide is an ingredient that is often used in pulp capping procedures. Calcium hydroxide can cause chronic inflammation and has weak antibacterial properties against Enterococcus faecalis. Epigallocatechin-3-gallate (EGCG) has anti-inflammatory properties and antibacterial properties against Enterococcus faecalis bacteria. Materials applied to the oral cavity were non-cytotoxic, biocompatible and antibacterial.

**Purpose:** To determine the inhibitory effect against Enterococcus faecalis bacterial growth and cytotoxic to fibroblast cells of calcium hydroxide and EGCG combination with various concentrations of EGCG solution 5 µg/mL, 10 µg/mL, 20 µg/mL.

**Method:** Calcium hydroxide mixed with EGCG with EGCG concentration in distilled water, at 5 µg/mL, 10 µg/mL, 20 µg/mL concentrations. The inhibitory test was tested using Petri dishes with Mueller-Hinton agar, using a dilution method. Observation of the inhibition zone at Petri dishes was carried out by measuring the diameter around the well using calipers. The cytotoxicity test was tested on BHK-21 fibroblast cells using the MTT assay test. The Formazan optical density test indicated the number of live cells.

**Result:** The result of the inhibitory test showed a significant difference between the calcium hydroxide-EGCG group. The cytotoxicity test showed that there was no significant difference in the percentage of living fibroblasts, after administration of the calcium hydroxide-EGCG combination.

**Keywords:** Calcium Hydroxide; Epigallocatechin-3-Gallate; Cytotoxicity; Fibroblasts; *Enterococcus faecalis*

## 1. Introduction

Caries is a localized progressive tooth decay, caused by bacteria that form complex polymicrobial biofilms. Caries that reach the dentine cause pulpal inflammation due to bacterial invasion into the pulp through the dentinal tubules [1,2]. The inflammatory process is the body's defense process when the body is injured due to bacteria, trauma, chemicals, or others [3]. The inflammatory process is not able to heal damage to the pulp because the pulp has limited self-healing abilities so adequate treatment is necessary [4]. Direct pulp capping is a treatment to prevent the entry of bacteria into the pulp, promote soft tissue healing, and repair hard tissue that has been exposed in the oral cavity so that the vitality of the pulp tissue can be maintained [1, 4].

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Calcium hydroxide ($Ca(OH)_2$) is a material that is widely used in pulp capping treatments. Calcium hydroxide has various advantages, which can trigger healing and the formation of reparative dentin, has bactericidal and bacteriostatic properties. Lack of calcium hydroxide is a weak antibacterial property against *Enterococcus faecalis* [5].

*Enterococcus faecalis* is commonly found in deep carious lesion cavities and is the most common cause of secondary infection in endodontic treatment [6, 7, 8]. Calcium hydroxide has weak antibacterial properties against *Enterococcus faecalis* and can cause chronic inflammation, in vivo cell necrosis, and superficial pulpal necrosis [4, 9, 10]. *Epigallocatechin-3-gallate* has antibacterial and anti-inflammatory effects that are expected to compensate for calcium hydroxide deficiency.

*Epigallocatechin-3-gallate* is the most active polyphenolic catechin component of green tea which has antibacterial, antioxidant and anti-inflammatory properties [11]. *Epigallocatechin-3-gallate* has been shown to inhibit the growth of *Enterococcus faecalis* [12]. *Epigallocatechin-3-gallate* (EGCG) shows an antioxidant effect by carrying out radical scavenging of reactive oxygen species (ROS) [13]. Materials used in dentistry must be biocompatible, so a cytotoxicity test is necessary [14]. Based on the background, it is necessary to conduct research on the combination of calcium hydroxide and EGCG in the inhibitory test on *Enterococcus faecalis* bacteria and the cytotoxicity test on fibroblast cells.

The EGCG-calcium hydroxide ratio used in this study was based on research conducted by Yuanita et al (2020) regarding the effect of calcium hydroxide combined with green tea extract and cocoa fruit peel extract, on the activity of MAPK P38 and reparative dentin [15]. The ratio of calcium hydroxide to distilled water in the study by Yuanita et al (2020) was 0.1 g : 0.1 mL [15]. The concentration of EGCG in distilled water was based on research conducted by Lee and Tan (2015), regarding the effect of EGCG on *Enterococcus faecalis* bacteria [12]. The study showed that EGCG was able to inhibit growth and kill *Enterococcus faecalis* bacteria, at concentrations of 5 μg/mL and 20 μg/mL. The ratio of calcium hydroxide and EGCG in this study was 0.1 g : 0.1 mL, with various concentrations of EGCG in distilled water (5 μg/mL, 10 μg/mL, 20 μg/mL). This study aimed to determine the inhibitory effect against Enterococcus faecalis bacterial growth and cytotoxic to fibroblast cells of calcium hydroxide and EGCG combination with various concentrations of EGCG solution 5 μg/mL, 10 μg/mL, 20 μg/mL.

## 2. Material and methods

The inhibition test and cytotoxicity test used calcium hydroxide (Hidróxido cálcio P.A., Biodinamica, Brazil) and *Epigallocatechin-3-gallate* (E4143 Sigma Aldrich, Germany). The growth inhibition test used *Enterococcus faecalis* ATCC 2921, in BHIB (Brain Heart Infusion Broth) media and Mueller-Hinton media. The equipment used included Petri dishes, branders and matches, wire loops, wire swabs, spreaders, test tubes and test tube racks, sterile rings (diameter 5 mm), glass slab, spatula cement, vibrator, incubator, analytical balance, tweezers, and calipers.

The cytotoxicity test of the combination of calcium hydroxide and EGCG on fibroblast cells used BHK-21 fibroblast cells, distilled water, Eagle Dulbecco's modification minimum essential medium (DMEM), phosphate buffer saline (PBS), trypsin EDTA, MTT powder, and dimethyl sulfoxide (DMSO). The equipment used in the cytotoxicity test included analytical balances, thick glass slabs, ring-shaped plastic molds (5 mm diameter & 2 mm height), glass beakers, cement spatulas, plastic filling instruments, $CO_2$ incubators, micropipettes, Petri dishes, 15 ml conical tubes, Eppendorf, 3 ml syringes, 0.2 μm pore minisart filters (Sartorius, Germany), a centrifuge, an inverted microscope, a 96-well microplate, a plate shaker, and an ELISA reader.

The inhibition and cytotoxicity test has been declared to be ethically appropriate by Universitas Airlangga Faculty of Dental Medicine Health Research Ethical Clearance Commission. Ethical clearance certificate number 233/HRECC.FODM/V/2022 (inhibition test) and 538/HRECC.FODM/VIII/2022 (Cytotoxicity test).

### 2.1. Inhibitory Test

Preparation of a combination sample of calcium hydroxide ($Ca(OH)_2$) and EGCG with various concentrations of EGCG 5 μg/mL, 10 μg/mL, 20 μg/mL (Table 1).

Table 1 shows repetition 1 for each group. Each group was repeated 5 times. groups 3, 4, and 5 (0.1 mL EGCG solution) with respective concentrations of EGCG 5 μg/mL, 10 μg/mL, 20 μg/mL were mixed with 0.1 g calcium hydroxide powder, using a cement spatula, based on a glass slab, for 1 minute.

Ten Petri dishes containing Mueller-Hinton agar were divided into 3 equal parts by marking a line using a marker on the back of the Petri dishes. Samples of pure EGCG, pure calcium hydroxide, and in combination calcium hydroxide and EGCG with different concentrations were prepared. The *Enterococcus faecalis* culture was swabbed using a wire swab evenly on the Mueller-Hinton agar medium in Petri dishes, then wells were made using a sterile ring with a diameter of 5 mm.

**Table 1** Weight of calcium hydroxide and EGCG ingredients

| Group | EGCG concentration (µg/mL) | Material Weight | | | |
|---|---|---|---|---|---|
| | | $Ca(OH)_2$ (gram) | EGCG Solution (µg/mL) | | *Enterococcus faecalis* (CFU/mL) |
| | | | EGCG powder (µg) | Sterile Aquadest (mL) | |
| 1 | - | 0.1 | - | 0.1 | $1.5x10^8$ |
| 2 | - | - | 0.5 | 0.1 | $1.5x10^8$ |
| 3 | 5 µg/mL | 0.1 | 0.5 | 0.1 | $1.5x10^8$ |
| 4 | 10 µg/mL | 0.1 | 1 | 0.1 | $1.5x10^8$ |
| 5 | 20 µg/mL | 0.1 | 2 | 0.1 | $1.5x10^8$ |

*Epigallocatechin-3-gallate* as positive control 1 was included in the first well. Calcium hydroxide as positive control 2 was added to the second well. A combination of calcium hydroxide and EGCG with an EGCG concentration of 5 µg/mL was added to the third well. A combination of calcium hydroxide and 10µg/mL EGCG was added to the fourth well. A combination of calcium hydroxide and 20 µg/mL EGCG was added to the fifth well.

Petri dishes that have been filled with samples in the wells with each test material are incubated for 2 x 24 hours in an incubator at $37^oC$. Observation of the inhibition zone on Petri dishes was carried out by measuring the diameter formed around the well using calipers in millimeters (mm) and then dividing it by 5 to get the average.

### 2.2. Cytotoxicity test

Preparation of samples of the combination of calcium hydroxide and EGCG according to Table 2. Each group is mixed, stirred until the sample is homogeneous, and put into the mold. The sample is awaited until setting and removed from the mold. Samples were immersed in DMEM media, incubated, and filtered with 0.2 µm minisart. 50 µL of sample filtering results will be added to each well for the cytotoxicity test.

The cell splitting/passage process was carried out to multiply cells taken from BHK-21 fibroblast primary cells with a total of 5 passages. BHK-21 fibroblast cells in the incubated Petri dishes were observed under an inverted microscope with 100x magnification to ensure that the fibroblast cells that had been planted in each well were the same, with an amount of 1-1.2 x $10^3$ cells/mL.

BHK-21 fibroblast cells with a density of 1-1.2 x $10^3$ cells/mL were distributed in 96 well microplates which were divided into 5 treatment groups and each well was filled with 50 µL sample filtering results according to group division. Microplates were incubated in a 5% $CO_2$ incubator at 37°C for 24 hours.

A mixed solution of MTT and PBS was added to each well. The microplate was incubated again in a 5% $CO_2$ incubator at $37^0C$ for 4 hours. All DMEM culture media in the wells and the test material were discarded. Each well on the microplate was added with 50 µL of dimethyl sulfoxide (DMSO) to dissolve the formazan crystals. The microplate was then stirred using a plate shaker for 5 minutes until the formazan crystals dissolved. The microplate is inserted into the ELISA reader to see the optical density value for each well.

**Table 2** Details of the weight of calcium hydroxide and EGCG ingredients

| Group | EGCG concentration (μg/mL) | Material Weight | | | | |
|---|---|---|---|---|---|---|
| | | $Ca(OH)_2$ (gram) | EGCG Solution (μg/mL) | | BHK-21 fibroblast cells (cells/mL) | DMEM Media (μL) |
| | | | EGCG powder (μg) | Sterile Aquadest (mL) | | |
| 1 | - | 0.1 | - | 0.1 | 1-1.2x$10^3$ | 600 |
| 2 | - | - | 0.5 | 0.1 | 1-1.2x$10^3$ | 600 |
| 3 | 5 μg/mL | 0.1 | 0.5 | 0.1 | 1-1.2x$10^3$ | 600 |
| 4 | 10 μg/mL | 0.1 | 1 | 0.1 | 1-1.2x$10^3$ | 600 |
| 5 | 20 μg/mL | 0.1 | 2 | 0.1 | 1-1.2x$10^3$ | 600 |

## 3. Result

The inhibition test of the combination of calcium hydroxide and EGCG on the growth of *Enterococcus faecalis* has been carried out. The inhibition ability of the combination of calcium hydroxide and EGCG can be seen from the diameter of the resulting inhibition zone (Figure 1). The diameter of the growth inhibition zone for *Enterococcus faecalis*, on the calcium hydroxide and EGCG mixture, can be seen in Table 3.

**Figure 1** Inhibition zone for each group

The results of the research in Table 3.1 show that all samples formed inhibition zones. The smallest average diameter of the inhibition zone in the control group 1 calcium hydroxide was 16.11 mm. The largest mean diameter was 26.61 mm for group 5.

Inhibition test results, for the combination of calcium hydroxide and EGCG on *Enterococcus faecalis* bacteria, were normally distributed and homogeneous, so it was followed by one way ANOVA analysis and Tukey HSD post-hoc test. The results of one way ANOVA analysis showed that the combination of calcium hydroxide and EGCG produced a significant inhibition zone on the growth for *Enterococcus faecalis*. The results of the Tukey HSD post-hoc test analysis showed that there were significant differences between the sample groups.

The cytotoxicity test of the calcium hydroxide-EGCG combination on fibroblast cells was carried out using the MTT assay method with the reading of the results through an ELISA reader with a wavelength of 595 nm (Figure 2). The results of observing and reading the absorbance values of this study through an ELISA reader divided into the treatment group and the control group can be seen in Table 4.

**Table 3** The mean diameter of the inhibition zone (mm) and the standard deviation

| Group | Number of Samples (n) | The Mean Diameter of The Inhibition Zone (mm) | Standard Deviation |
|---|---|---|---|
| 1 | 5 | 16.11 | 0.100 |
| 2 | 5 | 18.59 | 0.134 |
| 3 | 5 | 21.15 | 0.165 |
| 4 | 5 | 23.43 | 0.189 |
| 5 | 5 | 26.61 | 0.249 |

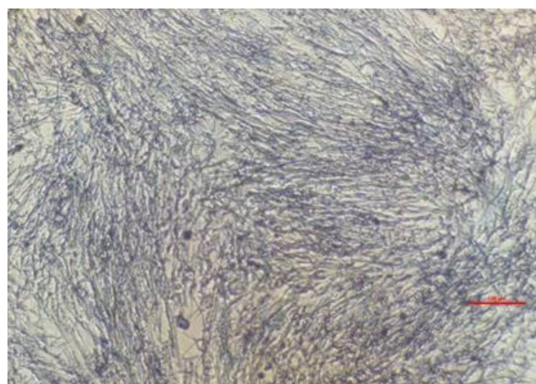

**Figure 2** The results of the MTT group 5 assay test

Table 4 shows that the highest percentage of living cells was in group 5, namely calcium hydroxide-EGCG with an EGCG concentration of 20 μg/mL of 98.704%. The lowest percentage of living cells from the research data was found in group 1, namely calcium hydroxide of 89.471%. Live fibroblast cells marked in purplish blue will be seen with the help of an ELISA reader to measure the number of living cells.

The results of analysis of research data on the cytotoxicity test of the calcium hydroxide-EGCG combination on fibroblast cells with a normal and homogeneous distribution fulfill the requirements for the one way ANOVA test. The results of the one way ANOVA analysis showed that there were no significant differences in all sample groups. This means that statistically, living fibroblast cells in the five sample groups did not show a significant difference in average values

**Table 4** Sample size, mean optical density, percentage of live fibroblasts and standard deviation.

| Group | Number of Samples (n) | Average | | Standard Deviation |
|---|---|---|---|---|
| | | Optical Density | Percentage of Living Cells | |
| 1 | 4 | 0.4733 | 89.471% | 0.05312 |
| 2 | 4 | 0.4818 | 91.361% | 0.05432 |
| 3 | 4 | 0.4992 | 95.032% | 0.05218 |
| 4 | 4 | 0.5105 | 97.624% | 0.06636 |
| 5 | 4 | 0.5163 | 98.704% | 0.06252 |

## 4. Discussion

Research on the inhibition test of the combination of calcium hydroxide and *Epigallocatechin-3-gallate* (EGCG) with various EGCG concentrations of 5 μg/mL, 10 μg/mL, and 20 μg/mL on the growth of *Enterococcus faecalis* has been carried out. The research data in Table 3 shows that the mean diameter of the smallest inhibition zone in group 1 (calcium hydroxide positive control) was 16.11 mm. The largest mean diameter of the inhibition zone was 26.61mm in

group 5, namely the combination of calcium hydroxide and EGCG with an EGCG concentration of 20 μg/mL. Data in group 5 showed that the combination of calcium hydroxide and EGCG at this concentration had a very strong inhibitory effect.

The results of one way ANOVA data analysis in Table 3 show that there are significant differences in all sample groups. Administration of a combination of calcium hydroxide and EGCG produced an inhibition zone against *Enterococcus faecalis.*

The results of research data analysis using the Tukey HSD post hoc test showed that there were significant differences in the mean diameter of the inhibition zone for *Enterococcus faecalis* growth between sample groups. There is a significant difference between groups 1 and 2 which are the control group. group 2 had stronger inhibition than group 1 based on the average diameter of the inhibition zone. The mean diameter of the inhibition zone for group 1 (calcium hydroxide control) was 16.11 mm, while the mean diameter of the inhibition zone for group 2 for the EGCG control was 18.59 mm. *Epigallocatechin-3-gallate* has a stronger inhibition power than calcium hydroxide because of the EGCG antibacterial mechanism through hydroxyl radical ions which can damage the lipid, protein and DNA components of bacteria so that it is more effective in inhibiting the growth of *Enterococcus faecalis* [12]. The antibacterial properties of calcium hydroxide are weaker than EGCG due to the antibacterial mechanism of calcium hydroxide by means of dissociation of calcium and hydroxyl ions which increase the environmental pH to become alkaline. Alkaline pH conditions cause damage to phospholipids in bacterial membranes so that bacterial metabolism and replication are disrupted. *Enterococcus faecalis* is able to survive at an alkaline pH produced by calcium hydroxide through a proton pump mechanism. *Enterococcus faecalis* when in an alkaline pH environment will pump protons through the cytoplasmic membrane to maintain the internal pH of the bacteria. This causes calcium hydroxide to be less effective against *Enterococcus faecalis* [16].

The mean diameter of the inhibition zones of the control groups 1 and 2 compared to the combination treatment group of calcium hydroxide and EGCG, namely groups 3, 4 and 5, had significant differences. groups 3, 4 and 5 had stronger inhibition than groups 1 and 2 based on the average diameter of the inhibition zone. The combination of calcium hydroxide and EGCG has a stronger inhibition because the antibacterial properties of calcium hydroxide synergize with the antibacterial properties of EGCG. The antibacterial properties of calcium hydroxide are obtained from the hydroxyl ions produced through the ionization process of $Ca(OH)_2$ into calcium ions and hydroxyl ions. These hydroxyl ions are able to change the pH of the environment to become alkaline [17]. The alkaline pH conditions produced by calcium hydroxide damage the phospholipids in the bacterial cell membrane. The alkaline pH conditions produced by calcium hydroxide also disrupt the pH gradient of the cytoplasmic membrane, causing protein denaturation [18]. The negative charge of EGCG binds to the positive charge on the *Enterococcus faecalis* cell membrane thereby damaging the lipid layer of the bacteria [19]. *Epigallocatechin-3-gallate* via the hydroxyl radical formed from the Fenton reaction. EGCG hydroxyl radicals enter *Enterococcus faecalis* cells and damage cellular components such as lipids, proteins, and DNA so that they can kill bacteria [12]. The alkaline pH condition produced by calcium hydroxide helps the performance of EGCG inhibition. *Epigallocatechin-3-gallate* can work optimally at alkaline pH [20].

The mean diameter of the inhibition zones in the 3$^{rd}$, 4$^{th}$, and 5$^{th}$ treatment groups showed a very strong inhibition based on the classification of the inhibition zones in the study of Ouchari et al. (2019, p. 3) which is more than 20 mm [21]. Between treatment groups 3, 4, and 5 showed significant differences. The mean diameters of the inhibition zones in the treatment groups 3, 4 and 5 showed an increase of respectively 21.15 mm, 23.43 mm and 26.61 mm. These results were in line with the increase in EGCG concentration in the combination of calcium hydroxide and EGCG which was directly proportional to the increase in the average diameter of the inhibition zone. The higher the concentration of EGCG in the combination of calcium hydroxide and EGCG, the stronger the inhibition of *Enterococcus faecalis* bacteria. This shows that the addition of EGCG in combination with calcium hydroxide can increase the inhibitory ability of calcium hydroxide against *Enterococcus faecalis* bacteria.

The cytotoxicity test of the calcium hydroxide-EGCG combination on fibroblast cells with EGCG concentrations in distilled water of 5 μg/mL, 10 μg/mL, 20 μg/mL has been carried out. The parameter used in this study is the parameter Inhibitory Concentration 50% (IC50). The IC50 parameter is the concentration of a substance that can inhibit cell proliferation up to 50% of the population [22]. In Table 4 it can be seen that living fibroblast cells with a value of more than 50% are present in all groups. Based on the IC50 parameter, it can be interpreted that all groups are not cytotoxic to fibroblast cells.

The results of calculating the percentage of living cells in Table 4 show that calcium hydroxide (Group 1) has the lowest average percentage of living cells (89.471%) compared to other sample groups. This is due to the high pH of calcium hydroxide which causes damage to the mitochondria present in fibroblast cells. Mitochondria in fibroblast cells will

balance these conditions by increasing the respiration process which causes an increase in the number of Reactive Oxygen Species (ROS). Prolonged high levels of ROS will induce cell damage [23]. In this study, the percentage of living cells in group 1, namely calcium hydroxide, had the lowest percentage compared to the percentage of living cells in the other sample groups, but the results of statistical calculations showed that there was no significant difference between the sample groups.

The results of calculating the percentage of living cells in Table 4 show that *Epigallocatechin-3-gallate* (EGCG) (Group 2) has an average percentage of living cells of 91.381%. This is because EGCG has antioxidant properties [24]. The antioxidant properties of EGCG bind free radicals in two ways, namely by binding directly and indirectly. *Epigallocatechin 3-gallate* (EGCG) exhibits antioxidant properties directly by binding to free radicals (ROS) via the -OH group. The -OH group binds to free radicals indirectly by regulating pathways that regulate ROS and enzyme clearance [25].

Table 4 shows that group 3, group 4, and group 5 had an average percentage of living cells, respectively, 95.032%, 97.624% and 98.704%. The mean percentage of living cells increased with increasing EGCG concentration in distilled water (5 µg/mL, 10 µg/mL, 20 µg/mL) in the calcium hydroxide-EGCG combination. This is because EGCG has antioxidant properties which can reduce free radicals (ROS) as a result of balancing the alkaline conditions of calcium hydroxide by mitochondria. The combination of calcium hydroxide-EGCG can increase the potential for fibroblast cells to survive. In this study it was proven that increasing the concentration of EGCG in distilled water (5 µg/mL, 10 µg/mL, 20 µg/mL) in the calcium hydroxide-EGCG combination caused the percentage of living cells to increase, although there was no statistically significant difference. The Limitation of this study is we only examine the killing ability of combination.

## 5. Conclusion

Based on the results of the study it can be concluded that the combination of calcium hydroxide and *Epigallocatechin-3-gallate* (EGCG) with EGCG concentrations in distilled water 5 µg/mL, 10 µg/mL, 20 µg/mL has a very strong inhibitory effect on the growth of *Enterococcus faecalis* bacteria which increases according to with increased concentrations of EGCG and not cytotoxic to fibroblast cells.

## Compliance with ethical standards

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### *Disclosure of conflict of interest*

The authors declare no conflict of interest.

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