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Original Article

The physical characteristics and antibacterial properties of a combination of ellagic acid and calcium hydroxide

[Características físicas y propiedades antibacterianas de una combinación de ácido elágico e hidróxido de calcio]

Intan Nirwana[1*], Titien Hary Agustantina[1], Elly Munadziroh[1], Syafira Hasnarani Putri Baskoro[2], Risyanuari Saufika[2], Nahdliyah Lifah Nur Islami[2], Imanina Akbarilly Putri[2], Naufal Zhaffrano Razan[2], Meircurius Dwi Condro Surboyo[3], Khairul Anuar Shariff[4]

[1]Department of Dental Materials, Faculty of Dental Medicine, Airlangga University, Surabaya, Indonesia.
[2]Bachelor of Dental Science Student. Faculty of Dental Medicine, Airlangga University, Surabaya, Indonesia.
[3]Department of Oral Medicine, Faculty of Dental Medicine, Airlangga University, Surabaya, Indonesia.
[4]Biomaterial Niche Area, School of Materials and Mineral Resource Engineering, Universiti Sains Malaysia, Pulau Pinang, Malaysia.
*E-mail: intan-n@fkg.unair.ac.id

Abstract

Context: Ellagic acid is a natural pomegranate compound with potent anti-inflammatory properties. A combination of ellagic acid and bone-stimulating material in bone remodelling plays a vital role in suppressing inflammation and resulting in bone formation. However, the characteristic of the mixture of ellagic acid and bone-stimulating material, like calcium hydroxide, needs to be established, especially its physical attributes and antibacterial properties.

Aims: To analyse the characteristics of three different ratios of combinations of ellagic acid and calcium hydroxide, including compound, compressive strength, acidity, calcium ion release and antibacterial properties of *Streptococcus mutans*.

Methods: Using distilled water solvent, the combination of ellagic acid and calcium hydroxide was made with three different ratios, 1:99, 3:97 and 5:95. Each combination was created as a sample mould with 4 mm in diameter. At the same time, the control group only had calcium hydroxide. The identified compound of ellagic acid was analysed using the compressive strength of the Fourier transform infrared (FTIR), the compressive strength using an autograph compression strength tester, and acidity and calcium release using Inductively Coupled Plasma Optical Emission Spectroscopy (ICP-OES), while the antibacterial properties used diffusion methods. The differences in each characteristic were analysed using a one-way ANOVA test and Tukey's HSD post hoc test, with $p<0.05$ considered significantly different.

Results: The combination of ellagic acid and calcium hydroxide showed a functional group of hydroxyls (-OH) and carbonate (CO_3^{2-}). The combination with a ratio of 1:99 showed a higher compressive strength, alkaline acidity, calcium release and higher inhibition to *Streptococcus mutans* than combination 3:97 and 5:95 ($p<0.05$).

Conclusions: The combination of ellagic acid and calcium hydroxide showed a promising characteristic for use in bone regeneration. The lower concentration of ellagic acid (1:99) showed better characteristics, including acidity, compressive strength and antibacterial properties to *Streptococcus mutans*.

Keywords: antibacterial; calcium hydroxide; ellagic acid; pulp capping.

Resumen

Contexto: El ácido elágico es un compuesto natural de la granada con potentes propiedades antiinflamatorias. La combinación de ácido elágico y material estimulante del hueso en la remodelación ósea desempeña un papel vital en la supresión de la inflamación y en la formación de hueso. Sin embargo, es necesario establecer las características de la mezcla de ácido elágico y material estimulante óseo, como el hidróxido de calcio, especialmente sus atributos físicos y propiedades antibacterianas.

Objetivos: Analizar las características de tres proporciones diferentes de combinaciones de ácido elágico e hidróxido de calcio, incluyendo el compuesto, la resistencia a la compresión, la acidez, la liberación de iones de calcio y las propiedades antibacterianas del *Streptococcus mutans*.

Métodos: Utilizando disolvente de agua destilada, se elaboró la combinación de ácido elágico e hidróxido de calcio con tres proporciones diferentes, 1:99, 3:97 y 5:95. Cada combinación se creó como un molde de muestra de 4 mm de diámetro. Al mismo tiempo, el grupo de control sólo tenía hidróxido de calcio. El compuesto identificado de ácido elágico se analizó mediante la resistencia a la compresión del infrarrojo por transformada de Fourier (FTIR), la resistencia a la compresión mediante un probador de resistencia a la compresión autógrafa, y la acidez y la liberación de calcio mediante espectroscopia de emisión óptica de plasma acoplado inductivamente (ICP-OES), mientras que para las propiedades antibacterianas se utilizaron métodos de difusión. Las diferencias en cada característica se analizaron mediante una prueba ANOVA unidireccional y la prueba post hoc HSD de Tukey, considerándose $p<0,05$ significativamente diferente.

Resultados: La combinación de ácido elágico e hidróxido cálcico mostró un grupo funcional de hidroxilos (-OH) y carbonato (CO_3^{2-}). La combinación con una proporción de 1:99 mostró una mayor resistencia a la compresión, acidez alcalina, liberación de calcio y mayor inhibición al *Streptococcus mutans* que la combinación 3:97 y 5:95 ($p<0,05$).

Conclusiones: La combinación de ácido elágico e hidróxido de calcio mostró unas características prometedoras para su uso en regeneración ósea. La menor concentración de ácido elágico (1:99) mostró mejores características, incluyendo acidez, resistencia a la compresión y propiedades antibacterianas frente a *Streptococcus mutans*.

Palabras Clave: antibacteriano; hidróxido de calcio; ácido elágico; recubrimiento de pulpa.

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AUTHOR INFO
ORCID: 0000-0003-3523-431X (IN)
0000-0001-6044-2722 (THA)
0000-0002-1969-2380 (EM)
0000-0002-6052-2287 (MDCS)
0000-0003-3331-2505 (KAS)

INTRODUCTION

Calcium hydroxide is a widely used pulp capping material and is considered the gold standard due to its antibacterial property, which stimulates dentinal bridges. Forming a dentin bridge can help maintain pulp vitality (Accorinte et al., 2008). Calcium hydroxide has also been shown to stimulate bone formation and is a bone-stimulating material (Kostyiuk et al., 2021). However, the alkaline nature of calcium hydroxide can lead to inflammation (Zarei et al., 2016), especially in dental pulp, and may result in pulp necrosis (Pallotta et al., 2010). Furthermore, calcium hydroxide has poor physical properties and is dissolved easily in saliva (Pribadi et al., 2020). To improve its efficacy, combining calcium hydroxide with a material with anti-inflammatory properties like ellagic acid and hydroxyapatite (Nirwana et al., 2021) and propolis (Pribadi et al., 2020) may enhance bone formation.

The ellagic acid is an active substance from pomegranate extract. It possesses a strong anti-inflammatory (Rogerio et al., 2008) due to its ability to reduce the production of pro-inflammatory cytokines (Yuniarti et al., 2018), inhibiting the nuclear factor *kappa* beta 1 (NFκB1) and heat shock protein 70 (HSP70) pathways (Saputera et al., 2022). In the biological process, ellagic acid also strongly binds to toll-like receptors (TLR), especially TLR2 and TLR4, to show an anti-inflammatory (Saputera et al., 2020). Some modification was done in ellagic acid to enhance its anti-inflammatory properties, especially osteogenesis ability, by combining it with bone-stimulating material like calcium hydroxide, hydroxyapatite or calcium phosphate.

A preliminary study combined ellagic acid and calcium hydroxide at 1:99 and 3:97 demonstrated non-toxicity and maintained fibroblast cell proliferation (Nirwana et al., 2021). Another research reported that 93% of ellagic acid can promote bone formation by reducing the expression of tumour necrosis factor α (TNF-α) and increasing the expression of interleukin 10 (IL-10), bone morphogenetic protein 4 (BMP-4) and osteopontin (OPN), increasing bone formation (Primasari et al., 2022). Additionally, ellagic acid proved to enhance bone regeneration by increasing the expression of vascular endothelial growth factor (VEGF), fibroblast growth factor 2 (FGF-2) and alkali phosphatase (ALP) (Nirwana et al., 2022).

Although there are reports of the biological effect of a combination of ellagic acid and bone-stimulating material, there is a lack of information on the characteristics of the mixture, especially ellagic acid and calcium hydroxide, including its acidity, calcium ion release, and antibacterial properties that are important for its use as a pulp capping material. The antibacterial properties are particularly relevant because ellagic acid strongly binds to the TLR2 and TRL4, which are widely expressed in the oral mucosa and play an essential role in dental pulp (Hasan et al., 2022), mucosa (D'Souza et al., 2013) and alveolar bone regeneration (Deng et al., 2020). Therefore, this study aimed to analyse the characteristics of a combination of ellagic acid and calcium hydroxide using FTIR. To identify the component, evaluate acidity, calcium ion release and its antibacterial effects on *Streptococcus mutans*.

MATERIAL AND METHODS

Materials

The materials used for this experiment were ellagic acid (90%, Xi'an Biof Bio-Technology, Shaanxi, China), calcium hydroxide (Sigma Aldrich, Merck, Darmstadt, Germany), and sterile water.

Mould design

The compression strength test was carried out by making a sample mould built from a 1 mL insulin syringe measuring 4 mm in diameter, measured with a ruler to 6 mm in height, and then marked. The bottom of the sample mould was given a celluloid strip and placed on a glass slab. The sample mould was fixed using red wax on the outside of the mould.

Ellagic acid and calcium hydroxide combination

The ellagic acid and calcium hydroxide combination was produced by mixing calcium hydroxide powder, ellagic acid powder and sterile water, according to Table 1. The ellagic acid and calcium hydroxide powder were mixed using a mortar and pestle for 10 seconds. The calcium hydroxide powder and ellagic acid powder mixture were mixed with sterile water and stirred using a cement spatula on a glass slab for 30 seconds.

The control group was prepared by mixing 0.2 g of pure calcium hydroxide powder with 0.2 mL of distilled water and stirring the mixture using a cement spatula on a glass slab for 30 seconds. A plastic filling instrument inserted the combination into the sample mould. The dough was turned over from the mould and then given a celluloid strip. Finally, it received a thin glass layer, resulting in a weight of 50 g, and left to set.

Table 1. The ellagic acid and calcium hydroxide combinations.

Group	CH powder (g)	EA powder (g)	Sterile water (mL)	Total (g)
Pure CH (control)	0.200	-	0.2	0.2
EA and CH (1:99)	0.198	0.002	0.2	0.2
EA and CH (3:97)	0.194	0.006	0.2	0.2
EA and CH (5:95)	0.190	0.010	0.2	0.2

EA: ellagic acid; CH: calcium hydroxide.

FTIR analysis

An FTIR test commenced to determine the functional groups of calcium hydroxide, ellagic acid and the combination (Saputera et al., 2021). Each sample test (0.2g) was secured in the sample holder by clamping it. The FTIR program, OMNIC (OMNIC spectra, Thermo Scientific, Massachusetts, USA) was switched on and awaited until a dialogue box showed the sample identity. The background was then collected to calibrate the FTIR tool and to produce a standard graphic from the sample without entering the sample. The standard sample graph was obtained. The FTIR results made graphs that can be read by matching the peak table.

Compressive strength

A compression strength test was performed using an autograph compression strength tester (Shimadzu AG-10 TE, Japan*)* (Ranjbar Omrani et al., 2021). Each sample was placed in the centre of the press with the vertical axis of the sample perpendicular to the plane. The Autograph test device was activated, and a pressure of 1 kN was slowly applied with a speed of 5 mm/min, pressing the sample until crushed. The pressure component of the device stopped automatically when the sample was destroyed, and then the numbers were recorded. The figures stated in kgF were converted to Newtons and then divided by the cross-sectional area to obtain the compression strength in Pascal units. The test performed on each group was replicated six times.

Acidity and calcium release

Each sample was dissolved in a buffer solution and then calculated using a pH meter (OHAUS ST3100 pH meter, USA) at three different times for one, three, and seven days. Calcium release was measured using Inductively Coupled Plasma Optical Emission Spectroscopy (ICP-OES).

Antibacterial test

The stock culture of *Streptococcus mutans* was cultures in brain heart infusion broth (BHIB) and incubated for 24 hours at 37°C with a concentration of 1.5 × 10^8 CFU/mL according to the Standard McFarland (Ahrari et al. 2015). Once the required concentration was achieved, the bacteria were cultured on blood agar. The calcium hydroxide or combination with ellagic acid was then applied to blood agar and incubated for 24 hours at 37°C. The antibacterial properties were measured by a clear zone created by each test material and presented in millimetres (mm) using a digital calliper.

Statistical analysis

The data of compression strength was presented as mean ± standard deviation. The one-way ANOVA test and Tukey's HSD post hoc test were used for analysing the compression strength differences in each group, with $p<0.05$ considered significantly different. The SPSS version 24 (IBM SPSS Statistic 24 for Mac, USA) was used for the analysis.

RESULTS

FTIR analysis

Calcium hydroxide has two functional groups: hydroxyl (^-OH) and carbonate (CO_3^{2-}). The FTIR test of calcium hydroxide showed the presence of ^-OH at the peaks 3639.90 cm^{-1}, 2323.82 cm^{-1} and 1793.61 cm^{-1}. The CO_3^{2-} groups were characterised by peaks at 1409.54 cm^{-1} and 873.10 cm^{-1} (Fig. 1).

Ellagic acid has two functional groups: CO_3^{2-} and sulphate (SO_4^{2-}). The FTIR test of ellagic acid showed the presence of CO_3^{2-}, marked by peaks at 1445.80 cm^{-1} and 810.341 cm^{-1}. SO_4^{2-} was characterised by peaks at 1194.79 cm^{-1}, 1052.63 cm^{-1}, and 641.50 cm^{-1} (Fig. 1).

The ellagic acid and calcium hydroxide combination has two functional groups: ^-OH and CO_3^{2-}. The ^-OH functional group was characterised by peaks at 3639.69 cm^{-1}, 1979.85 cm^{-1}, and 1701.03 cm^{-1}. CO_3^{2-} was characterised by peaks at 1414.51 cm^{-1}, 1111.94 cm^{-1}, and 874.43 cm^{-1} (Fig. 1).

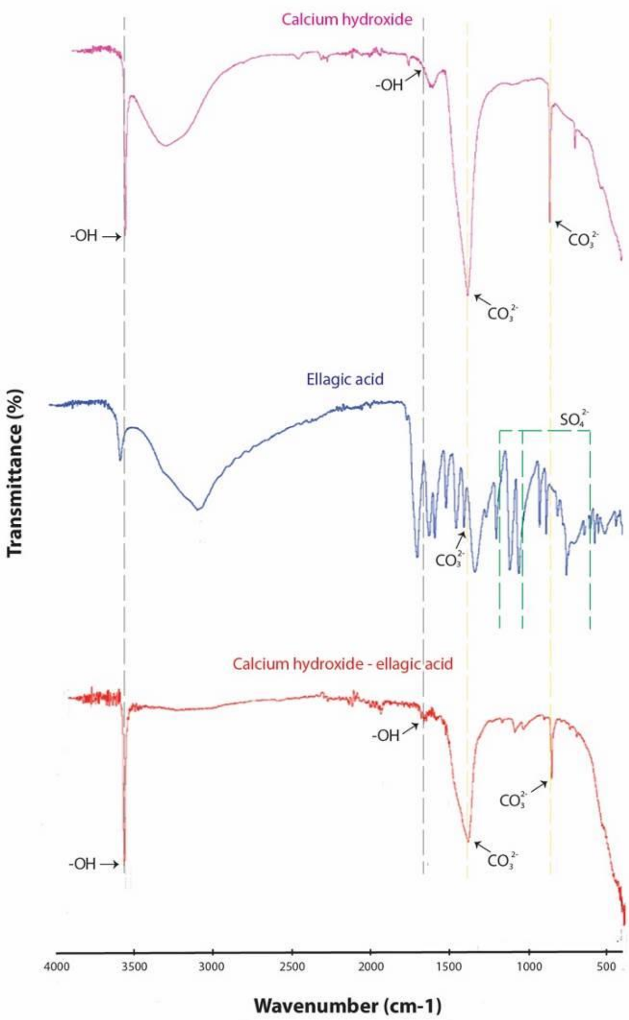

Figure 1. FTIR results analysis of calcium hydroxide, ellagic acid and a combination of both materials.

Table 2. The result of compression strength.

Group	Number of samples	Compressive strength (MPa)*
Pure CH (control)	6	0.79 ± 0.07[a]
EA and CH (1:99)	6	0.39 ± 0.10[a,b,c]
EA and CH (3:97)	6	0.34 ± 0.11[b]
EA and CH (5:95)	6	0.20 ± 0.007[c]

Data are presented as mean ± standard deviation *Same character indicated significant differences with Tukey HSD with $p<0.05$. EA: ellagic acid; CH: calcium hydroxide.

Compressive strength

The compression strength of pure calcium hydroxide (0.79 ± 0.07 MPa) is higher than the ellagic acid and calcium hydroxide ratio 1:99 (0.39 ± 0.10 MPa) ($p<0.05$). The ellagic acid and calcium hydroxide ratio 1:99 (0.39 ± 0.10 MPa) is higher than the ellagic acid and calcium hydroxide ratio 3:97 (0.34 ± 0.11 MPa) and the ellagic acid and calcium hydroxide ratio 5:95 (0.20 ± 0.007 MPa) ($p<0.05$) (Table 2).

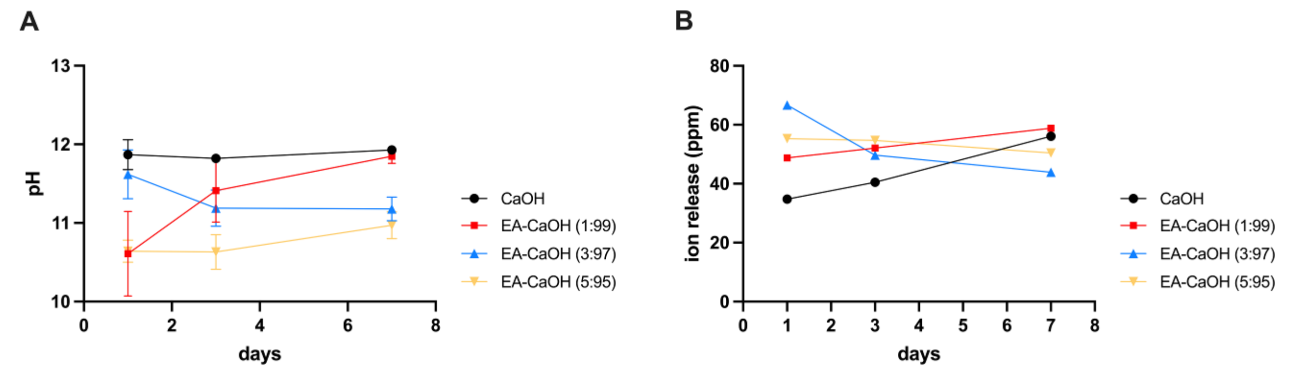

Figure 2. The pH value of a combination of ellagic acid and calcium hydroxide in three different observation times **(A)** and the calcium ion release **(B)**.

Data are presented as mean ± standard deviation.

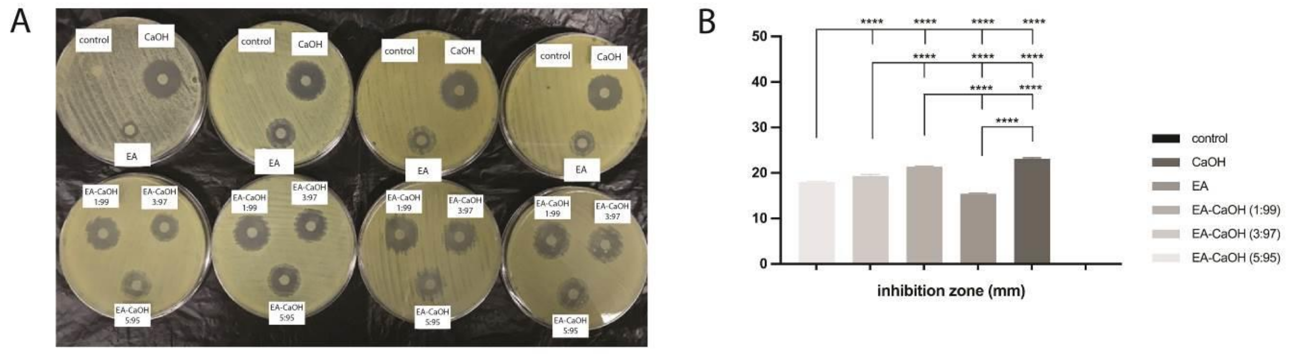

Figure 3. The diffusion test of antibacterial to *Streptococcus mutans*.

(A) The result of the diffusion test using blood agar. **(B)** The antibacterial is represented by a zone of inhibition (mm). Data are presented as mean ± standard deviation.Each test material was replicated as 4 replicates. ****p<0.05 with One-way ANOVA and Tukey HSD test. EA: ellagic acid; CaOH: calcium hydroxide.

Acidity and calcium release

The pH value of calcium hydroxide was stable during one, three, and seven days. The ellagic acid and calcium hydroxide ratios 1:99 and 5:95 increased the pH value after three and seven days, while the ratio 3:97 decreased (Fig. 2A). The calcium release, calcium hydroxide and ellagic acid and calcium hydroxide ratio 1:99 produced increased calcium release from one, three and seven days, while the ellagic acid and calcium hydroxide ratios 3:97 and 5:95 decreased from three and seven days (Fig. 2B).

Antibacterial

The combination of ellagic acid and calcium hydroxide antibacterial effect was more significant than ellagic acid alone ($p<0.05$). Conversely, the ellagic acid and calcium hydroxide mixture shows lower antibacterial properties than pure calcium hydroxide ($p<0.05$). Among the different ratios of ellagic acid and calcium hydroxide tested, the ratio of 1:99 showed a higher inhibition zone compared to the ratios of 3:97 and 5:95 ($p<0.05$) (Fig. 3).

DISCUSSION

The combination of ellagic acid and calcium hydroxide was stable at the point of this experiment. The presence of ellagic acid in the combination was shown by ^{-}OH and CO_3^{2-} in the peaks at 3639.69 cm^{-1}, 1979.85 cm^{-1} and 1701.03 cm^{-1}, which are within the hydroxyl range, i.e., between 3700–3600 cm^{-1}, 3550–3500 cm^{-1} and 3000–1700 cm^{-1}. CO_3^{2-} was included in the observation because it is in the carbonate bond range, which ranges between 1450–1410 cm^{-1} and 880–800 cm^{-1}. The CH contains ^{-}OH and CO_3^{2-} functional groups. ^{-}OH was detected on calcium hydroxide because calcium hydroxide includes the OH bond wave range resulting from hydrated inorganic compounds. Hydrated inorganic compounds come from the production process of the calcium hydroxide material with the addition of water. The water molecules are incorporated at 3800-3200 cm^{-1}, resulting from the stretching and bending of the O-H bond (Grunenwald et al., 2014), and CO_3^{2-} bonds at peaks 1409.54 cm^{-1} and 873.10 cm^{-1}. These two peaks are within the CO_3^{2-}

bond range, which ranges between 1450–1410 cm^{-1} and 880–800 cm^{-1}.

Ellagic acid is stable when combined with other materials, such as calcium hydroxide. Another experiment also showed that the ellagic acid could bind with calcium phosphate and not be lost when combining processes. The properties of ellagic acid were still strong (Saputera et al., 2021). The combination affected compression strength more than pure calcium hydroxide. The increased amount of ellagic acid decreased compression strength in combination form. This condition proved that the ellagic acid and calcium hydroxide (1:99) have higher compression strength than other ratios. This condition demonstrates that adding ellagic acid affects the decrease of compression strength following the research hypothesis. The acidity of the mixture can affect the reduction in the compressive strength of the ellagic acid and calcium hydroxide combination. Calcium hydroxide has an alkaline pH, while ellagic acid is weakly acidic. A previous research study showed that adding 5% pomegranate extract to calcium hydroxide can reduce the pH value (Cavalcanti et al., 2011).

This study used ellagic acid powder with a content of 90%, thus allowing a more significant pH decrease in the ellagic acid and calcium hydroxide combination. A material's acidic atmosphere can drastically reduce compression strength (Saghiri et al., 2013). Materials with lower acidity can cause changes in the microstructure of the material, which can increase porosity and affect the decrease in compressive strength. This is because the acidic atmosphere can inhibit the setting reaction, affect adhesion and increase dissolution in the material, affecting the material's mechanical properties, including the material microstructure (Deepthi et al., 2018). The higher the acidic atmosphere in the material mixture, the wider the material's porosity (Namazikhah et al., 2008). The above statement comes from the results of this study, i.e., the more ellagic acid is added to calcium hydroxide, the lower the compressive strength.

Differences in the ratio of ellagic acid and calcium hydroxide used caused the difference in pH in this study. The more ellagic acid used, the higher the pH obtained. Ellagic acid is acidic, so the combination increases pH and causes an increase in the release of hydroxyl ions. Increased secretion of hydroxyl ions will increase antibacterial activity because hydroxyl ions can damage the cell membrane of bacteria (Gandolfi, 2012). In bone regeneration, the release of ion calcium will be affected by tissue mineralisation because ion calcium is necessary for mineralisation, cell migration and cell differentiation. The accumulation of calcium ions can activate Ca-dependent ATPase, which plays a role in the mineralisation process (Silva et al., 2003).

This study represents a preliminary investigation into the characteristics and basic properties of ellagic acid and calcium hydroxide, explicitly focusing on their antibacterial properties. While we have confirmed the antibacterial properties in this preliminary study, it is essential to acknowledge certain limitations. Firstly, this research's preliminary nature may restrict our findings' generalizability. Further, to comprehensively elucidate the implications for human applications, additional in-vivo research is imperative. Such studies will enable a deeper understanding of the effects on human subjects and may uncover nuances not addressed in our preliminary in-vitro investigation. The data collected in this study regarding ellagic acid and calcium hydroxide characteristics, their antibacterial properties, and ion release serve as foundational knowledge for future research endeavours. These findings can guide subsequent studies, which may lead to the development of novel antibacterial applications or therapeutic interventions, like bone regeneration, and is also not limited to dental pulp regeneration.

CONCLUSION

The combination of ellagic acid and calcium hydroxide showed a promising characteristic for use in bone regeneration. The lower concentration of ellagic acid (1:99) showed more favourable characteristics, including acidity, compressive strength, and antibacterial properties to *Streptococcus mutants*.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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AUTHOR CONTRIBUTION:

Contribution	Nirwana I	Agustantina TH	Munadziroh E	Baskoro SHP	Saufika R	Islami NLN	Putri I A	Razan NZ	Surboyo MDC	Shariff KA
Concepts or ideas	x	x	x	x	x	x	x	x		
Design	x	x	x	x	x	x	x	x		
Definition of intellectual content	x	x	x	x	x	x	x	x		
Literature search	x	x	x	x	x	x	x	x	x	x
Experimental studies				x	x	x	x	x		
Data acquisition				x	x	x	x	x		
Data analysis	x	x	x						x	x
Statistical analysis	x	x	x						x	x
Manuscript preparation	x	x	x							
Manuscript editing									x	x
Manuscript review	x	x	x	x	x	x	x	x	x	

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