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Research Article

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Antimicrobial and cytotoxic properties of calcium-enriched mixture cement, Iranian propolis, and propolis with herbal extracts in primary dental pulp stem cells

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ABSTRACT

Objectives: In this study, natural substances were introduced as primary dental pulp caps for use in pulp therapy, and the antimicrobial and cytotoxic properties of these substances were investigated.

Materials and Methods: In this *in vitro* study, the antimicrobial properties of calcium-enriched mixture (CEM) cement, propolis, and propolis individually combined with the extracts of several medicinal plants were investigated against *Enterococcus faecalis*, *Escherichia coli*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus*. Then, the cytotoxicity of each substance or mixture against pulp stem cells extracted from 30 primary healthy teeth was evaluated at 4 concentrations. Data were gathered via observation, and optical density values were obtained using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) test and recorded. SPSS software version 23 was used to analyze the data. Data were evaluated using 2-way analysis of variance and the Tukey test.

Results: Regarding antimicrobial properties, thyme alone and thyme + propolis had the lowest minimum inhibitory concentrations (MICs) against the growth of *S. aureus*, *E. coli*, and *P. aeruginosa* bacteria. For *E. faecalis*, thyme + propolis had the lowest MIC, followed by thyme alone. At 24 and 72 hours, thyme + propolis, CEM cement, and propolis had the greatest bioviability in the primary dental pulp stem cells, and lavender + propolis had the lowest bioviability.

Conclusions: Of the studied materials, thyme + propolis showed the best results in the measures of practical performance as a dental pulp cap.

Keywords: Cell viability; Herbal; Primary teeth; Propolis; Pulpotomy; Stem cells

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No potential conflict of interest relevant to this article was reported.

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INTRODUCTION

Pulpotomy is one of the most common pulp treatments in primary teeth. Pulpotomy treatment is based on the principle that the root pulp is healthy and capable of healing after the removal of infectious coronary pulp [1]. The ideal material for a root pulp cap is bactericidal and bioviable in proximity to the pulp and adjacent structures. It should also hasten the healing of the root pulp and should not interfere with physiological root resorption. The most common materials used in pulpotomy are formocresol, calcium hydroxide, and mineral trioxide aggregate (MTA) [2-4]. Clinical and radiographic studies have indicated a success rate of pulpotomy with formocresol of 70% to 97%; however, an alternative substance has been sought due to the caustic nature, toxicity, and mutagenicity of formocresol [1]. Calcium hydroxide and MTA can repair damaged pulp tissue and stimulate calcified barrier formation [2,5]. However, some researchers have argued that the high pH produced by calcium hydroxide is toxic to pulp and causes chronic pulp inflammation and cell necrosis *in vivo*. Additionally, the success rate of calcium hydroxide as a pulpotomy material is low in primary teeth compared to permanent teeth [4,6]. The high cost of MTA has also prevented its widespread clinical application in pediatric dentistry [1]. Calcium-enriched mixture (CEM) cement (YektazistDandan Co., Tehran, Iran) is another alternative to overcome the limitations of formocresol and has shown favorable therapeutic results in various vital pulp treatments, including primary dental pulpotomy. In a randomized clinical trial, the application of CEM cement for pulpotomy of decayed primary molars demonstrated promising therapeutic results comparable to those obtained with MTA [7], but calcium hydroxide as a secondary product of this substance is less effective against *Enterococcus faecalis* and *Candida albicans* bacteria than against other common pathogens [8].

Given the increase in studies of natural materials as medicines or health enhancers, various natural substances, such as propolis, have been studied in this context. Propolis is a resin that is collected by bumblebees and contains more than 180 substances. Flavonoids, considered the most important active pharmacological component, are herbal compounds that have antibacterial, antiviral, antifungal, antioxidant, and anti-inflammatory properties [9,10]. The ethanol extract of propolis is superior to the water extract in its concentrations of flavonoids and phenolic components, antimicrobial, antioxidant, and anti-inflammatory properties [11]. Herbal extracts can be used in combination with propolis to increase the desirable traits of this natural compound, such as antioxidant properties. Thyme contains many flavonoids and can be used as an antibiotic and antioxidant source [12]. Origanum extract has antioxidant and antibacterial properties and is an inhibitor of enzyme activity [13]. Additionally, it has a healing and disinfecting effect due to the anti-inflammatory and anti-analgesic properties of thymol and carvacrol [14]. Berberine extract has widespread antimicrobial activity against oral pathogens and has anti-inflammatory properties, as demonstrated in laboratory and clinical studies [15-17]. Lavender has antimicrobial activity against oral pathogens and antibiotic-resistant bacteria and has potent antioxidant effects [18]. The anti-analgesic effects of this herbal extract have been repeatedly evaluated and confirmed [19].

As of today, few studies have been performed on Iranian propolis for the treatment of vital pulp, particularly pulpotomy. No studies have been performed to evaluate the effects of combining propolis with herbal extracts to enhance its desirable properties and use in pulpotomy treatment. Therefore, the present study was conducted to determine the antimicrobial and cytotoxic properties of CEM cement, Iranian propolis, and propolis

combined with thyme, origanum, lavender, and berberine herbal extracts in primary dental pulp stem cells. In this study, the ethanol extract of Iranian propolis was used. The null hypothesis was that no differences would exist in the antimicrobial and cytotoxic properties of these substances in primary dental pulp stem cells.

MATERIALS AND METHODS

Preparation of propolis and mixtures

The propolis used in this study was from the Devin region of Hamadan, Iran (Soren Tech Toos Company, Mashhad, Iran). The primary form of this material was solid, and the ethanol extract was prepared as follows.

First, the solid material was separated into fine pieces, and 10 g was added to 100 mL of 96% ethanol. Then, the compound was shaken in a shaker for 24 hours, and the insoluble particles were separated by filter paper. Next, 100 mL of 96% ethanol was again poured on the propolis residue, and the solution was placed in a shaker for 30 minutes. After repeating this step twice, the whole solution was vacuum distilled to obtain an ethanol extract of propolis.

In addition, the extracts of the studied plants, including thyme, origanum, lavender, and berberine (Adonis Gol Darou, Tehran, Iran) were each used in a 1 mg/mL solution in equal composition with propolis ethanol extract. CEM cement was also used as a 1 g/mL solution and prepared according to the manufacturer's recommendations. After preparation, the extracts were refrigerated at 2°C to 5°C in a dark container.

Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) for antimicrobial screenings

The MIC and MBC values of CEM cement, propolis, and propolis mixed with thyme, origanum, lavender, and berberine extracts were calculated using the dilution method in a liquid medium for standard *Escherichia coli* (ATCC 25922), *E. faecalis* (ATCC 29212), *Staphylococcus aureus* (ATCC 25923), and *Pseudomonas aeruginosa* (ATCC 27853) microorganisms. For this purpose, 100 µL of Mueller-Hinton agar medium and 100 µL of mixture were added to each well of sterile 96-well plates. Microdilution was used to obtain different concentrations. The bacterial suspension was added to each well with a dilution of 0.005 (0.5 McFarland). In the 12th well, 100 µL of molar medium and 100 µL of bacterial suspension were included as controls. The plates were incubated at 35°C for 24 hours, followed by point cultivation of the well contents. The concentrations of the first well with no bacterial growth and the well prior to that were documented as the MIC and MBC, respectively.

Selection of samples

The teeth were collected under guidelines approved by the ethics committee of Hamadan University of Medical Sciences (IR.UMSHA.REC.1397.49). Extracted pulp stem cells were used to investigate the cytotoxicity of the studied materials. The inclusion criteria included healthy primary teeth with no caries or previous restorations in different root resorption stages, collected from patients between 6 and 12 years old. The exclusion criteria were any systemic disease or tooth damage during removal. Two to 5 days before extraction of the primary teeth, patients underwent complete dental hygiene and prophylaxis training. Patients' mouths were rinsed with 0.2% chlorhexidine mouthwash once for 30 seconds after injection of anesthesia.

Stem cell extraction

Dental pulp stem cells were isolated from primary healthy teeth (n = 30). Immediately after tooth extraction, the remaining pulp was extracted with a spoon excavator or endo file with minimal trauma under sterile conditions and was immersed in a digestive solution. This solution contained phosphate-buffered saline with 1% penicillin-streptomycin, 3 mg/mL type I collagenase, and 4 mg/mL dispase. After 1 hour at 37°C, the solution was filtered on a 70-μm Falcon strainer. Then, the cells were placed in α-modified Eagle culture medium containing 20% fetal bovine serum (Gibco, Grand Island, NY, USA), 200 μM L-ascorbic acid 2-phosphate (Sigma-Aldrich, Schnelldorf, Germany), 100 μM glutamine, 10 U/mL penicillin to inhibit the growth of gram-positive bacteria, and 100 mg/mL streptomycin to prevent the growth of Gram-negative bacteria.

The flasks were incubated at 37°C and 5% CO_2. Approximately 6 to 8 days after the initial culture, when the cell density in the colonies reached around 80%–90%, cell passage was performed to double the culture, purify the pulp cells, and allow the cells to differentiate.

Evaluation of bioviability

The antimicrobial activity of each mixture was evaluated after preparing an equal ratio of propolis with each of the herbal extracts, and the cytotoxicity was evaluated in the following groups at 4 concentrations (lower than MIC [MIC−], MIC, MBC, and greater than MBC [MIC+]):

Group 1: Basal medium with propolis and lavender extract
Group 2: Basal medium with propolis and thyme extract
Group 3: Basal medium with propolis and origanum extract
Group 4: Basal medium with propolis and berberine extract
Group 5: Basal medium with propolis
Group 6: Basal medium with CEM cement (control group)

To assess the cytotoxicity, 5,000 cells were seeded in 96-well plates and were cultured for 24 and 72 hours, followed by adding the materials specified above. Then, the rate of cell proliferation was studied using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay. A spectrophotometer was used to read the absorbance of samples and blanks at a wavelength of 570 nm. Finally, 3 measurements were taken, and the mean was used to calculate the cytotoxicity of the mixture. The percentage of bioviability calculated using the Dahl index was classified as severe cytotoxicity (cell viability less than 30%), moderate cytotoxicity (30%–60%), slight cytotoxicity (60%–90%), or no cytotoxicity (greater than 90%) [20].

Statistical analysis

Two-way analysis of variance was used to evaluate and compare the cytotoxicity values of the 6 groups at the 4 concentrations at both 24 and 72 hours. If the combined groups showed statistically significant results, pairwise comparisons of the groups were performed separately for each of the 4 concentrations using the Tukey *post hoc* test. Data were analyzed using SPSS version 23 software (IBM Corp., Armonk, NY, USA), and p values of less than 0.05 were considered to indicate statistical significance.

RESULTS

Mesenchymal stem cells express surface markers such as integrin beta-1 (CD29), Thy-1 (CD90), and endoglin (CD105) but do not express sialomucin (CD34) or lymphocyte common

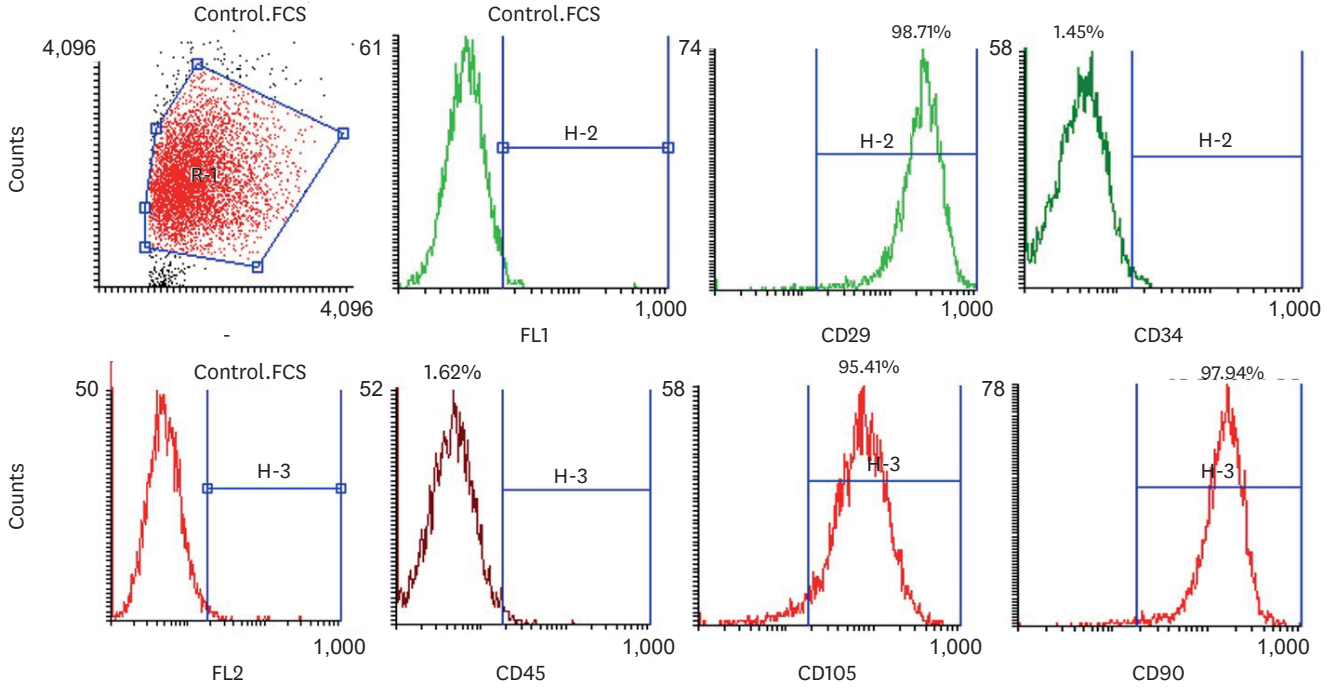

Figure 1. Flow cytometry analysis of cells extracted from primary dental pulp.
FCS, frame check sequence.

antigen (CD45). Flow cytometry analysis was used to evaluate the phenotypic profiles of surface markers and the stem cell nature of pulp tissue extract (**Figure 1**). The expression levels of CD29, CD90, and CD105 were 98.71%, 97.94%, and 95.41%, respectively, while those of CD34 and CD45 were 1.45% and 1.62%, respectively.

As shown in **Table 1**, thyme alone and thyme + propolis showed the greatest inhibitory activity (as indicated by the lowest MIC) and bactericidal activity (as indicated by the lowest MBC) against *S. aureus*, *E. coli*, and *E. faecalis*. Against *P. aeruginosa*, the lowest MIC and MBC values were observed in the CEM cement, followed by thyme alone and thyme + propolis. Origanum alone and berberine + propolis exhibited weaker inhibitory and bactericidal properties against *S. aureus* than any of the other substances. Propolis alone and berberine + propolis were similarly weakest against *E. coli*, whereas lavender + propolis and origanum + propolis

Table 1. Mean values of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) (mg/mL) against growth of different bacterial species by material

Material	Bacteria							
	Staphylococcus aureus		*Escherichia coli*		*Pseudomonas aeruginosa*		*Enterococcus faecalis*	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
Lavender	3.9	7.81	0.48	0.97	125	250	31.25	62.5
Thyme	0.00009	0.001	0.0001	0.0002	0.48	0.97	0.24	0.48
Origanum	15.62	31.25	0.97	0.48	31.25	62.5	62.5	125
Propolis	0.97	1.95	7.81	15.62	62.5	125	0.97	1.95
Lavender + propolis	0.97	1.95	0.97	1.95	125	250	31.25	62.5
Thyme + propolis	0.0001	0.0002	0.0001	0.0002	1.95	3.9	0.001	0.003
Origanum + propolis	0.24	0.48	0.48	0.97	125	250	1.95	3.9
Berberine + propolis	15.62	31.25	31.25	62.5	62.5	125	15.62	31.25
CEM cement	0.39	0.78	0.39	0.78	0.39	0.78	1.56	3.12

CEM, calcium-enriched mixture.

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Table 2. Bioviability values of the 6 groups at different concentrations in primary dental pulp stem cells at 24 and 72 hours

Time	Group	Concentration							
		MBC+	*p* value	MBC	*p* value	MIC	*p* value	MIC−	*p* value
24 hours	1	57.71 ± 1.82	0.616	58.69 ± 0.44	0.463	60.37 ± 2.33	0.523	60.97 ± 1.18	0.085
	2	57.08 ± 0.69	0.747	58.41 ± 0.84	0.862	59.71 ± 1.79	0.512	60.20 ± 2.97	0.067
	3	59.92 ± 0.52	0.998	61.18 ± 0.79	0.780	62.15 ± 1.23	0.328	64.01 ± 0.48	0.637
	4	29.77 ± 0.64	0.637	33.09 ± 1.11	0.739	37.15 ± 1.11	0.363	38.96 ± 0.63	0.817
	5	53.24 ± 0.58	0.900	56.42 ± 1.05	0.890	60.58 ± 0.84	0.862	63.03 ± 0.61	0.328
	6	40.50 ± 2.11	0.835	49.04 ± 2.26	0.872	49.95 ± 1.03	0.490	54.11 ± 0.64	0.637
72 hours	1	55.26 ± 0.27	0.363	57.48 ± 1.01	0.600	59.56 ± 0.59	0.510	60.73 ± 0.39	0.253
	2	54.99 ± 0.63	0.637	57.10 ± 0.83	0.862	58.73 ± 0.63	0.817	59.97 ± 0.22	0.463
	3	54.54 ± 1.20	0.417	58.38 ± 0.58	0.702	63.09 ± 3.19	0.472	66.10 ± 1.35	0.915
	4	28.57 ± 0.85	0.593	33.93 ± 0.53	0.567	37.50 ± 1.72	0.900	41.90 ± 1.30	0.230
	5	51.49 ± 1.25	0.817	55.26 ± 1.09	0.843	59.73 ± 1.20	0.417	63.75 ± 0.94	0.756
	6	39.92 ± 1.05	0.094	49.65 ± 1.28	0.312	54.54 ± 1.05	0.672	56.06 ± 1.69	0.659

Values are presented as mean ± standard deviation.
Group 1: basal medium with propolis and lavender extract. Group 2: basal medium with propolis and thyme extract. Group 3: basal medium with propolis and origanum extract. Group 4: Basal medium with propolis and berberine extract. Group 5: Basal medium with propolis. Group 6: Basal medium with CEM cement (control group).
MBC, minimum bactericidal concentration; MIC, minimum inhibitory concentration; CEM, calcium-enriched mixture.

displayed the highest MIC and MBC values of the compounds against *P. aeruginosa*. Finally, origanum had the highest MIC and MBC of the tested substances against *E. faecalis*.

The bioviability measurements (reported as means with standard deviations) of the groups at different concentrations in primary dental pulp stem cells at 24 and 72 hours are reported in **Table 2**. The MIC− concentration was associated with the maximum and the MBC+ concentration with the minimum mean bioviability in all groups at both 24 and 72 hours. As indicated in **Table 2**, at 24 and 72 hours, thyme + propolis had the greatest viability, followed by propolis alone, CEM cement, and origanum + propolis. Lavender + propolis had the lowest bioviability. According to the Dahl index, the cytotoxicity of propolis and thyme + propolis was low within 24 hours, while other groups were moderately cytotoxic in this time interval. Only the cytotoxicity of thyme + propolis was low at the 72-hour time interval. The distribution of the cytotoxicity of this mixture was normalized at any concentration at 24 and 72 hours based on Shapiro-Wilk normality test ($p > 0.05$).

The effects of group, concentration, and the interaction between group and concentration on bioviability were found to be statistically significant at both 24 and 72 hours. The mean (standard error) and results of pairwise comparisons of the mean bioviability values of groups at different concentrations at 24 and 72 hours are reported in **Table 3**. A *p* value less than 0.05 indicates that the mean values of the 2 groups differed to a statistically significant extent.

As shown in **Table 3**, the bioviability of lavender + propolis was significantly lower than that of the other groups at 24 and 72 hours ($p < 0.001$). Similarly, the bioviability of thyme + propolis was significantly greater than the bioviability of the others at 24 and 72 hours ($p < 0.001$). Finally, the mean bioviability of the berberine + propolis mixture was significantly lower than the bioviability of origanum + propolis, propolis alone, and CEM cement ($p < 0.001$).

DISCUSSION

Pulpotomy is a standard treatment in primary teeth for radicular pulp and tooth preservation. This study was designed to investigate the antimicrobial and cytotoxic properties of Iranian

Table 3. Pairwise comparisons of mean bioviability values of the 6 groups at different concentrations in primary dental pulp stem cells at 24 and 72 hours

Time	Group I-Group II	MBC+		MBC		MIC		MIC−	
		Mean diff (SE)	*p* value	Mean diff (SE)	*p* value	Mean diff (SE)	*p* value	Mean diff (SE)	*p* value
24 hours	Group 1-Group 2	−30.15 (1.01)	< 0.001	−28.09 (1.00)	< 0.001	−25.01 (1.21)	< 0.001	−25.04 (1.14)	< 0.001
	Group 1-Group 3	−23.47 (1.01)	< 0.001	−23.33 (1.00)	< 0.001	−23.43 (1.21)	< 0.001	−24.06 (1.14)	< 0.001
	Group 1-Group 4	−10.74 (1.01)	< 0.001	−15.95 (1.00)	< 0.001	−12.8 (1.21)	< 0.001	−15.15 (1.14)	< 0.001
	Group 1-Group 5	−27.95 (1.01)	< 0.001	−25.60 (1.00)	< 0.001	−23.22 (1.21)	< 0.001	−22.00 (1.14)	< 0.001
	Group 1-Group 6	−27.32 (1.01)	< 0.001	−25.32 (1.00)	< 0.001	−22.56 (1.21)	< 0.001	−21.23 (1.14)	< 0.001
	Group 2-Group 3	6.68 (1.01)	< 0.001	4.76 (1.00)	0.005	1.57 (1.21)	0.779	0.98 (1.14)	0.949
	Group 2-Group 4	19.41 (1.01)	< 0.001	12.14 (1.00)	< 0.001	12.21 (1.21)	< 0.001	9.90 (1.14)	< 0.001
	Group 2-Group 5	2.20 (1.01)	0.316	2.48 (1.00)	0.203	1.78 (1.21)	0.684	3.04 (1.14)	0.152
	Group 2-Group 6	2.83 (1.01)	0.126	2.76 (1.00)	0.132	2.45 (1.21)	0.383	3.81 (1.14)	0.051
	Group 3-Group 4	12.73 (1.01)	< 0.001	7.38 (1.00)	< 0.001	10.63 (1.21)	< 0.001	8.92 (1.14)	< 0.001
	Group 3-Group 5	−4.48 (1.01)	0.008	−2.27 (1.00)	0.275	0.21 (1.21)	1.000	2.06 (1.14)	0.492
	Group 3-Group 6	−3.85 (1.01)	0.024	−1.99 (1.00)	0.398	0.87 (1.21)	0.975	2.83 (1.14)	0.201
	Group 4-Group 5	−17.21 (1.01)	< 0.001	−9.65 (1.00)	< 0.001	−10.42 (1.21)	< 0.001	−6.86 (1.14)	0.001
	Group 4-Group 6	−16.58 (1.01)	< 0.001	−9.37 (1.00)	< 0.001	−9.76 (1.21)	< 0.001	−6.09 (1.14)	0.002
	Group 5-Group 6	0.63 (1.01)	0.987	0.63 (1.01)	0.987	0.28 (1.00)	1.000	0.66 (1.21)	0.993
72 hours	Group 1-Group 2	−25.97 (0.77)	< 0.001	−24.45 (0.76)	< 0.001	−25.59 (1.35)	< 0.001	−24.2 (0.91)	< 0.001
	Group 1-Group 3	−22.92 (0.77)	< 0.001	−21.33 (0.76)	< 0.001	−22.23 (1.35)	< 0.001	−21.85 (0.91)	< 0.001
	Group 1-Group 4	−11.36 (0.77)	< 0.001	−15.72 (0.76)	< 0.001	−17.04 (1.35)	< 0.001	−14.16 (0.91)	< 0.001
	Group 1-Group 5	−26.70 (0.77)	< 0.001	−23.55 (0.76)	< 0.001	−22.06 (1.35)	< 0.001	−18.84 (0.91)	< 0.001
	Group 1-Group 6	−26.42 (0.77)	< 0.001	−23.16 (0.76)	< 0.001	−21.23 (1.35)	< 0.001	−18.07 (0.91)	< 0.001
	Group 2-Group 3	3.05 (0.77)	0.018	3.12 (0.76)	0.014	3.36 (1.35)	0.202	2.35 (0.91)	0.174
	Group 2-Group 4	14.61 (0.77)	< 0.001	8.73 (0.76)	< 0.001	8.55 (1.35)	< 0.001	10.04 (0.91)	< 0.001
	Group 2-Group 5	−0.73 (0.77)	0.926	0.90 (0.76)	0.834	3.53 (1.35)	0.166	5.37 (0.91)	0.001
	Group 2-Group 6	−0.45 (0.77)	0.990	1.28 (0.76)	0.561	4.36 (1.35)	0.062	6.13 (0.91)	< 0.001
	Group 3-Group 4	11.57 (0.77)	< 0.001	5.61 (0.76)	< 0.001	5.19 (1.35)	0.022	7.69 (0.91)	< 0.001
	Group 3-Group 5	−3.77 (0.77)	0.004	−2.22 (0.76)	0.102	0.17 (1.35)	1.000	3.01 (0.91)	0.054
	Group 3-Group 6	−3.50 (0.77)	0.007	−1.84 (0.76)	0.222	1.00 (1.35)	0.972	3.77 (0.91)	0.013
	Group 4-Group 5	−15.34 (0.77)	< 0.001	−7.83 (0.76)	< 0.001	−5.02 (1.35)	0.027	−4.67 (0.91)	0.003
	Group 4-Group 6	−15.06 (0.77)	< 0.001	−7.44 (0.76)	< 0.001	−4.19 (1.35)	0.076	−3.91 (0.91)	0.010
	Group 5-Group 6	0.28 (0.77)	0.999	0.38 (0.76)	0.995	0.83 (1.35)	0.988	0.76 (0.91)	0.954

Group 1: Basal medium with propolis and lavender extract. Group 2: Basal medium with propolis and thyme extract. Group 3: Basal medium with propolis and origanum extract. Group 4: Basal medium with propolis and berberine extract. Group 5: Basal medium with propolis. Group 6: Basal medium with CEM cement (control group).
SE, standard error; MBC, minimum bactericidal concentration; MIC, minimum inhibitory concentration; CEM, calcium-enriched mixture.

propolis, CEM cement, and propolis with herbal extracts of thyme, origanum, lavender, and berberine in primary dental pulp stem cells. Against *S. aureus*, *E. faecalis*, and *E. coli*, the lowest MIC and MBC values were found with thyme and the thyme + propolis mixture. However, for *P. aeruginosa*, the lowest MIC and MBC measurements were found with extract of thyme, CEM, and the thyme + propolis mixture. Additionally, propolis + thyme, propolis alone, and CEM cement had the lowest toxicity and best bioviability measurements after 24 and 72 hours. The antibacterial activity of propolis has 2 important facets: propolis directly affects the microorganisms, potentially by altering their membrane permeability, while also stimulating the immune system against them [21]. High concentrations of kaempferide, drupanin, and p-coumaric acid in propolis promote its antibacterial effects against *S. aureus*, *Listeria*, and *E. faecalis* [22]. Numerous studies have shown that *E. faecalis* is an endodontic pathogen that is often found in stable lesions. This pathogen can bind to host tissues such as dentin and penetrate through dentinal tubules, forming biofilms and potentially surviving long-term with limited nutrients [23]. In a study by Jahromi *et al.* [24], propolis and calcium hydroxide showed similar inhibition of *E. faecalis* after 7 days. As reported by Madhubala *et al.* [25], propolis was similarly effective to a paste consisting of 3 antibiotics (ciprofloxacin, minocycline, and metronidazole) in inhibiting *E. faecalis* after 48 hours. Propolis has been shown to have excellent antibacterial activity against *S. aureus* and moderate antimicrobial activity against *P. aeruginosa* [26]. Another study using the agar diffusion method showed

that propolis concentrations up to 3.1 mg/mL had antibacterial activity against *E. faecalis* [27]. However, a study by Machado *et al.* [28] showed contradictory results; an evaluation of 50% (50 mg/mL) Chilean propolis extract demonstrated no significant antibacterial activity against *E. faecalis* in comparison with chlorhexidine and calcium hydroxide. The results of the present study indicate that thyme and thyme combined with propolis have greater antimicrobial properties than the other tested compounds against *E. faecalis*. In a study by Valera *et al.* [29], the antimicrobial properties of propolis were investigated against *E. coli* and another endotoxin. In that study, root canal irrigation with propolis was effective in fully removing *E. coli* and reducing the number of endotoxins, whereas the present study confirmed the antimicrobial properties of propolis on this bacterium that were augmented when propolis was combined with thyme. However, the results of studies related to propolis should be interpreted with caution because its compounds vary depending on geographic area and season of collection [30]. The results of the present study and its comparison with other studies show that thyme, alone and in combination with propolis, acts as an antimicrobial compound against gram-positive and -negative bacteria. This finding aligns with the study by Mohammadzadeh *et al.* [31], who found that the ethanol extract of propolis inhibited the growth of all tested bacterial species and had the greatest antibacterial effect against gram-positive species such as *S. aureus* and *S. epidermidis*.

Rezende *et al.* [32] investigated the antimicrobial properties of calcium hydroxide, propylene glycol, and propolis with calcium hydroxide on polymicrobial culture media collected from 16 necrotic primary molars with fistulas. The researchers concluded that the combination of the 2 materials had a stronger antibacterial effect than calcium hydroxide alone. In the present study, the thyme + propolis mixture also had stronger antibacterial activity than propolis alone, indicating that thyme reinforces the antibacterial effect of propolis.

Additionally, in the present study, the cytotoxic properties of the compounds on primary dental pulp stem cells were investigated using the MTT assay. MTT is a simple and reliable method for evaluating cytotoxicity. In the first study conducted on primary teeth, Ozório *et al.* [4] examined the response of pulp tissue in primary pig teeth following pulpotomy with propolis and calcium hydroxide. Microscopic examination of the pulp revealed that the hard tissue barrier had formed, and the pulp tissue was without inflammation in all groups. Given the potential for different responses in the primary teeth of animals and humans, clinical studies are recommended. Bretz *et al.* [33] investigated the effect of propolis on the healing of the dental pulp. In that study, the teeth of 25 rats were divided into 2 groups after dental pulp exposure: one treated with propolis and the other with calcium hydroxide. The results indicated no significant difference between these 2 materials in terms of dental pulp repair and lack of increase in blood vessels; both showed minimal inflammation with stimulation of the restorative dentin. The present study resembled previous *in vitro* studies [34,35] in which researchers investigated the biological response of stem cells to endodontic cements based on hydraulic calcium silicate that was confirmed for biologically-based endodontic procedures. In the present study, thyme combined with propolis was found to be the most promising pulp capping material, considering the antimicrobial properties and cytotoxicity of the tested compounds. Based on the Dahl index, the combination of thyme and propolis has low cytotoxicity at both 24 and 72 hours, while at 24 hours, propolis alone also has low cytotoxicity. The results indicated that the addition of lavender and berberine to propolis increases its cytotoxicity. Adding origanum to propolis makes little difference in this property, while the addition of thyme to propolis produces a significant improvement. Several studies have been conducted on the cytotoxic properties of these materials.

Mendonça *et al.* [36] stated that propolis has numerous medicinal properties and minimal adverse effects. They also noted that this material has no significant toxic properties, and their only concern was allergic reactions in patients with a history of similar responses and overuse of this material. Another interesting point regarding this substance is its cytotoxicity against cancer cells, as mentioned in some studies [37]. Jahromi *et al.* [38] investigated and compared the cytotoxic effects of Iranian propolis and calcium hydroxide on permanent dental pulp fibroblasts. They illustrated that cells exposed to propolis were more motile than cells exposed to calcium hydroxide. Thus, according to the similar results in the present study, one can conclude that propolis has an analogous effect on primary dental pulp stem cells. Parolia *et al.* [39] investigated the tissue response of human dental pulp following direct dental pulp capping with propolis, MTA, and Dycal (Dentsply Sirona, Charlotte, NC, USA); teeth treated with Dycal exhibited more pulp inflammation than teeth in the other 2 groups. However, propolis was comparable to MTA as a dental pulp capping material, indicating that propolis can be a good alternative to Dycal. In a study by Noorollahian *et al.* [10], radiographic evaluation of propolis-treated molars revealed extensive furcation and radicular radiolucency, and the authors stated that propolis is not a safe drug for pulpotomy of primary molar teeth. That result is inconsistent with the present study. In the Noorollahian *et al.* [10]'s study, the geographical source of the propolis was not mentioned, and a water extract of propolis was used. In contrast, the present study involved use of the ethanol extract, which has superior antimicrobial, antioxidant, and anti-inflammatory properties in terms of the concentrations of flavonoids and phenolic components [11]. Thyme was another compound in our study that showed favorable effects. An established high clinical success rate (94.1%) in the follow-up after use is due to the anti-inflammatory, antibacterial, and hemostatic properties of thyme constituents, such as thymol, flavonoids, carvacrol, and apigenin [40,41]. While the cytotoxic properties of propolis have been investigated in some studies, none have yet examined these properties on primary dental pulp stem cells. Similarly, no study has been conducted to compare the cytotoxic properties of propolis in combination with herbal extracts. In the present study, this property was also investigated at various concentrations, confirming that these combinations have bactericidal and bacteriostatic properties at those concentrations.

CONCLUSIONS

In this study, we investigated the antimicrobial and cytotoxic effects of propolis in combination with several herbal extracts. Based on the results, propolis combined with thyme extract can enhance these properties compared to the other materials investigated. As such, the null hypothesis was rejected. This result is important because the use of herbal materials in various dental treatments is supported. Thus, propolis combined with thyme may be a suitable candidate for use as a pulp cap in primary dental pulpotomy. However, *in vitro* studies should be performed on a wider variety of bacteria that are involved in dental infections, and *in vivo* studies should then be done to demonstrate the effectiveness of this compound and compare it with current materials. Due to the nature of the research, this issue has not been fully clarified, which can be considered a limitation of the study.

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