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Comparative Antimicrobial Activity and Cytotoxicity of Iranian Propolis Extract, Thymus Vulgaris Extract, Allium Sativum Extract, and Calcium-Enriched Mixture Cement for Dental Pulp Stem Cells of Primary Teeth

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ABSTRACT

This study compared the antimicrobial activity and cytotoxicity of Iranian propolis, Thymus vulgaris, and Allium sativum extracts, and calcium-enriched mixture (CEM) cement on human dental pulp stem cells (DPSCs) from primary teeth. In this in vitro study, the antimicrobial activity of the extracts and their combinations against Enterococcus faecalis, Escherichia coli, Pseudomonas aeruginosa, and Staphylococcus aureus was evaluated using agar-well diffusion, MIC (minimum inhibitory concentration), and MBC (minimum bactericidal concentration) assays. Cytotoxicity was assessed using the MTT assay. Data were analyzed using two-way ANOVA and the Tukey test ($\alpha = 0.05$). The combination of T. vulgaris and propolis demonstrated the lowest MIC values against most tested bacteria, while T. vulgaris alone was most effective against E. coli. CEM cement showed the highest antibacterial activity, with an effect comparable to that of T. vulgaris–propolis combination against S. aureus. Regarding cytotoxicity, CEM cement showed the highest cell viability, followed by propolis and the T. vulgaris–propolis combination, whereas A. sativum exhibited the lowest viability. Overall, the 1:1 combination of Iranian propolis and *T. vulgaris* demonstrated superior antimicrobial activity compared with CEM cement and other extracts. However, as this study was conducted in vitro, further in vivo studies are necessary to confirm the clinical applicability of these findings.

Keywords: Cell Survival, Pulpotomy, Stem Cells, Herbal, Propolis

INTRODUCTION

Dental caries is a multifactorial chronic infectious disease that is known as the most common chronic disease worldwide, especially in children. This condition is considered a public health dilemma and, despite preventive policies adopted in developed countries, it still affects 2.4 billion adults and 486 million children worldwide [1]. Apart from the pain and discomfort caused by dental caries, complications resulting from primary tooth caries can impair dental arch coordination, development of the orofacial system, esthetic dental appearance, and subsequently the children's self-confidence [2]. Conservative treatment of carious teeth to preserve their vitality and prolong their functional life until exfoliation has always been challenging. Pulpotomy is among the treatments performed for this purpose, which involves removal of the coronal pulp and leaving the healthy radicular pulp [3]. Since the introduction of this treatment, various capping materials have been proposed and investigated for placement over the remaining radicular pulp tissue, such as formocresol, ferric sulfate, calcium hydroxide, mineral trioxide aggregate (MTA), and calcium-enriched mixture (CEM) cement. In general, these materials are classified into three categories of devitalizing (formocresol), preservative (ferric sulfate), and regenerative (CEM cement, MTA, and calcium hydroxide) [4, 5]. Among the most important characteristics of a desirable pulp capping material are its biocompatibility (lack of mutagenicity, carcinogenicity, or cytotoxicity) and antimicrobial properties to help maintain pulp vitality and allow healing [6]. Formocresol, as the most commonly used pulp capping agent in pulpotomy, has significant clinical disadvantages despite its high long-term success. Its caustic nature, toxicity for pulp cells (necrotizing and immunogenic), systemic absorption, and potential for mutagenesis have limited the use of this substance and prompted efforts to explore alternative options [7, 8].

Although calcium hydroxide and MTA have been successful in overcoming some of the aforementioned limitations and have been specifically proposed for pulp regeneration and calcified barrier formation [9, 10], they still have some disadvantages. The high pH of calcium hydroxide (the main ingredient of MTA) can lead to cell necrosis and chronic pulp inflammation in the clinical setting [11, 12]. Calcium hydroxide is also ineffective against some microorganisms [11], and its success rate as a pulpotomy agent in primary teeth is lower than in permanent teeth [12]. Although recent results of MTA treatment have been promising and have shown a statistically

significant difference compared to formocresol, its high cost and lack of easy availability have prevented its widespread clinical use in pediatric dentistry [13].

CEM cement is another alternative material introduced to overcome the limitations and disadvantages of formocresol, which has shown favorable therapeutic results in various vital pulp therapy procedures, including pulpotomy of primary teeth [14]. The use of this material in a randomized clinical trial for pulpotomy of carious primary molars showed favorable therapeutic results comparable to those obtained with MTA [15]. However, calcium hydroxide, as a secondary product of this material, has a lower efficacy against Enterococcus faecalis (E. faecalis) and Candida albicans [16].

Currently, there is great emphasis on the use of natural substances, such as propolis [17]. This natural product produced by honey bees is rich in flavonoids, has antimicrobial, antiviral, antifungal, anti-inflammatory, and antioxidant properties, and has found widespread applications in dentistry including wound healing, use as a pulp capping agent, treatment of denture stomatitis, and resolution of tooth hypersensitivity [18].

Thyme or Thymus vulgaris (T. vulgaris) is also known to have antimicrobial properties according to various studies in this field [19, 20]. Comparable clinical and radiographic success of pulpotomy with Allium sativum (A. sativum) or garlic extract to MTA [21] and formocresol [22] has also been reported.

To date, few studies have been conducted on the use of Iranian propolis in vital pulp therapy, especially pulpotomy, and no study has been conducted to investigate the efficacy of a combination of propolis and plant extracts as a pulp capping agent. Obviously, in vitro studies are required before any specific combination is introduced for clinical application. Therefore, the present study was designed to assess the antimicrobial activity and cytotoxicity of CEM cement, Iranian propolis, T. vulgaris extract, A. sativum extract (garlic extract), and combination of propolis with plant extracts for human dental pulp stem cells (DPSCs) of primary teeth.

MATERIALS AND METHODS

This in vitro experimental study was conducted on human DPSCs of primary teeth obtained from the Iranian Biological Resource Center. Enterococcus faecalis (E. faecalis; ATCC 29212), Staphylococcus aureus (S. aureus; ATCC 25923), Escherichia coli (E. coli; ATCC 25922), and Pseudomonas aeruginosa (P. aeruginosa; ATCC 27853) were obtained from the Iranian Microbial and Fungal Culture Collection. The study was approved by the ethics committee of Shahid Beheshti University of Medical Sciences (IR.SBMU.DRC.REC.1401.091) and was conducted in accordance with ISO 10993-12:2021 [23].

Preparation of Materials

The 100% (v/v) extracts of Iranian propolis, T. vulgaris, and A. sativum extract were obtained from Adonis Goldaru (Tehran, Iran) with relevant certifications (HALAL-HACCP-ISO 9001-ISO 22000). According to the manufacturer, the extracts had been obtained with a microwave extraction rotor (AEG; Germany) with a frequency of 900 Hz.

To prepare CEM cement as instructed by the manufacturer, the powder and liquid were mixed and transferred into an empty 24-well plate to fabricate CEM cement discs with 20 mm diameter and 1 mm height, as described by Shi et al [24]. The discs were incubated at 37°C temperature and and 90% humidity for 24 hours to simulate in vivo conditions. Next, 2.5 mL of Dulbecco's modified Eagle's medium (DMEM) was added to each disc according to ISO 10993-12:2021 [23]. After 24 hours of incubation at 37°C temperature and 90 humidity, the obtained extract was collected and sterilized using a 0.22-µm syringe filter.

Preparation of Bacterial Suspensions

To defrost the bacteria, they were stored at −20°C for 24 hours, followed by storage at 4–8°C (in a refrigerator) for 3 hours. Finally, they were inoculated into brain heart infusion (BHI) agar, cultured, and incubated at 37°C for 24 hours.

To prepare a 0.5 McFarland standard microbial suspension, a 24-hour culture of the bacteria was used. Using a sterile loop, a quantity of bacteria was removed from single colonies (on solid BHI culture medium) and transferred (inoculated) to a glass tube containing sterile 0.9% saline solution, and a homogeneous microbial suspension was prepared using a vortex device. Then, 1 mL of the prepared suspension was transferred into a disposable cuvette, and its optical density (OD) was read at a wavelength of 600 nm using a spectrophotometer. The saline solution without bacterial inoculation was used as the control, and the microbial suspension with an OD between 0.08 and 0.1 was considered equivalent to 0.5 McFarland.

Assessment of Antibacterial Activity

Agar-well diffusion (well plate) method: The well plate or agar-well diffusion method was used to initially determine the microbial susceptibility of the studied aqueous and hydroalcoholic extracts (whether or not they had antibacterial effects). First, a sterile cotton swab soaked in 0.5 McFarland standard suspension of the bacteria was lawn-cultured on BHI agar solid culture medium (microbial plate). Then, each plate was divided into four hypothetical parts, and a well was created in each part using the circular end of a sterile glass Pasteur pipette. The bottom of the well was sealed using one drop of melted BHI agar medium, and then 50 µL of each extract (sterile) was inoculated into the wells using a sampler. Also, in this experiment, water (solvent for aqueous extracts) and 96% alcohol (solvent for hydroalcoholic extracts) were used as negative controls, and 0.2% chlorhexidine mouthwash was used as a positive control. This experiment was repeated three times, i.e., 3 separate plates with the same test and control groups. Finally, the plates were examined after 24 hours of incubation at 37°C to qualitatively examine the formation of growth inhibition zones and quantitatively measure their diameter (with a ruler in millimeters). In the negative control groups (water and 96% alcohol) and also in the aqueous extracts (except propolis extract on S. aureus), no growth inhibition zone was observed, indicating a lack of antibacterial effect. In the positive control groups, i.e., chlorhexidine and hydroalcoholic extracts, a growth inhibition zone was observed, indicating an antibacterial effect. Given the ineffectiveness of the aqueous extracts, the study was continued with hydroalcoholic extracts.

Determination of minimum inhibitory concentration (MIC): The MIC determination test was performed by the micro-dilution method in liquid medium using a colorimetric assay (resazurin dye). To perform this test, first, 100 µL of sterile BHI broth culture medium was

added to all wells of a sterile 96-well plate for microbial culture. Then, 100 µL of the substance (plant extracts and CEM) was added to the first well of the test row, and mixed completely with 100 µL of the culture medium that was already present in the well using a sampler. Next, 100 µL of the contents of this well was removed and added to the second well (containing 100 µL of culture medium). This process was repeated until the last well of the corresponding row (the 12^{th} well) such that the concentration of the studied substances in each well was half the concentration in the previous well. Subsequently, 10 µL of a 10^7 CFUs/mL bacterial suspension prepared from a fresh bacterial culture was added to all wells (except one row as a negative control for bacterial growth) (final concentration: 10^6). In fact, the wells of the negative control row for bacterial growth contained BHI broth culture medium and extract but without bacterial suspension. It should be noted that one row was also considered as a positive control for bacterial growth, the wells of which contained only BHI broth culture medium and bacterial suspension and without the presence of extracts. Then, the plates were incubated for 24 hours in an incubator at 37°C under aerobic conditions. To investigate and determine the effective antibacterial concentration, after 24 hours of incubation, 20 µL of 0.01% resazurin blue dye was added to all wells, and the plates were incubated at 37°C for 2 hours. Since the enzymatic activity of the growing bacteria causes the color of resazurin to change from blue to pink, the lowest concentration of the substance at which no color change to pink was observed (remained blue) was considered as the MIC.

Determination of minimum bactericidal concentration (MBC): To determine the MBC of the substances, samples were taken from all wells with antibacterial effect (indicating no bacterial growth) identified in the previous step, i.e. all blue wells before the MIC well and the MIC well itself, as well as one pink well (indicating bacterial growth) after the MIC well, with the help of a sterile loop, and cultured on BHI agar medium (3 replicates for each sample). The above plates were incubated at 37°C for 48 hours, and then the lowest concentration of the substance in which no bacterial growth occurred (no colony formation) was considered as the MBC.

Culture of Human DPSCs

The cell line used in this study was obtained from the cell bank of the Iranian Biological Resource Center. All materials, equipment and surfaces were sterilized before use using an autoclave, 70% alcohol and 37°C hot water bath. The materials used were brought to a suitable temperature for the cells using a 37°C hot water bath. Initially, the cells were cultured and passaged in a 75 mL cell culture flask in high-glucose nutrient DMEM containing 10% fetal bovine serum and 1% penicillin-streptomycin antibiotic, and were incubated with 5% CO_2 and 95% humidity at 37°C until the main experiments.

Assessment of Cell Viability and Cytotoxicity of the Materials

The methyl thiazolyl tetrazolium (MTT) colorimetric assay was used to investigate and compare the cytotoxicity of the studied substances and also to investigate their effect on the viability and proliferation of DPSCs, according to ISO 10993-5:2009. On the first day of the study, the cells in the logarithmic growth phase were carefully counted under completely sterile conditions using trypan blue dye and a hemocytometer slide, and 5000 cells were seeded in 100 µL of complete culture medium (DMEM containing serum and antibiotics) in each well of 96-well plates. The plates were then incubated for 24 hours in a cell culture incubator (temperature 37°C, 95% humidity, and 5% CO_2). On the second day of the study, after microscopic examination of the cells and confirmation of their health status, absence of contamination, and minimum cell density of 50% (50% confluence), 4 different concentrations of plant extracts and CEM, namely 25, 12.5, 6.25, and 3.125% v/v (obtained from microbial tests) were prepared in cell culture medium (DMEM containing serum) and separately added to the cells. It should be noted that the zero concentration of the substances (cell culture medium alone) was considered as the control group (normal conditions of cell growth and proliferation). In addition, wells containing culture medium without cells were considered as blank wells for background correction. Two identical series of treated plates were transferred to the incubator to examine viability, proliferation, and cytotoxicity 24 hours after contact (to examine acute cytotoxicity) and 72 hours after contact (to examine chronic cytotoxicity). At each time point (24 or 72 hours after contact), the plates corresponding to the test time were removed from the incubator and the medium on the cells in each well was slowly and carefully removed and replaced with cell culture medium (without serum and antibiotics) containing 10% MTT dye (100 µL per well) [13]. Furthermore, MTT solution was also added to cell-free wells as a reagent blank to account for background absorbance of the MTT solution. The plates were then placed in an incubator for 2 hours and after confirming the formation of formazan crystals with an inverted microscope, the MTT dye was removed from each well, and the same amount of dimethyl sulfoxide solvent was added. Then, the OD of the colored solutions was read by an ELISA reader. To calculate the percentage of cell viability, the mean OD of each group treated with the test substances was divided by the mean OD of the control group (non-treated cells, without cytotoxicity = 100% viability) and multiplied by 100. According to ISO 10993-5, a substance that causes a decrease in cell viability by more than 30% compared to the control group (100% viability) (the viability percentage falls below 70%) is considered cytotoxic.

Statistical Analysis

The normality of the data distribution was analyzed by the Kolmogorov-Smirnov and Shapiro-Wilk tests, and homogeneity of the variances was analyzed by the Levene test. The growth inhibition zones were analyzed using one-way ANOVA and Tukey's post hoc test. To compare the cytotoxic properties of different substances after 24 and 72 hours, the Kruskal-Wallis test was applied. For pairwise comparisons, the Mann-Whitney test was used. All statistical analyses were performed using SPSS 21 (SPSS Inc., IL, USA) at the 0.05 level of significance.

RESULTS

Growth Inhibition Zones

Table 1 presents the diameter of the growth inhibition zones of S. aureus and E. coli following exposure to different substances. As shown, in S. aureus culture, propolis-T. vulgaris combination created a growth inhibition zone equal to that of CEM cement. The smallest zone was caused by A. sativum. In E. coli culture medium, T. vulgaris extract resulted in the formation of the highest growth inhibition zone, which was larger than that caused by CEM cement. CEM cement and T. vulgaris-A. sativum combination were next in rank. The smallest inhibition zone was created by the propolis-A. sativum combination.

Table 1 Diameter of growth inhibition zones (mm) of S. aureus and E. coli following exposure to different substances

Bacteria	S. aureus				E. coli			
Substance	Mean	SD	Min.	Max.	Mean	SD	Min.	Max.
Propolis	21.00	1.000	20	22	9.67	2.082	8	12
T. vulgaris	18.67	.577	18	19	13.00	2.000	11	15
A. sativum	15.00	.000	15	15	11.67	1.528	10	13
A. sativum+ Propolis	21.00	.000	21	21	8.33	1.528	7	10
T. vulgaris+ Propolis	22.33	1.528	21	24	11.00	.000	11	11
A. sativum+ T. vulgaris	16.67	.577	16	17	12.00	1.000	11	13
A. sativum+ T. vulgaris+ Propolis	21.00	.000	21	21	11.00	.000	10	12
CEM	22.33	1.528	21	24	12.00	1.00	11	13
Water	.00	.000	0	0	0.00	0.00	0	0
Alcohol	.00	.000	0	0	0.00	0.00	0	0
Chlorhexidine	21.33	1.528	20	23	29.33	2.517	27	32
P value	<0.001				<0.001			

SD: Standard deviation; Min: minimum; Max: maximum

Table 2 presents the diameter of the growth inhibition zones of P. aeruginosa and E. faecalis following exposure to different substances. As shown, in P. aeruginosa culture, T. vulgaris extract resulted in the formation of the largest growth inhibition zone. The smallest was attributed to CEM cement. In E. faecalis culture, propolis extract resulted in the formation of the largest growth inhibition zone. Next in rank were T. vulgaris and propolis-T. vulgaris combination, and the values for both extracts were higher than that of CEM cement. The smallest value was related to A. sativum.

Table 2 Diameter of growth inhibition zones (mm) of P. aeruginosa and E. faecalis following exposure to different substances

Bacteria	P. aeruginosa				E. faecalis			
Substance	Mean	SD	Min.	Max.	Mean	SD	Min.	Max.
Propolis	10.00	.000	10	10	20.00	.000	19	22
T. vulgaris	11.67	1.528	10	13	15.00	.000	15	15
A. sativum	9.67	0.577	9	10	9.67	.577	9	10
A. sativum+ Propolis	9.67	0.577	9	10	14.00	1.00	13	15
T. vulgaris+ Propolis	10.00	0.000	10	10	15.00	.000	15	15
A. sativum+ T. vulgaris	11.00	1.000	10	12	11.00	.000	11	11
A. sativum+ T. vulgaris+ Propolis	10.00	0.000	10	10	14.00	1.00	13	15
CEM	8.00	1.00	7	9	13.00	1.00	12	14
Water	0.00	0.00	0	0	0.00	0.00	0	0
Alcohol	0.00	0.00	0	0	0.00	0.00	0	0
Chlorhexidine	19.33	1.155	18	20	18.67	.577	18	19
P value	<0.001				<0.001			

MIC and MBC Values

Table 3 shows the MIC and MBC values of the substances for the tested microorganisms. Regarding S. aureus and E. faecalis, the lowest MIC and MBC values were related to the propolis-T. vulgaris combination. Regarding E. coli, the lowest MIC and MBC values were related to T. vulgaris extract alone, and regarding P. aeruginosa, the lowest MIC and MBC values were related to T. vulgaris extract alone and the propolis-T. vulgaris combination.

Table 3 MIC and MBC values of the substances for the tested microorganisms

Substance	*P. aeruginosa*		*E. coli*		*S. aureus*		*E. faecalis*	
	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC
A. sativum hydroalcoholic extract	12.5	3.1	6.2	6.2	12.5	6.2	12.5	12.5
T. vulgaris hydroalcoholic extract	6.2	3.1	3.1	3.1	1.6	1.6	6.2	3.1
Propolis hydroalcoholic extract	12.5	3.1	12.5	12.5	1.6	0.8	6.2	3.1
Combination of A. sativum and T. vulgaris extracts	12.5	3.1	12.5	6.2	3.1	1.6	12.5	6.2
Combination of A. sativum and propolis	12.5	3.1	12.5	12.5	1.6	0.8	6.2	3.1
Combination of T. vulgaris and propolis	6.2	1.6	6.2	6.2	0.8	0.4	3.1	3.1
Combination of all three	12.5	6.2	6.2	6.2	3.1	0.8	6.2	3.1
CEM cement	50	25	-	-	3.1	1.6	-	-

MTT Test Results

Figure 1 shows the MTT test results of cell viability at 24 and 72 hours. In total, as shown, propolis yielded the highest, and A. sativum yielded the lowest percentage of cell viability. At 24 hours, the highest cell viability was found for CEM cement (92-100%) in all four concentrations tested. After that, propolis extract, propolis-T. vulgaris combination, and T. vulgaris alone at a concentration of 3.125% v/v were considered biocompatible due to a cell viability of over 70%. A. sativum extract and its combinations were considered cytotoxic due to a reduction of more than 30% in the number of viable cells in the culture medium. Given the presence of two independent variables, type of substance and concentration, and the quantitative nature of the dependent variable, two-way ANOVA was used. The results indicated significant effects of group, concentration, and their interaction on 24-hour MTT results (P<0.001). Given the significance of

the interaction between group and extract concentration, subgroup analysis was used to examine the effect of each of these factors on 24-hour MTT results. ANOVA showed that in relation to CEM cement, the concentrations studied did not have a significant effect on the 24-hour MTT results (P=0.092), but in the other groups (P<0.001) there was a significant effect. Also, the Welch test for the A. sativum group confirmed the effect of concentration (P=0.001).

At 72 hours, the highest cell viability was observed for the CEM cement (93-100%) at all four concentrations tested. After that, only propolis extract alone at a concentration of 3.125% v/v was considered biocompatible due to a cell viability of over 70%. The other extracts studied were cytotoxic at all four concentrations due to a reduction of more than 30% of viable cells in the culture medium. At 72 hours, two-way ANOVA showed significant effects of group, concentration, and their interaction on MTT test results (P<0.001). Given the significant interaction between group and extract concentration, subgroup analysis was performed to examine the effect of each of these factors on the72-hour MTT result. One-way ANOVA showed that in relation to CEM cement (P=0.761) and the combination of A. sativum + T. vulgaris (P=0.139), the studied concentrations did not have a significant effect on the 72-hour MTT result, but in other groups (P<0.001) there was a significant effect.

The Welch test showed a significant difference in the mean percentage of cell viability among the groups at 24 hours (P<0.001) and 72 hours (P<0.001). Pairwise comparisons by the Games-Howell test are presented in Tables 4 and 5.

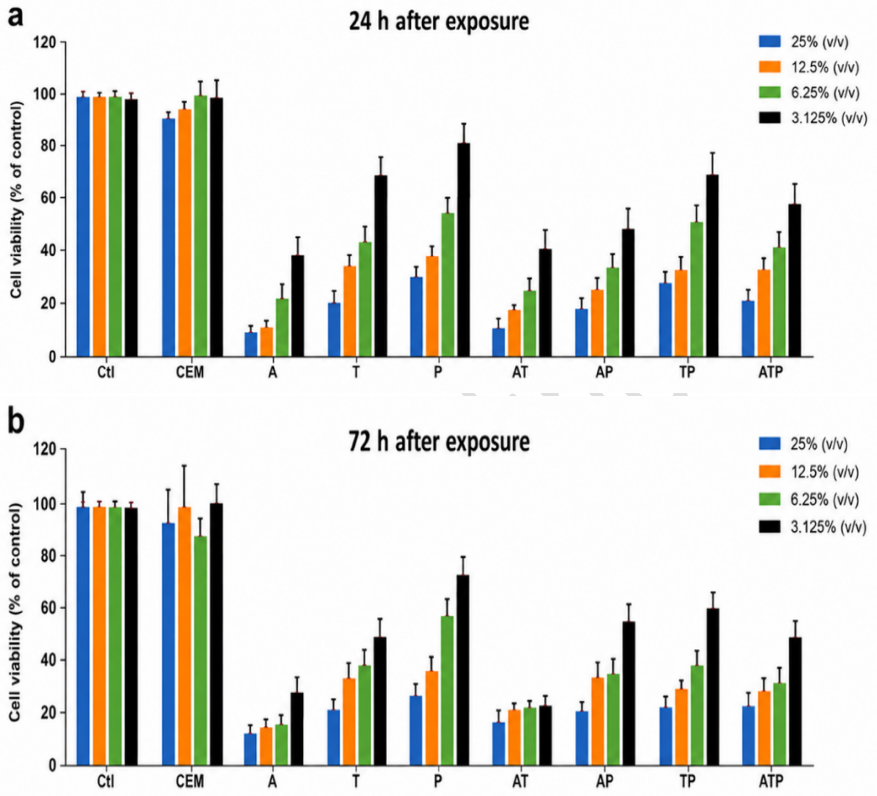

Fig. 1 Percentage of DPSC viability at (a) 24 and (b) 72 hours following exposure to different substances (Ctl=control, CEM=Calcium-Enriched Mixture, A=A. sativum, T=T. vulgaris, P=Propolis, AT=A. sativum + T. vulgaris, AP=A. sativum+ Propolis, TP=T. vulgaris + Propolis, ATP=A. sativum + T. vulgaris + Propolis). As shown, propolis yielded the highest and A. sativum yielded the lowest percentage of cell viability.

Table 4 Pairwise comparison of the groups regarding cell viability percentage at 24 hours

Group (I)	Group (J)	Mean difference	SE	P value
Control	CEM	3.36	4.72	.991
	A. sativum	79.02	5.57	.001
	T. vulgaris	58.59	6.80	.001
	Propolis	49.60	7.47	.001
	A. sativum + T. vulgaris	75.15	5.56	.001
	A sativum + propolis	68.95	5.63	.001
	T. vulgaris + propolis	54.25	6.97	.001
	A. sativum + T. vulgaris + propolis	60.75	6.07	.001
CEM	A. sativum	75.65	3.43	.000
	T. vulgaris	55.22	5.19	.000
	Propolis	46.24	6.04	.000
	A. sativum + T. vulgaris	71.78	3.41	.000
	A sativum + propolis	65.58	3.53	.000
	T. vulgaris + propolis	50.89	5.41	.000
	A. sativum + T. vulgaris + propolis	57.38	4.20	.000
A. sativum	T. vulgaris	-20.43	5.98	.058
	Propolis	-29.41	6.73	.010
	A. sativum + T. vulgaris	-3.87	4.51	.993

	A sativum + propolis	-10.07	4.61	.448
	T. vulgaris + propolis	-24.76	6.17	.018
	A. sativum + T. vulgaris + propolis	-18.27	5.14	.040
T. vulgaris	Propolis	-8.98	7.78	.958
	A. sativum + T. vulgaris	16.55	5.96	.188
	A sativum + propolis	10.35	6.03	.731
	T. vulgaris + propolis	-4.33	7.30	.999
	A. sativum + T. vulgaris + propolis	2.15	6.45	1.000
Propolis	A. sativum + T. vulgaris	25.54	6.72	.030
	A sativum + propolis	19.34	6.78	.170
	T. vulgaris + propolis	4.65	7.93	1.000
	A. sativum + T. vulgaris + propolis	11.14	7.15	.816
A. sativum + T. vulgaris	A sativum + propolis	-6.20	4.59	.905
	T. vulgaris + propolis	-20.89	6.16380	.062
	A. sativum + T. vulgaris + propolis	-14.40	5.12649	.171
A. sativum + propolis	T. vulgaris + propolis	-14.69	6.23422	.360
	A. sativum + T. vulgaris + propolis	-8.20	5.21094	.808
T. vulgaris + propolis	sativum + T. vulgaris + propolis	6.49167	6.63417	.984

Table 5 Pairwise comparison of the groups regarding cell viability percentage at 72 hours

Group (I)	Group (J)	Mean difference	SE	P value
Control	CEM	4.84	5.81	.989
	A. sativum	85.31	4.76	.008
	T. vulgaris	69.89	5.40	.002
	Propolis	55.58	7.23	.001
	A. sativum + T. vulgaris	81.83	4.68	.011
	A sativum + propolis	66.66	6.06	.001
	T. vulgaris + propolis	65.27	6.27	.001
	A. sativum + T. vulgaris + propolis	70.18	5.60	.001
CEM	A. sativum	80.47500	3.86749	.000
	T. vulgaris	65.05000	4.63006	.000
	Propolis	50.74167	6.67053	.000
	A. sativum + T. vulgaris	76.99167	3.75812	.000
	A sativum + propolis	61.82500	5.37987	.000
	T. vulgaris + propolis	60.43333	5.61932	.000
	A. sativum + T. vulgaris + propolis	65.34167	4.86143	.000
A. sativum	T. vulgaris	-15.42500	3.21755	.005
	Propolis	-29.73333	5.78024	.005
	A. sativum + T. vulgaris	-3.48333	1.74318	.561
	A sativum + propolis	-18.65000	4.22590	.013
	T. vulgaris + propolis	-20.04167	4.52680	.013
	A. sativum + T. vulgaris + propolis	-15.13333	3.54242	.014
T. vulgaris	Propolis	-14.30833	6.31594	.412
	A. sativum + T. vulgaris	11.94167	3.08522	.033
	A sativum + propolis	-3.22500	4.93337	.999
	T. vulgaris + propolis	-4.61667	5.19345	.991
	A. sativum + T. vulgaris + propolis	.29167	4.36219	1.000
Propolis	A. sativum + T. vulgaris	26.25000	5.70764	.013
	A sativum + propolis	11.08333	6.88453	.789
	T. vulgaris + propolis	9.69167	7.07322	.897
	A. sativum + T. vulgaris + propolis	14.60000	6.48747	.418
A. sativum + T. vulgaris	A sativum + propolis	-15.16667	4.12604	.052
	T. vulgaris + propolis	-16.55833	4.43373	.048
	A. sativum + T. vulgaris + propolis	-11.65000	3.42268	.077
A. sativum + propolis	T. vulgaris + propolis	-1.39167	5.87175	1.000
	A. sativum + T. vulgaris + propolis	3.51667	5.15114	.999
T. vulgaris + propolis	sativum + T. vulgaris + propolis	-4.90833	5.40074	.990

DISCUSSION

This study investigated the in vitro antimicrobial efficacy and cytotoxicity of Iranian propolis, T. vulgaris extract, A. sativum extract, and their combination in comparison with CEM cement on DPSCs of primary teeth. The aim was to evaluate whether these natural agents, alone or in combination, could serve as effective and biocompatible alternatives to conventional pulpotomy materials.

Among the tested materials, the combination of propolis and T. vulgaris demonstrated the highest antimicrobial activity against S. aureus, E. faecalis, and P. aeruginosa. This finding is consistent with a previous study by Esmaeilzadeh *et al.* [25], which also reported enhanced antimicrobial effects of this combination compared to either agent alone. Overall, T. vulgaris extract alone showed strong antibacterial activity, particularly against E. coli, which aligns with previous studies highlighting the antimicrobial potential of thyme due to thymol

and carvacrol [26-31]. These compounds are known to disrupt bacterial cell membranes and interfere with essential cellular processes [32, 33].

The ethanolic extract of T. vulgaris showed superior antimicrobial activity compared to its aqueous extract, which is in agreement with previous reports indicating minimal antibacterial effects in aqueous preparations due to the absence of key active compounds [29, 34]. Phytochemical analyses in previous studies have shown that aqueous extracts lack carvacrol and contain only trace amounts of thymol, which may explain their weaker antimicrobial activity. In the present study, only ethanolic extracts of A. sativum and propolis were used due to the lack of inhibitory effects observed in their aqueous forms.

Oliveira et al. [35] demonstrated that a brief 5-minute exposure to T. vulgaris extract significantly reduced bacterial colony counts. Nikolić *et al.* [30] also reported that the essential oil of T. vulgaris showed stronger antimicrobial activity than chlorhexidine and amoxicillin against several bacterial species. Similarly, many other studies have reported high antimicrobial activity of thyme against various oral pathogens [36, 37]. The clinical potential of thyme extract has also been investigated. Alolofi *et al.* [38] reported a high success rate (94.4%) for pulpotomy treatments performed using T. vulgaris extract, which was comparable to the success rate of formocresol.

CEM cement's mechanism of action involves the release of calcium hydroxide and the maintenance of an alkaline pH, which contribute to microbial inhibition [39]. Additionally, its composition, containing phosphate, calcium oxide, and calcium silicate, further enhances its antimicrobial properties [40]. In the present study, CEM cement was particularly effective against S. aureus.

Overall, the combination of propolis and T. vulgaris demonstrated strong antimicrobial activity and, in some cases, greater efficacy than CEM cement under the conditions of this in vitro study. While CEM was most effective against S. aureus, T. vulgaris and its combination with propolis showed stronger effects against E. coli and P. aeruginosa, and propolis was particularly effective against E. faecalis. These differences may be attributed to variations in bacterial cell wall structure and the susceptibility of Gram-positive and Gram-negative bacteria to specific bioactive compounds [30].

Gram-negative bacteria such as E. coli and P. aeruginosa appeared more sensitive to thyme, consistent with studies showing that thymol and carvacrol can penetrate the outer membrane of Gram-negative bacteria [41]. In contrast, propolis, rich in flavonoids and phenolic compounds, was more effective against Gram-positive bacteria such as E. faecalis, supporting its traditional endodontic use [42].

A. sativum extract showed the least antimicrobial activity. Although garlic contains allicin with known antibacterial effects, its efficacy depends strongly on concentration and preparation method [43]. Previous studies have reported variable inhibitory effects of garlic against different bacterial species [44-46], and the relatively low concentration used in this study may explain its limited activity. Allicin is known to primarily inhibit RNA synthesis, with additional effects on DNA and protein synthesis [47].

Cytotoxicity evaluation using the MTT assay at 24 and 72 hours showed that CEM cement had the highest cell viability (>90%) across all concentrations, followed by propolis and the propolis–thyme combination. According to ISO 10993-5, materials with cell viability above 70% are considered non-cytotoxic [48]. At 24 hours, both propolis and its combination with thyme maintained viability above this threshold at the highest concentration; however, after 72 hours, only propolis remained within the non-cytotoxic range.

These findings are consistent with previous studies reporting good biocompatibility of propolis and its ability to support odontogenic activity [49-51]. CEM cement also demonstrated excellent biocompatibility, with no significant reduction in cell viability over time, consistent with previous reports [52].

The cytotoxic profile of T. vulgaris was higher than propolis and CEM cement, particularly after prolonged exposure, which is in agreement with reports suggesting dose-dependent cytotoxic effects of thymol and carvacrol [53, 54]. Similarly, A. sativum showed the highest cytotoxicity, consistent with the known dose-dependent cytotoxic effects of allicin [55-57]. Although garlic showed limited antimicrobial and higher cytotoxic effects in this study, some clinical studies have reported favorable short-term pulpotomy outcomes with garlic compared to conventional agents [21, 22]. This suggests that its effects may depend strongly on concentration and formulation.

Future in vivo and long-term clinical studies are recommended to further evaluate the efficacy and safety of these natural agents, particularly propolis and T. vulgaris, as potential alternatives in pulpotomy procedures. Since the present investigation was conducted in vitro, the clinical applicability of these findings should be interpreted with caution until confirmed by in vivo studies. Additionally, further ex vivo studies on A. sativum and investigations into combinations of these agents with calcium-based cements are suggested.

CONCLUSION

The findings of this study suggest that the combination of Iranian propolis and T. vulgaris has strong antimicrobial activity and acceptable biocompatibility at an appropriate concentration (3.125% v/v), making it a promising candidate for use in pediatric pulp therapy. CEM cement continues to demonstrate superior biocompatibility and reliable antimicrobial effects. Garlic extract showed the least favorable results in both antimicrobial and cytotoxicity assays, but further studies may help optimize its formulation for clinical use.

Declarations Section

Ethics Approval and Consent to Participate

The study was approved by the ethics committee of Shahid Beheshti University of Medical Sciences (IR.SBMU.DRC.REC.1401.091) and was conducted in accordance with ISO-10-993-12-2021. The parents of patients consented to the use of their extracted teeth for research purposes.

Consent for Publication

Identified images or other personal or clinical details are not presented in this manuscript.

Author Availability of Data and Material

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing Interests

The authors declare that they have no competing interests.

Clinical Trial Number

Not applicable.

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Author Contributions

M.Ek. Study concept and design, critical revision of the manuscript for important intellectual content, administrative, technical, and material support and study supervision; M.T. Analysis and interpretation of data; A.A. acquisition of data and statistical analysis; Z.S. drafting of the manuscript. All authors have read and approved this manuscript.

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List of abbreviations

T. vulgaris: Thymus vulgaris

A. sativum: Allium sativum
E. faecalis: Enterococcus faecalis
S. aureus: Staphylococcus aureus
E. coli: Escherichia coli
CEM: Calcium-Enriched Mixture
DPSCs: Dental Pulp Stem Cells
MIC: Minimum Inhibitory Concentration
MBC: Minimum Bactericidal Concentration
MTT: Methyl Thiazolyl Tetrazolium
MTA: Mineral Trioxide Aggregate
BHI: Brain Heart Infusion
OD: Optical Density