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Original Article

Antimicrobial Activity of Methanolic Extracts of *Myrtus Communis L.* and *Eucalyptus Galbie* and their Combination with Calcium Hydroxide Powder against *Enterococcus Faecalis*

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KEY WORDS
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ABSTRACT

Statement of the Problem: The goal of endodontic therapy is the reduction or elimination of microorganisms from the root canal system. The use of intracanal medicament between treatment appointments is recommended in order to eliminate any remainang microorganisms in the pulp space.

Purpose: The aim of the present study was to investigate the antimicrobial activity of methanolic extracts of *Myrtus communisL.* and *Eucalyptus galbie*, their combination with calcium hydroxide powder; combination of calcium hydroxide powder with water, and ready-to-use calcium hydroxide paste with iodoform; against *Enterococcus faecalis.*

Materials and Method: After gathering the plants, their methanolic extracts were obtained by masceration method. The diameters of inhibition zone of all mentioned materials were determined by agar diffusion test. Minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC); and anti biofilm effect of the materials that showed antibacterial effect in agar diffusion test, were then evaluated by tube dilution test, and microtiter plate assay followed by colorimetric crystal violet methods, respectively.

Results: After 48 hours, both herbal extracts showed antimicrobial effect. However, combination of calcium hydroxide with extracts produced no zone of inhibition. The mean inhibition zone of *Eucalyptus* extract was more than that of *Myrtus*. However, the results of ANOVA test, showed that there was no significant difference between the antibacterial effect of *Eucalyptus galbie*, *Myrtus communisL.* and positive control (Cefoxitin) (p Value= 0.987). The MIC for both extracts were 12.5 mg/ml. MBC evaluation of the two methanolic extracts showed no bactericidal effect on *Enterococcus faeca*lis. Based on ELISA analysis, biofilm formation in response to different sub-MIC concentrations of both extracts was scored as weak to moderate.

Conclusion: The methanolic extracts of *Eucalyptus galbie* and *Myrtus communis L.* in combination with calcium hydroxide powder were not able to eliminate *Enterococcus faecalis* within 48 hours.

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Introduction

Pulp and periapical diseases are the most common cause of referring patients to dentist. These diseases are directly or indirectly associated with microorganisms [1]. In infected root canals, bacteria accumulate mainly in the form of biofilms. However, during the growth of biofilm, continuous detachment of bacteria to planktonic phase is also observed [2]. During the biofilm phase, bacteria considerably show resistance against the host immune system as well as external antimicrobial factors [3-4].

The main purpose of root canal treatment is elimination or apparent reduction of microorganisms [5]. It has been shown that obturation of the canals with positive cultures causes more undesirable treatment outcomes [6]. On the other hand, bacteria associated with endodontic treatment failure can be colonized in periapical tissue and the outer surface of the root and induce bone loss and root resorption [7]. Only in 50-70% of cases, root canal instrumentation with antimicrobial irrigation results in a bacteria-free root canal system [8]. Therefore, the use of an intra-canal medicament between treatment sessions is recommended for effective eradication of microorganisms [9]. Among various substances, calcium hydroxide has been the most widely accepted substance [1]. However, there are some disadvantages with the use of calcium hydroxide in endodontics. For instance, the anatomy of root canal system is in such a way that carrying calcium hydroxide to the canals and maintaining its high pH is a challenge. In addition, careful placement of calcium hydroxide into the canal is important. If calcium hydroxide were not in direct contact with microorganisms, its credibility would be questionable [10]. Furthermore, buffering potential of dentine can reduce the pH of calcium hydroxide [11]. Some microorganisms such as *Enterococcus faecalis* (*E. faecalis)* and *Candida albicans*, reported as the etiology of endodontic failure, are resistant to calcium hydroxide [12-13].

E. *faecalis* is a gram-positive, facultative anaerobic and opportunistic bacterium that plays a role in the failed root canal treatments [14-15]. E. *faeca*lis has a remarkable ability to attack the dentinal tubules [16]. This microorganism binds tightly to the collagen and resist against the typical canal irrigants [17]. It has been also shown that *E. faeca*lis is resistant to sodium hypochlorite under starved condition [6]. According to the total mentioned topics, many studies have been conducted to evaluate the effect of various intra-canal medicaments on *E. faecalis*.

To improve the antimicrobial effect of calcium hydroxide, some researchers have suggested the mixture of calcium hydroxide powder with antimicrobial solutions. Nevertheless, calcium hydroxide mixed with various chemicals such as chlorhexidine, IKI, iodoform, and silicone oil has not been able to remove *E. faecalis* completely [18-20]. On the other hand, adverse reactions to chemical substances have increased attention to natural products [21]. Considering the fact that there is not sufficient scientific evidence on the efficacy of medicinal plants, the World Health Organization (WHO) strongly recommended the assessment of pharmaceutical plants in order to increase their safety and efficacy [22]. In this study, two medicinal plants in combination with calcium hydroxide powder were examined *in vitro*. One of these plants was *Myrtus communis L.* from Myrtaceae family. It is evergreen and contains tannin, resin, camphor and 0.3 percent essence. The therapeutic effect of Myrtus is mostly due to the existence of essence in various parts, especially in the leaves of the plants that exhibits significant antibacterial and antifungal activities [23-24]. *Eucalyptus galbie* from Myrtaceae family was the other investigated plant. Volatile oils and tannins are commonly found as active ingredients of the plant's leaves that present antiseptic, antipyretic, expectorant, and decongestant properties. This plant is also used as antimicrobial and astringent medication [24].

Contradictory results exist on the antimicrobial efficacy of the mentioned extracts against *E. faecalis* [25-27]. Moreover, the effect is unclear in the presence of calcium hydroxide. Therefore, the aim of the present study was to evaluate *in vitro* antimicrobial activity of *Myrtus communis L.* and *Eucalyptus galbie* and their combination with calcium hydroxide powder against *E. faecalis*.

Materials and Method

Materials

Methanolic extract of Myrtus, methanolic extract of Eucalyptus, calcium hydroxide powder (Golchai, Tehran, Iran), ready-to-use calcium hydroxide paste with iodoform (Metapex, Korea) and *E. faecalis* suspension

(PTCC 1237) were employed in this study.

Preparation of calcium hydroxide paste

By mixing 180 mg of calcium hydroxide powder with 300 ml of distilled water, 60%paste was prepared until a creamy consistency. For mixture of calcium hydroxide powder with extracts, we tried to achieve the same consistency.

Making a crude extract from the plants

Plant specimens were collected in the native pastures located a few miles away from Kerman city. The plants were used after identification and verification of scientific names by Herbarium of School of Pharmacy at Kerman University of Medical Sciences. Initially fresh plants were kept in good condition (dark and dry with proper temperature) and dried thoroughly. Usable parts of the plant powdered in the mill containing a sieve with pore size of 200 micrometers. Then 200 grams of each plant powder was dissolved in 1000 ml of absolute methanol [28]. In this study, maceration method was used to prepare plant extracts. Then extracts were dried by use of a rotary evaporator (Heidolph, Germany) under vacuum. The extracts were kept in the refrigerator until the time of experiment.

Microbial tests

In order to evaluate antimicrobial effects of extracts, agar well diffusion method was used. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the materials that showed antimicrobial activity were then determined by tube dilution method [29].

Brain-heart infusion (BHI) broth medium

The medium formulation was in liquid form without agar. The amount of 37 g of brain heart infusion (BHI) powder was dissolved in one liter of distilled water. Then the solution was heated and was divided into tubes and finally was autoclaved at 121 °C with pressure of 15 pounds per square inch (psi) 15 minutes.

BHI agar medium

52 grams of the medium in one liter of purified water were mixed until it was evenly dispersed. It was heated to achieve a transparent medium. The medium was autoclaved (121 °C and pressure of 15 pounds/square inch /for 15 minutes) and then cooled to 45-50°C. The medium was then transferred to the plate.

Preparation of Bacterial Suspension

The lyophilized vials of the *E. faecalis* bacteria opened in sterile conditions, transferred to BHI broth liquid medium, and incubated for 24 h at 37°C. The turbidity of BHI containing *E.faecalis* was adjusted to 0.5 McFarland standard units (equivalent to 1.5 x 108 CFU/ ml bacteria). It was incubated at 37°C for 24 hours [29].

Preparation of extracts concentration

Dimethyl sulfoxide (DMSO) was used to dissolve the extracts powder. Initially, the concentration of 100 mg / ml was prepared from the powders and this solution was used for preparation of serial dilution of the extracts. The resulting solutions with specified concentrations were passed through 0.22 mm filters to ensure lack of microbial contamination of the extracts. Filtered extracts were kept in sterile tubes at -20 °C until the experiment [29].

Antimicrobial Effects of Plant Extracts

Agar Well Diffusion Method

The agar plate surface was inoculated by bacterial suspension with 0.5 McFarland turbidity (1.5x108 CFU/ ml). By use of a sterile cork borer, holes of 6mm in diameter were made in the seeded agar. Then in sterile condition, 20 ml of each of the extracts (the concentration 100mg/ml), combination of calcium hydroxide and extracts, ready to use calcium hydroxide paste containing iodoform and the solvent DMSO were introduced into the wells. The plates were incubated at 37°C for 48 hours [29]. Finally, the diameter of each zone of inhibition was measured with a caliper. DMSO and cefoxitin (30 mg / ml) were used as negative and positive controls, respectively. The data were analyzed using SPSS-16 software. One-way analysis of variance (ANOVA) was applied to determine the differences among the tested materials. The significance level was set at $p< 0.05$.

Assessing MIC and MBC of the extracts using broth dilution method

To determine the MIC for each extract, a series of 12 test tubes were used. Ten tubes were used for serial dilution of each extract; one tube as a positive control (containing culture medium with bacteria) and one tube were used as a negative control (containing culture medium alone). Different concentrations of stored solution of extracts were prepared by broth dilution method. Each tube contained one ml of BHI culture medium. Extract solution was added to the first tube to achieve 100 mg/ ml concentration and mixed completely, then 1 ml of

solution from the first tube was added to the second tube. Finally, 1ml was discarded from the last tube so that the dilution of each tube was half of the concentration of the previous one. 50 microliter of the prepared bacterial suspension was added to each tube. Following 48h incubation at 37°C, the tubes were examined in terms of turbidity caused by the growth of inoculated bacteria. The first tube with no observable turbidity was selected as the lowest concentration that prevents bacterial growth (MIC) and then 10µl of that tube was cultured on the BHI agar without plants extract and incubated for 48 h at 37°C. If *E. faecalis* growth was positive, this concentration was considered as MIC [29].

In order to determine MBC of the extracts, 10 microliter was taken from all tubes with no observable bacterial growth and spread on the surface of BHI agar containing no extract. After 48 hours of incubation at 37°C, cultured plates were controlled for the presence of microbial growth. The first concentration of extracts without any bacterial growth on agar was considered as MBC [29].

Assessing Anti-biofilm effect of plant extracts

The anti-biofilm effect of the extracts was measured using microtiter plate assay and colorimetric crystal violet methods [30]. 150µl of BHI broth was added to 96 well microtiter plates. Then, 150 microliters of each of the extracts was added to the first well and was completely mixed. 150 microliters of solution in the first well was transferred to the second well, this trend continued through the tenth well, and finally 150 microliter of the solution was removed from the tenth well. Thus, each well had one-half of the concentration of previous one. Finally, 50 microliter of the prepared bacterial suspension (with turbidity of 0.5 McFarland) was added to the wells. Plates were incubated at 37°C for 48 hours. Then contents of the wells discarded by flipping the microtiter plate and all the wells were washed three times, each time with 300µl of sterilized physiological serum to remove all planktonic bacteria. Microtiter plate dried at room temperature. 200ml of 96% ethanol was then added to each well in order to stabilize the bacteria attached to the inner surface of the wells (which are biofilm population) and after 5-10 minutes, ethanol removed from the wells and the plate dried at room temperatures. Each well was stained for 5 minutes by 250 µl of 0.1% crystal violet. The wells were rinsed off with gentle flow of water and finally the plates were dried. Biofilms were then visible on the sides of each well. Optical density was measured at 490 nm using an automated enzyme-linked immunosorbent assay (ELISA) plate reader. The mean percentage inhibition of biofilm for each sample was calculated and compared to control wells.

Results

Results of Agar Well Diffusion Method

The inhibitory effect of the extracts on the growth of *E. faecalis* has been shown. Table 1 shows the mean diameter of inhibition zone for each tested material. Results are the mean of three repetitions. According to the present data, the methanolic extracts of *Myrtus communis L.* and *Eucalyptus galbie* had antibacterial activity against *E. faecalis*. The highest diameter of inhibition zone was related to Eucalyptus extract (9.6 mm). Regarding calcium hydroxide powder mixed with distilled water, ready to use calcium hydroxide paste containing iodoform and mixture of calcium hydroxide with extracts, there was no growth inhibition zone. In addition, inhibition zone did not establish for DMSO, which indicates that the solvent had no effect on reducing the population of bacteria.

Table 1: The mean diameters of growth inhibition zones' values (mm) after 48 hours

Sample	Mean	Standard Deviation (SD)
Methanolic extract of Eucalyptus	9.63	0.32
Methanolic extract of Myrtus	7.6	0.36
Calcium hydroxide+distilled water	-	-
Calcium hydroxide paste containing iodoform	-	-
Calcium hydroxide + Eucalyptus	-	-
Calcium hydroxide + Myrtus	-	-
DMSO **	-	-
Cefoxitin ***	13.7	0.25

* Dash means no inhibitory zone is established.
** Negative control
*** Positive control

In addition, there were no statistically significant differences between the antibacterial effect of methanolic extract of Myrtus, eucalyptus and positive control (Cefoxitin) (p= 0.987).

The results of MIC evaluation of extracts

MIC is the minimum concentration of an antimicrobial compound that prevents the growth of bacteria and in its

Table 2: The adherence status of *E.faecalis* in the presence of Myrtus methanolic extract (sub- MIC)

Concentration (mg/ml)	Mean optical density (nm)	Adherence status
6.25	0.134	WA
3.12	0.164	WA
1.56	0.202	MA
0.78	0.232	MA
0.39	0.251	MA
0.19	0.271	MA
Positive control*	0.527	SA
Negative control**	0.084	-

NA:Non Adherent; WA:Weakly Adherent; MA:Moderately Adherent; SA:Strongly Adherent
*Culture medium+ Bacteria (without extracts)
**Myrtus ethanolic extract + culture medium

Table 3: The adherence status of *E.faecalis* in the presence of Eucalyptus methanolic extract (before MIC)

Concentration (mg/ml)	Mean optical density (nm)	Adherence status
6.25	0.117	WA
3.12	0.135	WA
1.56	0.151	WA
0.78	0.176	MA
0.39	0.187	MA
0.19	0.213	MA
Positive control*	0.525	SA
Negative control**	0.084	-

NA:Non Adherent; WA:Weakly Adherent; MA:Moderately Adherent; SA:Strongly Adherent
*Culture medium+ Bacteria (without extracts)
**Eucalyptus ethanolic extract + culture medium

absence, the bacterium is able to grow. The concentration of MIC for the two mentioned extracts in this study was 12.5 mg/ml.

The results of MBC evaluation of extracts

MBC or minimal bactericidal concentration is the minimum concentration of an anti-microbial agent that can destroy 99.9% of bacteria and when secondary culture is prepared from this concentration, less than 0.1% of the initial bacteria will grow. According to the results of the current study, the two methanolic extracts had no bactericidal effect on *E. faecalis* and only had bacteriostatic effect.

The results of anti-biofilm effect of extracts

The results revealed that *E. faecalis* in the absence of any of the herbal extracts had high binding strength to polystyrene surface of microtiter plate and biofilm formation. As shown in Table 2, *E.faecalis* in the presence of the sub-MIC dilution of Myrtus extract (0.19-6.25 mg/ml) was able to form biofilms and none of the concentrations was able to inhibit biofilm formation. In the concentrations of 3.12 and 6.25mg/ml of the extract, weak biofilm formation was observed while in the concentrations of 0.19 to 1.56 mg/ml, moderate biofilm formation was observed.

As shown in Table 3, none of the sub- MIC concentrations of Eucalyptus extract was able to prevent biofilm formation of *E.faeca*lis. At the concentrations of 6.25 to 1.56 mg/ml, the adherence to the surface of wells and ability for biofilm formation was weak while in the concentrations of 0.78 to 0.19mg/ml, the adhesion was moderate.

Discussion

In this study, *E.faecalis* established no growth inhibition zone after 48 hours of exposure to calcium hydroxide powder mixed with distilled water as well as ready to use calcium hydroxide paste containing iodoform. This result is in line with some previous studies that showed *E. faecalis* is resistant to calcium hydroxide therapy [31-34].

The low influence of calcium hydroxide can be related to factors such as low solubility, low diffusion rate, differences in alkaline potential of different formulations, high density of bacterial biofilms and *E. faecalis* escape from hydroxyl ions due to hiding in irregularities, and the canal isthmus [35]. Moreover, *E. faecalis* has a proton pump with the capacity to acidify the cytoplasm. It enables the microorganism to be resistant to killing by calcium hydroxide at or below pH= 11.1 [12].

The vehicles that are used for mixing calcium hydroxide, in addition to maintaining alkalinity, should preferably enhance the antimicrobial effects of calcium hydroxide and the ability of its diffusion into the dentinal tubules [36-37]. Sjögren *et al.* [9] showed that mixing calcium hydroxide powder with various materials such as saline, distilled water and anesthetics help maintenance of high alkaline pH for at least 7 days. Viscose vehicles such as glycerin, polyethylene glycol, and propylene glycol lead to very slow release of calcium and hydroxyl ions for a longer period. In the situations, that calcium hydroxide should be remained in canal for a long time they would be beneficial [38]. Oil-based vehicles such as camphorated paramonochlorophenol, eugenol, or olive oil have limited applications and their application has been reported in situations that very slow diffusion of hydroxyl ions is required [39].

Over the last decade, several studies have been investigated antibacterial effects, diffusion rate, and the

pH of calcium hydroxide powder in combination with different vehicles [20, 31, 39-41]. The discrepancy between reported results can be related to the various methods of the studies.

According to the literature, there are limited studies concerning the effects of plant extracts on endodontics microorganisms. In an *in vitro* study by Nourzadeh *et al.* [25], irrigation of infected root canals with methanolic extracts of *Eucalyptus galbie* and *Myrtus communis L*. exerted acceptable antimicrobial activity against *E. faecalis*. Another study used disk agar diffusion method and indicated that methanolic extracts of *Myrtus communis L*. exhibited significant antibacterial activity against *E. faecalis* [26]. In contrast, in the study by Mert *et al.* [42], methanolic extract of *Myrtus communis L.* did not show activity against *E. faecalis*. Conflicting results may be due to different methodologies. Moreover, geographic features such as location make changes in structural chemicals and effects of a medicinal plant [42].

In the present study, and in line with two previous studies [25-26], extracts of eucalyptus and Myrtus showed significant antimicrobial activity against *E. faecalis*. After 48 hours, the effectiveness of these extracts was considerably better than calcium hydroxide. Different compounds of Eucalyptus leaves such as essences, phenolic acids, and tannins compound of this plant can show inhibitory effects against microbial strains. Eucalyptus leaves essence has a significant amount of Cineol, which has a considerable antibacterial activity [43]. Myrtus is an herb that has been traditionally used as an antiseptic, anti-inflammatory, analgesic, anti-virus, and anti-bacterial agent [24]. The essence of this plant also contains significant amounts of cineole. Its antimicrobial agents are in the category of phenolic compounds and particularly phenolic acids. In addition, these compounds may produce complex with other biomolecules especially with tannins [24].

The mixture of extracts with calcium hydroxide powder inhibited the antibacterial effect of the extracts. It is possible that by mixing the extracts with calcium hydroxide powder, certain chemical reactions occur that might reduce the release of the active ingredients. On the other hand, since calcium hydroxide has alkaline pH and remains stable at high pH, herbal extracts may become unstable in this pH and lose their properties.

In this study, like many other studies, *E. faecalis* was used as microorganism for the study [32, 44]. This choice is mainly related to the difficulty of eliminating *E. faecalis* from infected root canals and dentinal tubules that can lead to refractory apical periodontitis [17]. This bacterium can withstand very high pH values through activation of proton pump and its enzymatic systems and survive for 12 months under starvation condition. In addition, its culture is relatively easy [12, 45].

In biofilm phase, bacteria show considerable resistance against host immune system and external antimicrobial agents. Bacteria in biofilms are 2 to 1000 times more resistant than planktonic phase [3-4]. Therefore, in this study the effects of herbal extracts on biofilm was evaluated and according to the obtained results, none of the sub-MIC dilutions of the eucalyptus and Myrtus extracts (0.19– 6.25 mg/ml) were able to inhibit biofilm formation by *E. faecalis*. However, at concentrations of 3.12 and 6.25 mg/ml of Myrtus extract and concentrations of 6.25 to 1.56-mg/ml of Eucalyptus, the bacteria showed weak adherence to the well wall and ability to form biofilm.

Conclusion

The results of the present study showed after 48 hours, the methanolic extracts of *Eucalyptus galbie* and *Myrtus communis L.* had antibacterial effects, and that there was no significant difference between the extracts and positive control (Cefoxitin) in inhibiting *E. faecalis* growth. The extracts in combination with calcium hydroxide powder were not able to eliminate *E. faecalis.*

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Conflict of Interest

None declared.

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