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Antimicrobial Effect of *Pinus longifolia* Roxb. ex.Lamb. and *Pinus eldarica* Medw. Fruits Hydroalcoholic Extract Against *Candida albicans* and *Enterococcus faecalis*

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ABSTRACT

As a result of the growing resistance of pathogenic microorganisms to antibiotics, researchers are working to identify novel herbal medicines as an alternative to pharmaceutical medicines and antibiotics. The antimicrobial property of the hydroalcoholic extract of *Pinus longifolia* and *P. eldarica* fruits was examined in vitro with respect to *Candida albicans* and *Enterococcus faecalis* pathogens. *P. longifolia* and *P. eldarica* fruit hydroalcoholic extracts were made for this laboratory work, and the extracts' minimum inhibitory concentration (MIC), minimum bacterial killing concentration (MBC), and minimum fungal killing concentration (MFC) were measured. For *E. faecalis* and *Candida albicans*, the MIC of *P. longifolia* hydroalcoholic extract was 12.5 and 6.25 mg/ml, respectively. The MIC for the extract and chlorhexidine combination was 0.78 mg/ml. In the case of *E. faecalis* and *C. albicans*, the MIC of *P. eldarica* fruit hydroalcoholic extract was 12.5 and 6.25 mg/ml, respectively. The MIC of the extract + chlorhexidine against *E. faecalis* and *C. albicans* was 1.56 and 0.78 mg/ml, respectively. In comparison to the extract alone, the extract and chlorhexidine yielded superior results. Therefore, the combination of chlorhexidine and hydroalcoholic extracts of *P. longifolia* and *P. eldarica* could be a candidate for producing antimicrobial and strong intracanal rinsing compounds, which requires further investigation, including cytotoxicity assays for human cells and interaction with dentin or other detergents.

Keywords: *Pinus longifolia, Pinus eldarica,* Hydroalcoholic extract, Antimicrobial effect, *Enterococcus faecalis*

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INTRODUCTION

Enterococcus faecalis is a bacterium responsible for root canal infections. *E. faecalis* is a facultative anaerobic gram-positive coccus implicated in the pathogenesis of both treated and untreated root canal infections. Treatment failure often occurs as a result of antibiotic resistance [1, 2]. The prolonged life of *E. faecalis* in the root canal system may result from its capacity to cling to dentin, penetrate dentinal tubules, and form structured biofilms, hence inducing bacterial resistance to intracanal antibacterial strategies [3]. Fungi are common causes of infection in the oral soft tissue. Among the fungal infections of the mouth, candidiasis is highly prevalent. The opportunistic yeast *C. albicans* is the most common candidal agent causing oral candidiasis [4]. Success in root canal treatment in the past was based on complete disinfection of the root canal with chemical and mechanical processes. However, it is very difficult to completely disinfect the root canal system using conventional chemical and physical methods [5]. Currently, agents like sodium hypochlorite, chlorhexidine, and calcium hydroxide are used to disinfect the root canal. Nonetheless, infection persists in a considerable proportion of root canals due to several factors, including the intricacy of the root canal system, limited access of substances to certain areas, the development of microbial biofilms, and the inactivation of antimicrobial agents near dentin [6]. Due to the present limits and drawbacks of existing antimicrobial medications, it is essential to seek novel antimicrobial molecules that exhibit high efficacy and low toxicity, particularly from natural sources like plants and their secondary metabolites[7]. Plant extracts are abundant in bioactive molecules and are crucial in drug discovery due to the variety of their chemical components, including flavonoids, polyphenols, and alkaloids [8]. Presently, 80% of the global population utilizes medicinal plants, sometimes in the form of extracts or isolated active chemicals, for illness treatment. This estimate derives from a study by the World Health Organization (WHO), indicating that over 80% of the global population depends on plants or their

derivatives [9]. The adverse effects of synthetic substances, the rise in drug resistance, and the high costs associated with medication synthesis have long been the development of herbal medicines a subject of attention [10]. Consequently, given that medicinal plants have been used for the treatment of diverse diseases for many years, they have garnered the interest of contemporary researchers [11]. Iran has significant potential for medicinal plant cultivation because of its 11 distinct climatic variations. Among the plants that have therapeutic properties, pine can be mentioned. Important and medicinal species of dark pine are *Pinus* L., *Abies* Mill., *Larix* Mill., and *Cedrus* Trew [13]. *P. longifolia* Roxb. ex.Lamb. is a beautiful tree with thick bark with deep cracks, which, in terms of the appearance of these cracks, divide the bark of the tree trunk into irregular pieces along the entire length of the stem. It has uneven, winding branches and needle-shaped, narrow, and long leaves, which are grouped in a common sheath of 3. The resulting resinous substance has a bitter, acrid taste and has laxative, carminative, diuretic, analgesic, and anthelmintic effects and can be used to relieve indigestion and treat inflammation. It has laxative and expectorant properties and is beneficial in treating asthma, chronic bronchitis, epilepsy, dermatological conditions, and pruritus. The antimicrobial and antioxidant properties of these plants have been shown [14, 15]. Tehran pine plant, scientifically known as (*P. eldarica* Medw.*)*, is a one-foot plant, 15-12 meters high, with light gray or gray-brown bark and green leaves 6-9 centimeters long, which are elongated. The branches are elongated, and the fruits are cones with black seeds 6-7 mm long. This pine is native to Eldar, Georgia, and is therefore known as the Tehran pine. It has been cultivated in Iran for several centuries and is adapted to favorable environmental conditions, growing well in humid, semi-humid, and semi-arid areas. Regarding the compounds present in the leaf and fruit essence of this plant, it can be used in the perfumery industry, as a disinfectant, insecticide, antibacterial, sedative, astringent, and anti-inflammatory agent [16-18]. Therefore, in this study, we investigated the antimicrobial effect of hydroalcoholic extract of *P. longifolia* and *P. eldarica* fruits on the pathogens *C. albicans* and *E.faecalis* of the dental root canal *in Vitro*.

MATERIALS AND METHODS

Study type, Plants, and Microbial Isolates Studied

In this laboratory work, numerous female cones of *P. longifolia* and *P. eldarica* were obtained from Isfahan's flower garden. *E. faecalis* ATCC 29212 and *C. albicans* PTCC 5027 were collected from the Iranian Industrial Microorganism Collection Center.

The plant materials were identified and authenticated by Dr. Bahadori, botanist at the Faculty of Pharmacy, Tehran University of Medical Sciences, Tehran, Iran. The correct taxonomic names are *P. roxburghii* Sarg. (syn. *P. longifolia*) and *P. eldarica*. Voucher specimens have been deposited in the Herbarium of the Faculty of Pharmacy, Lorestan University of Medical Sciences (LUMH), under accession numbers LUMH- 2215 and LUMH-2216, respectively.

Preparation of Hydroalcoholic extract of *P. longifolia* and *P. eldarica* Fruits

After collecting the cones of *P. longifolia* and *P. eldarica*, they were washed with distilled water. Then, they were completely dried in the shade away from direct sunlight and at room temperature. To facilitate extraction, they were powdered using an electric grinder. Extraction was performed by maceration (soaking). To prepare the extract, 100 grams of dried plant powder was weighed and poured into a sterile beaker and 500 ml of 98% ethanol and distilled water were added. Extraction was performed using a hydroalcoholic solvent consisting of ethanol and distilled water in a 70:30 (v/v) ratio. The lids of the beaker were completely closed with parafilm, and the plant and solvent mixture was allowed to stand in a shaking incubator at 40 °C for 3-4 days. The maceration temperature of 40 °C was chosen based on previous studies that showed that gentle heating can increase the solubility and penetration rate of phenolic and resinous compounds present in *Pinus* species, without causing significant degradation of thermolabile compounds [19]. Our preliminary experiments at room temperature (25 °C) showed lower extraction yields and resulted in incomplete dissolution of the plant matrix; therefore, 40 °C was chosen as a good balance between extraction efficiency and stability of active compounds. The maceration process was carried out for 3-4 days to achieve maximum extraction efficiency and to avoid prolonged contact with the solvent, which could cause oxidation.

Then, the obtained extracts were filtered using Whatman filter paper. The excess solvent was separated using a rotary evaporator. The remaining liquid containing the filtered extract was poured into sterile glass plates and placed in an oven at 45°C until completely dry [20]. The dried extracts were stored in amber vials at 4 °C until further use.

The Antimicrobial Effect of Hydroalcoholic Extract of *P. longifolia* and *P. eldarica* Against *E. faecalis* and *C. albicans*

The antibacterial efficacy of the extracts, chlorhexidine, sodium hypochlorite, and nystatin was examined using the well diffusion technique on the agar surface [21]. Initially, a microbial suspension with a turbidity corresponding to half McFarland (1.5×10^8 CFU/ml) was prepared. Subsequently, sterile swabs were employed to inoculate the surface of Mueller Hinton Agar for *E. faecalis* and Sabouraud Dextrose Agar (SDA) for *C. albicans*. After the surface of each plate was dried, the wells with a diameter of 5 mm were created at an appropriate distance from each other using a sterile glass Pasteur pipette. In these wells, different concentrations of extracts were poured separately, including 50 and 100 mg/ml, chlorhexidine 30 µl, sodium hypochlorite, and nystatin. The plates were kept at 37°C for 24 hours. After this period, the sensitivity of the studied isolates to the extracts, chlorhexidine, cerium hypochlorite, and nystatin was measured by measuring the diameter of the growth inhibition zone.

MIC of Hydroalcoholic Extracts of *P. longifolia* and *P. eldarica*

For MIC, MBC, and MFC determinations, the same standardized inoculum used in the well diffusion assay was applied, corresponding to 0.5 McFarland standard (approximately 1.5×10^8 CFU/ml for bacteria and 1.0×10^6 CFU/ml for yeasts), as recommended by CLSI guidelines. Antifungal and antibacterial susceptibility testing was conducted to ascertain the MIC of hydroalcoholic extracts from *P. longifolia* and *P. eldarica* fruits. This was achieved utilizing 25.5% sodium hypochlorite and chlorhexidine as a control for *E. faecalis*, nystatin as a control for *C. albicans*, and normal saline as a negative control, employing the micro broth dilution method in a 96-well microplate in accordance with the guidelines of the Institute of Laboratory and Clinical Standards (For bacteria M07/M100 and for fungi M27/M60) [22, 23]. To prepare medicinal dilutions of the

aforementioned extracts, in terms of the insolubility of the extract in water, it was dissolved in dimethylsulfoxide (DMSO), and a dilution of 1.6 mg/ml (1600 µg/ml) was obtained, then diluted with Mueller Hinton Broth (MHB) medium. After preparing serial dilutions of the extracts and drug, 100 µL of the extract and drug were poured into the wells of the microplate, and the same proportion of bacterial suspension was added. Then, they were incubated with a negative control that lacked the organism and a positive control that contained a bacterial suspension for 48 hours at 35 °C, and the MIC was determined visually. In all assays, extracts were dissolved in DMSO, with the final DMSO concentration kept below 1% (v/v). A DMSO-only control was included to confirm that DMSO had no inhibitory effect. All antimicrobial assays were performed in triplicate (n=3). Results are presented as mean ± standard deviation (SD). Serial dilutions of the extracts were prepared at concentrations of 1600, 800, 400, 200, 100, 50, 25, 12.5, 6.25, 3.125, and 1.56 mg/ml. Each assay was performed in triplicate (n = 3). The MIC endpoint was defined as the lowest concentration showing complete absence of visible turbidity after 48 h incubation at 35 °C, in accordance with CLSI M07/M27 guidelines.

MBC and MFC of Hydroalcoholic Extracts of *P. longifolia* and *P. eldarica*

To determine MBC and MFC, the MIC concentration and several concentrations higher than it are cultured on Mueller Hinton agar medium. After incubation for 24 hours at 37°C, the lowest concentration of extracts in which microbial strains did not grow was considered the MBC and MFC.

RESULTS

Determination of the Antimicrobial Effect of Hydroalcoholic Extract of *P. longifolia* and *P. eldarica* Fruits on *E. faecalis* and *C. albicans* by Well Diffusion Method

It should be noted that the well diffusion method has inherent limitations for plant extracts with hydrophobic components, as these compounds may not diffuse uniformly in agar media. Therefore, this method was applied only for preliminary qualitative screening, whereas the broth microdilution assay was considered the main quantitative method for determining antimicrobial activity. The antibacterial efficacy of the hydroalcoholic extract from the fruits of *P. longifolia* and *P. eldarica* against *E. faecalis* and *C. albicans*, as determined by the well diffusion technique on culture media, is shown in Table 1 and Figure 1. In comparison to the analyzed chemicals, the hydroalcoholic extract of *P. longifolia* combined with chlorhexidine exhibited the greatest halo diameter (18 mm) against *E. faecalis*. Hydroalcoholic extract of *P. longifolia* at 50 and 100 mg/ml had a smaller halo diameter than chlorhexidine and sodium hypochlorite. By examining the effect of *P. longifolia* hydroalcoholic extract against *C. albicans*, it was found that nystatin and chlorhexidine had the largest diameter of growth inhibition zone against *C. albicans*, with a size equivalent to 25 and 20 mm, respectively. *P. longifalia* hydroalcoholic extract at concentrations of 50 and, 100 mg/ml and in combination with chlorhexidine, had a smaller diameter of the growth inhibition zone than other compounds such as chlorhexidine, nystatin, and sodium hypochlorite. Thus, it was found that *P. longifolia* hydroalcoholic extract showed the greatest antimicrobial effect in combination with chlorhexidine against *E. faecalis*. The antimicrobial activity of *P. eldarica* hydroalcoholic extract against *E. faecalis* showed that, after chlorhexidine, the combination of extract with chlorhexidine had the largest diameter of the zone (19 mm) against *E. faecalis*. All inhibition-zone measurements were recorded in millimeters (mm) and are presented as mean ± SD from three independent replicates (n = 3).

The hydroalcoholic extract of *P. eldarica* at doses of 50 and 100 mg/ml exhibited a reduced growth inhibition zone diameter compared to chlorhexidine. The hydroalcoholic extract of *P. eldarica* exhibited antimicrobial action against *C. albicans*, demonstrating that the combination of the extract with chlorhexidine produced the biggest inhibition zone width (22 mm) after nystatin. Consequently, the hydroalcoholic extract of *P. eldarica*, when combined with chlorhexidine, exhibited antibacterial efficacy against *E. faecalis* and *C. albicans.*

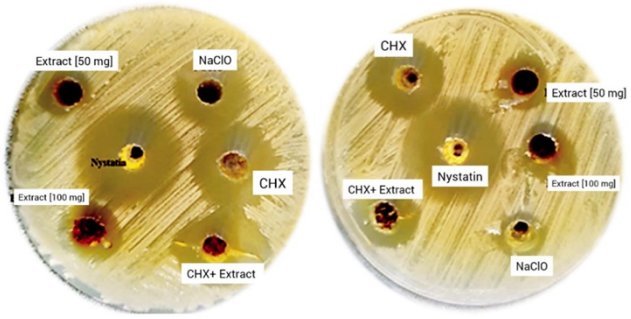

Fig. 1 Antimicrobial activity of hydroalcoholic extract of *P. longifolia* and *P. eldarica* fruits against *E. faecalis* and *C. albicans* by well diffusion method. Each assay was performed in triplicate (n = 3). Data represent mean ± SD. Statistical significance was determined using one-way ANOVA followed by Tukey's post hoc test ($p < 0.05$). Scale bar = 10 mm.

Table 1 Diameter of the microbiological inhibitory zone of the hydroalcoholic extract of *P. longifolia* and *P.eldarica* fruits against *E. faecalis* and *C.albicans* (mm)

Hydroalcoholic extract	Microbial strains	Nystatin (1mg/ml)	Sodium hypochlorite (2.5%)	Chlorhexidine(15ul) + hydroalcoholic extract(15ul)	Chlorhexidine (30ul)	Hydroalcoholic extract 50mg/ml	Hydroalcoholic extract 100 mg/ml
P. longifolia	*E. faecalis*	-	12 □ 0.0 (n = 3)	18 □ 1.0 (n = 3)	22 □ 0.0 (n = 3)	6 □ 3.0 (n = 3)	8 □ 2.0 (n = 3)
P. eldarica	*E. faecalis*	-	12 □ 0.0 (n = 3)	19 □ 1.0 (n = 3)	22 □ 1.0 (n = 3)	8 □ 2.0 (n = 3)	12 □ 1.0 (n = 3)
P. longifolia	*C. albicans*	25	18 □ 1.0 (n = 3)	13 □ 3.0 (n = 3)	20 □ 1.0 (n = 3)	10 □ 3.0 (n = 3)	13 □ 2.0 (n = 3)
P. eldarica	*C. albicans*	25	18 □ 2.0 (n = 3)	12 □ 2.0 (n = 3)	20 □ 1.0 (n = 3)	10 □ 3.0 (n = 3)	8 □ 2.0 (n = 3)

Data are expressed as mean ± standard deviation (SD) from three independent experiments (n = 3).

Determination of the MIC

The MIC of the hydroalcoholic extract of *P. eldarica* fruit against *E. faecalis* and *C. albicans* was 12.5 and 6.25 mg/ml, respectively, while the MIC for the extract + chlorhexidine combination was 0.78 mg/ml. MIC for the extract + chlorhexidine combination against *E. faecalis* and *C. albicans* was 1.56 and 0.78 mg/ml, respectively. In fact, similar to the results of the well diffusion method, the extract + chlorhexidine combination had better results than the extract alone. MIC for nystatin was 39 µg/ml, which indicated a stronger antifungal effect of this

combination compared to the compounds studied in our study. In general, compared to chlorhexidine, the extract + chlorhexidine combination also showed favorable results in terms of microbial inhibition

Table 2 MIC of hydroalcoholic extracts

Hydroalcoholic extract	Microbial strains	Nystati (µg/ml)	Sodium hypochlorite	Chlorhexidine + hydroalcoholicExtract (mg/ml)	Chlorhexidine (mg/ml)	Hydroalcoholi extract (mg/ml)
P. longifolia	*E. faecalis*	-	-	0.78 □ 0.0 (n = 3)	0.78 □ 2.0 (n = 3)	12.5 □ 1.0 (n = 3)
P. eldarica	*E. faecalis*	-	-	1.56 □ 1.0 (n = 3)	0.78 □ 2.0 (n = 3)	12.5 □ 3.0 (n = 3)
P. longifolia	*C. albicans*	39	-	0.78 □ 1.0 (n = 3)	0.78 □ 1.0 (n = 3)	6.25 □ 0.0 (n = 3)
P. eldarica	*C. albicans*	39	-	0.78 □ 2.0 (n = 3)	0.78 □ 0.0 (n = 3)	6.25 □ 1.0 (n = 3)

Data are expressed as mean ± standard deviation (SD) from three independent experiments (n = 3).

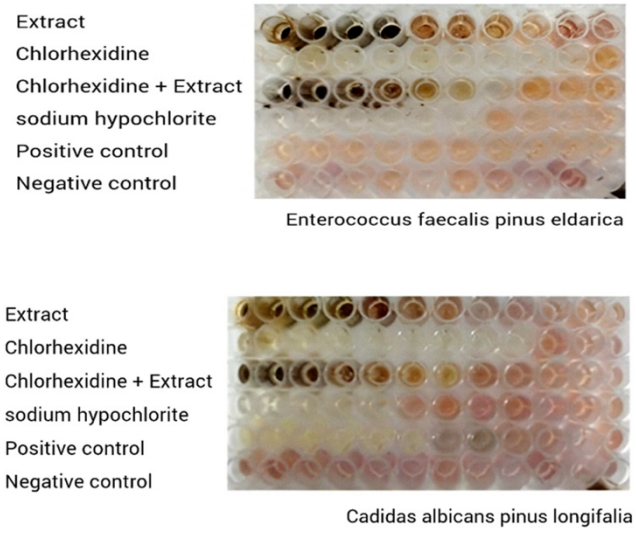

Fig. 2 MIC of hydroalcoholic extracts. Each assay was performed in triplicate (n = 3). Data represent mean ± SD. Statistical significance was determined using one-way ANOVA followed by Tukey's post hoc test ($p < 0.05$). Scale bar = 10 mm.

The Antimicrobial Effect of Hydroalcoholic Extract of *P. longifolia* and *P. eldarica* Fruits Using MBC and MFC

The findings of the present study on the antimicrobial efficacy of the hydroalcoholic extracts of *P. longifolia* and *P. eldarica* fruits, based on MBC and MFC values, are presented in Figure 3 and Table 3. The MBC and MFC values of the hydroalcoholic extract of *P. longifolia* fruit were 25 mg/mL and 12.5 mg/mL, respectively. However, when combined with chlorhexidine, the MBC and MFC values decreased to 3.125 mg/mL and 1.56 mg/mL, respectively. The MBC and MFC values for chlorhexidine alone were 1.56 mg/mL. Compared with chlorhexidine alone, the extract–chlorhexidine combination eradicated *C. albicans* at an equivalent concentration, while a higher concentration was required to eliminate *E. faecalis*. For *P. eldarica*, the MBC of the hydroalcoholic extract was 12.5 mg/mL. When combined with chlorhexidine, both the MBC and MFC values were reduced to 1.56 mg/mL. In contrast, chlorhexidine alone showed MBC and MFC values of 1.56 mg/mL. The combination demonstrated effective elimination of both *C. albicans* and *E. faecalis* at comparable concentrations. The lowest lethal concentration of nystatin was 39 µg/mL, indicating strong antifungal activity compared with the other agents evaluated in this study.

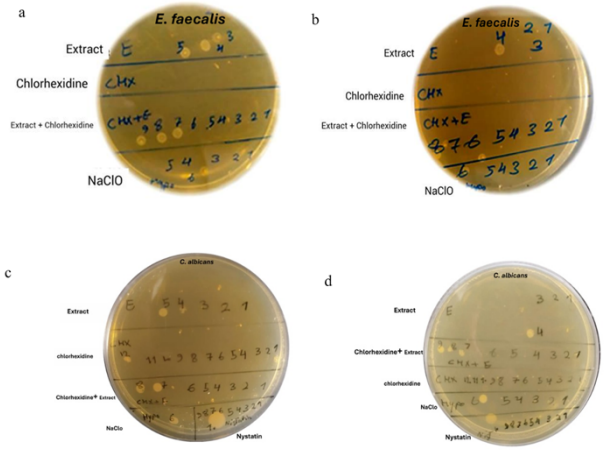

Fig. 3 Antimicrobial effect of extracts using MBC and MFCf . a and c: antimicrobial efficacy of hydroalcoholic extract of *P. longifolia* . b and d: antimicrobial efficacy of hydroalcoholic extract of *P. eldarica)*. Each assay was performed in triplicate (n = 3). Data represent mean ± SD. Statistical significance was determined using one-way ANOVA followed by Tukey's post hoc test ($p < 0.05$). Scale bar = 10 mm.

Table 3 MFC and MBC of hydroalcoholic extracts

Hydroalcoholic extract	Microbial strains	Nystatin (µg/ml)	Chlorhexidine+ hydroalcoholic Extract (mg/ml)	Chlorhexidine (mg/ml)	Hydroalcoholic extract (mg/ml)
P. longifolia	*E. faecalis*	-	3.125 □ 1.0 (n = 3)	1.56 □ 2.0 (n = 3)	25 □ 0.0 (n = 3)
P. eldarica	*E. faecalis*	-	1.56 □ 5.0 (n = 3)	1.56 □ 1.0 (n = 3)	12.5 □ 1.0 (n = 3)
P. longifolia	*C. albicans*	39	1.56 □ 1.0 (n = 3)	1.56 □ 0.0 (n = 3)	12.5 □ 4.0 (n = 3)
P. eldarica	*C. albicans*	39	1.56 □ 0.0 (n = 3)	1.56 □ 1.0 (n = 3)	12.5 □ 1.0 (n = 3)

Data are expressed as mean ± standard deviation (SD) from three independent experiments (n = 3)

DISCUSSION

Considering the fundamental problems of the commonly used routine compounds, such as the lack of effectiveness of conventional drugs, adverse drug reactions, and the increasing prevalence of microbial resistance, researchers have become increasingly interested in using medicinal plants and conducting further research in this field to find an effective herbal alternative to routine drugs. Because of the limited diffusibility of hydrophobic phytochemicals in agar, the well diffusion results should be interpreted as supportive, qualitative findings. The quantitative evaluation of antimicrobial activity in this study relied primarily on the broth microdilution method, which provides more reproducible and comparable data. Many studies have proven the effectiveness of some medicinal plants for the treatment of many diseases [24]. The current research aimed to examine the antibacterial efficacy of the hydroalcoholic extract of *P. longifolia* and *P. eldarica* fruits against the diseases *C. albicans* and *E. faecalis* in vitro.The study on the antimicrobial

effects using the well diffusion method demonstrated that the hydroalcoholic extract of *P. eldarica*, in conjunction with chlorhexidine, exhibited antimicrobial activity against *E. faecalis* and *C. albicans*. Conversely, the hydroalcoholic extract of *P. longifolia*, when combined with chlorhexidine, displayed antimicrobial activity solely against *E. faecalis*. The research findings indicate that the MIC of *P. longifolia* fruit hydroalcoholic extract against *E. faecalis* and *C. albicans* was 12.5 mg/ml and 6.25 mg/ml, respectively, but the MIC for the extract combined with chlorhexidine was 0.78 mg/ml. The MIC of the hydroalcoholic extract of *P. eldarica* fruit against *E. faecalis* and *C. albicans* was 12.5 mg/ml and 6.25 mg/ml, respectively. In contrast, the MIC of the extract combined with chlorhexidine against *E. faecalis* and *C. albicans* was 1.56 mg/ml and 0.78 mg/ml, respectively. Indeed, similar to the findings of the antibacterial characteristics investigation using the well diffusion technique, the combination of extract and chlorhexidine yielded superior results compared to the extract alone. The hydroalcoholic extract of *P. longifolia* fruit, when combined with a higher dose of chlorhexidine, had greater efficacy in eliminating *E. faecalis* compared to *C. albicans*. The hydroalcoholic extract of *P. eldarica* fruit, in conjunction with chlorhexidine, effectively eradicated *C. albicans* and *E. faecalis*, surpassing the efficacy of chlorhexidine alone at equivalent concentrations. A study conducted by Norouzi et al. in 2023 investigated the antifungal properties of *P. eldarica* and *P. longifolia* fruit extracts against *Candida* spp. isolated from patients with vulvovaginal candidiasis. The findings indicated that aqueous, ethanolic, and methanolic extracts exhibit weak anti-*Candida* effects, which contradicts the results of the current study [25]. The variance in findings from the study of plant extracts may stem from discrepancies in the extraction procedure, the kind of plant extract (aqueous, alcoholic, or hydroalcoholic), or the methodologies used to assess antimicrobial activities. A study conducted by Ghaffari et al. in 2021 revealed that lung cancer cells treated with *P. eldarica* essential oil exhibited a significant reduction in cell proliferation and an induction of apoptotic cell death, when compared to treatments with needles, bark, and pollen extract [26]. Research conducted by Sarvmeili et al. in 2016 assessed the cytotoxic effects of *P. eldarica* essential oil and extract on HeLa and MCF-7 cell lines, revealing that *P. eldarica* had significant potential as an active anticancer agent [27]. Consequently, based on the findings of prior research and our investigation, *P. eldarica* extract exhibits greater antibacterial and anticancer potential, likely attributable to its active constituents.

Research conducted by Khodadadnejad et al. sought to examine the antibacterial efficacy of 2.5% sodium hypochlorite, 10% case microemulsion, and 0.6% thyme microemulsion against *E. faecalis* after root canal obturation. The findings indicated that 2.5% sodium hypochlorite exhibited the most significant antibacterial efficacy against *E. faecalis*, resulting in the lowest colony development rate; conversely, 10% case microemulsion and 0.6% thyme microemulsion were positioned second and third, respectively [28]. In our research, the combination of extract and chlorhexidine was rated second in efficacy, behind chlorhexidine alone. In the 2015 study by Khatun *et al.,* following the green synthesis of silver oxide nanoparticles derived from *P. longifolia* leaf extract, the assessment of their antibacterial activity revealed a broad spectrum of antimicrobial efficacy against both gram-positive and gram-negative bacteria.

The antibacterial efficacy of silver nanoparticles synthesized using pine extract against *Staphylococcus aureus, Bacillus cereus*, and *Escherichia coli* has shown significant potential as an antimicrobial agent in the pharmaceutical, food, and cosmetic industries. Khatun *et al.* [15] reported that *P. longifolia* extract in combination with silver nanoparticles exhibited notable antibacterial activity. Similarly, our findings demonstrated substantial antimicrobial efficacy when the extract was combined with chlorhexidine.

A 2013 study by Vinothkumar et al. evaluated the antimicrobial efficacy of five plant extracts—*Terminalia chebula, Myristica fragrans, Aloe barbadensis, Curcuma longa,* and *Azadirachta indica* (neem)—against *C. albicans* and *E. faecalis* [29]. The findings indicated that neem extract was particularly effective in diminishing *E. faecalis* and *C. albicans* in tooth roots. In this investigation, extracts of *P. longifolia* and *P. eldarica* exhibited minimal antibacterial activity against *C. albicans* and *E. faecalis* pathogens; however, when combined with chlorhexidine, their antimicrobial efficacy was equivalent to that of chlorhexidine alone. In 2011, Radulescu et al. demonstrated that the essential oil derived from the young branches of *Picea abies* (L.) Karst. has antimicrobial properties, proving to be more efficient against fungal strains and gram-positive bacteria[30]. The current investigation, which evaluated the extracts against *C. albicans* and the gram-positive bacteria *E. faecalis*, revealed that the extracts exhibited little antibiotic activity. Iqbal et al. (2011) examined the essential oil derived from Kashfi pine leaves and its constituents, concluding that the predominant active component exhibits efficacy against *Staphylococcus aureus* and *Bacillus subtilis*, while demonstrating no activity against *Escherichia coli*, *Salmonella typhi*, and *Enterobacter* spp. [31]. The disparity in findings between this study and Radulescu's research may be attributed to the differing compounds examined: essential oil vs extract of *P. longifolia*. Besides the researched plant chemicals, such as essential oils and other extracts, variations in the microbial strains examined may also contribute to the discrepancies in the findings of different studies. This research used most resistant microbial strains, namely the *E. faecalis* strain, which is a biofilm builder and recognized as one of the resistant strains. It should be noted that the MIC, MBC, and MFC values obtained for the hydroalcoholic extracts were relatively high compared to standard antimicrobial agents such as nystatin or chlorhexidine. This difference is expected because crude plant extracts contain a mixture of active and inactive constituents at much lower effective concentrations than purified drugs. Moreover, in some cases, the MIC or MBC values of the extract + chlorhexidine combination were equal to those of chlorhexidine alone. In such instances, the interpretation refers to comparable or non-inferior antimicrobial efficacy rather than superior activity. These findings collectively suggest that the extracts can enhance or maintain antimicrobial potency when combined with chlorhexidine, potentially reducing the need for higher concentrations of synthetic agents.

Limitations of the present study include the the lack of cytotoxicity assessment, the lack of in vivo validation, and lack of data from animal models. These assessments will be necessary in future studies to complete the safety, efficacy, and clinical application of the extract. One of the weaknesses of the present study is that although the results showed that the combination of the extract with chlorhexidine had better effects compared to the MIC of the extract alone, in order to prove and precisely explain the nature of this effect (synergistic or simply additive), there is a need to report the FICI index and/or draw time-kill curves. The

lack of these assessments limits the interpretation of the results, and it is suggested that this issue be addressed in future studies.

CONCLUSION

The results of this study suggest that *P. longifolia* and *P. eldarica* hydroalcoholic extracts, when used in conjunction with chlorhexidine, are effective antimicrobial agents against certain significant organisms in the dental root canal. Furthermore, the findings indicated that the combination of chlorhexidine and *P. longifolia* and *P. eldarica* hydroalcoholic extracts could be considered a potential candidate for the production of potent antimicrobial and irrigating compounds in the canal, thereby enhancing the success rate of root canal treatment. However, further research *in vivo* and *in vitro* conditions is necessary.

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ETHICS

Our research project was approved by the Research Ethics Committee of Lorestan University of Medical Sciences and with the code of ethics IR.LUMS.REC.1403.313.

CONFLICT OF INTEREST STATEMENT

The authors declare that they have no competing interests.

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