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Original Article

In vitro comparison of antioxidant, cytotoxic, and antibacterial (against *Aggregatibacter actinomycetemcomitans*) effects of *Citrus reticulata*, *Olea europaea* extracts, and essential oils

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ABSTRACT

Background: This study compared the antioxidant, cytotoxic, and antibacterial effects of *Citrus reticulata (C. reticulata)* peel and *Olea europaea (O. europaea)* leaf hydroalcoholic extracts and essential oils.

Materials and Methods: In this *in vitro* study, the antibacterial effect of the *C. reticulata* and *O. europaea* extracts and essential oils was evaluated on *Aggregatibacter actinomycetemcomitans* (*A. actinomycetemcomitans*) by the agar diffusion test. Their minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were determined by the broth microdilution method. Their cytotoxicity against human gingival fibroblasts was evaluated by the methyl thiazolyl tetrazolium assay in 24 and 72 h, and their antioxidant effect was assessed by the 2,2-diphenyl-1-picrylhydrazyl assay. Data were analyzed by ANOVA and Tukey's test ($P < 0.05$).

Results: The *O. europaea* hydroalcoholic extract and *C. reticulata* essential oils had significant antibacterial effects on *A. actinomycetemcomitans*. The highest and lowest growth inhibition zones belonged to *C. reticulata* essential oils (40.0 ± 0.0 mm) and *O. europaea* extract (32.3 ± 2.2 mm), respectively. The MIC and MBC were 0.78 v/v% for *O. europaea* extract and 6.25% for *C. reticulata* essential oils. Unlike the essential oils (1%, 5%, and 10% v/v), none of the extracts showed significant cytotoxicity at low concentrations (1% v/v). All essential oils and extracts, particularly *O. europaea* and *C. reticulata* extracts, had significant antioxidant effects.

Conclusion: Of the tested materials, *O. europaea* extract had the highest potential for possible use as an organic mouthwash due to its strong antibacterial and antioxidant effects and absence of cytotoxicity in low concentrations.

Key Words: Antibacterial agents, antioxidants, citrus, cytotoxicity, essential oils, immunologic, *Olea*, plant extracts, volatile

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INTRODUCTION

Oral and dental conditions can cause pain, discomfort, and dysfunction and adversely affect the quality of life.[1] Periodontal diseases are chronic bacterial infections caused by the mutual effects of microbial biofilm and host inflammatory response, which result in gingival inflammation,

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periodontal destruction, and bone loss. No treatment would result in disease progression and subsequent tooth loss.[2] Gram-negative bacteria such as *Fusobacterium nucleatum, Aggregatibacter actinomycetemcomitans (A. actinomycetemcomitans), Prevotella intermedia, Treponema denticola, Bacteroides forsythus, and Porphyromonas gingivalis (P. gingivalis)* are the main pathogenic microorganisms responsible for development of periodontal diseases. *A. actinomycetemcomitans* is among the main culprits responsible for the development of periodontitis.[3]

Scaling and root planing are part of nonsurgical periodontal therapy during which supragingival and subgingival microbial plaque and calculus are removed.[4] However, due to incomplete debridement of some inaccessible areas, risk of recolonization of microorganisms still exists after scaling and root planing. Thus, adjunct treatments with chemical antimicrobial agents can decrease the recolonization of bacteria and the subsequent need for pocket surgery.[5]

Considering the increased resistance to antibiotics and the adverse side effects of most chemical antibacterial agents, there is an obvious need for safe, cost-effective, and efficient alternatives. Accordingly, researchers have been trying to formulate nonchemical and organic mouthwashes with high efficacy against periopathogenic microorganisms and minimal adverse side effects.[6] The role of some herbal extracts and essential oils in the prevention and treatment of oral conditions, growth inhibition of oral pathogens, and prevention of biofilm and dental plaque formation has been previously confirmed.[7]

Essential oils are synthesized in different parts of plants as secondary metabolites and can have strong antibacterial effects.[8] Essential oil is a thick hydrophobic liquid containing volatile compounds, whereas extract is obtained from inactive parts of plants using a proper solvent aiming to increase the concentration and purity of effective agents and their stability.[9]

Carrol *et al*.[10] tested 416 herbal species and showed that *Olea europaea* (*O. europaea*) extract had the lowest minimum inhibitory concentration (MIC) against *P. gingivalis*. The alcoholic extract[11] and essential oil of *Citrus reticulata (C. reticulata)* also have strong antibacterial activity against pathogens, particularly *Streptococcus mutans*.[12] However, no information is available regarding the antibacterial effects of *O. europaea* and *C. reticulata* extracts and essential oils on *A. actinomycetemcomitans*.

An optimal mouthwash should have no or minimal cytotoxic effects on oral cells and must not trigger local or systemic allergic reactions. Since oxidative stress plays an important role in the pathogenesis of periodontal diseases, materials with antioxidant effects can effectively decrease inflammation and tissue destruction.[13] Thus, this *in vitro* study aimed to compare the antioxidant, cytotoxic, and antimicrobial (against *A. actinomycetemcomitans*) effects of *C. reticulata* and *O. europaea* extracts and essential oils.

MATERIALS AND METHODS

In this *in vitro* study, *A. actinomycetemcomitans* (Aa jp2nov799; ATCC 700685) was obtained from the Oral Microbiology Laboratory of Shahid Beheshti Dental School for antimicrobial testing, and human gingival fibroblasts (HGF1 PI 1 [NCBI: C165]) were obtained from the Cell Bank of the Pasteur Institute of Iran. The *C. reticulata* peel, *O. europaea* leaf hydroalcoholic extracts, and *C. reticulata* peel essential oils were obtained from the Traditional Medicine Department of Tehran University of Medical Sciences.

Preparation of bacterial suspension

Initially, *A. actinomycetemcomitans* was stored at −20°C for 24 h and was then refrigerated at 4º–8°C for 3 h. It was then cultured on brain–heart infusion (BHI) agar. The bacterial culture was subsequently incubated at 37°C in an anaerobic jar (Gas-Pak A) for 48 h. A sterile loop was used to collect some single colonies from the solid BHI agar culture and transfer them into a glass test tube containing BHI broth. The contents of the tube were vortexed to obtain a homogenous microbial suspension. The optical density (OD) of 1 mL of the suspension was read at 600 nm wavelength spectrophotometrically (UV-visible spectrophotometer; UNICO, USA). The culture medium without the bacteria served as the control, and a microbial suspension with 0.08–0.1 OD was considered to have 0.5 McFarland standard concentration.

Assessment of antibacterial effects of extracts and essential oils

Agar well diffusion test

The agar well diffusion or the well-plate technique[14] was used for initial assessment of microbial susceptibility to the respective herbal extracts and

essential oils (presence/absence of antibacterial effects). For this purpose, a sterile cotton swab was dipped in 0.5 McFarland standard bacterial suspension to lawn-culture the bacteria on solid BHI agar plate. Each plate was then hypothetically divided into four segments, and in each segment, a well was created by the round end of a sterile Pasteur pipette. Next, 50 µL of each extract/essential oil (sterilized) was inoculated into each well by a sampler. Furthermore, 0.2% chlorhexidine (CHX) mouthwash served as the positive control. This test was repeated three times in three different plates with the same test and control groups. Finally, the plates were incubated at 37°C for 24 h in an anaerobic jar (Gas-Pak A) to qualitatively assess the formation of growth inhibition zones and quantitatively measure their diameter by a millimeter ruler.

Determination of minimum inhibitory concentration

The MIC of the extracts and essential oils was determined by the broth microdilution method using colorimetry (resazurin dye).[14,15] For this purpose, 100 µL of sterile BHI broth was added to all wells of a 96-well plate. Next, 100 µL of the respective essential oil/extract was added to the first well of the test row and mixed with the culture medium using a sampler. Next, 100 µL of the contents of this well was transferred to the second well, and this process was repeated until the 12th well. Next, 10 µL of bacterial suspension containing 10^7 colony-forming units (CFUs)/mL was added to all wells except for the negative control wells (final concentration of 10^6). The negative control wells contained BHI broth and essential oil/extract without bacterial suspension. One row was also considered the positive control and its wells contained BHI and bacterial suspension without any extract/essential oil. The plates were subsequently incubated at 37°C for 48 h under anaerobic conditions. Next, 20 µL of 0.01% resazurin blue dye was added to all wells, and the plates were incubated at 37°C for 3 h. The lowest concentration of extract/essential oil that did not show pink discoloration (remained blue) was recorded as the MIC of the respective extract/essential oil.

Determination of minimum bactericidal concentration

To determine the minimum bactericidal concentration (MBC) of the essential oils/extracts, samples were collected from all blue-color wells before the MIC well, and the MIC well itself and also all pink-color wells (indicating bacterial growth) after the MIC well by a sterile loop, and cultured on BHI agar (three repetitions for each specimen). The plates were incubated at 37°C for 72 h in an anaerobic jar, and the lowest concentration of extract/essential oil with no bacterial growth was recorded as the MBC of the respective extract/essential oil.

Assessment of cytotoxicity by the methyl thiazolyl tetrazolium assay

Cytotoxic effects of 1%, 5%, and 10% (v/v) extracts and essential oils were evaluated. Human gingival fibroblasts in the logarithmic growth phase were counted under sterile conditions using Trypan Blue dye and a hemocytometer and were seeded in each well of 96-well culture plates with a cell density of 5000 cells/well along with 100 µL of Dulbecco's modified Eagle's medium and antibiotic. The plates were then incubated at 37°C, 95% humidity, and 5% CO2 for 24 h.

On day 2, after ensuring the health status of the cells, no contamination, and a minimum of 50% confluence, different concentrations of the extracts/essential oils were separately added to the cells. For this purpose, two plates were considered for assessment of cell viability and proliferation and acute cytotoxicity after 24 h of exposure and cell viability and proliferation and chronic cytotoxicity after 72 h of exposure. The plates were incubated and, at each time point (24/72 h), the overlaying medium was gently removed, the well contents were rinsed with phosphate-buffered saline, and culture medium (without serum and antibiotic) containing 10% methyl thiazolyl tetrazolium (MTT) dye (Sigma-Aldrich) was added to each well in an amount of 100 µL/well. The plates were incubated for 3 h, and after ensuring the formation of formazan crystals under an inverted microscope, the MTT dye (Sigma-Aldrich) was removed and replaced with an equal amount of dimethyl sulfoxide (Sigma-Aldrich). The OD of the wells was then read by an ELISA reader, and the percentage of cell viability was calculated by dividing the mean OD of the treated cells in each group by the mean OD of the control wells (nontreated cells with 100% viability) and multiplied by 100. According to ISO-10993-5, a material causing over 30% reduction in cell viability percentage compared with the control group (resulting in <70% viability) is considered cytotoxic.

Assessment of antioxidant effects

The 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay (reduction of free radicals) was used for evaluation of antioxidant effects of essential oils and extracts. Antioxidant activity of a material results in discoloration of DPPH to pale, no color, or yellow color, depending

on the intensity of its activity. The intensity of discoloration can be measured spectrophotometrically at a wavelength of 490–520 nm. For this assay, 1 mM concentration of ascorbic acid (Vitamin C) in water was prepared to serve as a positive control due to its confirmed antioxidant effect. Next, 1%, 5%, and 10% (v/v) concentrations of essential oils and hydroalcoholic extracts were prepared. DPPH solution with 0.1 mM concentration in pure ethanol was then prepared. For the test groups, 50 µL of ascorbic acid and the respective essential oil/extract were added to each well of a 96-well plate (three repetitions for each concentration), and then 50 µL of DPPH dye was added to each well. For the blank groups, 50 µL of ascorbic acid and the respective essential oil/extract were added to each well of a 96-well plate (three repetitions for each concentration) and then 50 µL of pure ethanol was added to each well. For the negative control groups, 50 µL of pure ethanol was added to each well of a 96-well plate, and then 50 µL of DPPH dye was added to each well. The plates were incubated in the dark at room temperature for 30 min, and the OD of each well was read by an ELISA reader at 429 nm wavelength. The percentage of inhibition of DPPH free radicals was calculated using the following formula:

$$\text{Percentage of inhibition of DPPH free radicals} = \frac{ODc - (ODs - ODb)}{ODc} \times 100$$

where ODc is the OD of the negative control group, ODs is the OD of the test group, and ODb is the OD of the blank well.

Statistical analysis

The mean of three repetitions was reported for the MTT and DPPH assays. Initially, all test groups were compared with the control group one by one, and then the test groups were compared with each other pairwise. Data were analyzed by GraphPad Prism version 9 using one-way ANOVA and Tukey's test at 0.05 level of significance.

RESULTS

Antibacterial effects of the tested extracts and essential oils on *Aggregatibacter actinomycetemcomitans*

Well plate results

As shown in Figure 1, no growth inhibition zone was created by *C. reticulata* hydroalcoholic extract after 24 h, indicating the absence of antibacterial activity against *A. actinomycetemcomitans*. However, growth inhibition zones were observed around hydroalcoholic extracts of *O. europaea*, and the positive control group (0.2% CHX), indicating their antibacterial activity against *A. actinomycetemcomitans*. The largest growth inhibition zone belonged to CHX (mean of 42.3 ± 2.6 mm) followed by *C. reticulata* essential oils (mean of 40.0 ± 0.0 mm). The growth inhibition zones of extracts were smaller than those of essential oils [Table 1], and the smallest growth inhibition zone belonged to *O. europaea* extract (32.3 ± 2.2 mm).

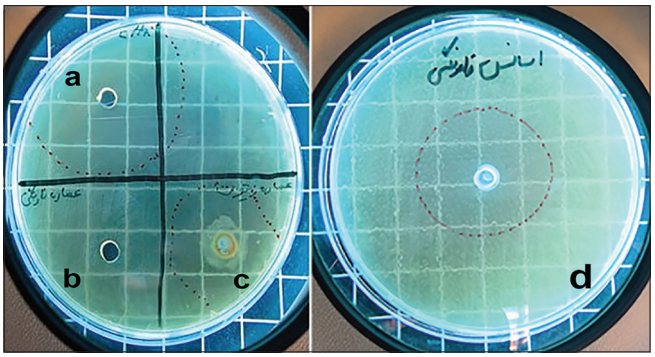

Figure 1: Assessment of antibacterial activity of the respective essential oils and extracts against *Aggregatibacter actinomycetemcomitans* by the well plate technique. (a) chlorhexidine, (b) *Citrus reticulata (C. reticulata)* extract, (c) *Olea europaea* extract, (d) *C. reticulata* essential oil.

Minimum inhibitory concentration results

The lowest MIC (=MBC) belonged to 0.0001% (v/v) CHX, followed by *O. europaea* extract (0.78%) and *C. reticulata* essential oil (6.25%). Table 2 presents the MIC and MBC of the extracts and essential oils.

Cytotoxicity result

At 24 h [Figure 2a], 1% *O. europaea* extract showed 71% cell viability, which was significantly different from the control group (100% viability). At 5% and 10% concentrations, the percentage of viability decreased to 40% and 30%, respectively, indicating their cytotoxicity. The difference in percentage of cell viability was significant between the control group and all concentrations of *O. europaea* extract ($P < 0.05$). The results for *C. reticulata* extract were that 1% and 5% concentrations had >70% cell viability, while 10% concentrations were cytotoxic. *C. reticulata* extract had no significant difference with the control group in terms of viability percentage. All essential oils at all tested concentrations were cytotoxic.

At 72 h [Figure 2b], 1% *C. reticulata* extracts were not cytotoxic (>70% viability), while other

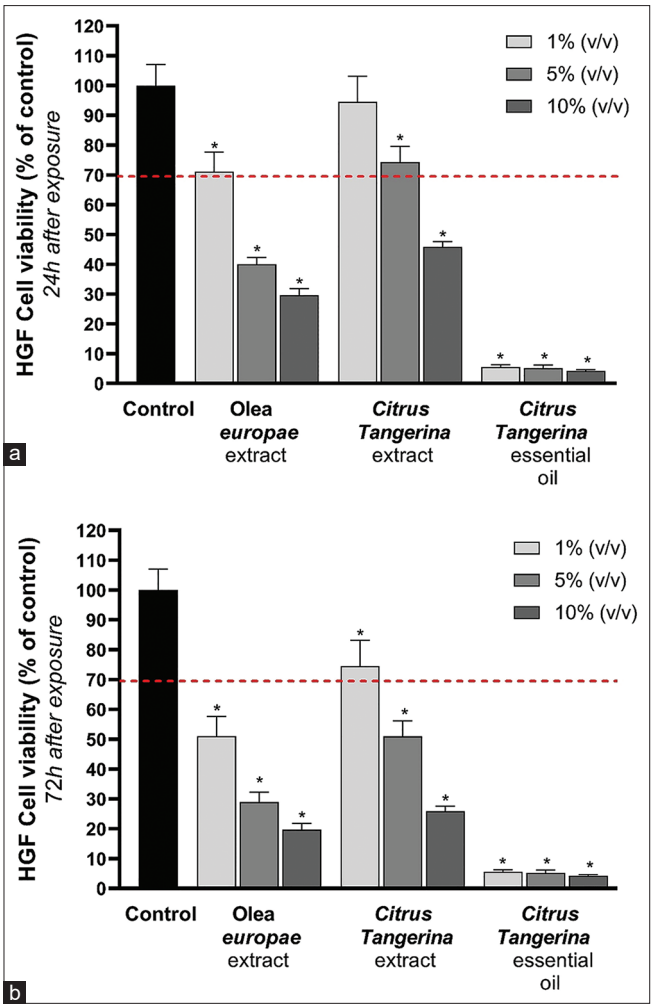

Figure 2: Quantitative assessment of the cytotoxic effects of extracts and essential oils on human gingival fibroblasts at 24 (a) and 72 (b) h. Stars over the columns indicate the presence of a significant difference with the control group ($P < 0.05$). Stars between two columns indicate the presence of a significant difference between the two groups ($P < 0.05$). HGF: Human gingival fibroblast.

tested materials showed cytotoxic effects, and this difference was statistically significant ($P < 0.05$). Cytotoxicity increased at 72 h compared with 24 h, indicating its time dependence. It was also dose dependent ($P < 0.05$).

Antioxidant effects

As shown in Figure 3, all essential oils and extracts had antioxidant effects. *C. reticulata* and *O. europaea* extracts in all three concentrations of 1%, 5%, and 10% (v/v) had higher antioxidant effects than the ascorbic acid. The antioxidant effects of 1%, 5%, and 10% *O. europaea* extract were 102%, 148%, and 165%, respectively; these values were 103%, 165%, and 155%, respectively, for the *C. reticulata* extract.

Table 1: Growth inhibition zones of extracts and essential oils

Extract/essential oil	Growth inhibition zone (mm), mean±SD
C. reticulata extract	-
O. europaea extract	32.3±2.2 (*P*=0.0028 compared to *C. reticulata* essential oil; *P*=0.0007 compared to CHX)
C. reticulata essential oil	40.0±0.0 (*P*=0.0028 compared to *O. europaea* extract; *P*=0.2717 compared to CHX)
CHX	42.3±2.6

CHX: Chlorhexidine; *C. reticulate: Camellia reticulate; O. europaea: Olea europaea*; SD: Standard deviation

Table 2: Minimum inhibitory concentration and minimum bactericidal concentration of the extracts and essential oils

Extract/essential oil	MIC=MBC (% v/v)
C. reticulata extract	-
O. europaea extract	0.78±0.0 (*P*<0.0001 compared to *C. reticulata* essential oil; *P*<0.0001 compared to CHX)
C. reticulata essential oil	6.25±0.0 (*P*<0.0001 compared to *O. europaea* extract; *P*<0.0001 compared to CHX)
CHX	0.0001±0.0

MIC: Minimum inhibitory concentration; MBC: Minimum bactericidal concentration; *C. reticulate: Camellia reticulate; O. europaea: Olea europaea*; CHX: Chlorhexidine

In 1% concentration, the antioxidant effect of both extracts was similar to that of the control group with no significant difference. Compared with extracts, *C. reticulata* essential oils in 1%, 5%, and 10% concentrations had significantly lower antioxidant effects than ascorbic acid (49%, 58%, and 70%, respectively, for *C. reticulata*).

DISCUSSION

This study compared the antioxidant, cytotoxic, and antimicrobial effects of *C. reticulata* and *O. europaea* extracts and essential oils. The results showed that the hydroalcoholic extract of *O. europaea* and the *C. reticulata* essential oils had antibacterial effects on *A. actinomycetemcomitans*. The hydroalcoholic extract of *C. reticulata* had no significant antimicrobial activity against *A. actinomycetemcomitans*. The largest growth inhibition zone belonged to CHX, followed by *C. reticulata* essential oils (40.0 ± 0.0 mm). The growth inhibition zones of extracts were smaller than those of essential oils, and the smallest growth inhibition zone belonged to *O. europaea* extract. The lowest MIC and MBC belonged to 0.0001% (v/v) CHX, followed by *O. europaea* extract (0.78%) and *C. reticulata* essential oil (6.25%). In general, Gram-positive bacteria are more susceptible to herbal extracts and essential oils than Gram-negative bacteria due to the

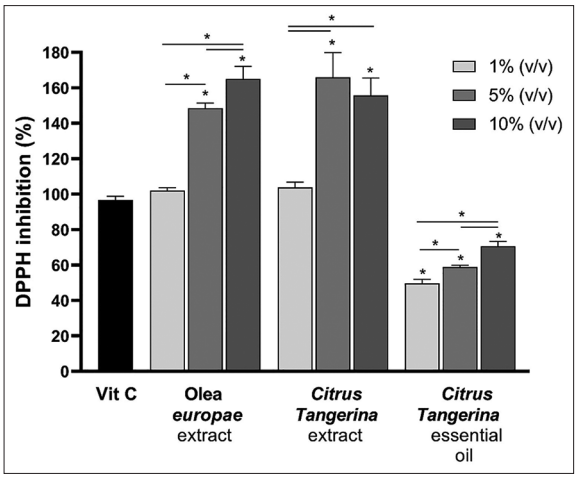

Figure 3: Antioxidant effects (percentage of inhibition of 2,2-diphentyl-1-picrylhydrazyl assay radicals) of ascorbic acid and the respective extracts and essential oils in 1%, 5%, and 10% concentrations; stars over the columns indicate the presence of a significant difference with the ascorbic acid control group ($P < 0.05$). Stars between two columns indicate the presence of a significant difference between the two groups ($P < 0.05$). DPPH: 2,2-diphenyl-1-picrylhydrazyl.

different cell wall compositions of Gram-negative and Gram-positive bacteria. The higher resistance of Gram-negative bacteria can be attributed to the presence of complex compositions of hydrophobic lipopolysaccharides in their external cell membrane, which are impermeable to hydrophilic compounds.[16]

The optimal antibacterial effects of different parts of citrus plants, especially *C. reticulata* peel on different bacteria, have been previously documented.[17-20] However, in the present study, the hydroalcoholic extract of *C. reticulata* had no significant antibacterial effect on *A. actinomycetemcomitans*. Furthermore, search of the literature by the authors yielded no study on MIC and MBC of *C. reticulata* essential oil for *A. actinomycetemcomitans*. In the current study, *C. reticulata* essential oil had antibacterial activity against *A. actinomycetemcomitans* with MIC = MBC of 6.25% (v/v); however, this value was lower than the MIC of CHX (0.0001%v/v). Similarly, Adham[21] showed no significant antibacterial effect of hydroalcoholic extract of *C. reticulata* on *Staphylococcus aureus*. Furthermore, Torshabi *et al*.[14] indicated optimal antibacterial effect of *C. reticulata* peel essential oil on *S. mutans* while its extract had no such antibacterial effect. The results of both of the abovementioned studies were in agreement with the present findings regarding *A. actinomycetemcomitans*. Pardo *et al*.[22] demonstrated that 100% concentration of *C. reticulata* peel essential oil had an antibacterial activity comparable to that of CHX against *Fusobacterium nucleatum*.

The methanolic extract of *O. europaea* has shown antibacterial activity against a wide range of oral pathogens.[10] In the present study, *O. europaea* extract showed antibacterial activity against *A. actinomycetemcomitans* with MIC = MBC of 0.78% (v/v). Karygianni *et al*.[15] showed that *O. europaea* had the highest inhibitory effects on streptococci, *Fusobacterium nucleatum, P. gingivalis*, and *Parvimonas micra*. Furthermore, Carrol *et al*.[10] demonstrated that the methanolic extract of *O. europaea* had antibacterial activity against *P. gingivalis* with a MIC of 64 μg/mL. Since the present study showed an optimal antibacterial effect of hydroalcoholic extract of *O. europaea* on *A. actinomycetemcomitans*, it may have the potential for incorporation in the formulation of mouthwashes. Golestannejad *et al*.[23] reported the MIC and MBC of ethanolic, methanolic, and hydroalcoholic extracts of *O. europaea* for *S. mutans* to be 12%–25%, 50%–75%, and 12%–25%, respectively. By an increase in extract concentration, acidic products and adhesion of *S. mutans* decreased, and the diameter of bacterial growth inhibition zones increased. Their results were in agreement with the present findings. To the best of the authors' knowledge, the MIC and MBC of *O. europaea* extract or essential oil for *A. actinomycetemcomitans* have not been evaluated in any previous study to compare our results.

Cytotoxicity assessments of the extracts and essential oils in the present study revealed that essential oils at all concentrations were cytotoxic at both 24 and 72 h and caused approximately 90% reduction in cell viability, which was significant and time dependent. The *O. europaea* extract in 1% concentration did not have cytotoxic effects at 24 h, but it was cytotoxic in all other concentrations and also at 72 h. All other concentrations and essential oils were cytotoxic. Ishfaq *et al*.[24] indicated no cytotoxic effect of *C. reticulata* peel essential oil on rabbit epithelial cells. Difference between their results and the present findings can be attributed to different concentrations of essential oils used. Another study showed that the cytotoxicity of essential oils often decreases by a reduction in their concentration, pointing to their dose-dependent cytotoxicity.[25] The *O. europaea* leaf extract in

1% concentration showed 71% viability at 24 h, but it was cytotoxic in higher concentrations. Its cytotoxicity was dose dependent. A systematic review by Carrol *et al*.[10] reported low cytotoxicity for methanolic extract of *O. europaea* leaf in MIC.

By an increase in concentration, the antioxidant effect of its essential oil increased. The *C. reticulata* peel extract in all three concentrations showed higher antioxidant activity than ascorbic acid, but its essential oil in all three concentrations had lower antioxidant activity than ascorbic acid. Goyal and Kaushal[26] reported 15%–70% antioxidant activity for *C. reticulata* peel essential oil in 200–2000 ppm concentrations, which was consistent with the present results. Furthermore, Frassinetti *et al*.[27] reported that *C. reticulata* essential oil in 200–1000 ppm concentrations inhibited DPPH by 20%–80%, confirming the present results. Shahzad *et al*.[28] reported 76.38%–76.75% antioxidant activity for 10%–20% concentrations of *C. reticulata* essential oil.

The *O. europaea* leaves prevent the activity of reactive species that take part in biochemical processes and protect human red blood cells against oxidative stress.[29] The present results indicated that the *O. europaea* leaf extract in all concentrations had higher antioxidant activity than the ascorbic acid. Hannachi *et al*.[30] showed high antioxidant activity of *O. europaea* leaf, pulp, and stone extracts using the DPPH assay.

In vitro design and the unavailability of *O. europaea* essential oil were among the limitations of this study. Future studies are required to assess the antibacterial effects of *Elettaria cardamomum, C. reticulata,* and *O. europaea* extracts and essential oils on other periopathogenic microorganisms. Moreover, the present *in vitro* results should be further confirmed in animal and clinical studies.

CONCLUSION

Of the tested materials, *O. europaea* extract had the highest potential for possible use as an organic mouthwash due to its strong antibacterial and antioxidant effects and absence of cytotoxicity in low concentrations.

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Conflicts of interest
The authors of this manuscript declare that they have no conflicts of interest, real or perceived, financial or non-financial in this article.

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