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Antimicrobial and cytotoxic activity of *Ferula gummosa* plant essential oil compared to NaOCl and CHX: a preliminary *in vitro* study

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Objectives: The usage of medicinal plants as natural antimicrobial agents has grown in many fields including dental medicine. The aim of this *in vitro* study was three-fold: (i) to determine the chemical compositions of the *Ferula gummosa* essential oil (FGEO), (ii) to compare the antimicrobial efficacy of the oil with sodium hypochlorite (NaOCl) and chlorhexidine (CHX), (iii) to assess the toxic behavior of FGEO in different concentrations compared to 5% NaOCl and 0.2% CHX. ***Materials and Methods:*** Gas chromatography/mass spectrometry (GC/MS) was used to determine the chemical compositions of the oil. The disk diffusion method and a broth micro-dilution susceptibility assay were exploited to assess the antimicrobial efficacy against *Enterococcus faecalis*, *Staphylococcus aureus, Streptococcus mitis*, and *Candida albicans*. The cytocompatibility of the FGEO was assessed on L929 fibroblasts, and compared to that of NaOCl and CHX. ***Results:*** Twenty-seven constituents were recognized in FGEO. The major component of the oil was β-pinene (51.83%). All three irrigants significantly inhibited the growth of all examined microorganisms compared to the negative control group. FGEO at 50 μg/mL was effective in lower concentration against *Enterococcus faecalis* than 5% NaOCl and 0.2% CHX, and was also more potent than 0.2% CHX against *Candida albicans* and *Staphylococcus aureus*. FGEO was a cytocompatible solution, and had significantly lower toxicity compared to 5% NaOCl and 0.2% CHX. ***Conclusions:*** FGEO showed a promising biological potency as a root canal disinfectant. More investigations are required on the effectiveness of this oil on intracanal bacterial biofilms. (*Restor Dent Endod* 2015;40(1):50-57)

Key words: Antimicrobial activity; Chlorhexidine; Cytocompatibility; *Ferula gummosa*; Root canal irrigant; Sodium hypochlorite

Introduction

Elimination of the microbial infection from the root canal space with aid of antimicrobial agents is considered as the most important phase to achieve successful endodontic treatment. However, specific organisms may still survive after careful chemo-mechanical preparation of the root canal system and cause persistent intraradicular infection.[1,2] Gram positive bacteria such as *Enterococcus faecalis* and some species of *Streptococci* and *Staphylococci* have been found with high prevalence in treated teeth with failed endodontic treatments.[2,3] *Candida albicans* is another commonly found pathogen in some cases of persistent endodontic infections.[2,4]

Sodium hypochlorite (NaOCl) is considered as the most widely used irrigant in root canal treatment due to its vigorous antibacterial activity and tissue dissolving ability.[5,6] However, the main drawbacks of NaOCl are its unpleasant taste and odor together with its high caustic effect in case of inadvertently extrusion to periapical region.[6,7]

Chlorhexidine (CHX) is another commonly used irrigant in endodontics with acceptable antibacterial activity. The inability of this irrigant to dissolve necrotic tissue remnants, and its relatively low antibacterial activity against gram-negative bacteria together with its cytoxicity, have made this agent as a supplement rather than a main irrigation solution in root canal treatment.[6,8,9] Considering the aforementioned disadvantages of these irrigants, the researchers are still continuing their efforts to find more compatible, cost-effective and efficient disinfectants.

At the present time, there is no available irrigant to be considered as an ideal choice alone. Recently, the tendency to investigate herbal remedies has been enhanced in many studies. The genus of *Ferula*, belonging to the family of Apiace, is consisted of 170 species and grows widely in central Asia, Mediterranean region and Northern Africa. *Ferula gummosa* (FG) is one of the most popular genus of Apiaceae which have been frequently used as herbal medicaments.[10,11] In recent years, various biological effects of FG have been revealed. This medicinal herb has shown to have favorable antimicrobial, anti-nociceptive, anti-inflammatory, anti-convulsant, anti-oxidant and anti-spasmodic activities.[12-17] It also has been demonstrated that FG fruit essential oil (FGEO) had strong effects on gram positive and gram negative bacteria, and also active against *Candida albicans*.[16]

The aims of this preliminary *in vitro* study were: (i) to determine the chemical compositions of FGEO for the better understanding of its bioactivity; (ii) to determine and compare the minimum inhibitory and microbicidal concentrations of FGEO, NaOCl and CHX against endodontic pathogens such as *Enterococcus faecalis*, *Streptococcus mitis*, *Staphylococcus aureus* and *Candida albicans*; (iii) to assess the cytocompatibility of FGEO in different concentrations on L929 mouse fibroblasts compared to that of 5% NaOCl and 0.2% CHX.

Materials and Methods

Preparation of the experimental solutions

FG oleo gum resin was purchased from Jokar Trading Company, Shiraz, Iran. This sample was collected from Firouz kooh, Iran in spring 2010 and identified by high performance thin layer chromatography (HPTLC) technique using standard samples. The voucher specimen (487 pm) of this plant was deposited at the Herbarium of the Department of Pharmacognosy, School of Pharmacy, Shiraz University of Medical Sciences, Iran. In order to obtain FGEO, 50 g of the oleo gum resin was soaked in 1 L fresh distilled water and then subjected to hydro-distillation for 4 hours using a clevenger-type apparatus. According to the yield of the essential oil, a concentration of 50 µg/mL of the FGEO was obtained. NaOCl in the regular concentration of 5% (equivalent to 5×10^4 µg/mL, Sigma-Aldrich Co., St. Louis, MO, USA) and CHX at 0.2% (equivalent to 2×10^3 µg/mL, Sigma-Aldrich Co.) were selected for further evaluations in this study. All experimental solutions were freshly prepared just before the commencement of the experiments.

Gas chromatography/mass spectrometry analysis

Gas chromatography/mass spectrometry (GC/MS) analysis was carried out by using Agilent 7890 gas chromatograph connected with a mass detector (Model 5975C, Agilent technologies, Santa Clara, CA, USA). For the purpose of analyzing the plant essential oil, the gas chromatograph was equipped with a HP-5MS capillary column (phenyl methyl siloxane, 30 m × 0.25 mm i.d., Agilent technologies). The oven temperature was programmed from 60℃ (0 min) to 250℃ at the rate of 5℃/min and then held for 10 min at 250℃. Helium was selected as the carrier gas while the flow rate was adjusted to 1 mL/min. Also, the mass spectrometer was set in EI mode at 70 eV. The interface temperature was adjusted to 280℃ and mass range was 30 - 600 m/z. The constituents of the oil were identified by comparison of Kováts retention indices (KI) and mass spectra of each ingredient with the known compounds from Willey (nl7) and Adams library data.[18]

Measurement of antimicrobial activity

Among persistent root canal microorganisms, three bacterial strains including *Enterococcus faecalis* (AGH04), *Streptococcus mitis* (ATCC 49456), *Staphylococcus aureus* (ATCC 25923) and one fungal strain, *Candida albicans* (ATCC 10231), were selected to include in this study.

For the primary evaluation of the antibacterial susceptibility, the bacterial disc diffusion method was applied according to Clinical Laboratory and Standard Institute (CLSI) instructions.[19] Briefly, the bacterial cultures (adjusted to 0.5 McFarland standards) were added to Muller-Hinton agar plates using a sterile swab. The paper discs which had been saturated with our experimental solutions (FGEO at a concentration of 50 µg/mL, 5% NaOCl and 0.2% CHX) were placed on the Mueller-Hinton agar surface together with a positive and negative control (100 µL/mL ampicillin [Merck, Darmstadt, Germany] and dimethyl sulfoxide [DMSO, Merck, Darmstadt], respectively). The plates were evaluated for the induced inhibition zones

after a 24 hour incubation period at 37℃. The antifungal disc diffusion procedure was performed in a same way but in Muller-Hinton agar medium with 2% glucose to support yeast growth. Besides, amphotericin B (Sigma Aldrich, 100 µL/mL) was regarded as a positive control in this phase. It is noteworthy that all experimental tests were performed in triplicate.

The minimum inhibitory concentrations (MICs) of the experimental solutions against each bacterium were measured by the micro-dilution broth method in accordance with the instructions presented by CLSI.[19] In short, ten-fold serial dilutions (up to 10^{-5}) of the experimental and control groups were prepared with Muller-Hinton broth in 96 well microplates. Then, the bacterial suspension containing 5×10^5 colony forming unit (CFU)/mL (equal to 0.5 McFarland, with optical density of 600 nm) was added to them. The plates were incubated for 24 hours at 37℃. Ampicillin was regarded as positive and DMSO as negative controls. All experiments were performed in triplicate. To determine the MICs against *Candida albicans*, ten-fold serial dilutions of each experimental and control solution were prepared by Roswell Park Memorial Institute (RPMI) 1640 medium. In summary, five colonies of the 24 hour cultured *Candida albicans* on sabouraud dextrose agar were transferred to normal saline (0.9%). Then, the suspension was diluted with RPMI 1640 medium to yield a final inoculum concentration approximately equal to 1×10^4 to 5×10^4 CFU/ml in each well of 96 well microplates. The inoculated plates were then incubated at 37℃ for 24 hours.

To measure the minimum bactericidal concentrations (MBCs), the media from the wells of no bacterial growth was cultured on tryptic soy agar. The same procedure was used to measure minimum fungicidal concentration (MFC), using sabouraud dextrose agar. The MBC and MFC values were characterized as the lowest concentration possessing a mortality rate of 98% of the microorganisms in the primary inoculums, and this occurred when less than 4 visible colonies can be detected after 24 hour incubation at 37℃ in agar plates.

The results obtained from disc diffusion test were analyzed using Two-way analysis of variance (ANOVA). Multiple comparison was performed using One-way ANOVA and *post hoc* Tukey's HSD test. A level of $p < 0.05$ was accepted as statistically significant.

Evaluation of the cytotoxicity

Cytocompatibility of the FGEO in different concentrations was evaluated by using MTT (3-[4,5 Dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide, Sigma-Aldrich Co.) colorimetric assay on L929 mouse fibroblasts and compared with that of 5% NaOCl and 0.2% CHX solutions. 35% hydrogen peroxide and culture medium served as the positive and negative controls. In brief, the L929 mouse fibroblasts in RPMI 1640 media were placed into a 96 well cell culture plate (at a density of approximately 1×10^4 cells/well) and incubated in a humidified atmosphere (5% CO_2, 95% air at 37℃) to reach about 80% confluence. After 24 hours, 100 µL of each sample which was previously incubated at 37℃ in RPMI 1640 media was transferred into each well. The medium was removed 24 hours after incubation and the wells were washed twice for 2-3 minutes with 200 µL of the fresh medium. A total of 25 µL of MTT solution was added to each well and incubated in a humidified atmosphere for 4 hours. Then, 100 µL of DMSO was added to each well. The absorption of the solution was read at a wavelength of 540 nm with the aid of an ELISA plate reader (PowerWave X52, BioTek Instruments, Potton, UK). All tests were performed in triplicate. The mean cell viability values were expressed as percentage of negative control.

One-way ANOVA and *post hoc* Tukey tests were used to evaluate the differences in the mean cell viability values of the experimental solutions. A level of $p < 0.05$ was accepted as statistically significant.

Results

Gas chromatography/mass spectrometry analysis

Yield of the essential oil was 32%. Twenty seven constituents were recognized by the GC/MS analysis, which represented 96.32% of the total oil (Table 1). The main component was β-pinene, and the minor components were α-pinene, δ3-carene, β-phellandrene and carvacrol methyl ether.

Antimicrobial measurement

Results obtained from the antimicrobial effectiveness of the test solutions are shown in Tables 2 and 3. According to the results of disc diffusion test, all three irrigants significantly inhibited the growth of all examined microorganisms compared to the negative control group. FGEO at 50 µg/mL was more effective than other medicaments against *Enterococcus faecalis* and *Candida albicans*. Further, it was significantly less effective against *Streptococcus mitis* than other microorganisms tested.

According to the results of microdilution susceptibility test, all solutions had the same inhibitory and bactericidal activity against each microorganism. The MICs and the MBCs of the plant essential oil were same for all test organisms. NaOCl was less potent against *Enterococcus faecalis* than other solutions. FGEO was effective in lower concentration against *Enterococcus faecalis* than NaOCl and CHX, and was also more potent than CHX against *Candida albicans* and *Staphylococcus aureus*.

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Table 1. Chemical composition of volatile oils obtained from FG

Number	Compounds	KI*	Percentage
1	Alpha Thujene	928.259	1.41
2	Alpha Pinene	937.070	6.44
3	Beta Pinene	991.189	51.83
4	Beta Myrcene	995.725	3.32
5	Delta 3-Carene	1015.898	5.47
6	O-Cymene	1027.852	2.29
7	Beta Phellandrene	1033.725	4.16
8	Trans Pinocarveol	1142.593	1.00
9	Myrtenol	1200.456	1.08
10	Fenchyl acetate	1224.154	2.08
11	Thymyl methyl ether	1237.238	1.05
12	Carvacrol methyl ether	1247.661	4.06
13	Unknown	1311.323	1.13
14	Unknown	1326.304	0.66
15	Alpha Terpinyl acetate	1353.502	1.72
16	Alpha Copaene	1381.206	0.65
17	Beta Elemene	1396.615	0.59
18	Cymene 2,5-dimethoxy-para	1425.728	0.68
19	Longifolene V1	1455.178	0.78
20	Beta Selinene	1492.435	0.56
21	Gamma Cadinene	1519.788	0.73
22	Delta Cadinene	1528.195	0.53
23	Unknown	1588.110	1.91
24	Guaiol	1604.155	2.28
25	Alpha Muurolol	1646.604	0.87
26	Bulnesol	1674.441	2.18
27	Guaiol acetate	1728.773	0.56

*The compounds have been sorted according to retention indices on HP-5 MS capillary column.
KI, Kováts retention indices.

Table 2. The mean size ± SD of the zones of inhibition in mm against the test organisms

Medicament	*Enterococcus faecalis*	*Streptococcus mitis*	*Staphylococcus aureus*	*Candida albicans*
Negative control	$5.00 \pm 0.71^{A,a}$	$6.00 \pm 0.00^{AB,a}$	$7.33 \pm 0.24^{B,a}$	$7.00 \pm 0.41^{B,a}$
5% NaOCl	$10.00 \pm 1.47^{A,b}$	$18.33 \pm 0.85^{B,bc}$	$29.33 \pm 1.03^{C,d}$	$19.00 \pm 1.08^{B,c}$
0.2% CHX	$13.00 \pm 0.71^{A,b}$	$21.66 \pm 1.25^{C,c}$	$18.33 \pm 0.62^{B,b}$	$11.33 \pm 1.25^{A,b}$
FGEO at 50 µg/mL	$22.50 \pm 1.87^{B,d}$	$17.50 \pm 0.41^{A,b}$	$22.50 \pm 0.82^{B,c}$	$25.00 \pm 1.41^{B,d}$
Positive control	$17.33 \pm 1.03^{A,c}$	$28.66 \pm 1.93^{B,d}$	$45.33 \pm 2.01^{C,e}$	$16.00 \pm 1.08^{A,c}$

Read horizontally, uppercase letters denote comparisons within each group. Read vertically, lowercase letters denote comparisons among groups. Having equal letters denotes lack of statistically significant difference ($p > 0.05$).
NaOCl, sodium hypochlorite; CHX, chlorhexidine; FGEO, *Ferula gummosa* fruit essential oil.

Table 3. Mean value of MICs and MBCs/MFCs of experimental groups against test organisms

Microorganism	FGEO (μg/mL)		CHX (μg/mL)		NaOCl (μg/mL)	
	MIC	MBC/MFC	MIC	MBC/MFC	MIC	MBC/MFC
Staphylococcus aureus	50	50	200	200	50	50
Streptococcus mitis	50	50	20	20	50	50
Enterococcus faecalis	50	50	200	200	5,000	5,000
Candida albicans	50	50	200	200	50	50

FGEO in concentration of 50 μg/mL, NaOCl in concentration of 50,000 μg/mL and CHX in concentration of 2,000 μg/mL were employed to start the experiment. Ten-fold serial dilutions up to five times were made to achieve the MICs, MBCs and MFCs
FGEO, *Ferula gummosa* fruit essential oil; CHX, chlorhexidine; NaOCl, sodium hypochlorite; MIC, minimum inhibitory concentration; MBC, minimum bactericidal concentration; MFC, minimum fungicidal concentration.

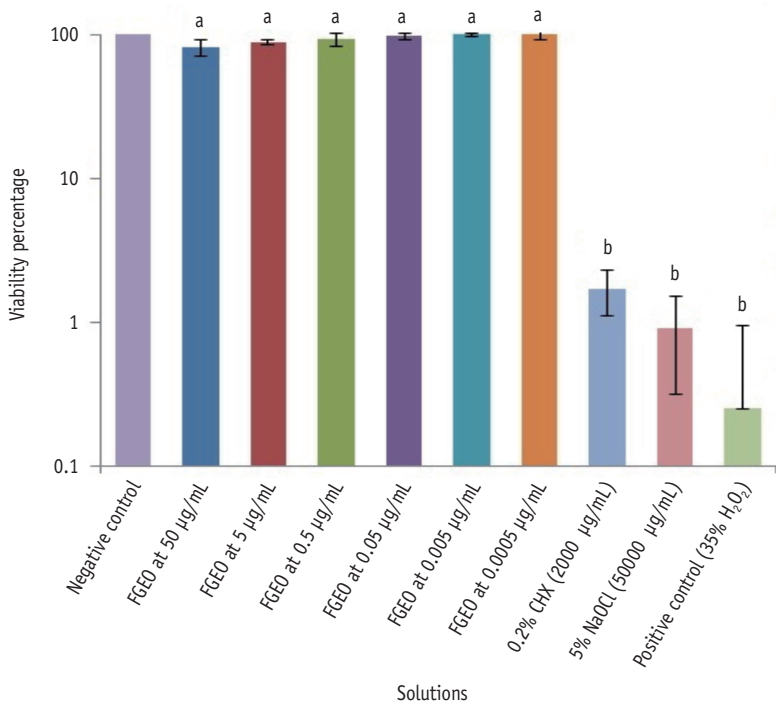

Figure 1. Mean viability (%) of the L929 fibroblasts when treated with different concentrations of *Ferula gummosa* essential oil (FGEO), 5% NaOCl and 0.2 % CHX solutions. Equal letter denotes lack of statistically significant difference ($p > 0.05$). The negative control group did not include in the statistical analyses. NaOCl, sodium hypochlorite; CHX, chlorhexidine.

Cytotoxicity assessment

Figure 1 shows the mean cell viability (%) of L929 fibroblasts when treated with different concentrations of FGEO, 5% NaOCl and 0.2% CHX for 24 hours. No statistical difference was observed between the viability of NaOCl and CHX group when they were compared with 35% H_2O_2 as a positive control. The full concentration of FGEO was able to keep 88% of the cell viability. There was a significant difference between the cytotoxicity of FGEO and NaOCl or CHX.

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Discussion

With a strong background in the history, the usage of herbal remedies by natural products has grown in many fields including dentistry. In this respect, many plants around the world were investigated in order to discover their potential application in endodontic therapy. Some of these plants are *Arctium lappa, Morinda citrifolia, Triphala, Green Tea Polyphenols, Liquorice, Syzygium aromaticum, Ocimum sanctum* and *Cinnamomum zeylanicum*.[20-24] The present study was designed to investigate the potency of FGEO to be used as a root canal disinfectant. It is noteworthy that this essential oil has a wide range of application in cosmetic industry particularly as a natural perfume ingredient. It is also used in clinical medicine, and marketed as a lotion for the treatment of *Acne vulgaris*.

In this study, we first analyzed the chemical components of FGEO to avoid the lack of standardization and to have better understanding of its mode of action. Then, we selected various microorganisms commonly found in endodontic failures for antimicrobial assessments. In the next phase, we assessed the cytocompatibility of this oil in different concentrations compared with NaOCl and CHX solutions as the two are frequently used irrigants in endodontics. Given it is established that NaOCl at concentrations of 0.5% to 6% and CHX at 0.1% to 2% are used in endodontic investigations, we selected 5% NaOCl and 0.2% CHX solutions for comparisons. However, dependent on clinical situation, other concentrations of these irrigants might be used in clinical settings.

In this study, the purchased sample of FG was first subjected to HPTLC analysis for the initial screening of the tested plant material and to confirm its nature. The results were initially confirmed matching them with the previously known standard samples. Further, GC/MS analysis was performed to determine all component ingredients of the oil. A total of 27 constituents were recognized in FGEO in which β-pinene was found as the major component, and α-pinene, δ3-carene, β-phellandrene and carvacrol methyl ether as minor components. Although the reports from the quantity of these components were different in previous researches our general findings about the detected constituents were consistent with them.[16,25-27] The discrepancy between these reports may attribute to the different methods of preparing the essential oil, the variable geographic areas, and the collection times of the plants. It is notable that the diversity in compositions of the oil prepared from various regions can be considered as an opportunity to benefit from various pharmacological activities of the same plant grown in different regions.

In the current study, the disk diffusion method and the broth microdilution susceptibility assay were exploited to evaluate the antimicrobial activity of the tested solutions against selected microorganisms. Although disk diffusion method has several drawbacks such as the lack of standardization in agar viscosity, inoculum density and concentration of the tested material, and does not precisely reflect the performance of the disinfectants *in vivo*, it is still the first step in estimation of antimicrobial efficacy.[28] In contrast, broth microdilution method is able to overcome some of the limitations of the disk diffusion method and it is capable to draw quantitative conclusions by determining the MIC values for antimicrobials.

In line with the findings of previous studies, we verified that FGEO had strong antimicrobial effects.[14,16] The rational to select *Enterococcus faecalis, Staphylococcus aurous, Streptococcus mitis* and *Candida albicans* as test organisms was based on the previous reports which linked these pathogens to persistent endodontic infections.[2-4] Our study results revealed that FGEO had acceptable antimicrobial activity against all test organisms. This activity might be related to the presence of β-pinene and α-pinene which had been previously known as excellent antimicrobial agents.[29] Among the minor components, myrtenol has also been recognized to have anti-inflammatory and anti-nociceptive activities.[30] Moreover, the component β-myrcene was previously known as a substance with analgesic and anti-inflammatory properties.[31]

The results of the current study showed that FGEO at 50 µL/mL inhibited the growth of all microorganisms with the inhibition zones of 17.5 - 25 mm in diameter. Other study observed that FGEO at concentration of 7 µL inhibited the growth of *Staphylococcus aureus* with the inhibition zone of 10 - 14 mm and *Candida albicans* with the inhibition zone of more than 14 mm.[16] Likewise, Eftekhar *et al*., reported that 25 µL of FGEO was effective to inhibit the growth of *Staphylococcus aureus* with 25 mm inhibition zone, and *Enterococcus faecalis* with 12 mm inhibition zone.[14] Such diversity of reports in disk diffusion results can be attributed to the difference in concentration of FGEO tested.

By using micro-dilution assay, our findings obtained by the disk diffusion test were partially confirmed. According to disc diffusion results, FGEO at a concentration of 50 µg/mL inhibited the growth of all organisms tested and was more effective than other medicaments against *Enterococcus faecalis* and *Candida albicans*. Furthermore, it was significantly less effective against *Streptococcus mitis* than other microorganisms tested. According to the results of microdilution susceptibility test, FGEO was found to have more potency (smaller MIC) against *Enterococcus faecalis* than NaOCl and CHX, although its effectiveness was similar to NaOCl against *Streptococcus mitis, Staphylococcus aureus* and *Candida albicans*. The oil was also potent in lower concentration compared to CHX against all organisms except *Streptococcus mitis*. It is notable that the partial discrepancy between the results of disk diffusion and microdilution susceptibility tests may attribute to the

difference in the level of each medicament's diffusibility.

Although there is no report in literature comparing the effectiveness of FGEO with NaOCl and CHX, the acceptable antibacterial properties of FGEO have been addressed previously. It has been shown that this oil inhibited the growth of *Staphylococcus aureus* and *Enterococcus faecalis* at concentrations of 3.125 µL/mL and 0.2 mg/ml, respectively.[25,32] Given, there is a variation in results which might be due to the diverse compositions of FGEO tested or different resistance level among the examined stains of microorganisms. Comparing the resistance level of test organisms against NaOCl, *Enterococcus faecalis* was found as the most resistant microorganism with the MIC and MBC values of 5,000 µg/mL. FGEO was the most potent solution against *Enterococcus faecalis* followed by CHX and NaOCl.

In present survey, an L929 cell line was employed to assess the cytocompatibility of FGEO. The reason to choose this cell line is because it is a well-characterized cell model and has been previously used to assess the cytotoxic effects of dental materials.[33,34] To the best of our knowledge, limited data is available in literature regarding the cytotoxicity of FGEO against specific cell lines. In an only found report by Gharaei et al., the FG leaf and flower extracts (at concentrations of 50 and 60 µL/mL, respectively) could resulted in 50% cell death on the human gastric cancer cell line (AGS).[35] In contrast, our findings revealed that FGEO at 50 µL/mL was able to keep more that 88% cell viability in L929 fibroblasts. The discrepancy between these results can be explained by the difference in the amount of cellular particle uptake between different cell types, and also different forms and compositions of herbal extracts used. Furthermore, the nature of the cell line tested (cancerous or normal) can extensively affect the cytotoxic results. From this point of view, a medicament with a high level of toxicity to cancerous cell lines while being compatible to normal cells is considered as a valuable antimicrobial agent.

Conclusions

Under the experimental conditions of this study, it can be concluded that FGEO is a cytocompatible solution and has a favorable antimicrobial effectiveness against endodontic pathogens. More investigations are needed to evaluate the effectiveness of FGEO on bacterial biofilms present in infected root canals.

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

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