Research Article

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Cymbopogon citratus essential oil: an active principle of nanoemulsion against *Enterococcus faecalis* root canal biofilm

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Aim: The objective was to formulate and characterize the nanoemulsion based on *Cymbopogon citratus* oil, intended for use in teeth infected root canal therapy. The investigation of the antioxidant and antibiofilm potential toward *Enterococcus faecalis* was aimed as well. **Materials & methods:** Characterization of oil (by GC/MS analysis) and nanoemulsion (by dynamic light scattering instrument), and determination of antibacterial (by microdilution assay), antibiofilm (by crystal violet assay) and antioxidant properties (by 2,2-diphenyl-1-picryl-hydrazyl-hydrate and thiobarbituric acid assay methods) were provided. Antibiofilm efficacy of irrigation procedure including nanoemulsion was screened on extracted teeth (by CFU counting assay). **Results:** Notable antibacterial and antibiofilm activity, both against forming and preformed biofilms of oil, was observed. Irrigation involved nanoemulsion showed remarkable antibiofilm potential. Both substances induced some antioxidant activity. **Conclusion:** Results encourage further research with the aim of application of the nanoemulsion in dental practice.

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Keywords: antibiofilm activity • *Cymbopogon citratus* essential oil • *Enterococcus faecalis* • infected teeth root canal • nanoemulsion

Incidence of *Enterococcus faecalis* in unsuccessfully treated root canals of the teeth is high and well-documented in endodontic literature [1,2]. It is due to capability of *E. faecalis* to invade dentin tubules of the teeth, to form biofilm within them and to survive exposure to commonly used endodontic antimicrobials, such as sodium hypochlorite (NaOCl), chlorhexidine and calcium hydroxide [3–6]. Although being routinely used, all of them may be responsible for the production of reactive oxygen species (ROS) [7–9]. Even though ROS lead to bacterial cell death they also have side effects, such as causing an inflammation, human cell aging and mutation [7]. If the above-mentioned common intracanal antimicrobials lack in their efficiency and/or surgical treatment wants to be avoided, antibacterial pastes, such as triple antibiotic paste (TAP), are used in the endodontic procedure [10–13]. TAP has been commonly successful in the total elimination of *E. faecalis* from the root canal [14], though its usage can contribute to antibiotic resistance [11].

Taking into account all the above-mentioned, discovering novel antimicrobial agents which are at the same time effective, possess the antioxidant potential and do not lead to bacterial resistance, represents a serious breakthrough in dental research. Many researchers, aware of the mentioned problems, are in search of alternative antimicrobials in nature. Therefore, exploring essential oils (EOs), as natural products of well-known antibacterial efficacy, with no documented resistance issues and with antioxidant potential [15,16], seems to be of special importance for the dental community. Some of the EOs have already been documented for their inhibitory potential against *E.*

faecalis [17]; moreover, our recent research showed the notable activity of *Cymbopogon martinii* EO against this intracanal pathogen [18]. The outcomes of our previous studies combined with the literature data documenting the EOs antioxidant potential [19,20], focused this investigation toward EO from other species of *Cymbopogon* genera, namely to a pleasant lime-scented *Cymbopogon citratus* EO [21]. Moreover, it is worth mentioning that it has a proven high antibacterial potential against numerous bacteria, including the oral ones [22] and even more the antibiotic-resistant strains [23]. In addition, *C. citratus* EO is enriched with antioxidant potential [19,20]. Recognized antibacterial and antioxidant activities encouraged the investigation of this particular EO for its possible application in dentistry, particularly against intracanal pathogens.

Therefore, the objective of this study was to investigate the antibacterial, antioxidant and antibiofilm potential of *C. citratus* EO toward *E. faecalis*, and to formulate and characterize nanoemulsion made of it, as a delivery system being appropriate for the endodontics application. Characterization of the nanoemulsion included not only the determination of its physicochemical attributes, but also the antibiofilm potential of its application in *ex vivo* root canals, as well as its *in vitro* antioxidant efficacy.

Materials & methods

Antibacterial agents

Cymbopogon citratus EO – origin & chemical characterization

Pure, commercial EO from leaves of *C. citratus* (DC.) Stapf. (Lemongrass oil), was purchased from Herba d.o.o, Belgrade, Serbia. TAP, used as a positive control, was composed of the powder of three antibiotics: Metronidazole (Orvagyl®, Galenika, Belgrade, Serbia, 400 mg), Ciprofloxacin (Ciprofloxacin®, Remedica LTD, Limassol, Cyprus, 200 mg) and Minocycline (Minocin®, Pfizer, NY, USA, 100 mg), mixed in the ratio 1:1:1. This mixture was further dissolved in sterile distilled water to provide the stock concentration of 1 mg/ml^{-1}.

Procedures for EO GC/FID and GC/MS analyses were described in detail by Marinković *et al.* [18]. The identification of the individual EO constituents was accomplished by comparing their spectra to those from available MS libraries (NIST/Wiley), as well as by comparing their experimentally determined retention indices (calibrated AMDIS), to the data from the literature [24].

C. citratus EO nanoemulsion – preparation & characterization

C. citratus nanoemulsion was prepared as a mixture of 2.5% of *C. citratus* EO, 2.5% of ethanol, 0.25% of Tween 80 and 94.75% (v/v) of distilled water. Emulsification was performed by addition of an organic phase (containing *C. citratus* EO and nonionic surfactant Tween 80 in ratio 10:1) to an aqueous phase (double distilled water and ethanol) during magnetic stirring (20 min at 500 r.p.m.) of this mixture at ambient temperature (~25°C). After that, the emulsion was subjected to 15 min of ultrasonification at 10% amplitude (Sonoplus, Ultrasonic homogenizer HD 2200, Bandelin Electronic, Berlin). Additionally, the EO-nanoemulsion negative control, involving all the constituents with exception of the EO, was provided. As a positive control, 0.5% NaOCl was used. The prepared emulsion was stored at 5°C for 28 days.

The particle size distributions and polydispersity indices of *C. citratus* EO nanoemulsion were measured using a dynamic light scattering instrument (Zetasizer Nano ZS, Malvern Instruments, Malvern, UK). This instrument determines the particle size from intensity–time fluctuations of a laser beam (632.8 nm) scattered from a sample at an angle of 90°. Each individual measurement was an average of 13 runs. To avoid multiple scattering effects, samples were diluted with distilled water (1:100) before measurements. The measurements were provided regularly: immediately after emulsion synthesis and after 14, 21 and 28 days of storage.

Antioxidant activity

2,2-diphenyl-1-picryl-hydrazyl-hydrate free radical scavenging assay & thiobarbituric acid assay

2,2-diphenyl-1-picryl-hydrazyl-hydrate (DPPH) assay, performed as previously explained [25], was used to evaluate radical-scavenging activity. On the other hand, thiobarbituric acid assay (TBA) assay, performed with commercial preparation of liposomes (l-α-phosphatidylcholine) and previously described by Vasilijević *et al.* [26], was used to measure the extent of lipid peroxidation (LP). *C. citratus* EO and EO nanoemulsion were tested, while ascorbic acid was used as a positive control. Tested concentrations ranges were 0.125–9000 μg ml^{-1}, 14–226 μg ml^{-1} and 3.125–200 μg ml^{-1}, for EO, nanoemulsion and positive control, respectively. Tested concentrations for EO-nanoemulsion were expressed through available EO concentration (active principle) in the nanoemulsion.

The absorbance (A) of remaining DPPH radical (DPPH assay) and of extent of LP (TBA assay) were measured spectrophotometrically at 517 and 532 nm, respectively (UV-6300 PC spectrophotometer, MRC, Scientific Instruments, Holon, Israel). The inhibition of DPPH/LP was calculated using the following equation:

$$I = 100\% \times (Acontrol - Atestsubstance) \div Acontrol$$

Where applicable, the concentration providing 50% inhibition (IC_{50}) was read off from the graph plotting scavenging activity as a function of test substance concentration. Experiments were performed twice in triplicate.

Microbiological assessment

Bacterial strains

The clinical isolates of *E. faecalis* EFc11, EFc12 and EFc13 were sampled from both symptomatic and asymptomatic infected root canals at the Department of Pediatric and Preventive Dentistry, School of Dental Medicine, University of Belgrade, Serbia [27]. Reference strains *E. faecalis* ATCC 29212 and ATCC 194303 were also included.

Antibacterial activity of C. citratus EO

Minimal inhibitory and bactericidal concentrations (MICs and MBCs) were determined by the resazurin-incorporated microdilution method using 96-well microtiter plates. MICs and MBC have been determined by the procedure previously described by Nikolić *et al.* [28], with only one modification: to increase EO solubility instead of dimethyl sulfoxide (DMSO) surfactant Tween 80 was used (EO: Tween 80 ratio was 1:1). Bacterial inoculums were 10^5 CFU ml^{-1}, while the tested concentration range of EO was 4–0.03125 μg ml^{-1}. The experiment was done in triplicate and repeated twice.

Antibiofilm activity of C. citratus EO

The impact of *C. citratus* EO on formation and eradication of biofilm was screened on *E. faecalis* ATCC 29212, used as a model organism. Crystal violet (CV) assay was performed as previously described by Stepanović *et al.* [29], but with slight modifications.

Concerning the effect on the biofilm formation, the bacterial cell suspensions (10^6 CFU ml^{-1}) and serially diluted antibacterial agent (MIC/8-MIC) were prepared separately in the Tryptic soy broth enriched with 2% glucose. By mixing 100 μl of bacterial inoculums and 100 μl of test agent dilutions into 96-well microtiter plates with flat bottom (Sarstedt, Germany), the final inoculums (5×10^4 CFU/well) and final concentrations ranging MIC/16–MIC/2 were adjusted. The plates were incubated at 37°C, for 24 h to allow biofilm formation with and without test agents. Following a biofilm formation, the medium was aspirated and planktonic cells have been removed by washing the medium twice with sterile saline. The cells were fixed with 200 μl of methanol/well, which was removed after 10 min, and the plates were air-dried for 30 min. The biofilm was stained in each well by adding 200 μl of 0.1% CV (Bio-Merieux, Marcy-l'Etoile, France). After 30 min, the wells were washed, while the remaining stain, bound to biofilms, was resuspended in 96% ethanol (200 μl per well). The absorbance (A) was read at 620 nm on Multiskan™ FC Microplate Photometer, Thermo Fisher Scientific™. Percentage of the inhibition of biofilm formation was calculated according to following equitation:

$$I = 100\% \times (Acontrol - Atreatment) \div Acontrol$$

Concerning the effect on the biofilm disruption, the bacterial cell suspensions (10^6 CFU ml^{-1}) prepared in the same medium was added into 96-well microtiter plates with flat bottom (200 μl/well) and incubated at 37°C, for 24 h, to allow biofilm preformation. Following a biofilm formation, the medium was replaced with the same volumes (200 μl/well) of medium containing test agents (MIC/4–MIC/2) were added and additional 24 h incubation at 37°C was provided. Following procedure was exactly the same as previously described, resulting in absorbance at 620 nm determination and percentage of biofilm disruption calculation.

For both inhibitions of formation and disruption of biofilms, the experiments were done in five replicas per treatment point and repeated twice. TAP was used as a positive control.

Table 1. Experimental irrigation procedures.

Irrigation procedure[†]	First irrigant solution[‡]	Intermediate irrigant solution[§]	The final irrigant solution
A – test procedure	NaOCl	Sterile saline	EO nanoemulsion[¶]
B – negative control	NaOCl	Sterile saline	EO-nanoemulsion negative control[#]
C – without the final irrigation	NaOCl	-	-

[†]Volume of each irrigant solution was set to be 2 ml.
[‡]NaOCl solution contained 0.5% of active Cl_2.
[§]Intermediate irrigation with sterile saline is commonly used in dentistry, in order to avoid any interaction between the irrigant solutions.
[¶]Nanoemulsion content: *Cymbopogon citratus* EO (2.5%, v/v), Tween 80 (0.25%, v/v), ethanol (2.5%, v/v), distilled water (added to 100% content).
[#]Nanoemulsion negative control content: Tween 80 (0.25%, v/v), ethanol (2.5%, v/v), distilled water (added to 100% content).
EO: Essential oil.

Antibiofilm activity of C. citratus EO nanoemulsion

Monitoring of antibiofilm potential of *C. citratus* EO nanoemulsion was provided by CFU-counting assay performed to monitor the biofilm disruption within the root canals of the extracted teeth [30]. The detailed procedure of biofilm cultivation and CFU determination has already been described in our recent publication [18]. In brief, it included root canal preparation at working length, its proper disinfection and sterilization by autoclaving of the extracted teeth. When sterility within the root canal was achieved and confirmed, *E. faecalis* inoculums (2×10^4 CFU/root canal in volume 20 µl) were initially introduced into root canals and repeated periodically every 48 h, along a 15 day time period. During the entire cultivation period, the root canal samples were incubated at 37°C. To monitor the efficiency of *C. citratus* EO nanoemulsion to enhance the antibiofilm activity, the following experimental design has been provided (Table 1). Experimental irrigation procedure is actually procedure A, including the biofilms treatment with NaOCl 0.5%, sterile saline and EO nanoemulsion. This procedure was compared with adequate controls (procedures B and C). Concerning irrigation procedure B, this involved successive irrigation with NaOCl, sterile saline and EO-nanoemulsion negative control. In addition, the irrigation involving NaOCl only was also provided (irrigation procedure C). After irrigation, sampling was provided by sterile paper point (#30) and values of log CFU ml^{-1} was determined by plating on blood agar, both applied as previously explained by Marinković *et al.* [18]. Intracanal biofilm disruption assay was performed in two individual experiments, in triplicate.

Statistical analysis

One-way analysis of variance test was used in SPSS 20.0 (IBM Corporation) statistical software. All p-values less than 0.05 were considered significant.

Results

C. citratus EO composition

Analysis of *C. citratus* EO revealed the abundance in oxygenated monoterpenes, and among them, citrals A (45.7%), B (31.6%) and geraniol (5.9%) were the major constituents (Table 2). Oxygenated monoterpenes (93.37%) comprised the major chemical group of the EO, followed by monoterpene hydrocarbons (3.41%), sesquiterpene hydrocarbons (2.45%) and oxygenated sesquiterpenes (0.36%).

Antioxidant activity of *C. citratus* EO

In the DPPH assay notable DPPH scavenging potential of the *C. citratus* EO was observed, with IC_{50} valuing 1.8 mg ml^{-1} (Figure 1A). The TBA assay also confirmed the notable antioxidant potential of the EO, ranging from 30.6 to 47.3% of inhibition, but the IC_{50} value could not be determined (Figure 1B).

Antibacterial activity of *C. citratus* EO

Results of the evaluation of antibacterial activity of *C. citratus* EO is shown in Table 3. Inhibitory potential of *C. citratus* EO ranged between 0.5 and 2 mg ml^{-1} and it was higher than that of positive control TAP. Bactericidal potential of both tested substances has been, in general, two-times lower in comparison to their inhibitory potential.

Antibiofilm activity of *C. citratus* EO

The CV assay revealed the significant inhibitory effect of EO on the biofilm formation (Figure 2A), as well as its disruptive potential on the preformed biofilm (Figure 2B, $p < 0.05$).

Table 2. Chemical composition of *Cymbopogon citratus* EO (% w/w).

RI	EO constituents	Composition (%)
904	α-Thujene	1.2
923	Camphene	1.7
1004	Limonene	0.6
1007	1,8-Cineol	0.9
1080	Linalool	1.6
1133	Citronellal	0.5
1142	Isoborneol	0.5
1163	*Trans*-isocitral	1.7
1170	α-Terpineol	0.6
1197	*Cis*-carveol	0.7
1210	Citronellol	0.4
1218	Neral (Citral-B)	31.6
1237	Geraniol	5.9
1248	Geranial (Citral-A)	45.7
1360	Geranyl acetate	3.3
1387	*Cis*-caryophyllene	1.4
1422	α-Humulene	0.4
1482	γ-Cadinene	0.7
1551	Caryophyllene oxide	0.4
Monoterpene hydrocarbons		3.41
Oxygenated monoterpenes		93.37
Sesquiterpene hydrocarbons		2.45
Oxygenated sesquiterpenes		0.36
Total identified		99.59
Identified constituents (n)		19

EO: Essential oil; RI: Retention index.

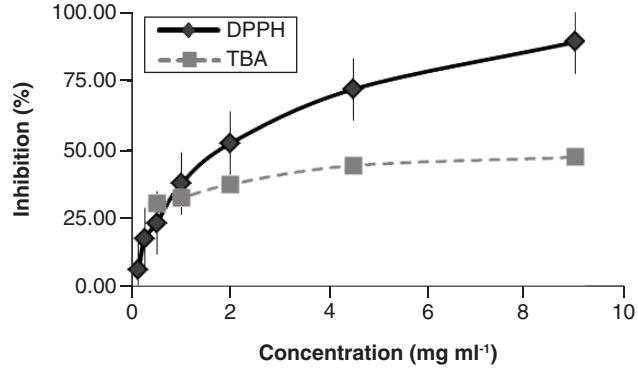

Figure 1. Antioxidative potential of the *Cymbopogon citratus* essential oil. Presented values are averages with standard deviations, obtained for two individual experiments performed in triplicates.
TBA: Thiobarbituric acid assay.

Table 3. Minimal inhibitory concentrations and minimal bactericidal concentrations of *Cymbopogon citratus* EO toward *Enterococcus faecalis* (mg ml^{-1}, average ± standard deviation).

E. faecalis	*C. citratus* EO		TAP	
Clinical isolate† /reference strain	MIC	MBC	MIC	MBC
Isolate EFc11	0.50 ± 0.00	1.00 ± 0.00	0.013 ± 0.00	0.025 ± 0.00
Isolate EFc12	1.00 ± 0.00	2.00 ± 0.00	0.025 ± 0.00	0.025 ± 0.00
Isolate EFc13	2.00 ± 0.00	4.00 ± 0.00	0.025 ± 0.00	0.050 ± 0.00
ATCC 29212	1.00 ± 0.00	2.00 ± 0.00	0.025 ± 0.00	0.050 ± 0.00
ATCC 194303	0.50 ± 0.00	1.00 ± 0.00	0.013 ± 0.00	0.025 ± 0.00

†Isolates of *E. faecalis* originated from infected root canals.
EO: Essential oil; MBC: Minimal bactericidal concentration; MIC: Minimal inhibitory concentration; TAP: Triple antibiotic paste.

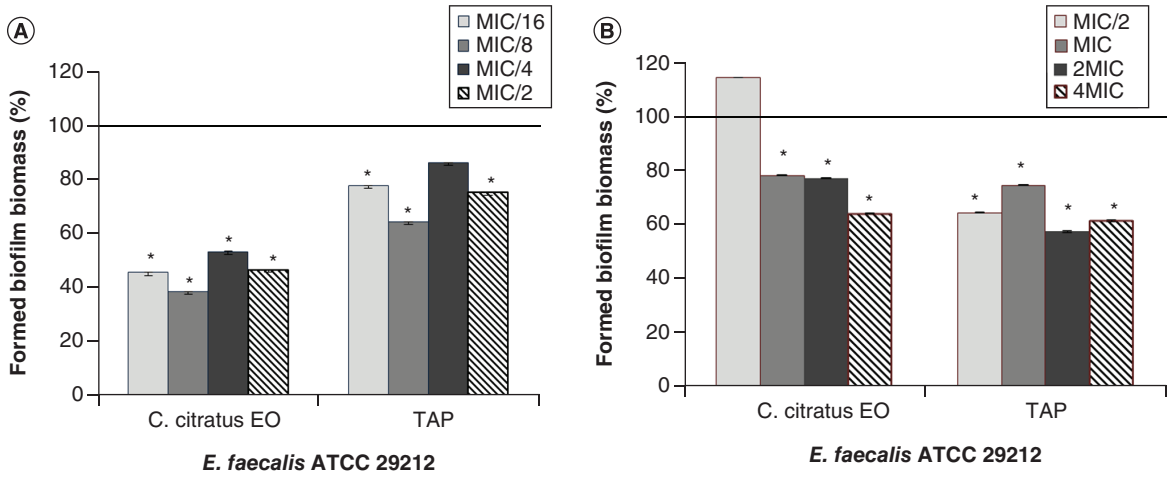

Figure 2. ***In vitro*** **effect of C.** ***citratus*** **essential oil in the crystal violet assay. (A)** On the biofilm formation and **(B)** on the pre-formed biofilm. The results are expressed as the average ± standard deviation of two individual experiments, each performed in five replicas. Statistical significance was tested using the one-way ANOVA.
*$p < 0.05$; statistical difference in the biofilm biomass compared with the untreated control (100% biofilm biomass).
EO: Essential oil; TAP: Triple antibiotic paste.

Table 4. Behavior of *Cymbopogon citratus* EO nanoemulsion NWD and PDI with time.

Day	0	14	21	28
NWD (nm)	95 ± 23	121 ± 8	127 ± 11	109 ± 24
PDI	0.17 ± 3	0.15 ± 1	0.15 ± 3	0.15 ± 2

EO: Essential oil; NWD: Number-weighted mean particle diameter; PDI: Polydispersity index.

Impact of *C. citratus* EO on the biofilm formation was higher than that of positive control (TAP) and ranged between 61.8% of inhibition at MIC/8 (0.125 mg ml^{-1}) and 47.2% at MIC/4 (0.25 mg ml^{-1}). On the other hand, disruption of preformed biofilm with the EO was somewhat lower than that obtained with TAP; the inhibitions ranged 22–36% and 25.5–42.6%, respectively. In addition, the only concentration that was used in both procedures (MIC/2, 0.5 mg ml^{-1}) induced the opposite effect, that is, inhibited the biofilm formation (53.8%), but also induced proliferation of already formed biofilm (stimulation 14%, Figure 2).

Formulation & characterization of *C. citratus* EO nanoemulsion

Immediately after emulsion synthesis, calculated number weighted mean particle diameter and the polydispersity index of the nanoemulsion were 95 ± 23 nm and 0.17 ± 3, respectively. Furthermore, the time dynamics of intensity weighted size distributions (Figure 3) and polydispersity indices (Table 4) indicated that the nanoemulsion remained stable during the whole storage period (4 weeks).

Antioxidant activity of *C. citratus* EO nanoemulsion

Both antioxidant assays revealed the low antioxidant activity of formulated nanoemulsion (Figure 4), which was expected, since the activity was presented in respect to concentrations of EO (active principle) within the nanoemulsion, being relatively low. The DPPH test showed scavenging potential with maximal inhibition determined at 12.9%, while inhibition of LP in the TBA assay was up to 24.0%. The fact that the inhibitory values for approximately similar concentrations of sole EO and the EO in the nanoemulsion (0.125 and 0.113 mg ml^{-1}, respectively) were similar, it confirmed that preparation of nanoemulsion did not affect the antioxidant activity of sole *C. citratus* EO.

The impact of irrigation procedure including *C. citratus* EO nanoemulsion on the *E. faecalis* biofilm cell viability

The outcomes of intracanal biofilm disruption assay, conducted in *ex vivo* teeth, confirmed the high inhibitory potential of irrigation procedure including *C. citratus* EO nanoemulsion toward *E. faecalis* intracanal biofilm (Δlog

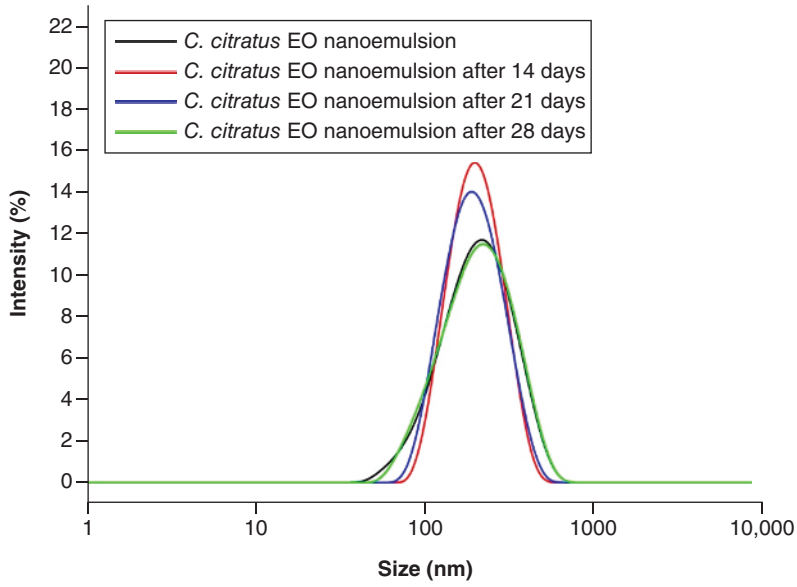

Figure 3. Time evolution of *Cymbopogon citratus* essential oil nanoemulsion size distribution.
EO: Essential oil.

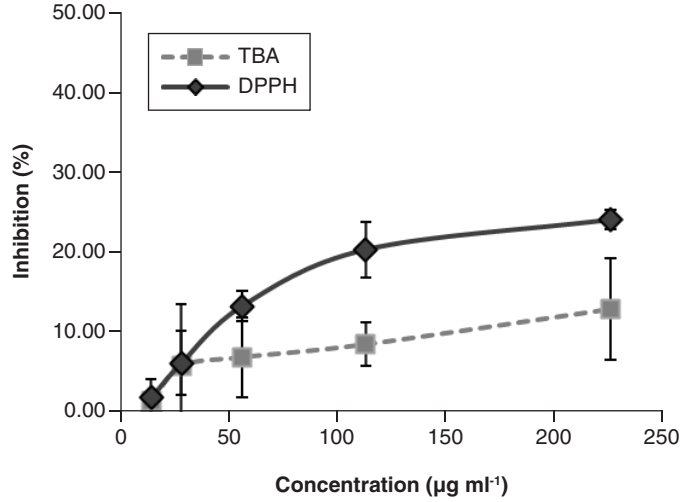

Figure 4. Antioxidative potential of the *Cymbopogon citratus* essential oil nanoemulsion. Presented values are averages with standard deviations, obtained for two individual experiments performed in triplicates.
TAP: Triple antibiotic paste.

Table 5. The impact of irrigation procedures (including or not *Cymbopogon citratus* EO nanoemulsion) on the *Enterococcus faecalis* biofilm cell viability within the root canals of extracted teeth.

Log CFU	Irrigation procedure A (NaOCl + sterile saline + *Cymbopogon citratus* EO nanoemulsion†)		Irrigation procedure B (NaOCl + sterile saline + negative control‡)		Irrigation procedure C (sole NaOCl§)	
	Before	After	Before	After	Before	After
Log CFU	5.92 ± 0.13	2.52 ± 0.66	5.98 ± 0.03	4.67 ± 0.03	6.60 ± 0.60	4.82 ± 0.13
Δ (log CFU)¶	3.4		1.31		1.78	

†Nanoemulsion content: *C. citratus* EO (2.5%, v/v), Tween 80 (0.25%, v/v), ethanol (2.5%, v/v), distilled water (added to 100% content).
‡Nanoemulsion negative control content: Tween 80 (0.25%, v/v), ethanol (2.5%, v/v), distilled water (added to 100% content).
§NaOCl solution contained 0.5% of active Cl_2.
¶The value of biofilm reduction after irrigation treatment, determined by subtraction of log CFU values before and after each irrigation. The results are expressed as the average ± standard deviation of two individual experiments performed in triplicates.
EO: Essential oil.

CFU 3.4, Table 5); the reduction was remarkably higher than those achieved with the final irrigation solution

without EO (negative control) and with the sole NaOCl (Δlog CFU were 1.31 and 1.78, respectively).

Discussion

In this study antioxidative, antibacterial and antibiofilm properties of the *C. citratus* EO have been investigated. The preparation and assessment of *C. citratus* EO nanoemulsion was particularly stressed, and involved *in vitro* determination of its antioxidative potential and inhibitory potential against *E. faecalis* biofilm within extracted teeth root canals. This was of our special interest as a kind of investigation that is a step toward the practical application of *C. citratus* EO in infected root canal therapy.

Biofilm of *E. faecalis* has been selected in accordance with scientific evidences confirming this pathogen as the most commonly isolated bacterial species in endodontic cases, which need retreatment [31,32]. This is related to its capabilities to invade and bind to collagen in dentinal tubules, to survive in prolonged periods of starvation until nutritional supply becomes available again [33] and to overcome activities of the commonly used intracanal antimicrobials [4,5,33]. Being aware of all the above-mentioned, in addition to findings that *E. faecalis* could be resistant to various antibiotics, including the TAP constituents [34–36], some authors have already pursued the possible alternatives among EOs [37,38]. These authors have demonstrated the efficiency of *Aloe vera*, *Zataria multiflora* and *Matricaria chamomilla* oils against *E. faecalis* within the root canals. Our previous study also suggested *C. martinii* and *Thymus zygis* EOs, as possible supportive antimicrobials against multispecies biofilm including *E. faecalis* [18].

Prescreening of the antibacterial properties of *C. citratus* EO in the microdilution assay, indicating a notable antibacterial potential, is in agreement with the findings of Bassole [39]. *In vitro* testing of the antibiofilm activity showed the high efficiency in inhibition of biofilm formation and in disruption of the preformed biofilm. In a recent study, Ortega-Ramirez *et al.* [40] also presented the high inhibitory impact of the same EO, but against biofilm formed by *Escherichia coli* strains. In this study comparison of effectivity against preformed and emerging *E. faecalis* biofilms pointed out the more pronounced activity during biofilm formation. A similar was also observed by Costa *et al.* [41] and Correa *et al.* [42], and could be explained by the fact that planktonic cells, being exposed during biofilm formation, are more vulnerable than the cells being protected by biofilm matrix [43,44]. Considering possible mechanism underlying the observed antibiofilm properties, the suggestions of Ortega-Ramirez *et al.* [40] and Gao *et al.* [45] seem to be even more interesting as they showed that *C. citratus* EO reduced the viability of the cells and disrupt the biofilm matrix, at least formed by *E. coli* and cross-kingdom pathogens *Staphylococcus aureus* and *Candida* spp., respectively.

Application of pure EO during a root canal treatment is limited due to its viscosity and hydrophobicity, which makes it hard to remove from the root canal prior to the final step of the infected root canal treatment – its obturation. Therefore, the formulation of the most appropriate EO delivery system intended for use in the root canal disinfection and with no impediment to the following step in the endodontic procedure is considered highly desirable. In this study, we designed the nanoemulsion–liquid dispersion of the *C. citratus* EO in an aqueous phase stabilized by the surfactant Tween 80. Concerning a fact that the emulsions are unstable systems, EOs-based nanoemulsion has required the evaluation of the stability throughout the storage time [46]. We monitored the basic parameters through several weeks and pointed out that the nanoemulsion was stable for up to 4 weeks.

Bearing in mind that the nanoemulsification of EOs increases the overall antimicrobial activity [47], this approach is valuable not only because of optimizing delivery in root canals but also due to antibacterial demands. Moreover, Bonferoni *et al.* [48] has already formulated specific *C. citratus* EO nanoemulsion characterized by the improved antimicrobial effect compared with that of the sole EO. The results provided by the screening of antibiofilm effect on extracted teeth showed remarkable potential of the nanoemulsion-involved irrigation against enterococcal biofilm established in root canals. It is worth to note that the reduction of log CFU provided by a procedure including EO nanoemulsion was >3 log, being defined by the National Committee for Clinical Laboratory Standards [49] as a border for considering the bactericidal activity. The fact that both of other irrigation procedures without the nanoemulsion (negative control and sole NaOCl) induced weak antibiofilm effect confirmed that EO nanoemulsion contributed remarkably to overall activity.

Bearing in mind that conventional endodontic treatments, including chlorhexidine, calcium hydroxide and also the NaOCl used in this study, contribute to ROS production consequently inducing oxidative stress [7–9], we had to focus also on the antioxidative potential. It was screened for both, the EO and EO-based nanoemulsion, in the DPPH and TBA assays. Results of DPPH assay pointed out the notable scavenging activity of the *C. citratus* EO, which was in agreement with previous studies [19,20]. Searching for the antioxidative potential in the TBA assay indicated that EO could additionally be protective on the cell membranes, due to its potential to reduce LP.

This is of special importance as this is the first report indicating potential of the *C. citratus* EO to prevent LP on the cell membranes. According to the facts that the EO concentration in the nanoemulsion is low, as well as that only EO could contribute to its antioxidative effect, both scavenging capacity and potential to inhibit LP of the EO-based nanoemulsion were expectedly lower. However, the antioxidative properties do exist and could be beneficial particularly following the NaOCl-induced ROS production [7] during the standard irrigation procedure.

In order to explain the background of the presented antibacterial, antibiofilm and antioxidant activities of the *C. citratus* EO and its nanoemulsion potential active compounds among *C. citratus* EO constituents were also explored. The fact that the EO is dominantly abundant with citrals A and B (i.e., geranial and neral), being characterized as active aldehydes, which could interfere with electron transfer and vital nitrogen-containing components, for example, proteins and nucleic acids of the bacteria [50], it seem that they could be of interest for the observed antimicrobial activity. Accordingly, the citrals could be responsible for the bacterial growth inhibition (i.e. for the observed antibacterial activity). Antibiofilm effect could also be attributed to citrals as a few recent studies confirmed their high antibacterial and antibiofilm activity against numerous bacterial species, including *E. faecalis* [51–54]. The recent study of Gao *et al.* [45] demonstrating that they possess remarkable potential to diminish all components of biofilm matrix (nucleic acid, proteins and carbohydrates) of combined pathogens *S. aureus* and *Candida* spp., additionally strengthen this claim.

On the other hand, several studies confirmed antioxidant potential of geraniol [55–58] other major *C. citratus* EO constituent. Interestingly, Widelska [59] suggested that other EO constituents, geranial and neral, have not been responsible for antioxidant activity of *C. citratus* EO; the author tested antioxidant activity of this EO along with sole neral/geranial, and explained that antioxidant potential of complete EO should be attributed to synergism of the neral/geranial with other EO constituents.

Conclusion

In this study we presented promising properties of the *C. citratus* EO nanoemulsion: reductive potential against *E. faecalis* biofilm formed in infected teeth root canals, and local tissue protective effect against oxidative stress induced by conventional irrigants. Presented results encourage further investigation in order to upgrade the *C. citratus* EO-based nanoemulsion, in order to formulate suitable adjuvant being useful in the future endodontic practice.

Future perspective

Bearing in mind that eradication *of E. faecalis* within infected root canal is recognized as highly problematic in dental practice, our results strongly encourage future studies, including the clinical ones, of new antimicrobials in the forms of the nanoemulsions based on EOs, being proved for remarkable antibacterial activity and no resistance issues. Encouraging results of *in vitro* and *ex vivo* assays presented in this study, provide a basis for the expectation that the EOs based nanoemulsions would successfully combat with *E. faecalis* and other pathogens of clinical relevance, within root canals *in vivo*. Moreover, beneficial antioxidant properties of the EOs additionally support their use as an active principle for preparation of the nanoemulsion, which should be protective against oxidative stress. Clinical confirmation of the ROS neutralization and bacterial elimination would be a serious breakthrough in the endodontics.

Authors expect that the future clinical studies would confirm these *in vitro* obtained results, enabling the usage of the EO-based nanoemulsion, as a part of common routine in dental practice.

Author contributions

All authors were responsible for the study conception and design. Authors J Marinković, B Nikolić, T Marković, M Radunović, J Ilić and M Bošković were responsible for data acquisition. All authors were responsible for data analysis. Authors J Marinković, B Nikolić and M Bošković were responsible for the manuscript drafting. All authors were responsible for the revision of the manuscript.

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Summary points

- *Cymbopogon citratus* essential oil (EO) has been chemically characterized and proved to possess notable antibacterial activity.
- EO proved highly efficient in the inhibition of biofilm formation as well as in disruption of the preformed biofilm.
- The most appropriate EO delivery system for the root canal disinfection was provided through formulation of EO-based nanoemulsion.
- Nanoemulsion characterization, involving determination of calculated numbers weighted mean particle diameters and polydispersity indices, pointed out that it remained stable up to 4 weeks.
- Bactericidal activity toward *Enterococcus faecalis* was confirmed with irrigation procedure including the EO nanoemulsion in *ex vivo* root canals.
- Antioxidative properties of EO and its nanoemulsion could be considered as beneficial, especially after irrigation procedures containing reactive oxygen species producer NaOCl.
- Citrals A and B (i.e., geranial and neral) may be responsible for observed antibacterial and antibiofilm properties of the EO and its nanoemulsion.
- Geraniol as well as citrals interactions with the other constituents of the EO may be responsible for the observed antioxidative potential.

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Ethical conduct of research

Ethical approval was granted by the Ethical committee of School of Dental Medicine, Belgrade University, on the date 07.03.2019. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

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