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Effect of *Lippia alba* and *Cymbopogon citratus* essential oils on biofilms of *Streptococcus* mutans and cytotoxicity in CHO cells

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ARTICLE INFO

Chemical compounds studied in this article:
List of up to 10 names of chemical compounds studied in the article. Geranial. Neral. Myrcene. Geraniol. (*E*)-Caryophyllene. trans-Verbenol. Geranyl acetate. cis-Verbenol. Germacrene D. 3
7-Dimethyl-2
6-octadiene-1-ol

Keywords:
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ABSTRACT

Background: Caries is a public health problem, given that it prevails in 60 to 90% of the school-age global population. Multiple factors interact in its etiology, among them dental plaque is necessary to have lactic acid producing microorganisms like Streptococcus from he Mutans group. Existing prevention and treatment measures are not totally effective and generate adverse effects, which is why it is necessary to search for complementary strategies for their management.
Aim: The study sought to evaluate the eradication capacity of *Streptococcus mutans* biofilms and the toxicity on eukaryotic cells of *Lippia alba* and *Cymbopogon citratus* essential oils.
Methodology: Essential oils were extracted from plant material through steam distillation and then its chemical composition was determined. The MBEC-high-throughput (MBEC-HTP) (Innovotech, Edmonton, Alberta, Canada) assay used to determine the eradication concentration of *S. mutans* ATCC 35668 strain biofilms. Cytotoxicity was evaluated on CHO cells through the MTT cell proliferation assay.
Results: The major components in both oils were Geraniol and Citral; in *L. alba* 18.9% and 15.9%, respectively, and in *C. citratus* 31.3% and 26.7%. The *L. alba* essential oils presented eradication activity against *S. mutans* biofilms of 95.8% in 0.01 mg/dL concentration and *C. citratus* essential oils showed said eradication activity of 95.4% at 0.1, 0.01 mg/dL concentrations and of 93.1% in the 0.001 mg/dL concentration; none of the concentrations of both essential oils showed toxicity on CHO cells during 24 h.
Conclusion: The *L. alba* and *C. citratus* essential oils showed eradication activity against *S. mutans* biofilms and null cytotoxicity, evidencing the need to conduct further studies that can identify their active components and in order to guide a safe use in treating and preventing dental caries.

1. Introduction

Dental caries is a physiological alteration of the minerals of the dental tissue and fluids surrounding it, where a broad group of biological, socio-economic, and cultural determinants interact (Fejerskov, 2004). Among the biological factors biofilm is implied (Marsh and Bradshaw, 1995); these is a matrix formed by proteinaceous material, extracellular polymers, and microbial communities that interact through dynamic systems, providing bacterial protection and resistance to antimicrobial agents between 100 and 1000 times greater with respect to bacteria in planktonic state (Jhajharia et al., 2015). The bacterial species, *Streptococcus mutans*, is highlighted for being the principal agent associated to dental caries due to its acidogenic and aciduric properties that permit it to adapt and survive environmental changes (Loesche, 1986).

Dental caries is a global public health problem (Giacaman et al., 2013) due to its magnitude, besides, it causes pain, scholar and labor absenteeism, nutritional difficulties, as well as phonation, aesthetic, and economic problems (Petersen et al., 2005). According to the World Health Organization, 60–90% of school-age children throughout the world have dental caries (http://www.who.int/mediacentre/factsheets/fs318/en/), 98% of adolescent population between 11 and

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13 years of age in Latin America (Bönecker and Cleaton-Jones, 2003) and Colombia; in spite of efforts by the Ministry of Health and Social Protection to prevent and treat this disease, the problem still remains given that 91.58% of the individuals between 12 and 79 years of age have presented dental caries at some point of their lives, 33.27% of children from 1.3 to 5 years of age, at their early age, have also presented antecedents of this disease.

(https://www.minsalud.gov.co/sites/rid/Lists/BibliotecaDigital/RIDE/VS/PP/ENT/abcsalud-bucal.pdf).

To control dental bacterial plaque (biofilm) and prevent caries, brushing and flossing continue being the most effective methods (Löe, 2000); in addition to fluorinated substances (Bratthall, 1996), mouthwash with Chlorhexidine, phenols, and quaternary ammonium derivatives (Twetman, 2004). However, these substances produce secondary effects like dental fluorosis (Jha et al., 2011), dental chromatism, and dysgeusia (Chlorhexidine) (Eriksen et al., 1985; Marinone and Savoldi, 2000), burning sensation (quaternary ammonium compounds and phenols), ulcerative lesions (hexedetine) (Enrile de Rojas and Santos-Alemany, 2005; Bascones and Morantes, 2006), and erosive effects on the enamel (phenols) (Bascones and Morantes, 2006). In this sense, it is necessary to broaden the research on new substances that permit controlling dental bacterial plaque, considering the possible adverse effects on the oral mucosa, given that these fulfill important functions, modulate the relationship between the tissue subjacent to the epithelium and the medium that washes its free surface is a barrier against mechanical damage (Chiego, 2007), avoids desiccation of the oral mucosa (Newman, 2003), and participates in immune response (Gómez and Campos, 2006). Natural products, as pure compounds or standardized extracts from plants, provide opportunities like new drugs due to their chemical diversity (Cos, 2006). Previous investigations have evidenced the antimicrobial activity of Lippia alba (L. alba) and Cymbopogon citratus (C. citratus) essential oils on S. mutans. (Almeida et al., 2013) demonstrated that C. citratus essential oils have antimicrobial activity and eradication of biofilms over S. mutans and Candida spp, and Nogueira et al. (2007) observed inhibition zones of 7.0 mmc of the *L. alba* essential oil over S. mutans cultures (Nogueira et al., 2007).

Considering these antecedents, the aim of this study was to generate information to support the future introduction of essential oils extracted from native plants from the Colombian Caribbean region as a therapeutic alternative in oral health and set the basis for further indepth assessment of oils The results reached are due to inter-institutional alliances promoted by CORPOICA within its 2014 five-year agenda to identify agro-industrial possibilities of aromatic plants in the Colombian Caribbean.

2. Materials and methods

2.1. Essential oils

Extraction of essential oils was carried out through steam distillation, from plant material harvested during the rainy season (October - November). The harvest was done manually from plant material that had not been subjected to fertilization plans or use of agrochemicals. The *C. citratus* essential oil was extracted in the Kankuamos Indian reservation in Atanquez, Sierra Nevada de Santa Marta (1200 MASL) in Colombia, from plant material cultivated on slightly acidic, highly fertile loam soil and 2% organic matter, with an extraction yield of 0.71%. The *L. alba* essential oil was extracted in the Natural Products laboratory at Universidad de Córdoba in Montería, Colombia from plants harvested at the Motilonia Research Center in Codazzi, Cesar (100 MASL), a region with slightly acidic, moderate fertility clay loam soils and 1% organic matter, with a yield of 0.5%. The plant material was classified in the José Cuatrecasas Herbarium at Universidad Nacional at Palmira, with Code 4952 20/10/2014 for *C. citratus* and 4863 18/10/2014 for L. alba.

2.2. Phytochemical analysis

The chemical composition of the essential oils was determined through gas chromatography with mass selective detector (GC-MSD); sample preparation was carried out through dilution in dichloromethane and direct injection of the samples into the chromatographic equipment, reconstructed total ion current, simple normalization, presumptive identification in APOLAR DB-5MS (60 m) and POLAR DB-WAX (60 m) Columns, with NIST library version 14; MASSLAB program.

2.3. Culture of S. mutans biofilms

Mono-microbial cultures of *S. mutans* ATCC 35668 strain biofilms were carried out by using the MBEC-high-throughput (MBEC-HTP) (Innovotech, Edmonton, Alberta, Canada) commercial system, described by Ceri et al. (1999). The protocol of use was adapted by the research group to culture *S. mutans* biofilms. The system was inoculated with a suspension corresponding to pattern 1,0 by McFarland, equivalent to 3×10^8 colony forming units (CFU) per mL suspended in 30 mL of brain-heart infusion (BHI; Scharlau Chemie S.A., Barcelona, Spain). With this inoculum, serial dilutions were conducted from 10^{-1} to 10^{-6} cultured in dish with BHI agar to establish the initial CFU count. Thereafter, the system was cultured on an orbital shaker platform (Unimax 1010, Heidolph®) at 110 rpm and 37 °C for 24 h, under controlled atmosphere with 5% CO_2. The biofilms formed on the lid pegs of the MBEC-HTP were washed with saline solution at 0.9% during 2 min to remove weakly adhered bacterial cells. To verify the formation of biofilms, four pegs were removed from the lid with the aid of a mosquito clamp and placed in 200 mL of saline solution at 0.9% in a sonic cleaner (MIDMARK Soniclean model M 150) during 30 min. With the resulting suspensions, serial dilutions were carried out from 10^{-1} to 10^{-6} and these were cultured in BHI agar plates adding 10% saccharose, for count of viable cells. To control growth of the planktonic culture, 20 μL of the planktonic culture were taken, diluted in 180 μL of saline solution at 0.9%, and seeded in triplicate in BHI agar.

2.4. Determination of the minimal biofilm eradication concentration

Susceptibility tests were conducted on a standard 96-well plate dish (Nunclon Delta Surface; Nunc, Roskilde, Denmark). Ethanol was used as a vehicle for the essential oils; after corroborating in a test tube the lack of inhibitory effect upon *S. mutans* at the highest concentration (10%), and replicas were made in the MBEC-HTP using it as control. Concentrations of 0.1 mg/100 mL, 0.01 mg/100 mL, and 0.001 mg/100 mL were used for the essential oils and Chlorhexidine at 0.5%, 1%, and 2% was used as positive control. Sterility (BHI) and growth controls, respectively, were placed in the first and last well of each row. The lid with the pegs was submerged in the micro-titration dish during 5 min and then the biofilms were washed twice by using a standard 96-well plate dishes with 180 μL of saline solution at 0.9% during 2 min. Thereafter, the biofilms were placed in another a standard 96-well plate dish (recovery dish), which had been prepared with 180 μL of BHI in each well and there they were sonicated during 30 min. The sonication products were diluted serially from 10^0 to 10^{-5} with saline solution at 0.9% and 10 μL were used to inoculate the BHI agar plates for viable cells count through the spot technique.

Each concentration of the essential oils was evaluated through eight replicates and the same amount of controls were performed, both positive (Chlorhexidine) and negative (sterile distilled water). The effect of each essential oil on the biofilms was determined through calculating the percentage of cells deaths:

$$[1-(\text{Mean CFU}_{\text{irrigant}}/\text{Mean CFU}_{\text{initial bacterial number}})] \times 100\%.$$

2.5. Cell viability through reduction of bromide from 3-(4,5-Dimethyltiazol-2-ilo)–2,5-diphenyltetrazol (MTT)

The test was conducted according to the method developed by Mosmann (1983), modified by Denizot and Lang (1986), with Chinese hamster ovary cells (CHO) and according to that stipulated in the 2009 ISO 10993 standard. A total of 10^3 cells per well were seeded, incubated during 24 h with 0.1 mg/100 mL, 0.01% mg/100 mL, and 0.001 mg/100 mL solutions of the *L. alba* and *C. citratus* essential oils in ethanol (Table 2). Methanol was used as cell death control. Upon finishing the test, absorbance was measured through ELISA technique at a 570-nm wavelength. The statistical analysis was performed in the SPSS- program; for each concentration, three triplicates or nine experimental units were conducted and the data are expressed as mean ± SEM. The comparison among the different concentrations and the control was analyzed via one-way ANOVA with a post-hoc examination with Dunnet's test. A statistically significant difference was considered if $p < 0.05$.

3. Results

3.1. Phytochemical composition of the oils analyzed

The major components in both essential oils were Geraniol and Neral (Citral), with a higher concentration of said compounds found in *C. citratus* (31.3% and 26.7%, respectively) than in *L. alba* (18.9% and 15.9%, respectively). A third high-concentration component was also identified for *L. alba*, corresponding to Geraniol (11.5%) and for *C. citratus* corresponding to Myrcene (15.6%). Other common compounds in both oils are (E) - β-Ocimene, (E)-Caryophyllene, cis-Verbenol, Curlone, trans-Verbenol, and Linalool (Table 1).

3.2. Biofilm eradication activity of essential oils through MBEC assay

Chlorhexidine, used as positive control, presented 100% mortality effect on the *S. mutans* biofilm in concentrations of 1% and 2%; and 99.4% mortality effect at a concentration of 0.5%, as expected. The diluting agent control (ethanol) produced a mortality logarithm of −1.9. The *C. citratus* essential oil presented a mortality logarithm of 1.3 at 0.1 mg/100 mL and 0.01 mg/100 mL concentrations, and a mortality log of 1.2 at 0.001 mg/100 mL concentration, while *L. alba* essential oil presented a mortality logarithm of 0.4 at 0.1 mg/100 mL and 0. 001 mg/100 mL concentrations, along with a mortality logarithm of 1.4 at 0.01 mg/100 mL concentration (Table 2).

"Concentration %" indicates the concentration of essential oil used. "% Mortal" percentage of non-viable bacteria with relation to the initial inoculum. "Log Mortal" is the effectiveness of the substance, according to the formula: Log10 (CFU/mm^2)=log10 [(X/B) (V/A) (D)+1]. Log10 Reduction=Mean Log10 Controls without treatment −Mean Log10 Pegs with treatment. Where: X=mean CFU; B=volume used on the dish (Ex. 0.02 mL) V=well volume (0.20 mL); A=surface area of the peg (46.63 mm^2); D=Dilution.

3.3. Cell viability through MTT assay

The MTT test on CHO cells evidences that none of the concentrations evaluated for the *L. alba* and *C. citratus* oils inhibit cell proliferation during a 24-h treatment, with significant differences in relation to the control with methanol (p=0.00), which inhibits most of the cells. On the contrary, higher cell proliferation is observed in the concentrations of essential oils than in the solvent only vehicle based on ethanol at 0.1%, indicating the role of the oils as protective agents of the CHO cells (Fig. 1).

Table 1
Relative amount (%) of the principal components present in *L. alba* and *C. citratus* essential oils.

Lippia alba		***Cymbopogon citratus***	
Identification	**Relative amount (%)**	**Identification**	**Relative amount (%)**
Geranial	18.9	Geranial	31.3
Neral	15.9	Neral	26.7
Geraniol	11.5	Myrcene	15.6
(E)-Caryophyllene	8.5	trans-Verbenol	3.8
Geranyl acetate	3.8	cis-Verbenol	3.0
Germacrene D	3.3	3,7-Dimethyl-2,6-octadiene-1-ol	3.0
Mono(2-ethylhexyl) phthalate	3.3	6-Methyl-5-hepten-2-ona	1.9
3,7-Dimethyl-2,6-octadiene-1-ol	3.1	Mono(2-ethylhexyl) phthalate	1.5
Limonene	2.8	Linalool	1.4
α-Guaiene	2.8	N.I.	1.2
α-Humulene	2.6	Tumerone	1.1
Nerol	2.4	3-(4-Methyl-3-pentyl)-furan	1.0
trans-Verbenol	2.3	N.I.	1.0
6-Methyl-5-hepten-2-one	1.9	(Z)-β-Ocimene	0.9
Cis-Verbenol	1.7	Geraniol acetate	0.9
δ-Guaiene	1.3	N.I.	0.7
Tumerone	1.3	(E)- β-Ocimene	0.6
β-Elemenone	1.2	N.I.	0.6
Linalool	1.1	Citronelol	0.6
Eugenol	1.1	N.I.	0.4
Caryophyllene oxide	1.1	2-Undecanone	0.4
Geranyl isobutyrate	0.9	ar-Tumerone	0.4
N.I.	0.8	Curlone	0.4
α-Cadinol	0.8	Eugenol	0.3
Myrcene	0.7	(*E*)-Caryophyllene	0.3
N.I.	0.7	α-Phellandrene	0.2
α-Phellandrene	0.6	2-Methyl−6-methylene-1,7-octadien-3-one	0.2
Curlone	0.6	trans-β-Bergamotene	0.2
1,8-Cineole	0.4	α-Zingibirene	0.2
(*E*)-β-Ocimene	0.4	β-Sesquiphellandrene	0.2
ar-Curcumene	0.4	Tricyclene	0.1
α-Zingibirene	0.4	Rose furan epoxide	0.1
β-Sesquiphellandrene	0.4		
ρ-Cymene	0.3		
(E)-Bisabolene	0.3		
Rose furan epoxide	0.2		
(E)-β-Farnesene	0.2		
α-Pinene	0.2		

N.I. Not identified.

4. Discussion

The phytochemical analysis obtained for *L. alba* was similar to that reported by (Olivero et al., (2009), who identified Geraniol and Citral as the principal components of the plant's essential oil, but at different concentrations (23.3% and 19.5%, respectively); also, Machado et al. (2014) identified a higher proportion of these phytochemicals (Citral 31.6% and Neral 25.5%). In turn, the same peculiarity occurs in the analysis obtained for *C. citratus* (major components: Myrcene, Neral, and Geraniol); for (De Oliveira et al., 2013), the levels were Neral 30.9% and Geraniol 42.9%, and for (Gbenou et al., 2013) Citral 27.04%, Neral 19.93%, and Myrcene 27.04%. This indicates that both plants belong to the "citral" chemotype composed of Neral and Geraniol, commonly identified in the region and which has antioxidant, antimicrobial, and bactericide properties (Espina et al., 2015; Stashenko et al., 2014).

The lower content of the main chemical compounds of essential oils observed in this test in relation to other studies is influenced by the

Table 2
Evaluation of the antimicrobial activity of *C. citratus* and *L. alba* essential oils.

	Concentration mg/100 mL	Mean CFU/mL	% Survival	% Mortality	Mortality Log
Lippia alba	**0.1**	**7812.5**	**39.1**	**60.9**	**0.4**
	0.01	**850**	**4.3**	**95.8**	**1.4**
	0.001	**8500**	**42.5**	**57.5**	**0.4**
Cymbopogon citratus	0.1	925	4.6	95.4	1.3
	0.01	925	4.6	95.4	1.3
	0.001	1375	6.9	93.1	1.2
Controls	**Concentration %**	**Mean CFU/mL**	**% Survival**	**% Mortality**	**Mortality Log**
Ethanol	1	1416537.5	7082.7	−6982.7	−1.9
Chlorhexidine	0.5	125	0.6	99.4	
	1	0	0.0	100	
	2	0	0.0	100	

culture's non-technical seeding conditions in the zone and the lack of production models that define the specific practices and conditions that maximize the production efficiency in the department of Cesar. This circumstance agrees with demands from the agricultural production chain of aromatic and seasoning.

plants and herbs at the national level like determination of agro-ecological conditions and development of strategies for agronomic management of medicinal and aromatic plants, as well as seasoning and similar herbs in Colombia, described in the SIEMBRA 2015 platform (http://www.siembra.gov.co/siembra/main.aspx). Silva et al. (2010) determined that environmental stress (urban contamination) can contribute to changes in the chemical composition of the *C. citratus* leaves, reduction of the synthesis of the more polar compounds and this may lead to changes in pharmacological and toxicological properties, besides citing other research that associates production, content, and composition of the oils with seasonality or weather conditions and phenology cycle; this demonstrates that the content and chemical composition of the plant oils depends sensibly on the geographic origin, crop conditions (nutrients, temperature, luminosity, urban contamination), age, segment of the plant used for the extraction, and other geo-botanic factors, which indicates that the safeness of the final product is highly influenced by the standardization of the production methods and crop conditions (an always weak aspect in field production of aromatic plants) to achieve raw material consistent with the results expected for the production of transformed article and its traceability. According to the aforementioned, it may be understood that within the description of oil production, besides the laboratory methodology implemented, a complete description must be presented of the agro-ecological conditions, geographic coordinates, management, and harvest time of the plant material.

Natural products provide opportunities like new medications, given their chemical diversity (Cos et al., 2006). Due to this, it is important to generate information that supports the introduction of essential oils from native plants as therapeutic alternative in oral health. Identification of concentrations to eradicate *S. mutans* biofilm of 0.1 mg/100 mL and 0.01 mg/100 mL for *C. citratus* essential oil, and of 0.001 mg/100 mL for *L. alba* essential oil, indicated in Table 1, is due to the safety and sensitivity of the method of micro-dilutions through the MBEC-HTP assay, which contrasts with the evaluation of the Antimicrobial activity of *C. citratus* tinctures on *S. mutans* strains isolated from patient saliva through the agar diffusion method conducted by Da Silva et al. (2012), where no positive results were obtained; however, (Nogueira et al. 2007) observed inhibition zones of 7.0 mm from the *L. alba* essential oil tested on *S. mutans* cultures.

This study obtained positive results of *C. citratus* essential oil,

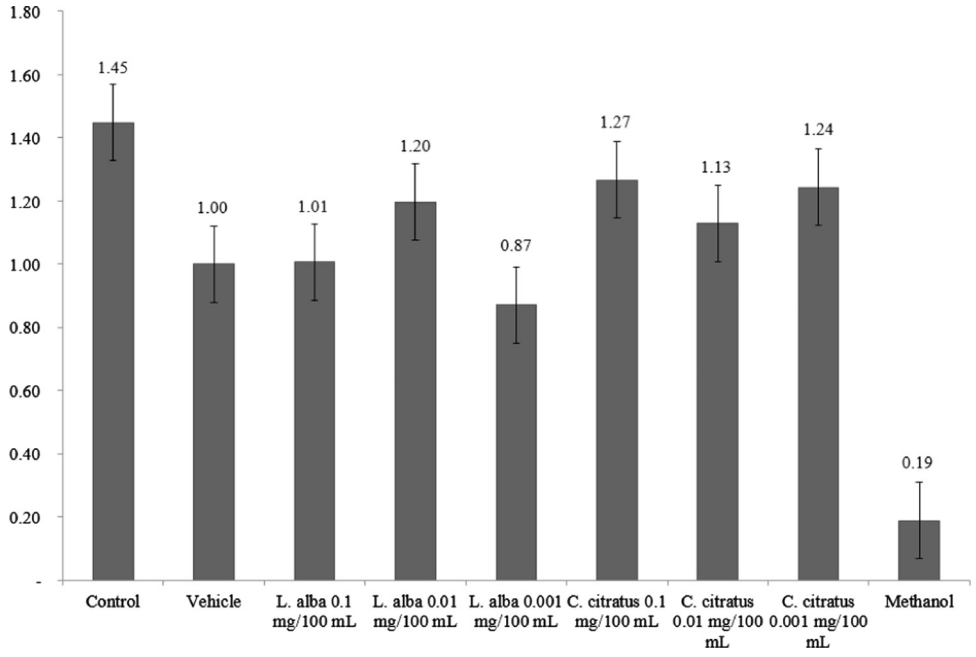

Fig. 1. Cytotoxicity on CHO cells of *L. alba* and *C. citratus* essential oils in three concentrations in relation to methanol. Strands indicate the standard deviation of the mean (n=1000 CHO cells).

similar to those reported by Almeida, et al. (2013), who, through micro-dilution, found antimicrobial activity and eradication of *S. mutans* ATCC 35668 biofilms at 0.5% concentration of the essential oil. The antibacterial activity of the oils may be explained according to diverse variables; (Vera et al., 2007) evidenced that no significant differences existed regarding the composition of bioactive phytochemicals and the biological activity of the different plant chemotypes.

Machado et al. (2014) highlighted the highest antimicrobial effectiveness in *L. alba* essential oils obtained during the period from December to February against the S. aureus species.

According to the results obtained during the cytotoxicity evaluation (Fig.1), these essential oils do not pose a risk of producing cell death or toxicity in CHO cells, which would allow advancing with other determinant preclinical trials. Consequently, studies should be conducted seeking to evaluate the antimicrobial activity of the *C. citratus* essential oil and its major components to determine if the eradication of biofilms found can be attributed to the complete oil, or to fractioned or associated components of said oil; besides, evaluating cytotoxicity tests in tissue cells of the oral cavity.

5. Conclusion

The minimal biofilm eradication concentration for *S. mutans* biofilms –MBEC- from the essential oils assessed was estimated with 95% effectiveness levels, in 0.1 mg/100 mL, 0.01 mg/100 mL for *C. citratus* and in 0.001 mg/100 mL for *L. alba*, without cytotoxicity and "citral" chemotype for both species, which favors antimicrobial activity. Further studies are recommended to standardize the production of the oil by technifying crop management to generate higher yield per kilogram of plant during oil extraction, which permits increasing the dilution of the oils being studied to favor their mass use, granting economic advantages to the treatment over chemical products to control dental caries. The results reached from MBEC techniques and cytotoxicity, call for continued research with essential oils, their components, and for the evaluation of their possible toxicity in oral cavity cells.

Ethical considerations and conflict of interest

Ethical compliance does not apply for this study and the authors present no conflicts of interest to declare. The manuscript represents valid work and not is being considered for publication elsewhere.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.jep.2016.10.044.

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Glossary

ATCC: American Type Culture Collection
BHI: brain-heart infusion
C. citratus: *Cymbopogon citratus*
CFU: colony forming units
CHO: Chinese hamster ovary cells
CORPOICA: Corporación Colombiana de Investigación Agropecuaria
ELISA: Méthode immuno-enzymatique
GC-MSD: gas chromatography with mass selective detector
L. alba: *Lippia alba*
MASL: meters above sea level
MBEC-HTTP: MBEC-high-throughput
MTT: 3-(4,5-Dimethyltiazol-2-ilo)-2,5-diphenyltetrazol
SEM: standard error of the mean
WHO: World Health Organization