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ORIGINAL ARTICLE

Anti-Biofilm and Hemolytic Effects of Cymbopogon citratus (Dc) Stapf Essential Oil

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Abstract

Objective: To perform chemical analysis and to evaluate the anti-biofilm and hemolytic effect of the essential oil of *Cymbopogon citratus*. **Material and Methods:** Gaseous chromatography coupled to mass spectrometer was performed for chemical characterization of the essential oil. To verify the antimicrobial action, the Minimum Inhibitory Concentration (MIC), Minimum Bactericidal Concentration (MBC) and Minimum Fungicidal Concentration (MFC) were determined. From MIC, MBC and MFC data, concentrations were established to verify the anti-biofilm effect and for the hemolysis test on human erythrocytes. A multispecies biofilm was developed in vitro and mouthwash applications were simulated to determine the inhibition of biofilm formation or its removal. Results were analyzed through ANOVA statistical test, complemented by the Tukey test, considering a significance level of 5%. **Results:** The major component of the essential oil is citral. MIC verified for *Streptococcus mutans* was 1mg / mL, while for *Candida albicans*, it was 125 µg/mL, presenting microbicidal effect for both microorganisms tested. The essential oil was able to inhibit biofilm formation ($p<0.001$), presenting non-toxic hemolysis percentage in concentration below 500 µg/mL. **Conclusion:** The essential oil of *Cymbopogon citratus* is antimicrobial, antibiofilm and non-toxic to human erythrocytes, representing a natural product with potential for use in Dentistry.

Keywords: Plants, Medicinal; Biofilms; Cymbopogon; Drug Evaluation, Preclinical.

Introduction

Biofilm is a three-dimensional structure that forms on several solid surfaces considered as substrate [1]. It begins with the formation of an adsorbed film of exopolymers, constituting a mucilaginous matrix, which favors the adhesion of the first colonizing species, mostly aerobic microorganisms, increasing in thickness and complexity with maturation [1,2].

The more developed the biofilm, the greater its organization, as well as the number of microbial species and the interaction between them, being more difficult for the biofilm to be removed or disorganized [1,3]. Not all individuals are able to perform the biofilm control in the best way, or even some temporary or permanent conditions prevent proper oral hygiene by dental brushing, making it necessary the use of substances for the chemical control of biofilm [4,5].

One of the medicinal plants most cultivated and consumed in traditional medicine is *Cymbopogon citratus*, popularly known as lemon grass [6-9]. This plant has activities such as anti-microbial, anti-inflammatory and anti-proliferative of tumor cells, which make it a potentially beneficial natural product for use in the health area [10,11].

However, each country has specific legislation to regulate and authorize research and marketing of plant-based products, whether medicines, cosmetics or foods [12]. In Brazil, regulation is carried out by the National Sanitary Surveillance Agency, which authorized the use of oral antiseptics manufactured with infusion of *Lippia sidoides* and the decoction of *Siryphnoden drom adstrigens*, with *Cymbopogon citratus* being indicated by this pharmacopoeia as a natural anxiolytic in the treatment of insomnia [13]. The European Union, through the Herbal Medicinal Products Committee (HMPC) standardizes indications, and *Cymbopogon citratus* has its indication through the Natural Sources of Flavorings compendium [14]. This procedure approximates the two regulations in countries under the jurisdiction of these agencies, valuing the popular knowledge and the local culture that already uses such products, requiring some tests to prove their safety less complex than for the production of new drugs of different origin [15].

For the development of new products for human health, research protocols should be followed to ensure clinical efficacy and safety. The research sequence for new drugs or therapeutic techniques follows a long and time-consuming path, starting from laboratory studies [16].

The aim of this work was to chemically characterize and to evaluate the antimicrobial and antibiofilm effects of the essential oil of *Cymbopogon citratus*, as well as to verify its toxicity and determine an ideal concentration for the development of a mouthwash solution formulated with its active pharmaceutical ingredient.

Material and Methods

The essential oil of *Cymbopogon citratus* was commercially obtained (Quinari Casa das Essências, Ponta Grossa, PR, Brazil), chemically analyzed and prepared for the determination of the Minimal Inhibitory Concentration (MIC) and the anti-adherent effect of biofilm at initial concentration of 2mg / mL. The MIC found was used as a parameter for the antibiofilm evaluation and hemolysis test.

Chemical Analysis

Chemical characterization was performed by chromatography using a gas chromatograph coupled to mass spectrometer (GCMS-QP2010 SE, Shimadzu Corporation, Kyoto, Japan) and capillary column (J & W Scientific Inc., Folsom, California, USA) with stationary phase composed of 5% phenyl and 95% dimethylpolysiloxane, measuring 30 m in length, 0.25 mm in internal diameter and 0.25 µm film thickness [17].

Minimum Inhibitory Concentration (MIC) Determination

MIC determination was performed by serial microdilution technique, by placing 100 µL of culture medium in the wells of plates, on which 100 µL of EO (essential oil) were added and serially transferred from well to well to achieve the desired dilutions [18]. The test was completed by adding 100 µL of previously adjusted inoculum.

Bacterial MIC was determined using BHI broth culture medium (Becton Dickinson GmbH, Heidelberg, Germany) for the growth of *Streptococcus mutans* UA159 and for fungal MIC using Sabouraud broth (Becton Dickinson GmbH, Heidelberg, Germany) for the development of *Candida albicans* ATCC 90029 strains. Assays were run in triplicate and plates were incubated at 37°C for 24 hours.

Controls of the strain viability were performed using medium only with inoculum, and positive using medium with inoculum and 0.12% chlorhexidine for bacteria and medium with inoculum and nystatin solution, for fungi. After that period, 50 µL of 1% TTC solution (2,3,5-triphenyl tetrazolium chloride) were placed in the wells of plates, which were again incubated for 24 hours at 37°C.

To determine the Minimum Bactericidal Concentration, 50 µL were collected from wells corresponding to MIC, MICx2 and MICx4 and dripped onto petri dishes containing BHI agar medium (Becton Dickinson GmbH, Heidelberg, Germany), incubated for 24 hours at 37°C in microaerophilia. For determination of the Minimum Fungicidal Concentration, 50 µL were collected from wells corresponding to MIC, MICx2 and MICx4 and dripped onto petri dishes containing Sabouraud Dextrose agar medium (Becton Dickinson GmbH, Heidelberg, Germany), incubated in aerobiosis for 24 hours at 37°C.

Anti-Biofilm Effect

Streptococcus mutans UA159 and *Candida albans* ATCC 90029 standard strains were used to determine the antibiofilm effect, which were inoculated using BHI broth (Becton Dickinson GmbH, Heidelberg, Germany) in an equal portion of Sabouraud broth (Becton Dickinson GmbH, Heidelberg, Germany) and enriched with 5% sucrose. Plates were prepared according to the type of biofilm to be evaluated, with recent growth (initial biofilm) and late growth (mature biofilm).

To simulate oral mouthwash applications, the concentrations defined by MIC, MICx2 and MICx4 and MICx8 were added to the wells of microdilution plates, remaining for 1 minute, followed

by washing and new addition of the culture medium and incubation. In the next step, plates were prepared for spectrophotometric reading (GloMax, Multi Detection System, Wisconsin, USA).

In order to simulate mouthwash applications and to evaluate if the best action of the oil occurs in the inhibition of formation, biofilm removal or in both situations, the pre-defined times were adopted:

G1 - Plates with Initial Biofilm Formation

The strains were inoculated and incubated for 2 hours in microaerophilia at 37ºC, after the wells of plates were washed with 3 baths of 150 µL of neutral pH phosphate buffered saline (PBS). After 50 µl of each culture medium were added to wells and then 100 µl of EO and test concentrations and chlorhexidine were added, waiting 1 minute (simulating the mouthwash application). After one minute, the wells were again washed with 3 times with PBS and filled with equal parts of culture medium (50 µL of BHI broth and 50 µL of Sabouraud broth), proceeding with a new incubation of 12 hours. Process was repeated until 48 hours have elapsed.

G2 - Plates with Formation of Mature Biofilm

The strains inoculated and incubated for 48 hours in microearophilia at 37ºC. The contents liquid present in the wells of plates were removed and received 3 baths with 150 µL of PBS, after placed 50 µl of each culture medium were added to wells and then 100 µl of EO and test concentrations chlorhexidine were added, waiting 1 minute (simulating the mouthwash application). After one minute, again the wells washed 3 times with PBS and filled with equal parts of culture medium (50 µL of BHI broth and 50 µL of Sabouraud broth), proceeding with a new incubation of 12 hours. Process repeated until 48 hours have elapsed.

Control of the strain viability (medium with inoculum only) and positive control (medium with inoculum and 0.12% chlorhexidine) were performed.

The preparation of plates for spectrophotometric reading respected the following protocol: after 48 hours of baths and incubations, plates were washed 2 times with 200 µl PBS and incubated again at 37°C for 45 minutes. Once dried, they received 110 µL of crystal violet solution (4%), waiting 45 minutes to stain the biofilm formed in the wells of plates. After the staining period, plates were washed 4 times with 200 µL distilled water and received 200 µL of 95% alcohol, waiting another 45 minutes. At the end of 45 minutes, 100 µl of contents present in the wells of plates were transferred to a new flat bottom plate and taken for reading on a plate reader (GloMax®-Multi Detection System, Wisconsin, USA) at wavelength 525 Nm.

The biofilm inhibition / removal percentage can be calculated by the formula:

$$\% = \frac{\text{Absorbance found - negative control absorbance}}{\text{positive control absorbance - negative control absorbance}} \times 100$$

After percentages were defined, groups were classified according to the degree of biofilm inhibition / removal [19]: 0 =≤ 25% (non-inhibition); 1 = >25% to ≥ 50% (weak inhibition); 2 = >50% to ≥75% (moderate inhibition); 3 = >75% to ≥100% (strong inhibition).

Results were analyzed through descriptive and inferential statistics, in which the hypothesis of inhibition of biofilm formation or removal was tested using ANOVA statistical tests, complemented by the Tukey test, considering a significance level of 5% ($p<0.05$).

Hemolytic Activity

Human erythrocytes were used to determine cell selectivity of *Cymbopogon citratus* oil, with approval from the Ethics Research Committee of the State University of Paraíba, Brazil (Protocol No. 53237916.2.0000.5187).

The cells were obtained from five healthy graduate dentistry students ranging in age from 25 to 30 years, who had not used antibiotic and anti-inflammatory agents in the last 30 days. Blood was collected by the same operator with 10 mL disposable syringes, measuring 25x7 in the morning, without previous fasting. The collected blood was centrifuged at 1500 rpm for 15 minutes. Erythrocytes were isolated and washed in phosphate buffered saline (PBS) for three times and suspended at 2% in PBS.

Erythrocytes suspensions were transferred to "U"-bottom microdilution plates and incubated 1:1 with *Cymbopgon citratus* essential oil at concentrations previously defined in the Minimum Inhibitory Concentration (MIC, MICx2, MICx4 and MICx8) tests. The samples were diluted in distilled water (DW) or in PBS under product's natural pH (pH 4) or neutralized pH with NaOH. Distilled water and saline were used as positive and negative controls, respectively.

After one hour of incubation, 70 µL of the supernatant were transferred to flat bottoms of microdilution plates and absorbance was measured (GloMax®-Multi Detection System, Wisconsin, USA). Hemolysis percentage was calculated by the formula [20].

$$\%H = \frac{\text{sample absorbance - negative control absorbance}}{\text{positive control absorbance - negative control absorbance}} \times 100$$

Hemolysis values up from 10% hemolysis was considered non-toxic to erythrocyte membrane, 10 to 49% are slightly toxic, 50 to 89% toxic, and 90 to 100% are highly toxic.

Results

Gas chromatography showed the presence of important phytochemicals in the essential oil of *Cymbopogon citratus*, all of the terpenes class, the majority being citral (Table 1).

Table 1. Phytochemicals Identified By Cg/Ms.

RT (min.)	Area %	Compound
6.357	0.19	
7.398	0.59	Hept-5-en-2-one<6-methyl->
7.534	0.53	Myrcene
14.316	0.49	Isogeranial
15.115	0.84	Isogeranial
17.784	43.48	2,6-octadienal,3,7-dimethyl-, (Z)-
18.319	0.62	Geraniol
19.121	53.27	Geranial

The data obtained in the antimicrobial evaluation allow defining the essential oil as antibacterial and antifungal, with bactericidal and fungicidal effect at concentrations determined as bacterial MIC of 1 mg / mL and fungal MIC of 125 µg / mL.

In the anti-biofilm evaluation, EO had inhibitory effect on biofilm formation at the concentrations evaluated ($p<0.05$) (Table 2).

Table 2. Determination of the anti-biofilm effect for G1.

Group Test	Absorbance (SD)	p-value
G1 CHX (µg/mL)		<0.0001
50	0.099 (±0.010)[a]	
25	0.161 (±0.038)[a]	
12.5	0.117 (±0.022)[a]	
6.25	0.127 (±0.022)[a]	
G1 EO (µg/mL)		<0.0001
1000	0.061 (±0.044)[a]	
500	0.108 (±0.021)[a]	
250	0.120 (±0.021)[a]	
125	0.177 (±0.050)[a]	
Negative Control	0.733 (±0.259)[b]	
Positive Control	0.240 (±0.174)[a]	0.0910

SD = Standard deviation; *Different letters indicate statistically significant differences.

However, there was no mature biofilm removing effect for the essential oil group, as observed in Table 3.

Table 3. Determination of the anti-biofilm effect for G2.

Group Test	Absorbance (SD)	p-value
G2 CHX (µg/mL)		<0.0001
50	0.279 (±0.110)[b]	
25	0.369 (±0.178)[b]	
12.5	0.874 (±0.089)[b]	
6.25	0.584 (±0.139)[b]	
G2 EO (µg/mL)		0.5559
1000	0.993 (±0.022)[a]	
500	0.994 (±0.020)[a]	
250	1.007 (±0.010)[a]	
125	0.977 (±0.043)[a]	
Negative Control	0.976 (±0.020)[a]	
Positive Control	0.240 (±0.174)[b]	<0.0001

SD = Standard deviation; *Different letters indicate statistically significant differences.

It is possible to define as "strong inhibition" the potential of inhibition of biofilm formation for EO, being as effective as chlorhexidine (Table 4).

Table 5 shows the mean absorbance values and hemolysis percentages for the different EO manipulations. At low concentrations, both EO + S (saline) samples promoted hemolysis lower the negative control and both EO + DW (distilled water) sample revealed no-toxicity against erythrocyte membrane.

Table 4. Results of biofilm inhibition percentage for G2.

Substance Test	%
EO (µg/mL)	
1000	89.9
500	87.8
250	86.6
125	80.3
CHX (µg/mL)	
50	89.2
25	79.6
12.5	88.4
6.25	84.6

Table 5 shows the mean absorbance values and hemolysis percentages for the different EO manipulations. At low concentrations, both EO + S (saline) samples promoted hemolysis lower the negative control and both EO + DW (distilled water) sample revealed no-toxicity against erythrocyte membrane.

Table 5. Absorbance values and hemolysis percentages.

Concentration (µg/mL)	Absorbance EO pH 4 + S	%	Absorbance EO + S	%	Absorbance EO pH 4 + DW	%	Absorbance EO + DW	%
4000	0.194±0.033	87.3	0.184±0.068	81.6	0.178±0.037	78.3	0.211±0.037	97.8
2000	0.121±0.029	44.1	0.151±0.046	62.2	0.116±0.020	41.5	0.172±0.056	74.7
1000	0.049±0.001	2.01	0.080±0.025	19.8	0.061±0.012	8.9	0.081±0.017	20.8
500	0.048±0.006	1.06	0.045±0.003	-0.8	0.055±0.013	5.5	0.052±0.012	3.4
250	0.067±0.038	12.4	0.039±0.002	-3.9	0.067±0.023	12.5	0.056±0.015	5.6
125	0.040±0.001	-3.7	0.036±0.004	-53.6	0.067±0.021	12.6	0.061±0.025	9.1

Discussion

The major component of the essential oil of *Cymbopogon citratus* is citral; an aldehyde formed by the geranial monoterpene in its cis and trans configurations. Other observed phytocompounds are myrcene and sulcatone, aromatic compounds that may contribute to the strong odor present in the EO. The findings corroborate literature regarding the chemical composition of the product, and quantities are variable regardless of phytocompound [21,22]. These variations may be related to differences in the period and site of collection of plants for the essential oil production [13].

The essential oil of *Cymbopogon citratus* demonstrated microbicidal effect on *S. mutans* and *C. albicans*, and no Colony Forming Unit was observed in Petri dishes used for MFC and MBC determination. The results obtained for MICs with EO can be considered very good [23], since they are at concentrations below 1mg / mL. Some authors consider substance with MIC less than 500 µg / mL as potent [24]. However, the effect on yeasts observed in this study can be considered moderate [25], since MIC was 125 µg / mL, being between 100 and 500 µg / mL, whereas the antibacterial effect was weak, with MIC of 1mg / mL. A previous study using dye formulation with *C. citratus* found no antibacterial effect [26].

Several authors corroborate the results described here for the antibacterial effect of the essential oil of *Cymbopogon citrates* [6], and it has been demonstrated that this substance may act as an enhancer of the antimicrobial effect of some antibiotics [27]. Interaction between *Streptococcus mutans* and *Candida albicans* has been reported in literature as increasing the adherence of the species to biofilm [28,29]. For this reason, the multispecies biofilm developed in the present study used these microorganisms as microbial association.

The present study observed antimicrobial effect of the essential oil of *Cymbopogon citratus*; however, the fungicide action occurred in a much lower concentration when compared to the bactericidal concentration. This may be due to the mechanism of action of the phytotherapeutic product, not fully elucidated, which provides toxicity to yeast cells more intensely than to bacterial cells studied here. Possibly, this phytotherapic has good microbicidal effects by the action of terpenes (citral in greater amount) present in it [27].

It is known that the use of mouthwash will occur mostly on epithelial cells of the oral mucosa. However, it is important to know the possibility of substance penetration into tissues and the toxic effects of the product. Since erythrocytes are cells of simple composition, in which it is possible to measure the action of drugs on the cell membrane [20], we opted for the initial investigation of the toxic potential of this phytomedicine on human erythrocytes.

Monoterpenes are capable of changing the fluidity of the cell membrane of erythrocytes and fibroblasts and, consequently, favor the penetration of substances inside the cells. However, depending on concentration and interactions with membrane receptors, it is not capable of causing cell damage [30-33]. This may justify the protective effect observed in the hemolysis test with the manipulation of the essential oil in saline solution and neutralized pH at concentrations below 500 µg / mL. The results suggest a change in the organization of the erythrocyte membrane, preventing the loss or gain of content to the medium, favoring the maintenance of the intact cell.

Conclusion

The essential oil of *Cymbopogon citratus* demonstrated antimicrobial effect on *Streptococcus mutans* and *Candida albicans*, and was able to inhibit the *in vitro* formation of multispecies biofilms, presenting low toxicity on the cell membrane of erythrocytes at concentrations lower than 500µg / mL.

Authors' Contributions: NBS designed the study, performed the experiment and wrote the manuscript. MLR and JML performed the experiment and wrote the manuscript. AMGV and ALC designed the study and reviewed the manuscript. RDC and LRCC performed the experiment and reviewed the manuscript.

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