European Journal of Medical and Health Sciences
Vol 5 | Issue 5 | October 2023
ISSN 2593-8339

RESEARCH ARTICLE

Evaluation of the Antibacterial and Anti-Adherent Activities of *Eucalyptus globulus* and *Eucalyptus citriodora* Essential Oils against *Pseudomonas aeruginosa* Strains

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ABSTRACT

Biofilm can be defined as a complex sessile microbiological ecosystem formed by one or more species of bacteria, fungi or protozoa. Among the microorganisms capable of forming biofilms is *Pseudomonas aeruginosa*, a gram-negative bacterium with extensive virulence factors and high resistance to antimicrobials, making it difficult to treat infections caused by it. In this sense, phytotherapy and essential oils are promising therapeutic alternatives to multi-resistant microorganisms. The aim of this study was to evaluate the antibacterial and anti-adherent activities of *Eucalyptus globulus* and *Eucalyptus citriodora* essential oils against clinical strains of *P. aeruginosa*, and to compare their antibiofilm effects with 0.12% chlorhexidine digluconate. To determine the Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of the oils, the broth microdilution technique was used in 96-well plates. To determine the Minimum Adherence Inhibitory Concentration (MAIC), the inclined glass tube technique was used in the presence of 5% sucrose, using proportions corresponding to the pure essential oil up to a dilution of 1:1024, and its positive control was 0.12% chlorhexidine digluconate. It was therefore concluded that the essential oils under study had an antibacterial effect against the strains tested, varying between bactericidal and bacteriostatic action, as well as demonstrating an effective anti-adherence effect that was superior to the positive control.

Keywords: Dentistry, Microbiology, Phytotherapy.

Submitted: August 28, 2023

Published: October 30, 2023

10.24018/ejmed.2023.5.5.1922

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1. Introduction

Biofilm can be defined as a complex sessile microbiological ecosystem formed by one or more species of bacteria, fungi or protozoa, isolated or in combination [1], [2]. It is estimated that more than 90% of microorganisms live in this form and can colonise almost any environment with favourable conditions [3]. Bacteria can live as free-living (or planktonic) cells or as sessile cells, also called biofilm, which indicates their chronic content of formation [4], [5].

This structure is embedded in a matrix of extracellular polymeric substances, responsible for the morphology, structure, cohesion and functional integrity of the biofilm, which adheres to a biotic or abiotic surface. It is well known that water is present in a large proportion of biofilm composition, filling up to around 97% of the matrix, while microorganisms make up around 3% of this organisation [1], [6], [7].

Biofilm can be combated by inhibiting its formation or by treating the biofilm that has already formed. The former occurs by blocking bacterial adhesion to a surface or by disrupting communication between these bacteria (Quorum sensing) through the prophylactic use of antibiotics and biocides. Treatment takes place through the mechanical removal of this biofilm, the use of antimicrobials and the replacement of biomedical devices [8]–[11].

Among the microorganisms capable of forming biofilm is *Pseudomonas aeruginosa*, a gram-negative bacillus-shaped bacterium with extensive virulence factors and intrinsically resistant to various antibacterials. This species is found mainly in implantable biomedical devices and is one of the main causes of nosocomial infections. Studies have also detected the significant presence of *P. aeruginosa* in the subgingival biofilm of patients with periodontitis and in endodontic canals [6], [12]–[16].

Pseudomonas aeruginosa has several mechanisms of resistance to antibacterial drugs, both natural and acquired, including the production of β-lactamase enzymes, which inactivate betalactam drugs such as penicillins, cephalosporins and carbapenems [17]–[20]. Infections caused by this bacterium are among the most feared, due to the difficulty of treatment resulting from the high resistance of this species, thus presenting a challenge for synthetic therapeutic options [21].

Thus, phytotherapy has become a promising therapeutic alternative for the treatment of multidrug-resistant microorganisms, given its many advantages such as lower cost to the population and health services, wide availability of raw materials, good popular acceptance, fewer side effects and better patient tolerance [22], [23]. Essential oils, also known as volatile oils or essences, originate from the secondary metabolism of plants and are complex mixtures of volatile, lipophilic, generally odourous and liquid substances [24], [25].

In addition, eucalyptus can be found in all regions of the Brazilian flora, with around 400 species known, which can be utilised from their stems for pulp extraction to their leaves, where their essential oils are extracted [26], [27]. These oils can be divided into three substantial groups, which differ according to their purpose: medicinal, industrial or perfumery [28].

The *Eucalyptus globulus* species is the main producer of oils for medicinal purposes, and its main component is the monoterpene cineol, which is widely used in pharmaceutical products as inhalants, flavourings and aromatisers for medicines and oral hygiene products. *Eucalyptus citriodora*, on the other hand, is the most exploited in the perfumery industry, with the predominant constituent being the monoterpene citronellal, used to flavour cleaning products [28]–[30].

Therefore, in view of the knowledge about the properties of essential oils against various microorganisms, this study aims to evaluate the potential anti-adherent activity of the essential oils of *Eucalyptus globulus* and *Eucalyptus citriodora* against the strain of *Pseudomonas aeruginosa*.

2. Objectives

2.1. General

Avaliar as atividades antimicrobiana e antiaderente dos óleos essenciais de *Eucalyptus globulus* e *Eucalyptus citriodora* contra a cepa de *Pseudomonas aeruginosa*.

2.2. Specific

- To analyse the Minimum Inhibitory Concentration (MIC) of the essential oils of *Eucalyptus globulus* and *Eucalyptus citriodora* against strains of *Pseudomonas aeruginosa*;
- To determine the Minimum Bactericidal Concentration (MBC) of the essential oils of *Eucalyptus globulus* and *Eucalyptus citriodora* against strains of *Pseudomonas aeruginosa*;
- To identify the Minimum Inhibitory Concentration (MIC) of the essential oils of *Eucalyptus globulus* and *Eucalyptus citriodora* against strains of *Pseudomonas aeruginosa*;
- Comparing the anti-adhesion effect of *Eucalyptus globulus* and *Eucalyptus citriodora* essential oils with 0.12% chlorhexidine.

3. Methodology

3.1. Place of Study

The laboratory tests were carried out in the Microbiology and Biochemistry laboratories of the Federal University of Campina Grande, Patos campus (CSTR), Paraíba state-Brazil.

3.2. Test Substances and Microorganisms

The essential oils of *Eucalyptus globulus* and *Eucalyptus citriodora* were purchased from Indústria Quinari® (Ponta Grossa-PR). To carry out the pharmacological tests, when necessary, the substances were solubilised in dimethylsulphoxide (DMSO) and diluted in distilled water. The concentration of DMSO used was less than 0.1% v/v. The project follows the rules of CGen—Conselho de Gestão do Patrimônio Genético (Genetic Heritage Management Council) and is registered on the SISGEN platform under protocol number A25230B. Three clinical strains of *Pseudomonas aeruginosa* (PA 101, PA 104 and PA 109) were used, which were maintained in Muller Hinton Agar (MHA) at a temperature of 4 °C, and 24-hour replicates in MHA incubated at 35 ± 2 °C were used for the tests. The inocula were obtained from overnight cultures in Muller Hinton liquid (MH) at 35 ± 2 °C and diluted in sterile saline solution to obtain a final concentration of approximately 1.5×10^8 CFU/mL, adjusted for turbidity by comparing with the 0.5 tube of the McFarland scale [31].

3.3. Culture Media

The culture media used in the tests to assess antimicrobial activity were MH medium and solid Muller Hinton Agar (AMH) medium. The culture medium was purchased from Difco® and prepared according to the manufacturer's instructions.

3.4. Determination of the Minimum Inhibitory Concentration (MIC)

A The MIC was determined using the microdilution technique in a 96-well plate with a U-shaped bottom. To each plate was added 100 µL of Mueller Hinton broth, doubly concentrated, and 100 µL of the essential oils of *Eucalyptus globulus* and *Eucalyptus citriodora* previously solubilised in DMSO and diluted in distilled water, respectively, at concentrations of 1024 to 16 µg/mL. The MIC was determined with 10 µL of the microorganisms in each

cavity, approximately 1.5×10^8 CFU/mL. The penultimate well containing 200 µL of the broth was inoculated with the microorganism suspension as the growth control, and the last well received only 200 µL of the broth as the negative control. The test was carried out in duplicate. The plates were incubated at 35 ± 2 °C for 24 hours [31]–[34].

3.5. Determination of the Minimum Bactericidal Concentration (MBC)

After reading the results, inoculations (10 µL) of three dilutions from the MIC were made into Mueller-Hinton broth medium (100 µL/cavity) in a sterilised microdilution plate for CBM determination. After incubation at 35 ± 2 °C for 24 hours, 20 µL of resarzurin was added. The assays were incubated at 35 ± 2 °C for a further 24 hours to confirm the concentration capable of inhibiting the total growth of the bacterial species, as verified by a non-change in the colour of the indicator dye [35], [36].

3.6. Determination of the Minimum Adhesion Inhibitory Concentration (MIC)

The Minimum Adherence Inhibitory Concentration (MIC) of the essential oils of *Eucalyptus globulus* and *Eucalyptus citriodora* was determined in the presence of 5% sucrose, according to Albuquerque *et al*. [37] with modifications, using proportions corresponding to the pure compound up to a dilution of 1:1024. After bacterial growth, the *Pseudomonas aeruginosa* strains were grown at 35 ± 2 °C in Mueller Hinton broth (DIFCO, Michigan, United States), then 0.9 mL of the subculture was poured into test tubes and 0.1 mL of the solution corresponding to the essential oil dilutions was added. Incubation was carried out at 35 ± 2 °C for 24 hours with the tubes tilted at 30°.

The reading was taken by visually observing the bacteria adhering to the walls of the tube after shaking it with an added fuchsin solution. The test was carried out in duplicate. The same procedure was carried out for the positive control, 0.12% chlorhexidine digluconate (Periogard®, Colgate-Palmolive Company, New York, USA). CIMA was considered to be the lowest concentration of the agent in contact with sucrose that prevented adherence to the glass tube.

4. Results

The results of the MIC study indicated that the lowest concentration of *E. citriodora* essential oil capable of inhibiting bacterial growth was 500 µg/mL for strain PA101, 62.5 µg/mL for strain PA104 and 1000 µg/mL for strain PA109. As for *E. globulus* oil, the MIC was 1000 µg/mL, 62.5 µg/mL and >1000 µg/mL respectively, as shown in Tables I and II.

With regard to the CBM study, the results shown in Tables III and IV reveal values for strain PA101 of >1000 µg/mL, for PA104 of 250 µg/mL, and for PA109 of 1000 µg/mL, referring to the essential oil of *E. citriodora*. At the same time, *E. globulus* oil showed CBM > 1000 µg/mL for PA101 and 1000 µg/mL for PA104. In addition, the action of a given oil in relation to the strains can be observed, so both essential oils showed a bacteriostatic effect against strains PA101 and PA104. In addition, *E. citriodora* oil showed a bactericidal effect against strain PA109, but no CBM was carried out for this strain with *E. globulus* oil, since its MIC was >1000 µg/mL.

TABLE I: Minimum Inhibitory Concentration (MIC in µg/mL) of E. citriodora Essential Oil against P. aeruginosa Strains

E. citriodora	
Strains	Concentration in µg/mL
PA101	500
PA104	62.5
PA109	1000

Source: Author (2023).

TABLE II: Minimum Inhibitory Concentration (MIC in µg/mL) of E. globulus Essential Oil against P. aeruginosa Strains

E. globulus	
Strains	Concentration in µg/mL
PA101	1000
PA104	62.5
PA109	>1000

Source: Author (2023).

TABLE III: Minimum Bactericidal Concentration (MBC in µg/mL) of E. citriodora Essential Oil against P. aeruginosa Strains

E. citriodora		
Strains	Concentration in µg/mL	Effect
PA101	> 1000	Bacteriostatic
PA104	250	Bacteriostatic
PA109	1000	Bactericidal

Source: Author (2023).

TABLE IV: Minimum Bactericidal Concentration (MBC in µg/mL) of E. globulus Essential Oil against P. aeruginosa Strains

E. globulus		
Strains	Concentration in µg/mL	Effect
PA101	>1000	Bacteriostatic
PA104	1000	Bacteriostatic
PA109	–	–

Source: Author (2023).

Finally, the CIMA experiments indicated that the lowest concentration of *Eucalyptus citriodora* essential oil capable of inhibiting the adhesion of *Pseudomonas aeruginosa* (PA104) to the tube wall was 1:8. The lowest effective concentration of Eucalyptus globulus essential oil was 1:128. Thus, both showed good efficiency against biofilm formation by this bacterium, with results superior to the control with 0.12% chlorhexidine digluconate, which inhibited biofilm formation up to a concentration of 1:4, as described in Tables V and VI respectively.

5. Discussion

According to Sartoratto *et al*. [38], the antimicrobial activity of essential oils is classified according to the MIC value, which can be strong when they have a MIC of up to 500 µg/mL, moderate for values between 600 and 1500 µg/mL and weak for above 1500 µg/mL. Thus, the results

TABLE V: Minimum Adherence Inhibitory Concentration (MIC) of E. citriodora Essential Oil against P. aeruginosa Strains

Eucalyptus citriodora								
Concentration	1:1	1:2	1:4	1:8	1:16	1:32	1:64	1:128
	–	–	–	–	+	+	+	+
Chlorhexidine digluconate 0.12%								
Concentration	1:1	1:2	1:4	1:8	1:16	1:32	1:64	1:128
	–	–	–	+	+	+	+	+

Legend: (–) No adhesion to the tube wall (+) With adhesion to the tube wall.
Source: Author (2023).

TABLE VI: Minimum Adherence Inhibitory Concentration (MIC) of E. globulus Essential Oil against P. aeruginosa Strains

Eucalyptus globulus								
Concentration	1:1	1:2	1:4	1:8	1:16	1:32	1:64	1:128
	–	–	–	–	–	–	–	–
Chlorhexidine digluconate 0.12%								
Concentration	1:1	1:2	1:4	1:8	1:16	1:32	1:64	1:128
	–	–	–	+	+	+	+	+

Legend: (–) No adhesion to the tube wall (+) With adhesion to the tube wall.
Source: Author (2023).

identified in this research validate that the antimicrobial activity of *E. citriodora* essential oil exhibits strong inhibition against *P. aeruginosa* strains PA101 and PA104, and moderate action for strain PA109. *Eucalyptus globulus* oil has a strong effect against strain PA104 and a moderate effect against strain PA101. As for strain PA109, it was not possible to determine its MIC using the methodology applied.

In fact, the results obtained from the essential oil of *E. citriodora* in this study are validated by the work of Bezerra *et al*. [39], who demonstrated the strong antibacterial and bacteriostatic effects against *P. aeruginosa* through the action of the monoterpene citronellal, which is the main compound in the essential oil, with the MIC corresponding to 512 μg/mL. In addition, similar results were obtained for the MIC of the same oil, being effective at the lower ratio of 1:8, and superior to the positive control.

In experiments carried out by Monteiro *et al*. [40] with *E. globulus* essential oil to verify its antibacterial potential against E. *coli*, *E. faecalis* and *S. aureus*, it was observed that the oil at its maximum concentration showed greater inhibition than the positive control with 0.12% chlorhexidine digluconate against the first two species, and similar to the control when used against *S. aureus*. In addition, at a concentration of 50%, it was as effective as the control against *E. faecalis*. These results confirm its antimicrobial action against the main bacteria in the oral cavity.

Studies in the literature have already demonstrated the antibacterial activity of *E. globulus* essential oil, for example, Merghni *et al*. [41] evaluated the antimicrobial effect of this oil and its main component, 1,8-cineole, against methicillin-resistant *S. aureus* strains, and then showed the effectiveness of both in inhibiting the development of biofilm by the bacteria analysed, as well as the high anti-quorum sensing activity of the oil, even at low concentrations. Thus, the aforementioned results found in the research carried out are supported by scientific findings documented in the literature.

According to Hafidh *et al*. [42], for a substance to be considered bactericidal, its MBC must be equal to or twice the MIC, while for it to be considered bacteriostatic, the MBC must be greater than twice the MIC. Therefore, the results found in this study indicate that the essential oil of *E. citriodora* has bactericidal potential against strain PA109 and bacteriostatic potential against strains PA101 and PA104. With regard to the essential oil of *E. globulus*, it was observed that it has a bacteriostatic action on the PA101 and PA104 strains.

In their study, Insuan and Chahomchuen [43] analysed the action of *E. citriodora* essential oil against the bacterial strains *Bacillus subtilis*, *Staphylococcus aureus*, *Staphylococcus intermedius*, *Escherichia coli* and *Pseudomonas aeruginosa*, which are of a different origin to the strains in this study. Using disc diffusion and microdilution techniques, they obtained positive results for antibacterial activity against all the species tested. The oil's effect against *P. aeruginosa* is notable for its bactericidal action, with its effect on the cell wall and membrane, since serious morphological changes were observed after a certain period of incubation with the oil.

Furthermore, a study by Resende *et al*. [44] evaluated the antimicrobial and antibiofilm potential of *E. globulus* essential oil against five bacterial strains, including *P. aeruginosa*, which is a different strain from the strains in this study. It was possible to verify that the oil had a bacteriostatic effect against this species, and also, using the technique in microplates with flat-bottomed wells, inhibited initial cell adherence, turning the classification from "very adherent" to "moderate adherence", with an inhibition rate of 75.85%. Also in this study, the effect of the oil associated with commercial antimicrobials was analysed using the disc diffusion technique, and it was observed that the oil had a synergistic effect when associated with ciprofloxacin and gentamicin, compared to the isolated effect of each antimicrobial.

Furthermore, with regard to the anti-adherence effect of *E. globulus* oil, this study corroborates the positive results obtained in studies by Matos [45] and Ramalho *et al*. [46], in which the minimum inhibitory concentration of adherence (MIC) of this oil against the *Staphylococcus aureus* and *Klebsiella pneumoniae* strains, respectively, was effective at a ratio of 1:8, similar to the success of 0.12% chlorhexidine digluconate in both experiments. On the other hand, in the same analyses, the essential oil of *E. citriodora* showed no anti-adherent activity against the bacteria tested at any concentration.

6. Conclusion

In short, using the methodology used, it can be concluded that the essential oils of *Eucalyptus globulus* and *Eucalyptus citriodora* showed an antibacterial effect against the strains of *Pseudomonas aeruginosa* tested, varying between bactericidal and bacteriostatic effects, as well as having an anti-adherent effect against these bacteria,

being superior to the positive control with 0.12% chlorhexidine digluconate, with *E. globulus* oil standing out as the most effective.

Conflict of Interest

The authors declare no conflict of interest.

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