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Original Research

Modulatory-antibiotic activity of the essential oil from *Eucalyptus citriodora* against MDR bacterial strains

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Abstract: The growing number of bacterial strains resistant to therapeutic agents has been surpassing the various antibiotics developed by the chemical and pharmaceutical industries. This problem has driven the development of research using agents with antimicrobial potential, with an emphasis on plant-derived natural products. This study evaluated the chemical compounds present in *Eucalyptus citriodora* essential oil (EOEc) cultivated in northeastern Brazil and its properties as an antibacterial agent and resistance modifier against methicillin-resistant *Staphylococcus aureus* (MRSA) and β-lactamase-producing strains. The EOEc was obtained using the hydrodistillation method, later analyzed by GC/MS, presenting a total of twelve compounds, with citronellal (65.45%); citronellol (14.87%); isopulegol (11.80%) and citronellyl acetate (2.51%) as its main constituents. The microdilution test was used to determine the minimum inhibitory concentration (MIC) and the bacterial resistance modulation of the essential oil. The EOEc did not present significant activity against the tested strains (MIC > 1000 µg mL^{-1}). However, when evaluating the capacity of the EOEc to modify the resistance of *S. aureus* and *E. coli* strains to different antimicrobials, synergistic effects were obtained with reduced MIC values for all tested antibiotics being obtained. The EOEc showed antimicrobial and β-lactam optimizing potential against resistant strains, presenting itself as a possible alternative for the use of these drugs at concentrations lower than those indicated against resistant strains.

Key words: Antimicrobial activity; MRSA; Medicinal plants; Modulation; Bacterial resistance.

Introduction

Although the chemical and pharmaceutical industries have produced a huge variety of different antibiotics in recent years, the emergence of bacterial strains resistant to most available therapeutic agents has increasingly been observed (1). This scenario has driven a constant search for alternative antimicrobial agents, among which, plant-derived natural products can be highlighted (2). Given this problem, the importance of research involving natural products with antimicrobial activity stands out. In addition, plant-derived natural products may also alter the effect of antibiotics, by increasing or reducing antibiotic activity (3).

The antimicrobial properties of essential oils have been demonstrated against a range of microorganisms including bacteria, protozoa and fungi (4). *Eucalyptus* oils are among the various essential oils reported in the literature with antibacterial function, this being due to good inhibitory results against pathogenic strains (5-7). The characteristics from this genus make it an excellent candidate for bioprospecting for the development of new antimicrobials.

Eucalyptus (Myrtaceae) species are among the most traded species in the world, with the *E. citriodora* oil being one of the most important ones in terms of commercial volume (8). The chemical composition of the *E. citriodora* essential oil has been evaluated in previous studies, according to which a predominance of the oxygenated monoterpenes citronellal, citronellol and isopulegol was observed (9-11). A broad spectrum of biological activities such as antimicrobial activity, insecticide,

herbicide, acaricide, as well as anti-inflammatory and analgesic effects have been reported (12-14).

Thus, the present study aims to identify the chemical compounds present in the *E. citriodora* essential oil cultivated in northeast Brazil and to evaluate the properties of this oil as an antibacterial agent and resistance modifier in multiresistant human pathogens.

Materials and Methods

Plant materials and oil analysis

The extraction of the essential oils was carried out in the Organic Chemistry Laboratory in the Professora Cinobelina Elvas Campus at the Federal University of Piaui. Fresh leaves (400 g) of *E. citriodora* were colleted from 12:30 h (Brasilia time), in county of Bom Jesus-PI, were subjected to hydrodistillation using equipment Tecnal, model TE-2761 in distilled water (500 mL). A voucher specimen was deposited in herbarium Graziela Barroso – UFPI/Teresina-PI, under registered number 28.835. The samples were steam distilled for three hours. The oils obtained were analyzed by GC/MS using a Shimadzu, QP5050A instrument under the following conditions: column dimethylpolysiloxane DB-1 fused silica capillary column (30 m × 0.25 mm × 0.25 μm); carried gas: helium (1 mL.min^{-1}); injector temperature: 250 °C; interface temperature: 230 °C; split ratio 27; column temperature: 35 °C – 180 °C at 4 °C.min^{-1}; then 180 °C – 250 °C at 10 °C.min^{-1}; mass spectra: electron impact 70 eV. Individual components were identified by spectrometric analysis using computer library MS searches (Wiley) and Kovats indices as a prelection aid. Visual spectra comparison data from the literature were used for confirmation (15).

Microbial strains

The strains used in this study were provided by the Microbiology Laboratory of the Federal University of Piauí. Multiresistant *Staphylococcus aureus* 23 (resistant to penicillin, ampicillin, oxacillin, tetracycline and vancomycin), *Staphylococcus aureus* 55 (MRSA-methicillin resistant), *Escherichia coli* ATCC® 35218 (β-lactamase producer) and enteropathogenic *Escherichia coli* 208 - EPEC (resistant to gentamicin, cephalothin, sulfamethoxazole-trimethoprim, ampicillin, chlortetracycline; F6, BFPa virulence factors) strains were used. The strains were grown in Brain Heart Infusion (BHI) broth at 37ºC for 24 h before the assays.

Minimal inhibitory concentration (MIC) determination and modulation activity

The minimum inhibitory concentration (MIC) was determined by the microdilution method in 96-well plates using BHI broth (16). Microorganism colonies were suspended in 0.9% saline solution and the suspension was adjusted by the spectrophotometric method at 625 nm to a final concentration of 10^6 CFU mL^{-1}. Serial dilutions of the oil solubilized in 10% DMSO in the range of 1000 – 3.9 μg mL^{-1} and antibiotics (ciprofloxacin, ceftriaxone, amoxicillin, gentamicin and vancomycin) in the range of 2500-2.4 μg mL^{-1} were performed. The 10% DMSO solution was included as a negative control. The plates were incubated at 37 ± 1 °C for 24 h. Bacterial growth was indicated by the addition of 20 μL of 0.01% Resazurin (Sigma–Aldrich) aqueous solution with incubation at 37 ± 1 °C for 2 h. The MIC values were identified as the lowest concentration in which no bacterial growth was visible. Evaluation of the essential oil as an antibiotic-resistance modulator was performed according to Coutinho and collaborators (17). The MIC values of the antibiotics were determined in the presence and absence of sub-inhibitory concentrations (125 μg mL^{-1}) of the essential oil. Plates were incubated as described above and each assay was performed in triplicates.

Statistical analysis

The test results were expressed as the geometric mean. A Two-Way analysis of variance (ANOVA) followed by Bonferroni post-hoc test was applied using the GraphPad Prism 5.0 software. Only results with $p < 0.05$ were considered significant.

Table 1. Constituents identification in *Eucalyptus citriodora* essential oil.

CONSTITUENTS	RT [a]	KI [b]	$IK_{simulated}$	% Composition
α-pinene	8.257	939	-	0.28
β-pinene	9.729	979	-	0.66
Bergamal	12.532	1056	1051	0.58
Rose oxide (*cis*)	14.668	1108		0.66
Rose oxide (*trans*)	15.295	1125	-	0.26
Isopulegol	16.060	1149	1146	11.80
Citronellal	16.616	1153	-	65.45
Isopulegol (*neo-iso-*)	17.006	1171	1171	0.79
Citronellol	19.226	1225	-	14.87
Menthol (8-hydroxy-*neo-*)	22.894	1330	1329	0.51
Citronellyl acetate	23.472	1352	-	2.51
Caryophyllene (*Z*)	25.840	1408	1408	0.28
Percentage total of identified constituents				98.65
Percentage total of unidentified constituents				1.35
Monoterpenes				11
Sesquiterpenes				1
Total of the identificated constituents				12

[a] Retention Time; [b] Kovats indices

Results

The chemical constituent analysis of the EOEc presented a 90.43% composition for monoterpenes and 8.22% for sesquiterpenes (Table 1). The main constituents are presented, in descending order, by the following constituents: citronellal (65.45%); citronellol (14.87%); isopulegol (11.80%), citronellyl acetate (2.51%); *neo-iso*-isopulegol (0.79%); *cis*-rose oxide (0.66%); β-pinene (0.66%); bergamal (0.58%); 8-hydroxy-*neo*-menthol (0.51%); α-pinene (0.28%); (*Z*)-caryophyllene (0.28%); *trans*-rose oxide (0.26%) (Fig. 1).

The EOEc did not show significant activity against the tested strains (MIC > 1000 μg mL^{-1}), with respect to its antimicrobial activity. However, the ability of the EOEc to modify *S. aureus* and *E. coli* resistance to different antimicrobials was also evaluated. Although the EOEc did not present direct antimicrobial activity, when associated with antimicrobial drugs it significantly enhanced the activity of some of these agents against the tested strains. In the assays with *S. aureus* MED 55 (MRSA), MIC reductions were observed for all tested antibiotics, with the most significant results being obtained for gentamicin (reduction from 250 to 19.67 μg mL^{-1}) followed by ceftriaxone (reduction from 250 to 62.50 μg mL^{-1}), ciprofloxacin (reduction from 78.75 to 0.50 μg mL^{-1}) and vancomycin (reduction from 6.19 to 2.41 g mL^{-1}) (Fig. 2). In assays with *S. aureus*, a significant MIC reduction was observed only for gentamicin (reduction from 79.42 to 31.49 μg mL^{-1}).

In the *E. coli* assays, a positive interaction between the EOEc and different antimicrobials was also observed (Fig. 3). *E. coli* 208 presented a significant reduction in MIC for amoxicillin (500 to 396.85 μg mL^{-1}) and gentamicin (62.5 to 7.81 μg mL^{-1}). For *E. coli* 35 a MIC reduction for all antimicrobials was observed, where for amoxicillin the reduction was from 99.21 to 31.12 μg mL^{-1}, for gentamicin this was from 24.73 to 12.39 μg mL^{-1} and for vancomycin the MIC reduced from 31.12 to 7.8 μg mL^{-1}.

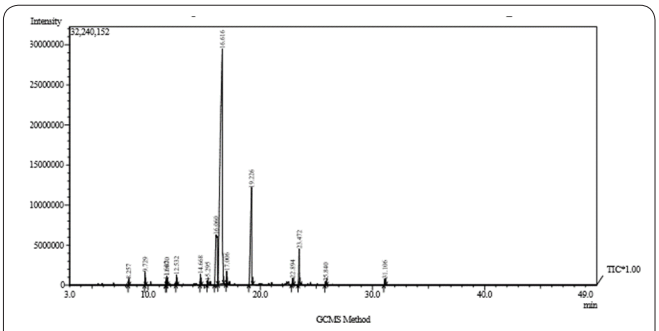

Figure 1. Chromatogram of the essential oil of *E. citriodora.*

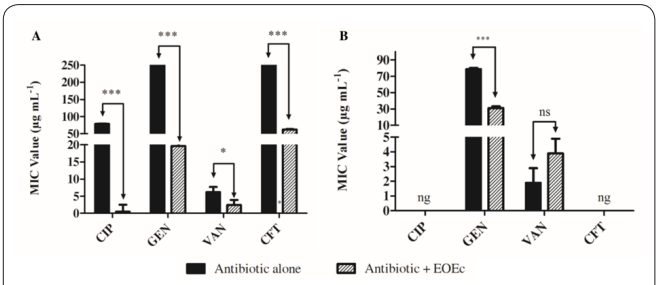

Figure 2. Modulatory activity of the Essential oil of *E. citriodora* on resistance of *Staphylococcus aureus* to antibiotics. A- *S. aureus* MED 55 (MRSA); B – *S. aureus* 23. *** – Statistically significant difference with P value < 0.001; ns – not statistically significant value with P > 0.05; ng – no growth; CIP – Ciprofloxacin; GEN – Gentamicin; VAN – Vancomycin; CFT – Ceftriaxone.

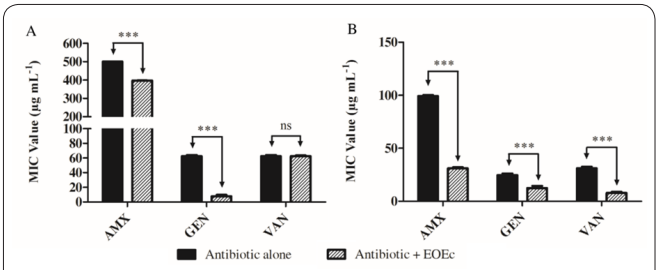

Figure 3. Modulatory activity of the Essential oil of *E. citriodora* on resistance of *Escherichia coli* to antibiotics. A- *E. coli* 208; B – *E. coli* 35. *** – Statistically significant difference with P value < 0.001; ns – not statistically significant value with P > 0.05; ng – no growth; AMX – Amoxicillin; GEN – Gentamicin; VAN – Vancomycin.

Discussion

The chemical composition of essential oils is mainly determined by the genetic components of the plant, however, it also depends on external factors such as variations in the plant physiological state, environmental and geographical conditions, harvesting, as well as conservation and extraction techniques (18,19).

Rajeswara Rao and collaborators (11) evaluated the phytochemical composition of the EOEc obtained by hydrodistillation of the leaves from plants grown in India, showing the presence of 31 compounds, with citronellal (70.3%), citronellol (8.8%) and isopulegol (6.7%) standing out. Cimanga and collaborators (20) reported the presence of 16 compounds in the EOEc obtained from specimens grown in the Democratic Republic of Congo and, similarly, the citronellal (72.7%) and citronellol (6.3%) were the main components. In studies using specimens grown in Brazil, 19 compounds were identified where the major components were citronellal (89.59%), citronellyl acetate (3.34%) and 1,8-cineole (2.87%). These data corroborate with the results found in this study with respect to the main EOEc components (21).

The antimicrobial activity of the *E. citriodora* essential oil against *S. aureus* and *E. coli* has been demonstrated in previous studies (22,23). Studies report the antimicrobial activity of the EOEc is associated with the promotion of cell lysis, as well as the inhibition of DNA and ATP metabolism in bacteria such as *E. coli* and *S. aureus* (24). The divergence between the present study and the literature may be due to differences in the phenotypic resistance profile of the bacterial strains used in the studies. Additionally, the oil is volatile and chemically unstable and may suffer changes in the presence of air, light, humidity and higher temperatures (24).

Moreover, reports addressing the antimicrobial activity of the EOEc major components exist, such as citronellal which may cause changes in cell membrane permeability through its interaction with functional membrane proteins (25). In a study by Horne and collaborates (26), citronellol was attributed the ability to damage the cell wall and the cytoplasmic membrane, causing cell lysis in *Streptococcus pneumoniae*. Mono-

terpene alcohols such as isopulegol may act by causing cytoplasmic membrane rupture and bacterial cell wall component loss, thus affecting its structure (27).

In addition to substances with direct antimicrobial activity, researchers have sought a complementary strategy for the use of existing drugs, these being adjuvants. These substances have little or no antimicrobial activity, however, these possess the ability to attenuate or even eliminate resistance mechanisms allowing existing drugs to regain their activity (28). Several studies have evaluated the potential of natural products as adjuvants in antimicrobial therapy, showing promising results (29-31).

Previous studies have evaluated the interaction of *E. citriodora* secondary metabolites and other substances for their actions against different microorganisms. Kirui and collaborators (32) found synergism in the *E. citriodora* and *Syzygium aromaticum* oil association against *S. aureus*, *E. coli*, MRSA and *Microsporum gypseum*. In other studies, the combination of the *E. citriodora* leaf methanolic extract and antimicrobials resulted in a synergistic effect with ciprofloxacin against *Pseudomonas aeruginosa* (33). Similarly, secondary metabolites from other *Eucalyptus* species have also exhibited synergistic effects when associated with antimicrobials. Chaves and collaborators (7) reported the *E. camaldulensis* essential oil presented a positive interaction when associated with amoxicillin and ampicillin against *S. aureus* and MRSA, and with cephalexin and cefuroxime against *E. coli*. Pereira and collaborators (34) reported a synergism in the association between the *E. globulus* essential oil extracts and gentamicin against *P. aeruginosa*.

The nature of the oil components, which are mostly monoterpenes, is among the factors that corroborate the modulatory activity presented by the EOEc. These compounds have been well reported in the literature to have the ability to interact with the plasma membrane, altering its permeability and other properties (25,26). Moreover, the hypothesis that this activity is associated with the interaction of the natural products with other microbial cell targets (e.g., efflux pumps) cannot be excluded (35).

Based on these results, *E. citriodora* essential oil is reported to present no antimicrobial activity against the tested multiresistant strains, however, the *in vitro* antimicrobial activity of different antimicrobials can be significantly increased in the presence of the oil. This demonstrates the combination of the *E. citriodora* essential oil and antimicrobials is very promising as it enables the use of these drugs at concentrations lower than indicated, which until then were ineffective. In addition, such use may mitigate possible toxic effects caused by high antimicrobial doses. However, further investigations are needed to evaluate the effect of combining the oil with other antimicrobials, and against other multiresistant bacterial species to ratify a synergistic effect, as well as to identify the substances involved in the modulatory activity and their respective mechanisms of action.

Conflict of interests

The authors declare that there is no conflict of interests regarding the publication of this article.

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