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Callistemon citrinus essential oil from Cameroon: potent inhibitor of bacterial respiratory pathogens without inducing resistance or persistence

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Abstract

Background: Antibiotic resistance, persistent cells in respiratory infections, and adverse effects associated with antibiotics underscore the urgent need to search for alternative treatments. Natural products such as essential oils derived from aromatic plants like *Callistemon citrinus* may be a promising avenue to explore. This study aimed to assess the antibacterial activity of *C. citrinus* essential oils from two Cameroonian regions against bacteria causing respiratory infections, investigate their possible modes of action, and evaluate the microbiological effects of continuous exposure at sub-inhibitory concentrations.

Methods: The antibacterial activity was determined by the broth microdilution method. Potential modes of action were investigated using assays to assess effects on the bacterial membrane, protein synthesis, and H+-ATPase pump activity. Continuous exposure experiments were performed for 08 days and compared with levofloxacin as a reference.

Results: *Callistemon citrinus* essential oil from the University of Dschang campus exhibited the strongest antibacterial activity, with minimum inhibitory concentration values ranging from 78.13 to 468.75 µg/mL. Tests on the mode of action revealed inhibition of protein synthesis in *Pseudomonas aeruginosa* and interference with H+-ATPase pump activity in *Staphylococcus aureus*, with no significant impact on the bacterial membrane. It is noteworthy that continuous exposure to *C. citrinus* essential oil did not induce resistance or persistence, unlike levofloxacin, which led to the emergence of resistant and persistent populations in *P. aeruginosa* and *S. aureus*, respectively.

Conclusion: *C. citrinus* essential oil exhibits potent antibacterial activity against major respiratory pathogens through various cellular targets, while minimizing the risk of resistance or persistence. These results highlight its potential as a natural candidate for the development of alternative therapies; however, further studies such as chemical characterization, cytotoxicity, and *in vivo* validation are needed.

Keywords: *Callistemon citrinus*; essential oil; antibacterial; respiratory infections.

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Background

Respiratory tract infections are diseases caused by infectious agents that affect the respiratory system [1]. They can be caused by bacteria, viruses, fungi, or parasites and, are mainly transmitted through inhalation or contact with infected bodily fluids or aerosols [2]. These infections can be classified into two types: upper respiratory tract infections and lower respiratory tract infections [3]. Respiratory infections of bacterial origin can be triggered by several bacteria, including *Streptococcus pyogenes*, *Streptococcus pneumoniae*, *Klebsiella pneumoniae*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa* [4–6]. Globally, respiratory infections are among the top causes of death. In Africa, lower respiratory tract infections are the leading cause of mortality [7], making them a serious public health concern [8]. Bacterial respiratory infections are typically treated with antibiotics such as amoxicillin and levofloxacin [9–11]. However, although this is the most commonly used treatment, it should be noted that the use of these antibiotics is not only beneficial but also has many disadvantages, including the occurrence of adverse effects and the increasingly observed generation of bacteria that are resistant [12,11] and persistent to antibiotics [13,14]. Research shows that these resistant and persistent bacteria are associated with antibiotic treatment failures and that persistent ones are the cause of recurrent infections [14,15]. Therefore, there is an urgent need to find alternative treatments that can prevent the emergence of such resistant and persistent bacteria and improve treatment.

According to estimates by the World Health Organization, about 80% of people worldwide are still dependent on traditional herbal medicines. This widespread use is largely due to their low cost, easy availability, and generally minimal side effects compared to conventional allopathic treatment [16]. Research indicates that about 50% of therapeutic pharmaceuticals available today are derived from natural sources, predominantly plants [17]. Medicinal and aromatic plants represent a significant portion of natural flora and are regarded as a vital source of raw materials across different sectors, including the pharmaceutical industry [18]. Essential oils derived mainly from aromatic plants are playing an increasingly important role in the formulation of alternative antimicrobial drugs due to their powerful biological activities [19] and their lower toxicity [20,21]. *Callistemon citrinus* (Curtis) Skeels (*C. citrinus*), commonly known as “lemon bottlebrush,” is an aromatic shrub from the Myrtaceae family that grows naturally in Australia [22]. While often used for decorative purposes, this plant is exploited for its therapeutic properties in ethnopharmacology and holds significant value in traditional medicine. In India, it is used for the treatment of gastrointestinal problems, pain, infectious diseases, and for its cough-relieving, bronchial-soothing, and insect-repelling properties [23]. In Australia, it is traditionally used to manage respiratory tract infections [24] ; in Uganda for the treatment of tuberculosis [25] and in Cameroon, to preserve stored food products [26]. Previously valued mainly for its insecticidal and antifungal effects [27,28,26,29–32], some scientific studies have shown that its essential oil also possesses cytotoxic, antioxidant, anti-inflammatory, and antibacterial properties [33,34,19,35–38]. Studies analyzing the chemical composition of its essential oil have identified eucalyptol as one of its major compounds [26,35,37,38]. However, despite these promising results, very few studies have investigated the antibacterial activity of *Callistemon citrinus* essential oil on bacteria that cause respiratory infections. To the best of our knowledge until now, no studies have explored its mode of action or the microbiological impact of prolonged exposure to sub-inhibitory concentrations of this essential oil on these bacteria.

Thus, this study aimed to evaluate the antibacterial activity of *Callistemon citrinus* essential oil sourced from two regions of Cameroon against several respiratory pathogens. It also sought to identify the likely modes of action of the most potent oil on *Pseudomonas aeruginosa* and *Staphylococcus aureus* NCTC 12973 and to analyze the microbiological effect of continuous exposure of these bacteria to sub-inhibitory concentrations of the essential oil.

Methods

Collection and identification of plant material

The *Callistemon citrinus* (staff) leaves used were collected in the campus of the University of Yaoundé I and the University of Dschang, located in the Central and Western regions of Cameroon, respectively, in early May 2024. The collected plants were identified at the Cameroon National Herbarium in comparison with Daniel DANG's material nº 565, registered at the herbarium under nº 25801/SRF/Cam.

Bacterial strains and culture media

A panel of six bacteria (Gram-negative and Gram-positive) was used in this study. These include 03 reference strains: *Staphylococcus aureus* NCTC 12973, *Streptococcus pneumoniae* HM 145, *Klebsiella pneumoniae* ATCC-Baa 1705 and 03 clinical isolates of *Staphylococcus aureus, Pseudomonas aeruginosa* and *Streptococcus pyogenes*. The bacterial strains were obtained from the Laboratory of Phytobiochemistry and Medicinal Plant Research at the University of Yaoundé I and the Referral Teaching Hospital of Yaoundé, Cameroon. Bacterial strains were maintained on agar plates at 4°C and sub-cultured onto appropriate fresh agar plates 24 h prior to each antibacterial test. Mueller-Hinton agar (MHA; Sigma) was used for bacterial activation and Mueller-Hinton broth (MHB; Sigma) was used for determining inhibition parameters, modes of action, and resistance induction testing. Chapman agar (CA; Sigma) and Cetrimide agar (CTA; Sigma) were used for the selection of resistant or persistent bacteria of *Staphylococcus aureu*s NCTC 12973 and *Pseudomonas aeruginosa* during the continuous exposure test, respectively.

Chemical reagents and antibiotics

All chemical reagents used in this study were of analytical grade and procured from Sigma-Aldrich (St. Quentin Fallavier). These included: Anhydrous sodium sulfate, Tween 80, Alamar Blue, Sodium chloride, Phosphate buffer, Bradford reagent, Potassium chloride and Glucose. Reference antibiotics used were Levofloxacin (REMEDIA; Simpex Pharma Pvt. Ltd, India) and Amoxicillin (Ospamox; Sandoz GmbH Kundl, Austria)

Essential oil extraction

C. citrinus leaves were dried for 10 days at room temperature and essential oil was extracted by hydro-distillation for 4 h using Clevenger-type apparatus. Each oil recovered was dried over anhydrous sodium sulfate (Na_2SO_4), stored in an amber colored flask and kept at 4°C until use. Essential oils extraction yields were determined according to the formula:

$$\text{Extraction yield } (\%) = \frac{Masse\ of\ essential\ oil\ (g)}{Masse\ of\ dry\ leaves\ (g)} \times 100$$

Evaluation of antibacterial activity

The microdilution method was carried out according to the Microplate Alamar Blue Assay, as previously described [39]. In each well of a 96-well microplate, 100 µL of Mueller Hinton Broth (MHB) culture medium was introduced. Then, 100 µL of a stock solution of essential oils (EOs) initially prepared at 20 mg/mL and antibiotics (Levofloxacin and Amoxicillin) initially prepared at 0.3 mg/mL were added to the wells containing MHB. A series of dilutions following a geometric progression with a ratio of 1/2 were performed. Each well received 100 µL of bacterial inoculum at 1.5×10^6 CFU/mL, prepared in physiological saline (0.9% NaCl solution). The plates were covered, sealed and incubated at 37°C for 24 h. Wells containing only broth were used as negative controls for sterility testing, whereas those containing broth and inoculum served as positive controls to evaluate bacterial growth. After the incubation period, 20 µL of a 0.02% resazurin salt solution were added to each well, and the plates were re-incubated for 20 min. Color change was then observed: a shift from blue to pink indicated a reduction of the indicator, thus signifying bacterial growth. The minimum inhibitory concentration (MIC) was defined as the lowest concentration of the samples at which no microorganism growth was observed. The minimum bactericidal concentration (MBC) of EOs was determined using subcultures. Fifty microliters (50 µL) of well contents with a concentration greater than or equal to MIC were introduced in 150 µL of MHB. These preparations were incubated at 37°C for 48 h. MBC was considered the lowest concentration of EOs that did not produce a color change after the addition of a 0.02% resazurin salt solution, as described above. Each serial dilution and subculture was performed in triplicate, and the assay was repeated twice.

The activity of essential oils against the different strains was classified according to the following scale: Outstanding activity: MIC ≤ 8 µg/mL; Excellent activity: 8 < MIC ≤ 40 µg/mL; Very good activity: 40 < MIC ≤ 128 µg/mL; Good activity: 128 < MIC ≤ 320 µg/mL; Average activity: 320 < MIC ≤ 625 µg/mL; Weak activity: 625 < MIC ≤ 1024 µg/mL; Not active: MIC values > 1024 µg/mL [40]. Meanwhile, for reference antibiotics used in this study were classified according to the following scale: Outstanding activity: MIC ≤ 2 µg/mL; Excellent activity: 2 < MIC ≤ 4 µg/mL; Very good activity: 4 < MIC ≤ 8 µg/mL; Good activity: 8 < MIC ≤ 32 µg/mL; Average activity: 32 < MIC ≤ 64 µg/mL; Weak activity: 64 < MIC ≤ 512 µg/mL; Not active: MIC > 512 µg/mL [40].

The MBC/MIC ratio was used to determine the bacteriological profile of the essential oils: an MBC/MIC ratio < 4 indicates a bactericidal effect and MBC/MIC ratio ≥ 4 indicates bacteriostatic effect [41].

Mode of action of Callistemon citrinus essential oil

The mode of action of *C. citrinus* essential oil was studied with that from the University of Dschang (Cc-UDs) on *Pseudomonas aeruginosa* and *Staphylococcus aureus* NCTC 12973.

Effect on bacterial membrane

The protocol described [42] with some modifications was used. Bacterial suspensions (1.5×10^6 CFU/mL) prepared in 0.9% saline were mixed with EO and MHB medium at concentrations equivalent to 1/2 MIC, MIC and 2 MIC in a microplate. The whole set was incubated at 37°C with stirring, and absorbance at 620 nm was then measured at 0 h, 1 h, 2 h, 4 h, 6 h and 8 h. Bacterial membrane lysis will be indicated by a decrease in absorbance at 620 nm. The positive control consisted of untreated bacterial suspensions. All tests were performed in triplicate, and the experiment was repeated twice. The absorbances (A) obtained at different incubation times (t) were used to plot the curves: A = f(t).

Effect on protein synthesis

To achieve this, we used the protocol described [43], with a few modifications. To tubes containing 9 mL of MHB, 0.5 mL of bacterial suspension (1.5×10^6 CFU/mL) prepared in 0.9% saline was added. 0.5 mL of EO was then added to each tube to obtain concentrations equivalent to 1/2 MIC, MIC and 2 MIC. The control tube received 0.5 mL of MHB broth. Tubes were subsequently incubated at 37°C for 24 h, then spun (4000 g; 4˚C) for 5 min. The resulting bacterial pellet was weighed and mixed with 5 mL phosphate buffer (pH =7.5). The whole was then vortexed for 15 seconds and centrifuged (4000 g; 4°C) for 10 min. After centrifugation, 50 µL of protein-containing supernatant was removed and added to 450 µL of distilled water and 500 µL of Bradford reagent. The whole set was then incubated in the dark for 15 min and absorbances read at 595 nm against blank. The blank was the lysis buffer (phosphate buffer, pH = 7.5). All assays were performed in triplicate and repeated two times.

Effect on H+-ATPase pumps

The effect on H+-ATPase pumps was examined by monitoring the acidification of the external medium in bacterial suspensions following glucose stimulation. This was done by measuring pH changes using an electrode, based on method described [44], with slight modifications. Bacterial strains at a concentration of 1.5×10^6 CFU/mL were cultured in 4 mL of MHB and incubated at 37°C with shaking for 24 h. After incubation, the bacterial cells were harvested by centrifugation (3000 g; 4°C) for 10 minutes, then washed with sterile distilled water and 50 mM KCl (pH 6.5). The washed cells were centrifuged again and resuspended in 100 mL KCl (50 mM; pH 6.5), followed by overnight incubation at 4 °C to deplete their carbon reserves. The carbon-depleted cells were then collected by centrifugation and resuspended in 100 mL of 50 mM KCl (pH 6.5) to reach a cell density of approximately 1.5×10^6 CFU/mL. EO was added to 4 mL of this bacterial suspension at concentrations corresponding to 1/2 MIC, MIC and 2 MIC. The mixture was thoroughly mixed and adjusted to a final volume of 5 mL with 50 mM KCl (pH 6.5). It was then incubated at room temperature with gentle shaking for 10 min. Afterward, 0.5 mL of 10% glucose was added, and the pH of the external medium was recorded every 15 min over a 75-min period. For positive control, EO was replaced with sterile distilled water. All experiments were conducted in duplicate and repeated twice. The recorded pH values were used to generate a pH versus time curve: pH = f(t).

Continuous exposure of bacterial strains to Callistemon citrinus essential oil and Levofloxacine

Pseudomonas aeruginosa and *Staphylococcus aureus* NCTC 12973 were continuously exposed to fixed and variable (increasing) sub-inhibitory concentrations of *C. citrinus* essential oil from the campus of the University of Dschang and levofloxacin (reference antibiotic). Fixed sub-inhibitory concentrations were 1/8 MIC, 1/4 MIC and 1/2 MIC, while increasing ones ranged from 1/8 MIC to 16 MIC. This test was, carried out in two phases as described [45] with a few modifications: the first phase involved continuous exposure of bacteria to fixed and increasing sub-inhibitory concentrations of *C. citrinus* EO and levofloxacin, known

as the exposure or “induction” phase; and the second was the non-exposure phase (in the absence of *C. citrinus* EO and levofloxacin), designed to assess the stability of the effect observed during the induction phase. Each phase lasted eight days and, every four days, inhibition parameters were determined by the microdilution method.

- 1st phase: induction phase or continuous exposure of bacteria

Pseudomonas aeruginosa and *Staphylococcus aureus* NCTC 12973 were grown every 24 h for 8 days in MHB medium containing *C. citrinus* EO and levofloxacin at fixed and increasing sub-inhibitory concentrations. When growth was visible in a tube (turbid tube), 50 µL of this culture was removed to inoculate a new corresponding tube containing 4950 µL of MHB medium with the test substance at the same sub-inhibitory or increasing concentration. On the other hand, if no bacterial growth was observed in a tube (that is the tube remained clear or non-turbid), the test was halted for that concentration, which was then designed as the new MIC. The control tube contained 4950 µL of MHB medium. After inoculation, all tubes were incubated at 37°C with agitation at 150 rpm.min^{-1}. On days 4 and 8, sample from each tube were plated onto selective solid media, namely Chapman and Cetrimide media for *Staphylococcus aureus* and *Pseudomonas aeruginosa* respectively, to determine the MIC during the exposure phase.

- 2nd Phase: Phase of continuous non-exposure of bacteria

After 8 days of exposure to the test substances (exposure phase), *Pseudomonas aeruginosa* and *Staphylococcus aureus* NCTC 12973 were cultured again every 24 h for an additional 8 days in MHB medium without *C. citrinus* essential oil and without levofloxacin (non-exposure phase) to verify the stability of the effect on these bacteria following their previous exposures. When bacterial growth was observed in a tube, 50 µL of the culture was transferred to a new tube containing 4950 µL of MHB medium only. These inoculated tubes were then incubated at 37°C with shaking at 150 rpm.min^{-1}. On days 4 and 8, samples from each tube were plated onto selective solid media, namely Cetrimide and Chapman media for *Pseudomonas aeruginosa* and *Staphylococcus aureus*, respectively, to determine the MICs during the non-exposure phase. All tests were performed in duplicate, and the experiment was repeated twice.

Statistical analysis

Statistical analysis was carried out using one-way ANOVA in GraphPad Prism 8.0. Results are presented as mean ± standard deviation (SD) from experiments performed in triplicate. Error bars represent the SD, and significant differences in multiple comparisons were identified using the Turkey’s test at a significance level of $p < 0.05$. Graphs and histograms were also generated using GraphPad Prism 8.0.

Results

Essential oils yield and color

The yields of essential oils obtained from the hydrodistillation of the leaves of *C. citrinus* coming from the University of Yaoundé I campus and the University of Dschang campus were 0.85% and 1.2% (w/w), respectively. Both essential oils were yellow in color.

Evaluation of antibacterial activity

The antibacterial activity of *C. citrinus* essential oils and reference antibiotics was evaluated by determining inhibition parameters, minimum inhibitory concentration (MIC) and bactericidal concentration (MBC), as well as the bacteriological profile by calculating the MBC/MIC ratio. The results obtained are summarized in Table 1. This table shows that both essential oils were active on certain strains tested and that the intensity of this activity varied from one strain to another. Of these essential oils, Cc-UDs was the most active, with MICs ranging from 78.13 to 478.75 µg/mL and MBCs ranging from 468.75 to 5000 µg/mL. The antibacterial activity of Cc-UDs essential oil was very good (40 < MIC ≤ 128 µg/mL) on *Staphylococcus aureus* NCTC 12973, *Pseudomonas aeruginosa* (78.13 µg/mL for both strains) and *Staphylococcus aureus* (117.19 µg/mL); good (128 < MIC ≤ 320 µg/mL) on *Streptococcus pneumoniae HM 145* and *Klebsiella pneumonia* ATCC-Baa 1705 (234.38 µg/mL for both strains) and moderate (320 < MIC ≤ 625 µg/mL) against *Streptococcus pyogenes* (468.75 µg/mL). Furthermore, with this essential oil, the MBC/MIC ratio ranged from 2 to 10.67, indicating that it had a bactericidal effect only on the *K. pneumoniae* ATCC-Baa 1705 and a bacteriostatic effect on all other strains tested. The essential oil of Cc-UY1 was less active, with MICs ranging from 937.5 to 3750 µg/mL and MBCs ranging from 1875 to 5000 µg/mL. The antibacterial activity of this oil was very good against *Staphylococcus aureus* (117.19 µg/mL); good against *Staphylococcus aureus* NCTC 12973 (234.38 µg/mL); weak (625 < MIC ≤ 1024 µg/mL) on *Streptococcus pneumoniae* HM 145 and *Pseudomonas aeruginosa* (937.5 µg/mL for both strains) and inactive (MIC > 1024 µg/mL) on *Streptococcus pyogenes* and *Klebsiella pneumonia* ATCC-Baa 1705 (3750 µg/mL for both strains). The calculation of the MBC/MIC ratio for this essential oil ranged from 1.33 to 16, reflecting a bactericidal effect of the latter only on *K. pneumoniae* ATCC-Baa 1705 and a bacteriostatic effect on the other strains. However, with this essential oil, the MBC and bacteriological profile could not be determined on *S. pneumonia* HM 145 and *S. pyogenes* at the concentrations tested. Levofloxacin and amoxicillin, used as reference antibiotics, showed variable antibacterial potency across different bacteria overall. Levofloxacin was more active with MICs ranging from 0.59 to 18.75 µg/mL and MBCs ranging from 0.59 to 28.13 µg/mL. The action of levofloxacin was outstanding (MIC ≤ 2 µg/mL) on *Staphylococcus aureus* NCTC 12973, *Pseudomonas aeruginosa* (0.59 µg/mL for both strains), *Staphylococcus aureus* and *Streptococcus pneumoniae* HM 145 (0.88 µg/mL for both strains); very good (4 < MIC ≤ 8 µg/mL) on *Streptococcus pyogenes* (4.69 µg/mL) and good (8 < MIC ≤ 32 µg/mL) on *Klebsiella pneumonia* ATCC-Baa 1705 (18.75 µg/mL). This antibiotic was bactericidal on all strains tested, except for *S. pyogenes* and *P. aeruginosa*, where it was bacteriostatic. For amoxicillin, MICs ranged from 0.88 to 75 µg/mL and MBCs ranged from 3.52 to 300 µg/mL. This antibiotic was not active (MIC > 512 µg/mL) against *K. pneumoniae* ATCC-Baa 1705 at the concentrations tested; had shown an average activity (32 < MIC ≤ 64 µg/mL) on *Streptococcus pyogenes* (37.5 µg/mL) and low activity (64 < MIC ≤ 512 µg/mL) on *Streptococcus pneumoniae* HM 145 (75 µg/mL). It had a bacteriostatic effect on other strains, except for *S. pyogenes*, for which the MBC could not be determined.

Mode of action of C. citrinus essential oil

Effect on bacterial membrane

Figure 1 below shows the relative absorbance at different concentrations as a function of time for Cc-UDs essential oil on *P. aeruginosa* and *S. aureus* NCTC 12973. It was observed that, in the presence of treatment, there was no significant reduction in relative absorbance at all concentrations tested throughout the study period compared to the positive control for both *P. aeruginosa* and *S. aureus*.

Effect on protein synthesis

The results in Figure 2 illustrate the effect of the presence of Cc-UDs essential oil at different concentrations on protein synthesis by *P. aeruginosa* isolate and *S. aureus* NCTC 12973. This figure shows that, in the presence of essential oil, there is a significant, dose-dependent reduction in protein synthesis in *P. aeruginosa* compared to the positive control. However, a non-dose-dependent and non-significant increase in protein synthesis compared to the positive control was observed in *S. aureus*.

Effect on H+-ATPase pumps

The effect of Cc-UDs essential oil on the activity of H+-ATPase pumps in *P. aeruginosa* and *S. aureus* NCTCC 12973 is shown in Figure 3. In *P. aeruginosa*, there was a gradual, non-significant decrease in pH over time until the end of the study, both in the absence and presence of Cc-UDs essential oil, at all concentrations tested. In contrast, in *S. aureus*, the difference in pH variation observed in the presence of Cc-UDs essential oil was significant compared to the positive control. From the start of the experiment, the presence of essential oil led to a steady increase in pH, reaching 6.74 at MIC and 6.84 at 2 MIC. At 1/2 MIC, the change was minimal, with pH rising only slightly from 6.50 to 6.57. In contrast, the positive control showed a consistent decline in pH, ending at 6.18.

Continuous exposure of bacterial strains to Callistemon citrinus essential oil and Levofloxacin

Continuous exposure of *P. aeruginosa* isolate and *S. aureus* NCTCC 12973 to Cc-UDs essential oil and levofloxacin had different effects on them, as shown in Figure 4. In *P. aeruginosa* isolate with levofloxacin, continuous exposure to the fixed sub-inhibitory concentration 1/2 MIC did not result in a significant change in MIC throughout the study period compared to the initial MIC. At fixed sub-inhibitory concentrations of 1/4 MIC and 1/8 MIC, there was also no significant change in MIC throughout the exposure phase and during the first 4 days of non-exposure, followed by a significant increase (23-fold) on day 8 of the non-exposure phase compared to the initial MIC. At the variable sub-inhibitory concentration 1/8 MICv, the MIC did not vary significantly until the 4th day of the exposure phase, followed by a sharp increase (57-fold) on the 8th day of exposure compared to the initial MIC, which then remained almost unchanged until the end of the non-exposure phase. Furthermore, with Cc-UDs EO on this isolate, whether with fixed sub-inhibitory concentrations 1/2 MIC, 1/4 MIC, 1/8 MIC, or variable 1/8 MICv, the MIC did not vary significantly compared to the initial MIC during all 8 days of exposure and all 8 days of non-exposure. However, in the *S. aureus* NCTCC 12973 with levofloxacin at a fixed sub-inhibitory concentration of 1/2 MIC, a gradual increase was observed during the exposure phase, with a significant increase (31-fold) recorded on day 8 compared to the initial MIC, after which the MIC began to gradually decrease, returning to the initial MIC on day 8 of the non-exposure phase. At 1/4 MIC, 1/8 MIC, and 1/8 MICv concentrations, there was a significant increase (39-fold) in MIC from the 4th day of the exposure phase compared to the initial MIC, which remained constant until the 8th day of exposure; it then began to gradually decrease, returning to the initial MIC on the 8th day of the non-exposure phase. Furthermore, regarding Cc-UDs EO on the *S. aureus* NCTCC 12973, no significant variation in MIC compared to the initial MIC was observed during the exposure and non-exposure phases for all sub-inhibitory concentrations tested.

Discussion

The present study evaluated the antibacterial activity of *Callistemon citrinus* essential oils from two regions of Cameroon against key bacteria responsible for respiratory infections, as well as their potential modes of action and the microbiological effect of continuous exposure at sub-inhibitory concentrations. Our results confirm the antimicrobial potential of *C. citrinus*, particularly the oil from the University of Dschang campus (Cc-UDs), which exhibited the lowest MICs (78.13 µg/mL) against *Staphylococcus aureus* NCTC 12973 and a clinical isolate of *Pseudomonas aeruginosa*. These findings are consistent with previous studies highlighting the antibacterial effects of *C. citrinus* essential oil against Gram-positive and Gram-negative bacteria [33,46,19,35,47,30,31]. However, our results suggest a higher level of antibacterial activity than previously reported, which may be attributed to differences in the chemical composition of the essential oil. Essential oils are known to inhibit the growth of a wide variety of pathogens due to the presence of natural compounds produced by plant organs, and their bioactive properties depend on the secondary metabolites they contain [48]. This chemical variability may be due to differences in geographical origin, environmental conditions, the state (fresh or dried) of the plant material, and even the extraction method. This reinforces the need for chemical characterization of this EO in order to identify the bioactive constituents responsible for this activity. Previous studies conducted in Cameroon on the chemical composition of this essential oil have revealed that it is mainly composed of 1,8-cineole, α-pinene, and α-terpineol [26,29]. At least two of these compounds have also been found to be major compounds in several other studies on this essential oil outside Cameroon [46,35,37,32]. In addition, the inhibitory activity of this essential oil may also be the result of a synergistic effect between its major and minor components [49].

The mechanisms by which plant extracts and compounds exert their effects on microbes are varied and complex. EOs can act on the cell wall, on membrane components, or penetrate the cytoplasm where they can act on organelles and nucleic acids or interfere with biochemical processes important for bacterial life [50]. The results of the bacteriolysis test revealed that Cc-UDs essential oil did not cause damage to the bacterial membrane of the strains tested, which could mean that this essential oil does not act directly on these bacteria in a way that leads to membrane lysis. Damage to the bacterial membrane ultimately leads to cell lysis, causing the cell contents to leak out and inducing the death of the bacterial cell [51]. This membrane lysis effect is generally observed with essential oils that have bactericidal potential, characterized by a high content of compounds with this effect, such as citronellol, citronellal, thymol, linalool and menthol [51,52]. However, given that the action of this essential oil did not cause

membrane lysis at the concentrations tested in this work, this suggests that it may instead have a bacteriostatic effect. Indeed, the determination of the bacteriological profile in this study of this EO on these two strains also showed that it is bacteriostatic. Furthermore, growth is a core property of bacteria, and it crucially depends on protein synthesis [53]. As shown in this study, the significant and dose-dependent reduction in protein content in the presence of EO on *Pseudomonas aeruginosa* compared to the positive control could indicate that EO of Cc-UDs acts on this bacterium by inhibiting protein synthesis. Plant compounds can inhibit protein synthesis by interfering with various stages of the process, including activation, initiation, disruption of peptide chain elongation, blockage of the ribosome's A site, misreading of the genetic code, and prevention of the binding of oligosaccharide side chains to glycoproteins [54]. It has been shown that terpinen-4-ol may block protein synthesis by inhibiting DNA synthesis [55]**.** Terpinen-4-ol and α-terpineol are two isomers of terpineol sharing the same chemical formula $C_{10}H_{18}O$ [56]. It has been reported that in *C. citrinu*s essential oil, α-terpineol is one of the major compounds, while terpinene-4-ol is among the minor compounds [26,29]. This could therefore explain the inhibitory effect on protein synthesis observed on *P. aeruginosa* in this study in the presence of this essential oil. Another target that plays a vital role in bacterial metabolism is H+-ATPase pumps. Plasma membrane H+-ATPases (H+-pumps) are the primary active transporters that translocate protons to the outside of each cell, providing the electrical and chemical energy that drives solute transport [57]. Thus, any inhibition of these pumps reduces the growth and survival of the bacteria [58]. In this study, acidification of the medium was significantly reduced with *S. aureus* in the presence of the essential oil (at MIC and 2 MIC), compared to the positive control where it increased with time. This suggests that inhibition of the plasma membrane proton pump could be one of the modes of action of this essential oil on this bacterium. *Pinus pumila* Pinecone essential oil, which contains α -terpineol and β-pinene among its major components, has demonstrated its action against *E. coli* through various mechanisms, including the reduction of ATPase activity [59].

Sub-inhibitory concentrations of antibiotics are commonly found in various body tissues of patients undergoing antimicrobial treatment. This can result from factors such as suboptimal dosages, poor drug pharmacokinetics, low drug potency, and/or poor patient adherence to treatment protocols [60]. Recent research indicates that these low concentrations of antibiotics can influence bacteria in three key ways: by selecting for resistance, generating genetic and phenotypic variability (resistant strains and persisters), and acting as signaling molecules [60]. To determine the impact of low concentrations of Cc-UDs EO and levofloxacin on *Pseudomonas aeruginosa* and *Staphylococcus aureus* NCTC 12973, we exposed them continuously to fixed and variable sub-inhibitory concentrations of EO and antibiotic. This study showed that during exposure to *P. aeruginosa*, in the presence of levo at 1/2 MIC, the MIC remained constant throughout the experiment, which could mean that it did not affect the physiology of this bacterium, either at the phenotypic or genotypic level. The significant increase of MIC (at 1/4 MIC and 1/8 MIC) on day eight of the non-exposure phase would indicate certain effects on this bacterium, but further investigation is needed to better understand this variability. During the exposure phase at 1/8 MICv, a notable 57-fold increase in MIC was observed on day eight, which then stabilized until the end of the experiment, suggesting an impact at the genetic level and likely resistance development in the presence of levo at this concentration. Additionally, for *S. aureus*, all tested concentrations of levo during the exposure phase led to a significant rise in MIC, which gradually declined back to the initial MIC level. This pattern may indicate that the strain regained sensitivity, possibly due to phenotypic changes and the emergence of persister cells. In contrast, continuous exposure to Cc-UDs essential oil, whether on *P. aeruginosa* or *S. aureus*, did not result in any genetic or phenotypic changes at any sub-inhibitory concentration tested. This suggests that the essential oil does not promote the development of resistant or persistent bacterial strains. This could indicate that exposure to this essential oil does not lead to the generation of resistant bacteria or persistent bacteria. This observation is of particular interest in the context of antimicrobial resistance, suggesting that *C. citrinus* essential oil may exert a lower selective pressure compared to conventional antibiotics. Two major differences distinguish persistence from resistance: first, antibiotic tolerance is not hereditary, as it is not caused by a genetic mutation; second, persistence is a transient state that disappears after antibiotic treatment is stopped [60].

However, some limitations should be acknowledged. First, the absence of GC-MS analysis prevents a direct correlation between antibacterial activity and chemical composition. Second, our study was limited to *in vitro* experiments; further *in vivo* investigations are required to confirm efficacy and safety. Third, no cytotoxicity assay was performed on mammalian cells, which is essential to assess potential therapeutic applications. Finally, the bacterial panel was restricted to five species; future studies should include a wider range of respiratory pathogens and resistant strains.

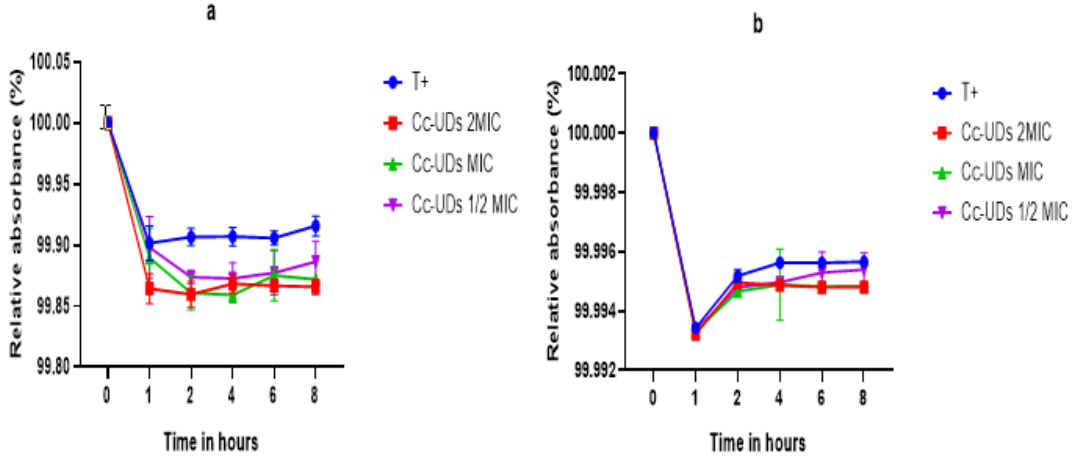

Figure 1. Effect of Cc-UDs essential oil on bacterial membrane of *P. aeruginosa* isolate (a) and *S.aureus* NCTCC 12973 (b).
T+: Positive control. Each point represents the mean ± SD; n = 3 (number of repetitions). The MIC of Cc-UDs EO was 78.124 µg/mL.

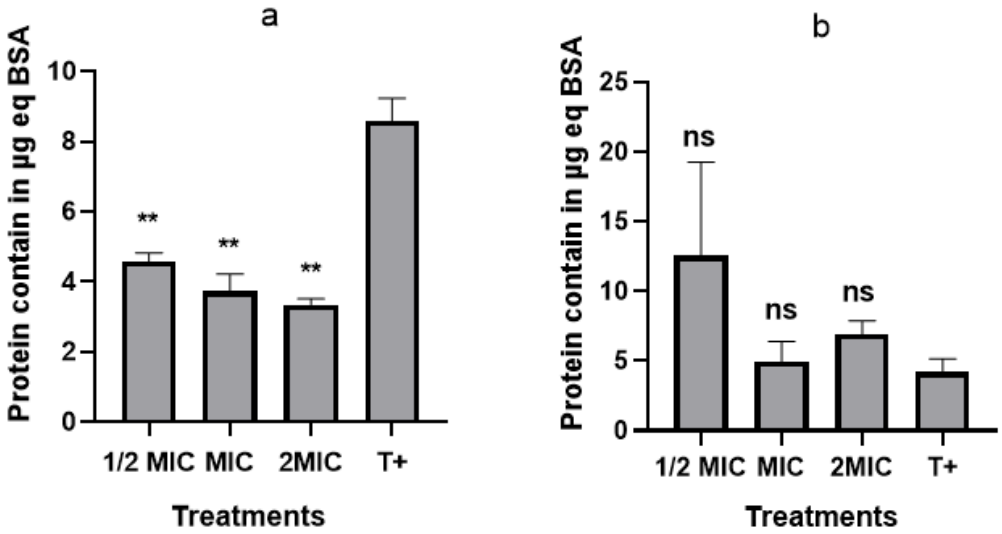

Figure 2. Effect of Cc-UD essential oil on protein synthesis of *P. aeruginosa* isolate (a) and *S. aureus* NCTCC 12973 strain (b).
T+: Positive control. Each bar represents the mean ± SD; n = 3 (number of repetitions). Significantly different from the positive control, * p < 0.05, ** p-values < 0.01, and *** p-values < 0.001; ns: non-significant. The MIC of Cc-UDs EO was 78.124 µg/mL.

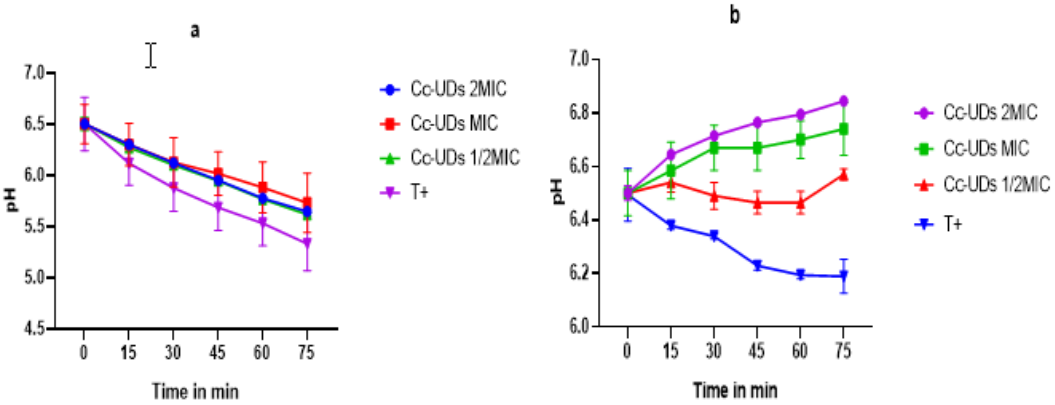

Figure 3. Effect of Cc-UDs essential oil on H+-ATPase pumps of *P. aeruginosa* isolate (a) and *S.aureus* NCTCC 12973 (b).
T+: Positive control. Each point represents the mean ± SD; n = 3 (number of repetitions). The MIC of Cc-UDs EO was 78.124 µg/mL.

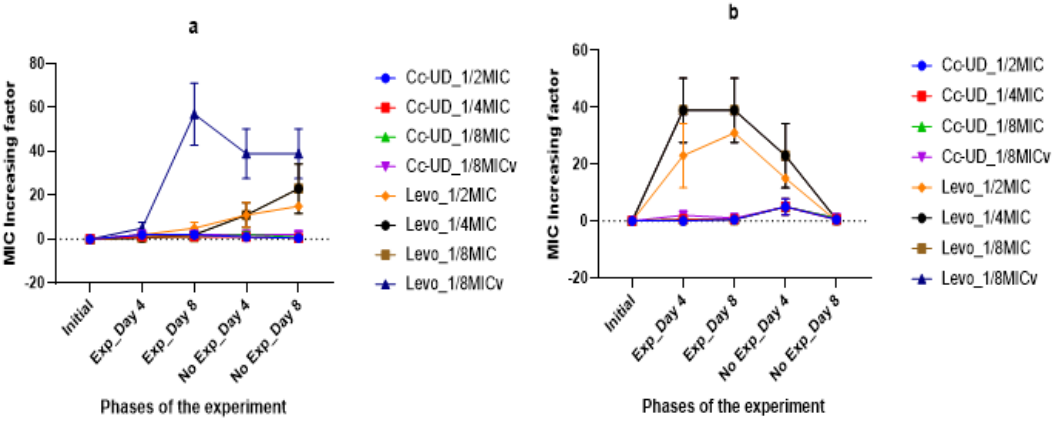

Figure 4. Effect of continuous exposure of *P. aeruginosa* isolate (a) and *S. aureus* NCTCC 12973 (b) to fixes and variable sub-inhibitory concentration of *C. citrinus* essential oil and levofloxacin.
Each point represents the mean ± SD; n = 3 (number of repetitions). The MIC of Cc-UDs EO was 78.124 µg/mL. Cc-UDs: *C. citrinus* from the University of Dschang campus; Levo: levofloxacin; Exp_Day 4: Day 4 of exposure phase; Exp_Day 8: Day 8 of exposure phase; No Exp_Day 4: Day 4 of non-exposure phase; No Exp_Day 8: Day 8 of non-exposure phase.

Table 1. Values of inhibition parameters (µg/mL) MIC and MBC, MBC/MIC ratio, and bacteriological profile of *C. citrinus* essential oils and reference antibiotics

Essential oils/Antibiotics	Inhibition parameters (µg/mL)	Bacterial strains					
		***S.a* NCTC 12973**	***S.a* isolate**	***S.pn* HM 145**	***S. py* Isolate**	***P.a* Isolate**	***K.p* ATCC-Baa 1705**
Cc-UY1	MIC	234.38	117.19	937.5	3750	937.5	3750
	MBC	2500	1875	N.D	N.D	3750	5000
	MBC/MIC	10.67	16	N.D	N.D	4	1.33
	Bacteriological profile	Bacteriostatic	Bacteriostatic	N.D	N.D	Bacteriostatic	Bactericide
Cc-UDs	MIC	78.13	117.19	234.38	468.75	78.13	234.38
	MBC	468.75	1250	937.5	5000	468.75	468.75
	MBC/MIC	6	10.67	4	10.67	6	2
	Bacteriological profile	Bacteriostatic	Bacteriostatic	Bacteriostatic	Bacteriostatic	Bacteriostatic	Bactericide
Levo	MIC	0.59	0.88	0.88	4.69	0.59	18.75
	MBC	0.59	0.88	0.88	18.75	14.06	28.12
	MBC/MIC	1	1	1	4	24	1.5
	Bacteriological profile	Bactericide	Bactericide	Bactericide	Bacteriostatic	Bacteriostatic	Bacteriostatic
Amox	MIC	3.52	0.88	75	37.5	1.76	N.D
	MBC	150	3.52	300	N.D	225	N.D
	MBC/MIC	42.67	4	4	N.D	128	N.D
	Bacteriological profile	Bacteriostatic	Bacteriostatic	Bacteriostatic	N.D	Bacteriostatic	N.D

S.a: *Staphylococcus aureus*; S.pn: *Streptococcus pneumoniae;* S. py: *Streptococcus pyogenes*; P.a: *Pseudomonas aeruginosa*; K.p: *Klebsiella pneumonia*; Cc-UY1: *Callistemon citrinus* from the University of Yaoundé I campus; Cc-UDs: *Callistemon citrinus* from the University of Dschang campus; Levo: Levofloxacin; Amox: Amoxicillin.

Conclusion

Callistemon citrinus essential oil, particularly that from the University of Dschang campus, demonstrated promising antibacterial activity against pathogens involved in respiratory infections. Its mode of action involves inhibition of protein synthesis in *P. aeruginosa* and disruption of H+-ATPase pumps in *S. aureus*. Importantly, continuous exposure did not induce resistance or persistence, in contrast to levofloxacin. These findings highlight *C. citrinus* essential oil as a potential candidate for the development of alternative antimicrobial agents. Nevertheless, chemical characterization, cytotoxicity testing, and in vivo validation are essential next steps before considering clinical applications.

Abbreviations

ATCC: American Type Culture Collection; *C. citrinus : Callistemon citrinus*; Cc-UD: *Callistemon citrinus* from the University of Dschang campus; Cc-YD: *Callistemon citrinus* from the University of Yaoundé 1 campus; CA: Chapman agar; CFU: Colony Forming Unit**;** CTA: Cetrimide agar*;* EO: Essential oil*;* EOs : Essential oils; *K. pneumoniae: Klebsiella pneumonia*; MBC: Minimal Bactericidal Concentration; MBCs : Minimal Bactericidal Concentrations**;** MHA: Mueller-Hinton agar*;* MHB: Mueller-Hinton broth; MIC: Minimal Inhibitory Concentration; MICs : Minimal Inhibitory Concentrations**;** NCTC : National Collection of Types Cultures; *P. aeruginosa: Pseudomonas aeruginosa*; *S. aureus : Staphylococcus aureus*; *S. pneumoniae: Streptococcus pneumoniae*; *S. pyogenes: Streptococcus pyogenes*

Authors' Contribution

MFYT designed the study, carried out the experiments, carried out data analysis and wrote the manuscript; ETF participated in conducting experiments and analyzing data; CFT participated in the study design and manuscript writing; ADT revised the manuscript; VBP and JPAA supervised the work; All authors have read, revised, and approved the final version of the manuscript.

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Conflict of interest

The authors declare no conflict of interest

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