

4,6,6-trimethylbicyclo[3.1.1]hept-3-ene: Analysis of the Inhibitory Effect of Monoterperene on *Pseudomonas aeruginosa* Strain

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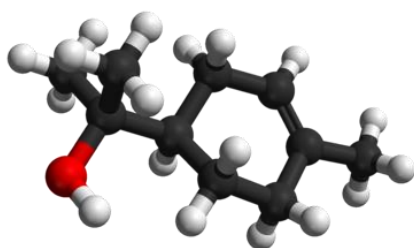
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Graphical Abstract



Abstract.

Pseudomonas aeruginosa is a ubiquitous gram-negative non-fermentative bacterial species that exhibits natural resistance to some antibiotics and antiseptics, in addition to having a high expression of virulence factors, being responsible for causing, mainly, opportunistic infections in the hospital environment. It affects the respiratory tract causing about 80% of hospital pneumonias, being able to reach skin, soft tissues, eyes, ears, bones and the urinary tract. The treatment of nosocomial infections caused by *P. aeruginosa* is based on several classes of drugs, such as: Cephalosporins, Carbapenems, Aminoglycosides, among others. However, studies point to the existence of multiresistant species, including reserve drugs, such as imipenem, thus generating a public health problem. In addition, this year the World Health Organization has released a list of ten challenging multi-resistant microorganisms that require new antibiotics, and secondly the species *Pseudomonas aeruginosa* carbapenem-resistant. Given this panorama of bacteria resistant to

multiple commercially available antibiotics, it is necessary to study new compounds with antibacterial activity. As a possibility to combat bacterial infections, the action of a natural product, the positive enantiomer of 4,6,6-trimethylbicyclo [3.1.1] hept-3-ene, also known as (+) - α -pinene, before the *Pseudomonas aeruginosa* strain ATCC 27853, using methodologies standardized by the Manual Clinical and Laboratory Standards Institute. Minimum Inhibitory Concentration (MIC), Minimum Bactericidal Concentration, and Nature Classification of Compound Effect were determined according to MBC/MIC ratio. The (+) - α -pinene was dissolved in 1% Tween 80, 5% DMSO and distilled water. In broth microdilution, the MIC was determined for the *P. aeruginosa* strain, at a concentration of 40 μ L/mL, being characterized as bacteriostatic and the concentration 4 times higher than MIC was demonstrated to be bactericidal. This experiment made it possible to observe the action of the phytoconstituent on the species of *Pseudomonas aeruginosa*, emphasizing the need for permanent studies to determine the mechanism of action and toxicity of (+) - α -pinene allowing its future use against opportunistic infections caused by *Pseudomonas aeruginosa*.

Introduction

Pseudomonas aeruginosa is a ubiquitous gram-negative non-fermentative bacterial species that exhibits natural resistance to some antibiotics and antiseptics, in addition to having a high expression of virulence factors, being responsible for mainly causing opportunistic infections in the hospital environment. It affects the respiratory tract causing about 80% of hospital pneumonias, being able to reach skin, soft tissues, eyes, ears, bones and the urinary tract [2]. The treatment of nosocomial infections caused by *P. aeruginosa* is based on several classes of drugs, such as penicillins, cephalosporins, carbapenems, aminoglycosides and quinolones. However, studies point to the existence of multiresistant species, including reserve drugs, such as imipenem, thus generating a public health problem [3-4].

In addition, this year the World Health Organization has released a list of ten multiresistant microorganisms that require new antibiotics, and secondly the species *P. aeruginosa* carbapenem-resistant. Therefore, in view of the challenge of developing new antibiotics for the growing number of super-resistant microorganisms in the hospital environment, it is necessary to research natural products with antibacterial activity to aid in the fight against superbugs [6]. Oily compounds derived from plants

are composed of several substances, including monoterpenes, which are hydrocarbons present in these natural products, which act as antimicrobial agents with therapeutic potential, highlighting the 4,6,6-trimethylbicyclo[3.1.1]hept-3-ene or also known as α -pinene, which can be observed in several proportions, including as major compound, as occurs in the oils of *Satiria trimera*, *Juniperus phoenicea*, *Cupressus sempervirens*, among other oils [8].

The objective of this study was to analyze the inhibitory effect of (+) - α - pinene against the strain *Pseudomonas aeruginosa* ATCC 27853, by determining the Minimum Inhibitory Concentration (MIC), Minimum Bactericidal Concentration (MBC) and classification of the nature of the effect of the compound according to the MBC/MIC ratio.

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Materials and Methods

The phytoconstituent, (+) - α -pinene, used in this experiment was obtained from Sigma-Aldrich do Brasil Ltda., And the solutions were prepared at the time of the tests, dissolving them first in 1% Tween 80 and DMSO in a ratio of up to 5%, and using sterile distilled water to achieve the desired concentrations.

The determination of Minimum Inhibitory Concentration was obtained by the broth microdilution technique with 96-well plates. The phytoconstituent concentrations used in this assay were 320, 160, 80, 40, 20, 10, 5, 2.5, 1.25, 0.625, 0.312 and 0.156 $\mu\text{L}/\text{mL}$. As a positive control, amikacin was used as control of sterility of the medium. Wells were used only with the culture medium, as well as the viability test of the bacterial strain with wells with culture medium plus bacterial inoculum [10]. The plates were aseptically closed and incubated at 35 ° C for 24 hours.

After the incubation period, the results were read with the addition of 20 μL of sodium resazurin solution (0.01%; w / v) (SIGMA), recognized as a colorimetric oxide-reduction indicator for bacteria. The experiments were performed in triplicate and the result was expressed by the arithmetic mean of the MICs obtained in the three trials [11].

The Minimum Bactericidal Concentration (MBC) was obtained by sowing aliquots of 20 μL of the dilutions corresponding to MIC and two immediately higher, CIMx2 and MICx4, from the contents of the wells of the microdilution plates, in Petri dishes containing Agar Müller-Hinton, which were scattered with the aid of a Drigalsky handle [12]. After sowing, the plates were incubated in an oven at 35 ° C for 24 hours. According to CLSI, CBM is considered the lowest concentration that prevented the visible growth of bacteria or allowed the formation of up to 3 Colony Forming Units (CFU). It is noteworthy that all experiments were performed in triplicate.

Results and Discussion

In this study, MIC was defined as the lowest concentration capable of visually inhibiting the bacterial growth observed in the orifices when compared to control growth [10].

Throughout the reading of the results, it can be identified that the phytoconstituent MIC was 40 $\mu\text{L} / \text{mL}$, twice the MIC equal to 80 $\mu\text{L}/\text{mL}$ and four times the MIC equal to 160 $\mu\text{L}/\text{mL}$. Meanwhile, the MIC of amikacin was 1 $\mu\text{g}/\text{mL}$, and therefore the species studied was sensitive to the positive control used in the study, since according to CLSI, *P. aeruginosa* strain is considered to be susceptible to amikacin if the MIC is present, if less than or equal to 18 $\mu\text{g}/\text{mL}$. In addition, evaluating the wells with the controls allowed to guarantee the safety of the results, since the feasibility of the studied strain was verified and the sterility of the culture medium was confirmed.

Then, the determination of the minimum bactericidal concentration (MBC) was performed, which, after reading the results, was 40 $\mu\text{L}/\text{mL}$, and therefore the (+) - α -pinene MIC was bacteriostatic, since in the inoculated plates it was possible to visualize the formation of three more colonies for the MIC, MICx2 concentrations of the phytoconstituent. In addition, for the concentration 160 $\mu\text{L}/\text{mL}$ there was no formation of visible colonies to the naked eye, being therefore bactericidal [12]. The MBC: MIC ratio was also applied, the result of which was 4: 1 (**Table 1**), characterizing the nature of the compound's effect as bacteriostatic [17].

Table 1: CIM and CBM values and classification of the nature of the antibacterial effect of 4,6,6-trimethylbicyclo[3.1.1]hept-3-ene [(+) - α - pineno]

Microorganism	(+)- α -pineno ($\mu\text{L}/\text{mL}$)		MBC:MIC	Effect
	MIC	MBC		
<i>Pseudomonas aeruginosa</i> ATCC 27853	40 $\mu\text{L}/\text{mL}$	160 $\mu\text{L}/\text{mL}$	4:1	Bacteriostática

This study is unprecedented in the evaluation of the antibacterial activity of the positive enantiomer of 4,6,6-trimethylbicyclo [3.1.1] hept-3-ene, (+) - α -pinene, against the bacterial strain of *P. aeruginosa* ATCC 27853, that there is no information in the research literature on the subject using the methodologies used for this phytoconstituent.

In the literature, Farias et al. Carried out a study with (+) - α -pinene in 2017, with concentrations ranging from 160 to 5 $\mu\text{L}/\text{mL}$, using the disk diffusion technique for *P. aeruginosa* ATCC 27853. For this strain there was no formation of an inhibition halo visible to the naked eye, so the researchers considered it resistant to all concentrations used. This shows the importance of performing other techniques for the evaluation of an organic compound, since in the present work, using the broth microdilution method, it was possible to determine the MIC (+) - α - pinene, 40 $\mu\text{L}/\text{mL}$, whereas for the disc-diffusion test this determination was not possible.

In 2010, researchers evaluated the oils of *Juniperus phoenicea* L. and *Cupressus sempervirens* L., which mostly contain α - pinene, however, in this study it was not possible to determine the phytochemical MIC for *P. aeruginosa* ATCC 27853, seen which, according to the authors, proved to be resistant [9].

Meanwhile, in 2013, in a study on the chemical composition and evaluation of antimicrobial properties of *Cupressus lusitanica* Mill. Essential oil, whose composition presents α - pinene, for bacterial strains, the researchers determined the MIC of the oil at 10% to 31.25 $\mu\text{g}/\text{mL}$ for *P. aeruginosa* ATCC 27853, and its CBM with the same value for the oil with > 10% concentration [19].

Conclusions

After the experiment, it can be concluded that 4,6,6-trimethylbicyclo [3.1.1] hept-3-ene presents antibacterial activity against the *P. aeruginosa* ATCC 27853 strain, according to the broth microdilution test, and that this action is bacteriostatic. Therefore, it is recommended to continue the studies on the mechanisms of action and toxicity of the compound so that, in the future, it can be used as a new therapeutic alternative against opportunistic infections caused by *Pseudomonas aeruginosa*.

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