

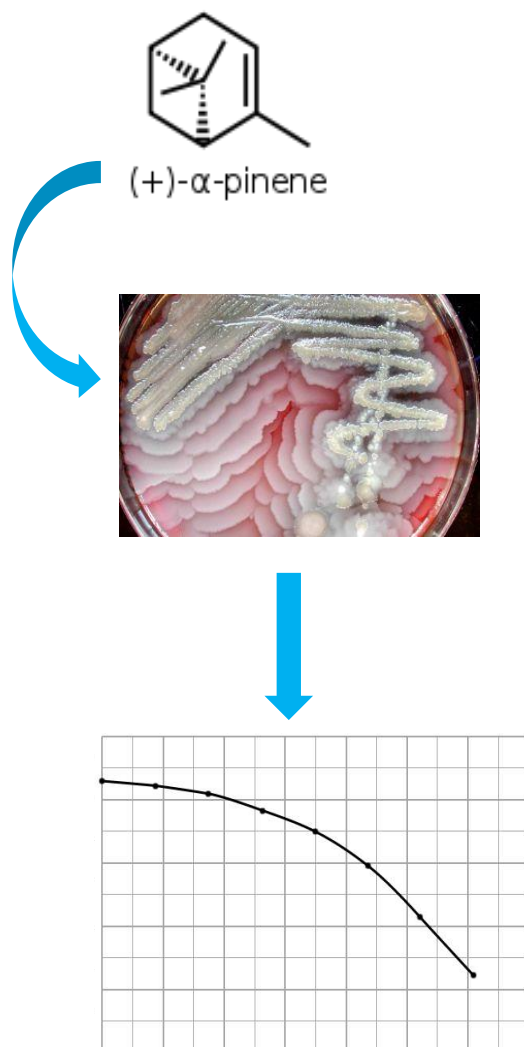
Antibacterial Potential Of The Alpha-pinene Positive Enantiomer Against The Strain *Proteus mirabilis*

Letícia de Sousa Eduardo (E-mail: leticialivesousa@gmail.com)^a, Ticiane Costa Farias (E-mail: ticiane_92@hotmail.com)^a, Gildoberg Nunes da Silva (E-mail: bergnunes22@gmail.com)^a, Francisca Patrícia da Silva Lopes (E-mail: patysilvasjp@hotmail.com)^a, Sávio Benvindo Ferreira (E-mail: saviobenvindo@gmail.com)^b.

^a Graduate Student, Center for Teacher Training (CFP), Federal University of Campina Grande (UFCG), Cajazeiras campus, Paraíba, Brazil.

^b Substitute Professor of Nursing Academic Unit, Center for Teacher Training (CFP), Federal University of Campina Grande (UFCG), Cajazeiras campus, Paraíba, Brazil.

Graphical Abstract



Abstract.

Essential oils are complex mixtures of volatile aromatic compounds derived from the secondary metabolism of plants, and which have several chemical components responsible for their therapeutic and organoleptic properties, among them, the class of terpenes, specifically alpha-pinene, a compound organic, which acts as an antibacterial agent. Among the multiresistant strains, the gram-negative species *Proteus mirabilis* is responsible for causing urinary tract infections. Thus, the present study aims to evaluate the antibacterial potential of (+) - alpha-pinene against the ATCC (American Type Culture Collection) standard strain of *Proteus mirabilis* ATCC 25933. Knowing the sensitivity of the bacterial strain, after the test of microdilution in broth, we intend to evaluate the antibacterial potential, ie the time by which the compound was able to act to eliminate the bacterial strain. For this, the present work used the methodology of Time Killing (Bacterial). In this sense, the strain was initially peeled into Mueller Hinton Agar (AMH) 24 hours prior to the test. For the analysis of the death curve in the time intervals 0, 2, 4, 8 and 24 hours of the MIC and MICx2 of the α -pinene, MIC of the amikacin and negative control, that is, the culture medium plus the bacterial inoculum. A 10 μ L aliquot of the well contents and diluted in a 0.9% physiological solution

was withdrawn, thereby forming a suspension, and an aliquot of 10 μ L of this new dilution was then withdrawn and plated on plates containing Mueller Hinton agar with the aid of a Drigalski handle. This procedure was repeated at times t0, t2, t4, t8 and t24. The plates were then incubated at $35 \pm 2^\circ \text{C}$ for 24 hours and the number of colony forming units (CFU) counted, adjusting with the dilution factor used in each procedure. It is worth noting that this work is a pioneer in the evaluation of the antibacterial activity of the positive enantiomer of α -pinene, considering that there are no reports in the literature of studies against this bacterial. The MIC of amikacin was shown to be able to totally inhibit the growth of the strain within the first two hours, and the alpha-pinene MIC inhibited the growth of *P. mirabilis* after 24 hours. Inhibition occurred progressively at times 2, 4 and 8 hours until its total inhibition at 24 hours. However, the MICx2 of the alpha-pinene was also able to inhibit the total growth of the strain, but in a less time, requiring 8 hours for its total effect. It is therefore observed that the test substance, (+) - alpha-pinene has therapeutic potential to treat infections resulting from this bacterial strain. It is hoped that this study may support the development of future research to better elucidate the mechanism of action, the clinical safety of the substance as well as the toxicity of the compound. Thus, it may be a naturally occurring compound used as a new therapeutic option in opportunistic infections caused by *Proteus mirabilis*.

Introduction

Research into new antimicrobial agents is necessary because of the emergence of resistant microorganisms and fatal opportunistic infections. Among the bacterial strains, the strain of *Proteus mirabilis*, a gram-negative anaerobic bacterial species responsible for causing opportunistic infections in the Urinary Tract stands out [1].

UTI is defined as a condition where the urinary tract is infected by pathogens that cause inflammation. It is one of the most prevalent pathologies in all age groups. It is particularly important in young, sexually active women because of the high prevalence. It is the major cause of sepsis in hospitalized patients. In fact, urinary tract infection can be considered as a syndromic diagnosis, which encompasses several clinical conditions such as asymptomatic bacteriuria, urethritis, cystitis, pyelonephritis, prostatitis, renal and peri-renal abscess, in various contexts of presentation [2-3].

The genus *Proteus* is divided into 5 species: *Proteus vulgaris*, *Proteus mirabilis*, *Proteus penneri*, *Proteus myxofaciens* and *Proteus hauseri*. These microorganisms are usually found in the intestinal microbiota of man and animals, soil and polluted water. The *Proteus mirabilis* strain is a Gram-negative bacterium, belonging to the family Enterobacteriaceae, where the movements of its flagella through the surface of the solid medium have a "veil" appearance [4].

In this context, essential oils are complex mixtures of volatile aromatic compounds derived from the secondary metabolism of plants and have several chemical components responsible for their therapeutic and organoleptic properties, among them there is a class called monoterpene such as alpha pinene [5].

In this way, searching for natural alternatives to combat multiresistant strains, essential oils such as: *Juniperus phoenicea*, *Salvia officinalis*, *Cupressus sempervirens*, *Mutellina purpurea*, *Thymus vulgaris*, which have monoterpene alpha-pinene, which has antibacterial activity [6]. -7]. In this sense, this research becomes relevant as it seeks a new therapeutic alternative, using the phytoconstituent in clinical practice, using it alone or in association with antibiotics, making it possible to effectively expand the arsenal to combat bacterial infections.

Thus, the development of research of this kind can contribute significantly to the development of the health field worldwide, finding more effective substances in the race against resistance and the appearance of pathogenic microorganisms. Therefore, it is intended with this study to evaluate the antibacterial potential of the alpha-pinene compound against *Proteus mirabilis* (ATCC 25933).

Materials and Methods

The laboratory tests were carried out from February to September 2017. The test substance, the phytoconstituent (+) - alpha-pinene obtained from Sigma-Aldrich do Brasil Ltda and acquired with its own resources. It is noteworthy that the compound was prepared at the time of the tests, using two solvents in the dissolution of the phytoconstituent, such as: 1% Tween 80 and Dimethyl sulfoxide (DMSO) in a proportion of 5%, in addition to the sterile distilled water to reach the concentrations.

In this context, the gram-negative *Proteus mirabilis* ATCC 25933 standard strain was used for the antimicrobial tests in the antibacterial assay using the Time-Kill method. Therefore, in the execution of the tests, it used the culture media Müller-Hinton Agar and Müller-Hinton Broth (HIMEDIA, India). Before using them, the media were solubilized in distilled water and autoclaved at 121° C for 15 minutes.

In addition, after the incubation period, the bacterial inoculum was prepared, where a direct suspension, in saline, of selected isolated colonies was made. The suspension was then adjusted to show turbidity similar to the McFarland 0.5 scale, which corresponds to 1×10^8 CFU/mL [8].

For assay performance, the strains were first primed on Mueller Hinton Agar 24 hours prior to the test. For the analysis of the death curve of *P. mirabilis* in the time interval $t = 0$ hour, $t = 2$ hours, $t = 4$ hours, $t = 8$ hours et $t = 24$, of the MICs and MICx2 of the α -pinene, ampicillin MIC and negative control (culture medium + bacterial inoculum). An aliquot of 10 μ L was withdrawn from the well contents and seeded on plates containing Müller Hinton agar with the aid of a Drigalski loop. This procedure was repeated at times t_0 , t_2 , t_4 , t_8 and t_{24} . The plates were then incubated at 35 ± 2 ° C for 24 hours and the number of colony forming units (CFU) counted, adjusting with the dilution factor used in each procedure. The results were presented in log 10 CFU/mL as a function of time to verify the rate and extent of antibacterial activity at the various concentrations of (+) - α - pinene.

All experiments were performed in triplicate and the results were expressed as the mean \pm standard error of the mean (e.p.m). Differences between groups were assessed by the paired t-test. Differences were considered significant when $p < 0.05$.

Results and Discussion

Currently, there are several methods to evaluate the antibacterial activity of natural products, and the most known methods include agar diffusion method, macrodilution method and microdilution. Thus,

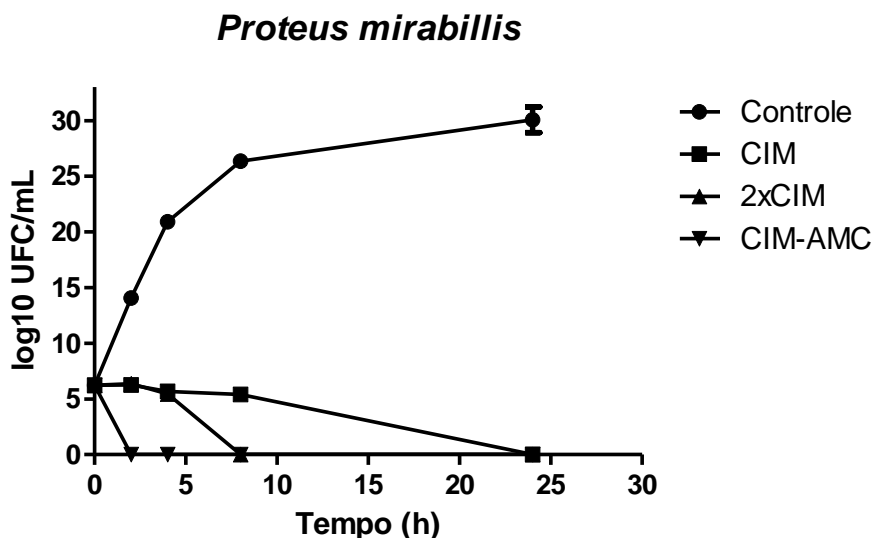
a screening was performed to evaluate the activity of alpha-pinene against the strain of *P. mirabilis* by disc diffusion methodology with concentrations ranging from 160 to 5 $\mu\text{L/mL}$ and found that there was no halo formation of visible inhibition to the naked eye, so the researchers considered it resistant to all concentrations used [9].

This evidences the need to use different methodologies to evaluate a phytoconstituent, since another study using broth microdilution methodology was able to determine the MIC of alpha-pinene, which was 80 $\mu\text{L/mL}$, whereas for the disc- this determination was not possible [10].

Thus, by attempting to evaluate the antibacterial potential of the compound, i.e. the time by which it begins to develop its effect against the strain, the kinetics methodology of death was employed and it was found that the ampicillin MIC was able of totally inhibiting the growth of the strain between the first two hours, which was already expected, since this antibiotic has a good action spectrum, that is, they act reaching a large number of microorganisms at the therapeutic doses.

As for the alpha-pinene MIC, it was found that it inhibited the growth of *P. mirabilis* after 24 hours. Inhibition occurred progressively at times 2, 4 and 8 hours until its total inhibition at 24 hours. However, the MICx2 of alpha-pinene was also able to inhibit the total growth of the strain, but in a shorter time, thus requiring only 8 hours for its total effect, as represented in **Graphic 1**.

Graphic 1: Death curve of the *P. mirabilis* strain (ATCC 25933) against the action of the (+) - α -pinene MIC and MICx2 concentrations and positive and negative controls.



A study using *S. officinalis* essential oil had alpha-pinene in its composition and found that the growth of the *P. mirabilis* strain was inhibited after 1h of contact with concentrations of 5 and 10 $\mu\text{L/mL}$, but the bacteria recovered its growth after 24h [11]. Proving that the compound acted faster, however, it was not able to permanently eliminate the bacterial species. Another research developed to evaluate the antimicrobial effects of the different isomers and enantiomers of the monoterpenes as (+) α -pinene and (+) β -Pinene against the gram-negative bacteria species, *Proteus vulgaris* and *C. albicans* fungus, where it was possible to verify that Time-Kill curves showed that (+) - α -pinene and (+) - β -pinene were highly toxic to *C. albicans*, killing 100% of the inoculum in 1 h. In contrast, the bactericidal effect occurred after 6h. In combination with commercial antimicrobials, ciprofloxacin plus (+) - α -pinene or (+) - β -pinene showed synergistic activity against *P. vulgaris* strain, whereas an indifferent effect against all fungi was detected when amphotericin B was combined with positive pinene enantiomers [12].

It is worth noting that this work is a pioneer in the study of the antibacterial effect of the positive enantiomer of α -pinene against the species of *Proteus mirabilis* ATCC 25933, since there is no information in the literature of research on the subject, using the methodologies used for this phytoconstituent.

Conclusions

According to the methodology used, it is concluded that the positive enantiomer of alpha-pinene has therapeutic potential to act against the bacterial species of *Proteus mirabilis* (ATCC 25933). In addition, due to the pioneering nature of the present study, there was a shortage of research that used the kinetic methodology of death, which interfered in the deepening of the discussions. Therefore, it is suggested that further studies conducted new studies using other methodologies, in order to determine more precisely the antimicrobial activity and the existence of possible toxic effects of this phytoconstituent. In addition, it is expected that this study may contribute to the development of future research related to the study of antimicrobial activity of natural products.

References

1. Armbruster CE, Mobley HLT. *Merging mythology and morphology: the multifaceted lifestyle of Proteus mirabilis*, Nat Rev Microbiol., 10, 743–754, 2012. DOI:: 10.1038/nrmicro2890.
2. Costa LC; Belém LF; Silva PMF, et al. Infecções urinárias em pacientes ambulatoriais: prevalência e perfil de resistência aos antimicrobianos. RBAC, vol. 42(3): 175-180, 2010.
3. Ficher KN, Araújo AAN, Houly SGB et al. Síndrome da urina roxa na bolsa coletora: relato de caso por Streptococcus agalactiae e revisão da literatura. J Bras Nefrol 2016;38(4):470-472
4. Imelouane, BH, Amhamdi JP, Wathelet M, Ankit K. Chemical Composition and Antimicrobial Activity of Essential Oil of Thyme (*Thymus vulgaris*) from Eastern Morocco. International Journal of Agriculture and Biology 2009 Vol.11 No.2 pp.205-208 .
5. Ramdani I M, Lograda T, Silini H. Antibacterial Activity of Essential oils of Juniperus phoenicea from Eastern Algeria. Journal of Applied Pharmaceutical Science Vol. 3 (11), pp. 022-028, November, 2013. DOI: 10.7324/JAPS.2013.31105
6. Ravichandran PK, Manoj P. Study on the growth inhibitory effects of Puromycin and Doxorubicin on tumor cell lines (A549, HepG2, HT29 and K562) using Cell Titer-Glo and Alamar blue (Resazurin) based cell viability assays. Indian Journal of Research in Pharmacy and Biotechnology, 2(1), 2014.
7. Clinical Laboratory Standards Institute (CLSI). Metodologia dos Testes de Sensibilidade a Agentes Antimicrobianos por Diluição para Bactéria de Crescimento Aeróbico: Norma Aprovada - Sexta Edição. M7-A6 Vol. 23 No. 2 Substitui a Norma M7-A5 Vol. 20 No. 2 .
8. Klepser, ME, Ernst EJ, Lewis RE, Ernst ME, Pfaller MA. Influence of test conditions on antifungal time-kill curve results: proposal for standardized methods. Antimicrobial Agents and Chemotherapy, v. 42, p. 1207-1212, 1998.
9. Farias TC, Eduardo LS, Ferreira SB et al. Screening Antibacteriano Do (+)-A – Pineno Frente A Cepas Bacterianas Gram Negativas. II Congresso Brasileiro de Ciências da Saúde, 2017, Campina Grande. Anais II CONBRACIS. Campina Grande - PB: Realize, 2017. v.1.
10. Farias, TC, Eduardo LS, Ferreira SB et al. Atividade Antibacteriana In Vitro Do (+) – A – Pineno Para A Espécie *Proteus mirabilis*. V Congresso Nacional de Saúde e Meio Ambiente, 2017, João Pessoa. V CINASAMA. João Pessoa-PB, 2017. v.1.

11. Khalil R, Li Zheng-Guo. Antimicrobial activity of essential oil of *Salvia officinalis* L. collected in Syria. African Journal of Biotechnology Vol. 10(42), pp. 8397-8402, 8 August, 2011. DOI: 10.5897/AJB10.2615.
12. Silva ACR, Lopes PM, Azevedo MMB et al. Biological Activities of α -Pinene and β -Pinene Enantiomers. Molecules, 17, 6305-6316, 2012.