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MODULATIVE AND ADAPTIVE EFFECT OF (+)-ALPHA-PINENE IN FRONT OF COMMERCIAL ANTIMICROBIALS IN *Staphylococcus aureus* STRAINS

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ABSTRACT

The discovery of new molecules with antimicrobial activity and the understanding of the mechanisms of action involved are important strategies against multiresistant pathogens.Given this perspective, the positive α-pinene enantiomer appears as an alternative to combat them, as it inhibited the growth of microorganisms, including strains of *Staphylococcus aureus*, which gives it the possibility of its use as an isolated antimicrobial agent or in combination with other drugs.The aim of this study is to evaluate the sensitivity profile of *S. aureus* ATCC 25923 strain against clinical antimicrobials associated with (+) - α-pinene and how it behaves after successive exposures to subinhibitory concentrations of phytoconstituent.MIC was determined according to the Clinical Laboratory Standards Institute, the study of the modulating effect of (+)-α-pinene on the activity of antibiotics of clinical use in *S. aureus* strains, and the analysis of the adaptation of strain to the monoterpene tested.As a result, it was observed that for penicillin, rifampicin and nitrofurantoin there was no final change in adaptation to phytoconstituent; For vancomycin, cefoxitin, ciprofloxacin and gentamicin, the effect was considered indifferent, as no change in sensitivity was observed.Meanwhile, for the other antimicrobials the strain studied was resistant.It wasnoticed through this study that, after successive exposures to subinhibitory concentrations, the *S.aureus* strain suffered from bacterial stress and acquired resistance, consequently presenting a reduction in the efficacy of antimicrobials, which can be noticed by the increase of MIC against the phytoconstituent, as well as phenotypic changes visualized in cultures subjected to incubation.

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INTRODUCTION

Bacterial resistance is a major challenge in world health, affecting both developed and emerging countries, as there is a significant increase in the number of bacterial strains increasingly resistant to available antibiotic therapy. This makes it difficult to control infections in a hospital environment, resulting in longer hospital stays, the need to use more expensive medicines and health services, and increased mortality of patients, especially the most vulnerable, such as the elderly, children, immunosuppressed, patients admitted to the intensive care unit (ICU), among others (SANTOS, 2004; REIS *et al*., 2013).

With the advancement of studies on herbal medicines, the use of plants as natural sources of new compounds that have the potential to combat bacterial infections has been discussed, in an attempt to overcome the obstacles to the treatment of diseases caused by bacterial resistance (WANNMACHER, 2004). Looking back over the decades from the 1940s to the mid-1960s, we can find several discoveries of antimicrobials developed from experiments with natural products that have proven to be effective agents in the treatment of gram-positive bacteria of antimicrobials: β-lactams, aminoglycosides, tetracyclines, macrolides, peptides, among others (BRUNTON *et al*., 2019). Thus, the discovery of new molecules with antimicrobial activity, as well as the understanding of the

mechanisms of action involved, are important strategies against multiresistant pathogens. Given this perspective, the positive α-pinene enantiomer appears as an alternative to combat them, as it inhibited the growth of microorganisms, including strains of *Staphylococcus aureus*, which gives it the possibility of its use as an isolated or antimicrobial agent. in combination with other drugs (DHAR, 2014; EDUARDO *et al*., 2014). Therefore, this research aims to evaluate the modulating effect of (+) - α-pinene on the activity of synthetic antimicrobials, as well as to investigate whether *S. aureus* strain develops cross-resistance to synthetic antimicrobials after exposure to subinhibitory concentrations of phytochemicals.

MATERIALS AND METHODS

Experimental Site: The laboratory tests were performed at the Laboratory of Microbiology, Parasitology and Pathology of the Teacher Training Center of the Federal University of Campina Grande, campus of Cajazeiras-PB.

Tested Substance: Phytoconstituent (+) - α - pinene (+ AP) was obtained from the company Sigma-Aldrich do Brasil Ltda., Located in São Paulo, Brazil, acquired with its own resources.Solutions were prepared at the time of testing by first dissolving them in 1% Tween 80 and 5% DMSO, and using sterile distilled water to achieve desired concentrations (CLEELAND; SQUIRES, 1991).

Microorganism: The tests were performed on the *Staphylococcus aureus* ATTC (American Type Culture Collection) 25923 strain.The strain was kept on Müeller-Hinton agar (AMH) at a temperature of 4 °C until preparation of the inoculum for testing.

Commercial Antimicrobials: For the modulation and adaptation tests, discs containing the commercial antimicrobial (ATM) were used: CPM - Cefepime 30 µg, CFO - Cefoxitin 30µg, CIP - Ciprofloxacin 5µg, CLO - Chloramphenicol 10µg, CLI - Clindamycin 2µg, ERI - Erythromycin 15 µg, GEN - Gentamicin 10 µg, NIT - Nitrofurantoin 300 µg, OXA - Oxacillin 1 µg, PEN - Penicillin G 10 µg, RIF - Rifampicin 5 µg, SUT - Sulfazotrim 23,75 / 1,25 µg, TET - Tetracycline 30 µg and VAN - Vancomycin 30 µg. The discs were purchased from Cecon®, São Paulo - SP.

Culture Mediums: In the performance of the tests to evaluate the phytoconstituent antibacterial activity, the culture media used were Mueller-Hinton Agar (MHA), Müeller-Hinton Broth (MHB) and BHI Broth (Brain and Heart Infusion Broth) (HIMEDIA, India).Prior to use, the media was solubilized in distilled water and autoclaved at 121ºC for 15 minutes, according to the manufacturer's guidelines.

Bacterial Inoculum: After the incubation period, the inoculum was prepared by making a direct suspension in saline of selected isolated colonies.Then, the suspension was adjusted to show turbidity similar to the McFarland 0.5 scale, which corresponds to 1 x 10^8 CFU/mL (CLSI, 2015).A sterile cotton swab was plunged into the adjusted suspension, rotated several times and tightened firmly against the inner wall of the tube above liquid level to remove any excess inoculum.The dry surface of the Müeller-Hinton Agar (AMH) plate was inoculated by rubbing the swab across the sterile surface of the medium.The procedure was repeated by rubbing three more times, rotating the plate approximately 60° each time to ensure even distribution of the inoculum.At the end, the swab was run around the edge of the agar plate twice, closing the inoculum.

Determination of Minimum Inhibitory Concentration (MIC): MIC determination was performed by the sterile 96-well plate microdilution with cap (CLEELAND; SQUIRES, 1991; ELOFF, 1998; HADACEK;GREGER, 2000).In each well of the plate, 100 µL of double concentrated Müeller-Hinton Broth (MHB) was added.Then 100 µL of the (+)-α-pinene solution at the initial concentration of 10 µL/mL were dispensed into the wells of the first line of the plate.And by means of a serial dilution of two, concentrations of 5, 2.5, 1.25, and 0.625 µL/mL were obtained, so that in the first line of the plate was found the highest concentration and Lastly, the lowest concentration.Finally, 10 µL of the bacterial inoculum was added to the wells, each column of the plate corresponding to one strain.In addition, a medium sterility control was performed, in which 200 µL of HCM were placed in a hole in the absence of bacterial suspension.In parallel, the same procedure was performed for the standard antibacterial Amikacin. The plates were aseptically closed and incubated at 35 ± 2 °C for 24 hours for reading. After the incubation time, the results were read. 20 µL of sodium resazurin solution (0.01%; w/v) (SIGMA), recognized as a colorimetric oxide-reduction indicator for bacteria, was added.MIC was defined as the lowest concentration capable of visually inhibiting the bacterial growth observed in the orifices when compared to the control growth.The experiments were performed in triplicate and the result was expressed by the arithmetic mean of the MICs obtained in the three trials (SANTOS; HAMDAN, 2005).

Study of the Modulating Effect of (+)-α-Pinene: The modulating action of monoterpene on the antimicrobial activity of antibiotics for clinical use on standard strains of *S. aureus* ATCC 25923 was determined by the modified disc diffusion method (BAUER, 1966; OLIVEIRA, 2006). Cultures containing approximately 1.5 x 10^8 CFU/mL were sown on the surface of Müeller-Hinton Agar (AMH). Commercial discs of antimicrobial agents (ATB) were applied to the surface of the medium and to each disc were added 20 µL of the MIC (+)-α-pinene (+AP), previously determined. To evaluate the effect of the +AP-ATM combination, ATM disks without the addition of +AP were tested. After incubation of the plates at 35 ° ± 2 °C for 24 hours, the diameter of the microbial growth inhibition halos was read.The halo diameters formed by each AP+ATM association were compared to those determined by the ATM alone.A synergistic effect was considered when the combination showed an increase in inhibition halo diameter ≥ 2 mm;antagonistic effect when the diameter of the halos determined by the combination was smaller than that of the isolated ATM;and indifferent effect, when the combination demonstrated an increase in the ATM halo diameter <2mm (CLEELAND; SQUIRES, 1991).

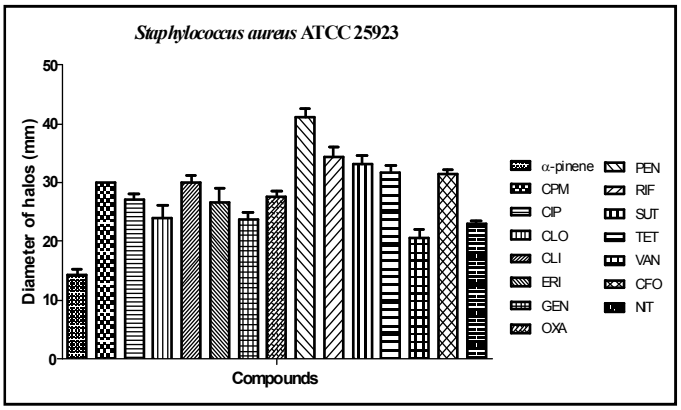
Adaptation of *Staphylococcus aureus* to Monoterpene (+)-α-Pinene: To investigate the effect of subinhibitory concentrations of the positive α-pinene enantiomer on the sensitivity profile of the *Staphylococcus aureus* ATCC 25923 strain, the adapted experimental protocol of ULTEE *et al.* (2000) and MCMAHON (2007) was used. Initially, bacterial pealing was performed and incubated At 35 ± 2°C for 24 hours. After that, the microorganism pre-exposure tests were performed to the phytoconstituent and the commercial ATM

by the disc diffusion method, besides the 96-hole microdilution microdilution method, according to the previous experiments to evaluate the amikacin and +AP MIC. Petri dishes as well as the microplate were incubated at 35 ± 2 ° C for 24 hours. The following day, the results were read by measuring the halo diameters obtained in the pre-exposure experiment, as well as obtaining the concentrations of amikacin and 4xCIM, 2xCIM, MIC, MIC/2 and MIC/4 of the AP. Then, an aliquot of the sample, subcultured in MHB with the concentration of the CIM/2 phytoconstituent, was plated on an MHA plate free of any antibacterial product, which was incubated again for 24 hours at 35 ± 2 °C. called 1st exposure.It is noteworthy that to perform this culture, inocula were prepared from these strains adjusted to the 0.5 McFarland scale tube.At this point, the suspensions of the new strains supposedly adapted to the products were obtained. In this phase, as well as in the subsequent experiments (2nd and 3rd exposures), the analysis of the new sensitivity profile of these adapted strains against the standard antimicrobials was performed, performing the disc diffusion assays and determining their sensitivity to these adapted strains(BAUER, 1966). A microorganism control was performed in parallel, where in CMH the products were not added and the microorganism grew without any change in sensitivity to the products.Thus, one can compare the sensitivity profile of both treatment groups and confirm whether or not there was an adaptation of *S. aureus* strains to the tested product.The experiment was performed in triplicate and the results expressed as mean and standard error.

Statistical Analysis: All experiments were performed in triplicate. Results were subjected to statistical treatment using GraphPad Prism 5.0 software (GraphPad Software, Inc., San Diego, CA). Data were submitted to analysis of variance (ANOVA) and expressed as mean + standard deviation. Differences were assessed by paired t-test. Differences were considered significant when $p< 0.05$.

RESULTS AND E DISCUSSION

Since it is of an oily nature, the preparation of the dilution of the phytoconstituent was carried out with the aid of surfactants, which, according to the literature, these DMSO and Tween 80 solvents do not have antimicrobial activity for the concentrations used, 5% and 1%, respectively (BRITO *et al*., 2017; SILVA*et al*., 2017). After carrying out the experiments, differences in the mean diameter of the inhibition halos (HI) can be observed before and after the association with the phytochemical, so that the strain of *S. aureus* ATCC 25923 proved to be sensitive to all antibiotics before and after the association with (+)-α-pinene, that is, the association with antimicrobials with the phytoconstituent did not lead to a qualitative change in the sensitivity profile of the microorganism. Therefore, in figure 1, it was possible to trace the sensitivity profile of *S. aureus* for monoterpene and for commercial antimicrobials. For the tested strain, the following values of the diameters of the growth inhibition halos were found: (+) - α-pinene: 14.33 mm (+ 1.53 mm), cefepime: 30.00 mm (+ 0.00 mm), ciprofloxacin: 27.00 mm (+ 1.73 mm), chloramphenicol: 24.00 mm (+ 3.46 mm), clindamycin: 30.00 mm (+ 2.00 mm), erythromycin: 26.67 mm (+ 4.16 mm), gentamicin: 23.67 mm (+ 2.08 mm), oxacillin: 27.67 mm (+ 1.53 mm), penicillin G: 41.00 mm (+ 2.65 mm), rifampicin: 34.33 mm (+ 3.06 mm), sulfazotrim: 33.00 mm (+ 2.65 mm), tetracycline: 31.67 mm (+ 2.08 mm), vancomycin: 20.67 mm (+ 2.31 mm), cefoxitin: 31.33 mm (+ 1.53 mm) and nitrofurantoin: 23.00 mm (+ 1.00 mm). Then, the antimicrobial activity modulation test associated with the phytoconstituent was carried out. The results of this test were plotted in Figure 2, which shows a comparison between the mean sizes of HI pre and post-association with (+) - α-pinene and its association with the ATMs under study.



α-pinene: (+)-alpha-pinene, CPM: Cefepime 30µg, CIP: Ciprofloxacin 5µg, CLO: Chloramphenicol 10µg, CLI: Clindamycin 2µg, ERI: Erythromycin 15 µg, GEN: Gentamycin 10 µg, OXA: Oxacillin 1µg, PEN: Penicillin G 10 µg, RIF: Rifampicin 5 µg, SUT: Sulfamethoxazole + Trimethoprim 23.75/1.25 µg, TET: Tetracycline 30 µg, VAN: Vancomycin 30 µg, CFO: Cefoxitin 30µg, NIT: Nitrofurantoin 300 µg.

Fig. 1. *Staphylococcus aureus* ATCC 25922 sensitivity profile against monoterpene (+) - α-pinene and commercial antimicrobials

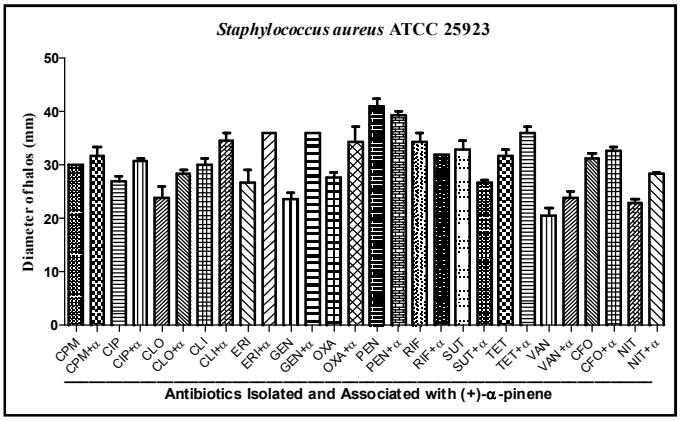
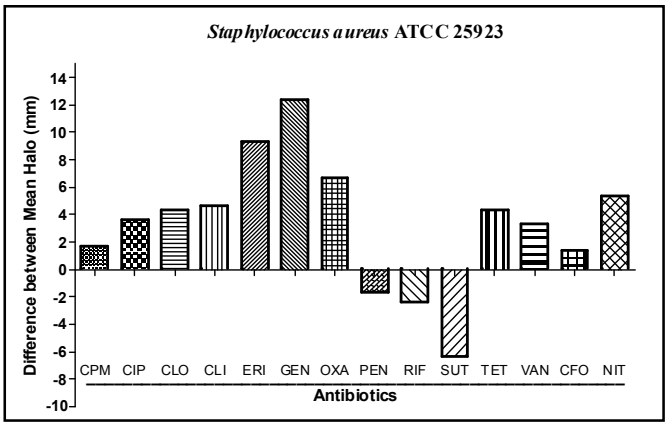


Figure 2. Comparison of the sizes of the diameters of zones of inhibition of bacterial growth pre and post-exposure (+) - α-pinene and their association with commercial antimicrobial against *S. aureus* ATCC 25923

After the association, the values of the diameters of the growth inhibition halos were changed to: cefepime: 31.67 mm (+2.89 mm), ciprofloxacin: 30.67 mm (+1.56 mm), chloramphenicol: 28.33 mm (+1.53 mm), clindamycin: 34.67 mm (+2.31 mm), erythromycin: 36 mm (+0.00 mm), gentamicin: 36 mm (+0.00 mm), oxacillin: 34.33 mm (+5.13 mm), penicillin G: 39.33 mm (+1.16 mm), rifampicin: 32 mm (+0.00 mm), sulfazotrim: 26.67 mm (+1.16 mm), tetracycline: 36.00 mm (+2.00 mm), vancomycin: 24.00 mm (+2.00 mm), cefoxitin: 32.67 mm (+1.16 mm) and nitrofurantoin: 28.33 mm (+0.58 mm). When analyzing the gross values of the tests, a decrease in the growth inhibition halos (HI) is identified after the addition of (+)-α-pinene for ATM ATM penicillin G, rifampicin and sulfazotrim; an increase in HI for ATMs ciprofloxacin, chloramphenicol, clindamycin, erythromycin, gentamicin, oxacillin, tetracycline, vancomycin and nitrofurantoin; and for

ATMs cefepime and cefoxitin, there were no changes in the diameters of HI. Then, the difference between the HI averages before and after exposure to the phytochemicals was subjected to statistical analysis (Figure 3).



CPM – Cefepime 30µg, CIP – Ciprofloxacina 5µg, CLO – Cloranfenicol 10µg,CLI – Clindamicina 2µg, ERI – Eritromicina 15 µg, GEN – Gentamicina 10 µg, OXA – Oxacilina 1µg, PEN – Penicilina G 10 µg, RIF – Rifampicina 5 µg, SUT – Sulfametoxazol + Trimetoprim 23,75/1,25 µg, TET – Tetraciclina 30 µg, VAN – Vancomicina 30 µg, CFO – Cefoxitina 30µg, NIT – Nitrofurantoína 300 µg.

Figure 3. Difference between the mean diameter of the growth inhibition halos of commercial antimicrobials before and after association with (+) - α-pinene against the strain of *S. aureus* ATCC 25923

This study appears to be a pioneer in the evaluation of the resistance profile and the modulating effect of (+)-α-pinene against strains of *Staphylococcus aureus*, considering that there are no reports in the literature on studies on this theme.
In the literature, there are studies using essential oils extracted from plants that have the racemic mixture of α-pinene, either as a major phytochemical constituent or not. In addition, research has evaluated the activity of the positive and negative enantiomers of α-pinene against fungal strains and Methicillin-Resistant *Staphylococcus aureus* (MRSA) (SILVA *et al*., 2012). Researchers evaluated the in vitro antimicrobial activity of (+)-α-pinene against *S. aureus* ATCC 25932, in which the researchers carried out the determination of MIC, characterization of bacterial activity, whether bactericidal or bacteriostatic, and construction of the bacterial death curve, demonstrating that the phytoconstituent is a promising natural product to combat the gram-positive strain (EDUARDO *et al*., 2018). One study described the activity of eugenol, β-pinene and α-pinene against *S. aureus* ATCC 13150, *S. aureus* ATCC 6538, *S. aureus* ATCC 25932 and *S. aureus* ATCC LB 126, using the broth microdilution method to determine MIC and subsequently studied the bacterial death curve. The authors found values for bactericidal MICs, whose obtained death curves showed the eradication of bacterial colonies in a 24-hour period (LEITE *et al*., 2007).

Researchers addressed the activity of *Mutellina purpurea* essential oil and its isolated major phytochemical constituent, α-pinene, against strains of *S. epidermidis* ATCC 12228 and ATCC 35984. Concentration values for oil and phytochemicals capable of eradicating biofilm formation were determined. In view of the data found, the researchers concluded that both had a good activity against the biofilms formed, however with a preponderance of the response of *M. purpurea*, when compared with the effect of the isolated phytoconstituent. Thus, the authors suggested that α-pinene may be responsible for the antibacterial activity of the oil, however, that the synergistic effect between α-pinene and the other phytocontents of *M. purpúrea* has better antimicrobial potential (SIENIAWSKA, 2013). Another study described the antimicrobial activity of the essential oil *Rosmarinus officinalis* L. isolated and in association with aminoglycosides against multi-resistant strains of *S. aureus* ATCC 25923 and *E. coli* ATCC 10.536. In this study, evaluating the gram-positive strain, the researchers found changes in the bacterial sensitivity profile, showing MIC values for amikacin of 39.1 µg/mL (intermediate) and, after the association, amikacin + phytoconstiuinte presented MIC of 9.8 µg/mL (sensitive). The same happened with gentamicin, which had a MIC of 5.0 µg/mL (intermediate) and, after the association, gentamicin + phytochemicals presented a MIC of ≤ 1.2 µg/mL (sensitive). The MIC found for the isolated oil was ≥ 1,024 µg/mL (BARRETO *et al*., 2014).

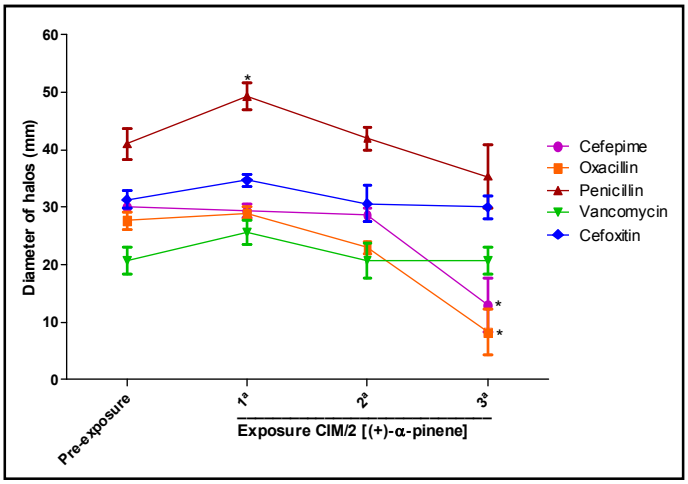


Figure 4. Modulation of the adaptive response of *Staphylococcus aureus* against antimicrobials that act on the cell wall after exposure to CIM/2 of (+)-α-pinene

Figure 4 shows the modulation of the adaptive response of *S. aureus* to antimicrobials that act on the cell wall after exposure to CIM/2 of (+)-α-pinene, so that cefepime, as well as oxacillin , tested separately, showed significant difference only from the 2nd to the 3rd exposure, that is, after 3 exposures the strain decreased its sensitivity to antimicrobials, becoming resistant. Meanwhile, for penicillin, there was an increase in the sensitivity of the micro-organism to the drug at the 1st exposure, but then, at the 2nd and 3rd exposures, the micro-organism returned to the sensitivity profile it presented before the exposure, concluding that the modulation occurred without final modification. Regarding vancomycin and cefoxitin, its effects were considered indifferent, since there was no change in sensitivity. Others working in the literature demonstrate the therapeutic potential of combining the ability of natural compounds. A study was developed on the antimicrobial activity of Tanreqing - a product made up of five Chinese herbs that is widely used in traditional Chinese medicine when used to treat respiratory tract infections - in association with commercial antimicrobials against methicillin-resistant strains of *S. aureus* ATCC 43300. In this work, using the broth microdilution method, the researchers determined the MIC of vancomycin alone, which was 2.5 µg/mL, but realized that when adding Tanreqing, the MIC of vancomycin reduced to 1.25 µg/ml. In addition, another stage of the research was the evaluation of the death curve, in which the authors used subinhibitory concentrations of the antimicrobials alone

without finding significant activity, however, when combining the two antimicrobials (½ vancomycin CIM associated with ½ CIM Tanreqing), a synergistic effect capable of eradicating MRSA in 24 hours was perceived (YANG, 2018). A group of researchers reported in their work that resistance to cephalosporins and penicillins occurs through the hydrolysis of the peptide bond in the beta-lactam ring, inactivating the drug. This mechanism induces the production of beta-lactamases, which may be related to changes in the blaZ plasmid gene. In addition, another mechanism cited by the authors is the reduction of the affinity between the PBP bonds expressed by the mecA gene and the resistance of *S. aureus* to vancomycin is expressed through the vanA gene, which promotes a change in the cell wall (MAYERS, 2009).

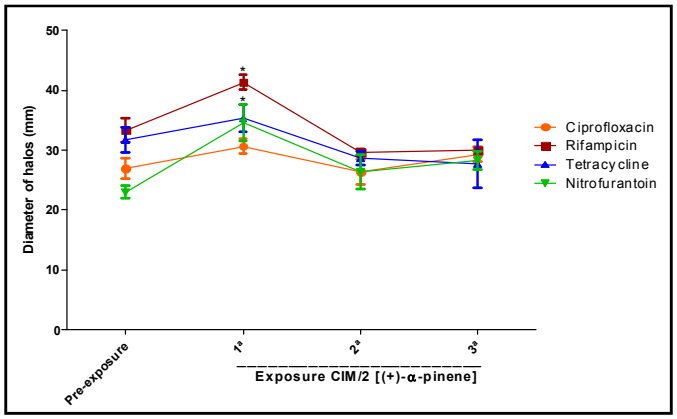


Figure 5. Modulation of the adaptive response of *S. aureus* against antimicrobials that act on bacterial nucleic acid after exposure to CIM/2 of (+)-α-pinene

In Figure5, it is possible to observe the modulation of the adaptive response of *S. aureus* against antimicrobials that act on bacterial nucleic acid after exposure to CIM/2 of (+)-α-pinene. After the first exposure, rifampicin and nitrofurantoin, there was an increase in his sensitivity to the drug. However, at the 2nd and 3rd exposure, the microorganism returned to the initial sensitivity profile, concluding that the modulation occurred without final modification. Meanwhile, it was possible to observe that both ciprofloxacino and tetracycline had their effects were considered to be indifferent, since they did not present any change in sensitivity. A study investigated the association between phytotherapics and antimicrobials in the prevention and control of biofilms formed by strains of *S. aureus* SA 1199B, with the aim of observing possible action of these natural products as adjuvants in inhibiting biofilm, as a resistance modifying agent or in combination able to change the permeability of the bacterial cell wall, thus improving the therapeutic activity of the commercial antimicrobial. In addition, the study sought to assess whether subinhibitory concentrations of antibiotics can affect responses or alter bacterial gene expression, thus providing the emergence of resistance/tolerance to currently available antimicrobial therapies. Thus, the researchers determined the MIC of the antimicrobials (ciprofloxacin, erythromycin and tetracycline) and of five herbal medicines alone and then in combination against the gram-positive strain, the results of which showed that all herbal medicines associated with the antibiotics under study had good control of the biofilm formation, in particular the combination of ciprofloxacin and quinine (ABREU *et al.*, 2016). Researchers also evaluated the resistance profile and modulating effect of the essential oil *Croton grewioides*, as well as the racemic mixture of α-pinene (which is part of the oil previously mentioned as a major compound), against *S. aureus* strain. After the experiment, the researchers found that α-pinene modulated tetracycline activity, so that the MIC found for the isolated antimicrobial was 32 µg/mL, while the MIC for the association between tetracycline and the phytochemical was 1 µg/mL, and the MIC of the antibiotic with Croton grewioides was 0.5 µg/mL. Therefore, the associations between natural products showed a reduction of 32x and 64x respectively. However, this modulating effect was not seen with norfloxacin. Therefore, this study concluded that both the essential oil of *Croton grewioides*, as well as α-pinene were presented as potential adjuvants of tetracycline, acting, probably in the efflux pump and modulating bacterial resistance (MEDEIROS *et al.*, 2017). It is reported in the literature that the main mechanisms of resistance of *S. aureus* strains to ciprofloxacin are related to mutations in the grlA/grlB and gyrA/gyrB genes, as well as may involve the efflux of antimicrobials through NorA membrane proteins (CAMPION *et al.*, 2004). For rifampicin, when performing the molecular characterization of antimicrobial resistant strains of *S. aureus* in a university hospital, researchers observed that most of the strains studied had mutations in the rpoB gene (ZHOU *et al.*, 2012).

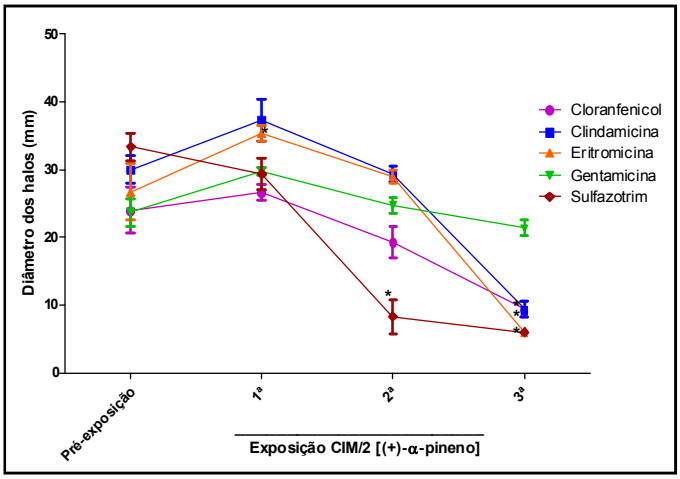


Figure 6. Modulation of the adaptive response of S. *aureus* against antimicrobials that act on protein and bacterial folate synthesis after exposure to CIM/2 of (+)-α-pinene

Figure 6 shows the modulation of the adaptive response of *S. aureus* against antimicrobials that act on protein and bacterial folate synthesis after exposure to MIC/2 of (+)-α-pinene. For erythromycin, the strain became more sensitive after the first exposure and then became resistant at the 2nd and 3rd exposures. Meanwhile, for both chloramphenicol and clindamycin, it was possible to observe a decrease in the diameter of the halo, only at the 3rd exposure, thus making the strain resistant to the drug. Sulfazotrim showed a statistically significant reduction at the 2nd exposure and that was maintained at the 3rd exposure, so, at the end of the protocol, the strain was considered resistant to the drug. Regarding gentamicin, its effect was considered indifferent, since there was no change in the sensitivity profile. One study evaluated the synergism between natural products, *Camellia sinensis*, *Juglans regia*, and *Hippophae rhamnoides,* which have monoterpenes in their composition including α-pinene, associated with commercial antimicrobials against a strain of *S. epidermidis* using the disk-diffusion method. The results of the research showed synergism after association with cephalexin and erythromycin, the latter being also responsible

for demonstrating resistance reversal (ABIDI *et al*., 2015). Mcmahon *et al*. (2008) analyzed the effect of exposure to sublethal concentrations of oil extracted from *Melaleuca alternifolia*, which has monoterpenes mostly in its composition, in strains of the genus *Staphylococcus* in the profile of sensitivity to antimicrobials for therapeutic use. It was observed that, for strains of S. aureus, there was an increase in resistance to antimicrobials, including vancomycin and chloramphenicol, after exposure to concentrations of essential oil, which was reversed in the absence of the compound. This result is divergent from that found in this research, since for vancomycin there was no change in the sensitivity profile and for chloramphenicol there was an irreversible resistance to the antimicrobial. This divergence may be due to the methodology and microorganism or not be exactly the same in the two surveys. In addition, the monoterpenes present in the essential oil of the comparative study are different from the (+)-alpha-pinene used in this research, suggesting that microorganisms react differently according to the type of monoterpene used. In the literature, it is possible to find how the increased expression of the NorA efflux pump in *S. aureus* confers resistance to chloramphenicol and fluoroquinolones. In addition, erythromycin resistance can be conferred by mutating the 50S subunit binding site genes (BRUNTON *et al*., 2009).

Conclusion

The present study was able to corroborate the antimicrobial activity of + AP against the strain of *S. aureus* ATCC 25923 present in the literature. It was found that, after successive exposures to subinhibitory concentrations, the strain *S. aureus* ATCC 25923 suffered a stress that led to resistance induction for 6 tested antimicrobials. This effect results in a reduction in the efficacy of antimicrobials, which can be perceived by the increase in the minimum inhibitory concentration compared to the phytochemical, as well as phenotypic changes seen in cultures subjected to incubation. However, the other ATMs did not show final modification during the resistance induction test. The potential of phytochemicals as a future antimicrobial agent in combined therapies was also evidenced, since it was possible to observe a synergistic effect in the associations between phytochemicals with erythromycin and gentamicin. In addition, although an antagonistic effect was observed in the combination with rifampicin, penicillin G and sulfamethoxazole/trimethoprim, and an indifferent effect in the associations of (+)-α-pinene with the other antimicrobials, it is noteworthy that, in this study, only the disk-diffusion method. Therefore, other protocols must be applied to better evaluate these results. It is recommended to expand the studies to understand the mechanisms of action involved in the biological activity of the phytochemicals, as well as possible toxic effects.

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