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Research Article

Antibacterial Activity of the Monoterpene Linalool: Alone and in Association with Antibiotics Against Bacteria of Clinical Importance

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ABSTRACT
Antibacterial activity studies of new molecules, either alone or in combination with existing antibiotics, are of great importance considering the resistance acquired by microorganisms in recent times. Linalool is a phyto-constituent found in the essential oils of various plant species. It is a monoterpene widely used in perfumery, cosmetics, and the food industries. Our objective was to determine the pharmacological effects produced on the bacterial strains *Staphylococcus aureus*, and *Pseudomonas aeruginosa* when combining standard antibiotics with linalool. The Minimum Inhibitory Concentration (MIC) was calculated using microdilution technique, where the linalool concentrations varied from 2 to 1024 µg /mL. Combinations with standard antibiotics were analyzed by the checkerboard method where the fractional inhibitory concentration (FIC) indices were calculated. Linalool, Imipenem, and Ciprofloxacin showed respective MIC antibacterial activities against *S. aureus* of 1024, 4, and 2 µg/mL. In *S. aureus*, the linalool with Imipenem association showed a synergistic effect (FIC = 0.0625); while with ciprofloxacin, the linalool showed additivity (FIC = 0.75). In *P. aeruginosa*, the Imipenem/linalool association was synergistic for both the ATCC and clinical strains (FIC = 0.0625). The association of *linalool* with ciprofloxacin was indifferent. We conclude that Linalool associated with existing standard antibiotics may increase antibacterial effectiveness, resulting in synergistic activity against bacterial strains of clinical importance. This makes the molecule potentially important for production of new, therapeutically effective drugs against resistant microorganisms.

Key words: natural products, antibacterial activity, synergism, linalool.

INTRODUCTION

In the 21st century, given the growing number of multiresistant bacterial strains, and resistance exchanges between different species, bacterial resistance has become a critical challenge, (Ex.: *Neisseria gonorrhoeae, Pneumocystis pneumoniae, Pseudomonas aeruginosa and Staphylococcus aureus*)[1,2]. As a global public health problem, the theme was proposed by the World Health Organization (WHO) in 2011, and emphasized on World Health Day as a controlling target among global strategies to ensure safe healthcare. Attention was also drawn to the challenges of implementing immediate actions to control the spread of resistant microorganisms in order to minimize the progressive deterioration of therapies handling such cases.

Among the pathogens considered important in relation to bacterial resistance, one might highlight *S. aureus* and *P. aeruginosa*. Infections caused by these pathogens are a clinical challenge, due to adaptations under the selective pressures of intense antimicrobial use; *S. aureus* has achieved a great ability to develop resistance[3], and *P. aeruginosa* is already characterized by limited susceptibility to any number of antimicrobial agents[4].
Because of the great resistance that microorganisms, such as *S. aureus* and *P. aeruginosa*, have acquired to a wide range of antibiotics in recent years, the search for new compounds has been the subject of intensive research. The fight against this emerging problem of pathogenic organism resistance has in the present day employed two divergent approaches: the development of completely new antibiotics, and/or combinations of substances

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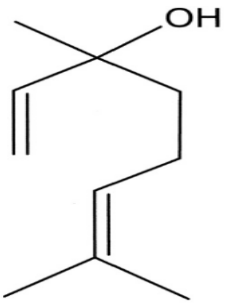

Figure 1: Quemical structure of Linalool

already in use[5]. The adoption of combination therapy often occurs in cases where the etiological character is poly-microbial, making it difficult to achieve mono-therapeutic healing[6].

Linalool (Figure 1), 3,7-Dimethyl-1,6-octadien-3-ol, is a widely used monoterpene in perfumery, cosmetics, and the food industries. It has been used as the starting compound for several important syntheses, such as linalyl acetate. It has been used successfully as a sedative, and has anticonvulsant, hypnotic, and hypothermic properties, affecting the central nervous system as a depressant. It is also being analyzed for its bactericidal and fungicidal properties. More studies on its properties are necessary[7,8].

The compound is a constituent of the essential oils of various plants of the Brazilian flora, such as rosewood (*Aniba roseadora*), several species of the *Piper* and *Croton* genres, Coriander (*Coriandrum sativum*), Tangerine (*Citrus reticulata*), and the Bergamot variation (*Citrus bergamia*), as well as other citrus fruits, and even the basils; (*Ocimum basilicum*) and (*Ocimum gratissimum*)[9,10,11,12].

Based on the above, this study aimed to observe the effects of combinations of linalool with standard antibiotics used in clinical practice against strains of *S. aureus* and *P. aeruginosa*.

MATERIALS AND METHODS

Linalool

Linalool was acquired commercially from Sigma-Aldrich.

Antibiotics

The antibiotics used in this work were Imipenem and Ciprofloxacin acquired commercially from Sigma-Aldrich, as based on the sensitivity profile of the strains.

Bacterial Strains

As follows four bacterial strains were used: 2 strains of *Staphylococcus aureus* (ATCC 6538 and M-177*)*, and 2 strains of *Pseudomonas aeruginosa* (ATCC 25853 and 1662339). The strains of clinical origin used were provided by the clinical analysis Laboratory of Hematology in João Pessoa- PB-Brazil. All other microorganism strains were obtained from the Laboratory of Mycology collection. Bacteria were kept on Nutrient Agar (NA) slants at 4°C. Inoculum was obtained from overnight cultures grown on NA slants at 37°C, and diluted in sterile saline solution (NaCl 0.85% w/v) to provide a final concentration of approximately 10^6 colony forming units per mL (CFU mL^{-1}), and adjusted according to turbidity at 0.5 McFarland tube scale.

Determination of Minimum Inhibitory Concentration (MIC)

The microplate bioassay was used to determine the minimum inhibitory concentrations (MIC) for (Imipenem, Ciprofloxacin, and the linalool). For this purpose, 96-well plates were prepared by dispensing 100 µL of double strength Nutrient Broth (NB) inoculated with the bacteria into each well prior to the assay. Aliquots (100 µL) of each compound (at its respective concentrations) were transferred into six consecutive wells. The highest substance concentration (1024 µg/mL) solution was added to the first well with the smallest concentration (2 µg/mL) in the antepenultimate well. The penultimate and the last wells containing 200 µL of the NB were respectively inoculated with the microorganism suspension, and Imipenem (100 µg/mL), being the negative control and positive controls. The microplate was aseptically sealed, and incubated at 37 °C for 24 h[13,14]. The antibacterial activity was detected using colorimetric method adding 20 µL of resazurin (0.1 g/100 mL) aqueous staining solution to each well at the end of the incubation period. The MIC was defined as the lowest sample concentration able to inhibit the bacterial growth as indicated by resazurin staining[15]. All experiments were carried out at least twice with consistent results.

Association studies using the checkerboard method

The strains were tested using the microdilution checkerboard technique[16]. Suspensions of 10^6 CFU/mL were prepared and distributed into micro-titer plates containing various concentrations of the different drugs. The inoculated plates were incubated at 37°C for 24h, and then evaluated for bacterial growth. In order to determine the activity of the drug combinations, fractional inhibitory concentration (FIC) indices were calculated as FIC^A + FIC^B, where FIC^A and FIC^B represent the minimum concentrations that inhibited bacterial growth for drugs A, and B, respectively: FIC^A = MIC^A combination/MIC^A alone, and FIC^B = MIC^B combination/MIC^B alone. A mean FIC index was calculated based on the following equation: FIC index = FIC^A + FIC^B, interpretation as follows: synergistic (≤0.5), additive (>0.5 but <1), indifferent (≥1 but <4), or antagonistic (≥4.0).

RESULTS

The results of the linalool/standard antibiotics association study are shown in Tables 1 and 2.

Observing Table 1, the respective MIC results for linalool and Imipenem each used alone against the *S. aureus* strains tested were 1024 and 4 $\mu g.mL^{-1}$. Linalool in combination with Imipenem reduced the MIC of these compounds to 32 and 0.125 µg/mL, respectively. The FIC was 0.0625, and the compound associations were characterized as synergistic for the tested *S. aureus* strains. Regarding ciprofloxacin, the MIC of the antibiotic alone was 2 µg/mL. For the linalool/ciprofloxacin association, we observed that the MIC decreased to 512 $\mu g.mL^{-1}$ for linalool, and the MIC of ciprofloxacin decreased to 0.5 $\mu g.mL^{-1}$, resulting in an FIC of 0.75 (indicating additivity).

Table 1: Antibacterial activity of the isolated compounds and in combination against *S.aureus* strains

Compounds		*S.aureus* ATCC 6538			*S.aureus* M-177		
		MIC (µg/mL)	FIC (Index)	Type of interaction	MIC (µg/mL)	FIC (Index)	Type of interaction
Alone	LNL	1024			1024		
	IM	4			4		
	CP	2			2		
Association	LNL+IM	32 / 0.125	0.0625	Synergism	32 / 0.125	0.0625	Synergism
	LNL+CP	512 / 0.5	0.75	Additivity	512 / 0.5	0.75	Additivity

MIC: Minimum Inhibitory Concentration; FIC: Fractional Inhibitory Concentration Index; LNL= Linalool; IM=Imipenem; CP= Ciprofloxacin

Table 2: Antibacterial activity of the isolated compounds and in combination against P.*aeruginosa* strains

Compounds		*P.aeruginosa* ATCC 25853			*P.aeruginosa* 1662339		
		MIC (µg/mL)	FIC (Index)	Type of interaction	MIC (µg/mL)	FIC (Index)	Type of interaction
Alone		1024			1024		
		4			4		
	CP	2			2		
Association	LNL+IM	32/ 0.125	0,0625	Synergism	32 / 0,125	0,0625	Synergism
	LNL+CP	256 / 2	1,25	Indifferent	1024/ 2	2	Indifferent

MIC: Minimum Inhibitory Concentration; FIC: Fractional Inhibitory Concentration Index; LNL= Linalool; IM=Imipenem; CP= Ciprofloxacin

In Table 2, we observe that for the standard and clinical isolate strains of *P. aeruginosa*; the linalool MIC was 1024 $\mu g.mL^{-1}$, for Imipenem it was 4 $\mu g.mL^{-1}$, and for Ciprofloxacin it was 2 $\mu g.mL^{-1}$ (compounds used alone). Linalool, in association with Imipenem was classified as synergistic for the clinical isolate and standard strains. Ciprofloxacin, in combination with linalool for the strain ATCC was classified as indifferent (FIC=2).

DISCUSSION

The combined use of antimicrobial agents is a routine clinical practice; always seeking an increase in the drug's therapeutic role[17]. Studies on aspects of plant derivatives and the possibility of synergism with conventional antimicrobial drugs are common[18]. Antibiotics interacting synergistically in combinations with herbal extracts against resistant microbial strains are a new strategy for treating infections which allows the use of antimicrobial drugs that when used alone are not effective on certain bacterial strains[19]. Studies with combinations of natural products from plants (or phytochemicals) together with synthetic drugs are still limited, but the results are often positive.

In this study, we evaluated antibacterial activity of linalool (a monoterpene found in many essential oils) /antibiotic associations in combination with antibiotics used in clinical practice against strains of hospital importance. The results showed that linalool, either alone or in combination with Imipenem (resulting in synergism), displayed antibacterial activity against *S. aureus* and *P.aeruginosa* strains. For the *P. aeruginosa* strains tested, the linalool combination with ciprofloxacin was indifferent.

Mossa et al[20] documented the synergism of linalool and α-terpineol from *Melaleuca leucodendron* when combined with ampicillin and kanamycin. Bassolé et al[21] found FIC indices ranging from 0.11 to 2.47 for paired combinations of *L. multiflora*, *Mentha* x *piperita,* and *O. basilicum* essential oils. All of the paired combinations had synergetic effects; inhibiting *E. faecalis*, *L. monocytogenes*, and *E. coli*. Combinations of *L. multiflora* with *Mentha x piperita*, or *O. basilicum* had synergetic effects inhibiting *S. typhimurium,* and *S. dysenteria*.

There are a few generally accepted mechanisms of antimicrobial interaction that produce synergism. They include sequential inhibition of a common biochemical pathway, inhibition of protective enzymes, and the use of cell wall agent activity to enhance the uptake of other antimicrobials[22].

Based on the hypothesis of Pei et al[23], we suggest that the synergetic effects observed might be amplified due to increases in one of three factors which determine a monoterpene's antimicrobial character: its lipophilic properties, its functional groups' potencies, and the paired combination's resulting aqueous solubility[24].

CONCLUSION

This work showed that linalool is able to potentiate the antibacterial activity of existing clinical antibiotics thru synergistic interactions; the molecule could be an alternative for the production of new drugs which are effective against multiresistant microorganisms.

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DECLARATION OF INTEREST

The authors declare no conflicts of interest.

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