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Original article

Effect of *Lippia origanoides* H.B.K. essential oil in the resistance to aminoglycosides in methicillin resistant *Staphylococcus aureus*

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Abstract

Introduction: Methicillin resistant *Staphylococcus aureus* (MRSA) is a common cause of hospital and community-acquired infections worldwide.
Methodology: In this study the essential oil of *Lippia origanoides* H.B.K., Verbenaceae, was assayed for its modifying drug activity against a MRSA strain by microdilution method.
Results: A significant potentiating effect between this oil and the aminoglycosides tested was verified. The MIC values for neomycin and amikacin alone were 2500 and 788 μg/mL, respectively. These were reduced to 248 and 78 μg/mL when they were associated with the essential oil against MRSA strain. A similar synergism was observed when the oil was changed for chlorpromazine, an efflux pump inhibitor, suggesting the involvement of resistance mediated by efflux system.
Conclusions: The results indicated that *L. origanoides* H.B.K. can be a source of secondary metabolites to be used in association with aminoglycosides in the antibiotic chemotherapy against illnesses caused by MRSA.

Keywords: MRSA; *Staphylococcus aureus*; Antibiotic activity; Synergism; *Lippia origanoides*

Introduction

Staphylococcus aureus is a common etiologic agent of infectious illnesses worldwide and it exhibits a strong tendency to acquire resistance to several antibiotics. It is the case of methicillin resistant *Staphylococcus aureus* (MRSA) strains which show resistance to methicillin and oxacillin, two antibiotics resistant to inactivation by beta-lactamases. MRSA strains make an altered penicillin binding protein (PBP2a) with lower affinity to beta-lactam antibiotics [1]. This protein is encoded by *mecA* gene located on a mobile genetic element named staphylococcal cassette chromosome *mec* (SCC*mec*), containing several insertion sequences which allow the insertion of additional resistance genes and acquisition of multi-drug resistance phenotype [2]. MRSA have been isolated from hospital environment with a high frequency [3,4], but the isolation of MRSA from patients with staphylococci infections acquired in the community it is

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increasingly common [5–7]. In the United States it was estimated that 23.4% of the population were colonized with *S. aureus*, with a MRSA prevalence estimated at 2.6% [8].

The beta-lactam antibiotics are drugs of first choice for treatment of staphylococci infections, but in the most severe cases caused by MRSA have been necessary a combined chemotherapy with other drugs, e.g. glycopeptids and aminoglycosides, for a successful therapeutic [9]. Aminoglycosides are antibacterial agents that act by impairing bacterial protein synthesis through binding to ribosomal subunit 30S, interfering with the binding of mRNA and leading to the synthesis of anomalous proteins which insert on plasma membrane changing your permeability [10]. In *S. aureus*, resistance to aminoglycosides is frequently related with enzymatic inactivation [11], but has been showed that it can be mediated by membrane transporter proteins which are able to pump the antibiotic to the extracellular medium, decreasing the drug concentration into bacterial cell [12–15]. The knowledge about involvement of efflux pumps in multi-drug resistant strains isolated from clinical samples, has motivated the looking for synthetic or natural compounds that can act how efflux pump inhibitors [16–18]. In this sense, natural products from vegetable origin have been investigated for its ability of increase the effectiveness of antibiotics currently used against multi-drug resistant microorganisms [19–23].

Lippia origanoides H.B.K. (Verbenaceae) is an aromatic plant commonly used as culinary seasoning and in traditional medicine as remedy for gastrointestinal disorders and respiratory diseases treatment [24]. The essential oil obtained of *L. origanoides* (LOEO) and its major constituents, carvacrol and thymol, exhibited antigenotoxic effect in bacterial cells, showing that this plant can be an important source of compounds with application in cancer chemoprevention [25]. Also, has been verified that it shows antioxidant activity and low toxicity [26,27], as well as antimicrobial activity against several pathogen groups, including bacteria, fungi, virus and evolutionary forms of protozoa [28–31]. In the present study was tested the hypothesis that the essential oil of *L. origanoides* could act as a modifier of the resistance to aminoglycosides in MRSA, a biological activity still not investigated.

Material and methods

Plant material and essential oil extraction

The aerial parts of *L. origanoides* H.B.K. were collected at the Garden of Medicinal and Aromatic Plants of the Universidade Federal do Piaui (UFPI), in Teresina, Piaui, Brazil. Voucher specimen was identified and deposited at the Graziela Barroso Herbarium in UFPI under internal control number TEPB09205. Essential oil was obtained by hydro distillation for 5 h from air-dried leaves using the Clevenger apparatus, leading to ca. 4.6% yield (dry weight basis).

Strains and drugs

The clinical methicillin resistant *S. aureus* SA10 strain isolated from rectal swab was used in all assays. The resistance profile of this MRSA strain it was determined by diffusion disk method. The *S. aureus* ATCC 25923 was used as a positive control. The strains were maintained on Nutrient Agar (Himedia, India) slant at 4 °C and prior to assay the cells were grown overnight at 37 °C in Brain Heart Infusion (BHI, Himedia, India). Neomycin, Amikacin and Chlorpromazine (CPZ) were obtained from Sigma Chemical Corp., St. Louis, MO, USA. All drugs were dissolved in sterile water.

Drug susceptibility test

Stock solution of the LOEO was prepared by dissolving 10 mg of the compound in 1 mL of Dimethylsulfoxide (DMSO – MERCK), thus starting with an initial concentration of 10 mg/mL. The resulting solution was then diluted to 1024 μg/mL in sterile water. The minimal inhibitory concentration (MIC) of the LOEO, antibiotics and Chlorpromazine were determined by the microdilution assay in BHI broth with suspensions of 10^5 CFU/mL. The essential oil and drug concentrations ranged of 1024–8 μg/mL [32]. MIC was defined as the lowest drug concentration in which there was not bacterial growth. For evaluation of LOEO as antibiotic resistance modulator, MICs of the antibiotics were determined in the presence of LOEO (128 μg/mL) and Chlorpromazine (8 μg/mL) at sub-inhibitory concentrations [33]. The antibiotic concentrations ranged of 5000–2.4 μg/mL. The plates were incubated at 37 °C for 24 h. Differences ranging only one point in the MIC were considered not significant.

Statistical analysis

All experiments were performed in triplicate and the results were normalized by calculation of geometric average values. The error deviation and standard deviation of the geometric average were revealed. Differences between treatment with antibiotic alone and with antibiotic-LOEO (or antibiotic-CPZ) association were examined using two-way analysis of variance (ANOVA) and $P < 0.05$ was considered statistically significant.

Results and discussion

The resistance profile obtained for the strain studied is showed on Table 1. It was identified as MRSA because it presented resistance to Oxacillin. Besides, it was resistant to eighteen drugs belonging to six antibiotic classes.

Previous studies have verified a significant inhibitory effect of *L. origanoides* essential oil, as well its main compounds isolated, on *S. aureus*, but they have tested disks impregnated with the oils by diffusion disk method [27,34] or application of one oil drop on agar by the drop agar diffusion method [30]. However the antimicrobial activity of essential oils has shown to be dependent on several factors, such as, the target organism, origin, method of oil extraction and the procedure used in the tests of antibacterial activity [35]. At the present study was utilized the microdilution method and the MIC value obtained for both MRSA and *S. aureus* ATCC 25923was ≥1024 μg/mL,

Table 1
Resistance profile obtained for the MRSA strain SA10.

Antibiotic classes	Resistance to
Beta-lactam	Cpt Cpx Cfd Oxa Pen Amp Amo
Fluorquinolone	Mfx Cip Lev Nal
Macrolide	Ery Cla Azi
Lincosamine	Cli
Tetracycline	Tet
Aminoglycoside	Neo Gen Ami

Cpt = Cephalothin, Cpx = Cephalexin, Cpd = Cefadroxil, Pen = Penicillin G, Amp = Ampicillin, Amo = Amoxicillin, Oxa = Oxacillin, Ery = Erythromycin, Cla = Clarithromycin, Azi = Azithromycin, Cli = Clindamycin, Mfx = Moxifloxacin, Cip = Ciprofloxacin, Lev = Levofloxacin, Nal = Nalidixic acid, Tet = Tetracycline, Neo = Neomycin, Gen = Gentamicin, Ami = Amikacin.

indicating that the LOEO did not showed antibacterial activity against these strains at clinically relevant concentrations tested [36].

On the other hand, addition of LOEO to the growth medium at 128 μg/mL did cause a 10-fold decrease in the MIC for Neomycin (2500–248 μg/mL) and for Amikacin (788–78 μg/mL), demonstrating a synergistic effect of LOEO with aminoglycosides against the MRSA strain (Fig. 1). No effect was observed on tests with *S. aureus* ATCC 25923 (data not shown). The modulation of resistance to aminoglycosides by products extracted from other plants in MRSA has been verified in previous studies [33,36,37]. But, as far we know natural products from *L. origanoides* having a synergistic effect with aminoglycosides or other antibiotics have not been previously reported.

A significant MIC reduction for Neomycin and Amikacin was also verified when CPZ was added to the growth medium at 8 μg/mL (Fig. 1). Phenotiazines, such as Chlorpromazine, probably act on the plasma membrane of bacteria, affecting efflux pumps [38–40]. Thus, the decrease in the MIC in the presence of CPZ indicates the involvement of efflux pump in the resistance to these antibiotics in the MRSA strain tested at the present study. This result suggesting that LOEO is a putative inhibitor of efflux pumps, although additional studies are needed to confirm such mechanism.

Several studies have showed that essential oils obtained from *L. origanoides* they are rich in hydrophobic compounds such as monoterpenes [27,29,30,34]. The chemical composition of the LOEO tested in the present study was investigated in a previous research which verified that its majority compounds were the monoterpenes Carvacrol (37.3%), Thymol (22.4%) and γ-Terpinene (10.9%) [28]. Since these compounds are highly lipophilic they are able to accumulate in the bacterial membrane plasma [41], causing the loss of their integrity, increasing its permeability mainly to K^+ and H^+ ions [42,43], loss of cytoplasmic content, dissipation of the proton-motive force, lysis and cell death [44].

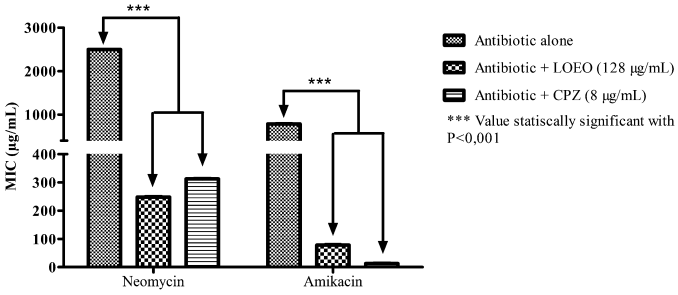

Fig. 1. Geometric means of MIC values shown by antibiotics in the absence and presence of *Lippia origanoides* H.B.K. essential oil (LOEO) and Chlorpromazine (CPZ) for *Staphylococcus aureus* MRSA.

It is interesting that the any efflux pumps are dependent of proton-motive force, as is the case of Qac protein occurring in *S. aureus* strains which are capable of extruding aminoglycosides out of the bacterial cell [9,14]. It is possible that the reduction in the MIC for the aminoglycosides in the LOEO presence could be occurred due the intercalation of its lipophilic components in the plasma membrane, leading to an increase in the cell permeability to the antibiotic molecules, and/or inhibition of efflux systems, increasing the intracellular concentration of the drug [16,45].

Besides its use in the treatment of MRSA infections, aminoglycosides such as Amikacin, Kanamycin and Streptomycin, are second-line drugs used in the tuberculosis therapy, however they can cause adverse reactions, e.g. ototoxicity and nephrotocity, which can lead to discontinuation of treatment or restrict its use in elderly individuals and patients with chronic kidney disease [46]. In addition, nephrotoxic agents such as aminoglycosides often require close monitoring of serum drug concentrations and creatinine levels, which contributes to increase the total cost of therapy [47]. Therefore, the design of pharmaceutical formulations based on combined use of aminoglycosides with LOEO or with its phytochemicals isolated could be an alternative for reducing the therapeutic concentrations of these drugs, minimizing its adverse effects and decreasing the costs of treatment.

Conclusion

The present study provided strong evidence of the synergistic effect of *L. origanoides* H.B.K. essential oil in combination with aminoglycoside antibiotics against MRSA, but further studies are warranted to elucidate the compounds related with the synergism. The results indicated that *L. origanoides* H.B.K. can be a source of secondary metabolites to be used in association with aminoglycosides in the antibiotic chemotherapy against illnesses caused by MRSA.

Conflict of interests

No conflict of interests was disclosed.

Acknowledgments

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