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Inhibition of the NorA multi-drug transporter by oxygenated monoterpenes

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ABSTRACT

The aim of this study was to investigate intrinsic antimicrobial activity of three monoterpenes nerol, dimethyl octanol and estragole, against bacteria and yeast strains, as well as, investigate if these compounds are able to inhibit the NorA efflux pump related to fluoroquinolone resistance in *Staphylococcus aureus*. Minimal inhibitory concentrations (MICs) of the monoterpenes against *Staphylococcus aureus*, *Escherichia coli* and *Candida albicans* strains were determined by micro-dilution assay. MICs of the norfloxacin against a *S. aureus* strain overexpressing the NorA protein were determined in the absence or in the presence of the monoterpenes at subinhibitory concentrations, aiming to verify the ability of this compounds act as efflux pump inhibitors. The monoterpenes were inactive against *S. aureus* however the nerol was active against *E. coli* and *C. albicans*. The addition of the compounds to growth media at subinhibitory concentrations enhanced the activity of norfloxacin against *S. aureus* SA1199-B. This result shows that bioactives tested, especially the nerol, are able to inhibit NorA efflux pump indicating a potential use as adjuvants of norfloxacin for therapy of infections caused by multi-drug resistant *S. aureus* strains.

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1. Introduction

The prevalence of infectious diseases caused by multi-drug resistant microorganisms has increased dramatically worldwide [1,2] despite of the wide range of available antimicrobial agents. Infections caused by methicillin resistant *Staphylococcus aureus* (MRSA) and *Escherichia coli* resistant to cephalosporin of third-generation and fluoroquinolones are commonly acquired in hospitals and communities of all countries of the world [3,4]. Fluoroquinolones have been proposed as a possible alternative to vancomycin therapy against methicillin resistance *S. aureus* (MRSA) infections [5], however resistance to these antibacterial agents has become common and widespread [6–8].

Fluoroquinolones are able to binding to the complexes formed between DNA and DNA gyrase or topoisomerase IV, inactivating these bacterial enzymes, leading to a rapid inhibition of DNA replication [9] Bacterial resistance to fluoroquinolones occur due to mutation in one or more genes encoding these target enzymes or by expression of multidrug efflux pumps capable of actively removing fluoroquinolones from bacterial cell [10–12], as NorA efflux protein overexpressed by SA1199-B strain tested in the present study [13,14].

The knowledge about resistance mediated by efflux pumps has motivated the search for efflux pump inhibitor (EPI) compounds which could recover the efficacy of current antibiotics [15]. In this sense, synthetic or natural products from vegetable origin have been investigated for its ability to act how EPI [16–20].

Essential oils from plants are rich in bioactive compounds, such

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as terpenes and terpenoids, which have showed promising results as potential therapeutic agents [21–23]. Monoterpenes, the main constituents of essential oils [24], have been reported for their antifungal, anti-aflatoxin and antioxidant activities [25]. The aim of the present study was to evaluate if monoterpenes nerol, estragole and dymethil-octanol, are potential intrinsic antimicrobial agents and/or efflux pump inhibitors of the NorA multi-drug transporter of *S. aureus.*

2. Materials and methods

2.1. Strains and drugs

The intrinsic antimicrobial activity of the monoterpenes was tested against Gram-positive (*S. aureus* ATCC 25923, SA1199, SA1199-B and SA10), Gram-negative (*E. coli* ATCC 25922) or yeast (*Candida albicans* NEWP031) strains. The inhibitory effect on NorA activity was performed with *S. aureus* SA1199-B strain which over-express the *norA* gene encoding NorA. NorA can efflux hydrophilic fluoroquinolones and other drugs such as DNA-intercalating dyes [13]. Bacterial strains were maintained on Brain Heart Infusion Agar (BHIA, 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). The yeast strain was maintained on Sabouraud Dextrose Agar (SDA, Himedia, India) slant at 4 °C and prior to assay the cells were grown for 24 h at 37 °C in Sabouraud Dextrose Broth (SDB, Himedia, India).

Oxygenated monoterpenes 3,7-dimethyl-octan-1-ol, (2Z)-3,7-dimethylocta-2,6-dien-1-ol (nerol), and 1-methoxy-4-(prop-2-en-1-yl)benzene (estragole), norfloxacin and ethidium bromide were obtained from Sigma Chemical Corp., St. Louis. Antibiotics and ethidium bromide were dissolved in sterile water.

2.2. Log P estimation

Estimation of the Log *P* was performed using the MarvinSketch 6.2.2 (Chemaxon), by the Phys method. Log *P* has been calculated for the uncharged molecule due to the typical range of pKa for aromatic hydroxyl is 8.0–10.0.

2.3. Evaluation of the intrinsic antimicrobial activity

Stock solutions of nerol, 3,7-Dimethyl-1-octanol and estragole were prepared by dissolving 10000 μg of each monoterpene in 1 mL of dimethyl sulfoxide, thus starting with an initial concentration of 10000 μg/mL. This stock solution was then diluted in sterile distillated water to obtain the test solution (1024 μg/mL). Minimal inhibitory concentrations (MICs) of monoterpenes were determined by micro-dilution assay in BHI broth 10% with bacterial suspensions of 10^5 CFU/mL and monoterpene solutions ranging from 8 to 512 μg/mL. Microtiter plates were incubated at 37 °C for 24 h, then 20 μL of resazurin (0.01% w/v in sterile distilled water) was added to each well to detect bacterial growth by color change from blue to pink. MICs were defined as the lowest concentration at which no bacterial growth was observed.

Antifungal assays were performed by micro-dilution method in SDB double concentrated with yeast suspension of 10^5 CFU/mL and monoterpene solutions ranging from 8 to 512 μg/mL. Microtiter plates were incubated at 37 °C for 24 h. The MIC was defined as the lower concentration of the monoterpene solution able to inhibit the visible growth. Inhibition of the fungal growth was confirmed transferring an aliquot from each well of the MIC test microtiter plate to a Petri dish containing SDA and checking cell viability after incubation at 37° for 24 h.

2.4. Evaluation of the NorA efflux pump inhibition

For evaluation of the monoterpenes as modulators of fluoroquinolone resistance, MICs of the norfloxacin for SA1199-B strain were determined in the presence or absence of each compound at sub-inhibitory concentrations (1/8 MIC, 1/4 MIC or 1/2 MIC). Antibiotic or ethidium bromide (EtBr) concentrations ranged from 0.125 to 128 μg/mL. Microtiter plates were incubated at 37 °C for 24 h and readings were performed with resazurin as previously described.

2.5. Statistical analysis

All experiments were performed in triplicate and results were normalized by calculation of geometric average values. Error deviation and standard deviation of the geometric average were revealed. Differences between treatment with antibiotics alone or associated with monoterpenes were examined using one-way analysis of variance (ANOVA). Differences mentioned above were analyzed by Bonferroni posttest and $p < 0.05$ were considered statistically significant.

3. Results

Chemical structures and Log P values of the oxygenated monoterpenes tested are presented in Table 1. MICs found to every compound against *S. aureus*, *E. coli* and *C. albicans* strains are presented in Table 2. Monoterpenes tested did not present activity against all *S. aureus* strains [26]. On the other hand, the nerol showed a weak inhibitory activity (512 μg/mL) against *E. coli* e *C. albicans* strains.

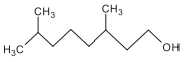
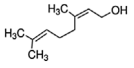
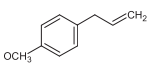
Addition of oxygenated monoterpenes to the growth medium at sub-inhibitory concentrations caused a decrease in the MIC for norfloxacin against SA1199-B (Figs. 1–3). The nerol and 3,7-dimethyl-octan-1-ol enhanced the antibiotic activity of norfloxacin against SA1199-B in a concentration-dependent manner. On the other hand, a modulatory effect was also verified when antibiotics were replaced by ethidium bromide, a well-known substrate of NorA protein (Fig. 4).

4. Discussion

Essential oils have been proposed as a natural source of compounds with antibacterial activity against multi-drug resistant bacteria, as well as, a natural source of compounds able to inhibit bacterial resistance mechanisms [27–29]. In this work we investigate the intrinsic antibacterial activity of three oxygenated monoterpenes and their potential as EPI of the NorA efflux pump which is related with resistance to hydrophobic fluoroquinolones in *S. aureus* SA1199-B.

In respect to intrinsic antimicrobial activity, only nerol was active against *E. coli* ATCC 25923 and *Candida albicans* NEWP031.

Table 1
Chemical structure and Log P estimation for oxygenated monoterpenes tested.

Monoterpene	Structure	Log P
3,7-dimethyl-octanol		3,12
(2*Z*)-3,7-dimethyl-2,6-octadien-1-ol (nerol)		3,02
1-allyl-4-methoxybenzene (estragole)		2,89

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