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Original Article

Citral, a monoterpenoid aldehyde interacts synergistically with norfloxacin against methicillin resistant *Staphylococcus aureus*

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ABSTRACT

Background: *Staphylococcus aureus* (SA), is a major human pathogen causing wide range of clinical infections, which has been further complicated by drug resistance like methicillin resistant *S. aureus* (MRSA), vancomycin intermediate *S. aureus* (VISA)/vancomycin resistant *S. aureus* (VRSA), etc. The present study was aimed at determining anti-staphylococcal potential of citral against drug resistant clinical isolates alone and in combination with antibiotics.

Purpose: To assess the potential of citral in combination with norfloxacin in treating drug resistant infections of SA.

Study design: In the present study, synergistic interaction of citral and norfloxacin against drug resistant SA strains was evaluated. Further the efficacy and possible mechanism of action of the combination was also evaluated using *in vitro* and *in vivo* assays.

Method: The anti-staphylococcal activity of each of the monoterpene and the antibiotic was determined in terms of MIC and the effective concentration of both compounds in combination was obtained by checkerboard assay. *In vivo* efficacy and oral acute toxicity was evaluated in Swiss albino mice model. To understand the mechanism of action, time-kill curve, bacteriolysis, leakage, membrane depolarization, salt tolerance and ethidium bromide efflux assays were performed.

Results: Citral was found effective against clinical isolates of SA with MIC values ranging from 75 to 150 μg ml^{-1} exhibiting bacteriostatic activity. Citral interacted synergistically, reducing MIC of norfloxacin up to 32-folds with FICI ≤ 0.50. Citral did not affect cell wall, but could damage cell membrane, inhibit efflux pump and affect the membrane potential. Citral could reduce the staphylococcal load of spleen and liver tissues in a dose-dependent manner which was further reduced when used in combination with norfloxacin. Citral did not exhibit any mortality or morbidity up to 500 mg kg^{-1} body weight and found to prolong the post-antibiotic effect of norfloxacin.

Conclusion: Based on these observations, citral could be a lead candidate phytomolecule for further developing it into an anti-staphylococcal agent. The observations of combination study will help in reducing the burden of antibiotics leading to delayed resistance development.

Introduction

Staphylococcus aureus is a major human pathogen that causes a wide range of clinical infections especially in human skin and soft tissues (Davies and Davies, 2010). SA is associated with a high mortality rate and puts an ample cost and resource burden on health care systems (Naber, 2009).

SA is mostly responsible for hospital acquired methicillin MRSA (HA-MRSA) causing an array of site specific infections in hospitalized patients, including bloodstream, pneumonia, surgical site, and urinary tract

Abbreviations: Nor, norfloxacin; SA, *Staphylococcus aureus*; FICI, fractional inhibitory concentration index; CFU, colony forming unit; MIC, minimum inhibitory concentration; h, hour; MRSA, methicillin resistant *S. aureus*; PAE, post antibiotic effect; MHA, Muller Hinton Agar; i.v, *intra venous*; CLSI, clinical and laboratory standard institute; CCCP, carbonyl cyanide *m*-chlorophenyl hydrazine; VISA, vancomycin intermediate *S. aureus*; VRSA, vancomycin resistant *S. aureus*; DMSO, dimethyl sulfoxide; $DiSC_3$-3,3′, dipropylthiadicarbocyanine iodide; FR, fold reduction; CI, combination index; DRI, dose reduction index; MDR, multi drug resistance

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infections (Raygada and Levine, 2009). MRSA is difficult to treat with existing antibiotics like ß-lactams, macrolide and quinolones types of antimicrobial drugs. The MRSA strains have showed to be resistant toward many other antibiotics leading to multidrug resistant strains (Lowy, 2003). Due to acquired resistance, sometimes single antibiotic does not produce the desired effects, in such cases combination of drugs often workouts to be a better option (Worthington and Melander, 2013).

Phytochemicals are well known for their potential as antibacterials when used alone and as potentiators of antibacterial drugs in clinical use. Phytochemicals often act through different mechanisms than conventional antibiotics and could, therefore be useful in the treatment of resistant bacteria (Abreu et al., 2012). Many phytomolecules such as reserpine, gallic acid, piperine, thymol have been reported to possess either anti-bacterial activity or synergize with the antibiotics leading to the reduced effective concentration (Gonzalez-Lamothe et al., 2009). Essential oils and their components have been reported to enhance the efficacy of antibiotics when used in combination (Langeveld et al., 2013; Hamoud et al., 2014).

Citral (3,7-dimethyl-2,6-octadienal) is an open-chain monoterpene aldehyde present in the essential oils of several medicinal plants including *Backhousia citriodora* F. Muell (Myrtaceae) (90–98%), *Ocimum basilicum* L.(Lamiaceae) (55–99%), *Cymbopogon citratus* (Grasses) (65–80%) and *Citrus sinensis* Osbeck (Rutaceae) (0.7–3%) (Shi et al., 2016). It can be present in two geometric stereoisomeric forms: the *E*-isomer is known as geranial or citral A and the *Z*-isomer is known as neral or citral B (Saddiq and Khayyat, 2010). Citral has been reported earlier for its antibacterial action against SA (Kim et al., 2011; Vimal et al., 2013; Espina et al., 2015) but there have been no reports on its antibacterial activity against drug resistant clinical isolates of SA in combination with clinically used antibiotics. Further its antibacterial mode of action has also not been determined so far. The aim of the present study was to determine anti-staphylococcal effect of citral against drug resistant clinical isolates of SA alone as well as in combination with antibiotics. *In vivo* efficacy, toxicity and understanding possible mode of action of citral on its own was also the objective of this study.

Material and methods

Chemicals and reagents

Citral ($C_{10}H_{16}O$) used in the study was obtained from CARL ROTH (Karlsruhe, Germany) with purity ≥95% (catalogue no. 5937.1) and antibiotics used in the study were procured from Sigma-Aldrich (St. Louis, Missouri, USA).

Bacterial strain

SA strain is said to be resistant (MRSA) if MIC of methicillin ≥ 16 μg ml^{-1} and is said to be sensitive if MIC of methicillin ≤8 μg ml^{-1} whereas it is said to be resistant (VRSA) if MIC vancomycin ≥16 μg ml^{-1}, is said to be intermediate if MIC vancomycin is between 4–8 μg ml^{-1} and is said to be sensitive if MIC vancomycin ≤2 μg ml^{-1} (Zurita et al., 2010; CLSI, 2009).

Clinical isolates (MRSA) with MIC 1000 μg ml^{-1} of oxacillin (marker of methicillin resistance) were procured from the clinical microbiology laboratory of Sanjay Gandhi Post Graduate Institute of Medical Sciences (SGPGIMS), Lucknow, India. SA MTCC-96 was procured from the Microbial Type Culture Collection, Chandigarh, India, and used for *in vivo* assays in the mice model. Mueller–Hinton agar/broth (MHA/MHB) and brain heart infusion agar/broth (BHIA/ BHIB) were used to grow the bacteria.

Statistical analysis

One-way analysis of variance was used to analyze the mean values obtained for the treatment and vehicle groups. Tukey's test was used to compare the treatment and vehicle groups, and statistical significance was set at $p \leq 0.001$.

Anti-staphylococcal activity

Determination of the MIC

The antibacterial activity of citral was determined by the broth microdilution assay using 96 'U' -bottom micro-titre plates as per CLSI guidelines (CLSI, 2015). Experimental MIC determinations were performed in triplicate to rule out any error during the procedure. An antibiotic norfloxacin (nor) was used as the positive control.

Interaction study of citral with clinically used antibiotics

The interactions of citral with clinically used antibiotics including tetracycline, erythromycin, penicillin, streptomycin and nor against MRSA isolates were assessed through checkerboard method described previously by Sun et al. (2008). The interaction of each combination tested was based on the fractional inhibitory concentrations FIC which was summed to derive the FIC index, which indicated the interaction when the index values were the following: FICI ≤ 0.5 = synergy, FICI > 4.0 = antagonism and FICI > 0.5–4 = no interaction (Iten et al., 2009). The FICI, was defined as the following equation: FICI = FIC-A + FICB = $C_A^{comb}/MIC_A^{alone} + C_B^{comb}/MIC_B^{alone}$, where MIC_A^{alone} and MIC_B^{alone} are the MICs of drugs A and B when acting alone and C_A^{comb} and C_B^{comb} are concentrations of drugs A and B at the iso-effective combinations, respectively.

Further the combination index and dose reduction index of the combinations were calculated as described by Chou (2006). The isobologram was plotted for combinations using MIC values as described by Pillai et al. (2013).

Bacterial killing assay

The *in vitro* bactericidal activity of citral in combination with nor against clinical isolate of SA (MRSA-ST 2071) was studied by using MIC, 2MIC and 4MIC in accordance to the method described by Klepser et al. (1998). Each analysis was done in triplicate with a control without test sample. Time–kill curves were derived by plotting $\log_{10}$ colony-forming units CFU ml^{-1} against time (h).

Bacteriolysis and cytoplasmic leakage assay

Bacterial cells were cultured as previously described by Carson et al. (2002) and bacteriolysis assay was performed. For loss of 260 nm absorbing material, assay was performed as previously described by Oonmetta-aree et al. (2006). Each analysis was done in triplicate with a control without test sample.

Loss of salt tolerance

The ability of bacterial cells treated with combination of citral (15.625 μg ml^{-1}) and nor (9.375 μg ml^{-1}) and NaCl to grow on MHA (HiMedia, Mumbai, India) was investigated according to Carson et al. (2002) with slight modifications. Suspensions of bacteria (10^6 CFU ml^{-1}) were prepared in MHB (HiMedia, Mumbai, India). They were treated with 0.5MIC, MIC and 2MIC of the combination [citral (15.625 μg ml^{-1}) and nor (9.375 μg ml^{-1})] and 1% DMSO. In other groups, each concentration was combined with 7.5% NaCl and the control was set up without combination. The test tubes were incubated in a shaker at 37 °C and samples were removed at intervals, serially diluted and plated on MHA. After 24 h of incubation, the CFU ml^{-1} were determined in the plates and compared with the control.