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Evaluation of *in vitro* growth-inhibitory effect of carvacrol and thymol combination against *Staphylococcus aureus* in liquid and vapour phase using new broth volatilization chequerboard method

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ABSTRACT

Carvacrol and thymol, both plant-derived volatile compounds, have extensively been studied individually as well as in combination with other agents for their antimicrobial activity in liquid phase. However, in contrast to well-established assays for testing of antimicrobial combinatory effects in liquid media, there are no standardized methods for evaluation of interactions between volatile compounds in vapour phase. The objective of this study was to verify new broth volatilization chequerboard method by testing the combination of carvacrol and thymol and to determine *in vitro* inhibitory effect of these compounds in liquid and vapour phase against twelve *Staphylococcus aureus* strains. The new method, based on combination of standard microdilution chequerboard and new broth volatilization tests allowing calculation of fractional inhibitory concentrations (FICs), was used. Combination of carvacrol and thymol produced the additive antimicrobial effect against all strains tested. In several cases, they reached ΣFIC values lower than 0.6, which can be considered as a strong additive interaction. The best result was found in vapour phase against one standard strain at combination of 128 μg/mL of carvacrol and 16–256 μg/mL of thymol (ΣFIC = 0.51) and in liquid phase against one clinical isolate at combination of 256 μg/mL of carvacrol and 256 μg/mL of thymol (ΣFIC = 0.53). The study verified that the new technique is suitable for simple and rapid high-throughput combinatory antimicrobial screening of volatile compounds simultaneously in vapour and liquid phase and that it allows determination and comparison of MIC and FIC values in both, liquid and solid media.

1. Introduction

Staphylococcus aureus is an important pathogen responsible for broad spectrum of diseases, ranging from food poisoning, mild skin and soft tissue infections to highly serious diseases such as endocarditis and osteomyelitis [1]. Currently, the global spread of methicillin-resistant *S. aureus* (MRSA) is one of the most serious public health challenges worldwide. It acquires resistance to all *β*-lactam agents as well as to other groups of antibiotics such as macrolides, fluoroquinolones, aminoglycosides, and glycopeptides [2,3]. Since *S. aureus* is commonly found on respiratory tract mucosa, the antibiotic inhalation could be one of its possible treatments. For example, dry-powder vancomycin and combination of fosfomycin and tobramycin are currently in late-stage development for supporting inhalation therapy and treatment of MRSA in cystic fibrosis patients [4].

The increase in bacterial resistance to antibiotics has also revived the interest in plant products as alternative antimicrobial agents to control pathogenic microorganisms [5]. Plants produce secondary metabolites, which serve them as strong defence against predators and microbial pathogens due to their biocidal properties [6]. Their defence never rely on one particular class of compounds and secondary metabolites occur always as mixtures in plants. Thus, synergistic and antagonistic effects can either significantly enhance or reduce activities of single compounds [7]. Essential oils (EOs) are typical example of such complex mixtures, whereas many of them produce antimicrobial synergy [6]. Several experiments focused on combinatory action of EOs

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and their volatile constituents as well as on combinatory effects between phytochemicals and antibiotics have previously been conducted against numerous bacteria including *S. aureus*. Various methods such as chequerboard, time-kill curve, and e-test assays have been used for evaluation of their antimicrobial combinatory interactions [8,9]. However, due to the specific physico-chemical properties of EOs such as high volatility and hydrophobicity, these conventional methods face specific problems. Because of the low solubility of these compounds in water based media (*e.g.* in broth), the surfactants are usually added, whereas high volatility causes a risk of active substances losses by evaporation [10]. Furthermore, the transition of vapours of EOs and their constituents may affect the results of microplate assays [11].

In contrast to well-established assays for testing of antimicrobial combinatory effects in liquid media, there are no standardized methods for determination of interactions between volatile compounds in the gaseous phase. Disk volatilization assay is probably the most frequently used method for evaluation of combinatory activities of plant volatile vapours. Interactions of EOs in the gaseous phase have previously been measured using this method by several researchers [12–14]. The tests were carried out in Petri dishes, where solidified medium was exposed to the vapours of EO combinations by placing an impregnated disc on the lid of the dish. After incubation, zones of microorganism growth inhibition were measured on the agar surface. Subsequently, these zones were compared with the zones of inhibition of individual compounds [13], or the fractional inhibitory concentrations (FICs) were calculated [14]. However, this assay based on modification of standard agar disk diffusion test is not appropriate for minimum inhibitory concentration (MIC) determination [15] and suffers from the lack of automation [16]. On the other hand, the advantages of the microdilution procedure include the generation of MICs, reproducibility, economy of reagents and space that occurs due to the miniaturization of the test [15,16]. Since our new broth microdilution volatilization assay performed in 96-well microtiter plates allows determination of MIC values in both liquid and vapour phases [17], it has the potential to be modified for evaluation of combinatory effects of volatiles using chequerboard design and allowing determination of FIC indices.

Carvacrol and its isomer thymol are one of the most extensively studied EO constituents. They are phenolic monoterpenoids, commonly present in EOs of *Origanum* and *Thymus* species [5], which are used as antiseptics in pharmacology, agriculture, cosmetics and food industry [18]. Beside the multiple biological properties [19], they also possess wide spectrum of antibacterial activity, including their anti-staphylococcal effects [5,20]. Several studies investigating the combinatory effects of carvacrol and thymol against various pathogenic microorganisms have previously been performed in liquid phase. Their interactions against *S. aureus* were also evaluated using chequerboard assay and calculation of fractional areas [20–22]. Nevertheless, the obtained results of studies mentioned above differ significantly, whereas synergistic [22], antagonistic [21], and additive effects [20] have been observed. In contrast to above mentioned papers showing their interactions in liquid media, there are no reports on combinatory effects of carvacrol and thymol in the vapour phase.

Therefore, the main aim of this study was to determine an *in vitro* inhibitory effect of carvacrol and thymol combination against twelve *S. aureus* strains simultaneously in vapour and liquid phase using broth volatilization chequerboard assay – a new method based on combination of standard microdilution chequerboard and new broth volatilization tests [17] allowing calculation of FIC values.

2. Materials and methods

2.1. Chemicals

Carvacrol (97%, CAS 499-75-2), thymol (99%, CAS 89-83-8), oxacillin (86.3%, 7240-32-2), and thiazolyl blue tetrazolium bromide (MTT) were purchased from Sigma-Aldrich (Prague, CZ). Dimethyl sulfoxide (DMSO) was obtained from Penta (Prague, CZ).

2.2. Bacterial strains and culture media

In this study, twelve *S. aureus* strains, including antibiotic-resistant and sensitive forms were used. American Type Culture Collection (ATCC) standard strains 25,923, 29,213, 33,591, 33,592, 43,300, and BAA 976 were purchased from Oxoid (Basingstoke, UK) on ready-to-use bacteriological Culti-Loops, and clinical isolates (SA 1–6) were obtained from The Motol University Hospital (Prague, CZ). The identification of clinical isolates was performed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry as it is described in Rondevaldova et al. [23]. Cation-adjusted Mueller-Hinton (MH) broth (Oxoid, Basingstoke, UK) equilibrated to pH 7.6 with Trizma base (Sigma-Aldrich, Prague, CZ) and MH agar (Oxoid, Basingstoke, UK) were used as cultivation and assay media.

Stock cultures of bacterial strains were cultivated in broth medium at 37 °C for 24 h prior the testing. Turbidity of the bacterial suspension used for inoculation of both, lid and plate, was adjusted to 0.5 McFarland standard using Densi-La-Meter II (Lachema, Brno, CZ) to get the final concentration of 10^7 CFU/mL.

2.3. Broth volatilization chequerboard method

The broth microdilution volatilization method [17], modified according to the chequerboard assay design [24], was used for assessment of combinatory antimicrobial effect of carvacrol and thymol in vapour and liquid phase. The experiments were performed in white 96-well immunoplates (total well volume = 400 μL) covered by tight-fitting lids with flanges designed to reduce evaporation (SPL Life Sciences, Naechon-Myeon, Republic of Korea). Initially, 30 μL of agar was pipetted into every flange on the lid (with exception of outer most wells) and inoculated with 5 μL of bacterial suspension. The lid layout is shown in Fig. 1a. Subsequently, both, carvacrol and thymol were dissolved in DMSO, and diluted in the broth medium to initial concentrations of 2048 μg/mL (with maximum concentration of DMSO 1%) DMSO did not inhibit the growth of bacteria in broth and agar media. Assay plate preparation and serial dilutions were performed by the automated pipetting platform Freedom EVO 100 equipped with four-channel liquid handling arm (Tecan, Mannedorf, CH). In combinations, six two-fold serial dilutions of thymol from horizontal rows were subsequently cross-diluted vertically by six two-fold serial dilutions of carvacrol. The initial concentration used for both thymol and carvacrol was 2048 μg/mL. After that, plates were inoculated by bacterial suspensions. Each plate also contained sterility and growth control. Oxacillin was used as a positive control for verification of susceptibility of *S. aureus* strains in broth media. The outer most wells were not used to prevent edge effect. The plate layout is shown in Fig. 1b. After the inoculation, plate and lid were fasten together by clamps (Lux Tool, Prague, CZ), with handmade wooden pads for better fixing (Fig. 2) and incubated for 24 h at 37 °C.

MICs and combinatory effect in both liquid (in plate) and vapour (on lid) phase were evaluated by visual assessment of bacterial growth after colouring of metabolically active bacterial colony with 25 μL of MTT dye when the interface of colour change from yellow and purple (relative to that of colours in control wells) was recorded in agar and broth (Figs. 3a, b). MICs were defined as the lowest concentration that visually inhibited growth of bacteria compared with the compound free growth control and expressed as in μg/mL. The final MIC value presented in this work is the average of MICs obtained from three independent experiments performed in triplicate. The MICs of independent experiments varied in maximum range of three-dilutions.

Combinatory effect of volatile compounds was determined based on fractional inhibitory concentration indices (ΣFIC). For combination of compound A (thymol) and compound B (carvacrol), the ΣFIC is calculated according to the following equation: $\Sigma FIC = FIC_A + FIC_B$, where $FIC_A = MIC_{A\ (in\ combination\ with\ B)} / MIC_{A\ (alone)}$, and $FIC_B = MIC_{B\ (in}$