Antibacterial effect of plant volatiles against *Pseudomonas aeruginosa* assessed by using broth microdilution volatilization method

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Abstract. Plant-derived compounds (carvacrol, cinnamaldehyde, eugenol, 8-hydroxyquinoline, thymol, and thymoquinone) were assessed for their *in vitro* growth-inhibitory effect against *Pseudomonas aeruginosa*. For this purpose, the broth microdilution volatilization method evaluating antibacterial activity simultaneously in liquid and vapour phase was used. This recently developed screening method is performed in 96-well microtiter plates, it combines principles of standard broth microdilution assay with disc volatilization test. The results showed 8-hydroxyquinoline and thymol as the most effective antibacterial agents with the lowest minimum inhibitory concentration 256 µg/mL for both compounds.

1. Introduction

*Pseudomonas aeruginosa* is the most common bacterial pathogen that easily colonizes respiratory system and causes airway infections associated with cystic fibrosis [1]. Respiratory diseases belongs to the major causes of morbidity and mortality, which pose the highest risk to children under five years, elderly and immuno-compromised individuals especially in low-income countries [2]. For example in Indonesia, more than 80,000 people died because of lower respiratory infections [3]. Timely antibiotic remedy can reduce fatal consequences of these infections. Besides a systematic antibiotic treatment, an inhalation therapy is possible way of the cure for such diseases. This approach based on direct delivery of the antibacterial agents to the site of infection in the respiratory system maximizes their efficacy and simultaneously restricts systemic exposure and associated toxicity [4]. Moreover, inhalation of therapeutics prevents the degradation of active compounds in the gastrointestinal tract [5].

Due to multidrug-resistance of *P. aeruginosa* strains, there are difficulties in the cure of the infections caused by this bacteria. It was observed that *P. aeruginosa* is resistant to at least three antibiotics from the group beta-lactams, carbapenem, aminoglycoside, and fluoroquinoline [6]. Therefore, there is need to search for new possibilities of antimicrobial agents. Natural substances including plant volatile compounds are of great potential for development of novel antimicrobial drugs, because of the wide range of chemical diversity. Medicinal plants contain a huge spectrum of such components, thus they can be effective in treatment of bacterial infections [7-9]. In contrast to well established methods for antimicrobial susceptibility testing in liquid media, there are no standardized assays for determination of microbial sensitivity to volatile compounds in vapour phase e.g. in accordance with Clinical and Laboratory Standards Institute (CLSI) or European Committee on Antimicrobial Susceptibility Testing (EUCAST) [10,11]
In this study we tested antibacterial activity of carvacrol, cinnamaldehyde, eugenol, 8-hydroxyquinoline, thymol, and thymoquinone as representatives of various classes of antimicrobially effective plant-derived compounds against *P. aeruginosa*. For this purpose, new screening method based on the broth microdilution volatilization was used that was designed for simple and rapid simultaneous determination of antibacterial potential of plant volatile compounds in the liquid and the vapour phase at different concentrations.

2. Material and methods

2.1. Chemicals

The plant volatile compounds: carvacrol (97 %, CAS 499-75-2), cinnamaldehyde (99 %, CAS 14371-10-9), eugenol (99 %, CAS 97-53-0), 8-hydroxyquinoline (99 %, CAS 148-24-3), thymol (99 %, CAS 89-83-8), and thymoquinone (99 %, CAS 490-91-5); antibiotic: ciprofloxacin (98 %, 85721-33-1) were purchased from Sigma-Aldrich (Prague, Czech Republic).

2.2. Bacterial strains and culture media

The standard strain of *Pseudomonas aeruginosa* ATCC 27853 was used. The cultivation and assay media were Mueller-Hinton (MH) broth and Trypton Soya Broth agar complemented by horse blood in concentration of 1%. The pH of broth was equilibrated to final value of 7.6 using Trizma® base (Sigma-Aldrich, Prague, Czech Republic). The bacterial strain and cultivation media were purchased from Oxoid (Basingstoke, UK).

Stock culture of *P. aeruginosa* was cultivated in MH broth at 37 °C for 24 h prior the testing, and then the turbidity of the bacterial suspension 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. The susceptibility of *P. aeruginosa* to ciprofloxacin was checked as positive antibiotic control [10].

2.3. Antimicrobial assay

The antibacterial potential of plant volatiles in liquid and vapour phase was determined using a broth microdilution volatilization method [12]. The experiments were performed in 96-well immune plates, covered by tight-fitting lids with flanges designed to reduce evaporation (SPL Life Sciences). Briefly, 30 µL of agar was pipetted into every flange on the lid, except the outermost flanges, and inoculated with bacterial suspension (5 µL). In the second part of this method, each sample of plant volatile compounds was dissolved in DMSO (Sigma-Aldrich) at maximum concentration of 1%, and diluted in broth medium. Seven two-fold serially diluted concentrations of samples starting from 1,024 µg/mL were prepared for all compounds. The plates were then inoculated with bacterial suspensions using a 96-pin multi-blot replicator (National Institute of Public Health). The wells containing inoculated and non-inoculated broth were prepared as growth and purity controls simultaneously. Finally, clamps (Lux Tool) were used for fastening the plate and lid together, with the handmade wooden pads for better fixing. The microtiter plates were incubated at 37°C for 24 h. The minimum inhibitory concentrations (MICs) were evaluated by visual assessment of bacterial growth after colouring of a metabolically active bacterial colony with thiazolyl blue tetrazolium bromide dye (MTT) (Sigma-Aldrich) at a concentration of 600 µg/mL when the interface of colour change from yellow and purple (relative to that of colours in control wells) was recorded in broth and agar. The MIC values were determined as the lowest concentrations inhibiting bacterial growth compared with the compound-free control and expressed in µg/mL. The DMSO assayed as the negative control at concentration of 1% did not inhibit the
bacterial strain tested either in broth or agar media. All experiments were carried out in triplicate in three independent experiments and results were expressed as median/modal MICs values.

3. Results and discussion

The results of in vitro growth-inhibitory effect of plant volatile compounds against *P. aeruginosa* in liquid and vapour phase using the broth microdilution volatilization method are summarized in Table 1. All volatiles tested exhibited certain degree of antibacterial effect at least in one medium. Their effectiveness varied substantially ranging from 256 to 1,024 µg/mL in both media.

In liquid phase, the lowest MIC values was observed for 8-hydroxyquinoline (256 µg/mL) followed by carvacrol (512 µg/mL). Cinnamaldehyde, eugenol, and thymol shown only weak antibacterial activity with MIC value 1,024 µg/mL. In vapour phase, the most active compound was found thymol with MIC 256 µg/mL. Other substances, carvacrol, cinnamaldehyde, and 8-hydroxyquinoline possessed moderate inhibitory effect with MIC 512 µg/mL, and thymoquinone (1,024 µg/mL). Interestingly, some compounds were more effective in vapour phase, for example MIC of thymol was four times lower on agar medium, thus it could be beneficial aspect in development of inhalation preparations for cure of respiratory diseases.

| Compounds              | Growth medium/MIC (µg/mL) | broth | agar     |
|------------------------|---------------------------|-------|---------|
| Carvacrol              |                           | 512.00| 512.00  |
| Cinnamaldehyde         |                           | 1024.00| 512.00  |
| Eugenol                |                           | 1024.00| >1,024.00|
| 8-Hydroxyquinoline     |                           | 256.00| 512.00  |
| Thymol                 |                           | 1024.00| 256.00  |
| Thymoquinone           |                           | >1,024.00| 1024.00|
| **Positive antibiotic control** |                     |       |         |
| Ciprofloxacin          |                           | 0.25  | 2.00    |

Table 1. Antibacterial activity of plant volatile compounds and antibiotics in liquid and vapour phase against pneumonia causing bacteria

The results of our assay are in correspondence with those obtained by other authors using standard broth microdilution method for various strains of *P. aeruginosa*, e.g. the MIC values previously observed for carvacrol (1,000 µg/mL) and eugenol (500 µg/mL) [13,14]. However results of other compounds previously tested are varying when MIC values of cinnamaldehyde, thymol, and thymoquinone were 125; 400; 1,56 µg/mL, respectively [15-17]. The differences in susceptibility of individual strains to antibacterial agents may be responsible for various results observed in our study and in the literature.

According to the results of cytotoxic effect of these volatiles observed in our previous study [12], carvacrol, eugenol, and thymol were found as non-toxic to the human lung cells, whereas cinnamaldehyde, 8-hydroxyquinoline, and thymoquinone were evaluated as toxic.
4. Conclusion
Considering the effectiveness of individual compounds tested, 8-hydroxyquinoline and thymol were identified as the most active agents against *P. aeruginosa* in liquid and vapour phase, respectively. Due to the non-toxic potential of thymol and the antibacterial effect of its vapours, it is suggested that practical potential of thymol in the inhalation therapy could be promising, however further research focused on *in vivo* evaluation will be necessary to be carried out in order to verify its potential practical use.

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