Natural Terpenes Influence the Activity of Antibiotics against Isolated *Mycobacterium tuberculosis*

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Significance of the Study

- The study revealed the synergistic effect of combinations of *R*-limonene, *S*-limonene, myrcene, sabinene, and β -elemene with first-line tuberculostatic antibiotics against isolated *Mycobacterium tuberculosis*. Hence, such combinations might be promising for treating resistant *M. tuberculosis*.

Key Words

Antimycobacterial antibiotics · Minimum inhibitory concentration · Terpenes · Synergistic activity · Tuberculosis

Abstract

Objective: In this study, we aimed to describe the influence of natural terpenes on the antimycobacterial activity of first-line tuberculostatic drugs against isolated *Mycobacterium tuberculosis*. **Materials and Methods:** The natural terpenes used in this study were *R*-limonene, *S*-limonene, myrcene, sabinene, α -pinene, and β -elemene. The values of the minimum inhibitory concentration (MIC) for these terpenes, as well as for combinations of terpenes with tuberculostatic antibiotics (ethambutol, isoniazid, and rifampicin), were determined using a tube log₂ dilution method in the range of 125–0.059 μ g/mL. **Results:** *S*-limonene had a strong synergistic effect with all tested antibiotics (MIC decreased from 16 to 0.475 μ g/mL for ethambutol, from 16 to 0.237 μ g/mL for rifampicin, and

from 32 to 0.475 μ g/mL for isoniazid). Combinations of myrcene, *R*-limonene, β -elemene, and sabinene with tuberculostatic antibiotics resulted in a decreased MIC of the antibiotics (from 3.9 to 0.475 μ g/mL for ethambutol, from 15 to 0.475 μ g/mL for isoniazid, and from 0.475 to 0.237 μ g/mL for rifampicin) while combinations of α -pinene with ethambutol and isoniazid resulted in increased MIC values (from 16 to 125 μ g/mL for ethambutol, and from 32 to 125 μ g/mL for isoniazid). Rifampicin had a synergistic increase in activity with all the tested compounds. **Conclusions:** Our study showed that terpenes enhance the activity of tuberculostatic antibiotics.

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Introduction

The World Health Organization reported that there was a 3.3% rise in new cases of tuberculosis (TB) in 2015 [1]. It was also estimated that 20% of previously treated

cases involved multidrug-resistant (MDR)-TB, and that, in this group, 9.7% were extensive drug-resistant (XDR)-TB [1]. Drug resistance in *Mycobacterium tuberculosis* (Mtb) is caused by the sequential accumulation of mutations in the genes encoding the targets of tuberculostatic antibiotics [2]. More importantly, the active transmission of genotypes of several strains circulating worldwide is the cause of increased resistance [2]. Molecular fingerprinting methods enable the identification of MDR-TB and XDR-TB, which constitute 0.5% of the circulating strains in Japan and 40% in Russia, respectively [2]. XDR-TB is the biggest threat because it is a form of the disease that is nearly untreatable [1] and may develop as a multi-system disease [3].

The natural terpenes are known for their antimicrobial properties, and their detrimental effects on the structure and function of the microbial membranes and cell walls are thought to be evidence of antimicrobial action [4]. Combinations of essential oils and their constituents have been reported to have synergistic, additive, or inhibiting activity with antimicrobial interactions against several microorganisms [5]. A high antimycobacterial activity of thymol and carvacrol has been described against Mtb and *Mycobacterium bovis* [4]. Synergistic in vitro interactions between oleanolic acid and isoniazid, rifampicin, or ethambutol against Mtb have also been described [6].

In this study, we aimed to describe the influence of *R*-limonene, *S*-limonene, myrcene, sabinene, α -pinene, and β -elemene on the antimycobacterial activity of first-line tuberculostatic drugs, i.e. isoniazid (an inhibitor of fatty acid synthesis), rifampicin (an inhibitor of RNA polymerase), and ethambutol (an inhibitor of arabinose transferases involved in cell wall biosynthesis).

Materials and Methods

Standards and Media

The standard antibiotics, rifampicin, isoniazid, and ethambutol, and also the terpenes (with the exception of sabinene) were purchased from Sigma-Aldrich (Munich, Germany). Gas chromatography-mass spectrometry (GC-MS) was used to verify the purity of the terpenes: 98% for α -pinene, 97% for *R*-limonene, 98% for *S*-limonene, 97% for β -elemene, and 95% for myrcene. Middlebrook medium 7H9 supplemented with Middlebrook oleic albumin dextrose catalase growth supplement (OADC enrichment) was obtained from BD Difco™ (Franklin Lakes, NJ, USA). Analytical grade *n*-hexane and acetonitrile were obtained from Polish Reagents (Gliwice, Poland), *tert*-butyl-methyl ether was purchased from Acros Organics (part of Thermo Fisher Scientific, Belgium). The helium 5.0 used for the GC-MS analysis was 99.999% pure, supplied by PGNiG (Warsaw, Poland). The commercially available carrot-seed essential oil was purchased from Primavera (Germany).

Isolation of Sabinene

Sabinene used in the susceptibility testing was isolated from commercially available *Daucus carota*-seed essential oil, as described previously [7]. Separation was carried out with Spectrum high-performance counter-current chromatography (HPCCC) equipment (Dynamic Extraction Co., Ltd. Slough, UK). The solvent system used for the separation was a mixture of *n*-hexane, acetonitrile, and *tert*-butyl-methyl ether in the ratio 2:1:0.1 (v/v), in the reverse-phase mode. A sample of 280 mg of essential oil was used for semipreparative separation, and continuously monitored with a UV detector at 210 nm. The 1-min fractions collected from the beginning of the run were monitored by GC-MS performed with a Shimadzu gas chromatograph, GC-2010 Plus, coupled with a Shimadzu QP2010 Ultra mass spectrometer. A fused-silica capillary column ZB-5 MS (length 30 m, internal diameter 0.25 mm) with a film thickness of 0.25 mm (Phenomenex) was used. The retention indices were determined in relation to a homologous series of *n*-alkanes (C8–C24) under the same operating conditions. Compounds were identified using a computer-supported spectral library (<http://www.massfinder.com>), mass spectra of reference compounds, and MS data from the literature [8].

Antimycobacterial Assay

The isolated strain of Mtb (attenuated) was donated by the National Institute of Tuberculosis and Lung Diseases, Warsaw, Poland. The minimum inhibitory concentration (MIC) for the tested terpenes, antibiotics, and combinations of terpenes and antibiotics were determined in a liquid medium, in a tube log₂ dilution test [9]. The antibiotics: rifampicin, isoniazid, and ethambutol, with known tuberculostatic activity, were used as a reference. Mtb was grown in roller bottles at 37°C in Middlebrook 7H9 liquid medium supplemented with oleic acid, bovine albumin fraction V, dextrose, and catalase (OADC enrichment). The stock solutions of terpenes and antibiotics were prepared in dimethyl sulfoxide, and then diluted in Middlebrook 7H9 broth supplemented with OADC enrichment. The final concentrations of tested compounds were in the range of 0.059–125 µg/mL.

The MIC values for terpenes and antibiotics alone were determined after 24 h of exposure to the tested substances in a bacterial suspension (equivalent to a McFarland No. 1 standard). For the determination of MICs of the combinations of antibiotics and terpenes, the antibiotics were added to the medium. Next, all samples were inoculated in 12 µL of Mtb cultures kept in the incubator for 12 h to equilibrate, and dissolved with broth to obtain the turbidity of the suspensions comparable to McFarland No. 1 standard. Turbidity of the suspensions was measured using a nefelometer (BD Phoenix Spec., USA). The tested terpenes were then added to each vial containing antibiotic and Mtb cultures, grown, and collected after 24 h of exposure at 37°C. The concentration of terpenes in the samples was as follows: α -pinene, 16 µg/mL; *S*-limonene and *R*-limonene, 64 µg/mL; β -elemene, 2 µg/mL; sabinene, 32 µg/mL; and myrcene, 32 µg/mL. Because the bacteria inoculum could cause traces of turbidity, the sample inoculated with Mtb and kept at 4°C for 12 h was used as a negative control. The second control contained the highest concentration of DMSO used in the samples, in order to eliminate the bactericidal effect of the solvent. The third, positive, control for bacteria growth contained only medium inoculated with Mtb.

The assessment of the activity of antibiotic-terpene combinations was done according to *x/y* methodology [10]. Briefly, *x* rep-

represents the MIC value of a drug-terpene combination, while y represents the lowest MIC value obtained with any of the single compounds used within the combination tested. An x/y quotient of <0.5 in the case of a 2-compound combination indicates enhanced drug action. Respective x/y quotients were interpreted as follows: no synergistic effect (–), $x/y \geq 0.5$; moderate synergistic effect (+), $x/y \leq 0.5$; significant synergistic effect (++), $x/y \leq 0.1$; and high synergistic effect (+++), $x/y \leq 0.05$ (Table 1).

Results

The HPCCC technique used in this work enabled the separation of sabinene from carrot-seed essential oil. Sabinene was present in fractions collected between 58 and 61 min. GC-MS evaluation confirmed the 99% purity of the target compound.

The MIC values obtained for terpenes and antibiotics tested alone as well as in combination are presented in Table 1. The cyclic monoterpene limonene showed the lowest antimycobacterial activity, regardless of the stereoisomers (MIC 64 $\mu\text{g/mL}$). The acyclic monoterpene myrcene and the bicyclic monoterpenes sabinene and α -pinene had higher activity than the limonenes (MIC range 16–32 $\mu\text{g/mL}$). The lowest MIC value was obtained for β -elemene (2 $\mu\text{g/mL}$). The investigated strain had a similar sensitivity to terpenes and antibiotics. Ethambutol and rifampicin inhibited bacterial growth at a 16- $\mu\text{g/mL}$ concentration, and isoniazid at a 32- $\mu\text{g/mL}$ concentration. Of the combinations of terpenes with first-line tuberculostatic antibiotics, *S*-limonene had a high synergistic effect with all antibiotics (MIC range 0.237–0.475 $\mu\text{g/mL}$). Rifampicin showed a high synergistic increase in activity with every compound tested, except for β -elemene (MIC range 0.237–0.475 $\mu\text{g/mL}$). Combinations of myrcene, *R*-limonene, and sabinene with antibiotics decreased the MIC for the antibiotics, while combinations of α -pinene with ethambutol and isoniazid had no synergistic effect (Table 1). Except for α -pinene, all the studied compounds enhanced the susceptibility of *Mtb* to the effects of the antimycobacterial drugs, but differences in sensitivity to the drugs used were observed.

Discussion

In this study, we tested the influence of natural terpenes on an isolated *Mtb* strain. The results revealed a similar sensitivity to the terpenes and antibiotics tested. What is more, a significant enhancement of antibiotic activity in the presence of terpenes was obtained. The MIC values

Table 1. The influence of terpenes on antibiotics activity

Compounds	MIC, $\mu\text{g/mL}$	Enhancement of drug activity (and respective x/y quotients) ^a
Ethambutol	16	
Rifampicin	16	
Isoniazid	32	
α -Pinene alone	16	
α -Pinene + ethambutol	125	– (7.81)
α -Pinene + isoniazid	125	– (7.81)
α -Pinene + rifampicin	0.475	+++ (0.03)
β -Elemene alone	2	
β -Elemene + ethambutol	0.475	+ (0.24)
β -Elemene + isoniazid	0.475	+ (0.24)
β -Elemene + rifampicin	0.237	+ (0.12)
Myrcene alone	32	
Myrcene + ethambutol	3.9	+ (0.24)
Myrcene + isoniazid	0.95	+++ (0.03)
Myrcene + rifampicin	0.475	+++ (0.03)
<i>S</i> -limonene alone	64	
<i>S</i> -limonene + ethambutol	0.475	+++ (0.03)
<i>S</i> -limonene + isoniazid	0.475	+++ (0.01)
<i>S</i> -limonene + rifampicin	0.237	+++ (0.01)
<i>R</i> -limonene alone	64	
<i>R</i> -limonene + ethambutol	0.95	++ 0.06
<i>R</i> -limonene + isoniazid	15	+ (0.47)
<i>R</i> -limonene + rifampicin	0.475	+++ (0.03)
Sabinene alone	32	
Sabinene + ethambutol	3.9	+ (0.24)
Sabinene + isoniazid	1.95	++ (0.06)
Sabinene + rifampicin	0.475	+++ (0.03)

^a An x/y quotient of <0.5 in the case of a 2-drug combination indicates enhanced drug action. –, ≥ 0.5 ; +, ≥ 0.5 ; ++, ≥ 0.1 ; +++, ≥ 0.05 .

obtained for *R*-limonene, *S*-limonene, myrcene, sabinene, α -pinene, and β -elemene tested alone suggest that acyclic, monocyclic, and/or bicyclic structure and the number of double bonds have no significant influence on the antimycobacterial activity of monoterpenes. Similar findings were reported in a previous study [11] but a higher activity for aromatic and/or oxygenated monoterpenes was obtained than for the corresponding carbonyl compounds [12]. Equally important, quantitative structure-activity relationship (QSAR) studies revealed that the number of conjugated carbons, the number of phenolic and hydroxyl groups, and the number of acceptor atoms of hydrogen bonds are the most important structural descriptors in the antimycobacterial activity of terpenes [4].

Despite having the highest MIC, limonene showed tuberculostatic activity. Several studies have proved that lim-

onene interacts with the cytoplasmic membranes of bacteria, resulting in a loss of membrane integrity, the dissipation of the proton-motive forces, and the inhibition of the respiratory enzymes [13]. What is more, a *D*-limonene, organogel-based nanoemulsion was found to cause irreversible damage to the cytoplasmic membranes of bacteria other than *Mycobacterium* [14]. In our studies, myrcene was more active than limonene, but Gallucci et al. [15] report that it was not active against slime-producing/non-slime-producing staphylococci, and that this is associated with its low aqueous solubility. This low aqueous solubility could limit the dose required to inhibit the growth of staphylococci, but in the case of Mtb, the amount of myrcene dissolved in the aqueous medium was sufficient to penetrate the cell wall barrier. Our previous study showed that monoterpenes caused morphological changes in mycobacterial cells, suggesting that they may affect the cell wall synthesis/maintenance pathways, with the consequences being changes in cell permeability and also microbial death [16]. In this study, the best antimycobacterial activity was observed for sesquiterpene, β -elemene. This several-times lower MIC value may be explained by higher lipophilicity ($\log_p = 6.1$) compared to the other studied substances, and a greater affinity to lipophilic structures in the mycobacterial cell wall and phospholipid bilayer [4]. Vik et al. [17] observed very good antimycobacterial activity for geranylgeraniol, which has a similar lipophilicity ($\log_p = 6.6$) to β -elemene. Furthermore, in the QSAR model, the lipophilicity descriptor octanol/water ($\log_p$) showed a high contribution to the activity of terpenes against Mtb [4].

One would expect that the MIC values obtained for the control antibiotics would have been the lowest, but antibiotics tested alone showed activity comparable with that of α -pinene, sabinene, and myrcene. The low bacteria sensitivity to antibiotics is characteristic for isolated strains of Mtb, which usually acquires resistance to ≥ 1 tuberculostatic drugs [18–20]. What is more, Mtb strains possess natural resistance due to the rich composition of mycolic acids in the mycobacterial cell wall, which is highly lipophilic and responsible for a low permeability to most antibiotics [4, 10]. The lipophilic properties of natural terpenes are responsible for their affinity to the phospholipid bilayer. The study performed on isolated bacterial membranes suggests that cell membranes may be a site of action of terpenes, and that antibacterial activity is a result of their lipophilic properties, the potency of their functional groups, and their aqueous solubility [21]. Terpenes change the permeability of the outer membrane of bacteria, but their absorption is also determined by the permeability of the cell wall [4]. The changed permeability of the cell wall

alters the absorption and activity of antibiotics. In our study, the best results were obtained with combinations of terpenes with rifampicin, which is a lipophilic compound that inhibits DNA-dependent RNA polymerase, by forming a stable steric block with the enzyme and subsequently inhibiting a transcription elongation [22]. Rifampicin works on a genetic material level. A less significant increase in activity was observed for isoniazid and ethambutol under the influence of terpenes. These antibiotics act at the cell wall level. Isoniazid, when activated in vivo, can oxidize or acylate protein groups that are part of the synthesis of mycolic acids. It leads to the inhibition of the elongation of fatty acids during the synthesis of mycolic acids, by interacting with the NADH-dependent enoyl-ACP reductase, while ethambutol inhibits arabinosyltransferase and interferes with the biosynthesis of the arabinogalactan [23]. These differences in drug susceptibility caused by terpenes confirm that the site of action of terpenes may be the cell membrane [24]. The drugs interfering with cell envelope formation are less efficient in combination with terpenes because the terpene disrupts the cell wall/membrane [25]. In the case of rifampicin, terpenes enable better penetration into the cell. The significant increase in the activity of rifampicin in combination with terpenes indicates that the resistance acquired by the examined strain was not associated with gene mutation. Also studies on Mtb clinical isolates resistant to tuberculostatic drugs demonstrated that not only classical gene mutations play role in resistance. Additional mechanisms that contribute to drug resistance in mycobacteria are: drug-modifying and -inactivating enzymes and efflux-related mechanisms [25]. Because the combinations of antibiotics with terpenes increase the bacterial susceptibility to antibiotics the drug modifications probably is not a reason of resistance. Decreased membrane permeability alone is also unlikely to contribute in significant antibiotic resistance [25] and it was shown that Mycobacteria extrude many drugs via active efflux systems. The active efflux is an immediate stress response to the presence of noxious agents [26]. No specific gene was associated with extrusion of isoniazid via an efflux pump [26], however in case of rifampicin several efflux systems (Rv2333, DrrB, DrrC, Rv0842, BacA, and EfpA) were indicated [27]. Terpenes can modulate the membrane proteins and receptors in a nonspecific manner [28], but some naturally occurring lipophilic alkaloids, terpenes, and flavonoids have been described as efflux pump inhibitors [29]. The terpene carnosic acid from *Rosmarinus officinalis* and the diterpene totarol from *Cupressus nootkatensis* inhibit the efflux pump NorA in *Staphylococcus aureus* [30]. This kind of

interaction of terpenes with efflux pumps would make mycobacteria more susceptible to antimycobacterial drugs and may contribute to the increased activity of tuberculostatic antibiotics tested in combination with terpenes.

Conclusions

This study revealed that natural terpenes with high lipophilicity inhibited the growth of mycobacteria to a greater extent. Equally important, *R*-limonene, *S*-limo-

nene, myrcene, sabinene, and β -elemene increased the antimycobacterial activity of tuberculostatic antibiotics due to inhibition of the natural mechanisms of mycobacteria resistance.

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