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Morphological Changes in the Overall *Mycobacterium tuberculosis* $H_{37}Ra$ Cell Shape and Cytoplasm Homogeneity due to *Mutellina purpurea* L. Essential Oil and Its Main Constituents

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Key Words

Mycobacterium tuberculosis $H_{37}Ra$ · *Mutellina purpurea* · Tuberculosis · Minimal inhibitory concentration · Terpenes · Essential oil

Abstract

Objective: The aim of this study was to evaluate the antimycobacterial activity of the essential oil (EO) of *Mutellina purpurea* L. and its main constituents against the *M. tuberculosis* $H_{37}Ra$ strain. ***Materials and Methods:*** The *M. purpurea* EO was obtained by hydrodistillation, while its main constituents were purchased. The minimal inhibitory concentration values were determined by the log2 dilution method. Visualization of the effects of the tested substances on *M. tuberculosis* was performed using a transmission electron microscope (TEM). Mathematical shape descriptors such as area, circularity, aspect ratio and roundness were calculated to describe morphological changes in bacterial cell shape. ***Results:*** The EO of *M. purpurea* and all substances tested in this experiment showed a significant antimycobacterial activity. The most active was α-pinene followed by bisabolol and myrcene (8, 16 and 32 μg/ml, respectively). The EO and limonene exhibited the same antimicrobial activity (64 μg/ml). The TEM images and shape descriptors showed significant changes in the overall tuberculosis cell shape and cytoplasm homogeneity (uniformity and consistency) ***Conclusions:*** In this study, the low molecular weight compounds of mono- and sesquiterpenes penetrated/destabilized the complex mycobacterial cell wall and decreased its viability. There is a need for further experiments to explain the mechanism of action of these small particles.

Introduction

According to the WHO, tuberculosis (TB) is second only to HIV/AIDS as the greatest killer worldwide due to a single infectious agent [1]. In the recent years, 310,000 people worldwide became sick with multi-drug-resistant TB (resistant to isoniazid and rifampicin) and extensively drug-resistant TB with additional resistance to fluoroquinolones and injectable drugs (amikacin, kanamycin, capreomycin) [1]. Therefore, efforts are required to search for new molecules to prevent the spread of the disease [2].

Natural products contribute significantly as a source for the derivation of lead compounds and the development of drugs that are introduced into the market [3]. Traditional knowledge applications and its use of plant

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extracts in medicinal practices provide an excellent database for the potential identification of sources that may provide lead compounds with bioactive properties. In the field of anti-TB agents, a number of ethnobotanical surveys on potential medicinal plants have been reported from various parts of the world [4–7]. A large group of aromatic plants are among those with antimicrobial activity [8]. The pharmacological properties of aromatic plants are partially attributed to essential oils (EOs) [9, 10]. *Mutellina purpurea* L. (syn. *Ligusticum mutellina*) is a perennial, aromatic Apiaceae plant, which was described by Dudek et al. [11] as an antitubercular plant listed among *Cetraria islandica, Glechoma hederacea, Mentha arvensis, Plantago lanceolata, Plantago major*, *Polypodium vulgare*, *Pulmonaria officinalis* or *Verbascum thapsus.*

Based on the results of our earlier findings on the antibacterial activity of the *M. purpurea* EO [12], the present investigation was undertaken to evaluate the antimycobacterial activity of the EO of this plant and its main constituents against the *M. tuberculosis* H_{37}Ra strain.

Materials and Methods

Extraction and Analysis of Plant Material and EO

M. purpurea L. herb was collected in the Medicinal Plants Garden of the Medical University of Lublin in the vegetation season of 2012. The voucher specimen was deposited at the Department of Pharmacognosy with the Medicinal Plant Unit, Medical University, Lublin, Poland (ES2012MP). The EO hydrodistillation and gas chromatography/mass spectrometry analysis of EO were described previously [12].

Standards and Media

Unless otherwise stated, all chemicals and standards (α-pinene, bisabolol, limonene and myrcene) were obtained from Sigma-Aldrich (Munich, Germany). Commercial standards of streptomycin and isoniazid were purchased from Sigma-Aldrich (Munich, Germany). Middlebrook medium 7H9 supplemented with OADC (oleic acid, bovine albumin fraction V, dextrose and catalase) enrichment was obtained from BBL/Becton-Dickinson, USA.

Antimycobacterial Assay

The values of the minimal inhibitory concentration (MIC) for the tested substances (EO, α-pinene, myrcene, limonene and bisabolol) were determined by the log2 dilution method [13]. *M. tuberculosis* H_{37}Ra were grown in roller bottles at 37°C in Middlebrook 7H9 liquid medium supplemented with OADC enrichment (Difco). The stock solutions of EO and its main compounds were prepared in dimethyl sulfoxide. The highest concentration of dimethyl sulfoxide used in the determination of the MIC did not exceed 1% and had no influence on the bacterial viability. In order to determine the mycobacterial susceptibility, standard antibiotics control was performed at the same time. Antibiotics with known tuberculostatic activity (streptomycin and isoniazid) were used as a reference. The strain of *M. tuberculosis* H_{37}Ra (attenuated) donated by the Scientific Research Pulmonology Institute, Warsaw, Poland, was used in this study. Cultures were stored on Löwenstein-Jansen agar at 4°C and then grown on Middlebrook 7H9 medium supplemented with OADC (BBL/Becton-Dickinson) to 0.25 optical density at 600 nm (equivalent to a McFarland No. 1 standard). Then cultures were divided into 50-ml aliquots and kept in the incubator for 24 h to equilibrate. Turbidity of the suspensions was measured using a nephelometer (BD Phoenix Spec.; Becton-Dickinson). Then the tested substances (EO, α-pinene, myrcene, limonene and bisabolol) were added to each vial containing the cultures, grown and collected after 24 h of exposure. The control was treated as the vials containing the tested substances.

A transmission electron microscope (TEM; Tecnai G2 20 X-TWIN companies; Fei Company, Hillsboro, Oreg., USA) was used to visualize the effects of the tested substances on *M. tuberculosis.* Preparations from the mycobacterial cells were made on formvar-carbon grids.

2-Dimensional Shape Analysis of Mycobacterial Cells

Mathematical shape descriptors are much more effective in describing the shape than semantic characterization; thus, basic 2-dimensional cell shape calculations were performed for quantitative characteristics of morphological changes in bacteria.

Analysis of the TEM images was performed with the ImageJ program (National Institutes of Health, Bethesda, Md., USA). Shape descriptors and their formulae were used in the following calculations: area (μm^2); circularity $4\pi \times (\text{area}/\text{perimeter}^2)$, where a value of 1.0 indicates a perfect circle, and a value of 0.0 indicates an increasingly elongated shape; aspect ratio (major axis/minor axis) describes the proportional relationship between width and length; roundness $4 \times (\text{area})/\pi \times (\text{major axis})^2$ is the inverse of the aspect ratio. Ten images of the single cells were analyzed both for control and each evaluated substance. Statistical data were computed using Excel (Microsoft Corp.)

Table 1. The MIC values of the EO and its main constituents

Substances tested	MIC, μg/ml
EO	64
α-Pinene	8
Bisabolol	16
Limonene	64
Myrcene	32
Izoniazid	32
Streptomycin	32

Results

The *M. purpurea* EO and its main constituents tested in this experiment showed antimycobacterial activity described as significant (<100 μg/ml). The MIC values are presented in table 1. The most active was α-pinene followed by bisabolol and myrcene with MIC values of 8, 16

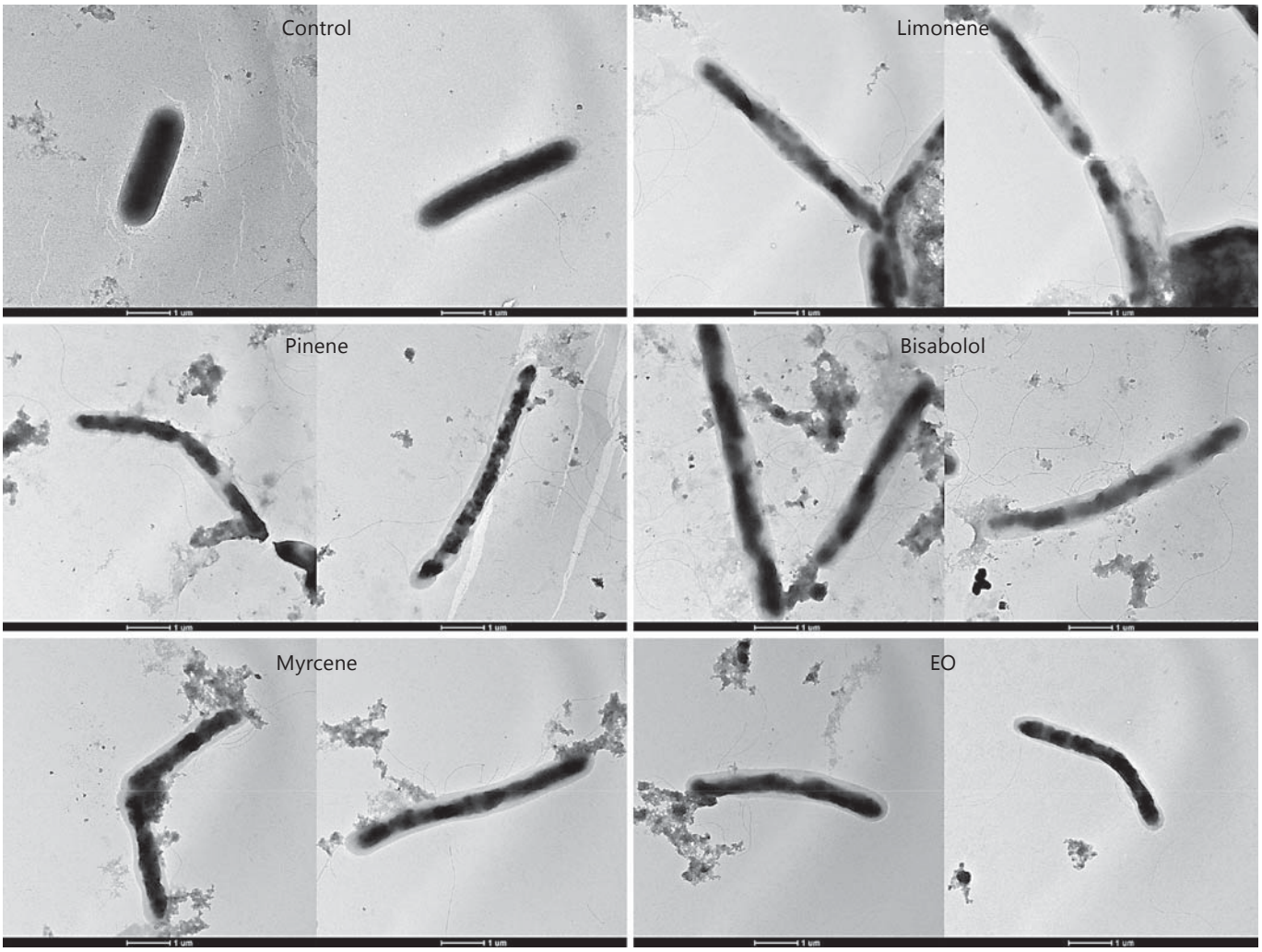

Fig. 1. The TEM visualization of *M. tuberculosis*. Images show the influence of α-pinene, myrcene, limonene, bisabolol and EO on bacterial cell morphology.

and 32 μg/ml, respectively. EO and limonene exhibited the same antimicrobial activity (MIC value 64 μg/ml). The TEM images showed significant changes in the overall TB cell shape and cytoplasm homogeneity (fig. 1–3).

Discussion

In this study, we verified the previously reported [11] antimycobacterial activity of *M. purpurea*. The direct estimation of MIC of antibacterial activity was supported by the assessment of mycobacterial cell morphology, using the mathematical shape descriptors. The obtained results showed that the main constituents of *M. purpurea* EO (α-pinene, myrcene, limonene and bisabolol) had significant antimicrobial activity, greater than that of the whole EO. Therefore the EO activity could be attributed to several compounds, that represents 26% of *M. purpurea* EO. The MIC obtained for α-pinene was twice lower than the MIC of bisabolol, 3 times lower than the MIC of myrcene and antibiotics (izoniazid and streptomycin) and 4 times lower than the MIC of the whole EO and limonene. Based on these results, it could be expected that the biggest changes in cell morphology will be observed for α-pinene and then for bisabolol. However, MIC values for α-pinene and bisabolol were not directly reflected in morphological changes in bacterial cell shape. The differences were not statistically significant in all parameters.

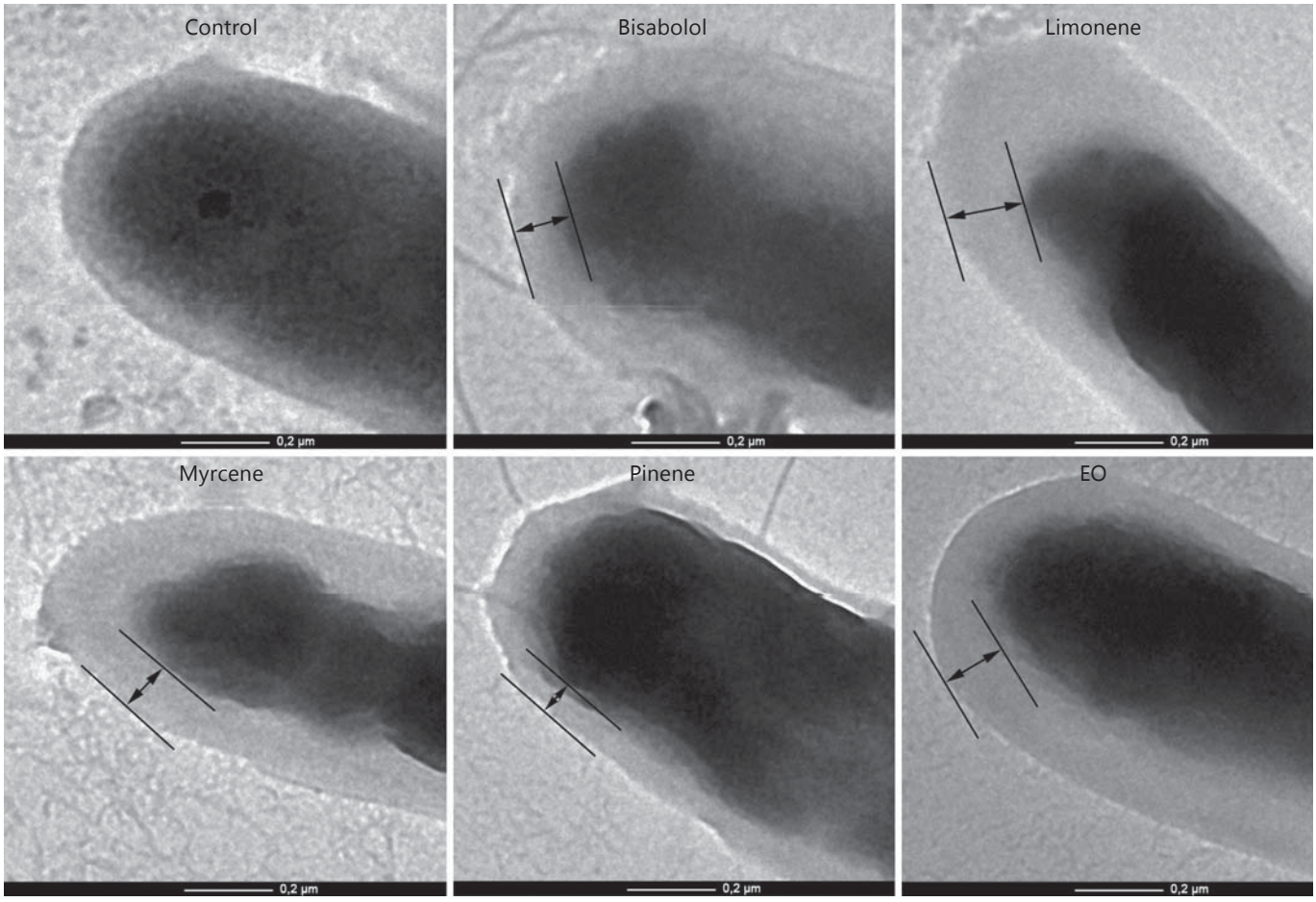

Fig. 2. The influence of *M. purpurea* EO and its main constituents on the cytoplasm homogeneity (uniformity and consistency) and cell wall thickness of *M. tuberculosis*. The differences in the cell wall thickness are marked with arrows.

The areas covered by both control and experimental cells were virtually the same (average 2.56 μm^2). The other descriptors showed statistically significant differences ($p < 0.05$) between control and cells treated with almost all tested substances. The circularity and roundness parameters were lower for experimental cells compared to control cells. Only in case of bisabolol were these differences not statistically significant. The aspect ratio parameter was higher for all experimental cells; however, this difference was statistically significant only for limonene, myrcene and α-pinene. The mathematical shape descriptors indicate that bacteria are thinner and longer when exposed to the tested compounds. What is more, filamentation of the investigated mycobacterial cells was observed. Filamentation is the anomalous growth of bacteria, in which cells continue to elongate but do not divide. Oxidative stress, nutrient limitation, DNA damage and antibiotics exposure are some of the stress conditions to which bacteria respond, altering their DNA replication and cell division. Filamentous bacteria have been considered to be overstressed, sick and dying members of the population [14]. Goldberg and Morgan [15] observed that sublethal or noninhibitory concentrations of streptomycin caused a marked change in the morphology of cells of 18- to 24-hour cultures of *Erwinia amylovora*. A brief study of this phenomenon by electron microscopy showed that following the treatment of the cells with streptomycin a series of nodules appeared in the cytoplasm. The observation in *E. amylovora* may be similar to that occurring in *M. tuberculosis* where shrinkage of the cell wall emphasizes solid material remaining within the cell treated with tested substances.

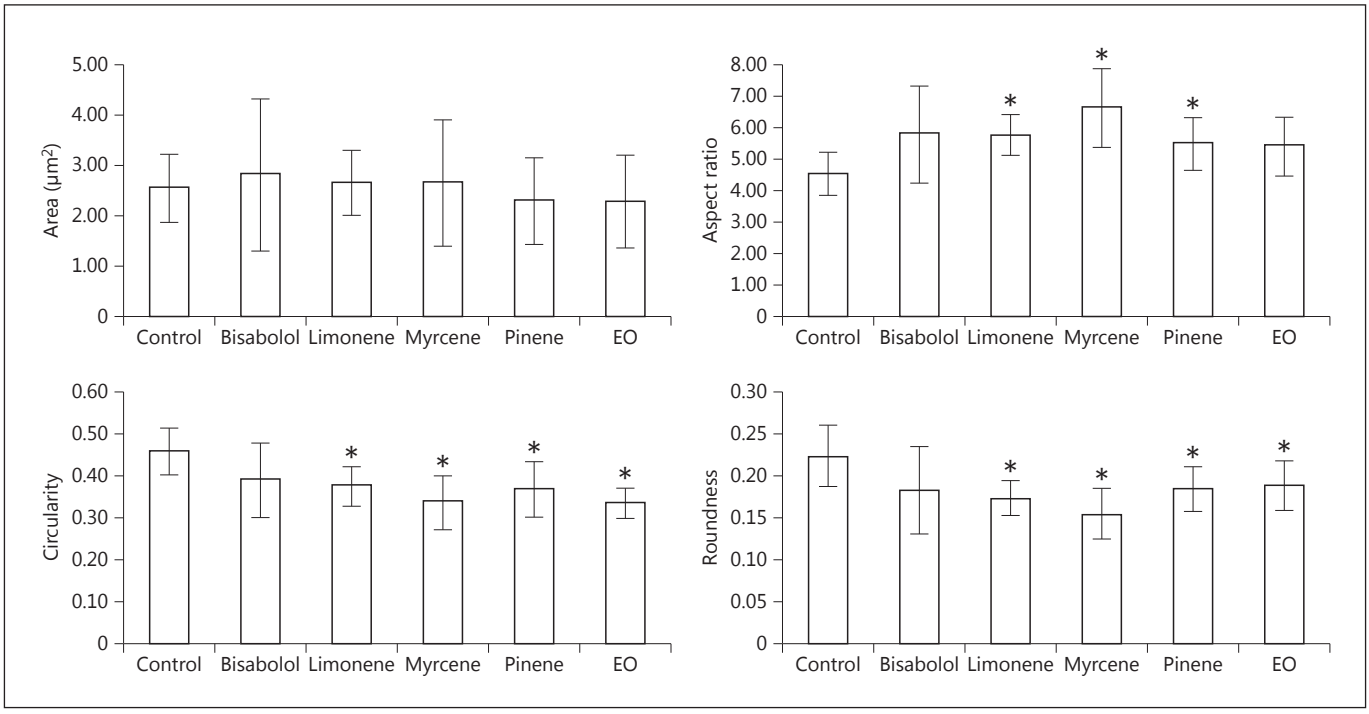

Fig. 3. The bar graphs of *M. tuberculosis* cell shape changes. Bars marked with an asterisk show statistically significant data ($p < 0.05$).

The previous literature data concerning antimycobacterial activity are limited only to MIC values [16–21]. The activity of several *Salvia* extracts against *M. tuberculosis* $H_{37}Ra$ was estimated as 196 µg/ml to the MIC [16]. The cumin and cinnamon EOs showed a MIC of 12.5 µg/ml against the reference strain $H_{37}Rv$ [17]. However, the MIC value does not suggest any mechanism of action of EOs or active isolated compounds. Mathematical shape descriptors may be useful in the case of mycobacteria, because the shape of the mycobacterial cells is maintained by the cell wall. The changes observed in this study may suggest that the tested substances (except bisabolol) affect the cell wall synthesis/maintenance pathways.

In this study, the synergism of action of constituents of EO was not observed. This is in opposition to the results obtained for *M. purpurea* EO and α-pinene tested against *Staphylococcus epidermidis*, where the EO had a lower MIC value than α-pinene [12]. The differences in activity of EO and isolated compounds may be caused by different ways of interaction with bacterial cells and a different sensitivity of tested microorganisms. These findings highlight the need for evaluation of additional features or parameters that may indicate the mechanism of action of the active ingredients. The results presented in this paper show the new approach to detection and measurement of *M. tuberculosis* morphological changes.

Conclusion

In this study, a low molecular weight compound of mono- and sesquiterpenes penetrated/destabilized the complex mycobacterial cell wall and decreased its viability. However, there is a need of further experiments to explain the mechanism of action of these small particles.

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