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Antimycobacterial natural products from Moroccan medicinal plants: Chemical composition, bacteriostatic and bactericidal profile of *Thymus satureioides* and *Mentha pulegium* essential oils

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ABSTRACT

Objective: To evaluate the susceptibility of *Mycobacterium aurum* and *Mycobacterium smegmatis in vitro* to the essential oils obtained from two medicinal plants: *Thymus satureioides* (*T. satureioides*) and *Mentha pulegium* (*M. pulegium*), and to study their chemical composition.

Methods: The aerial parts of *T. satureioides* and *M. pulegium* (leaves and stems) were hydro-distillated using a Clevenger-type apparatus and essential oils were analyzed and identified by gas chromatography-mass spectrometry. Antimycobacterial screening of essential oils was performed on the basis of the inhibition zone diameter by disc diffusion method against two mycobacterial strains whereas the minimal inhibitory concentration and minimal bactericidal concentration were determined by using the micro-dilution method.

Results: Chemical analysis of their aerial part's essential oils gave as major compounds, borneol (34.26%), carvacrol (31.21%) and thymol (3.71%) for *T. satureioides* and R(+)-pulegone (75.48%), carvone (6.66%) and dihydrocarvone (4.64%) for *M. pulegium*. Thereafter their antimycobacterial effect evaluation, using the micro-dilution method, indicated that minimal inhibitory concentration values of *T. satureioides* essential oil ranged from 0.062% to 0.015% (v/v) and from 0.125% to 0.031% (v/v) for *M. pulegium* respectively against *Mycobacterium aurum* and *Mycobacterium smegmatis*.

Conclusions: It is clearly evident from the results obtained that the Moroccan medicinal plants have great potential to be used as anti-tuberculosis agents. These findings may help scientists to undertake several research projects to discover useful natural product as new anti-tuberculosis drug.

1. Introduction

The infectious killer disease, tuberculosis (TB), is the leading death cause worldwide from a single human pathogen. It still remains a great public health problem and comes at the second rank of death causes by infectious diseases worldwide.

Mycobacterium tuberculosis (*M. tuberculosis*), discovered by Robert Koch in 1882, is the usually responsible organism which typically affects the lungs (pulmonary TB), but can also affect other sites (extrapulmonary TB) [1]. In addition, the AIDS epidemic and the emergence of bacilli multi-resistant to antibiotics aggravates the impact of this disease. Indeed, HIV and Koch's bacillus make a dangerous combination, each of these two infectious agents helping the growth of the other [2].

According to the latest World Health Organization estimates, there were 9.0 million new TB cases in 2013 and 1.5 million TB deaths (1.1 million among HIV-negative people and 0.4

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million among HIV-positive people) [1]. These alarming statistics indicate the devastating nature of TB.

The majority of *Mycobacterium* species are resistant to the most widely used therapeutic agents in the treatment of tuberculosis which is hydrazide of isonicotinic acid (isoniazid) [3]. Thus, there is an urgent need to search for and develop new, effective and affordable anti-TB drugs. Hence, recently growing interest has focused on naturally occurring molecules; in particular plant oils and crud extracts which have been used for a wide variety of purposes for many years.

The objective of this work is to explore opportunities, for drug discovery by researchers, which are offered by Morocco represented by its rich culture, traditions and natural biodiversity. So, this investigation was aimed to study the chemical composition of *Thymus satureioides* (*T. satureioides*) and *Mentha pulegium* (*M. pulegium*) essential oils, followed by a screening of the antimycobacterial activity. Furthermore, the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were determined.

2. Materials and methods

2.1. Plant material

The plants used in this work were *T. satureioides* and *M. pulegium*, freshly harvested and collected respectively from Ljoukak (30°59′50.365″ N, 8°9′45.13″ W, altitude 1 210 m) and Oued Laou (35°26′24″ N, 5°4′48″ W, altitude 69 m). The botanical authentication was confirmed at the National Institute of Medicinal and Aromatic Plants, Morocco.

2.2. Essential oils extraction

The aerial parts of *T. satureioides* and *M. pulegium* (leaves and stems) were hydro-distillated using a Clevenger-type apparatus to recover the essential oils for 3 h. The distilled essential oils were kept in dark at 4 °C until further use.

2.3. Gas chromatography-mass spectrometry (GC–MS) analysis conditions

The essential oil was analyzed using GC–MS (Polaris Q ion trap MS). Hence, analyses were performed on a Hewlett-Packard (HP 6890) gas chromatograph (flame ionization detector), equipped with a 5% phenyl methyl silicone HP-5 capillary column (30 m × 0.25 mm × film thickness 0.25 μm). The temperature was programmed from 50 °C after 5 min initial hold to 200 °C at 4 °C/min. Chromatography carrier gas was N_2 (1.8 mL/min); split mode was used with a flow of 72.1 mL/min and a ratio of 1/50; temperature of injector and detector was 250 °C, and final hold time was 48 min. The machine was led by a computer system type "HP Chem Station", managing its functioning and allowing to follow the evolution of chromatographic analyses. Diluted samples (1/20 in methanol) of 1 μL were injected manually.

2.4. Microbial strains

The essential oils of *T. satureioides* and *M. pulegium* were tested for their antimycobacterial activity against two reference microbial strains.

Mycobacterium aurum A+ (*M. aurum*) is a non-pathogenic *Mycobacterium* species with a generation time of approximately 6 h. This strain was used as a model to evaluate the effect of active substances on the growth of *M. tuberculosis*.

Mycobacterium smegmatis mc2-155 (*M. smegmatis*) is a non-pathogenic atypical mycobacterial strain with a generation time of approximately 3 h.

These strains were maintained in 20% glycerol at −20 °C and sub-cultured before use. The mycobacteria were cultivated at 37 °C on Sauton's medium for 48–72 h [4,5]. The turbidity was adjusted at 10^6 UFC/mL (estimated by absorbance at 600 nm).

2.5. Disc diffusion method

A primary antimycobacterial screening was performed using the disc diffusion method according to National Committee for Clinical Laboratory Standards [6]. Briefly, Petri dishes containing Sauton agar culture medium were inoculated with a previously prepared mycobacterial inoculum. The discs (filter paper, 6 mm of diameter) placed in the center of each plate were impregnated with 5 μL of each essential oil. Petri dishes were placed at 4 °C for 2 h to allow a better diffusion of molecules and then incubated at 37 °C for 48–72 h.

The antimycobacterial activity was evaluated by measuring the diameter of inhibition zone in millimeters. All experiments were conducted in triplicates.

2.6. Determination of MIC

The MIC values, which represent the lowest essential oil concentration that completely inhibits the growth of mycobacteria, were performed in 96 well-microplate using the microdilution assay according to the protocol previously described by Bouhdid *et al.* [7] with slight modifications.

Due to the immiscibility of essential oils with water and thus the culture medium, each essential oil was serially diluted in Sauton broth supplemented with agar 0.15% (w/v), used as an emulsifier, in which the final concentration of the essential oil was between 8.000% and 0.007% (v/v) for *T. satureioides* and *M. pulegium*. The 12th well was considered as growth control (it contained only the culture medium and strain). Then, 50 μL of bacterial inoculum was added to each well at a final concentration of 10^6 CFU/mL. After incubation at 37 °C for 48–72 h, 10 μL of rezasurin were added to each well as mycobacterial growth indicator. After further incubation at 37 °C for 2 h, the bacterial growth was revealed by the change of coloration from purple to pink [7]. Experiments were carried out in triplicates to minimize experimental error.

2.7. Determination of MBC

The MBC was determined by inoculating 3 μL from each negative well, which were spotted on Sauton plates and incubated at 37 °C for 48–72 h. The MBC corresponded to the lowest concentration of the essential oil at which the incubated microorganism was completely killed [8]. Tests were performed in triplicates.

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