SUPPLEMENATRY MATERIAL

"*In vitro*" activity of *Melaleuca cajuputi* against mycobacterial species

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Abstract

The increasing incidence of resistance in tuberculosis and in atypical mycobacterial infections has prompted the search for alternative agents. We explored the antimycobacterial activity of *Melaleuca cajuputi* essential oil against tubercular and non tubercular mycobacterials isolates. The good activity observed towards *M. cajuputi* indicated that this essential oil might represent a promising antimicrobial agents, particularly in the management of microbial resistance.

Key Words: Tuberculosis; Melaleuca; Resistance; Bacteria

Experimental section

M. cajuputi was collected in September 2017 in Vietnam in PhongDien District, ThuaThien Hue Province, Vietnam **(PhongDien District: 16°29'15.68" N 107°17'20.00" E).** Plant taxonomist authenticated the plant, and the sample was kept in the Herbarium SASSA of the Department of Chemistry and Pharmacy, University of Sassari, with voucher specimen number ML0125MCJV.

The essential oil sample was obtained by hydrodistillation in a Clevenger type apparatus for 4 hours following an established protocol [European Pharmacopeia Council of Europe, 2002].

The part of the plant used to obtain the essential oil was the leaves and the amount of plant material used in the extraction was 5 Kg.

The yield of EO ranged between 0.01% (2 litres/1000 kg : 0.2%) (v/w) and 0.04% (3 litres/1000 kg:0.3%) (v/w). The extraction was carried out in triplicate and the EO obtained was dried over anhydrous sodium sulfate and then stored at –20°C until analyzed**.**

Gas chromatography–mass spectrometry (GC–MS) analysis was carried out using an Agilent Technologies model 7820A coupled to an Agilent 5977E MSD detector. The chromatographic separation was performed on a silica capillary column (60 m x 0.25 mm, film thickness 0.25µm) (Agilent). The following temperature programme was used: 50°C increased to 135°C at a rate of 5.0 °C/min held for 1 min, then increased to 225°C at a rate of 5°C/min and held for 5 min, finally increased to 260°C at a rate of 5°C/min and held for 10 min. Helium was used as the carrier gas.

Identification of the individual components was performed of their retention times with those of authentic samples and/or by comparison of their mass spectra with those of published data (Nist Library Mass spectra) or on the interpretation of the EI-fragmentation of the molecules.

Gas chromatography–flame ionization detector (GC–FID) analysis was carried out using a Hewlett-Packard Model 5890A GC equipped with a flame ionization, using the same conditions and column of GC-MS analysis. The quantization of each compound was expressed as absolute weight percentage using internal standard and response factors. The detector response factors (RF_S) were determined for key components relative to 2,6-dimethylphenol and assigned to other components on the basis of functional group and/or structural similarity.

Retention indexes

A hydrocarbon mixture of *n*-alkanes (C9-C22) was analyzed separately under the same chromatographic conditions used on the HP-5MS and the VF-Wax capillary columns in order to

calculate the retention indexes with the generalized equation by Van del Dool and Kartz (1963), $I_x = 100[(t_x - t_n)/(t_{n+1} - t_n) + n]$, where t is the retention time, x is the analyte, n is the number of carbons belonging to the alkane that elutes before the analyte and $n + 1$ is the number of carbons belonging to the alkane that elutes after the analyte.

Antimycobacterial activity

Antimycobacterial activity of *M. cajuputi* EO has been tested against twenty-two microorganisms: sixteen strains of *Mtb* (*Mtb* H37Rv and clinical isolates of *Mtb* indicated from1 to 15) and six strains of NTM (*M. abscessum-1, M. abscessum-2, M. simiae, M. avium, M. gordonae-1* and *M. gordonae-2*). All mycobacteria strains were collected from Laboratory of Mycobacteriology, University of Sassari. Except *Mtb* H37Rv, all mycobacterial species were clinical isolates. *Mtb* H37Rv and *Mtb-2* were susceptible to all first antitubercular drugs, while the remaining strains, both *Mtb* that NTM, had one or more resistances. Five *Mtb* strains were Multi-Drug-Resistant (MDR), MDR TB is TB that does not respond to at least isoniazid and rifampicin, the 2 most powerful anti-TB drugs.

The antimycobacterial activity of *Melaleuca* was assessed by the Resazurin Microtiter Assay (REMA) as described in Palomino et al [Palomino et al., 2002]. Briefly, 100 µl 7H9 was dispensed in each well of a sterile 96-well plate, and serial twofold dilutions of essential oil were prepared directly on the plate by adding 100 µl of the working solution of drug to achieve the final concentration. For all mycobacteria strains the EO concentration range used was 16-0.5% (v/v). The inoculum was prepared from the 7H9 growth, adjusted to a McFarland tube scale 1. The suspension was diluted 1:10 and 100 µl was added to each well. The plates were covered, sealed in plastic bags, and incubated for 7 days at 37ºC. After the final visual reading, 30 µl of 0.02% resazurin was added to each well and re-incubated overnight. A change in color from blue (oxidized state) to pink (reduced) indicated bacterial growth and Minimal inhibitory concentration (MIC) was defined as the lowest drug concentration that prevented the color change.

Table S1: Compounds, expressed as area percentage, identified in the *Melaleuca cajuputi* EOs

Retention index	Compound	Area percentage (%)
930	α-thujene	3.93
939	(-)-α-pinene	9.12
956	(+)-camphene	0.16
979	(-)-1S-β-pinene	5.87
991	β-myrcene	0.73
1003	α-phellandrene	0.36
1011	δ-3-carene	3.62
1017	α-terpinene	1.51
1026	o-cymene	1.9
1029	(+)-limonene	4.42
1031	1,8-cineole	23.59
1060	γ-terpinene	4.74
1089	terpinolene	2.41
1097	Linalool	2.01
1110	Myrcenol	0.08
1177	terpinen-4-ol	1.24
1189	α-terpineol	4.91
1221	cis-geraniol	0.09
1375	α-ylangene	0.46
1377	α-copaene	0.32
1391	β-elemene	0.12
1419	β-caryophyllene	5.32
1440	α-guaiene	0.16
1450	cis-muurola-3,5-diene	0.1
1455	α-humulene	4.76
1484	γ-selinene	0.5
1485	α-amorphene	1.16
1490	α-elemene	0.19
1493	δ-selinene	0.94
1498	eremophilene	0.19
1498	α-selinene	2.09
1508	7-epi-α-selinene	1.89
1512	δ-amorphene	0.79
1523	δ-cadinene	0.67
1524	1,2,4a,5,8,8a-hexahydro-4,7 -dimethyl 1(1methylethyl)naphthalene	0.19
1546	α-calacorene	0.46
1547	selina-3,7(11)-diene	0.39
1583	caryophyllene oxyde	0.12
1600	Guaiol	1.86
1632	γ-eudesmol	1.5
1638	Hinesol	0.16

1654	α-eudesmol	2.92
1666	bulsenol	0.52

Traces of: a-fenchene, a-sabinene, pseudolimonene, p-cymenene, (e)-β-ocimene, fenchol, borneol.

Table S2: First line drugs profile of the Streptomycin, Isoniazid, Rifampicin, Ethambutol and MIC values of *Melaleuca* EOs against *Mtb* strains.

	First line drugs profile				**MIC % v/v**
Mtb strains	**Streptomycin**	**Isoniazid**	**Rifampicin**	**Ethambutol**	***Melaleuca* EOs**
Mtb H37Rv	S[1]	S	S	S	16%
Mtb-1[3]	S	R[2]	R	R	≤0.5 %
Mtb-2	S	S	S	S	8%
Mtb-3	R	R	S	R	16%
Mtb-4[3]	S	R	R	R	8%
Mtb-5[3]	R	R	R	R	8%
Mtb-6	S	R	S	R	8%
Mtb-7	S	S	R	R	16%
Mtb-8	S	S	S	S	8%
Mtb-9	S	R	S	R	≤0.5%
Mtb-10[3]	S	R	R	R	2%
Mtb-11	S	R	S	R	2%
Mtb-12	S	R	S	R	2%
Mtb-13	S	R	S	R	1%
Mtb-14[3]	R	R	R	R	8%
Mtb-15	R	R	S	R	1%

[1]Susceptible; [2]Resistant; [3]Multi-Drug-Resistant *Mtb* strains (*Mtb* H37Rv was the reference strain, *Mtb* strains from 1 to 15 were clinical strains)

Table S3: Drugs profile of the azithromycin (AZT), ciprofloxacin (CIP), amikacin (AMK), levofloxacin (LVX), moxifloxacin (MXF), rifabutin (RFB), linezolid (LZD) and MIC values of *Melaleuca* EOs against *NTM strains.*

NTM strains	**Drugs profile**							**MIC v/v %**
	AZT	**CIP**	**AMK**	**LVX**	**MXF**	**RFB**	**LZD**	***Melaleuca*EOs**
M. abscessum-1	R	R	R	R	R	R	S	4%
M. abscessum-2	R	R	R	R	R	S	R	≤0.5%
M. simiae	S	R	R	R	R	R	R	2%
M. avium	R	S	S	S	S	S	R	2%
M. gordonae-1	R	S	S	S	S	S	R	≤0.5%
M. gordonae-2	R	S	S	S	S	S	R	1%