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Composition of Three Essential Oils, and their Mammalian Cell Toxicity and Antimycobacterial Activity against Drug Resistant-Tuberculosis and Nontuberculous Mycobacteria Strains

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Tuberculosis (TB) is the most ancient epidemic disease in the world and a serious opportunistic disease in HIV/AIDS patients. The increase in multidrug resistant *Mycobacterium tuberculosis* (MDR-TB, XDR-TB) demands the search for novel antimycobacterial drugs. Essential oils (EOs) have been widely used in medicine and some EOs and their major components have been shown to be active against *M. tuberculosis.* The aim of this work was to evaluate the antimycobacterial and cell toxicity activities of three EOs derived from *Salvia aratocensis*, *Turnera diffusa* and *Lippia americana*, aromatics plants collected in Colombia. The EOs were isolated by hydrodistillation and analyzed by GC/MS techniques. The EOs were tested against 15 *Mycobacterium* spp using a colorimetric macrodilution method and against mammalian Vero and THP-1 cells by MTT. The activity was expressed as minimal concentration in µg/mL that inhibits growth, and the concentration that is cytotoxic for 50 or 90% of the cells (CC_{50} and CC_{90}). The major components were *epi*-α-cadinol (20.1%) and 1,10-di-*epi*-cubenol (14.2%) for *Salvia aratocensis*; drima-7,9(11)-diene (22.9%) and viridiflorene (6.6%) for *Turnera diffusa*; and germacrene D (15.4%) and *trans*-β- caryophyllene (11.3%) for *Lippia americana*. The most active EO was obtained from *S. aratocensis*, with MIC values below 125 µg mL^{-1} for *M. tuberculosis* Beijing genotype strains, and 200 to 500 µg mL^{-1} for nontuberculous mycobacterial strains. The EOs were either partially or non toxic to Vero and THP-1 mammalian cells with CC_{50} values from 30 to >100 µg mL^{-1}, and a CC_{90} >100 µg mL^{-1}. The EOs obtained from the three aromatic Colombian plants are an important source of potential compounds against TB. Future studies using the major EO components are recommended.

Keywords: antimycobacterial, essential oil, tuberculosis, multidrug-resistant, Beijing genotype, mammalian cell toxicity.

Human TB is a contagious disease caused mainly by *Mycobacterium tuberculosis*, an aerobic bacterium that establishes its infection typically in the lungs [1]. About one-third of the world's population is currently infected and 10% of the infected patients could develop a clinical disease, especially those with diverse types of immunodeficiency (i.e. HIV infection, transplant patients, cancer chemotherapy, under nutrition). TB is the leading cause of death worldwide from a single human pathogen and it is estimated that TB active cases occur in 7-8 million people annually and produce 3 million deaths per year [1,2].

New TB drugs are necessary for several reasons: 1. to improve the current treatment by shortening the total duration of treatment and/or by providing more widely spaced and intermittent treatment; 2. to improve the treatment of multi-drug resistant (MDR) TB and extremely drug-resistant (XDR) TB; 3. to provide more effective treatment to latent tuberculosis infection; and 4. to abolish side effects, especially hepatotoxicity that in some cases forces an untimely treatment termination [3-5]. Natural products are a source of potentially active molecules against several types of pathogens, such as parasites, bacteria, viruses and fungi [6-8].

The study of aromatic plants in Colombia is supported by the enormous biodiversity of the country [9]. The pharmaceutical properties of aromatic plants are partially attributed to their EO composition. EOs have demonstrated activity against a wide spectrum of pathogenic microorganism such as *Candida albicans*, *Salmonella*

typhyimurium, *Escherichia coli* and *Herpes simplex* virus [10]. Some EOs have shown important activity against *M. tuberculosis* [11], including those from garlic (active at 1 μg mL^{-1}), *Laurus* spp (active at 6.25 μg mL^{-1}), *Thymbra spicata* (active at 128 μg mL^{-1}), *Thymus vulgaris* (active at 50 μg mL^{-1}) and *Achyrocline alata* (active at 62.5 μg mL^{-1}) [11-15].

Of the EOs used in this study that of *Turnera diffusa* has been reported to have antibacterial activity against Gram-positive and Gram-negative bacteria [16]. *T. diffusa* and *Lippia americana* oils have been characterized in previous studies, the major constituents identified being β-pinene, β-caryophyllene oxide, cadinene, α-cadinol, 1,8-cineol, opoplenone, cadalene, *epi*-cubenol, and cadin-4-en-1-ol [17-19]

The aim of this work was to evaluate the antimycobacterial and cell toxicity activities of the EOs derived from *S. aratocensis*, *T. diffusa* and *L. americana*, three aromatic plants of Colombia. The EOs were obtained by microwave-assisted hydrodistillation and analyzed by GC/MS techniques. They were isolated with yields of 0.5%, 0.7% and 0.5%, w/w, respectively. Tables 1 to 3 show their chemical compositions. The major components of the EO from *S. aratocensis* were *epi*-α-cadinol (20.1%) and 1,10-di-*epi*-cubenol (14.2%), of *T. diffusa* drima-7,9(11)-diene (22.9%) and viridiflorene (6.6%), and of *L. americana* *trans*-β-caryophyllene (11.3%) and germacrene D (15.4%).

The EO activities against the panel of 15 *Mycobacterium* strains are presented in Table 4. The EO from *S. aratocensis* was the most active, followed by that of *T. diffusa* with MIC values below 125 μg mL^{-1} for *M. tuberculosis* strains (good activity below 100 μg mL^{-1}); nontuberculous mycobacteria were more resistant to the three EOs with MIC values between 200 and 500 μg mL^{-1}; *M. tuberculosis* H37Rv and *M. chelonae* were the most susceptible strains.

The time kill curves of *S. aratocensis* and *T. diffusa* against *M. tuberculosis* H37Rv and *M. chelonae* are shown in Figure 1. Isoniazid and rifampin presented bactericidal activity against *M. tuberculosis* H37Rv at concentrations equivalent to 0.5-fold above the respective MIC; isoniazid was bacteriostatic against *M. chelonae* (data not shown). *S. aratocensis* and *T. diffusa* were bactericidal against both strains at concentrations equivalent to 2-fold above the respective MIC.

The EOs were either partially or non toxic to Vero and THP-1 mammalian cells with CC_{50} activities in the range from 30 to >100 μg mL^{-1} and CC_{90} activities >100 μg mL^{-1}. The EO from *T. diffusa* was the least active, being non-toxic to THP-1 cells at the maximal concentration used in this study.

Table 1: Chemical composition of the essential oil from *Salvia aratocensis* COL N° 516326. Vernacular name: Velero.

Peak number	I_k-DB-5MS	Compound	Relative amount (%)
1	929	Tricyclene	0.3
2	973	Sabinene	0.1
3	980	β-Pinene	0.1
4	978	1-Octen-3-ol	0.1
5	1033	Limonene	0.1
6	1035	β-Phellandrene	0.1
7	1037	1,8-Cineole	0.1
8	1047	Benzene acetaldehyde	0.1
9	1100	Linalool	0.1
10	1180	Borneol	0.2
11	1185	*cis*-Pinocamphone	0.1
12	1202	Myrtenal	0.3
13	1290	Bornyl acetate	0.1
14	1329	Myrtenyl acetate	1.0
15	1343	δ-Elemene	0.3
16	1356	α-Cubebene	0.3
17	1387	α-Copaene	0.5
18	1396	β-Bourbonene	2.0
19	1398	β-Elemene	0.8
20	1420	α-Gurjunene	0.3
21	1422	α-Cedrene	0.1
22	1437	*trans*-β-Caryophyllene	5.1
23	1444	β-Copaene	1.0
24	1459	*trans*-β-Farnesene	2.0
25	1472	α-Humulene	2.1
26	1478	γ-Muurolene	4.0
27	1490	γ-Curcumene	1.1
28	1497	Germacrene D	3.4
29	1503	Valencene	1.0
30	1505	Viridiflorene	1.9
31	1510	Bicyclogermacrene	1.0
32	1518	Germacrene A	1.0
33	1532	γ-Cadinene	9.3
34	1537	*cis*-Calamenene	1.7
35	1545	10-*epi*-Cubebol	1.1
36	1550	α-Cadinene	1.0
37	1563	Elemol	3.3
38	1574	Germacrene B	0.5
39	1598	Caryophyllene oxide	1.5
40	1610	Gleenol	1.1
41	1632	1,10-di-*epi*-Cubenol	14.2
42	1667	*epi*-α-Cadinol	20.1
43	1675	*neo*-Intermedeol	1.0
44	1677	α-Cadinol	1.4
45	1699	α-Bisabolol	1.2

The need to counter bacterial resistance is obvious and pressing. Plants produce antibacterial metabolites as part of their chemical defense strategy to protect themselves against microbes in their environment [20]. Some species of soil bacteria, such as *Streptomyces*, are plant pathogens and these are taxonomically related to *Mycobacterium* species [20]. This study represents the first phase of ongoing research to identify new agents, which are safe and effective, for the treatment of TB both MDR and XDR as nontuberculous mycobacterial isolates.

Terpenes play an important role in the defense of many types of organisms, such as plants, fungi and certain marine organisms [21,22]. Terpenes have been evaluated for their *in vitro* antimycobacterial activity [23], especially thymol, with an antimycobacterial MIC of 100 μg mL^{-1} [13]. Other terpenes, such as carvacrol and α-pinene, have given MICs against *M. tuberculosis* H37Rv of 64 and 128 μg mL^{-1}, respectively [14]. Carvacrol was active too against *M. avium* strains, with a MIC of 72.2 μg mL^{-1} [24]. In addition, the antitubercular activity of citronellol, nerol and geraniol has been evaluated with MICs between 64-128 μg mL^{-1} [23]. Thus, the activity of the EOs examined

Table 2: Chemical composition of the essential oil from *Turnera diffusa* COL N° 516293. Vernacular names: Damiana, Hierba de la pastorcita, Hierba del ahorcado, Hierba del venado, Malva blanca, Orégano cimarrón, Rompecamisas macho, Turnera, Oreganillo, Peludillo, Rompecamisa macho, Yerba del pastor, Yerba del venado.

Peak number	I_k-DB-5MS	Compound	Relative amount (%)
1	1021	Δ^3-Carene	0.5
2	1029	*p*-Cimene	2.1
3	1033	Limonene	0.2
4	1062	γ-Terpinene	0.3
5	1188	Terpinen-4-ol	0.4
6	1201	α-Terpineol	0.3
7	1249	Monoterpenoid, $C_{10}H_{16}$	1.1
8	1265	Monoterpenoid, $C_{10}H_{16}O$	0.5
9	1291	Thymol	0.2
10	1301	Carvacrol	0.4
11	1312	*iso*-Ascaridole	0.5
12	1380	α-Ylangene	0.4
13	1388	Sesquiterpene, $C_{15}H_{24}$	0.5
14	1398	β-Elemene	2.7
15	1433	*trans*-β-Caryophyllene	5.2
16	1451	Alloaromadendrene	1.7
17	1484	Drima-7,9(11)-diene	22.9
18	1488	γ-Gurjunene	1.0
19	1496	Sesquiterpene, $C_{15}H_{24}$	0.6
20	1499	Valencene	5.5
21	1502	β-Selinene	5.9
22	1504	γ-Patchoulene	1.0
23	1507	Viridiflorene	6.6
24	1509	α-Selinene	3.1
25	1527	δ-Cadinene	0.6
26	1533	7-*epi*-α-Selinene	0.6
27	1585	Longipinocarvone	0.6
28	1595	Caryophyllene oxide	3.6
29	1603	Sesquiterpene, $C_{15}H_{24}$	2.2
30	1607	Sesquiterpene, $C_{15}H_{24}$	0.9
31	1631	*neo*-Intermedeol	1.0
32	1638	Sesquiterpene, $C_{15}H_{24}$	0.5
33	1642	Sesquiterpene, $C_{15}H_{24}$	0.6
34	1648	Sesquiterpene, $C_{15}H_{24}$	0.7
35	1662	Sesquiterpene, $C_{15}H_{24}$	0.9
36	1669	Sesquiterpene, $C_{15}H_{24}$	1.4
37	1678	Hydroxy-isolongifolene	1.2
38	1696	Zierone	0.6
39	1822	Dihydrokaranone	14.4

could be attributed to their terpenoid content [25], Similarly, EOs that were characterized by high levels of sesquiterpenes, such as γ-cadinene, (*Z*)-*β*-farnesene, *γ*-muurolene, spathulenol, hexahydrofarnesyl acetone and *α*-selinene, exhibited antifungal and antibacterial activity [25].

Possibly, the antibacterial activity of terpenes is due to a perturbation of the microorganism lipid fraction of the plasma membrane, which results in alterations of membrane permeability and in leakage of intracellular materials, and as such impairs the energy metabolism and influences genetic material synthesis [26,27]. Antimicrobial activity against *Mycobacterium* species depends on how efficiently the cell wall constitutes an efficient permeability barrier. The natural resistance to a wide range of antibiotics is often thought to be at least partially related to the poor penetration of solutes across the cell wall, It is also well known that the mycobacterial cell wall is extremely rich in lipids, principally in the form of very long-chain fatty acids (mycolic acids), which account for 30 to 60% of the weight of the wall. Such a thick layer of lipidic material obviously acts as a barrier to penetration of hydrophilic solutes. This fact can explain the differences between the low MICs obtained with

Table 3: Chemical composition of the essential oil from *Lippia americana* COL N° 516287. Vernacular names: Barelago, Belita, Mamoncillo, Mano de Dios, Mastranto, Oreganito, Oreganito macho, Orégano macho, Varita blanca.

Peak number	I_k-DB-5MS	Compound	Relative amount (%)
1	932	α-Thujene	0.8
2	939	α-Pinene	1.4
3	975	Sabinene	6.6
4	987	β-Pinene	0.5
5	993	β-Myrcene	0.6
6	1029	*p*-Cymene	1.1
7	1035	Limonene	1.5
8	1037	β-Felandrene	2.4
9	1051	*trans*-β-Ocimene	0.5
10	1063	γ-Terpinene	2.4
11	1189	Terpinen-4-ol	0.3
12	1253	Carvone	0.7
13	1345	δ-Elemene	0.7
14	1359	α-Cubebene	0.9
15	1387	α-Copaene	0.8
16	1397	β-Bourbonene	1.3
17	1398	β-Elemene	3.2
18	1440	*trans*-β-Caryophyllene	11.3
19	1446	β-Copaene	0.6
20	1464	γ-Muurolene	0.5
21	1472	α-Humulene	2.6
22	1476	γ-Gurjunene	1.3
23	1502	Germacrene D	15.4
24	1507	β-Selinene	1.7
25	1514	Bicyclogermacrene + α-Selinene	5.5
26	1528	γ-Cadinene	0.5
27	1529	δ-Cadinene	1.6
28	1551	α-Cadinene	0.6
29	1560	Germacrene B	5.5
30	1593	Espathulenol	2.6
31	1601	Caryophyllene oxide	1.8
32	1632	1,10-di-*epi*-Cubenol	3.3
33	1637	10-*epi*-γ-Eudesmol	1.7
34	1656	*epi*-α-Cadinol	2.1
35	1673	α-Cadinol	0.6
36	1679	*Neo*-Intermedeol	0.7

Table 4: Inhibitory effect of three EOs on the growth of some *Mycobacterium* species (results expressed in μg mL^{-1}).

***Mycobacterium* strains**	**Minimum Inhibitory Concentration**		
	EOs		
	Salvia aratocensis	*Turnera diffusa*	*Lippia americana*
***M. tuberculosis* H37Rv**	62.5	62.5	99.2
***M. tuberculosis* ATCC 35837**	49.6	62.5	62.5
***M. tuberculosis* ATCC 35838**	125	125	125
***M. tuberculosis* ATCC 35822**	99.2	125	125
***M. tuberculosis* ATCC 35820**	62.5	78.7	78.7
M. tuberculosis* Beijing MTB 985	62.5	62.5	62.5
M. tuberculosis* Beijing MTB 2556	125	62.5	62.5
M. tuberculosis* Beijing MTB 4000	62.5	125	62.5
M. tuberculosis* Beijing MTB 1472	62.5	62.5	62.5
M. chelonae* MNT 1407	79	198.4	198.4
M. abscessus* MNT 1512	315	397	>500
M. fortuitum* MNT 1073	397	397	>500
M. intracellulare* MNT 1408	315	500	>500
M. terrae* MNT 1080	397	500	>500
M. szulgai* MNT 1100	99.2	198.4	125

*Internal Code from Instituto Nacional de Salud (Colombia). Good activity is a MIC value below 100 μg mL^{-1} for crude extracts and 1 μg mL^{-1} for pure compounds.

M. tuberculosis strains and the high MIC values with nontuberculous mycobacterial species [28]. The EOs and their components are an interesting source of new antimycobacterial agents. EOs can be useful for the development of new biocidal agents for environmental mycobacterial control, for purifying air, personal hygiene,

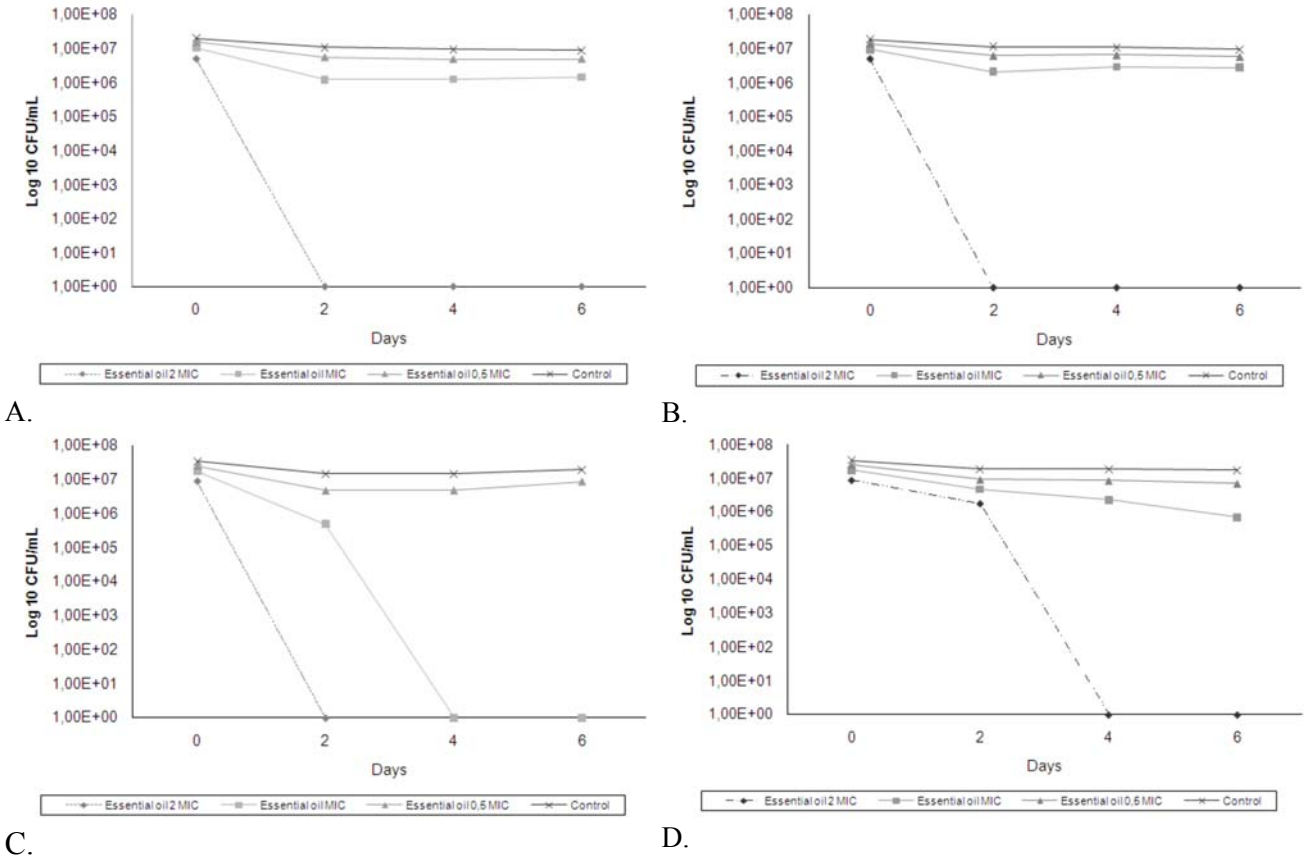

Figure 1: Antimycobacterial time kill curves of essential oils at concentrations 0.5-, 1-, and 2-fold above the respective MIC; determinations are shown as $\log_{10}$ CFU (Colony Formation Units) against time and expressed as the mean. A bactericidal effect can be seen by a 3 $\log_{10}$ (99.9% killing) decrease in CFU at the time specified compared with the control **A.** Effect of *Salvia aratocensis* oil on kinetic growth curve of *Mycobacterium tuberculosis* H37Rv, **B.** Effect of *Turnera diffusa* oil on kinetic growth curve of *M. tuberculosis* H37Rv, **C.** Effect of *S. aratocensis* oil on kinetic growth curve of *M. chelonae*, **D.** Effect of *T. diffusa* oil on kinetic growth curve of *M. chelonae* MNT 1407.

Table 5: Cytotoxic effects of three EOs on Vero and THP-1 cells (results expressed in μg mL^{-1}).

Eos	Toxicity (μg mL^{-1})			
	Vero cells		THP-1 cells	
	CC_{50}±DS*	CC_{90}/DS*	CC_{50}/DS	CC_{90}/DS
Salvia aratocensis	40.0±4.6	>100	63.1±3.0	>100
Turnera diffusa	88.1±1.2	>100	>100	>100
Lippia americana	30.2±0.8	>100	40.1±5.9	>100

*concentration required for 50% and 90% (CC_{50} and CC_{90}) cell killing.

and even for internal use via oral consumption. Antimicrobial activity of essential oils against the mycobacterial cell wall has not been demonstrated and so new experiments should be directed to check this effect. It would be very important to evaluate the individual components of *S. aratocensis* and *T. diffusa* EOs, as well as their interactions in checkerboard assays, in order to determinate the active principles.

Experimental

***Plant material*:** The voucher numbers, the region of plant collection, vernacular and botanical names appear in Tables 1, 2 and 3. The taxonomic identification of the species was performed at the Herbario Nacional de Colombia (COL), Natural Sciences Institute, Faculty of Sciences, Universidad Nacional de Colombia (Bogotá), where voucher samples are deposited.

EO extraction: The EOs were extracted from plant leaves and stems (300 g) by microwave-assisted hydrodistillation (30 min, 250 mL water), using a Clevenger-type distillation apparatus and a Dean-Stark distillation trap in a domestic microwave oven (Kendo, MO-124, 2,5 GHz, 800 W), as described elsewhere [29]. Sodium sulfate (Merck, Darmstadt, Germany) was added as a drying agent to the decanted essential oil. After extraction, the volume of essential oil obtained in both extractions was measured and the essential oil stored in hermetically sealed glass containers with rubber lids and maintained at a temperature lower than 4°C until used.

EOs analysis: An aliquot (50 μL) of each essential oil, along with the internal standard (*n*-tetradecane, 4 μL) were dissolved in dichloromethane to a final volume of 1.0 mL. For chromatographic analysis, this solution (1.0 μL) was injected into an Agilent Technologies 6890 Plus gas chromatograph [Agilent Technologies, splitless injector (split ratio 1:50), a 7863 automatic injector, and a MSChemStation G1701-DA data system, that included the spectral libraries Wiley138K, NIST 2002 and QUADLIB 2004]. A fused-silica capillary column DB-5MS (J&W Scientific, Folsom, CA, USA), 60 mm x 0.25 mm I.D. x 0.25 μm d_f, was employed. The oven temperature was

programmed from 45°C (5 min) to 150°C (2 min) at 4°C min^{-1}, then to 250°C (5 min) at 5°C min^{-1}, and finally, to 275°C (15 min) at 10°C min^{-1}. The ionization chamber and transfer line temperatures were kept at 230 and 285°C, respectively. Compound identification was based on chromatographic (Kovàts indices) and spectrometric (MS interpretation and comparison with those of standards and of databases) criteria, and using standard terpene compounds.

Microorganisms and culture methods: The *Mycobacterium* species used were obtained from the Laboratorio Micobacterias, Instituto Nacional de Salud, Bogotá, Colombia: *M. tuberculosis* H37Rv (ATCC 27294), their resistant variants (ATCC 35837 ethambutol resistant, ATCC 35838 rifampin resistant, ATCC 35822 isoniazid resistant and ATCC 35820 streptomycin resistant), four strains of *M. tuberculosis* Beijing genotype belonging to the Colombia National Study of Drug resistance, and 6 clinical isolates from humans with mesotherapy associated outbreak caused by nontuberculous mycobacteria (NTM): *M. chelonae*, *M. abscessus*, *M. fortuitum*, *M. intracellulare*, *M. terrae* and *M. szulgai*. All organisms were maintained at 37°C in Lowestein-Jensenn medium until log phase growth.

Mammalian cells: Vero cells (ATCC) and human acute monocytic leukemia cell line THP-1 cells (ATCC) were cultured in RPMI 1640 medium plus 10% heat inactivated fetal calf serum (hiFCS) at 37°C and 5% CO_2-95% air mixture.

Antimycobacterial activity: The antimycobacterial activity of the essential oils was evaluated following the macro-dilution protocol, described by Abate *et al*. and Bueno-Sánchez *et al*. [11,30]. A microorganism suspension was prepared at a concentration of about $2x10^6$ CFU (Colony Formation Units) mL^{-1} and further diluted 1:20 in Middlebrook 7H9 (Becton Dickinson and Co, Sparks MD, USA) medium supplemented with 10% OADC (oleic acid-albumin-dextrose-catalase) (Becton Dickinson and Co,, Sparks MD, USA) and 0.001% Tween 80 (Sigma, New Jersey, USA); 1 mL of the bacterial suspension was added to each glass tube with a cap, together with the EOs diluted to final concentrations from 31.25 to 500 µg mL^{-1}, adjusted to a final 2 mL volume. After incubation for about 7 days for *slow growing mycobacteria* (M. tuberculosis*, M. abscessus*, *M. intracellulare*, *M. terrae* and *M. szulgai*) and 5 days for rapid growing mycobacteria (*M. chelonae* and *M. fortuitum*), 100 µL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) (5 mg mL^{-1}) (Sigma, New Jersey, USA) with 20% Tween 80 (Sigma, New Jersey, USA) were added to the glass tubes. A violet color indicates bacterial growth. The results were read in the following day. For standard tests, the MIC values of rifampin, isoniazid, streptomycin and ethambutol (Sigma, New Jersey, USA) were determined each time. The minimum inhibitory concentration (MIC) of each oil corresponded to the lowest concentration at which the bacteria tested did not show growth. Susceptibility testing was performed 3 times. The results are expressed as the mean obtained from 3 different assays.

Antimycobacterial time kill curves: Time-kill curves were used to measure the bactericidal activity of EOs with the lowest MIC values. Bactericidal activity was measured in glass tubes each containing 2 mL of Middlebrook 7H9 medium supplemented with OADC and Tween 80 at concentrations 0.5-, 1-, and 2-fold above the respective MIC; *M. tuberculosis* H37Rv and *M. chelonae* were used. The final concentration of mycobacteria was approximately 10^6 CFU mL^{-1}. Samples were taken each 2 days until the sixth day and were serially diluted in sterile distilled water in order to avoid any significant carry over and plated into Lowestein-Jensenn tubes with screw caps; tubes were incubated at 37ºC in an Incubator Shaker Model G25 (New Brunswick Scientific Co, New Jersey, USA). Isoniazid and rifampin were used as control drugs. The time-kill curve assay was carried out according to the recommendations of CLSI [31].

Toxicity to mammalian cells: The cell toxicity was tested using the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide)] reduction assay following the protocol described by Escobar *et al*.[32]. Briefly, Vero and THP-1 cells were treated with each essential oil (0-100 µg/mL) for 72 h. A solution of MTT (10 ng/mL) was added for 4 h and formazan crystals were dissolved with DMSO. The optical density (OD) was measured using a microplate reader at a wavelength of 580 nm. The cell toxicity was expressed as the concentration required for 50% and 90% (CC_{50} and CC_{90}) cell kill. These were calculated by sigmoidal regression analyses (Msxlfit, ID Business Solution, Guildford, UK).

Statistical analysis: MIC results were analyzed using Excel program and are expressed as geometric mean (GM). The results of kill-kinetic determinations are shown graphically by plotting $\log_{10}$ CFUs against time and expressed as the mean. A bactericidal effect can be seen by a 3 $\log_{10}$ (99.9% killing) decrease in CFU at the time specified.

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