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ARTÍCULO ORIGINAL

Anti-tubercular activity of eleven aromatic and medicinal plants occurring in Colombia

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Introduction. Human tuberculosis is a contagious-infectious disease mainly caused by *Mycobacterium tuberculosis*. Although regimens exist for treating tuberculosis, they are far from ideal. Development of effective strategies for treatment of human tuberculosis has posed a challenge, considering the increase in infections associated with the human immunodeficiency virus and immunocompromised patients. Essential oils -volatile, aromatic oil extracts from plants- have been used in traditional treatment of many diseases; however careful investigation of these oils has not been undertaken with respect to treatments of tuberculosis.
Objective. The *in vitro* antitubercular activity of essential oils from 11 medicinal plants grown in Colombia were assessed for efficacy as new medications (phytomedicines) for treatment of *M. tuberculosis* H37Rv.
Material and methods. Essential oil extraction and analysis were performed as described Stashenko et al. (2004). Minimal inhibitory concentrations were determined by a colorimetric macrodilution method, following the protocol described by Abate et al. (1998). Isoniazide and rifampin were used as control treatments. Bactericidal and bacteriostatic activity was measured using the method developed by the Clinical and Laboratory Standards Institute consigned in the M26-A protocol.
Results. Essential oils from *Achyrocline alata* and *Swinglea glutinosa* were the most active with minimal inhibitory concentrations of 62.5±0.1 and 100±36 μg ml^{-1}, respectively. Carvacrol, thymol, *p*-cymene, 1,8-cineole, limonene, and β-pinene were the major components ,most often identified in the 11 plant extracts of essential oils. Time-kill curve assays demonstrated the bacteriostatic activity of these essential oils.
Conclusions. The essential oils from *A. alata* and *S. glutinosa* plants, and the components identified therein, are candidates as potential phytotherapeutic agents for human tuberculosis control.

Key words: *Mycobacterium tuberculosis*, tuberculosis, anti-infective agents; plants, medicinal; phytotherapy, Colombia

Actividad antituberculosa de plantas colombianas

Introducción. La tuberculosis es una enfermedad infectocontagiosa causada por *Mycobacterium tuberculosis*. Aunque existen protocolos para su tratamiento, no son ideales. Actualmente, el desarrollo de estrategias terapéuticas efectivas ha tomado nuevos rumbos, considerando el incremento de pacientes positivos para el virus de la inmunodeficiencia humana. Los medicamentos basados en plantas medicinales se usan ampliamente en la medicina tradicional para el tratamiento de diversas afecciones. Los aceites esenciales obtenidos de plantas medicinales presentan amplia actividad antimicrobiana, sin embargo, existen pocos estudios que reporten la actividad antituberculosa de los mismos.
Objetivo. Evaluar la actividad antituberculosa *in vitro* de 11 aceites esenciales provenientes de plantas medicinales que crecen en Colombia, los cuales podrían ser candidatos para el desarrollo de futuros fitofármacos.

Materiales y métodos. La extracción y el análisis de los aceites esenciales se realizó bajo la metodología desarrollada por Stashenko *et al*.. La obtención de la concentración inhibitoria mínima se llevó a cabo por un método colorímetrico de macrodilución en caldo descrito por Abate y *et al*.; la isoniacida y la rifampicina se usaron como medicamentos control. La actividad bactericida y bacteriostática se determinaron mediante el protocolo M26-A del *Clinical and Laboratory Standards Institute*.

Resultados. Los aceites esenciales provenientes de las plantas *Achyrocline alata* y *Swinglea glutinosa* fueron los más activos con concentraciones inhibitorias mínimas de 62,5±0,01 y 100±36 µg ml^{-1}, respectivamente. Carvacrol, timol, *p*-cymene, 1,8-cineole, limoneno, y β-pineno fueron los componentes mayoritarios identificados en los 11 aceites. Los ensayos de curva de letalidad evidenciaron que ambos aceites son bacteriostáticos.

Conclusiones. Los aceites esenciales obtenidos de las plantas *A. alata* y *S. glutinosa*, así como sus componentes, son candidatos potenciales como fitoterapéuticos para el control de la tuberculosis.

Palabras clave: *Mycobacterium tuberculosis*, tuberculosis, agentes antiinfecciosos, plantas medicinales, fitoterapia, Colombia

Human tuberculosis (TB) is a contagious infectious disease mainly caused by *Mycobacterium tuberculosis*. It is an aerobic pathogenic bacterium that usually establishes its infection in the lungs (1). About one third of the world's population is currently infected with *M. tuberculosis*. Fully 10% of those infected will develop clinical disease, particularly those who also have the human immunodeficiency virus (HIV) infection. TB is the leading cause of death worldwide from a single pathogen, claiming more adult lives than diseases AIDS, malaria, diarrhea, leprosy, and all other tropical diseases combined (2). The World Health Organization (WHO) estimates that active cases of tuberculosis afflict seven to eight million people annually, and lead up to three million deaths per year (3).

Although regimens exist for treating tuberculosis, they are far from ideal. Treatment usually involves a combination of the drugs isoniazid (INH) and rifampin (RMP), which must be administered for at least six months, or a combination of pyrazinamide and ethambutol (EMB) (or streptomycin), which are used only in the first two months of treatment (4). Because adherence to this regimen is extremely difficult, WHO recommends a program of directly observed treatment, short-course (DOTS), in which health care workers watch as each patient ingests the medicine (4). Approximately 21% of the world's TB patients were treated under DOTS in 1998. Therefore, inconsistent or partial treatment is common and has led to the development and spread of drug-resistant strains. Consequently, shorter, simpler therapeutic and prophylactic regimens must be developed to increase adherence. In addition, new drugs are necessary to combat the increasing number of multi-drug-resistant TB strains (MDR-TB) and extensively drug-resistant (XDR-TB) strains. A greater understanding of the molecular mechanisms of drug action and drug resistance will assist and promote the development of newer compounds (1,4).

Plant-based drugs have been used worldwide in traditional medicines for the treatment of a variety of diseases. Approximately 60% of the world population relies on medicinal plants for its primary healthcare. These medicinal plant species serve as a rich source of many biologically active compounds, although very few plant species have been thoroughly investigated for their medicinal properties (5). Interest in phytomedicine has been renewed during the last decade and now, many medicinal plant species are being screened for pharmacological activities (6). Aromatic plants have been used since ancient times for their preservative and medicinal properties, as well as to impart aroma and flavor to food. Hippocrates,

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the 'father of medicine', prescribed aromatic fumigations (7).

The pharmaceutical properties of aromatic plants are partially attributed to essential oils. Essential oils are natural, complex, multi-component systems composed mainly of terpenes along with a few non-terpene components (7). The ancient Egyptians used aromatic plants in embalming to stop bacterial growth and prevent decay, an effect largely attributed to their essential oil content. Strong *in vitro* evidence indicates that essential oils can act as antibacterial agents against a wide spectrum of pathogenic bacterial strains (7). Some terpenes, such as citronellol, nerol and geraniol have shown moderate antimycobacterial activity (8). The aim of the present study is to evaluate the antimycobacterial activity of essential oils derived from 11 species of aromatic and medicinal plants, grown in Colombia. If effective, these substances pose as potential candidates for the development of new medication for treatment of TB.

Material and Methods

Plant material

The voucher numbers, the geographic region of plant collection, vernacular names and botanical names are listed in Table 1. The taxonomic identification of the botanical species was performed by José Luis Fernández at Herbario Nacional de Colombia, Natural Sciences Institute, Faculty of Sciences, Universidad Nacional de Colombia, Bogotá; where desiccated samples of each plant remain as permanent vouchers.

Essential oils extraction

The essential oils were extracted from a 300 g sample of plant leaves and stems 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) (9). Sodium sulfate (Merck, Darmstadt, Germany) was added as a drying agent to the decanted essential oil.

Essential oils analysis

An aliquot (50 µL) of each essential oil, along with the internal standard (*n*-tetradecane, 4 µL) was dissolved in dichloromethane to reach a final volume of 1.0 mL. For chromatographic analysis, 1.0 µL of this solution was injected into an Agilent Technologies 6890 Plus gas chromatograph (Agilent Technologies, Palo Alto, CA, USA.), equipped with an Agilent Technologies 5973N mass selective detector, a split/splitless injector (split ratio 1:50), a 7863 automatic injector, and a MSChemStation G1701-DA data system. The available spectral libraries included the WILEY 138K, NIST 2002 and QUADLIB 2004. A fused-silica capillary column DB-5MS (J&W Scientific, Folsom, CA, USA) of 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°C and 285°C, respectively. Compound identification was based on comparisons with standard terpenic compounds by (1) chromatographic (Kovàts indices) criteria and (2) spectroscopic data and their comparison with known standards and with extant databases.

Antimycobacterial activity

The essential oil antimycobacterial activity was evaluated according to the macrodilution protocol, described by Abate et al. (1998) (10). The *M. tuberculosis* H37Rv strain (ATCC 27294) was cultured at 37°C in Lowestein-Jensenn medium until log phase growth; then a cell suspension was prepared at a concentration of about $2x10^6$ UFC mL^{-1} and further diluted 1:20 in Middlebrook 7H9 (Becton Dickinson and Co., Sparks MD, USA) medium. The later was 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). One mL of the bacterial suspension was added to each tube (capped, glass) together with the diluted essential oils diluted. The final concentrations of the essential oils ranged from 31.25 to 500 µg ml^{-1} and adjusted to a final 2 mL-volume. After a 7-day incubation, 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,

Table 1. Plant information, main compounds and minimum inhibitory concentration (MIC, µg ml^{-1}) against M. tuberculosis H37Rv, of the essential oils studied.

Sample N°	Botanical name	Collection site	Vernacular name[a] and *Voucher* number	Part of the plant used for essential oil extraction and oil yield	Essential oil main compounds	MIC[b] ± s[c], µg ml^{-1} (n[d] = 3)
1	*Lippia origanoides* (Fam. Verbenaceae)	Pedregal, Nariño	*Orégano de monte*" COL.520285, " *Thymol* chemotype	Leaves and stems; 3.1%	1. Thymol (54.5%) 2. *p*-Cymene (10.0%) 3. γ-Terpinene (5.0%) 4. Thymyl acetate (4.8%) 5. β-Myrcene (2.8%) 6. *trans*-β-Caryophyllene (2.4%) 7. Methyl thymyl esther (1.9%) 8. Carvacrol (1.7%) 9. α-Terpinene (1.6%) 10. α-Humulene (1.5%) (14)	125 ± 0.01
2	*Cananga odorata* Fam. Annonaceae)	Bucaramanga, Santander	"*Ylang-ylang*", introduced to Colombia, presumably from Philippines	Fresh, fully mature yellow flowers; 1.2%	1. Linalool (20.7%) 2. Benzyl acetate (20.6%) 3. Benzyl benzoate (14.2%) 4. Germacrene D (10.2%) *5. p*-Methyl anisole (6.8%) 6. Cinnamyl acetate (5.1%) 7. Geranyl acetate (2.9%) 8. α–Humulene (2.8%) 9. (*E,E*)-Franesene (2.4%) 10. Benzyl salicylate (2.3%) (15, 16)	300 ± 217
3	*Swinglea glutinosa* (Fam. Rutaceae)	Bucaramanga, Santander	"*Limón africano*", introduced to Colombia, COL.521530	Fruit peel; 0.7%	1. β-Pinene (49.6%) 2. α-Pinene (12%) 3. Sabinene (11.0%) 4. Bicyclosesquiphellandrene (8.1%) 5. Limonene (4.4%) 6. 1,8-Cineol (3.0%) 7. Terpinen-4-ol (2.7%) 8. *trans*-β-Caryophyllene (1.5%) 9. γ-Terpinene (1.4%) 10. α-Terpineol (1.2%) (18)	100 ± 36
4	*Hyptis mutabilis* (Fam. Lamiaceae))	Villavicencio, Meta	"*Mastranto*" COL.512275	Leaves and stems; 0.3%	1. Fenchone (17.1%) 2. 1,8-Cineol (12.6%) *3. trans*-β-Caryophyllene (10.9%) 4. Bicyclogermacrene (8.7%) 5. Germacrene D (6.2%) 6. Limonene (4.8%) 7. α-Pinene (3.8%) 8. β-Pinene (3.7%) 9. β-Boubonene (3.4%) 10. Spathulenol (3.0%)	125 ± 0.01
5	*Piper auritum* (Fam. Piperaceae)	Cali, Valle del Cauca	"*Anisillo*" COL.512209	Leaves; 2.3%	1. Safrol (91.3%) 2. Myristine (4.8%) 3. Methyl eugenol (0.8%) 4. α-Terpinolene (0.6%) 5. γ–Terpinene (0.5%) 6. *p*-Cymene (0.2%) 7. *trans*-β-Caryophyllene (0.3%) 8. Eugenol (0.2%) 9. Elemicine (0.2%) 10. Spathulenol (0.2%)	400 ± 220
6	*Lippia origanoides* (Fam. Verbenaceae)	Los Santos, Santander	"*Orégano de monte*", COL.516294, *Caryophyllene* chemotype	Leaves and stems; 1.5%	*1.trans*-β-Caryophyllene (11.3%) *2. p*-Cymene (11.2%) 3. α-Phellandrene (9.9%) 4. Limonene (7.2%) 5. 1,8-Cineol (6.5%) 6. α-Humulene (6.0%) 7. Borneol (3.1%)	400 ± 220

					8. Camphene (2.6%) 9. α-Pinene (2.3%) 10. Caryophyllene oxide (2.2%) (15)	
7	*Achyrocline alata* (Fam. Asteraceae)	Nariño Potosí,	"*Viravira*" COL.519601	Leaves and stems; 0.3%	1. Thymol (24.0%), *2. trans*-β-Caryophyllene (13.3%), *3. p*-Cymene (3.2%), 4. α-Pinene (2.6%), 5. α-Humulene (2.4%), 6. Thymyl acetate (2.3%), 7. Viridiflorene (2.3%), 8. Caryophyllene oxide (2.2%), 9. α-Bisabolol (1.7%). 10. α-Eudesmol (1.6%)	62.5±0.01
8	*Lippia origanoides* (Fam. Verbenaceae)	Piedecuesta, Santander	"*Orégano de monte*", COL.516290, *Carvacrol* chemotype	Leaves and stems; 4.4%	1. Carvacrol (46.2%) *2. p*-Cymene (12.0%) 3. Thymol (9.9%) 4. γ-Terpinene (9.5%) 5. α-Terpinene (2.7%) 6. β-Myrcene (2.5%) *7. trans*-β-Caryophyllene (2.0%) 8. α-Thujene (1.5%) 9. α–Humulene (1.2%) 10. Terpinen-4-ol (1.1%) (15)	160 ± 72
9	*Lippia alba* (Fam. Verbenaceae)	Venadillo, Tolima	"*Pronto alivio*", COL.520287 *Carvone* chemotype	Leaves and stems; 2.5%	1. Carvone (50.3%) 2. Limonene (30.2%) 3. Piperitone (6.1%) 4. Bicyclosesquiphellandrene (3.5%) 5. β-Bourbonene (1%) 6. Piperitone (3.1%) 7. β-Myrcene (0.8%) 8. Dihydrocarvone (0.8%) 9. Carveol (0.8%) 10. Cubebol (0.5%) (9)	200 ± 72
10	*Piper bogotense* (Fam. Piperaceae)	Ipiales, Nariño	"*Matico*" COL.519590	Leaves; 0.2%	1. Sesquisabinene hydrate (14.2%) 2. α-Phellandrene (13.7%) 3. α-Pinene (8.7%) 4. Limonene (5.3%) 5. Linalool (4.7%) *6. p*-Cymene (4.4%) 7. β–phellandrene (3.4%) 8. δ-Cadinene (3.4%) 9. α-Bisabolol (3.5%) *10.trans*-β-Caryophyllene (3.1%)	130 ± 95
11	*Lippia alba* (Fam. Verbenaceae)	Bucaramanga, Santander	"Pronto alivio", COL.512272 *Citral* Chemotype	Leaves and stems; 1.6%	1. Geranial (31.5%), 2. Neral (23.8%), 3. Geraniol (7.9%), *4. trans*-β-Caryophyllene (5.8%), 5. Geranyl acetate (3.6%), 6. Limonene (2.5%), 7. 6-Methyl-5-hepten-2-one (2.1%), *8. cis*-Verbenol (1.9%), *9. trans*-Verbenol (1.5%), 10 α-Humulene (1.4%) (17)	130 ± 95
INH	Isoniazid	--	--	--	--	0.19±0.07
RIF	Rifampin	--	--	--	--	0.3±0.21

[a] – Vernacular name in Colombia
[b] Minimum Inhibitory Concentration (μg ml^{-1})
[c] Standard error of the mean
[d] Number of assays

USA) was added to the glass tubes. A violet color indicated bacterial growth. The tubes were evaluated for color change on day 8. For standard tests, the MIC values of rifampin and isoniazid (Sigma, New Jersey, USA) were determined each time. The acceptable minimum inhibitory concentration (MIC) ranges of these drugs were 0.50-0.03 µg ml^{-1}, respectively. The 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 were expressed as the mean of the three tests. Results were expressed as geometric mean (GM) ± standard error of the mean (*s*) of the MICs.

Antimycobacterial time kill curves

Time-kill curves were used to measure the bactericidal activity of essential oils with lowest MICs values. Bactericidal activity was measured in glass tubes, each containing 2 mL of Middlebrook 7H9 (Becton Dickinson and Co., Sparks MD, USA) medium. The medium was 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) at concentrations 0,1-, 2-, and 5-fold above the respective MIC. The final concentration of mycobacteria was approximately 10^6 CFU mL^{-1}. Samples were taken every two days until the sixth day and were serially diluted in sterile distilled water to avoid significant carryover, and then plated in Lowestein-Jensenn tubes with screw caps. The tubes were incubated at 37°C 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 done according to the recommendations of the Clinical and Laboratory Standards Institute (11).

Data analysis

MICs results are expressed as geometric mean (GM) ± standard error of the mean (S.E.M). The results of kill-kinetic determinations are shown graphically by plotting $\log_{10}$ CFUs against time and expressed as mean ± standard error of the mean (S.E.M). A bactericidal effect can be seen by a 3 $\log_{10}$ (99.9% killing) decrease in CFU at the time specified.

Results

The results of the essential oil antimycobacterial screening and the composition of each oil are presented in table 1. All essential oils showed activity against *Mycobacterium tuberculosis* H37Rv. Only the *Achyrocline alata* essential oil exhibited a pronounced antimycobacterial activity below 100 µg ml^{-1} (62.5 µg ml^{-1}). Other essential oils presented a moderate activity (MIC between 100 and 200 µg ml^{-1}). The major components of essential oils evaluated are listed in table 1. Carvacrol, thymol, *p*-cymene, 1,8-cineole, limonene, and β-pinene were the most commonly identified, the major components of *A. alata* were thymol, *trans*-β-caryophyllene, *p*-cymene and α-phellandrene. Figure 1 shows the interaction between essential oils MIC and *M. tuberculosis* strain H37Rv. As shown, the *A. alata* essential oil possessed the lowest MIC, in comparison with the others.

The time kill curves of *A. alata* essential oils, *Swinglea glutinosa* essential oils and the control drugs isoniazid and rifampin are shown in the figures 2 to 5. In comparison with the bactericidal activity of isoniazid and rifampin at concentrations equivalent 0.5-fold above the respective MIC, the essential oils were bacteriostatic.

Discussion

Historicall, plants have been used worldwide in traditional medicines for the treatment of diseases. Today, approximately two-thirds to three-quarters of the world's population are estimated to rely on medicinal plants as their primary source of medicines (12). The current study represents the first phase of ongoing research to identify new, safe and effective agents for the treatment of TB. *Lippia origanoides* and *Lippia alba* plants of the Verbenaceae family already have many ethno-pharmacological applications. Both species possess several chemotypes (13,14). *Cananga odorata* (ylang-ylang) trees are an introduced species and have been widely distributed as ornamentals in Colombian cities. Ylang-ylang essential oil pharmacological effects have

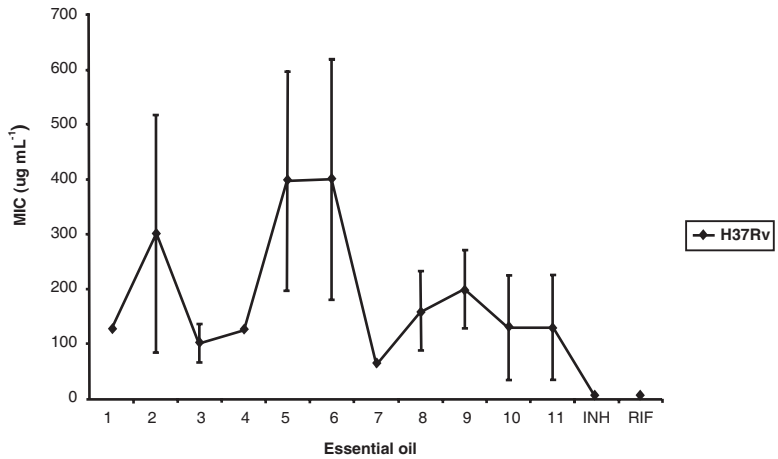

Figure 1. Interaction between essential oils MIC with *M. tuberculosis* H37Rv. Values are reported as mean ± S.E.M. *Achyrocline alata* (sample 7) and *Swinglea glutinosa* (sample 3) produced MICs below 100 µg/mL. Isoniazide (INH) and rifampin (RIF) were used as controls.

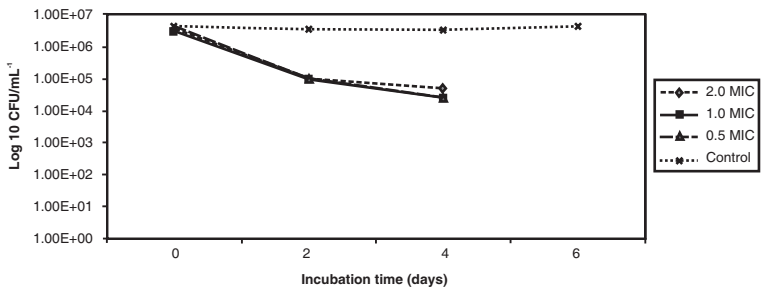

Figure 2. Time kill curve of isoniazide against *M. tuberculosis* H37Rv. Values are reported as mean ± S.E.M. Concentrations at 0.5-, 1-, and 2-fold levels of the respective MIC indicate the bactericidal activity in comparison with the control.

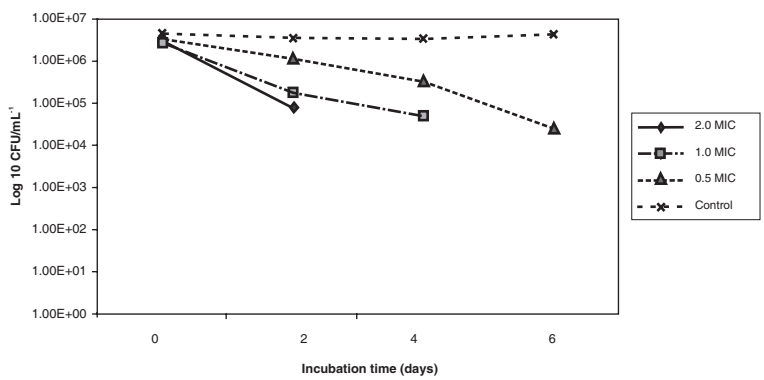

Figure 3. Time kill curve of rifampin against *M. tuberculosis* H37Rv. Values are reported as mean ± S.E.M. Concentrations at 0.5-, 1-, and 2-fold above the respective MIC present bactericidal activity in comparison with the control.

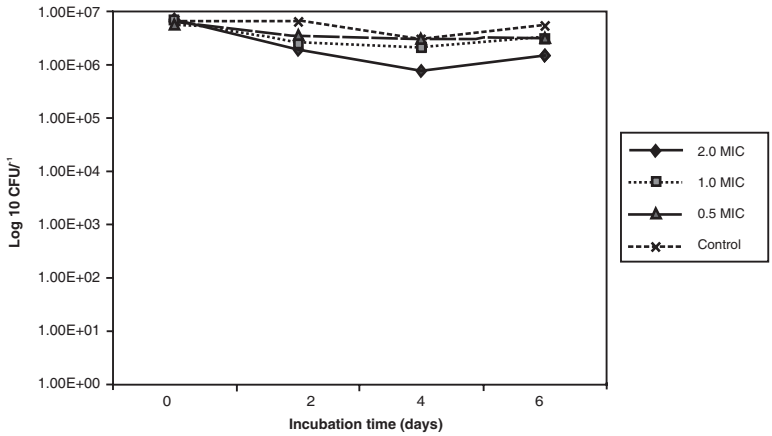

Figure 4. Time kill curve of *S. glutinosa* against *M. tuberculosis* H37Rv. Values are reported as mean ± S.E.M. Concentrations at 0.5-, 1-, and 2-fold levels of the respective MIC indicate the bacteriostatic activity in comparison with the control.

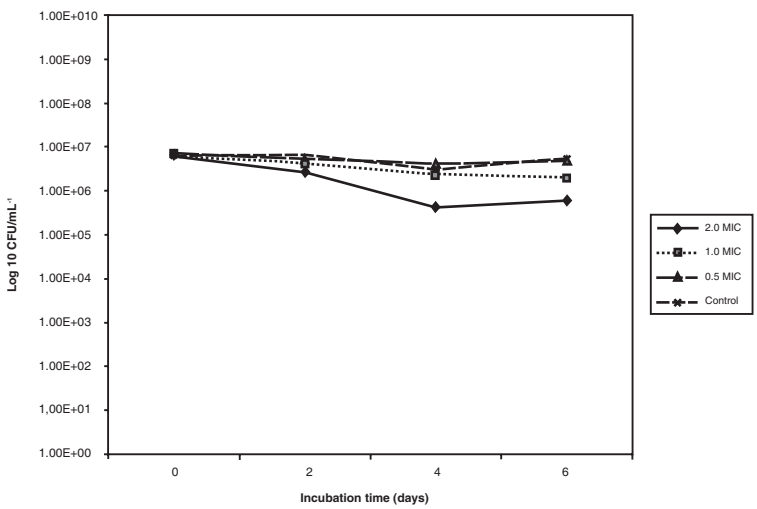

Figure 5. Time kill curve of *A. alata* against *M. tuberculosis* H37Rv. Values are reported as mean ± S.E.M. Concentrations at 0.5-, 1-, and 2-fold levels of the respective MIC indicate the bacteriostatic activity in comparison with the control.

been thoroughly investigated (15-17). *Swinglea glutinosa* (Rutaceae family) is a shrub planted in cities and countryside as a natural fence. Its extract is used in agriculture in natural preparations of biocides (18,19). *Piper auritum* and *Piper bogotense* of the family Piperaceae are widely distributed in Colombia and serve a variety of applications in folk medicine. *Hyptis mutabilis* (family Asteraceae) and *Achyrocline alata* (family Lamiaceae) play similar roles (20, 21).

The activity of essential oil from *Achyrocline alta* is probably due to the high concentration of terpenoids (22), which have been evaluated for their *in vitro* antimycobacterial activity (8,23). More specifically, thymol has an antimycobacterial activity with a MIC of 100 µg mL^{-1} (24). Other terpenes as carvacrol and α-pinene have shown a MIC against *M. tuberculosis* H37Rv of 64 and 128 µg mL^{1} respectively (25). In addition, the antitubercular activity of citronellol, nerol and

geraniol has been evaluated with MICs between 64-128 µg ml^{-1} (8).

In many essential oils, the antimicrobial activity is due to the presence of isoprenes such as monoterpenes, sesquiterpenes or related alcohols and phenols (26). The lipophilic character of their hydrocarbon skeleton and the hydrophilic character of their functional groups are of main importance in the antimicrobial action of essential oil components (26) which have high antimicrobial and antifungal activities (26). Possibly, the antibacterial activity of terpenes is due to a perturbation of the microorganism lipid fraction of the plasma membrane, and results in alterations of membrane permeability and in leakage of intracellular materials (27). This effect may be a consequence of the interaction between the major and minority components of essential oil. It was demonstrated that the physical properties significantly influenced the actions of the individual components, increasing or reducing antimicrobial efficacy (26).

The essential oil concentrations decrease substantially in broth and agar media, when incubated under open conditions. This decrease is caused primarily by evaporation and adherence on plastic surfaces (28). For these reasons, broth dilution assays must be carried out under sealed conditions to prevent evaporative loss, and glass materials must be used in place of plastics to prevent loss by absorption. In the current study, the antimycobacterial assays performed by a macrodilution method in glass tubes corrected these problems and consequently reproducible results were obtained. In addition, the screening method permitted the detection of resistance by *M. tuberculosis* to INH, RMP and even for EMB (10,29).

Some authors use Tween 80 in the broth medium to enhance oil solubility (30). In the current study, MIC of Tween 80 against *M. tuberculosis* H37Rv determined in the antimycobacterial assay (0.99%) was much higher than the concentration used for solubilizing oil (0.001%). Therefore, the addition of Tween 80 to the culture medium did not alter the MIC values obtained.

The essential oils and their volatile components may provide an important source of new anti-mycobacterial agents. Evaluations in checker-board assays of the individual components and their interactions of *A. alata* and *S. glutinosa* essential oils are necessary in order to determinate the active principles and toxicity of these complex mixes. Performing the macrodilution method with capped glass tubes is an alternative tool for obtaining more reproducible MIC values by controlling the essential oil high volatility.

Conflict of interest

The authors are in agreement with the results published in this article and claim no conflicts of interest with the same.

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References

1. **Ducati RG, Ruffino-Netto A, Basso LA, Santos DS.** The resumption of consumption-a review on tuberculosis. Mem Inst Oswaldo Cruz. 2006;101:697-714.

2. **Zumla A, Grange J.** Tuberculosis. BMJ. 1998; 316: 1962-4.

3. **Chang Blanc D, Nunn P.** Incentives and disincentives for new anti-tuberculosis drug development. Geneva: World Health Organization; 1999.

4. **National Institute of Allergy and Infectious Diseases.** NIAID Global Health Research Plan for HIV/AIDS, malaria, and tuberculosis. Bethesda: National Institute of Allergy and Infectious Diseases; 2001.

5. **Heinrich M, Gibbons S.** Ethnopharmacology in drug discovery: an analysis of its role and potential contribution. J Pharm Pharmacol. 2001;53:425-32.

6. **Gautam R, Saklani A, Jachak SM.** Indian medicinal plants as a source of antimycobacterial agents. J Ethnopharmacol. 2007;110:200-34.

7. **Edris AE.** Pharmaceutical and therapeutic potentials of essential oils and their individual volatile constituents: a review. Phytother Res. 2007;21:308-23.

8. **Cantrell CL, Franzblau SG, Fischer NH.** Anti-myco-bacterial plant terpenoids. Planta Med. 2001;67:685-94.

9. **Stashenko EE, Jaramillo BE, Martinez JR.** Comparison of different extraction methods for the analysis of volatile secondary metabolites of *Lippia alba* (Mill.) N.E. Brown, grown in Colombia, and evaluation of its *in vitro* antioxidant activity. J Chromatogr A. 2004;1025:93-103.

10. **Abate G, Mshana RN, Miorner H.** Evaluation of a colorimetric assay based on 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) for rapid detection of rifampicin resistance in *Mycobacterium tuberculosis*. Int J Tuberc Lung Dis. 1998;2:1011-6.

11. **Clinical and Laboratory Standards Institute.** Methods for determining bactericidal activity of antimicrobial agents; approved guideline. CLSI document M26-A. Wayne, Pennsylvania: Clinical and Laboratory Standards Institute; 1999.

12. **Newton SM, Lau C, Wright CW**. A review of antimycobacterial natural products. Phytother Res. 2000;14:303-22.

13. **Hennebelle T, Sahpaz S, Dermont C, Joseph H, Bailleul F.** The essential oil of *Lippia alba*: analysis of samples from French overseas departments and review of previous works. Chem Biodivers. 2006;3:1116-25.

14. **Stashenko EE, Ruiz C, Muñoz A, Castañeda M, Martínez J.** Composition and antioxidant activity of essential oils of *Lippia origanoides* H. B. K. grown in Colombia. Nat Prod Commun. 2008;3:563-5.

15. **Stashenko EE, Torres W, Martínez J.** A Study of compositional variation in the essential oil of ylang-ylang (*Cananga odorata*) Hook. Fil. et Thomson, forma genuina) during flower development. J High Resolut Chromatogr. 1995;18:101-4.

16. **Stashenko EE, Quiroz N, Martínez J**. HRGC/FID/NPD and HRGC/MSD Study of Colombian ylang-ylang (*Cananga odorata*) oils obtained by different extraction techniques. J High Resolut Chromatogr. 1996;19:353-8.

17. **Durán D, Monsalve L, Martínez J, Stashenko EE**. Estudio comparativo de la composición química de aceites esenciales de *Lippia alba* provenientes de diferentes regiones de Colombia, y efecto del tiempo de destilación sobre la composición del aceite. Scientia et Téchnica. 2007;13:435-8.

18. **Weniger B, Robledo S, Arango GJ, Deharo E, Aragon R, Munoz V, *et al.*** Antiprotozoal activities of Colombian plants. J Ethnopharmacol. 2001;78:193-200.

19. **Stashenko EE, Martínez JR.** Sampling volatile compounds from natural products with headspace/solid-phase micro-extraction. J Biochem Biophys Methods. 2007;70:235-42.

20. **García-Ríos A, Leyva M, Martínez J, Stashenko EE.** Determinación de la composición química y actividad antioxidante *in vitro* del aceite esencial de *Piper auritum* Kunth (Piperaceae) difundida en la costa colombiana. Scientia et Téchnica. 2007;13:439-42.

21. **Castañeda M, Muñoz A, Martínez J, Stashenko EE.** Estudio de la composición química y actividad biológica de los aceites esenciales de diez plantas aromáticas colombianas. Scientia et Téchnica. 2007;13:165-6.

22. **Billo M, Cabalion P, Waikedre J, Fourneau C, Bouttier S, Hocquemiller R, *et al.*** Screening of some New Caledonian and Vanuatu medicinal plants for antimycobacterial activity. J Ethnopharmacol. 2005; 96: 195-200.

23. **Vik A, James A, Gundersen LL.** Screening of terpenes and derivatives for antimycobacterial activity; identification of geranylgeraniol and geranylgeranyl acetate as potent inhibitors of *Mycobacterium tuberculosis in vitro*. Planta Med. 2007;73:1410-2.

24. **Jiménez-Arellanes A, Martínez R, García R, León-Díaz R, Luna-Herrera J, Molina-Salinas G, *et al.*** *Thymus vulgaris* as a potencial source of antituberculous compounds. Pharmacologyonline. 2006;3:569-74.

25. **Kilic T.** Analysis of essential oil composition of *Thymbra spicata* var. spicata: antifungal, antibacterial and antimycobacterial activities. Z Naturforsch [C]. 2006;61:324-8.

26. **Koroch A, Juliani R, Zygadlo A.** Bioactivity of essential oils and their components. In: Berger R, editor. Flavours and fragrances. Heidelberg: Springer-Verlag; 2007. p. 87-115.

27. **Trombetta D, Castelli F, Sarpietro MG, Venuti V, Cristani M, Daniele C, *et al.*** Mechanisms of antibacterial action of three monoterpenes. Antimicrob Agents Chemother. 2005;49:2474-8.

28. **Inouye S, Tsuruoka T, Uchida K, Yamaguchi H.** Effect of sealing and Tween 80 on the antifungal susceptibility testing of essential oils. Microbiol Immunol. 2001;45:201-8.

29. **Martin A, Morcillo N, Lemus D, Montoro E, Telles MA, Simboli N, *et al.*** Multicenter study of MTT and resazurin assays for testing susceptibility to first-line anti-tuberculosis drugs. Int J Tuberc Lung Dis. 2005;9:901-6.

30. **Hammer KA, Carson CF, Riley TV.** *In vitro* activity of essential oils, in particular *Melaleuca alternifolia* (tea tree) oil and tea tree oil products, against *Candida* spp. J Antimicrob Chemother. 1998;42:591-5.