Vrije Universiteit Brussel

In vitro antimycobacterial activity of medicinal plants Lantana camara, Cryptolepis sanguinolenta, and Zanthoxylum leprieurii

Tuyiringire, Naasson; Taremwa Mugisha, Ivan; Tusubira, Deusdedit; Munyampundu, Jean-Pierre; Mambo Muvunyi, Claude; Vander Heyden, Yvan

Published in:
Journal of Clinical Tuberculosis and Other Mycobacterial Diseases

DOI:
10.1016/j.jctube.2022.100307

Publication date:
2022

License:
CC BY-NC-ND

Document Version:
Final published version

Link to publication

Citation for published version (APA):
Tuyiringire, N., Taremwa Mugisha, I., Tusubira, D., Munyampundu, J-P., Mambo Muvunyi, C., & Vander Heyden, Y. (2022). In vitro antimycobacterial activity of medicinal plants Lantana camara, Cryptolepis sanguinolenta, and Zanthoxylum leprieurii. *Journal of Clinical Tuberculosis and Other Mycobacterial Diseases*, *27*, [100307]. https://doi.org/10.1016/j.jctube.2022.100307

General rights
Copyright and moral rights for the publications made accessible in the public portal are retained by the authors and/or other copyright owners and it is a condition of accessing publications that users recognise and abide by the legal requirements associated with these rights.

- Users may download and print one copy of any publication from the public portal for the purpose of private study or research.
- You may not further distribute the material or use it for any profit-making activity or commercial gain
- You may freely distribute the URL identifying the publication in the public portal

Take down policy
If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

Download date: 06. Jun. 2022

Journal of Clinical Tuberculosis and Other Mycobacterial Diseases 27 (2022) 100307

Contents lists available at ScienceDirect

Journal of Clinical Tuberculosis and Other Mycobacterial Diseases

journal homepage: www.elsevier.com/locate/jctube

In vitro antimycobacterial activity of medicinal plants *Lantana camara*, *Cryptolepis sanguinolenta*, and *Zanthoxylum leprieurii*

Naasson Tuyiringire [a,b,*], Ivan Taremwa Mugisha [c], Deusdedit Tusubira [d], Jean-Pierre Munyampundu [e], Claude Mambo Muvunyi [f], Yvan Vander Heyden [g]

[a] *Pharm-BioTechnology and Traditional Medicine Centre (PHARMBIOTRAC), Department of Pharmacy, Mbarara University of Science & Technology, P. O. Box 1410, Mbarara, Uganda*
[b] *School of Nursing and Midwifery, College of Medicine and Health Sciences, University of Rwanda, KG11 Ave, 47St/P.O. Box 3286, Kigali, Rwanda*
[c] *Institute of Applied Health Sciences, Clarke International University, P.O. Box 7782, Kampala, Uganda*
[d] *Department of Biochemistry, Mbarara University of Science and Technology, P. O. Box 1410, Mbarara, Uganda*
[e] *School of Science, College of Science and Technology, University of Rwanda, KN 67 Street Nyarugenge, P.O. Box 3900, Kigali, Rwanda*
[f] *College of Medicine and Health Sciences, University of Rwanda, KG11 Ave, 47St/P.O. Box 3286, Kigali, Rwanda*
[g] *Department of Analytical Chemistry, Applied Chemometrics and Molecular Modelling, Vrije Universiteit Brussel (VUB), Laarbeeklaan 103, B-1090 Brussels, Belgium*

ARTICLE INFO

Keywords:
Medicinal plants
Minimum inhibitory concentration
In vitro activity
L. camara
C. sanguinolenta
Z. leprieurii
M. tuberculosis
M. smegmatis
Resazurin microtiter assay

ABSTRACT

Background: Imperative need exists to search for new anti-TB drugs that are safer, and more effective against drug-resistant strains. Medicinal plants have been the source of active ingredients for drug development. However, the slow growth and biosafety level requirements of *M. tuberculosis* culture are considerable challenges. *M. smegmatis* can be used as a surrogate for *M. tuberculosis.* In the current study, preliminary phytochemical screening and antimycobacterial activity evaluation of crude methanolic extracts of medicinal plants against *M. smegmatis*, and two *M. tuberculosis* strains, were conducted.

Materials and Methods: Crude methanolic extracts, obtained from the leaves of *L. camara*, roots of *C. sanguinolenta,* and stem barks of *Z. leprieurii*, were tested for antimycobacterial activity against *M. smegmatis* (mc^2155), pan-sensitive (H37Rv), and rifampicin-resistant (TMC-331) *M. tuberculosis*, using visual Resazurin Microtiter Assay (REMA) on 96 well plates. Preliminary qualitative phytochemical screening tests were performed using standard chemical methods.

Results: The three methanolic extracts inhibited mycobacterial growth in vitro. They were more active against rifampicin-resistant strain with MICs of 176, 97, and 45 μg/mL for *L. camara*, *C. sanguinolenta*, and *Z. leprieurii* extracts, respectively. The lowest activity was observed against *M. smegmatis* with MICs of 574, 325, and 520 μg/mL, respectively. Against H37Rv, activity was intermediate to those of TMC-331 and mc^2155. However, *L. camara* extract showed the same activity against H37Rv and *M. smegmatis*. Preliminary phytochemical analysis revealed alkaloids, flavonoids, phenolic compounds, saponins, tannins, and terpenoids.

Conclusions: Leaves of *L. camara*, roots of *C. sanguinolenta*, and stem barks of *Z. leprieurii* exhibit antimycobacterial activity against *M. smegmatis*, pan-sensitive, and rifampicin-resistant *M. tuberculosis*. This offers the possibilities for novel therapeutic opportunities against TB including multidrug-resistant TB. Further investigations on safety and mechanisms of action are required. These studies could be done using *M. smegmatis* as a surrogate for the highly pathogenic *M. tuberculosis*.

1. Background

Human tuberculosis (TB) represents one of global public health burdens [1], despite the availability of treatment [2]. The disease is the cause of death from a single infectious agent for 1.6 million deaths and 10 million new cases in 2018 [1] globally. This deadly disease results from an infection with *Mycobacterium tuberculosis* (*M. tb*) [3,4]. *Mycobacterium tuberculosis* is a member of the *Mycobacterium tuberculosis*

* Corresponding author.
E-mail addresses: ntuyiringire@std.must.ac.ug (N. Tuyiringire), imugisha@ymail.com (I. Taremwa Mugisha), dtusubira@must.ac.ug (D. Tusubira), mpundupeter@hotmail.com (J.-P. Munyampundu), clmuvunyi@gmail.com (C. Mambo Muvunyi), Yvan.Vander.Heyden@vub.be (Y. Vander Heyden).

https://doi.org/10.1016/j.jctube.2022.100307

Available online 3 March 2022
2405-5794/© 2022 The Author(s). Published by Elsevier Ltd. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

complex (MTBC) [3,5,6] characterized by slow growth, and subsequent latent infection [3]. The World Health Organization (WHO, 2019) report indicates that one-fourth of the world population is latently infected with TB [1]. The increase in drug-resistant strains and co-infections is a major factor in the mortality and morbidity of TB [1,2].

M. tuberculosis became resistant as early as the introduction of streptomycin as first anti-TB drug. Resistance to streptomycin led to introduction of a combinatory therapy [2]. The first combination of anti-TB drugs comprised streptomycin and *para*-aminosalicylic acid. Currently, the World Health Organization recommends a two-phase treatment of TB. The first phase is a 2-month initiation with a cocktail of four first-line anti-TB agents: isoniazid, rifampicin, ethambutol, and pyrazinamide. This phase is followed by a 4-month continuation phase with isoniazid and rifampicin [7]. Early establishment of anti-TB combination therapy did not stop the emergence of bacterial resistance [1]. A crucial factor in minimizing the development of resistance is patient adherence. Patient adherence is negatively impacted by the length and complexity of the treatment regimen, and by the numerous associated adverse drug reactions [2,7,8]. Furthermore, the global spread of drug-resistant *M. tuberculosis* strains is promoted by differences in the quality of public health systems and in the availability of high-quality anti-TB drugs [7]. Therefore, there is an urgent need for new anti-TB drugs that are safer, more effective against drug-resistant strains, and synergistic with current drugs.

Medicinal plants have been the source of natural products, which were lead compounds in the discovery and development of drugs for many diseases [9,10]. In Uganda, medicinal plants with anti-mycobacterial activity have been reported [11–14]. Despite interest in developing new therapeutics for TB, the nature of *Mycobacterium tuberculosis*, the causative agent of TB, places significant barriers to progress. *M. tuberculosis* is a slowly-growing organism, which must be handled under strict containment laboratory to minimize the risk of contamination to personnel and the environment [15]. Anecdotal experience revealed that getting fresh colonies of *M. tuberculosis* for research is hard and time consuming. Potential model systems are required in the search for new anti-TB drugs.

Mycobacterium smegmatis mc^2155 is widely used as a model system to study *M. tuberculosis* [16]. This a non-pathogenic, fast-replicating mycobacterium. Both *M. smegmatis* mc^2155 and *M. tuberculosis* share significant similarities in their genomes, including genes associated with mycolic-acid-rich cell walls [16–19].

In the current study, the antimycobacterial activity of leaves of *Lantana camara* (*L. camara*), roots of *Cryptolepis sanguinolenta* (*C. sanguinolenta*), and stem barks of *Zanthoxylum leprieurii* (*Z. leprieurii*) were tested against (i) a standard laboratory-adapted virulent strain [20] or pan-sensitive *M. tb* (ATCC 27294/H37Rv) [21]; (ii) a model for multi-drug resistant (MDR) *M. tb* strain, a rifampicin-resistant (ATCC 35838/TMC-331) [22], and (iii) an avirulent surrogate organism of *M. tb*, *M. smegmatis* (a nontuberculous mucobacterium, NTM) (ATCC 19420^{TM} / mc^2155) [18,23] using resazurin microtiter assay (REMA) [24]. Additionally, preliminary qualitative phytochemical tests were conducted. This study aimed to carry out preliminary phytochemical screening and to determine antimycobacterial activity against *M. smegmatis*, a model system to study *M. tuberculosis*, as a starting point for further research to elucidate the mechanisms of action of anti-mycobacterial medicinal plants. It is part of a larger research study on metabolomic approaches to elucidate the mechanisms of action of antimycobacterial medicinal plants. In that study on the one hand, the methanolic extracts of the plants will also be analyzed to identify the possibly active compounds/agents, which are responsible for the anti-mycobacterial activity. On the other hand, the secondary metabolites from cultures of mycobacteria, treated with interesting plant extracts, will be analyzed in comparison with untreated cultures. The goal is to identify the molecular targets of the methanolic plant extracts. This holistic approach is faster, and possibly more efficient, than the classical ethno pharmacological approach, where the screening for activity is followed by fractionation, isolation of active agent, optimization steps and the determination of the mechanism of action. The combination of the two processes would help to obtain an anti-TB drug that could join the modern medicine pool.

2. Materials and methods

2.1. Plant materials

Hundreds of mature and healthy leaves of *L. camara* were collected at (Latitude: − 0.6141°S, Longitude:30.6514°E, Altitude: 1416 m) in Kakyeka, Mbarara Municipality, Southwestern Uganda. Stem barks of *Z. leprieurii* were collected at (Latitude: 0.1155°N, Longitude: 32.1302°E, Altitude: 1170 m) in Kabango, Buyijja, Buwama, Mpigi District, Central Uganda. Roots of *C. sanguinolenta* were collected at (Latitude: 0.1118°N, Longitude: 32.1247°E, Altitude: 1185 m) in Kabango, Buwama, Mpigi District, Central Uganda. Plant collection was done by a team of the principal investigator, botanist and research assistant. The collected plant materials were kept in black plastic bags for transport. A shoot of each plant with roots, leaves and flowers was collected, and used for identification by a taxinomist. A voucher specimen of each plant was deposited in the herbarium of Makerere University, Kampala, Uganda with accession numbers 50,923 for *C. sanguinolenta*, 50,924 for *L. camara* and 50,925 for *Z. leprieurii*. Roots of *C. sanguinolenta* were washed with tap water, and all parts of the plant materials were dried on clean sheets, in open air, and protected from direct exposure to sunlight. Plant materials were turned regularly to avoid fermentation and rotting. The moisture was measured to ensure the dryness (the required dryness is between 8 and 10%). The dried plant materials were separately powdered using a blender and the powder was weighed into a bucket for immediate extraction. The remaining powder was stored in glass jars wrapped in newspaper, and kept in a refrigerator at 4 °C.

2.2. Extraction of plant materials

All plant materials were extracted using 99 % methanol (Trust Chemicals Uganda, Kampala, Uganda) as previously described [11,12,14]. The powder of each plant material was separately soaked in 99 % methanol (937 g *L. camara* powder in 5 L methanol, 374 g *C. sanguinolenta* in 748 mL methanol, and 291 g *Z. leprieurii* in 800 mL methanol). The suspensions were all mixed with an IKA mixer (IKA®, Germany) at 16 revolutions per minute (rpm) for 3 h, and left for 48 h for better dissolution. Extracts were filtered using Whatman No.1 filter paper (Buckinghamshire, Uk) and concentrated using a rotary evaporator with a bath water at 40 °C, 20 rpm under reduced pressure. The extracts were frozen in a freezer at −80 °C and freeze-dried to remove the remaining solvent and water. The dried methanolic extracts obtained from each plant were air-dried, weighed and packed in glass bottles with proper labeling for future use and kept at 4 °C. To evaluate its bacteriological quality, a drop of extract solution has been inoculated to the agar/MacConkey plates and incubated at 36 °C overnight. There was no growth.

2.3. Extracts and drug preparation for antimycobacterial activity

Stock solutions of plant extracts were prepared in dimethyl sulfoxide (DMSO) at a concentration of 12.5 mg/mL and sterilized using a 0.2 μm Whatman disposable syringe filter (Buckinghamshire, UK). The final concentration of DMSO was<2%. DMSO was initially diluted to 2% in 7H9 Middlebrrok broth (Difco Laboratories, Detroit, MI). The 6.25 μg/mL stock solutions of isoniazid and rifampicin were prepared in sterile water and sterilized using a 0.2 μm disposable syringe filter. Two fold serial dilutions of extracts/drugs were made using 7H9 Middlebrook broth (Difco Laboratories).

2.4. Test organisms preparation and identification

Two standard laboratory *M. tuberculosis* strains, a pan-sensitive (ATCC 27294/H37Rv) and a rifampicin-resistant ATCC 35,838 (TMC-331^{TM}), and one NTM strain, *M. smegmatis* mc^{2}155/ATCC 19420^{TM}, were used. The strains were initially sub-cultured on mycobacterial growth indicator tubes (MGIT) 960 system (Difco Laboratories) as previously described [25]. The MGIT 960 system, a fully auto- mated system, can test simultaneously up to 960 vials via the fluorescence in each vial every 60 min by a photodetector. The amount of fluorescence is inversely proportional to the oxygen level in the culture medium. Thus indicating the consumption of oxygen due to the growth of inoculated organisms in the vials [25]. Therefore, the system detects and quantifies mycobacteria in each tube.

Upon detectable growth, mycobacteria were confirmed using smear microscopy using a Ziehl Neelsen stain for acid-fast bacilli (AFB) staining and visualization, and an Olympus CX21 microscope (Olympus, Tokio, Japan). The contamination was assessed by plating few drops of MGIT content on blood agar (BA) plates (Oxoid, Hampshire, UK) and incubated for 24 h at 37 °C. Two hundred microliter of the confirmed growth was then subcultured on Löwenstein-Jensen (LJ) slants (Difco Laboratories). These were monitored regularly, and growth identification was confirmed using colonial appearance, confirmed by AFB smear, and molecular methods. Molecular characterization included (i) immunochromatographic assay, detection of immunogenic protein (Rv 1980c or MPT64)[26] using MPT64Ag rapid kit (SD Bioline, South Korea) to differentiate MBTC from NTM, and (ii) a genoType common mycobacterium (CM), known as HAIN, for drug susceptibility tests (MTBDR-Plus Version-2, HAIN Lifescience, Nehren, Germany) [27]. The contamination was again assessed as stated above. Fresh cultures of *M. tuberculosis* strains and *M. smegmatis* were obtained by subculturing them on LJ slants for three to five weeks and one week, respectively.

2.5. Preparation of Middle Brook 7H9 broth medium

Middle Brook 7H9 broth medium (Difco Laboratories) supplemented with glycerol (0.2%) and 10% (v/v) OADC (oleic acid, albumin, dextrose, catalase) (Difco Laboratories) was prepared according to the manufacturer instructions. Briefly, 4.9 g of 7H9 powder was dissolved in 900 mL distilled water, 2 mL glycerol was added and mixed well. The solution was autoclaved at 121 °C for 10 min in a Priorclave 40 (London, UK). After cooling to room temperature, 10% (v/v) OADC was added. Blood agar plates were used to check Middle Brook 7H9 broth medium contamination.

2.6. Preparation of inoculum

M. tuberculosis pan-sensitive strain (H37Rv/ATCC 27,294 TMC-102 ^{TM}), rifampicin resistant ATCC 35,838 (TMC-331^{TM}) and *M. smegmatis* (mc^2 155/ATCC 19420^{TM}) were sub-cultured and grown on Lowenstein Jensen medium (LJ) for three to five weeks (*M. tuberculosis* strains) and 1 week (*M. smegmatis*). The inocula were prepared following the Clinical and Laboratory Standards Institute (CLSI) recommendations [27] standardized with few modifications. Fresh colonies were suspended in saline solution to obtain the scales of McFarland no.1, equivalent to 3.2 × 10^6 colony forming units (CFU)/mL. The inoculum was prepared by diluting the bacterial suspension at a ratio of 1:20 in Middle Brook 7H9 broth medium (5 μL of suspended bacteria in 100 μL of 7H9). The suspension was well vortexed in a vial and allowed to settle. The diluted suspension (100 μL) was used to inoculate each well of the plate [27] and a drop of this inoculum was cultured on a blood agar plate for 48 h to check for possible contamination..

2.7. Anti-mycobacterial activity tests and determination of minimum inhibitory concentrations

The antimycobacterial activity of methanolic crude extracts of *L. camara*, *C. sanguinolenta*, and *Z. leprieurii* was tested using the REMA. The susceptibility test was performed in 96 microplates (wells) using resazurin as an indicator of cell viability or growth inhibition [24].

Working solutions of the tested extracts were diluted in Middle Brook 7H9 broth supplemented with OADC to obtain final sample concentrations ranging from 0.012 to 6.25 mg/mL. Rifampicin (RFP) and isoniazid (INH) were dissolved in distilled water and further diluted in 7H9 broth to a concentration range of 0.006 to 3.125 μg/mL. The two first-line anti-TB drugs were used as positive control drugs, whereas extract/drug-free medium with strain suspensions were used as negative controls. One hundred microliter of Middle Brook 7H9 broth was dispensed into each well of a 96 microtiter plate. Then, one hundred microliter of working solutions of extract (6.25 mg/mL)//drug (3.125 μg/mL) was added and twofold serially diluted by transferring 100 μL from the first well to the next, up to tenth well. The excess 100 μL was removed from the tenth well. Finally, 100 μL of 7H9 Middle Brook broth mixed with inoculum was added from the first to eleventh well. The 11th well contains the broth without the intervention (fertility control/growth control), while the 12th contains broth without inoculum and intervention (sterility control). The final volume in each well was 200 μL.

Each extract/drug concentration was assayed in triplicate. Each microtiter plate was then sealed with parafilm, and incubated for 10 days for *M. tuberculosis* strains and 2 days for *M. smegmtis* at 37 °C at a normal atmosphere. After the incubation period, 25 μL of resazurin 0.02% w/v was added to each well and incubated at 37 °C for 24 h for color development (from blue to pink). The visual MIC was defined as the lowest drug/extract concentration that prevented the color change of resazurin reagent from blue to pink. The blue color in the well was interpreted as there was no mycobacterial growth and pink color was scored as growth occurrence [24].

2.8. Preliminary phytochemical screening tests

Preliminary qualitative phytochemical tests for the presence of secondary metabolites were done as described in [28]. Briefly,.

2.8.1. Test for phenolic compounds

Ferric chloride (Iron III chloride) test was carried out.The methanolic extract was put in a clean tube, and then a few drops of neutral 5% ferric chloride solution was added. A dark-green color indicates the presence of phenolic compounds.

2.8.2. Test for terpenoids

Approximately 2 mL extract solution was mixed with 2 mL chloroform and 2 mL concentrated sulfuric acid. The formation of a reddish-brown color shows the presence of terpenoids.

2.8.3. Test for alkaloids

2 mL 2% sulfuric acid (H_2SO_4) was added to 0.2 g crude methanolic extract. The mixture was warmed above a flame for two minutes, and filtered with Whatman No.1 filter paper. A few drops of Dragendrof's reagents were added to the filtrate. The appearance of an orange-red precipitate indicates the presence of alkaloids.

2.8.4. Test for flavonoids

Few drops 10% sodium hydroxide (NaOH) was mixed with 0.2 g extract and then a few drops concentrated hydrochloric acid (HCl were added). Change of the yellow colored solution into a colorless solution confirms the presence of flavonoids [29].

2.8.5. Test for saponins

5 mL methanolic plant extracts were dissolved in 5 mL distilled water and vigorously shaken. Formation of a persistent foam indicated the presence of saponins.

2.8.6. Test for tannins

2 mL methanolic plant extracts were dissolved in 2 mL distilled water. Few drops of 0.1 M iron III chloride ($FeCl_3$) were added. Formation of a dark green coloration indicates the presence of tannins.

2.9. Data analysis

GraphPad Prism 5.01 statistical software (GraphPad Software Inc, California, USA) was used for descriptive data analysis. The MIC results are presented as mean (Standard deviation, SD) values of three replicates. The analysis of variance (ANOVA) analysed the significant difference of antimycobacterial activity of the three medicinal plants, on each strain at 95% Confidence Interval (CI).

2.10. Ethical consideration

The Research and Ethics Committee of the Faculty of Medicine, Mbarara University of Science and Technology, and the Uganda National Council of Science and Technology approved the study. Approval/reference numbers are 06/10–18 and HS422ES respectively.

3. Results

3.1. Qualitative phytochemical screening tests

The qualitative phytochemical tests of *L. camara*, *C. sanguinolenta* and *Z. leprieurii* (Table 1) showed the presence of alkaloids, flavonoids, phenolic compounds, saponins, tannins and terpenoids. The intensity of color and the persistence of foam (saponins) varied for different plant extracts. *L. camara* showed the highest content of saponins (persistence of foam) and *C. sanguinolenta* the highest color intensity when testing for the presence of alkaloids. Preliminary phytochemical screening does not inform about the active compounds. However, it gives basic information about the phytochemicals present in the plant extracts. Further, a deep phytochemical analysis is required to identify the active compounds and their mechanisms of action.

3.2. Antimycobacterial activity of crude methanolic extracts

Three medicinal plants were tested for antimycobacterial activity against three strains of mycobacteria; a non-tuberculosis *M. smegmatis* (mc^2155), a pan-sensitive *M. tuberculosis* (H37Rv), and a rifampicin-resistant *M. tuberculosis* (TMC-331). Two standard anti-TB drugs, isoniazid (INH) and rifampicin (RFP), were used for comparison. The antimycobacterial activity varied considerably depending on the plant and the strain tested (Table 2). Each plant showed significantly different activities against the three strains (P-values < 0.05, ANOVA). Further it was observed that the activities of the different plants against a *M. tuberculosis* strain were significantly different (P-values < 0.05, ANOVA), while against the *M. smegmatis* strain is was not the case. All methanolic plant extracts showed higher activity against the rifampicin-resistant (TMC-331) strain with MICs of 176, 97, 45 μg/ml for *L. camara*, *C. sanguinolenta*, and *Z. leprieurii*, respectively. The activity against *M. smegmatis* was the least with MICs of 574, 325, and 520 μg/ml, respectively. However, *L. camara* showed similar activity against *M. smegmatis* and H37Rv strains (Fig. 1). An intermediate activity was observed against H37Rv when compared to TMC-331 and *M. smegmatis*. However, standard anti-TB drugs, isoniazid and rifampicin, were more active against *M. smegmatis* strain, with MICs of 0.03, 0.04 μg/mL for INH and RFP, respectively. Isoniazid showed less activity against the rifampicin-resistant strain (TMC-331) with MIC of 0.09 μL/mL, while RFP showed no growth inhibition against TMC-331, even not at the highest concentration used (3.125 μg/mL).

Table 1
Results of preliminary phytochemical screening tests.

Test type	*L. camara*	*C. sanguinolenta*	*Z. leprieurii*
Dragendrof's test (Alkaloids)	+	++	+
Test for flavonoids	+	+	+
Ferric chloride test (Phenolic compounds)	+	+	+
Test for saponins	++	+	+
Test for tannins	+	+	+
Test for terpenoids	+	+	+

+: Presence, ++: Higher compared to other plants (*L. camara* has more saponins compared to *C. sanguiolonta* and *Z. leprieurii*. *C. sanguiolonta* has more alkaloinds than *L. camara* and *Z. leprieurii*.

4. Discussion

In this study, we investigated the antimycobacterial activity of methanolic extracts from the leaves of *L. camara*, roots of *C. sanguinolenta* and stem barks of *Z. leprieurii* against two strains of *M. tuberculosis* (H37Rv and TMC-331), and one *M. smegmatis* (mc^2155) strain. The results showed that all methanolic extracts at the tested doses have significant different antimycobacterial activity against the tested mycobacteria strains, which indicates that these plants may contain one or a mixture of active ingredients responsible for the antimycobacterial activity. Different types of secondary metabolites, which are potentially resoponsible for antimycobacterial activity, have been identified (Table 1) and reviewed in [30]. There is only limited knowledge on the specific mechanisms of action of those plant extracts on mycobacteria. Specific molecular targets are not well known. However, the general possible mechanisms of action of antibacterial effects, from the literature, are summarized in Table 3. Further investigations are needed to identify and quantify the active ingredients of the three medicinal plant extracts. All methanolic extracts showed higher activity against the TMC-331 strain with MICs of 176, 97, and 45 μg/mL for *L. camara*, *C. sanguinolenta*, and *Z. leprieurii*, respectively (Fig. 1). The lowest activity was shown against *M. smegmatis*,with MICs of 574, 325, and 520 μg/ml, respectively. Against H37Rv, the activity was intermediate to those of TMC-331 and *M. smegmatis*. However, *L. camara* showed similar activity against H37Rv and *M. smegmatis*. The results of our study

Table 2
Minimum inhibitory concentrations (in μg/mL ± STDEV, n = 3) of total methanolic plant extracts of *L. camara*, *C. sanguinolenta*, and *Z. leprieurii* and the standard drugs (Isoniazid and Rifampicin). *P-values* originate from ANOVA.

	Strains			
	H37Rv	TMC-331	mc^2155	P-value
L. C	574 ± 196	176 ± 33	574 ± 196	0.036
C. S	130 ± 56	97 ± 0.000	325 ± 113	0.048
Z. L	144 ± 49	45 ± 0.000	520 ± 230	0.010
P-value	0.006	0.004	0.290	
INH	0.06 ± 0.280	0.09 ± 0.0005	0.03 ± 0.014	
RFP	0.16 ± 0.056	>3.125	0.04 ± 0.0005	

L.C*: L. camara;* C.S*: C. sanguinolenta;* Z.L*: Z. leprieurii,* INH: isoniazid, RFP: rifampicin, H37Rv: pan-sensitive *M. tuberculosis,* TMC-331: rifampicin-resistant *M. tuberculosis* and mc^2155: *M. smegmatis*. STDEV: Standard deviation.

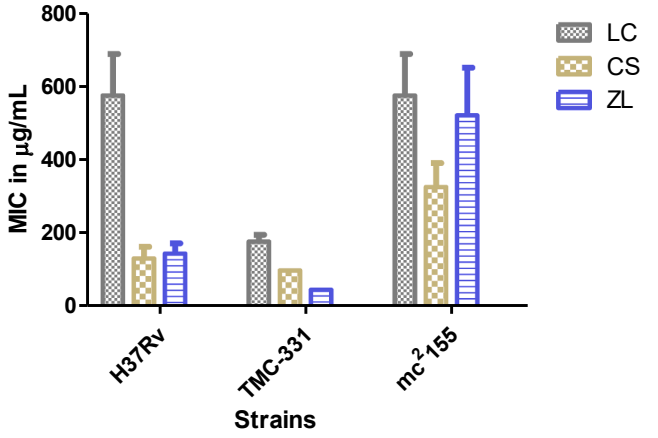


Fig. 1. Minimum inhibitory concentrations (in µg/mL) of methanolic plant extracts of *L. camara* (LC), *C. sanguinolenta* (CS), and *Z. leprieurii* (ZL) against *M. tuberculosis* strains (H37Rv and TMC-331), and *M. smegmatis* (mc^{2}155).

Table 3
Possible mechanisms of action of phytochemicals and metabolites on antibacterial activity.

Secondary Metabolites	Possible mechanisms of antibacterial activity	References
Alkaloids	Morphological changes and cellular breakdown in bacteria. Targetting DNA topoisomerase enzymes and inhibit bacterial growth.	[30,39]
Flavonoids	Inhibition of nucleic acid synthesis, of cytoplasmic membrane function, of energy metabolism, of the attachment and biofilm formation, of the porins on the cell membrane, alteration of the membrane permeability, and attenuation of the pathogenicity.	[40]
Phenolic compounds	Interactions with bacterial cells surface, hence bacteriual breakdown.	[41]
Terpenoids, including saponins	Inhibition of the activity, not only in electron transport chain but also in other sites of energy metabolism. Disruption of bacterial cell membrane integrity.	[42–44]
Tannins	Inhibition of extracellular microbial enzymes, deprivation of the substrates required for microbial growth or direct action on microbial metabolism through inhibition of oxidative phosphorylation, iron deprivation by iron chelation, inhibition of cell wall synthesis, disruption of the cell membrane, and inhibition of fatty acid biosynthetic pathways. Acting as quorum sensing inhibitors and attenuating the gene expression of several virulence factors such as biofilms, enzymes, adhesins, motility, and toxins.	[45,46]

comply with previous studies [11,12,14], but with notable differences. Previous studies did not include *M. smegmatis* as a model for the *M. tuberculosis* study. This NMT strain showed no significant different susceptibility to the three plants. They all indicated that *L. camara* and *C. sanguinolenta* exhibited higher activity against TMC-331with MICs of 15 and 156 µg/ml, respectively than against H37Rv with MIC of 20 and 260 µg/mL [11,12]. For *Z. leprieurii* the MIC for TMC-331 (75.3 µg/ml) was higher than that of H37Rv (47.5 µg/ml). The antimycobacterial activity reported for *L. camara* [11] was significantly higher compared to the results in the present study as well as those from another study (unpublished data in [31]), which reported similar results to ours. The difference from previous studies might be associated with the method used to determine the MICs. Resazurin Microtiter Plate Assay (REMA) is simple, rapid, cheap, and sensitive compared to agar dilutions, prevoulsly used in [11,12]. Besides, the incubation period and inoculum size may also affect the results. Thus, a standardized method needs to be established for antimycobacterial susceptibility tests of medicinal plants with REMA. The antimycobacterial activity of the three plants was significantly lower than that of standard drugs (Table 2), which may be explained by the fact that the methanolic extracts contains many diverse compounds; some of which may affect the activity of the active compounds. If purified, they may show higher activity as reported for *Z. leprieurii* [14]. The content of active compounds may also differ from one plant to another. The preliminary phytochemical tests have revealed the presence of different major phytochemical components. This valuable step needs deep chemical analysis to identify and quantify the active compounds in each plant extract. The elucidation of the mechanisms of action of the three plants is required. Using systems biology methods such as metabolomics would help to identify the molecular targets of these plants. The method could explain the different susceptibility profiles of H37Rv, TMC-331, and mc^{2}155 towards their treatment with methanolic extracts of the three medicinal plants [32,33]. An ongoing study by our team looks at the metabolic fingerprinting of the methanolic extracts. Besides, the analysis of extracts from mycobacteria cultures with/without plant extracts treatment, will be conducted [30].

H37Rv, TMC-331, and mc^{2}155 responded differently to the methanolic extracts of *L. camara*, *C. sanguinolenta*, and *Z. leprieurii*. Both H37Rv and TMC-331 are pathogens that cause TB in humans. The higher acivity observed for TMC-331 compared to H37Rv can be explained by a phenomenon known as fitness cost associated with acquired resistance [34]. The fitness cost manifests during reduced growth rate, competitive ability, and virulence [35]. A drug-resistant strain may grow slower under usual culture conditions. From anecdotal experience, it was observed that the TMC-331 was slower compared to the H37Rv under the same culture conditions. Nevertheless, the results showed that the three medicinal plants could be a good source of drugs for MDR TB since rifampicin resistance and *M. smegmatis* are good indicators of multidrug resistance in *M. tuberculosis* [36]. In a screening of BSL-1 Mycobacterium spp. against a battery of TB drugs in [37], *M. smegmatis* (ATCC607) exhibited good agreement with its drug susceptibility against the TB drugs. The study was conducted under a low-nutrient culture medium (0.5% Tween 80 in Middlebrook 7H9 broth). The authors in [37] recommended evaluating new molecules with the MIC values of < 6.25 mg/mL against *M. smegmatis* (ATCC607) in bacterial growth inhibitory activity (MIC) assays against Mtb strain(s). The results of our study (Fig. 1) comply with the MICs values of < 6.25 mg/mL. However, the low-nutrient culture medium defined above needs to be explored for further investigations.

The results in (Fig. 1) show the lower activity of extracts against *M. smegmatis* (mc^{2}155) compared to other strains. We suggest that the lower activity against *M. smegmatis* results from the fact that it was grown under a rich-nutrient culture medium. However, the fact that mc^{2}155 is susceptible to methanolic extracts of the above-mentioned medicinal plants indicates that it can be used as a surrogate for H37Rv for further investigations on in *vivo* efficacy, safety, and mechanisms of action [18,23]. The use of *M. smegmatis* mc^{2}155 is advantageous as it grows fast and can be handled easily. It can be safely used in a standard biosafety Level II facility [19], unlike H37Rv and TMC-331, which require biosafety level III facilities [27,38].

5. Conclusions and future considerations

This study showed that leaves of *L. camara*, roots of *C. sanguinolenta* and stem barks of *Z. leprieurii* exhibit antimycobacterial activity against *M. smegmatis*, pan-sensitive *M. tuberculosis*, and rifampicin-resistant *M. tuberculosis*. This offers the possibilities for novel therapeutic opportunities against tuberculosis including multidrug-resistant TB. However, further investigations on in *vivo* efficacy, safety and mechanisms of action are required. These studies can follow the isolation of active compound or the preparation of standardized plant extracts and could be done using *M. smegmatis* as a surrogate for the highly

pathogenic *M. tuberculosis*. A standardized plant extract can also be tested for in vivo efficacy and safety studies. This would even be a better option if the extract contains many compounds that are synergistically active against *M. tuberculosis*.

An untargeted fingerprinting of the plant methanolic extracts will help identifying the active ingredients from those plants. The results from this analysis may shed light on possible further investigations.

Author Contributions

NT conceived the research idea. NT collected, extracted plant materials, and performed phytochemical screening tests. NT and ITM conducted antimicrobial activity study. JPM, TD, CMM and YVH monitored and mentored the proposal. NT wrote the draft manuscript. All authors revised, edited and approved the final manuscript.

Funding

This study is part of the Ph.D. program that is funded by the World Bank and the Vrije Universiteit Brussel (VUB).

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

The authors express their gratitude to the Vrije Universiteit Brussel (VUB) and World Bank for their funding through Global Minds Scholarship and the PHARMBIOTRAC-Africa Center of Excellence (ACE II), Mbarara University of Science and Technology, respectively.

References

[1] World Health Organization. Global tuberculosis report 2019. Geneva: 2019. https://doi.org/https://apps.who.int/iris/handle/10665/329368.
[2] Torfs E, Piller T, Cos P, Cappoen D. Opportunities for overcoming mycobacterium tuberculosis drug resistance: Emerging mycobacterial targets and host-directed therapy. Int J Mol Sci 2019;20:2868. https://doi.org/10.3390/ijms20122868.
[3] G. Delogu M. Sali G. Fadda The biology of mycobacterium tuberculosis infection Mediterr J Hematol Infect Dis 5 1 2013 e2013070 10.4084/mjhid.2013.070.
[4] Kaufmann, S.H.E.; Hahn H. Mycobacteria and TB. Issues in Infectious Diseases, Vol 2. Basel, Karger; 2003. https://doi.org/doi: 10.1159/000066884.
[5] Ehrt S, Warner DF, Arlehamn CSL, Lewinsohn D, Sette A, Walzl G, et al. Mycobacterium Tuberculosis Metabolism and Host Interaction : Mysteries and Paradoxes and Host Interaction : Mysteries. Curr Top Microbiol Immunol 2013; 374:163–88. https://doi.org/10.1007/82_2012_299.
[6] P.P. Salvatore Y. Zhang Tuberculosis: Molecular Basis of Pathogenesis 2017 Elsevier Inc. New York 10.1016/B978-0-12-801238-3.95697-6.
[7] Gygli SM, Borrell S, Trauner A, Gagneux S. Antimicrobial resistance in Mycobacterium tuberculosis: Mechanistic and evolutionary perspectives. FEMS Microbiol Rev 2017;41:354–73. https://doi.org/10.1093/femsre/fux011.
[8] Vilariça AS, Diogo N, André M, Pina J. Adverse reactions to antituberculosis drugs in in-hospital patients: Severity and risk factors. Rev Port Pneumol 2010;16: 431–51. https://doi.org/10.1016/S0873-2159(15)30040-4.
[9] Veeresham C. Natural products derived from plants as a source of drugs. J Adv Pharm Technol Res 2012;3:200–1. https://doi.org/10.4103/2231-4040.104709.
[10] Cowan MM. Plant products as antimicrobial agents. Clin Microbiol Rev 1999;12(4): 564–82.
[11] Kirimuhuzya C, Waako P, Joloba M, Odyek O. The anti-mycobacterial activity of Lantana camara a plant traditionally used to treat symptoms of tuberculosis in South-western Uganda. Afr Health Sci 2009;9:40–5. https://doi.org/10.4314/ahs.v9i1.7101.
[12] Kirimuhuzya C, Bunalema L, Waako P, Tabuti JRS, Orodho J, Magadula JJ, et al. Efficacy of Cryptolepis sanguinolenta root extract on slow-growing rifampicin resistant Mycobacterium tuberculosis. J Med Plants Res 2012;6:1140–6. https://doi.org/10.5897/JMPR10.856.
[13] J. AO, P. O, J. BT, N. O, J.j M, C. K. Indigenous knowledge of communities around Lake Victoria Basin regarding treatment and management of tuberculosis using medicinal plants. Int J Med. Med Sci 2014;6(1):16–23. https://doi.org/10.5897/IJMMS09.374.
[14] Bunalema L, Fotso GW, Waako P, Tabuti J, Yeboah SO. Potential of Zanthoxylum leprieurii as a source of active compounds against drug resistant Mycobacterium tuberculosis. BMC Complement Altern Med 2017;17:4–9. https://doi.org/10.1186/s12906-017-1602-x.
[15] Kumar A, Chettiar S, Parish T. Current challenges in drug discovery for tuberculosis. Expert Opin Drug Discov 2017;12(1):1–4. https://doi.org/10.1080/17460441.2017.1255604.
[16] Mohan A, Padiadpu J, Baloni P, Chandra N. Complete Genome Sequences of a Mycobacterium smegmatis Laboratory Strain (MC2 155) and Isoniazid-Resistant (4XR1/R2) Mutant Strains. Genome Announc 2015;3:4–5. https://doi.org/10.1128/genomeA.01520-14.Copyright.
[17] Hentschel J, Burnside C, Mignot I, Leibundgut M, Boehringer D, Ban N. The Complete Structure of the Mycobacterium The Complete Structure of the Mycobacterium smegmatis 70S Ribosome. Cell Reports 2017;20(1):149–60. https://doi.org/10.1016/j.celrep.2017.06.029.
[18] Chaturvedi V, Dwivedi N, Tripathi RP, Sinha S. Evaluation of Mycobacterium smegmatis as a possible surrogate screen for selecting molecules active against multi-drug resistant Mycobacterium tuberculosis. Evaluation of Mycobacterium smegmatis as a possible surrogate screen for selecting molecules active against multi-drug resistant Mycobacterium tuberculosis 2007;53(6):333–7. https://doi.org/10.2323/jgam.53.333.
[19] Andreu N, Soto CY, Roca I, Martín C, Gibert I. Mycobacterium smegmatis displays the Mycobacterium tuberculosis virulence-related neutral red character when expressing the Rv0577 gene. FEMS Microbiol Lett 2004;231:283–9. https://doi.org/10.1016/S0378-1097(04)00008-4.
[20] Barczak A, Domenech P, Boshoff HM, Reed M, Manca C, Kaplan G, et al. In Vivo Phenotypic Dominance in Mouse Mixed Infections with Mycobacterium tuberculosis Clinical Isolates. J Infect Dis 2005;192(4):600–6. https://doi.org/10.1086/432006.
[21] Nguta JM, Appiah-Opong R, Nyarko AK, Yeboah-Manu D, Addo PGA. Current perspectives in drug discovery against tuberculosis from natural products. Int J Mycobacteriology 2015;4(3):165–83. https://doi.org/10.1016/j.ijmyco.2015.05.004.
[22] Falzon D, Jaramillo E, Schunemann HJ, Arentz M, Bauer M, Bayona J, et al. WHO guidelines for the programmatic management of drug-resistant tuberculosis: 2011 update. Eur Respir J 2011;38(3):516–28. https://doi.org/10.1183/09031936.00073611.
[23] Arthur PK, Amarh V, Cramer P, Arkaifie GB, Blessie EJS, Fuseini M-S, et al. Characterization of Two New Multidrug-Resistant Strains of Mycobacterium smegmatis: Tools for Routine In Vitro Screening of Novel Anti-Mycobacterial Agents. Antibiot (Basel, Switzerland) 2019;8(1):4. https://doi.org/10.3390/antibiotics8010004.
[24] Palomino J-C, Martin A, Camacho M, Guerra H, Swings J, Portaels Francoise. Resazurin microtiter assay plate: Simple and inexpensive method for detection of drug resistance in Mycobacterium tuberculosis. Antimicrob Agents Chemother 2002;46(8):2720–2. https://doi.org/10.1128/AAC.46.8.2720-2722.2002.
[25] Hillemann D, Richter E, Rusch-Gerdes S. Use of the BACTEC mycobacteria growth indicator tube 960 automated system for recovery of mycobacteria from 9,558 extrapulmonary specimens, including urine samples. J Clin Microbiol 2006;44(11): 4014–7. https://doi.org/10.1128/JCM.00829-06.
[26] Andersen P, Askgaard D, Ljungqvist L, Bennedsen J, Heron I. Proteins released from Mycobacterium tuberculosis during growth. Infect Immun 1991;59(6): 1905–10.
[27] Woods GL, Brown-Elliott BA, Conville PS, et al. Susceptibility Testing of Mycobacteria, Nocardiae, and Other Aerobic Actinomycetes. 3rd ed. Wayne (PA): Clinical and Laboratory Standards Institute; March; 2018.
[28] Patil US, Deshmukh OS. Preliminary phytochemical screening of six medicinal plants used as traditional medicine. Int J Pharma Bio Sci 2016;7:77–81.
[29] M.A. Hossain K.A.S. AL-Raqmi Z.H. AL-Mijizy A.M. Weli Q. Al-Riyami Study of total phenol, flavonoids contents and phytochemical screening of various leaves crude extracts of locally grown Thymus vulgaris. Asian Pac J Trop Biomed 3 9 2013 705 710 10.1016/S2221-1691(13)60142-2.
[30] Tuyiringire N, Deyno S, Weisheit A, Tolo CU, Tusubira D, Munyampundu J-P, et al. Three promising antimycobacterial medicinal plants reviewed as potential sources of drug hit candidates against multidrug-resistant tuberculosis. Tuberculosis 2020; 124:101987. https://doi.org/10.1016/j.tube.2020.101987.
[31] Antimycobacterial MA. Activity of Alkaloids from Medicinal Plants. Mbarara University of Science and Technology; 2019.
[32] Sieniawska E, Sawicki R, Golus J, Georgiev MI. Untargetted metabolomic exploration of the mycobacterium tuberculosis stress response to cinnamon essential oil. Biomolecules 2020;10:357. https://doi.org/10.3390/biom10030357.
[33] Tuyiringire N, Tusubira D, Munyampundu J-P, Tolo CU, Muvunyi CM, Ogwang PE. Application of metabolomics to drug discovery and understanding the mechanisms of action of medicinal plants with anti-tuberculosis activity. Clin Transl Med 2018; 7:29. https://doi.org/10.1186/s40169-018-0208-3.
[34] Vogwill T, MacLean RC. The genetic basis of the fitness costs of antimicrobial resistance: A meta-analysis approach. Evol Appl 2015;8(3):284–95. https://doi.org/10.1111/eva.12202.
[35] Gagneux S, Long CD, Small PM, Van T, Schoolnik GK, Bohannan BJM. The competitive cost of antibiotic resistance in Mycobacterium tuberculosis. Science (80-) 2006;312(5782):1944–6.
[36] Namouchi A, Cimino M, Favre-Rochex S, Charles P, Gicquel B. Phenotypic and genomic comparison of Mycobacterium aurum and surrogate model species to Mycobacterium tuberculosis: implications for drug discovery. BMC Genomics 2017;18:530. https://doi.org/10.1186/s12864-017-3924-y.
[37] Lelovic N, Mitachi K, Yang J, Lemieux MR, Ji Y, Kurosu M. Application of Mycobacterium smegmatis as a surrogate to evaluate drug leads against Mycobacterium tuberculosis Application of Mycobacterium smegmatis as a

surrogate to evaluate drug leads against Mycobacterium tuberculosis. J Antibiot (Tokyo) 2020;73(11):780–9. https://doi.org/10.1038/s41429-020-0320-7.

[38] Long Q, Zhou Qi, Ji L, Wu J, Wang W, Xie J. Mycobacterium smegmatis genomic characteristics associated with its saprophyte lifestyle. J Cell Biochem 2012;113 (10):3051–5. https://doi.org/10.1002/jcb.24199.

[39] García MT, Carreño D, Tirado-Vélez JM, Ferrándiz MJ, Rodrigues L, Gracia B, et al. Boldine-Derived Alkaloids Inhibit the Activity of DNA Topoisomerase I and Growth of Mycobacterium tuberculosis. Front Microbiol 2018;9. https://doi.org/10.3389/fmicb.2018.01659.

[40] Xie Y, Yang W, Tang F, Chen X, Ren L. Antibacterial activities of flavonoids: structure-activity relationship and mechanism. Curr Med Chem 2015;22:132–49. https://doi.org/10.2174/0929867321666140916113443.

[41] Bouarab-Chibane L, Forquet V, Lantéri P, Clément Y, Léonard-Akkari L, Oulahal N, et al. Antibacterial Properties of Polyphenols: Characterization and QSAR (Quantitative Structure-Activity Relationship) Models. Front Microbiol 2019;10. https://doi.org/10.3389/fmicb.2019.00829.

[42] Guimarães AC, Meireles LM, Lemos MF, Guimarães MCC, Endringer DC, Fronza M, et al. Antibacterial Activity of Terpenes and Terpenoids Present in Essential Oils. Molecules 2019;24(13):2471. https://doi.org/10.3390/molecules24132471.

[43] Barbosa ADP. AN OVERVIEW ON THE BIOLOGICAL AND PHARMACOLOGICAL ACTIVITIES OF SAPONINS. Int J Pharm Pharm Sci 2014;6:47–50.

[44] Mandal P, Sinha Babu SP, Mandal NC. Antimicrobial activity of saponins from Acacia auriculiformis. Fitoterapia 2005;76:462–5. https://doi.org/10.1016/j.fitote.2005.03.004.

[45] Farha AK, Yang Q-Q, Kim G, Li H-B, Zhu F, Liu H-Y, et al. Tannins as an alternative to antibiotics. Food Biosci 2020;38:100751. https://doi.org/10.1016/j.fbio.2020.100751.

[46] Scalbert A. Antimicrobial properties of tannins. Phytochemistry 1991;30:3875–83. https://doi.org/10.1016/0031-9422(91)83426-L.