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Anti-Mycobacterial Activity of Medicinal Plant Extracts Used in the Treatment of Tuberculosis by Traditional Medicine Practitioners in Uganda

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Abstract

Tuberculosis (TB) remains a public health challenge and one of the leading causes of death worldwide. TB is preventable and curable. However, treatment of tuberculosis has continued to be difficult as a result of rapid increase of multidrug and extensively drug resistant strains of *Mycobacterium tuberculosis*. Medicinal plants have for centuries been traditionally used in treatment of tuberculosis and similar ailments. They possess antimicrobial properties which render them a new hope as a source of novel bioactive leads in the development of antimycobacterial agents. In this study, 2 plant species commonly used traditionally in Uganda for treatment of tuberculosis, *Zanthoxylum leprieurii* and *Rubia cordifolia* were screened for *in vitro* antimycobacterial activity against *Mycobacterium tuberculosis* strains; pan sensitive MTB H37Rv, Rifampicin resistant TMC 331 strain and two wild strains (one rifampicin resistant and another one rifampicin susceptible). Antimycobacterial activity of aqueous, ethanolic and methanolic plant extracts was determined using Resazurin Microtiter Assay (REMA). Both plant extracts exhibited significant *in vitro* antimycobacterial activity against all strains of *Mycobacterium tuberculosis*. Minimum inhibitory concentrations (MIC) of methanolic crude extracts of both plants ranged from 23.4 μg/mL to 187.5 μg/mL. Comparatively, methanol extracts of both plants possessed superior antimycobacterial activity against all *Mycobacterium tuberculosis* strains. Our findings indicated that both plants exhibited activity against susceptible and re-

sistant strains of *Mycobacterium tuberculosis.* While antimycobacterial activity of *Z. leprieurii* confirms results from previous studies, activity of the extracts of *R. cordifolia* is reported for the first time in East Africa. Further studies aimed at determining the effects of combination of these plant extracts and standard anti-TB drugs should be carried out.

Keywords

Tuberculosis, Antimycobacterial Activity, Medicinal Plants, *Zanthoxylum leprieurii*, *Rubia cordifolia*

1. Introduction

Tuberculosis (TB) is ranked among the ten leading causes of death in Africa and remains a major global health problem. In 2020, World Health Organization (WHO) reported 10 million people to have acquired tuberculosis and about 1.3 million HIV negative and 0.2 million HIV positive people to have died from it globally. In 2020, 90,000 people were estimated to have developed TB in Uganda, out of which 7400 died from it [1]. Emergence of Multi-Drug Resistant (MDR) (470 cases in 2020 in Uganda), Extensively Drug Resistant (XDR) (5 cases) and lately totally drug resistant (TDR) strains together with HIV co-infection has amplified the problem of mycobacterial disease management [1] [2].

Tuberculosis treatment is mainly by chemotherapy involving combination of a complex regimen of five drugs for a period of 6 months in drug susceptible patients. The period is usually increased up to 8 months in drug resistant tuberculosis (DR TB) patients. Treatment of drug resistant TB is by the use of second-line drugs, which are expensive and are associated with severe adverse effects thus resulting into high levels of non-compliance [3] [4]. Additionally, drug interactions between anti-TB drugs especially rifampicin with some antiretroviral drugs (ARVS) has also complicated the management of TB in HIV positive individuals [5].

Medicinal plants have been traditionally used to treat tuberculosis and may offer a new hope as source of bioactive molecules for developing alternative medicines for the mycobacterial diseases [6] [7]. WHO has stated that 80% of the world's population depends on traditional medicine (TM) for primary health care [8] [9]. Furthermore, it has been reported that 170 of the 194 WHO Member States use traditional and complementary medicine (T&CM) [10]. Marrying the traditional knowledge with modern science may provide an innovative and valuable tool for affordable, safe, novel and effective therapies [11].

In recent past, efforts have been done to rediscover the traditional knowledge of plant-based therapeutics used to treat mycobacterial diseases with plant species in different parts of the world including Uganda. Surveys have been undertaken in the region to explore this traditional knowledge [3] [12]. In a question-

naire-based interview of 40 Traditional Medicine Practitioners (TMPs) of Mpigi and Butambala districts in Uganda, Bunalema and colleagues reported 90 plant species used to treat tuberculosis traditionally. Out of 90 plant species, 4 priority plants were mentioned. These included: *Zanthoxylum leprieurii*, *Rubia cordifolia*, *Piptadeniastrum africanum*, and *Albizia coriaria* which were most mentioned by TMPs [12]. Despite plants having played an integral role in treatment of many ailments in Uganda, scientific analyses of their benefit are still greatly inadequate [13]. This study was therefore aimed at evaluating the antimycobacterial activity of two plants commonly used by TMPs in the treatment of tuberculosis in Uganda (*Zanthoxylum leprieurii* and *Rubia cordifolia*). This was the starting point of a bigger study aimed at evaluating cytotoxicity and the effect of combination of these plant extracts with conventional anti-tuberculosis drugs.

2. Materials and Methods

2.1. Plant Material Collection

Zanthoxylum leprieurii bark of a mature plant (approximately 5 kg) was collected from Mabira forest in Buikwe district, Central Uganda. *Rubia cordifolia* was picked from Kagamba village in Ntungamo District, Southwestern Uganda. Both mature plant materials were collected with the help of a Botanist. They were air dried under the shed, powdered separately using a blender into fine powder. They were stored in dark containers, wrapped in black polythene bags until required for extraction.

2.2. Extraction of Plant Materials

2.2.1. Aqueous Extraction

Powdered plant materials (200 g) were heated in 800 mL of distilled water to boiling point before filtration by gravity using Whatman No. 1 filter paper [14]. The filtrate was then concentrated using a freezer at −80˚C and freeze-dried (lyophilized) (Freeze Dryer). The lyophilized material was then air-dried, measured and packed in glass amber bottles. They were labelled and stored at 4˚C for future use.

2.2.2. Methanol and Ethanol Extraction

The powdered plant materials were extracted using 80% methanol and ethanol [15] [16]. The powdered plant parts each weighing 200 g was extracted with 1000 mL of the respective extraction solvents by cold maceration at room temperature (24˚C) for 72 h with frequent agitation [14]. The filtrate was concentrated using a rotary vacuum evaporator including water bath set at a temperature of 45˚C. The concentrate was frozen at −80˚C and then lyophilized using Freeze Dryer. The extracts were stored as stated above.

2.3. Antibiotics and Chemicals

Rifampicin and Isoniazid (positive control drugs) (*Sigma Aldrich Chemie GmbH, Steinheim, Germany*) were obtained from MSF-Epicentre Mbarara Research

Centre, Uganda. Stock solutions were prepared using distilled water at a concentration of 8 µg/mL and 4 µg/mL, respectively. They were sterilized by filtration through 0.2 µM millipore filter membrane syringe. The stock solutions of the plant extracts were prepared in dimethyl sulfoxide (DMSO) at a concentration of 6 mg/mL. Working solution was prepared by a 2-fold dilution of the stock solutions in Middlebrook 7H9 broth.

2.4. Bacterial Strain and Identification

Strains: H37Rv (ATCC 27294): Susceptible to both rifampicin and isoniazid. TMC 331 (ATCC 35838): Rifampicin resistant and isoniazid susceptible (both used internationally as the standard laboratory *Mycobacterium tuberculosis* strains), 2 clinical isolates from study participants, one resistant to rifampicin (tested using GeneXpert) and susceptible to isoniazid (confirmed using Genotype MTBDR plus version 2.0 (HAIN Lifescience GmbH, Nehren, Germany)) and another one susceptible to both drugs. The isolates were retrieved from −80˚C, and allowed to thaw through −20˚C, 4˚C, room temperature and 37˚C before they were inoculated on Lowenstein-Jensen (LJ) slants. These were then inoculated on LJ media for 3 weeks to obtain fresh colonies, and also on blood agar (BA) for up to 48 h at 37˚C to check for possible contamination. The identification of the H37Rv and TMC 331 strains were confirmed using the routine methods; colonial appearance, Ziehl-Neelsen (ZN) on the LJ colonies, MPT64Ag rapid (SD Bioline, South Korea), and HAIN for DST (MTBDR-Plus Version-2.0, HAIN Lifescience GmbH, Nehren, Germany).

2.5. Preparation of the Inoculum

Fresh sub cultures of Mycobacterial strains were made on LJ medium for 3 weeks. The inoculum was prepared in Middle Brook 7H9 broth (Difco, Detroit, Mich.) supplemented with glycerol (0.2%) and 10% (v/v) OADC (oleic acid, albumin, dextrose, catalase; Difco) using fresh colonies, adjusted to 1.0 McFarland's standard (3×10^6 CFU/mL) using a densitometer [17]. The inoculum was further diluted 1:20 in 7H9 media for the test (diluting 1 mL of the suspended strain in 19 mL of 7H9 media). The MTB inoculum (100 µL) was used to inoculate each well except well 12 (sterility check). A drop of this inoculum was cultured on a BA plate for 48hours to check for possible contamination. The plates were very well covered, and incubated at 37˚C for 7-days.

2.6. Anti-Mycobacterial Activity Test and Minimum Inhibitory Concentration Determination

The antimycobacterial activity was determined using the Resazurin Microtiter Assay (REMA) [18] [19]. Briefly, 100 µL of 7H9 broth were dispensed in each well of a sterile 96-well microtiter plate except for the last row. To column 1, 100 µL of respective crude extracts or drugs were added (with concentrations ranging from 3000 µg/mL to 5.9 µg/mL in well 10). The contents in the well were

mixed by pipetting at least 6-times. Serial two-fold dilutions were prepared in the plate there by discarding 100 µL after column 10. One hundred microliters of the inoculum was added to each well, except well 12 (a sterile control). A growth (negative) control was included in column 11 (extracts/drug free medium with strain suspension). The plates were well covered and incubated at 37˚C for 7 days. The susceptibility test was done in 96 well microtiter plates using 0.02% v/v of resazurin dye as an indicator of cellular viability or growth inhibition. After 7 days of incubation, the plates were removed from the incubator and 25 uL of the resazurin dye solution added to all the wells. The plates were again covered with aluminum foil and incubated at 37˚C for 24 hours for color development. The extracts were considered active (have inhibitory activity) for the well of the plate with unchanged blue color and if the color of the reagent changed to pink, the extract was considered not active against MTB strain. The minimum inhibitory concentration (MIC) of the test compound was interpreted as the lowest concentration of the extract/drug that prevents a change in color of resazurin dye [20].

3. Results

In this study, we determined the *in vitro* antimycobacterial activity of aqueous, ethanolic and methanolic extracts of *Zanthoxylum leprieurii* (bark) and *Rubia cordifolia* (stem and leaves) against pan sensitive MTB H37Rv, Rifampicin resistant TMC 331 strain and two wild strains (one rifampicin resistant and another one rifampicin susceptible). The minimum inhibitory concentration (MIC) values of crude extracts are shown in **Table 1** below. The MICs of *Zanthoxylum leprieurii* aqueous, ethanolic and methanolic extracts were 750 µg/ml, 187.5 µg/mL and 187.5 µg/mL against pan sensitive MTB H37Rv, 375 µg/mL, 187.5 µg/mL and 187.5 µg/mL against Rifampicin resistant TMC 331, 187.5 µg/mL, 46.9 µg/mL and 187.5 µg/mL against wild MTB strain (rifampicin resistant) and 46.9 µg/mL, 93.8 µg/mL and 23.4 µg/mL against rifampicin sensitive MTB wild strain respectively. The MICs of *Rubia cordifolia* were 750 µg/mL for both aqueous and ethanolic extracts and 187.5 µg/mL for methanolic extracts against MTB H37Rv strain and 1500 µg/ml, 750 µg/ml and 187.4 µg/mL against MDR TMC 331 strain. Methanol extracts of both plants showed higher anti-mycobacterial activity against all the four strains compared to other solvents. Aqueous extracts on the contrary had the least activity against *M. tuberculosis* strains used in the study. Comparatively, *Z. leprieurii* extracts were more active against all the *M. tuberculosis* strains than *R. cordifolia* extracts. The MIC of all crude extracts for each plant exhibited significantly low antimycobacterial activity compared to the two standard anti-TB drugs used; rifampicin and isoniazid. However, rifampicin susceptible wild strain of *M. tuberculosis* was comparatively the most susceptible to all extracts of the study plants with MICs ranging from 23.4 - 187.5 µg/mL. Rifampicin as a positive control was inactive on both rifampicin resistant reference (TMC 331) and wild strains.

Table 1. Minimum Inhibitory Concentration values of crude plant extracts against MTB H37Rv, TMC 331, and two clinical wild strains.

Plant/Drug	Extract	MICs (μg/mL)			
		MTB H37Rv	TMC 331 strain	Wild strain (Rif. Resistant)	Wild strain (Rif. Sensitive)
Z. leprieurii	Aqueous	750	375	187.5	46.9
Z. leprieurii	Ethanolic	187.5	187.5	46.9	93.8
Z. leprieurii	Methanolic	187.5	187.5	187.5	23.4
R. cordifolia	Aqueous	750	1500	375	750
R. cordifolia	Ethanolic	750	750	187.5	187.5
R. cordifolia	Methanolic	187.5	187.5	187.5	46.9
Rifampicin		0.5	>4.0	>4.0	0.06
Isoniazid		0.25	0.13	0.13	0.03

4. Discussion

Tuberculosis has been a major public health problem for developing countries including Uganda. Due to emergence of MDR, XDR and recently, totally drug resistant (TDR) strains of *Mycobacterium tuberculosis,* there is an urgent need of finding newer, effective and safer anti-mycobacterial compounds to combat this global problem.

From literature survey carried out on medicinal plants used for treatment of tuberculosis in Uganda, two priority plants were selected. In the present study, aqueous, ethanolic and methanolic extracts of selected priority anti-tuberculosis medicinal plants in Uganda (*Zanthoxylum leprieurii* and *Rubia cordifolia*) [12], were found to have activity against drug susceptible reference strain MTB H37Rv, Rifampicin resistant reference (TMC 331) strain and two clinical strains (one rifampicin resistant and another one rifampicin susceptible). The rifampicin resistant strains were found to be resistant to rifampicin.

The results of the present study are in conformity with findings of previous studies done both locally and elsewhere in the world. Previous studies have reported anti-mycobacterial activity of *Zanthoxylum leprieurii* crude extracts and its active compounds [21] [22] [23] [24]. Bunalema *et al.*, in 2017 reported that methanolic extract of *Z. leprieurii* exhibited very significant activity (MIC of 47.5 μg/mL and 75.3 μg/mL) against *M. tuberculosis* H37Rv and rifampicin resistant strains respectively [21]. Tuyiringire and others reported MICs of 144 μg/mL and 45 μg/mL for 99% methanolic extracts of *Z. leprieurii* against H37Rv and Rifampicin resistant (TMC-331) strains respectively [23]. In a study by Oloya and others, MICs of 195 μg/mL and 293 μg/mL for methanolic extracts of *Z. leprieurii* were reported against H37Rv and MDR-TB strains respectively [24]. Methanolic extracts of *Rubia cordifolia* equally exhibited moderately high anti-mycobacterial activity against all *M. tuberculosis* strains used. Similar or higher activity was reported by Makgatho *et al.*, in a study carried out in South Africa to evaluate *R. cordifolia* anti-mycobacterial activity [25]. Crude 80% etha-

nolic and methanolic extracts of both plant species had their inhibitory concentrations ranging as low as 23 μg/mL to 750 μg/ml. This activity is not only comparable to studies that have studied similar plants under investigation but also other different medicinal plants with significant antimycobacterial activity [16] [25] [26] [27]. Methanol extracts demonstrated higher activity compared to other solvents. This could be as a result of several compounds that are extracted by 80% methanol, which is found to be an effective extraction solvent [21] [28]. This could be due to the presence of high levels of polar compounds in plants that are soluble in solvents with higher polarity like methanol and ethanol. The difference in antimycobacterial activities of the different extracts is an indication of the corresponding extraction yield. This implies that methanol resulted in a higher extraction yield followed by ethanol and then water. Thus the extraction efficiency with these two medicinal plants favors highly polar solvents [29]. The antimycobacterial activity of standard drugs was superior to that of the crude plant extracts because the latter contains a mixture of bioactive constituents of which some might antagonize the effects of others. Isolation of active molecules increases the activity of these compounds as reported for *Zanthoxylum leprieurii* [21], *Zanthoxylum capense* [30] and *R. cordifolia* [25].

5. Conclusion

This study demonstrates that stem bark of *Z. leprieurii* and *R. cordifolia* (stems and leaves) exhibit antimycobacterial activity against both susceptible and rifampicin resistant strains. The findings provided scientific justification for the use of *Rubia cordifolia* in the management of tuberculosis by Traditional Medicine Practitioners in Uganda. Even though the findings support traditional use of these medicinal plants, their interaction with standard anti-TB drugs should be ascertained since it is not uncommon to find TB patients combining both conventional and traditional medicines.

Author Contributions

Conceptualization, Moses Mpeirwe; Data curation, Moses Mpeirwe and Ivan Taremwa; Methodology, Moses Mpeirwe; Supervision, Patrick Orikiriza, Patrick Ogwang, Crispin Sesaazi and Joel Bazira; Writing—original draft, Moses Mpeirwe; Writing—review & editing, Moses Mpeirwe, Ivan Taremwa, Patrick Orikiriza, Patrick Ogwang and Joel Bazira.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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