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Combination Activity of Standard Antituberculosis Drugs and Extracts of Medicinal Plants Commonly Used in Traditional Treatment of Tuberculosis in Uganda

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Abstract

Introduction: Resistance to antituberculosis drugs and adverse drug reactions remain the leading causes of tuberculosis therapeutic failure globally. Despite the increasing acceptance of medicinal plant use in combination with conventional antituberculosis drugs in treatment of tuberculosis (TB) in Uganda, there is paucity of knowledge on their combination effect. **Aim:** This research aimed to determine combination activity of standard antituberculosis drugs with extracts of *Zanthoxylum leprieurii* Guill. & Perr. and *Rubia cordifolia* L., the two common antituberculosis medicinal plants in Uganda, against pansensitive (H37Rv) and multi-drug resistant (MDR) *Mycobacterium tuberculosis* strains. **Materials and Methods:** Two reference MTB strains (H37Rv and MDR strain) were inoculated on Middlebrook 7H11 medium containing a combination of standard antituberculosis drugs and methanol extracts of *Z. leprieurii* and *R. cordifolia* at varying concentrations. The number of colonies on the plates was observed and counted weekly for up to 8 weeks. *In vitro* combination activity was determined using proportion method. Mean percentage inhibition was calculated for the reduction of number of colonies on drug-extract combination medium in relation to drug-extract-free control medium. **Results:** Drug-extract combinations showed good combination activity against *Mycobacterium tuberculosis* strains when compared with individual standard anti-TB drugs. This was more exhibited against MDR strain. There was however a reduction in per-

centage inhibition when extracts were combined with ethambutol and streptomycin against H37Rv strain. **Conclusions:** *Zanthoxylum leprieurii* and *Rubia cordifolia* in combination with standard anti-TB drugs exhibited increased *in vitro* activity against *Mycobacterium tuberculosis,* especially MDR-TB strain. This justifies the local use of these plants in traditional treatment of tuberculosis especially in resistant cases in Uganda.

Keywords

Combination Activity, Medicinal Plants, *Zanthoxylum leprieurii, Rubia cordifolia*, Standard Antituberculosis Drugs

1. Introduction

Tuberculosis (TB) is a communicable disease that has caused ill health to humanity for many years. TB was the leading cause of death worldwide from a single infectious agent until 2020, when the coronavirus (COVID-19) pandemic emerged. According to the World Health Organization (WHO), approximately 10.6 million people fell ill with TB in 2021, which represents a 4.5% increase from 10.1 million in 2020. The disease also claimed 1.6 million lives globally, with the burden mainly confined within 30 countries, which account for over 86% of all cases [1].

Although TB treatment is effective, its duration is long, and anti-TB drugs can cause serious side effects. These factors, among others, confer poor adherence of patients to chemotherapy, and patients frequently abandon treatment. This, consequently, may result in secondary resistance to anti-TB drugs and eventual spread of drug-resistant bacilli. Ultimately, this leads to primary resistance (presence of resistant *Mycobacterium tuberculosis* in patients with no prior history of treatment) [2].

The emergence of multidrug-resistant (MDR) and extensively drug-resistant (XDR) TB continues to be a public health threat. In 2021, the World Health Organization estimated that the proportion of people with MDR-TB to be 3.6% and 18% among new and previously treated cases respectively. In the same year, 25,038 new cases of XDR were detected globally [1]. The standard short-course treatment of TB requires a combination of anti-TB drugs with a duration of six months. This regimen has been found to give an 85% - 90% success rate in 100% complaint TB patients. Patients with MDR-TB are treated with a regimen of four to five anti-TB drugs for a period of greater than 15 months after a negative culture is obtained [3]. This long duration, accompanied by frequent serious adverse effects, leads to poor compliance and cessation of treatment [4] [5].

Medicinal plants have for centuries been used as a source of medicine and in Uganda, traditional medicine use is found to be between 60% - 80% [6] [7]. Many plant species have broadly been studied for their activity against *Myco-*

bacterium tuberculosis. Recently, a number of studies have reported that native Ugandan plants used traditionally for respiratory diseases and other ailments have antimycobacterial activity [8]-[12]. It has been reported that quite a number of people in Africa use both medicinal plants and conventional medications concurrently without any knowledge of possible interactions [13]. The deficiency of knowledge of interactions between medicinal plants and conventional drugs and as well as the lack of reporting of traditional medicine use to healthcare practitioners can pose a serious risk to patient safety and treatment outcome [14] [15]. It has also been noted that even in some of the world-class hospitals in Africa, traditional medicine is found to be used concurrently with conventional therapies [16].

Bunalema and colleagues reported that medicinal plants, *Zanthoxylum leprieurii* Guill. & Perr. and *Rubia cordifolia* L. have been extensively used by traditional medicine practitioners (TMPs) for the treatment of tuberculosis either singly or in combination with standard anti-tuberculosis drugs [8]. This study therefore aimed to investigate the *in vitro* activity of extracts of *Zanthoxylum leprieurii* and *Rubia cordifolia* in combination with standard anti-TB drugs against two MTB strains (pansensitive H37Rv and MDR-TB strain).

2. Materials and Methods

2.1. Plant Collection

Zanthoxylum leprieurii stem bark of a mature plant was collected from Mabira forest, Najjembe Division, Lugazi Municipality, Buikwe district, Central Uganda (Latitude: 0.404703, Longitude: 33.006252). *Rubia cordifolia* was picked from Kagamba village in Ntungamo District, southwestern Uganda (Latitude: -0.81052, Longitude: 30.16245). Both plants were collected with the help of a botanist. A voucher specimen for *Z. leprieurii* was deposited in the herbarium of Makerere University, Kampala, Uganda with accession number 50,925. Permission to collect plant parts from Mabira forest was obtained from National Forest Authority (NFA), Kampala, Uganda. Collected plant parts were air dried under the shed for approximately 2 weeks and powdered separately using a blender into fine powder. They were stored in dark containers and wrapped in black polythene bags until required for extraction.

2.2. Extraction

The powdered plant materials were extracted using 80% methanol [17] [18]. The powdered plant parts, each weighing 200 g, were extracted with 1000 mL of the extraction solvent by cold maceration at room temperature (24°C) for 72 h with frequent agitation [19]. The filtrate was concentrated using a rotary vacuum evaporator including a water bath set at a temperature of 45°C. The concentrate was frozen at −80˚C and then lyophilized using a freeze dryer. The lyophilized material was then air-dried, measured and packed in glass amber bottles. They were labeled and stored at 4°C for future use.

2.3. Mycobacterium Strains

Two standard strains of *Mycobacterium tuberculosis* were used: the reference pan-sensitive (ATCC 27294/H37RV) and a rifampicin-resistant ATCC 35838/TMC-331) strain. These strains were provided by the Mycobacteriology BSL-3 Laboratory, Department of Microbiology, College of Health Sciences, Makerere University, Uganda. They were subcultured on Middlebrook 7H11 agar for 2 weeks. Freshly growing colonies were carefully scraped from the surfaces of the media. The colonies were suspended in 10 mL of normal saline. The turbidity of the suspension was adjusted to that of a McFarland standard number 1 by either adding more cells to the suspension or diluting with normal saline.

2.4. Reagents and Antibiotics

Rifampicin (RMP), Isoniazid (INH), Ethambutol (EMB) and Streptomycin (STR) were used as standard antibiotics. A stock concentration of 100 µg/ml of each drug was prepared using sterile distilled water except for rifampicin. In addition, Dimethyl sulfoxide (DMSO) at a concentration of 0.5% v/v was used as a solvent to prepare both rifampicin and 10,000 µg/ml stock concentration of test plant extracts [20].

2.5. Preliminary Study

The stock methanol extracts were diluted using DMSO and incorporated into Middlebrook 7H11 medium (Difco Laboratories, Detroit, MI, USA) supplemented with 10% oleic acid, albumin, dextrose and catalase (OADC) (Becton Dickinson) to attain concentrations of 50, 100, 500 and 1000 µg/mL. The results from this preliminary study were used to establish the extract concentrations to be combined with standard antituberculosis drugs. Extract-free medium with MTB was used as a positive control, and extract-free medium without MTB was used as a negative control for the assay. The previously prepared *Mycobacterium tuberculosis* strains (1 MacFarland standard) were inoculated onto each plate except the negative control. All plates were incubated at 37°C for 8 weeks. The number of colonies was enumerated weekly.

2.6. Drug-Extract Combination Assay

The *in vitro* antimycobacterial combination activity of *Zanthoxylum leprieurii* and *Rubia cordifolia* methanol extracts with RMP, INH, EMB and STR were determined by Agar proportion method with slight modification using 7H11 medium as described by Prabasiwi Nur Fauziyah, 2017 [21]. The effect of the combination was determined using proportianl method by calculating the mean percentage inhibition reduction in a number of colonies on drug-extract medium compared to extract-free control medium. The standard extract concentrations used in this assay were 500, 100 and 50 µg/mL for both plant extracts. The standard antituberculosis drug concentrations used in combinations were 1

μg/mL, 0.2 μg/mL, 7.5 μg/mL and 2 μg/mL for rifampicin, isoniazid, ethambutol and streptomycin, respectively [22]. Two series of stock solutions were prepared by diluting each extract and standard drug in DMSO and sterile water, respectively, except for the *Z. leprieurii* extract, which was diluted in DMSO. The concentrations of the stock solutions used were 10,000 μg/mL for extracts and 100 μg/mL for standard drugs. To make a 1:500 combination of rifampicin and the extracts, 1.25 mL of 10,000 μg/mL extract stock solution and 0.25 mL of 100 μg/mL rifampicin stock solution were added to 23.5 mL of sterile Middlebrook 7H11 medium and shaken gently until mixed homogenously. The same procedure was repeated for the rest of the combinations as summarized in **Table 1** below. The entire 25 mL of the extract-drug-medium solution was poured onto a 90 mm petri dish and allowed to cool at room temperature.

A total of 100 μL of MTB strains (1 McFarland standard) was inoculated onto each of the petri dishes containing extract-drug-medium at varying concentrations, except for the negative controls (uninoculated medium with no extract and no drug). This activity assay was done in duplicate to increase data reliability. After inoculation, the petri dishes were sealed using parafilm and incubated at 37°C. Positive controls (media with no extracts and no drugs) were also inoculated and incubated in the same conditions as above. The number of colonies was observed and counted weekly for up to 8 weeks.

3. Results

The study was performed as an antimycobacterial assay. Data collected were

Table 1. Summary of the preparation of the combination of *Zanthoxylum leprieurii* and *Rubia cordifolia* extracts with standard antituberculosis drugs.

Standard Drug	Standard drug stock concentration (μg/mL)	Drug stock volume (mL)	Extract stock concentration (μg/mL)	Extract stock volume (mL)	Media volume (mL)	Total volume (mL)	Final drug-Extract concentration
Rifampicin	100	0.25	10,000	1.25	23.5	25	1:500
Rifampicin	100	0.25	10,000	0.25	24.5	25	1:100
Rifampicin	100	0.25	10,000	0.125	24.625	25	1:50
Isoniazid	100	0.05	10,000	1.25	23.7	25	0.2:500
Isoniazid	100	0.05	10,000	0.25	24.7	25	0.2:100
Isoniazid	100	0.05	10,000	0.125	24.825	25	0.2:50
Ethambutol	100	1.875	10,000	1.25	21.875	25	7.5:500
Ethambutol	100	1.875	10,000	0.25	22.875	25	7.5:100
Ethambutol	100	1.875	10,000	0.125	23.0	25	7.5:50
Streptomycin	100	0.5	10,000	1.25	23.25	25	2:500
Streptomycin	100	0.5	10,000	0.25	24.25	25	2:100
Streptomycin	100	0.5	10,000	0.125	24.375	25	2:50

MTB colony counts on 7H11 media for 8 weeks. The collected data were then processed using the proportion method, which resulted in a percentage inhibition as shown in Table 2 for the preliminary study and Table 3 for the extract-drug combination assay.

Starting with the preliminary study (individual extract antimycobacterial activity), the methanol extract of *Zanthoxylum leprieurii* exhibited 100% growth inhibition against both H37Rv and MDR *Mycobacterium tuberculosis* strains at concentration of 100 µg/mL and above. Both extracts showed preferentially high inhibition rates against MDR strain. A concentration of 500 µg/mL was used as the highest concentration for both *Z. leprieurii* and *R. cordifolia* extracts for the combination study because it gave 100% growth inhibition for both MTB strains. The results indicated a dose-response relationship, thus justifying the lowering of extract concentrations to 50 µg/mL in the combination assay.

The results indicated an additive effect when rifampicin was combined with both plant extracts against the H37Rv strain. Specifically, both plant extracts exhibited complete (100%) inhibition at a lower concentration of 50 µg/mL against the pansensitive MTB strain when combined with rifampicin. An increased potency for the combination of ethambutol with the extracts against the MDR-TB strain was observed. In contrast, the results indicated a decrease in potency for the combination of rifampicin and isoniazid against MDR-TB isolates. Noticeably, *Z. leprieurii* extract alone exhibited 100% growth inhibition at 50 µg/mL, while its combination with isoniazid at a concentration of 100 µg/mL yielded 94% and 95% inhibition against H37Rv and MDR-TB, respectively. There was a remarkable increase in the percentage inhibition of extract-drug combinations against MDR-TB compared to individual single-standard anti-tuberculosis

Table 2. Percentage inhibition of different plant extract concentrations against pansensitive MTB (H37Rv) and multidrug-resistant (MDR) *Mycobacterium tuberculosis*.

		***Mycobacterium tuberculosis* strains**			
Botanical Name	**Concentration (µg/mL)**	**H37Rv**		**MDR**	
		Mean CFU*	**Mean % Inhibition**	**Mean CFU***	**Mean % Inhibition**
Control (-)	-	0		0	
Control (+)	-	480	-	17	-
Zanthoxylum leprieurii Guill. & Perr.	50	91	81	02	88
	100	0	100	0	100
	500	0	100	0	100
	1000	0	100	0	100
Rubia cordifolia L.	50	45	91	6	65
	100	105	78	0	100
	500	0	100	0	100
	1000	0	100	0	100

**Colony Forming Unit.*

Table 3. Percentage inhibition for different combinations of standard antituberculosis drugs and extracts against H37Rv and MDR-TB strains.

Standard Drugs	Methanol Extract	concentration in combination (µg/mL)	H37Rv		MDR	
			Mean CFU	Mean % Inhibition	Mean CFU	Mean % Inhibition
Control (+)	**-**	**-**	**282**	**-**	**152**	-
	-	-	0	100	107	29
	Z. leprieurii	1:500	0	100	0	100
	Z. leprieurii	1:100	0	100	0	100
Rifampicin	*Z. leprieurii*	1:50	0	100	22	86
	R. cordifolia	1:500	0	100	0	100
	R. cordifolia	1:100	0	100	17	89
	R. cordifolia	1:50	0	100	57	63
Control (+)	**-**	**-**	**776**	**-**	**516**	**-**
	-	-	118	85	491	5
	Z. leprieurii	0.2:500	0	100	0	100
	Z. leprieurii	0.2:100	48	94	26	95
Isoniazid	*Z. leprieurii*	0.2:50	174	76	46.5	91
	R. cordifolia	0.2:500	0	100	0	100
	R. cordifolia	0.2:100	4.5	99	40	92
	R. cordifolia	0.2:50	2	99	194	62
Control (+)	**-**	**-**	**273**	**-**	**157**	**-**
	-	-	0	100	98	38
	Z. leprieurii	7.5:500	0	100	0	100
	Z. leprieurii	7.5:100	0	100	0	100
Ethambutol	*Z. leprieurii*	7.5:50	13.5	95	0	100
	R. cordifolia	7.5:500	4	99	0	100
	R. cordifolia	7.5:100	20	93	0	100
	R. cordifolia	7.5:50	57	79	0	100
	-	-	0	100	76	52
	Z. leprieurii	2:500	0	100	0	100
	Z. leprieurii	2:100	1	99	0	100
Streptomycin	*Z. leprieurii*	2:50	10	96	3	99
	R. cordifolia	2:500	0	100	0	100
	R. cordifolia	2:100	0	100	0	100
	R. cordifolia	2:50	3.5	99	0	100

drugs. Individual standard anti-TB drugs showed a growth inhibition ranging from as low as 5% to 52% against MDR-MTB strain, whereas on combination with both extracts, ethambutol and streptomycin exhibited complete inhibition (100%) against the same strain.

4. Discussion

Tuberculosis treatment is intrinsically associated with major challenges, such as complex and lengthy treatment. These have a direct impact on patient compliance and treatment outcome. Recently, there has been another concern regarding the increasing incidence of MDR and XDR tuberculosis [21]. As a result of the growing burden of drug-resistant genotypes of *Mycobacterium tuberculosis* coupled with TB-HIV coinfection, the burden of TB is now quite difficult to manage. Therefore, there is a global imperative to discover and develop new and efficacious antituberculosis agents, which should be an urgent biomedical priority [23] [24]. The plants selected in the current study are commonly used in Ugandan traditional medicine to treat tuberculosis and related ailments and have also been studied for their antimycobacterial activity against different strains of *Mycobacterium tuberculosis* [4] [12] [25]. However, little is known about the effects of combination of medicinal plants and conventional antituberculosis drugs.

This study is the first to report on the combination effect of *Zanthoxylum leprieurii* and *Rubia cordifolia* with standard antituberculosis drugs in Uganda. Previous studies have only focused on the antimycobacterial activities of these plants. It has been demonstrated that when used in combination, drugs with significantly low efficacy have therapeutic effects, even against drug-resistant strains [3]. Drug combination treatment has long been employed as a standard treatment strategy for many chronic diseases, including tuberculosis. With an objective to elucidate any potential synergistic interactions of the two commonly used medicinal plant extracts with standard first-line anti-TB drugs, we evaluated the antimycobacterial activity (percentage inhibition) of *Z. leprieurii* and *R. cordifolia* methanol extracts in combination with rifampicin, isoniazid, ethambutol and streptomycin against *M. tuberculosis* (H37Rv and MDR) strains.

Rifampicin alone exhibited 100% and 29% growth inhibition at 1 μg/mL against pansensitive H37Rv and MDR strains, respectively. On combination with *Z. leprieurii* and *R. cordifolia* extracts at a concentration of 50 μg/mL, an increase in growth inhibition was observed at 86% and 63% respectively against MDR-MTB. When each of the extracts at a concentration of 50% μg/mL was combined with isoniazid at 0.2 μg/mL, an additive effect was observed against the MDR-strain, resulting in 91% and 62% inhibition by *Z. leprieurii* and *R. cordifolia* against the MDR strain, respectively. The mechanism contributing to this synergistic effect against MDR strains is not yet known; however, there is a likelihood that since isoniazid inhibits mycolic acid synthesis, these plant extracts

inhibit cell wall synthesis [3] [26].

All anti-TB drugs used in this study, rifampicin at 1 μg/mL, isoniazid at 0.2 μg/mL, ethambutol at 7.5 μg/mL and streptomycin at 2 μg/mL, exhibited a growth inhibition of not more than 52% against the MDR strain. However, a synergistic effect was observed when the standard drugs were combined with both extracts at even lower concentrations. They exhibited mycobacterial growth inhibition of between 62% and 100% against the MDR strain. This finding was consistent with other standard anti-TB drugs studied against MDR strains. This is in tandem with other previous studies that indicated that extracts of *Z. leprieurii* and *R. cordifolia* inhibited the growth of the MDR strain of *Mycobacterium tuberculosis* [4] [25] [27].

This study demonstrated that extracts of *Z. leprieurii* and *R. cordifolia* possess significant *in vitro* antimycobacterial activities against both pansensitive (H37Rv) and multidrug-resistant (MDR) MTB strains. Additionally, the extracts exhibited additive and/or synergistic interactions when combined with standard anti-tuberculosis drugs, more remarkably against MDR-MTB. This implies that pure compounds of these plants have the capability of reducing the doses and duration of treatment when combined with conventional anti-TB drugs. This may result in a multitude of advantages, ranging from preventing the emergence of resistance, increased compliancy and general reduction in global tuberculosis burden.

5. Conclusion

This study demonstrated that *Zanthoxylum leprieurii* Guill. & Perr. and *Rubia cordifolia* L. extracts increase inhibitory activity of standard anti-tuberculosis drugs, especially against MDR-TB strain. This study verified local use of these plants in the traditional treatment of tuberculosis in Uganda. However, the use of these medicinal plants as traditional anti-tuberculosis remedies in combination with standard anti-TB drugs should be done cautiously, especially on individuals with susceptible tuberculosis, since not all extract-drug combinations showed good *in vitro* combination effects. These plants' cytotoxicity and intracellular activities should be determined to further justify their traditional use either singly or in combination with conventional anti-tuberculosis drugs.

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Authors' Contributions

MM developed the concept and proposal; MM and KK collected, sorted the data;

MM analyzed the data and wrote the manuscript; JB, DS, PO supervised the study and reviewed the final manuscript. All authors reviewed and approved the final manuscript.

Ethics Approval and Consent to Participate

Approval was sought from Institutional Review Board of Mbarara University of Science and Technology. Permission to collect plant parts from Mabira forest was obtained from National Forest Authority (NFA), Kampala, Uganda.

List of Abbreviations

MDR-TB	Multidrug-Resistant Tuberculosis
MTB	*Mycobacterium tuberculosis*
TB	Tuberculosis
TMPs	Traditional Medicine Practitioners
WHO	World Health Organization
XDR-TB	Extensively Drug-Resistant Tuberculosis

Conflicts of Interest

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

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