EVALUATION OF ANTIMYCOBACTERIAL ACTIVITY OF *Zanthoxylum leprieurii* Guill. & Perr. AND *Rubia cordifolia* L. AND THEIR INTERACTION WITH CONVENTIONAL ANTI-TUBERCULOSIS DRUGS USED FOR THE TREATMENT OF TUBERCULOSIS IN UGANDA

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SEPTEMBER, 2024

DECLARATION

I, Moses Mpeirwe, hereby declare that this thesis is due to my own knowledge, effort and it has never been submitted by any other researcher or a student for the award of a degree in any university or institution of higher learning.

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This thesis has been submitted with my approval as the student's supervisor.

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DEDICATION

This work is dedicated to my late father,

Mr. Fred Kashaija (RIP)

Who was a constant, though short-lived, inspiration throughout my early days of childhood.

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"To God be the Glory"

LIST OF ABBREVIATIONS

ARVS	Antiretroviral Therapy
CCM	Complete Culture Media
DMSO	Dimethyl sulfoxide
DOT	Directly Observed Treatment
HIV	Human Immunodeficiency Virus
INH	Isoniazid
LJ	Lowenstein-Jensen
MDR-TB	Multi-Drug Resistant Tuberculosis
MIC	Minimum Inhibitory Concentration
MTB	*Mycobacterium tuberculosis*
MTT	Microculture Tetrazolium
OADC	Oleic acid Albumin Dextrose Catalase
PZA	Pyrazinamide
REMA	Resazurin Microtiter Assay
RIF	Rifampicin
TB	Tuberculosis
TMP	Traditional Medicine Practitioner
WHO	World Health Organization
XDR-TB	Extensive Drug Resistant Tuberculosis

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ABSTRACT

Background: 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 emergence of multidrug resistant (MDR) and extensively drug resistant (XDR) strains of *Mycobacterium tuberculosis* (MTB). Medicinal plants have for centuries been traditionally used in treatment of tuberculosis and similar ailments globally. They possess antimicrobial properties which render them a new hope as a source of novel bioactive leads in the development of antimycobacterial agents. Additionally, 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.

Methods: Two plant species commonly used traditionally for the treatment of tuberculosis in Uganda, *Zanthoxylum leprieurii* Guill. & Perr. and *Rubia cordifolia* L. 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). We also determined the combination activity of both medicinal plant extracts at varying concentrations and standard antituberculosis drugs against H37Rv and MDR (TMC 331) strains. The cytotoxic effect of the active plant extracts was determined against the mouse BALB/C monocyte-macrophage (J774.2) cells using Microculture Tetrazolium (MTT) assay.

Results: Both plant extracts exhibited significant *in vitro* antimycobacterial activity against all MTB strains. Minimum inhibitory concentrations (MIC) of methanolic crude extracts of both plants ranged from 23.4μg/mL to 187.5μg/mL. Comparatively, methanol extracts of both plants possessed superior antimycobacterial activity against all the strains. Drug-extract combinations showed good combination activity against *Mycobacterium tuberculosis* strains when compared with individual standard anti-TB drugs especially against MDR-MTB strain. All extracts exhibited moderate to weak cytotoxicity against J774.2 mouse macrophage monocyte cell line in a dose dependent manner.

Conclusions: Our findings indicated that both plants exhibited activity against susceptible and resistant 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. *Zanthoxylum leprieurii* and *Rubia cordifolia* in combination with standard anti-TB drugs exhibited increased *in vitro* activity against *Mycobacterium tuberculosis* especially MDR-MTB strain. This verifies the local use of these plants in traditional treatment of tuberculosis in Uganda.

CHAPTER 1: INTRODUCTION

1.1 Background

Tuberculosis (TB) remains a major global health problem. It is caused by *Mycobacterium tuberculosis*. TB is ranked the ninth leading cause of death worldwide and the leading cause from infectious agents, ranking above HIV/AIDS. In 2022, 10.6 million people acquired tuberculosis and about 1.3 million people died from it globally(WHO, 2023). Of the new TB cases estimated globally, Africa accounted for 25%. Uganda had 94,000 new TB cases and 1,900 deaths and is one of the top 30 high burden TB countries. Emergence of Multi-Drug Resistant (MDR) (762 cases in 2022 in Uganda), Extensively Drug Resistant (XDR) (1 laboratory confirmed case) and lately totally drug resistant (TDR) strains together with HIV co-infection has amplified the problem of mycobacterial disease management(Daletos et al., 2015, WHO, 2023).

Tuberculosis treatment is mainly by chemotherapy involving combination of a complex regimen of at least four drugs for a period of 6 months in drug susceptible patients. The period is usually increased up to 24 months in drug resistant tuberculosis (DR-TB) patients depending on the clinical outcome. 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(Orodho et al., 2011, Wright et al., 2006). 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(Camp et al., 2006).

Medicinal plants have traditionally been used to treat tuberculosis and may offer a new hope as source of bioactive molecules for developing alternative medicines for the mycobacterial diseases(Singh et al., 2015, Gupta et al., 2010b). The World Health Organization (WHO) has stated that 80% of the world's population depends on traditional medicine (TM) for primary health care(Hiremath and Taranath, 2013, Gupta et al., 2017). Additionally, it has been reported that 170 of the 194 WHO Member States use traditional and complementary medicine (T&CM)(WHO, 2019). Combining the traditional knowledge with modern science may provide an innovative and valuable tool for affordable, safe, novel and effective therapies(Patwardhan, 2005). Natural products have played and continue to play an important role in the drug discovery process. For a long period of human existence, Natural products were the only therapeutic option available for use by sick people or to maintain a healthy state. Statistics from studies carried out by different researchers continue to bring to light the potentials and untapped reservoir of molecules with therapeutic effects (Cragg and Newman, 2005). As a result, a number of drugs like chloroquine, morphine and artemisinin derivatives have been derived from different plant sources (Chen et al., 2006).

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% (Logiel et al., 2021, WHO, 2002). Many plant species have broadly been studied for their activity against *Mycobacterium tuberculosis*. Recently, a number of studies have reported that native Ugandan plants used traditionally for respiratory diseases and other ailments have antimycobacterial activity

(Bunalema et al., 2014, Tabuti et al., 2010, Obakiro et al., 2020, Oloya et al., 2022, Tuyiringire et al., 2020). 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 (Van Wyk et al., 1997). 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 (Vieira and Huang, 2012, Butterweck and Derendorf, 2012). 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 (Hübsch et al., 2014).

Traditionally, plant-based medicines have been extensively used in different parts of the world for treating mycobacterial diseases. In recent past, there has been enormous efforts to rediscover the traditional understanding of plant-based remedies used to treat mycobacterial diseases with plant-based medicines in different parts of the world including Uganda. Surveys have been undertaken in the region to explore this traditional knowledge(Bunalema et al., 2014, Orodho et al., 2011). In a questionnaire-based interview of 40 Traditional Medicine Practitioners (TMPs) of Mpigi and Butambala districts of Uganda, Bunalema and colleagues reported 90 plant species used to treat tuberculosis traditionally. Out of 90 plant species, 2 priority plants were frequently mentioned by TMPs; *Zanthoxylum leprieurii* and *Rubia cordifolia* (Bunalema et al., 2014). We therefore aimed at evaluating the antimycobacterial activity of *Zanthoxylum leprieurii* Guill. & Perr. and *Rubia cordifolia* L. and their combination effects with standard anti-tuberculosis drugs.

1.2 Problem statement

The rapid emergence of MDR-TB, XDR-TB and of late total drug resistant (TDR) Mycobacterium tuberculosis strains(Pandya et al., 2012, Streicher et al., 2012), compels an urgent need to find new TB treatment agents derived from new medicinal leads. As a result of this together with the adverse effects of currently available anti-TB drugs, intensive efforts have to be put in place to search for new and effective therapeutic agents of tuberculosis. Some of the notable adverse effects with most MDR-TB and XDR-TB drugs include hepatotoxicity with ethionamide, ototoxicity and nephrotoxicity with aminoglycosides, and dysglycemia with gatifloxacin(Shehzad et al., 2013).

It is estimated that up to 80% of the population in Africa, Asia and Latin America depend mainly on Traditional medicine (TM) for their healthcare needs, involving mainly the use of plant extracts (Organization, 2003). Local communities in Uganda have for centuries used medicinal plants and continue to use them for the treatment of tuberculosis. It is also not uncommon to find conventional anti-TB drugs being used in combination with medicinal herbs for the treatment of TB, despite that, the interaction between the two has not been ascertained. Despite the increasing acceptance of traditional medicine use in our communities, the indigenous knowledge on traditional remedies is not sufficiently documented. The slow pace in anti-mycobacterial drug discovery in addition to the rapid emergence and spread of resistant *Mycobacterium tuberculosis* strains calls for urgent and continuous search for new agents that may arrest this scourge. To this end, the search for new effective and affordable antimycobacterial drugs motivated us to study

antimycobacterial activity of the commonly used medicinal plants in the traditional treatment of tuberculosis in Uganda.

1.3 Study Justification

Medicinal plants have long been affordable option being used for treatment of tuberculosis in local communities in Uganda. The two medicinal plants to be studied are widely used by TMPs especially in the central districts of Uganda (Bunalema et al., 2014). An immense number of medicinal plants have been reported in literature to be having antimycobacterial activity. In our quest for natural and effective antimycobacterial agents, two medicinal plants commonly used locally were selected to be validated for their antimycobacterial activities against MTB strains. Since the current treatment regimen is prescribed for quite a long time (at least 6 months), which negatively affects adherence and compliance, the current study proposes to acquire an understanding on the effects of combining conventional anti-TB drugs with plant extracts, which may result into shortened treatment duration.

1.4 Research questions

What is the anti-mycobacterial activity of commonly used indigenous plants by TMPs in Uganda?

What is the effect of the combination of standard anti-TB drugs and plant extracts?

What are the cytotoxic effects of the active extracts?

1.5 Research objectives

1.5.1 General objective

The aim of the study was to evaluate the anti-mycobacterial activity of *Zanthoxylum leprieurii* Guill. & Perr. and *Rubia cordifolia* L. and their interaction with conventional anti-tuberculosis drugs used in the treatment of TB in Uganda.

1.5.2 Specific objectives

1. Determined *In Vitro* activity of anti-mycobacterial plant extracts using Resazurin Microtiter Assay (REMA).
2. Determined the combination activity of conventional anti-TB drugs and extracts of *Zanthoxylum leprieurii* and *Rubia cordifolia*.
3. Determined the cytotoxic effect of the active plant extracts against mouse BALB/C monocyte-macrophage (J774.2) cell line using the MTT assay.

CHAPTER 2: LITERATURE REVIEW

2.1 Background

Tuberculosis (TB) remains one of the most important global health problems. It is more prevalent today than any other time in human history. *Mycobacterium tuberculosis* (MTB), the microorganism that causes TB, uses diverse strategies to live in a variety of lesions and evade immune responses (Koul et al., 2011). It causes ill health among millions of people each year and is responsible for substantial deaths worldwide. TB predominates in Asia and Africa with over 80% cases. Although the greatest numbers of TB patients reside in densely populated countries of Asia, its highest incidence is found in the WHO region of Africa (Zager and McNerney, 2008)

Emergence of increased multi-drug resistant (MDR) and extensively drug-resistant (XDR) strains has further exacerbated TB burden. As a result, diverse research groups worldwide are directing their efforts on finding novel anti-tuberculosis agents that provide greater effectiveness, less toxicity and possibly being co-adjuvants in the conventional TB treatment regimens.

The ability of *Mycobacterium tuberculosis* to remain dormant or viable within the host cells for many years with the potential to be reactivated allows it to escape the immune system of the host (Meena and Rajni, 2010). An individual infected with MTB has a 5-10% risk of developing active TB disease. The TB latent infection is one of the greatest hindrances in the cure and prevention of TB. Reactivation of latent TB is prerequisite for disease development mainly in the immunocompromised individuals notably those co-infected with HIV or with diabetes (Koul et al., 2011).

A proportion of Ugandans use herbal-based medicines as their treatment for a variety of infections. Among the plants used locally are those with anti-tuberculosis properties. However, there is still a huge scientific knowledge gap on the effectiveness of these herbal remedies used locally.

2.2 Epidemiology of Tuberculosis

Tuberculosis is ranked the ninth leading cause of death worldwide and the leading cause from infectious agents, ranking above HIV/AIDS. In 2022, 10.6 million people acquired tuberculosis and about 1.3 million people died from it worldwide; therefore, the disease remains a global scourge as shown in figure 1 below. Of the new TB cases estimated globally, Africa accounted for 25%. Uganda had 94,000 new TB cases and 1,900 deaths and is one of the high TB/HIV burden countries(WHO, 2023). Most of the persons with clinically active tuberculosis (>95%) and nearly all of those who die from it reside in developing countries (Bhargava and Bhargava, 2020). Drug resistant TB still imposes a continuous threat to the management of tuberculosis, with 176,600 cases of MDR-TB emerging in 2022.

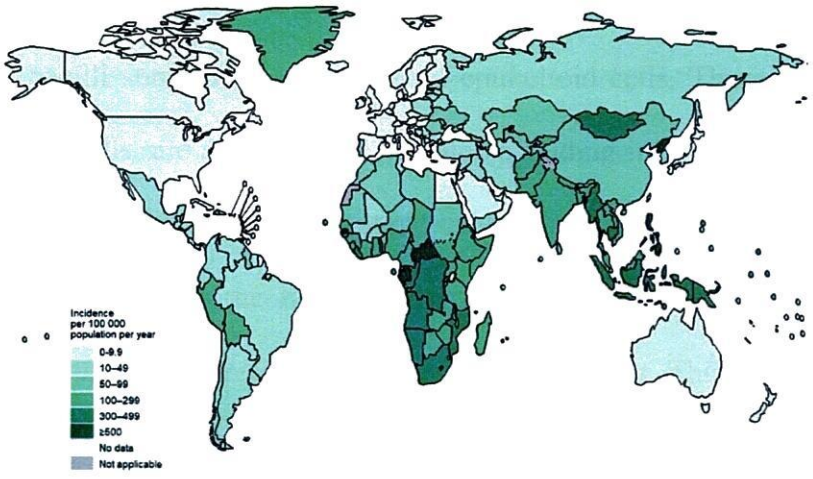


Figure 1. Estimated TB incidence rates, 2022(WHO, 2023).

Most TB cases and deaths largely occur among men; however, TB remains one of the top three killers of women worldwide. There were an estimated 378,000 TB deaths among HIV-negative women in 2016, with additional 115,000 among HIV-positive women. HIV/AIDS exposes an individual 50-100 times to develop active TB, and 50-60% of persons with latent TB and HIV will develop the active form of the disease (Jiménez-Arellanes et al., 2014). In the developing countries, TB is the leading cause of death in persons living with HIV (Date and Modi, 2015). In 2016, there were an estimated 374,000 deaths among HIV-positive people (Gatechompol et al., 2019).

2.3 Pathogenesis of Tuberculosis

Infection is established when a person inhales droplet nuclei containing tubercle bacilli which reaches the alveoli of the lungs. Most of these bacilli are inhibited, though some may multiply intracellularly and will be released when macrophages die (Weiss and Schaible, 2015). The live mycobacteria are phagocytosed by alveolar macrophages in the lungs and induce a localized proinflammatory response, which leads to recruitment of mononuclear cells from neighboring blood vessels. These cells are the building blocks for the granuloma, which is the hallmark of the disease. The granuloma consists of a central area of giant cells containing tubercle bacilli surrounded by a zone of epithelioid cells. These giant cells, called Langhans' giant cells, are an important pathologic finding in tuberculous lesions. This tissue response then enters the containment phase of the infection, during which there are no signs of the disease and the host does not transmit the infection to others. Containment fails after there is suppression of immunity of the host. This comes evident especially in situations of malnutrition, old age or underlying infections like HIV or

diabetes. Under such circumstances, the center of the granuloma undergoes caseation and spills viable, infectious bacilli into the air pathways. This results to the development of a productive cough, which facilitates the aerosol spread of infectious bacilli (Hussain, 2007).

2.4 Immunology of Tuberculosis

The macrophages, after the initial entry of M. tuberculosis, release inflammatory cytokines and chemokines when then serve as a signal for infection. Both mononuclear and polynuclear cells (specifically monocytes and neutrophils) migrate to the focal site of the infection. However, they are not able to destroy the bacteria. Chen and colleagues state that the bacilli resist the bactericidal mechanisms of the macrophage by penetrating phagosome-lysosome fusion. They multiply in the phagosome and cause macrophage death (Chen et al., 2006).

The released bacilli multiply extracellularly and are phagocytosed by other macrophages, which are incapable of controlling the growth of *M. tuberculosis*. Dendritic cells with engulfed bacilli mature migrate to the regional lymph nodes and induce $CD4^+$ and $CD8^+$ cells against mycobacterial antigens (Bodnar et al., 2001). The specific immune responses mediate the production of prime T cells, which travel back to the focus of the infection, guided by the infected macrophages and other cells. The collective accumulation of macrophages, T cells, dendritic cells, fibroblasts, endothelial cells and stromal cells results into the formation of a granuloma at the site of infection (Gonzalez-Juarrero et al., 2001). Tubercle bacilli are walled off from the rest of the lung tissue as a result of granuloma formation. This helps in limiting bacterial spread and provides microenvironment for interactions among macrophages and other cells of the host's immune system and the

cytokines produced by these cells. The CD+ cells recognize infected macrophages presenting antigens from MTB and kill them (Wolf et al., 2008). However much the infection progression is stopped, some resistant bacilli capable of surviving the depriving conditions generated by the host escape death. These bacilli enter a state of dormancy/latency and persist by avoiding elimination by the immune system (Hingley-Wilson et al., 2003).

2.5 TB and HIV Co-infection

World Health Organization (2017) stated that there was a high incidence of TB among HIV infected individuals. People with HIV infection are at an increased risk of rapid progression of a recently acquired infection, as well as of re-activation of latent infection compared to HIV negative individuals. Raja (2004) affirmed that TB is an opportunistic infection occurring among HIV-positive people globally(Raja, 2004).

TB and HIV infections are both intracellular and known to have a profound effect on the progression of each other. HIV-1 is now globally recognized as the leading risk factor for TB (Walker et al., 2013). HIV infection brings about the reduction in CD4+ T cells, which a vital role in the immunity of TB. Besides the reduction in numbers, HIV also causes functional abnormality of CD4+ and CD8+ cells. Similarly, TB infection also accelerates the progression of HIV disease from asymptomatic infection to advanced HIV (AIDS) then eventually to death. TB is the commonest cause of death in HIV infected people in Africa and a third of all TB deaths are in HIV- positive patients (Chaisson and Martinson, 2008). A potent activator of HIV replication within T cells is TNF-α, which is produced by activated macrophages within granulomas as a response to tubercle bacilli infection. TB

diagnosis in HIV infected individuals is usually difficult due to nonspecific clinical features. The most widely used method for detection of acid bacilli is microscopic examination of sputum smears, however, is of limited use. This is because about 50% of HIV-TB cases are negative by acid-fast staining and chest radiographs are normal in up to 20% of AIDS patients (Raja, 2004).

2.6 Anti-tuberculosis pharmacological therapy

Current drug resistance to existing treatment regimens is urgently driving the need to develop new and novel anti-TB drugs. The intrinsic challenge in producing drugs against this pathogenic bacterium is also recognized partly because the microorganism has an unusually thick, waxy cell wall that is hard to penetrate. Getting any drugs through this barrier, although possible, is tough (Zhang and Amzel, 2002).

Management of TB is mainly by chemotherapy involving a complex regimen of five drugs used in combinations, with treatment duration of up to 8 months. These drugs are found to be associated with serious side effects. The Directly Observed Therapy, Shot Course (DOTS) strategy, which was initiated by WHO for management of TB to improve compliance and hinder the development of resistance against existing drugs, seems to be failing (Young et al., 2020). The XDR MTB strains, which are resistant to both first- and second-line anti-TB drugs, are proving to be impossible to treat more so in cases of HIV/AIDS co-infection. Riccardi and Pasca (2014) noted that in patients infected with XDR-TB, the chances of successful treatment are quite low, supporting the need for urgent discovery of novel principles with activity against strains resistant to second-line anti-TB drugs(Riccardi and Pasca, 2014).

Treatment of MDR-TB requires the use of second line drugs, which are exceedingly expensive, and with even more serious side effects. This has resulted into high levels of non-compliance among patients and could be the main factor that evokes the emergence of XDR TB strains. In addition, reported drug interaction between anti-TB drugs, mainly rifampicin, with antiretroviral drugs (ARVS) especially nevirapine, has complicated the management of TB in HIV-positive patients (Regazzi et al., 2014). A meta-analytic study revealed that MDR-TB treatment success is 54% and that of XDR ranges from 40% to 19%, both with 15% to 35% deaths (Migliori et al., 2013).

To counteract the shortcomings of conventional treatment of TB, a large proportion of patients in Africa, and East Africa more so, seek remedy from Traditional Medical Practitioners (TMPS) who use crude herbal preparations. Orodho and colleagues (2011) cited that there have been claims in the East Africa region of TMPS being able to treat the symptoms of TB and that there has been knowledge gap with regard to documentation and scientific validation of the medicinal plants(Orodho et al., 2011).

2.6.1 Why is there a need for new TB drugs?

Inadequate, incomplete or improperly supervised treatment regimens, incorrect prescriptions, and co-infection with HIV are responsible for the emergence of resistant strains of MTB. However much TB can be treated with the current anti-TB drugs, treatment is complex and long involving four drugs for two months and two more drugs for at least another four months. Directly Observed Treatment (DOT) as promoted by the WHO to

improve compliance with long regimen can improve cure rates. However, it is demanding for patients and labor intensive for health staff.

Interrupted treatment of TB will result in the transmission of disease. Many patients with TB only adhere to treatment until symptoms have resolved and stop. Patients usually associate the disease with illness and therefore, the need to continue treatment. The premature discontinuation of anti-TB drugs results inn relapse, development of drug resistance and at times death ensues. This phenomenon is a threat not only to patients but the community as well.

In HIV/TB co-infection, it is difficult to take drugs to treat both diseases simultaneously. Interaction between some antiretroviral drugs (ARVS) and antimycobacterial drugs has been reported, making treatment of the dual infection more challenging. It is imperative that the TB treatment is taken regularly as the health care provider advises. If the anti-TB drugs are not taken regularly, the bacilli are more likely to develop resistance to the drugs. Multi-drug resistant TB and XDR-TB are much more difficult to treat as well as being more expensive (Organization, 2013). There is therefore a compelling need to develop new and more effective TB drugs, which are active against MDR-TB and XDR-TB and can shorten the duration of treatment.

2.7 Newly Approved Anti-TB Drugs and Drug Candidates in the Pipeline.

The global burden of tuberculosis (TB) has necessitated the development of new drugs, especially in light of rising cases of multidrug-resistant (MDR-TB) and extensively drug-resistant TB (XDR-TB). Traditional first-line treatments, such as isoniazid, rifampicin, ethambutol, and pyrazinamide, are becoming increasingly ineffective against resistant strains, prompting the need for novel anti-TB drugs with new mechanisms of action. Recently approved anti-TB drugs include; Bedaquiline (BDQ), Delamanid (DLM) and Pretomanid (PA-824).

Bedaquiline is a novel anti-tuberculosis drug specifically designed to treat multidrug-resistant tuberculosis (MDR-TB). It was developed by Janssen Pharmaceuticals and approved by the U.S. Food and Drug Administration (FDA) in 2012, marking it as the first new TB drug approved in over 40 years(Mahajan, 2013). Bedaquiline represents a significant advancement in TB treatment, especially for patients with limited treatment options due to resistance to standard first-line drugs(Andries et al., 2005). Clinical trials have shown that bedaquiline significantly improves treatment outcomes in MDR-TB patients compared to standard care without bedaquiline. In a pivotal study, patients receiving bedaquiline had higher rates of sputum culture conversion (an indicator of bacterial clearance) and improved cure rates compared to those on standard regimens alone(Diacon et al., 2014).

Delamanid (DLM) is a modern anti-tuberculosis (TB) drug developed specifically for the treatment of multidrug-resistant tuberculosis (MDR-TB). It was developed by Otsuka Pharmaceutical Co., Ltd., and received approval from the European Medicines Agency

(EMA) in 2014 for use in adults with MDR-TB(Ryan and Lo, 2014). Delamanid inhibits the synthesis of mycolic acids, essential components of the mycobacterial cell wall. By disrupting mycolic acid synthesis, delamanid compromises the integrity of the bacterial cell wall, leading to bacterial death. This mechanism of action is particularly potent against both actively replicating and dormant forms of *Mycobacterium tuberculosis*(Matsumoto, 2006). Studies have shown that delamanid can improve treatment outcomes, particularly in patients with extensively drug-resistant TB (XDR-TB), who have limited therapeutic options(von Groote-Bidlingmaier et al., 2019).

Pretomanid (PA-824) is a novel anti-tuberculosis drug developed for the treatment of highly drug-resistant tuberculosis (TB), including multidrug-resistant (MDR-TB) and extensively drug-resistant TB (XDR-TB). Developed by the non-profit organization TB Alliance, Pretomanid was approved by the U.S. Food and Drug Administration (FDA) in 2019 as part of a novel regimen with bedaquiline and linezolid (BPaL) for the treatment of adults with pulmonary XDR-TB or treatment-intolerant or non-responsive MDR-TB(Keam, 2019). Pretomanid has dual mechanisms of action. Under aerobic conditions, it inhibits mycolic acid synthesis, essential for the mycobacterial cell wall, leading to cell wall disruption. Under anaerobic conditions, it releases reactive nitrogen species, including nitric oxide, which have potent bactericidal activity, particularly against non-replicating TB bacteria(CK, 2000).

The approval of bedaquiline, delamanid, and pretomanid represents a significant advancement in the treatment of drug-resistant TB. The continued development of new

drug candidates like sutezolid, TBA-7371, Q203, BTZ-043, and OPC-167832 offers hope for shorter, safer, and more effective TB therapies(Edwards and Field, 2022). However, challenges related to drug resistance, safety, and access must be addressed to fully realize the potential of these new treatments in the global fight against TB.

2.8 Interactions between standard anti-tuberculosis drugs and herbal medicines and their implications.

The cornerstone of TB management involves the use of standard anti-TB drugs, which include isoniazid, rifampicin, ethambutol, and pyrazinamide. Despite the availability of standard anti-TB drugs, the emergence of drug-resistant strains (MDR-TB and XDR-TB) and the long duration of treatment pose significant challenges in effective TB management(Zumla et al., 2013). The use of medicinal plants alongside conventional anti-TB drugs has garnered attention due to their potential to enhance treatment efficacy, reduce side effects, and shorten therapy duration. However, the combination of these therapies raises concerns about drug-herb interactions, safety, and the need for scientific validation. Medicinal plants have been used traditionally to treat respiratory ailments, including TB, due to their antimicrobial, anti-inflammatory, and immune-boosting properties(Gurib-Fakim, 2006). The rationale for integrating medicinal plants with standard anti-TB drugs has been to synergize antimicrobial effects, reduce on drug toxicity and immune modulation. Some medicinal plants exhibit direct antimycobacterial activity that can enhance the efficacy of conventional drugs. plant extracts can potentiate the effects of rifampicin and isoniazid by enhancing drug penetration into mycobacterial cells. Herbs such as *Andrographis paniculata* have been noted to boost the antimycobacterial effects of pyrazinamide, particularly in drug-resistant TB strains, by altering bacterial cell wall

synthesis (Gupta et al., 2010a, Mangwani et al., 2020). Herbal remedies such as milk thistle (*Silybum marianum*) are known for their hepatoprotective properties, potentially mitigating drug-induced liver damage(Adetuyi et al., 2021). Certain plants, such as Echinacea and ginseng, have immunomodulatory effects that could help in boosting the host's immune response against TB. Herbs like Echinacea can modulate immune responses, enhancing the host's ability to fight TB when used alongside standard drugs(Hu et al., 2005, Block and Mead, 2003).

While the combination of medicinal plants and anti-TB drugs holds promise, there are significant challenges. Many medicinal plants interact with the cytochrome P450 enzyme system, affecting the metabolism of anti-TB drugs and potentially leading to subtherapeutic levels or increased toxicity. For instance, St. John's Wort induces CYP3A4, which may reduce rifampicin efficacy(Hermann and von Richter, 2012, Colalto, 2010). More still, variations in herbal medicine preparation, dosage, and purity pose significant barriers to the safe and effective integration of herbal medicines with conventional TB treatment. The addition of herbal therapies may complicate treatment regimens, potentially impacting patient adherence and overall treatment success(Colalto, 2010). The combination of standard anti-TB drugs and medicinal plants offers a promising avenue to improve TB treatment outcomes, especially in the context of drug resistance and adverse drug reactions. However, the potential for herb-drug interactions, toxicity, and lack of standardization underscores the need for further research to validate the safety and efficacy of these combinations.

2.9 Drug discovery against Tuberculosis (TB) from Natural Products.

Despite the availability of effective treatments, the rise of drug-resistant TB has necessitated the search for new drugs(WHO, 2023). Natural products have been instrumental in the development of anti-TB drugs, offering structurally diverse compounds with unique mechanisms of action(Newman and Cragg, 2016). Historically, natural products have played a crucial role in TB drug discovery, providing essential leads for the development of effective therapeutics. In this review, we provided an examination of past and present efforts in TB drug discovery from natural sources and discussed the challenges and future directions in this field.

2.9.1 Early Natural Product-Derived Anti-TB Drugs

Natural products have historically been a rich source of anti-TB agents, with significant discoveries in the mid-20th century. **Streptomycin**, discovered in 1944 from *Streptomyces griseus*. streptomycin was the first antibiotic effective against TB. This discovery revolutionized TB treatment and marked the beginning of the antibiotic era(Waksman, 1944). **Rifampicin**, derivatived of rifamycins produced by *Amycolatopsis rifamycinica* (formerly *Streptomyces mediterranei*), rifampicin was introduced in the 1960s and remains a cornerstone drug in TB therapy(Floss and Yu, 2005). **Cycloserine and Capreomycin**: These second-line drugs, derived from *Streptomyces orchidaceus* and *Streptomyces capreolus*, respectively, were identified in the 1950s and 1960s and are used primarily in MDR-TB treatment(Steiner, 1967).

The future of TB drug discovery from natural products lies in integrating modern technologies with traditional natural product research. These technologies enable the manipulation of biosynthetic pathways to produce novel natural products with enhanced anti-TB properties(Newman and Cragg, 2016). Using natural products in combination with

existing drugs could improve efficacy and reduce resistance development. However, natural product discovery slowed due to difficulties in isolating novel compounds, complex synthesis processes, and challenges in drug formulation(Fernandes et al., 2022).

2.9.2 Status of anti-tuberculosis drugs from medicinal plants.

While natural products, including those from medicinal plants, have historically contributed to drug discovery, none of the existing first-line or second-line anti-TB drugs are directly derived from medicinal plants(Newman and Cragg, 2016). Most currently approved anti-TB drugs are synthetic or derived from microorganisms rather than medicinal plants. Isoniazid, Rifampicin, Ethambutol, and Pyrazinamide are synthetic compounds or derived from microbial sources, such as rifampicin from *Amycolatopsis rifamycinica*(Floss and Yu, 2005). No clinically approved anti-TB drugs have been directly sourced from medicinal plants; however, significant research is underway to explore plant-derived compounds that may have therapeutic potential against TB(Tuyiringire et al., 2020). Despite the promising preclinical data, translating plant-derived compounds into clinically approved TB drugs faces several hurdles. Many plant-derived compounds, like curcumin, suffer from poor bioavailability, which limits their therapeutic efficacy(Moballegh Nasery et al., 2020). While some compounds show anti-TB activity, their safety profiles at effective doses are often suboptimal, requiring extensive modification or formulation adjustments(Dartois and Rubin, 2022).

2.10 Medicinal plants traditionally used in the treatment of tuberculosis in Uganda.

Traditional medicine continues to be a crucial component of healthcare, especially in rural areas where access to modern medical facilities is limited. Various medicinal plants have

been traditionally used to treat TB or alleviate its symptoms, often in combination with conventional therapies.

A summary of the medicinal plants for traditional treatment of tuberculosis adapted from a survey carried out by Bunalema et al. in 2014 is presented in Table 1.

Table 1. Medicinal plant species reported by TMPs for the treatment of TB, local names and frequency of mention. (L-Leaves, SB-stem bark, F-fruit, RT-woot bark, WP-Whole plant, S-seeds, H-husks, B-bulb)(Bunalema et al., 2014)

Plant name	**Family**	**Local name (Luganda)**	**Part used**
Zanthoxylum leprieurii Guill. & Perr.	*Rutaceae*	Munyenye	SB
Piptadeniastrum africanum (Hook. f.) Brenan	*Fabaceae*	Mpewere	SB
Albizia coriaria Welw.ex Oliv.	*Fabaceae*	Mugavu	SB
Rubia cordifolia L.	*Rubiaceae*	Kasalabakesi	L/WP
Dracaena steudneri Engl.	*Asparagaceae*	Kajjolyenjovu	SB
Canarium schweinfurthii Engl.	*Burseraceae*	Muwafu	SB, S
Callistemon citrinus (Curtis) Skeels	*Myrtaceae*	Mwambala butonya	L/SB
Combretum molle R.Br. ex. G. Don.	*Combretaceae*	Ndagi	SB
Erythrina abyssinica Lam.	*Fabaceae*	Eggirikiti	SB
Phaseolus vulgaris L.	*Fabaceae*	Bijanjaro	H
Hibiscus fuscus Garcke	*Malvaceae*	Lusaala	L
Garcinia buchananii Baker	*Clusiaceae*	Musaali	SB
Blighia unijugata Baker	*Sapindaceae*	Enkuza nyana	SB
Mangifera indica L.	*Anacardiaceae*	Muyembe	SB
Toddalia asiatica (L.) Lam.	*Rutaceae*	Kawule	L
Prunus africana (Hook.f.) Kalkman	*Rosaceae*	Ntaseesa	SB
Entada abyssinica Steud. ex A. Rich.	*Fabaceae*	Mwolola	SB
Ribes uva-crispa L.	*Grossulariaceae*	Entuntunu	L
Myrica kandtiana Engl.	*Myricaceae*	Enkikimbo	F/L/SB/RB
Spathodea campanulata P. Beauv.	*Bignoniaceae*	Kifabakazi	SB

Ficus glumosa Delile	*Moraceae*	Muwo	SB
Celosia trigyna L.	*Amaranthaceae*	Kakubaggiri	L
Eucalyptus spp.	*Myrtaceae*	Kalitunsi	L
Vernonia amygdalina Delile	*Asteraceae*	Mululuza	L
Lantana camara L.	*Verbenaceae*	Kayukiyuki	L/WP
Aspilia africana (Pers.) C.D. Adams	*Asteraceae*	Makaayi	RB/L
Carica papaya L.	*Caricaceae*	Papaali	L
Triumfetta flavescens Hochst. ex A. Rich.	*Malvaceae*	Luwugula	S
Momordica foetida Schumach.	*Cucurbitaceae*	Bombo	L
Gnaphalium purpureum L.	*Asteraceae*	Omuya	L
Zingiber officinale Roscoe	*Zingiberaceae*	Ntangawuzi	L
Milicia excelsa (Welw.) C.C. Berg	*Moraceae*	Muvule	L
Rhus vulgaris Meikle	*Anacardiaceae*	Kakwanskwanso	SB/L
Albizia spp.	*Fabaceae*	Ennongo	SB
Bidens pilosa L.	*Asteraceae*	Sere	L
Warburgia ugandensis Sprague	*Canellaceae*	Abaki	SB
Acacia spectabilis A. Cunn. ex Benth.	*Fabaceae*	Gasiya	L
Allium sativum L.	*Amaryllidaceae*	Katungulu chumu	F
Trichilia dregeana Sond.	*Meliaceae*	Sekoba	SB
Plectranthus barbatus Andrews	*Lamiaceae*	Ekibankulata	L
Tithonia diversifolia (Hemsl.) A. Gray	*Asteraceae*	Ekimyula	SB
Indigofera emarginella Steud. ex A. Rich.	*Fabaceae*	Olutunga nsonzi	L and F
Cinnamomum zeylanicum Blume	*Lauraceae*	Mudalasini	SB
Azadirachta indica A. Juss.	*Meliaceae*	Neem tree	L
Maytenus senegalensis (Lam.) Excell	*Celastraceae*	Naligwalimu	L and F
Zanthoxylum chalybeum Engl.	*Rutaceae*	Ntale ya ddungu	SB
Syzygium cumini (L.) Skeels	*Myrtaceae*	Jambula	SB
Ficus natalensis Hochst.	*Moraceae*	Omutuba	SB
Aloe vera (L.) Burm. f.	*Xanthorrhoeaceae*	Kigaji	L

Pseudospondia microcarpa (A. Rich.) Engl.	*Anacardiaceae*	Muziru	SB
Lippia grandifolia Hochst. ex A. Rich	*Verbenaceae*	Olugumaguma	L
Sapium ellipticum (Hochst.) Pax	*Euphorbiaceae*	Omusasa	SB
Coffea canephora Pierre ex A. Froehner	*Rubiaceae*	Emwanyi	S/SB
Chaetacme aristata Planch.	*Ulmaceae*	Embutami	L
Persea americana Mill.	*Lauraceae*	Ovacado	SB
Desmodium salicifolium (Poir.) D.C.	*Fabaceae*	Enkolimbo	L
Fleurya aestuans (L.) Gaudich. ex Miq.	*Urticaceae*	Munyango	L
Solanum incanum L.	*Solanaceae*	Entengo ennene	L
Kalanchoe glaucescens Planch. ex benth	*Crassulaceae*	Ekiyondo ekyeru	L
Bridelia micrantha (Hochst.) Baill.	*Euphorbiaceae*	Katazamitti	SB
Phyllanthus reticulatus Poir.	Phyllanthaceae	Mutulika	L
Tetradenia riparia (Hochst.) Codd	*Lamiaceae*	Ekyewamala	L
Antiaris toxicaria Lesch.	*Moraceae*	Kirundu	SB
Moringa oleifera Lam.	*Moraceae*	Moringa	f
Acacia hockii De Wild.	*Fabaceae*	Kasana	L
Solanum spp.	*Solanaceae*	Katunkuma	L/F
Cymbopogon citratus D.C. ex Stapf	*Poaceae*	Kisubi	L
Phaseolus lunatus L.	*Fabaceae*	Kayindiyindi	L
Plumbago zeylanica L.	*Plumbaginaceae*	Musajja abanda	L
Ageratum conyzoides L.	*Asteraceae*	Namirembe	WP
Morinda lucida Benth.	*Rubiaceae*	Kabaja nsayi	SB
Podocarpus usambarensis Pilg.	*Podocarpaceae*	Kamusenene	L
Vernonia cinerea (L.) Less.	*Asteraceae*	Kayayana	L
Alangium chinense (Lour.) Harms	*Cornaceae*	Omusiisa	SB
Steganotaenia araliacea Hochst.	*Apiaceae*	Omuwanula	SB
Kigelia africana (Lam.) Benth.	*Bignoniaceae*	Omusa	L
Alchornea cordifolia (Schumach. & Thonn.) Müll. Arg.	*Euphorbiaceae*	Luzibaziba	L

Cassia didymobotrya Frensen.	*Fabaceae*	Omucuula	L
Crotalaria agatiflora Scheinf.	*Fabaceae*	Kasamba ndege	L
Centella asiatica (L.) Urb.	*Apiaceae*	Kabbo kabakyala	L
Citropsis articulata (Willd. ex Spreng.) Swingle & M. Kellerm.	*Rutaceae*	Akatimbolo	F
Argemone mexicana L.	*Papaveraceae*	Akatovu akeeru	F/WP
Phyllanthus amarus Schumach. & Thonn.	*Phyllanthaceae*	Kabalila mugongo	L
Hyptis suaveolens (L.) Poit.	*Lamiaceae*	Kawetale	L
Catharanthus roseus (L.) G. Don	*Apocynaceae*	Sekajja	L
Lippia javanica (Burm f.) Spreng.	*Verbenaceae*	Kisumuluzo	L
Pycnanthus angolensis (Welw.) Warb.	*Myristicaceae*	Olunaaba	SB
Parkia filicoidea Welw. ex Oliv.	*Fabaceae*	Jjoge	SB
Trimeria grandifolia (Hochst.) Warb.	*Salicaceae*	Nabumba	SB
Aristolochia elegans Mast.	*Aristolochiaceae*	Nabuseero	WP

2.11 Plants used in this Research for Antimycobacterial Activity

The study species will be *Zanthoxylum leprieurii* Guill. & Perr. and *Rubia cordifolia* L. These species were mentioned by traditional medicine practitioners in a study carried out in the central districts of Uganda to be used in the treatment of tuberculosis and respiratory tract related infections (Bunalema et al., 2014).

Zanthoxylum leprieurii Guill. & Perr.

Taxonomy of *Zanthoxylum leprieurii* (Mabberley, 2017)

Kingdom: Plantae
Subkingdon: Tracheobionta
Superdivision: Spermatophyta
Division: Magnoliopsida
Class: Magnliopsida
Subclass: Rosidae

Order: Sapindales
Family: Rutaceae
Genus: Zanthoxylum
Species: *Zanthoxylum leprieurii* Guill. & Perr.

Locally known as Munyenye in Luganda, is a medium sized tree up to 15-24 m high and up to 40 cm in diameter at base. It can grow in wet and dry forest vegetation (Adesina, 2005). Like any other African *Zanthoxylum* species, *Zanthoxylum leprieurii* is distinguished by its classical taxonomic characteristics such as the anatomical and morphological features of the fruits, leaves and roots. The stem is a conical woody prickle-bearing outgrowth up to 6 cm long. Leaves are alternating with 8-16 leaflets, 15-55 cm long. It occurs in rain forests and savanna woodland, from sea level to about 2000 m altitude. It was formally known under the generic name *Fagara* Linn. Zanthoxylum currently belong to the sub-family of Rutoideae, tribe Zanthoxyleae in the family of Rutaceae (Adesina, 2005). Zanthoxyllum contains about 549 species distributed globally, qualifying it to be the largest genus in *Rutaceae* family (Patiño Ladino and Cuca Suárez, 2010). The crosscutting characteristic is that the trunks, branches, leaf stalks and inflorescence of all the species are covered by prickles commonly described as spines (Waterman, 1985) as cited by Fogang and others in 2012(Fogang et al., 2012a). In Africa, *Zanthoxyllum leprieurii* is traditionally used in the treatment of HIV/AIDS, malaria, urinary infections, rheumatic pain and is used as an antiseptic (Lamorde et al., 2010). Additionally, *Z. leprieurii* is used traditionally in the treatment of fevers, coughs, colds, toothache (Reisch et al., 1985) and some fungal infections (Ngane et al., 2000). Species of *Zanthoxylum* were used for treatment of a wide range of ailments by traditional healers throughout West and Central Africa. Some of these disorders include toothache, urinary

and venereal diseases, rheumatism and lumbago (Fogang et al., 2012b). Local communities of Western province of Cameroon use dried fruits of Zanthoxylum leprieurii, especially in two famous soups called 'nkui' and 'nah poh' as cited by Ngoumfo and others in 2010(Ngoumfo et al., 2010). Tabuti and others (2010), Bunalema and colleagues (2017) stated that stem bark of *Z. leprieurii* is used in the management of tuberculosis and respiratory tract related infections in Ugandan communities(Tabuti et al., 2010, Bunalema et al., 2017). The genus *Zanthoxylum* is very well known its diverse chemistry, particularly by the presence of alkaloids, aromatic and aliphatic amides, sterols and phenylpropanoids-lignans and cumarins (Nooreen et al., 2019). Secondary metabolites, which are well distributed in abundance within the *Zanthoxylum* species, include benzophenanthridines and acridones among others (Patiño Ladino and Cuca Suárez, 2010).



Figure 2: *Zanthoxylum leprieurii.*

***Rubia cordifolia* L.**

Taxonomy of *Rubia cordifolia*(Mabberley, 2017)

Kingdom: Plantae
Subkingdom: Tracheobionta
Supervision: Spermatophyta
Division: Magnoliophyta
Class: Magnoliopsida
Subclass: Asteridae
Order: Gentianales

Family: Rubiaceae
Genus: Rubia
Species: *Rubia cordifolia* L.

Commonly known as Indian Madder (trade name- manjistha) and Kasalabakesi in Luganda is a species of flowering plant in the coffee family of Rubiaceae. *Rubia cordifolia* is an important medicinal plant often used in the traditional treatment of different ailments. It is distributed in hilly areas of India up to 3750 m. It is a perennial, prickly climber with a stem of up to 12 m long. It has very variable leaves, which are ovate lanceolate. Flowers are fragrant, minute, whitish or greenish yellow (Ved et al., 2002).

Rubia cordifolia has been grown for a red pigment derived from its roots. The extracts and phytochemicals of *Rubia* plants have drawn sufficient attention due to their potent bioactivities (Badyal et al., 2003). Roots of *Rubia* plant species have been reputed for their satisfactory efficacy and thus being used for the treatment of cancers, tuberculosis, rheumatism, hematemesis, metrorrhagia, epistaxis and menoxenia in the Chinese traditional medicine (Zhao et al., 2011).

Rubia cordifolia, is highly important plant in Ayurvedic system of medicine used for the treatment of a number of skin diseases (Dev, 2006). Rubia cordifolia roots are used internally in the treatment of abnormal uterine bleeding, internal and external hemorrhage, bronchitis, kidney and gall bladder stones, dysentery and so many others. The roots can also be used to lower blood pressure (Verma et al., 2016). Phytochemical constituents identified in *Rubia cordifolia* were; anthraquinone constituents: munjistin, purpurin and pseudopurpurin (Akhtar et al., 2006).

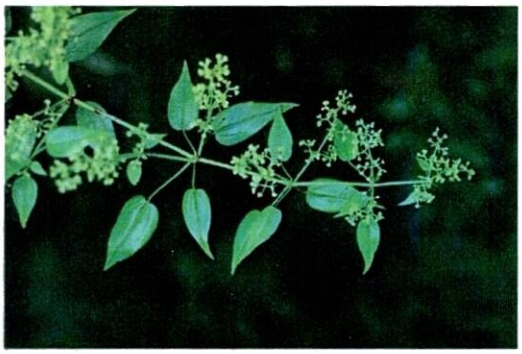


Figure 3: *Rubia cordifolia*

2.12 Phytochemical profiles of *Zanthoxylum leprieurii* and *Rubia cordifolia.*

The biological activities and toxicity of plants are primarily influenced by the composition of their secondary metabolites, commonly referred to as phytochemicals. Phytochemicals are generally non-nutritive compounds produced by plants as a response to various biotic and abiotic stressors. In Zanthoxylum species, several classes of phytochemicals have been identified, including flavonoids, coumarins, phenolic acids, terpenoids, saponins and alkaloids, with alkaloids being the most frequently reported among these classes(Kosh-Komba et al., 2017, Mbaze et al., 2007, Tuyiringire et al., 2022, Kusuda et al., 2006, Oloya et al., 2022). It has been used traditionally to treat various ailments such as malaria, pain, and infectious diseases. The plant's therapeutic effects are closely linked to its diverse phytochemical composition.

Alkaloids: Alkaloids are among the most significant bioactive compounds in *Zanthoxylum leprieurii,* contributing to its analgesic, antimicrobial, and anti-inflammatory properties. They are present in most species of Zanthoxylum and are largely present in all plant organs, being abundant in the stem and root bark. (Patiño et al., 2012). Key alkaloids identified include skimmianine and dictamnine, which exhibit notable antimicrobial activity against various pathogens. Skimmianine, in particular, has been shown to have significant effects on pain modulation, which corroborates the traditional use of the plant for pain relief. Alkaloids also have been found to possess anti-cancer and anti-trypanosomal properties(Eze et al., 2020).

Flavonoids: Flavonoids, such as quercetin and kaempferol derivatives, are abundant in the leaves and bark of *Zanthoxylum leprieurii*. These compounds are known for their strong antioxidant activities, which help protect the body against oxidative stress and inflammation. Flavonoids also contribute to the plant's antimicrobial properties by disrupting the cell walls of bacteria and fungi.(Okagu et al., 2021, Yoro et al., 2020).

Tannins are polyphenolic compounds found in high concentrations in the bark of *Zanthoxylum leprieurii*. They have astringent properties and contribute to the plant's ability to treat gastrointestinal issues and skin conditions. Additionally, tannins exhibit antiviral activity, anti-sickle cell activity making them valuable in the treatment of viral infections and sicle cell(Tatiana et al., 2020).

Saponins: Saponins in *Zanthoxylum leprieurii* are known for their immunomodulatory and inti-inflammatory effects. They enhance the permeability of bacterial cell membranes, leading to increased susceptibility of pathogens to antibiotics. Saponins also possess cytotoxic properties, which can impact their therapeutic index and selectivity in medical applications(Kplé et al., 2023, Kuete and Efferth, 2015).

Rubia cordifolia, commonly known as Indian Madder, is a perennial climbing herb belonging to the Rubiaceae family. It is widely used in traditional Asian medicine for its blood-purifying, anti-inflammatory, and antimicrobial properties. Several studies have documented that *Rubia cordifolia* exhibits a wide range of pharmacological activities, including anti-inflammatory, anticancer, antitumor, antioxidant, antibacterial, anti-platelet aggregation, anti-nephrotoxic, anti-urolithiasis, hepatoprotective, and neuroprotective effects(Rawal et al., 2004, Joy and Nair, 2008, Luo et al., 2022). When *Rubia cordifolia* is combined with honey, it is reported to be effective against vitiligo and diabetic foot ulcers(Ojha et al., 1994). The plant's medicinal value is attributed to its diverse array of phytochemicals, particularly anthraquinones(Dave and Ledwani, 2012). Other phytochemicals present in *Rubia cordifolia* are; alkaloids, cardiac glycosides, tannins, flavonoids and phenols(Kannan et al., 2009). Anthraquinones are key compounds found in

Rubia cordifolia, including notable examples such as alizarin, munjistin, rubiadin, purpurin, techoquinone, and xanthopurpurin. So far 28 anthraquinones have been isolated from *R. cordifolia*(King Suxian, 1992, Dave and Ledwani, 2012).

CHAPTER 3: METHODS AND MATERIALS

3.1 Study site

The study was carried out at Mbarara University of Science and Technology pharmacy laboratory, MSF-Epicentre Mbarara Research Centre Laboratory and Makerere University College of Health Sciences (both at Immunology and Mycobacteriology laboratories). MSF-Epicentre is an MSF satellite dedicated to epidemiology and research. Epicentre Uganda has a laboratory located within the Epicentre facility with a biosafety level 3. The laboratory is equipped to do parasitology, mycobacteriology (biosafety level 3 to perform cultures), microbiology (biosafety level 2, molecular biology; classical PCR and real-time qPCR and GeneXpert), biochemistry and hematology. It has a cold chain capacity for -20 and -80 degress storage. Mycobacteriology laboratory of Makerere University, College of Health Sciences is a tuberculosis culturing laboratory based at the Medical School. It is a College of American Pathologists (CAP) accredited fully functional Biosafety level (BSL)-3 culture facility.It is a site laboratory for most of the multinational tuberculosis research initiatives. Immunology laboratory of Makerere University, College of Health Sciences is equipped with the latest technology equipment and performs a quite a number of tests and procedures. These include cytotoxicity assays, flow cytometry, cell sorting, cell & tissue culture and Luminex assays among others.

3.2 Study design

The study was an experimental study design. Two plant species were studied, that was *Zanthoxylum leprieurii* Guill. & Perr. and *Rubia cordifolia* L.

3.3 Plant Collection and Extraction

3.3.1 Plant material 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) as shown in the figures 4 and 5. 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.



Fig. 4. showing collection of stem bark of *Z. leprieurii* from Mabira forest. Photo A: student standing near *Z. leprieurii* tree, photo B: field guide with a panga used to remove stem bark, and photo C depicts stem bark collection.



Fig. 5. showing collection of R. cordifolia from Kagamba village in Ntungamo district. Photo A: student standing with a field guide demonstrating the plant, photo B: collection the plant, and photo C depicts drying of the plant parts.

3.3.2 Preparation of extracts

The powdered plant materials were extracted using 80% methanol, 80% ethanol and distilled sterile water as extraction solvents (Nguta et al., 2016, Gemechu et al., 2013). For methanol and ethanol extraction, the powdered plant parts, each weighing 200 g, were extracted with 1000 mL of the respective solvent by cold maceration at room temperature (24°C) for 72 hours with frequent agitation(Swami et al., 2008). The filtrate was concentrated using a rotary vacuum evaporator including a water bath set at a temperature of 45°C under reduced pressure. The concentrate was freeze-dried 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.

3.3.3 Aqueous Extraction

Powdered plant materials (500g) were heated in 1000 ml of distilled water to boiling point before filtration by gravity using Whatman No. 1 filter paper. The filtrate was then concentrated using a bio-freezer at -80° C and lyophilized (Benchtop Freeze Dryer). The lyophilized material was stored at 4°C for future use.

3.4 Mycobacterium strains

Two standard strains of *Mycobacterium tuberculosis* were used: the reference pan-sensitive $H_{37}Rv$ (ATCC 27294) and a rifampicin-resistant TMC-331 (ATCC 35838) strain. These strains were provided by the Mycobacteriology BSL-3 Laboratory, Department of Microbiology, College of Health Sciences, Makerere University, Uganda. Then we also used two wild strains for antimycobacterial activity determination; one was rifampicin sensitive and another one was rifampicin resistant. 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 using a densitometer by either adding more cells to the suspension or diluting with normal saline.

3.5 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 (Pitaloka and Sukandar, 2018).

3.6 *In vitro* antimycobacterial assay

3.6.1 Bacterial strain and identification

Strains: $H_{37}Rv$ (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 48h at 37°C to check for possible contamination. The identification of the $H_{37}Rv$ 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).

3.6.2 Preparation of the inoculum

Fresh sub cultures of Mycobacterial strains were made on LJ medium for 3 weeks. The inoculum was prepared in MiddleBrook 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 ($3x10^6$ CFU/mL) using a densitometer(Ramos et al., 2008). 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 48 hours to check for possible contamination. The plates were very well covered, and incubated at 37^0C for 7-days.

3.6.3 Anti-mycobacterial activity test and Minimum Inhibitory Concentration determination.

The antimycobacterial activity was determined using the Resazurin Microtiter Assay (REMA) (Singh et al., 2012, Palomino et al., 2002). 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 for the extracts). 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/drug 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 prevented a change in color of resazurin dye(Coban et al., 2004). The tests were performed in duplicates and mean MICs determined.

3.7 Drug-Extract Combination Activity Determination.

3.7.1 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.

3.7.2 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 (Fauziyah et al., 2017). The plant 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(WHO, 2018). Two series of stock solutions were prepared by diluting each standard drug and extract in sterile water and DMSO respectively, except for rifampicin, 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 2 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. 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. All tests were run in duplicates to increase reliability of results.

Table 2. 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 dr Extract concent
ampicin	100	0.25	10,000	1.25	23.5	25	1:5(
ampicin	100	0.25	10,000	0.25	24.5	25	1:1(
ampicin	100	0.25	10,000	0.125	24.625	25	1:5
niazid	100	0.05	10,000	1.25	23.7	25	0.2::
niazid	100	0.05	10,000	0.25	24.7	25	0.2:1
niazid	100	0.05	10,000	0.125	24.825	25	0.2:
hambutol	100	1.875	10,000	1.25	21.875	25	7.5::
hambutol	100	1.875	10,000	0.25	22.875	25	7.5:1
hambutol	100	1.875	10,000	0.125	23.0	25	7.5:
ptomycin	100	0.5	10,000	1.25	23.25	25	2:5(
ptomycin	100	0.5	10,000	0.25	24.25	25	2:1(
ptomycin	100	0.5	10,000	0.125	24.375	25	2:5

3.8 Cytotoxicity Screening of the Active Plant Extracts of *Zanthoxylum leprieurii* and *Rubia cordifolia*

3.8.1 Mouse BALB/C Monocyte-Macrophage (Line J774.2)

3.8.1.1 Cell Maintenance

Mouse BALB/C Monocyte-Macrophage (Line J774.2) was used. The maintenance of the cells was performed according to protocols obtained from Freshny (1987). All cell culture procedures were carried out in a laminar flow cabinet containing UV light and was disinfected using 70% ethanol before use. The cells were grown aseptically in 75 cm^3 tissue culture flasks using filter sterilized 10% Complete Culture Medium (CCM), which comprised of Dulbecco's Modified Eagle's Medium (DMEM), containing 10% fetal calf serum and supplemented with antibiotics (penicillin, 10 000 U/ml, streptomycin sulphate, 10 000 U/ml).

The culture flasks were examined for color changes and turbidity of the media every two days. The cells were examined microscopically for cell growth. Cells were harvested by trypsinization when the culture was 80% confluent and divided into two separate flasks. More media was then added to each culture flask and incubated at 37°C in a 5% CO_2 humidified incubator until required for experimental use.

3.8.2 Trypan Blue Exclusion Assay

Cell viability was determined using the Trypan Blue Dye Exclusion method (Patterson Jr, 1979). The test is based on the ability of an intact cell membrane to exclude certain dyes such as safranin, eosin, Congo red and trypan blue.

Cell suspensions was prepared by diluting 10 µl of cell suspension and 10 µl of 0.2% trypan blue dye. After 5 minutes, a drop of suspension was placed on a hemocytometer, covered with a coverslip and examined by a light microscope at x400 magnification. Colorless cells were harvested if the dye exclusion test showed a viability count of greater than 80%.

3.8.3 MTT Assay

The cytotoxic effect of the active plant extracts on mouse BALB/C monocyte-macrophage (J774.2) cells was measured using microculture tetrazolium (MTT) assay (Mosmann, 1983).

Prior to use in the assay, cells were grown to 80% confluence and synchronized by incubation in the culture media for 4 hours. The cells (10000 cells/well) were then incubated with various concentrations of the active plant extract solutions (50 µg/mL, 100 µg /ml, 500 µg /ml, 1000 µg /mL and 2000 µg /mL) at 37°C in a 5% CO_2 humidified incubator for 48 hours. After the 48h treatment with the plant extracts, aliquots of 20 µL of

a 5 mg/mL MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide solution were added to the wells and microtiter plates incubated for 4 hours at 37°C in a humidified incubator with 5% CO_2. Thereafter, an aliquot of 100 µL of Dimethyl Sulphoxide (DMSO) was added to the wells to dissolve formazan crystals that formed in metabolically active cells and incubated for 1 hour(Morgan, 1998). The plates were read at 570 nm on a plate reader and data was entered and analyzed using Microsoft Excel. The percentage growth inhibition was determined using the following equation:

$$\textbf{Percentage Cell Viability} = \frac{(\textbf{Absorbance of treated cells})}{(\textbf{Absorbance of positive control})} \times \mathbf{100}$$

Graphs illustrating percentage cell viability against extract concentrations were generated using the results. The CC50 value, indicating the cytotoxic concentration of plant extracts that reduced J774.2 cell viability to 50%, was estimated from the graph using linear regression equation (Y=aX+b).

We also determined Selectivity Index (SI) of the extracts of *Zanthoxylum leprieurii* and *Rubia cordifolia*. Selectivvity Index of a plant extract is a measure of its safety and effectiveness. It indicates how selectively the extract targets the pathogenic cells (in this case *Mycobacterium tuberculosis*) compared to normal cells. A higher SI value suggests that the extract is more effective against the target cells with minimal toxicity to normal cells. The SI was calculated using the following formula:

$$\textbf{Selectivity Index } (\textbf{SI}) = \frac{\textbf{CC50 of normal cells}}{\textbf{MIC of target cells}}$$

3.9 Ethical clearance

The study was approved by the Faculty of Medicine Research Committee (FRC) and Research Ethics Committee (REC) of Mbarara University of Science and Technology with the approval number: 03/02-19.

CHAPTER 4: RESULTS

4.1 Anti-mycobacterial activity test and Minimum Inhibitory Concentration determination

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 $H_{37}Rv$, Rifampicin resistant TMC 331 strain and two wild strains (one rifampicin resistant and another one rifampicin susceptible). The mean Minimum Inhibitory Concentration (MIC) values of crude extracts are shown in Table 3 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 as indicated in figure 6. below. 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 3. Minimum Inhibitory Concentrations (in µg/mL ± STDEV, n = 2) of total crude plant extracts of *Z. leprieurii* and *R. cordifolia* and the standard drugs (Rifampicin and Isoniazid)

Plant/Drug	**Extract**	**Mean MICs (µg/mL)**			
		MTB $H_{37}Rv$	**RR-TMC 331 strain**	**Wild strain (Rif. Resistant)**	**Wild strain (Rif. Sensitive)**
Z. leprieurii	Aqueous	750±0	375±0	187.5±0	46.9±11.3
Z. leprieurii	Ethanolic	187.5±24.8	187.5±0	46.9±25.3	93.8±0
Z. leprieurii	Methanolic	187.5±27.6	187.5±0.0	187.5±0.0	23.4±0.0
R. cordifolia	Aqueous	750±0	1500±0	375±21.2	750±0
R. cordifolia	Ethanolic	750±0	750±0	187.5±0	187.5±0
R. cordifolia	Methanolic	187.5±28.3	187.5±0	187.5±0	46.9±11.3
Rifampicin		0.5±0.4	>4.0±0	>4.0±0	0.06±0.06
Isoniazid		0.25±0.14	0.13±0	0.13±0	0.03±0

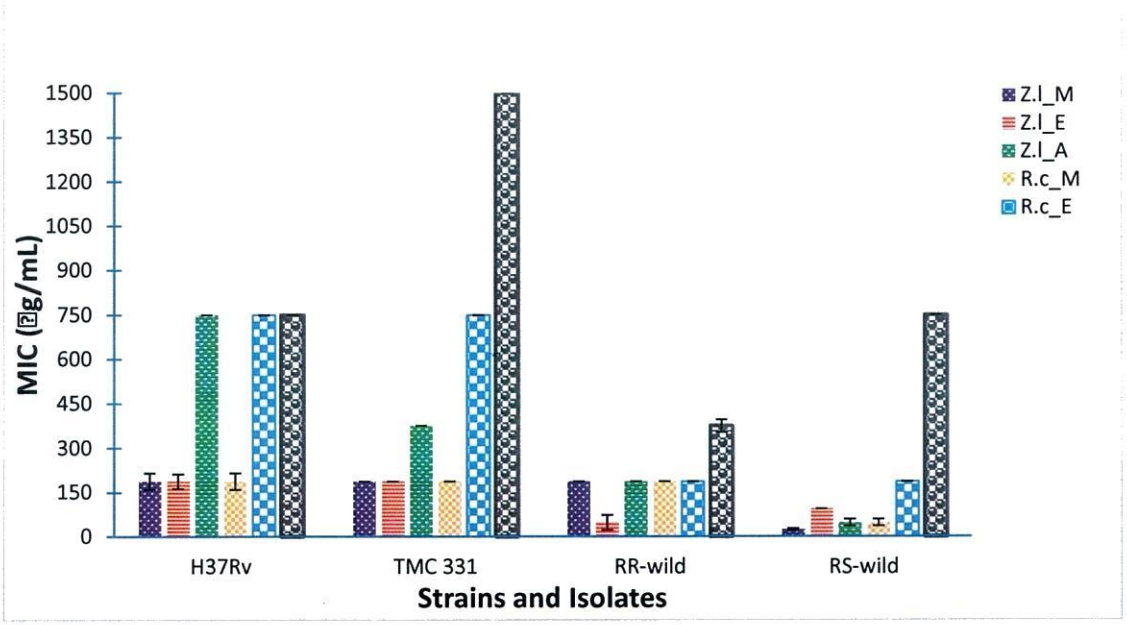


Fig. 6. Graph showing minimum inhibitory concentrations (µg/mL) (STDEV) of methanol, ethanol and aqueous extracts of *Z. leprieurii* and *R. cordifolia* against MTB strains (H_{37}Rv and TMC-331), and wild isolates, one rifampicin resistant and another one sensitive.

4.2 Drug-Extract Combination Assay

The study was performed as an antimycobacterial assay. Data collected were 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 4 for the preliminary study and Table 5 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.

Table 4. 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.*

The was total inhibition of $H_{37}Rv$ on combining rifampicin with methanol extracts of the two plants. 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. Combination of the plant extracts with ethambutol and streptomycin exhibited an enhanced growth inhibition against MDR-MTB strain at tested concentrations.

Table 5. Percentage inhibition for different combinations of standard antituberculosis drugs and extracts against H37Rv and MDR-TB strains.

			$H_{37}Rv$				MDR			
Standard Drugs	**Methanol Extract**	**concentration in combination (µg/mL)**	**1**	**2**	**Mean CFU**	**Mean % Inhibition**	**1**	**2**	**Mean CFU**	**Mea Inhib**
Control (+)	-	-	**309**	**255**	**282±27**	-	**171**	**133**	**152±**	-
Rifampicin	-	-	0	0	0±0	100±0	98	116	107±9	29±
	Z. leprieurii	1:500	0	0	0±0	100±0	0	0	0±0	10(
	Z. leprieurii	1:100	0	0	0±0	100±0	0	0	0±0	10(
	Z. leprieurii	1:50	0	0	0±0	100±0	37	7	22±15	87
	R. cordifolia	1:500	0	0	0±0	100±0	0	0	0±0	10(
	R. cordifolia	1:100	0	0	0±0	100±0	12	22	17±5	89
	R. cordifolia	1:50	0	0	0±0	100±0	48	66	57±9	63±
Control (+)	-	-	**709**	**843**	**776±67**	-	**441**	**591**	**516±**	-
Isoniazid	-	-	101	135	118±17	85±1	438	544	491±53	5±
	Z. leprieurii	0.2:500	0	0	0±0	100±0	0	0	0	10(
	Z. leprieurii	0.2:100	96	0	48±48	94±7	0	52	26±26	95
	Z. leprieurii	0.2:50	0	348	174±174	79±21	93	0	46.5±46.5	89±
	R. cordifolia	0.2:500	0	0	0±0	100±0	0	0	0	10(
	R. cordifolia	0.2:100	0	9	4.5±4.5	99±0.6	0	80	40±40	93
	R. cordifolia	0.2:50	4	0	2±2	99±1	48	340	194±146	61±
Control (+)	-	-	**298**	**248**	**273±25**	-	**182**	**132**	**157±25**	-
Ethambutol	-	-	0	0	0±0	100±0	115	81	98±17	38
	Z. leprieurii	7.5:500	0	0	0±0	100±0	0	0	0	10(
	Z. leprieurii	7.5:100	0	0	0±0	100±0	0	0	0	10(
	Z. leprieurii	7.5:50	25	2	13.5±11.5	95±4	0	0	0	10(
	R. cordifolia	7.5:500	3	5	4±1	98±1	0	0	0	10(
	R. cordifolia	7.5:100	9	31	20±11	93±5	0	0	0	10(
	R. cordifolia	7.5:50	36	78	57±21	79±10	0	0	0	10(
			298	**248**	**273±25**		**182**	**132**	**157±25**	
Streptomycin	-	-	0	0	0±0	100±0	54	98	76±22	52±
	Z. leprieurii	2:500	0	0	0±0	100±0	0	0	0	10(
	Z. leprieurii	2:100	0	2	1±1	99±1	0	0	0	10(
	Z. leprieurii	2:50	8	12	10±2	96±1	0	3	1.5±1.5	99
	R. cordifolia	2:500	0	0	0±0	100±0	0	0	0	10(
	R. cordifolia	2:100	0	0	0±0	100±0	0	0	0	10(
	R. cordifolia	2:50	7	0	3.5±3.5	99±1.2	0	0	0	10(

In contrast, the results indicated a decrease in the combination activity for the combination of rifampicin and isoniazid with extracts against MDR-TB isolates in comparison to the plant extracts alone. 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 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.

4.3 Cytotoxicity

Following the promising antimycobacterial activity, the cytotoxic effect of the active plant extracts; *Zanthoxylum leprieurii and Rubia cordifolia* on mouse BALB/C monocyte-macrophage (J774.2) after 48 hours is shown in Figure 7. We performed the *in vitro* cytotoxicity screening using the MTT assay to measure the damage caused by the antimycobacterial plant extracts on normal living cells. The motivation was to determine whether the plant extracts could be used for therapeutic purposes without excessive damage to host cells.

The major objective of the cytotoxicity assay was to determine which of the plant extracts that had positive antimycobacterial activity were not toxic to J774.2 cell lines. The effect was observed after 48 hours at various extract concentrations (31.2 μg/mL, 62.5 μg/mL, 125 μg/mL, 250 μg/mL, 500 μg/mL, 1000 μg/mL and 2000 μg/mL) for both ethanolic and methanolic extracts. After 48 hours, both extracts of *Z. leprieurii* and *R. cordifolia* showed a decrease in cell viability for the J774.2 cell line (Figure 7). As the concentration of the extracts increased, cell viability of J774.2 decreased in a dose dependent manner. The cytotoxic concentration at 50% (CC50) values of the extracts ranged from 85.6 μg/mL to

600 µg/mL, as presented in Table 6. Methanol and ethanol extracts of Z. leprieurii and R. cordifolia demonstrated low selectivity indices as shown in Table 7.

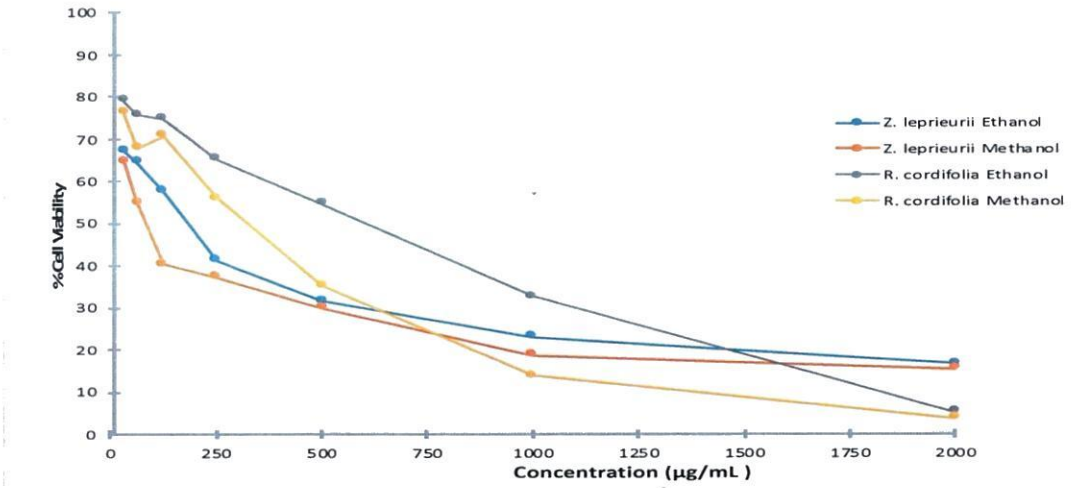


Figure 7. Cell viability of the methanol and ethanol plant extracts of *Zanthoxylum leprieurii* and *Rubia cordifolia* on the BALB/C monocyte macrophage cell line (J774.2).

Table 6. Cytotoxicity evaluation (expressed as CC_{50}) of the plant extracts on J774.2 cells

Botanical name	**Solvent used**	**CC_{50} (µg/mL)**
Zanthoxylum leprieurii	Methanol	85.6
	Ethanol	149.9
Rubia cordifolia	Methanol	224.7
	Ethanol	600

Table 7. Selectivity Indices of Zanthoxylum leprieurii and Rubia cordifolia crude extracts

Plant	**Solvent**	**MIC (µg/mL) on $H_{37}Rv$**	**CC_{50} (µg/mL)**	**SI**
Zanthoxylum leprieurii	Methanol	187.5	85.6	0.46
	Ethanol	187.5	149.9	0.80
Rubia cordifolia	Methanol	187.5	224.7	1.20
	Ethanol	750	600	0.80

CHAPTER 5: DISCUSSION

5.1 Plant Extraction

Three different solvents were used to extract the active ingredients from the stem bark of *Zanthoxylum leprieurii* and *Rubia cordifolia* whole plant. Assessing the composition of the plant extracts is important in exposing specific phytochemicals that are responsible for their therapeutic property. The solvents that we employed to extract the plant compounds in this study were water, ethanol and methanol. The most commonly used extraction solvent by traditional medicine practitioners is water. Water usually does not dissolve the intermediate polar to nonpolar compounds of plant extracts(Visht and Chaturvedi, 2012, Masoko and Nxumalo, 2013). The success of determining the biologically active secondary metabolites largely depends on the type of extraction solvent used. It is therefore critical to use solvents which will extract all active compounds, covering all range of polarity.

Methanol extracts exhibited higher antimycobacterial activity compared to other solvents. This could be as a result of several compounds that are extracted by methanol, which is found to be an effective extraction solvent (Bunalema et al., 2017, Chatha et al., 2006). This could be as a result of the presence of high levels of polar secondary metabolites 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 (Truong et al., 2019). Different studies have reported that methanol extracts have the best extract yield(Masoko et al., 2008, Visht and Chaturvedi, 2012, Tuyiringire et al., 2022). In the present study, it was indeed confirmed

that methanol was the best extraction solvent resulting in a greater yield and subsequent antimycobacterial activity of the methanol extracts.

5.2 Anti-mycobacterial activity and Minimum Inhibitory Concentration

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 a previous study that surveyed on medicinal plants used for treatment of tuberculosis in Uganda, two medicinal plants that were frequently mentioned were selected. In the present study, aqueous, ethanolic and methanolic extracts of 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(Bunalema et al., 2017, Oloya et al., 2021, Tuyiringire et al., 2022). 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 (Bunalema et al., 2017). 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 (Tuyiringire et al., 2022). 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 (Oloya et al., 2022). *Rubia cordifolia* methanolic extracts 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 determine *R. cordifolia* anti-mycobacterial activity (Makgatho et al., 2017). Crude 80% ethanolic 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 (Gemechu et al., 2013, Makgatho et al., 2017, Komape et al., 2017, Nyambuya et al., 2017). 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* (Bunalema et al., 2017), *Zanthoxylum capense* (Luo et al., 2013)*and R. cordifolia* (Makgatho et al., 2017).

5.3 Drug-Extract Combination Assay

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 (Fauziyah et al., 2017). 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 (Chinsembu, 2016, Koul et al., 2011). 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* (Tuyiringire et al., 2020, Bunalema et al., 2017). However, there is no published literature about the effects of combination of these 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 (Garg et al., 2022). 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 (Garg et al., 2022, Haas and Belknap, 2018).

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* (Mpeirwe et al., 2023, Bunalema et al., 2017, Makgatho et al., 2017).

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, we observed an enhanced inhibition of MDR-MTB when standard anti-TB drugs, more remarkably ethambutol and streptomycin were combined with methanol extracts of *Z. leprieurii* and *R. cordifolia*. 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.

5.4 Cytotoxicity Screening of the Active Plant Extracts

According to Zirihi and colleagues (2005) and Kigondu and colleagues (2009), the plant extracts can be considered non-cytotoxic if their CC50 values is greater than 20 µg/mL (> 20 µg/mL)(Zirihi et al., 2005) (Kigondu et al., 2009). However, some studies have reported moderate and/ or high cytotoxicity of plant extracts on both normal and cancerous cells at values even above 100 µg/mL(Paraskeva et al., 2008). *Zanthoxylum leprieurii* has exhibited comparable cytotoxicity on a panel of cancer cells (liver cancer cell line, colon cancer and prostate cancer) with IC_{50} values ranging from 17 µg/mL to 88 µg/mL(Misra et al., 2013). In the present study, cell viability decreased with increasing extract concentrations for both plants in a dose dependent manner. Aqueous extracts of *Rubia cordifolia* exhibited similar patterns of moderate decrease in cell viability against monkey kidney cell line MA-104(Sun et al., 2016). Based on the findings reported in this study, there is a need to further investigate *in vivo* antimycobacterial activity and cytotoxicity of these plant extracts.

Selectivity index values help determine whether a plant extract could be a viable candidate for therapeutic use, especially in treating infectious diseases such as tuberculosis. Both the methanol (0.46) and ethanol (0.80) extracts of *Zanthoxylum leprieurii*, and the ethanol extract of *Rubia cordifolia* (0.80), exhibited low SI values, indicating that these extracts are relatively non-selective. They have comparable or higher toxicity to normal cells (J774.2 cell line) than their antimycobacterial efficacy. This suggests limited therapeutic potential due to the high likelihood of adverse effects on normal cells. The methanol extract

of *Rubia cordifolia* (SI = 1.20) had a slightly better selectivity, suggesting some level of preferential toxicity towards *Mycobacterium tuberculosis* compared to normal cells. However, an SI slightly above 1 still indicates that while it may be more effective against pathogens, there remains a notable level of cytotoxicity to healthy cells.

The selectivity of plant extracts is heavily influenced by their phytochemical profiles. For instance, *Zanthoxylum leprieurii* contains alkaloids, flavonoids, tannins, and saponins, which have been reported to possess antimicrobial properties but may also be cytotoxic to mammalian cells. The cytotoxic effects could stem from these compounds interacting with essential cellular components of both normal and pathogenic cells, thereby reducing selectivity(Okagu et al., 2021, Tuyiringire et al., 2022, Misra et al., 2013). Similarly, Rubia cordifolia is known to contain anthraquinones, naphthoquinones, and flavonoids, which are effective antimicrobial agents but can also be cytotoxic at higher concentrations or with prolonged exposure(Wen et al., 2022). The presence of these potent compounds explains why the extracts can inhibit microbial growth but also cause significant damage to healthy cells, resulting in moderate to low SI values. The extraction solvent plays a significant role in the profile of bioactive compounds. Methanol and ethanol are polar solvents that extract a broad range of bioactive compounds, including highly potent secondary metabolites. Methanol is particularly efficient in extracting both polar and semi-polar compounds, often leading to extracts with higher cytotoxicity(Ng et al., 2020). Some plants contain compounds that are inherently cytotoxic to a broad range of cells. This is often a desired feature in the context of antimicrobial or anticancer therapies, but it poses challenges in achieving selectivity. For instance, saponins and tannins, present in both *Zanthoxylum*

leprieurii and *Rubia cordifolia*, can cause membrane disruption leading to cell lysis, which affects both microbial and mammalian cells(Patra and Saxena, 2009, Wina et al., 2005).

CHAPTER 6: CONCLUSIONS AND RECOMMENDATIONS

6.1 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. *Zanthoxylum leprieurii* Guill. & Perr. and *Rubia cordifolia* L. extracts increase inhibitory activity of standard anti-tuberculosis drugs especially against MDR-MTB strain. This study verified local use of these plants in the traditional treatment of tuberculosis in Uganda. Their combination with conventional anti-TB therapy may enhance treatment outcomes, reduce drug resistance and improve MDR-TB patient care. 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 effect. Both plant extracts possessed moderate cytotoxicity on J744.2 cell line in a dose dependent manner. The low to moderate selectivity indices observed for *Zanthoxylum leprieurii* and *Rubia cordifolia* extracts indicate the need for careful evaluation of their therapeutic potential. These results highlight the importance of balancing efficacy with safety in the development of plant-based therapies. Based on the findings of the current study, there is a compelling need to determine the in vivo antimycobacterial and cytotoxic activities of these plant extracts.

6.2 Recommendations

We recommend that in vivo determination of antimycobacterial and cytotoxic activities of these promising medicinal plants be studied.

Cytotoxicity effects of the combination of standard antituberculosis drugs and these plant extracts should be evaluated especially in animal models.

We also recommend that studies to obtain dosage forms and determine the toxicities of the active extracts be carried out.

Exact mechanisms of action for these plant extracts should be elucidated.

Some of these highlighted recommendations form basis of our future aspirations to further the understanding of these promising antimycobacterial traditional therapies.

Integrating herbal medicines into TB care must be approached cautiously, with a focus on evidence-based practice, patient education, and close monitoring.

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LIST OF APPENDICES

Appendix 1: Standard Operating Procedures (SOPs)

1.1 Resazurin Microtiter Assay (REMA) SOP

Introduction

This standard operating procedure (SOP) describes the procedure for colorimetric redox indicator assay methods for *M. tuberculosis* isolates grown from conventional culture. This indirect method is based on the reduction of a colored indicator added to liquid culture medium on a microtiter plate after i*n vitro* exposure of *M. tuberculosis* isolates to anti-TB agents.

Purpose

The purpose of this SOP is to determine the activity of different drugs and other antimycobacterial agents on Mycobacterium tuberculosis (M.tb).

Principle

Colorimetric methods for detecting drug/extract activity in *M. tuberculosis* are based on the reduction of an oxidation-reduction indicator added to a liquid culture medium after *M. tuberculosis* has been exposed *in vitro* to different antimycobacterial agents. Activity/resistance is detected by a change in color of the oxidation-reduction indicator, which is directly proportional to the number of viable mycobacteria in the medium.

Samples

Pure cultures of acid-fast bacilli grown on solid media and liquid medium are used for indirect microscopic observation drug susceptibility assay.

Confirm the growth as *M. tuberculosis* using mycobacterial antigen 64(MPT64) or any other standard methods being practiced in the local laboratory.

Equipment and material

- 7H9 broth: Middlebrook 7H9 (Becton Dickinson)
- Oleic acid dextrose catalase (OADC) (10 × 20 ml Becton Dickinson)
- Glycerol
- Casitone (pancreatic digest casein Bacto Casitone)
- 96-well plate with lid: (box of 50 plate-Becton Dickinson)
- Filter 0.2 µm: Acrodisc Syringe filter (50 filters-Pall Life Sciences)
- Syringes for filter: 2 or 5 ml
- Distilled water (DW)
- Micropipettes: 1000 µl/200 µl
- Tips + tips with filter (1000 µl/200 µl)
- Multichannel pipette
- Resazurin dye: Resazurin sodium salt (Sigma®, USA)
- Analytic balance
- Incubator 37°C
- Autoclave

- Test extracts and Drugs: Rifampicin (RIF), isoniazid (INH).

Preparation of inoculum

Inoculum from growth on solid medium

Older cultures may result in unreliable susceptibility test results.

- Take a loop full of bacterial growth with a sterile loop and put it in a sterile vial with glass beads just covering the bottom of the vial with 2.5 ml of 7H9-S broth (try not to take any medium when removing growth)
- Vortex the vial for at least 1 min to break the clumps until a fairly turbid suspension is obtained
- Allow it to stand for 5 min
- Transfer the supernatant to a new sterile vial and leave it to sediment for 15 min
- Transfer the supernatant to a new sterile vial
- Compare the turbidity of the suspension using the scale McFarland 1.0 standard
- Set the turbidity of inoculum as per McFarland 1.0 standard using 7H9-S
- Make a 1:20 dilution of inoculum with 7H9-S broth

Inoculum from a liquid medium

- Transfer 2.5 ml of the pellet of culture to a sterile vial containing glass beads
- Vortex for at least 1 min until a fairly turbid suspension is obtained
- Allow it to stand for 5 min
- Transfer the supernatant to a new sterile vial and leave it to sediment for 15 min

- Transfer the supernatant to a new sterile vial
- Compare the turbidity of the suspension using the scale McFarland 1.0 standard
- Set the turbidity of inoculum as per McFarland 1.0 standard using 7H9-S
- Make a 1:20 dilution of inoculum with 7H9-S broth

REMA Procedure

In a 96-well flat-bottom microtiter plate there is enough space to test either four isolates against the two drugs or two isolates against four drugs, in six 2-fold dilutions of each drug.

- Add 100 µl of 7H9-S broth to all wells of the columns 2-11 from rows B to G
- Add 100 µl of the working solution of the test drugs/extracts to wells starting from B2 up to B9 depending on the number of drugs or isolates.
- With a multi-channel pipette make two-fold serial dilutions (transfer 100 µl) from rows B to G (columns 2-9) discarding the last 100 µl after mixing in row G
- Add 100 µl of 7H9-S broth to wells B11-E11; these will represent the negative and sterility controls on the test
- Inoculate plate with 100 µl of final culture suspension in respective culture wells including its respective growth control (GC) (+)
- Add 200 µl of sterile DW to all the outer wells left without broth; these will prevent evaporation during incubation of the plate.
- Cover the plate with lid, seal properly with parafilm from all sides or in zip lock bags and incubate at 37°C for 7 days.

Preparation of resazurin dye

Prepare a resazurin solution at 0.02% in sterile DW, sterilized by 0.2 μm syringe filter. The dye solution must be stored in dark at 4°C for 2 weeks.

Color development and interpretation of results

After 7 days incubation, the plates are taken from the incubator and 30 μl of the resazurin dye solution is added to all the wells and the plate again sealed and incubated **overnight** for color development.

A change in color from blue to pink means a growth of the isolates at that concentration of the drug. For better interpretation of the results, the color must be compared to the color present in the Growth Control (GC) well.

The minimal inhibitory concentration

Minimal inhibitory concentration (MIC) of each drug is interpreted as the lowest concentration of the antibiotic that prevents a change in color of the resazurin dye.

Positive and negative controls

The positive control should show positive growth and the negative control (NC) should show no growth within the incubation protocol period. If NC shows a growth, investigate procedures, could be a cross-manipulation and check all reagents for possible source of contamination.

1.2 MTT Cell Viability Assay-SOP

Introduction:

MTT Cell Viability Assay provides a simple method for determination of cell number using standard microplate absorbance readers. Determination of cell growth rates is widely used in the testing of drug action and cytotoxic agents, and screening other biologically active compounds.

Several methods can be used for such determinations, but indirect approaches using fluorescent or chromogenic indicators provide the most rapid and large-scale assays. Among such procedures, the MTT assay developed by Mossman is still one of the most versatile and popular assays.

Principle:

The MTT assay involves the conversion of the water soluble MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) to an insoluble formazan. The formazan is then solubilized, and the concentration determined by optical density at 570 nm.

Materials:

- MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MW = 414)
- SDS sodium dodecyl sulfate (MW = 288)
- Phosphate-buffered saline (PBS) pH 7.4
- Sterile HCl,
- 0.01 M solution dimethylsulfoxide (DMSO)

- Cell culture incubator
- ELISA plate reader
- Shaker
- Cell culture medium
- Dimethyl sulfoxide (DMSO)

Experimental Protocol

Reagent Preparation

1. Prepare a 12 mM MTT stock solution by adding 1 mL of sterile PBS to one 5 mg vial of MTT. Mix by vortexing or sonication until dissolved. Occasionally there may be some particulate material that will not dissolve; this can be removed by filtration or centrifugation. Each 5 mg vial of MTT provides sufficient reagent for 100 tests, using 10 µL of the stock solution per well. Once prepared, the MTT solution can be stored for four weeks at 4°C protected from light.

2. Add 10 mL of 0.01 M HCl to one tube containing 1 gm of SDS. Mix the solution gently by inversion or sonication until the SDS dissolves. Once prepared, the solution should be used promptly. Each tube makes sufficient solution for 100 tests, using 100 µL per well.

Procedure

1. Seed cells homogeneously in tissue culture plates at a density of 10,000 cells per well and let them attach overnight for 24 hours.

2. Change medium the next day, and then add various concentrations of the plant extracts (50 µg/ml, 100 µg/ml and 1000 µg/ml). Incubate for 48 hours.
3. Add aliquots of 20 µl of a 5 mg/ml MTT solution to the wells.
4. Incubate microtiter plates for 4 hours at 37^0C in a humidified incubator with 5% CO_2.
5. Thereafter, an aliquot of 100 µl of Dimethyl Sulphoxide (DMSO) or SDS will be added to the wells to dissolve formazan crystals that formed in metabolically active cells and incubated for 1 hour or 4 hours when SDS is used.
6. Mix each sample again using a pipette
7. The plates will be read at 570 nm on ELISA plate reader

Appendix 2: Publications

Scientific Research Publishing

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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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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*.

Botanical Name	Concentration (µg/mL)	*Mycobacterium tuberculosis* strains			
		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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Appendix 3. Research Ethics Committee Approval.

MBARARA UNIVERSITY OF SCIENCE AND TECHNOLOGY
P.O. Box 1410, Mbarara Uganda. Tel: +256 485433795, Fax: +256 4854 20782
RESEARCH ETHICS COMMITTEE
E-mail: sec.rec@must.ac.ug

Our Ref: MUREC 1/7 **Date:** March 25, 2019

Mr. Moses Mpeirwe
Postgraduate student

Re: **"Evaluation of antimycobacterial activity of *Zanthoxylum leprieurii* and R*ubia cordifolia* and their interaction with conventional anti-tuberculosis drugs used in treatment of tuberculosis in Uganda"03/02-19**

Mbarara University of Science & Technology
P. O. Box 1410, Mbarara
RESEARCH ETHICS COMMITTEE
24 MAR 2019
APPROVED
VALID UNTIL DATE SHOWN ABOVE

Type: [√] Initial Application
[ ] Protocol Amendment
[ ] Letter of Amendment (LOA)
[] Continuing Review
[ ] Other, specify: _____

Reference is made to the above protocol which was ~~resubmitted to the Research Ethics~~ Committee for reconsideration and approval under expedited review process.

It is noted that you have addressed all the concerns earlier raised by the Committee.

I am pleased to inform you that your study has been approved for a period of one year from **March 25, 2019** up to **March 24, 2020.**

As Principal Investigator of the research, you are responsible for fulfilling the following requirements of approval:

1. All co-investigator must be kept informed of the status of the research.
2. Changes, amendments, and addenda to the protocol or the consent form must be submitted to the REC for review and approval **prior** to the activation of the changes. The REC application number assigned to the research should be cited in any correspondence.
3. Reports of unanticipated problems involving risks to participants or other must be submitted to the REC. New information that becomes available which could change the risk: benefit ratio must be submitted promptly for REC review.
4. Only approved consent forms are used in enrolment of participants. All consent forms signed by subjects and/or witness should be retained on file. The REC may conduct audits of all study records, and consent documentation may be part of such audits.
5. Regulations require review of an approved study not less than once per 12-month period. **Therefore, a continuing review application must be submitted to REC eight weeks prior to the above expiration date of March 24, 2020 in order to continue the study beyond the approved period.** Failure to submit a continuing review application in timely fashion may result in suspension or termination of the study, at which point new participants may not be enrolled and currently enrolled participants must be taken off the study.

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6. You are required to register the research protocol with the Uganda National Council for Science and Technology (UNCST) for final clearance to undertake the study in Uganda.

The following is the list of documents approved in the application:

Document	Language	Version
Proposal	English	Version 2
Protocol form	English	Version 2
Data Collection tools	English	**March 2019**

I wish you all the best.

Dr. Francis Bajunirwe
CHAIR,
MUST RESEARCH ETHICS COMMITTEE

RESEARCH ETHICS COMMITTEE
MBARARA UNIVERSITY OF SCIENCE AND TECHNOLOGY
P.O. BOX 1410, MBARARA

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MBARARA UNIVERSITY OF SCIENCE AND TECHNOLOGY
FACULTY OF MEDICINE RESEARCH COMMITTEE

Ref: DMS 6

January 29, 2019

Mr Moses Mpeirwe
2017/MPhD/103/PS
Department of Microbiology
MUST

Dear Mr Mpeirwe

Submitted Proposal "Evaluation of Antimycobacterial Activity of *Zanthoxylum leprieurii* and *Rubia cordifolia* and their Interaction with Conventional anti-tuberculosis Drugs Used for the Treatment of Tuberculosis in Uganda

Reference is made to the above protocol you re-submitted to the Faculty Research Committee (FRC) for review.

The Committee reviewed and approved your protocol.

You are now advised to proceed to produce enough copies of the Protocol Form and Research Proposal for eventual submission to the Institutional Research Ethics committee. The Secretary to that Committee will advise you on the number of copies you are to submit.

Please remember to attach a letter from the Head of Department or your supervisor when submitting.

Sincerely

Associate Professor Paul Alele
Chairperson, Faculty Research Committee

Copy: Drs Joel Bazira and Duncan Sesaazi,. Supervisor