

Anti-mycobacterial activity and acute toxicity of *Erythrina abyssinica*, *Cryptolepis sanguinolenta* and *Solanum incanum*

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DECLARATION

I **Bunalema Lydia** declare that this dissertation has never been submitted to any other University or Academic Institution for purposes of getting an academic award. All the information in this dissertation is based on my observations.

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DEDICATION

I would like to dedicate this piece of work to my mum and dad George and Sarah Matovu and to my husband Dr. J. Wasswa.

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LIST OF ABBREVIATIONS

TB	Tuberculosis
WHO	World Health Organization
CDC	Center for Disease Control
XDR	Extensively Drug Resistance
MDR	Multi Drug Resistance
HIV	Human Immune Virus
AIDS	Acquired Immune Deficiency Syndrome
MIC	Minimum Inhibitory Concentration
JCRC	Joint Clinical Research Center
DMSO	Dimethyl sulfoxide

ABSTRACT

Tuberculosis (TB) kills approximately two million people annually. Efforts to treat the disease have been made much more difficult due to development of drug resistant TB strains (MDR and XDR TB) and co-infection with HIV AIDS. There is an urgent need therefore, to search for and develop new, inexpensive and effective anti-TB drugs. Extracts from three plants, *Solanum incanum*, *Cryptolepis sanguinolenta* and *Erythrina abyssinica* used in traditional medicine to treat TB symptoms were screened for anti-mycobacterial properties against a Rifampicin resistant, pan sensitive and *Mycobacteria avium* strains. In addition, the acute toxicity profile and phytochemistry of the active extracts were studied.

The chloroform extract of *E. abyssinica* was the most active on *M. avium* wild strain and the rifampicin resistant strain (MIC= 0.3 and 0.39 mg/ml respectively). Against the pansensitive strain the methanol total crude extract was most active (MIC= 0.2 mg/ml). *C. sanguinolenta* total crude methanol extract was also active against the three strains of mycobacteria; however *S. incanum* did not show activity on any of the strains. Toxicity studies showed that the two plants had an LD₅₀ of more than 500mg/kg body weight and thus considered to be safe.

E. abyssinica extracts and *C. sanguinolenta* total crude extract contained alkaloids, terpenoids, tannins and flavones. Saponins and phenols were not detected in all extracts.

C. sanguinolenta and *E. abyssinica* have potential to be developed into new anti-TB drugs. The results have also validated traditional knowledge from the local people regarding the use of these species to treat TB.

CHAPTER ONE

INTRODUCTION

1.1 Background

1.1.1 Current status of Tuberculosis

Tuberculosis (TB) is one of the leading causes of morbidity and mortality globally. The global mortality rate stands at two million deaths per year with one third of the world's population infected with the bacilli (Centre for Disease Control (CDC), 2005; Sanjay, 2004; World Health Organisation (WHO), 2007). It is estimated that 9.2 million new cases are diagnosed every year. According to the World Health Organization (WHO), the incidence of tuberculosis in African countries more than doubled between 1990 and 2005 and is taking an upward trend. (WHO,2008). According to Chaisson and Martinson (2008), Africa carries 29% of the world's disease burden and 34% of the world's total death rate. Uganda ranks 15th among the world's 22 countries with a high tuberculosis burden; with an estimated incidence of 355 cases per 100,000 people per year and mortality of 84 deaths per 100,000 people per year (WHO, 2008).

The emergence of drug resistant strains of *Mycobacterium tuberculosis*, is one of the major reasons contributing to the rise in global incidence of tuberculosis since 1980. Multi Drug Resistant (MDR) forms are defined as *M. tuberculosis* strains resistant to atleast Rifampicin and Isoniazid: the first line drugs used in treatment of tuberculosis. Extensively Drug Resistant TB (XDR) is tuberculosis caused by strains resistant to first line drugs, to fluoroquinolones and at least one of three injectable second-line drugs; capreomycin, kanamycin, and amikacin (Lawn and Wilkison, 2006).

According to Zignol *et al.*, (2006), more than 420,000 TB cases world wide are due to MDR strains of *M. tuberculosis*; 40,000 of these occur in Africa. In Uganda, 0.7% of all new cases of tuberculosis are multi drug resistant (WHO, 2008). MDR TB treatment requires the use of second-line drugs (SLDs) that are less effective, more toxic, and more costly than the first-line isoniazid and rifampin-based regimens (CDC, 2005). This has made efforts to control tuberculosis much more difficult. The situation is made worse by co-infection with HIV among TB patients. Autopsy studies have shown that 50% of the 40 million HIV-infected individuals die of tuberculosis (Corbett *et al.*, 2003). Treatment of TB patients co-infected with HIV/AIDS has been associated with treatment failure, relapses, acquired drug resistance in addition to drug interactions that increase the risk of toxicity (Peloquin *et al.*, 1996; Chan and Iseman, 2002).

1.1.2 Natural products in drug discovery and development

Natural products have continued to provide new and important leads in the drug discovery process (Balunas and Kinghorn, 2005). Natural products or their semi synthetic derivatives have indeed provided novel drug leads for tuberculosis therapy (Shu, 1998). Examples of such compounds include Streptomycin and Kanamycin from *Streptomyces griseus* and capreomycin isolated from *S. capreolus* (Copp, 2003; Shu, 1998). Rifampicin is a semi-synthetic drug that has been derived from Rifamycin a product of *Amiclatopsis mediterranei* (Tribuddharat and Fennewald, 1999).

The plant kingdom can be looked at as an important source of new drugs for the treatment of tuberculosis because of its enormous chemical diversity (Gautam *et al.*, 2007). Several drugs have been derived from medicinal plants and some of these include quinine from cinchona tree,

codeine and morphine from *Papaver somniferum* and the artemisinin derivatives from *Artemisia annua* (Chin *et al.*, 2006).

In 2007, an ethno botanical survey to identify plants used to treat tuberculosis in the Lake Victoria basin was carried out (Okemo, *et al.*, unpublished). Medicinal plant species were reportedly used by traditional practitioners to treat TB. However, in Uganda though the plants have been reported to be widely used by traditional healers in TB treatment, their efficacy against *Mycobacterium tuberculosis* and safety have not been scientifically validated. This study investigated the efficacy and toxicity of *Solanum incanum*, *Cryptolepis sanguinolenta* and *Erythrina abyssinica* against *Mycobacterium tuberculosis*.

1.2 Statement of the problem

Among infectious diseases, tuberculosis is one of the leading killers of adults in the world today. The incidence of tuberculosis is exacerbated by the emergence of drug-resistant strains (MDR and XDR) and HIV co-infection (Furin, 2007). Available treatment regimens are lengthy and complex, inviting problems of non-adherence and inadequate response. In the case of MDR TB, second line drugs used are more toxic and expensive while XDR TB is virtually untreatable. HIV/AIDS patients presenting with tuberculosis stand a risk of drug adverse reactions as a result of possible drug-drug interactions. On the other hand a number of traditional medicinal plants have been reported to treat tuberculosis, however their efficacy and safety remains unknown. This study was conducted to determine the efficacy and safety of some of the plants that have been suggested in treatment of TB symptoms by traditional healers.

1.3 Objectives

1.3.1 General objective

To determine the anti-mycobacterial activity and safety of extracts from the traditional medicinal plants *Erythrina abyssinica*, *Cryptolepis sanguinolenta* and *Solanum incanum*.

1.3.2 Specific Objectives

- i) To determine the anti-mycobacterial activity of *Erythrina abyssinica*, *Cryptolepis sanguinolenta* and *Solanum incanum*.
- ii) To determine the acute toxicity profile of crude extracts found to possess anti-mycobacterial activity in mice.
- iii) To determine the compounds found in some of the crude extracts which have anti-mycobacterial activity.

1.4 Justification of the study

Trends in the incidence of tuberculosis together with the development of multi-drug and extensively drug resistant strains of tuberculosis raises the need to intensify the search for more efficient drugs to combat this disease (CDC Report, 2005; Corbett *et al.*, 2003). There are widespread claims by some traditional healers that TB can be treated using herbs. However these claims have no scientific justification mainly because, Uganda has one of the least published literature on plants screened for anti-mycobacterial activity (Kirimuhuzya *et al.*, 2009). The situation is further complicated by an increase in loss of biodiversity within the country (Bumpi and Kayondo, 2009). The results of this study will go along way to authenticate the claims by

traditional healers and will as well enrich the databases on plants with anti-mycobacterial activity that can be used in drug discovery.

1.5 Significance of the study

This study will provide information on the safety and efficacy of *E. abyssinica*, *C. sanguinolenta* and *S. incanum*. This information could lead into promotion or discourage further use of the plants. Research elsewhere in the world has been done on anti-mycobacterial agents derived from natural products especially plants, however little scientific work has been done on such plants in Uganda. This study adds knowledge and identifies plants that could be sources of lead compounds for new tuberculosis drug development. The study was therefore aimed at answering the following questions:-

- i) Does *Erythrina abyssinica*, *Cryptolepis sanguinolenta* and *Solanum incanum* have antimycobacterial activity?
- ii) If any of the crude extracts of the two plants is active, what is their acute toxicity profile in mice?
- iii) What are the classes of compounds contained in *Erythrina abyssinica*, *Cryptolepis sanguinolenta* and *Solanum incanum* ?

CHAPTER TWO

LITERATURE REVIEW

2.1 Etiology of tuberculosis

Tuberculosis (TB) is caused by different tubercle bacilli from the genus *Mycobacterium* (Sensi and Grassi, 2003). The genus contains over 50 species including *Mycobacterium tuberculosis*, the most common pathogen for humans, *M. africanum* common in West Africa and *M. bovis* that causes infection in animals but can also infect humans (Gautam *et al.*, 2007; Adeniyi *et al.*, 2004). Other members of *Mycobacteria tuberculosis* complex include *M. microti*, *M. pinnipedii* and *M. bovis*. Further more *M. avium*, *M. scrofulaceum* and *M. intracellurae* are potentially pathogenic species that have been reported to cause opportunistic infections in HIV/AIDS patients. Other species, *M. chelonae*, *M. vaccae* and *M. fortuitumare* are important causes of cutaneous, pulmonary and nosocomial infections (Newton *et al.*, 2000; Adeniyi *et al.*, 2004).

Mycobacteria are non motile rod shaped obligate aerobes that stain weakly with dyes (Bruton *et al.*, 2007). They are characterized as acid fast bacteria because once stained by dyes or stains, they will remain stained after treatment with acidified organic material (Madison, 2001). One of the major characteristics of *mycobacteria* is that it is slow growing and may remain inactive in the host for a long period. This characteristic contributes to its virulence (Tripathi *et al.*, 2004). Secondly, the cell wall of *mycobacteria* is 60% lipophilic containing lipids like mycolic acids and wax D; this hinders antibacterial agents from penetrating the cell (Some *mycobacteria* reside in macrophages thus adding yet another penetration barrier for the anti TB agents) (Tripathi *et al.*, 2004).

2.2 Epidemiology

Tuberculosis is one of the leading causes of illness and mortality worldwide; it has a mortality rate and incidence rate of approximately 2 million deaths per year and 9.2 million new cases respectively (CDC, 2005; WHO, 2007). WHO (2004) estimates show that one third of the world's population is infected with the mycobacterium that causes TB and that 5,000 people die daily from TB.

According to Adeniyi *et al.*,(2004), 95% of the world's TB burden is in developing countries. Sub-Saharan Africa has the highest incidence rate of 29% of the world's burden and 34% of the world's total death (Chaisson and Martinson, 2008). Between 1990 and 2005, the average incidence of TB in Africa more than doubled from 149 to 343 per 100,000 population and is still rising at a rate of 3-4% annually (WHO, 2007).The high incidence rate, morbidity and mortality of TB in Africa has led WHO to declare TB an emergence in the continent. HIV AIDS, resistance of TB to first line drugs, poverty and overcrowding are some of the reasons that explain the increase in Africa (Tripathi *et al.*, 2004).

Uganda ranks 15th among the world's 22 countries with the highest TB burden (WHO, 2007). It has approximately 112,000 new TB cases per year, an estimated incidence of 355 cases per 100,000 populatios and mortality of 84 deaths per 100,000 pop/year.

2.3 Treatment of Tuberculosis

Effective treatment of TB involves targeting the multiple populations of bacteria that reside in the host (Bryrne *et al.*, 2007). The World Health Organisation in its new strategy to stop TB recommends short course chemotherapy as a standardized TB treatment regimen. Each regimen

has an initial phase of about two months as the intensive phase followed by a choice of several options for the continuation phase of either four or six months (American Thoracic Society *et al.*, 2003). For new TB cases, treatment involves use of isoniazid (INH), rifampicin (RMP) and pyrazinamide (PZA) plus either streptomycin or ethambutol for the first two months followed by four months of isoniazid and rifampicin (Fattorini *et al.*, 2007). However, in cases where acid fast bacilli smear and culture are negative, a regimen with isoniazid, streptomycin, rifampicin and pyrazinamide has been recommended (Tripathi *et al.*, 2004).

The lengthy therapy results in poor patient compliance and the emergency of drug resistance (Bryne *et al.*, 2007). In order to improve adherence and cure rates WHO recommends the use of Directly Observed Treatment shortcourse (DOTs) in which patients are observed ingesting their medicine by a trained personnel (American thoracic society *et al.*, 2003). Several studies have confirmed that DOTs has significantly improved adherence and treatment completion (Jasmer *et al.*, 2004; Nahid *et al.*, 2006).

Treatment of patients with MDR-TB is much more difficult and relies extensively on second-line drugs that include fluoroquinolones, ethionamide, the aminoglycosides capreomycin, cycloserine, para-aminosalicylic acid, and clofazimine. These agents have poorer activity than the first-line drugs and greater tendency to cause adverse reactions. Fluoroquinolones such as moxifloxacin and levofloxacin have considerable activity against *M. tuberculosis* and are preferred in the treatment of all MDR-TB cases, unless resistance to this class is also demonstrated as the case is in Extensively Drug Resistant TB strains (XDR). XDR-TB is that which is resistant to any fluoroquinolone, and at least one of three injectable second-line drugs

(capreomycin, kanamycin, and amikacin), in addition to MDR-TB (Nachega and Chaisson 2003; Furin, 2007).

Previously discovered chemical structures in the curtailment of TB include; thiolactoycin and tryptanthin which have demonstrated significant activity against MDR TB. However lack of *in-vivo* toxicity profile has greatly hindered their use in humans (Tripathi *et al.*, 2004).

2.4 Challenges in the control of TB

One of the major challenges in TB control is the development of resistance that is Multi Drug Resistant TB (MDR TB) and Extensively Drug Resistant TB (XDR TB). MDR TB is TB that is resistant to at least rifampicin and isoniazid while XDR TB is TB that is resistant to all the first line drugs, any flouroquinolone and one of the injectable second line drugs (Fattorini *et al.*, 2007). The annual global burden of MDR TB ranges from 300,000-600,000 cases according to CDC (2006) and 40,000 of which occur in Africa (Zignol *et al.*, 2006). In Uganda, 0.7% of all new cases are multi drug resistant TB cases (WHO, 2008).

MDR TB treatment requires the use of second-line drugs (SLDs) that are less effective, more toxic, and costly than first-line isoniazid- and rifampin-based regimens (CDC, 2005). In addition, therapy should be based on individual drug susceptibility testing including residual first line and second line drugs (Fattorini *et al.*, 2007). If MDR TB is not well managed, XDR TB will eventually develop which poses a much more powerful hindrance for management of TB since it is virtually untreatable (CDC, 2006).

2.5 *In-vitro* assays for evaluation of anti tubercular activity

Anti-mycobacterial activity of plant extracts is usually done by culturing mycobacteria in ranging types of Agar and broth based media (Newton *et al.*, 2000). These methods are: Agar well/disc diffusion method, Macro and micro dilution method and Micro plate alamar blue assay.

The Agar well/disc diffusion method is one of the most commonly used methods (Newton *et al.*, 2000). In this method, wells or micro discs are impregnated with the drug extract and placed on to the inoculated medium; zones of inhibition are then measured after a period of incubation (Parish and Stoker, 1998). The major disadvantage with such a method is that hydrophilic compounds may not diffuse thus can be missed owing to the fact that mycobacteria cell wall is lipophilic (Gautam *et al.*, 2007; Connel and Nikaido, 1994). Secondly, these are non quantitative methods but only indicative of whether there is activity or not (Newton *et al.*, 2000).

In the macro and micro dilution method known concentrations of the extracts are tested on the bacteria in agar media (Pauli *et al.*, 2005). The method allows for quantification and determination of minimum Inhibitory concentration (Gautam *et al.*, 2007). The medium is supplemented with oleic acid, albumin, dextrose and catalase (OADC supplement Difco) (Pauli *et al.*, 2005). The major disadvantage with the method is that it requires at least 18 days to visibly detect growth of the colonies (Gautam *et al.*, 2007).

Micro broth dilution method is used in the determination of Minimum Inhibitory Concentration. In this method serial dilutions of the extract are placed in different test tubes containing inoculated broth (Suffredin *et al.*, 2004). Quantification is done by visualizing turbidity in the test tube. This poses a disadvantage of misinterpretation due to the tendency of mycobacteria to clump and also crude extracts may impart some turbidity to the medium (Gautam *et al.*, 2007).

The use of a redox indicator dye (alarmar blue) makes this test not only rapid but also sensitive (Gautam *et al.*, 2007). Micro plate alamar blue assay (MABA) can be read visually without necessarily using instrumentation (Franzblau *et al.*, 1998). The reduced form of the dye can also be quantified calorimetrically by measuring absorbance at 570nm (Pauli *et al.*, 2005)

2.6 Role of Natural products in drug development

The ability of plants to synthesize aromatic substances and secondary metabolites has been of importance in drug development (Cowan, 1999). Some of the most important compounds of plants with medicinal uses are alkaloids, tannins, flavonoids, sterols and phenols (Edeoga *et al.*, 2005). These compounds can be extracted from plants by use of different solvents ranging from alcohols, chloroform, ether, hexane and water. Alcohols and water solvents extract the polar components of the plant while hexane and ether extract the non polar compounds (Cowan, 1999).

Natural products or their semi synthetic derivatives have in recent years provided novel drug leads in tuberculosis chemotherapy (Shu, 1998). Examples of such compounds include Streptomycin and Kanamycin from *Streptomyces griseus* (Copp, 2003), Capreomycin isolated from *S. capreolus* (Shu, 1998). Rifampicin is a semi synthetic drug that has been derived from rifamycin a product of *Amycolatopsis mediterranei* that has been in natural environments for a long time (Tribuddharat and Fennewald, 1999).

Like wise the plant kingdom continues to provide new and important leads against various pharmacological targets (Balunas and Kinghorn, 2005). Several drugs have been derived from medicinal plants. These include quinine from cinchona tree, codeine and morphine from *Papaver somniferum* and artemether, artemisinin, an antimalarial agent isolated from *Artemisia annua*

(Chin *et al.*, 2006). Owing to the plant kingdom's enormous chemical diversity, it can be looked at as an important source of new TB agents (Gautam, *et al.*, 2007).

A number of anti-mycobacterial compounds have been isolated from plants used in traditional medicine though they have not so far yielded comparable potency to those from micro organisms (Newton *et al.*, 2000). According to Gautam *et al.*, (2007), 70% of 365 plant species in India have shown anti-mycobacterial activity and chemical derivatives like Indicanine B and C, have been isolated from *Erythrina variegata* and *E. indica* (Waffo, *et al.*, 2000).

2.7 Ethnobotany and pharmacology of *Erythrina abyssinica*, *Cryptolepis sanguinolenta* and *Solanum incanum*

The study species in this project were *Erythrina abyssinica*, *Cryptolepis sanguinolenta* and *Solanum incanum*. These species were mentioned by traditional healers in Uganda to be used in the treatment of tuberculosis symptoms and chest related infections according to an ethno botanical survey that was done by Okemo *et al.*, (un published). The berry, stem bark and Root bark of *S. incanum*, *E. abyssinica* and *C. sanguinolenta* respectively were mentioned to be the parts most used (Okemo, *et al.*, un published).

Previous studies on *E. abyssinica* have shown that the stem bark as well as the root bark of the plant have weak antibacterial activity against *Bacillus subtilis* and *Staphylococcus aureus* (Mageresi *et al.*, 2008; Hamill *et al.*, 2003). Antifungal studies on the methanol and chloroform extracts of the stem bark and leaf of *E. abyssinica* showed activity against *Candida albicans*, the leaf chloroform extract had the highest activity (Hamill *et al.*, 2003). However a study done by Hamza *et al.*, (2006) indicated that the methanol extract of *E. abyssinica* showed no activity against *Candida* sp and he attributed this to the use of different methodologies and solvents.

The genus *Erythrina* produces an enormous array of alkaloids, phenolics and chalcones (Copp, 2003). One of the structures that have been elucidated from *E. gibbosa* includes an isoflavonoid compound called Erythrabyssin II that occurs in the root bark of the plant. This compound was found to have weak antibacterial activity against *S. aureus* and *B. subtilis* while its derivatives have significant activity against mycobacteria (Copp, 2003; Waffo et al., 2000). Additionally, *E. indica* contains indicanine B which also inhibits growth of *M. smegmatis* with an MIC of 18.5µg/ml.

Anti plasmodial activity of *E. abyssinica* has also been evaluated in a study done by Yenesew *et al.*, (2004). The ethyl acetate extract of the stem bark of the plant was found to be active against the chloroquine-sensitive (D6) and chloroquine-resistant (W2) strains of *Plasmodium falciparum*. A chalcone and a flavonone (5-deoxyabyssinin (II)) were responsible for the activity.

Hygroscopic crystals isolated from the fruit of *S. incanum* by Mbaya and Mohammed (1976) showed antibacterial activity against both Gram-positive and Gram-negative bacteria. In addition, the crystals were also active against *Candida* sp but even more active against dermatophytes. The methanol extract of the leaf were shown to be active against *C. glabrata* and *C. tropicalis* (Hamza *et al.*, 2006). The extracts of *S. incanum* and *S. nigrum* have been found to show cytotoxicity effects against human hepatoma cells. The compound solamargin has been isolated from *S. nigrum* and it is responsible for the above activity (Al- Fatimi *et al.*, 2007).

The climbing liana *C. sanguinolenta* has long been used in treatment of malaria by traditional healers in Africa. Two alkaloids responsible for the anti malarial effect have been isolated and identified as cryptolepine and isocryptolepine from the root bark of the plant. Cryptolepine had

IC₅₀ in the range of 0.2 to 0.6 μ MSC while isocryptolepine had IC₅₀ of about 0.8 μ MSC (Frappier *et al.*, 1995).

Cryptolepine, has been evaluated for anti-mycobacterial activity against fast growing mycobacteria namely *M. fortuitum*, *M. smegmatis*, *M. phlei*, *M. abscessus* and *M. aurium* using the micro dilution method. The compound was found to be active on all the above mycobacteria (Gibbons *et al.*, 2003). However studies on the slow growing *M. tuberculosis*, the leading cause of TB in humans has not been done. This is one of the objectives of this study.

The same alkaloid has been evaluated for its anticancer effects and according to Ansha and Goonderman (2002) cryptolepine was cytotoxic to V79 cells and a number of cancer cell lines.

The antibacterial and antifungal activities of *C. sanguinolenta* have been demonstrated in a study by Silva *et al.*, (1999).

2.8 Toxicity studies

Toxicity tests are carried out on animals in order to determine how toxic a chemical is and also to determine the starting dose in humans. Animals such as mice, rats, guinea pigs, rabbits, dogs, cats or monkeys (laboratory animals) are used. The animal tests that may be performed include acute, sub-acute and chronic toxicity tests as well as some special tests for effects like carcinogenicity and teratogenicity, as described by Ghosh (1984).

Acute toxicity studies are those carried out within 24 hours with single dose administration of the drug to two species, one rodent (mice or rats) and one non rodent (rabbits). It is aimed at establishing the therapeutic index (LD₅₀/ED₅₀). The higher the index the safer the compound (Gosh 1984). A chemical is considered to be extremely toxic if it has LD₅₀ of 1mg/kg and practically non toxic if it has an LD₅₀ of 1500mg/ kg and above (Gosh 1984).

Sub acute tests are tests in which animals are dosed daily for two to three weeks. The animals that are preferably used in such a test are rats and dogs. The starting dose should be around the expected therapeutic levels and this should be increased stepwise every two to three days until toxic signs are observed. The purpose of this test is to determine the maximum tolerated dose and nature of toxic reactions (Gosh 1984).

Chronic tests are those in which animals are dosed daily for a period of six months. Two species are used one rodent (rat) and another non rodent usually a dog or monkey. During the course of the tests several parameters are measured and these include; body weight, food intake, haematology, renal function pulse rate and blood pressure. At the end of the test all animals are sacrificed and autopsy performed (Gosh 1984).

Results of the tests in animals can then be extrapolated onto human beings. However, man is generally six times as sensitive as the dog, and ten times as sensitive as the rat to the toxic effects of drugs (Ghosh 1984). There are also metabolic variations and all these have to be put into account (Ghosh, 1984). Tests for cytotoxicity may be done using lower organisms such as brine shrimp, mosquito larvae or cell lines. Special tests may also be done to determine teratogenic effects of the drug and also whether the chemical is carcinogenic or not (Gosh 1984).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study design

This study was conducted using an experimental design. Three plant species were studied that is *Erythrina abyssinica* (Lam) (Eggirikiti in Luganda), *Cryptolepis sanguinolenta* (Kafulu in Luganda) and *Solanum incanum* (Ruiz & Pav) (Entengotengo in Luganda). The stem bark of *E. abyssinica* and fruits of *S. incanum* were collected from Mukono district while the root bark of *C. sanguinolenta* were collected from Kayunga district. Crude extracts were then prepared and tested *in-vitro* on three strains of mycobacteria. Susceptibility tests and minimum inhibitory concentration for the active extracts were determined. Acute toxicity tests for the most active extracts were also performed.

3.2 Selection criteria

Study plants were selected following three main criteria: these three plants have been mentioned by four traditional healers in the districts of Mukono and Kayunga to be used in the treatment of TB. Secondly the plants have never been worked on for their anti-mycobacterial activity in Uganda. Thirdly other species from the same genera elsewhere in the world have shown significant activity against mycobacteria.

3.3 Plant collection and identification

Fruits of *S. incanum*, stem bark of *E. abyssinica* and root bark of *C. sanguinolenta* were harvested from Mukono and Kayunga districts. To ensure quality of collected material, only plants judged as mature were harvested during a rainy season. A Parataxonomist identified the

collected specimens; voucher specimens were prepared, scientifically identified and kept in the Makerere University herbarium.

TABLE 1: List of the medicinal plants used in the bioassay with their voucher numbers

Voucher number	Scientific name	Local name	Family	Part collected	Collected from
JRST784	<i>Erythrina abyssinica</i>	Eggirikiti	Papilionaceae	Stem bark	Mukono 1220m 00°20N 32°44E
JRST786	<i>Solanum incanum</i>	Ntengontengo	Solanaceae	fruit	Mukono 193m 00°11N 32°17E
JRST778	<i>Cryptolepis sanguinolenta</i>	Kafulu	Periplocaceae	root bark	Kayunga 1057 m 1°12.877'N 32°52.471'E

3.4 Drying and pulverizing

The plant parts were dried under shade inside a room to avoid direct sunshine that could degrade some of the compounds in the plants. They were also spread out and regularly turned over to avoid fermenting and rotting. The dried parts were pulverized using a wooden pestle and mortar. The powder was weighed using an analytical scale and stored at room temperature.

3.5 Extract preparation

The total crude methanol extract was prepared by soaking the powder (500g) of each dried plant in methanol (1000ml) for seven days; then 500g of the plant powder was serially extracted by

soaking in ether (1000ml), followed by chloroform (1000ml) and lastly methanol (1000ml) in the order of increasing polarity of the solvents for three days each with occasional shaking. Whatman's filter paper no 1 was used for filtering to obtain the crude extract. The crude solution was then concentrated to a minimum volume by a rotary evaporator (Büchi Labortechnik AG, Switzerland) at 40°C and reduced pressure. The concentrated crude extracts were allowed to evaporate to constant weight at room temperature. The drying, extraction and concentration processes were done in the Phytochemistry laboratory in the Department of Pharmacology and Therapeutics Makerere University College of Health Sciences.

3.6 Mycobacterial tests

These tests were done in a level three level safety laboratory at Joint Clinical Research Center (JCRC) located in Mengo Kampala. The three preserved strains of *Mycobacteria* used were obtained from JCRC. They included a rifampicin- resistant strain (TMC 331strain) as a good indicator of MDR, a fully susceptible strain (H37Rv) as a control and *Mycobacterium avium* (MA) a wild strain from a Ugandan patient to represent mycobacteria other than tuberculosis strains (MOTT).

3.6.1 Growth media

Middle brook 7H10 agar (Becton Dickinson Company (DifcoTM), 7 Loveton Circle, Sparks, Maryland, USA; Lot No. 8175150) supplemented with oleic acid-albumin- catalase (OADC) (Becton Dickinson Company Lot 8136781) was used for reviving and culturing the mycobacteria for sensitivity testing. It was prepared by adding dehydrated medium (19.0g) to purified water (900ml) containing glycerol (15.0ml). The mixture was stirred well to dissolve and afterwards

autoclaved at 121°C for 10 minutes. Oleic acid-albumin catalase (100ml) was aseptically added to the medium after cooling to 45°C. No adjustment for PH was made.

3.6.2 Preparation of inoculum for drug sensitivity testing

Preserved strains of mycobacteria were revived on Middle brook 7H10 agar, prior to anti tuberculosis susceptibility testing. Cells were scraped from freshly growing colonies (three weeks old) on Middle brook 7H10 plates and introduced into saline (10ml). Bacterial suspensions with 0.5 McFarland standard turbidity equivalents to 10^8 CFU were prepared by dilution with saline. The mixture was vortexed for 30 seconds in a glass bottle containing glass beads and the particles allowed to settle (Parish and Stroker, 1998).

3.6.3 Bioassay protocol for susceptibility tests

The disc diffusion method was used to determine susceptibility as described by Parish and Stroker (1998).

3.6.3.1 Preparation of the drugs/ extracts

The dried crude extracts (1 g) were each dissolved in analytical grade methanol (20ml) to give a concentration of 50 mg/ml. Extracts were sterilized using 0.2 μ m single use filters. For rifampicin, a stock solution of 5.0 mg/ml was prepared by dissolving 0.1g in 10ml of methanol. A stock solution of 2.5mg/ml of isoniazid was prepared by dissolving 0.1 g in 20ml of distilled water.

3.6.3.2 Preparation of biodiscs

A concentration of 20 µg for each of the drugs and extracts was used per disc, for the general susceptibility tests so that for the extracts each disc contained 10mg of the extract; 0.05mg/disc of isoniazid and 0.1mg/disc for rifampicin. The discs were left in a hood to dry for 24 hours.

3.6.3.3 Procedure

The culture medium was sterile Middle brook 7H10 agar placed in 90mm diameter Petri dishes with quadrants. In each quadrant of the Petri dish, 5.0 ml of the medium was put. The solidified medium in the quadrants was inoculated using a swab. A rifampicin impregnated disc was placed in the first quadrant with a concentration of 0.1mg/ml. In the second quadrant isoniazid impregnated disc containing 0.05mg/ml was placed. The third quadrant had an extract impregnated disc containing 10mg. Finally the fourth quadrant contained a blank disc as a negative control.

All the tests for the extracts and the three strains of mycobacteria were done in triplicate. The Petri dishes were then left in the hood overnight to allow diffusion of the extracts and drug and then sealed with a carbon dioxide-permeable tape. These were then incubated at 37°C in a carbon dioxide incubator for four weeks. The sensitivity of *M. tuberculosis* and *M. avium* to the extracts and the drug was determined by measuring the zones of inhibition surrounding the disc using a millimeter scale.

3.6.4 Determination of the Minimum Inhibitory Concentration (MIC)

Microtitre plate method was used in the determination of MIC. Serial dilutions of the drugs/extracts were used to determine the Minimum Inhibitory Concentration of the drug or extract, using Middle brook 7H9 as the medium (Parish and Stroker, 1998).

3.6.4.1 Preparation of the medium (Middle brook 7H9)

The powder (4.7g) was suspended in purified water (900ml) containing glycerol (2ml) and autoclaved at 121°C for 10 minutes. OADC enrichment (100mls) (Becton Dickinson Company, Lot 8136781) was aseptically added to the medium when cool for enrichment. The medium was from Becton Dickinson Microbiology Systems of Becton Dickinson Company (Difco™), 7 Loveton Circle, Sparks, Maryland, USA; Lot No. 5123072.

Procedure

The procedure followed was that described by Parish and Stroker (1998) with some modifications. Middle brook 7H9 broth (100µl) was dispensed into all the wells of a sterile 96-well microtitre plate. In the first column, rifampicin (100 µl) at a concentration of 50mg/ml was added using a pipettor. The drug was mixed well by sucking up and down six times using a pipetter. 10 fold dilutions were made up to column 10 by pipetting 100 µl from column 1 and adding it to column 2 and then taking 100 µl from column 2 into column 3. The procedure was repeated up to column 10. From column 10, 100 µl were discarded instead of placing it into column 11. With a pipettor 5 µl of bacteria (10^4 - 10^5 CFU/ml) were dispensed in columns 1 to 11. Plate 12 was left blank as a sterility control. The procedure was repeated for the remaining drug/extracts in rows 2-5. The plates were incubated at 37°C for three weeks. The MIC tests for

the three strains of mycobacteria were done in duplicate. The lowest concentration with no visible turbidity was taken to be the minimum inhibitory concentration.

3.7 Acute toxicity tests

Acute toxicity tests on the most active extracts were carried out on white albino mice, *Mus musculus* as described by Ghosh (1984) with a few modifications. Total crude extracts of *C. sanguinolenta* and *E. abyssinica* were worked on. A pilot study was carried out on pairs of mice of both sexes aged four weeks, after fasting them overnight. Widely separated doses of 50,200,500, 800 and 1000 mg/kg were orally administered using a gastro intestinal tube to the mice to determine approximate lethal and non lethal dose ranges. From the pilot study the approximate LD₅₀ was found to be at 800mg/kg.

The actual study involved selecting five different groups with five mice in each. These were orally administered with 700,750,800,850 and 900mg/kg body weight as a single dose. Mice (*Mus musculus*) of both sexes, for each concentration were used and they were fasted overnight before giving them the extracts orally using a gastro intestinal tube. The control group was given DMSO which was the solvent used to dissolve the extracts. Observations were made and recorded after 24 hours. LD₅₀ was then determined through plot of a graph. The tests were done from the animal house in the Department of Pharmacology and Therapeutics.

3.8 Qualitative phytochemical testing

In the most active extracts qualitative tests for Terpenoids, Tanins, flavones and alkaloids were carried out as described by Edeoga *et al.*, (2005).

Terpenoids were tested for by adding chloroform (1ml) to the extract (1ml) and then an equal volume of concentrated sulphuric acid was added. Formation of a bluish red coloration indicated presence of terpenoids.

Tannins were tested for by boiling the dried powdered extract (0.5g) with water (20ml) in a test tube. 2ml of 0.1M FeCl_3 were added. Formation of a blue black coloration indicated presence of tannins.

Flavones were tested for by adding ammonium solution (5ml) to 1ml of aqueous filtrate of the extract followed by addition of sulphuric acid (2ml). A yellow coloration indicated presence of flavones.

Alkaloids were tested for by mixing 50g of the powder with 250ml of 1% sulphuric acid. It was allowed to stand and then filtered. 10mls of the filtrate was shaken and added to Meyer's reagent. Formation of a white precipitate indicated presence of alkaloids.

3.9 Data analysis

The numerical data from the replicated investigations is presented in form of tables and histograms. Statistical analysis involved use of the statistics computer program, Graph pad prism version 5.0. For toxicity studies, probit was plotted against log dose while the standard deviation was calculated for the concentrations.

3.10 Ethical considerations

Ethical approval was sought from the Research and Ethics Committee of the Faculty of Medicine and the Uganda National Council for Science and Technology (NS 141). Protection of the investigators was ensured by carrying out the work in collaboration with, and under the guidance

of the Mycobacteriology laboratory staff at the Joint Clinical Research Centre, Mengo in Kampala, who had the necessary expertise in handling *M. tuberculosis*. Additionally, the necessary protective wears including respirators and gloves as well as safety cabinets were used, to minimize the risk of exposure to *M. tuberculosis*.

Guidelines for the handling of Laboratory animals were followed. Animals were sacrificed under general anesthesia (Ghosh, 1984).

CHAPTER FOUR

RESULTS

4.1 Yields from extractions

The yields were calculated as percentages of mass yield. The extract yields with the different solvents are indicated in table 2. The total crude extracts had the highest yield for all the plants while the ether extracts had the lowest. *E. abyssinica* total crude extract had the highest yield while the ether extract of the same plant had the lowest yield of 0.01% and could not allow further study.

TABLE 2: **The percentage yield of crude extracts from *E. abyssinica*, *S. incanum* and *C. sanguinolenta* using three solvents**

Plant Species	Extract/solvent			
	Chloroform	Methanol	Ether	Methanol total crude
<i>E.abyssinica</i>	1.0	3.3	0.01	4.5
<i>S. incanum</i>	1.5	1.6	1.2	2.0
<i>C. sanguinolenta</i>	3.1	3.7	2.5	3.9

4.2 Anti-mycobacterial activity

Rifampicin did not show activity against *M. avium* complex and the rifampicin resistant strain (TMC 331) however it showed a zone of inhibition of 26mm for H37Rv (a pan sensitive strain) at a concentration of 0.1 mg. Isoniazid cleared the quadrant for two strains at a concentration of 0.05mg. *S. incanum* total crude extract did not show any activity on any of the three extracts and therefore serial crude extracts were not worked on. *E. abyssinica* total crude extract showed the highest activity on the

pan sensitive strain (H37Rv) and the rifampicin resistant strain (TMC331). However the chloroform and ether extracts of *C. sanguinolenta* were not active on the Pan sensitive strain and TMC 331. There were only two extracts active on *M. avium* and these were; *E. abyssinica* total crude and chloroform extracts. The negative control showed no activity. Details of the results are shown in table 3.

TABLE 3: The antimycobacterial activity of ether, methanol, chloroform and Total crude extracts of *E. abyssinica*, *S. incanum* and *C. sanguinolenta* against the rifampicin resistant strain (TMC), pan sensitive (H37RV) and *M. avium* (MA) strains by the disc diffusion method. (The results were based on 3 experiments)

Extract/Strain	Zones of inhibition (mm) \pm standard deviation		
	H37RV	TMC 331	MA
<i>E. abyssinica</i> total crude methanol extract	23.0 \pm 2.0	21.0 \pm 1.0	8.3 \pm 0.7
<i>E. abyssinica</i> chloroform extract	15.0 \pm 2.0	14.0 \pm 2.0	10.0 \pm 0.0
<i>E. abyssinica</i> methanol extract	17.0 \pm 1.0	17.0 \pm 1.0	6.0 \pm 0.0
<i>C. sanguinolenta</i> total crude extract	10.0 \pm 1.0	11.0 \pm 1.0	6.0 \pm 0.0
<i>C. sanguinolenta</i> ether extract	6.0 \pm 0.0	6.0 \pm 0.0	ND
<i>C. sanguinolenta</i> chloroform extract	6.0 \pm 0.0	6.6 \pm 0.6	ND
<i>C. sanguinolenta</i> methanol extract	10.6 \pm 0.6	6.0 \pm 0.0	ND
<i>S. incanum</i> total crude extract	6.0 \pm 0.0	6.0 \pm 0.0	6.0 \pm 0.0
Isoniazid	cleared	Cleared	6.0 \pm 0.0
Rifampicin	26.0 \pm 0.0	6.0 \pm 0.0	6.0 \pm 0.0
Negative control	6.0 \pm 0.0	6.0 \pm 0.0	6.0 \pm 0.0

ND denotes for not done.

The serial extracts for *C. sanguinolenta* (ether, chloroform and methanol) were not tested on *M. avium* because it's total crude methanol extract was not active on the strain.

4.3 Minimum Inhibitory Concentration of *E. abyssinica* and *C. sanguinolenta*

The chloroform extract of *E. abyssinica* had the lowest MIC value against *M. avium* wild strain and the rifampicin resistant strain. This was followed by the methanol extract of the same plant with MIC of 0.59mg/ml. The total crude methanol extract of *E. abyssinica* had the lowest MIC value against H37Rv. The total crude extract of *C. sanguinolenta* showed activity against both the pan sensitive and rifampicin resistant strain of *M. tuberculosis*. The detailed results are shown in table 4. Rifampicin was not active on the rifampicin resistant strain (TMC) and *M. avium* wild strain while isoniazid was not active on *M. avium*.

TABLE 4: The minimum inhibitory concentration (mg/ml \pm SD) of the methanol, chloroform and total crude extracts of *E. abyssinica* and *C. sanguinolenta* using the microbroth dilution method.

Extract/Strain	H37RV	TMC 331	MA
<i>E. abyssinica</i> total crude extract	0.20 \pm 0.0	1.17 \pm 1.1	0.20 \pm 0.0
<i>E. abyssinica</i> methanol extract	2.35 \pm 2.2	0.59 \pm 0.6	0.59 \pm 0.0
<i>E. abyssinica</i> chloroform extract	1.17 \pm 0.8	0.20 \pm 0.0	0.15 \pm 0.1
<i>C. sanguinolenta</i> total crude extract	1.17 \pm 0.0	1.56 \pm 0.0	
Rifampicin	0.25 \pm 0.0		
Isoniazid	0.13 \pm 0.0	0.25 \pm 0.0	

4.4 Acute toxicity

Behavioral changes observed during the first six hours of administration of the *C. sanguinolenta* total crude extract included decreased motility, sedation, frequent urination and tremors. The mice which received the *E. abyssinica* total crude extract were sedated and had decreased motility. In the control group no peculiar changes in behavior were observed. Details of the behavioral changes are shown in table 5.

Table 5: Behavioral changes observed during acute toxicity studies of the extracts from *E. abyssinica* and *C. sanguinolenta* in the *Mus musculus* mice.

Characteristic	CKVT	EEVT
Increased motor activity	-	-
Tremors	+	-
Pilo erection	-	-
Decreased motor activity	+	+
Sedation	+	+
Analgesia	-	-
Lacrimation	-	-
Diarrhea	-	-

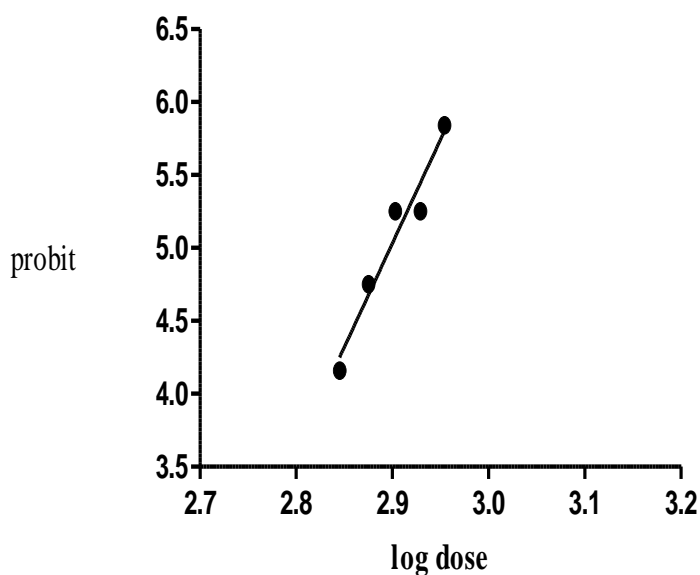
Table 6 shows results obtained after a single dose administration of the total crude extracts of *C. sanguinolenta* and *E. abyssinica* to *M. musculus*. No mice died in the first six hours of drug administration, however during preliminary studies mice which were administered with a dose of 1000mg/kg died after four hours. Increase in dose of extract is proportional to increase in death of the mice within the dose range of 600- 900mg/kg.

TABLE 6: The effect of increased dose of the extract of *C. sanguinolenta* and *E. abyssinica* total crude extracts on the survival of white albino mice

Dose (mg/Kg)	Log dose	No of mice dead	No of mice Alive	% death	probit
<i>C. sanguinolenta</i>					
700	2.845	1	4	20	4.16
750	2.875	2	3	40	4.75
800	2.903	3	2	60	5.25
850	2.929	3	2	60	5.25
900	2.954	4	1	80	5.84
<i>E. abyssinica</i>					
600	2.778	1	4	20	4.16
700	2.845	2	3	40	4.75
800	2.903	2	3	40	4.75
850	2.929	3	2	60	5.25
900	2.954	4	1	80	5.84

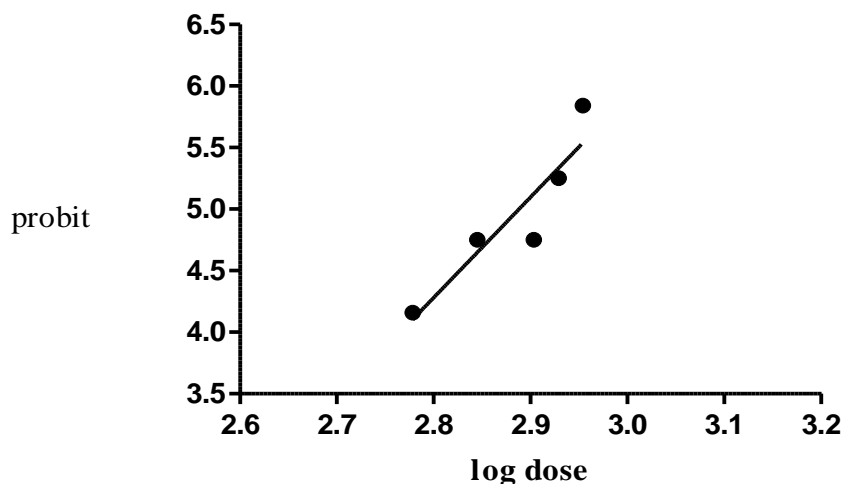
From graph 1 which is a plot of the probit against log dose administered, the LD₅₀ (Probit 5) of the *C. sanguinolenta* methanol crude extract was found to correspond to a log dose of 2.88 which is 758.6 mg/kg body weight.

Graph 1 A plot of probit against log dose for *C. sanguinolenta*



From graph 2 which is a plot of probit against log dose administered, the LD₅₀ of *E. abyssinica* crude extract was found to correspond to a log dose of 2.89 which is 776.2mg/kg body weight.

Graph 2: A plot of probit against log dose for *E. abyssinica*



4.5 Phytochemical analysis

Phytochemical qualitative tests carried out on the extracts showed that none of the extracts had phenols. Flavones were found in all the four crude extracts while terpenoids were present in all crude extracts except *C. sanguinolenta*. Tannins were found in only *E. abyssinica* total crude extract. Saponins and phenols were not detected in all the extracts.

TABLE 7: Compounds present in *C. sanguinolenta* and *E. abyssinica* crude extracts

Extract	alkaloids	tannins	terpenoids	flavones
<i>C. sanguinolenta</i> total crude extract	+	+	-	+
<i>E. abyssinica</i> total crude extract	+	+	+	+
<i>E. abyssinica</i> methanol extract	+	-	+	+
<i>E. abyssinica</i> chloroform extract	-	-	+	-

+ denotes present - denotes not present

CHAPTER FIVE

DISCUSSION

5.1 Anti-mycobacterial activity

The study showed that extracts from two of the three plants had anti-mycobacterial activity. The activity was found in total crude, chloroform and methanol extracts of *E. abyssinica* and *C. sanguinolenta*. All the extracts were active against the rifampicin resistant strain of *M. tuberculosis*. The methanol and chloroform extracts of *E. abyssinica* were actually more active on the rifampicin resistant strain than the Pan sensitive strain of *M. tuberculosis*. This is important because rifampicin resistance is a good indication of multi drug resistant TB and so the extracts may also be active on MDR TB.

The potency of the extracts against the pan sensitive strain as compared to the standard drugs used was low. This may be attributed to the fact that isoniazid and rifampicin were in a pure state as compared to the extract. The wild strain of *M. avium* that was isolated from a Ugandan patient seemed to be resistant to both isoniazid and rifampicin and so the impure plant extracts possess an advantage over the two pure drugs.

Previous studies have reported several plants with anti-mycobacterial activity (Waffo *et al.*, 2000; Newton *et al.*, 2000; Pauli *et al.*, 2005; Gautam *et al.*, 2007; Kirimuhuzya *et al.*, 2009). Studies had mostly reported activity in the plant families of Asteraceae, Lamiaceae, Fabaceae and Apiaceae among others (Gautam *et al.*, 2007). The plants in this study that showed activity belong to the families of Fabaceae and Periplocaceae. Periplocaceae is one of the families that have least been researched on for anti-mycobacterial activity (Gautam *et al.*, 2007; Pauli *et al.*, 2005).

The findings in this study report a lower activity compared to above mentioned studies. This may attributed to the fact that different *in-vitro* methods are used and also because in some of the studies the pure compounds were tested.

Previous studies done on the anti-mycobacterial activity of the renowned anti-malarial climbing liana *C. sanguinolenta* by Gibbons *et al.*, (2003) are in agreement with the findings of this study though activity was on fast growing non virulent mycobacteria. This is therefore a confirmation that the plant extract besides being active on the fast growing mycobacteria strains, is also active on the slow growing virulent strains of mycobacteria which were used in this study. Their study was on an alkaloid (cryptolepine) isolated from the plant. This explains why the minimum inhibitory concentrations of *C. sanguinolenta* in this study are much lower compared to that reported by Gibbons *et al.*, (2003).

The results of anti-mycobacterial activity for *E. abyssinica* are comparable to findings by Waffo *et al.*, (2000) on *E. indica*, a species from the same genus. In their study Indicanine B, an isoflavonoid from *E. indica* showed anti-mycobacterial activity against *M. smegmatis* with an MIC value of 18.5µg/ml. This is a higher activity as compared to the MIC value for *E. abyssinica*. This could be because the pure compound was tested against *M. smegmatis*, a rapidly growing a virulent saprophytic mycobacterium. This is however the first study to report activity of *E. abyssinica* on different mycobacteria. Further studies are needed to isolate and identify the anti-mycobacterial compounds in *E. abyssinica*.

5.2 Acute toxicity

In this study the total crude extracts from *E. abyssinica* and *C. sanguinolenta* affected behavior of white albino mice. The plant extracts had LD₅₀s within the safe range according to Gosh (1984). Behavioral changes that were made included sedation and decreased motor activity for both the extracts at doses higher than 500mg/kg body weight. The observations could have been CNS-related rather than enzymatic due to the decreased motility. *C. sanguinolenta* treated mice showed signs of tremors and frequent urination at very high doses (1000mg/kg). This suggests that the plant could have some diuretic effects however more research is needed to confirm this.

Acute toxicity studies on *C. sanguinolenta* in this study were contrary to what was done by Ansha *et al.*, (2009) where the LD₅₀ was found to be about 3000mg/kg. The difference in the values could be attributed to the fact that they used rats which are bigger animals compared to the mice used in this study. Further more water extracts were tested in their study while the methanol extract was used in this study and lastly tragacanth was used as the solvent during their study while DimethylSulphoxide was the solvent used in this study.

However they agree that there was prolonged sleep in the animals and also that there could be some CNS disturbances. A study done by Ansha *et al.*, (2008) concluded that *C. sanguinolenta* could synergize with hypno-sedatives or other CNS depressants and therefore caution needs to be taken in the concomitant administration of the plant with other CNS depressants.

Ansha *et al.*, (2009) also agrees that the extract is safe for use at doses less than 500mg/kg. In vitro studies have reported cytotoxicity at the molecular level. However this may not be reflected *in vivo* (Ansha and Gooderham, 2002).

This is the first study that has been done concerning *E. abyssinica* toxicity. However in a study that was done on *E. americana* by Garín-Aguilar *et al.*, (2000), similar observations were made.

5.3 Phytochemical testing

Phytochemical tests of the four extracts that were active on mycobacteria showed presence of alkaloids, terpenoids, tannins and flavones. The total crude extract of *E. abyssinica* contained all the compounds while the methanol extract contained alkaloids, terpenoids and flavones. This could be the possible explanation for the high activity of the *E. abyssinica* extracts on mycobacteria.

The chloroform extract of *E. abyssinica* contained only terpenoids which could explain why the extract was the most active on *M. avium* and the Rifampicin resistant strain. According to Copp (2003), secondary metabolites of terpenoids origin lead the number of natural products with reported anti-mycobacterial activity due to their lipophilic nature and therefore ability to penetrate the mycobacterial cell wall.

The genus *Erythrina* has long been known to contain a vast array of alkaloids (Copp 2003). *E. abyssinica* has been found to contain flavonones by Yenesew *et al.*, (2004). This is in agreement with the findings of this study. However on the contrary, saponins were found to be present in the plant in a study done by Kareru *et al.*, (2008). The difference could be attributed to the different methods of preparation of the extracts and methods used in the detection of the compounds.

C. sanguinolenta total crude extract contained tannins, alkaloids and flavones. Presence of alkaloids in *C. sanguinolenta* is in agreement with a study done by Gibbons *et al.*, (2003) in

which an alkaloid cryptolepine was identified and isolated. Though saponins and flavones have been identified in *C. sanguinolenta* total crude extract, the compound which could have caused the anti-mycobacterial activity could have been the cryptolepine alkaloid but more studies are needed to confirm this. On the contrary, tannins and flavonoids were not identified in the root bark of *C. sanguinolenta* in a study done by Tona *et al.*, (1998). This may be attributed to the different methods of preparation of the crude extracts.

The group of compounds that could have caused sedation and decreased motility in the mice for both extracts could have been alkaloids as portrayed in a study done by Dos Santos *et al.*, (2005). In their study done on rats, an alkaloid that was identified as isoquercitrin increased sleeping in the barbiturate- and diazepam-induced sleeping time. In another study done by Garín-Aguilar *et al.*, (2000) on *E. americana*, alkaloid fractions were tested on rats and there was decreased aggressive behavior.

5.4 Limitations

One species of rodents was used in acute toxicity studies however the results may not be true for other species. Additionally, acute toxicity measures lethality within 24 hours and there is no information on the long term effects of the plants.

There were also problems associated with dissolving the total crude extracts because they contain both the polar and non polar compounds and this is precisely why DMSO had to be used as the solvent in acute toxicity tests. The yield for the ether extract of *E. abyssinica* was so small that it could not be worked on.

Water extract was not used because it is cumbersome to deal with.

5.5 Conclusions

The results of this study have further shown that there is potential to develop new compounds against multi drug resistant TB from *Erythrina abyssinica* and *Cryptolepis sanguinolenta*. This therefore verifies their use in the treatment of tuberculosis by traditional practitioners and further emphasizes that there is a strong positive correlation between the anti-mycobacterial activity results and traditional knowledge on plants used for TB and TB-related diseases in ethnomedicine.

The plant extracts were found to be relatively safe for use as far as lethality is concerned however more studies on the toxicity of the plants are needed before declaring them completely safe for use in humans.

The plant extracts contained alkaloids, tannins, terpenoids and flavones.

5.6 Recommendations

The active constituents of the plants should be isolated, identified and characterised. These should then be tested on the resistant strains of mycobacteria. Further research on the toxicity of *E. abyssinica* should be ventured into since this was practically the most active of the plants.

C. sanguinolenta and *E. abyssinica* should be conserved by communities where they are found.

Efforts should also be made to cultivate the two plants species.

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