EVALUATION OF ANTI-TRYPANOSOMAL ACTIVITY OF EXTRACTS OF SELECTED MELIACEAE PLANT SPECIES BY *IN VITRO* AND *IN VIVO* ASSAYS

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DECLARATION

This thesis is my original work and has not been presented for award of a degree in any other university or any other award.

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This thesis is dedicated to my dear husband Dr. Joseph Mahiri, my daughter Damaris Mahiri, my sons, Lance Chacha and Francis Mahiri.

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LIST OF ABBREVIATIONS AND ACRONYMS

AAT	Animal African Trypanosomiasis
ANOVA	Analysis of variance
BBB	Blood-brain barrier
Bwt	Body weight
COSY	Correlated Spectroscopy
CPDD	Consortium for Parasitic Drug Development
CCl_4	Tetrachloromethane
CNS	Central Nervous System
1-D	One dimensional
2-D	Two dimensional
^{13}C NMR	Carbon 13 nuclear magnetic resonance
^{1}H NMR	Proton nuclear magnetic resonance
DCM	Dichloromethane
DMSO	Dimethylsulfoxide
DNDi	Drugs for Neglected Diseases initiative
EATRO	East Africa Trypanosomiasis Research Organisation
EDTA	Ethylene diamine tetra acetic acid
EtOAc	Ethylacetate
FAO	Food and Agriculture Organization
HAT	Human African Trypanosomiasis
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HMBC	Heteronuclear Multiple Bond Connectivity
HMQC	Heteronuclear Multiple-Quantum Coherence
HPLC	High Performance Liquid Chromatography
IACUC	Institutional Animal Care and Use Committee
ICIPE	International Centre of Insect Physiology and Ecology
i.p	intraperitoneally
IR	Infra red
KALRO	Kenya Agricultural and Livestock Research Organisation

KARI	Kenya Agricultural Research Institute
KEMRI	Kenya Medical Research Institute
KETRI	Kenya Trypanosomiasis Research Institute
LC-MS	Liquid chromatography-Mass spectroscopy
LD_{50}	Half maximum lethal dose
MEM	Minimum essential media
MeOH	Methanol
MIC	Minimum inhibitory concentration
MS	Mass Spectroscopy
MSD	Mean survival time in days
NMR	Nuclear Magnetic Resonance
NMRI	Nuclear Magnetic Resonance Imaging
NOESY	Nuclear Overhauser Effect Spectroscopy
PCV	Packed cell volume
PDPs	Product Development Partnerships
PI	Post infection
PPPs	Public Private Partnerships
PTLC	Preparative Thin Layer Chromatography
R&D	research and development
SAM	*S*-Adenosylmethionine
SEM	Standard error of mean
TLC	Thin layer Chromatography
TRC	Trypanosomiasis Research Centre
UV-VIS	Ultra violet visible spectroscopy
VLC	Vacuum Liquid Chromatography
WHO	World Health Organization

ABSTRACT

African human trypanosomiasis (HAT) and African animal trypanosomiasis (AAT) are vector-borne parasitic diseases, which are among the most neglected diseases in the world. They cause major health and economic problems in rural sub-Saharan Africa. Chemotherapy, the main means of controlling the disease is limited due to parasite resistance and toxicity of the current anti-trypanosomal drugs. The development of a vaccine has been thwarted by antigenic variation of the parasite. Thus, the use of natural products is one of the strategies being explored to address some of the problems encountered with allopathic chemical drugs. The main objective of the current research was to evaluate the anti-trypanosomal activity of methanol and aqueous extracts of selected Meliaceae species through *in vitro* and *in vivo* assays against *Trypanosoma brucei rhodesiense, Trypanosoma brucei brucei* and *Trypanosoma evansi.* Methanol and aqueous extracts of five Meliaceae plant species: *Trichilia emetica, Toona ciliata, Azadirachta indica*, *Turraea mombassana* and *Melia azedarach* were tested for *in vitro* anti-trypanosomal activity, acute toxicity and *in vivo* efficacy using mice as animal models. Sequential extraction of powdered plant samples in hexane, ethyl acetate, methanol and water afforded the crude extracts. *In vitro* assays were carried out in 96-well microtitre plates and melarsoprol and suramin were used as the positive controls. The results revealed that the minimum inhibitory concentrations (MICs) of MeOH extracts were significantly lower than those of the aqueous extracts. The MeOH extracts of *M. azedarach* stem bark, *A. indica* stem bark and *T. emetica* root bark (MIC 9.11±3.44, 9.93±2.88 and 9.11±3.44 µg/ml respectively) showed the best anti-trypanosomal activity against *T. b. rhodesiense*; the MeOH extract of *T. emetica* root bark (MIC 9.93±2.88 µg/ml) showed the best anti-trypanosomal activity against *T. b. brucei.* The methanol extracts of *M. azedarach* root bark, *T. emetica* root bark and *A. indica* stem bark (9.11±3.44, 9.11±3.44 and 9.93±2.88 µg/ml respectively) showed the best anti-trypanosomal activity against *T. evansi*. Based on the strength of *in vitro* anti-trypanosomal activity, four extracts were selected and subjected to acute toxicity tests in mice. It was established that the MeOH extract of *A. indica* stem bark and the aqueous extract of *T. mombassana* leaves were safe in mice at dose levels of 100, 200 and 400 mg/kg body weight. The LD_{50} values of *T. emetica* root bark and *M. azedarach* root bark were 707.95±229.25 and 229.09.54±95.54 mg/kg body weight respectively. Two extracts (*A. indica* stem bark and the *T. mombassana* leaves) were subjected to *in vivo* efficacy tests using *T. b. rhodesiense* infected mice. Melarsoprol and suramin at doses of 3.6 and 5 mk/kg bwt respectively were used as positive controls. The infected-untreated group served as negative control. The study showed that the MeOH extract of *A. indica* stem bark at 200 and 400 mg/kg and the aqueous extract of *T. mombassana* leaf at 400 mg/kg reduced parasitemia levels, prevented body weight loss and reduced decline in packed cell volume ($p < 0.05$) in mice. Chromatographic fractionation of the methanol extract of *A. indica* stem bark led to the isolation of nimbin (**55)** and 1-detigloyl salannin (**84**). *Toona ciliata* stem extract yielded bis(2-ehtylhexyl) phthalate (**85)** and bis(2-methylheptyl) phthalate (**86).** Nimbin (**55**) displayed the highest *in vitro* antiptrypanosomal activity with MIC values of 9.74±0.93, 11.90±0.02 and 11.89±0.01 µg/ml against *T. b. rhodesiense, T. b. brucei* and *T. evansi* respectively. Bis(2-ehtylhexyl) phthalate (**85**) showed mild activity against the three strains (MIC > 146 µg/ml). In conclusion, the study established that *A. indica* stem bark and *T. mombassana* leaves have *in vitro* and *in vivo* anti-trypanomal activities and can be considered portential sources of new anti-trypanosomal compounds. Based on the findings of the current study, it is recommended that extracts of *A. indica* and *T. mombassana* may be used as alternative remedies for African trypanosomiasis.

CHAPTER ONE: INTRODUCTION

1.1 Background Information

African trypanosomiasis is a parasitic infection caused by single-celled protozoa of the genus Trypanosoma that are primarily transmitted by the bite of infected tsetse flies. Human African trypanosomiasis is caused by two subspecies of *Trypanosoma brucei*: *T. b. gambiense* and *T. b. rhodesiense* while animal African trypanosomiasis is caused by *T. b. brucei*, *T. congolense, T. vivax, T. evansi* and *T. equiperdum* (Brun *et al*., 1998; Swallow, 2000; Patrick *et al*., 2012). Both human and animal trypanosomiases negatively affect the whole economy of Africa by weakening the health of human and animals (John *et al*., 2012). The parasite lives in the mid-gut of the fly (procyclic form), whereupon it migrates to the salivary glands for injection to the mammalian host on biting. The parasite lives within the bloodstream (bloodstream form) where it can re-infect the fly vector after biting (Roditi and Lehane, 2008). Later, during a *Trypanosoma brucei* infection, the parasite may migrate to other areas of the host. An infection caused by *T. brucei* may be transferred from human to human via bodily fluid exchange, primarily blood transfer (Roditi and Lehane, 2008). Both human and animal trypanosomiases affect the central nervous system. The development of late stage sleeping sickness takes decades to occur in West African (Gambianse) sleeping sickness and a patient may only suffer from mild fatigue due to the occasional increases in parasitic load in blood (Hoet *et al*., 2004).

In contrast, the typical East African (Rhodesiense) sleeping sickness is far more virulent and can develop into late stage sleeping sickness within weeks and untreated patients can die within weeks or months of infection (Kuzoe, 1993; Molyneux *et al*., 1996). The escalating costs of initiating and maintaining tsetse control campaigns coupled by unavailability of vaccine as a result of antigenic variation of the parasite have led to the vast tsetse infested areas of sub-Saharan Africa to almost completely rely on the use of anti-trypanosomal drugs (Geerts and Holmes, 1998). However, the few chemoprophylactic and chemotherapeutic anti-trypanosomal drugs currently in use suffer from several drawbacks including resistance, toxicity, restriction to parenteral

administration, lack of efficacy in some cases and unaffordable prices (Legros *et al*., 2002; Delespaux *et al*., 2008). Based on these setbacks, the search for alternative compounds for the treatment of trypanosomiasis is an urgent and important task (Lun *et al*., 1993). One possible source of such compounds lies in the use of natural products that demonstrate potent trypanocidal effects.

The fascination of natural products, mostly as crude preparations from plants with known medicinal properties, goes back to ancient times (Newman *et al*., 2003). Several established human antiprotozoal drugs have their origins in nature, such as quinine, an alkaloid from *Cinchona* sp. (Rubiaceae) and artemisinin, a sesquiterpene lactone from *Artemisia annua* (Asteraceae) used to treat malaria, or emetine, an alkaloid from *Cephaelis ipecacuanha* (Rubiaceae) used to treat amoebiasis. Additionally, these antiprotozoal plant-derived compounds have been used as leads to develop other semi-synthetic or synthetic drugs with better efficacy, safety or pharmacokinetic profiles (Tagboto *et al*., 2001). The use of herbal remedies in the treatment of trypanosomiasis is potentially promising with some plants such as *Morinda lucida* and *Azadirachta indica* having been demonstrated to be potent trypanocides (Asuzu and Chineme, 1990; Nok *et al*., 1993; Nok *et al*., 2001). Pharmacologically active compounds of plant origin can provide an alternative to chemically synthesized drugs to which many infectious microorganisms have become resistant (Akinpelu *et al*., 2006).

The family of Meliaceae contains a wide range of floral and fruit structures, and although investigated for many years, it remains a source of great source of pharmacologically important phytochemicals (Huang *et al*., 2007). A study by Githua *et al*. (2010) on *in vitro* screening of some Meliaceae species against *T. b. rhodesiense* revealed that MeOH extracts had encouraging levels of trypanocidal properties. Based on recommendations made by Githua *et al*.(2010), the current study aimed at investigating both *in vitro* and *in vivo* anti-trypanosomal activities of polar (MeOH and aqueous) extracts of selected Meliaceae plant species against *T. b. rhodesiense*, *T. b. brucei* and *T. evansi*. Chromatographic fractionation of an active MeOH extract *A.*

indica stem bark led to the identification of nimbin which was active against all the three trypanosome strains used in the current study.

1.2 Statement of the Problem

African Trypanosomiasis is among the top contenders for the most neglected diseases in the world. Publications reporting the activity of plant extracts on African trypanosomes and isolated anti-trypanosomal natural products are not abundant compared to other protozoal diseases such as malaria. Chemotherapy, the main means of controlling the disease is under threat due to parasite resistance and toxicity of the anti-trypanosomal drugs. The poor prospect for a vaccine due to antigenic variation of the parasite is further compounded by the unwillingness of the pharmaceutical industry to develop new compounds because of uncertain and unprofitable market or perhaps the localized nature of the disease to Africa. The approved drugs for trypanosomiasis have to be administered through parenteral route thus requiring medical and veterinary facilities and specialized staff which often do not exist in rural sub-Saharan Africa. Adverse effects associated with the use of the existing trypanocidal drugs are severe, sometimes life-threatening. For instance, the drug melarsoprol causes a serious reactive encephalopathy in 5-10 % of the cases, half of which are fatal. Other common side effects include vomiting, abdominal colic and peripheral neuropathy. There are also increasing reports of treatment failures. Therefore, there is an urgent need for new drugs against African trypanosomiasis which are safe, effective and orally administered.

1.3 Justification of the Study

Pharmaceutical research in natural products represents a major strategy for discovering and developing new drugs. Nature with its numerous plant species is a potential source of such drugs since it contains countless quantity of molecules with a great variety of structures and pharmacological activities (Newman *et al*, 2003). The diversity of natural products with anti-protozoal activities has been illustrated by several research groups which cover molecules that are mainly active on the trypanosomes responsible for HAT and AAT. The majority of these compounds have been tested for their *in vitro* activity on bloodstream trypomastigotes of African trypanosomes. However, only a few natural

compounds have been evaluated for *in vivo* activity in animals and none have been assessed clinically. In this sense, screening of natural products against trypanosomes should allow discovery of new trypanocidal lead compounds (Dardonville *et al*., 2004). A Study on some Meliaceae species led to the isolation and structural characterization of several limonoids that showed encouraging levels of *in vitro* activities (Githua *et al*., 2010), indicating that compounds with the limonoid skeleton with some fine structural characteristics might be good candidates for development into anti-trypanosomal drugs. These plants are readily available in Kenya and in Africa suggesting a number of potential practical sources for anti-protozoal screening, which could eventually lead to the development of a drug, that is formulated and standardized based on the active anti-trypanosomal constituent.

1.4 Research Questions

1. Which part(s) of *Trichilia emetica, Toona ciliata, Azadirachta indica, Turraea mombassana* and *Melia azedarach* show anti-trypanosomal activity against *Trypanosoma brucei rhodesiense, Trypanosoma evansi* and *Trypanosoma brucei brucei?*
2. What are the safe dose levels of the identified anti-trypanosomal extracts in 1 above in laboratory animal models?
3. What is the pharmacological performance of the identified anti-trypanosomal extracts in 2 above in laboratory animal models?
4. Which compounds/fractions are responsible for the anti-trypanosomal activity of the extracts identified in 3 above?
5. What are the chemical structures of the pure constituents of the active plant extracts identified in 4 above?

1.5 Hypothesis of the Study

Trichilia emetica, Toona ciliata, Azadirachta indica, Turraea mombassana and *Melia azedarach* contain phytochemicals with *in vitro* and *in vivo* anti-trypanosomal activity against *Trypanosoma brucei rhodesiense, Trypanosoma brucei brucei* and *Trypanosoma evansi.*

1.6 Objectives of the Study

1.6.1 General Objective

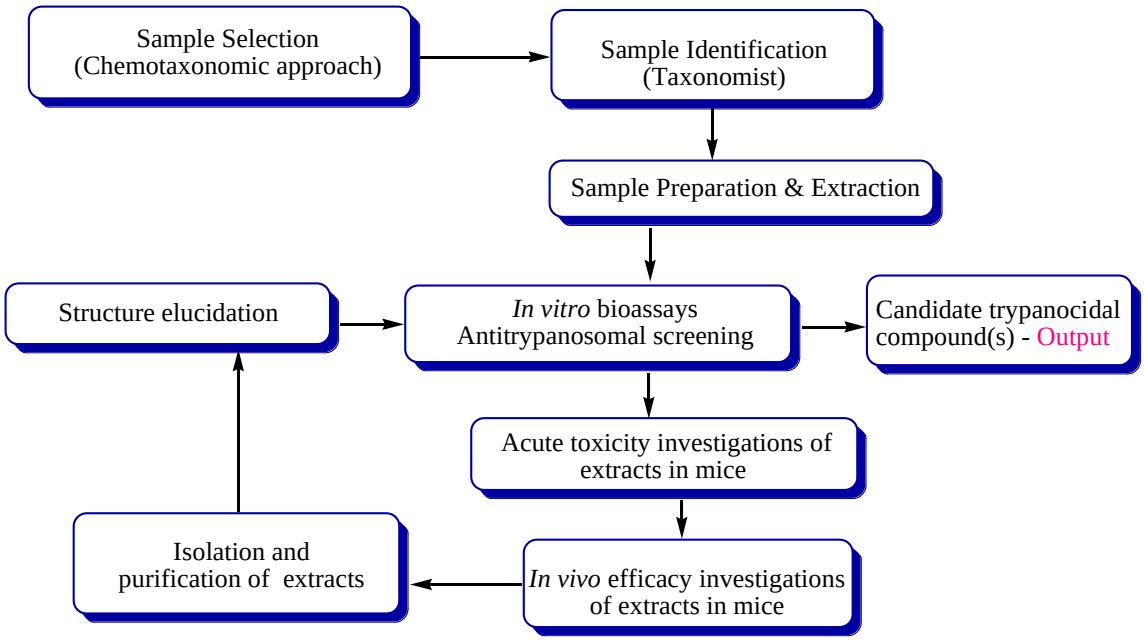
The overall objective of the present study was to evaluate the anti-trypanosomal activity of extracts of *Trichilia emetica, Toona ciliata, Azadirachta indica, Turraea mombassana* and *Melia azedarach* through *in vitro* and *in vivo* assays using *Trypanosoma brucei rhodesiense, Trypanosoma brucei brucei* and *Trypanosoma evansi.*

1.6.2 Specific Objectives

1. To undertake *in vitro* screening of the extracts of root barks, leaves and stem barks of *Trichilia emetica, Toona ciliata, Azadirachta indica, Turraea mombassana* and *Melia azedarach* against *T. b. rhodesiense, T. evansi* and *T. b. brucei*
2. To evaluate acute *in vivo* toxicity of the antitrypanosomal extracts identified in 1 above in mice
3. To determine the *in vivo* efficacy of the active extracts in 2 above against *T. b. rhodesiense* using mice.
4. To undertake *in vitro* bioassay-guided isolation and purification of anti-trypanosomal constituents of the active extracts and fractions in 3 above.
5. To identify the structures of the isolated constituents of the active extracts in 4 above.

1.7 Schematic Presentation of the Study Outline

A schematic presentation of the steps which were followed in the study to achieve the expected outcome is shown in scheme 1.



Scheme 1: Procedure followed to achieve the expected outcome of the study

CHAPTER TWO: LITERATURE REVIEW

2.1 African Trypanosomiasis

African trypanosomiasis is a group of parasitic infections caused by single-celled protozoan parasites of the genus *Trypanosoma* that are primarily transmitted by the bite of infected tsetse flies. The disease negatively affect the whole economy of sub-Saharan Africa by weakening both human and animals health (John *et al*., 2012). Human African trypanosomiasis (HAT), also known as sleeping sickness, affects humans and is caused by *Trypanosoma brucei rhodensiense* in eastern Africa and *Trypanosoma brucei gambiense* in western Africa, with some overlaps between the two trypanosomes in central Africa (WHO, 2000). On the other hand, animal African trypanosomiasis (AAT), also known as nagana, is a disease of vertebrate animals which is caused by *T. b. brucei*, *T. congolense* and *T. vivax*, *T. evansi* and *T. equiperdum* (Brun *et al*., 1998; Swallow, 2000; Patrick *et al*., 2012). Trypanosomes have a glycoprotein coat that is encoded by genes that are antigenically distinct thus making the parasite able to engage an immune-evasive process of antigenic variation (Borst *et al*., 1998).

2.1.1 Human African Trypanosomiasis (HAT)

Human African trypanosomiasis (HAT) ranks among the top contenders for title of greatest neglected diseases of mankind (Stich *et al*., 2003). HAT represents a major public health threat in Africa and is considered a main obstacle for development of rural regions of the tsetse endemic parts of the African continent (Simarro *et al*., 2008). Unfortunately, since this diseas persists almost exclusively in the most marginalized communities of undeveloped countries, very little resource is spent to lighten their tremendous social and economic burden. The disease was largely controlled in the 1960's, but it re-emerged in the 1980's and currently 60 million people are exposed to it (WHO, 2013). Since 1997, WHO has been raising awareness of this most neglected disease, favouring the establishment of national control programs and the involvement of public and private partnerships (Stich *et al*., 2003). These efforts have significantly reduced the incidence of HAT in endemic countries by implementation of surveillance and drug availability.

2.1.2 African Animal Trypanosomiasis (AAT)

African animal trypanosomiasis (AAT) is a disease of vertebrate animals. The disease is caused by trypanosomes of several species in the genus *Trypanosoma: T. b. brucei, T. congolense, T. vivax, T. evansi* and *T. equiperdum.* Previous studies have led to the subdivision of the *T. congolense* species into several types which can be distinguished by isoenzymatic differences and molecular techniques. These are designated as *T. congolense* savannah type, *T. congolense* Tsavo type, *T. congolense* forest type, *T. congolense* Kilifi type (Majiwa *et al*., 1985; 1993). *Trypanosoma congolense, T. vivax* and, to a lesser extent *T. b. brucei*, are the major pathogenic species of African cattle (Morrison, 2001). *Trypanosoma congolense* is considered the most important cause of AAT in East Africa, and *T. vivax* in West Africa (Stephen, 1986). The disease is most significant in cattle but can cause serious losses in pigs, camels, goats, and sheep. Infection of cattle by one or more of the three African animal trypanosomes, results in sub-acute, acute, or chronic disease characterized by intermittent fever, anaemia, occasional diarrhea, and often terminates in death (Gilbert *et al*., 2001).

2.1.3 African Trypanosomes

African trypanosomes, the causative agents of African trypanosomiasis are protozoan parasites of the genus *Trypanosoma* that live and multiply extracellularly in blood and tissue fluids of their mammalian hosts and are transmitted by the bite of infected tsetse flies (*Glossina* sp.).

2.1.3.1 Human African Trypanosomes

Human African trypanosomiasis is caused by a haemoflagellate protozoan belonging to the species *T. brucei* (genus *Trypanosoma*, order Kinetoplastida) (Cox, 2004). Of the three subspecies of *T. brucei*, only two are infectious to humans (*T. b. gambiense* and *T. b. rhodesiense*), while *T. b. brucei* causes infection in wild and domestic animals (Barrett *et al*., 2003). *Trypanosoma b. gambiense* and *T. b. rhodesiense* (Figure 1) are morphologically indistinguishable. The trypanosome is a highly pleomorphic organism, frequently showing in a single blood smear a variety of forms ranging from slender-bodied organisms with a long free flagellum, reaching a length of 30 mm or more, to

fatter, stumpier forms without a free flagellum which average about 15 mm in length. Sporadic cases of human infection with other trypanosome species have been reported (Truc *et al*., 1998; Joshi *et al*., 2005), but, at least in one of the cases, infection could be ascribed to a mutated apolipoprotein L1 found in the serum of the patient (Lun *et al*., 2009), which is a component of the trypanolytic factor that normally protects humans from animal trypanosome infection (Pays and Vanhollebeke, 2008).

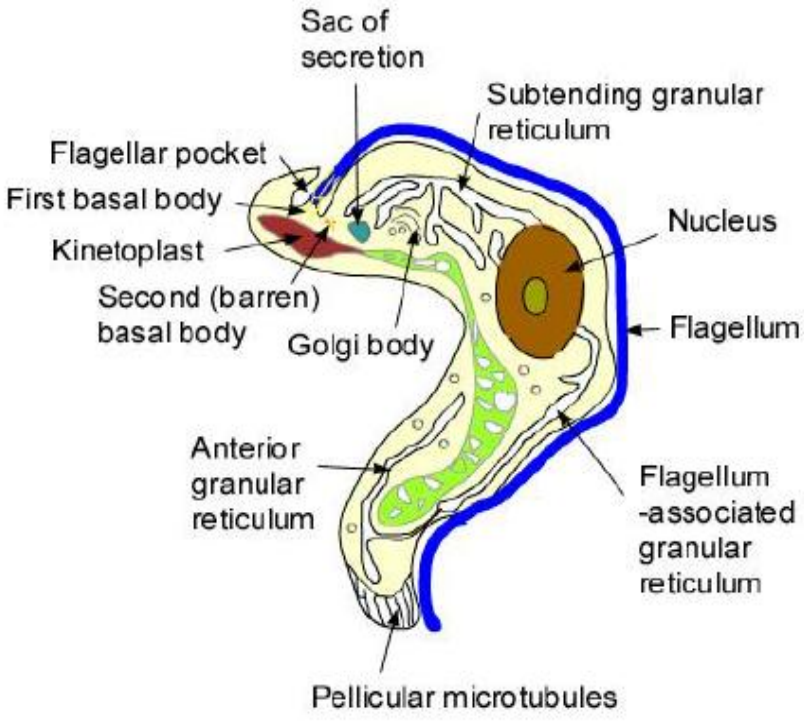


Figure 1: The structure of *Trypanosoma brucei rhodesiense* trypanosome

The two forms of HAT differ greatly (Welburn *et al*., 2001). *Trypanosoma b. gambiense* is responsible for more than 90% of reported cases of HAT and causes a chronic form of the illness, which can last for months or years before major symptoms arise. On the other hand, *T. b. rhodesiense* causes an acute form that usually leads to the patient's death within weeks or few months (Brun *et al*., 2009). However, exceptions to these rules, with acute Gambian disease and chronic Rhodesiense trypanosomiasis cases, are observed (Garcia *et al*., 2006). Moreover, despite most *T. b. gambiense*

infections being fatal in absence of treatment, human trypano-tolerance, with self-resolving and asymptomatic chronic infections, has been postulated (Checchi *et al*., 2008). Other differences between the Rhodesiense and Gambiense forms lie in their clinical features and the chemotherapy protocols used, in their epidemiology and transmission and, therefore, in the control strategies applied (Fèvre *et al*., 2006).

2.1.3.2 African Animal Trypanosomes

African animal trypanosomiasis is caused by protozoa in the family Trypanosomatidae genus *Trypanosoma*. *Trypanosoma congolense* resides in the subgenus *Nannomonas*, a group of small trypanosomes with medium-sized marginal kinetoplasts, no free flagella, and poorly developed undulating membranes. In East Africa, *T. congolense* is considered to be the single most important cause of AAT. This trypanosome is also a major cause of the disease in cattle in West Africa. Sheep, goats, horses, and pigs may also be seriously affected. In domestic dogs, chronic infection often results in a carrier state.

Trypanosoma vivax is a member of the subgenus *Duttonella*, a group of trypanosomes with large terminal kinetoplasts, distinct free flagella, and inconspicuous undulating membranes. *Trypanosoma vivax* is a large (18-26 μm long) monomorphic organism that is very active in wet-mount blood smears. Cattle, sheep, and goats are primarily affected. Although this organism is considered to be less pathogenic for cattle than *T. congolense*, it is nevertheless the most important cause of AAT in West African cattle. This trypanosome readily persists in areas free of tsetse flies (for example, in Central and South America and in the Caribbean), where it is transmitted mechanically by biting flies or contaminated needles, syringes, and surgical instruments.

Trypanosoma b. brucei resides in the subgenus *Trypanozoon. Trypanosoma b. brucei* is an extremely polymorphic typanosome occurring as short, stumpy organisms without flagella, long slender organisms with distinct flagella, and intermediate forms that are usually flagellated. Horses, dogs, cats, camels and pigs are very susceptible to *T. b.*

brucei infection. Infection of cattle, sheep, goats and sometimes pigs results in mild or chronic infection. Studies have shown, however, that this organism is widespread in East and West Africa and that it can cause serious disease and high mortality in cattle, sheep, and goats (Moulton and Sollod, 1976).

Trypanosoma evansi is a typically monomorphic protozoan and has only long-slender trypamastigote form. Its size is between 15-36 µm in length (Stevens and Brisse, 2004). However, *T. evansi* is distinguished from *Trypanosoma b. brucei, T. congolense* and *T. vivax* by its distinctive kinetoplas DNA (kDNA), which show a lack of mini-circle sequence heterogeneity and an absence of maxicircles (Stevens and Brisse, 2004).

2.1.4 The disease vector

Tsetse flies (*Glossina spp*.) are found mostly in Africa. They are the biological and/or mechanical vector of trypanosomes and constitute a potent and constant threat to humans and livestock over much of sub-Saharan Africa (Gooding and Krafsur, 2004). Thirty-one species and subspecies of these tsetse flies have been identified. Only a few species are vectors of human sleeping sickness but all are potential vectors of animal trypanosomiasis. The historical classification of tsetse, based on morphological criteria, divides the species into three groups (Newstead *et al*., 1924). The fusca group flies (subgenus *Austenina*) tend to occur in the lowland rainforests of West and Central Africa. The palpalis group (subgenus *Nemorhina*) is found in the riverine galleries of West and Central Africa but can extend into savannah regions between river systems; *G. palpalis* and *G. tachinoides* are important AAT vectors in this group. The morsitans group (subgenus *Glossina*) occurs in a variety of savannah habitats lying between the forest edges and desert and includes several important vectors of AAT including *Glossina morsitans* spp., *G. pallidipes* and *G. austeni*.

Tsetse feed exclusively on blood; they are holometaboulos insects with females giving birth to full-grown larvae which rapidly pupate in the soil. Their longevity, mobility and frequent feeding make these flies highly efficient vectors, but the low rate of population growth means that even small increases in mortality rate can result in population decline

and even extinction (Hargrove, 2003). Tsetse flies can fly at speeds of up to 25 km per hour, but they usually fly more slowly and only for short periods of time, up to 50 minutes and usually rest more than 23 hours per day in trees to avoid desiccation. The tsetse fly is very sensitive to environmental conditions - it will not survive in areas that are too hot, too dry, or too high. When the tsetse flies suck blood, development of trypanosomes in them depends on the species of *Trypanosoma*. *T. vivax* only colonizes the proboscis, *T. congolense* and *T. simiae* the midgut and the proboscis, whereas *T. b. gambiense, T. b. rhodesiense* and *T. b. brucei* develop in different regions of the intestine. The metacyclic infectious forms are found in the salivary glands of the tsetse fly.

2.1.5 The host of tsetse flies

Wildlife, particularly, warthog, bush pig, duiker, bush buck, kudu, buffalo and monitor lizard are the natural hosts of tsetse and may acquire prolonged, symptomless trypanosome infections. Livestock exhibit a range of susceptibilities to infection, from refractory to highly vulnerable (Bourn *et al*., 2001). However, the wildlife in Africa generally tolerates infection and often serves as a reservoir for human and livestock-infective trypanosomes (Taylor and Authie, 2004). Monkeys, rats, mice, guinea pigs and rabbits can also be infected by trypanosomes; ruminants, wild equidae, lions, leopards and wild pigs can serve as carriers (OIE, 2005). Susceptibility of cattle to trypanosomiasis depends on breed, age, behaviour, previous exposure and health status (Murray *et al*., 1994). The indigenous zebus are trypanosusceptible and West African *Bos taurus* breeds are trypano-tolerant. Exotic imported ruminants (improved dairy cattle) are more severely affected than local genotypes (Taylor and Authie, 2004).

Tsetse flies locate portential hosts through olfactory and visual cues. To locate hosts beyond their visual range, the fly is activated by the host's odor and flies upwind until it comes to the vicinity of the host, where visual cues such as shape, size color contrast, movement and close range stimuli may mediate alightment on the host (Gikonyo *et al*., 2000; Gikonyo *et al*., 2003). Differential response to colours of different reflectants

enable them to locate hosts, blue being particularly attractive. Different species prefer different regions of the body of the host for bloodsucking. Recent studies have shown that brown and fawn coloured cattle were more likely to be infected than cattle of other colours (Carty, 2002).

2.1.6 Disease burden

Both human and animal African trypanosomiasis negatively impact on human, animal health and productivity, livestock production and limits land utilization.

2.1.6.1 Human African Trypanosomiasis disease burden

Tsetse flies (*Glossina* spp), occupy an estimated area of 10 400 000 km^2 of agricultural land in Africa and are estimated to cost Africa an annual income of 4.5 billion USD (Tesfaye *et al*., 2012). Indeed, successive African governments from colonial times and the African Union (AU) have acknowledged that human and animal trypanosomiasis need to be controlled to improve health and economic development of Africa (Wilson, 1963; Simarro *et al*., 2008). A total of 36 countries are classified by the World Health Organization (WHO) as endemic for HAT (Simarro *et al*, 2008; WHO, 2012). A number of these countries have however not reported a single case of the disease in more than 10 years (WHO, 2012) and according to Stuart *et al*. (2008), HAT is a significant public health problem in only 20 countries. However, this restricted number of countries may mask the fact that the disease is a threat to an estimated 50-60 million people who live in foci where active transmission is possible (Stuart *et al*., 2008; WHO, 2012). The significance of this threat is amplified by the fact that a majority of the affected people are exposed to the tsetse fly menace in the course of eking out a livelihood in agriculture, fishing animal husbandry or hunting (Simarro *et al*., 2008; WHO, 2012).

2.1.6.2 Animal African Trypanosomiasis disease burden

African animal trypanosomiasis, according to the Food and Agriculture Organization of the United Nations (FAO), is probably the only disease which has profoundly affected the settlement and economic development of a major part of the African continent.

Animal African trypanosomiasis affects the health and productivity of livestock. It occurs in 37 sub-Saharan countries covering about 9 million km^2, an area which corresponds approximately to one-third of the Africa's total land area (Mattioli *et al*., 2004). An estimated 45 to 60 million cattle and tens of millions of small ruminants are at risk from trypanosomiasis (Chadenga, 1994; Gilbert *et al*., 2001). FAO estimates that about three million cattle die each year due to AAT. Other valuable livestock, such as camels, also suffer from trypanosomiasis (FAO, 2000).

Direct costs due to AAT involve decreased livestock productivity (mortality, fertility, milk yield, ability to work as traction animals) to which can be added expenditure on controlling the disease (Shaw, 2004). Thirty five million doses of trypanocides are administered each year to protect livestock in tsetse infected areas (Sones, 2001). Direct losses due to trypanosomiasis are estimated to amount to between US$ 1-1.2 billion each year whereas the indirect impact of AAT on agriculture in sub-Saharan Africa exceeds this amount. A pondered evaluation extrapolated for the total tsetse-infested lands values the total losses, in terms of agricultural Gross Domestic Product, at US$ 4.75 billion per year (FAO, 2000). The overall impact extends to the restricted access to fertile and cultivable areas, imbalances in land use and exploitation of natural resources and compromised growth and diversification of crop-livestock production systems (Mattioli *et al*., 2004). The presence of tsetse flies and animal trypanosomiasis in much of Africa south of the Sahara also had a major influence on the agricultural systems. Large areas of tropical Africa are unsuitable for livestock production due to presence of tsetse flies (Murray and Gray, 1984). In some Central African countries like the Republic of Gabon, the Republic of Congo, the Democratic Republic of Congo and southern Cameroon there are still extensive areas of relatively undeveloped land. Only trypano-tolerant breeds of domestic livestock can be kept in these areas without chemoprophylaxis.

2.1.7 African Trypanosomiasis Distribution

The distribution of trypanosomiasis in Africa corresponds to the existence of tsetse flies (*Glossina* spp) and comprises currently an area of 8 million km^2 between 14 degrees North and 20 degrees South latitude (WHO, 2013).

2.1.7.1 Human African Trypanosomiasis Distribution

The geographical distribution of both gambiense and rhodesiense HAT is uneven. Environmental modifications and human or livestock population movements can, however, result in shifts in geographical location and extent (WHO, 2013). Human African trypanosomiasis transmission is restricted to the African continent (Figure 2), but around 50 cases per year are diagnosed elsewhere in people who had travelled to affected regions (Sinha *et al*., 1999; Ripamonti *et al*., 2002; Lejon *et al*., 2003). Endemic foci of the disease have a discrete distribution, correlated to the presence of the tsetse flies. There are nearly 300 active foci identified, confined to an area that stretches south of the Sahara and north of the Kalahari Desert (Barrett *et al*., 2003). *Trypanosoma b. gambiense* infection is found in west and central Africa: Angola, Democratic Republic of the Congo and Sudan are the most affected countries by the Gambian form, with more than 1,500 new cases per year reported up to 2004 (WHO, 2006). The Democratic Republic of the Congo has the most people at risk (36 million) and the largest area at risk (790 000 km^2), but South Sudan and Angola also have sizeable populations at risk.

In West Africa, the areas of greatest endemicity are classified as at moderate risk and are located in central Cote d'Ivoire and coastal Guinea (Figure 2). The estimates are somewhat more precise for *T. b. gambiense* than for *T. b. rhodesiense*, since the presence of other infected humans, rather than animal reservoirs, is a key determinant in the former; nonetheless, some sensible extrapolations can be made, leading to an estimate of 12.3 million people at risk for contracting *T. b. rhodesiense* over an area of 0.171 million km^2, with 88% at low to very low risk and 12% (around 1.5 million people) at moderate to high risk (Figure 2) (Simarro *et al*., 2012). *Trypanosoma b.*

rhodesiense is found in the eastern and southern part of the continent. Malawi, Uganda and United Republic of Tanzania have been identified (WHO, 2013) as the countries with the highest incidence of this HAT form (50–1,500 cases per year). Uganda is the only country in Africa known to be affected by both *T. brucei* subspecies, but the distribution of these parasites is, at least for the time being, separate (Picozzi *et al*., 2005).

Compared to other parasitic diseases like malaria or worm infections, incidence of HAT is lower, but its potential to give rise to devastating epidemics as soon as active surveillance is abandoned makes this illness a major health priority in endemic countries (Cattand *et al*., 2001). Systematic control programs established by European authorities resulted in efficacious intervention in the big epidemics that occurred between the end of the 19th and the beginning of the 20th century, bringing the disease to a nearly elimination by the 1960s (Pépin and Méda, 2001; Maudlin, 2006). After the colonial era, however, the number of cases increased rapidly, due to lack of surveillance and awareness from local government, but also to poverty, political instability, wars and displacement of populations (Smith *et al*., 1998; Cattand, 2001; Brun *et al*., 2009).

It is estimated that, a total of 60 million people in 36 African countries are continuously exposed to the risk of infection by one of the two forms of HAT, but only 3-4 million are under surveillance (Cattand *et al*., 2001). For this reason, accurate epidemiological data for sleeping sickness are difficult to collect and reported incidence of the illness is often considered an underestimate (Fèvre *et al*., 2008; Welburn *et al*., 2009). Despite this uncertainty, at the end of the 20th century, WHO estimated an annual number of cases of at least 300,000 (40,000 – 50,000 deaths), of which only 13% were identified and treated (WHO, 2006). Fortunately, improvement in control policies and new international initiatives has led, during the last decade, to a steady decline of total cases, currently estimated to be 50,000 – 70,000 (WHO, 2006; Barrett, 2006).

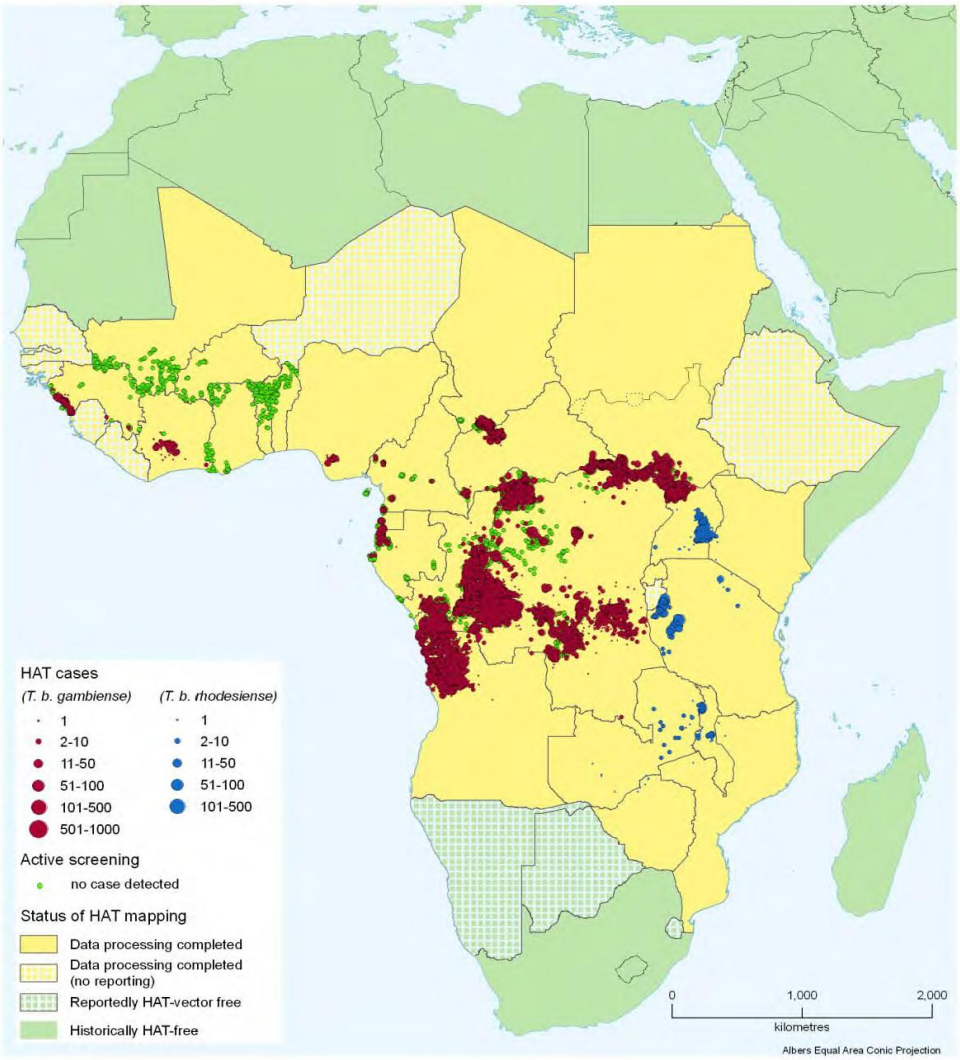


Figure 2: Distribution of human African trypanosomiasis (HAT) cases, 2000–2009 (source WHO, 2013)

2.1.7.2 African Animal Trypanosomiasis distribution

Trypanosomes can be found wherever the tsetse fly vector exists. Tsetse flies are endemic in Africa between latitude 15° N and 29° S, from the southern edge of the Sahara desert to Zimbabwe, Angola and Mozambique (Figure 3). Trypanosomes, particularly *T. vivax*, can spread beyond the "tsetse fly belt" by transmission through mechanical vectors. *T. vivax* is also found in South and Central America and the Caribbean, areas free of the tsetse fly. The distribution of the tsetse-transmitted African trypanosomes is governed by that of their tsetse vectors, which infest an area of sub-

Saharan Africa that extends from the southern edge of the Sahara desert (lat. 15°N) to Angola, Zimbabwe, and Mozambique (lat. 20 °S). Of the three African animal trypanosomes, only *T. vivax* occurs in the Western Hemisphere in at least 10 countries in the Caribbean and South and Central America (Hoare, 1972; Vickerman *et al*., 1977; Kirchhoff *et al*., 1999; Barrett *et al*., 2003).

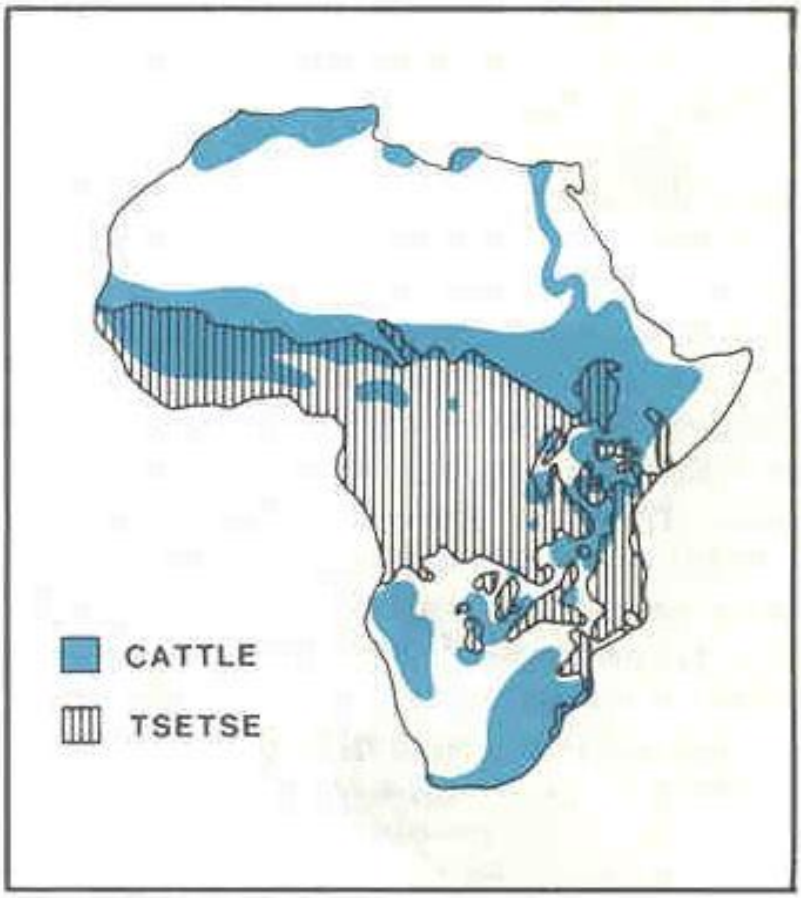


Figure 3: Distribution of major cattle production areas and tsetse-infested zones in Africa (source FAO, 2000)

2.1.8 Transmission and infection

African trypanosomes, both human and animal, are mainly transmitted by infected tsetse flies (*Glossina spp*).

2.1.8.1 Transmission and infection of Human African Trypanosomiasis

The infective stage is the metacyclic trypomastigote, which lives within the salivary gland of the tsetse fly. Infection occurs when an individual is bitten by an infected tsetse fly. Important vectors include *G. morsitans, G. pallidipes, G. fuscipes* and *G. palpalis*. The metacyclic trypomastigotes rapidly transform into bloodstream trypomastigotes within the extracellular spaces in the subcutaneous tissue. The trypomastigotes eventually find their way into the bloodstream and the lymphatics, where they continue the replication cycle. Invasion of the cerebrospinal fluid also occurs, but does not contribute to the life cycle. The tsetse fly becomes infected when it ingests the trypomastigote while taking a blood meal from an infected individual. The trypomastigote transform into the procyclic trypomastigote and after several cycles of cell division it migrates to the insect's salivary glands, where it differentiates further into the epimastigote form. Epimastigotes develop within the salivary gland into metacyclic trypomastigotes, the infective stage for the mammalian host (Barret *et al*., 2003). Wild animals and cattle are main reservoir hosts for *T. b. rhodesiense*. For *T. b. gambiense* the main reservoir are humans (Welburn *et al*., 2009).

2.1.8.2 Transmission and infection of African Animal Trypanosomiasis

Animal African trypanosomes (*T. congolense*, *T. vivax* and *T. brucei*) replicate in the tsetse fly and are transmitted through tsetse saliva when the fly feeds on an animal. The three main species of tsetse flies for transmission of trypanosomes are *Glossina morsitans*, which favors the open woodland of the savanna; *G. palpalis*, which prefers the shaded habitat immediately adjacent to rivers and lakes; and *G. fusca*, which favors the high, dense forest areas. Trypanosomiasis is also mechanically transmitted by tsetse and other biting flies through the transfer of blood from one animal to another. The most important mechanical vectors are flies of the genus *Tabanus*, but *Haematopota*,

Liperosia, *Stomoxys*, and *Chrysops* flies have also been implicated. In Africa, both *T. vivax* and *T. b. brucei* have spread beyond the "tsetse fly belts" (Clausen *et al*., 1993), where transmission is principally by *Tabanid* and *Hippoboscid* flies.

2.1.9 Life cycle of African trypanosomes

The life cycle of African trypanosomes consists of three distinct stages within both, the mammalian host and the insect vector (Figures 4 and 5).

2.1.9.1 Life cycle of the Human African trypanosomes

The life cycle, which is similar for *T. b. rhodesiense* and *T. b. gambiense* (Figure 4), starts when an infected tsetse fly takes its blood meal from the mammalian host and inoculates the metacyclic trypomastigote form of the parasite present in its saliva. The trypanosome quickly transforms into long slender trypomastigotes and proliferates by binary fission at the site of the bite for a few days leading to an inflammatory chancre. The parasites, then spread to the draining lymph nodes and the early or first haemolymphatic stage of infection (bloodstream), through which they reach other organs such as the liver, heart spleen and the endocrine system (Enanga *et al*., 2002). After a few weeks (*T. b. rhodesiense*) or several months (*T. b. gambiense*) trypanosomes cross the blood-brain barrier (BBB) to invade the central nervous system (CNS) through mechanisms that are poorly understood (Enanga *et al*., 2002). At this stage, the patient is said to be in the meningoncephalitic, the second stage or late stage of infection. When parasitaemia in the host increases, long slender trypomastigotes transform into non-dividing, short-stumpy trypomastigotes, which are taken up by the tsetse fly where they complete their life cycle. In the insect’s mid-gut, trypamosomes transform into procyclic form and after two or three weeks, they migrate to the salivary glands. Here they undergo other transformations into metacyclic forms ready to be injected into a susceptible vertebrate host during the next blood meal.

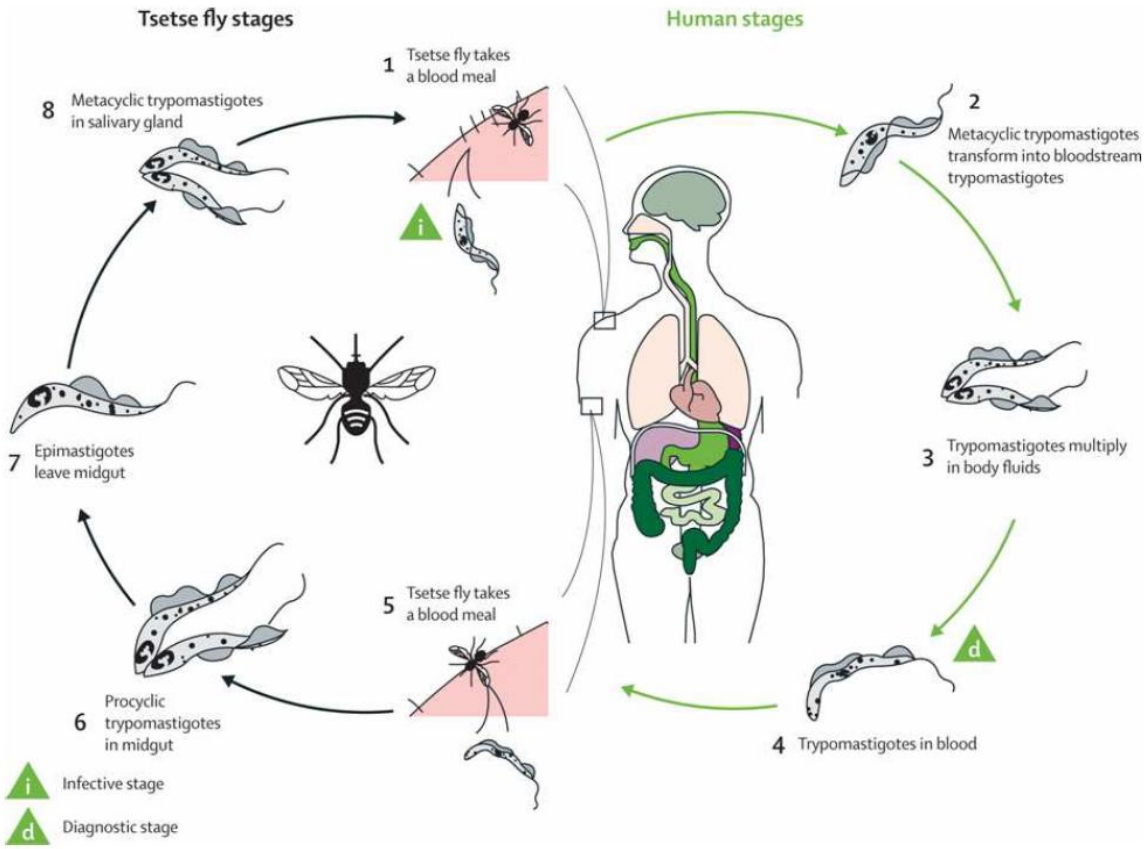


Figure 4: Schematic representation of the life cycle of Trypanosoma brucei in the mammalian and tsetse fly vector (source Blum *et al*., 2008).

2.1.9.2 Life cycle of the African Animal trypanosomes

During their feeding on a trypanosome-infected animal, tsetse flies ingest the bloodstream form trypanosomes that have to go through a number of morphological, biochemical and physiological transformations within the fly to become infectious (metacyclic forms) again for the mammalian host. For *T. vivax*, the entire cycle takes place in the proboscis whereas for *T. congolense*, the development occurs in the midgut and probosci. Once the final infectious metacyclic trypanosomes are present in the tsetse fly, they will be injected in the dermis of the mammalian host at each blood-feeding event (Figure 5). Within the mammalian host, the trypanosomes transform, start replication and are transported to the draining lymph node through the afferent lymphatic system. Then through the efferent lymphatic system, parasites reach the

thoracic lymph duct and finally enter the blood circulation where they continue to replicate (Akol and Murray, 1986). While *T. congolense* is preferentially localized in small blood vessels and capillaries, *T. vivax* is also seen in the tissues of their host. The duration of the pre-patent period generally ranges from 1 to 3 weeks depending on the species and strain of trypanosomes and on the immune status of the host (Clausen *et al*., 1993).

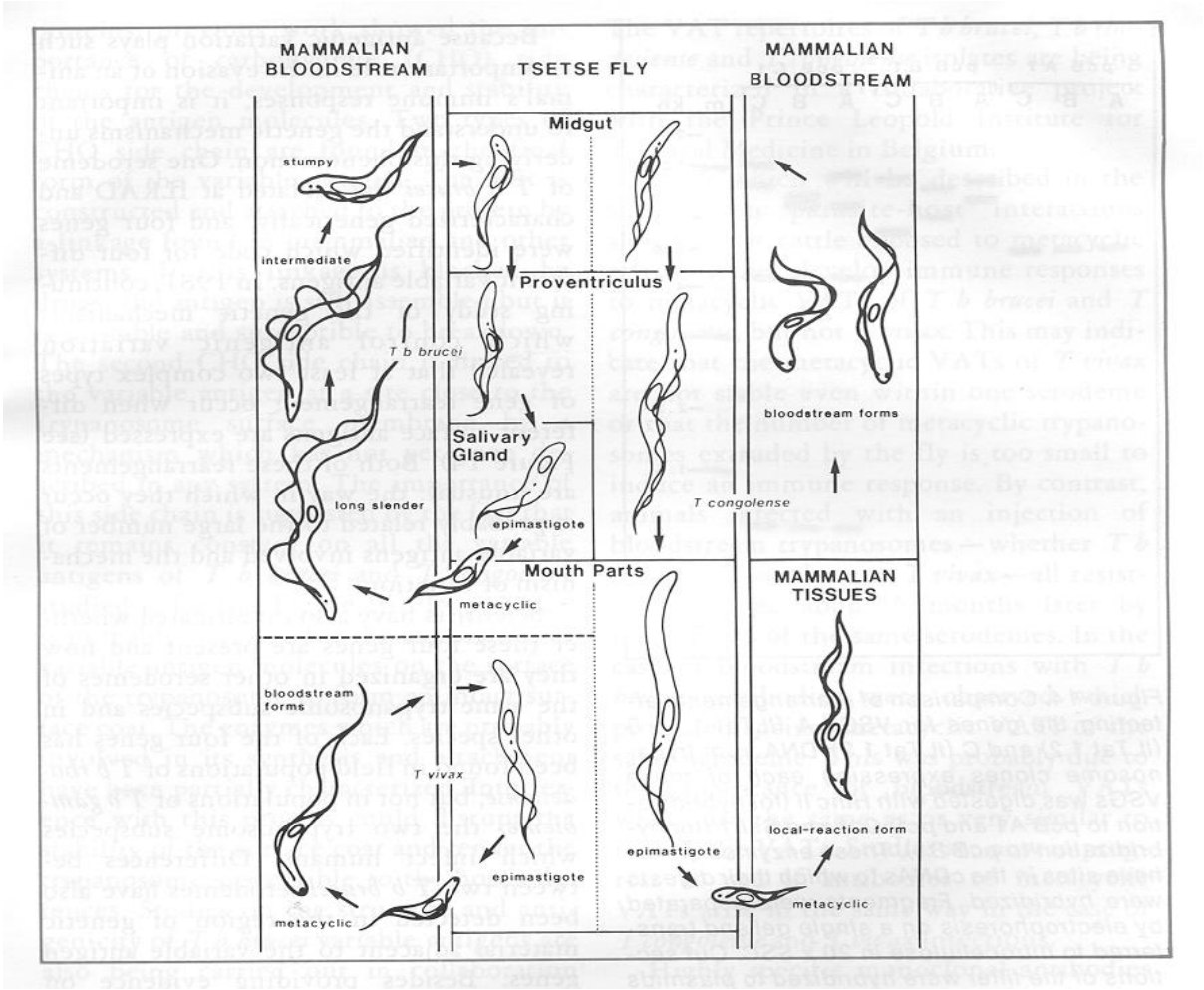


Figure 5: Life cycle of *T. b. brucei*, *T. congolense* and *T vivax* (source Peacock *et al*., 2012).

2.1.10 Clinical manifestation

The first sign of trypanosomiasis may be a localized swelling (chancre) at the site of the fly bite, but this usually remains unnoticed. Initially the trypanosomes are present extracellularly in the subcutaneous tissue at the site of the bite of the tsetse fly and give rise to the chancre. Most cases of trypanosomiasis are chronic, but acute disease, which may be fatal, can also occur.

2.1.10.1 Clinical manifestation of Human African Trypanosomiasis

In the first stage trypanosomes enter the bloodstream and multiply there. This stage is accompanied by fever and lymphoid hyperplasia leading to enlargement of the spleen and especially of the cervical lymph nodes. The second stage involves central nervous invasion associated with intermittent fever. Trypanosomes in the cerebrospinal fluid produce diffuse meningoencephalitis. The central nervous system lesions are accompanied by headache, apathy, wasting of musculature, tremors, inability to walk and eventually to somnolescence, paralysis, coma and death, usually after a course of 1-3 years (Mehlhorn *et al*., 2001).

2.1.10.2 Clinical manifestation of African Animal Trypanosomiasis

The primary clinical signs are an intermittent fever, signs of anemia, lymphadenopathy and weight loss. Animals lose condition and become progressively emaciated. Milk yield may be decreased in dairy animals. Neurological signs, dependent edema, cardiac lesions, diarrhea, keratitis, lacrimation, appetite loss and other clinical signs have also been reported. Effects on reproduction include abortions, premature births and perinatal losses, as well as testicular damage in males (Barrett *et al*., 2003). Deaths are common among chronically infected animals, and animals that recover clinically may relapse when stressed. Sudden deaths have been reported in small ruminants infected with *T. vivax*. Trypanosomes can cause immunosuppression, and concurrent infections may complicate this disease. An acute hemorrhagic syndrome has been reported among cattle infected with *T. vivax* in Africa. Affected animals have enlarged lymph nodes and signs of severe anemia, and they develop widespread visceral and mucosal hemorrhages, particularly in the gastrointestinal tract. In one outbreak, the main

hemorrhagic sign was bleeding from the ears. Weight loss can be severe. This syndrome can be rapidly fatal (Barrett *et al*., 2003).

2.1.11 African Trypanosomiasis control strategies

Tsetse fly eradication and chemotherapy are the only effective African trypanosomiasis control methods available. The strategies for the disease can target the vector, the animal reservoir or simply the human reservoir. The control of trypanosomiasis involves reduction of parasite transmission from the reservoir to the vector and onto reservoirs or hosts.

2.1.11.1 Human African Trypanosomiasis control strategies

Control of both forms of HAT (Gambianse and Rhodesiense) in a location requires understanding of the environment for transmission by tsetse flies, the main reservoir species in the area, the human interactions with the environment, tsetse habitats and reservoir species (WHO, 2013). For *T. b. gambiense* form, where humans represent the main reservoir, active case-finding and treatment of the infected patients are the strategies adopted to interrupt transmission (Simarro *et al*., 2008; Brun *et al*., 2009). For *T. b. rhodesiense*, however, the control of infection in livestock (and, to a minor extent, in wildlife) through the use of curative or prophylactic trypanocides or application of insecticides to the animals plays a crucial role in its control (Fèvre *et al*., 2006; Simarro *et al*., 2008).

2.1.11.2 African Animal Trypanosomiasis control strategies

Several approaches to tsetse control have been used with varying degrees of success (Molyneux *et al*., 1996). Discriminative bush clearing, extensively used in early tsetse fly eradication campaigns, has been locally useful because it eliminates the breeding places of the tsetse. But, to be completely effective, bush clearing requires ecologically unacceptable destruction of vast areas of bush and forest. It is still a useful procedure when used locally in conjunction with other control methods. Game elimination, and thus elimination of the main source of blood meals for the tsetse, was used in early eradication campaigns (Molyneux *et al*., 1996). This was an ineffective and wasteful

procedure. Sterilization of male tsetse received considerable attention in the 1980's. Early problems with breeding of the male flies have been overcome, and field trials have been done in both East and West Africa to determine the effectiveness of this approach in vector control (Hargrove, 2003). In limited trials, this procedure reduced fly populations. Ground and aerial spraying with insecticides and the use of synthetic pyrethroids on cattle have lowered fly densities in some areas, but widespread use requires considerable international cooperation and expense. Widespread application of insecticide has the tremendous disadvantage of also eradicating many other arthropods, several of which are desirable. The introduction of odor-baited targets impregnated with insecticides was promising as a means of reducing the tsetse fly (Hargrove, 2003). The use repellency technique has currently attracted attention of researchers (Mathew *et al*., 2015).

2.1.12 Drugs used for African Trypanosomiasis and their limitations

2.1.12.1 Drugs used for HAT and their limitations

The drugs currently used for the treatment of sleeping sickness are suramin (**1**), pentamidine (**2**), melarsoprol (**3**), eflornithine (**4**), and nifurtimox (**5**) (Figure 6). Suramin (**1**) was introduced in the early 1920's and to this day remains the drug of choice for treatment of the early phase of *T. b. rhodesiense* infections. The mode of action is still a complete mystery (Fairlamb, 2003). Pentamidine (**2**) was first introduced in 1949. The drug is only used as the secondline drug when therapy with suramin (**1**) is contraindicated (Pépin *et al*., 1994). The mechanism of action is not well understood. It is known that the drug is taken up by at least three transporters (Carter, 1993; De Koning, 2001) and then binds to negative-charged cellular components, and disrupts the structure of kinetoplasts DNA (Shapiro *et al*., 1990).

Melarsoprol (**3**) was introduced in 1949 for the treatment of late-stage sleeping sickness. The drug causes a serious reactive encephalopathy in 5-10 % of the cases, half of which are fatal (Pépin *et al*., 1994). Other common side effects include vomiting, abdominal colic and peripheral neuropathy. The mechanism of action could be the

combination of trypanothione depletion and the inhibition of trypanothione reductase (Fairlamb, 2003). Eflornithine (**4**) is the drug of choice for treatment of late-stage sleeping sickness caused by *T. b. gambiense*. The drug is not recommended for *T. b. rhodesiense* infections. The inhibition of polyamine biosynthesis by eflornithine (**4**) triggers a wide range of downstream biochemical effects, but opinion has been divided as to which of these are responsible for the trypanocidal effect (Heby, 2003). Nifurtimox (**5**) is currently registered for the treatment of chagas disease. Side effects are extremely common and 50 % of patients are unable to complete a full course of treatment. Nevertheless, nifurtimox (**5**) has been used in the treatment of late-stage sleeping sickness where eflornithine (**4**) or melarsoprol (**3**) are ineffective (Bray, 1994).

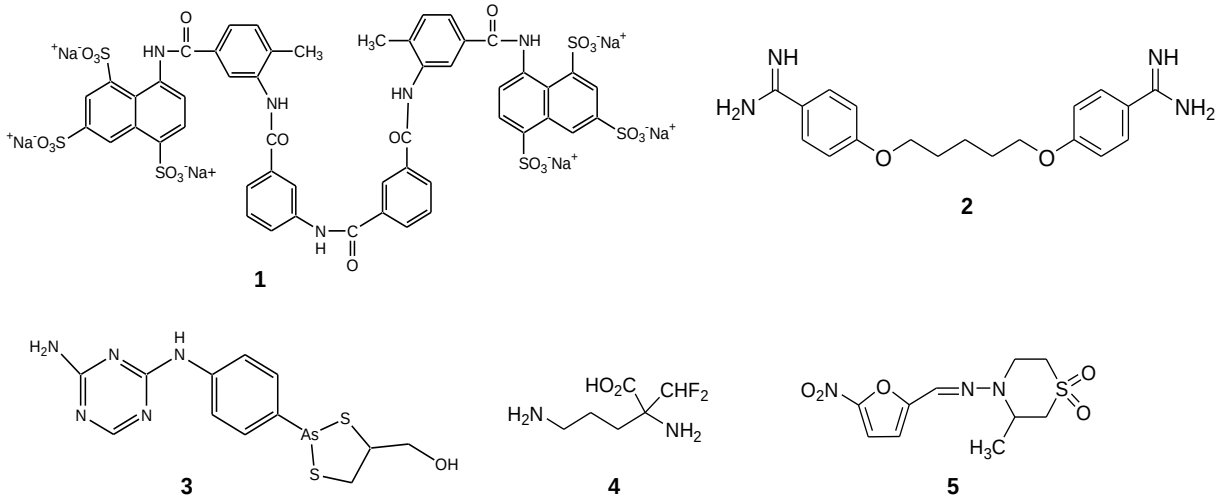


Figure 6: Chemical structures of current drugs used for HAT

2.1.12.2 Potential drugs for HAT

All the currently available HAT treatments have various shortcomings as highlighted above. The development of new drugs for HAT and other NTDs was however hardly being addressed in the period before year 2000 because these diseases do not offer good returns for the immense financial investments required to bring a drug to the market (Trouiller *et al*., 2002; Adams and Brantner, 2006; Chirac and Toreele, 2006). Since the

year 2000, public private partnerships (PPPs) which are also called product development partnerships (PDPs), were formed to address the drug research and development (R&D) needs of NTDs with funding from public and philanthropic organizations and other donors (Chatelain and Ioset, 2011). As a result various compounds are now at different stages of the drug discovery and development (Figure 7).

Fexinidazole

Fexinidazole **(6)** is a nitroheterocyclic molecule that has shown promising activity against *T. brucei* strains (Kaiser *et al*, 2011) and visceral leishmaniasis (Wyllie *et al*., 2012). Fexinidazole was originally shown to have anti-trypanosomal activity by the drug company Hoechst in the 1980s but its development was, for unknown reasons, not pursued to clinical trials. It has, however, been re-discovered through compound mining and subsequent screening of at least 700 nitroheterocycles by the Drugs for Neglected Diseases initiative (DNDi) (Torreele *et al*., 2010). The *in vitro* activity of fexinidazole and its metabolites fexinidazole-sulfoxide and fexinidazole-sulfone are indicated by IC_{50} values ranging from 0.7 to 3.3μM against both drug-sensitive and drug-resistant *T. brucei* spp (Kaiser *et al*., 2011). Fexinidazole was subsequently shown to cure *T. b. rhodesiense* and *T. b. gambiense* acute and chronic mouse models (Kaiser *et al*., 2011), was well tolerated in humans during phase I clinical trials (Torreele *et al*., 2010). Apart from the compound's Ames test result being positive and the need for high dose rates to achieve *in vivo* efficacy, the available pharmacological data are all promising (Maser *et al*., 2012), justifying optimism that fexinidazole might complete the drug development program successfully.

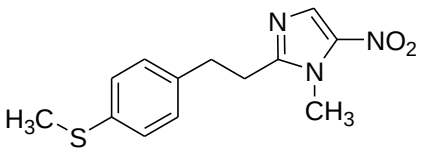
Benzoxaboroles

The oxaboroles are a promising new class of antimicrobials that contain boron in their structure. Screening an oxaborole library against *T. brucei* at Scynexis (Research Triangle Park, NC) and further activity based structural modifications led to the selection of oxaborole SCYX-7158 **(7)** as a clinical drug candidate for second stage

HAT (Jacobs *et al*., 2011; Maser *et al*., 2012). The compound has an IC_{50} against *T. b. rhodesiense* and *T. b. gambiense* strains between 0.2-1 μM. SCYX-7158 also cures mouse models of both 1st and 2nd stage HAT (Jacobs *et al*., 2011). Phase 1 clinical trials were initiated in 2012 by DNDi (Maser *et al*., 2012), ensuring that the HAT pipeline will not be empty even should an unexpected failure of fexinidazole occur.

Novel Diamidines

The Consortium for Parasitic Drug Development (CPDD) has focused on the diamidines class of compounds in their search for potential new therapeutic agents for neglected tropical diseases such as HAT. This was largely influenced by the success of pentamidine against *T. b. gambiense* since 1941 (WHO, 2012). Continued interest in diamidines as antiparastic agents led the synthesis of novel aromatic diamidines. Six of these aromatic diamidines, including furamidine DB75 **(8)**, were more active against *T. b. rhodesiense* than pentamidine (Das and Boykin, 1977). Despite their improved activity against trypanosomes, the new diamidines were, like pentamidine, not well absorbed after oral administration due to their positively charged amidine moieties. This was considered a significant limitation because of the need to develop easy to use oral drugs that would be more appropriate for the resource and infrastructure poor areas where HAT is endemic (Etchegory *et al*., 2001; Wilson *et al*., 2008).



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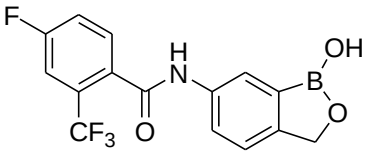

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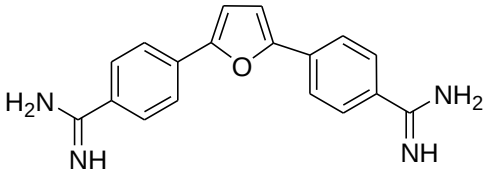

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Figure 7: Chemical structures of new drugs being developed for HAT

2.1.12.3 Drugs used for Animal African Trypanosomiasis and their limitations

Trypanocidal drugs remain widely available and most relied on by farmars in sub-Saharan Africa. The use of trypanocides is often the first control method tried by farmers when their cattle develop symptoms of the disease (Geerts *et al*., 2001). It has been estimated that about 35 million doses of trypanocides are administered each year to an approximately 45 - 60 million cattle at-risk of trypanosomiasis (Kristjanson *et al*., 1999; Sones, 2001). Trypanocides are popular because farmers can directly treat and, if successful, cure their own animals without relying on the efforts of others. The main compounds used for the treatment of AAT are Diamidines (diminazene aceturate), Phenanthridium (homidium and isometamidium salts), Quinoline-pyrimidine (quinapyramine salts) and Napthalidines (suramine). These drugs have been on the market for over 40 years and several generic forms of them from a wide range of companies have become available on the African market (Holmes *et al*., 2004).

Figure 8 shows the structures of the current drugs for AAT. Diminazine aceturate **(9)** is the most widely used chemotherapeutic drug for AAT. The drug is effective against all three African animal trypanosomes (Holmes *et al*., 2004). Homidium bromide **(10)** is used as preventive and therapeutic agent. It is effective against *T. congolense* in cattle and *T. vivax* in pigs, small ruminants and horses. However it is not advised to use it because of the carcinogenic activity of ethidium bromide (McCann *et al*., 1975; Desquesnes *et al*., 2011). Pyrithidium bromide **(11)**, an analogue of homidium bromide, is useful in the prophylaxis of *T. vivax* and *T. congolense* infections in cattle, sheep, and goats and can give protection for up to 6 months. Quinapyramine **(12)** is recommended as therapeutic treatment against *T. congolense* in camels. Quinapyramine dimethylsulphate is suggested as prophylactic use for *T. vivax* in equines and *T. brucei* in pigs (WHO, 2006; Holmes *et al*., 2014). Isometamidine **(13)** can be used as preventive and therapeutic agent for *T. vivax* and *T. congolense* in cattle and small ruminants and *T. brucei* in equines. Protection against trypanosomiasis of up to 6 months can be achieved with the use of isometamidium. Suramine **(14)** is recommended

as a therapeutic and prophylactic agent for *T. evansi* in camels and equines. Melarsomine **(15)** is preferred as therapeutic agent for *T. evansi* in camels, but also in horses. Although melarsomine has been scarcely used in other animal species, some studies have demonstrated its efficacy but only with higher doses (0.5 - 0.75 mg/kg) in goats, pigs, cattle and buffalos (Dia *et al*., 2007; Gutierrez *et al*., 2008). Of the drugs available only melarsomine hydrochloride and diminazene aceturate are considered safe for use in all animal species.

Despite the continuous demand for trypanocides by livestock keepers, is not considered sufficient to justify investment by large pharmaceutical companies in the development and licensing of new animal trypanocides; the cost of which is estimated from 200 to 800 million dollars. Manufacturers of drugs do not consider treatment of trypanosomiasis to offer profitable potential (Witola *et al*., 2005) and thus investment in drugs against these diseases is low. The unwillingness of pharmaceutical companies coupled with the appearance of resistance to the existing trypanocidal drugs (Zhou *et al*., 2004; Witola *et al*., 2005) it is necessary to search for new drugs.

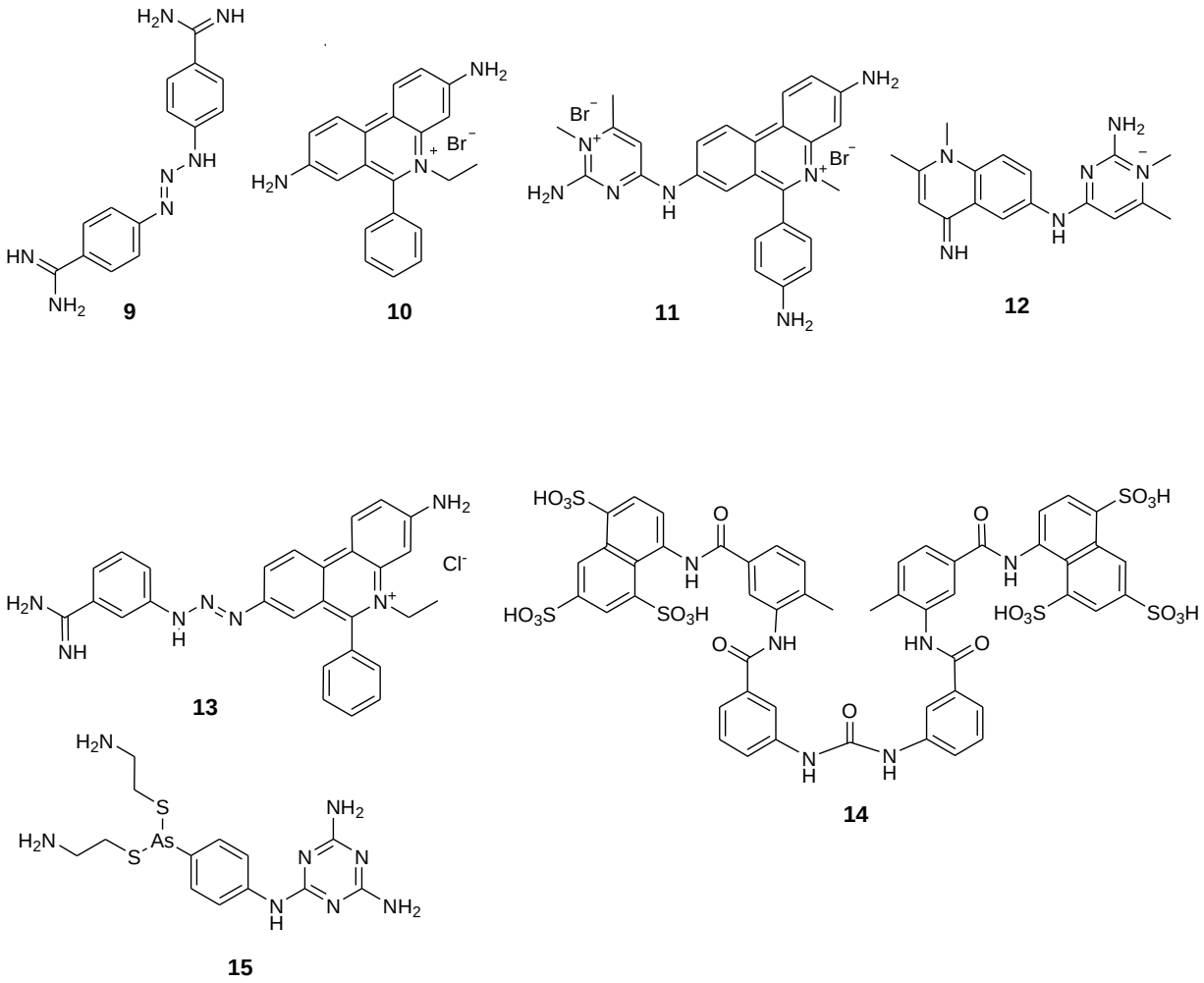


Figure 8: Chemical structures of drugs used for African Animal Trypanosomiasis

2.2 Medicinal plants with anti-trypanosomal activity

Several medicinal plants have been reported for the treatment of human and animal trypanosomiasis (Clayton and Wolf, 1993). The use of decoctions from medicinal plants in the treatment of trypanosomiasis dates as far back as ancient Egypt, Greece, Mediterranean, India, Assyria and China (Trease and Evans, 1989). However, inspite of the possible role of phytochemicals as trypanocides (Asuzu and Chimeme, 1990; Mbaya *et al*., 2007; 2009a; 2010), some of the secondary metabolites in the extracts have been reported to be toxic in nature (Mbaya *et al*., 2007).

2.2.1 Medicinal plants with *in vitro* anti-trypanosomal activity

Table 1 shows a summary of medicinal plants with *in vitro* anti-trypanosomal activity. In this method, trypanosomes were propagated in specialized media assayed with extracts of medicinal plants at various concentrations (Atawodi *et al*., 2002; 2003; Mbaya *et al*., 2010). This was followed by incubation at 37˚C and parasitaemia determination (Kamanzi *et al*., 2004; Nok, 2004; Particia *et al*., 2005; Shuaibu *et al*., 2008).

Table 1: Some plants with *in vitro* anti-trypanosomal activity

Trypanosome	Medicinal plant	Plant part	Reference
T. b. brucei	*Khaya senegalensis*	Flower, stem bark and root	Atawodi *et al*., 2003
	Terminalia avicennoides	Stem bark and flower	Atawodi *et al*., 2003
	Prosopis africana	Flower	Atawodi *et al*., 2003
	Sterculia setigera	Flower	Atawodi *et al*., 2003
	Piliostigma reticulatum	Flower	Atawodi *et al*., 2003
	Anogeissus leiocarpus	Flower	Atawodi *et al*., 2003
	Sclerocary birrea	Stem bark	Atawodi *et al*., 2003
	Commiphora kerstngii	Stem bark	Atawodi *et al*., 2003
	Azadirachta indica	Stem bark	Mbaya *et al*., 2010
	Securidaca longependuncullata	Root bark	Aderbauer *et al*., 2008
	Lawsonia inermis	stem bark	Wurochekke *et al*., 2004
	Mitragyna ciliata	stem bark	Ogbunugafor *et al*., 2008
	Cassia sieberiana	Leaves, twigs	Sara *et al*., 2004
T. cruzi	*Bachris trimera*	Stem bark	Particia *et al*., 2005
	Prunus domestica	Stem bark	Particia *et al*., 2005
	Sambucus canadensis	Stem bark	Particia *et al*., 2005
	Tanacetum parthenium	Stem bark	Particia *et al*., 2005
	Matricaria chamomilla	Stem bark	Particia *et al*., 2005
	Piper regnellii	Stem bark	Particia *et al*., 2005
	Stryphnodendron adstringens	Stem bark	
T. b. rhodesiense	*Hymenocardia acida*	Leaves	Sara *et al*., 2004
	Pericocopsis laxiflora	Leaves	Sara *et al*., 2004
	Trichilia emetica	Leaves, stem bark and root	Kamanzi *et al*., 2004
	Strychnos spinosa	Leaves	Kamanzi *et al*., 2004
	Albizia zygia	Stem bark	Kamanzi *et al*., 2004
	Enantia polycarpa	Stem bark, flowers	Kamanzi *et al*., 2004
T. congolence	*Khaya senegalensis*	Stem bark	Atawodi *et al*., 2003
	Terminalia avicennoides	Stem bark	Shuaiba *et al*., 2008
	Anogeissus leiocarpus	Stem bark	Shuaiba *et al*., 2008

2.2.2 Medicinal plants with *in vivo* anti-trypanosomal activity

A summary of medicinal plants reported to have *in vivo* anti trypanosomal activity against humoral trypanosomes is shown in Table 2. *Trypanosoma brucei* group such as *T. b. brucei*, *T. evansi*, *T. b. rhodesiense* and *T. brucei gambiense* were classified as humoral (Losos and Ikede, 1972; Mbaya *et al*., 2009b). For *T. cruzi*, however, it exists in two forms, trypomastigote in the blood (haemic) and amastigote (humoral) intracellularly in the tissues (Losos and Ikede, 1972).

Table 2: Some plants with *in vivo* anti-trypanosomal activity

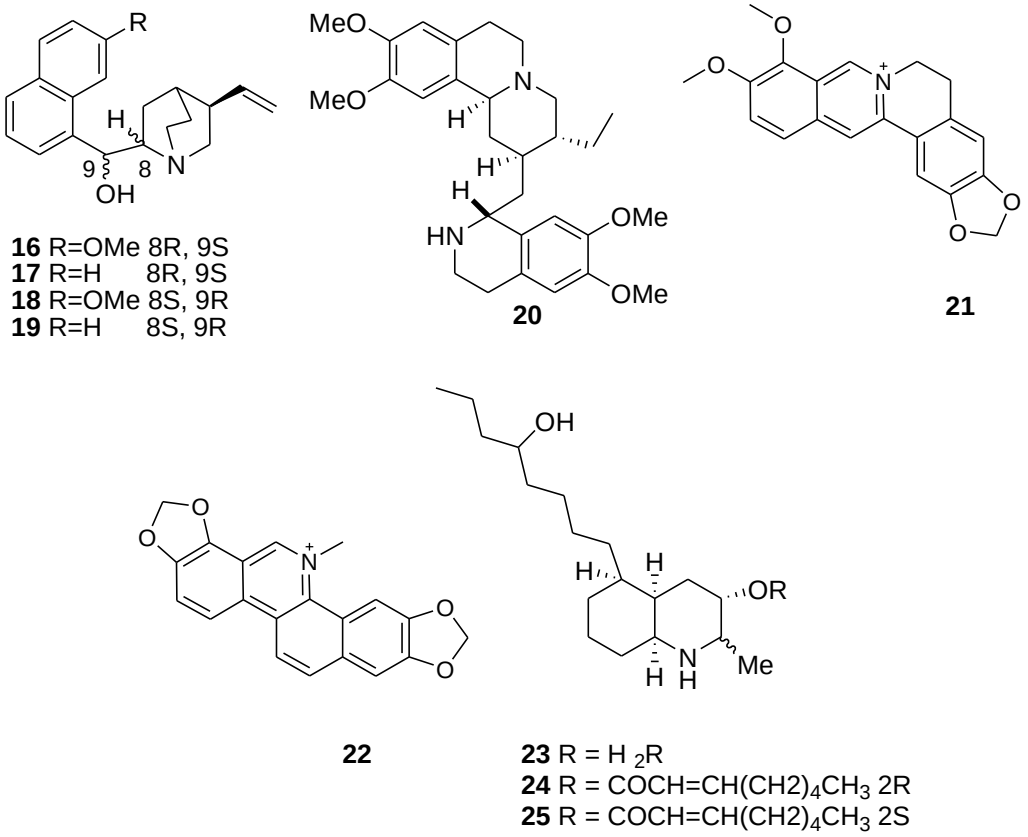
Trypanosomes	Medicinal plant	Part tested	Reference
T. b. brucei	*Guiera senegalensis*	Leaves	Aderbauer *et al*., 2008
	Securidaca longependuncullata	Root	Aderbauer *et al*., 2008
	Solonecio angulatus	Flower	Nibret *et al*., 2009
	Crotalaria phillipsiae	Twigs	Nibret *et al*., 2009
	Holarrhena africana	Leaf	Nwodo *et al*., 2007
	Garcinia lucida	Stem bark	Jean *et al*., 2007
	Mamordica balsamina	Pulp	Abubakar *et al*., 2005
	Aloe vera	Pulp	Abubakar *et al*., 2005
	Annona senegalensis	Leaves, root	Ogbadoyi *et al*., 2007
	Allium sativum	Pulp	Nok *et al*., 1996
	Mitragyna ciliata	Root	Ogbunugafor *et al*., 2008
	Lawsonia inermis	Root	Atawodi et al., 2003
T. evansi	*Aristolochia bacteolata*	Leaves	Samia *et al*., 2006
T. b. rhodesiense	*Butryrospermum paradoxum*	Stem bark	Mbaya *et al*., 2007
	Artemisia annua	Stem bark	Mbaya *et al*., 2009a
	Morinda lucida	Leaf	Asuzu and Chimeme, 1990
	Azadirachta indica	Stem bark	Mbaya *et al*., 2010
	Aristolochia albida	Leaf	Nok *et al*., 2005
T. congolence	*Butyrospermum paradoxum*	Stem bark	Mbaya *et al*., 2007
	Allium sativum	Pulp	Nok *et al*., 1996

2.3 Isolated compounds with anti-trypanosomal activity

Natural products derived from plants and animals continue to be the major source of medicines for man and the basis of many pharmaceutical drugs. In the area of tropical medicine in particular, widely used antiprotozoal drugs are derived from natural products.

2.3.1 Alkaloids

The quinoline alkaloids from Cinchona bark (Rubiaceae) (quinidine (**16**), cinchonine (**17**), quinine (**18**), cinchonidine (**19**)) have significant trypanocidal activity against *T. b. brucei*. Emetine (**20**), an isoquinoline alkaloid from *Cephaelis ipecacuanha* (Rubiaceae), which has been used in the treatment of amoebiasis, was very trypanocidal. Berberine (**21**) and sanguinarine (**22**), two quaternary benzylisoquinoline alkaloids found in a number of plant families, have shown some activity against *T. b. brucei* (Merschjohann *et al*., 2001). Several alkaloids isolated from marine organisms such as sponges, ascidians and tunicates have been evaluated for their antitrypanosomal activity (Kirsch *et al*., 2000). Lepadins **23**, **24** and **25**, isolated from a *tunicate* species from the genus *Didemnum*, possess an unusual decahydroquinoline skeleton and show significant and selective antitrypanosomal activity *in vitro*. Compounds **24** and **25**, two diastereoisomers, displayed an IC_{50} on *T. b. rhodesiense* bloodstream trypomastigotes (Kirsch *et al*., 2000). The structures of some alkaloids with anti-trypanosomal activity are shown in Figure 9.



16 R=OMe 8R, 9S
17 R=H 8R, 9S
18 R=OMe 8S, 9R
19 R=H 8S, 9R

20

21

22

23 R = H $_2$R
24 R = COCH=CH$(CH_2)_4CH_3$ 2R
25 R = COCH=CH$(CH_2)_4CH_3$ 2S

Figure 9: Structures of alkaloids with anti-trypanosomal activity

2.3.2 Phenolic derivatives

Compound **26** was potently trypanocidal *in vitro* for *T. b. brucei* bloodstream forms only when combined with glycerol (IC_{100} without glycerol 250 μM; IC_{100} with 4 mM glycerol, 0.03 μM). Compound **26** was also shown to be highly non-toxic *in vivo* and able to cross the blood–brain barrier (Yabu *et al.*, 1998). A recent study by the same researchers has shown that treatment with **26** alone could also have therapeutic efficacy in mice if higher doses and a longer treatment are applied. A dose of 100 mg/kg body weight of **26** given intraperitoneally for four consecutive days or 400 mg/kg given orally for eight consecutive days were necessary to cure mice (Yabu *et al.,* 1998). Figure 10 shows the structures of some trypanocidal phenolic derivatives.

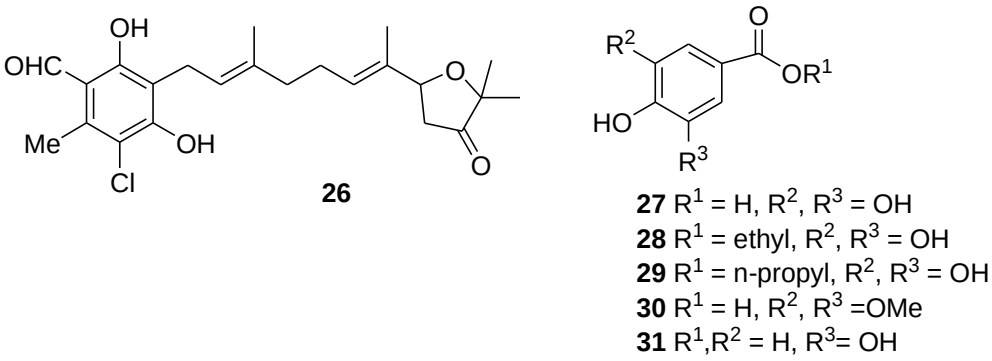


Figure 10: Structures of phenolic derivatives with anti-trypanosomal activity

Other simple phenolic compounds which are widely distributed in plants have been tested for their antitrypanosomal activity as well. Gallic acid (**27)**, gallic acid esters such as ethyl gallate (**28)** and *n*-propyl gallate (**29)**, syringic acid (**30)** and protocatechuic acid (**31)** were all found to have mild trypanocidal activities.

2.3.3 Quinones

It has been reported that quinones, especially 1, 4-naphthoquinones such as plumbagin (**32)** can induce oxidative stress in trypanosomes (Yardley *et al.,* 1996). Diospyrin (**33)**, a bis-naphthoquinone, isolated from the bark of *Diospyros montana* (Ebenaceae), diospyrin dimethyl ether (**34)** and its hydroquinone form **35** were active against *T. b. brucei* (Yardley *et al.,* 1996) (Figure 11).

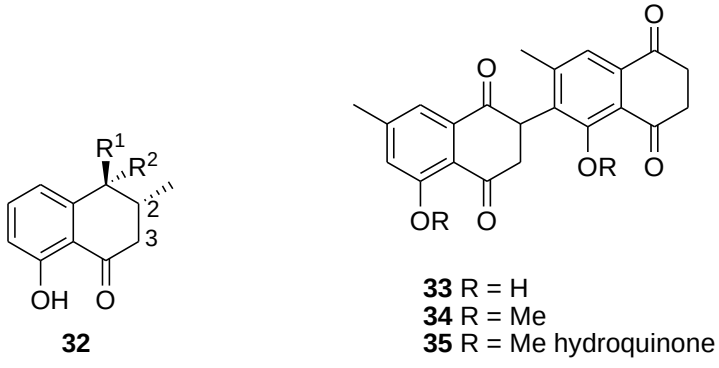


Figure 11: Structures of quinones with anti-trypanosomal activity

2.3.4 Terpenes

Mikus *et al*. (2000) evaluated the effect of several mono- and sesquiterpenes, which are frequently present in essential oils, on the viability of *T. b. brucei* bloodstream forms. Only the natural norisoprenoid **36** and its 1-epimer **37** were active on *T. b. brucei* bloodstream trypomastigotes (Busch *et al.,* 1998). Ilimaquinone (**38)** and pelorol (**39)**, two sesquiterpenes substituted with a quinone or hydroquinone moiety, were isolated from a tropical marine sponge *Dactylospongia elegans* and exhibited weak effects on the mammalian stage of *T. b. brucei* (Goclik *et al*., 2000).

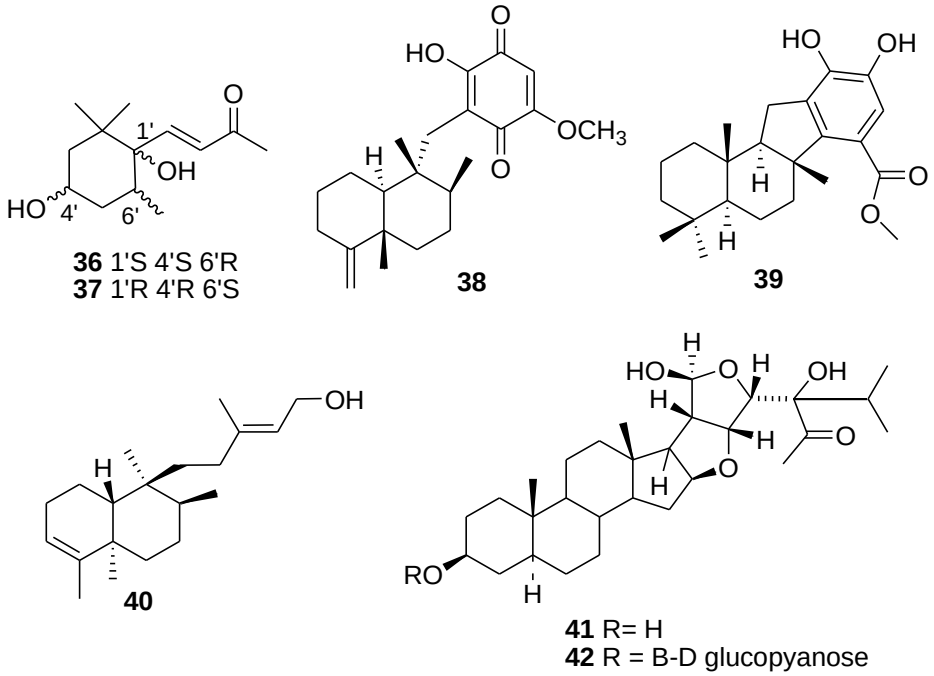


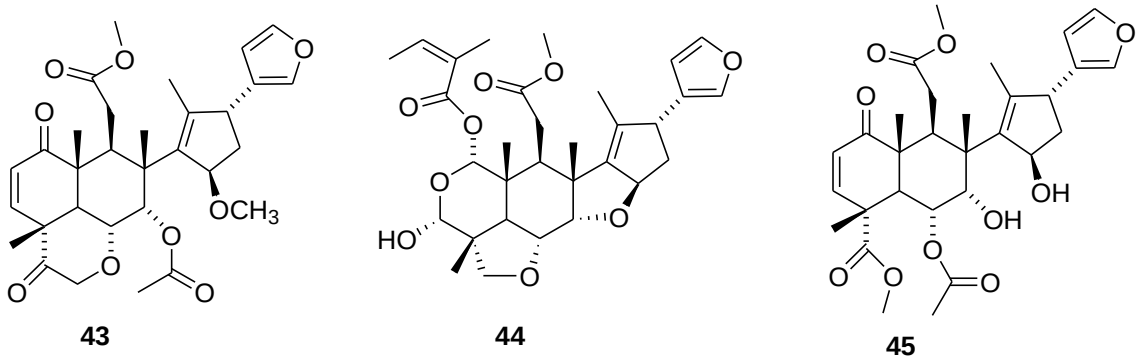
Figure 12: Structures of terpenes with anti-trypanosomal activity

A diastereoisomer of the diterpene kolavenol (**40**) was isolated from rootbark extract of *Entada abyssinica* (Leguminosae), a plant traditionally used in Uganda to treat sleeping sickness. It showed trypanocidal activity with an IC_{50} value of 8.6 μM against *T. b. rhodesiense* (Freiburghaus *et al.,* 1998). Two stigmastane-type steroids, vernoguinosterol (**41**) and vernoguinoside (**42**), isolated from the stem bark of *Vernonia guineensis* (Asteraceae), exhibited significant inhibitory activities against four strains of

T. b. rhodesiense mammalian trypomastigotes with IC_{50} values in the range of 5–10 μM (Tchinda *et al.,* 2002) (Figure 12).

2.3.5 Limonoids

Bitter tetranortriterpenoids, known as limonoids, are biosynthetically related to the quassinoids that are produced by species of Meliaceae. One well known representative from this family is *Azadirachata indica*, the neem tree, widely used as an antiplasmodial plant in Asia. Githua *et al*. (2010) identified three tetranotriterpenoids (**43-45**) (Figure 13) from *A. indica* leaves that were tested against *T. b. rhodesiense* procyclic forms (KETRI 3438). The minimum inhibitory concentration (MIC) values were 6.9 ± 0, 15.6 ± 0 and 7.8 ± 0 μg/ml, respectively. The mode of action of these natural products has not been established.



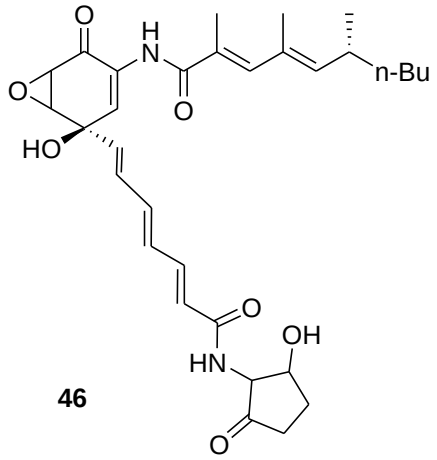
43 **44** **45**

Figure 13: Structures of limonoids with anti-trypanosomal activity

2.3.6 Miscellaneous metabolites

Manumycin (**46)**, an antibiotic produced by *Streptomyces* microorganisms, is potently active *in vitro* against the growth of both bloodstream and procyclic forms of *T. b. brucei* (IC_{50} = 1.5 and 0.4 μM, respectively). Compound **46** is well tolerated *in vivo*, but fails to cure experimental trypanosomiasis in mice when administered intraperitoneally (Ali *et al.*, 1999). Sinefungin (**47)**, a natural nucleoside produced by *Streptomyces grizeolus* and *S. incarnatus*, is a structural analog of *S*-adenosylmethionine (SAM) **48**. It

has been shown that **47** is a strong inhibitor of SAM-dependent transmethylation reactions. Compound **46** potently inhibits the *in vitro* growth of *T. b. rhodesiense* (Bacchi *et al*., 1995). Structures of **46 – 47** are shown in Figure 14.



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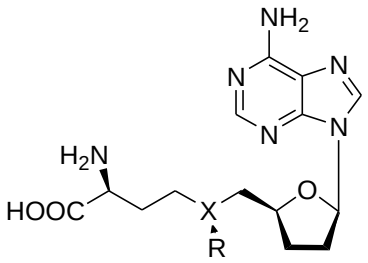

47 X = CH R = NH_2
48 X = S^+ R = CH_3

Figure 14: Structures of miscellaneous metabolites with anti-trypanosomal activity

2.4 Reported pharmacological activities of the Meliaceae species of plants

The Meliaceae family is a flowering family of plants of the order Sapindales, comprising 51 genera and about 1400 species of trees and (rarely) shrubs, native to tropical and subtropical regions (Huang *et al*., 1996). Most members of the family have large compound leaves, with the leaflets arranged in the form of a feather, and branched flower clusters. The fruit is fleshy and colored or a leathery capsule. Being this large, it contains a wide range of floral and fruit structures, and although investigated for many years, it remains a source of great taxonomic difficulty. Chemically, Meliaceae is characterized by its synthesis of modified triterpenes known as limonoids (Buchanan and Hallsall, 1970; Huang *et al*., 1996). This group of compounds has driven a lot of interest because of their high activity over the behaviour and physiology of several phytophagous insect species (Buchanan and Hallsall, 1970). Over 300 limonoids have been isolated to date and they are more abundant in the Meliaceae than in any other family (Huang *et al*., 1996). These are modified triterpenes with or derived from a precursor with a 4,4,8-trimethyl-17-furanyl steroid skeleton **(49)** (Figure 15).

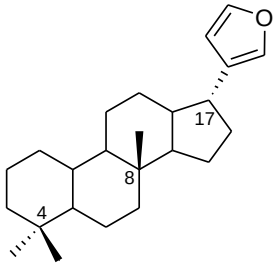

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Figure 15: Structure of a 4,4,8-trimethyl-17-furanyl steroid skeleton

2.4.2 *Melia azedarach*

Melia azedarach is a small to medium deciduous tree attaining a height up to 45 m tall; bole fluted below when old, up to 30-60 (max. 120) cm in diameter, with a spreading crown and sparsely branched limbs. It is grown as an ornamental avenue tree and sometimes as a shade tree in coffee and tea plantation.

2.4.2.2 Ethnomedicinal uses of *M. azedarach*

Melia azedarach is a well known ethnomedicinal tree used in Ayurveda (Deepika and Yash, 2013). Table 3 portrays some of the uses of different parts of *M.azedarach* in traditional system of medicine.

Table 3: Some of the ethnomedicinal uses of *M. azedarach*

Plant part	Ethnomedicinal uses	Reference
Stem bark	Antidiarrhoeal, deobstruent, diuretic	Joy *et al.*,1998
	Rheumatic pain, used in fever to relieve thirst, nausea, vomiting and general debility, loss of appetite, stomachache	Rahmatullah *et al.*, 2010
	Bark decoction is used as a remedy for fever aches and pains	Kokwaro *et al.*, 2009
	Bark decoction is used as a remedy for fever aches and pains	Sen *et al.*, 2010
	Bark paste is used to treat piles,used as lotion on ulcers, syphilitic	
Root Bark	As anthelmintic, used to treat malaria	Dharani *et al.*, 2010 Handa *et al.*, 2006
Leaves	insecticide, Antidiarrhoeal, deobstruent, diuretic	Joy *et al.*, 1998
	fodder and are highly nutritious, Skin diseases like scabies, brushing teeth, loosening pain of tooth, rheumatic pain, fever, insecticide	Rahmatullah *et al.*, 2010
	applied externally on burns, used as mouth wash for gingivitis; pyrexia and bloody piles	Khan *et al.*, 2011
	snake bite	Handa *et al*., 2006
	anemia, eczema and measles, jaundice, malaria and to expel parasitic worms. Decoction is used as astringent and stomachic	Sen *et al.*, 2010
Seed	expectorant, anthelmintic and aphrodisiac and are useful in helminthiasis, typhoid fever, pain in the pelvic region and scrofula	Sen *et al*., 2010
Seed oil	antiseptic for sores and ulcers. It is also used rheumatism and skin diseases such as ring worm and scabies. Internally the oil is useful in malaria fever and leprosy	Khan *et al*., 2011

2.4.2.1 Phytochemicals isolated from *M. azedarach*

Preliminary phytochemical screening of *M. azedarach* showed the presence of number of organic molecules such as terpenoids, flavonoids, steroids, acids, anthraquinones, alkaloids, saponins, tannins and phytosterols (Rishi *et al*., 2003; Suresh *et al.,* 2008). A variety of compounds have been isolated from *M. azedarach* (Figure 16). Kampherol **(50)**, quercetin **(51)**, campesterol **(52)**, β-sitosterol **(53)** and other related metabolites were isolated from the leaf extract (Sen and Batra, 2012). Chemical constituents of seeds include β-sitosterol (**53)**, daucosterol (**54**) and other related metabolites (Carpinella *et al*., 2005). The main constituents of stem bark and root bark are liminoids and terpenoids respectively (Kumar *et al*., 2003).

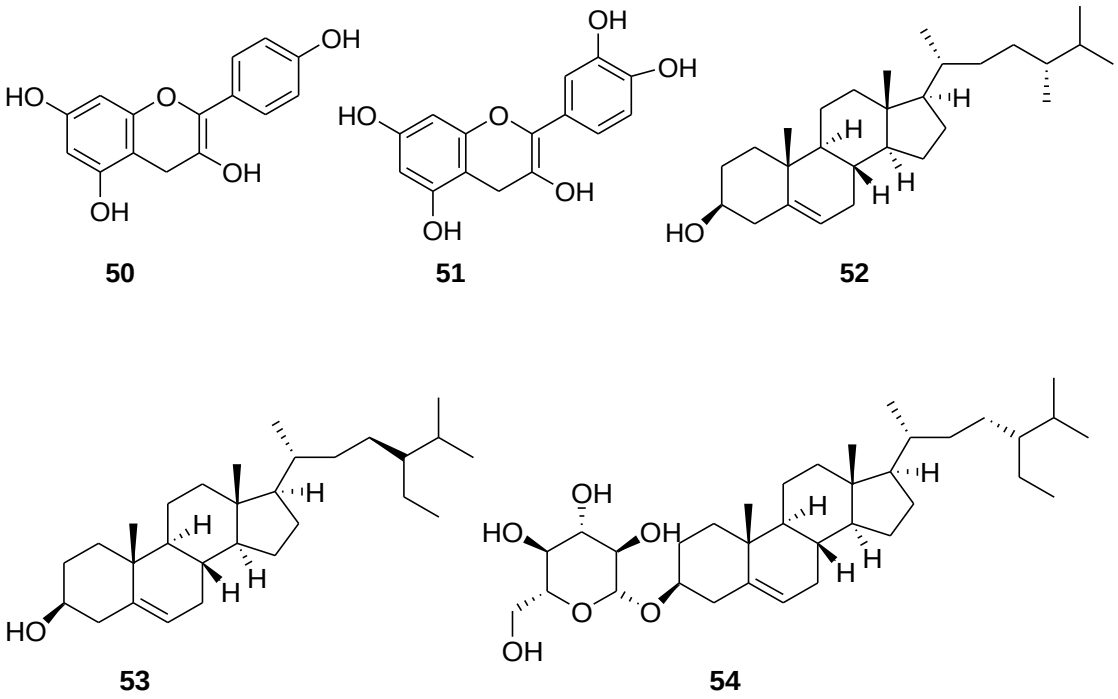


Figure 16: Structures of some phytochemicals from *M. azedarach*

The methanolic leaf extract of *M. azedarach* is used as an anti-inflammatory, hypocholesterolemic cancer preventive, nematicide, insecticide, hepatoprotective, antihistaminic properties. Hydro-alcoholic extract of *M. azedarach* roots exhibited significant anti-implantation and anti-progestational activity and devoid of estrogenic/anti-estrogenic activity (Vishnukanta *et al.*, 2009). The stem bark was found to exhibit antioxidant activity. Some of the reported pharmacological activities of different parts of *M. azedarach* are summarized in Table 4.

Table 4: Some of the reported pharmacological activities of *M. azedarach*

Plant extract	Pharmacological activity	Disease/organism	Reference
leaf, seed	Larvicidal	*Busseola fuscala, Spodoptera littoralis*	Gebre *et al*., 1999
seed	Pubicidal, anti oviposition, biting deterrency, anti oviposition, anti hatchability, anti feedant	*Anopheles stephensi, Culex quinque fasciatus, Orseolia oryzae, 3Mythimna separata, Erias vitella*	Nathan *et al*., 2006 Gajmer *et al*., 2002
Fruit	Antifeedant, pubicidal,anti oviposition	*Liriomyza huidobrensis*	Banchio *et al*., 2003
seed, fruit	Antifeedant	*Pseudaletia unipunctata, Trachiaplusia ni, Spodoptera eridania, Plutella xylostells*	Akhtar, *et al*., 2008 Charleston *et al*., 2005
leaf, seed	Antihelminthal	*Haemonchus contortus*	Akhtar *et al.*, 2004
leaf	Hepatoprotective action	CCl_4 induced hepatic damage, Paracetamol induced hepatic damage	Rajeswary *et al*., 2011 Ahmed *et al*., 2008
Root	Anti-Inflammatory activity	Carageenan induced paw edema	Vishnukanta. 2010
leaf, seed	Antilithiatic	Ethylene glycol-Induced Nephrolthiasis	Tina *et al*., 2006
Leaf	Antiulcer activity	Aspirin induced ulcers in Albino rats	Bahuguna *et al*., 2009

2.4.3 *Azadirachta indica*

Azadirachta indica (neem) is a small to medium-sized tree, usually evergreen, up to 15 (30 max.) m tall, with a round, large crown up to 10 - 20 m in diameter. Bole branchless for up to 7.5 m, up to 90 cm in diameter, sometimes fluted at base; bark moderately thick, with small, scattered tubercles, deeply fissured and flaking in old trees, dark grey outside and reddish inside, with colourless, sticky foetid sap.

2.4.3.2 Ethnomedicinal uses of *A. indica*

All parts of *A. indica* tree have been used medicinally for centuries. It has been used in Ayurvedic medicine for more than 4000 years due to its medicinal properties (Brototi *et al*., 2011). The medicinal utilities have been described, especially for leaf, fruit and bark (Thakur *et al*., 1981). Neem oil and the bark and leaf extracts have been therapeutically used as folk medicine to control leprosy, intestinal helminthiasis, respiratory disorders, constipation and also as a general health promoter (Kirtikar *et al*., 1935). The oil used for the treatment of rheumatism, chronic syphilitic sores and indolent ulcer has also been evident (Kirtikar *et al*., 1975). Neem oil finds use to control various skin infections (Chopra *et al*., 1956). Bark, leaf, root, flower and fruit together cure blood morbidity, biliary afflictions, itching, skin ulcers, burning sensations and pthysis. Some of the

medicinal attributes of various parts of neem (Ketkar *et al*., 1995) as mentioned in ayurveda have been summarized in Table 5.

Table 5: Some of the ethnomedicinal uses of *A. indica*

Plant part	Ethnomedicinal uses	Reference
Leaf	Leprosy, eye problem,epistaxis, intestinal worms, anorexia, biliousness, skin ulcers.	Thakur *et al*., 1981, Kirtikar *et al*., 1935
Bark	Analgesic, alternative and curative of fever.	Thakur *et al*., 1981
Flower	Bile suppression, elimination of intestinal worms and phlegm itching, skin ulcer, burning sensation and fruit together leprosy	Ketkar *et al*., 1995
Fruit	Relieves piles, intestinal worms, urinary disorder, epistaxis, phlegm, eye problem, diabetes, wounds and leprosy	Thakur *et al*., 1981
Root, bark, leaf	Blood morbidity, biliary afflictions	Ketkar *et al*., 1995
Twig	Relieves cough, asthma, piles, phantom, tumour, intestinal worms, spermatorrhoea, Obstinate urinary disorder, diabetes	Ketkar *et al*., 1995
Gum	Effective against skin diseases like ringworms, scabies, wounds and ulcers	Kirtikar *et al*., 1935
Seed pulp, oil	Leprosy and intestinal worms	Kirtikar *et al*., 1935

2.4.3.1 Phytochemicals isolated from *A. indica*

Biologically active principles isolated from different parts of the plant (Figure 17) include: nimbin (**55**), nimbolide (**56**), gedunin (**57**), mahmoodin (**58**), azadirachtin (**59**), salannin (**60**) catechin (**61**), epicatechin (**62**), margolone (**63**), margolonone (**64**), isomargolonone (**65**), azadirone (**66**) and many other derivatives of these principles (Kumar *et al*., 2010). The compounds have been divided into two major classes: isoprenoids and others. The isoprenoids include diterpenoids and triterpenoids containing protomeliacins, limonoids, azadirone and its derivatives, gedunin and its derivatives, vilasinin type of compounds and csecomeliacins such as nimbin, salannin and azadirachtin. The nonisoprenoids include proteins (amino acids) and carbohydrates (polysaccharides), sulphurous compounds, polyphenolics such as flavonoids and their glycosides, dihydrochalcone, coumarin, tannins and aliphatic compounds (Kumar *et al*., 2010). Although a large number of compounds have been isolated from various parts of neem, a few of them have been studied for biological activity as portrayed in Table 6.

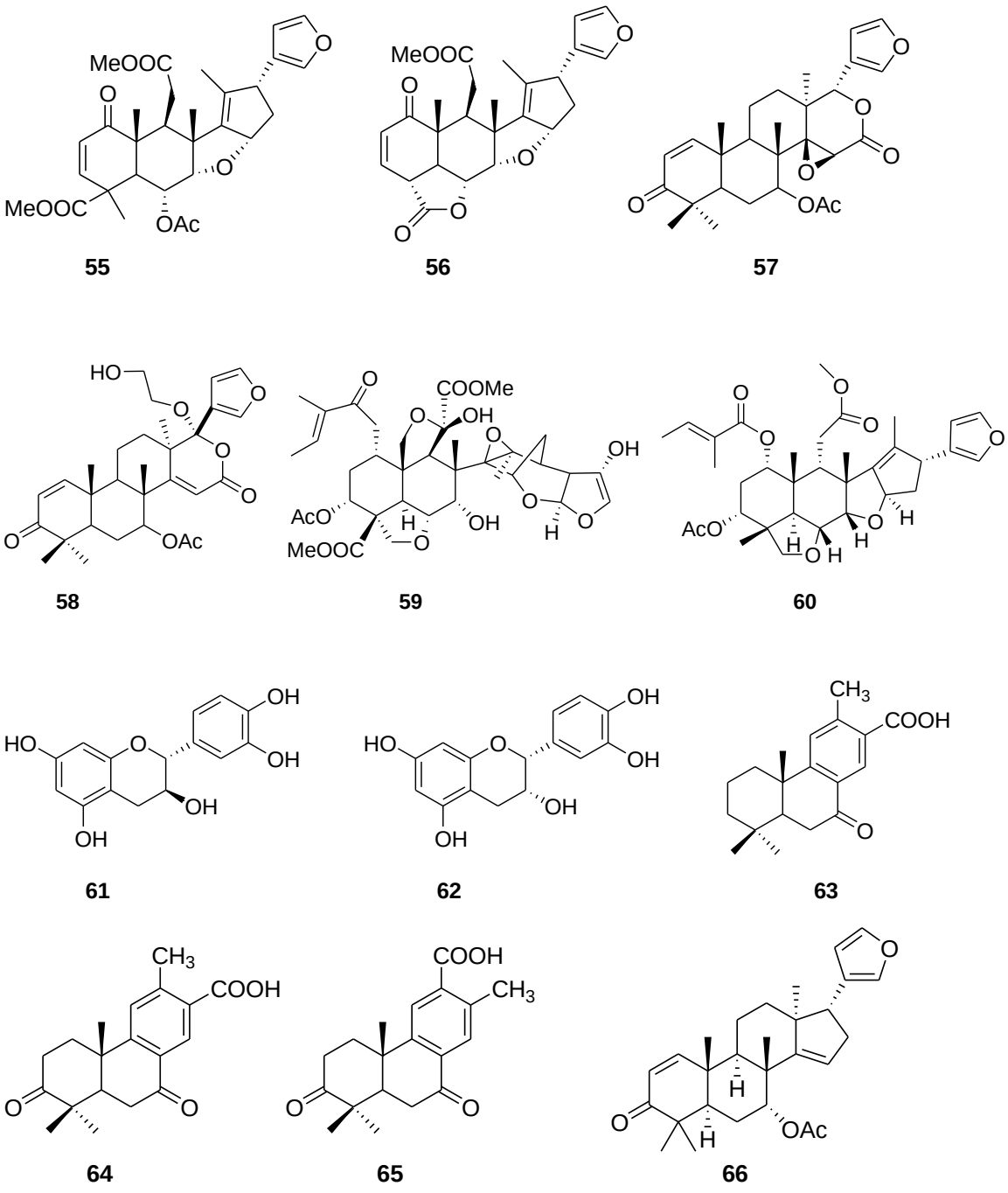


Figure 17: Stuctures of some phytochemicals from *A. indica*

Table 6: Pharmacological activity of some *A. indica* compounds

Plant part	Compound	Pharmacological activity	Reference
Leaf	Cyclic trisulphide and cyclic tetrasulphide	Antifungal	Pant *et al*., 1986
	Polysaccharides	inflammatory	Pant *et al*., 1986
Seed oil	Nimbidin	Anti-inflammatory	Bhargava *et al*., 1970
		Antiarthritic	Pillai, 1981
		Antipyretic	David, 1969
		Hypoglycaemic	Pillai, 1981
		Antigastric ulcer	Pillai, 1984; Pillai *et al*., 1978
		Spermicidal	Sharma *et al*., 1959
		Antifungal, Antibacterial	Murthy *et al*., 1958
		Diuretic	Bhide *et al*., 1958
	Sodium nimbidate	Anti-inflammatory	Bhargava *et al*., 1970; Pillai, 1981
	Nimbin	Spermicidal	Sharma *et al.*, 1959
	Nimbolide	Antibacterial	Rochanakij *et al*., 1985
		Antimalarial	Khalid *et al*., 1989
	Gedunin	Antifungal	Rao *et al*., 1977
		Antimalarial	
	Mahmoodin	Antibacterial	Khalid *et al*., 1989
Seed	Azadirachtin	Antimalarial	Khalid *et al*., 1989
Bark	Gallic acid	Anti-inflammatory	Khalid *et al.*, 1989
	(–) epicatechin and catechin	immunomodulatory	Van der Nat *et al.*, 1991
	Margolone, margolonone and isomargolonone	Antibacterial	Ara *et al.*, 1989
	Polysaccharides GIa	Antitumour	Fujiwara *et al*., 1982
	Polysaccharides GIIa	Anti-inflammatory	Fujiwara *et al.*, 1984
	NB-II peptidoglycan	Immunomodulatory	Vander Nat *et al.*, 1989

Extracts of different parts of the tree have been used as traditional medicine for the treatment of various diseases (Kirtikar, 1935). However, reported pharmacological activity of various extracts of *A. indica* include: Abortifacient, analgesic, anthelminthic, antibacterial, antiyeast, antiulcer, antifertility, antifilarial, antifungal, antihyperglycemic, anti-inflammatory, antiviral, antimalarial, diuretic, antinematodal, antipyretic, antispasmodic, insecticidal, antispermatogenic, antitumor,

hypercholesteremic, hypoglycaemic, immunomodulator (Ketkar *et al*., 1995; Kumar *et al*., 2010).

2.4.4 *Toona ciliata*

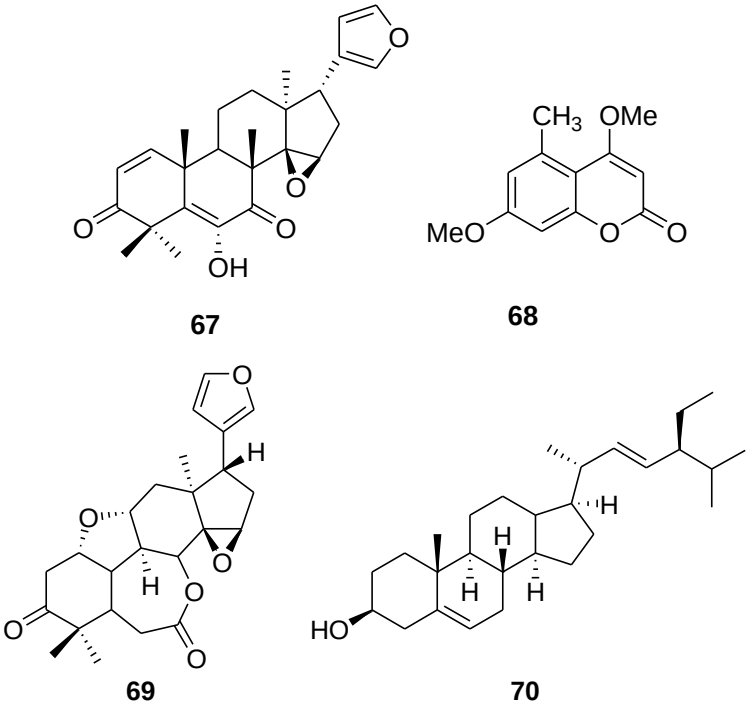
Toona ciliata is a large deciduous tree with a spreading crown, commonly attaining a height of 20-30 m and a girth of 1.8 - 3 m. The bark is dark grey or reddish-brown, smooth up to middle age, afterwards rough, with shallow reticulate cracks exfoliating in irregular woody scales. Leaves are 30-50 cm long, on young trees up to 90 cm long, usually imparipinnate, sometimes paripinnate by the abortion of the terminal leaflet. Seeds are pale brown, very light, winged at both ends, 1.3 - 1.5 cm long including the wing (Orwa *et al*., 2009).

2.4.4.2 Ethnomedicinal uses of *T. ciliata*

Various parts of *T. ciliata* are used medicinally as an astringent and tonic, to treat dysentery and to heal wounds (Orwa *et al*., 2009). Plant leaves used traditionally from the ancient time as anti-ulcer activity, analgesic activity, antifungal activity, antimicrobial, activity, anti-tumor activity, anti-feeding activity, insect-repellent, insectisidal, antiviral and molluscicidal activity (Kumar *et al*., 2012).

2.4.4.1 Phytochemicals isolated from *T. ciliata*

Phytochemical studies on *Toona ciliata* revealed the presence of cedrelone **(67)**, isolated from the benzene extract of heartwood (Gopalakrishnan *et al*., 2000). Limonoids i.e.Toonaciliatins was reported from leaves and stem of *Toona ciliata* (Lio *et al*., 2007). Siderin (**68**), a natural coumarin was isolated from the methanolic extract of the leaves of the plant (Veiga *et al*., 2007). Toonafolin (**69**), a tetranortriterpenoid lactone was isolated from the ether extract of leaves and stigmasterol **(70)** isolated from chloroform extracts of leaves of the plant (Karus and Grimminger, 1981). Figure 18 shows the chemical structures of some phytochemicals from *T. ciliata*.



67 **68** **69** **70**

Figure 18: Structure of some phytochemicals from *T. ciliata*

Tetratriterpenoids such as cedrelone **(67)** showed antifungal activity (Govindac *et al*., 2000). The petrol ether, dichloromethane and methanol extract of the stem bark of *Toona ciliata* showed antibacterial activity. Siderin **(68)**, a major coumarin from the plant, exhibited significant *in vitro* antibacterial activity (Chowdhary *et al*., 2003b). Chloroform extract of the leaves of plant *Toona ciliata* also showed inhibitory activity against the bacteria *Staphylococcus aureus*, *Bacillus subtilis*, *Micrococcus luteus* and *Salmonella Setubal* (Bibi *et al*., 2011). Ethanolic extract of the heartwood of the plant showed analgesic activity. The petroleum ether extract and column fraction of the bark of *Toona ciliata* exhibited antitumor activity (Chowdhary *et al*., 2003a). Some of the previous pharmacological investigations reported on *Toona ciliata* are summaried in Table 7.

Table 7: Some pharmacological activities of *T. ciliata*

Plant part	Extract/compound	Pharmacological activity	Reference
leaf	aqueous, acetone and methanol	antioxidant	Shama *et al*., 2009
heartwood	ethanol	anti-ulcer, analgesic	Malairajan *et al*., 2007 Malairaja *et al*., 2006
powdered wood	cedrelon	antifungal	Govindac *et al.*, 2000
stem bark	petrol ether, dichloromethane and methanol; siderin	antibacterial	Bibi *et al*., 2011
	12 alpha-hydroxystigmast -4-en-3-one	antitumor	Chowdhary *et al*., 2003

2.4.5 *Trichilia emetica*

Trichilia emetica is an evergreen tree, usually up to 21 m tall but occasionally reaching 30 m, trunk swollen at the base, sometimes becoming fluted with age. Bark grey-brown or red-brown with fine, shallow striations and smallish scales. Branches erect or partly spreading, producing a pyramid-shaped crown when young, oval to rounded and dense when mature with a diameter sometimes exceeding 15 m.

2.4.5.2 Ethnomedicinal uses of *T. emetica*

Trichilia emetica is native to the Savannah belt and open woodland of Africa. In West Africa, the pounded bark is used as an external application to treat parasitic skin infections and inflammation. The plant is purgative, and the bitter root extract is administrated as an anema for this purpose. An infusion of stem bark is used as purgative in many parts of Africa. Decoction of fresh leaf twigs is drunk in colic, convulsions and fever. *Trichilia emetica* is also used for liver ailments in folk medicine of Mali. A decoction of the roots is taken in hepatic disorders, as a remedy for colds, pneumonia and as a diuretic (Kokwaro, 1976; Malgras, 1992). In Senegal, *T. emetica* is also used in skin diseases, as a tonic, to stimulate bronchial secretion and as an antiepileptic (Oliver-Bever, 1986). The roots decoction is drunk in the mornings and evenings to treat jaundice (Aké Assi and Guinko, 1991). A small glassful of the decoction is consumed daily for three days against intestinal worms (Aké Assi and Guinko, 1991). The leaf decoction is used against malaria and scabies; the stem and leaf

decoction is used against intestinal, cutaneous or mouth infections. In Eastern Africa, the fruit is used as a diuretic.

Trichilia emetica is also used against poisoning, hepatitis, ulcer, dysmenorrhoea, asthma, cirrhosis and internal worms (Kokwaro, 1976). The plant is used as purgative, antiepileptic, antipyretic, general tonic and for bronchial inflammation (Iwu 1993). The infusion of *T. emetica* is used against headache and as lotion on burns (Burkill, 1997). Leaf and roots decoctions are used for bathing against insomnia (Neuwinger, 2000). In Mali, an ethnopharmacological survey of different uses of *T. emetica* reported the main uses of the plant: against malaria (23.8%), abdominal pain (19.2%), dermatitis (7.7%), haemorrhoids (6.2%), jaundice and chest pain (5.4%) (Togola *et al.*, 2005). The seed oil has medicinal properties and is applied externally to treat rheumatism. Poultices made from leaves or fruit and are applied to bruises and eczema.

2.4.5.1 Phytochemicals from *T. emetica*

Phytochemical screening of extract and fractions of *Trichilia emetica* revealed the presence of phenolics and flavonoids, fats, resins and tannins (Iwu, 1993). The methanol extract of *T. emetica* contains alkaloids, cardiac glycosides, saponins, flavonoids and tannins (Hussein *et al*., 1999a). The leaf and root bark extracts contain coumarins (Hussein *et al*., 1999b). Some of the chemical constituents of *T. emetica* include trichilin A **(71)** and dregeanin **(72)** whose structures are shown in Figure 19.

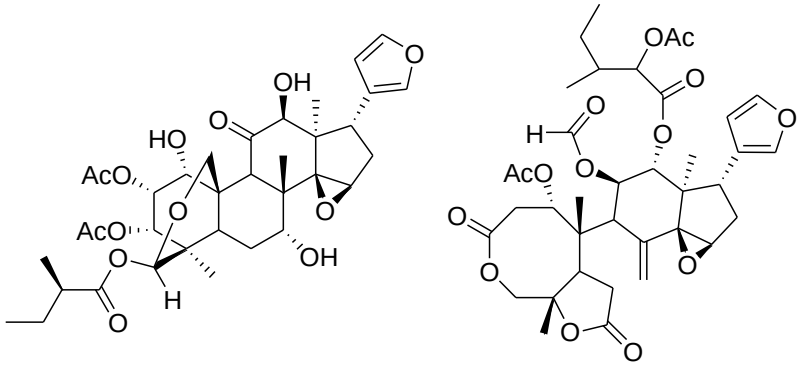


Figure 19: Structures of some phytochemicals from *Trichilia emetica*

Some limonoids from *Trichilia emetica* have a wide range of biological activities including insect antifeedant and growth regulation properties, antifungal, bactericidal and antiviral activities (Champagne *et al*., 1992). *Trichilia emetica* leaf extract showed inhibitory activity against cyclooxygenase (McGaw *et al*., 1997). Previous studies have demonstrated the antimalarial (El Tahir *et al.*, 1999; Traoré-Kéita *et al.*, 2000) and anti-schistosomiasis activities (Sparg *et al*., 2000). The polyphenolic components from diethyl ether fraction of the roots decoction presented a clear protective action against CCl_4-induced hepatic damage in the rat and an antioxidant activity *in vitro* (Germanò *et al*., 2001; Germanò *et al*., 2005; Germanò *et al*., 2006). The dried aqueous extract of roots demonstrated effective antipyretic activity on yeast-induced hyperthermia in rats (Sanogo *et al*., 2001). Some polysaccharides with wound healing and complement fixation ability have been isolated (Diallo *et al.*, 2003) from *T. emetica.* The leaf methylene chloride extract presented a good *in vitro* antitrypanosomal activity on *T. b. brucei* (Hoet *et al.*, 2004).

The aqueous extracts of leaves and roots bark of *T. emetica* showed effective anti-inflammatory activity with oedema inhibition (Sanogo *et al*., 2006). The crude ethanol extract of *T. emetica* exhibited promising antiplasmodial and antitrypanosomal activities against *T. b. rhodesiense* (Kamanzi *et al*., 2004). Dichloromethane extract of leaf exhibited antiplasmodial activity and a good binding activity to the GABA (A)-benzodiazepine receptor, while water and methanol extracts of the same plant did not show any activity (Bah *et al*., 2007). An aqueous extract of the leaves showed antifungal properties against a number of plant pathogens (Mashungwa and Mmolotsi, 2007). The plant polysaccharides at the dose levels of 50 mg/kg, demonstrated a significant cough-suppressive effect on chemically induced cough (Sutovská *et al*., 2009).

2.4.6 *Turraea mombassana*

Turraea mombassana is a shrub up to 4 metres tall, sometimes scrambling; first-year branchlets densely puberulous with spreading hairs, second-year more sparsely so, purplish-brown or reddish. Leaves are mostly in fascicles.

2.4.6.2 Ethnomedicinal uses of *T. mombassana*

There are few previous reports on ethnomedicinal uses of *T. mombassana*. However, in East Africa, the plant is used as a remedy for fever associated with malaria and other fevers (Kokwaro, 2009). Other plants species in the genus have been reported to show potential in ethnomedine. Table 8 summarizes some of the reported ethnobotanical uses of some plant species in the genus *Turraea*.

Table 8: Some of the reported ethnobotanical uses of some plant species in the genus *Turraea*

Plant species	Plant part	Medicinal uses	Reference
Turraea obtusifolia Hochst.	leaf,bark root bark	used in traditional medicine to treat stomach and intestinal ailments and as a drastic purge	Leistner, 2000
Turraea. mombassana Hiern ex C.DC.	root	decoction drunk for malaria and other fevers	Kokwaro, 2009; Irungu *et al.*, 2014
Turraea. roka (Forsk.) Chiov.	bark	infusion used as remedy for pneumonia	Kokwaro, 1993
	root	decoction used for colds, diuretic, purgative, and treatment of jigger soles	Kokwaro, 1993
Turraea. subcordata Gurke.	root	decoction is taken as an anthelmintic and is remedy for gonorrhoea	Kokwaro, 1993; Irungu *et al.*, 2014
Turraea nilotica Kotschy & Peyr	root	decoction taken for stomach upset and toothache.	Kokwaro, 1993; Irungu *et al.*, 2014

2.4.6.3 Phytochemicals from *T. mombassana*

Limonoids, the main constituents of the genus Turraea, have demonstrated different pharmacological activities (Adul *et al*., 1993). Although there are few previous phytochemical investigations reported on *T. mombassana*, literature search revealed phytochemical information of other members of the genus. However, previous phytochemical studies on root bark of *Turraea mombassana* led to the isolation of limonoids mombasone (**73**) and mombasol (**74**) (Fig. 8). From the root bark of *Turraea robusta* limonoids mzikonol (**75**), mzikonone (**76**), azadirone (**63**) and 1,2-dihydroazadirone (**77**); triterpenoids turranolide (**78**) and butyrospermol (**79**) were isolated (Rajab *et al*., 1988; Bentley *et al*., 1992). Phytochemical investigation on the root and stem bark of *Turraea nilotica* led to isolation of dihydroniloticin (**80**), nilotin (**81**), niloticin (**82**), and piscidinol A (**83**) (Mulholland and Taylor, 1988; Bentley *et al*., 1995) (Figure 20).

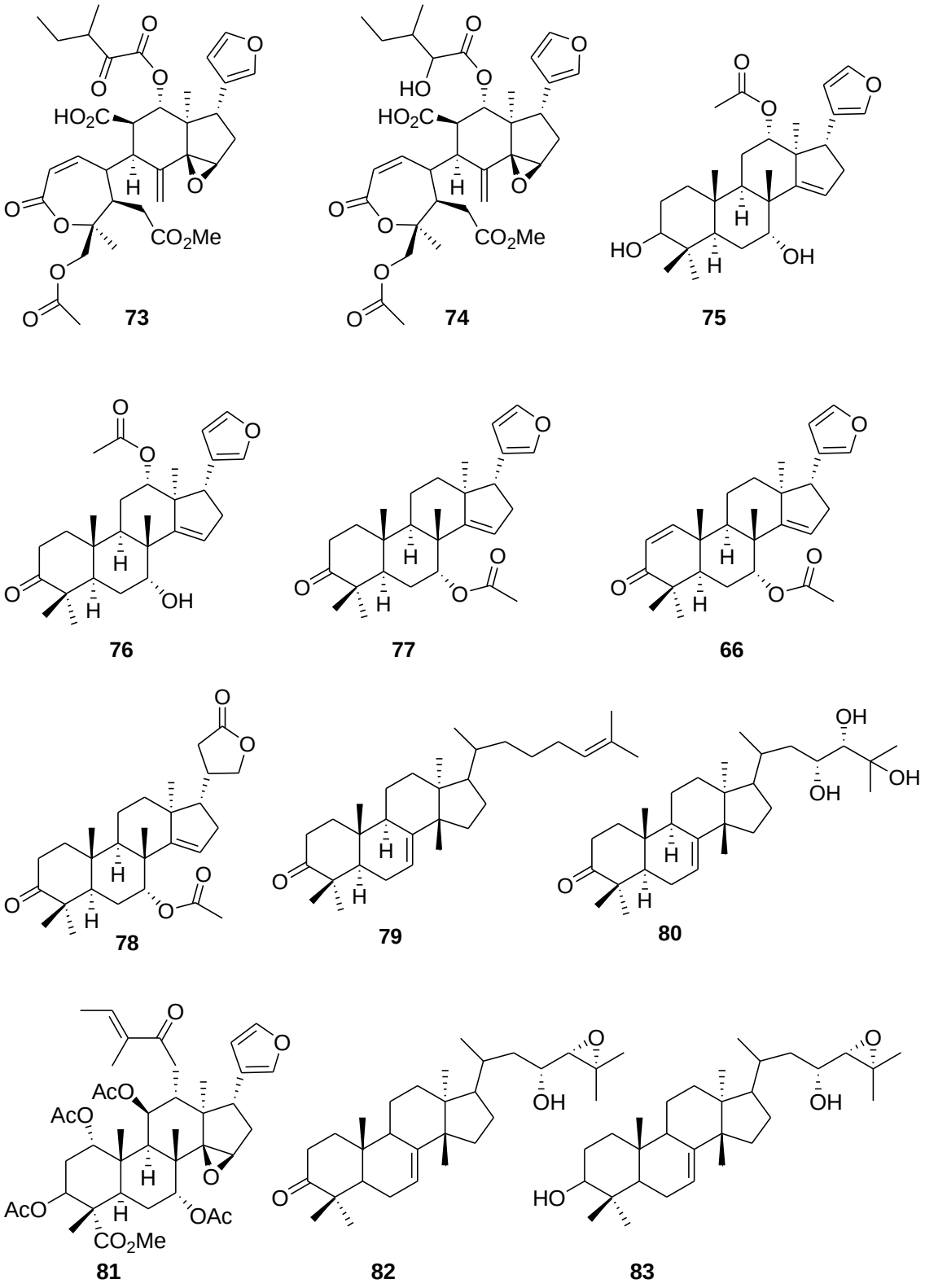


Figure 20: Structures of compounds isolated from some *Turraea* species

CHAPTER THREE: MATERIALS AND METHODS

3.1 Study design

The study design was analytical experimental. It involved *in vitro* and *in vivo* assays, chromatographic fractionation of active extracts and spectral analysis of isolated compounds which were mainly laboratory based.

3.2 Plant materials

The following five plant species of the Meliaceae family were used for the study: *Trichilia emetica* Vahl, *Toona Ciliata* M. Roem, *Azadirachta indica* A. Juss, *Turraea mombassana* Hiem ex C.DC and *Melia azedarach* L (Figure 21).

3.3 Plant collection and identification

Leaves, stem barks and root barks of the selected Meliaceae plant species were collected from Kenyatta University (Nairobi), Pwani University (Kilifi in the North Coast of Kenya), Shimba Hills National Park (Kwale in South coast of Kenya) (Figure 22). The plant species were identified by a taxonomist and voucher specimens deposited at the herbarium of the department of Pharmacy & Complementary/ Alternative Medicine, Kenyatta University and also at the National Museums of Kenya, Nairobi. Table 9 lists the plant samples and the locations where they were collected.

Table 9: Sample collection locations and voucher numbers

Plant species	Location	Voucher specimen No.
Trichilia emetica Vahl	Kenyatta University, Nairobi County	EN/01/2012
Toona Ciliata M. Roem	Kenyatta University, Nairobi County	EN/02/2012
Melia azedarach L.	Kenyatta University, Nairobi County	EN/03/2012
Turraea mombassana Hiem ex C.DC	Shimba Hills National Reserve, Kwale County	EN/04/2012
Azadirachta indica A. Juss	Pwani University, Kilifi County	EN/05/2012

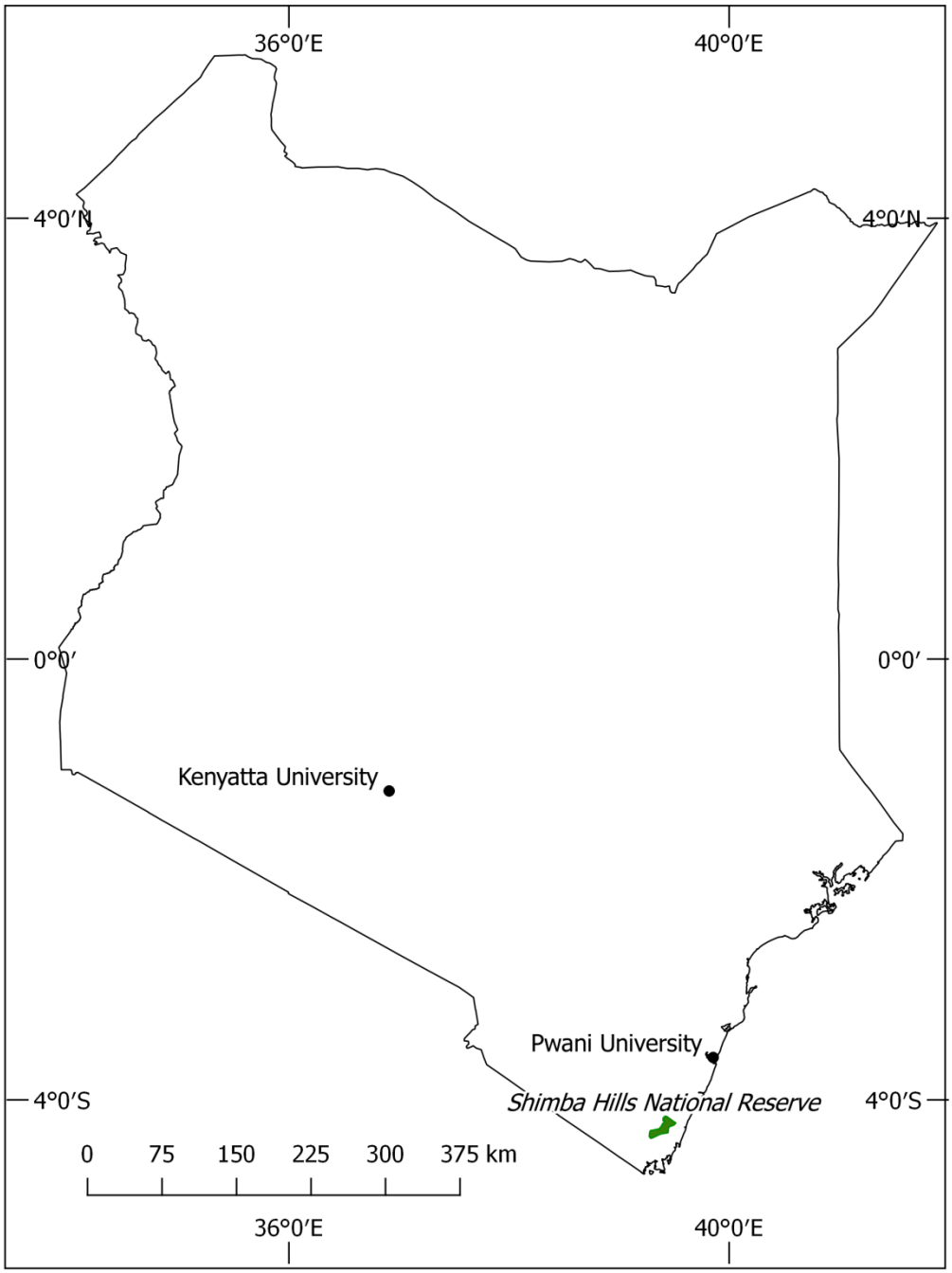


Figure 21: A map of Kenya showing locations of sample collection

Turraea mombassana

Trichilia emetica

Azadirachta indica

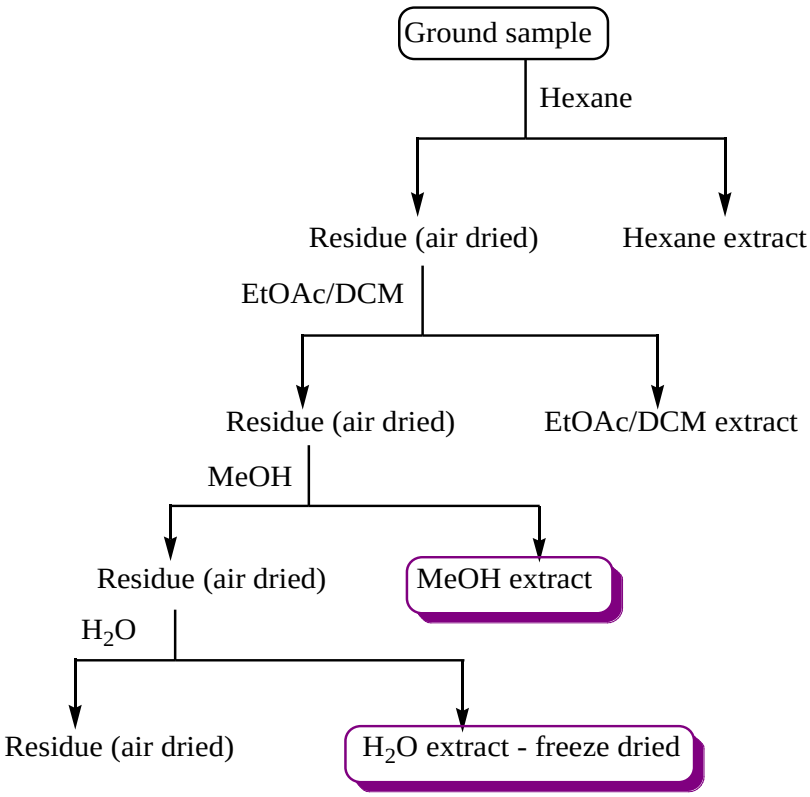
Melia azedarach

Toona ciliata

Figure 22: Photographs of aerial parts of selected Meliaceae species

3.4 Sample processing and extractions

Sample preparations were done at the department of Pharmacy and Alternative/Complementary Medicine, Kenyatta University. Extraction of the samples was done at the Centre for Traditional Medicine and Drug Discovery, Kenya Medical Research Institute (KEMRI). Air dried leaves, stem barks and root barks were ground into fine powders separately using a grinding machine. Each sample was extracted sequentially at room temperature for 36 hours using hexane, ethyl acetate, methanol and water respectively. The crude alcoholic extracts were concentrated *in vacuo* and then partitioned between water and dichloromethane/ethyl acetate. The dichloromethane /ethyl acetate phases were concentrated *in vacuo* while the aqueous phases and water extracts were freeze-dried. The dry extracts were weighed and then kept at -20˚C until required for assays. Scheme 2 gives an overview of the sequential extraction of the plant samples.



Scheme 2: Sequential extraction of the plant samples

3.5 *In vitro* assays

The *in vitro* antitrypanosomal assays to evaluate the ability of crude extracts, fractions and pure compounds to inhibit growth of *T. b. rhodesiense* (KETRI 3438), *T. b. brucei* (EATRO 2400) and *T. evansi* (KETRI 2454) bloodstream forms was carried out at Biotechnology Research Institute - Kenya Agricultural and Livestock Research Organisation (KALRO) formerly the Trypanosome Research Centre – Kenya Agricultural Research Institute (TRC – KARI), Muguga, according to the method by Raz *et al*. (1997). The Alamar Blue assay was used to determine drug sensitivity of African trypanosomes (*T.b. rhodesiense* and *T.b. gambiense*) *in vitro*. In this method, the active component, resazurin (blue in colour) is reduced to bright pink fluorescent resorufin in the presence of live parasites or cells. Wells containing active compounds are easily identified as they remain blue in colour.

3.5.1 The trypanosomes

Trypanosoma b. rhodesiense (KETRI 3438), *T. b. brucei* (EATRO 2400) and *T. evansi* (KETRI 2454) bloodstream forms were used in the present study. *Trypanosoma b. rhodesiense* (KETRI 3438) strain was isolated from a patient in Malaba (Kapalong), Kenya, in 1997. *Trypanosoma b. brucei* (EATRO 2400) was isolated from a bovine in central Nyanza, Kenya in 1964. The strain was established to be sensitive to suramin at 50 mg/kg and resistant at 2 mg/kg; *Trypanosoma b. brucei* (EATRO 2400) is sensitive to melarsoprol at 25, 5 and 3.6 mg/kg bwt (Murray, 1981). The 12^{th} passage of this strain was used in the present experimental study. *Trypanosoma evansi* (KETRI 2454) was isolated from a camel in Marsabit – Kulal in 1979. The strain is sensitive to suramin between the dosages of 5 and 1mg/kg bwt. *Trypanosoma evansi* (KETRI 2454) is sensitive to melasorprol at dosage range of 5 and 1mg/kg bwt (Olaho *et al*., 1993).

3.5.2 Preparation of test materials for *in vitro* assays

The first stock solutions of crude extracts, fractions and compounds were prepared in distilled water for the water-soluble samples at 20 mg/ml or in DMSO (at 1 mg/100 µl) for the water-insoluble samples. For the extracts, the solutions were further diluted in

minimum essential medium (MEM) to give 4, 2 and 1 mg/ml stock solutions. Solutions of isolated compounds were further diluted in MEM to give 100, 50 and 25 µg/ml stock solutions.

Figure 23: A photograph showing sample preparations and MEM supplementation

3.5.3 *In vitro* assays for anti-trypanosomal activity

The assays were carried out in 96-well (Corning Glass Works, Corning, New York) microtitre plates with a lid arranged in a matrix of 8 rows A - H and 12 columns 1 – 12. Each extract, fraction or compound was evaluated in two-fold serial dilutions and each concentration was tested in triplicate (Figure 24). For the preliminary screening, each extract, fraction or compound was tested once. For the more detailed study of the methanol extracts of *A. indica* stem bark, *T. emetic*a root bark, *M. azedarach* root bark and the aqueous extract of *T. mombassana* leaves, each extract was tested independently at least twice.

Fresh dilutions of the extracts, fractions or compounds in MEM were prepared each time. Parasite culture preparations were carried out asceptically in a biological safety

cabinet. The minimum essential medium (MEM) with Earle's salts was supplemented with 25mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 1g/l additional glucose and 10ml/l MEM non-essential amino acids (100×). The medium was further supplemented with 0.2 mM 2-mercaptethanol, 2mM sodium pyruvate, 0.1mM hypoxanthine and 15% heat inactivated horse serum. The medium was warmed to room temperature and aliquots (100 µl) were added to each well of the 96-well in rows A to G (Figure 24).

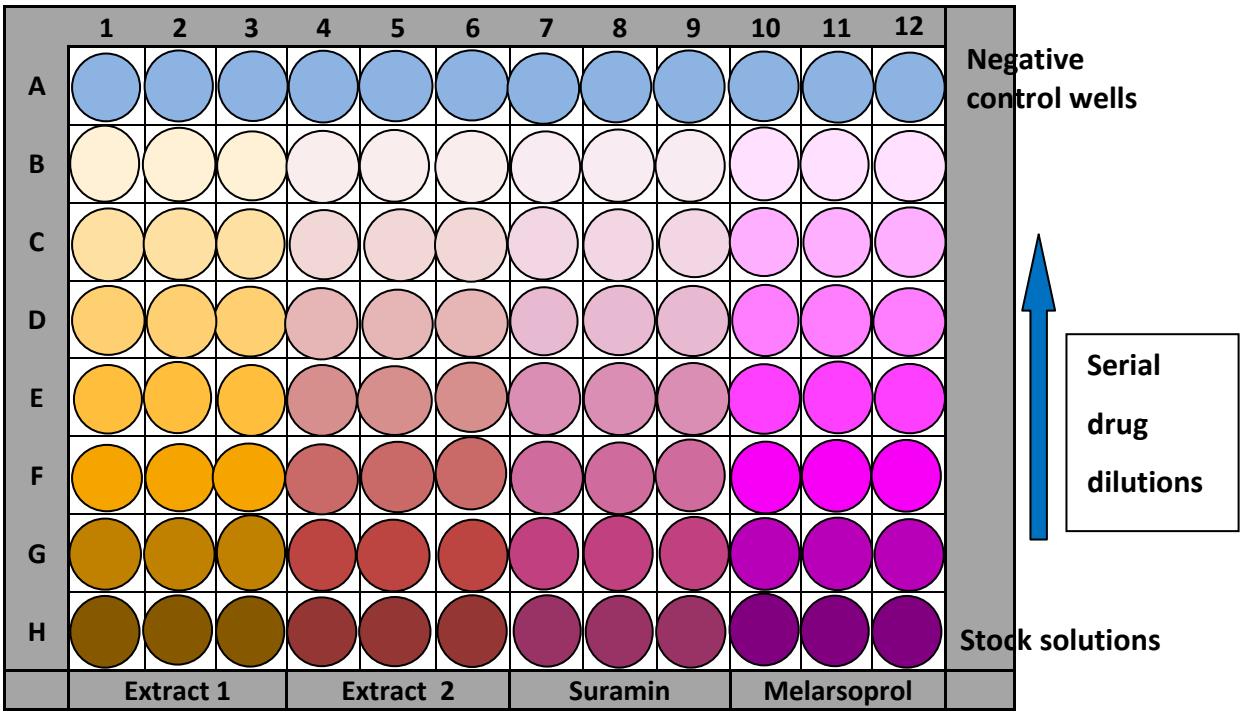


Figure 24: Diagrammatic representation of 96-well microtitre plate and drug dilutions

Further aliquots (100 µl) of the medium containing the extract, fraction or compound was added to three adjacent wells for each drug in row H. Four drugs were tested in this way on each plate (drug 1; columns 1- 3, drug 2; columns 3- 6, drug 3; columns 7- 9 and drug 4; columns 10-12). For each assay, melarsoprol and suramin were tested as the positive controls. The negative control consisted of a row of wells with medium and trypanosomes but no drug.

Serial dilutions were carried out by transferring 100 µl from wells of row H to wells of row G by a multi - channel pipette. After mixing, 100 µl were transferred from wells of row G to wells of row F and the process repeated until to the wells of row B. The 100 µl which were removed from the wells of row B were discarded. Each drug was evaluated in triplicate at seven concentrations in twofold dilution steps such that for every test the highest concentration was in row H and the lowest in row B. 100 µl of a suspension containing 1×10^4 bloodstream forms of trypanosomes were added to all the wells in all the rows. Wells of row A served as negative contol with medium and trypanosomes but no drug. The plates were incubated at 37 ˚C in a humified atmosphere containing 5 % CO_2 for 72 hours and the number of motile parasites was counted using a hemocytometer.

3.5.4 Determination of minimum inhibition concentration (MIC)

Each plate was examined with an inverted microscope to determine the minimum inhibitory concentration (MIC) which is the concentration at which no cell with a normal morphology and/or motility was found in comparison to the negative control cultures. In every row the highest dilution of either standard or samples with no motile trypanosomes of normal shape was determined. The concentration in this well was defined as the minimum inhibitory concentration (MIC), obtained using the equation:

MIC*D*2n = Cx,

Where,

MIC = Minimum Inhibitory Concentration

D = Dilution of extract by medium in wells of row H (maximum concentration)

n = Steps of dilution to the first well with no living/motile trypanosomes

Cx = Concentration of stock solution

* = Multiplication

Figure 25: A photograph showing examination of microtitre plates

3.6 *In vivo* assays

In vivo antitrypanosomal assays to evaluate the efficacy of crude extracts against *T. b. rhodesiense* (KETRI 3438) was carried out at Institute of Biotechnology - Kenya Agricultural and Livestock Research Organisation (KALRO). All experimental protocols and procedures used on animals during the study were reviewed and approved by the institutional animal care and use committee (IACUC) (Appendix 1).

3.6.1 Experimental animals

Randomly selected healthy female Swiss white mice 6-8 weeks old and weighing between 20 - 25 g were obtained from Biotechnology Research Institute, KALRO small animal breeding unit. They were dewormed using injectable ivermectin at 0.1ml per mouse and were housed in cages designed for mice. The animals were acclimatized for 14 days during which the animals were maintained on a diet of commercial mice pellets (mice pellets®, Unga Ltd, Nairobi, Kenya) and water was provided *ad* libitum. Donor mice were immunosuppressed using cyclosphosphamide at 300 mg/kg intraperitoneally for 3 consecutive days before infection.

Figure 26: Experimental mice in cages

3.6.2 Determination of *in vivo* acute toxicity

Twenty five mice were randomly grouped into five groups of five animals per group, with each group receiving treatment of the following concentration of the test compounds intraperitoneally (i.p.), daily for four days: Group i (100 mg/kg body weight (bwt); Group ii (200 mg/kg bwt); Group iii (400 mg/kg bwt); and Group iv (800 mg/kg bwt). Group v was given water only. Animals were initially observed closely for a period 10-15 min following administration of the test drug for signs of acute toxicity, including the hypotensive response (dyspnea and lethargy) elicited by test compound at its various doses. The mice were then closely monitored throughout the 4 days of administration. Changes in posture, breathing, food intake and water consumption patterns of the treated mice were observed and recorded on a daily basis for the remaining period of the experiment. Excessive weight loss and changes of packed cell volume of more than two-fold compared with the control group of mice over the 2 weeks period was considered a key indicator of declining health due to drug toxicity.

Initial preliminary acute toxicity tests of the selected extracts were carried out at a single dose of 1600 mg/kg body weight of the animals. The extract-treated groups which recorded 100 % mortality of animals at the initial single dose of 1600 mg/kg body weight were subjected to a repeat test of a single dose at 800 mg/kg body weight. The results obtained from the preliminary tests formed the basis for the actual acute toxicity tests reported in the current study.

3.6.3 Determination of LD_{50}

The final LD_{50} of the extracts was calculated by the method of Miller and Tainter (1944). Following their method, the percentage mortalities of test samples were converted into probits using the Finney's table (Table 15). The mortality values of 0 and 100 % were corrected before the determination of the corresponding probits using the formula (Ghosh, 1984; Al-Ali *et al*., 2008):

Corrected % formula for 0 and 100 % mortality:

For 0 % dead: 100 (0.25/n)

For 100 % dead: 100 (n-0.25/n); Where *n* is number of animals in each group.

Table 10: Transformation of percentages to probits

%	0	1	2	3	4	5	6	7	8	9
0	-	2.67	2.95	3.12	3.25	3.36	3.45	3.52	3.59	3.66
10	3.72	3.77	3.82	3.87	3.92	3.96	4.01	4.05	4.08	4.12
20	4.16	4.19	4.23	4.26	4.29	4.33	4.36	4.39	4.42	4.45
30	4.48	4.50	4.53	4.56	4.59	4.61	4.64	4.67	4.69	4.72
40	4.75	4.77	4.80	4.82	4.85	4.87	4.90	4.92	4.95	4.97
50	5.00	5.03	5.05	5.08	5.10	5.13	5.15	5.18	5.20	5.23
60	5.25	5.28	5.31	5.33	5.36	5.39	5.41	5.44	5.47	5.50
70	5.52	5.55	5.58	5.61	5.64	5.67	5.71	5.74	5.77	5.81
80	5.84	5.88	5.92	5.95	5.99	6.04	6.08	6.13	6.18	6.23
90	6.28	6.34	6.41	6.48	6.55	6.64	6.75	6.88	7.05	7.33

The probit values were then plotted against log-doses and the dose corresponding to probit 5 was determined as the LD_{50}.

The standard error (SE) of LD_{50} was then calculated using the formula:

$$\text{Approx. SE of } LD_{50} = \frac{(\log LD_{84} - \log_{16})}{\sqrt{2n}} \quad \text{..... (a)}$$

Where *n* is number of animals in each group.

Deriving from the outcome of the acute toxicity studies, dosage ranging from 100 – 400 mg/kg body weight was used throughout the efficacy experiments reported in the current study.

3.6.4 *In vivo* efficacy determination assays

In vivo antitrypanosomal efficacy of methanol extract of *A. indica* stem bark and aqueous extract of *T. mombassana* leaf was tested against *T. b. rhodesiense* (KETRI 3438).

3.6.4.1 Trypanosomes and infection of donor mice

Trypanosoma b. rhodesiense (KETRI 3438) was used for the *in vivo* efficacy studies. For propagation of the isolate, cryopreserved *T. b. rhodesiense* isolate (KETRI 3438) was obtained from the Biotechnology Research Institute (KALRO) trypanosome bank. The stabilate was thawed at room temperature; the content suspended in cold phosphate-saline-glucose (PSG) buffer pH 8.0 and injected intraperitonially into immunosuppressed donor mice for multiplication. At the first peak of parasitaemia, donor mice were anaesthesized using carbon dioxide, blood was collected from the heart of the mice using a syringe containing ethylene diamine tetra acetic acid (EDTA) as anticoagulant. The parasitaemia count was quantified using a haemocytometer and multiplied by haemocytometer factor 10^4. The blood was diluted appropriately with phosphate saline glucose (PSG) buffer to obtain the required inoculum dose.

3.6.4.2 Determination of *in vivo* efficacy

Thirty five mice were randomly divided into seven groups of five mice each. Each experimental mouse was intra-peritonially injected 0.2 ml of PSG buffer containing an

inoculum dose of 1×10^4 trypanosomes/ml of *T. b. rhodesiense* (KETRI 3438) from donor mice. The mice were then treated as follows: Group i; i.p. treatment of 100 mg/kg body weight of extract, 24 hours post-infection (PI) for four days; Group ii ; i.p. treatment with 200 mg/kg bwt of extract from 24 hours PI for four days; Group iii; i.p. treatment with 400 mg/kg bwt of extract from 24 hours PI for four days; Group iv; intraperitoneally treated using Suramin (5 mg/kg bwt) from 24 hours PI for three days (positive control I), Group v; intraperitoneally treated using melarsoprol (3.6 mg/ml) from 24 hours PI for three days (positive control II), Group vi; infected and given water *ad-libitum* with no extract (negative control), Group vii; uninfected and untreated (blank group) (Table 11). Group vii was included in the study for purposes of reference especially on the survival of the mice throughout the experimental period (60 days).

Table 11: Experimental design for *in vivo* efficacy determination

Group	Drug	Dose (mg/kg)	Treatment days
i	Extract	100	4
ii	Extract	200	4
iii	Extract	400	4
iv	Melarsoprol	3.6	3
v	Suramin	5.0	3
vi	Negative control	Infected untreated	-
vii	Blank	Uninfected untreated	-

The *in vivo* efficacy was determined based on the changes in the levels of parasitaemia, packed cell volume (PCV) and weights of the animals during the experimental period. The Kaplan Mayer curve was drawn to compare the effects of drugs on the survival time of the animals during the experimental period. For the treated groups, the mice were monitored by direct microscopy for trypanosomes for sixty days post-infection to confirm the status of cure.

Figure 27: Collection of infected blood from the heart of the donor mice

3.6.4.3 Parasitaemia examination

A drop of blood was collected from the mouse tail, placed on a clean slide, covered with a cover slip and the parasitaemia score correlated to a score sheet (Hebert and Lumsden, 1976) which involves daily use of wet smear to detect microscopic parasites using the rapid matching method. The parasitaemia examination was done daily during five days after the first treatment, then every two days for the remaining days of the experimental period (60 days). To assess the anti-trypanosomal effect of the extracts, the level of parasitaemia (expressed as log of absolute number of parasites per millimeter of blood) in the animal was compared to that of the control animals. Animals that survived to the end of the experiment, with no parasite in their blood sample were considered as cured.

3.6.4.4 Determination of Packed Cell Volume (PCV)

Packed cell volume (PCV) is the fraction of whole blood volume that consists of red blood cells (RBCs). Blood was obtained by bleeding tail vein of mice and filling three-

quarters full heparinized capillary tubes with 50-60 µl of the blood. After being sealed at their end, the tubes were centrifuged at 10000 rpm for 5 minutes in a haematocrit centrifuge in order to separate the RBCs from plasma and leave a band of buffy coat consisting of WBCs and platelets. The height of the red blood cell column was measured by use of haematocrit reader and compared to the total height of the column of the whole blood. The percentage of the total blood volume occupied by RBC mass was the PCV.

Figure 28: Equipment for determination of packed cell volume (PCV)

3.6.4.5 Mean Survival Time

The survival time for each mouse was recorded by daily checking the cages for cases of any mortality, the number of dead mice recorded until the end of the sixty days observation period. The average survival time was then calculated.

3.6.5 Data Analysis

Data for every experimental variable including the parasitaemia levels, body weight and packed cell volume were then entered and managed using Microsoft Excel (version 2007). Statistical analysis was performed using Statistical Package for Social Science (SPSS) version 21. Values of the data obtained were summarized and expressed as mean ± standard error of mean. The significant differences of the mean of parasitaemia, body weight, packed cell volume of mice from the negative control group, melarsoprol treated group, suramin treated group and the extracts treated groups at different dosages

were compared by one way ANOVA followed by Student Newmann Keul's test (SNK). P values less than 0.05 were considered significant.

3.7 Isolation of Compounds from the Active Extracts

Isolation of compounds from extracts was carried out using column chromatography and preparative thin layer chromatography (PTLC). The isolation process was monitored by TLC.

3.7.1 General Methods

Isolation of anti-trypanosomal compounds was carried out at the Centre for Traditional Medicine and Drug Research (CTMDR), KEMRI. The isolation of constituents was carried out using chromatographic separation techniques, particularly column chromatography, preparative thin layer chromatography (PTLC) and preparative high performance liquid chromatography (HPLC). Thin layer chromatography (TLC) was employed throughout the isolation and purification processes to monitor the separation processes. Column chromatography, packed with silica gel was employed to fractionate the crude extracts using appropriate solvent systems. Preparative thin layer chromatography and Preparative high performance liquid chromatography (HPLC) was employed to purify and isolate active constituents' difficult mixtures. Crystallisation and re-crystallisation of isolated solid compounds was carried out with suitable solvents.

3.7.1.1 Thin Layer Chromatography

Analytical thin layer chromatography was performed on aluminium plates pre-coated with Silica gel 60 F_{254} with 0.2 mm thickness. Visualization of the TLC spots was carried out under UV light 254 or 366 nm, spraying with vanillin sulphuric acid (1.0%) and the plate was kept in oven at 110 °C for 10 minutes for visualization. Preparative thin layer chromatography was carried out on prepared TLC plates.

3.7.1.2 Preparation of Plates for Preparative Thin Layer Chromatography (PTLC)

The glass plates of 20 × 20 cm were used for PTLC study. Silica gel G/UV254 with fluorescence indicator (Mecherey-Nagel) was used as a stationary phase and distilled water as a solvent for preparation of slurry. TLC plates of 20 × 20 cm were prepared with the help of spreader, having provisions for varying the thickness of silica gel layer from 0.25 mm to 2 mm. Slurry of silica gel was prepared in distilled water and applied as a thin layer of 0.5 mm on plates, allowed to air dry and then activated in oven for 1 hr at 110°C.

3.7.1.3 Column Chromatography

Glass columns wet packed with silica gel (70 – 230 mesh ASTM, Mercherey-Nagel) were used for column chromatography to fractionate the crude extracts and further purify the fractions. Different solvent systems were used depending on individual extract or fraction.

3.7.1.4 Preparative High Performance Liquid Chromatography (HPLC)

Preparative high performance liquid chromatography (HPLC) was carried carried out at Tianjin University of Traditional Chinese Medicine, China. It was performed on Beckmann system Gold chromatograph fixed with a Beckmann Ultrasphere ODS reverse phase column (250 × 10mm) and Beckman data processor using water/acetonitrile or methanol/water systems for elution of the compounds.

3.8 Structure Elucidation

Structure elucidation of each purified compound was done using: Ultraviolet-visible (UV-VIS), proton (^{1}H) and carbon (^{13}C) nuclear magnetic resonance (NMR). The data obtained was compared with literature values to confirm structural features of the isolated compounds.

3.8.1 Nuclear Magnetic Resonance (NMR) Spectral Analysis

Nuclear magnetic resonance (NMR) spectral analysis of the isolated compounds was carried out at Tianjin University of Traditional Chinese Medicine, China. ^{1}H-NMR and ^{13}C-NMR spectral analyses were recorded in CD_3OD at 400 MHz on a Bruker Spectrometer. Deuterated $CDCl_3$ was used as the solvent. Chemical shifts were given at δ (ppm) values with TMS (δ 0) as an internal standard. Coupling constants (*J*) were reported in Hz. Resonances were designated as *s* – singlet, *d* – doublet, *t* – triplet, *q* – quartet and *m* – multiplet.

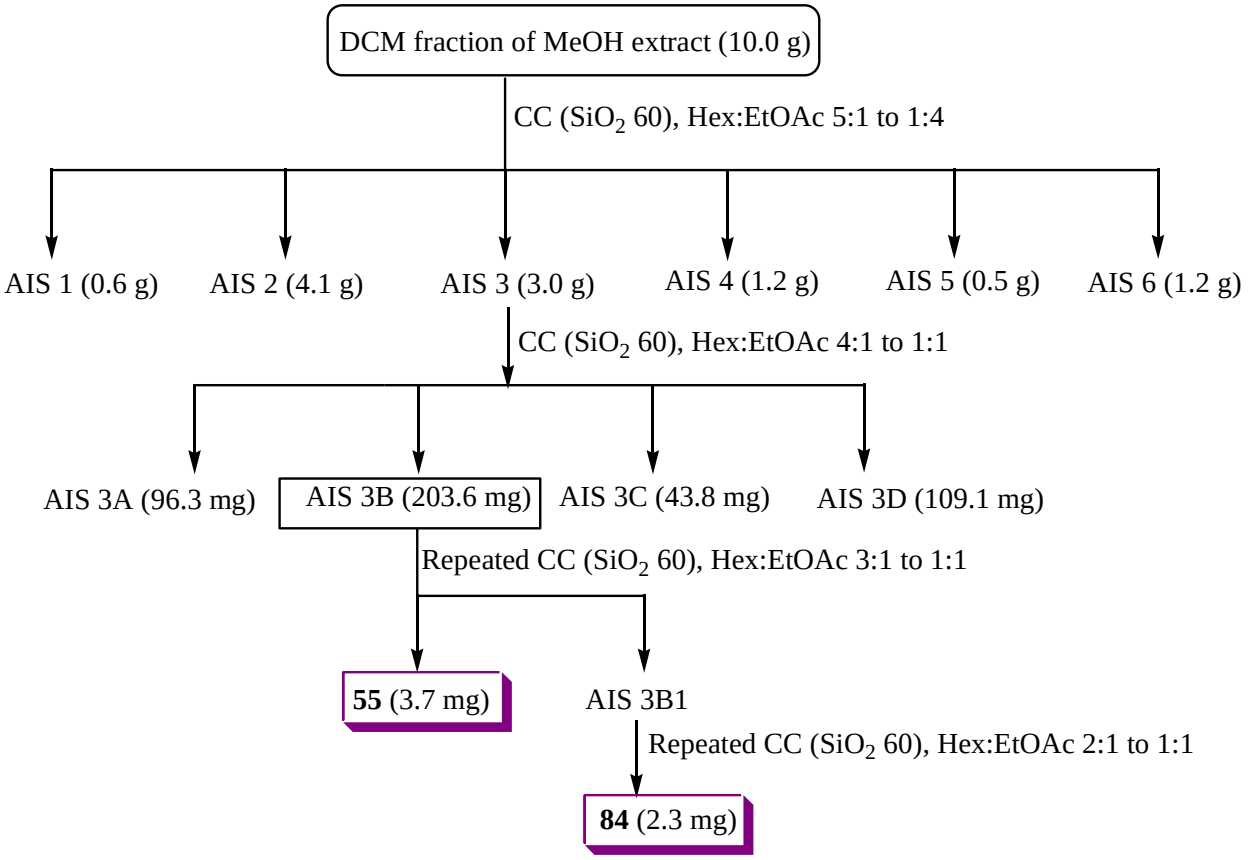
3.9 Extraction and Isolation of Compounds from *A. indica*

3.9.1 Extraction of *A. indica* stem bark

The ground stem bark of *A. indica* (1.9 kg) was soaked sequentially at room temperature for 36 hours each, using hexane, ethyl acetate, methanol and water, respectively. The resulting crude methanol extract (97.6 g) was concentrated *in vacuo* and then partitioned between water and dichlomethane (DCM) three times. The DCM phases were combined and concentrated *in vacuo* while the aqueous phases and water extracts were freeze-dried.

3.9.2 Isolation of compounds from *A. indica* stem bark

Scheme 3 shows the schematic presentation of the isolation process of compounds from the stem bark of *A. indica*. Dichloromethane fraction of the methanol extract of *A. indica* (10.0 g) was subjected to silica gel column chromatography, eluted with a gradient of Hex: EtOAc (5:1 to 1:4), to yield 106 fractions of 50 ml each. TLC analysis led to the combination of these fractions into six major fractions: Fractions 3 -16 (AIS 1), 17 – 29 (AIS 2), 30 – 47 (AIS 3), 48 – 75 (AIS 4), 76 – 94 (AIS 5) and 95 – 106 (AIS 6).



Scheme 3: Isolation of compounds from the MeOH extract of *A. indica* stem bark

Fraction AIS 3 (3.0 g) was chromatographed on a silica gel column (Hex: EtOAc from 4:1 to 1:1) to obtain four sub-fractions (AIS 3A−3D). Sub-fraction AIS 3B (203.6 mg) was subjected to repeated silica gel column chromatography, eluted with a gradient of Hex: EtOAc (4:1 to 1:1) to yield nimbin (**55)** (3.7 mg) as white needle-like crystals: chemical formula $C_{30}H_{36}O_9$; m.p. 197 - 199 °C; ^{1}H and ^{13}C NMR (400 MHz, $CDCl_3$) data are shown in Table 25 (section 4.5).

Repeated column chromatography of sub-fraction 3B1 (Scheme 3), eluted with a gradient of Hex : EtOAc (2:1 and 1:1) afforded compound (**84)** (2.3 mg) as a clear oil, which was partially identified as 1-detigloyl salannin ($C_{29}H_{36}O_8$) after comparing its proton NMR data (Table 26) with literature. The ^{13}C NMR was not obtained since a

higher resolution (600 – 1000 MHz) was required to analyse the small quantity of the compound obtained.

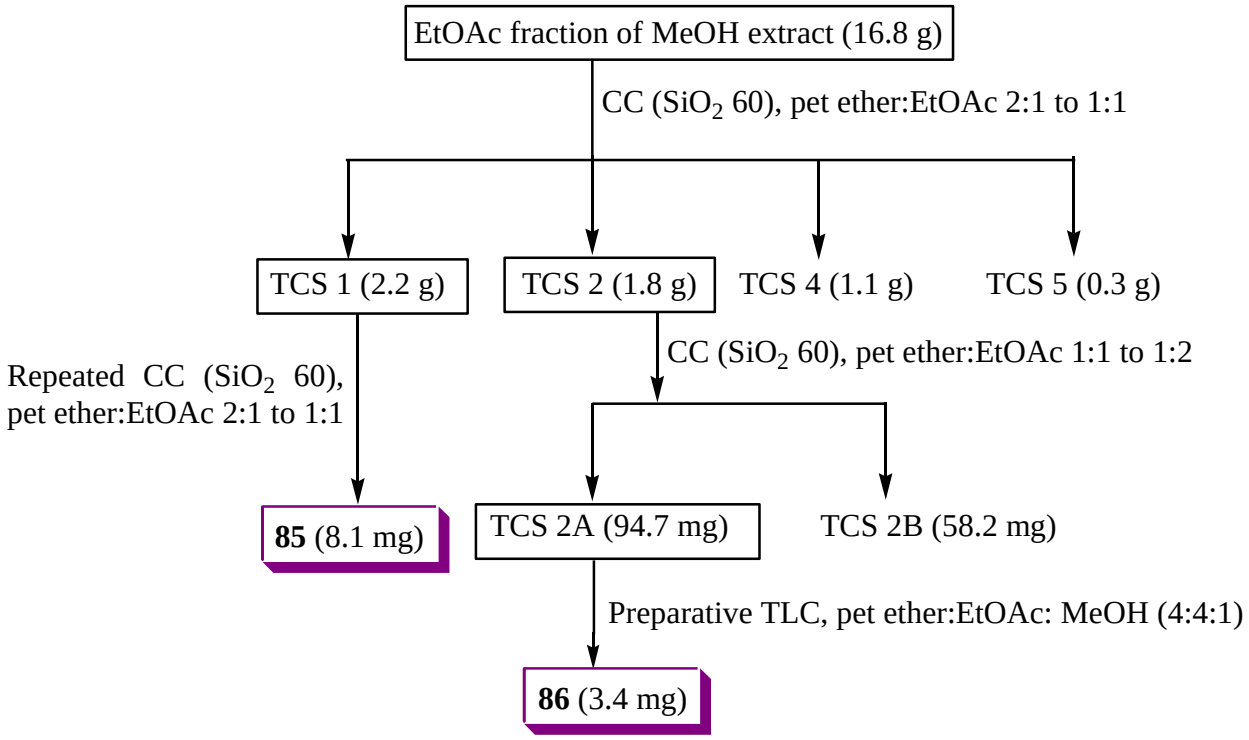
3.10 Extraction and Isolation of Compounds from *T. ciliata*

3.10.1 Extraction of *T. ciliata* stem bark

The ground stem bark of *T. ciliata* (1.8 kg) was soaked sequentially at room temperature for 36 hours each, using hexane, ethyl acetate, methanol and water, respectively. The resulting methanolic extract (124.7 g) was concentrated *in vacuo* and then partitioned between water and ethyl acetate three times. The ethyl acetate phases were combined and concentrated *in vacuo* while the aqueous phases and water extracts were freeze-dried.

3.10.2 Isolation of Compounds from *T. ciliata* stem bark

Ethyl acetate fraction of the methanol extract of *T. ciliata* (16.8 g) was subjected to silica gel column chromatography, eluted with a gradient of petroleum ether: EtOAc (2:1 to 1:4), to yield 50 fractions of 100 ml each. TLC analysis led to the combination of these fractions into four major fractions: Fractions 8 -15 (TCS 1), 16 – 25 (TCS 2), 26 – 36 (TCS 3) and 37 – 50 (TCS 4). Scheme 4 summarizes the isolation process of compounds from the stem bark of *T. ciliata.*



Scheme 4: Isolation of compounds from the stem bark of *T. ciliata*

Fraction TCS 1 (2.2 g) was subjected to repeated column chromatography, eluted with a gradient of pet ether: EtOAc (2:1 to 1:1) to obtain bis(2-ethylhexyl) phthalate (**85)** (8.1 mg) as light yellow oil: chemical formula $C_{24}H_{38}O_4$; ^{1}H and ^{13}C NMR (400 MHz, $CDCl_3$) data are shown in Table 27.

Sub-fraction TCS 2 (1.8 g) was subjected to silica gel column chromatography, eluted with a gradient of pet ether − EtOAc (1:1 to 1:2) to yield sub-fractions TCS 2A and TCS 2B. Preparative TLC of sub-fraction TCS 2A (94.7 mg) developed using pet ether: EtOAc: MeOH (4:4:1) afforded bis(2-methylheptyl) phthalate (**86)** (3.4 mg) as colourless oil: chemical formula $C_{24}H_{38}O_4$; ^{1}H and ^{13}C NMR (400 MHz, $CDCl_3$) data are shown in Table 28.

3.10 Physical and Spectroscopic Data of Isolated Compounds

Nimbin (**55**): white neede-like crystals; chemical formula $C_{30}H_{36}O_9$; m.p. 197 - 199 °C; 1H NMR (400 MHz, $CDCl_3$), Appendix 3; δ(ppm): 7.33 (1H, *m*, H-21), 7.22 (1H, *m*, H-23), 6.34 (2H, *m*, H-3, 22), 5.89 (1H, *d*, J = 10.2 Hz, H-2), 5.57 (1H, *m*, H-15), 5.22 (1H, *dd*, J = 12.4, 3.0 Hz, H-6), 4.07 (1H, *d*, J = 2.8 Hz, H-7), 3.75 (3H, *s*, H-28-CO_2Me), 3.67 (3H, *s*, H-12-CO_2Me), 3.62 – 3.68 (2H, *m*, H-17,5), 2.91 (1H, *dd*, J = 16.2, 5.2 Hz, H-11), 2.87 (1H, *t*, J = 4.0 Hz, H-9), 2.19 – 2.26 (2H, *m*, H-16,11), 2.06 (3H, *s*, H-$COCH_3$), 2.01 (1H, *m*, H-16), 1.69 (3H, *s*, H-18), 1.37 (3H, *s*, H-29), 1.35 (3H, *s*, H-30), 1.28 (3H, *s*, H-19). ^{13}C NMR (100 MHz, $CDCl_3$) Appendix 8; δ(ppm): 201.7 (C-1), 174.6 (C-28), 173.7 (C-12), 170.7 (C-$COCH_3$), 147.6 (C-3), 146.2 (C-14), 143.0 (C-23), 139.0 (C-21), 135.1 (C-13), 126.8 (C-20), 126.0 (C-2), 110.5 (C-22), 87.1 (C-15), 84.6 (C-7), 68.7 (C-6), 53.1 (C-28-CO_2Me), 51.7 (C-12-CO_2Me), 49.5 (C-17), 48.0 (C-10), 48.0 (C-4), 47.1 (C-8), 41.5 (C-16), 41.6 (C-5), 38.6 (C-9), 34.3 (C-11), 21.0 (C-$COCH_3$), 17.2 (C-29), 16.7 (C-30), 16.7 (C-19), 12.9 (C-18).

1-detigloyl salannin (**84**): Chemical formula $C_{29}H_{38}O_8$; ^{1}H NMR (400 MHz, $CDCl_3$), Appendix 8; δ(ppm): 7.35 (1H, *m*, H-21), 7.31 (1H, *m*, H-23), 6.36 (1H, *m*, H-22), 5.44 (1H, *m*, H-15), 4.98 (1H, *m*, H-3), 4.87 (1H, *m*, H-1), 4.18 (1H, *d*, J = 3.0 Hz, H-7), 4.00 (1H, *dd*, J = 12.7, 3.3 Hz, H-6), 3.60 - 3.67 (3H, *m*, H-17, 28), 3.30 (3H, *s*, H-CO_2Me), 2.76 (1H, *d*, J = 12.7 Hz, H-5), 2.73 (1H, *m*, H-9), 2.12-2.37 (6H, *m*, H-2,11,6), 2.10 (3H, *s*, H-$COCH_3$), 2.06 (1H, *s*, OH), 1.67 (3H, *s*, H-18), 1.31 (3H, *s*, H-29), 1.23 (3H, *s*, H-30), 0.99 (3H, *s*, H-19). Structural identity of compound (**84**) was not conclusive since the ^{13}C NMR spectral data was not obtained.

Bis(2-ethylhexyl) phthalate (**85):** light yellow oil; chemical formula $C_{24}H_{38}O_4$; ^{1}H (400 MHz, $CDCl_3$); Appendix 9; δ(ppm): 7.69 (2H, *dd*, *J* = 5.7, 3.3 Hz, H-3, H-6), 7.52 (2H, *dd*, *J* = 5.6, 3.2 Hz, H-4, H-5), 4.20 (4H, *dd*, *J* = 11.2, 5.8 Hz, H-1', H-1"), 1.65 – 1.70 (2H, *m*, H-2', H-2"), 1.23 – 1.33 (16H, *m*, C-3', C-3", C-4', C-4", C-5', C-5", C-7', C-7"), 0.86 – 0.96 (12H, *m*, C-6', C-6", C-8', C-8"). ^{13}C NMR (100 MHz, $CDCl_3$) Appendix 13; δ(ppm): 167.8 (2 C=O), 132.5 (C-1, C-2), 130.9 (C-3, C-6), 128.8 (C-4, C-5), 68.2

(C-1', C-1"), 38.7 (C-2', C-2"), 30.4 (C-3', C-3"), 28.9 (C-4', C-4"), 23.8 (C-7', C-7"), 23.0 (C-5', C-5"), 14.0 (C-6', C-6"), 11.0 (C-8', C-8").

Bis(2-methylheptyl) phthalate (**86):** colourless oil; chemical formula $C_{24}H_{38}O_4$; ^{1}H (400 MHz, $CDCl_3$); Appendix 14; δ(ppm): 7.72 (2H, *dd*, 5.8, 3.0 Hz, H-3, H-6), 7.54 (2H, *dd*, 5.7, 3.3 Hz, H-4, H-5), 4.23 (4H, *m*, H-1', H-1"), 1.69 (2H, *m*, H-2', H-2"), 1.34 (4H, *m*, C-3', C-3"), 1.32 (4H, *m*, H-5', H-5"), 1.28 (8H, *m*, H-4', H-4", H-6', H-6"), 0.92 (12H, *m*, H-7', H-7", H-8', H-8"). ^{13}C NMR (100 MHz, $CDCl_3$) Appendix 18; δ(ppm): 167.8 (2 C=O), 132.5 (C-1, C-2), 130.9 (C-4, C-5), 128.8 (C-3, C-6), 68.2 (C-1', C-1"), 38.8 (C-2', C-2"), 30.4 (C-5', C-5"), 29.7 (C-3', C-3"), 28.9 (C-4', C-4"), 23.0 (C-6', C-6"), 14.1 (C-7', C-7"), 11.0 (C-8', C-8").

CHAPTER FOUR: RESULTS AND DISCUSSION

4.1 Plant Extracts Yields

The sequential solvent extractions afforded crude extracts whose yields are summarized in Tables 12 and 13. Methanol extracts recorded the highest yields for all the plant species studied compared to the other solvent extracts. Hexane extracts, on the other hand recorded the lowest yields. Among the methanol extracts, *T. emetica* root bark recorded the highest yield (7.51 %) while *T. mombassana* stem bark gave the lowest yield (4.51 %). The yields from the EtOAc and the aqueous extracts were comparable.

Table 12: Yields of hexane and ethyl acetate extracts of the Meliaceae species

Plant Species	Plant part	Quantity (kg)	Hexane extract		EtOAc extract	
			Yield (gm)	Yield (%)	Yield (gm)	Yield (%)
Trichilia emetica	L	1.85	19.28	1.04	41.86	2.26
	Sb	1.53	15.25	1.00	40.37	2.64
	Rb	1.27	13.83	1.05	39.04	3.07
Toona ciliata	L	1.73	12.09	0.70	33.71	1.95
	Sb	1.95	10.72	0.55	28.40	1.46
	Rb	1.80	12.15	0.68	34.92	1.94
Melia azedarach	L	1.86	16.90	0.91	39.72	2.14
	Sb	1.71	12.52	0.73	34.61	2.02
	Rb	1.80	13.69	0.76	37.50	2.08
Turraea mombassana	L	1.05	9.40	0.90	25.90	2.47
	S	1.37	10.38	0.76	27.03	1.97
	R	0.95	8.50	0.89	22.17	2.33
Azadirachta indica	L	1.50	13.82	0.92	34.05	2.27
	Sb	1.93	16.90	0.88	38.92	2.02
	Rb	1.20	11.42	0.95	36.40	3.03

L - leaves; Sb - stem bark; S - stem; Rb - root bark; R - root; Hex - hexane; EtOAc - ethyl acetate

Table 13: Yields of methanol and aqueous extracts of the Meliaceae species

Plant Species	Plant part	Quantity (kg)	MeOH extract		H_2O extract	
			Yield (gm)	Yield (%)	Yield (gm)	Yield (%)
Trichilia emetica	L	1.85	103.41	5.59	46.25	2.50
	Sb	1.53	98.94	6.47	37.26	2.44
	Rb	1.27	95.32	7.51	36.80	2.90
Toona ciliata	L	1.73	94.50	5.46	28.24	1.63
	Sb	1.95	112.10	5.75	32.60	1.67
	Rb	1.80	105.60	5.87	24.65	1.37
Melia azedarach	L	1.86	126.32	6.79	32.75	1.76
	Sb	1.71	118.40	6.92	30.58	1.79
	Rb	1.80	124.65	6.93	32.95	1.83
Turraea mombassana	L	1.05	56.29	5.36	24.46	2.33
	S	1.37	61.80	4.51	27.83	2.03
	R	0.95	43.93	4.62	22.51	2.37
Azadirachta indica	L	1.50	94.76	6.32	30.15	2.01
	Sb	1.93	97.62	5.06	32.98	1.71
	Rb	1.20	85.30	7.11	27.90	2.33

L - leaves; Sb - stem bark; S - stem; Rb - root bark; R - root; MeOH - methanol; H_2O - water

4.2 *In vitro* Anti-trypanosomal Activities of the Extracts of Meliaceae Species

4.2.1 *In vitro* Anti-trypanosomal Activity against *T. b. rhodesiense* KETRI 3438 strain

In vitro screening involved propagation of *T. b. rhodesiense* KETRI 3438 strain in MEM media assayed in extracts of the Meliaceae species at various concentrations. Table 14 gives a summary of the *in vitro* anti-trypanosomal activities of the extracts against *T. b. rhodesiense* KETRI 3438 strain. The activities were classified as: (i) active with MIC values ≤19 µg/ml: (ii) moderately active with MIC values between 19 and 56 µg/ml; (iii) mildly active with MIC values between 56 and 167 µg/ml; and (iv) inactive with MIC values ≥167 µg/ml (Hoet *et al*., 2004).

Of the extracts tested for *in vitro* anti-trypanosomal activity against *T. b. rhodesiense* KETRI 3438 strain, seven extracts (25.0 %) showed strong activity (MIC values 9.11±3.44 – 13.67±0.98 µg/ml), eight extracts (28.6 %) showed moderate activity (MIC

values 24.82±0.57 – 51.98±1.21 µg/ml), ten extracts (35.8 %) showed mild activity (MIC values 61.10±1.40 – 125±0.00) and one extract (3.6 %) was inactive. For the methanol extracts, five extracts out of the fifteen extracts i.e the leaves, stem bark and root bark of *M. azedarach* (MIC 9.11±3.44 – 10.42±2.60 µg/ml), the stem bark of *A. indica* (MIC 9.93±2.88 µg/ml) and the root bark of *T. emetica* (MIC 9.11±3.44 µg/ml) showed the best anti-trypanosomal activity against *T. b. rhodesiense* KETRI 3438 strain. Two aqueous extracts, *T. mombassana* leaves and *A. indica* stem bark (MIC 12.04±2.28 and 13.67±0.98 µg/ml, respectively) showed the best activity.

Table 14: Anti-trypanosomal activity (MIC) of methanol and aqueous extracts of the Meliaceae species against *T. b. rhodesiense* KETRI 3438 strain

Plant Species	Plant part	MIC ($\overline{X}$ µg/ml ± SE)	
		MeOH	H_2O
Azadirachta indica	L	51.98±1.21	62.50±0.00
	Sb	9.93±2.88	13.67±0.98
	Rb	62.50±0.00	125.00±0.00
Toona ciliata	L	74.39±1.93	61.10±1.40
	Sb	125.00±0.00	–
	Rb	64.00±1.50	51.98±1.21
Melia azedarach	L	10.42±2.60	51.98±1.21
	Sb	9.11±3.44	62.50±0.00
	Rb	9.11±3.44	24.82±0.57
Turraea mombassana	L	25.99±0.61	12.04±2.28
	S	203.06±0.00	51.98±1.21
	R	61.10±1.40	25.99±0.61
Trichilia emetica	L	43.21±0.99	117.18±1.82
	Sb	61.10±1.40	–
	Rb	9.11±3.44	64.00±1.50
Melarsoprol		0.003±0.001	
Suramin		1.54±0.19	

L - leaves; Sb- stem bark; S- stem; Rb- root bark; R- root; MeOH - methanol; H_2O - water; $\overline{X}$ = mean; SE = standard error

4.2.2 *In vitro* Anti-trypanosomal Activity against *T. b. brucei* EATRO 2400 strain

Trypanosoma brucei brucei (EATRO 2400) was propagated in MEM and assayed in extracts of leaf, stem bark and root bark of the Meliaceae species at various concentrations. The results obtained (Table 15) showed that out of the twenty four extracts tested for *in vitro* anti-trypanosomal activity against *T. b. brucei* EATRO 2400 strain, eight extracts (33.3 %) showed strong activity (MIC values 9.93±2.88 – 18.88±3.25 µg/ml), seven extracts (29.2 %) showed moderate activity (MIC values 24.09±1.56 – 54.68±3.11 µg/ml), eight extracts (33.3 %) showed mild activity (MIC values 56.35±1.12 – 109±0.82) and one extract (4.2 %) was inactive. For the methanol extracts, the leaves, stem bark and root bark of *T. emetica* (MIC 9.93±2.84 - 14.65±0.98 µg/ml), the root bark and stem bark of *M. azedarach* (MIC 12.04±2.88 and 13.67±0.63 µg/ml, respectively) and stem bark and root bark of *A. indica* (MIC 16.25±0.92 and 18.88±3.25 µg/ml, respectively) showed the best anti-trypanosomal activity against *T. b. brucei* EATRO 2400 strain. Of the eleven aqueous extracts tested against *T. b. brucei* EATRO 2400 strain, only one extract (*T. mombassana* leaves) showed high activity (MIC 13.04±0.06 µg/ml).

Table 15: Anti-trypanosomal activity (MIC) of methanol and aqueous extracts of the Meliaceae species against *T. b. brucei* EATRO 2400 strain

Plant Species	Plant part	MIC ($\overline{X}$ µg/ml ± SE)	
		MeOH	H_2O
Azadirachta indica	L	48.17±0.11	88.52±3.01
	Sb	16.25±0.92	26.69±2.35
	Rb	18.88±0.25	46.22±0.95
Toona ciliata	L	–	109.35±0.82
	Sb	58.59±1.91	–
	Rb	42.13±2.25	–
Melia azedarach	L	13.67±0.63	27.34±1.96
	Sb	24.09±1.56	58.59±2.91
	Rb	12.04±2.28	54.68±3.11
Turraea mombassana	L	56.35±1.12	13.04±0.06
	S	218.71±3.65	109.35±0.82
	R	–	67.18±2.08
Trichilia emetica	L	15.92±0.16	88.71±1.65
	Sb	14.65±0.98	–
	Rb	9.93±2.84	–
Melarsoprol		0.005±0.002	
Suramin		1.66±0.10	

L - leaves; Sb- stem bark; S- stem; Rb- root bark; R- root; MeOH - methanol; H_2O - water; $\overline{X}$ = mean; SE = standard error

4.2.3 *In vitro* anti-trypanosomal activity against *T. evansi* KETRI 2454 strain

Trypanosoma evansi (KETRI 2454) was propagated in MEM and assayed in extracts of leaf, stem bark and root bark of the Meliaceae species at various concentrations. The results obtained (Table 16) show that out of the twenty nine extracts tested for *in vitro* anti-trypanosomal activity against *T. evansi* KETRI 2454 strain, eight extracts (27.6 %) showed strong activity (MIC values 9.18±0.18 – 16.13±0.58 µg/ml), eight extracts (27.6 %) showed moderate activity (MIC values 22.13±0.14 – 52.08±1.40 µg/ml), ten extracts (34.5 %) showed mild activity (MIC values 56.10±2.07 – 125.00±0.00) and three extracts (10.3 %) were inactive.

Table 16: Anti-trypanosomal activity (MIC values) of methanol and aqueous extracts of the Meliaceae species against *T. evansi* KETRI 2454 strain

Plant Species	Plant part	MIC ($\overline{X}$ µg/ml ± SE)	
		MeOH	H_2O
Azadirachta indica	L	22.13±0.14	36.67±0.98
	Sb	9.97±0.44	23.97±0.87
	Rb	32.70±0.09	51.98±1.21
Toona ciliata	L	74.90±0.03	–
	Sb	73.54±0.18	250.00±0.00
	Rb	64.00±1.50	117.18±2.82
Melia azedarach	L	13.06±1.17	10.42±2.60
	Sb	9.21±1.44	62.50±0.00
	Rb	10.07±0.16	16.13±0.58
Turraea mombassana	L	52.08±1.40	31.25±0.00
	S	147.19±1.32	302.04±2.04
	R	56.10±2.07	58.59±2.01
Trichilia emetica	L	26.51±1.70	31.25±0.00
	Sb	14.65±0.98	38.07±1.81
	Rb	9.18±0.93	87.00±0.50
Melarsoprol		0.003±0.00	
Suramin		1.51±0.13	

L - leaves; Sb- stem bark; S- stem; Rb- root bark; R- root; MeOH - methanol; H_2O - water; $\overline{X}$ = mean; SE = standard error

For the methanol extracts, the leaves, stem bark and root bark of *M. azedarach* (MIC 9.11±3.44 – 10.42±2.60 µg/ml), the root bark of *T. emetica* (MIC 9.11±0.93 µg/ml) and stem bark of *A. indica* (MIC 9.97±0.44 µg/ml) showed the best anti-trypanosomal activity against *T. evansi* KETRI 2454 strain. For aqueous extracts tested against the strain, two extracts, the leaves and root bark of *M. azedarach showed* the best activity (MIC 16.13±0.58 and 10.42±2.60 µg/ml, respectively.

4.2.4 Discussion on the *in vitro* anti-trypanosomal activities of the Meliaceae species

The variation in the percentage yields of the extracts of hexane, ethyl acetate, dichloromethane, methanol and aqueous solvents reflects the presence of different proportions of constituents of different polarity, with relatively polar methanol-soluble constituents being dominant.

The *in vitro* anti-trypanosomal activities exhibited by extracts of the Meliaceae species are in agreement with previous findings of other researchers with some variations in the levels of activity and the plant part studied. For instance, studies by Kamanzi *et al*. (2004) found that *T. b. rhodesiense* was most sensitive to the extract of *Trichilia emetica* stem bark *in vitro*. In another similar study, Hoet *at al*. (2004) reported that the *T. emetica* leaf extract showed high activity against *T. b brucei*. In the current study, it the root bark extract of *T. emetica* was found to be most active against *T. b. rhodesiense*, *T. b. brucei* and *T. evansi* blood stream forms.

Anti-trypanosomal activity of *A. indica* reported in the present study is comparable to a report by Mbaya *et al*. (2010) which showed that *A. indica* stem bark possesses impressive *in vitro* trypanocidal effect against *T. b. brucei*. A similar study on the leaf extract of *A. indica* by Githua *et al*. (2010) showed that the extract was active *in vitro* against procyclic forms of *T. b. rhodesiense*. The anti-trypanosomal effect exhibited by *A. indica* extracts may be attributed to the presence of some secondary metabolites like flavonoids, tannins, alkaloids, saponins, steroids, cyano-glycosides and terpenoids (Kagira *et al*., 2006; Kumar *et al*., 2010).

A report on anti-trypanosomal activity of *T. ciliata* by Githua *et al*. (2011) showed that crude methanol and chloroform extracts of *T. ciliata* exhibited good anti-trypanosomal *in vitro* activities against *T. b. rhodesiense* procyclic forms as well as siderin, a limonoid isolated from the methanol extract. In the present study however, the focus was on methanol and aqueous extracts of *T. ciliata* which showed mild *in vitro* activity

against *T. b. rhodesiense*, *T. b. brucei* and *T. evansi* blood stream forms. The observed differences between the results in the current study and those of other researchers may be attributed to the chemotypic variations in the chemical composition of plants according to the geographical area and the time or season of collection.

Literature search on extracts of *T. mombassana* leaf and *M. azedarach* root bark revealed that the present study is the first to show *in vitro* anti-trypanosomal activities of their extracts. Previous reports on the phytochemistry of *M. azedarach* root bark showed the presence of limonoids such as azedarachtin-A, azedarachtin-B (Kumar *et al*., 2003). However, the major constituents of the methanol extract of the *M. azedarach* were flavanoids such as quercetin. Thus, the observed *in vitro* activities of the methanol extract of *M. azedarach* root bark may be attributed to the presence of these flavanoids and limonoids (Ana *et al*., 2010).

4.3 Acute Toxicity of the Active Extracts in Healthy Mice

The results from the *in vitro* anti-trypanosomal activity assays (Table 14) revealed that eight extracts from the selected plant species were active (MIC ≤ 19 µg/ml). These were leaves and roots of *T. mombassana* (MIC 12.04±2.28 and 13.67±0.98 µg/ml, respectively), stem bark and root bark of *A. indica* (MIC 9.93±2.88 and 18.88±3.25 µg/ml, respectively), leaves, stem bark and root bark of *M. azedarach* (MIC 10.42±2.60, 9.11±3.44 and 9.11±3.44 µg/ml, respectively), root bark of *T. emetica* (MIC 9.11±3.44 µg/ml) These findings lay down useful groundwork for isolating and characterizing the specific compounds with promising anti-trypanosomal potentials that could be the bases for downstream development and use.

4.3.1 *In vivo* Acute Toxicity of Aqueous Leaf Extract of *T. mombassana*

The effects of aqueous extracts of *T. Mombassana* leaves at three doses (100, 200 and 400 mg/kg) following intraperitoneal treatment in healthy mice (Figure 29). However, preliminary toxicity tests at a single dose of 1600 mg/kg indicated that the aqueous extract of *T. mombassana* leaf was safe in healthy mice.

4.3.1.1 General behaviour of mice treated with aqueous leaf extract of *T. mombassana*

No toxic symptoms or mortality were observed in any group of experimental animals, which lived up to 14 days after the administration of aqueous leaf extract of *T. mombassana* at daily doses of 100, 200 and 400 mg/kg for four consecutive days. The behavioural patterns of animals were observed initially for the first 1 h, followed by 6 h and then 24 h after the administration of the extracts. All animals in the control group and extract-treated groups were normal and did not display observable effects on the skin, breathing, food intake, water consumption, postural patterns and hair loss.

4.3.1.2 Effect of aqueous leaf extract of *T. mombassana* on body weight of mice

There were no significant differences on different days ($p > 0.05$, SNK tests) in body weight changes of animals in all experimental groups. In all groups, animals exhibited a normal increment in body weight throughout the experimental period (Figure 29).

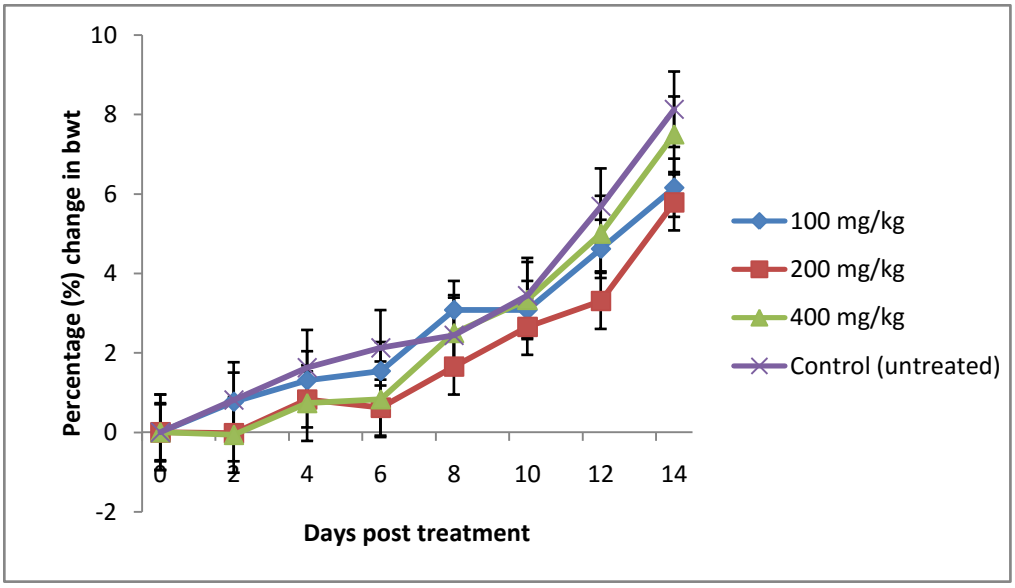


Figure 29: Trends of percentage change in body weight in mice treated intraperitoneally with aqueous extract of *T. mombassana* leaf; values are mean ± SE; SE = standard error; n = 5.

4.3.1.3 Effect of aqueous leaf extract of *T. mombassana* on packed cell volume

The aqueous leaf extract of *T. mombassana* at all the doses investigated in the present study did not have a significant change ($p > 0.05$) on the packed cell volume (PCV) of the experimental mice throughout the experimental period (Figure 30).

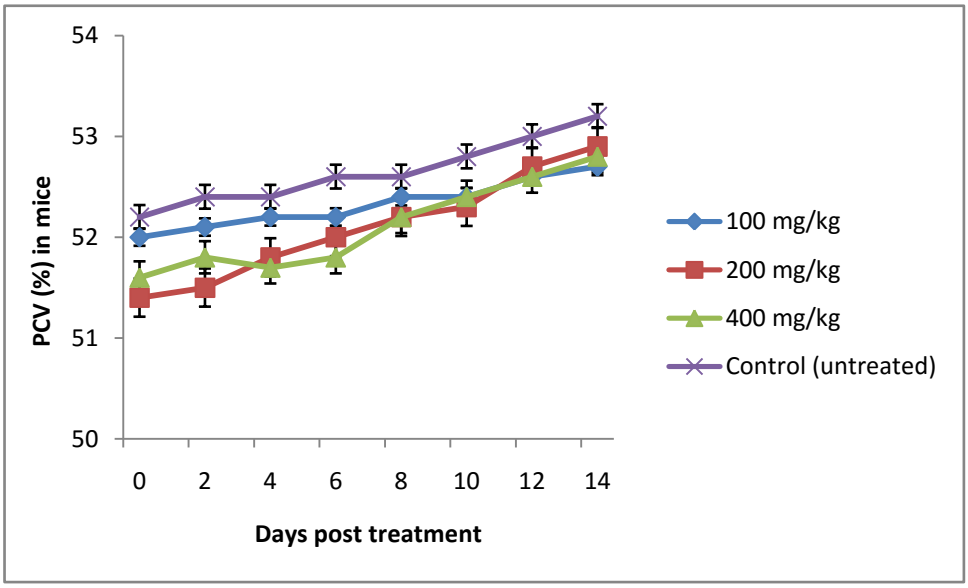


Figure 30: Trends of packed cell volume in mice treated intraperitoneally with aqueous extract of *T. mombassana* leaf; values are mean ± SE; SE = standard error; n = 5.

4.3.2 *In vivo* Acute Toxicity of Methanol Extract of *A. indica* stem bark

Effects of intraperitoneal treatments of methanol extract of *A. Indica* stem bark at three doses (100, 200 and 400 mg/kg) on healthy mice is highlighted in Figure 31. However, preliminary toxicity tests at a single dose of 1600 mg/kg indicated that the extract was safe in healthy mice.

4.3.2.1 General behaviour of mice treated with MeOH extract of *A. indica* stem bark

No animal mortality was observed during the 14 days of experimentation. Animals in all groups did not show any observable changes in feeding, water consumption, effects on the skin, breathing, loss of hair and postural patterns.

4.3.2.2 Effect of methanol extract of *A. indica* stem bark on body weight of mice

Figure 31 shows the trends of percentage change in body weight of the experimental animals treated intraperitoneally with methanol stem bark extract of *A. indica*. There was a similar trend in changes of body weight in all groups throughout the experimental period. All animals in all groups exhibited a normal increment in body weight. A slight decline in body weight of animals treated with the extract on day 2 post treatment was however noted. This observation may be attributed to stress among the animals due to the intraperitoneal treatment with the extracts.

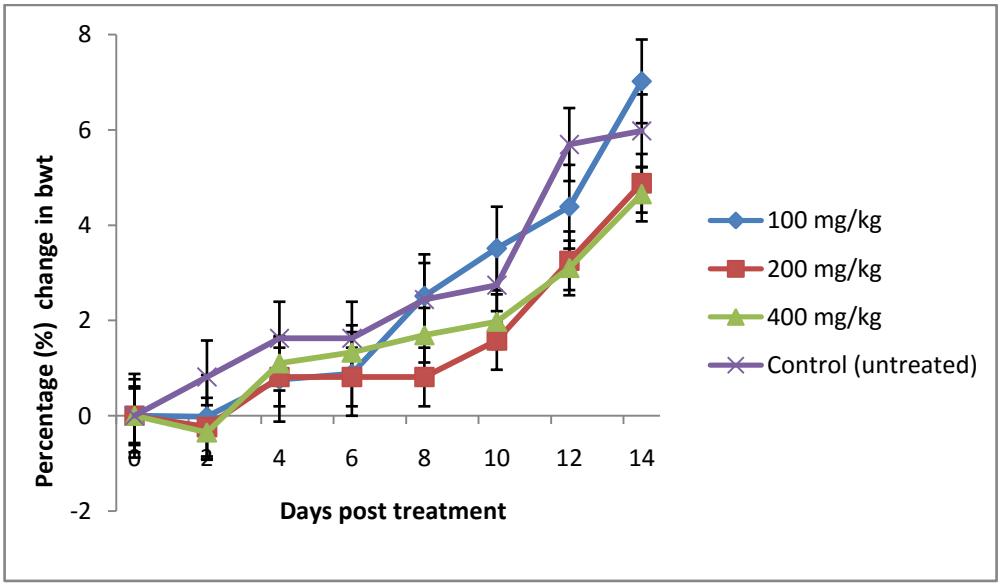


Figure 31: Trends of percentage change in body weight in mice treated intraperitoneally with methanol extract of *A. indica* stem bark; values are mean ± SE; SE = standard error; n = 5.

4.3.2.3 Effect of Methanol Extract of *A. indica* stem bark on Packed Cell Volume of mice

The methanol extract of *A. indica* stem bark at all the doses investigated in the present study did not significantly alter the packed cell volume (PCV) of the experimental mice ($p > 0.05$) throughout the experimental period (Figure 32) as compared to the control group (untreated) in healthy mice. The trends in PCV levels of animals in all the groups during the experimental period were similar indicating that the methanol extract of *A. indica* stem bark did not affect the packed cell volume of mice. There was a steady but gradual increase in PCV levels in all groups.

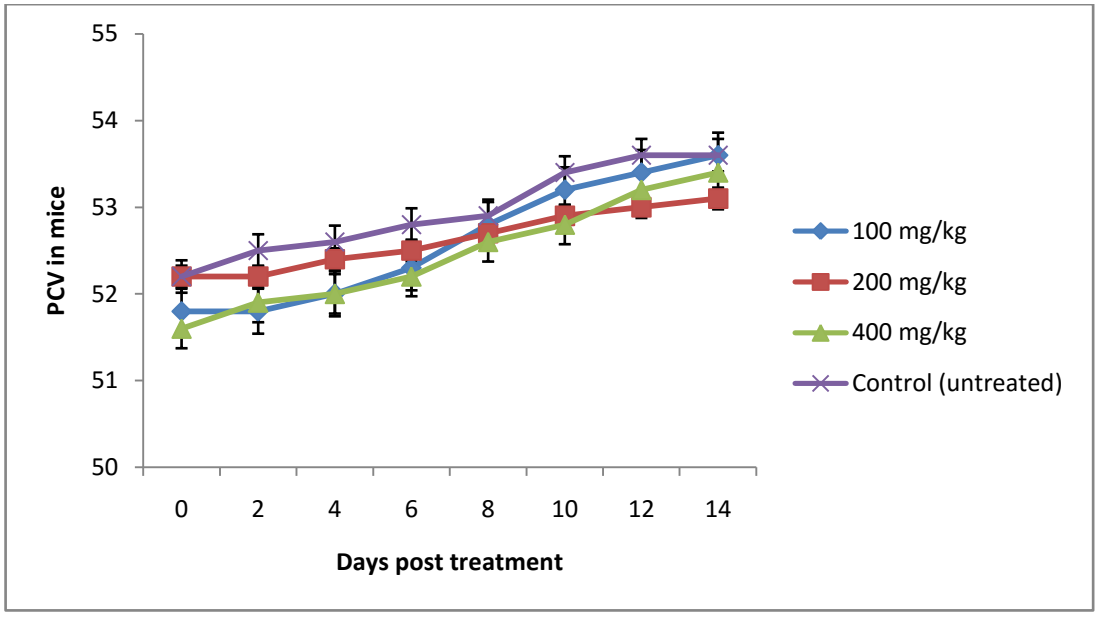


Figure 32: Trends of packed cell volume in mice treated intraperitoneally with methanol extract of *A. indica* stem bark; values are mean ± SE; SE = standard error; n = 5.

4.3.3 *In vivo* Acute Toxicity of Methanol Extract of *Trichilia emetica* root bark

During the preliminary tests, all the experimental animals treated intraperitoneally with the methanol extract of *T. emetica* root bark at a single dose of 1600 mg/kg body weight died within the first 24 h after treatment as compared to the control group which survived upto the end of the experimental period (14 days). The results indicated high

level of toxicity of the extract hence the need to establish the LD_{50} of the extract. LD_{50} was evaluated using the method of Miller and Tainter (Miller and Tainter, 1944; Al-Ali *et al*., 2008). Table 17 shows the percentage of animals (and the corresponding probits from Finneys Table) that had died at each dose level (see Table 10, Section 3.6.3).

Table 17: Results of the lethal doses of the methanol extract of *T. emetica* root bark for the determination of LD_{50} after intraperitoneal injection in mice (n=5)

Group	Dose (mg/kg)	Log dose	% dead	*corrected %	Probits
1	200	2.30	0	2.50	3.04
2	400	2.60	20	20.00	4.16
3	800	2.90	60	60.00	5.25
4	1600	3.20	100	97.50	6.96
5	Control (untreated)	-	0	2.50	3.04

*Corrected % formula for 0 and 100 values is given in the text.

The probit values were plotted against log-doses and the dose corresponding to probit 5, i.e., 50%, was determined as 2.85. The inverse $\log_{10}$ of 2.85 is 707.95, and therefore, the LD_{50} of the methanol extract of *T. emetica* root bark was estimated to be 707.95 mg/kg (Figure 33).

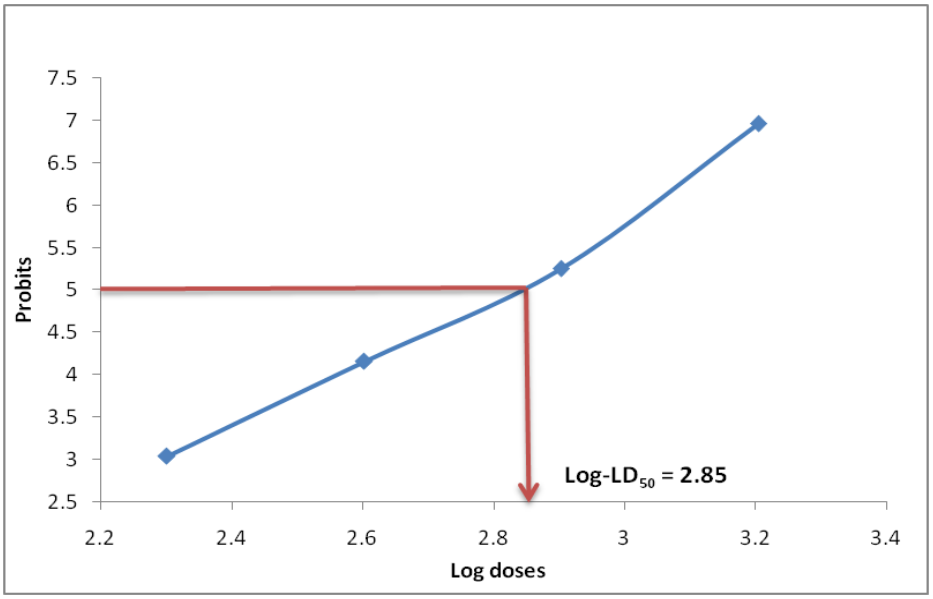


Figure 33: Plot of log-doses versus probits for the calculation of LD_{50} of methanol extract of *T. emetica* root bark administered intraperitoneally in mice

The SE of LD_{50} calculated from formula "(**a**)" shown in section 3.6.3 was 229.25. Therefore, LD_{50} of the methanol extract of *T. emetica* root bark when given intraperitoneally is 707.95±229.25 mg/kg, with 95 % confidence interval of 478.70 – 936.20 mg/kg.

4.3.4 *In vivo* acute toxicity of methanol extract of *Melia azedarach* root bark

Results from preliminary tests showed that all the experimental animals treated intraperitoneally with the methanol extract of *M. azedarach* root bark at a single dose of 800 mg/kg body weight died within the first 24 h after treatment as compared to the control group which survived upto the end of the experimental period (14 days). The results indicated high level of toxicity of the extract hence the need to establish the LD_{50} of the extract. LD_{50} was evaluated using the method of Miller and Tainter (1944) and elaborated by Al-Ali *et al*. (2008).

The percentage of animals that had died at each dose level was transformed to probits as shown in Table 19. The percentage dead for 0 and 100 values were corrected before the determination of probits using the formular "(**a**)" shown by Al-Ali A *et al*. (2008).

Table 18: Results of the lethal doses of the methanol extract of *M. azedarach* root bark for the determination of LD_{50} after intraperitoneal injection in mice (n=5)

Group	Dose (mg/kg)	Log dose	% dead	*Corrected %	Probits
1	100	2	0	2.5	3.04
2	200	2.30	40	40	4.75
3	400	2.60	80	80	5.84
4	800	2.90	100	97.5	6.96
5	Control (untreated)	-	0	2.5	3.04

- = not determined; *n* =5; *Corrected % formula for 0 and 100 valuesis given in the text.

The probit values were plotted against log-doses and then the dose corresponding to probit 5, i.e., 50%, was determined as 3.26 and LD_{50} = 220.09 mg/kg (Figure 34).

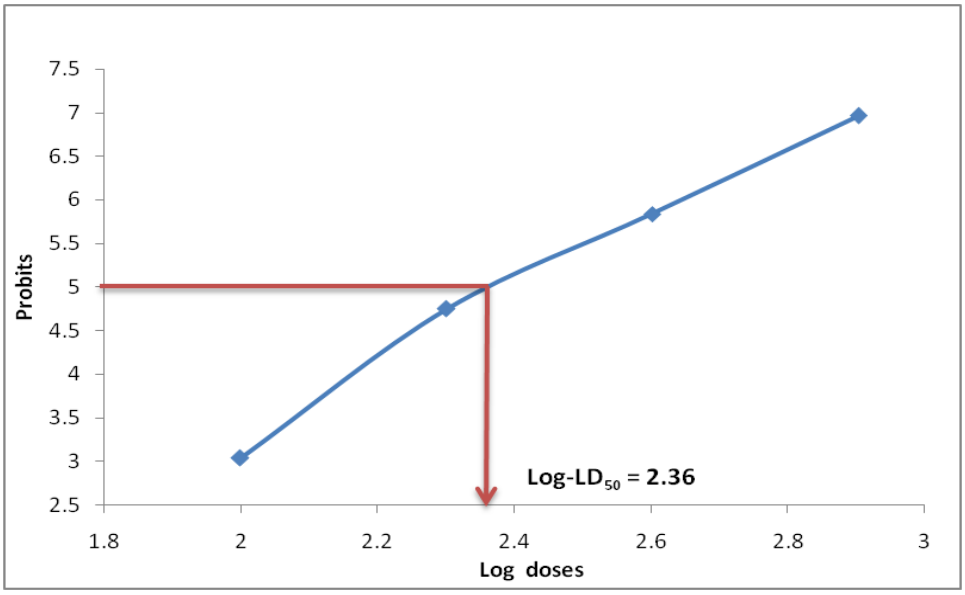


Figure 34: Plot of log-doses versus probits for the calculation of LD_{50} of methanol extract of *M. azedarach* root bark administered intraperitoneally in mice

The SE of LD_{50} calculated using formula "(**a**)" as shown earlier in the text (Section 3.6.3) was 95.54. Thus, LD_{50} of the methanol extract of *M. azedarach* root bark when given intraperitoneally is 229.09.54±95.54 mg/kg, with 95 % confidence interval of 133.55 – 324.63 mg/kg.

4.3.5 Discussion on the Acute Toxicity of the Active Extracts in healthy mice

Results from the current study indicate that the methanol extract of *A. indica* stem bark did not have toxic effects to mice at all dose levels tested. This finding is in agreement with results reported by other researchers that *A. indica* bark extract did not show any toxic effects in mice treated with as much as 200 mg/kg body weight extract (Udeinya, 1993; Ngure *et al.*, 2009; Pravin *et al*., 2011).

Similarly, *T. mombassana* leaf extract did not have any toxic effect on mice at the doses ranging between 100 – 1600 mg/kg body weight. This is consistent with a previous report by Nyangacha *et al.* (2011) that found no toxic effect or mortality in mice treated orally with methanol extract of *T. mombassana* as a single dose of 5000mg/kg body weight.

Trichilia emetica extract was found in the present study to be toxic when given intraperitoneally with a calculated LD_{50} of 707.95 ± 229.25 mg/kg body weight. Similar results were obtained in a study by Konaté *et al*. (2014) who reported that the LD_{50} of *T. emetica* extracts administered intraperitoneally was 568.5 mg/kg. On the other hand, oral intake of the extract of *T. emetica* was found to be only slightly toxic (Djoupo *et al*., 2015). In that study, some signs of toxicity were observed but no deaths were recorded after oral treatment of animals with *T. emetica* extract. The variation in the findings of these studies indicates that toxicity depends on the route of administration.

The calculated LD_{50} (229.09±95.54 mg/kg body weight) of *M. azedarach* root bark extract when administered intraperotoneally were comparable with a previous report by Zakir-Ur-Rahman *et al*. (1991) who found LD_{50} of 395 mg/kg body weight. The toxicity of extracts of *M. azedarach* may be attributed to the presence of toxic constituents including tetranortriterpenes, meliatoxins A_1, A_2, B_1 and B_2 (Peter *et al*., 1983). In that study, toxicity and pathological results confirmed that the meliatoxins were primarily responsible for most but not all of the effects resulting from ingestion of the extracts.

4.4 *In vivo* Anti-trypanosomal Efficacy of the Active Extracts

Aqueous extract of the leaves of *T. mombassana* (MIC 12.04±2.28 µg/ml), methanol extracts of the stem bark of *A. indica* (MIC 9.93±2.88 µg/ml), root bark of *M. azedarach* (MIC 9.11±3.44 µg/ml) and root bark of *T. emetica* (MIC 9.11±3.44 µg/ml) showed promising antitrypanosomal activities against *T. b. rhodesiense* KETRI 3438 strain (Table 14). However, in the acute toxicity studies, methanol extracts of the root bark of *M. azedarach* (LD_{50} = 229.09.54±95.54 mg/kg) and the root bark of *T. emetica* (LD_{50} = 707.95±229.25 mg/kg) demonstrated were found to be unsafe to healthy mice, unlike the methanol extract of *A. indica* stem bark and the aqueous extract of *T. mombassana* leaf. Thus, the extracts of *A. indica* stem bark, and the aqueous extract of *T. mombassana* leaves were selected for further efficacy investigations.

4.4.1 *In vivo* Anti-trypanosomal Efficacy of aqueous extract of *T. mombassana* leaves

Effects of intraperitoneal treatments of three doses (100, 200 and 400 mg/kg body weight) of the *T. mombassana* extract on parasitaemia, packed cell volume (PCV) and body weight of *T. b. rhodesiense* infected mice are highlighted in Table 19.

4.4.1.1 Effect of *T. mombassana* leaf extract on parasitemia of *T. b. rhodesiense* infected mice

The results indicated that there was no significant difference in parasitemia levels in animals treated with 400 mg/kg extract and the reference drugs (melarsoprol and suramin) on days 7, 49 and 56 ($p > 0.05$). Animals treated with melarsoprol and suramin did not show any parasites throughout the experimental period. The negative control (infected-untreated) group showed presence of parasites 5 days after infection with all the mice being positive by day 7. There was a delay in the commencement of parasite appearance in the extract treated groups with parasites being detected in some of the animals on day 7. During the experimental period, there were fluctuations in the level of parasitaemia of the extract-treated groups which remained at relatively low levels compared to the negative control (infected-untreated) group especially at higher doses of the extract (Table 19).

Table 19: Effect of aqueous extract of *T. mombassana* leaves on parasitemia of *T. b. rhodesiense* (KETRI 3438) infected mice

Treatment group	Parasitaemia level (log number)/mL							
	D7	D14	D21	D28	D35	D42	D49	D56
100 mg/kg	4.80 ± 1.96^{bc}	7.62 ± 0.11^{c}	8.64 ± 0.00^{c}	8.90 ± 0.08^{c}	8.90 ± 0.00^{c}	9.00 ± 0.00^{c}	–	–
200 mg/kg	2.64 ± 1.66^{abc}	7.14 ± 0.11^{c}	8.34 ± 0.26^{c}	8.70 ± 0.19^{c}	8.70 ± 0.21^{c}	8.85 ± 0.09^{c}	8.93 ± 0.00^{c}	9.00 ± 0.00^{c}
400 mg/kg	1.62 ± 0.42^{ab}	4.56 ± 1.88^{b}	4.80 ± 1.97^{b}	5.22 ± 2.09^{b}	5.16 ± 2.11^{b}	4.35 ± 1.25^{b}	2.90 ± 0.25^{ab}	3.00 ± 0.32^{ab}
3.6 mg/kg Mel	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}
5 mg/kg Sur	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}
Negative control	6.06 ± 1.54^{c}	7.86 ± 0.36^{c}	8.70 ± 0.15^{c}	8.93 ± 0.10^{c}	9.00 ± 0.00^{c}	–	–	–
p-value	0.023	<0.001	<0.001	<0.001	<0.001	0.012	0.002	0.020

Values are mean ± S.E; S.E = standard error ; n = 5; D = day; Mel = melarsoprol; Sur = suramin; negative control = infected untreateated; – implies that mice were dead; values with the same superscript(s) down the column do not differ significantly

The best results were obtained with the extract at 400 mg/kg which was, however, less effective compared to melarsoprol and suramin. Comparison with the negative control revealed that the 400 mg/kg extract kept parasitaemia at a significantly low level ($p < 0.05$) from day 7 to the rest of the experimental period. Among the extract treated groups, parasitemia levels in animals treated with 400 mg/kg were maintained at a significantly lower level ($p < 0.05$) on day 14 through day 56 compared to animals treated with 200 mg/kg. Similarly, parasitemia levels for 400 mg/kg was significantly lower on days 7 through day 42 compared to the 100 mg/kg. Parasitemia levels of extract at lower doses (100 and 200 mg/kg) did not differ significantly during day 7 through 35. There was no significance difference in parasitaemia levels between the extract at lower doses and negative control group on days 7 through 35.

4.4.1.2 Effect of aqueous extract of *T. mombassana* leaves on packed cell volume (PCV) of *T. b. rhodesiense* infected mice

The changes in PCV levels for the different groups involved in the study are portrayed in Table 20. Throughout the study period, animals in the negative control (infected-untreated) group showed significant decrease in PCV levels compared to the positive

control (melarsoprol and suramin treated) groups and the extract treated groups especially at higher doses (Table 20).

Table 20: Effect of aqueous leaf extract of *T. mombassana* on packed cell volume of *T. b. rhodesiense* (KETRI 3438) infected mice

Treatment group	Packed cell volume (PCV(%)) of mice							
	D7	D14	D21	D28	D35	D42	D49	D56
100mg/kg	51.60±1.29^{ab}	44.00±1.38^{ab}	44.80±1.24^{b}	42.33±1.14^{ab}	42.00±2.53^{b}	39.50±1.50^{a}	–	–
200 mg/kg	50.40±0.93^{ab}	45.00±1.11^{ab}	45.40±0.55^{b}	43.60±2.42^{ab}	44.20±2.51^{b}	42.67±2.98^{ab}	41.00±1.90^{a}	44.00±0.00^{a}
400 mg/kg	51.10±1.10^{ab}	45.60±1.92^{ab}	47.40±1.12^{b}	46.75±2.91^{bc}	45.75±1.65^{bc}	47.50±3.13^{bc}	49.60±0.67^{bc}	50.33±0.67^{bc}
3.6 mg/kg Mel	54.40±0.87^{b}	52.60±0.60^{c}	52.80±1.16^{c}	51.70±1.46^{c}	51.80±0.75^{c}	52.00±1.61^{c}	53.40±0.93^{c}	54.80±0.93^{c}
5 mg/kg Sur	52.80±0.92^{ab}	49.40±1.50^{bc}	51.40±0.68^{c}	51.20±1.88^{c}	50.60±0.66^{c}	50.80±0.80^{bc}	52.60±0.98^{c}	51.60±0.98^{bc}
Negative control	49.60±0.51^{a}	42.20±2.40^{a}	38.60±2.54^{a}	35.33±1.67^{a}	32.00±1.00^{a}	–	–	–
p-value	0.030	0.008	<0.001	0.007	<0.001	0.012	<0.001	0.009

Values are mean ± SE; SE = standard error; n = 5; D = day; Mel = melarsoprol; Sur = suramin; negative control = infected untreateated – implies that mice were dead; values with the same superscript(s) down the column do not differ significantly at $p< 0.05$

The PCV values in the infected-untreated group started dropping from day 7 and remained lower than the values of the extract treated groups and the positive control groups. However, there was a gradual decrease in the mean PCV levels in all the extract treated groups from day 7. The extract at 400 mg/kg and melarsoprol were comparable in prevention of decline in PCV levels among animals on day 7, 28, 35, 42, 49 and 56 (Table 20). At lower doses (100 and 200 mg/kg), however, *T. mombassana* leaf extract differed significantly with the positive control groups in preventing a drop in PCV levels throughout the study period. Trends on the changes of PCV levels in animals during the experimental period indicated a steady and continuous decline in the negative control as compared to the extract at higher doses.

4.4.1.3 Effect of aqueous extract of *T. mombassana* leaves on body weight of *T. b. rhodesiense* infected mice

Figure 35 depicts the trend in percentage change in body weight of *T. b. rhodesiense* (KETRI 3438) infected mice treated intraperitoneally with aqueous extract of *T.*

mombassana leaf. Mice in the positive control groups (melarsoprol and suramin treated) exhibited normal increments in body weights throughout the experimental period compared to the negative control (infected-untreated) and the extract treated groups. On day 7 after infection, a significant decline in body weight was observed in all trypanosome-infected animals except those in the positive control groups. Thereafter, notable body weight improvement was observed in the groups treated with the 400 mg/kg of the *T. mombassana* extract.

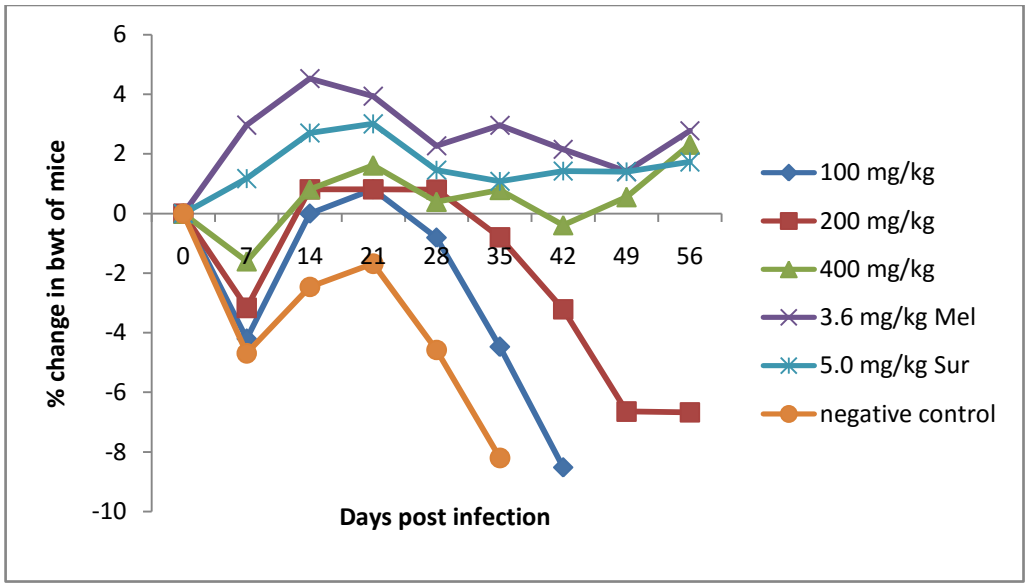


Figure 35: Trends of percentage change in body weight of *T. b. rhodesiense* (KETRI 3438) infected mice treated intraperitoneally with aqueous extract of *T. mombassana* leaves; values are mean ± SE; n = 5; negative control = infected untreated; Mel = melarsoprol; Sur = suramin

4.4.1.4 Effect of aqueous leaf extract of *T. mombassana* on mean survival time of *T. b. rhodesiense* infected mice

Table 21 summarizes the effect of aqueous extract of *T. mombassana* leaves on mean survival time of *T. b. rhodesiense* (KETRI 3438) infected mice. Results showed that all the mice treated with the reference drugs, melarsoprol (3.6 mg/kg) and suramin (5.0 mg/kg), survived throughout the period of the study (60.00 ± 0.00 days). A similar observation was made with the blank control (uninfected-untreated) group, which

survived throughout the period (60.00 ± 0.00 days), confirming that the deaths of the animals in the extract treated groups and the negative control (infected-untreated) group were as a result of the infection. The mean survival time of mice treated with extract at 400 mg/kg was comparable with the positive control (melarsoprol and suramin) groups as well as the blank. There was however, a significant difference between the survival times of animals at 400 mg/kg and the negative control. Among the animals treated with the extract, 200 mg/kg did not differ significantly with 400 mg/kg. The impact of the extract at 400 and 100 mg/kg differed significantly in prolonging the survival periods of mice (Table 21). The results indicated a clear dose-response effect, with the extract at 400 mg/kg giving the highest mean survival time as compared to the groups treated with the lower doses.

Table 21: Effect of aqueous extract of *T. mombassana* leaves on mean survival time of *T. b. rhodesiense* (KETRI 3438) infected mice

Treatment group	Number of mice			Percentage of mice cured	Mean survival time (days ± SE)
	treated	survived	cured		
100 mg/kg	5	0	0	0	36.20 ± 3.44^{a}
200 mg/kg	5	0	0	0	43.80 ± 4.89^{ab}
400 mg/kg	5	3	2	40	53.40 ± 4.28^{bc}
3.6 mg/kg Mel	5	5	5	100	60.00 ± 0.00^{c}
5 mg/kg Sur	5	5	5	100	60.00 ± 0.00^{c}
Negative control	5	0	0	0	31.40 ± 3.50^{a}
Blank					60.00 ± 0.00^{c}

SE = standard error; Mel = melarsoprol; Sur = suramin; negative control = infected untreated; blank = uninfected untreated; values with the same superscript(s) do not differ significantly at $p<0.05$

The Kaplan Meier plot of mean survival time (Figure 36) shows the fraction of the animals surviving at a given time throughout the experimental period. All the animals in experimental groups survived up to day 14. By day 21, 100 % survival rate was observed in the groups treated with reference drugs (3.6 mg/kg melarsoprol and 5.0 mg/kg suramin) and the blank (uninfected-untreated) group as compared to the extract

treated groups at various doses and the negative (infected-untreated) control (Figure 36). By the end of the experimental period, melarsoprol, suramin treated and the blank groups recorded 100 % survival of the animals as compared to the extract treated groups and the negative control groups. Among the extract treated groups, only the animals treated with the extract at 400 mg/kg survived up to end of the experiment (60 %).

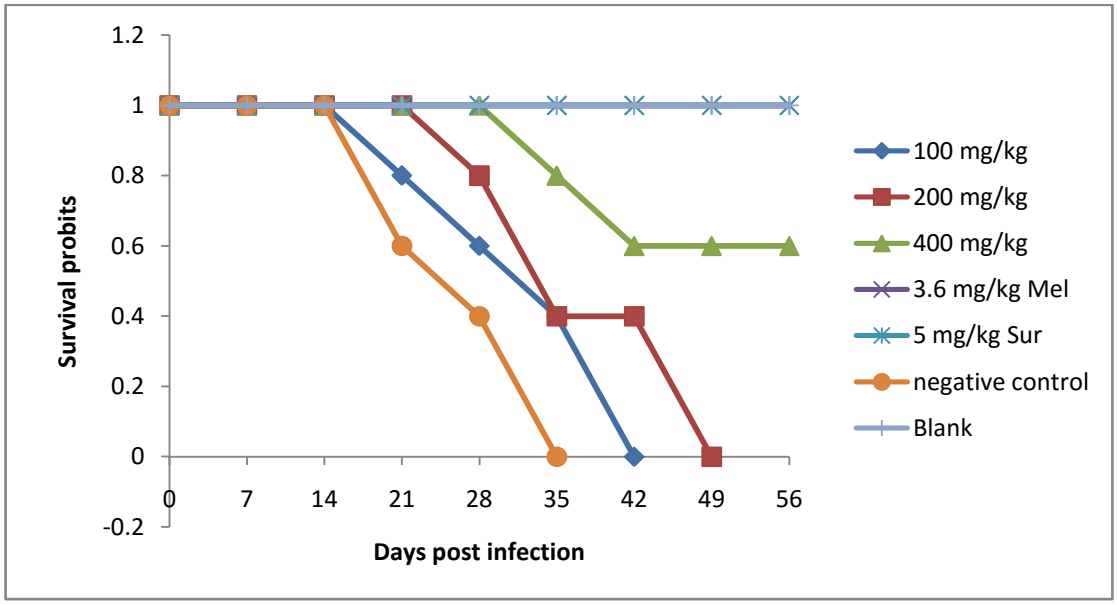


Figure 36: Kaplan Meier plot of mean survival time of *T. b. rhodesiense* (KETRI 3438) infected mice treated intraperitoneally with aqueous extract of *T. mombassana* leaves; values are mean ± SE; n = 5; negative control = infected untreated; blank = uninfected untreated; Mel = melarsoprol; Sur = suramin

4.4.2 *In vivo* Antitrypanosomal Efficacy of Methanol Extract of *A. indica* stem bark

Effects of intraperitoneal treatments of MeOH extract of *A. indica* stem bark at three doses (100, 200 and 400 mg/kg body weight) on parasitaemia, body weight and packed cell volume (PCV) of *T. b. rhodesiense* infected mice are highlighted in Table 22.

4.4.2.1 Effect of stem bark Extract of *A. indica* on Parasitemia of *T. b. rhodesiense* infected mice

The results indicated that there were significant differences in the parasitaemia levels between the various treatment groups throughout the experimental period (Table 22). Animals treated with melarsoprol and suramin did not develop parasites throughout the experimental period. There was a delay in the commencement of parasite appearance in the extract treated groups. For instance, at optimum dose of 400 mg/kg, parasites had not been detected on day 7 (Table 22).

Table 22: Effect of methanol extract of *A. indica* stem bark on parasitemia of *T. b. rhodesiense* (KETRI 3438) infected mice

Treatment group	Parasitemia level (log number)/mL							
	D7	D14	D21	D28	D35	D42	D49	D56
100 mg/kg	3.90 ± 1.76^{ab}	6.48 ± 1.64^{b}	6.00 ± 1.62^{bc}	6.72 ± 1.71^{c}	6.96 ± 1.75^{c}	5.90 ± 1.75^{b}	5.40 ± 1.29^{c}	4.80 ± 1.96^{c}
200 mg/kg	2.88 ± 1.62^{ab}	4.44 ± 1.82^{ab}	5.04 ± 2.07^{b}	5.10 ± 1.09^{bc}	5.08 ± 2.16^{bc}	3.00 ± 1.07^{b}	2.80 ± 0.80^{b}	3.00 ± 1.07^{bc}
400 mg/kg	0.00 ± 0.00^{a}	1.62 ± 0.32^{a}	1.62 ± 0.32^{ab}	1.38 ± 0.14^{ab}	1.68 ± 0.80^{ab}	1.74 ± 0.41^{ab}	1.80 ± 0.18^{ab}	1.74 ± 0.41^{ab}
3.6 mg/kg Mel	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}
5 mg/kg Sur	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}
Negative control	6.60 ± 1.66^{b}	8.34 ± 0.11^{b}	8.64 ± 0.29^{c}	8.48 ± 0.28^{c}	8.55 ± 0.28^{c}	9.00 ± 0.00^{c}	–	–
p - value	0.002	<0.001	<0.001	<0.001	0.003	0.013	0.031	0.042

Values are mean ± SEM; SE = standard error; n = 5; D = day; Mel = melarsoprol; Sur = suramin; negative control = infected untreateated; – implies that mice were dead; values with the same superscript(s) down the column do not differ significantly at $p< 0.05$

Parasitaemia levels in animals treated with *A. indica* stem bark extract at all doses remained at relatively low levels compared to negative control especially at higher doses of the extract. The best results were obtained with the extract treated group at 400 mg/kg which did not show significant difference with melarsoprol and suramin treated groups. Compared with the negative control, the 400 mg/kg extract kept parasitaemia at a significantly low level ($p < 0.05$) throughout the experimental period. At 200 mg/kg, *A. indica* stem bark extract kept the parasitemia levels at a significantly low level on days 21 and 42 ($p < 0.05$) compared to the infected-untreated control. Parasitemia level in animals treated with 100 mg/kg extract recorded a significant difference with the

negative control on day 42 while the animals in the later group survived. Among the extract treated groups, parasitemia level of the 400 mg/kg treated group remained at a significantly lower level ($p < 0.05$) on day 21 compared to the 200 mg/kg treated group and on days 14, 28 and 35 compared to the 100 mg/kg treated group. Parasitemia levels of animals in the 100 mg/kg and 200 mg/kg extract treated groups did not differ significantly during the experimental period.

4.4.2.2 Effect of stem bark extract of *A. indica* on packed cell volume of *T. b. rhodesiense* infected mice

Table 23 shows a summary of the packed cell volume (PCV) level changes in different groups involved in the study. During the study period, animals in the negative control (infected-untreated) group showed significant decrease in PCV levels compared to the positive control (melarsoprol and suramin treated) groups and the 400 mg/kg extract treated groups (Table 23). Packed cell volume (PCV) values in the negative control group started declining and remained lower than the values of the extract treated groups and the positive control groups. There was no significant difference in the prevention of PCV reduction in extract treated animals at 400 mg/kg and the positive control groups (melarsoprol and suramin) throughout the study period ($p > 0.05$). Among the extracts, all the three doses used in the study were comparable in prevention of PCV level decline in the animals (Table 23).

Table 23: Effect of methanol extract of *A. indica* stem bark on packed cell volume of *T. b. rhodesiense* (KETRI 3438) infected mice

Treatment group	Packed cell volume (PCV(%)) of mice							
	D7	D14	D21	D28	D35	D42	D49	D56
100mg/kg	47.00±0.71^{a}	45.40±2.73^{ab}	44.20±4.47^{ab}	43.80±2.99^{ab}	43.00±3.30^{ab}	48.33±3.61^{b}	45.33±4.11^{a}	48.00±0.00^{a}
200 mg/kg	49.80±1.87^{a}	45.60±2.20^{ab}	48.60±2.23^{ab}	50.20±1.11^{b}	46.20±2.87^{ab}	51.00±3.22^{b}	50.67±2.21^{bc}	49.67±3.39^{a}
400 mg/kg	50.00±1.24^{ab}	49.60±1.81^{ab}	48.80±1.24^{ab}	50.40±0.81^{b}	50.00±2.35^{b}	50.40±2.75^{b}	51.20±1.84^{bc}	51.00±1.10^{ab}
3.6 mg/kg Mel	52.40±1.69^{b}	51.80±1.21^{b}	53.20±0.37^{b}	53.60±0.80^{b}	52.80±0.86^{b}	54.80±0.49^{b}	52.80±0.37^{c}	52.90±0.58^{b}
5 mg/kg Sur	52.00±1.05^{b}	52.60±0.51^{b}	51.40±0.51^{b}	52.20±1.16^{b}	53.20±0.86^{b}	55.20±0.73^{b}	54.60±0.75^{c}	53.80±0.80^{b}
Negative control	47.40±1.03^{a}	43.00±1.34^{a}	42.00±1.76^{a}	40.75±4.96^{a}	39.67±2.85^{a}	37.50±2.21^{a}	–	–
p-value	0.029	0.005	0.017	0.008	0.006	0.018	0.039	0.043

Values are mean ± SE; SE = standard error; n = 5; D = day; Mel = melarsoprol; Sur = suramin; negative control = infected untreateated; – implies that mice were dead; values with the same superscript(s) down the column do not differ significantly at $p < 0.05$

4.4.2.3 Effect of stem bark extract of *A. indica* on body weight of *T. b. rhodesiense* infected mice

Figure 37 shows the trends of percentage (%) change in body weight of *T. b. rhodesiense* (KETRI 3438) infected mice treated intraperitoneally with methanol extract of *A. indica* stem bark. Mice in the positive control groups exhibited a significant increment in body weight throughout the experimental period compared to the infected-untreated control and the extract treated groups. The 400 mg/kg extract treated group and positive control groups showed comparable but significant increment in body weight compared to the 100, 200 mg/kg and the negative control.

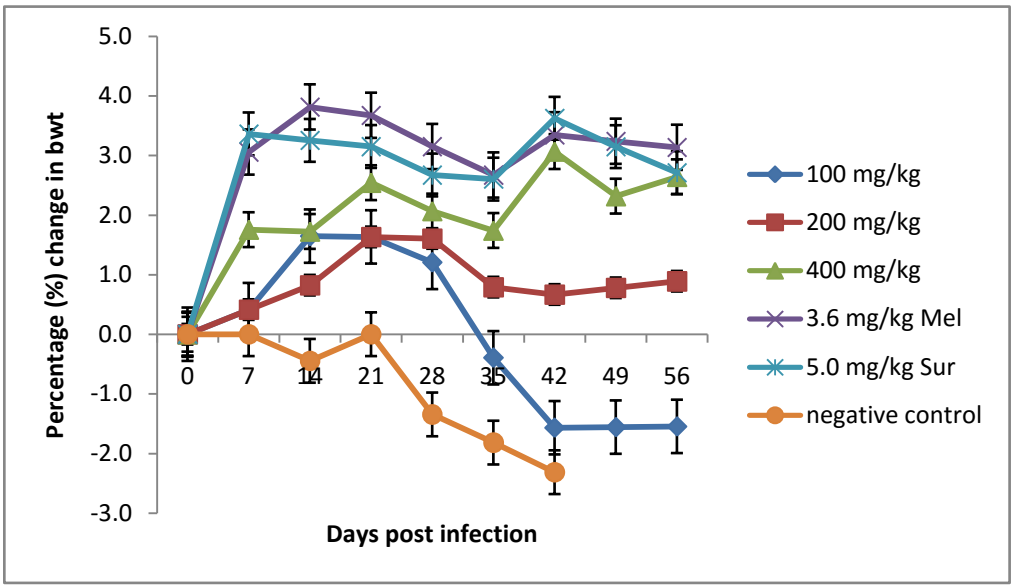


Figure 37: Trends of percentage (%) change in body weight of *T. b. rhodesiense* (KETRI 3438) infected mice treated intraperitoneally with methanol extract of *A. indica* stem bark; values are mean ± SE; n = 5; negative = infected untreated; Mel = melarsoprol; Sur = suramin

4.4.2.4 Effect on Mean Survival Time of *T. b. rhodesiense* infected mice

The results revealed that the blank (uninfected untreated) group and the groups treated with melarsoprol (3.6 mg/kg) and suramin (5 mg/kg) survived throughout the period of the study (60.00 ± 0.00) compared to groups that received the extracts, and the negative control (Table 24). The survival times between the extract treated animals at higher doses (200 and 400 mg/kg) differed significantly with the negative control group. Among the extract treated groups, the animals treated with the extract at 400 mg/kg had the highest mean survival time of 59.6±0.40 days but did not differ significantly with the group treated with the extract at 200 mg/kg (51.8±5.14 days). Animals treated with the extract at various dose levels survived for longer time compared to the infected-untreated control (33.00±3.08 days). At lower dose (100 mg/kg) however, the extract was comparable to the negative control.

Table 24: Effect of methanol extract of *A. indica* stem bark on mean survival time of *T. b. rhodesiense* (KETRI 3438) infected mice

Treatment group	Number of mice			Percentage of mice cured	Mean survival time (days ± SE)
	treated	survived	cured		
100 mg/kg	5	1	1	20	46.2 ± 5.44^{ab}
200 mg/kg	5	3	2	40	51.8 ± 5.14^{bc}
400 mg/kg	5	4	4	80	59.6 ± 0.40^{c}
3.6 mg/kg Mel	5	5	5	100	60.0 ± 0.00^{c}
5 mg/kg Sur	5	5	5	100	60.0 ± 0.00^{c}
Negative control	5	0	0	0	33.0 ± 3.08^{a}
Blank					60.0 ± 0.00^{c}

SE = standard error; Mel = melarsoprol; Sur = suramin; negative control = infected untreated; blank = uninfected untreated; values with the same superscript(s) do not differ significantly at $p<0.05$

Plotting Kaplan Meier plot of mean survival time (Figure 38) revealed that all the animals in the blank (uninfected-untreated) group, melarsoprol and suramin treated groups recorded 100 % survival. Among the extract treated groups, the animals treated with the extract at higher dose of 400 mg/kg recorded the highest survival rate (80 %) compared to the extract treated groups at lower doses of 200 mg/kg and 100 mg/kg (60 and 20 %, respectively). However, all the extract treated animals had a higher survival rate compared to the infected-untreated control group.

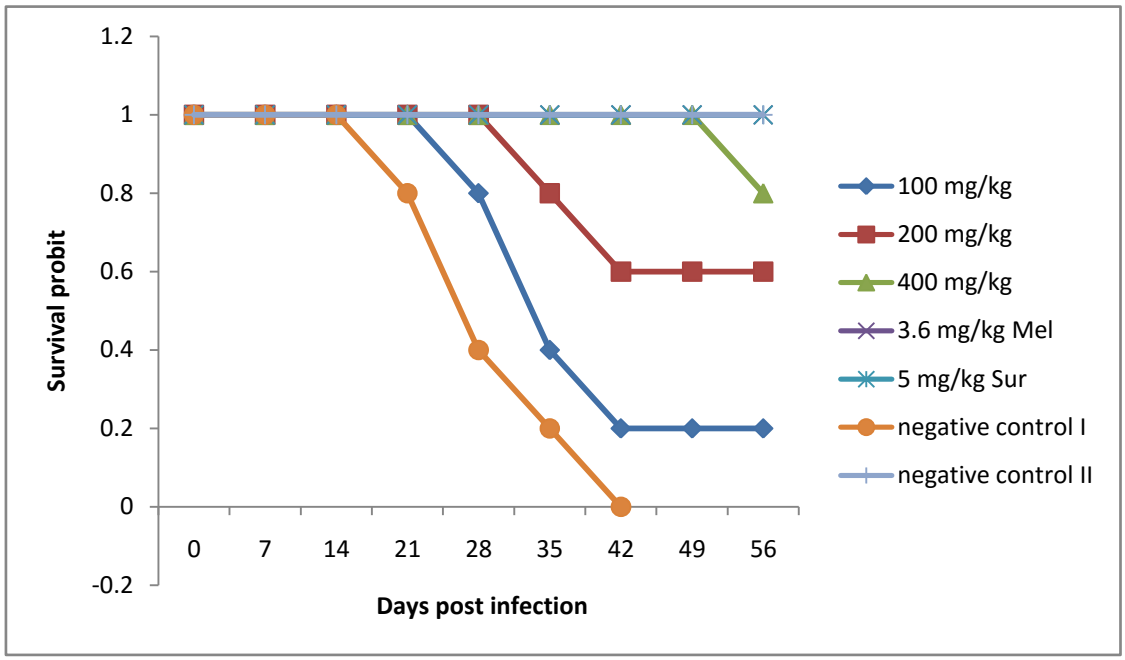


Figure 38: Kaplan Meier plot of mean survival time of *T. b. rhodesiense* (KETRI 3438) infected mice treated intraperitoneally with methanol extract of *A. indica* stem bark; values are mean ± SEM; n = 5; negative control = infected untreated; blank = uninfected untreated; Mel = melarsoprol; Sur = suramin.

4.4.3 Discussion on *in vivo* Anti-trypanosomal Efficacy of Active Extracts

Results from *in vivo* efficacy study revealed that the extract of *A. indica* stem bark significantly prevented the establishment of parasitaemia in *T. b. rhodesiense* infected mice as compared to the negative control indicating the presence of antitrypanosomal constituents in the extract. Similarly, the extract significantly reduced weight loss and decline in packed cell volume (PCV) associated with parasitaemia. Packed cell volume and weight loss are common and critical features in the pathogenesis of African trypanosomiasis contributing to morbidity and mortality (Kagira *et al*., 2006). Infected mice treated with *A. indica* extract in the current study showed significantly higher levels in PCV compared to the negative control (infected untreated) group which can be ascribed to an enhanced resistance of erythrocyte haemolysis. The ability to protect erythrocytes from haemolysis can be attributed to the presence of polyphenols in extracts of *A. indica* (Kagira *et al*., 2006). The ability of the extract to extend survival

period of infected mice was dose-dependent, indicating that the anti-trypanosomal activity of the extract can be improved by using a higher dose or by using isolated active compounds in the extract. These findings are in agreement with a previous study which reported that *A. indica* exhibited encouraging *in vivo* trypanocidal activity with a reduction in the level of *T. brucei* parasitemia in mice (Mbaya *et al*., 2010). This anti-trypanosomal effect may be attributed to specific limonoid and flavonoid constituents of *A. indica* stem bark (Kumar *et al*., 2010).

Anti-trypanosomal effect of *A. indica* extract against trypanosome infection can further be deduced from the weight status of the extract-treated animals as compared to the negative control (infected untreated) animals. The body weight improvement was consistent with the observation made on parasitaemia among the extract treated animals. This observation indicates that the extract treated animals could feed better (as a result of their improved physical state) than those in the negative control group and resist weight loss that is usually associated with trypanosomiasis.

Similarly, aqueous extract of *T. mombassana* leaf prevented weight loss and decline in PCV of *T. b. rhodesiense* infected mice as compared to negative control. Although there are few previous phytochemical reports on *T. mombassana,* reports on isolation of compounds from the root bark revealed the presence of limonoids such as mombasone and mombasol (Irungu *et al*., 2009). Studies have shown that other plants in the genus Turraea constitute of phytochemicals which demonstrated different pharmacological activities (Adul *et al*., 1993). The active components in the extract of *T. mombassana* leaf might be any of these secondary metabolites. There is therefore, a need to investigate the phytochemistry of the leaf of *T. mombassana* to unravel the compounds responsible for the trypanocidal activity.

4.5 Isolation and Identification of Constituents of the Active Extracts

In vivo efficacy study results (Section 4.4) confirmed that methanol extract of *A. indica* stem bark and the aqeous extract of *T. mombassana* leaves were potential sources of new drugs for the treatment of trypanosomiasis. The extracts significantly suppressed the development of parasitemia in *T. b. rhodesiense* infected mice, rendering them as candidates for isolation of antitrypanosomal lead compounds. Towards this end, fractionation and characterization of the extracts using chromatographic and spectroscopic techniques was undertaken to isolate pure active components responsible for the antitrypanosomal activity. This section describes the spectroscopic data and *in vitro* antitrypanosomal activity of the compounds obtained after isolation.

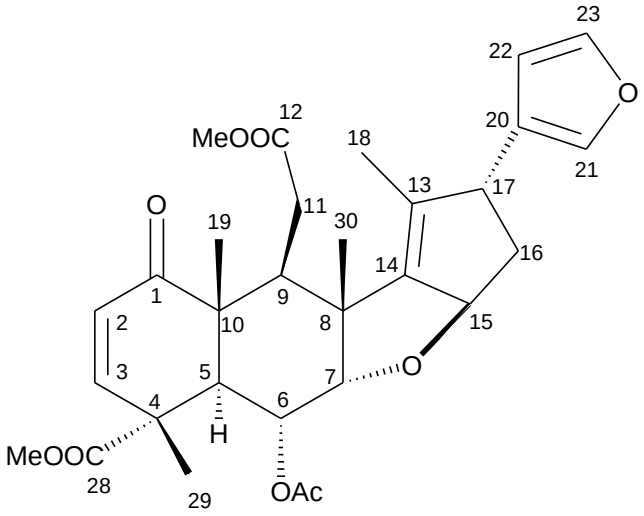
4.5.1 Isolation and identification of constituents of methanol extract of *A. indica* stem bark

Isolation of compounds from the methanol extract of *A. indica* was achieved through colunm chromatography which yielded two compounds. One was identified as nimbin **(55)** and the other as 1-detigloyl salannin (**84**), based on their ^{1}H and ^{13}C NMR data. Compound **84** showed ^{1}H NMR spectrum that was close to that of salanin (**60**).

4.5.1.1 Nimbin (55)

Sub-fraction AIS 3B which contained three spots on TLC plate, was subjected to repeated silica gel column chromatography, eluted with a gradient of Hex: EtOAc (4:1 to 1:1) to yield nimbin **(55)** as white needle-like crystals. The ^{13}C NMR spectral analysis revealed the presence of 30 carbon atoms (Appendix 7).

Both the ^{1}H and ^{13}C NMR spectra (Table 25, Appendices 3 – 7) established a limonoid skeleton whose furan ring resonances appeared at δH 7.33 (1H, *m*; H-21), 6.34 (1H, *m*; H-22), 7.22 (1H, *m*; H-23) and δC 139.0, 110.5 and 143.0 corresponding to C-21, C-22 and C-23, respectively. The presence of four singlets at δH 1.28, 1.35, 1.37 and 1.69 each integrating for three protons indicated presence of four tertiary methyls groups with the corresponding carbon signals at δC 16.7, 16.7, 17.2 and 12.9, respectively.



55

Figure 39: The structure of nimbin **(55)**

Carbonyl carbons C-12 and C-28 appeared at signal resonance at δC 173.7 and 174.6, respectively (Rojatkar *et al.,* 1998). The two methyl groups attached to the ester oxygen were confirmed by the presence of two singlets at δH 3.67 and 3.75 intergrating for six protons. An acetate singlet at δH 2.06 intergrating for three protons and a proton at δH 5.22 (1H, *dd; J* = 12.4, 3.0) revealed the presence of the acetoxy group at C-6 corresponding to a carbon signal at δC 68.7. A triplet at δH 2.87 (1H, *J* = 4.0) appeared at δC 38.6 (C-9). Resonances at δC 135.1 and δC 146.2 were typical of a C-13/C-14 double bond (Rojatkar *et al.*, 1998). The C-2/C-3 double bond appeared at δC 126.0 and δC 147.6 with the corresponding proton signals at δH 5.89 (1H, *d*; *J* = 10.2) and δH 6.34 (1H, *d*; *J* = 10.1). Based on the similarity of the ^{1}H and ^{13}C NMR data for compound **(55)** with those previously reported (Athar *et al*., 2012), the structure was identified as nimbin, chemical formula $C_{30}H_{36}O_9$.

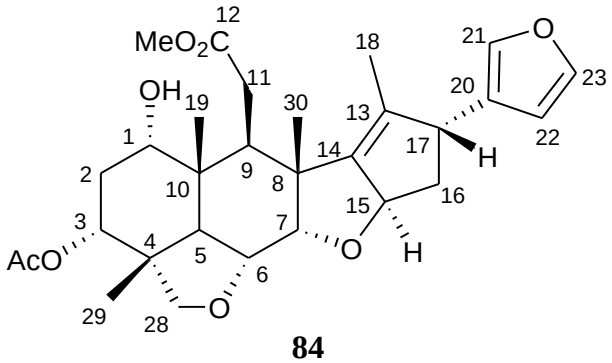
Table 25: ^{1}H and ^{13}C NMR (400 MHz, $CDCl_3$) data for nimbin **(55)**

Position	δ_H (obs) (J in Hz)	δ_C (obs)	δ_H (lit) (J in Hz)	δ_C (lit)
1	-	201.7	-	201.7
2	5.89 (1H, *d*, J = 10.2)	126.0	5.88 (1H, *d*, J = 10.2)	126.0
3	6.34 (1H, *d*, J = 10.1)	147.6	6.34 (1H, *d*, J = 10.2)	147.6
4	-	48.0	-	47.9
5	3.62 – 3.67 (1H, *m*)	41.6	3.62 – 3.69 (1H, *m*)	41.6
6	5.22 (1H, *dd*, J = 12.4, 3.0)	68.7	5.22 (1H, *dd*, J = 12.4, 2.8)	68.7
7	4.07 (1H, *d*, J = 2.8)	84.6	4.05 (1H, *d*, J = 2.6)	84.5
8	-	47.1	-	47.0
9	2.87 (1H, *t*, J = 4.0)	38.6	2.86 (1H, *t*)	38.5
10	-	48.0	-	48.0
11*a*	2.91 (1H, *dd*, J = 16.2, 5.2)	34.3	2.90 (1H, *dd*, J = 16.2, 5.2)	34.3
11*b*	2.19 – 2.26 (1H, *m*)		2.18 – 2.25 (1H, *m*)	
12	-	173.7	-	173.7
13	-	135.1	-	135.1
14	-	146.2	-	146.1
15	5.57 (1H, *m*)	87.1	5.56 (1H, *m*)	87.1
16*a*	2.19 – 2.26 (1H, *m*)	41.5	2.18 – 2.25 (1H, *m*)	41.6
16*b*	2.01 (1H, *m*)		2.01 (1H, *m*)	
17	3.62 – 3.68 (1H, *m*)	49.5	3.62 – 3.69 (1H, *m*)	49.4
18	1.69 (3H, *s*)	12.9	1.67 (3H, *s*)	12.9
19	1.28 (3H, *s*)	16.7	1.28 (3H, *s*)	16.8
20	-	126.8	-	126.8
21	7.33 (1H, *m*)	139.0	7.33 (1H, *m*)	139.0
22	6.34 (1H, *m*)	110.5	6.34 (1H, *m*)	110.5
23	7.22 (1H, *m*)	143.0	7.23 (1H, *m*)	143.0
28	-	174.6	-	174.6
29	1.37 (3H, *s*)	17.2	1.36 (3H, *s*)	17.2
30	1.35 (3H, *s*)	16.7	1.35 (3H, *s*)	16.6
$COOCH_3$	3.75 (3H, *s*)	53.1	3.74 (3H, *s*)	53.1
$COOCH_3$	3.67 (3H, *s*)	51.7	3.66 (3H, *s*)	51.7
$OCOCH_3$	-	170.7	-	170.7
$OCOCH_3$	2.06 (3H, *s*)	21.0	2.04 (3H, *s*)	21.0

Literature data source: Athar *et al*., 2012; Rojatkar *et al.,* 1998.

4.5.1.2 1-detigloyl salannin (84)

Compound (**84**), a derivative of salannin, was obtained after subjecting sub-fraction 3B1 (Scheme 3) to repeated column chromatography. Its structure was partially identified as 1-detigloyl salannin (**84**) after comparing its ^{1}H NMR data (Table 26) with literature (Mohamed *et al*., 1992; Kumar *et al*., 2010). However, ^{13}C NMR was not obtained since a higher resolution (600 – 1000 MHz) was required to analyse the small quantity of the compound obtained. Thus, *in vitro* anti-trypanosomal determination could not be performed.



84

Figure 40: Structure of 1-detigloyl salannin (**84**)

^{1}H NMR spectrum of 1-detigloyl salannin (**84)** (Appendix 9) indicated salannin basic structure whose furan ring resonances appeared at δH 7.35 (1H, *m*; H-21), 6.36 (1H, *m*; H-22), 7.20 (1H, *m*; H-23). Lack of tigloyl group in the structure was illustrated by the absence of the characteristic tigloyl resonances at δH 6.96, 1.95 and 1.82 corresponding to H-3', H-5' and H-4' respectively. The presence of four singlets at δH 1.67, 0.99, 1.31 and 1.23 each integrating for three protons indicated presence of four tertiary methyls groups at H-18, 19, 29 and 30 respectively. The 3-acetyl group was shown by the presence of a singlet resonance at δH 2.10 intergrating for three protons. A singlet at δH 3.30 showed possible methoxy protons attached to carbonyl C-12. A proton at δH 5.44 (1H, *m*) revealed the presence of the acetoxy group at C-6. A singlet proton at δH 2.06 indicated the presence of the C-1 hydroxyl group. Based on the similarity of the proton

NMR spectral features of compound (84) with those in literature, the structure was proposed to be 1-detigloyl salannin, chemical formular $C_{29}H_{38}O_8$. However, 600 or 800 MHz ^{13}C NMR facility was required to confirm its exact identity. Infra-red spectroscopy was required to confirm the free hydroxyl group at C-1.

Table 26: ^{1}H NMR (400 MHz, $CDCl_3$) data 1-detigloyl salannin (**84**)

Position	**$\delta_{H\ (obs)}$ (J in Hz)**	**$\delta_{H\ (lit)}$**
1	4.87 (1H, *m*)	4.85 (*m*)
2	2.12-2.37 (2H, *m*)	2.12-2.34 (*m*)
3	4.98 (1H, *m*)	4.96 (*m*)
5	2.76 (1H, *d*, J = 12.5)	2.79 (*d*, J =12.7)
6	4.00 (1H, *dd*, J =12.6, 3.1)	4.00 (*dd*, J =12.7,3.3)
7	4.18 (1H, *d*, J =3.0)	4.18 (*d*, J =3.0)
9	2.73 (1H, *m*)	2.75 (*m*)
11	2.12-2.37 (2H, *m*)	2.12-2.34 (*m*)
15	5.45 (1H, *m)*	5.44 (*m*)
16	2.12-2.37 (2H, *m*)	2.12-2.34 (*m*)
17	3.60 - 3.67 (1H, *m*)	3.58-3.70 (*m*)
18	1.67 (3H, *s*)	1.67 (*s*)
19	0.99 (3H, *s*)	0.99 (*s*)
21	7.35 (1H, *m*)	7.33 (*m*)
22	6.36 (1H, *m)*	6.33 (*m*)
23	7.30 (1H, *m*)	7.26 (*m*)
28	3.60 - 3.67 (2H, *m*)	3.58-3.70 (*m*)
29	1.31 (3H, *s*)	1.30 (*s*)
30	1.23 (3H, *s*)	1.22 (*s*)
CO_2CH_3	3.30 (3H, *s*)	3.29 (*s*)
$COCH_3$	2.10 (3H, *s*)	2.12 (*s*)
C-1 OH	2.06 (1H, *s*)	2.04 (*s*)

Literature data source: Mohamed *et al.*, 1992.

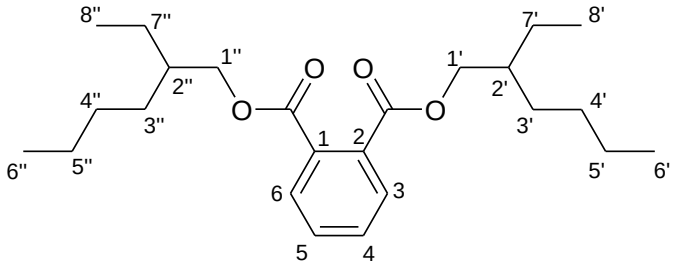
4.5.2 Isolation and identification of constituents of methanol extract of *T. ciliata* stem bark

Two structurally related phthalates, bis(2-ethylhexyl) phthalate (**85**) and bis(2-methylheptyl) phthalate (**86**) were isolated from methanol extract of *T. ciliata* stem bark through colunm chromatography and PTLC techniques. Structures of the two

compounds were established based on the comparison between the ^{1}H and ^{12}C NMR observed and the literature data.

4.5.2.1 Bis(2-ethylhexyl) phthalate (85)

Repeated column chromatography, eluted with a gradient of petroleum ether: EtOAc (2:1 to 1:4), four fractions. Fraction TCS 1 of the column chromatography fractions was then subjected to repeated column chromatography, eluted with a gradient of pet ether: EtOAc (2:1 to 1:1) to obtain compound **85** as light yellow oil.



85

Figure 41: Structure of bis(2-ethylhexyl) phthalate **(85)**

The ^{1}H-NMR spectrum of compound **85** (Table 27, Appendices 9 – 12) was typical of an ortho-phthalate (Rao *et al*., 2000), with the aromatic protons being revealed by resonances at δH 7.70 (*dd*, *J* =5.7, 3.3 Hz, H-3, H-6) and 7.52 (*dd*, *J* = 5.6, 3.2 Hz, H-4, H-5) intergrating for four protons. The signals at δH 4.20 (*dd*, *J* = 11.2, 5.8 Hz) revealed the presence of two equivalent OCH_2 groups at H-1' and H-1". The two protons H-2 and H-2' were shown by a multiplet signal at δH 1.65 – 1.70. Eight aliphatic CH_2 resonances at H-3', H-3", H-4', H-4", H-5', H-5", H-7' and H-7" were observed as a multiplet at δH 1.23 – 1.33 which intergrated for sixteen protons. A multiplet resonance observed at δH 0.86 – 0.96 was due to the four terminal methyl groups (H-6', H-6", H-8' and H-8") intergrating for twelve protons. The above data, thus, indicated two 2-ethylhexyl ester moieties (Amani *et al*., 2013).

The ^{13}C-NMR spectrum of compound **85** (Table 27, Appendix 13) showed twelve signals due to twenty four carbon atoms confirming the symmetry of the compound. The two ester carbonyl groups were observed at δC 167.8 whereas resonance at δC 132.5 was due to the quaternary aromatic atoms C-1' and C-2". The resonances at δC 130.9 and 128.8 were due to aromatic carbons (C-3, C-6) and (C-4, C- 5), respectively. The signal at δC 68.2 was due to resonance at C-1 and C-1'. The carbon methine atoms (C-2', C-2") were observed at δC 38.7 whereas the four pairs of aliphatic CH_2 carbons (C-3', C-3"), (C-4', C-4"), (C-5', C-5") and (C-7', C-7") were revealed by signals at δC 30.4, 28.9, 23.0 and 23.8 respectively. The two pairs of the terminal methyl groups (C-6', C-6") and (C-8', C-8") appeared at δC 14.0 and δ C 11.0 respectively. Comparison between the observed and the literature data (Amade *et al*., 1994; Rao *et al*., 2000; Sajan *et al*., 2005; Amani *et al*., 2013) confirmed the structure of compound (**85**) as bis(2-ethylhexyl) phthalate; chemical formula $C_{24}H_{38}O_4$.

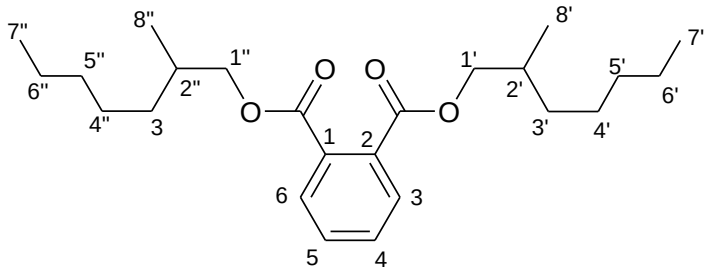
Table 27: ^{1}H and ^{13}C NMR (400 MHz, $CDCl_3$) data for Bis(2-ethylhexyl) phthalate **(85)**

Position	**δ_H (obs) (J in Hz)**	**δ_C (obs)**	**δ_H (lit)**	**δ_C (lit)**
C-1, C-2	-	132.5	-	132.4
C-3, C-6	7.69 (2H, *dd*, J = 5.7, 3.3)	130.9	7.68 (2H, *dd*, J = 5.8, 3.3)	130.9
C-4, C-5	7.52 (2H, *dd*, J = 5.6, 3.2)	128.8	7.51 (2H, *dd*, J = 5.7, 3.3)	128.8
C-1', C-1"	4.20 (4H, *dd*, J = 11.2, 5.8)	68.2	4.20 (4H, *dd*, J = 11.1, 5.7)	68.0
C-2', C-2"	1.65 – 1.70 (2H, *m*)	38.7	1.62 – 1.70 (2H, *m*)	38.7
C-3', C-3"	1.23 – 1.33 (4H, *m*)	30.4	1.24 – 1.31 (4H, *m*)	30.4
C-4', C-4"	1.23 – 1.33 (4H, *m*)	28.9	1.24 – 1.31 (4H, *m*)	28.9
C-5', C-5"	1.23 – 1.33 (4H, *m*)	23.0	1.24 – 1.31 (4H, *m*)	23.0
C-6', C-6"	0.86 – 0.96 (6H, *m*)	14.0	0.84 – 0.95 (6H, *m*)	14.1
C-7', C-7"	1.23 – 1.33 (4H, *m*)	23.8	1.24 – 1.31 (4H, *m*)	23.6
C-8', C-8"	0.86 – 0.96 (6H, *m*)	11.0	0.84 – 0.95 (6H, *m*)	11.0
2 C=O	-	167.8	-	167.7

Literature source: Sajan *et al*., 2005; Rao *et al*., 2000; Amade *et al*., 1994; Amani *et al*., 2013.

4.5.2.2 Bis(2-methylheptyl) phthalate (86)

Column chromatography of sub-fraction TCS 2 (Section 4.3.3.1) eluted with a gradient of pet ether : EtOAc (1:1 to 1:2) to yielded sub-fractions TCS 2A and TCS 2B. Preparative TLC of sub-fraction TCS 2A [pet ether: EtOAc: MeOH (4:4:1)] afforded compound **86** as a colourless oil.



86

Figure 42: Structure of bis(2-methylheptyl)phthalate **(86)**

The ^{1}H NMR of compound **86** (Table 28, Appendices 14 – 17) showed the presence of two terminal methyls (at C-7', C-7") and the two secondary methyls (at C-8', C-8") appeared at δH 0.92 integrating for twelve protons. The aromatic protons (H-3, H-6) and (H-4, H-5) appeared at δH 7.65 and 7.45, respectively. The methylene protons at H-1' attached to the ester appeared at δH 4.23 as multiplet. The other methylene protons at C-3', C-3", C-4', C-4", C-5', C-5", C-6' and C-6" appeared at δH 1.28 – 1.34 as multiplet intergrating for sixteen protons. The two protons at H-2 and H-2' were revealed by a multiplet signal at δH 1.69.

The ^{13}C NMR spectrum (Table 28, Appendix 18) confirmed the presence of ester carbonyl which appeared at δC 167.8 while the methylene attached to ester oxygen appeared δC 68.1. The three pairs of aromatic carbons (C-1, C-2), (C-3, C-6) and (C-4, C-5) appeared at δC 130.9, 128.8 and 132.4, respectively. Resonances at C-2' and C-2" were observed at δC 38.8. Four pairs of CH_2 carbons at (C-3', C-3"), (C-4', C-4"), (C-5', C-5") and (C-6', C-6") appeared at δC 29.7, 28.9, 30.4 and 23.0 respectively. The two terminal methyl carbons appeared at δC 14.1 while the methyl carbons attached to C-2'

and C-2" appeared at δC 11.0. On the basis of comparison of these data with reported literature (Cakir *et al*., 2003; Rameshthangam *et al*., 2007), compound (**86)** was determined to be bis(2-methylheptyl)phthalate, chemical formula $C_{24}H_{38}O_4$.

Table 28: ^{1}H and ^{13}C NMR (400 MHz, $CDCl_3$) data for Bis(2-methylheptyl) phthalate **(86)**

Position	**δ_H (obs) (J in Hz)**	**δ_C (obs)**	**δ_H (lit)**	**δ_C (lit)**
C-1, C-2	-	132.5	-	132.4
C-3, C-6	7.72 (2H, *dd*, J = 5.8, 3.0)	128.8	7.70 (2H, *dd*, J = 5.7, 3.1)	128.8
C-4, C-5	7.54 (2H, *dd*, J = 5.7, 3.3)	130.9	7.52 (2H, *dd*, J = 5.7, 3.2)	130.9
C-1', C-1"	4.23 (4H, *m*)	68.2	4.20 (4H, *m*)	68.1
C-2', C-2"	1.69 (2H, *m*)	38.8	1.68 (2H, *m*)	38.7
C-3', C-3"	1.34 (4H, *m*)	29.7	1.36 (4H, *m*)	29.8
C-4', C-4"	1.28 (4H, *m*)	28.9	1.28 (4H, *m*)	28.9
C-5', C-5"	1.32 (4H, *m*)	30.4	1.32 (4H, *m*)	30.3
C-6', C-6"	1.28 (4H, *m*)	23.0	1.28 (4H, *m*)	22.9
C-7', C-7"	0.92 (6H, *m*)	14.1	0.92 (6H, *m*)	14.1
C-8', C-8"	0.92 (6H, *m*)	11.0	0.92 (6H, *m*)	10.9
2 C=O	-	167.8	-	167.8

Literature source: Rameshthangam *et al*., 2007; Cakir *et al*., 2003

The ^{13}C NMR spectroscopic data for compounds **85** and **86** were similar except the presence of a resonance at δC 29.7 (C-3', C-3") in spectrum of compound **86** which lacked in the spectrum of compound **85** while a signal at δC 23.8 (C-7', C-7") observed in the spectrum **85** lacked in that of **86**.

4.5.3 *In vitro* anti-trypanosomal activity of isolated compounds

Nimbin **(55)** and bis(2-ethylhexyl) phthalate **(85)** isolated from *A. indica* and *T. ciliata,* respectively, were subjected to *in vitro* anti-trypanosomal assays using *T. b. rhodesiense* and *T. evansi*. The results were summarized in Table 29.

Table 29: Anti-trypanosomal activity (MIC values) of isolated compounds

Compound	MIC ± SE (µg/ml)		
	T. b. rhodesiense	*T. b. brucei*	*T. evansi*
Nimbin (**55**)	10.74 ± 0.93	11.90 ± 0.02	11.89 ± 1.01
Bis(2-ethylhexyl) phthalate (**85**)	149.00 ± 2.30	163.24 ± 3.11	146.50 ± 2.70
Melarsoprol	0.003 ± 0.001	0.005 ± 0.002	0.003 ± 0.001
Suramin	1.54 ± 0.09	1.66 ± 0.10	1.51 ± 0.13

Values are mean ± SE; SE = standard error.

According to the definition of anti-trypanosomal activity (Hoet *et al*., 2004), nimbin (**55**) was active against bloodstream forms of *T. b. rhodesiense*, *T. b. brucei* and *T. evansi* (MIC values of 10.74±0.93, 11.90±0.02 and 11.89±1.01 µg/ml, respectively). Bis(2-ethylhexyl) phthalate (**85**) showed mild trypanocidal activity against the three trypanosome isolates (MIC values 146.50 ± 2.70 - 173.24 ± 3.11 µg/ml). In a previous study, however, bis(2-ethylhexyl) phthalate (**85**) demonstrated to be a cathepsin B inhibitor, potentially useful for the treatment of inflammatory joint disease, invasion of cancer and other diseases related to disorder of cathepsin B (Hoang *et al*., 2008).

4.5.4 Discussion on Isolation and Structure Elucidation of pure compounds

All compounds isolated in the present study had been previously described. Nimbin (**55**), a limonoid was previously isolated from different parts of *A. indica* (Rojatkar *et al.,* 1998; Athar *et al*., 2012). 1-detigloyl salanin (**84**) was isolated from seed kernel of *A. indica* (Kumar *et al*., 2010). Bis(2-ethylhexyl) phthalate (**85**) was previously isolated from *Sterculia guttata* seeds, marine *Pseudomonas* strain, *Penicillium olsonii* and *Aloe vera* Linne (Amade *et al*., 1994; Lee *et al*., 2000; Hoang *et al*., 2008). Bis(2-methylheptyl) phthalate **(86)** was previously isolated from *Pongamia pinnata* leaves (Rameshthangam *et al*., 2007) and *Poncirus trifoliate* fruits (Cakir *et al*., 2003). However, the present study reports the presence of the two phthalates (**85** and **86**) from *T. ciliata* for the first time.

Among the isolated compounds, only those in sufficient amounts were subjected to the *in vitro* assays. Of these, nimbin (**55**) showed the highest activity against the three

trypanosome strains used in the current study. However, its relatively lower activity compared to the extract suggests synergistic or additive effects of other constituents.

Thus, results of the current study lays down some significant groundwork for large-scale bioassay-guided isolation of key anti-trypanosomal constituents from the active extracts. To date, isolation of specific constituents was undertaken by column chromatography and preparative thin layer chromatography (PTLC), which could target mainly the major constituents. The minor constituents (which appear to primarily contribute to the activities of the extracts) could not be isolated in sufficient amounts for anti-trypanosome activity screening. Access to facilities such as preparative high pressure liquid chromatography (HPLC), HPLC-linked Mass Spectrometer (LC-MS) and higher resolution (600 or 800 MHz) nuclear magnetic resonance (NMR) would be important in isolating and characterizing constituents and blends primarily responsible for the activity of the extracts and fractions.

In summary, *in vitro* antitrypanosomal assays showed that five methanol extracts (*A. indica* stem bark; *M. azedarach* leaves, stem and root barks; and *T. emetica* root bark) and two aqueous extracts (*T. mombassana* leaves and *A. indica* stem bark) were active (MIC ≤ 19 µg/ml) against *T. b. rhodesiense*. Seven methanol extracts (*M. azedarach* leaves and root bark; *A. indica* stem bark; and *T. emetica* leaves, stem and root barks) and one aqueous extract (*T. mombassana* leaves) were active (MIC ≤ 19 µg/ml) against *T. b. brucei*. For the assays against *T. evansi* strain, six methanol extracts (*M. azedarach* leaves, stem and root barks; *A. indica* stem bark; and *T. emetica* stem and root barks) and two aqueous extracts (*M. azedarach* leaves and root bark) were active (MIC ≤ 19 µg/ml). There was a clear indication that methanol-soluble constituents were responsible for the observed antitrypanosomal activities of the extracts. Among the plant species, methanol extracts of *M. azedarach* and *T. emetica* exhibited the highest activities against the three trypanosome strains used in the study. However, *in vivo* antitrypanosomal activity evaluation of these extracts was thwarted by their toxicity in healthy mice (LD_{50} 229.09.54±95.54 and 707.95±229.25 mg/kg, respectively).

Methanol extract of *A. indica* stem bark and and aqueous extract of *T. mombassana* leaves were demonstrated to be safe and efficacious especially at optimum dose levels of 400 mg/kg. *In vivo* anti-trypanosomal efficacy tests further revealed that the MeOH extract *A. indica* stem bark was more active than the aqueous extract of *T. mombassana* leaves. Notebly, MeOH extract *A. indica* stem bark was comparable with the standard drugs (melarsoprol and suramin) in preventing weight loss, decline in packed cell volume and establishment of parasitaemia in *T. b. rhodesiense* infected mice.

Chromatographic fractionation of extracts led to isolation of nimbin (**55**) and 1-detigloyl salannin (**84**) from the MeOH extract *A. indica* stem bark whereas bis(2-ethylhexyl) phthalate (**85**) and bis(2-methylheptyl) phthalate (**86**) were obtained from MeOH *T. ciliata* stem bark. *In vitro* anti-trypanosomal screening of isolated compounds revealed that nimbin (**55**) was active against *T. b. rhodesiense, T. b. brucei* and *T. evansi.*

The current study has therefore, demonstrated the *in vitro* antitrypanosomal activities of methanol and aqueous extracts of *T. emetica, A. indica, M. azedarach* and *T. mombassana* as well as the *in vivo* antitrypanosomal activities of methanol extract of *A. indica* and aqueous extract of *T. mombassana*. Isolation of the active constituents from these extracts could provide lead compounds for development of anti-trypanosomal drugs.

CHAPTER FIVE: CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusions

The pharmacological investigations reported in the current study comprised of evaluation of the anti-trypanosomal activity of extracts of plant parts of five species from the Meliaceae family, namely *Trichilia emetica* Vahl, *Toona ciliata* M. Roem, *Azadirachta indica* A. Juss, *Turraea mombassana* Hiem ex C.DC and *Melia azedarach* L. using *in vitro* and *in vivo* assays. From the results obtained and discussion thereto, the following conclusions can be made.

i. Five extracts, methanol extracts of *A. indica* stem bark, *M. azedarach* leaves and root bark, *T. emetica* root bark and aqueous extract of *T. mombassana* leaves, showed *in vitro* anti-trypanosomal activitites against all the three trypanosome strains used in the current study (*T. b. rhodesiense*, *T. b. brucei* and *T. evansi*).

ii. Dose determination toxicity studies showed that methanol extract of *A. indica* stem bark and aqueous leaf extract of *T. mombassana* were not toxic at dose levels of 100, 200 and 400 mg/kg body weight in mice. However, methanol extracts of the root bark of *M. azedarach* and the root bark of *T. emetica* demonstrated lethality potential in healthy mice for the tested doses (LD_{50} = 229.09.54±95.54 and 707.95±229.25 mg/kg, respectively).

iii. *In vivo* efficacy assays showed that methanol extract of *A. indica* stem bark and aqueous extract of *T. mombassana* leaf reduced parasitemia levels, prevented body weight loss and prevented decline in packed cell volume levels of mice infected with *T. b. rhodesiense*. Aqueous extract of *T. mombassana* leaves was effective *in vivo* at 400 mg/kg while the methanol extract of *A. indica* stem bark was effective at 200 and 400 mg/kg.

iv. Chromatographic fractionation and isolation of the methanol extract of *A. indica* stem bark yielded two compounds nimbin (**55**) and 1-detigloyl salanin (**84**) whereas *Toona ciliata* stem bark yielded two compounds, bis(2-ethylhexyl) phthalate (**85)** and bis(2-methylheptyl) phthalate **(86)**.

v. Nimbin (**55**), isolated from methanol extract of *A. indica* stem bark showed *in vitro* antitrypanosomal activtity against *T. b. rhodesiense, T. b. brucei* and *T. evansi.* Bis(2-ethylhexyl) phthalate (**85**) isolated from *T. ciliata* showed mild activity.

5.2 Recommendations

Based on the conclusions made in the current study, the following recommendations can be made.

i. Kenyatta University through Chandaria Business Inovation and Incubation Centre and the Department of Pharmacy & Complementary/Alternative medicine should standardize extracts of *A. indica* stem bark and *T. mombassana* to be utilized as drugs for trypanosomiasis.

ii. Communities in tsetse endemic regions of Kenya should consider using extracts of *A. indica* stem bark and *T. mombassana* leaves as alternative remedies for the management of trypanosomiasis.

iii. The recommended safe and efficacious dose range of *A. indica* stem bark extract is 200 – 400 mg/kg whereas *T. mombassana* leaves should be used at 400 mg/kg.

iv. Despite demonstrating *in vitro* anti-trypanosomal activities, extracts of *T. emetica* and *M. azedarach* root barks should not be used for the management of African trypanosomiasis at dose levels ≥100 mg/kg.

5.3 Suggestions for further research

i. From the *in vitro* anti-trypanosomal assays, methanol extracts of *T. emetica* and *M. azedarach* root barks exhibited anti-trypanosomal potential against *Trypanosoma brucei brucei, Trypanosoma brucei rhodesiense* and *Trypanosoma evansi.* Further investigation on their safety should be carried out to establish the maximum tolerated dose for *in vivo* efficacy tests.

ii. In the current study, methanol extract of *A. indica* stem bark and aqueous extract *T. mombassana* leaf were investigated for *in vivo* anti-trypanosomal efficacy against *T. b. rhodesiense*. It is therefore important to investigate *in vivo* anti-trypanosomal efficacy of these extracts against *T. b. brucei* and *T. evansi*.

iii. Large scale bioassay-guided fractionation of extracts of *A. indica* stem bark and *T. mombassana* leaves should be performed to obtain sufficient quantities of their constituents for *in vitro* and *in vivo* anti-trypanosomal assays.

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APPENDICES

Appendix 1: A letter of approval for *in vivo* assays

KENYA AGRICULTURAL RESEARCH INSTITUTE

Trypanosomiasis Research Centre, MUGUGA

P.O. BOX 362, KIKUYU, 00902, TEL: 020-2700604, 2700654, FAX: 066-32397

KIKUYU

Email:Centre@ Kari-trc.org, trccd@kari-trc.org

Ref: C/TR/4/325/150 4th March 2014

KARI-TRC Animal Care and Use Committee (IACUC)

M/S Everlyne Nafuna Wanzala (Principal Investigator, PI)

Dear Everlyne

RE: IACUC Approval for in vivo screening of Meliaceae extracts

The Institutional Animal Care and Use Committee (IACUC) of the Kenya Agricultural Research Institute-Trypanosomiasis Research Centre (KARI-TRC) has reviewed your proposal entitled **"Identification of anti-trypanosomal constituents of some Meliaceae species and screening of selected structural variants by in vitro and in vivo assays"**. The committee has found that the methods described for the in vivo component of your study are appropriate and are consistent with its recommendations as well as National (KVA) standards of animal welfare. The committee has therefore resolved to support your project. The committee will hold you (the PI) personally responsible to ensure that high standards of animal welfare are observed at all times of the project. The committee may make impromptu visits to ensure compliance with its regulations.

Yours Sincerely

Charles Otieno

Ag IACUC Chairman, KARI-TRC

Appendix 2: A letter of award of Science Technology and Inovation Research Grant

REPUBLIC OF KENYA

NATIONAL COUNCIL FOR SCIENCE AND TECHNOLOGY

Telephone: 254-020-2213471, 2241349
254-020-310571, 2213123, 2219420
Fax: 254-020-2213215
When replying please quote
secretary@ncst.go.ke

P.O. Box 30623-00100
NAIROBI-KENYA
Website: www.ncst.go.ke

Our Ref: **NCST/ST&I/RCD/4th Call PhD/164**

Date: **2nd October, 2012**

Wanzala Evelyne Nafula
Kenyatta University
P.O. Box 43844
NAIROBI

RE: SCIENCE, TECHNOLOGY AND INNOVATION RESEARCH GRANT (PhD)

I'm pleased to inform you that, you have been awarded the Science, Technology and Innovation (ST&I) grant for your **PhD** research proposal.

National Council for Science and Technology (NCST) has approved an amount of Kenya shillings. **one million, eighty three thousand, six hundred and sixty (Ksh. 1,083,660)** towards your proposal entitled "**Identification of antitrypanosomal constituents of some meliaceae species and screening of selected structural variants by in vitro and in vivo assays**" The approved grant is for research costs only and must not be used for payment of tuition fees.

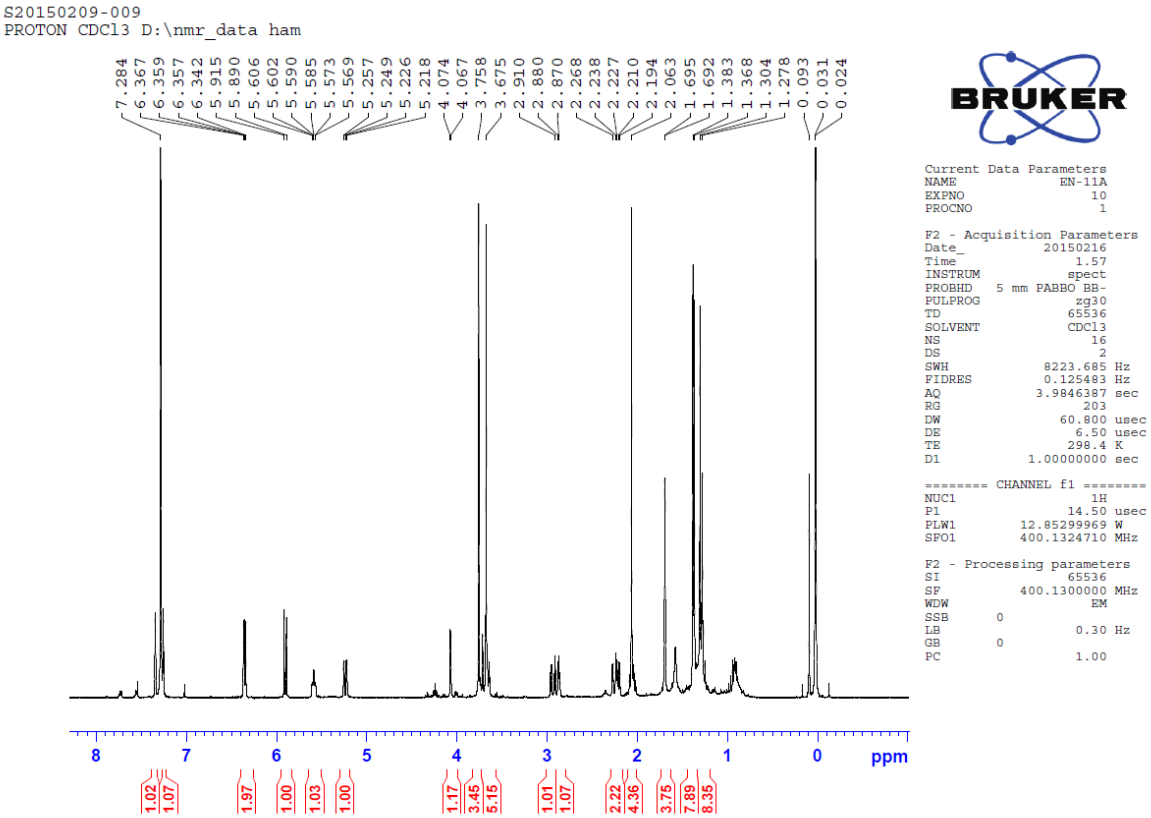
Find the enclosed ***Research Grant Contract Form (NCST/ST&I/CONTRACT/FORM 1C)*** that should be duly completed and returned to NCST. You should attach a copy of your ***national identity card and acceptance letter. Your contract must be accompanied by an abstract of your project, not more than 500 words and a recent digital passport size photograph. A soft copy of your abstract and passport size photograph must be submitted in (MS Word format) to the email: - postgraduate@ncst.go.ke***

Your signed contract form, acceptance letter and abstract should reach us not later than 12th October, 2012 for further action.

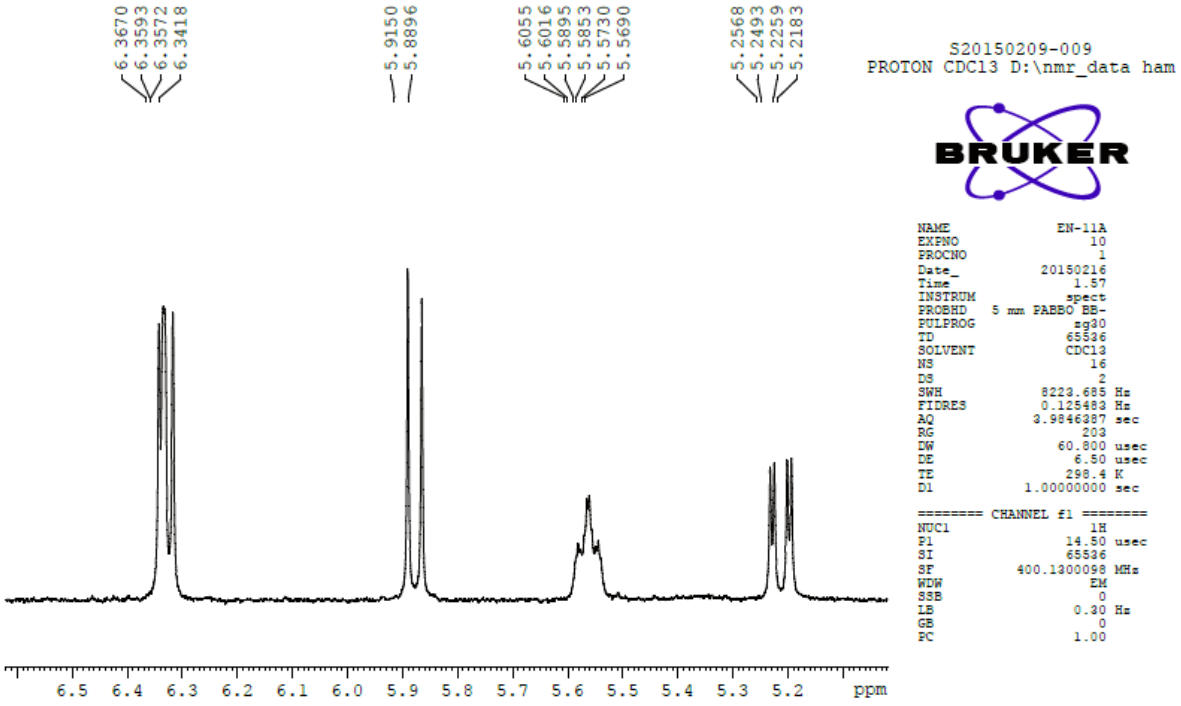
PROF. SHAUKAT ABDULRAZAK, Ph.D, FSB, FASI, MBS.
SECRETARY/CEO

cc: Vice Chancellor
Kenyatta University

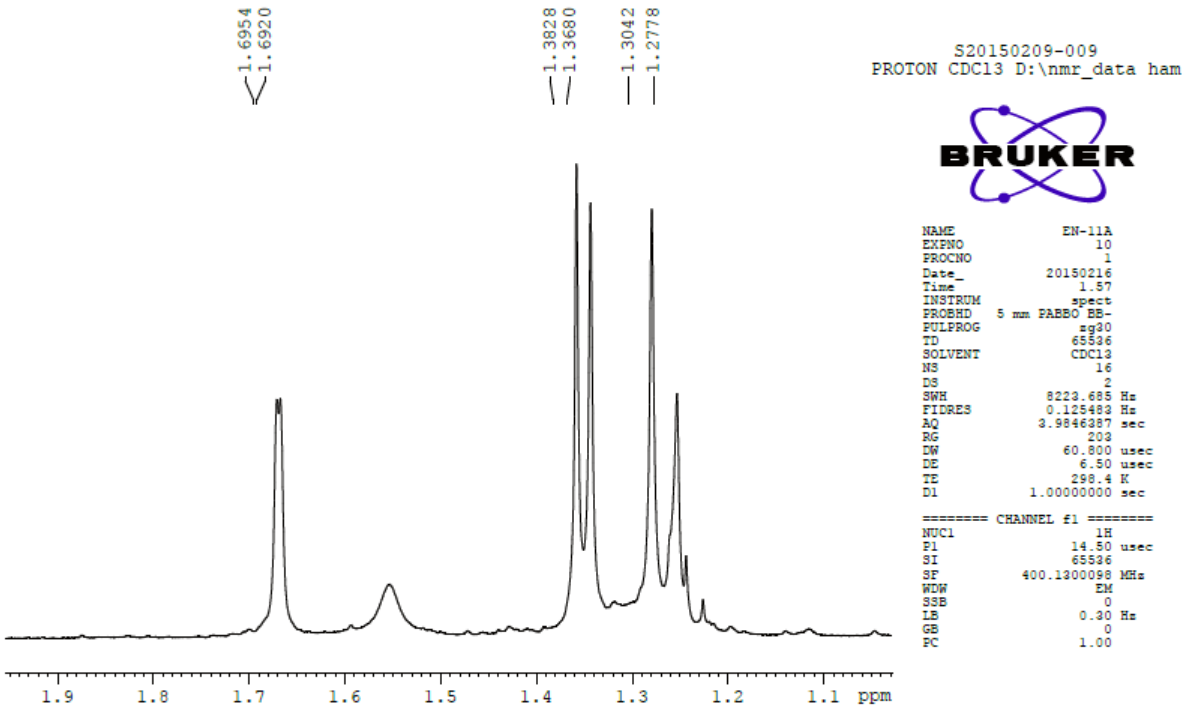
Appendix 3: ^{1}H NMR (400 Hz, $CDCl_3$) spectrum of nimbin **(55)**



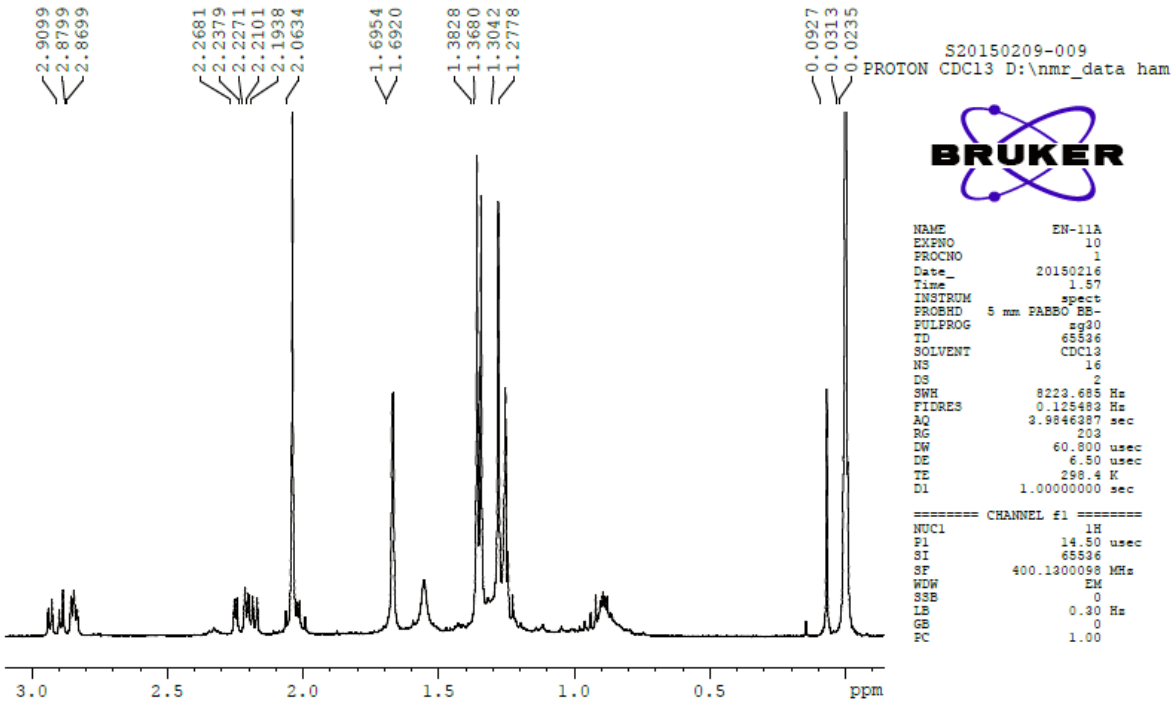
Appendix 4: Section A of ^{1}H NMR (400 MHz, $CDCl_3$) spectrum of nimbin **(55)**



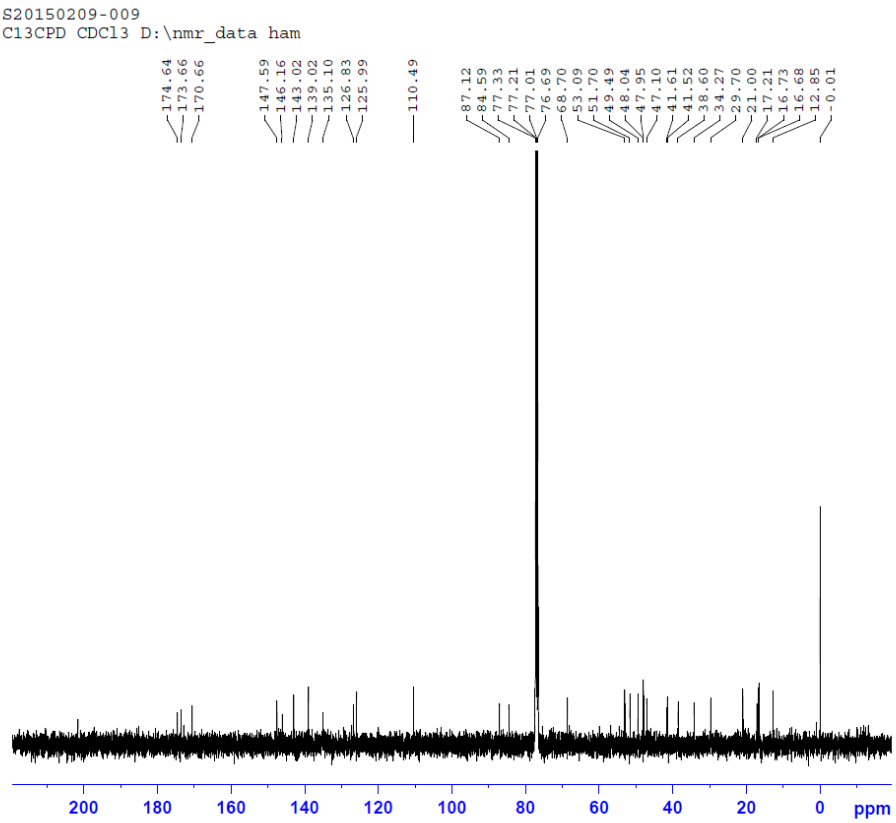
Appendix 5: Section B of ^{1}H NMR (400 MHz, $CDCl_3$) spectrum of nimbin **(55)**



Appendix 6: Section C of ^{1}H NMR (400 MHz, $CDCl_3$) spectrum of nimbin **(55)**



Appendix 7: ^{13}C NMR (100 MHz, $CDCl_3$) spectrum of nimbin **(55)**

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Current Data Parameters
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EXPNO                     11
PROCNO                     1

F2 - Acquisition Parameters
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Time                    2.57
INSTRUM                spect
PROBHD    5 mm PABBO BB-
PULPROG                zgpg30
TD                      65536
SOLVENT                 CDCl3
NS                       1024
DS                          4
SWH                 24038.461 Hz
FIDRES                0.366798 Hz
AQ                    1.3631988 sec
RG                        203
DW                     20.800 usec
DE                      6.50 usec
TE                      299.0 K
D1                  2.00000000 sec
D11                 0.03000000 sec

======== CHANNEL f1 ========
NUC1                      13C
P1                       9.65 usec
PLW1               49.65900040 W
SFO1              100.6228293 MHz

======== CHANNEL f2 ========
CPDPRG2                waltz16
NUC2                      1H
PCPD2                   90.00 usec
PLW2               12.85299969 W
PLW12               0.34196001 W
PLW13               0.27699000 W
SFO2              400.1316005 MHz

F2 - Processing parameters
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WDW                          EM
SSB          0
LB                       1.00 Hz
GB           0
PC                       1.40
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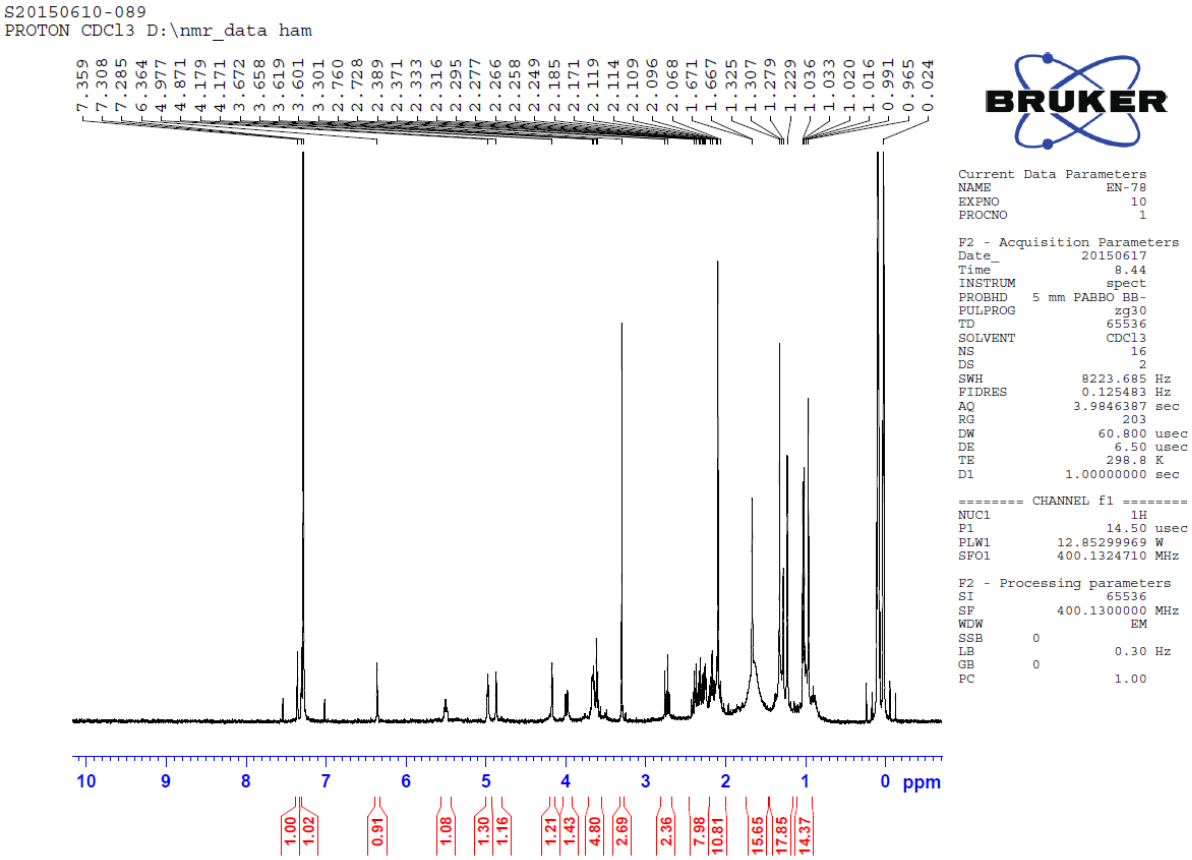
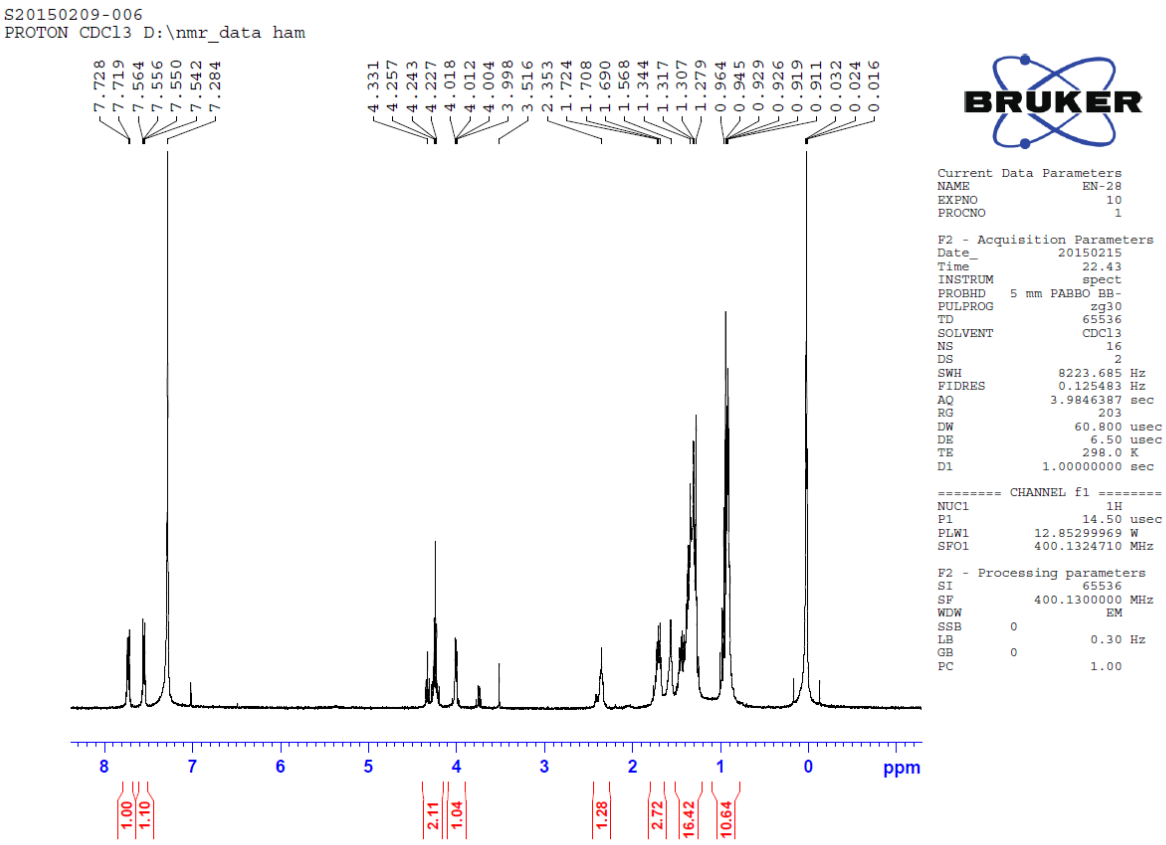
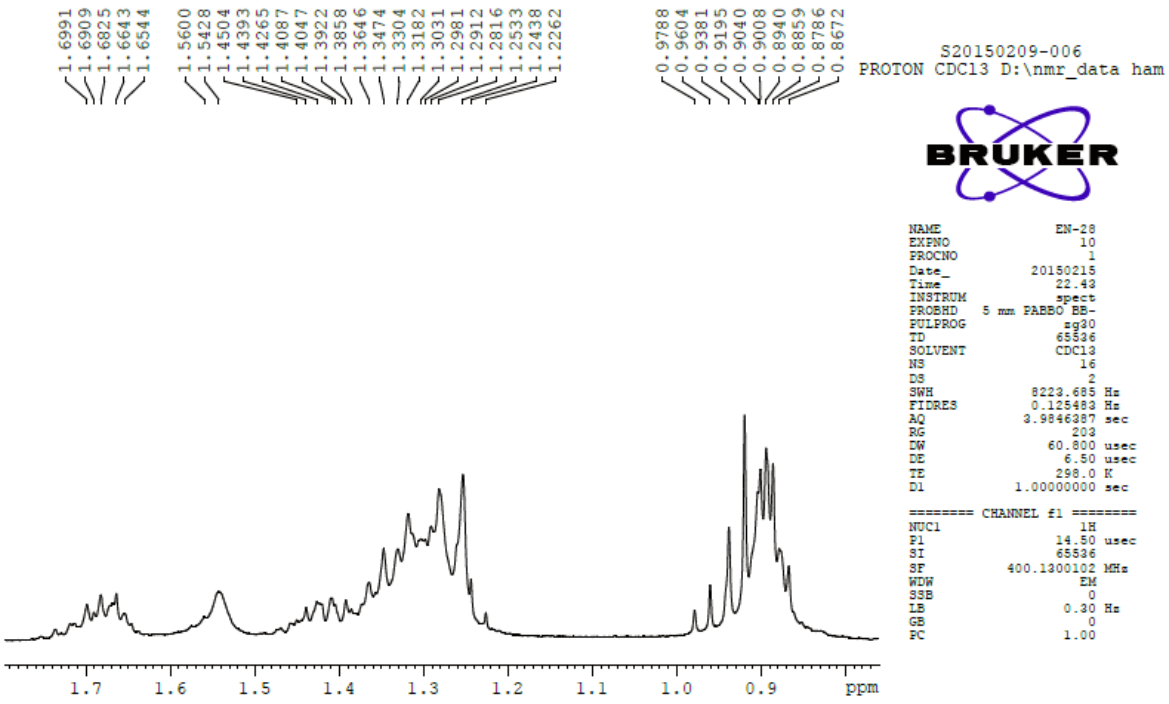
Appendix 8: ^{1}H NMR (400 MHz, $CDCl_3$) spectrum of 1-detigloyl salannin (**84**)



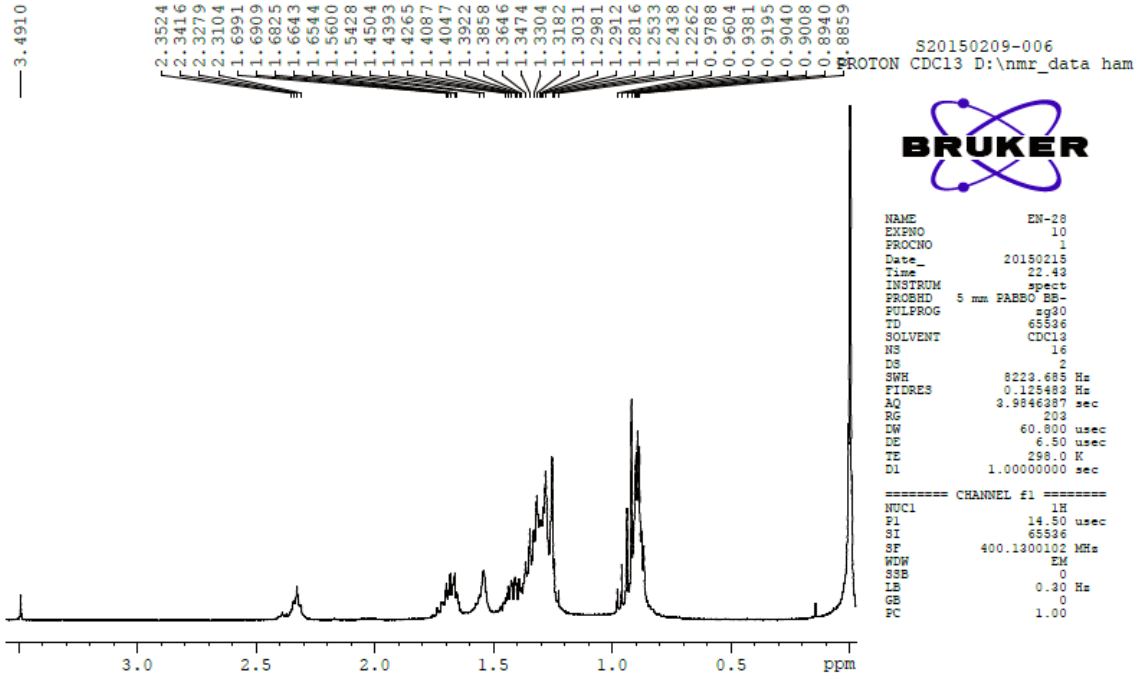
Appendix 9: ^{1}H NMR (400 MHz, $CDCl_3$) spectrum of bis(2-ethylhexyl) phthalate (**85**)



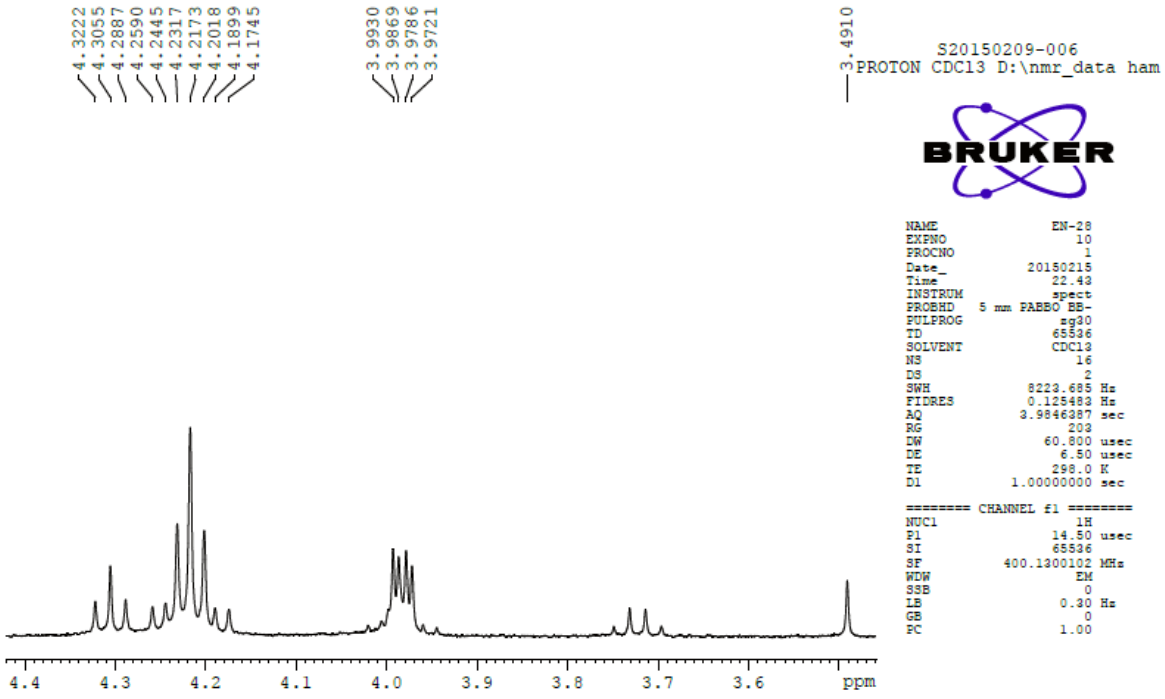
Appendix 10: Section A of ^{1}H NMR (400 MHz, $CDCl_3$) spectrum of bis(2-ethylhexyl) phthalate (**85**)



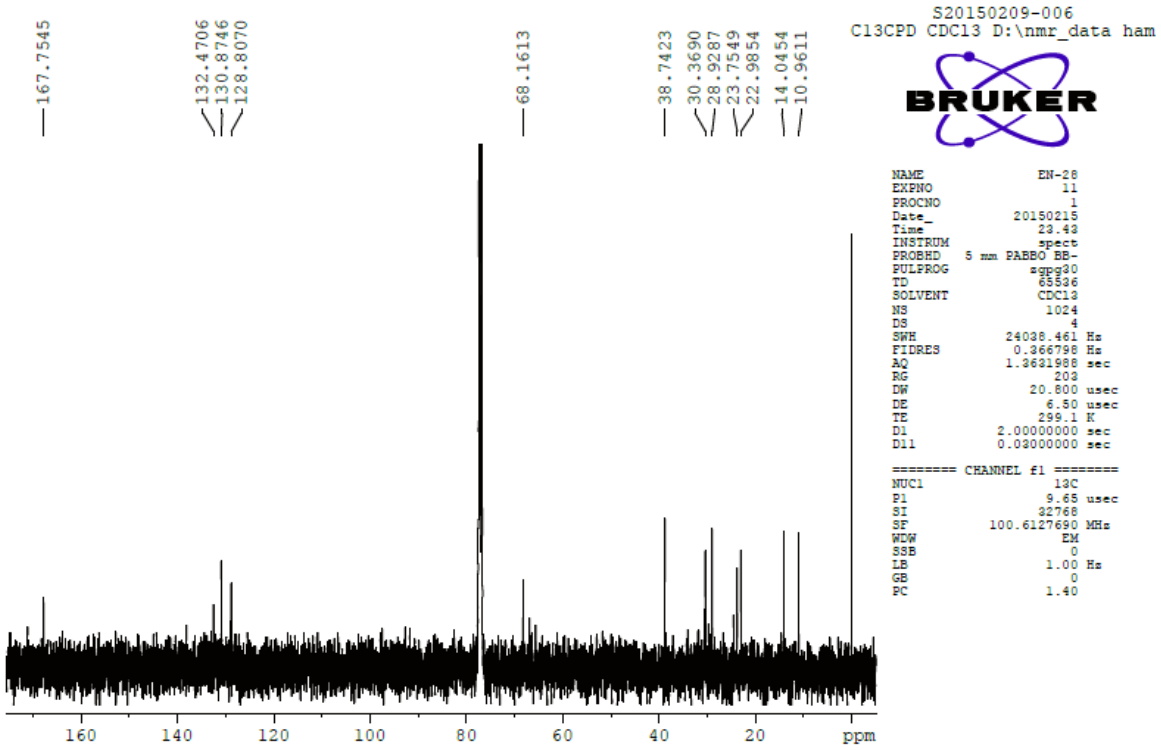
Appendix 11: Section B of ^{1}H NMR (400 MHz, $CDCl_3$) spectrum of bis(2-ethylhexyl) phthalate (**85**)



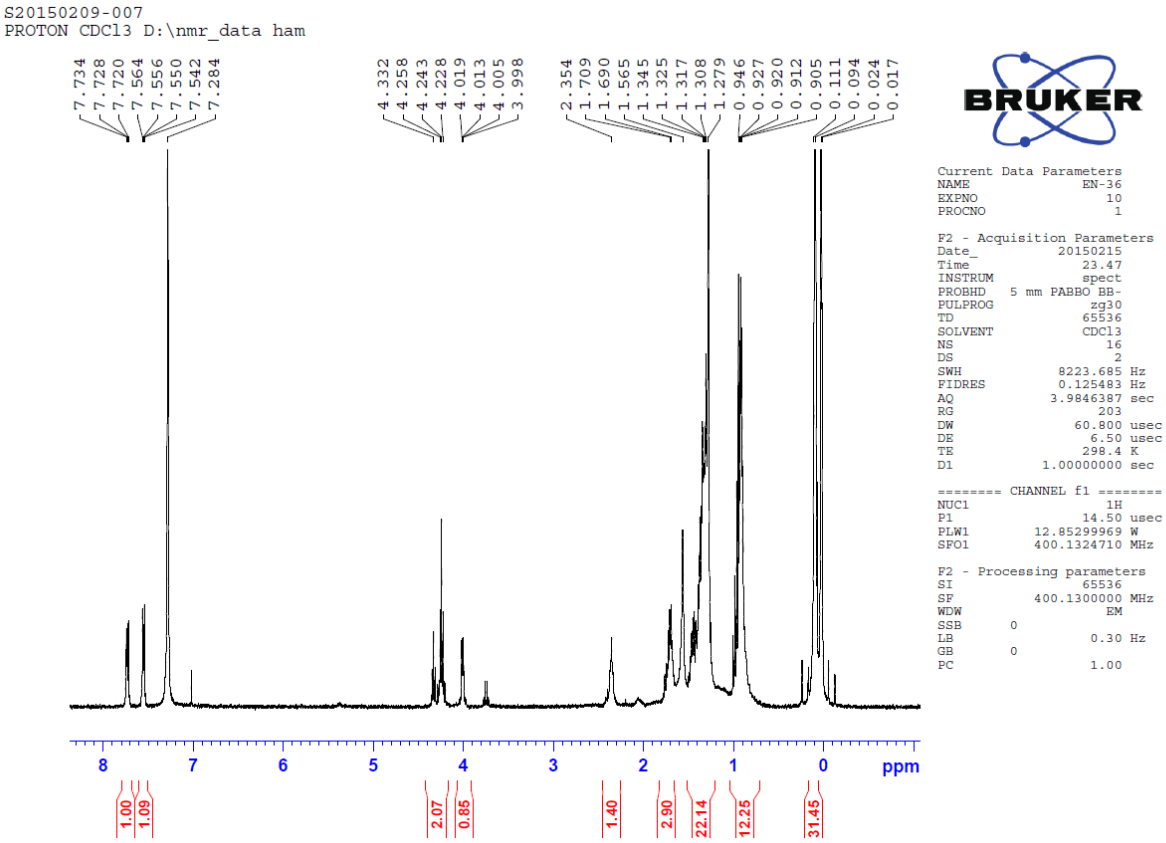
Appendix 12: Section C of ^{1}H NMR (400 MHz, $CDCl_3$) spectrum of bis(2-ethylhexyl) phthalate (**85**)



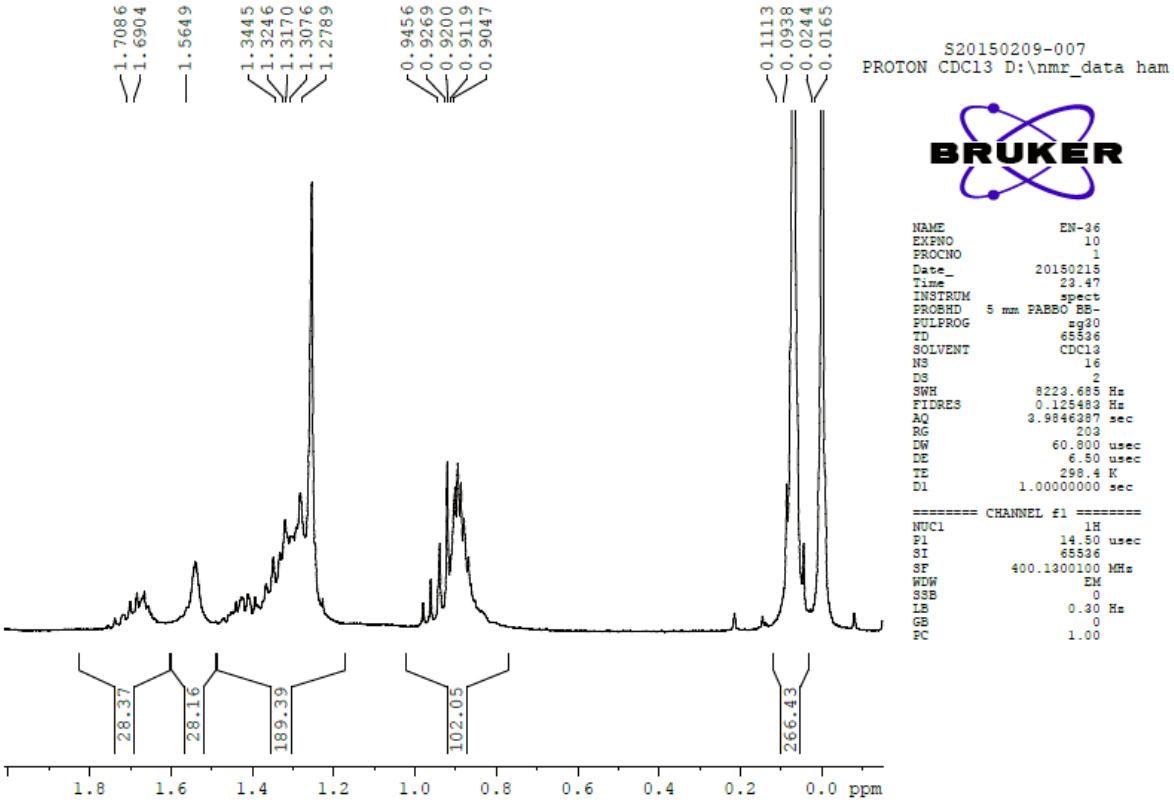
Appendix 13: ^{13}C NMR (100 MHz, $CDCl_3$) spectrum of bis(2-ethylhexyl) phthalate (**85**)



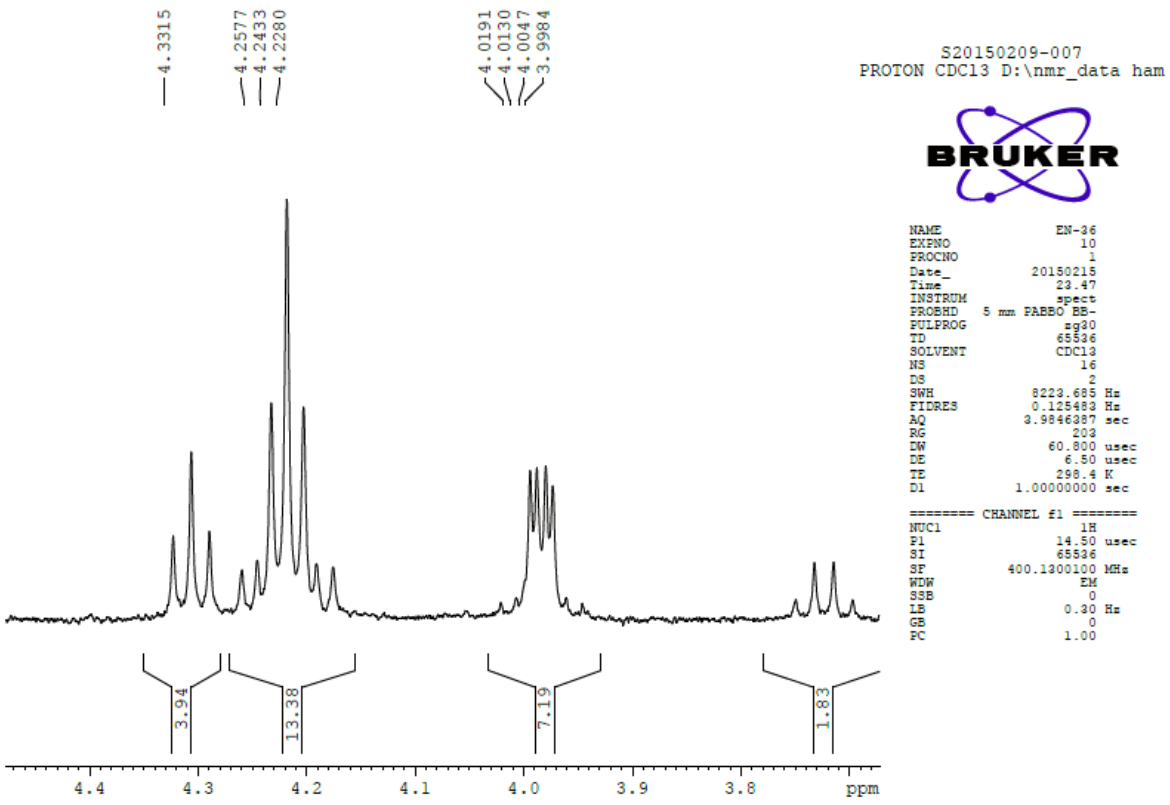
Appendix 14: ^{1}H NMR (400 MHz, $CDCl_3$) spectrum of bis(2-methylheptyl) phthalate **(86)**



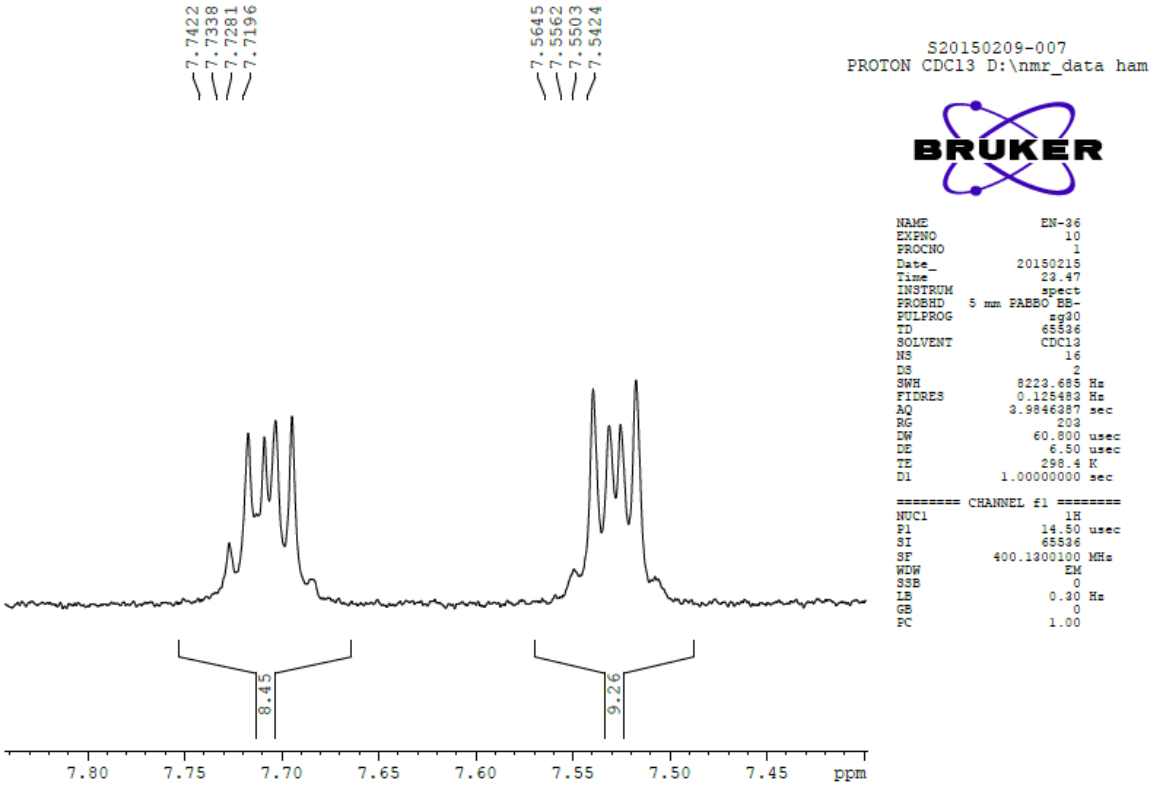
Appendix 15: Section A of ^{1}H NMR (400 MHz, $CDCl_3$) spectrum of bis(2-methylheptyl) phthalate **(86)**



Appendix 16: Section B of ^{1}H NMR (400 MHz, $CDCl_3$) spectrum of bis(2-methylheptyl) phthalate **(86)**



Appendix 17: Section C of ^{1}H NMR (400 MHz, $CDCl_3$) spectrum of bis(2-methylheptyl) phthalate **(86)**



Appendix 18: ^{13}C NMR (100 MHz, $CDCl_3$) spectrum of bis(2-methylheptyl) phthalate **(86)**