Isolation and characterization of antimicrobial components of the root of a Zambian medicinal tree *Terminalia mollis*

By

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(UNZA)

A dissertation submitted in partial fulfilment of the requirements for the degree of Master of Science in Chemistry

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DECLARATION

I, GAVIN MWELWA, hereby declare that this dissertation submitted for the partial fulfilment for the award of the Master of Science Degree in Chemistry at the University of Zambia, is my work and has not been previously submitted for a degree or any examination at any university or college.

Signed………………………….. **Date………………………**

APPROVAL

This dissertation of Gavin Mwelwa has been approved as fulfilling the requirements for the award of a Master of Science degree in Chemistry by the University of Zambia.

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ABSTRACT

T. mollis tree extracts have been used by THPs worldwide, in Africa and specifically in Zambia to treat various diseases in man for example urinary tract infections, sexually transmitted infections, chest infections and gastrointestinal diseases etc. *T. mollis* belongs to the genus *Terminalia,* and the family name is *Combretaceae.*

Most micro-organisms have developed resistance against commonly used antimicrobial drugs in modern clinical practice. This problem has necessitated the need for research into plants that have been used by THPs to treat various diseases affecting man. Plants provide a wide range of phytochemicals that inhibit micro-organism growth. Several phytochemicals have been isolated from plants which include alkaloids, steroids, tannins, saponins and flavonoids to name a few. Among the phytochemicals isolated are beta carboline alkaloids which are present in the genus *Terminalia.* Beta carboline alkaloids have been found to possess antimicrobial activity

The genus *Terminalia*, has not been exhaustively investigated for the presence of antimicrobial compounds and not all species of micro-organisms have been tested against those antimicrobial compounds that have been isolated from this genus. This research intended to investigate the antimicrobial properties of the whole root of the tree species *T. mollis* found in Zambia with a view to isolate and characterize some of its bioactive phytochemicals. The research involved identification, collection and processing of two separate samples of the whole root of *T. mollis* tree, consisting the root wood and root bark from two different regions in Zambia i.e. Lusaka and Mufulira (low and high rainfall regions) and then preparing of crude extracts from the root wood and root bark. The powdered plant material from the root bark, Soxhlet extraction method and methanol as solvent were adopted for the extraction process after conducting a method and solvent optimization experiment because they produced the highest yield of crude *T. mollis* extract. Metabolites (alkaloids) from crude extracts were obtained by acid extraction then separating using chloroform. The separated metabolites were purified using open tubular and stop cock column chromatography, eventually 4 fractions were obtained and labelled A1, A2, B1 and B2. *In vitro* antimicrobial screening, using non-sterile test method for detection of the test micro-organisms, involved testing of crude extracts and the fractions obtained from chromatography on selected micro-organisms namely *Staphylococcus aureus, Streptococcus pyogens, Corynebacterium diphtheriae, Escherichia coli, Salmonella typhi* and *Candida albicans*. The stock solution of the crude extracts and fraction A1 showed inhibition of growth of all the test micro-organisms while the rest of the fractions only showed activity against *Streptococcus pyogens* and *Corynebacterium diphtheria.* MIC was determined using agar streak plate dilution method and the MIC value of the crude extracts and fraction A1 was found to be 117.18 µg/mL against *Streptococcus pyogens* and *Corynebacterium diphtheria.* Spectroscopic analysis involving ^{13}C- and ^{1}H- Nuclear Magnetic Resonance (NMR) indicated that all the fractions from CC had impurities.

It is recommended that further purification and characterization be carried out. It is possible that the compounds contained in the fractions could be new molecules that have never been identified before.

DEDICATION

This is dedicated to my children Gavin Mwelwa Junior, Natasha Mwelwa and to my mother.

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LIST OF ABBREVIATIONS/SYMBOLS

Bi^+	Bismuth ion
BiO^+	Bismuth oxide ion
CHF	Chloroform
°C	Degrees Celsius
CC	Column chromatography
^{13}C-NMR	Carbon-13 Nuclear Magnetic Resonance
DCM	Dichloromethane
DMSO	Dimethylsulfoxide
$FeCl_3$	Ferric chloride
g	Gram
H_2O_2	Hydrogen peroxide
HCl	Hydrochloric acid
OH	Hydroxide ion
HIV/AIDS	Human immune-deficiency virus/acquired immune deficiency syndrome
Fe^{3+}	Iron (III) ion
H_2SO_4	Sulphuric acid
$HgCl_2$	Mercury chloride
^1H-NMR	Proton Nuclear Magnetic Resonance
kg	Kilogram
KI	Potassium iodide
LC-MS	Liquid chromatography - mass spectroscopy
LC	Liquid chromatography
mg/mL	milligram per milliliter
MeOH	Methanol
MIC	Minimum Inhibitory Concentration
µg/mL	micrograms per milliliter
NH_3	Ammonia
R_f	Retention factor
STIs	Sexually transmitted infections
T. mollis	*Terminalia mollis*

THPs	Traditional health practitioners
UTIs	Urinary tract infections
UNZA	University of Zambia
UV	Ultra violet
%	Percentage

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CHAPTER ONE

1.0 Introduction

1.1 Background

1.1.1 History of medicinal plants

Through history, man has relied so much on medicinal plant use for curing and preventing illnesses. It is well known that plants are an essential source of medicines. The use of medicinal plants as medicines in their raw form and also the passing on of this unscientific knowledge from generation to generation is known as traditional medicine. This type of medicine is sometimes referred to as folk medicine because man has gradually developed it.

Globally, Traditional Health Practitioners (THPs) still use herbal medicines for curing various ailments that affect man. The most common and widely used are traditional African, Chinese, Korean, Islamic, acupuncture, ancient Iranian, Unani, Siddha, Ayurveda and herbal medicines[1]. Some aspects of traditional medicine incorporate animal and mineral based medicines as well as spiritual, manual and exercises[2].

Plants are a crucial resource even for the modern conventional medicines which are in wide use in clinical practice today[3] and man has learnt through trial and error that many plants can cure ailments[4,5]. In developing countries, traditional medicine form a major part of many prescriptions and while in developed countries, traditional medicine pharmacies have also come into existence[1,].

In Zambia, 70% of the population accesses traditional medicine therefore a document to regulate and guide research in traditional, complementary and alternative Medicine[5] was developed. In addition there is an act of parliament which provides for guidance for the chemistry and pharmaceutical data for complete scientific evaluation and marketing of quality, safe and efficacious traditional medicine in Zambia[6]. These documents legitimize THPs use of whole or parts of plants for treatment of diseases affecting humans provided that they are safe.

It is estimated that 80% of the world population uses parts of plants for treatment of various diseases[7,8]. Examples of drugs obtained from plants are digoxin from digitalis species[9], quinine and quinidine from

cinchona species[10], vincristine and vinblastine from *Catharanthus roseus*[11], atropine from *Atropa belladona*[12], morphine and codeine from *Papaver somniferum*[13].

In Africa, herbal medicines have been used from time immemorial, most people on this continent prefer using herbal preparations as prescribed by THPs over the conventional medicines. Among the various reasons for this preference is the belief that herbal preparations may have fewer side effects and are relatively cheaper to acquire[14].

In Zambia, a large number of indigenous plants are used by contemporary THPs for treatment of various human ailments and diseases such as diarrhoea, cough, STIs, infected sores etc.

1.2 Bacterial resistance

With the coming of conventional medicine to treat microbial infections, the problem of antimicrobial resistance has also emerged over the years as a major global problem[15]. There are several conventional drugs available for treatment of bacterial infections. For example, ciprofloxacin[16], Roxthromycin[17], and sulfadiazine[18]. However, a number of bacteria have developed resistance to many of the currently used drugs. For example, some strains of *Staphylococcus aureus* are resistant to amoxicillin[19]; *Vibrio cholerae* are resistant to tetracycline[20]; *Mycobacterium tuberculosis* is resistant to isoniazid and streptomycin[21].

1.3 Plant phytochemicals

Plants produce a wide variety of both toxic[22] and non-toxic[23] phytochemicals or secondary metabolites[24] both co-existing in the same plant depending on the habitat in which they grow[25]. These phytochemicals are concentrated in various plant parts such as leaves, stem, roots, hard wood and the bark of the plant in varying quantities[26]. Some of the phytochemicals have been harnessed to cure diseases caused by micro-organisms such as bacteria, viruses, fungi, parasites etc. These phytochemicals may be used by plants for various metabolic functions as well as to protect themselves from environmental threats, e.g., sunlight or herbivores. Plants produce these toxic phytochemicals in order to hinder attack[27].

At sub-toxic levels to man but toxic to micro-organisms[28], phytochemicals can produce useful medicinal properties which man has been harnessing and, at the same time, investigating environmental conditions

that influence significant quantities to be present in a plant. Rainfall pattern, humidity, soil type, average temperatures, altitude, among many other environmental factors, influence quantities of phytochemicals in plants[27]. Phytochemicals in these plants need to be isolated and identified, then their potency determined so that they can eventually be developed into useful medicines[29,30]. The cinchona bark (*Cinchona rubra*), has been used for thousands of years to treat malaria. Quinine is an alkaloid derived from *Cinchona rubra,* is used to treat malaria[31] while the alkaloid, quinidine from the same plant is used to treat cardiac arrhythmias[32]. *Colchicum autumnale* is another plant from which colchicine, an alkaloid, is derived and used to treat gout[33]. Digoxin and digitoxin, both having steroidal ring systems as part of their chemical structures, are drugs used to treat congestive heart failure and are derived from the plant *Digitalis purpurea L*[34]. Hyoscyamine[35] and atropine are both alkaloids and are used as anticholinergic drugs, they are derived from the plants *Datura stramonium L* and *Amanita muscaria* respectively[36]. They are used to treat Parkinson's disease, myopia and bradyarrhythmias[37]. Pilocarpine, an alkaloid, is another drug derived from the plant *Pilocarpus microphyllus* used to treat glaucoma[38]. Curare, an alkaloid derived from a South American plant of the family *Logiaceae*, is a muscle relaxant used during surgical procedures[39].

Among the many plants used for their antimicrobial properties are American and Asian *ginseng*[40], which have many other uses which include anti-stress and anticancer[41] properties. Garlic (*Allium sativum*) contains allicin. It has been used for its antimicrobial properties against *Escherichia coli*, *Candida albicans*, *Entamoeba histolytica* and *Giardia lamblia*[42]. The leaves of olive leaf (*Origanum vulgare*) were found to have antimicrobial effects against a wide range of bacteria and this was attributed to elenoic acid present in the plant[43]. Papaya (*Carica papaya*) from the family *Caricaceae*, both the ripe and unripe fruit, was revealed to have antimicrobial activity against *Trichimonas vaginalis*, *Staphylococcus aureus*, *Escherichia coli* and *Salmonella* species, which was attributed to the sap of the plant which contains numerous phytochemicals[44]. The leaves and flowering tops of tarragon (*Artemisia dracunculus*), from the plant family *Asteraceae* contain *caffeic acid* which has antimicrobial activity against *Shigella* species, *Listeria monocytogens*, *Pseudomonas aeroginosa* and *Escherichia coli*[45]. These parts also contain coumarins all of which have antimicrobial properties[46]. The leaves of tea tree oil (*Melaleuca alterniflora cheel*) from the family *Myrtaceae*, have been used against *Enterococcus faecalis* and yeasts (*Candida albicans*)[47]. The flowing tops of thyme (*Thymus vulgaris L*) from the family *Labiatae*, like those of *tarragon* have been found to have antimicrobial activity against

Escherichia coli bacteria[48]. Flowering tops from these two plants have shown antimicrobial activity either taken orally or applied topically. They also contain thymol which has antimicrobial activity and is used on bandages and as the main excipient in mouth washes such as listerine[49].

With this background on phytochemicals and their sources from different plant parts, this research project intended to investigate the antimicrobial properties of the whole root of the tree species *T. mollis* found in Zambia with a view to isolate and characterize some of its bioactive phytochemicals.

1.4 Statement of the problem

Studies conducted elsewhere (Asia and in Africa namely South Africa, Tanzania and Nigeria in particular) indicate that *T. mollis* roots have inhibitory effects against micro-organisms.

The genus *Terminalia*, has not been exhaustively investigated for the presence of antimicrobial compounds and not all species of micro-organisms have been tested against those antimicrobial compounds that have been isolated from this genus.

This research focuses on isolation of antimicrobial compounds present in the genus *Terminalia.* The specific tree species to be investigated is *T. mollis* and this will involve testing the isolated antimicrobial compounds on selected micro-organisms.

In Zambia, there is no record of scientific data done on *T. mollis* tree with the only information available being the one relating to its use in traditional medicine by THPs. This is done on a purely empirical basis. Information on traditional use of the plant was obtained by verbatim from the THPs and that, it is used to treat infectious diarrhoeal diseases such as traveler's diarrhoea , UTIs like urethritis, STIs like gonorrhoea and it is also used to treat chest infections like common cough.

T. mollis is a plant commonly and easily found among THPs especially when there is a disease which involves the urinary tract, *T. mollis* is among the medicinal plants of first choice that THPs recommend.

There are other trees that are also well known among THPs but they recommend *T. mollis* because of its wide distribution and relative ease of finding it in the wild. The other reason THPs claim for its

preference is that it covers a broad range of infectious diseases compared to other medicinal plants used to treat the same diseases.

1.5 Aim

To investigate the antimicrobial properties of the whole root of the Zambian *T. mollis* tree.

1.6 Objectives

The specific objectives of the study were to:

1. Evaluate antimicrobial activity *in vitro* and determine MIC of the crude extracts and antimicrobial compounds isolated from the whole root of the Zambian *T. mollis* tree, against *Candida albicans, Salmonella typhi, Staphylococcus aureus, Escherichia coli, Streptococcus pyogens* and *Corynebacterium diphtheriae*.
2. Fractionate the antimicrobial components from bioactive extracts using chromatographic techniques.
3. Identify and characterize the isolated antimicrobial components using spectroscopic techniques.

1.7 Research questions

1. Is it possible to evaluate antimicrobial activity *in vitro* and determine MIC of the whole root of the Zambian *T. mollis* tree against the micro-organisms?
2. Is it possible to fractionate the antimicrobial components from bioactive extracts?
3. Is it possible to identify and characterize the isolated antimicrobial components?

1.8 Significance of the study

This study will contribute to the existing knowledge of medicinal properties of the indigenous Zambian medicinal tree *T. mollis* locally known as *Mulama*. It may possibly provide lead compounds for further research to address the need for new drugs for resistant micro-organism strains.

CHAPTER TWO

2.0 Literature review

2.1 Plants belonging to the family, *Combretaceae*

Combretaceae family is a large family consisting herbs, shrubs and trees. It consists of 20 genera and 600 species of plants which are widely distributed in tropical and subtropical areas such as Africa, South America and Asia. The largest genera are *Combretum* consisting of 370 species and *Terminalia* consisting of 200 species[50]. Several genera belonging to the *Combretaceae* family have been used by THPs in the treatment of many diseases such as rheumatoid arthritis, diabetes, diarrhoea, malaria, ring worms, gonorrhoea etc[51]. *Terminalia* genus are widely used in many traditional medicines, among them, Asian and African[52].

2.2 Isolation of phytochemicals present in the plant family *Combretaceae*

Screening of plants belonging to the *Combretaceae* family has shown that some of them contain alkaloids, phenols, flavonoids, saponins, steroids and tannins among other plant constituents and with different biological properties[53]. It is because of these phytochemicals and their biological properties that man has been harnessing the plant kingdom in order to find a lasting solution to the many ailments that affect him[54]. In order to maximize herbal medicines that are useful for therapeutic purposes, there is need to isolate and identify their bioactive phytochemicals and standardize them because this is an essential requirement for quality control, dose determination and scientific validation[55].

2.3 Medicinal potential and biological activities of the genus *Terminalia*

Although there are many plant species belonging to the *Combretaceae* family that are used by THPs for treatment of various diseases, the plant genus *Terminalia* is amongst the most widely used for traditional medicinal purposes worldwide because it possess biological activity[56]. From the genus *Terminalia* are different species of *Terminalia* which have been studied for their biological activities and these include *T. arjuna* which was found to have benefits in the treatment of some cardiovascular diseases[57]. The Indian species of *T. chebula* has a broad range of medicinal uses which include its use in wound healing and treatment of cardiovascular complications like ischemic heart disease[58]. The *T. chebula* plant extracts have been used to cure several ailments such as fever, cough, diarrhoea, gastroenteritis, skin diseases, candidiasis, UTIs and wound infections[56]. *T. sericea*, *T. phanerophlebia*, *T. gazensis*, *T.*

brachystemma, and *T. sambesiaca* were found to possess antiprotozoal, antiviral, anti-diarrhoea, analgesic, antimalarial, anti-oxidant, anti-inflammatory and anticancer activities[48,55].

2.4 Antimicrobial activities of *T. mollis* and the genus *Terminalia*

T. mollis tree extracts have been investigated for antimicrobial properties against only a few strains of bacteria and fungus[56]. This shows the need to investigate its antimicrobial properties and the phytochemicals of *T. mollis* responsible for the antimicrobial activity. This will broaden the scope of knowledge about the micro-organisms that are susceptible to *T. mollis* antimicrobial phytochemicals[56].

Studies on the extracts of the plant root, stem and leaves of the *T. mollis* species in Tanzania showed antimicrobial activity against bacterial species including *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*[57], *Klebsiella pneumoniae*, *Salmonella typhi*[58], *Bacillus anthracis* and fungal species including *Candida albicans*, *Aspergillus flavus* and *Cryptococcus neoformans*[61]. *T. mollis* plant extracts were also found to have activity against the micro-organism *Proteus mirabilis*[62].

There are many scientific reports on other plants belonging to the genus *Terminalia*, such as studies on *T. chebula ritz*, *T. brachystemma*, *T. prunioides* and *T. brownie*. These reports show that these species possess antimicrobial activity[63–66].
However, many plants belonging to this genus haven't been exhaustively subjected to studies that rigorously examine this important genus for their medicinal potential[67].

2.5 *T. mollis* species: Morphology, taxonomy and geographical distribution

In the *Combretaceae* family is *T. mollis* species and *mollis* is the specific name of the plant. Taxonomical classification describes *T. mollis* as belonging to the phyla, *Myrtiflorae* of the order *Myrtales*.

T. mollis is a medium to large semi-deciduous tree with deeply fissured dark grey bark. It can grow up to 15 m high and is rarely with a straight trunk, with the crown tapering to oval and has a layered appearance. It has broad ovate leaves, which are arranged alternate to the midrib of the leaf and are clustered at the end of the branches. The leaves are elliptic, broad and large measuring up to 37 cm long and 19 cm wide, slightly hairy above and densely hairy beneath. It forms a dense foliage and an intense

shade. The flowers are whitish-cream, being 8-17 cm long spikes from the leaf axils and the fruit is winged and yellowish green and of up to 12 cm long and 5.5 cm wide. The seed is large with 2 wings which is typical of many *Terminalia* species[53,68]. The *Terminalia* species are widely distributed in Asia specifically in southern Asia, Himalayas, Australia and Madagascar. In Africa, *T. mollis* is found in West Africa from Ghana through Cameroon to south Sudan through to Angola and Zambia. The tree is resistant to drought, fire and termites once established. In Zambia, it is widely distributed in all provinces and, in the Southern and Lusaka provinces, the locals call it *Mulama* and on the Copperbelt, Northern and Muchinga provinces, it is called *Umulama*.

2.6 Phytochemical constituents and medicinal potential of *T. mollis* species

Several studies have been done to try to isolate phytochemicals and this has revealed various constituents some of them having antimicrobial activity. Among the phytochemicals isolated are tannins, terpenoids, alkaloids and flavonoids just to name a few[69,70]. Accumulation of these phytochemicals in different parts of plants such as roots, leaves, stem, flowers, fruits and seeds is different and also dependent on environmental factors such as weather, soil type, climatic conditions etc[65,68,69].

For instance, *T. mollis* possesses norharmine and this justifies why it has been used to treat depression as this compound stimulates the brain[71]. South American shamans also use plants containing tetrahydroharmine like *T. mollis* for its hallucinogenic properties as it stimulates the brain[72]. Harmaline and Harmine have been used for their biological activity such as acetyl cholinesterase inhibitory activity, antioxidant and anti-inflammatory activities[73]. Harmol was also found to have anticancer properties and it has been used in traditional medicine for cancer treatment[74]. Harmalol has been used in traditional medicine because of its antitumor activity[75]. The components in *T. mollis* possessing antimalarial activity were isolated and determined to be ellagic acid and nitidine[76] (Figure 2.1).

Three polyphenolic compounds have been isolated from *T. mollis*, e.g., chebulagic acid, chebulanin, and chebulinic acid. Chebulanin functions as an anti-inflammatory agent[65].

2.7 General biological activities of *T. mollis* species

T. mollis has been used for medicinal properties which include antitrypanosomal, antioxidant and anti-inflammatory [77].

The leaves and flowers of *T. mollis* have been used to treat malaria and were evaluated *in vitro* and determined to possess antiplasmodial activity and hence its justification in its use in treatment of malaria although it was also revealed to possess cytotoxic activity [78]. *T. mollis* has also been used as an antioxidant to slow down the aging process like memory loss and also to improve cognitive behavior[56]. In another report, *T. mollis* stem and root extracts have been shown to possess anti-HIV activity against HIV type 1(HIV-1, III B strain)[67]. It has also been used for its analgesic and anticancer properties[52,79]. It has been revealed that *T. mollis* is used in Southern Africa for treatment of bilharzia[67], intestinal worms, tuberculosis, cholera and dysentery[65,77].

2.8 Isolated phytochemicals from *T. mollis* possessing antimicrobial activities

Alkaloids contained in the genus *Terminalia* are known as beta carboline alkaloids[80]. Some of these are harmane[81] (Figure 2.1), norharmane, tetrahydroharmine, harmine, harmaline, harmol, harmaalol and harmalan[82]. In one research, harmine was found to have the most effective antimicrobial activity against *Proteus vulgaris*, *Bacillus subtilis* and *Candida albicans*[83]. In general, the beta carbolines all possess antimicrobial activity[84]. Norharmine showed

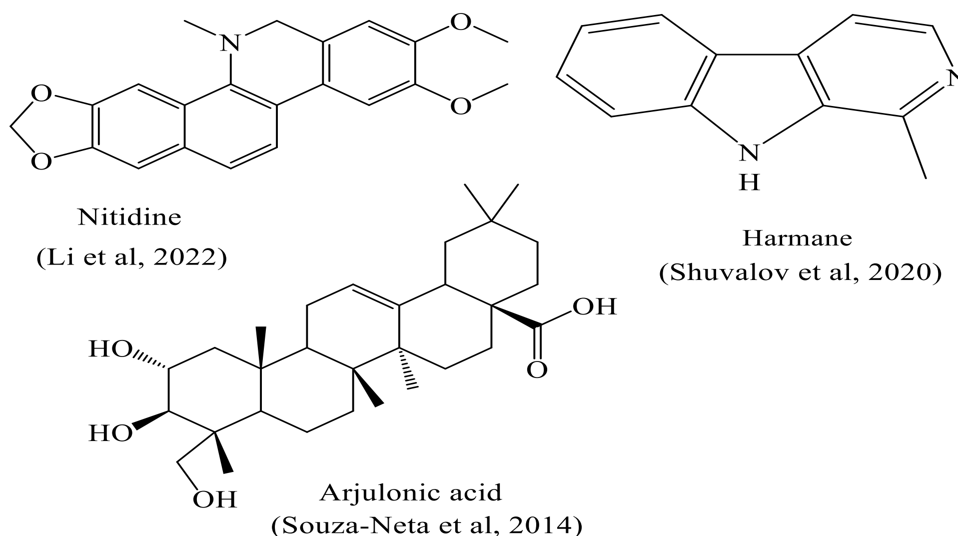


Figure 2.1: Chemical structures of some phytochemicals present in *T. mollis*.

antimicrobial activity against some species of *Pseudomonas aeruginosa*, *Klebsiella oxytoca* and *Escherichia coli*[85]. Tetrahydroharmine has also been used for its antimicrobial properties against viruses[12]. In one research, harmaline and harmine were found to possess antimicrobial activity against *Escherichia coli*, *Xanthomonas oryzae* and *Xanthomonas axonopodis*[86]. It has also been shown that harmol has antimicrobial activity against *Penicillium digitatum*[87]. Harmaalol was also shown to have antimicrobial activity against *Penicillium digitatum* and *Botrytis cinerea*[88].

Punicalagin was also found to have activity against *Candida* strains, *Cryptococcus gatty*[89] and *Candida parapsilosis* it also inhibits growth of *Colletotrichum goeosporioides*[90,91] and *Staphylococcus aureus*[91]. Terchebulin and terflavin showed growth inhibitory effects against *Neisseria gonorrhoea*[92]. Friedelin has antimicrobial activity against *Candida albicans*[93] while epicatichin has antimicrobial activity against *Candida krusei*, *Candida glabrata* and *Candida tropicalis*[94].

Ursolic acid, terminolic acid, arjungenin and quadranoside II exhibited good activity against *Staphylococcus aureus*, *Escherichia coli* and *Enterococcus faecalis*[65,95]. Oleanolic acid showed good growth inhibition against *Bacillus subtilis*, *Staphylococcus aureus*, *Listeria monocytogenes* and *Candida* species[96,97]. Catechins have been found to possess interesting antimicrobial effects[98]. For example, the bactericidal compound, epigallocatechin-gallate was shown to cause damage to bacterial membranes[99]. Elagitannins were also found to possess antimicrobial activity against *Candida albicans* and *Cryptococcus neoformans*[100]. Arjunolic acid[101] (Figure 2.1), is a naturally occurring chiral pentacyclic triterpenoid saponin and a constituent of the bark of *T. mollis* and has been shown to possess antimicrobial, anti-inflammatory and hepato-protective activity[102].

CHAPTER THREE

3.0 Materials and methods

3.1 Procedure for collection and identification of plant materials

Samples of *T. mollis* plant consisting the root wood and root bark of weight 10.2 kg were collected from Lusaka (Figure 3.1) within UNZA and 4.6 kg from Mufulira in Mipambe forest area. These were collected between the months of December 2013 and January 2014. The samples were collected from a region of high rainfall pattern (Mufulira) and low rainfall pattern (Lusaka) because this has an influence on the concentration of phytochemicals present in the various parts of the plant[27,65,68,69].

3.2 Procedure for Identification of plant materials

Branches with leaves and pods (with seeds inside) were collected from a single tree in Lusaka (Figure 3.1) and another similar sample was collected from another tree in Mufulira. Both samples of *T. mollis* collected were identified at the Department of Biological Sciences, UNZA, by a Taxonomist. The voucher specimens were kept in the Department of Biological Sciences at UNZA, in the Herbarium and were given specimen numbers GVM-001 and GVM-002 for future reference.

Figure 3.1: *T. mollis* tree at UNZA

3.3 Procedure for processing of plant materials

The whole root was cleaned with a brush and the bark was separated. Then the root wood was chopped into small pieces of length 5 cm using a machete and an axe. The small wood pieces and the root bark were dried in a shade at room temperature on a laboratory bench for 10 days in the Department of Chemistry laboratory at UNZA.

The dried whole root and bark pieces were ground to a fine powder using a heavy duty grinder, Brook Disintegrater-AG-EM08-308, and sieved using a sieve of size 8 (pore size 0.5 mm) in the Department of Animal Science and Nutrition, School of Agricultural Sciences at UNZA. The dried powdered *T. mollis* material was weighed and labelled appropriately as 4.1 kg root wood, 3.4 kg root bark from Lusaka and 3.0 kg root wood and 0.7 kg root bark from Mufulira. These samples were stored in airtight amber colored glass containers and kept in a dark room at room temperature in the research laboratory housed in the Department of Chemistry, School of Natural Sciences at UNZA.

3.4 Procedures for preparation of crude *T. mollis* root extracts[103,104]

The preparation of extracts was done employing protocols from literature.

In this research, two methods of extraction and a variety of polar and non-polar solvents were considered for the preparation of crude *T. mollis* extracts. Initially, cold percolation and Soxhlet extraction were used as experiments for preparation of extracts. The solvents selected were methanol, ethanol, diethyl ether, acetone, dichloromethane and distilled cold water.

3.4.1 Procedure for method and solvent optimization[103,104]

The method and solvent optimization procedure was done by adopting extraction protocols reported in literature.

The main aim of this analytical study was focused on the amounts of extracted compounds by maintaining the same extraction conditions while using different extraction solvents for each of the extraction methods (i.e. Soxhlet and Cold percolation).

Cold percolation extraction method were set at one extraction time i.e. 24 hours and Soxhlet extraction method at 8 hours, and in each method using the solvents methanol, ethanol, diethyl ether, dichloromethane and acetone in separate experiments.

A general full factorial design with two factors (amount extracted and type of extractor solvent) was implemented.

The Soxhlet extraction method presented a good yield of components in extract. This design showed that methanol produced the highest yield in grams from the root bark of the Lusaka and Mufulira plants (13.97 g and 3.07 g respectively) as compared to the other solvents.

The method and solvent selected in this research was the one which produced the highest yield of extract. In this experiment, Soxhlet extraction and methanol were eventually adopted for the experiment because they gave the highest yield of crude extract.

3.4.2 Cold percolation extraction method[103,104]

Aliquots of 15.0 g of dried powdered *T. mollis* plant material were extracted using 100 mL of different solvents (e.g., methanol, ethanol, diethyl ether, acetone, dichloromethane and distilled cold water).

In a typical experiment, 15.0 g of dried powdered plant material was soaked in 100 mL of solvent (methanol, ethanol, DCM diethyl ether, acetone and distilled water) in a stoppered Erlenmeyer flask. The flasks were shaken on a mechanical shaker, (labotec model 20.2) for 24 hours at room temperature. The mixture was then filtered by suction filtration using Whatman filter paper No 1. The clear filtrate was evaporated to dryness on a rotavapor (Figure 3.2) (Buchi Rotavapor R II, and Vacuum Controller V-850) at 60°C (methanol, ethanol and distilled cold water), 40°C (DCM) or 20°C (diethyl ether and acetone). The rotavapor-dried residues were then left in a vacuum desiccator over anhydrous calcium chloride overnight. They were later weighed, appropriately labelled and stored in airtight amber colored bottles at temperatures between 2 and 8°C.

3.4.3 Soxhlet extraction method[102]

Aliquots of 20 g of dried powdered plant material were extracted by use of the solvents methanol, ethanol, diethyl ether, acetone, DCM.

In a typical experiment, 20.0 g of dried powdered plant material was introduced into a thimble, which was placed into a Soxhlet apparatus in readiness for extraction, and then 200 mL of solvent were introduced into the apparatus. The plant material was subjected to 8 hours Soxhlet extraction on heating mantles set at temperatures ranging from methanol at 70℃, ethanol at 80℃, DCM at 50℃ diethyl ether at 35℃ and acetone at 60℃. The resulting solutions were then filtered while hot by suction filtration using Whatman filter paper No 1. The clear filtrates were then evaporated to dryness on a rotavapor (Buchi Rotavapor R II, and Vacuum Controller V-850) (Figure 3.2) at appropriate water bath temperatures ranging from methanol and ethanol at 60℃, dichloromethane (DCM) at 60℃ and diethyl ether and acetone at 60℃.

The rotavapor-dried residues were then left in a vacuum desiccator over anhydrous calcium chloride overnight and were later weighed, appropriately labelled and stored in airtight amber colored bottles at temperatures between 2 and 8℃.

Figure 3.2: Crude extract in the drying process in a rotavapor.

3.5 Procedures for phytochemical analysis tests[105-105]

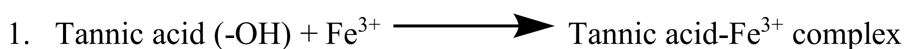
The procedure for phytochemical analysis was done by employing protocols from literature.

The dried powdered plant material was subjected to phytochemical tests to ascertain the presence of alkaloids, flavonoids, glycosides, saponins, tannins and steroids.

3.5.1 Test for tannins (ferric chloride test)

0.1 g of powdered plant material was boiled in 2 mL of distilled water in a test tube on a heating mantle for 5 minutes. The solution was filtered while hot after which 2 drops of 0.1 % $FeCl_3$ were added and the reaction was observed.

This reaction involved polyphenolic compounds or tannins. Polyphenolic compounds contain the OH (hydroxyl) groups which react with $FeCl_3$ to form tannate-Fe^{3+} complex (Figure 3.3), thus from light brown-green color to intense brown-black color.



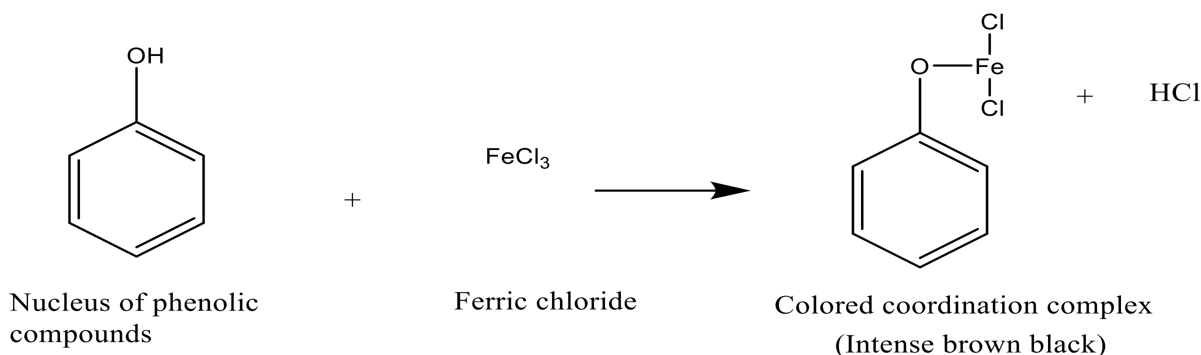


Figure 3.3: Principle mechanism of $FeCl_3$ test for phenolic (tannins) compounds.

3.5.2 Test for Saponins (foam test)

0.3 g of powdered plant material was suspended in 3 mL of distilled water in a test tube and warmed after which the mixture was shaken vigorously and the reaction was observed.

Saponins contain hydroxyl as polar groups and also steroids/triterpenoids as non-polar groups (Figure 3.4). A compound containing both polar and non-polar groups is surface active therefore when shaken vigorously with water, saponin compounds form miscellanea, where polar groups face outwardly and non-polar groups face inwardly. This is what is called foam formation.

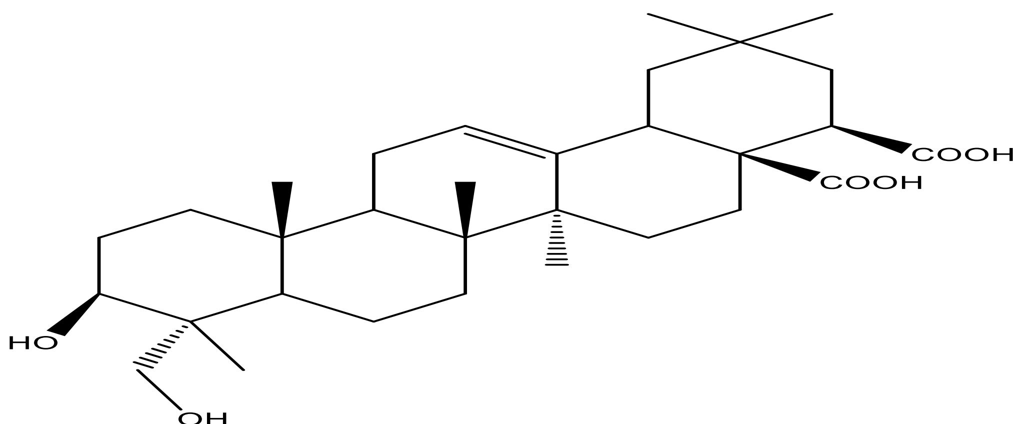


Figure 3.4: Hederagenin a saponin with hydroxyl polar group and Steroid non-polar group.

3.5.3 Test for triterpenoids/steroids (Salkowski's test)

0.1 g of the powdered plant material was suspended in 2 mL anhydrous chloroform and filtered. The filtrate was mixed with concentrated Sulphuric acid carefully so that the acid formed a lower layer and the interface was observed.

3.5.4 Test for steroids (Liebermann-Bourchard reaction)

0.1 g of powdered plant material was dissolved in a 3 mL mixture of hydro-alcoholic solvent (mixture of 1 mL methanol and 2 mL water) then the extract was evaporated to dryness in a porcelain capsule. The residue was then extracted with 0.5 mL chloroform, then this was evaporated to dryness on the rotavapor. To this, was added 4 drops of acetic anhydride then followed by addition of 2 mL concentrated H_2SO_4 from the side wall of the test tube and the reaction was observed.

The steroid/terpene screening test is based on the compounds in the powdered *T. mollis* plant material to form concentrated Sulphuric acid colors in solvents of acetic anhydride by sulfonation and removal of sulfuric acid from the cholesterol/steroid molecule leading to a purple color for terpenes and blue color for steroids (Figure 3.5) present in the plant extract. Cholesterol and its sulfate derivatives undergo sulfonation at various positions accompanied by skeletal rearrangements which is followed by elimination of SO_3H group as H_2SO_4 leading to generation of a new bond. When this desaturation is repeated it leads to polyenes and ultimately formation of aromatic steroids. Linearly conjugated polyene cations form too slowly and appear blue and account for the Liebermann-Bourchard reaction. For the

Salkowsky (Sulfuric acid) reaction, the polyene cation generates considerable cholesta-3.5-diene which gives a purple coloration.

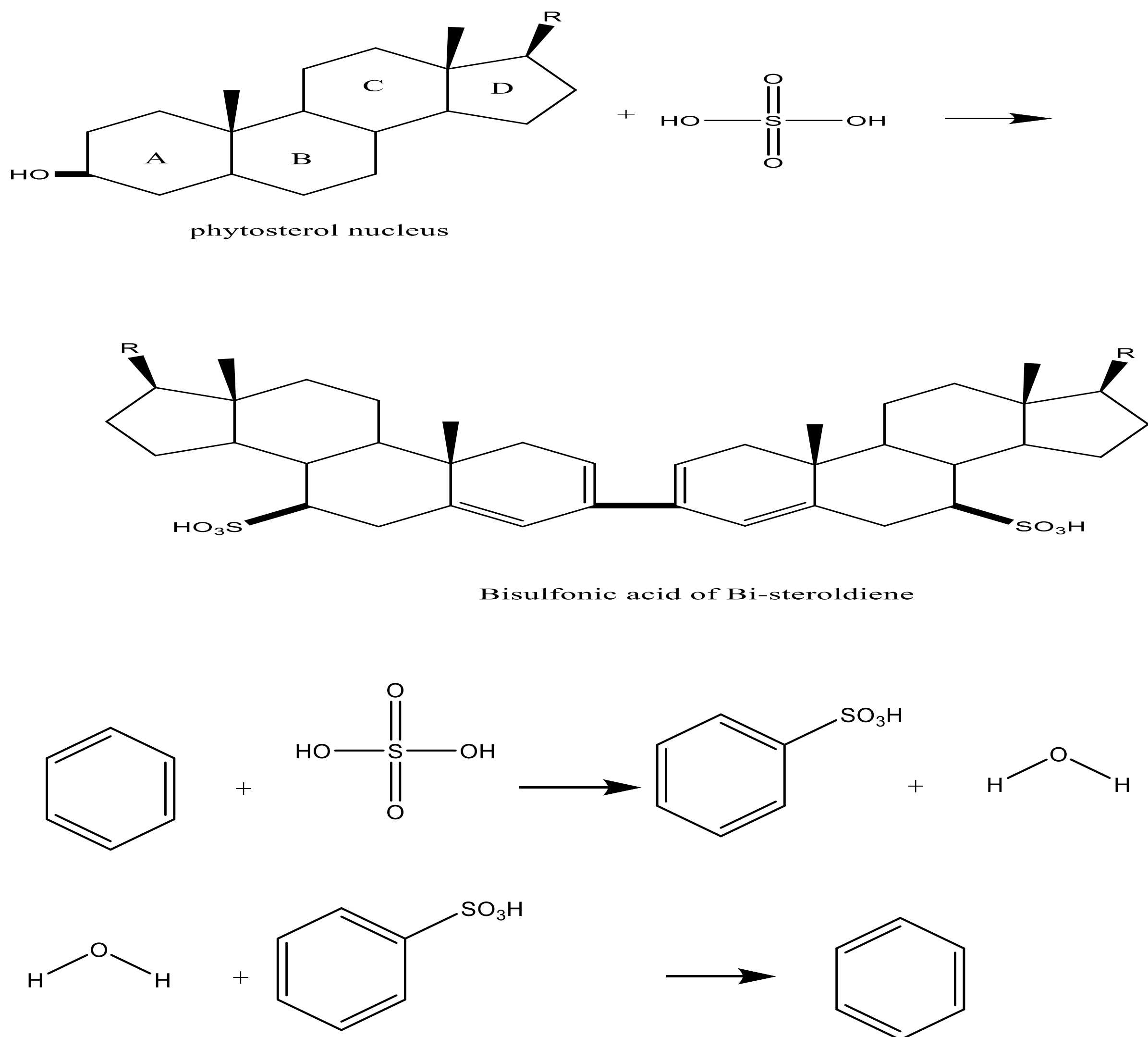


Figure 3.5: Principle mechanism for the Salkowski's and Liebermann-Bourchard test for steroids/triterpenes.

3.5.5 Test for flavonoids (Shinoda reagent test)

0.1 g of powdered plant material was suspended in 2 mL methanol, then, to this, was added 2 fragments of magnesium turnings. After 5 minutes, 2 mL concentrated HCl was added and the reaction was observed.

Flavonoids are polar because of the sugar (glycon) attached to the molecule, in the flavonoids test methanol is added in order to make the flavonoids more polar. When magnesium turnings and

hydrochloric acid are added this causes reduction of the flavonoids causing the color change to pink (Figure 3.6).

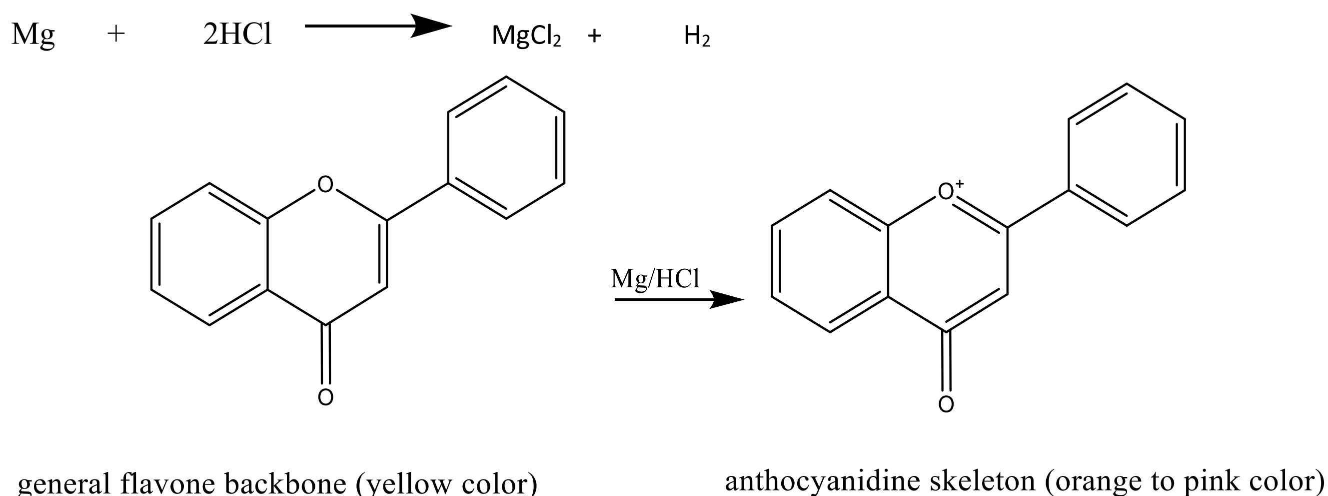


Figure 3.6: Principle mechanism for Shinoda reagent test for flavonoids.

3.5.6 Test for alkaloids (Mayer's reagent test and Dragendorff's test)

0.3 g of powdered plant material was suspended in 3 mL distilled water, then, to this, was added 2 mL of 1% HCl on a steam bath. Mayer's reagent was added to this solution and the reaction was observed. In another test tube, 0.3 g of powdered plant material was suspended in 3 mL distilled water, then, to this, was added 2 mL of 1% HCl on a steam bath. Dragendorff's reagent was added and the reaction was also observed.

Mayer's reagent (reconstitution)

$HgCl_2$ — 1.36 g in 60 mL distilled water

KI — 5 g in 10 mL distilled water, H_2O_2 — 30 mL

Then 3 drops were added to the suspension of plant material and the reaction was observed.

Dragendorff's reagent (reconstitution)

The Dragendorff's reagent has two solutions.

$Bi(NO_3)_3.5H_2O$ — 8 g in 20 mL liquid NH_3

KI — 27.2 g dissolved in 50 mL distilled water

To the suspension of powdered plant material, 3 drops of Dragendorff's reagent were added and the reaction was observed.

In the reaction of Mayer's reagent when added to the neutral or slightly acidic alkaloid solution, the nitrogen atom of the alkaloid donates a lone pair of electrons which is shared with the potassium ion, also known as a coordinate covalent bond, to form dipotassium tetraiodomercurate complex which is whitish-cream colored and appears as a whitish-cream colored precipitate (Figure 3.7).

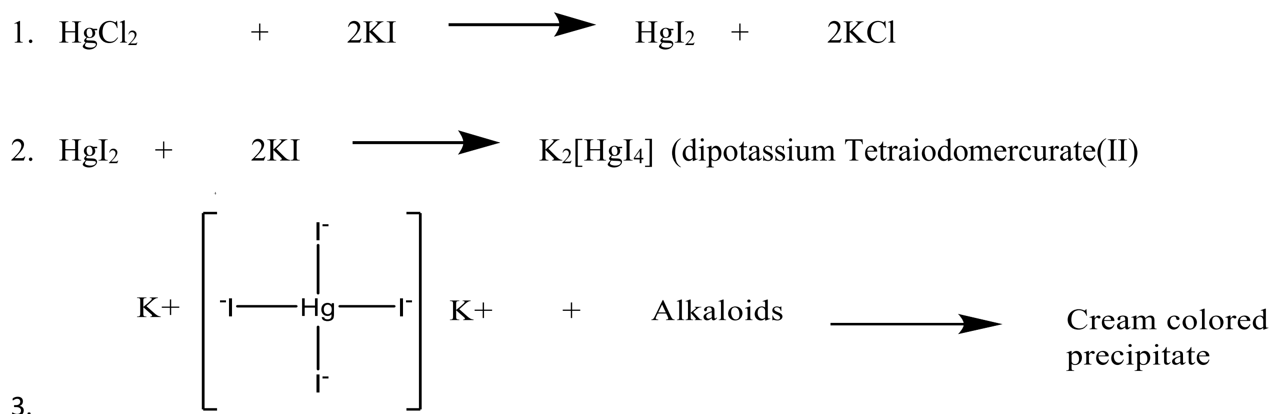


Figure 3.7: Principle mechanism for test for alkaloids (Mayer's reagent test).

In the reaction of Dragendorff's reagent when added to the slightly acidic alkaloid solution, the nitrogen atom of the alkaloids, formed a covalent coordination bond with K^+ ion metal. This reaction was observed as a reddish-orange precipitate. This precipitate was as a result of the complex formed from potassium-alkaloid. In the preparation of Dragendorff's reagent, bismuth nitrate was dissolved in hydrochloric acid which protected hydrolysis reaction because salts of bismuth are easily hydrolyzed by producing BiO^+ ion. Therefore Bi^{3+} ion reacts with potassium iodide producing the reddish-orange precipitate of Bismuth (III) iodide (Figure 3.8). This precipitate dissolved in excess potassium iodide producing potassium tetraiodobismuthate.

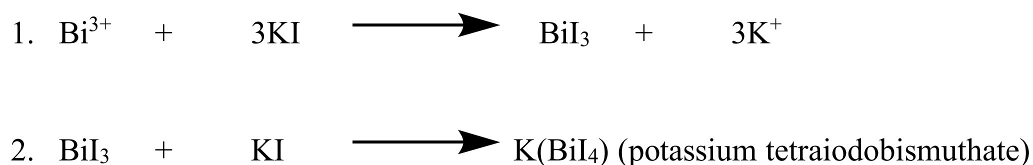


Figure 3.8: Principle mechanism for test for alkaloids (Dragendorff's test).

3.5.7 Tests for glycosides (Keller-Killiani test)

0.1 g of the powdered plant material was dissolved in 2 mL distilled water in a test tube, then shaken and filtered. The filtrate was then extracted with 3 mL of chloroform and evaporated to dryness on the rotavapor. The dried powder was dissolved in 3 mL glacial acetic acid and then 2 drops of $FeCl_3$ were added. Then, to this, were added 2 mL of concentrated H_2SO_4. The color change was observed (Figure 3.9).

Glycosides + Glacial acetic acid + $FeCl_3$ + H_2SO_4 $\rightarrow \rightarrow \rightarrow$ Brown ring at solution interface free from red.

Figure 3.9: Principle mechanism for test for glycosides (Keller-Killiani Test).

The glycosides are composed structurally of a sugar (glycon) and non-sugar (aglycon) moiety and the test is based on specific action of the acid hydrolysis of deoxy-sugars like digitoxin (glycoside) that is transformed initially by alkaline hydrolysis from acetyldigitoxin then transformed to digitoxigenin (aglycon) and 3-digitoxose (sugar residue) and eventually cymorose hence the color change.
The fractions obtained from CC were also subjected to phytochemical tests using the methods described under section 3.5.

3.6 Thin layer chromatography (TLC)[108]

The procedure for analytical TLC was done employing protocols from literature.
The optimum solvent system to be employed for gravity column chromatography was determined by analytical TLC experiments using silica gel pre-coated TLC chromatoplates. A number of solvent systems were attempted, including ethyl acetate: methanol (MeOH), and chloroform (CHF): methanol. The 4.5: 5.5 (chloroform: methanol) was found to be optimum as it gave better separation on the TLC plate. The fractions of *T. mollis* extracts obtained from column chromatography were also subjected to TLC in order to ascertain purity of the fractions.

3.7 Isolation of alkaloids from crude *T. mollis* extract[109]

3.7.1 Procedure for extraction of alkaloids[109]

The procedure for extraction of alkaloids was done employing protocols from literature.

A 5 g powdered crude extract was dissolved in 5 mL methanol and then, to this solution, was added slowly 2 mL of 3% w/v tartaric acid with continuous stirring upon addition. The solution was then filtered using silica gel (Buchner funnel and a filter paper on which was placed 10 mm silica gel). The filtrate was collected in a separate collection vessel (Erlenmeyer flask) and tested for alkaloids and the test was positive. The residue was washed 3 times using 2 mL of 3% w/v tartaric acid into a separate collection vessel (Erlenmeyer flask). The collected solution from the residue was tested for presence of alkaloids and the results were negative. And it was then evaporated to dryness on the rotavapor, labelled and stored at room temperature in the laboratory and no further experiments were done on this sample.

The filtrate which had been collected in an Erlenmeyer flask and had tested positive for alkaloids was basified with concentrated ammonia (25%. density 0.89) to pH 9-10 (using a pH meter) over ice cubes. Upon addition of the concentrated ammonia there was formation of a precipitate which was subsequently extracted in a separating funnel with 30 mL chloroform.

The precipitate which had been extracted with 30 mL chloroform was then dried using anhydrous sodium sulphate. The sodium sulphate was removed by decanting then testing whether the resultant solution had traces of sodium sulphate by using Barium chloride of which there were no traces. The resultant solution was then subjected to further drying to dry powder in the rotavapor. Afterwards the dried powder was tested for presence of alkaloids and the test was positive then it was labelled and placed in a desiccator (24 hours) over anhydrous calcium chloride for maximum drying.

3.8 Isolation of alkaloids by gravity CC[110–116]

3.8.1 Procedure for preparation of sample before fractionation[117]

The procedure for preparation of sample was done by adopting protocols reported in literature.

Ether was used for defatting of crude plant extracts prior to CC.

Gravity CC using 2 procedures i.e. Open tubular and Stop cock chromatography were used as the procedures to fractionate the compounds using silica gel as the stationary phase while the mobile phase used was 4.5 : 5.5 (chloroform : methanol).

3.8.2 Procedure for packing of the column for column CC[118]

The procedure for packing of column for CC was done by employing protocols from literature.

Wet packing technique was used to pack the column. There were 3 phases undertaken in order to carry out this column chromatography namely column packing with stationary phase, column loading with the sample and finally elution of the column using the mobile phase.

3.8.3 Procedure for preparation of the column[118]

The procedure for preparation of the column was done by employing protocols from literature.

Two column chromatography procedures were used. The first procedure involved elution of the crude samples in a 25 mL open tubular column and the second procedure involved elution of the semi-pure samples obtained through a 50 mL stop cock column. The samples collected were then subjected to TLC and then the plates were sprayed with Dragendorff's reagent for location of alkaloids.

3.8.4 Procedure for open tubular column chromatography[119]

The procedure for running of open tubular column chromatography was done by employing protocols from literature.

In the first procedure, a 25 mL open tubular column was selected and a pinch-size piece of glass wool was rolled up into a small ball like shape but not tightly compressed and it was introduced into the column just about above the curvature region at the bottom of the column. Then, just above the wool the column was filled with sand up to the level above the curvature region of the taper. The sand was levelled up on top to ensure a uniform length and width of the stationary phase in the column. After packing the sand, a small volume (3 mL) of chloroform was introduced into the column just above the sand and the column was tapped gently so as to remove any air bubbles. After this, the slurry for stationary phase was introduced into the column which was added briskly so as to avoid formation of air bubbles and also by tapping gently on the column to remove the bubbles seen. The powdered sample was dissolved in 1 mL mobile phase and loaded as a narrow band on top of the stationary phase (ratio of silica gel : sample, 30 : 1 using a 25 mL column) and 1 mL eluents were collected in ten different test tubes. These fractions were later subjected to TLC in order to ascertain their purity. In this case, all the ten fractions collected were presumed to be semi-pure and were subjected to further purification using a 50 mL stop cock chromatography column.

3.8.5 Procedure for TLC[120]

The TLC procedure was done by adopting protocols reported in literature.

Before subjecting the ten samples to further purification using a 50 mL stop-cock column, the following procedure was done:

The R_f values of the ten fractions were compared using TLC in the twin-trough chamber and appropriate fractions with similar R_f values were combined. This was after the fractions were located using short wave UV light of 254 nm of a Camag UV box.

The procedure conducted was that the fractions from CC were applied as small spots on the TLC plate using 2 μL small capillary tubes. 2 aluminium-backed silica gel TLC plates (10 cm x 10 cm) of 0.25 mm thickness were used and on each plate were 5 spots (each plate with fraction spots was labelled as 1-5 and 6-10 with the distance between spots being 1.5 cm.

The TLC plates were then placed in the saturated twin-trough chromatographic chamber with the appropriate solvent systems: chloroform: methanol: ammonia (60: 60: 1). The developed plates were examined under ultra-violet light (254 nm) using a Camag UV box. After comparing R_f values fractions 3 - 5 were combined as were 8 - 10 while fractions 1, 2, 6 and 7 didn't show any spots on TLC and no further purification was done on them. Fractions labelled 3-5 were combined because after comparing their R_f values, they were ascertained to be similar and hence it was concluded that they were the same metabolite and this same conclusion was also made for the fractions labelled 8-10.

Eventually, two combined sets of fractions were obtained and subjected to CC using 50 mL stop cock columns.

Having obtained two combined sets of fractions from open tubular CC, the following procedure was performed using a 50 mL stop cock chromatography column and two different columns were set up for the samples:

3.8.6 Procedure for stop cock column chromatography[121]

The procedure for running of stop cock CC was done by employing protocols from literature.

In the second procedure, a 50 mL stop-cock column was selected and glass wool was packed using the same method as described under open tubular column but introduced into the column just about above the taper region of the column above the stop cock tap.

The method for packing the stop cock column was done as described under open tubular column however, the ratio of silica gel: sample was 100:1.

After the stationary phase had been introduced and allowed to settle for 5 minutes and ensuring that the top level of the stationary phase had been established and was level, the tap of the stop-cock was opened to allow drainage of solvent which was above the stationary phase to a level were the stationary phase and mobile phase were level but not to allow the stationary phase to become dry. 1 cm of sand was added to the top of the stationary phase at this stage and it was also made level, then the sample solution was loaded slowly to the top of the sand from the center using a glass pipette. At this stage, the tap of the column was opened so as to allow the sample to move from the upper layer of the sand to just below the sand so as to just touch the surface of the stationary phase then the tap at the bottom was closed again while the top layer of the sand was kept wet by the sample solution which at this point they were almost level. At this stage the column was ready to be run.

3.8.7 Procedure for loading of crude *T. mollis* extract onto the column[118]

The procedure for loading of sample on to column was done by employing protocols from literature.

Wet application method was employed in which an equivalent sample to make the appropriate ratios of silica gel: sample (30:1 in the case for open tubular column and 100:1 in the case for stop cock column) was dissolved in 1 mL mobile phase and loaded as a narrow band on top of the column.

Then this was mixed in 1:1 ratio with silica gel by continuously stirring for 10 minutes. The 4.5 : 5.5 (chloroform : methanol) solvent system was found to be optimum as it gave better resolution on TLC and was used as mobile phase during elution of the column.

3.8.8 Procedure for elution of the sample[118]

The procedure for elution of the sample was done by employing protocols from literature.

Isocratic elution technique was used during the procedure of the experiment. Without disturbing the stationary phase the mobile phase was eluted slowly by pouring from the side wall of the glass column. The solvent was added throughout the experiment while the tap was turned on to initiate the movement of the mixture. By the end of the experiment, 4 eluates of 25 mL aliquots in 4 dry Erlenmeyer flasks were collected and labelled A1, A2, B1 and B2.

3.8.9 Procedure for locating of sample in the fractions[120]

The procedure for location of sample on TLC plates was done by employing protocols from literature.

TLC was done on the samples A1, A2, B1 and B2 in a chromatography twin-trough chamber.

Visualization of spots on TLC plates was done as described in subsection 3.8.5

The fractions A1, A2, B1 and B2 were applied as single spots on the TLC plate however, the elution of fractions in this step was followed by phytochemical screening, evaluation of antimicrobial activity and eventually spectroscopic characterization after drying to powder in the rotavapor.

3.9 Procedure for evaluation of antimicrobial activity of crude extracts[112-115]

The procedure for evaluation of antimicrobial activity of crude extracts was done by employing protocols from literature.

The extracts and fractions isolated from *T. mollis* were evaluated for *in vitro* antimicrobial activity against selected micro-organisms (*Staphylococcus aureus*, *Streptococcus pyogens*, *Corynebacterium diphtheriae*, *Salmonella typhi*, *Escherichia coli* and *Candida albicans*) using the non-sterile test method of examination for the presence of test micro-organisms at a concentration of 30 mg/mL by agar streak plate dilution.

3.9.1 Experiment design

Different concentrations of the extracts were evaluated on the test micro-organisms in order to determine the MIC. The *in vitro* assays also employed positive controls such as chloramphenicol and

amphotericin B. The incorporation of the positive controls was done to ensure that the micro-organisms selected were susceptible to the antimicrobial agents and the results were compared with the activity of the components from *T. mollis*. The micro-organisms treated with DMSO constituted the negative control. This was to ensure that the solvent system used had no antimicrobial effects on the selected micro-organisms (the negative control neither had any antimicrobials nor plant extracts inoculated into them).

3.9.2 Procedure for preparation of culture media

Culture media was prepared according to the methods as given by the manufacturer on the product label. Procedure for weighing and measuring of agar

250 g of powdered Nutrient agar was weighed out on a precision balance with sensitivity of 10 mg and dissolved in 800 mL of required volume of deionized water in an adequate measuring cylinder and the resultant suspension was thoroughly mixed with a stirring rod until dissolved and also gently heated. Afterwards, the solution was poured into a 1000 mL conical flask. Then the volume was made up to 1000 mL. The pH was checked using a pH meter and adjusted to pH 7.0. The container was labelled, stopped with steel wool and aluminium foil.

The weighing, measurement and preparation method of Sabouraud dextrose agar was also done as described under Nutrient agar.

3.9.3 Procedure for sterilization[125]

The procedure for sterilization process was done by employing protocols from literature.

The final volumes of the solutions were then placed in an Autoclave for sterilization at a temperature of 121°C for 45 minutes.

3.9.4 Procedure for preparation of positive and negative controls for culture media[126]

The procedure for preparation of growth and negative controls was done by employing protocols from literature.

To the plant sample petri dishes, immediately after sterilization, and while hot, 15 mL of culture media was poured into petri dishes using a micro pipette under a direct draft-free hood. Then, to these, were

added 0.1 mL crude extracts and mixed by swirling around with the 15 mL of hot culture media in the petri dish and allowed to solidify.

To the positive control petri dishes were added 0.1 mL of the antimicrobials chloramphenicol and amphotericin B (initially diluted as ratio 1:1, powder: deionized water). The negative control petri dish contained DMSO inoculated into culture media without the addition of any antimicrobials or crude extract.

Chloramphenicol was used as a positive control for bacterial species and Amphotericin B was used as positive control for the fungal species.

Culture test organisms, *Staphylococcus aureus*, *Streptococcus pyogens*, *Corynebacterium diphtheriae*, *Escherichia coli*, *Salmonella typhi* and *Candida albicans* were obtained from the Department of Microbiology and Pathology, School of Veterinary Medicine at UNZA.

3.9.5 Procedure for inoculation of micro-organisms onto culture media[127]

The procedure for inoculation of micro-organisms onto culture media was done by employing protocols from literature.

A wire loop was held into the very edge of a transparent hot Bunsen flame in order to sterilize it and, after cooling, it was used to scoop the micro-organism, from the petri dish and then inoculated by streaking method[124] into the petri dishes containing the agar. These micro-organisms were maintained on petri dishes of agar which were then incubated overnight for 24 hours in an oven at a temperature of 37°C. The negative and positive controls were also loaded into the oven.

The fungal micro-organisms (*Candida albicans*) were inoculated onto the petri dishes containing culture media Sabouraud dextrose agar while the bacterial micro-organisms were inoculated onto the petri dishes containing Nutrient agar.

The same procedure of inoculation of micro-organisms onto culture media as described under subsection 3.9.5 was used to inoculate the micro-organisms onto the petri dishes containing the negative control and eventually were all incubated overnight for 24 hours in an oven at a temperature of 37°C.

After incubation and upon examination for micro-organism growth the lowest concentration that caused micro-organism growth was noted and recorded.

Examination of depletion or increase in bacterial population was done using non-sterile test method for specific organisms and was used to identify presence of micro-organisms. The process involved overnight culturing of the micro-organisms on pure culture then examination of bacterial growth 24 hours afterwards to see whether there was growth or non-growth of the micro-organisms.

The procedures and methods described under 3.9 were repeated for preparation of culture media and determination of antimicrobial activity of the fractions from CC.

3.10 Procedure for determination of Minimum Inhibitory Concentration (MIC)[117,119] .

The procedure for determination of MIC was done by employing protocols from literature.

Agar streak plate dilution method was used to determine MICs of the bioactive crude extracts and was repeated to determine MICs for the fractions obtained from CC against all the selected micro-organisms.

3.10.1 Preparation of extract solutions for determination of MIC[117,119]

The procedure for preparation of serial dilutions was done by employing protocols from literature.

The stock solution of crude extracts for determination of MIC was prepared by dissolving 30 mg of the extract in 1.0 mL of DMSO. The stock solution was then serially diluted with DMSO sequentially to 1: 2, 1: 4,…. 1 : 512 etc. dilutions. For instance,1.0 mL of stock solution was diluted with 1.0 mL of DMSO to give a dilution of 1 : 2; 1.0 mL of this was then diluted with 1.0 mL of DMSO to give a dilution of 1 : 4, and so on until all the other dilutions containing extract solutions of 1 : 8, 1 : 16, 1 : 32, 1 : 64, 1 : 128, 1 : 256 and 1 : 512 were obtained. The concentrations were labelled in chronological order as No. 1 for concentration 1 : 2, No. 2 for 1 : 4, No. 3 for 1 : 8, and No. 3 for 1 : 16 etc. The same method described above under subsection 3.10 was repeated to make serial dilutions for the fractions obtained from CC.

Table 3.1 shows a typical example of serial dilutions of the hot methanolic crude *T. mollis* extracts which were done on both the Lusaka and Mufulira plant materials and this was done using DMSO as solvent for dilutions. Similar serial dilutions were employed on the fractions obtained from CC.

Table 3.1: serial dilutions of crude hot methanolic crude *T. mollis* extracts.

Extract dilutions	Concentration ratio	Concentration in µg/mL
Stock solution	1:1	30,000
No.1	1:2	15, 000
No.2	1:4	7,500
No.3	1:8	3,750
No.4	1:16	1,875
No.5	1:32	937.5
No.6	1:64	468.75
No.7	1:128	234.38
No.8	1:256	117.18
No.9	1:512	58.59
No.10	1:1024	29.29
No.11	1:2048	14.62
No.12	1:4096	7.31
No.13	1:8192	3.65
No.14	1:16384	1.82
No.15	1:32768	0.91
No.16	1:65536	0.45

3.11 Spectroscopic characterization.

Spectroscopic analysis was done on the fractions A1, A2, B1 and B2 which included LC-MS, ^{1}H- and ^{13}C- NMR.

CHAPTER FOUR

4.0 Results and discussion

4.1 Results of processing of plant materials

Table 4.1 illustrates results of masses of *T. mollis* root wood and root bark from Lusaka and Mufulira plants in kilograms. Results are presented as mean value (average) of double measurements with standard deviation in brackets.

Table 4.1: Quantity of powdered plant material from the *T. mollis* plants from Lusaka and Mufulira

	Root wood powder in kg and SD	**Root bark powder in kg and SD**
	3.55 (0)	2.05 (0)

Key: kg- kilograms, SD- standard deviation

Table 4.2 shows masses in grams of crude *T. mollis* extracts from the powdered root wood and root bark plant material in grams, from Lusaka and Mufulira after processing using two different extraction procedures and solvents. The procedure and solvent adopted were the ones that produced the highest yield of hot methanolic crude *T. mollis* extract. Results are presented as mean value (average) of double measurements with standard deviation in brackets.

Table 4. 2: Quantities of hot methanolic crude *T. mollis* extracts obtained after processing.

Mass of root wood powder in g and SD	**Mass of Root bark powder in g and SD**	**Solvent**	**Cold percolation**		**Soxhlet extraction**	
			Mass of root wood in g and SD	**Mass of root bark in g and SD**	**Mass of root wood in g and SD**	**Mass of root bark in g and SD**
574 (0)	338 (0)	Ethanol	1.05 (0)	1.91 (0)	2.63 (0)	3.15 (0)
565 (0)	333 (0)	DCM	1.15(0)	1.02 (0)	3.12 (0)	3.12 (0)
562 (0)	338.5 (0)	Methanol	2.10 (0)	2.14 (0)	4.63 (0)	8.52 (0)
620.5 (0)	335.5 (0)	Water	0.58 (0)	0.80 (0)	-	-
616 (0)	340.5 (0)	Diethyl ether	1.63 (0)	2.28 (0)	1.66 (0)	2.80 (0)
587 (0)	343 (0)	Acetone	1.67 (0)	1.79(0)	2.12 (0)	2.11 (0)

Key: g- grams, SD- standard deviation

4.2 Results of TLC on crude extracts

Dragendorff's reagent was sprayed on TLC plates containing fractions obtained from CC in order to identify the presence of alkaloids which appeared as dark spots on the TLC plates (Figure 4.1).

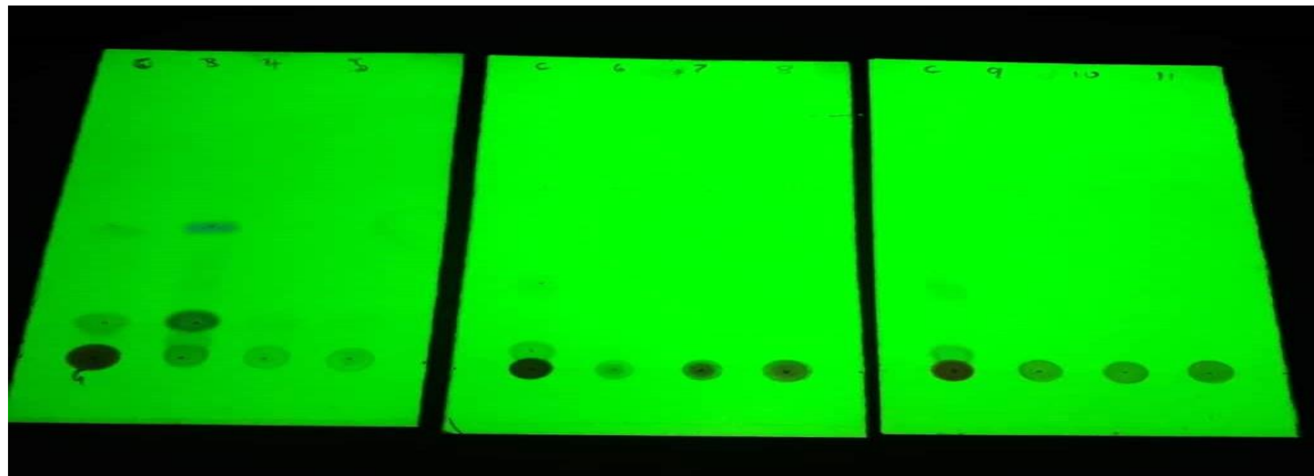


Figure 4.1: 3 normal phase TLC plates with spots of crude extracts viewed under UV-light (254 nm) - Silica gel 60 F_{254} plates, Manufacturer-Merck KGeA, Darmstadt, Germany

4.3 Results of evaluation of antimicrobial activity of the crude extracts

On the petri dishes clear arrears represent arrears of no bacterial growth on the underside of the petri dish identified by a number 1-5 while the colored spots represent areas of micro-organism growth identified by a number (Figure 4.2) and they were all labelled with numbers according to the species of bacteria streaked.

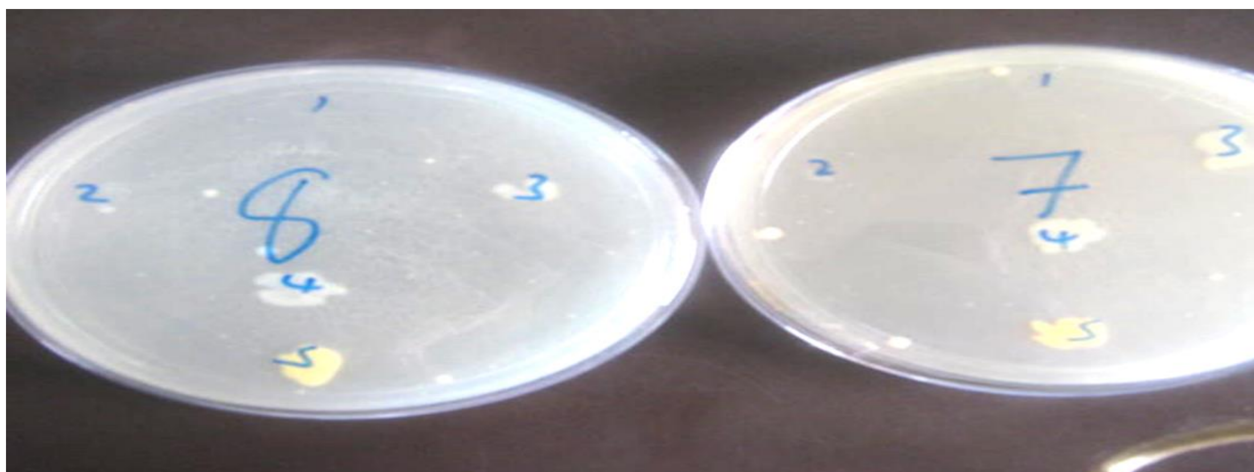


Figure 4.2: The petri-dishes were numbered according to serial dilution they contained (1 to 16).

Table 4.3 shows powdered hot methanolic crude *T. mollis* extracts at stock solution 30 mg/mL inhibited growth of all the micro-organisms. The tables of results for Lusaka and Mufulira plants were merged because they were similar.

Table 4. 3: *In vitro* antimicrobial effect of the powdered hot methanolic crude extracts of the stock solution.

Bacteria/fungi	Effect of stock solution (30mg/mL) on micro-organism growth	Growth controls		
		A	B	C
Staphylococcus aureus	-ve	-ve	+ve	+ve
Streptococcus pyogens	-ve	-ve	+ve	+ve
Corynebacterium diphtheriae	-ve	-ve	+ve	+ve
Salmonella typhi	-ve	-ve	+ve	+ve
Escherichia coli	-ve	-ve	+ve	+ve
Candida albicans	-ve	+ve	-ve	+ve

Key: (+ve) : Growth, (-ve): No growth; A-chloramphenicol (positive control), B-amphotericin B (positive control) and C-DMSO (negative control).

Table 4.4 summarizes *in vitro* antimicrobial activity of powdered hot methanolic crude *T. mollis* extract serial dilutions. The table illustrates the dilutions that inhibited micro-organism growth from the highest to the lowest (1-8). The lowest serial dilution that inhibited micro-organism growth was used as the MIC. The tables of results for Lusaka and Mufulira plants were merged because they were similar.

Table 4. 4: *In vitro* antimicrobial activity of serial dilutions of powdered hot methanolic crude extracts.

Bacteria/Fungi	Extract concentration gradient				Growth controls		
	1	**2-7**	**8**	**9-16**	**A**	**B**	**C**
Staphylococcus aureus	-ve	+ve	+ve	+ve	-ve	+ve	+ve
Streptococcus pyogens	-ve	-ve	-ve	+ve	-ve	+ve	+ve
Corynebacterium diphtheriae	-ve	-ve	-ve	+ve	-ve	+ve	+ve
Salmonella typhi	-ve	+ve	+ve	+ve	-ve	+ve	+ve
Escherichia coli	-ve	+ve	+ve	+ve	-ve	+ve	+ve
Candida albicans	-ve	+ve	+ve	+ve	+ve	-ve	+ve

Key: (+ve) : Growth, (-ve) : No growth; A-chloramphenicol (positive control), B-amphotericin B (positive control) and C-DMSO (negative control).

4.4 Results of determination of MIC for crude extracts

Table 4.5 shows MIC value of the powdered hot methanolic crude *T. mollis* extract which was found to be 117.18 µg/mL against *Streptococcus pyogens* and *Corynebacterium diphtheriae*. Results are presented as mean value (average) of double measurements with standard deviation in brackets. The tables of results for Lusaka and Mufulira plants were merged because they were similar.

Table 4. 5: Results of the MIC and SD of the crude extract.

Micro-organism	MIC 117.18 µg/mL and SD (0)	Growth controls		
		A	B	C
Staphylococcus aureus				
Streptococcus pyogens				
Corynebacterium diphtheriae				
Salmonella typhi				
Escherichia coli				
Candida albicans				

Key: A-chloramphenicol (positive control), B-amphotericin B (positive control) and C-DMSO (negative control); MIC – Minimum Inhibitory Concentration; SD – standard deviation.

	Growth
	No Growth

4.5 Results of phytochemical Analysis

4.5.1 Test for tannins (Ferric chloride test).

When 2 drops of 0.1 % $FeCl_3$ (ferric chloride) were added to the solution containing powdered *T. mollis* plant material the color change was an indication of the presence of tannins.

4.5.2 Test for Saponins (foam test).

When the solution containing powdered *T. mollis* plant material was shaken vigorously, the formation of a stable and intense foam was an indication of the presence of Saponins.

4.5.3 Test for Steroids/Triterpenoids (Salkowski's test).

Upon mixing carefully with concentrated Sulphuric acid so that acid formed the lower layer the coloration at the interface and a confirmatory test using Liebermann-Bouchard reagent on both the Lusaka and Mufulira plant species was indicative of the presence of steroids and triterpenoids.

4.5.4 Test for steroids (Liebermann-Bourchard reaction).

Upon addition of acetic anhydride followed by addition of concentrated Sulphuric acid down the side of the test tube a layer was formed underneath. The color change was indicative of presence of triterpenoids and steroids in fractions A1 and A2.

4.5.5 Test for flavonoids (Shinoda reagent test).

Upon addition of magnesium turnings and HCl acid to the solution containing *T. mollis* and upon observation for 5 minutes the color change was an indication of the presence of flavonoids in fraction B1.

4.5.6 Test for alkaloids (Mayer's reagent test and Dragendorff's reagent test).

Upon testing the powdered *T. mollis* plant material using Mayer's reagent the color change was an indication of the presence of alkaloids and testing the powdered *T. mollis* plant material using Dragendorff's reagent in another separate test tube, and upon observation the color change was also an indication of the presence of alkaloids.

When the TLC containing fractions A1 and A2 plates were sprayed with Dragendorff's reagent as test for alkaloids and observed under UV light (254 nm), the alkaloids were observed as dark spots and shown as confirmation.

4.5.7 Tests for glycosides (Keller-killiani test).

Upon addition of glacial acetic acid, $FeCl_3$ and then concentrated H_2SO_4 to the solution in the test tube containing the fractions from CC, the color change at the solution interface indicated the presence of a deoxy-sugar, characteristic of cardenolides or glycosides.

4.5.8 Summary of phytochemical results of powdered plant material

Table 4.6 illustrates phytochemical screening and confirmation of initial results for the *T. mollis* powdered plant material from Mufulira which showed the presence of all the phytochemicals tested, while for the plant material from Lusaka showed presence of some phytochemicals and absence of steroids (Salkowski's test) but later tested positive when a confirmatory (Liebermann-Bourchard reaction) test was done. The reasons behind initial negative results for steroids using the Salkowski's test on the Lusaka plant material was that, the plant material was colored and that the presence was low therefore the color change was not discernible. The tables of results for Lusaka and Mufulira plants were merged because they were similar.

Table 4. 6: phytochemical screening results of *T. mollis* plant material.

Phytochemicals	Alkaloids	Steroids	Tannins	Saponins	Flavonoids	Glycosides
Presence	+++	±	++	+	+	++

Key: − = absent; ± = low present; + = present; ++ = abundant; +++ = very abundant

4.5.9 Summary of phytochemical test results of fractions from CC

Table 4.7 illustrates phytochemical screening results for the fractions obtained from CC. Fractions A1 and A2 showed presence of steroids and alkaloids and fraction A2 also showed presence of tannins, while fractions B1 showed presence of tannins and flavonoids, fraction B2 showed presence of tannins. Glycosides were present in all the fractions.

Table 4. 7: The presence and relative abundance of phytochemicals in the fractions from CC.

Test	**A1**	**B1**	**B2**	**A2**
Tannins	-	++	+	++
Saponins	-	+	-	-
Steroids/Triterpenoids	+	-	-	+
Flavonoids	-	+	-	-
Alkaloids	+++	-	-	+
Glycosides	++	+	+	+

Key: − = absent; + = present; ++ = abundant; +++ = very abundant

4.6 Results of isolation and purification of alkaloids

Ten fractions (Figure 4.3) were collected from open tubular CC procedure and were subsequently subjected to stop cock CC procedure for further purification and eventually four fractions were collected and dried to powder. TLC of the fractions from open tubular CC, fractions labelled 3-5 were combined and fractions labelled 8-10 were also combined because they showed similar R_f upon visualization. Fractions labelled 3-5 were combined and also 8-10 were combined resulting into two different sets of combined fractions because each set of combined fractions was presumed to be the same compound.

Key: F: Fraction = from F1-F7.

Figure 4.3: Ten fractions were collected from Open tubular CC and four fractions from Stop cock CC procedure.

4.7 Summary of results of Masses of fractions obtained from CC

Table 4.8 summarizes masses in grams of four dried powdered fractions obtained from stop cock CC, labelled A1, A2 B1 and B2. The dried powdered fractions obtained from CC were subjected to phytochemical screening, spectroscopic analysis, antimicrobial activity evaluation and MIC determination.

Table 4. 8: Masses in grams of isolated fractions from CC.

No	Fractions	Mass (g)
1	A1	0.598
2	B1	0.414
3	B2	1.076
4	A2	1.724

Key: g: grams

4.8 Results of TLC on fractions from CC

Detection of developed TLC plates (Figure 4.4) was done using UV-light at wavelength 254 nm. The dark spots are the sample spots while the blue spots are the fluorescent material.

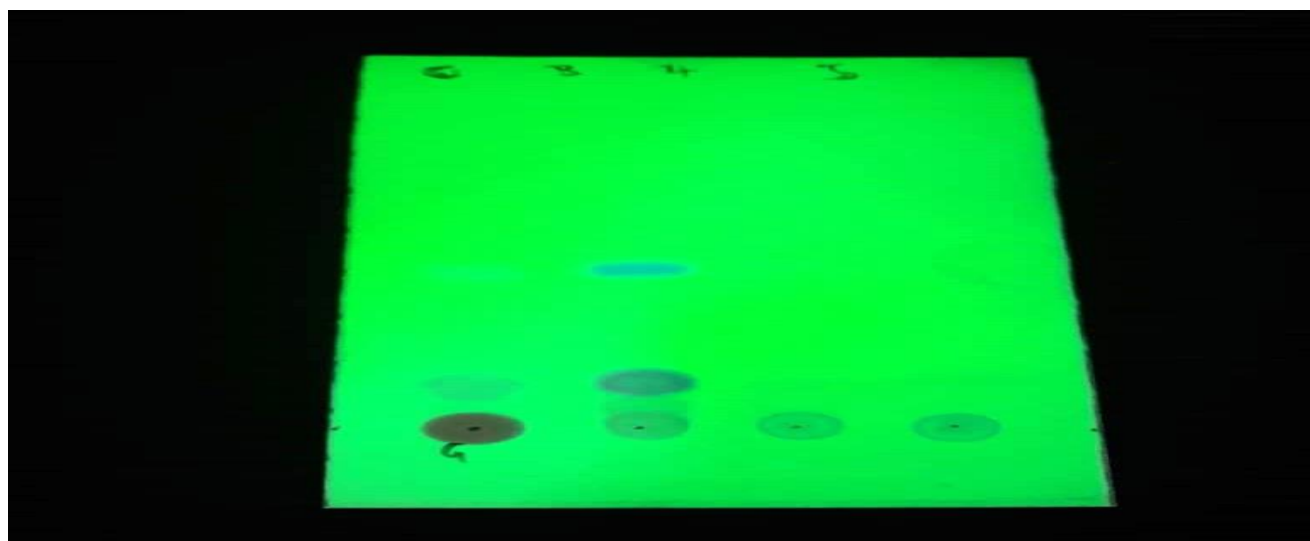


Figure 4.4: A normal phase TLC plate- Silica gel 60 F_{254}, Manufacturer-Merck KGeA, Darmstadt, Germany.

4.9 Results of antimicrobial activity of stock solution of fractions from CC

Table 4.9 illustrates i*n vitro* antibacterial activity of fractions from CC at stock solution concentration 30 000 µg/mL and this shows that fraction A1 had antimicrobial activity against all the test micro-organisms while the rest of the fractions had antimicrobial activity against only two micro-organisms namely *Streptococcus pyogens* and *Corynebacterium Diphtheriae*.

Table 4. 9: *In vitro* antimicrobial activity of fractions from CC.

Bacteria/fungi	Fractions				Growth controls		
	A1	B1	B2	A2	A	B	C
Staphylococcus aureus	-ve	+ve	+ve	+ve	-ve	+ve	+ve
Streptococcus pyogens	-ve	-ve	-ve	-ve	-ve	+ve	+ve
Corynebacterium diphtheriae	-ve	-ve	-ve	-ve	-ve	+ve	+ve
Salmonella typhi	-ve	+ve	+ve	+ve	-ve	+ve	+ve
Escherichia coli	-ve	+ve	+ve	+ve	-ve	+ve	+ve
Candida albicans	-ve	+ve	+ve	+ve	+ve	-ve	+ve

Key: (+ve): Growth, (-ve): No growth; A-chloramphenicol (positive control), B-amphotericin B (positive control) and C-DMSO (negative control).

4.10 Results of determination of MIC on the fractions from CC

Table 4.10 shows MIC value of fraction A1 against *Staphylococcus aureus, Candida albicans* and *Escherichia coli* which was found to be 30 000 µg/mL and against *Salmonella typhi* it was found to be 234.38 µg/mL.

MIC value for fractions A2, B1 and B2 was found to be 117.18 µg/mL against only *Streptococcus pyogens* and *Corynebacterium diphtheriae.*

The fractions A2, B1 and B2 showed no inhibitory activity against the micro-organisms *Staphylococcus aureus, Escherichia coli* and *Candida albicans* at stock solution concentrations 30 000 µg/mL.

Table 4. 10: MIC values of fraction A1 against the selected micro-organisms.

Bacteria/fungi	MIC value (µg/mL)	Growth controls		
		A	B	C
Staphylococcus aureus	30,000	-ve	+ve	+ve
Streptococcus pyogens	117.18	-ve	+ve	+ve
Corynebacterium diphtheriae	117.18	-ve	+ve	+ve
Salmonella typhi	234.38	-ve	+ve	+ve
Escherichia coli	30,000	-ve	+ve	+ve
Candida albicans	30,000	+ve	-ve	+ve

Key: (+ve) : Growth; (-ve) : No growth; A-chloramphenicol and B-amphotericin B (positive control), C-DMSO (negative control); MIC – Minimum Inhibitory Concentration.

4.11 Spectroscopic analysis of data.

Spectroscopic analysis on the fractions A1, A2, B1 and B2 which included ^{13}C- and ^{1}H-NMR and LC-MS showed that the fractions obtained were not pure.

It is possible that the impurities might been introduced into fractions A1, A2, B1 and B2, when the ten fractions from open tubular CC were mixed by combining fractions 3-5 to obtain one set of combined fractions and fractions 8-10 to obtain the other set of combined fractions, prior to subjecting both sets to stop cock CC.

CHAPTER FIVE

Conclusion

In this research the hot methanolic crude extracts of the whole root of the Zambian *T. mollis* tree was found to have antimicrobial activity *in vitro* against the selected micro-organisms.

It was possible in this research to find MIC value of the crude extract of the whole root of the Zambian *T. mollis* tree and that it was also possible to isolate antimicrobial metabolites from the bioactive extracts using chromatographic techniques

The MIC value for the powdered crude hot methanolic *T. mollis* extract and fraction A1 obtained from CC was found to be 117.18 µg/mL against *Streptococcus pyogens* and *Corynebacterium diphtheria.*

The MIC value for *T. mollis* against *Candida albicans* was not reported elsewhere however, the MIC values which were found to be lethal in Tanzania against *Staphylococcus aureus, Escherichia coli* and *Salmonella typhi* ranged from 26.3 to 101.3 µg/mL[64].

These results show that the MIC value in this research on *Staphylococcus aureus, Escherichia coli* and *Candida albicans* was higher than those for the same micro-organisms reported in Tanzania.

Recommendations

The main challenge was inadequate sample size to work on and carry out all the experiments that were required according to the objectives therefore, for future work it is recommended that more starting sample of the whole root of *T. mollis* tree is obtained so that sufficient quantities are available for downstream analysis. Planning and budgeting for all the laboratory reagents and chemicals must also cater for the samples collected.

Thus, it is recommended that further purification and characterization be carried out. It is possible that the compounds contained in the fractions could be new molecules that have never been identified before.

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APPENDIX A: APPROVAL LETTER FOR PROTOCOL

THE UNIVERSITY OF ZAMBIA
DIRECTORATE OF RESEARCH AND GRADUATE STUDIES

Great East Road Campus | P.O. Box 32379 | Lusaka10101 | Tel: +260-211-290 258/291 777
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APPROVAL OF STUDY

IORG No. 0005376
NASRECREC IRB No. 00006465
REF NO. NASREC: 2024-NOV-011

2nd December, 2024

Mr Gavin Mwelwa
P.O. Box 32379
LUSAKA

Dear Mr Mwelwa

RE: "ISOLATION AND CHARACTERIZATION OF ANTIMICROBIAL COMPONENTS OF THE ROOT OF A ZAMBIAN MEDICINAL TREE, *TERMINALIA MOLLIS*"

Reference is made to your protocol captioned above. The NASREC resolved to approve this study and your participation as Principal Investigator for a period of one year.

REVIEW TYPE	ORDINARY REVIEW	APPROVAL NO. NASREC-2024-NOV-011
Approval and Expiry Date	Approval Date: 2nd December, 2024	Expiry Date: 1st December, 2025
Protocol Version and Date	Version - Nil.	1st December, 2025
Information Sheet, Consent Forms and Dates	• English.	To be provided
Consent form ID and Date	Version - Nil	To be provided
Recruitment Materials	Nil	Nil
Other Study Documents	Questionnaire.	

Specific conditions will apply to this approval. As Principal Investigator it is your responsibility to ensure that the contents of this letter are adhered to. If these are not adhered to, the approval may be suspended. Should the study be suspended, study sponsors and other regulatory authorities will be informed.

CONDITIONS OF APPROVAL

- No participant may be involved in any study procedure prior to the study approval or after the expiration date.
- All unanticipated or Serious Adverse Events (SAEs) must be reported to NASREC within 5 days.
- All protocol modifications must be approved by NASREC prior to implementation unless they are intended to reduce risk (but must still be reported for approval). Modifications will include any change of investigator/s or site address.
- All protocol deviations must be reported to NASREC within 5 working days.
- All recruitment materials must be approved by NASREC prior to being used.
- Principal investigators are responsible for initiating Continuing Review proceedings. NASREC will only approve a study for a period of 12 months.
- It is the responsibility of the PI to renew his/her ethics approval through a renewal application to NASREC.
- Where the PI desires to extend the study after expiry of the study period, documents for study extension must be received by NASREC at least 30 days before the expiry date. This is for the purpose of facilitating the review process. Documents received within 30 days after expiry will be labelled "late submissions" and will incur a penalty fee of K500.00. No study shall be renewed whose documents are submitted for renewal 30 days after expiry of the certificate.
- Every 6 (six) months a progress report form supplied by The University of Zambia Natural and Applied Sciences Research Ethics Committee as an IRB must be filled in and submitted to us. There is a penalty of K500.00 for failure to submit the report.
- When closing a project, the PI is responsible for notifying, in writing or using the Research Ethics and Management Online (REMO), both NASREC and the National Health Research Authority (NHRA) when ethics certification is no longer required for a project.
- In order to close an approved study, a Closing Report must be submitted in writing or through the REMO system. A Closing Report should be filed when data collection has ended and the

study team will no longer be using human participants or animals or secondary data or have any direct or indirect contact with the research participants or animals for the study.

- Filing a closing report (rather than just letting your approval lapse) is important as it assists NASREC in efficiently tracking and reporting on projects. Note that some funding agencies and sponsors require a notice of closure from the IRB which had approved the study and can only be generated after the Closing Report has been filed.

- A reprint of this letter shall be done at a fee.

- All protocol modifications must be approved by NASREC by way of an application for an amendment prior to implementation unless they are intended to reduce risk (but must still be reported for approval). Modifications will include any change of investigator/s or site address or methodology and methods. Many modifications entail minimal risk adjustments to a protocol and/or consent form and can be made on an Expedited basis (via the IRB Chair). Some examples are: format changes, correcting spelling errors, adding key personnel, minor changes to questionnaires, recruiting and changes, and so forth. Other, more substantive changes, especially those that may alter the risk-benefit ratio, may require Full Board review. In all cases, except where noted above regarding subject safety, any changes to any protocol document or procedure must first be approved by NASREC before they can be implemented.

Should you have any questions regarding anything indicated in this letter, please do not hesitate to get in touch with us at the above indicated address.

On behalf of NASREC, we would like to wish you all the success as you carry out your study.

Yours faithfully,

Dr. Mususu Kaonda

VICE-CHAIRPERSON
THE UNIVERSITY OF ZAMBIA NATURAL AND APPLIED SCIENCES RESEARCH ETHICS COMMITTEE - IRB

cc: Director, Directorate of Research, Innovation and Development
Assistant Director (Research), Directorate of Research, Innovation and Development
Assistant Registrar (Research), Directorate of Research, Innovation and Development