College of Medicine

INVESTIGATION OF ANTIMYCOBACTERIAL AND ANTIVIRAL ACTIVITIES OF EXTRACTS AND COMPOUNDS ISOLATED FROM MALAWIAN MEDICINAL PLANTS

By

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DECLARATION

I, the undersigned hereby declare that this thesis is my own original work which has not been submitted to any other institution for similar purposes. Where other people's work has been used, acknowledgements have been made.

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CERTIFICATE OF APPROVAL

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DEDICATION

I dedicate this thesis to my late father (B.B. Ngonda), brother (Jimmy) and sister (Joyce).

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ABSTRACT

Infectious diseases accounts for approximately one-half of all the deaths that occurs in tropical countries including Malawi. Some of these infectious diseases are caused by microorganism such as mycobacteria and viruses. However, the threat of antimicrobial resistance is growing at an alarming rate and the situation has been aggravated in these developing countries due to so many factors including presence of multidrug resistants' strains and over prescriptions. Therefore, the purpose of present study was to investigate the phytochemical constitutes and compounds isolated from *Aeschynomene nyassana, Euphorbia whyteana, Euphorbia cooperi, Flueggea. virosa, Phyllanthus amarus, Ericae milanjiana* and *Rhus acuminatissima* medicinal plants, determine their antimicrobial activities and cytotoxic properties. Ethnobotanical survey was conducted and several methods were used to identify and confirm the selections of plants understudy. Search engine on several online databases (Google Scholar, PubMed, MEDLINE, Scopus, Cochrane Library, and Science Direct) were used to identify medicinal plants with antiviral and antimycobacterial activities. The plants found were then matched with published and unpublished ethnobotanical survey data and database on the Flora of Malawi, Chewa Medical Botany by Brian Morris, Useful Plants of Nyasaland by Jessie Williamson and other sources. Seven plants, were identified for this research and these were extracted twice with solvents such as methanol, ethyl acetated and dichloromethane. Part of the methanol/water extracts obtained were subjected to alkaloid extraction scheme while the other part was subjected to column chromatograph. 1,3,6 – tri(3,4,5-trihydroxyphenyl) acetyl ester - β-D-glucopyranose, 1,2,3,4,6 – penta(3,4,5-trihydroxyphenyl) acetyl ester - β-D- glucopyranose, 1,2,3,6 – tetra(3,4,5-trihydroxyphenyl) acetyl ester - β-D- glucopyranose, 2,3,6 – tri(3,4,5-trihydroxyphenyl) acetyl ester - β-D- glucopyranose, compounds were obtained from Hexane: Ethyl acetate 50%; 80%; 90% and Ethyl acetate: Methanol 5% and the compounds were identified from the NMR spectra while Betulinic acid was obtained as a white amorphous

powder, soluble in methanol, having been eluted with methylene chloride: methanol 9:1 from the silica gel column. The NMR spectral data was compared in reference literature and showed to be very similar to those of Betulinic acid compound. Benzoxylanthaquinone was identified by direct comparison of the spectroscopic data with those published literatures. The compound, Cyclanoline, greyish in colour was obtained from alkaloid extraction scheme and identified by direct comparison of the spectroscopic data with those published literatures. Mass spectra(ESI-MS) were obtained with a Thermo-Finningan LCD DECA mass spectrometer and HRESIMS spectra were measured with a FTHRMS-Orbitrap (Thermo-Finnigan) mass spectrometer. The compound E5 m/z 695.33, yellowish in colour was obtained from alkaloid extraction scheme and identified by direct comparison of the spectroscopic data with those published literatures. Mass spectra(ESI-MS) were obtained with a Thermo-Finningan LCD DECA mass spectrometer and HRESIMS spectra were measured with a FTHRMS-Orbitrap (Thermo-Finnigan) mass spectrometer. The resultant crude extracts, fractions and compounds were used in the phytochemical screening and antimicrobial analysis. The phytochemical analysis involved spectrophotometric screening for pro/antioxidant properties of the crude extracts, fractions and compounds using HPTLC, DPPH, FRAP, Reducing power, Crocin bleaching assay, Cupric reducing antioxidant capacity and Nitric oxide radical scavenging activities. Cell viability and cytotoxicity studies were conducted using the Trypan blue dye exclusion, (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) MTT and Mammalian macrophage cytotoxicity assays while heavy metals analysing was performed using atomic absorption spectrometry. The antimycobacterial activities of crude extracts, fractions and compounds were assessed using microplate alamar blue assay. Compounds obtained were further assessed for drug susceptibility against resistant *Mycobacterium tuberculosis* using the BACTEC™ MGIT 320 system. The results of the review study identified 50 different families of medicinal plants comprising 90 plants species that are frequently used by traditional healers to treat various

diseases in Malawi such as sexually transmitted infections, diarrhoea/dysentery, skin infections/rash, respiratory infection, cold/cough, fever, tuberculosis and herpes zoster. Furthermore, these medicinal plants also demonstrated some anti-viral and antimycobacterial activities. The compounds/compound groups identified in this study were categorized into eight distinct groups of flavonoids, alkaloids, saponins, phenolics, lignins, xanthones, proteins and peptide. In phytochemical analysis, results of crude extracts, fractions and compounds showed moderate DPPH scavenging activity, a dose dependent decrease in NO scavenging activity except for *R. acuminatissinia* while *A. nyassana* revealed reducing power ability that was significantly greater than standard Ascorbic acid. In HPTLC analysis, DPPH active spots showed pale-yellow coloured spots for the 4 plants understudy while the phenolic active blue colour spots were observed to be more visible on *R. acuminatissinia* and *E. milanjiana.* In PBMC viability analysis, the lymphocytes treated with *Rhus acuminatissima* and *Ericae milanjiana* had the lowest cell percent viability. The plants understudy also demonstrated a decrease in percent viability with increased time for all the plants extracts except for *E. milanjiana* and *A. nyassana* which showed some stimulative effects. *A. nyassana* and *Euphorbia whyteana* demonstrated relatively high cytotoxicity as compared to *E. milanjiana, R. acuminatissima* and standard drug, Doxorubicin. In heavy metal analysis, all the plants understudy displayed the least levels of toxic metals concentration in the order of Cadmium<Chromium<Lead. In the antimycobacterial analysis, *F. virosa* and *E. milanjiana* crude extracts displayed Minimum Inhibitory Concentration of 128 ug/ml and 512 ug/ml respectively while A. *nyassana* ANX fraction showed MIC of 256 ug/ml for *M. smegmatis*. *E. cooperi, E. milanjiana* and *E. whyteana* crude extracts displayed MIC value of 512 ug/ml while *E. whyteana* W1 fraction had MIC value of 252 ug/ml for *M. ulcerans.* The selectivity index (SI) values of plants understudy ranged from <0.082 – 2.20 and considerably good SI were observed in *F. virosa, E. whyteana* crude extracts and *E.* whyteana W1 fraction at 2.20, 0.625

and 0.985 respectively. In synergistic antimycobacterial analysis, the synergistic drug action showed Betulinic acid (EM8), 1,2,3,6 – tetra(3,4,5-trihydroxyphenyl) acetyl ester - β-D-glucopyranose (FV8) and Cyclanoline (C5) exhibited synergistic antimycobacterial activity at a final concentration of 18 ug/ml. The good synergistic results (susceptibility) were observed in combined EM8+INH and FV8+INH while a moderate synergistic result was observed in C5+INH indicating an additive effect of the combination. However, antagonistic activity (resistance) was observed in combined EM9+ETH and E1+ETH. Therefore, it can be concluded from the results that all the plants understudy showed considerable antimycobacterial activities against *M. tuberculosis, M. ulcerans* and *M. smegmatis*. The compounds obtained from the plants understudy also demonstrated synergistic properties with the first-line tuberculosis drugs, rifampicin; isoniazid ethambutol and streptomycin. Consequently, this study recommends that further studies to be undertaken in order to determine the mechanism of action of the novel compounds in order to unravel its exact potential to inhibit several pathogenic microbes especially resistant *M. tuberculosis* and *M. ulcerans* and also to evaluate whether its true pharmacological activities exist. Currently, an all-oral and less toxic treatment regimen of Buruli ulcer is been sought after and encouraged by WHO.

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ABBREVIATIONS AND ACRONYMS

AIDS	Acquired Immunodeficiency Syndrome
ART	Antiretroviral Therapy
CMI	Cell-mediated immune
CSRM	Certified reference materials
DNA	Deoxyribonucleic acid
DPPH	2,2-Diphenyl-1-picrylhydrazyl
DR	Death Receptors
EMB	Ethambutol
EPIs	Efflux pump inhibitors
FADD	Fas-associated protein with death domain
FAO	Food and Agriculture Organization
GC	Growth control
H_2O_2	Hydrogen peroxide
HIV	Human Immunodeficiency Viruses
HMBC	Heteronuclear Multiple Bond Correlation
HMQC	Heteronuclear Multiple Quantum Coherence
HPTLC	High-performance thin-layer chromatography
IN	Integrase
INH	Isoniazid
LCMS	Liquid Chromatograph Mass Spectra
LJ	Lowenstein-Jensen
LOO-	Lipid peroxide
MDR-TB	Multidrug-resistant tuberculosis
MP-AES	Microwave Plasma-Atomic Emission Spectrometer System
MTHUO	Malawi Tradition Healers Umbrella Organisation
NMR	Nuclear magnetic resonance
NO	Nitric oxide
NRTIs	Nucleoside Reverse Transcriptase Inhibitors

O_2	Singlet oxygen
O_2-	Superoxide ion
O_3	Non-radical ozone
OADC	Oleic Acid, Bovine Albumen, Dextrose and Catalase
OH-	Hydroxyl ion
OS	Oxidative stress
PBMCs	Peripheral Blood Mononuclear Cells
PGL	Persistent generalized lymphadenopathy ()
PR	Protease
RIF	Rifampicin
RNA	Ribonucleic acid
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute
RT	Reverse transcriptase
STR	Streptomycin
TB	Tuberculosis
TLRs	Toll-like receptors
TNF	Tumour necrosis factor
WHO	World Health Organization

CHAPTER 1: INTRODUCTION

1.1 Background

The use of medicinal plants as a source of medicine is a vital component in the primary health care system of most developing countries. These countries have a large population of people that live in rural areas with limited access to health facilities (1). According to the World Health Organization (WHO), more than 80 % of the world's population relies on traditional medicine for their primary healthcare needs (2). The World Health Organisation also estimates that there are over 2,000 plants that have been identified and recommended for use as herbal medicine globally (2). Information about these herbal plants is usually passed on informally between generations without documentation. The field of ethnobotanical research has expanded greatly in recent years as the value of this type of research has come to be more widely recognised. There is a resurgence of public interest in herbal remedies and this has been attributed to several factors including the popular belief that they are effective, high cost and side effects of most modern drugs, have dissatisfactory results from orthodox pharmaceuticals and the belief that herbal medicines might be effective in the treatment of certain diseases where conventional therapies and medicines have proven to be ineffective or inadequate (3). Globally, it is estimated that about 25 % of all modern medicines are either directly or indirectly derived from natural products (2). There are over 252 drugs that are considered as basic and essential by the WHO, of which 11 % are exclusively originating from plants, while the majority of synthetic drugs used globally have plant precursors (4).

1.2 Problem Statement

Natural products such as plant extracts found either in pure compounds or standardized extracts provide unlimited opportunities for new drug discoveries because of their unmatched availability of chemical diversity. Some of the phytochemical constituents present in plant extracts have been reported to possess medicinal uses (5,6). Phytochemical and pharmacological investigations of several plants have already led to the isolation of some of the natural antimicrobial compounds. The development of new products from natural sources is also encouraged because it is estimated that 300,000 plant species exist in the world where only 15 % of them have been evaluated to determine their pharmacological potential (7).

Malawi has a rich heritage in traditional medicine, hence there is a need to make strides towards the production of drugs based on medicinal plants through the establishment of information on the secondary metabolites associated with antimicrobial properties. Malawians widely use traditional medicine as a form of primary health for curing, preventing and protecting themselves against various ailments. Several factors such as high illiteracy levels among the rural majority (who make up about 80 % of the population) and limited access to modern health facilities are attributed to the dependence of traditional medicine. Malawi, just like any other developing country, drug-resistant pathogens continue to affect the implementation of the primary health care programme as most of the resources are spent on increasingly expensive alternative drugs. Therefore, it is not only important to supplement modern drugs with effective local herbs for economic reasons, but also for the fact that there is an acute shortage of drugs in most of the health facilities in developing countries (8).

1.3 Traditional medicine and medicinal plants

Traditional medicine refers to the totality of all the knowledge and practices whether explicable used or not, used in diagnosis, prevention and elimination of physical, mental or social imbalance and relying exclusively on practical experience and observations handed down from generation to generation whether verbal or written (9).

Since prehistoric time, human beings have used plants for disease prevention and control. It is believed that through trial and error, early man acquired the knowledge on the utilization of plant for disease prevention and therapeutic purposes (10). Several studies suggest that human beings were using medicinal plants during the early civilisations of China, India, Egypt and Sumeria (11). Gurib-Fakim (12) suggests even earlier use of medicinal plants noting that that physical evidence gathered from the Shanidar IV burial site of Neanderthal man discovered in Iraq suggests that the use of medicinal plants goes as far back as 60000 BCE.

1.3.1 Traditional medicine and medicinal plants in the world

It is estimated that more than 35,000 plants species are being used for medicinal use around the world (13). There has also been an increase in the use of herbal medicine and medicinal products and many newer products are being introduced into the global market. Herbal remedies are widely being embraced in many developed countries because of the belief that they promote a healthier lifestyle (14). In developed countries such as the United States, it is estimated that plant drugs comprise of up to 25 % of the total drugs, while in China and India is over 80 % of the total drugs. Consequently, the booming sales of herbal medicines are representing a substantial proportion of the global drug market which is currently approaching $ 60 billion US dollars (3,15–17).

1.3.2 Traditional medicine and medicinal plants in Africa

African traditional medicine is the oldest, and perhaps the most assorted, of all therapeutic systems. Africa is considered to be the cradle of mankind with a rich biological and cultural diversity marked by regional differences in healing practices (18). In African, most of the communities look at traditional medicine as complementary healthcare and not as an alternative to modern medicine. Information about the tradition of collecting, processing and applying plants and plant-based medications in African societies have been passed down informally from generation to generation (12). Therefore, herbal medicines have often maintained their popularity for historical and cultural reasons. Traditional medicine, with medicinal plants as their most important constituent, is sold and sometimes available in marketplaces or prescribed by traditional healers in their homes (19). Medicinal plants are the most easily accessible health resource available to the community in most parts of Africa due to a limited number of health facilities. Furthermore, they are most often the preferred option for the patients. Traditional healers are considered to offer information, counselling, and treatment to patients and their families in a personal manner as well as having an understanding of their patient's environment. The driving force of the majority of users appears to be the holistic belief that the health of body, mind and spirit are related and that this should be taken into account by whoever cares for their health (20).

1.3.3 Traditional medicine and medicinal plants in Malawi

Malawi is one of the developing countries in the world with more than 65 % of its population living below the poverty line. Due to the absence of adequate healthcare facilities especially in the rural areas, most of the people continue to rely on herbal medicine to meet their primary health care needs. This can also be attributed in part to the widespread belief in the effectiveness of many

traditional therapies and high illiteracy levels amongst the rural majority who make up about 80% of the population (21). Malawians have always been using traditional medicine as a cure and preventive or protective measure well before colonization. The traditional doctors in Malawi have been practising for centuries and are commonly known as Singánga. They play a significant role in the healthcare system so much that they treat about 60-70 % of patients.

In Malawi, traditional healers are more available in the communities than biomedical practitioners. In 1986 a study which was conducted by Msonthi and Seyani (22) indicated that the physician to patient ratio was at 1:50,000. Compared to 2011 where the ratio had grown to one physician per 62 000 people, about twelve times higher than what WHO recommended in its ratio of 1:5000 (23). Although, the Msonthi and Seyani study had reported a low ratio of 1:138 for traditional healers and patients. The unconfirmed reports from four traditional healer's association namely; Herbalist Association of Malawi, Traditional Health Practitioner Research Council of Malawi, Traditional Medicine Council of Malawi and Chizgani Ethnomedicine Council of Malawi (Associations under Malawi Tradition Healers Umbrella Organisation-MTHUO) show that the number of traditional healers registered with the associations has drastically increased.

1.4 Botanical information on plants used by traditional healers for treatment of infectious diseases in Malawi

1.4.1 Aeschynomene nyassana

Aeschynomene nyassana plant belongs to the family Fabaceae which has about 751 genera and about 19,000 known species (24). Figure 1.1 shows the flower, leaves and a whole *A. nyasanna* plant. *A. nyassana* is a small shrub that grows from a woody rootstock. It grows with several erect

stems and is usually 15 - 300 cm tall. Leaves are 3 - 25 cm long, pinnate with 7 - 40 pairs of leaflets that are 6 - 24 mm long oblong to ovate-oblong with mucronulate at the apex. It has a hairless stipule 7 - 24 mm long. The Inflorescences terminal has many-flowers and branches that can grow up to 35 cm long. Flowers are pale yellowish with purple veining and wings longer than the keel. Pods are in 1 - 2 segments, obovate, elliptic or semi-circular, flat and papery, hairless or slightly pubescent on the margins.

(a) Flower of *A. nyassana*

(b) Leaves of *A. nyassana*

(c) A. nyassana plant

Figure 1.1: *Aeschynomene nyassana* plant

1.4.2 Erica milanjiana

Ericae milanjiana as shown in Figure 1.2, is an endemic species at Mulanje mountain within the family Ericaceae. Ericaceae, comprises of 126 genera and some 4,000 species (24). Ericaceae is made up mostly of shrubs and small trees, and its members are widely distributed, extending into the subarctic and along mountain chains through the tropics. A large percentage of the family's

species are cultivated, including azaleas, rhododendrons, mountain laurel, blueberries, and the low shrubs of the genus Erica.

Figure 1.2: *Ericae milanjiana* plant

1.4.3 Flueggea virosa

Flueggea virosa plant belongs to the family Phyllanthaceae which contains 57 genera of flowering plants and approximately 2000 species (25). The genus Flueggea comprises 15 species which occurs in the tropics and subtropics of both hemispheres, and sometimes extending into warm temperate zones. However, only *Flueggea virosa* species occurs in tropical Africa. *Flueggea virosa* is commonly found in a wide variety of habitats, in forest edges, bushland, grassland, woodland and thickets. The species sometimes grows on termite mounds and rocky slopes. *F. virosa* as shown in Figure 1.3 is deciduous, much-branched shrub or small tree that can grow up to 4 m tall. Branches are erect while lower branches are often with a thorny end. Leaves are distichously alternate, simple and petiole is 3 – 6 mm long, grooved above, narrowly winged.

Inflorescences are an axillary fascicle, many-flowered in male plants, few-flowered in female plants. Flowers are unisexual, regular and sweet-scented while pedicel can grow up to 9 mm long. Fruits are somewhat fleshy slightly 3-lobed, globose capsule, 3 – 5 mm in diameter. Seeds are ovoid, 2 – 3 mm long, shiny and yellowish-brown.

(a) *F. virosa* plant

(b) Leaves and stem of *F. virosa*

(c) Fruits of *F. virosa*

Figure 1.3: Flueggea virosa

1.4.4 Euphorbia whyteana

Euphorbia whyteana plant belongs to the family Euphorbiaceae, which contains some 7,500 species in 300 genera (26). *E. whyteana* as shown in Figure 1.4, is a hairless perennial herb with annual stems that can grow up to 30 cm high from a woody rootstock. The family consists of annual and perennial herbs and woody shrubs or trees, as well as a few climbers. Leaves are normally 30 mm long, rounded and apiculate at the apex, they are numerous, sessile, spreading to

reflexed and linear-lanceolated. Although species of the family grow throughout the world, except in cold alpine or arctic regions, most of them are found in temperate or tropical regions.

Figure 1.4: *Euphorbia whyteana*

1.4.5 Euphorbia cooperi

Euphorbia cooperi plant belongs to family Euphorbiaceae, which contains some 7,500 species in 300 genera (26). More than 30 species of the genus Euphorbia have been recorded from Malawi, Figure 1.5 shows that *E. cooperi* is a spiny, succulent tree can grow up to 7 m while the trunk can grow up to 3m. Branches are deeply divided into heart-shaped segments, green, leafless, usually 4 -angled, with spines 7 mm long. Flowers are bisexual and yellowish green. Fruit have a 3 -lobed capsule.

(a) *E. cooperi* plant

(b) Leaves of *E. cooperi*

Figure 1.5: *Euphorbia cooperi* plant

1.4.6 Rhus acuminatissima

Rhus acuminatissima belongs to the family Anacardiaceae, commonly known as the cashew family or sumac family has 83 genera and about 860 known species (24). Members of the Anacardiaceae bear fruits that are drupes and, in some cases, produce urushiol, an irritant. *Rhus acuminatissima* is a small tree often straggling and can grow up to 9 metres in length. The leaves are 3 -foliolate while the leaflets are broadly elliptic, obtuse as shown in Figure 1.6. The flowers are in loose panicle and small yellowish-green in colour.

Figure 1.6: *Rhus acuminatissima* plant

1.4.7 Phyllanthus amarus

Phyllanthus amarus plant belongs to family Euphorbiaceae, which contains some 7,500 species in 300 genera (26). It is an annual herb found in shady places among other common weeds and can grow up to 30 - 60 cm in height and blooms with yellow flowers as shown in Figure 1.7.

(a) : Leaves of *P. amarus*

(b) Stems and fruits of *P. amarus*

Figure 1.7: *Phyllanthus amarus* plant

1.5 Ethno medical information on medicinal plant understudy

The genera Aeschynomene, Ericae, Flueggea, Euphorbia, Phyllanthus and Rhus comprise several species that are used in indigenous medicine for the treatment of various diseases (27). A study by Kayambazinthu *et al* (28) which was commissioned by the Food and Agriculture Organization (FAO) indicated that *A. nyassana* is used to promote healing and to reduce pain in HIV/AIDS patients in Malawi. Another study by Morris and Msonthi (27), reported in the book "Chewa Medical Botany: A Study of Herbalism in Southern Malawi" show that *A. nyassana* root infusion is used for enema, venereal diseases and anthelmintic. Table 1.1 shows that, in Malawi, other plants, such as *E. cooperi* is for the treatment of rheumatism, chronic cough and *Ericae milanjiana* for general ailments and they, are also used to treat various ailments.

Table 1.1: Ethnomedical information on Aeschynomene, Ericae, Flueggea, Euphorbia, Phyllanthus and Rhus genera

Species	Part(s) of plant	Ailment(s) treated / traditional uses	Place(s) where practiced	References
F. virosa	Leaves Roots	Invigorate body Treat lactation disorder Abortion Epilepsy, convulsion, rectal and uterine prolapsed Treat toothache	Central Africa Republic Burundi and Tanzania Uganda Tanzania Madagascar	(29)
Rhus acuminatissima	Roots	Heart complaints, stimulate blood circulation, rheumatism, mental disorders, sexually transmitted infections.	Malawi	(27)
Ericae milanjiana	Leaves	General ailment	Malawi	(27)
Aeschynomene nyassana	Roots	Promote healing, Reduce pain Venereal diseases Anthelmintic, Enema	Malawi	(27,28)
Euphorbia whyteana	Leaves Roots	Infectious diseases, respiratory ailments, cough, cancer, tumours, warts, asthma, earache, neuralgia, rheumatism, toothache	All countries	(30).
Euphorbia cooperi	Stems	Rheumatism, chronic cough	Malawi	(27)
Phyllanthus amarus	Stems Leaves	Hepatoprotective, Anti-inflammatory, Anticancer, Diuretics, Nephroprotective, Antioxidant, Antihyperglycemic, Antibacterial, Antihypercholesterolemic, Antiviral	India	(31)

1.6 Hypothesis of the study

Medicinal plants that are used by traditional healers to treat infectious diseases contain phytochemical compounds that are responsible for antimicrobial activity against common infectious pathogens.

1.7 General and specific objectives

The broad aim of the study was to investigate some of the medicinal plants used by traditional healers to treat viral and mycobacterial infections in Malawi. The specific objective includes:

i) To extract phytochemical compounds from medicinal plants

ii) To isolate and characterize secondary metabolites from medicinal plants,

iii) To identify biologically active compounds & obtain their biological efficacy data relevant to infectious diseases,

iv) To evaluate the immunological and cytotoxic activity of herbal compounds.

1.8 Thesis outline

The outline of the thesis is as follows: Chapter 1 has dealt with the introduction, Chapter 2 deals with literature review; Chapter 3 describes the materials and general methods used to achieve the objectives of this study; Chapter 4 provides results and discussions of the findings of this study; Chapter 5 provides the conclusions and Chapter 6 provides recommendations for further study.

CHAPTER 2: LITERATURE REVIEW

2.1 Human Immunodeficiency Virus and Tuberculosis

Syndemics/Co-infection of tuberculosis and Human Immunodeficiency Virus (HIV) acts synergistically to magnify their burden causing considerable morbidity and mortality in human beings. HIV infection speeds up the progression of Tuberculosis (TB) from latent to active while TB infection accelerates the progress of HIV infection (32). According to the WHO (2019), TB is the leading cause of death amongst people living with HIV, accounting for about 251 000 deaths in 2018 and that is a third of the total Acquired Immunodeficiency Syndrome (AIDS) deaths. The WHO (2019) further reports that of this number, Africa accounted for about 84 % of the TB/HIV deaths, and these were mostly from the sub-Saharan Africa region. Researchers have shown that there is substantial overlap in drug toxicities and drug-drug interactions when cotreating HIV and TB. In particular, the rifamycin derivatives induce the hepatic cytochrome P450 enzyme system, resulting in increased metabolism and decreased serum levels (34).

2.1.1 Mycobacteria

Mycobacterium is a genus of family Actinobacteria with over 190 species belonging to a class of Gram-positive bacteria with high guanine and cytosine contents in their DNA. Mycobacteria are normally between 0.2 - 0.6 µm wide and 1.0 - 10 µm long and they are either straight or slightly curved rods in shape (35). Most of the mycobacteria are whitish or cream coloured but some have carotenoid pigments that are bright yellow or orange (36). The cell wall contains high levels of mycolic acids with 3-hydroxy fatty acids (total 60 to 90 carbons) with an invariant C 26 aliphatic side chain at the C2 position (37). The high proportion of mycolic acids causes the mycobacteria cell wall to be highly hydrophobic and acid-alcohol-fast (37). In general, mycobacteria grow relatively slowly and are divided into two groups based on the

growth rate. The rapid growers will show colonies in 4 - 5 days while slow growers show colonies in 7 - 10 days and these are normally pathogen species (38).

2.1.1.1 Non-Pathogenic Mycobacteria

Non-pathogenic mycobacteria species are increasing with more and more new species being identified and described. Some of the non-pathogenic species are used in the biocatalysis field, for instance, to modify steroid nucleus or degrade steroid side-chain to produce pharmacologically active products in the steroid biotransformation process (35).

2.1.1.1.1 Mycobacterium smegmatis

M. smegmatis is a rapidly growing non-pathogenic mycobacterium that is usually used as a model in TB experiments to compare pathogenic *M. tuberculosis* and *M. avium* (39). *M. smegmatis* is a gram-positive bacterium, characterized by an inner cell membrane and a thick cell wall, however, it has some unique qualities that are divergent from most gram-positive bacteria (Sander et al., 2002). *M. smegmatis* contain some similar structural features to *M. tuberculosis*, however, it grows much faster in comparison to the latter (40). *M. smegmatis* is a non-motile obligate aerobic organism which requires unique fatty acid biosynthesis to produce the mycolic acids present in the cell wall (41). The outer lipid bilayer, a thick biological membrane is used to protect the organism by making them intrinsically resistant to many antibiotics, acidic and alkaline compounds. Furthermore, the membrane also serves as an effective permeability barrier (42).

2.1.1.2 Pathogenic Mycobacteria

Most of the pathogenic mycobacteria species attract more attention from researchers than non-pathogenic species due to their threat to public health. *M. tuberculosis*, which causes human

tuberculosis, is the most notorious mycobacteria in this family (43). It is a member of a complex that has at least nine members: *M. tuberculosis sensu stricto*, *M. africanum, M. canetti, M. bovis, M. caprae, M. microti*, *M. pinnipedii*, *M. mungi,* and *M. orygis* (44). It requires oxygen to grow, does not produce spores, and it is nonmotile (44, 45).

Other Pathogenic mycobacteria are *M. leprae which* cause leprosy, a serious disease that causes dreaded damage and lesions to human skin. There also other Pathogenic mycobacteria species that are *M. avium* complex, *M. marinum* (46), *M. ulcerans* (47), *M. chelonae, M. fortuitum* and *M. abscessus* (46), which may also cause infections in human.

2.1.1.2.1 *Mycobacterium ulcerans*

Mycobacterium ulcerans causes Buruli ulcer, a disabling skin infection that is the third most common mycobacterial infection in immunocompetent hosts after tuberculosis and leprosy (48). *M. ulcerans* causes a spectrum of diseases varying from non-ulcerating subcutaneous nodules and large plaque to ulcers that can progress to devastating necrotic lesions (49). A distinctive feature of *M. ulcerans* is the production of a macrocyclic polyketide called mycolactose which plays a critical role in bacterial pathogenicity (49). Although mycolactone is cytotoxic and proapoptotic (47), recent data suggest that it plays a major role in allowing *M. ulcerans* to evade host innate immunity by suppressing inflammatory processes (47).

2.1.1.2.2 *Mycobacterium tuberculosis*

Human tuberculosis is mainly caused by an acid-fast bacterium known as *M. tuberculosis*. The cell envelope of *M. tuberculosis* contains a polypeptide layer, a peptidoglycan layer, and free lipids. Additionally, there is also a complex structure of fatty acids such as mycolic acids that appear glossy (50). The *M. tuberculosis* cell wall contains three classes of mycolic acids: alpha-

keto- and methoxymycolates. The cell wall also contains lipid complexes including acyl glycolipids and other complexes such as free lipids and sulfolipids. There are porins in the membrane to facilitate transport. Beneath the cell wall, there are layers of arabinogalactan and peptidoglycan that lie just above the plasma membrane (51).

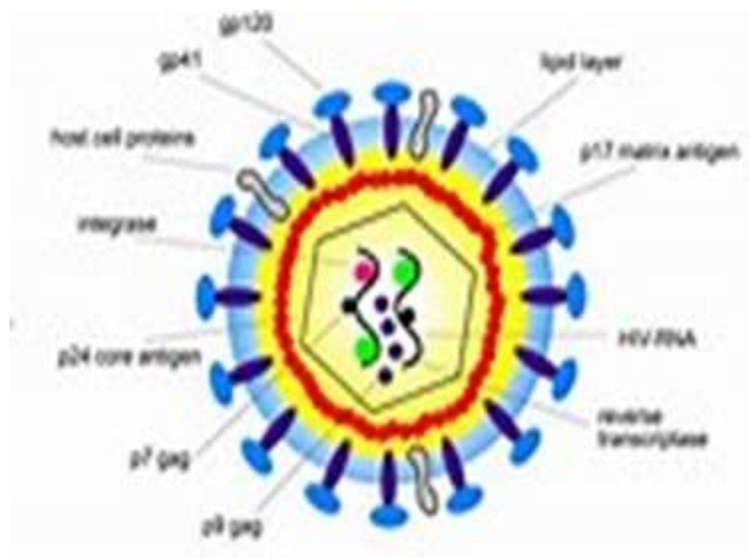
2.1.2 Viruses

Viruses are not cells and they completely dependent on their cellular hosts for the machinery of energy production and synthesis of macromolecules. The virus particle contains only one type of nucleic acid, either Deoxyribonucleic acid (DNA) or Ribonucleic acid (RNA), never both, and differs from non-viral organisms by having two clearly defined phases in their life cycle. In the first phase (the transmission phase) on the outside a susceptible cell, the virus particle is metabolically inert. In the second phase (the reproductive phase) on the inside the cell, the viral genome exploits the metabolic pathways of the host to produce progeny genomes and viral proteins that assemble to form new infectious virus particles called virions. The primary criteria for the delineation of virus families are the kind of nucleic acid that constitutes the genome, either DNA or RNA, with DNA viruses being the predominant group.

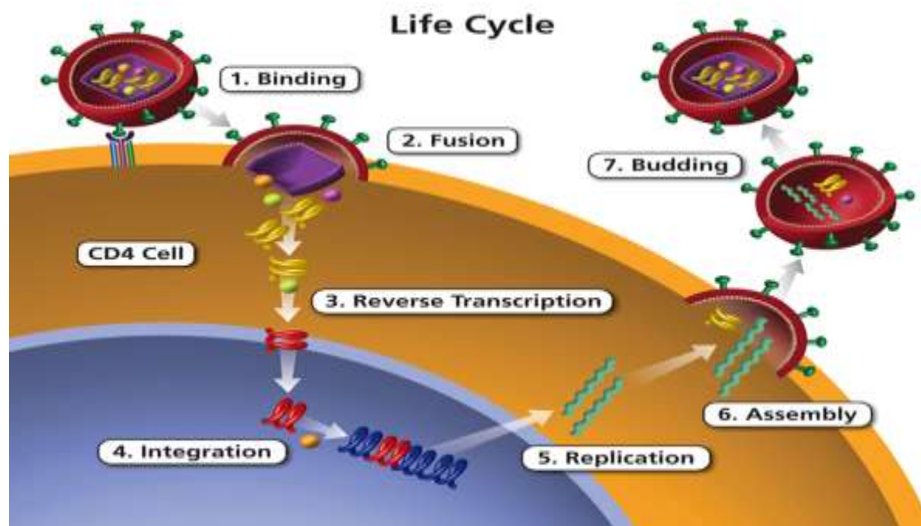
2.1.2.1 Human Immunodeficiency Virus (HIV)

Human Immunodeficiency Viruses (HIV-1 and HIV-2) are RNA viruses, belonging to the family of retroviruses. The genome of retroviruses consists of duplicate copies of positive single-stranded RNA. Once a cell has become infected with a retrovirus the viral genetic information will be transformed from RNA to DNA catalysed by viral enzyme reverse transcriptase. The name retrovirus is derived from this unique event, which is opposite to the normal process where RNA is transcribed from DNA. The virion is almost spherical and is about one ten-thousandth of a millimetre across (ca. 100 nm) (52). The virus is enveloped by a

lipid bilayer that is derived from the infected host cell. The outer surface is studded with surface glycoproteins (gp120) that are anchored to the virus via interactions with the transmembrane protein (gp41). These surface proteins play a crucial role when HIV binds to and enters the host cells. A shell of the matrix protein (p17) lines the inner surface of the viral membrane, and a conical capsid core particle constructed out of the capsid protein (p24) which is located in the centre of the virus. The capsid particle encapsulates has two copies of the viral genome, stabilized by the nucleocapsid protein (p7), and contains three essential virally encoded enzymes: protease (PR), reverse transcriptase (RT), and integrase (IN) (53) as shown in Figure 2.1.



(a) Structure of HIV



(b) HIV life cycle

Figure 2.1: Schematic drawing of the mature HIV-virion and HIV life cycle

2.2 Pathophysiology of Buruli ulcers, Tuberculosis & HIV/AIDS

Studies have shown that HIV infection complicates the management of Buruli ulcers patients. The weakened immune system makes the clinical progression of Buruli ulcer more aggressive, and as a result, the treatment outcomes are generally poor. Similarly, *M. tuberculosis* up regulates HIV-1 replication in acutely or chronically infected T cells or macrophages (54, 55), as well as ex vivo in lymphocytes and alveolar macrophages from HIV patients (56, 57). Furthermore, studies have shown that the depletion of CD4+ T cells as a result of AIDS, contributes to the increased risk of reactivation of latent TB and susceptibility to new *M. tuberculosis* infection, consequently, CD8+ T cells play a part in the control of latent TB (58, 59). Other mechanisms have also shown up-regulation of *M. tuberculosis* entry receptors on macrophages in individuals with HIV (60). Therefore, HIV manipulation of macrophage bactericidal pathways, deregulate chemotaxis, and tip Th1/Th2 balance (61). It has also been revealed that HIV impairs tumour necrosis factor (TNF)-mediated macrophage apoptotic response to *M. tuberculosis* and hence facilitates bacterial survival (62).

2.2.1 Pathophysiology of Tuberculosis

During the 19th century, Tuberculosis (TB) was also known as the White Plague which inflicted indescribable misery on the human race (63). Tuberculosis is a chronic infectious disease caused by an organism called *Mycobacterium tuberculosis* and it is transmitted through coughing, sneezing, or if the person inhales the infected droplet. It can be considered as primary or secondary infection depending on the recovery of the client from the communicable infection (64). According to a study conducted by Knechel, the progression of tuberculosis has several stages: Latent Tuberculosis, Primary Pulmonary Tuberculosis, Primary Progressive Tuberculosis and Extrapulmonary Tuberculosis (65). The most fatal location of infection is the central nervous system and its infection to the bloodstream. Other locations may include the lymphatic system, the bones and joints and at times the genitourinary system (64). The pathophysiology of *Mycobacterium tuberculosis* is presented in Figure 2.2.

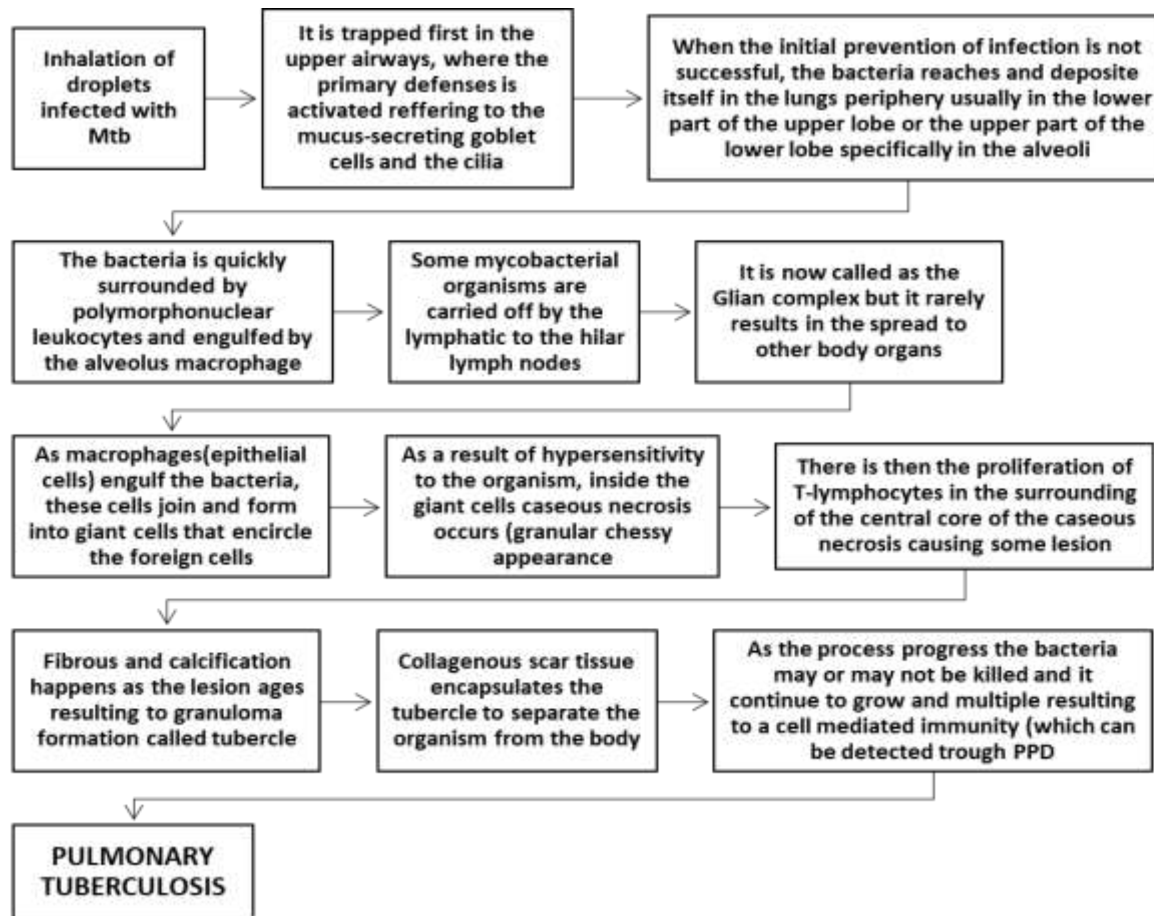
2.2.2 Pathophysiology of Buruli ulcers

Buruli ulcer infection is named after the Buruli district in Uganda, a region from where many of the early cases were described in literature (48). Buruli ulcer often starts as a painless swelling (nodule) and initially present as a large painless area of induration (plaque) or a diffuse painless swelling of the legs, arms or face (oedema). Local immunosuppressive properties of the mycolactone toxin enable the disease to progress with no pain and fever. Without treatment or sometimes during antibiotics treatment, the nodule, plaque or oedema will ulcerate within 4 weeks with the classical, undermined borders. Occasionally, the bones are affected causing gross deformities (47). Buruli ulcer treatment requires a combined antibiotic regimen (rifampicin and streptomycin-clarithromycin) and this is key to prevention of complications that can arise from severe skin ulcerations (47). The World Health Organization (WHO) introduced Buruli ulcer initiative in 1998 and this has increasingly drawn attention to research

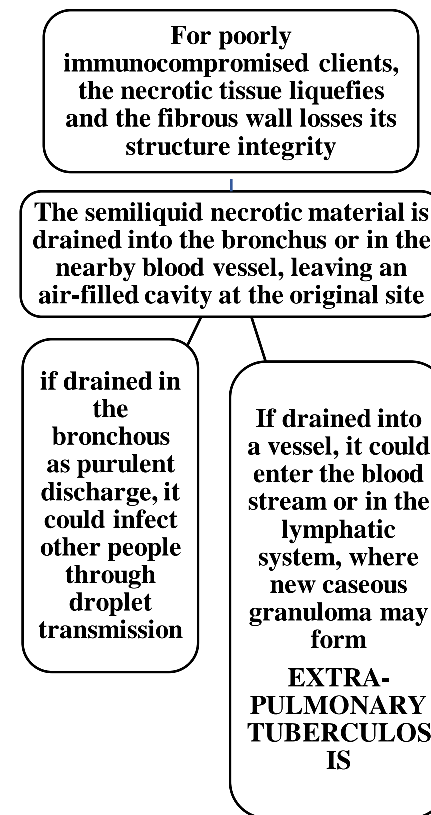
efforts for identification of treatment and control of Buruli ulcer. Schematic diagram of pathophysiology of Buruli ulcers is present in Figure 2.3.

2.2.3 Pathophysiology of Acquired Immune Deficiency Syndrome

Acquired immune deficiency syndrome (AIDS) is caused by the human immunodeficiency virus (HIV). The infection causes progressive destruction of the cell-mediated immune (CMI) system, primarily by eliminating CD4+ T-helper lymphocytes. Decreased immunity leads to opportunistic infections that are triggered by organisms that do not cause infections in healthy individuals. Research has also shown that HIV directly damages certain organs like the brain. And HIV infection passes through a series of steps or stages before it turns into AIDS. These stages include Seroconversion illness, Asymptomatic infection, Persistent generalized lymphadenopathy (PGL), Symptomatic infection and AIDS stage which is characterized by CD4 T-cell count below 200 cells/mm^3. The schematic diagram of pathophysiology of AIDS is presented in Figure 2.4.



(a) Schematic diagram of pathophysiology of Pulmonary tuberculosis



(b) Schematic diagram of pathophysiology of Extrapulmonary tuberculosis

Figure 2.2: Pathophysiology of *Mycobacterium tuberculosis*

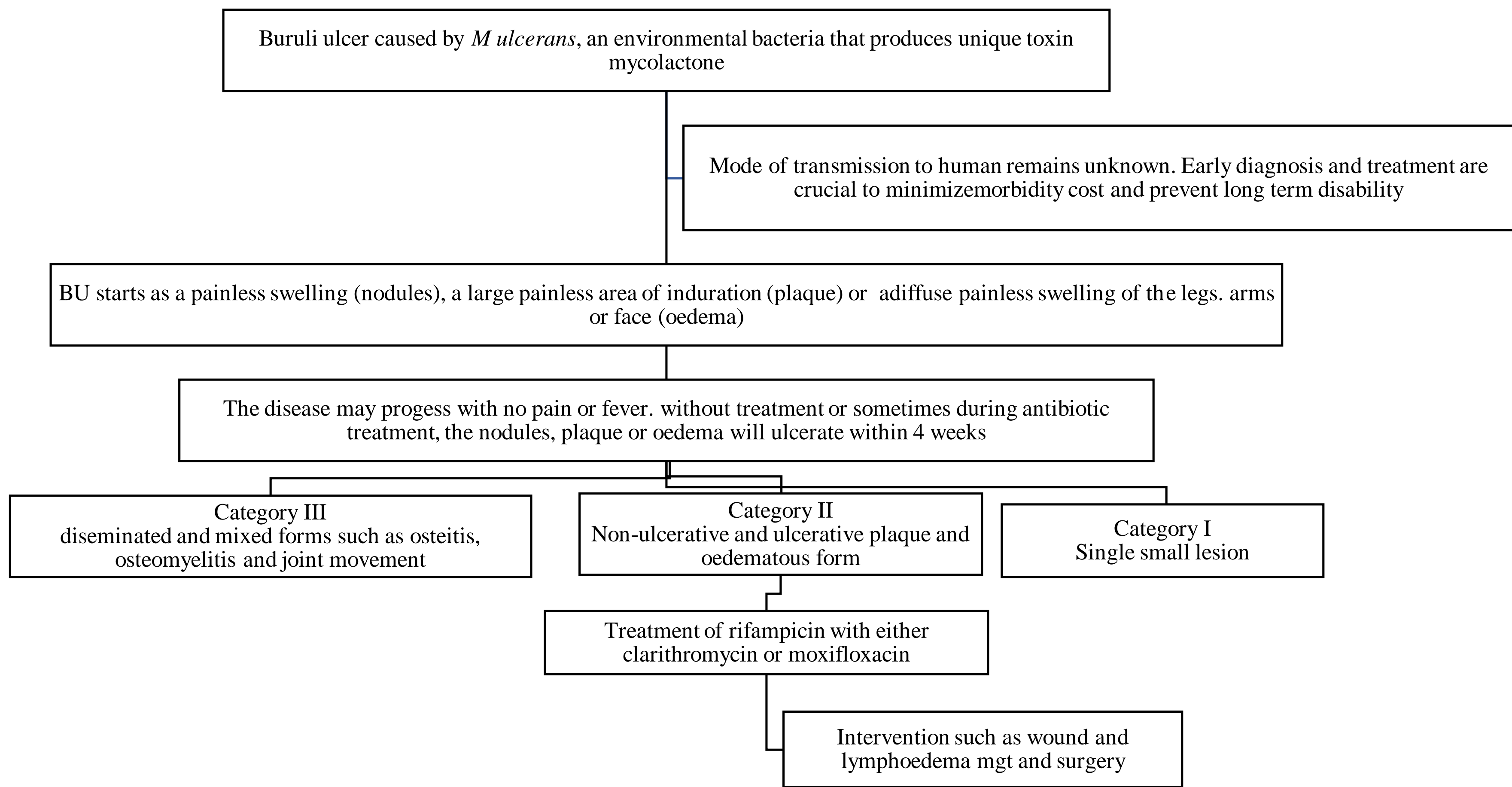


Figure 2.3: Schematic diagram of pathophysiology of Buruli ulcers

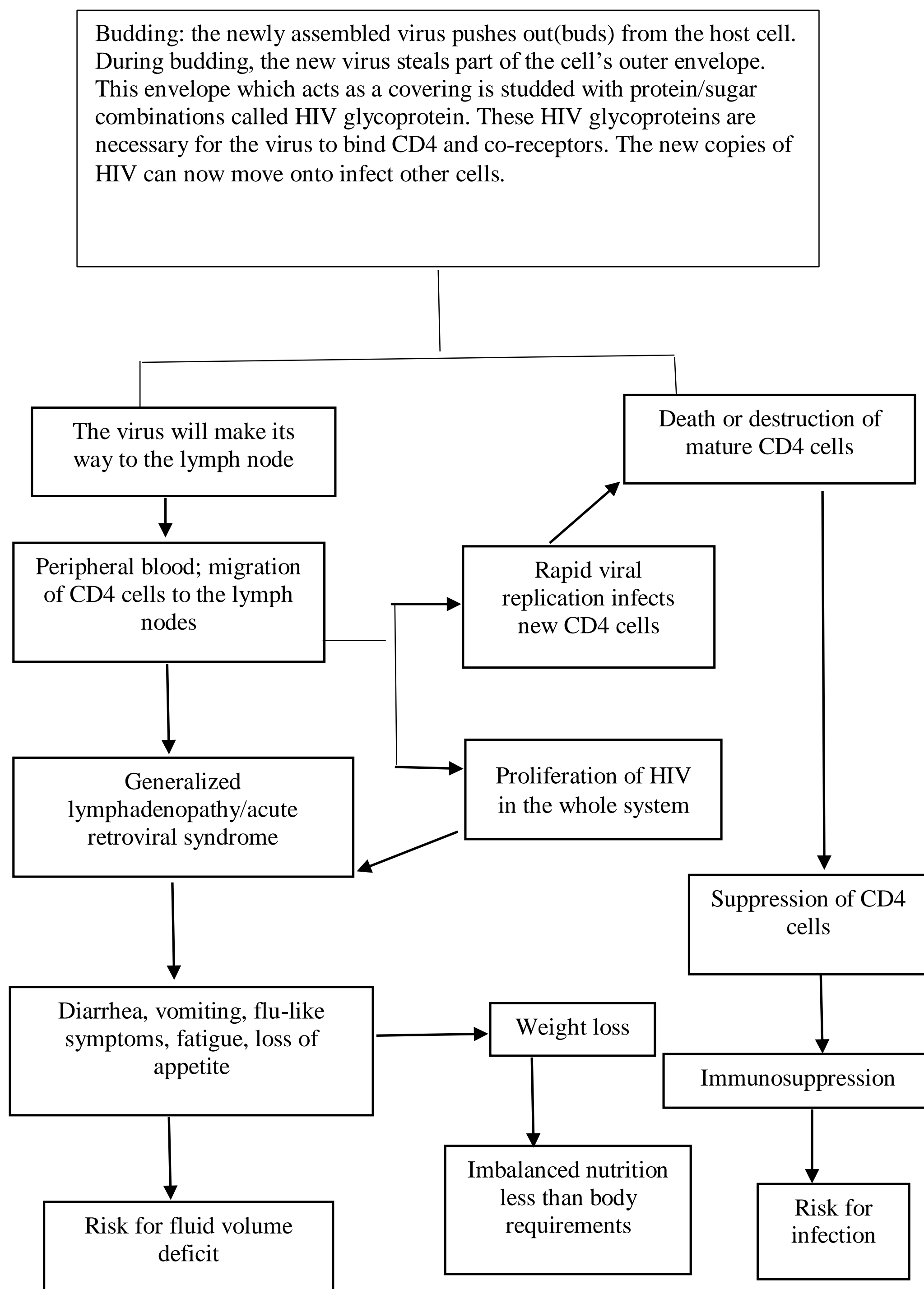


Figure 2.4: Schematic diagram of pathophysiology of Acquired Immune Deficiency Syndrome

2.3 Mechanism of actions of herbal compound

2.3.1 Reactive oxygen species

Reactive oxygen species (ROS) are chemically reactive molecules containing O_2 and represent a broad category of molecules, including a collection of radical [hydroxyl ion (OH-, superoxide ion (O_2 -) nitric oxide (NO-) and non-radical ozone (O_3), singlet oxygen (O_2), lipid peroxide (LOO-), hydrogen peroxide (H_2O_2)] that are oxygen derivatives (66). Oxygen derivatives are normally formed in different reactions and become very reactive (67). Superoxide and peroxide molecules are formed respectively from the reduction reaction of oxygen while the hydroxyl radical is formed by the reaction between superoxide and hydrogen peroxide or reduction of peroxide catalyzed by ferrous ions (68). Superoxide reacts with nitric acid to form peroxinitrite while hydrogen peroxide can react with chloride to form hypochlorite. Reactive nitrogen species (RNS) contain nitrogen and derived from nitric oxide and superoxide (68) as shown in Figure 2.5.

Endogenous ROS are produced in the mitochondria as by-products of normal cellular respiration and their increase is related to defective oxidative phosphorylation (69). Leukocytes are also the source of ROS. Exogenous sources of ROS include chemical compounds as well as pathologies and systemic infections (70).

In the normal state, when ROS/RNS are low in concentration it allows minimal oxidative damage that contributes to physiological signalling through redox reaction (71). However, it could be said that diseases and environmental factors contribute to excess ROS/RNS production resulting in oxidative damage (72) Oxidative Stress (OS) occurs when the reactive species are produced more than the antioxidants. Under OS conditions, cellular components are damaged and various disorders in the organism are evident (73). Reactive oxygen species

are known to induce DNA damage in the form of modification of all bases (primary guanine via lipid peroxyl or alkoxyl radicals), production of base-free sites, deletions, frameshifts, DNA cross-links through covalent binding to MDA, and chromosomal rearrangements (74). Reactive oxygen species can also cause gene mutations such as point mutation and polymorphism (75).

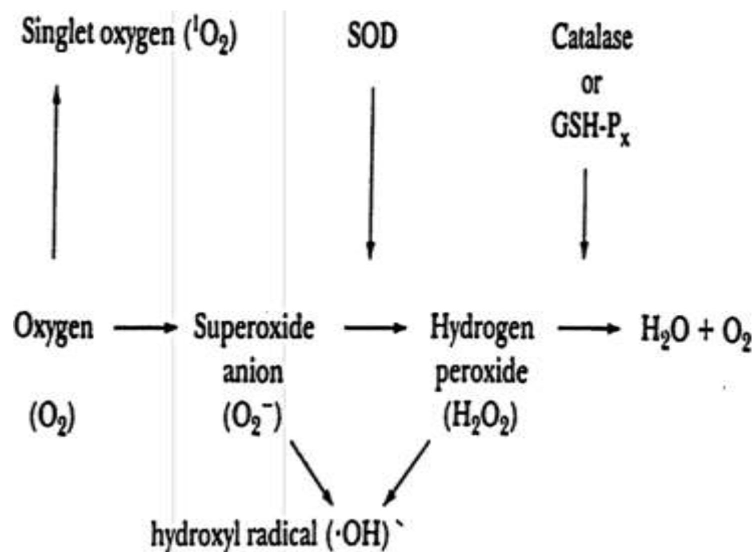


Figure 2.5: Reactive oxygen species

2.3.2 Oxidative stress and DNA damage

Oxidative damage to mitochondrial DNA occurs in all aerobic cells rich in mitochondria and the possible causes of DNA damage include abortive apoptosis, infection and oxidative stress (76). DNA damage is often induced by oxidative stress rather than being the result of other processes such as defective apoptosis and these may accelerate the process of apoptosis, also known as programmed cell death (77). Oxidative stress has also been associated with high frequencies of single and double DNA strand breaks (78).

2.3.3 Reactive oxygen species and antioxidants

An antioxidant is a molecule that inhibits the oxidation of other molecules by controlling or preventing excess free radicals which are ROS and RNS (79). Antioxidant defence system mechanism includes three levels of protection: prevention, interception and repair. For prevention, Antioxidant fight at the first line against an oxidative insult. This can be an antioxidant enzyme with metal ions, iron and copper ions co-factor binding to the chain reaction of free radicals to prevent its propagation (79). The second line of defence, the interception has to deactivate the process of free radicals' formation and conducts to non-radical molecules. During free radical-induced damage, antioxidants repair the damages. Oxidative stress plays a vital role in the aetiology and progression of major human degenerative diseases which has triggered enormous and worldwide interest in endogenous and exogenous antioxidants as shown in Figure 2.6 (80). Endogenous antioxidants include both enzymatic and non-enzymatic antioxidants while exogenous antioxidants are supplementary substances mostly found in plants.

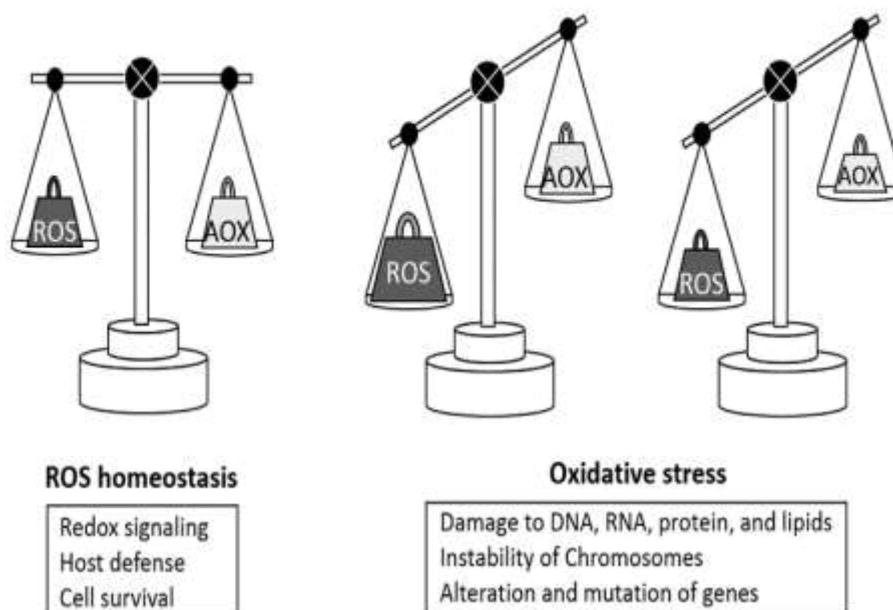


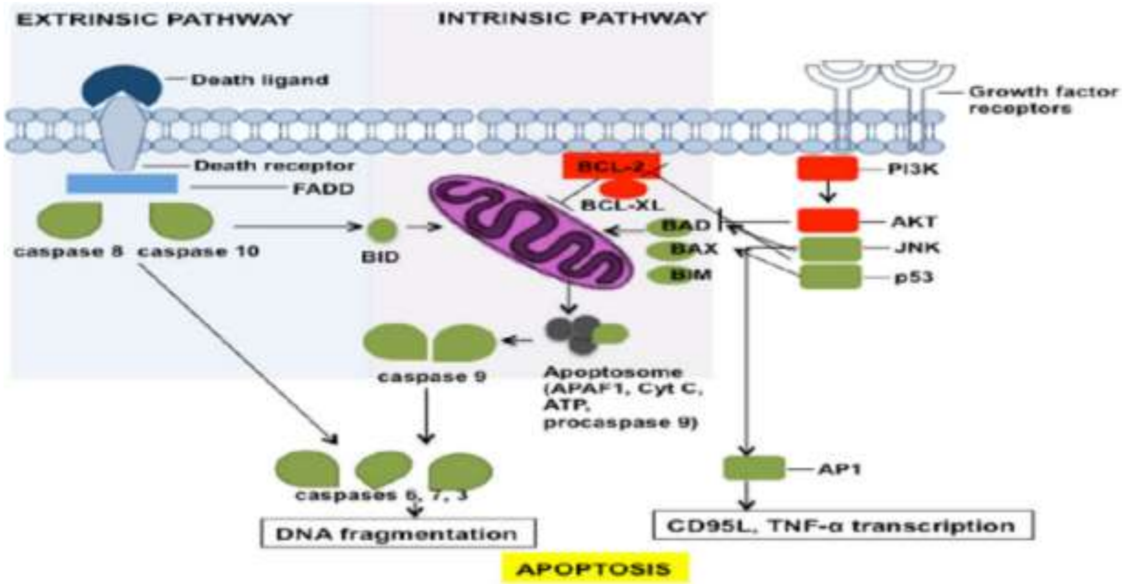
Figure 2.6: ROS homeostasis and oxidative stress

2.3.4 Oxidative stress and apoptosis

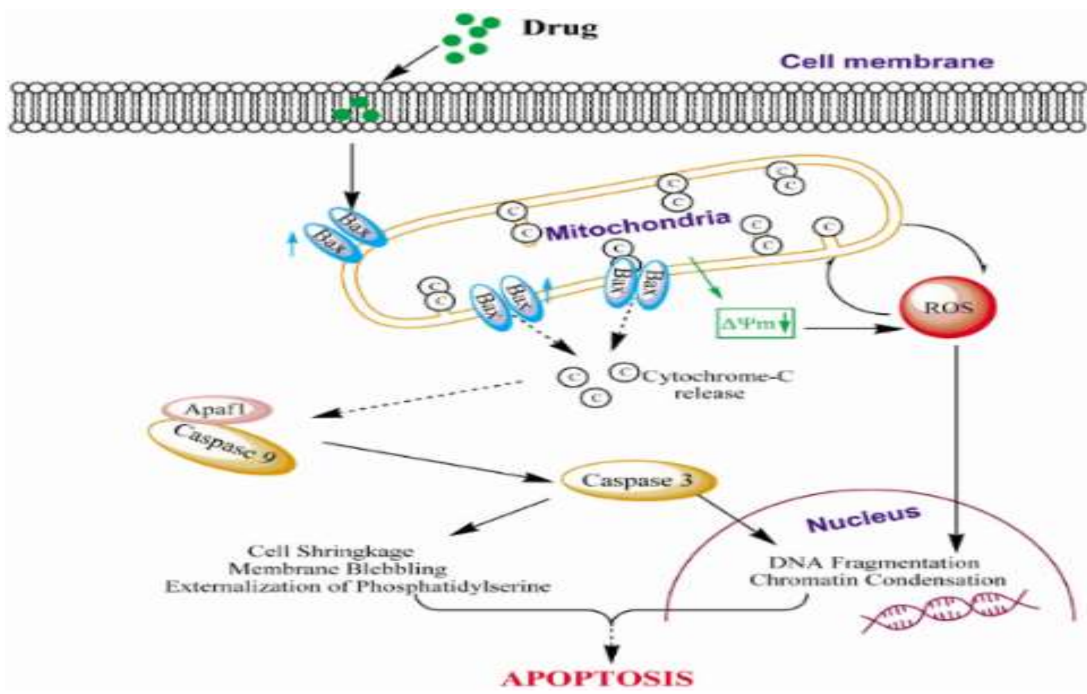
Apoptosis is a non-inflammatory response to tissue damage characterized by a series of morphological and biochemical changes and is important in both physiological and pathological conditions (81). Mitochondria play a crucial role in the mechanism of apoptosis (82). The integrity of the mitochondria is established by the presence of cytochrome c in the inner membrane space (82). Two pathways (intrinsic and extrinsic) exist in the process of caspase activation in mammalian cells as shown in Figure 2.7 (83).

The intrinsic pathway, Bax forms homo-dimers in the presence of an apoptotic signal, opening a channel that translocates cytochrome c from the intermembrane space to the cytoplasm. The proapoptotic BcL-2 interfere with Bax function by forming a heterodimer with Bax, closing the channel and inhibiting cytochrome c translocation. In the cytosol, cytochrome c bind to apoptotic protease activating factor 1 (Apaf- 1) to form a multiprotein complex named apoptosome (84). The fusion of this multiprotein complex named apoptosome results in the activation initiator caspase 9 which activates effector caspase 3, 6 and 7 (85).

The extrinsic pathway is activated by cytokines and triggered by the ligation of death receptors (DR). Death receptor stimulation, in turn, leads to receptor trimerization, recruitment of adaptor molecules such as Fas-associated protein with death domain (FADD) and activation of the initiator caspase-8, which propagates the death signal to effector caspases such as caspase-3 (86).



(a)

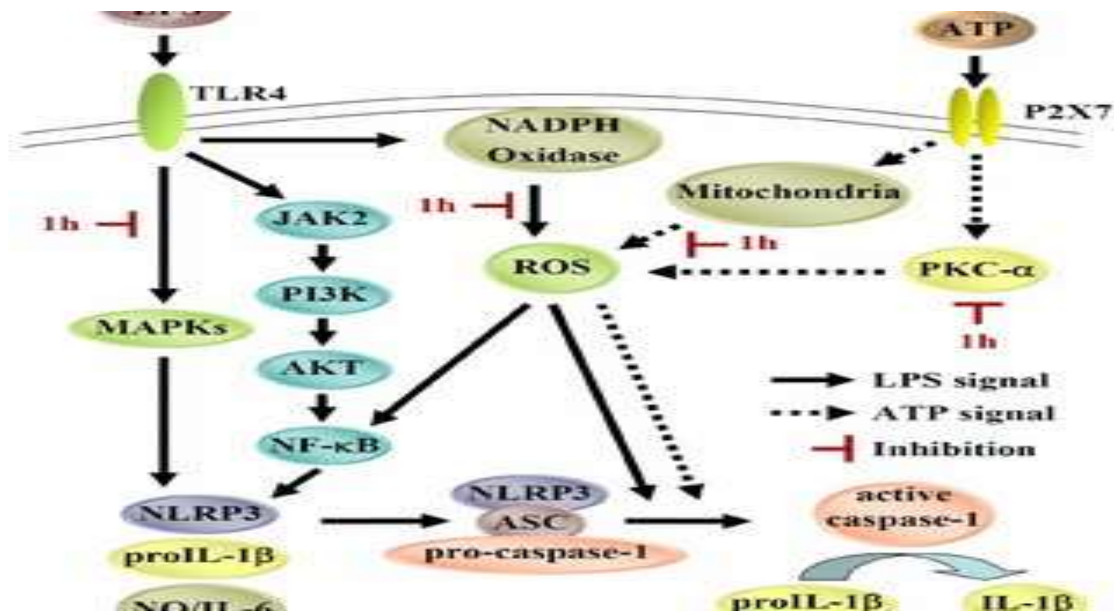


(b)

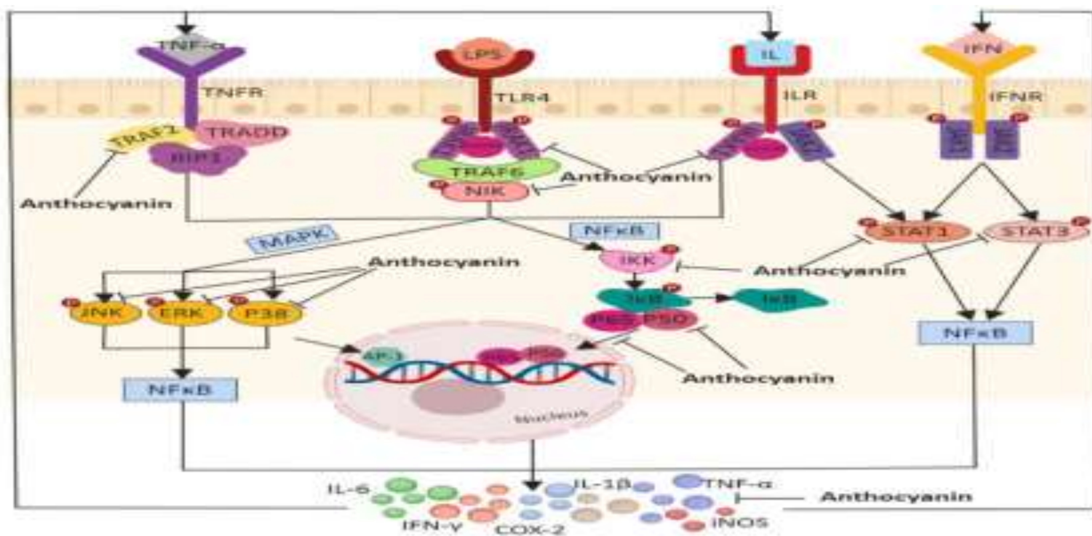
Figure 2.7: Apoptosis pathways

2.3.5 Inflammation

Inflammation is the immune system response to fight against external pathogens or internal injury. The inflammatory response is triggered by innate immune receptors that recognize pathogens and damaged cells. Inflammation normally begins at a localized area, and then spreads rapidly to the periphery accompanied with inflammatory cytokines released in the circulation system, particularly IL-1, IL-6 and TNFα as shown in Figure 2.8 (87). The major function of cytokines is to stimulate immune cell proliferation and differentiation, which leads to termination of invading pathogens or repairing damaged tissue. The cytokines perform these functions by activating downstream signalling pathways through interaction with their receptors on the cell membrane. Some of these cytokines exert their function as pro-inflammatory such as IL-1, IL-6 and TNFα, whereas others have anti-inflammatory activities, for example, IL-4 and IL-13. The inflammatory pathway consists of inducers, sensors, mediators and target tissues. The inducers initiate the inflammatory response and are detected by sensors such as Toll-like receptors (TLRs) expressed on specialized cells such as macrophages, dendritic cell and mast cells. When activated, they secrete inflammatory radiators such as cytokine TNF alpha, IL-1B, chemokines-CCL2, CXCL-8, bioactive amine e.g. histamine, bradykinin and inflammatory lipids.



(a)



(b)

Figure 2.8: Inflammatory pathway

2.3.6 TNF-mediated responses

The proinflammatory cytokine tumour necrosis factor (TNF) plays an important role in diverse cellular events such as septic shock, induction of other cytokines, cell proliferation, differentiation, necrosis and apoptosis (88). TNF-mediated responses involve the binding of

TNF to TNF-R1 which leads to the recruitment of TRADD (TNF-R1-associated death domain protein) into the receptor complex and this can activate three pathways. The first pathway involves activation of NF-kB which result in TRADD recruiting TNF receptor-associated factor (TRAF2) and the death domain, Receptor interacting protein (RIP). TRAF2 recruit's protein kinase IKK which is then activated by RIP. IKK phosphorates IKβα which release NF-kB to translocate to the nucleus to act as a transcriptional activator of genes involved in cell survival, proliferation, inflammation and anti-apoptotic factors (89). The second pathway involves the activation of Mitogen-activated Protein Kinase (MAPK). TNF induces activation of p38-MAP kinase signalling through activation of ASK1 and MEKK1 leading to phosphoration of MKK7 which activates Jun-NH2 terminal kinase (JNK), which is translocated to the nucleus and activates the AP-1 transcription factor to induce cell differentiation and proliferation genes. The third pathway involves the induction of cell death signalling. TNF induces cell death through TRADD recruiting FAS associated protein with death domain (FADD) which recruits caspase 8, a protease that activates caspase 3 leading to apoptosis as shown in Figure 2.9 (88).

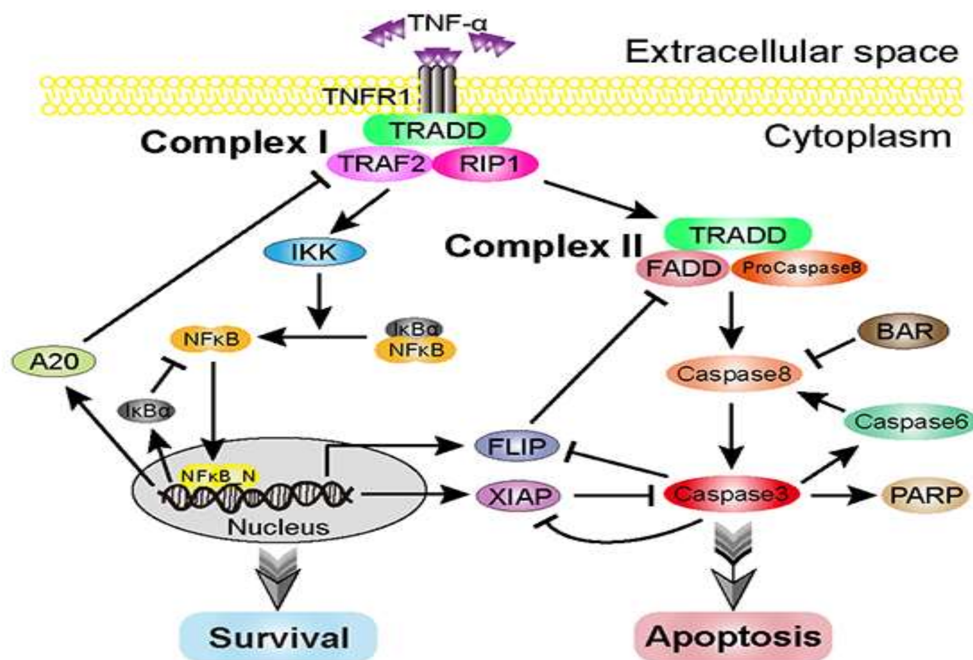


Figure 2.9: TNF mediated pathways

2.4 Resistance to Drugs

2.4.1 HIV and TB Drug resistance

The emergence of multidrug-resistant strains, which are both difficult and very costly to treat, poses an additional public health hazard and further roadblock in effective control of infectious diseases such as TB and HIV as shown in Figure 2.10. HIV multi-class drug resistance typically occurs when a virus that is resistant to one drug acquires resistance to another drug and it is possible for a virus acquires multiple drug-resistance mutations at the same time (90). Antiretroviral therapy (ART) generally works well in keeping the virus suppressed and the patient healthy as long as the virus is not resistant against the drugs used (90). Drug-resistant HIV strains can be transmitted from one patient to another and the newly infected patient may carry a drug-resistant virus even though they are not yet used antiretroviral drugs as shown in Table 2.1 (91).

Multidrug-resistant tuberculosis (MDR-TB) is defined as tuberculosis which shows high-level resistance to both isoniazid and rifampicin, with or without resistance to other anti-TB drugs (92). However, MDR-TB treatment currently requires four or five drugs and at least isoniazid (INH) and rifampin (RIF), often with considerable side effects, for at least 18 months (93). Resistance to isoniazid is due to mutations at one of two main sites, in either the katG or inhA genes (94) while resistance to rifampicin is nearly always due to point mutations in the rpo gene in the beta subunit of DNA-dependent RNA polymerase as shown in Table 2.2 (92).

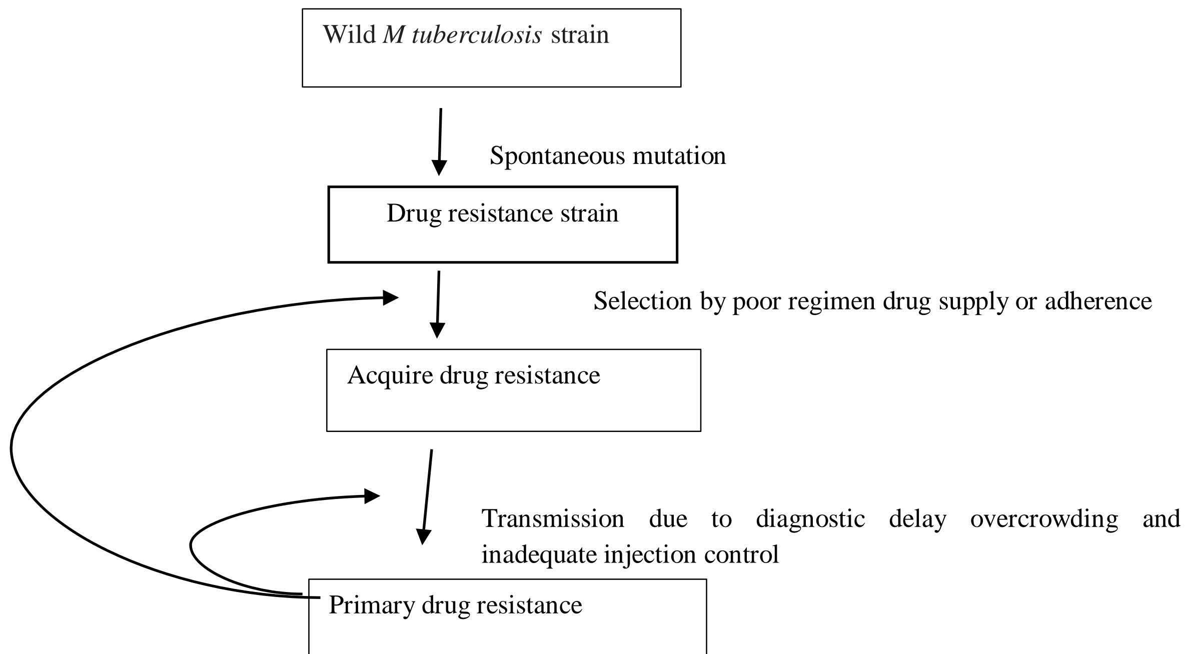


Figure 2.10: Drug resistance scheme

Table 2.1: FDA approved *Mycobacterium tuberculosis* drugs, Mechanism of action, gene involved in drug resistance**

Drug (year of discovery)	MIC ug/ml	Gene(s) involved in resistance	Gene function	Role	Mechanism of action	Mutation frequency
Isoniazid (1952)	0.02–0.2	katG inhA	Catalase-peroxidase Enoyl ACP reductase	Pro-drug conversion Drug target	Inhibition of mycolic acid biosynthesis and other multiple effects	50–95 8–43
Rifampicin (1966)	0.05–1	rpoB	β subunit of RNA polymerase	Drug target	Inhibition of RNA synthesis	95
Pyrazinamide (1952)	16–50 (pH 5.5)	pncA	Nicotinamidase /pyrazinamidase	Pro-drug conversion	Depletion of membrane energy	72 –97
Ethambutol (1961)	1–5	embB	Arabinosyl transferase	Drug target	Inhibition of arabinogalactan synthesis	47–65
Streptomycin (1944)	2–8	rpsL rrs gidB	S12 ribosomal protein 16S rRNA rRNA methyltransferase (G527 in 530 loop)	Drug target Drug target Drug target	Inhibition of protein synthesis	52-59 8–21
Amikacin/ kanamycin (1957)	2–4	Rrs	16S rRNA 16S rRNA	Drug target	Inhibition of protein synthesis	76
Capreomycin (1960)		tlyA	2'-O-methyltransferase			
Quinolones (1963)	0.5–2.5	gyrA gyrB	DNA gyrase subunit A DNA gyrase subunit B	Drug target	Inhibition of DNA gyrase	75–94
Ethionamide (1956)	2.5–10	etaA/ethA inhA	Flavin monooxygenase	Pro-drug conversion Drug target	Inhibition of mycolic acid synthesis	37 56
PAS (1946)	1–8	thyA	Thymidylate synthase	Drug activation	Inhibition of folic acid and iron metabolism	36

**This information is based on the Food and Drug Administration (FDA) http://www.fda.gov

Table 2.2: FDA approved ARV drugs used in the treatment of HIV infections and their potential side-effects**

<table>
<tr><th>Brand</th><th>Generic Name</th><th>Dosage administration</th><th>Mechanism of Action</th><th>Adverse Reaction</th><th>Approval</th></tr>
<tr><td colspan="6">Multiclass combination</td></tr>
<tr><td>Atripla</td><td>Efavirenz, fumarate emtricitabine tenofovir disoproxil</td><td>One tablet once daily taken orally on an empty stomach</td><td></td><td>Lactic acidosis/severe hepatomegaly with steatosis post treatment exacerbation of hepatitis B, diarrhoea, nausea, fatigue</td><td>12-Jul-06</td></tr>
<tr><td colspan="6">Nucleoside Reverse Transcriptase Inhibitors (NRTIs)</td></tr>
<tr><td>Combivir</td><td>Lamivudine and zidovudine</td><td>One tablet twice daily</td><td rowspan="3">-modification of the RT enzyme allowing it to discriminate between NRTIs and analog substrates, leading to a reduced incorporation of the NRTIs into the DNA chain
-eliminating the NRTI incorporated into the viral DNA</td><td>Hematologic toxicity, lactic acidosis, exacerbation of hepatitis B, diarrhoea, nausea, fatigue, headache,</td><td>27-Sep-97</td></tr>
<tr><td>Retrovir</td><td>Zidovudine, Azidothymidine</td><td>Tablets, capsules and syrup 600mg/day</td><td>Hematologic toxicity, myopathy, lactic acidosis, headache, malaise, nausea, anorexia, vomiting,</td><td>19-Mar-87</td></tr>
<tr><td>Zerit</td><td>Stavudine</td><td>Dose of 30-40mg taken every 12hrs</td><td>Lactic acidosis/severe hepatomegaly with steatosis, pancreatitis, headache</td><td>24-Jun-94</td></tr>
<tr><td colspan="6">Nonnucleoside Reverse Transcriptase Inhibitors (NNRTIs)</td></tr>
<tr><td>Viramune XR</td><td>Nevirapine</td><td>One tablet daily</td><td>prevent HIV from using reverse transcriptase to make copies of itself,</td><td>Life-threatening (including fatal) hepatotoxicity and skin reaction</td><td>25-Mar-11</td></tr>
<tr><td colspan="6">Protease Inhibitors (PIs)</td></tr>
<tr><td>Prezista</td><td>Darunavir</td><td>Dose of 800mg taken with ritonavir 100mg once daily with food</td><td>PIs prevent cleavage of the two proteins Gag and GagPol by binding to the active site of the PR.</td><td>Drug-induced hepatitis, skin reaction, new onset diabetes mellitus, fat redistribution, immune reconstitution syndrome</td><td>23-Jun-06</td></tr>
<tr><td colspan="6">Fusion inhibitors</td></tr>
<tr><td>Fuzeon</td><td>Enfuvirtide</td><td>Dose of 90mg(1ml) injected twice daily</td><td>block the gp41-mediated fusion of the HIV-1 and CD4+ T-cell membrane</td><td>Injection site reaction, hypersensitivity, immune reconstitution syndrome</td><td>13-Mar-03</td></tr>
<tr><td colspan="6">Entry Inhibitors – CCR5 co-receptor antagonist</td></tr>
<tr><td>Seizentry</td><td>Maraviroc</td><td>Tablet 150-600mg taken twice daily</td><td>block the binding of the gp120 to the co-receptors CCR5 or CXCR4</td><td>Hepatotoxicity, immune reconstitution syndrome</td><td>6-Aug-07</td></tr>
<tr><td colspan="6">HIV Integrase strand transfer inhibitors</td></tr>
<tr><td>Isentress</td><td>Raltegravir</td><td>Dose 400mg film-coated tablet taken twice daily</td><td>prevent the virus from incorporating its DNA into the host genome</td><td>Severe potentially life threatening and fatal skin reaction, immune reconstitution syndrome</td><td>12-Oct-07</td></tr>
</table>

**This information is based on the Food and Drug Administration (FDA) http://www.fda.gov

CHAPTER 3: MATERIALS AND METHODS

3.1 Ethnobotanical Survey: Review of medicinal plants used to treat HIV/AIDS and TB

3.1.1 Search criteria

The present study is a review of Malawian medicinal plants reported to possess anti-HIV activity. Articles published in both national and international journals were considered. Articles published online (1990-2019) were selected from the national databases and international databases (Google Scholar, PubMed, MEDLINE, Scopus, Cochrane Library, and Science Direct). The references of the identified articles were searched for additional sources of information. The used keywords were Traditional medicinal plants with anti-HIV activity, Malawian herbal plants, Anti-HIV activity, Medicinal plants and AIDS, and these were searched electronically. Firstly, research article titles were analysed to assess its appropriateness with prominence being given to plant species, phytocompounds and stated mode of action. Medicinal plants with anti-HIV activities from other African countries provided valuable information. The medicinal plants identified from this search were further compared with published and unpublished ethnobotanical survey data and database on Flora of Malawi, "Chewa Medical Botany by Brian Morris" and "Useful Plants of Nyasaland by Jessie Williamson" and duplicated articles were omitted. Each article in English was independently screened.

3.1.2 Inclusion and exclusion criteria

The search output evaluations were performed in four steps. Firstly, literature information on the plant species under study should indicate that it is used to cure infectious diseases or recognised HIV infection-related symptoms such as weight loss, skin rashes, body malaise and diarrhea by traditional healers. Secondly, plants belonging to the same genus with popular

traditional use and anti-HIV activity with known in vitro assay. Thirdly, the plant should have records information indicating that it was identified by a Botanist. Lastly, suitable studies were critically inspected for inclusion in the review based on stated inclusion and exclusion criteria. The inclusion criteria were 1- published articles or thesis reporting on the anti-HIV activity of medicinal plants, 2- details of the study design of anti-HIV assay used, 3- the studied plants should have displayed anti-HIV activity.

3.1.3 Methodological Appraisal

The PRISMA checklist (2009) was used to conduct the review as shown in Figure 1

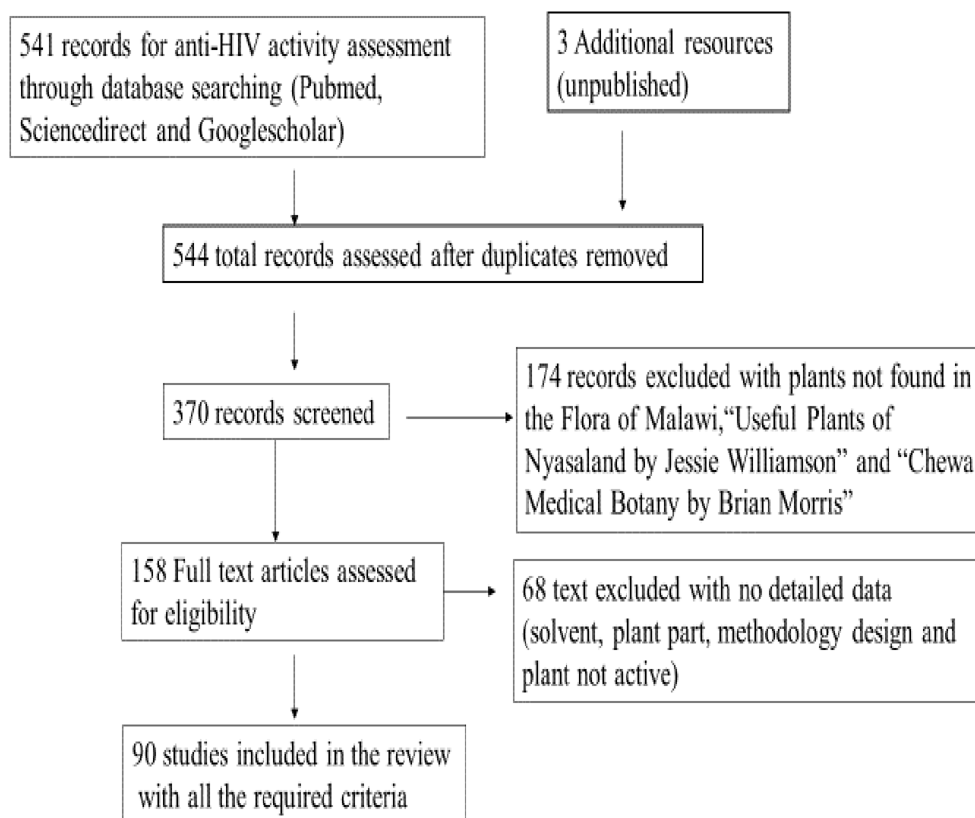


Figure 3.1: Flow chart of the literature search on medicinal plants with proven anti-HIV potential

3.2 Sample collection and preparation

3.2.1 Sample collection

Seven medicinal plants namely, *Aeschynomene nyassana* Taub., Voucher No. 129, B. Morris, Zomba (15°15′36.9″S and 35°19′38.7″E), *Euphorbia whyteana* Baker f. Voucher No. 5770, J.D. Chapman, Mulanje (15°54′54.2″S and 35°21′31.4″E), *Euphorbia cooperi,* var. calidicola, Zomba (15°15′36.9″S and 35°19′38.7″E), *Ericae milanjiana* Bolus, Voucher No. 6167, O. Hillard, Mulanje (15°54′54.2″S and 35°21′31.4″E), *Rhus acuminatissima* (15°54′54.2″S and 35°21′31.4″E), *Flueggea virosa* (Roxb. Ex Willd.) Voigt, Dedza (14°07′33.2″S and 34°10′28.4″E) and *Phyllanthus amarus* (16°02'0.67" S and 34°48'3.28”E) were collected from the Central and Southern part of Malawi with permission obtained from Malawi Traditional Healers Umbrella Organisation and the Forestry Departments. These medicinal plants were further identified by Mr Edwin Kathumba at the National Herbarium and Botanical Gardens of Malawi and voucher specimens deposited in the same herbarium. Two medicinal plants, *Ericae milanjiana and Euphorbia whyteana* were collected at higher altitude of rocky Mulanje mountain hence the need to assess heavy metals presence in this study.

3.2.2 Preparation of crude extracts, fractions and compounds

The collected medicinal plants were shade-dried at room temperature and then grounded into a fine powder. The powdered materials were further dissolved in methanol/ethyl acetate/dichloromethane/water twice for 48 hours period. The resultant mixtures were filtered using a vacuum system and the filtrates concentrated using Rota vapour (Buchi B-100). The crude extracts were then dried at room temperature, weighed and stored in a closed container for future use. For the fractions, 5 grams of methanol/ethyl acetate/dichloromethane/water extracts were fractionated using alkaloid extraction scheme with solvents such as n-hexane and dichloromethane to obtain *A. nyassana* ANX, *E. milanjiana* EM21, *A. nyassana* A9, *E.*

whyteana W1, *E. whyteana* WX fractions as shown in Figure 3.2. While for the compounds, methanol/ethyl acetate/dichloromethane/water extracts (20 g) were subjected to column chromatography (Si gel 70–230 mesh) eluting with hexane (1.5 L) then with hexane-dichloromethane (9:1, 4:1, 7:3, 3:2, 1:1, 3:7; 1.5 L each) and dichloromethane-ethyl acetate (9:1, 4:1, 7:3, 3:2, 1:1, 3:7; 1.5 L each) finally with ethyl acetate-methanol 5%, collecting fractions of 500 mL each as shown in Figure 3.3. For antimicrobial analysis, the resultant compounds were dissolved in 100 % Dimethyl sulfoxide (DMSO)/water before being diluted in media to make the final concentration exposed to cells less than 0.1 % (v/v).

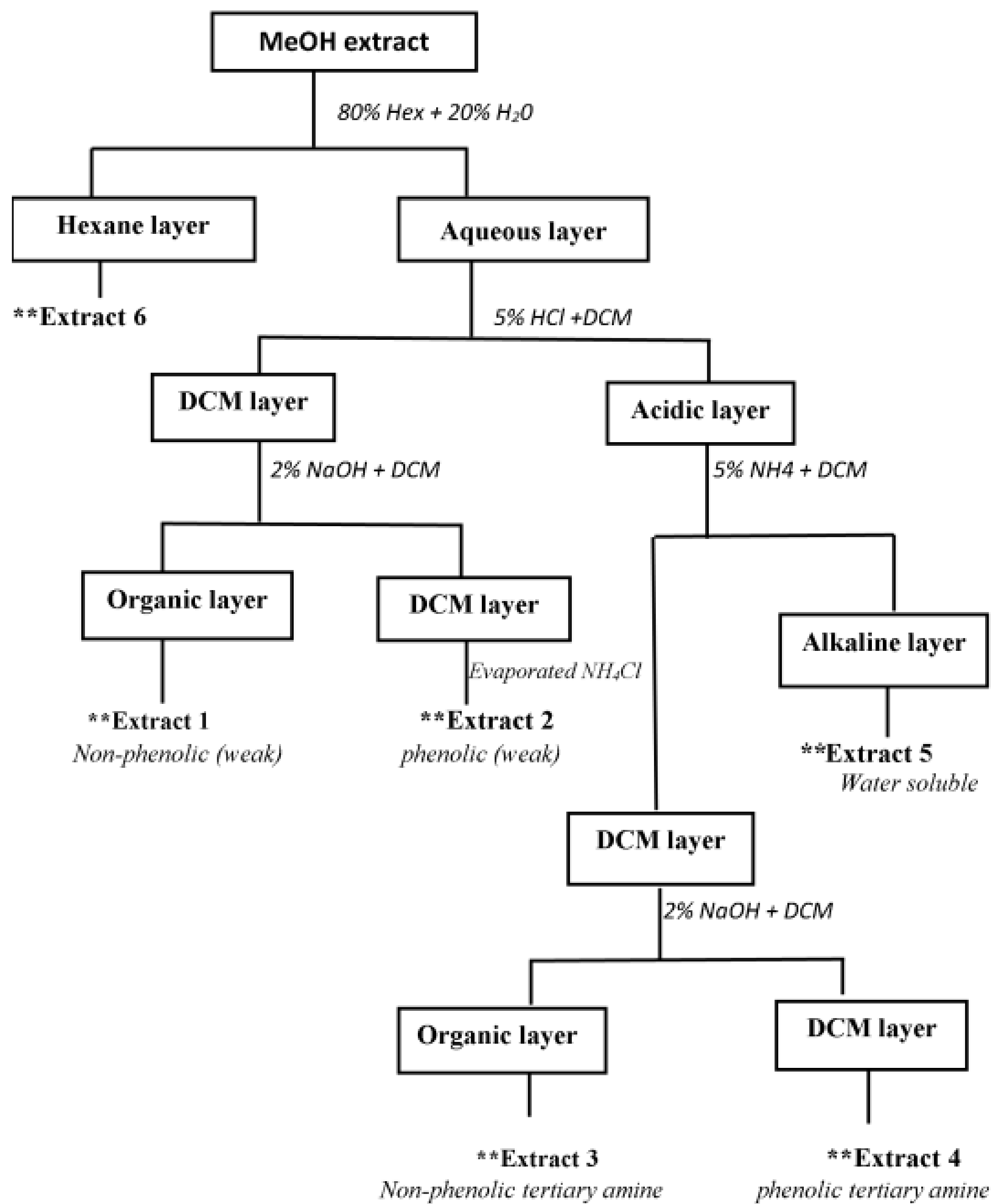
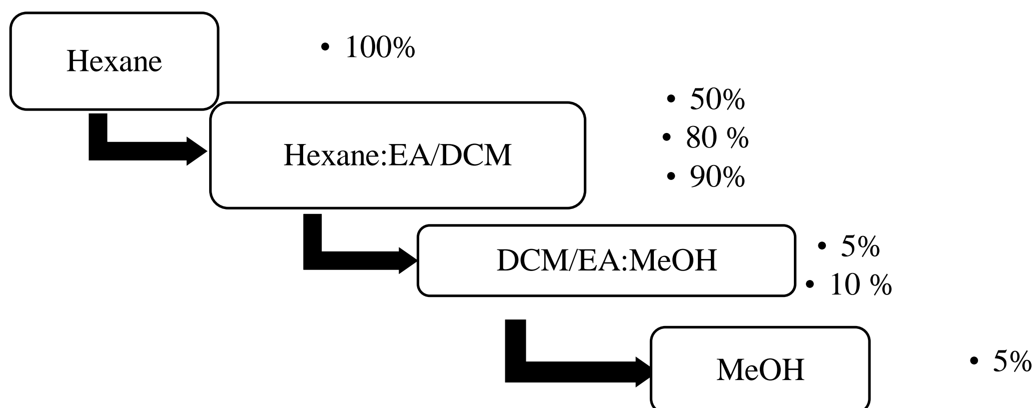


Figure 3.2: Extraction scheme for alkaloids



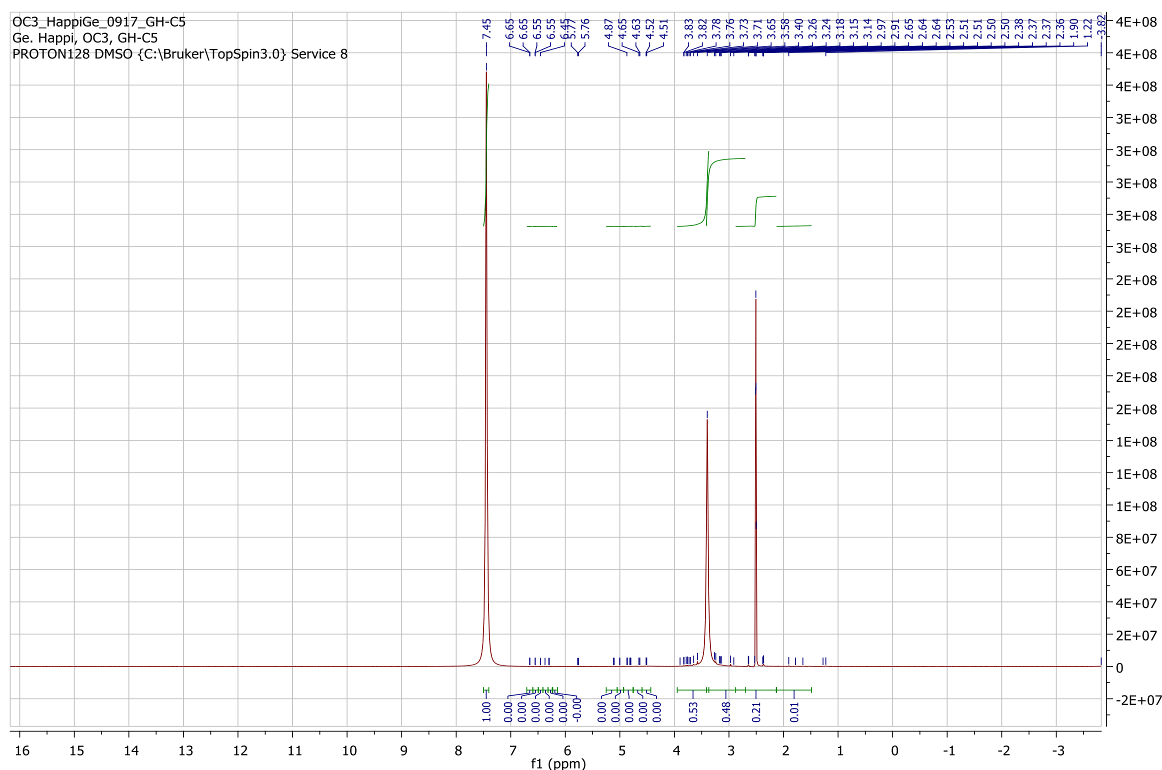
(a)

(b)

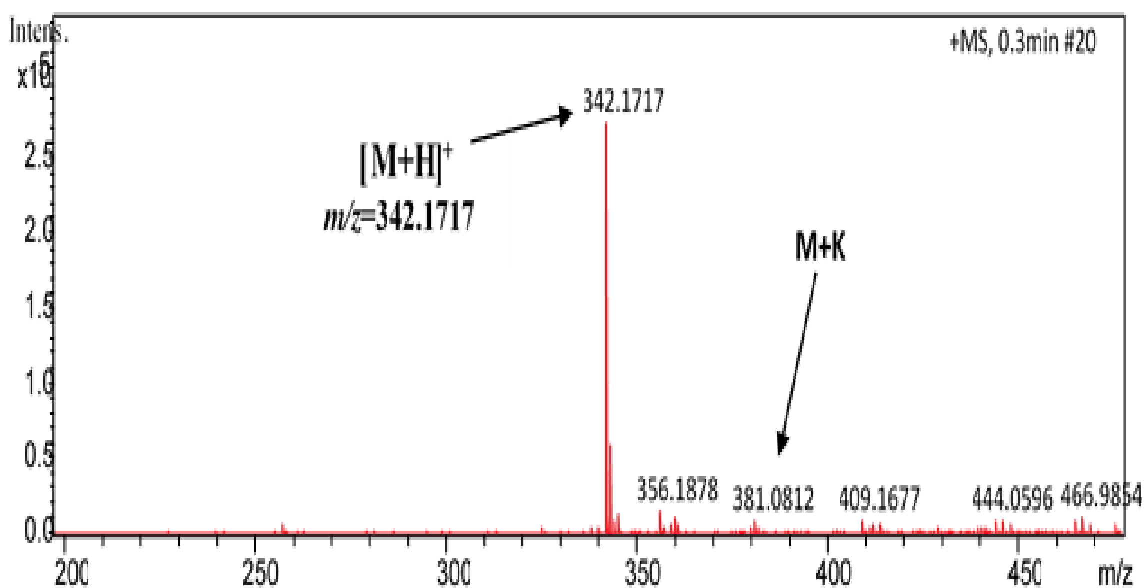
Figure 3.3: Column chromatography protocol and set up

Compound 1: Cyclanoline (Figure 3.5), greyish in colour was identified by direct comparison of the spectroscopic data with those published literatures. Mass spectra (ESI-MS) were obtained with a Thermo-Finningan LCD DECA mass spectrometer and HRESIMS spectra

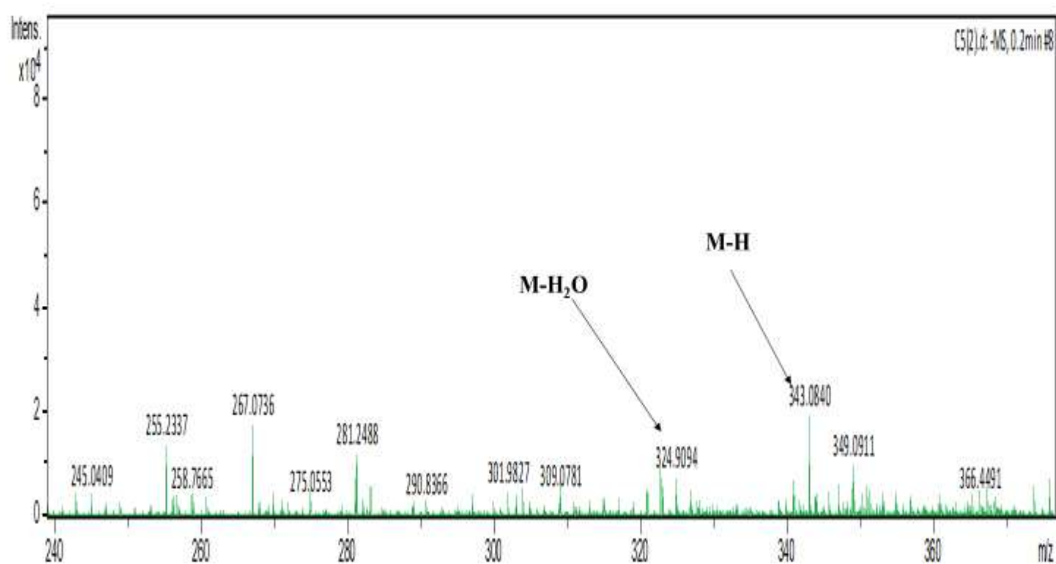
were measured with a FTHRMS-Orbitrap (Thermo-Finnigan) mass spectrometer. In the ESI positive ion mode, the analysis yielded a molecular ion peak at *m/z*= 342.1717[M+H] and a fragment ion peak at *m/z*=381.0812[M+K]. This was supported in the ESI negative ion mode showing fragment ion peak at *m/z*=343.0840[M-H] and fragment ion peak as a result of a loss of water molecule at *m/z*=324.9094[M-H_2O] as shown in Figure 3.4. ^{1}H NMR (500 MHz, DMSO) showed integration peaks δ 6.65 (d, J = 2.5 Hz), 6.55 (d, J = 1.8 Hz, 1H), 6.30 (d, *J* = 2.9 Hz, 3H), 6.45 (s), 5.11 (s), 5.00 (d, *J* = 1.0 Hz, 2H), 4.64 (d, J = 9.0 Hz), 4.51 (d, J = 6.1 Hz), 3.70(s), 3.67 (dd, J = 54.1, 23.9 Hz), 3.40 (s), 3.15 (d, J = 6.3 Hz), 3.13 (dd, *J* = 13.6, 8.0 Hz), 2.94 (d, J = 29.5 Hz) as shown in Table 3.1.



(a) ^{1}H NMR spectrum



(a) LCMS ESI positive ion mode



(b) LCMS ESI positive ion mode

Figure 3.4: The mass spectrum of Cyclanoline. The molecular ion (M+) is at 342.1717 m/z and the base peaks ESI negative ion mode at 324.9094 m/z is due to loss of water molecules and in ESI positive ion mode at 381.0812 is due to gain of potassium

Table 3.1: [1]H proton Data of Cyclanoline

C	δ H ppm
1	5.11 (s)
2	
3	3.67 (dd, J = 54.1, 23.9 Hz) 3.40
4	3.15 (d, J = 6.3 Hz) 2.94 (d, J = 29.5 Hz)
5	6.45 (s)
6	
7	
8	6.55 (d, J = 1.1 Hz, 5H)
9	3.40 (s) 3.13 (dd, *J* = 13.6, 8.0 Hz)
10	6.65 (d, J = 2.5 Hz)
11	6.30 (d, J = 4.2 Hz, 5H)
12	
13	
14	4.64 (d, *J* = 9.0 Hz) 4.51 (d, *J* = 6.1 Hz)
15	
16	
17	
N-CH3	3,40
CH3	3.70
OH	5.00 (d, *J* = 1.0 Hz, 2H)

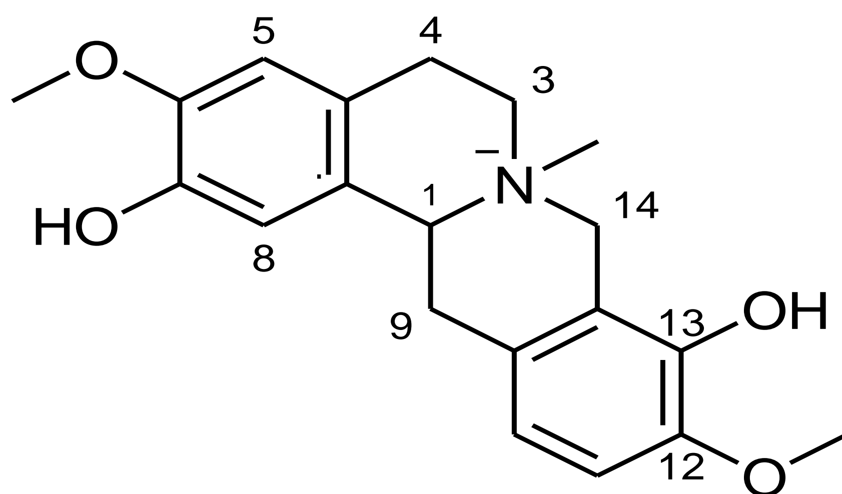


Figure 3.5: Structure of Cyclanoline

Compound 2: The water soluble alkaloid compound E5 yellowish in colour was identified by direct comparison of the spectroscopic data with those published literatures. Mass spectra (ESI-

MS) were obtained with a Thermo-Finningan LCD DECA mass spectrometer and HRESIMS spectra were measured with a FTHRMS-Orbitrap (Thermo-Finnigan) mass spectrometer. In the ESI negative ion mode, analysis yielded a molecular ion peak at *m/z*= 695.3346[M-H] and a fragment ion peaks at *m/z*=677.0022[M-H_2O], *m/z*=623.9983[M-4H_2O] and *m/z*=515.9941[M-10H_2O] as shown in Figure 3.6. E5 ^{1}H NMR (500 MHz, DMSO) showed integrations peaks at δ 14.15 (s, 8H), 14.01 (s, 8H), 13.82 (s, 8H), 13.66 (s, 8H), 13.57 (s, 8H), 13.03 – 12.93 (m, 20H), 12.82 (s, 7H), 12.71 (s, 7H), 12.62 – 12.51 (m, 19H), 12.39 (s, 7H), 12.22 (d, *J* = 16.9 Hz, 12H), 12.11 (s, 6H), 12.07 (s, 6H), 12.01 (s, 6H), 11.95 (s, 6H), 11.88 (s, 6H), 11.83 (s, 6H), 11.74 (d, *J* = 12.4 Hz, 9H), 11.60 (d, *J* = 12.9 Hz, 9H), 11.45 (s, 5H), 11.38 (s, 5H), 11.21 (d, *J* = 19.7 Hz, 9H), 10.80 (s, 4H), 10.41 (s, 3H), 10.36 (s, 3H), 10.04 (s, 3H), 9.88 (s, 3H), 8.30 (d, *J* = 13.7 Hz, 4H), 6.68 (d, *J* = 17.4 Hz, 3H), 2.14 (d, *J* = 228.7 Hz, 3H).

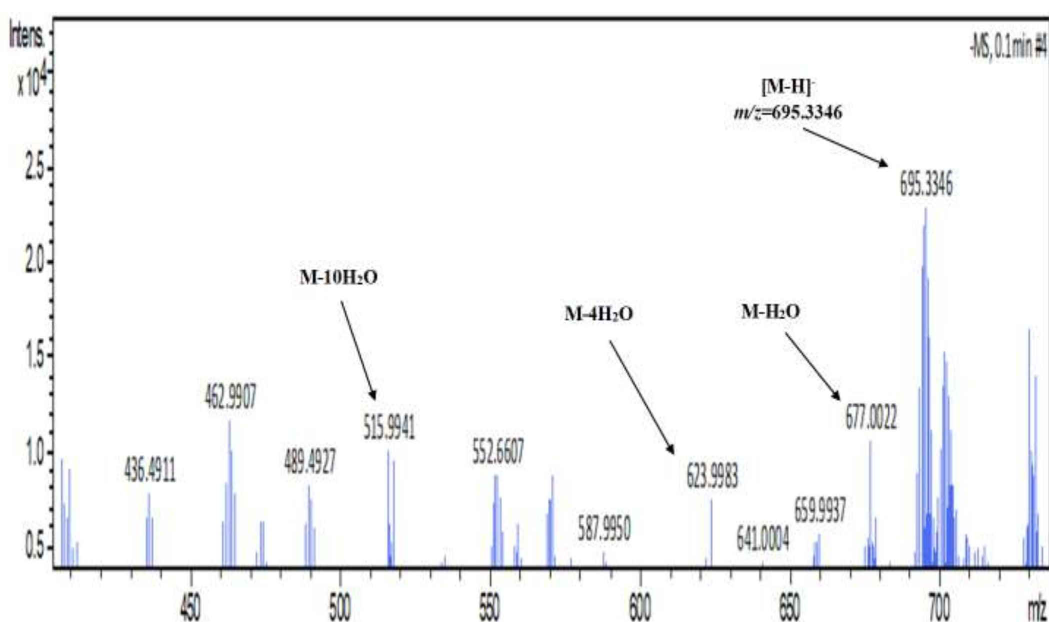


Figure 3.6: The mass spectrum of E5. The molecular ion (M+) is at 694.3346 m/z and the base peaks at 677.0022, 623.9983 and 515.9941 m/z are due to loss of one, four and ten water molecules respectively.

Compound 3: Betulinic acid (Figure 3.7), was obtained as white amorphous powder, soluble in methanol, having been eluted with methylene chloride: methanol 9:1 from the silica gel column. The negative electrospray ionization mode ESIMS showed a deprotonated ion [M-H] - at 455 and m/z 911 [2M-H] −with a strong intensity as shown in Figure 3.8. Since the carboxyl group is a strong proton donor, the deprotonated ion m/z: 455 [M] - can be generated more easily than protonated species [Shin et al., 1999]. The compound was therefore identified as Betulinic acid in comparison with authentic sample. The ^{13}C-NMR showed peaks at δ 36.92 (C-22), 178.64 (C-28), 108.72 (C-29), 13.68 (C-27), 78.28 (C-3), 56.08 (C-17), 55.46 (C-5), 50.59 (C-9), 49.05 (C-19), 42.16 (C-14), 48.10 (C-18), 38.53 (C-1), 38.66 (C-4), 40.51 (C-8), 31.90 (C-16), 34.18 (C-7), 36.73 (C-10), 25.48 (C-12), 15.22 (C-25), 38.25 (C-13), 18.13 (C-30), 15.31 (C-24), 150.59 (C-20), 18.03 (C-6), 14.69 (C-26), 27.19 (C-23), 26.63 (C-2), 30.30 (C-15), 20.68 (C-11) and 29.43 (C-21) giving a total of 30 carbon signals for Betulinic acid.

The ^{1}H NMR spectrum of Betulinic acid showed resonance for olefinic methylene proton at 4.73 and 4.61 ppm, a carbonyl proton at 3.14 ppm, a vinyl methyl singlet at 1.713 ppm which was found to be coupled to one of the two methylene proton (H 29, 4.61) indicating the presence of an isopropenyl group as well as a lupane skeleton. The spectrum also displayed vinyl methyl singlet at 1.713 ppm and five methyl singlets at 1.024, 0.99, 0.97, 0.88 and 0.77 ppm.

The ^{13}C-NMR spectra of the isolated compound showed that there were 6 methyl carbons, 11 methylene carbons, 6 methine carbons, 1 carbonyl carbon and 6 quaternary carbons. All these put together account for 30 carbon signals characteristic of Betulinic acid. The ^{13}C-NMR spectroscopic data of the isolated compound was further compared with others obtained from literature (Mahato and Kundu 1993). The HMBC spectrum of compound 3 showed correlations

between the carbonyl proton, H-3 (3.15 ppm) and the carbon atoms C-24 (15.31 ppm) and C-23 (27.19 ppm) as well as between the olefinic methylene protons: H-29a (4.73 ppm), H-29b (4.61 ppm) and the carbon atoms C-30 (18.13 ppm) and C-19 (49.05 ppm). Several other correlations were observed from the HMBC spectrum of compound 3 that were helpful in the assignment of quaternary carbon atoms. Several cross-peaks were observed from the COSY spectrum of compound 3 including those between H-29a (4.68 ppm) and H-30 (1.68 ppm), H-3 (3.15 ppm) and H-2 (1.55 ppm), H-19 (2.40 ppm) and H-18 (1.37 ppm) as well as that between H-21a (1.92 ppm) and H22a (1.37 ppm).

The spatial arrangement of the olefinic methylene protons (H-29a and H-29b) was assigned based on their coupling constants. H-29a (4.68 ppm) was observed as a doublet (J = 2.5 Hz) in the 1H spectrum of compound 3, hence suggesting its coupling with H-30. This supports its position that is trans to C-30. H-29b (4.55 ppm) was observed as a triple doublet (J= 2.7 Hz, 2.6 Hz, 1.3 Hz). This suggested its coupling with H-19 (J = 2.7 Hz), H-30 (J = 2.6 Hz) and H-18 (J = 1.3 Hz). H-29b was therefore assigned the position that is cis to C-30. The comprehensive literature survey and comparison with different spectral data identified the compound isolated in this research to be betulinic acid.

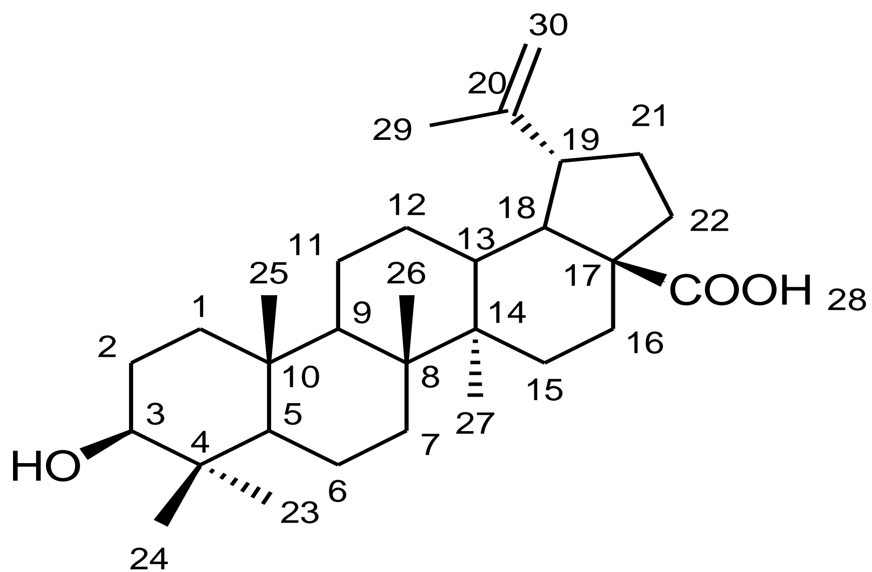
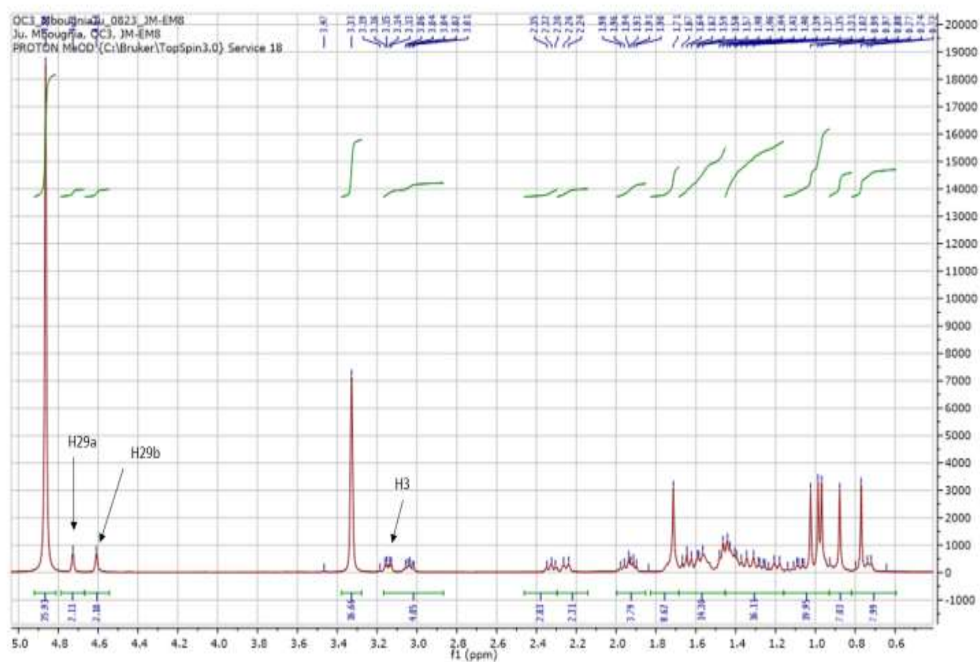
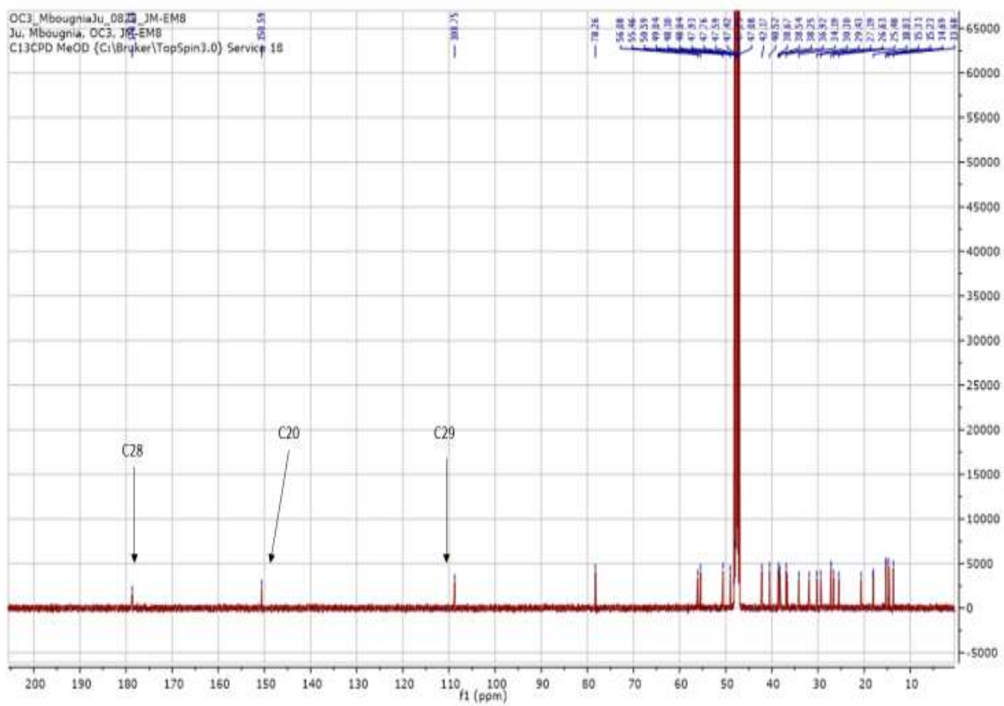


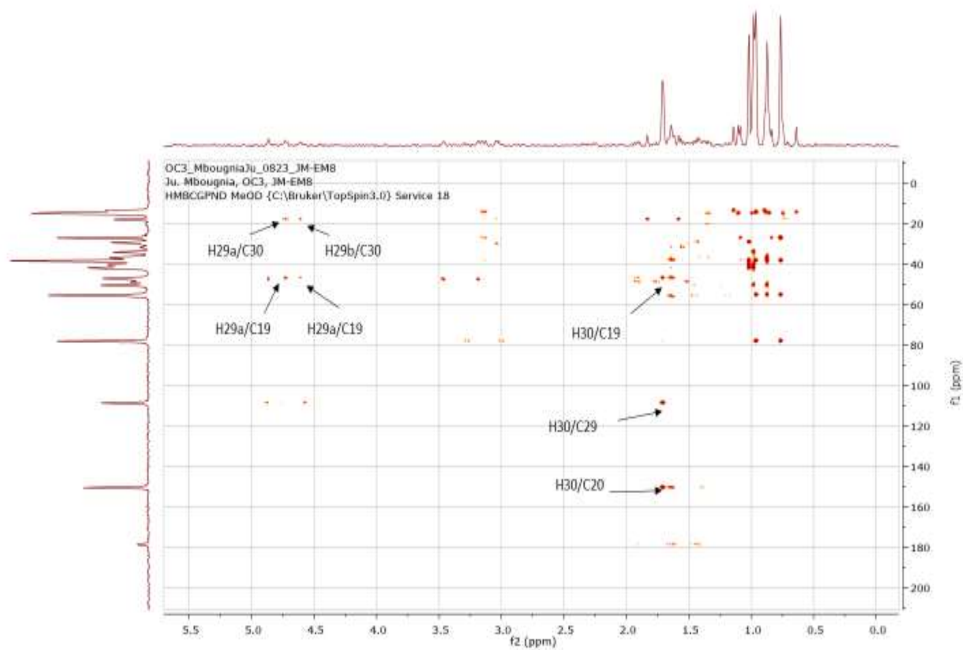
Figure 3.7: Structure of Betulinic acid



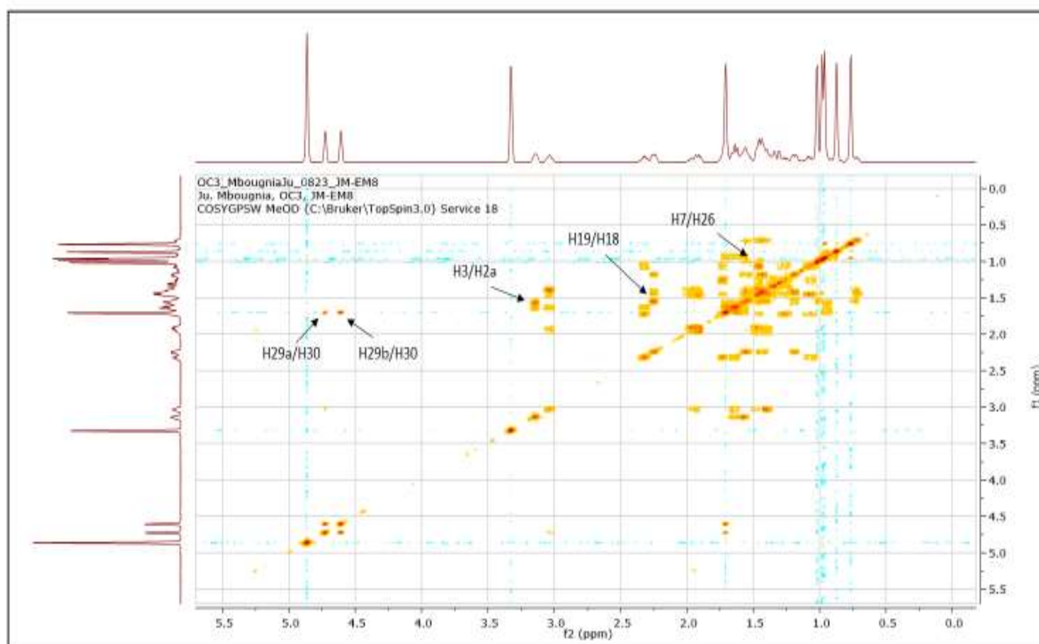
(a) ^{1}H NMR spectrum



(b) ^{13}C NMR spectrum

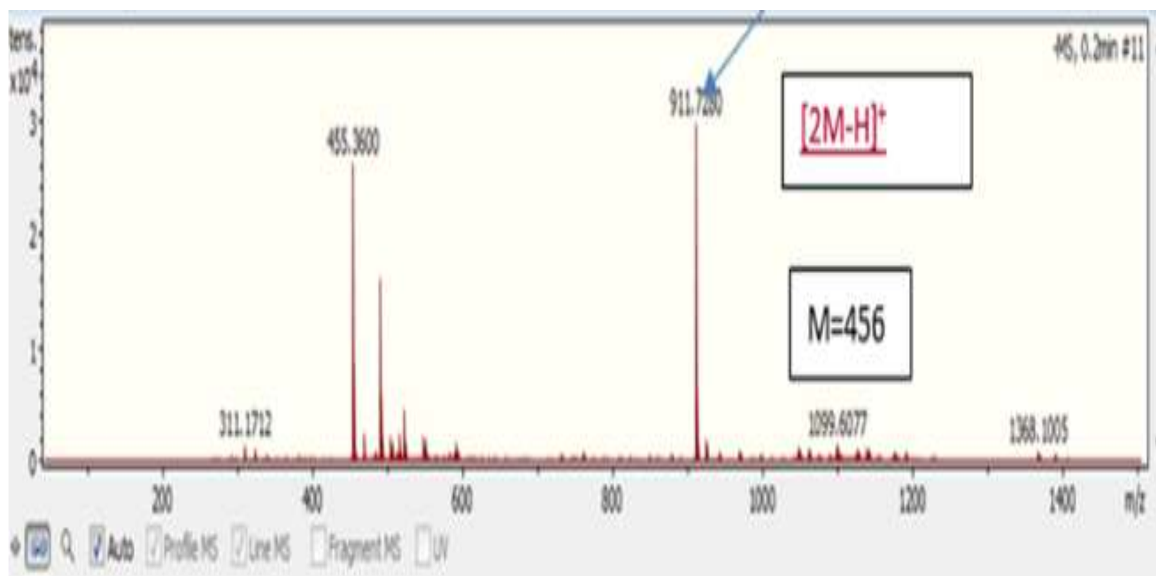


(c) ^{1}H-^{13}C HMBC spectrum



(d) ^{1}H-^{1}H COSY spectrum

Figure 3.8: Betulinic acid Spectrums; (a) 1H NMR spectrum; (b) 13C NMR spectrum; (c) 1H-13C HMBC spectrum; d) 1H-1H COSY spectrum



(e) LCMS ESI positive ion mode

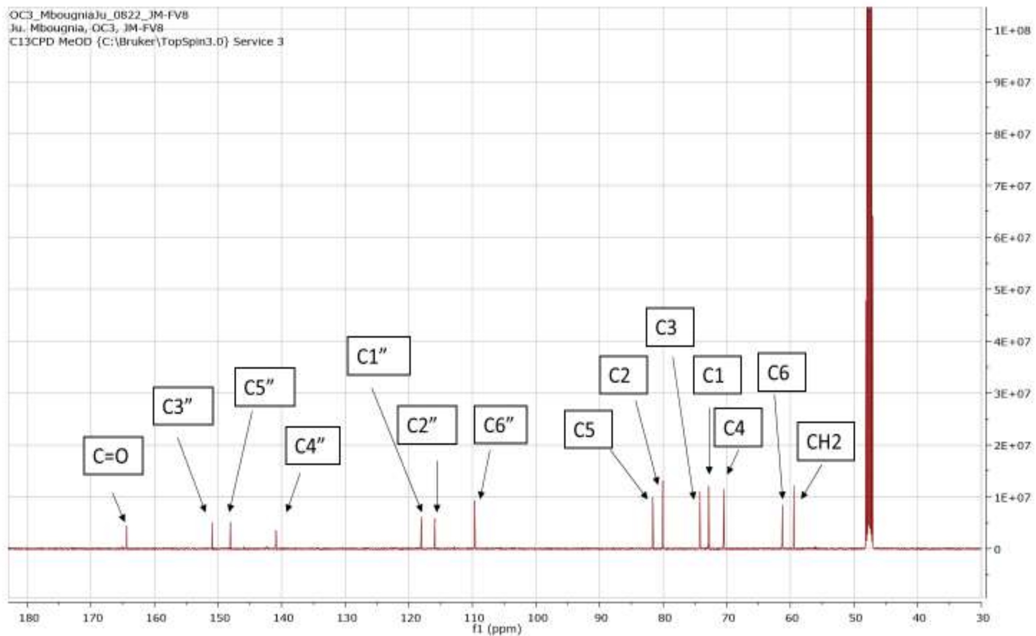
Figure 3.9: The mass spectrum of Betulinic acid. The molecular ion (M+) is at 456.3600 m/z and the base peak at 911.7230 m/z is due to two molecular ions minus hydrogen atom

Table 3.2: ^{1}H, ^{13}C NMR, ^{1}H-^{1}H COSY and HMBC Data of Betulinic Acid

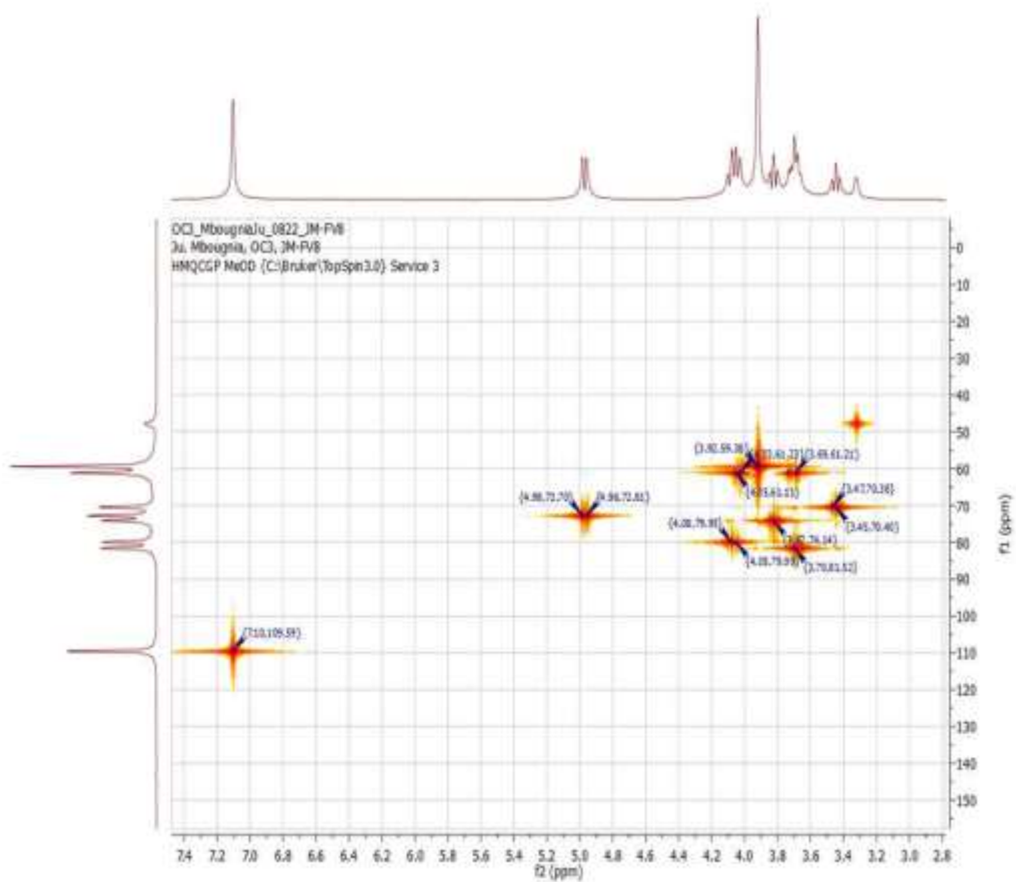
Carbon	**δ 13C (ppm)**	**δ 1H (ppm)**	**HMBC**	**COSY**
C-1a C-1b	38.53 (d, *J* = 15.8 Hz),	1.67 0.90 (d, *J* = 25.0 Hz, 7H),		
C-2a C-2b	26.63	1.62 1.58 (ddd, *J* = 60.6, 36.9, 10.3 Hz, 14H),		
C-3	78.28	3.13(dd, *J* = 11.4, 4.8 Hz, 1H)		
C-4	38.66			
C-5	55.46	0.72		
C-6a C-6b	18.03 (d, *J* = 12.3 Hz),	 1.40		
C-7	34.18	1.43		
C-8	40.51			
C-9	50.59	1.59		
C-10	36.73			
C-11	20.68	1.43 (dt, *J* = 28.3, 12.9 Hz, 6H),		
C-12a C-12b	25.48	1.64 1.09		
C-13	38.25	1.71		
C-14	42.16			
C-15a C-15b	30.30	1.94 (dt, *J* = 14.4, 8.3 Hz, 4H), 1.90		
C-16	31.90	2.25 (d, *J* = 12.9 Hz, 2H)	C-15, C-17	
C-17	56.08			
C-18	48.10	1.35		
C-19	49.05	2.33 (t, *J* = 10.5 Hz, 2H),		H29a, H29b, H30
C-20	150.59			H30
C-21a C-21b	29.43	2.24 1.31		
C-22	36.92	1.84		
C-23	27.19	0.97		
C-24	15.31	0.88		
C-25	15.22 (d, *J* = 10.2 Hz).	0.77	C-1; C-5; C-9	
C-26	14.69			
C-27	13.68	0.99	C-8; C-13; C-14; C-15	
C-28	178.64			
C-29a C-29b	108.72	4.73 4.61	C-19; C-29 C-19; C-29	H30
C-30	18.13	1.84	C-19; C-20; C-30	H29a, H29b

Compound 4; I, II, III, IV: 3,4,5-trihydroxyphenyl) acetyl ester and β-D-glucopyranose. The ^{13}C NMR data (Table 3.3) of compound 4III revealed the presence of a galloyl moiety [δC 164.35 (C=O), 150.96 (C-3'), 148.66 (C-5'),140.89 (C-4'), 118,04 (C-1'), 115.90 (C-2'), 109.84 (C-6'), 59.40 (CH2) and six oxygenated carbon signals at δC 81.66 (C-5), 80.03 (C-2),

74.23 (C-3), 72.85 (C-1), 70.51(C-4), 61.26 (C-6) for glucopyranose. The ^{1}H NMR spectrum displayed several signals between 3.00 and 4.00 ppm, suggesting the presence of sugar in the molecule. Furthermore, it also exhibited a typical resonance arising from six aromatic protons at 7.10 ppm and non-equivalent proton signals at 4.86 ppm. In ^{1}H-^{1}H COSY spectrum, the H1 signal (4.97 ppm) was correlated to H-6 which in turn is coupled to H-5 indicating C-5 to be non-substituted. For compound 4III, the ^{1}H NMR spectrum showed peaks at 4.97 (dd, J=10.4, 5.8Hz, 1H), 4.08 (dd, J = 10.2, 5.5 Hz, 1H), 3.82 (dd, J=5.8Hz, 1H), 3.46 (m), 3.68 (m), 3.72(s), 4.03(s) and 3.68 (dd, J=7.0, 1.6Hz, 1Hz) indicating a trisubstituted ring with the C-6 also being substituted. Consequently, compound 4III is 1,2,3,6 – tetra(3,4,5-trihydroxyphenyl) acetyl ester - β-D- glucopyranose. For compound 4IV, the ^{1}H NMR spectrum showed peaks at 4.97 (s), 4.08 (dd, J = 10.2, 5.5 Hz, 1H), 3.82 (dd, J=5.8Hz, 1H), 3.46 (m), 3.68 (m), 3.72 (s), 4.03 (s) and 3.68 (dd, J=7.0, 1.6Hz, 1Hz) indicating a trisubstituted ring with the C-6 also being substituted. Therefore, compound 4IV is 2,3,6 – tri(3,4,5-trihydroxyphenyl) acetyl ester - β-D-glucopyranose. For compound 4II, the ^{1}H NMR spectrum showed peaks at 4.96 (dd, J = 10.0, 5.2 Hz, 1H), 4.08 (dd, J = 10.2, 5.5 Hz, 1H), 3.82 (dd, J=5.8Hz, 1H), 3.47 (dd, J = 8.7, 5.5 Hz, 1H), 3.68 (m), 3.72 (s), 4.03 (s) and 3.68 (dd, J=7.0, 1.6Hz, 1Hz) indicating a tetrasubstituted ring with C-6 also being substituted. Therefore, compound 4II is 1,2,3,6 – tetra (3,4,5-trihydroxyphenyl) acetyl ester - β-D- glucopyranose. For compound 4I, the ^{1}H NMR spectrum showed peaks at 4.97 (dd, J=10.4, 5.8Hz, 1H), 4.08 (m), 3.82 (dd, J=5.8Hz, 1H), 3.46 (m), 3.68 (m), 3.72 (s), 4.03 (s) and 3.68 (dd, J=7.0, 1.6Hz, 1Hz) indicating disubstituted ring with C-6 also being substituted. Therefore, compound 4I is 1,3,6 – tri(3,4,5-trihydroxyphenyl) acetyl ester - β-D-glucopyranose.



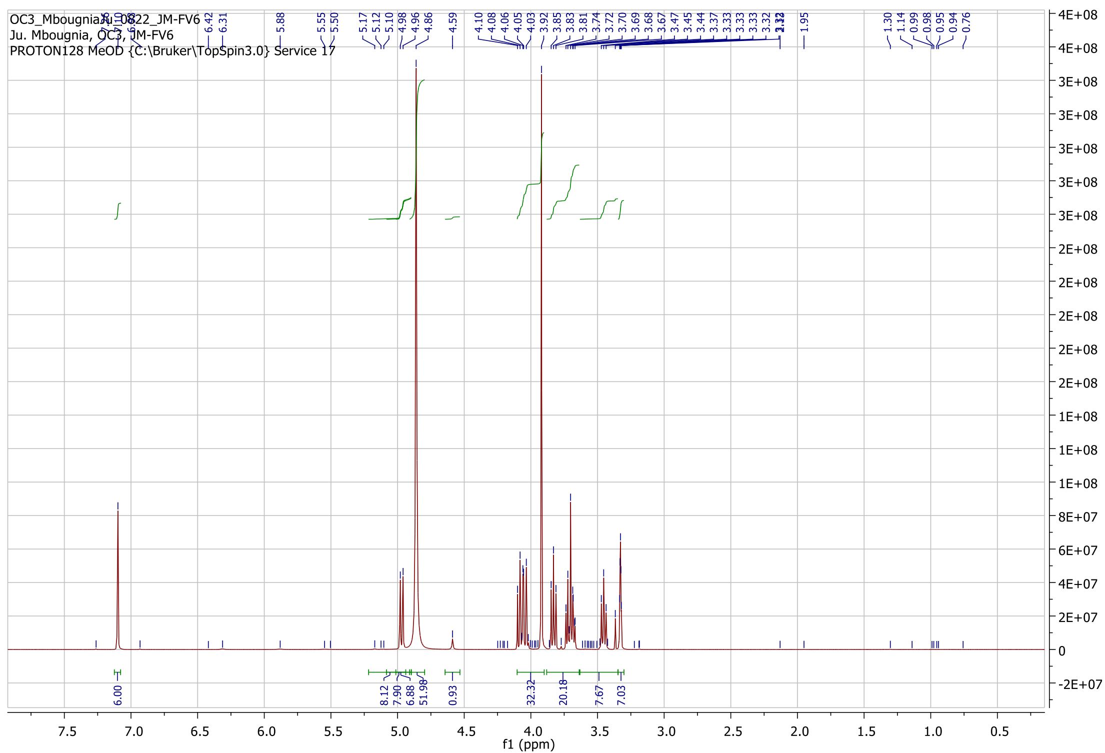
(a) ^{13}C NMR spectrum



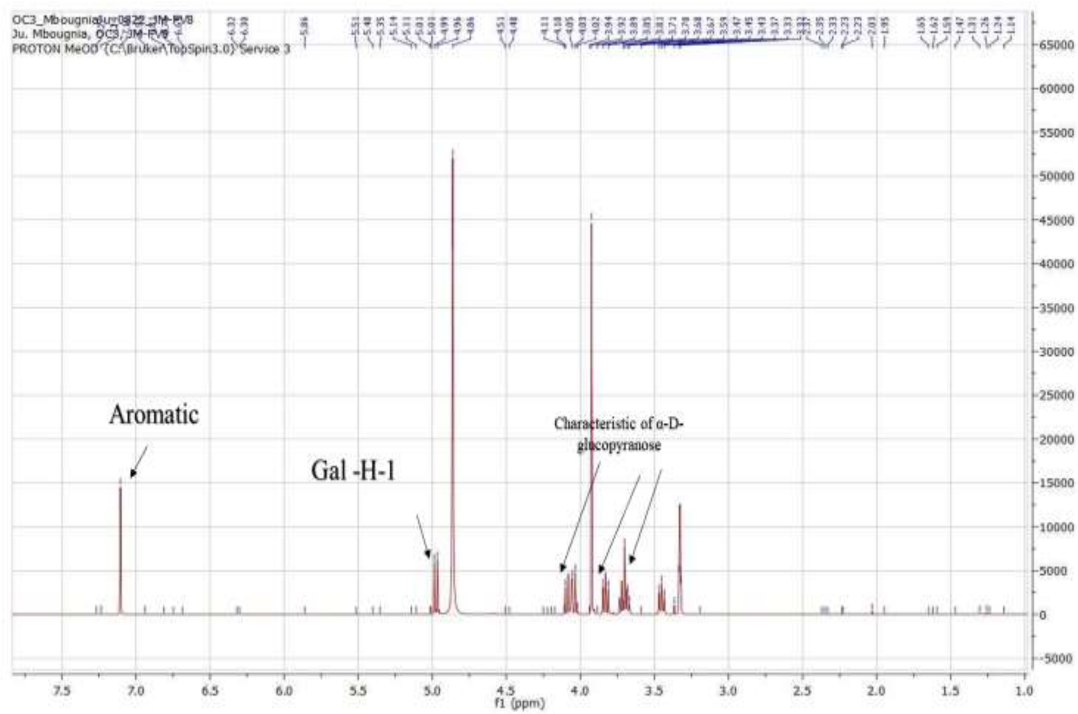
(b) ^{1}H-^{13}C HMQC spectrum

(c) ^{1}H NMR spectrum (II)

(d) ^{1}H NMR spectrum (IV)



(e) ^{1}H NMR spectrum (I)



(f) ^{1}H NMR spectrum (III)

(g) ^{1}H-^{1}H COSY spectrum

Figure 3.10: β-D-glucopyranose and(3,4,5-trihydroxyphenyl) acetic acid; (a) ^{13}C NMR spectrum; (b) 1H-13C HMQC spectrum; (c)1H NMR spectrum (II); (d) 1H NMR spectrum (IV); (e) 1H NMR spectrum (I); (f)1H NMR spectrum (III); (g) 1H-1H COSY spectrum

(a) β-D-glucopyranose

(b) TaG= (3,4,5-trihydroxyphenyl) acetic acid

Figure 3.11: Structures of β-D-glucopyranose and(3,4,5-trihydroxyphenyl) acetic acid

I. 1,3,6 – tri(3,4,5-trihydroxyphenyl) acetyl ester - *β*-D-glucopyranose: R_1 = TaG; R_3 = TaG; R_5 = TaG

II. 1,2,3,4,6 – penta(3,4,5-trihydroxyphenyl) acetyl ester - β-D- glucopyranose: R_1 = TaG; R_2 = TaG; R_3 = TaG; R_4 = TaG; R_5 = TaG

III. 1,2,3,6 – tetra(3,4,5-trihydroxyphenyl) acetyl ester - β-D- glucopyranose: R_1 = TaG; R_2 = TaG; R_3 = TaG; R_5 = TaG

IV. 2,3,6 – tri(3,4,5-trihydroxyphenyl) acetyl ester - β-D- glucopyranose: R_2 = TaG; R_3 = TaG; R_5 = TaG

Table 3.3: ^{1}H and ^{13}C NMR Data of 3,4,5-trihydroxyphenyl acetyl ester and β-D-Glucopyranose

	C	Mult	δ C ppm	δ H ppm (III)	δ H ppm (IV)	δ H ppm (II)	δH ppm (I)
3,4,5-trihydroxyphenyl acetyl ester							
	1	CH	118.04				
	2	CH	115.90	7.10 (s)			
	3	CH	150.96	4.86 (s)			
	4	CH	140.89	4.86 (s)			
	5	CH	148.66	4.86 (s)			
	6	CH	109.64	7.10 (s)			
	7	CH_2	59.37	3.91			
	C=O		164.35				
β-D-Glucopyranose							
	1	CH	72.76	4.97 (dd, *J* = 10.4, 5.8 Hz, 1H)	4.98 (s)	4.96 (dd, *J* = 10.0, 5.2 Hz, 1H).	4.97 (d, *J* = 4.7 Hz, 1H),
	2	CH	80.03	4.08 (dd, J = 10.2, 5.5 Hz, 1H)	4.07 (dd, J = 8.6, 4.6 Hz, 1H),	4.07 (dd, *J* = 9.2, 4.1 Hz, 1H).	4.07 (m)
	3	CH	74.11	3.82 (dd, J=5.8Hz, 1H)	3.82 (dd, *J* = 23.5, 14.2 Hz, 1H).	3.82 (d, *J* = 9.2 Hz, 1H).	3.82 (d, *J* = 6.3 Hz, 2H),
	4	CH	70.39	3.46 (m)	3.45 (m)	3.47 (dd, *J* = 8.7, 5.5 Hz, 1H)	3.45 (m)
	5	CH	81.545	3.70 (s)	3.70 (s)	3.70 (m)	3.70 (m)
	6	CH_2	61.26	3.68 (dd, J=7.0, 1.6Hz, 1Hz) 4.03 (s)	3.68 (dd, J=7.0, 1.7Hz, 1Hz 4.03 (s)	3.68 (dd, *J* = 7.0, 1.6 Hz, 1H). 4.03 (d, *J* = 7.3 Hz, 1H).	3.68 (dd, *J* = 7.0, 1.7 Hz, 1H).

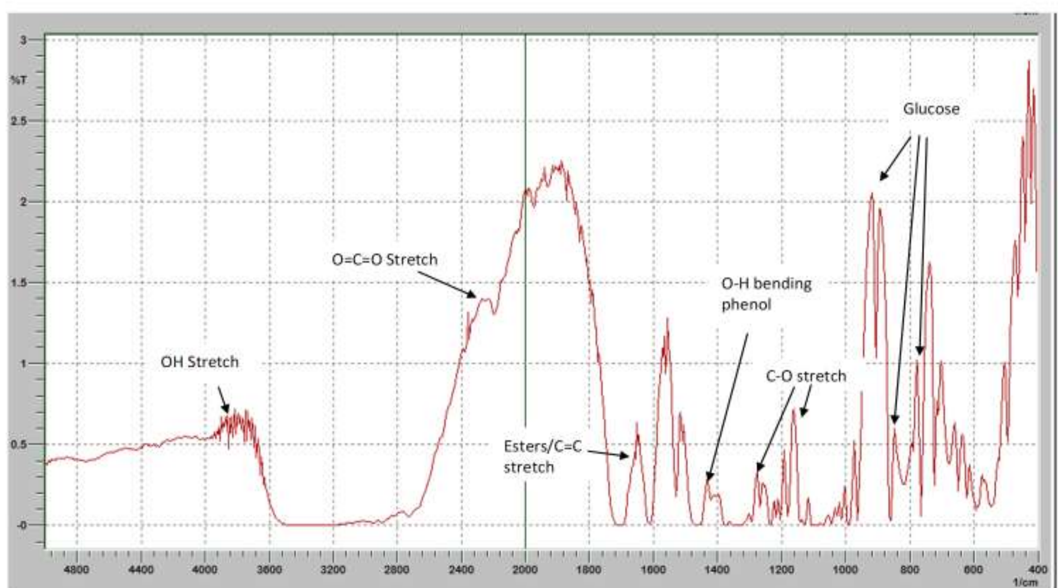


Figure 3.12: IR spectrum of 1,2,3,6 – tetra(3,4,5-trihydroxyphenyl)acetyl ester - β-D- glucopyranose

The spectrum obtained indicated an intense absorbance at 3600-4000 cm-1 attributed to O-H stretch vibrations in alcohol (96). Polyphenols present in the plant extract is attributable to this absorbance value (97). The sharp shoulder peaks at 2200-2400 cm-1 is characteristic of O=C=O and small absorbance peak at 1600-1733 cm-1 is attributed to the carbonyl C=O stretch in carboxylic acids, while the carbonyl stretch in ketones could be responsible for the absorbance peak at 1653.8 cm-1. Small peak at 1440 cm-1 is due to O-H bending phenol while small peaks at 1280 and 1160 are due to C-O stretch. The set of peaks at 790, 860 and 950 are characteristics of glucose molecule as shown in Figure 3.11.

Compound 5: 2-(6-Oxido-3-oxo-3H-xanthen-9-yl) benzoate (Benzoxylanthaquinone). Benzoxylanthaquinone as shown in Figure 3.13 was identified by direct comparison of the spectroscopic data with those published literatures. Mass spectra (ESI-MS) were obtained with a Thermo-Finningan LCD DECA mass spectrometer and HRESIMS spectra were measured with a FTHRMS-Orbitrap (Thermo-Finnigan) mass spectrometer. In the ESI positive ion

mode, the analysis yielded a molecular ion peak at m/z= 329.0812 [M+H] and a fragment ion peak at m/z=679.1385 [2M+Na] as shown in Figure 3.12.

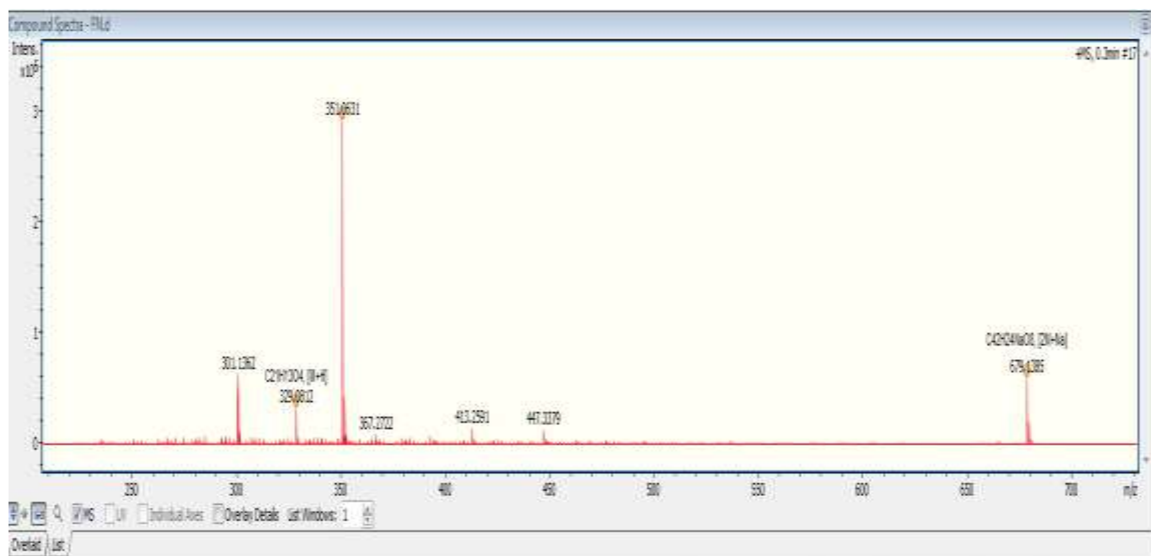


Figure 3.13 The mass spectrum of Benzoxylanthaquinone The molecular ion (M+) is at 328.0812 m/z and the base peak at 679.1385 m/z is due to two molecular ion plus sodium atoms

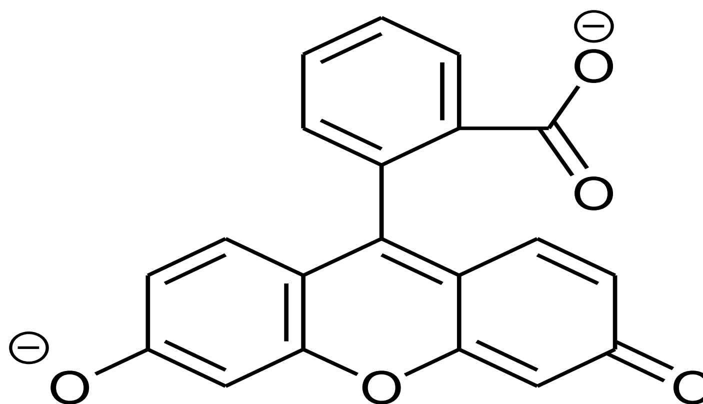


Figure 3.14: Structure of Benzoxylanthaquinone

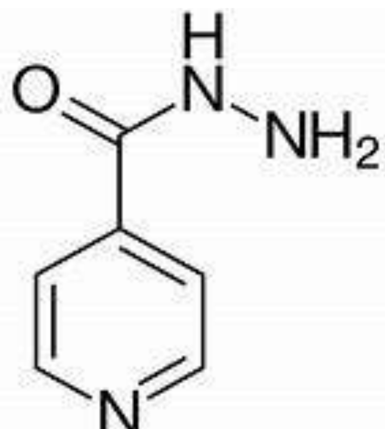
3.2.3 Preparation of drugs

Growth supplement (0.8 ml) containing a mixture of OADC- Oleic Acid, Bovine Albumen, Dextrose and Catalase was added to five 7 ml BBL™ MGIT™ tube labelled GC (growth control), STR (Streptomycin), INH (Isoniazid), RIF (Rifampicin), EMB (Ethambutol) to provide essential substrates for the rapid growth of Mycobacteria. 100 µl of BBL™ MGIT™ SIRE (Streptomycin, Isoniazid, Rifampicin, Ethambutol) prepared aseptically according to the

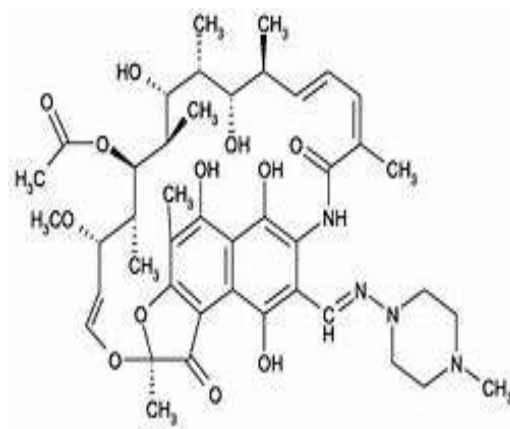
manufacturers' instruction was added to corresponding labelled BBL™ MGIT™ tube followed by addition of 0.5 ml of 1 % Mycobacterium suspension. Mycobacterium suspension was prepared by pipetting 0.1 ml Middlebrook 7H9 Broth containing Mycobacterium adjusted to 0.5 McFarland standard into 10 ml sterile saline aseptically. The BACTEC MGIT™ 320 system (BD, New York-U.S. A) was then loaded following the manufacturer's instructions and incubated at 37 °C. Streptomycin at 1.0 µg/ml, isoniazid at 0.1 µg/ml, rifampicin at 1.0 µg/ml and ethambutol at 5.0 µg/ml served as the positive controls against growth control tube. The procedure was repeated using compounds at 9.0 ug/ml in place of SIRE.

(a) SIRE supplements

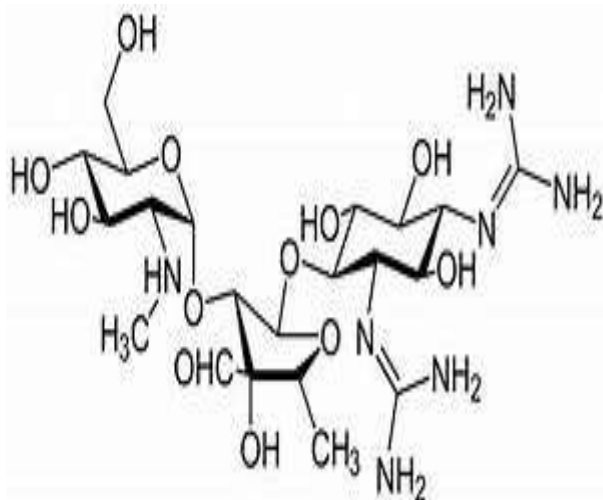
(b) SIRE drugs



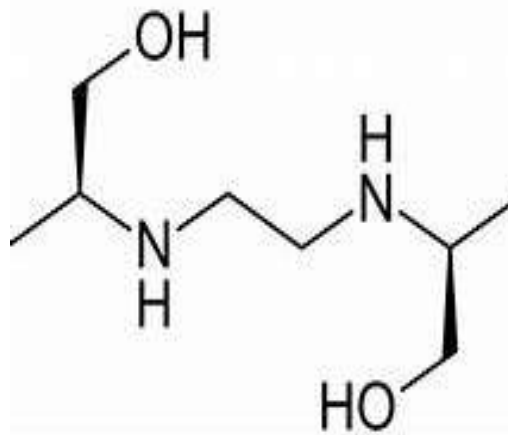
(c) Structure of Isoniazid



(d) Structure of Rifampicin



(e) Structure of Streptomycin



(f) Structure of Ethambutol

Figure 3.15 SIRE supplements and drugs

3.3 Analysis of heavy metals in medicinal plants understudy

3.3.1 Preparation of 1000 ppm stock and standard solutions

The stock solutions of zinc, manganese, iron, copper, lead, chromium and cadmium of 1000 ppm were prepared by dissolving; 1.2450 g of zinc oxide (ZnO), 3.6077 g of manganese chloride ($MnCl_2.4H_2O$), 1.5980 g of lead nitrate ($Pb(NO_3)_2$), 4.8400 g of iron (111) chloride ($FeCl_3.6H_2O$), 3.7980 g. of copper nitrate ($Cu(NO_3)_2.3H_2O$), 7.6960 g of chromium nitrate ($Cr (NO_3)_2 .9H_2O$) and 2.1032 g of cadmium nitrate ($Cd (NO_3)_2$) in deionized water and then diluted to 1 litre in a volumetric flask respectively. And to prepare 100 ppm of the sample, 10 ml of the standard $FeCl_3.6H_2O$, ZnO, $MnCl_2.4H_2O$, $Pb (NO_3)_2$, $Cr (NO_3)_2.9H_2O$ and $Cd (NO_3)_2$ stock solutions were pipetted into 100 ml flasks and deionized water added to the calibration mark. The standard curves for copper, zinc, chromium, cadmium, iron, manganese and lead were prepared as follows: 0.00 ppm, 2.5 ppm, 5 ppm, 7.5 ppm and 10 ppm. Blank and standard solutions were also prepared.

Table 3.4: Concentration values of standard solutions and correlation coefficients of calibration curves

No	Metal	Concentration of intermediate Standard(mg/L)	Concentration of working standard(mg/L)	Correlation Coefficients of calibration curves (R2)
1	Lead	10	0.0, 2.5, 5.0, 7.5 and 10.0	0.934
2	Chromium	10	0.0, 2.5, 5.0, 7.5 and 10.0	0.997
3	Cadmium	10	0.0, 2.5, 5.0, 7.5 and 10.0	0.9707
4	Zinc	10	0.0, 2.5, 5.0, 7.5 and 10.0	0.8899
5	Manganese	10	0.0, 2.5, 5.0, 7.5 and 10.0	0.9984
6	Iron	10	0.0, 2.5, 5.0, 7.5 and 10.0	0.9991
7	Copper	10	0.0, 2.5, 5.0, 7.5 and 10.0	0.9979

3.3.2 Digestion of samples preparation

The standard procedure for the determination of toxic metals as described in the Association of Official Analytical Chemists (AOAC) (2016) was used in the preparation of samples for analysis (98). Accurately, weighed 2 grams of the sample was transferred into a silica crucible and put in a furnace for ashing at 450 °C for 3 hours and then 5 ml HCl was added to the crucible while ensuring that all the acid was in contact with the ash. The crucible containing acid solution was constantly kept on a hot plate and digested to obtain a clean solution. The final residue was dissolved in 0.1M HNO_3 solution and made up to 50 ml in a conical flask. Working standard solutions were prepared by diluting the stock solution (prepared solution), with 0.1M Nitric acid to check the linearity (99).

Table 3.5: Mean±SD and %RSD values for Cu, Fe, Mn, Zn, Pb, Cr and Cd in samples

Metal type		***E. milanjiana***		***E. whyteana***		***R. acuminatissima***		**A. nyassana**	
		L	***R***	***L***	***R***	***L***	***R***	**L**	**R**
Lead	Mean	2.55±0.4	2.67±0.2	3.05±0.05	3.21±0.1	3.12±0.08	3.32±0.3	3.36±0.3	3.26±0.0
	%RSD	1.57	1.64	2.54	2.17	1.70	5.79	3.45	4.21
Chromium	Mean	0.43±0.03	0.72±0.09	0.41±0.2	0.22±0.06	-	-	-	-
	%RSD	4.43	6.97	9.07	9.21				
Cadmium	Mean	-	-	0.23±0.04	-	0.16±0.04	-	0.31±0.04	0.15±0.04
	%RSD			7.10		9.69		9.99	6.92
Copper	Mean	0.66±0.002	0.70±0.09	0.65±0.01	0.64±0.007	0.65±0.003	0.64±0.002	0.70±0.04	0.66±0.02
	%RSD	5.39	7.78	5.11	3.56	13.95	9.22	6.32	18.16
Zinc	Mean	1.77±0.4	0.60±0.2	1.03±0.5	0.82±0.2	0.69±0.01	0.68±0.2	1.79±0.1	0.87±0.1
	%RSD	2.86	5.70	3.41	3.39	3.24	9.347	24.98	16.0
Iron	Mean	2.25±0.05	2.34±0.1	2.25±0.1	1.92±0.1	2.19±0.05	2.53±0.2	1.39±0.3	2.63±0.6
	%RSD	2.43	6.72	5.91	1.7	25.25	8.32	4.08	9.26
Manganese	Mean	2.33±0.02	2.70±0.06	1.46±0.03	1.65±0.1	2.62±0.06	1.87±0.02	1.26±0.1	1.67±0.02
	%RSD	1.06	1.95	7.61	14.6	2.06	2.52	28.1	21.68

All the experiments were done in triplicates. P values < 0.05 were regarded as significant.

3.3.3 Optimization for digestion procedure

The optimum condition for digestion of a sample is the one that requires minimum reagent consumption, clear digestion solution, minimum digestion time reflection, ease of simplicity and absence of undigested sample materials. In this study, to prepare a clear colourless solution for analysis, different digestions were carried out using the mixture of HNO_3 and $HClO_4$ acid, by varying parameters such as the volume of the acid mixtures, digestion time and temperature (100).

Table 3.6: Recovery Tests for the optimized procedure for the samples

Metal		***E. milanjiana***		***E. whyteana***		***R. acuminatissima***		***A. nyassana***	
		L	***R***	***L***	***R***	***L***	***R***	**L**	**R**
Lead	Amount spiked (mg in 0.5 g)	0.0021	0.0028	0.0077	0.0031	0.0016	0.0017	0.0081	0.0063
	Amount recovered (mg in 0.5 g)	0.0018	0.0025	0.0074	0.0028	0.0011	0.001	0.0078	0.0058
	% Recovery	85.7	89.3	96.1	90.3	97.4	58.8	96.3	92.1
Chromium	Amount spiked (mg in 0.5 g)	0.0006	0.005	0.0036	0.0026	0.00182	0.0016	0.0067	0.0078
	Amount recovered (mg in 0.5 g)	0.0005	0.0045	0.0035	0.0025	0.0018	0.0015	0.0065	0.0069
	% Recovery	83.3	90	97.2	96.2	98.9	93.7	97.0	88.5
Cadmium	Amount spiked (mg in 0.5 g)	0.009	0.0010	0.0019	0.0017	0.00097	0.0039	0.0099	0.0087
	Amount recovered (mg in 0.5 g)	0.008	0.0009	0.0017	0.0015	0.00091	0.0036	0.0094	0.0084
	% Recovery	88.8	90	89.5	88.2	93.8	92.3	94.9	96.6
Copper	Amount spiked (mg in 0.5 g)	0.0124	0.0138	0.0093	0.0018	0.0015	0.0015	0.0128	0.0128
	Amount recovered (mg in 0.5 g)	0.0120	0.0135	0.0091	0.0016	0.0011	0.0010	0.0124	0.0123
	% Recovery	96.0	97.8	97.8	88.8	73.3	66.6	96.8	96.1
Zinc	Amount spiked (mg in 0.5 g)	0.025	0.0041	0.0082	0.0125	0.0062	0.0725	0.00145	0.00196
	Amount recovered (mg in 0.5 g)	0.022	0.0038	0.0078	0.0122	0.0044	0.0719	0.00132	0.00172
	% Recovery	88	92.7	95.1	97.6	70.9	99.1	91.0	87.8
Iron	Amount spiked (mg in 0.5 g)	0.009	0.0078	0.0075	0.0095	0.0097	0.0065	0.0057	0.0076
	Amount recovered (mg in 0.5 g)	0.0084	0.0071	0.0061	0.0079	0.0077	0.0048	0.0039	0.0062
	% Recovery	93.3	91.0	83.1	83.1	79.4	73.8	68.4	81.5
Manganese	Amount spiked (mg in 0.5 g)	0.0048	0.0036	0.0068	0.0054	0.013	0.029	0.071	0.008
	Amount recovered (mg in 0.5 g)	0.0027	0.003	0.0051	0.0034	0.009	0.027	0.069	0.0076
	% Recovery	56.3	83.3	75.0	62.9	69.2	93.1	97.1	95.0

All the experiments were done in triplicates. P values < 0.05 were regarded as significant.

3.3.4 Determination of metal content by MP-AES

3.3.4.1 Preparation of calibration curve

Stock solutions of each metal ion were prepared by dissolving the calculated amounts in 100ml of distilled water. Calibration curves were prepared to determine the concentration of the metals in the sample solution. The working standard solutions of each metal were prepared from an intermediate solution. Calibration curve for each metal ion was prepared by plotting the absorbance against the concentration.

3.3.4.2 Determination of metal contents

The samples were analysed using the Agilent 4100 Microwave Plasma-Atomic Emission Spectrometer System (MP-AES) for heavy metals; Pb, Cd, Zn, Cr, Cu, Fe and Mn. To avoid any possible contamination of the samples, all necessary precautions were followed as per AOAC guidelines. Determination of metal contents was done using MP-AES and concentration of the metal ions present in the sample were plotted against the absorbance. Cu, Fe, Mn, Zn, Pb, Cr and Cd were analysed in triplicates. The same procedure was used for digested blank solutions and the spiked samples.

3.3.5 Method Validation

3.3.5.1 Precision and accuracy

Accuracy and precision are probably the most often used terms to express the extent of errors in given analytical results. Analytical results must be evaluated to decide on the best values to report and to establish the probable limits of errors of these values for an effective experiment (101). In this study, the precision of an analytical procedure was expressed as the variance, relative standard deviation and percentage relative standard deviation of a series of measurements. While the precision of the results was evaluated by percentage relative standard

deviation of three samples (n = 3). On the other hand, the validity and accuracy of the measurements were determined by analyzing spiked samples.

3.3.5.2 Validation of optimized procedures

Spiking method used in digestion procedure for Cu, Fe, Mn, Zn, Pb, Cr and Cd analysis was adopted due to the absence of certified reference materials (CSRM). Accordingly, the efficiency of the optimized procedure was checked by adding different volumes of standard solutions containing 10 mg/L of each metal element into a 0.5 g plant sample. The spiked samples were then digested in the same way as the original sample. Then the digests were transferred into 50 ml volumetric flask and diluted to the mark with deionized water. Finally, the solutions were analysed for metal concentration using MP-AES. Recovery was calculated using the following equation:

$$Recovery\ percent = \frac{Amount\ after\ spike - Amount\ before\ spike}{Amount\ added} \times 100$$

3.4 Phytochemical screening

3.4.1 Determination of flavonoids contents

Aluminium chloride method was used to determine flavonoid contents according to standard procedures (102). About 0.5 ml of 1 mg/ml methanol extract was mixed with 0.5 ml of 2% aluminium chloride, then it was allowed to stand at room temperature for 60 minutes. Absorbance was then measured at 420 nm using the PerkinElmer Victor X3 Multimode plate reader. The total flavonoid content was evaluated as quercetin equivalents (mg/g) using the following equation based on the calibration curve, $y = 0.3812x + 0.1257$, $R^2 = 0.9583$, where y was the absorbance and x was the concentration.

3.4.2 Determination of total phenolics

Total phenolic contents were evaluated with Folin-Ciocalteu's phenol reagent according to standard procedures (103). Five millilitres of the extract solution were mixed with 5 ml Folin-Ciocalteu reagent which was previously diluted with water (1:9 v/v). The mixture was allowed to stand for 5 minutes, then 4 ml of 7 % Na_2CO_3 solution was added. The tubes were vortexed for 15 seconds and allowed to stand for 30 min at 40 °C for the development of colour. Absorbance was then measured at 765 nm using the PerkinElmer Victor X3 Multimode plate reader. The extracts were evaluated at a final concentration of 0.1 mg/ml. Total phenolic content was expressed as mg/g gallic acid equivalent (GAE) using the following equation based on the calibration curve: y = 0.3947x 0.0423, R^2 = 0.981, where y was the absorbance x was the concentration.

3.4.3 Saponins determination

The saponins content in the plant extracts was determined according to standard procedures (104). Ten grams of the powdered plant sample was placed in 200 ml of 20 % ethanol. The suspension collected was heated for 4 hours in a water bath at 55 °C while continuously being stirred. The mixture was filtered and the residue was re-extracted twice as above. The resultant combined extracts were reduced in a water bath at 90 °C to about 40 ml. The final concentrate was added to 20 ml diethyl ether in a 250 ml separator funnel and shaken vigorously. The layer of ether solution was discarded, while the purification process was repeated. 60 ml of n-butanol was added to the combined plant extract extracts. It was washed twice with 10 ml of 5 % aqueous sodium chloride. The solution that remained was heated in a water bath to evaporate the solvents and then the sample was dried in the oven to a constant weight. The saponins content was determined according to the equation:

$$Amount\ of\ saponins\ (mg/g) = \frac{Weight\ of\ residue}{Weight\ of\ sample}$$

3.4.4 Alkaloids determination

The alkaloids content in the plant extracts was determined according to standard procedures (105). Five grams of the powdered plant sample was weighed into 200 ml of 20 % acetic acid in ethanol and allowed to stand for 5 hours. The extracts were filtered and concentrated using a water bath at 55 °C to approximately one-quarter of the original volume. Then dropwise, concentrated ammonium hydroxide solution was added into the resultant extract until precipitation was complete. The precipitate collected after allowing the solution to settle was washed with dilute ammonium hydroxide solution and then filtered. The residue of the crude alkaloid was weighed and calculated according to the equation:

$$Amount\ of\ alkaloid\ (mg/g) = \frac{Weight\ of\ precipitate}{Weight\ of\ sample}$$

3.5 Antioxidant assays

3.5.1 DPPH scavenging activity

The DPPH (2,2-Diphenyl-1-picrylhydrazyl) radical-scavenging activity of the methanol extracts was determined according to standard procedures (106). DPPH free radical scavenging assay was performed using 96-micro-well flat plates. Stock solutions of the extracts were prepared as 1 mg/ml in methanol. Each well was filled in with 200 µl extract of different concentrations (3.13 - 25.0 µg/ml). Then, 5 µl of the DPPH solution (2.5 mg/ml in methanol) was added to each well. Ascorbic acid was used as standard control while a blank was prepared by mixing DPPH and methanol. Three replicates were made for each test sample. After 30 minutes of incubation at room temperature in the dark, the optical density of each well was read using PerkinElmer Victor X3 Multimode plate reader at wavelength 517 nm, and results were expressed as percentage antioxidant activity using the following equation: 100 – [(Sample Optical density (OD) – Sample background OD)/ (DPPH only OD) × 100]. The IC50 values were calculated by plotting a linear regression, where the abscissa represented the

concentration of the tested plant extracts and the ordinate represented the average percent of scavenging capacity from three replicates.

3.5.2 Nitric oxide-scavenging activity

The nitric oxide radical scavenging assay was determined according to standard procedures (107). The extracts were prepared from a 1 mg/mL stock solution of methanol and serially diluted to make concentrations (1.56−50 µg/mL). Griess reagent was prepared by mixing equal amounts of 1 % sulphanilamide in 2.5 % phosphoric acid and 0.1 % naphthylethylene diamine dihydrochloride in 2.5 % phosphoric acid immediately before use. A volume of 0.5 mL of 10 mM sodium nitroprusside in phosphate buffered saline was mixed with 1 mL of the different concentrations of the methanol extracts (1.56−50 µg/mL) and incubated at 25 °C for 180 mins. The freshly prepared Griess reagent was mixed with an equal volume of the plant extracts. Control samples were prepared by mixing equal volume of a buffer prepared similarly to the test samples but without the extracts. The colour tubes containing methanol extracts at the same concentrations with no sodium nitroprusside was also prepared. Then, 150 µL of the reaction mixture was transferred to a 96-well plate and absorbance was measured at 546 nm using a PerkinElmer Victor X3 Multimode plate reader. Ascorbic acid was used as the positive control. The percentage nitric oxide scavenging activity of the methanol extracts and ascorbic acid were calculated using the following formula:

$$Nitric\ oxide\ scavenging\ activity\ (\%) = \frac{A\ control - A\ test}{A\ control} \times 100$$

3.5.3 Reducing power assay

The reducing power of the plant extracts was determined according to standard procedures (108). Different amounts of plant extracts (3.125 - 50 µg/ml) in methanol were prepared and mixed with 2.5 ml of 0.2 M phosphate buffer (pH 6.6) and 2.5 ml potassium ferricyanide (1 %

w/v). The mixture was incubated at 50 °C for 20 minutes, followed by the addition of 2.5 ml of trichloroacetic acid (10 % w/v) and the mixture centrifuged at 3000 rpm for 10 minutes. About 2.5 ml of the supernatant was mixed with an equal volume of distilled water and 0.5 ml of $FeCl_3$ (0.1 % w/v) and the absorbance was measured at 700 nm. Ascorbic acid was used as positive controls.

3.5.4 Ferric-Reducing Antioxidant Power Assay (FRAP)

The FRAP assay was performed a previously described, with minor modifications (109). The samples (1.98 to 250 µg/mL) and Ascorbic acid (0.078 µg/mL to 10 µg/mL) were prepared by dissolving in double-distilled deionized water. Freshly prepared FRAP reagent (20 mL acetate buffer, 2 mL TPTZ solution, 2 mL ferric chloride solution and 2.4 mL autoclaved dH_2O) was heated to 37 °C and used in the analysis. 8 µl of sample and 240 µl FRAP reagent were added to 96 well plates, mixed and incubated for 10 minutes. Absorbance readings were obtained at 595 nm and plotted as (Sample Optical density (OD) – Sample background OD-blank) against compound or extract concentration.

3.5.5 Cupric reducing antioxidant capacity (CUPRAC)

This assay was performed as previously described (110). The samples (1.98 to 250 µg/mL) and Ascorbic acid (0.078 µg/mL to 10 µg/mL) were prepared by dissolving in double-distilled deionized water. The sample (50 µl) and 150 µl CUPRAC reagent consisting of 10 mM Copper(II)chloride, 7.5 mM neucoproine (AEC Amersham) and ammonium acetate buffer (1 % (v/v) acetic acid, 0.1 M ammonium acetate, pH 7) were mixed and incubated for 30 minutes at room temperature before absorbance readings were obtained at 450 nm. An experiment blank and sample background controls were also included. The data was plotted as (Sample Optical density (OD) – Sample background OD-blank) against compound or extract concentration.

3.5.6 Crocin bleaching assay using 2,2′-azobis (2-amidonopropane) hydrochloride (AAPH)

This assay was performed according to a previously described method (111). Fifty microliters of the samples (1.98 to 250 µg/mL) and ascorbic acid (0.078 µg/mL to 10 µg/mL) were added to 100 µl crocin solution and 100 µl of AAPH solution. These were mixed and incubated for 1 hour at room temperature before absorbance readings were obtained at 450 nm. The absorbance readings were plotted as (Sample Optical density (OD) – Sample background OD-blank) against compound or extract concentration.

3.5.7 Nitrite and nitrate detection by colorimetric assay

The effect of extract and isolated compound on NO production was studied using a NO assay colorimetric kit (Calbiochem, CA, USA). In aqueous solution, NO is rapidly converted to nitrate and nitrite. Hence, for accurate determination of the total NO generated, both nitrate and nitrite levels must be monitored. Spectrophotometric quantitation of nitrite using only the Griess reagent does not measure nitrate. Therefore, the NADH-dependent enzyme nitrate reductase is used to convert the nitrate to nitrite before quantitation using the Griess reagent. NO was measured from PBMCs by plating cells in 96 well plates at 2.0×10^5 cells/well. Cells were pre-incubated for 1 h with non-cytotoxic concentrations of the crude extract (25 µg/mL) and labdane diterpenoid (50, 25 and 10 µM) before stimulating for NO with a non-cytotoxic concentration of PHA-P, 25 µg/mL and further incubating for 24 h. After the 24 h incubation, cell culture supernatant (50 µl) was collected and incubated with 1 U/µl of nitrate reductase in the presence of 0.2 mM NADH and 50 mM MOPS buffer, pH 7.0. After 20 min, Griess reagent was added and further incubated for 5 min at room temperature. The colour was read at 550 nm (Multiskan Ascent; Thermo Labsystems; MA, USA). A standard curve was generated,

using freshly prepared 0–100 μM potassium nitrate dissolved in assay buffer, to quantitate unknown nitrite in samples.

3.6 High-Performance Thin Layer Chromatography (HPTLC)

High-performance thin-layer chromatography (HPTLC) was performed on a silica gel glass plate (20 × 20 cm, Silica gel 60 F254, Merck) according to standard procedures [Sahgal et al., 2009]. The extracts were dissolved in ethyl acetate and were directly deposited on glass silica gel. TLC plates were developed in a sandwich TLC chamber with Ethyl acetate/Methanol/Water (10:1.35:1) solvent mixture as a mobile solvent. The profiles of the separated spots were sprayed with 90:10 methanol/sulphuric acid reagent after visualization under UV (366 nm) and visible light respectively. The plates were further examined for DPPH active spots. After 25 min, the pale-yellow spots on purple background indicated spots antioxidant activity. A separate run of the plates was stained with Folin-Ciocalteu's reagent and heated at 80 ^{o}C/10 min. The plates were further examined for phenolic active spots. After 25 min, the blue colour spots indicated the presence of phenolic compounds.

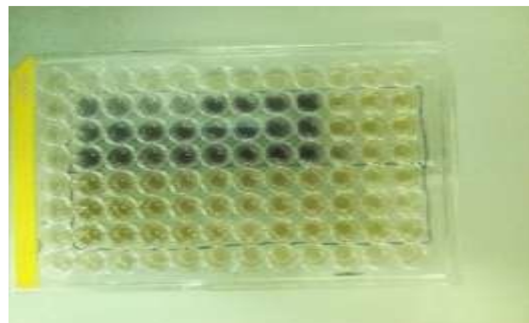
3.7 In vitro cell viability and cytotoxic evaluation

3.7.1 PBMC viability using MTS

Ethical approval for obtaining blood samples from consenting donors was granted by the University of Malawi College of Medicine Research and Ethics Committee (P.11/17/2309). Freshly isolated healthy peripheral blood mononuclear cells (PBMCs) from HIV person were suspended in complete RPMI 1640 (Sigma, MO, USA) medium (containing antibiotics and fetal bovine serum). Cells were plated in 96 well plates (Corning Incorporated, Corning, USA) at 5×10^5 cells per well and treated with the compound at final concentrations of 1.95, 3.9, 7.8, 15.6, 31.25, 62.5, 125 and 250 μg/mL. For plant extracts, cells were inoculated with 250 and

500 µg/ml of the plant extracts from 1 mg/mL stock solution. All the treatments were performed in triplicate and mean (± standard error) was calculated. The number of viable cells was detected after 24, 48 and 72 h using 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) solution (Promega Corporation, WI, USA). The plates were read at 490 nm (reference wavelength of 630 nm). The percentage viability was calculated relative to an untreated control of cells only and the CC_{50} values were determined using GraphPad Prism (GraphPad Software Inc. CA, USA).

(a) Blood sample



(b) Microplates

(c) Blood separation

(d) Blood collection

Figure 3.16: Specimen collection

3.7.2 Macrophage RAW 264.7 cell lines and culture conditions

The macrophage RAW 264.7 cells were used in the cytotoxic evaluation of plant extracts and fractions. The cells were grown (from -81 aliquot) and maintained in T-25 flasks (Corning Incorporated, USA) using complete Dulbecco modified Eagle Media (DMEM) (Sigma Aldrich, St Louis MI, USA). The media was supplemented with 10 % Fetal bovine serum (Sigma Aldrich, St Louis MI, USA) and 0.01 % Gentamycin sulfate (Sigma Aldrich, St Louis MI, USA). Cells were kept at 37 °C for 72 hrs in 5 % CO_2 incubator and observed daily. At approximately 90 % confluence, the cells were trypsinized and concentrated at 1800 rpm for 5 minutes. The pellet was re-suspended and diluted to get 10^4 cells per well.

3.7.3 Mammalian macrophage cytotoxicity assay

The cytotoxic evaluation of crude extracts and fractions were performed using macrophage RAW 264.7 cells as previously described method (Dzoyem et al. 2014). Cells were seeded at 100 ul in 96-well cell culture plate (Costar, USA) followed by incubation overnight to allow cell adhesion. Ten microliters of serially diluted crude extracts and fractions were added in

triplicate after 24 hrs of seeding then incubated for 48 hrs in a humidified atmosphere at 37 °C in 5 % CO_2. Ten microliters of the stock solution of Resazurin (0.15 mg/ml in PBS) were added to each well, gently mixed and further incubated for an additional 4hrs. Fluorescence was measured at an excitation and an emission wavelength of 530 and 590 nm respectively in a Magelan infinite M200 fluorescence multi-well plate reader (Tecan). Dose-response curve was constructed in order to determine the median cytotoxic concentration (CC50) by using the GraphPad version 5.0 Software.

3.8 Antimycobacterial analysis

3.8.1 Mycobacterium smegmatis and Mycobacterium ulcerans

Non-pathogenic, fast-growing *Mycobacterium smegmatis* (Figure 2b) and pathogenic slow-growing *Mycobacterium ulcerans* (12/97) (Figure 2a) were obtained from the Antimicrobial and Biocontrol Agents Unit, Faculty of Science, University of Yaoundé 1, Cameroon. *M. smegmatis* was cultured on Mueller Hinton agar plate at 37 ºC for 2 days. *M. ulcerans* were maintained on Lowenstein-Jensen slopes and cultured on enriched media comprising of Middlebrook 7H9 (Difco) supplemented with 10 % oleic acid albumin dextrose-catalase (OADC), 10 % FBS, 0.05 % Tween 80 (sigma), and 0.5 % glycerol. Then the colonies were transferred to sterile saline (0.9 % w/v NaCl) to obtain a suspension comparable to 0.5 McFarland turbidity standard.

(a) *M. ulcerans*

(b) *M. smegmatis*

Figure 3.17: *Mycobacterium ulcerans* and *Mycobacterium smegmatis*

3.8.2 Microplate alamar blue assay

The Microplate alamar blue assay was performed as previously described method (112). Briefly, 100 ul of the enriched broth was added to each well of the sterile flat-bottom 96-well plate. And 100 ul of the extracts and fractions were introduced to the wells in the first row and mixed thoroughly followed by serial two-fold dilution to create a concentration range of 0.8192 -512 ug/ml and 0.4096 -256 ug/ml respectively. The positive control drug, streptomycin was

prepared directly in the microtiter plate. Bacterial inoculums were prepared from cultures in Middlebrook 7H9 broth containing glycerol and OADC growth supplement. One hundred microliters of the inoculum were added to each well. The turbidity was adjusted to the equivalent of 0.5 McFarland No. 1 ($1x10^8$ CFU/ml). A sterile control comprising of medium only and a growth control consisting of cells and growth medium were also included for each microtiter plate. Evaporation during incubation period was minimized by the addition of media to all the perimeter wells of the 96-well microtiter plates. The covered microtiter plates were sealed in aluminum foil and incubated at 37 °C. After 7 days for *M. ulcerans* and 72 hours for *M. smegmatis*, 30 ul of the mixture of 1:1 ratio of 10 % tween 80 and Resazurin solution was added to each well and the plates were further incubated overnight. Resazurin reagent becomes fluorescent when metabolized by cells, allowing quantification of cell viability by measuring their metabolic function. A colour change from blue to pink indicated mycobacterial growth and the MIC was taken as the highest dilution of the crude extracts and fraction or positive control drug prevented colour change from blue to pink.

3.8.3 Mycobacterium tuberculosis

3.8.3.1 Specimens

Sputum specimens were collected from five patients with old chronic cases, those suspected to have MDR TB at Zomba Central Hospital and were put in a cool box and transported to the laboratory for analysis. At the laboratory, the sputum samples were macroscopically examined for adequate quantity and contained mucoid or mucopurulent material.

3.8.3.2 Specimen processing and identification

All specimens were processed within 24 hr of specimen collection and standard N-acetyl-L-cysteine-NaOH method for digestion, decontamination, and concentration (113). Samples were

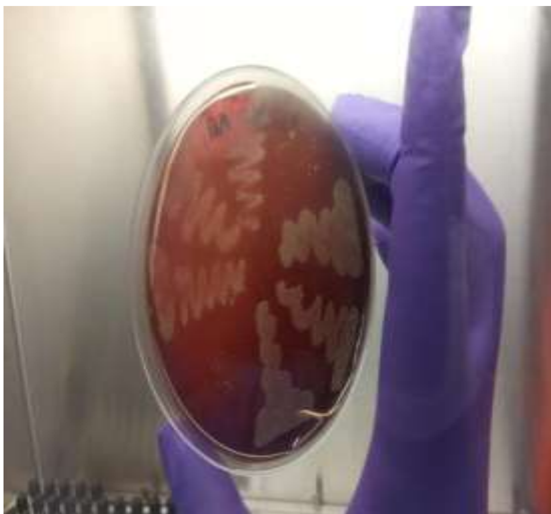
concentrated by centrifugation at 3,200 × g for 20 min. The supernatant was discarded, and the final sediment of specimens was suspended in phosphate buffer (pH 6.8) (113). This suspension was then used for making smears for acid-fast staining with the Ziehl-Neelsen and for culturing in liquid and solid media. The samples with microscopy positive of more than 10 acid-fast bacilli (AFB) per microscopic field (scored as "+++") were used in the analysis (113). The rest of the remaining sediment was resuspended in 3 ml of sterile distilled water and then used to inoculate the culture medium used in both drug susceptibility tests. The culture was grown on both solid and liquid media. An immunochromatographic test with mouse monoclonal anti–MPT64 from SD Bioline MPT64 was also used to identify the Mtb complex (74). Monoclonal antibodies are produced from hybridomas obtained by the fusion of P3UI myeloma cells with spleen cells of mice immunized with an MPB64 antigen (112). Briefly, 100 µl of the growth suspension was added in the cassette and incubated for 15 minutes at room temperature. The presence of a control band alone indicates a negative result, whereas the presence of two-colour bands indicates a positive result.

Inoculation of culture media for primary isolation. The Bactec MGIT 320 system for liquid and solid medium on Lowenstein-Jensen (LJ) slant was used. The media were inoculated using the established individual laboratory standard operating procedure (SOP). For MGIT medium, standard recommended procedures were followed (manufacturer's recommendations and the MGIT manual by FIND (114). Cultures grown on Lowenstein-Jensen (LJ) medium were used for drug susceptibility testing no later than 14 days after the first appearance of colonies on the slant. Colonies were scraped from the medium with a sterile loop. A suspension adjusted to be equivalent to a 0.5 McFarland standard was prepared by using glass beads to ensure homogeneity and then diluted 1:5 before inoculating 0.5 ml of the suspension into the MGIT

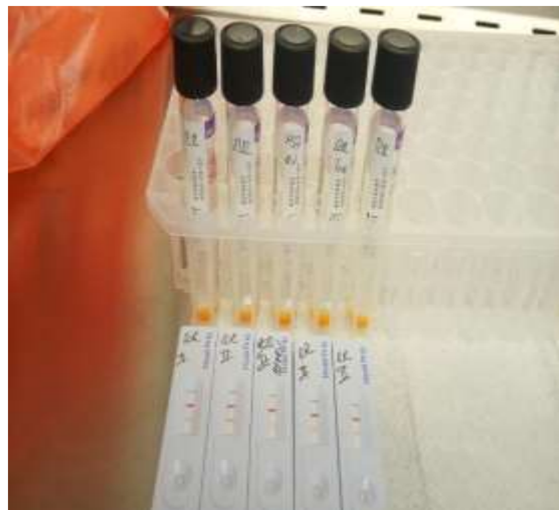
set. All inoculated sets were loaded into the BACTEC MGIT 320 instrument within 8 hours of inoculation.

3.8.3.3 Direct DST procedure

The direct DST procedure of 4-to 21-day protocol used in the analysis and the control was diluted 1:10 and an antimicrobial mixture of polymyxin B, amphotericin B, nalidixic acid, trimethoprim, and azlocillin (PANTA) (Becton Dickinson Diagnostic Systems, Sparks, MD) and growth supplements OADC were added to MGIT tubes with drugs and the control to suppress contamination and promote growth.



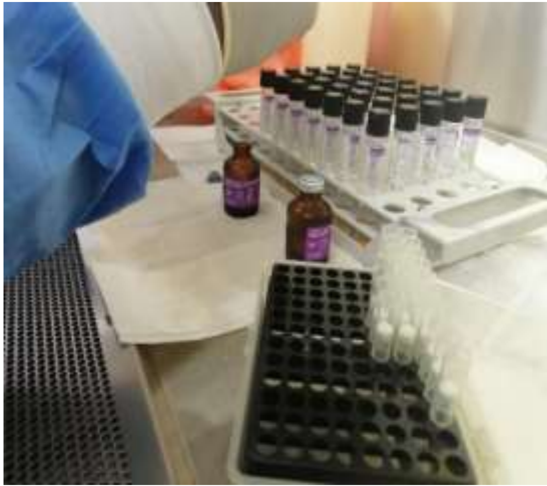
(a) Culture of Mtb resistant strain



(b) Confirmation of Mtb resistant strain

(c) Testing of Mtb resistant strain

(d) Sample preparation


(e) Sample preparation

(f) Loading of samples

Figure 3.18: Sample preparation, loading, testing and confirmation of tests on Mtb resistant strain

3.9 Statistical analysis

GraphPad Prism 8 (GraphPad Software Inc. CA, USA) and Microsoft Excel were used in the analysis. All parameters were tested in triplicate. The value expressed as mean±standard deviations and ANOVA were used to determine the significant differences amongst groups. Data for all experiments were presented as the mean ± standard deviation (n = 3-6). Significant differences were estimated using GraphPad Prism 8 (GraphPad Software Inc. CA, USA).

CHAPTER 4: RESULTS AND DISCUSSIONS

4.1 Review of Malawian medicinal plants that have reported anti-HIV activities

Table 4.1.1 below shows a detailed summary of the plant species, vernacular names, traditional use, active constituents, pharmacological activities and their references while Table 4.1.2 shows a summary of compound groups identified with anti-HIV activities.

Table 4.1.1: A summary of Malawian medicinal plants reported to possess Anti-HIV Activities

PLANT SPECIES	VERNACULAR NAME	TRADITIONAL USE	ACTIVE CONSTITUENTS	PHARMACOLOGICAL ACTIVITY
Abrus precatorius L (Fabaceae) (115)	Nsiyapita, Ntimba, Mkadanyulungwe	Aphrodisiac, purgative, malaria, asthenia, asthma, respiratory tract infections, convulsions, inflammation, antivenom, sexually transmitted infections.		Inhibits HIV-1 protease Interacts with ribosome function in the infected cell and inhibits viral protein synthesis
Adansonia digitata L (Bombaceae) (116)	Mlambe, Mlonje	Antimicrobial, antimalarial, diarrhoea, anaemia, asthma, antiviral, antioxidant, anti-inflammatory.	Flavonoid Glycosides, Proanthocyanidin Epicatechin	Inhibits HIV-1 reverse transcriptase and protease
Aerva lanata (Amaranthaceae) (117)		Anti-inflammatory, headache, skin disease, dissolve kidney, and gall bladder stones, uterus clearance after delivery.		Inhibits HIV-1 reverse transcriptase
Aeschynomene nyassana (Fabaceae) (118)	Nakajomba, Kanyata, Mvulala	Enema, sexually transmitted infections, oedema, menstrual problems		Personal communication
Albizia gummifera (Fabaceae) *Albizia amara* (Fabaceae) *Albizia antunesiana* (Fabaceae) (119)	Mtanganga, Chikwani	Bacterial infections, skin diseases, malaria, stomach pain	Vitalboside A, vitalboside-A-2-methylglucuronate, Lupeol, Lupenone	Inhibits HIV-1 reverse transcriptase
Aloe vera (L.) Burm.f. (Xanthorrhoeaceae) (120)	Alovera	Bacterial/fungal infections, boost appetite/immunity, cough, diarrhoea, herpes zoster, tuberculosis		Anti-HSV-2 activity
Argemone mexicana L. (Papaveraceae) (121)	Doza, Lichongwe, Mkumujalaga	Cryptococcal meningitis		Anti-HIV activity
Artemisia annua, (Asteraceae) (122)		Smallpox, infectious disease, stomach ache	Artemisinin	Anti-HIV activity

Asparagus spp (Liliac). *Asparagus africanus* Lam (Liliac) (123)	Mtsitsilamanda, Kankande, Mgalambuti	Aphrodisiac, anaemia, diarrhoea, dysentery, antispasmodic, sterility, stomach ache, ulcers, digestive, rheumatism, diabetes	Saponins, Tannins Flavonoids	Anti-HIV Activity
Aspilia pluriseta Schweinf. (Compositae)	Chamasala	Infection, rheumatism, fevers, malaria.	Thiarubrine-A, dithiacyclohexadienepo lyacetylen	Anti-HIV Activity
Bersama abyssinica Fresen. (Melianthaceae) (116)	Mkanga, Chiwindu, Mtutumuko	Gonorrhoea, epilepsy, leprosy, haemorrhoids.	Mangiferin Cardiac glycosides, Sterols,	Target the interaction between the viral envelope glycoprotein gp120 and the CD4 receptor**.**
Bidens pilosa L (Asteraceae) (124)	Chinomba, Kanzota, Kaliputi	Malaria, diarrhoea, dysentery, hypertension, analgesic, asthenia, diuretic, arthritis, kidney disorders,		Inhibits viral core protein p-24 expression
Boerhavia coccinea Mill. (Nyctaginaceae) *Boerhavia erecta* L. (Nyctaginaceae) (125)	Damata, Nambenawo	Infectious diseases	Boeravinone, Coccineon	Inhibits HIV-1 integrase
Bridelia micrantha (Euphorbiaceae) (126)	Mpasa, Msopa	Burns, wounds, conjunctivitis, painful eyes, constipation, gastric ulcers, cough, headache, rheumatism, painful joints, dysentery, malaria, sexually transmitted infections, stomach ache, Anthelmintic, antidiabetic, antimicrobial, anticonvulsant, antidiarrhoeal, antinociceptive, antioxidant, antiplasmodial	Flavonoids, Tannins Friedelin, Taraxerone, Epifriedelinol, Taraxerol, Gallic acid, Ellagic acid Sitosterol	Inhibits RNA-dependent-DNA polymerase activity of HIV-1 reverse transcriptase.
Burkea africana (Myrtaceae) (127)	Mkalati,	Antioxidant, antidiarrhoeal, anticonvulsant, antimicrobial, antimycobacterial	Saponins, Tannins, Cardiac glycosides, Flavonoids,	Anti-HIV Activity
Carica papaya (Caricaceae) (128)	Papaya	Antimicrobial, antiparasite, haemorrhoids, hypertension, wound healing, antivenom,	Triterpenes, Sterols,	Anti-HIV Activity

		sickle cell anaemia, malaria, abortifacient, antifertility, cancer, mental disorder	Glycosides, Flavonoids, Tannins	
Cassia abbreviate Oliv. (Caesalpiniaceae) (116)	Mchalamira, Mkwapukwapu, Mlumanyame	Backache, abdominal pains, diarrhoea, constipation, ulcers, tooth ache, fever, syphilis, gonorrhoea, general blood cleanser, appetite enhancer, reducing HIV levels		Anti-HIV activity
Centella asiatica (L.) Urb. (Apiaceae) (116)	Namsanganya, Tengwe	Gonorrhoea, syphilis, diabetes, fever, leprosy, wound healing, gastro-intestinal ailments, asthma, neurosis	Asiatic acid, Asiaticoside, Madecassic acid, Terminolic acid, Quercetin, Luteolin, Kaempferol,	Anti-HIV activity.
Cleome gynandra (Cleomaceae) (116)		Oral candidiasis		Anti-HIV activity
Combretum adenogonium Steud. ex A. Rich (Combretaceae) *Combretum molle* (Combretaceae) (116)	Kadale, Msimbiti, Chinama, Kakungi	Eye disorder, leprosy, haemorrhoids, diarrhoea, dysentery, sexually transmitted infections	Gallotannin, Ellagitannin, Pentacyclic Triterpene glucosides namely punicalagin, arjunglucoside, sericoside.	Inhibits RNA-dependent-DNA polymerase activity of HIV-1 reverse transcriptase.
Crinum macowani Baker (Amaryllidaceae) (129)	Mfutukulu	Acne, backache, cold, fever, blood cleansing, boils, glandular swelling, kidney and bladder diseases, rheumatism, wounds, tonic, tuberculosis, sexually transmitted infections		Anti-HIV activity
Dichrostachys cinerea (Fabaceae) *Dichrostachys cinerea* (L.) Wight & Arn. (Fabaceae)	Chipangala, Naphangale, Mphangala	Dysentery, leprosy, headache, antiparasitic, anthelmintic, aphrodisiac purgative, diuretic, epilepsy, antivenom, toothache, analgesic, laxative, sexually transmitted infections, coughs, elephantiasis, toothache,	Friedelin, Friedlan-3-Ol, Sitosterol, Amyrin, Octacosanol, Hentricontanol, Coumarins Imperatorin, Marmesin, Aesculetin	Inhibits HIV-1, 2 reverse transcriptase

Dodonaea viscosa (Sapindaceae) (116)	Nandolo, Mlaka, Nkangamani	Cold, fever, malaria, piles, skin infections, sore throat, wounds		Inhibits HIV-1 replication
Elaeodendron transvaalense (Burtt Davy) R.H. Archer (Celastraceae) (116)		Pain, sores, sexually transmitted infections, gastro-intestinal disorders, microbial infections, genitourinary system disorders.	Phenolic compounds, Coumarins, Flavonoids, Saponins Stilbenoids, Tannins, Triterpenoids	Anti-HIV activity
Elephantorrhiza goetzei (Harms) (Fabaceae) (116)	Nkhumba, Chitete, Chikundulima	Pain, sores, sexually transmitted infections, gastro-intestinal disorders, microbial infections, genitourinary system disorders.	Phenolic compounds, Coumarins, Flavonoids, Saponins Stilbenoids, Tannins, Triterpenoids	Inhibits/protects 50% of the monolayer cells against destruction by the virus
Eleusine indica (Gramineae) (116)	Chinsangwi, Chigombe	Antioxidant, antimicrobial, anticancer	Phenolic compounds	Antiviral activity
Emilia coccinea (Sims) G. Don (Compositae) (116)	Kaliwendo, Nambenawo	Infectious diseases, ulcers, craw-craw, ringworm, fever, convulsions in children	Alkaloids, Phenolics, Flavonoids, Terpenoids, Cardiac glycosides	Anti-HIV activity
Erythrina abyssinica (Fabaceae) (116)	Mlindimila, Mtambe	Ethnoveterinary medicine, bacterial infection, skin diseases		Inhibits HIV-1 reverse transcriptase
Euphorbia hirta L. (Euphorbiaceae) *Euphorbia tirucalli* L. (Euphorbiaceae) *Euphorbia whyteana* L. (Euphorbiaceae) (116)	Nakameso, Nakatobwa Mkhadze Nakachechi	Infectious diseases, female disorders, respiratory ailments, cough, cancer, tumors, warts, asthma, earache, neuralgia, rheumatism, toothache	Tannins, Triterpenes, Phytosterols, Polyphenols, Flavonoids	Inhibits HIV-1, 2 reverse transcriptase.
Ficus sycomorus L. (Moraceae) (116)	Mkuyu, Mtundu, Chikujumba	Infectious diseases	Various volatile compounds	Inhibits HIV-1 and HIV-2
Flacourtia indica (Burm. f.) Merr. (Salicaceae) (116)	Ndawa, Songoma, Mtema	Snakebite, arthritis, STI, cough, pneumonia, bacterial throat infection.	Phenolic glycosides, Lignin, Coumarins B-Sitosterol, Tannins, Polysaccharides, Flavonoids, Sugars, Alkaloids, Terpenoids	Inhibits/protects 50% of the monolayer cells against destruction by the virus
Flueggea virosa, (Phyllanthaceae) (116)	Mponbona, Mkulangondo	Cough, tuberculosis, fever, pain, diabetes, contraceptive.	Alkaloids- virosaine	Inhibits HIV-1 reverse transcriptase

Garcinia speciose (Clusiaceae) *Garcinia edulis* (Clusiaceae) *Garcinia buchananii* Bak. (Clusiaceae) *Garcinia livingstonei* T. Anderson (Clusiaceae) (130)	Mpimbi, Mtundira	Cryptococcal meningitis, diarrhoea, herpes simplex/zoster, skin rash, tuberculosis	Protostanes, Garcisaterpenes A&C Garceduxanthone, Pentacyclic Triterpenoids, Friedelin, Lupeol, Lupeol acetate.	Inhibits HIV-1 reverse transcriptase Inhibits HIV-1 protease
Harrisonia abyssinica (Rutaceae) (131)	Msangalasa	Dizziness, Insomnia, nausea, vomiting, bubonic plague, swollen testicles, induce abortion, tuberculosis, Fever, sexually transmitted infections		Anti-HIV Activity
Hypericum perforatum (St. John's wort) (Hypericaceae) (132)		Antitumor, anti-inflammatory, antibacterial, antifungal, antidepressant, antiviral/antiretroviral	Hypericin	Inhibits non-human retrovirus and HIV-1 RTase
Hypoxis hemerocallidea (African potato) (Hypoxidaceae) (129)		Cancer, blood cleansing, boost appetite/immunity, wounds, tuberculosis, vaginal and oesopharyngeal candidiasis,	Hypoxoside,	Inhibits HIV-1 reverse transcriptase
Jatropha curcas (Euphorbiaceae) (72)	Msatsimanga, Msapatonje	Wound healing, fever, jaundice, rheumatism, lymphocytic leukemia	Corilagin	Anti-HIV activity
Kigelia africana (Lam.) Benth. (Bignoniaceae) (116)	Mvunguti	Infectious diseases, HIV, fainting, anaemia, sickle-cell epilepsy, respiratory ailments, hepatic and cardiac disorders, nutritional illnesses, wasting, leprosy, impetigo,	Alkaloids, Saponins, Terpenoids, Phenolic Flavonoids, Cardiac glycosides, Steroids	Inhibits HIV-1 reverse transcriptase
Lannea schweinfurthii Engl. (Anacardiaceae) (30)	Chiumbu	Infectious diseases.		Anti-HIV Activity

Leonotis nepetifolia L. R.Br. (Lamiaceae) (116)	Nlongandundu Mnyambalame	Infectious diseases, HIV		Inhibits HIV-1 (strain III b) and HIV-2
Maesa lanceolata (Myrsinaceae) (133)	Mangachule, Mkakama	Flu, antihelmenthic, appetizer, stomach ache, sexually transmitted diseases, malaria, backache, arthritis	Maesasaponin	Anti-HIV Activity
Melia azedarach (Meliaceae) (116)	Matholisa	Diarrhoea, malaria, skin infections, genitourinary tract infections	Lectins, Polypeptides Sugar-containing compounds:	Inhibits HIV-1 Reverse transcriptase Antiviral activity against herpes simplex virus type 1 (HSV-1) by inhibiting specific infected-cell polypeptides (ICPs) produced late in infection.
Momordica charantia (Cucurbitaceae) *Momordica balsamina L* (Cucurbitaceae) *Momordica foetida* (Cucurbitaceae) (134)	Likulypsa Chikhaka, Tungwi	Antioxidant	Ribosome-inactivating proteins (RIPs), lectins	Inhibits HIV 1 RT infection and replication Anti-HIV activity
Monotes africanus (Dipterocarpaceae) (135)	Mkakatuku, Mkalakate	Toothache	6,8-diprenylaromadendrin 6,8-diprenylkaempferol	Blocks HIV-1 replication at the entry step
Morella salicifolia (Myricaceae) (119)		Boost appetite/immunity cryptococcal meningitis, diarrhoea, herpes simplex, tuberculosis		Inhibits HIV-1 replication and protease
Moringa oleifera Lam. (Moringaceae) (136)		Antimicrobial, antiparasitic, anti-cancer, inflammation, colic, diuretic, anaemia, hypertension, aphrodisiac, fever, purgative.		Inhibits HIV-1 reverse transcriptase
Musa acuminate (Musaceae) *Musa paradisiaca* (Musaceae) (116)	Nthochi	diarrhoea (unripe), dysentery, menorrhagia, diabetes, antilithic, antiulcerogenic hypoglycemic, hypolipidemic, antioxidant, anti-inflammation, pain, snakebite	BanLec, Jacalin-related lectin	Blocks HIV entry Inhibits HIV-1 replication

Myrothamnus flabellifolia Welw. (Myrothamnaceae) (116)	Chanasa, Chisoni	Chest complaints, wounds, cough, influenza, mastitis, backaches, kidney disorders, hemorrhoids, abdominal pains, scurvy, halitosis, gingivitis.	Polyphenols, Gallotannins 3,4,5-tri-Ogalloylquinic acids.	Inhibits HIV-1 reverse transcriptase Polyphenols protect cell membranes against free radical-induced damage;
Ozoroa reticulata (Baker f.) R. Fern. & A. Fern (Anacardiaceae) (116)	Namasira, Masimya	Infectious diseases, kidney and liver complaints, chest pain, diarrhoea, schistosomiasis, ulcers and hernias, otitis, colic, dysentery, muscle pains, fever, hypertension, throat infections.	6-pentadecylsalicylic acid, Anacardic acid, Ginkgoic acid	Inhibits HIV-1
Phyllanthus amarus (Phyllanthacaea) *Phyllanthus reticulatus* Poir. (Phyllanthacaea) (137)		Anti-parasitic, fever, sexually transmitted infections, cancer, diuretic, dysentery, diarrhoea, analgesics, hypertension, inflammation, respiratory tract infection, jaundice, liver disorder, ulcers kidney disorder	Phyllanthin Hypophyllanthin	Inhibits HIV-mediated cell fusion, Inhibits HIV-1 Reverse Transcriptase, Inhibits HIV-1 integrase Inhibits HIV-1 protease
Piliostigma thonningii (Schum.) Milne-Redh. (Fabaceae) (138)	Chitimbe, Msekese	Cough		Anti-HIV, HSV-1 and 2 activities
Prunus Africana (Hook. f.) Kalkman (Rosaceae) (116)		Fevers, malaria, stomach pain wound dressing, arrow poison, purgative, HIV, kidney disease, appetite stimulant, gonorrhoea.	Ferulic acid, n-docosanol, lauric acid, myristic acid, β-sitostenone, β-sitosterol	Inhibits HIV-1 reverse transcriptase
Pseudolachnostylis maprouneifolia (Phyllanthaceae) (139)	Msolo, Likulpsya	Stomach ache, cathartic, pneumonia, diarrhoea, sexually transmitted infections, cough tuberculosis, anaemia, leprosy, snakebite antidote, fever,		Inhibits HIV-1 reverse transcriptase
Psidium guajava (Myrtaceae) (140)	Guwava	Dysentery, diarrhoea, sexually transmitted infections, respiratory tract infections.	Procyanidine B Saponins	Inhibits HIV-1 reverse transcriptase Prevents HIV-1 envelop proteins (env) mediated virus entry. Prevents gp41 6-HB formation
Pterocarpus angolensis (Fabaceae) (116)	Mlombwa	Aching ear, menorrhagia, infertility amongst women, eye		Anti-HIV activity

		infections, wounds and psoriasis sexually transmitted infections, piles, amenorrhoea, haematuria and bilharzias, Antibacterial, diarrhoea, heavy menstruation, nose bleeding, headache, parasitic worms, sores and skin problems, antifungal, anthelmintic, Anti-inflammatory		
Peltophorum africanum (Ceasalpiniaceae) (116)		Abdominal pains, cough, diarrhoea, dysentery, wounds dysmenorrhea, infertility, sore throat, toothache, tuberculosis,		Inhibits HIV-1 reverse transcriptase
Pyrenacantha kaurabassana (Icacinaceae) (52)	Mchende, Chitupa	Stomach ache, sexually transmitted infections, roundworms, rheumatism	Xanthones hexadecahydrochrysen-3-ol	Anti-HIV activity
Rauvolfia caffra (Apocynaceae) (141)	Chiwimbi, Mwimbi	Coughs, gastrointestinal disturbances, skin infections, hypertension, diarrhoea, dysentery, scabies, worm infections, malaria	Indole alkaloid	Anti-HIV Activity
Ricinus communis (Euphorbiaceae) (126)	Msatsi, Mbalika	Abortifacient, abscesses, cough, antihelmentic, arthritis, asthma, dermatitis, diarrhoea, fever, flu, toothache, tuberculosis, wounds.		Interacts with ribosome function in the infected cell and inhibits viral protein synthesis
Rhoicissus tridentate (Vitaceae) (116)	Mpesa, Mpete	Eye infections, sexually transmitted infections		Inhibits HIV-1 reverse transcriptase
Rhus chirindensis Baker f. (Anacardiaceae) *Rhus acuminatissima* (Anacardiaceae) (116)		Heart complaints, stimulate blood circulation, rheumatism, mental disorders, sexually transmitted infections.	Triterpenoids: Rhuscolide -A, Moronic acid .	Anti-HIV activity *Rhus acuminatissima* weak activity against HIV protease
Senna occidentalis (Fabaceae) (142)		Cough, diarrhoea, jaundice, malaria		Inhibits HIV-1 reverse transcriptase
Securidaca longipedunculata Fresen. (Polygalaceae) (116)	Bwazi, Chosi	Stomach complaints, cough, tuberculosis, wound dressing,	Caffeoylquinic acids: 3,4,5-tri-O-caffeoylquinic acid,	Anti-HIV and anti-SIV activities. Binds irreversibly to gp120 and inactivates virus.

		rheumatism, syphilis, diarrhoea, typhus.	4,5-di-O-caffeoylquinic acid, caffeic acid, synapoic acid	
Solanum americanum L (Solanaceae) (143)	Mthungwi	Boost appetite/immunity		Antiviral activity
Steganotaenia araliacea (Apiaceae) (52)	Mpoloni, Mpandanjobvu	Dysentery, diarrhoea, Sore throats, colds, antivenin, Rodent poison, insect repellent, asthma, antidiuretic	Flavonoids, Saponins	Anti-HIV Activity
Syzygium cordatum Hochst. ex Krauss (Myrtaceae) *Syzygium guineense* Willd (Myrtaceae) (144)	Nyowe, Nanyole, Katope	Malaria, diarrhoea, stomach ache, purgative, analgesic, menstrual disorders, wounds, respiratory tract infections.		Anti-HIV activity Inhibits HIV-1 reverse transcriptase
Terminalia chebula Retz. (Combretaceae) *Terminalia sericea* (Combretaceae) (116)	Napini, Mpini	Venereal diseases, diarrhoea, dysentery, colic, pneumonia, cough, skin diseases, schistosomiasis, problems with menstruation	Chebulagic acid Punicalin	Inhibits HIV reverse transcriptase and integrase Inhibits RNA-dependent-DNA polymerase activity of HIV-1 reverse transcriptase.
Toddalia asiatica (Rutaceae) (145)	Msangalusi, Ncheula	Stomach problems, malaria, Cough, chest pain, food poisoning, sore throat, Antioxidant, Antidiabetic	Nitidine Magnoflorine	Inhibits of HIV-1 reverse transcriptase
Vernonia stipulacea Klatt (Asteraceae) *Vernonia amygdalina* Delile (Asteraceae) (116)	Mfutsa, Msanagusanga	Diarrhoea, fever, flu, contraceptive, rheumatic diseases, ascariasis, hepatitis, malaria, diabetes, worms, tonsillitis, STI, measles, skin problems, chicken pox	Gallic acid, Chlorogenic acid, Dicaffeoyl acids, Quercetin, Vernolide, Octahydrovernodalin, Vernonioside A3, Vernodalol,	Inhibits HIV-1 reverse transcriptase and protease
Ximenia americana L. (Olacaceae) (116)	Mpinji, Mtengere, Mtundu	Contagious diseases, stomach complaints, placenta expulsion, internal parasitism,	Proanthocyanidins, Triterpenoid, Saponin.	Inhibits HIV-1 replication
Warburgia salutaris G. Bertol. Chiov.		Malaria, cold, cough, sexually transmitted infections	Muzigadial, furan,	Anti-HIV Activity

(Canellaceae) (116)			warburganal, polygodial	
Ziziphus mucronata Willd. (Rhamnaceae) (116)	Kankande, Msawu wathengo	Diarrhoea, dysentery, stomach ulcers, fever, anti-peristalsis, skin disease, anti-inflammatory, menorrhagia, infertility	Flavonoids Mucronine D, Sanjoinine compounds	Inhibits HIV-1 reverse transcriptase and protease

Table 4.1.2: A summary of some compound groups identified with anti-HIV activities

Compound group	Example	Mode of action	Species/ Family
Alkaloids (146)	Nitidine Magnoflorine	Inhibits human lymphoblastoid cell killing by HIV-1 in in vitro XTT- based. Anti-HIV assay	*Toddalia asiatica* (Rutaceae)
Quinones (132)	Hypericin	Inhibitory activity against non-human retrovirus and HIV-1 RTase	*Hypericum perforatum*
Lignans (147)	Phyllanthin Hypophyllanthin	Good anti-HIV activity in vitro	*Phyllanthus amarus* (Phyllanthacaea)
Xanthones (148)	Mangiferin	Inhibit virus replication within cells.	*Bersama abyssinica* Fresen. (Melianthaceae)
Flavonoids (135)	6,8-diprenylaromaden-drin 6,8-diprenylkaempferol	Anti-HIV-1 integrase activity	*Monotes africanus* (Dipterocarpaceae)
Phenolics (116)	Chebulagic acid Punicalin	Anti-HIV integrase and reverse transcriptase activity	*Terminalia chebula* Retz. (Combretaceae)
Proteins (134)	Ribosome-inactivating proteins (RIPs)-MAP30 (Momordica Anti-HIV Protein), α- and β-momorcharins	Inhibit HIV replication in acutely and chronically infected cells	*Momordica charantia* (Cucurbitaceae)
Peptide (116)	Meliacine	Antiviral activity against herpes simplex virus type 1 (HSV-1) by inhibiting specific infected-cell polypeptides (ICPs) produced late in infection	*Melia azedarach* (Meliaceae)
Saponins (133)	Maesasaponin	Moderate anti-HIV-1 activity	*Maesa lanceolate* (Myrsinaceae)

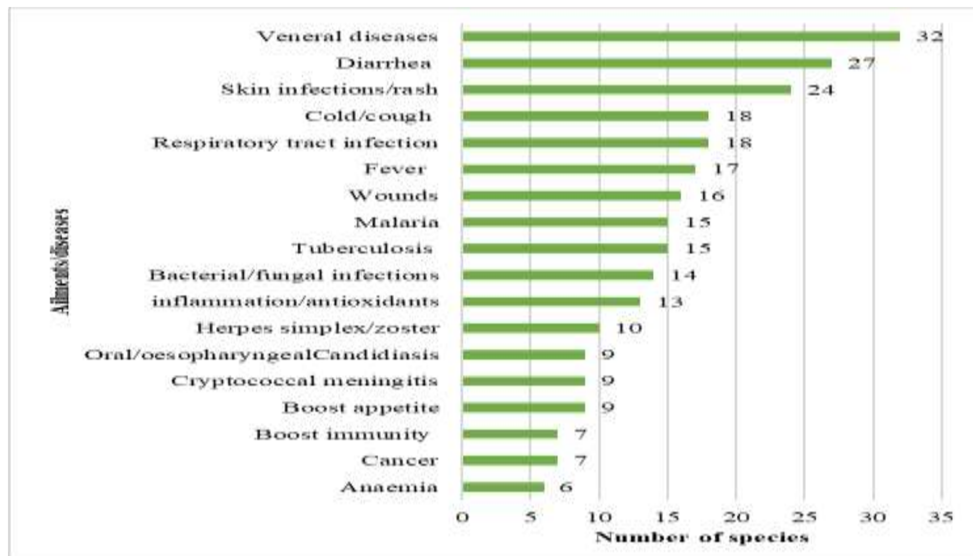


Figure 4.1.1: Major ailment and disease categories and plant species reported. Most species were reported in more than one ailment and disease category

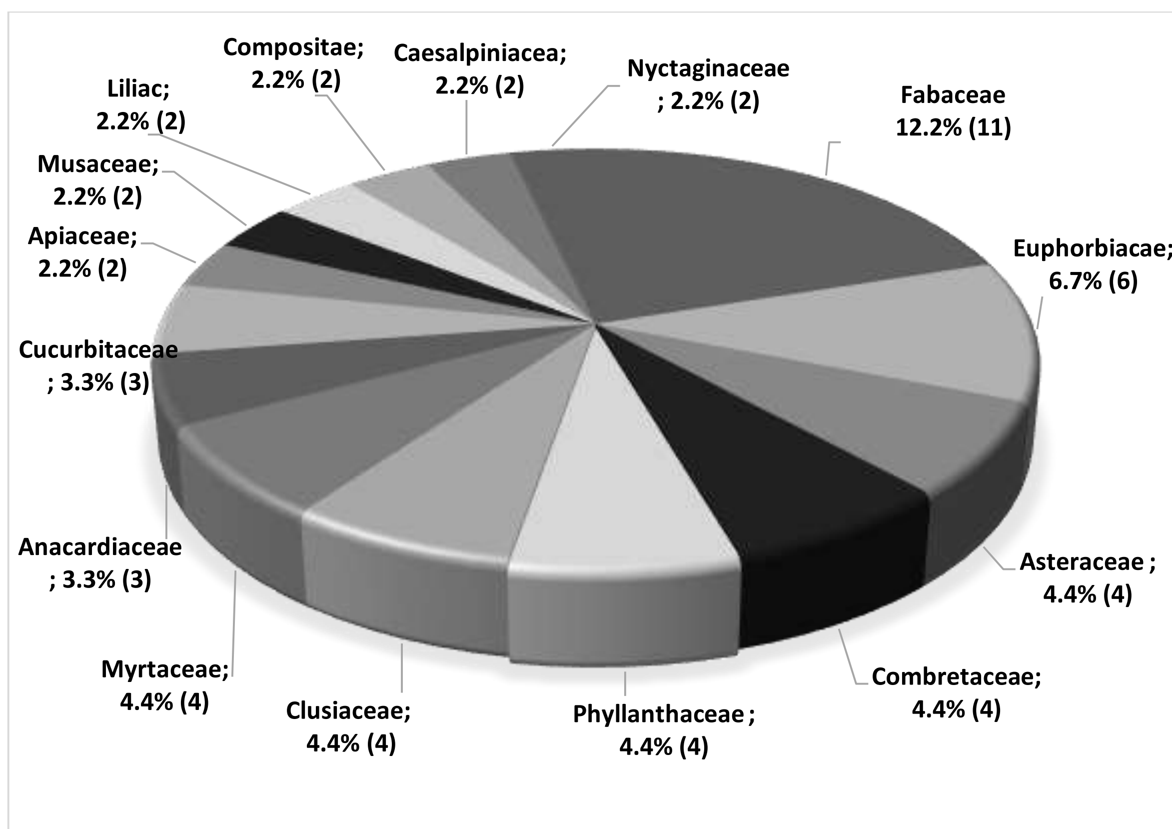


Figure 4.1.2: Families with the largest number of medicinal plants (more than 2 species) used to treat and manage HIV/AIDS opportunistic diseases in Malawi

In the present study, the 90 plants listed in Table 4.1.1 are frequently used by local Malawians as traditional medicine for the treatment of various ailments and these have demonstrated anti-HIV activities. These plants are identified to belong to 50 families which included Fabaceae (12.2 %), Euphorbiaceae (6.7 %), Myrtaceae (4.4 %), Combretaceae (4.4 %), Asteraceae (4.4 %), Clusiaceae (4.4 %), and Phyllanthaceae (4.4 %) being the most represented families, whose species are well-known to have various pharmacological activities as shown in Figure 4.1.2. By analysing and comparing the information, several compounds and groups of compounds have been identified and categorized into eight distinct groups. These included flavonoids active compounds (6, 8-diprenylaromadendrin and 6, 8-diprenylkaempferol) isolated from *Monotes africanus,* and are known to blocks HIV-1 replication at the entry step. Alkaloids active compounds (Nitidine and Magnoflorine) isolated from *Toddalia asiatica* also known to inhibit human lymphoblastoid cell. Quinones active compound, Hypericin isolated from *Hypericum perforatum* known for its inhibitory activity against non-human retrovirus and HIV-1 RTase. Protein compounds from *Momordica charantia* reported to have Ribosome-inactivating proteins (RIPs) - MAP30 (Momordica Anti-HIV Protein), α- and β-momorcharins which inhibit HIV replication in acutely and chronically infected cells. Terpenoid and terpenoid glycosides compound (Sericoside) from *Combretum molle* reported inhibiting RNA-dependent-DNA polymerase activity of HIV-1 RT. Phenolic compounds Chebulagic and Punicalin from *Terminalia chebula* that exhibits anti-HIV integrase and reverse transcriptase activity. Lignans compounds Phyllanthin and Hypophyllanthin from *Phyllanthus amarus* reported having good anti-HIV activity in vitro. Xanthones compound, Mangiferin isolated from *Bersama abyssinica* inhibit virus replication within cells (Table 4.2). Most of these plant species are reported (Figure 4.1.1) to treat various major ailment and diseases including venereal diseases (32), diarrhoea/dysentery (27), skin infections/rash (24), respiratory infection (18), cold/cough (18), Fever (17), tuberculosis (15) and Herpes zoster (10). Most of these

species were reported in more than one ailment and disease category. Some of the plants reviewed in the study included *Phyllanthus amarus* from Phyllanthaceae, *Adansonia digitata* from Bombaceae, Monotes africanus from Dipterocarpaceae and *Terminalia sericea* from Combretaceae families respectively. These plants are well studied with promising results around the world, well known for their use in the management of opportunistic infections and the management of asymptomatic patients in the earliest stage of AIDS. Scientifically, they have proven to possess anti-inflammatory, immunostimulant, antiviral, antimicrobial, anticancer and antioxidant activities. The study further reviewed several assays mechanism for an anti-HIV plant evaluation. Some of the commonly used assays for the selected plants included HIV-1 Reverse Transcriptase (RT) assay, HIV-1 Integrase (IN) assay, HIV-1 Protease (PR) assay, HIV-1 p24 assay, Cell-based assays infected with isolated HIV strains or pseudovirions, NF-κB activation assay, Hela-Tat-Luc assay and Hela-Tet-ON-Luc assay. In this study, it was observed that HIV-1 Reverse Transcriptase (RT) assay is the widely used assay for anti-HIV activities evaluation for the plants including local medicinal plants *Dichrostachys cinerea, Pterocarpus angolensis, Bersama abyssinica* and *Albizia gummifera*. Another commonly used assay in this review study is HIV-1 Protease (PR) assay which contains HIVII PR HIV-FRET (fluorescence resonance energy transfer) and the recombinant HIV-1 protease solution (116). The assay was reported for evaluation of several plants including *Ziziphus mucronata, Adansonia digitate* and *Abrus precatorius*. HIV-1 Integrase (IN) assay was reported also for anti-HIV evaluation. The plants reported for this assay included *Monotes africanus* and *Terminalia chebula.* Few studies reported having used HIV-1 p24 assay, which is an enzyme-linked immunosorbent assay that detects and quantify HIV-1 p24 core protein. HIV-1 inhibition is determined by a decrease in viral p24 antigen levels measuring absorbance at 450 nm (116). The plants reported to have used this assay for anti-HIV evaluation included *Bidens pilosa* and *Momordica charantia. Bersama abyssinica* anti-

HIV activity evaluation was done using the Cell-based assay. The review observed that many research studies confirmed that opportunistic infections are the main target of medicinal plants used by traditional healers. It was also noted that the ability of plants to have simultaneous activities is being exploited by traditional healers, who often make mixtures of different plants with same effects, potent or synergistic effects, to maximize the chance to heal patients. However, the uncertain outcomes of these combinations could result in toxicity and may lead to intoxications.

4.2 Levels of Heavy metals in four Malawian medicinal plants used for the treatment of infectious diseases

Table 4.2.1: Profile of heavy metals in E. *milanjiana*, *E. whyteana*, *R. acuminatissima* and *A. nyassana* medicinal plants

Plants	Part	Metal Element						
		Copper	Zinc	Cadmium	Chromium	Iron	Manganese	Lead
E. milanjiana	Leaves	0.66±0.002	1.77±0.4	-	0.43±0.03	2.25±0.05	2.33±0.02	2.55±0.4
	Roots	0.70±0.09	0.60±0.2	-	0.72±0.09	2.34±0.1	2.70±0.06	2.67±0.2
E. whyteana	Leaves	0.65±0.01	1.03±0.5	0.23±0.04	0.41±0.2	2.25±0.1	1.46±0.03	3.05±0.05
	Roots	0.64±0.007	0.82±0.2	-	0.22±0.06	1.92±0.1	1.65±0.1	3.21±0.1
R. acuminatissima	Leaves	0.65±0.003	0.69±0.01	0.16±0.04	-	2.19±0.05	2.62±0.06	3.12±0.08
	Roots	0.64±0.002	0.68±0.2	-	-	2.53±0.2	1.87±0.02	3.32±0.3
A. nyassana	Leaves	0.70±0.04	1.79±0.1	0.31±0.04	-	1.39±0.3	1.26±0.1	3.36±0.3
	Roots	0.66±0.02	0.87±0.1	0.15±0.04	-	2.63±0.6	1.67±0.02	3.26±0.0
WHO recommended		3.00	27.40	0.30	2.00	20.00	400	10.00

Herbal samples analysis was carried out using atomic absorption spectrometry interfaced with a computer. Copper, zinc, cadmium, chromium, iron, manganese and lead metal elements were analysed. All the experiments were done in triplicates. P values < 0.05 were regarded as significant.

As shown in Table 4.2.1, comparatively high levels of copper were observed in *Ericae milanjiana* (0.70 ± 0.09 ppm) and *A. nyassana* (0.70 ± 0.04 ppm) respectively while the lowest levels were found in *R. acuminatissima* roots (0.64 ± 0.002 ppm) and *E. whyteana* (0.64 ± 0.007 ppm). The permissible limit set by FAO/WHO in edible plants is 3.00 ppm and the limit for herbal medicine has not been established by WHO while in China, the permissible limit for copper is 20 ppm. Studies have shown that high levels of copper are known to cause dermatitis and irritation of the upper respiratory tract (150

Zinc is an essential trace element which plays an important role in various cell processes including normal growth, bone formation, behavioural response, brain development and wound healing

(Jabeen et al., 2010). Comparatively High levels of zinc were found in *A. nyassana* (1.79 ± 0.1 ppm) and *Ericae milanjiana* leaves (1.77 ± 0.4 ppm) as compared to the lowest levels in *Ericae milanjiana* roots (0.60 ± 0.2 ppm). The permissible limit of zinc set by FAO/WHO in edible plants is 27.4 ppm while the limit for herbal medicine has also not been established by WHO.

Studies have shown that cadmium intoxication causes both acute and chronic poisoning effect on the kidney, liver and immune system (149). For most of the plants in this study, cadmium was found to be within the WHO limit of 0.3 ppm with *A. nyassana* leaves being at the limit level of 0.31 ± 0.04 ppm but the lowest levels were also observed in *A. nyassana* roots 0.15± 0.04 ppm. Chromium chronic exposure may result in liver, kidney and lung damage and toxic intake of the same causes skin rash, nose irritations, bleeds, upset stomach, kidney and liver damage, nasal itch and lungs cancer (151. For most of the plants in this study, chromium was found to be within Canada's permissible limit of 2 ppm.

Iron is an essential component of haemoglobin and also plays an important role in oxygen and electron transfer in human and animal bodies (150). And in this study high levels of iron were found in *A. nyassana* roots (2.63 ± 0.6 ppm) while the lowest levels were found in *A. nyassana* leaves (1.39 ± 0.3 ppm).

Comparatively High levels of manganese were found in *Ericae milanjiana* roots (2.70 ± 0.06 ppm) and *R. acuminatissima* leaves (2.62 ± 0.06 ppm). However, the limit of manganese in herbal medicine has not been established by WHO (151). Comparatively High levels of lead were found

in *A. nyassana* leaves (3.36±0.3 ppm) while the lowest was observed in *E. Ericae milanjiana* leaves (2.55 ± 0.4 ppm).

Lead is a non-essential trace element having no known function in neither the human body or in plants. They are known to induce various toxic effects in the human body at low dose with such as lead poisoning resulting in symptoms such as anaemia, headache, convulsions and chronic nephritis of the kidney, brain damage and central nervous disorder (112). All the plants showed that lead levels were within the WHO limit of 10 ppm.

Consequently, it was observed from the present study that the concentration of heavy metals in the plant under study was within the WHO permissible range except for *A. nyassana* which showed limit levels of toxic element, cadmium of 0.31 ± 0.04 ppm. Again, all the medicinal plants under study had least levels of toxic metals concentration of the order Cadmium< Chromium<Lead. Lead was shown to be the most abundant metal while cadmium was the least abundant in all the plants under study. It can be noted from the results that sample preparation procedure used was effectively applied for the acid digestion of the herbal medicine samples. The percentage recovery of heavy metals from most of the samples was within the acceptable range of 88 to 103 % (152).

Medicinal plants or their products may be harmful to the human body if they are improperly used or handled, and some may cause serious adverse effects when they are taken excessively or under inappropriate circumstances. Therefore, it is recommended that a successful treating material should exhibit its therapeutic effect without causing serious side effects or exerting toxicity to the body cells and organs.

4.3 In Vitro Antioxidant Activities and HPTLC Fingerprint Analysis of five Malawian Medicinal Plants

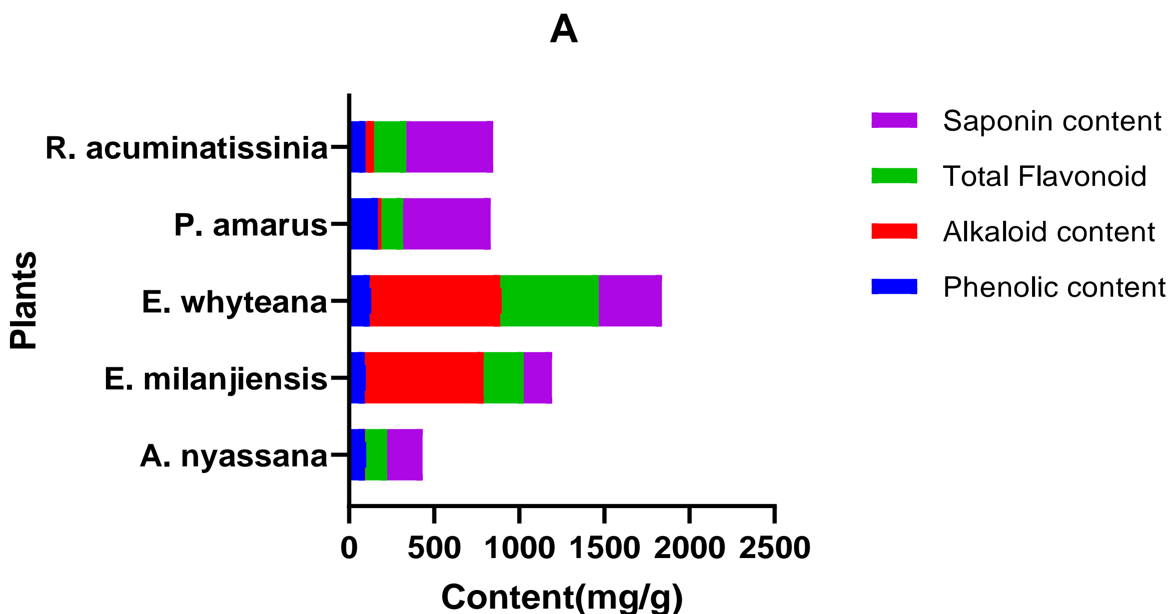
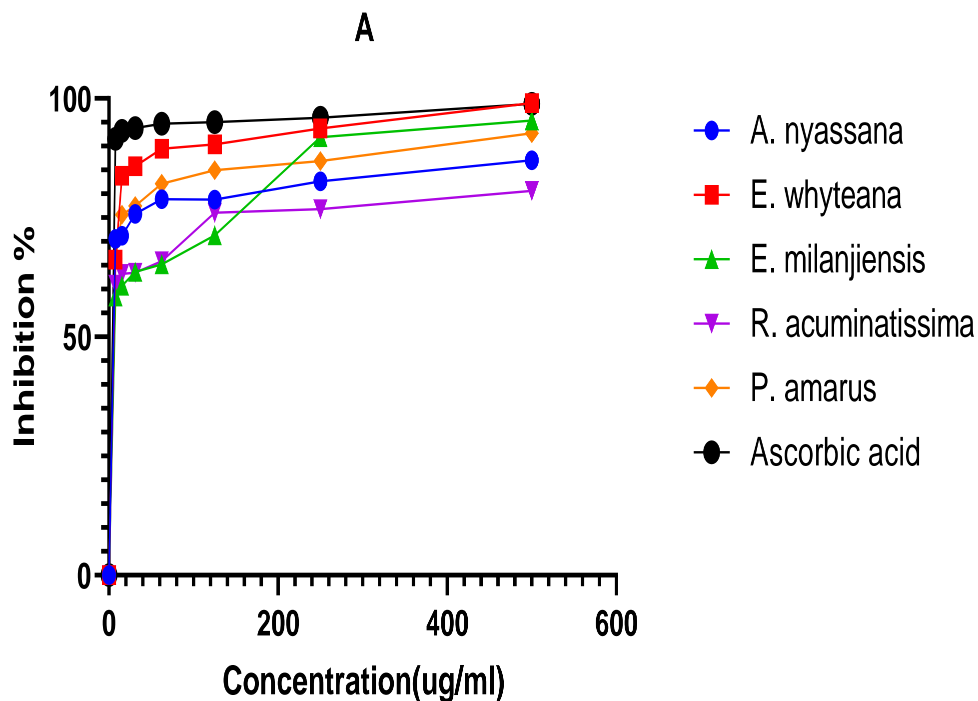


Figure 4.3.1: Phytochemical contents for the five medicinal plants were evaluated. [1]

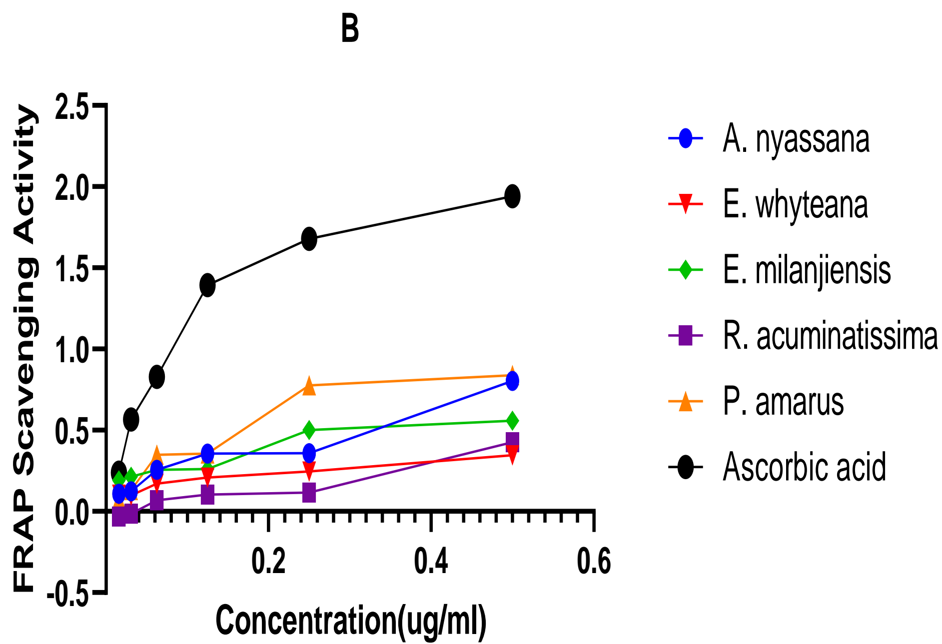
As shown in Figure 4.3.1, significant amounts of saponins ($p < 0.05$) were quantitatively indicated from *P. amarus* (514.24 mg/g) and *R. acuminatissinia* (509.90 mg/g) as compared to 209.9, 370.82 and 167.60 mg/g indicated from *A. nyassana*. *E. whyteana* and *E. milanjiana* respectively. Saponins are known to have characteristics that include bitterness, the formation of foams in aqueous solutions, hemolytic activity, antifungal and cholesterol-binding properties (153) Significant amounts of flavonoids ($p < 0.05$) were also indicated in *E. whyteana* (579.46 mg/g) as compared to 127.51 mg/g indicated from *A. nyassana*. Literature indicates that flavonoids decrease free radicals by chelating radical intermediate compounds and through quenching, up-regulating

[1] Aluminium chloride method was used to assess the flavonoids contents, Folin-Ciocalteu reagents method was used to evaluate the total phenolic contents, Harborne method was used to measure the alkaloids content and also saponins contents were also evaluated. The samples were analysed in triplicate presented as mean ± standard deviation (SD)

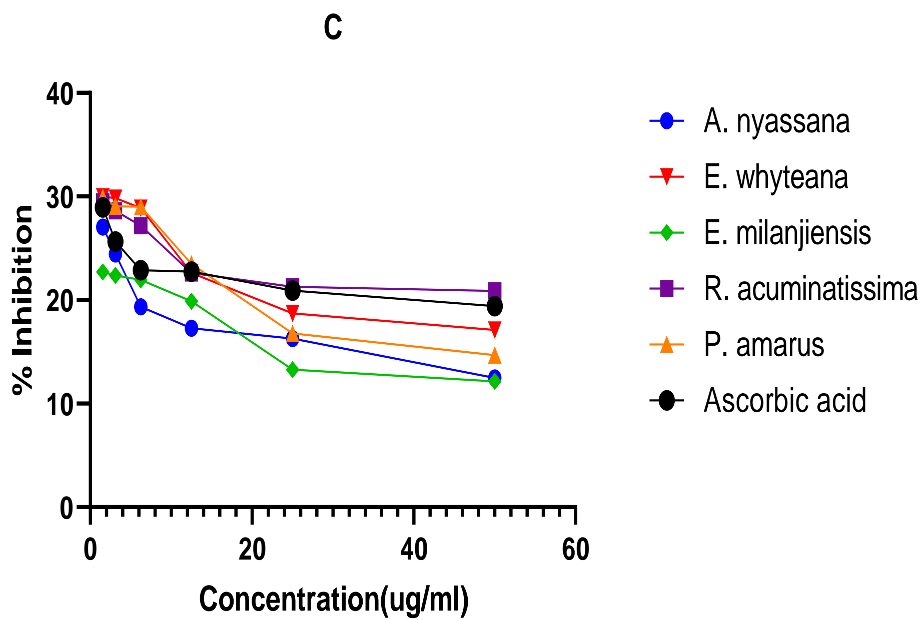
or protecting antioxidant defences (154). There were more alkaloids compounds ($p < 0.05$) indicated from *E. whyteana* (766.90 mg/g) and *E. milanjiana (*698.48 mg/g) respectively as compared to the other plants. Alkaloids have been shown to exhibit marked physiological activity when administered to animals and also they are reported to have analgesic activities (155). Significant amounts of phenolic compounds ($p < 0.05$) were quantitatively indicated from all the 5 plants under study. Literature indicates that phenolic compounds can readily donate a hydrogen atom to the radical and this could be responsible for the DPPH radical scavenging activity reported in the study (107).



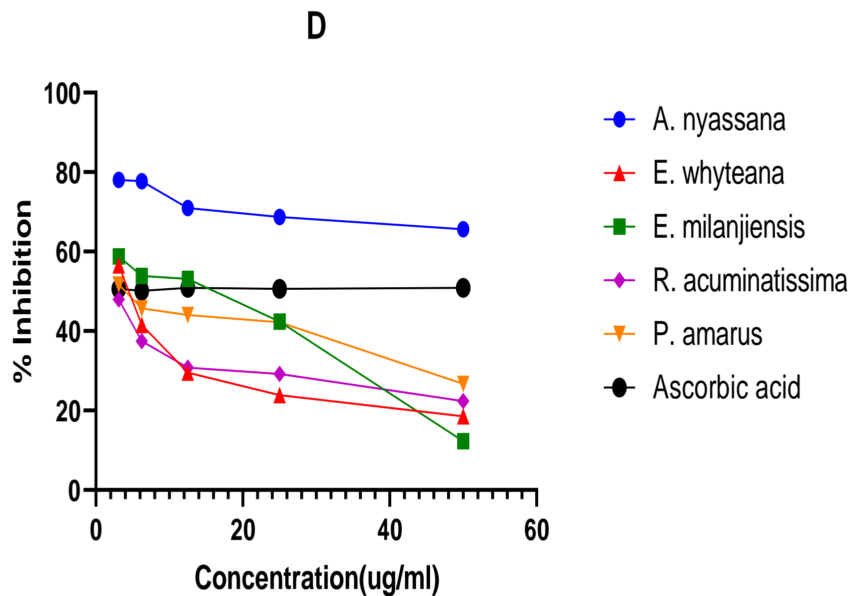
(a) DPPH Assay



(b) FRAP Scavenging activity



(c) Nitric oxide scavenging activity



(d) Reducing power

Figure 4.3.2: The analysis of 1,1-diphenyl-2-picryl hydrazyl (DPPH) free radical scavenging, the ferric ion reducing capacity (FRAP), Nitric oxide scavenging and Reducing power activities are shown in a, b, c and d respectively

In Figure 4.3.2 (a), the results of DPPH scavenging activity demonstrated an inhibition percent of above 80 (87.7, 99.6, 95.7, 81.1, and 94.3) at a higher concentration of 500 ug/ml, and also showed considerably higher inhibition percent of above 50 (70.5, 65.8, 58.3, 62.6 and 67.0) at a lower concentration of 7.8 ug/ml for *A. nyassana, E. whyteana, E. milanjiana, R. acuminatissinia* and *P. amarus* respectively. However, it can be observed that Ascorbic acid had stronger activity as compared to the plants under study and this might be attributed to the additive or synergistic effects of polyphenols making the antioxidant activity of the extracts weaker than that of the isolated bioactive compounds (156).

In Figure 4.3.2 (b), the results of FRAP scavenging activity showed increased absorbance with increased concentration trend for all the plants. The absorbance increased from 0.1 to 0.8, 0.1 to 0.3, 0.2 to 0.5, -0.03 to 0.4, 0.07 to 0.8 and 0.2 to 1.9 for *A. nyassana, E. whyteana, E. milanjiana, R. acuminatissinia, P. amarus* and Ascorbic acid respectively at a concentration range of 0.16 - 0.5 ug/ml. Literature indicates that compounds with iron-chelating ability can act as powerful antioxidants in most of the times. The iron ions are known to catalyse the conversion of less reactive species such as lipid peroxides to more reactive species such as hydroxyl, peroxyl/alkoxyl radicals. It is worth noting that the release of iron by cellular damage can accelerate oxidative damage to tissues (157).

In Figure 4.3.2 (c), the nitric oxide scavenging activity for the plant extracts and ascorbic acid demonstrated the scavenging activity of less than 50 % inhibition for all the plants understudy. *P. amarus, R. acuminatissinia, E. whyteana*, and Ascorbic acid showed lower percentage inhibition of 29.8, 29.4, 29.2 and 28.9 respectively at a concentration of 50 µg/ml. A dose-dependent decrease in NO scavenging activity was observed for all the extracts except *R. acuminatissinia* at all the tested concentrations. In the human body, nitric oxide is generated from the amino acid, L-arginine, by vascular endothelial cells, phagocytes and certain cells of the brain. Nitric acid is classified as free radical, because of its unpaired electron and normally displays important reactivity with certain types of proteins and other free radicals. It becomes adverse when it reacts with superoxide radical, forming a highly reactive peroxynitrite anion hence being toxic (158).

In Figure 4.3.2 (d), the results for reducing power activity displayed an inverse relationship between concentration and percentage inhibition of the plant extracts. *A. nyassana, E. milanjiana,*

E. whyteana, and ascorbic acid showed reducing power of 78.0, 58.8, 56.5 and 50.8 % respectively at 50 ug/ml concentration. Increased absorbance of the reaction mixture indicates an increased reducing power of the plant extracts. The reducing properties of plant extracts have been shown in literature to exert antioxidant action that breaks the free radical chain through a donation of a hydrogen atom to the compound (159).

Table 4.3.1: The IC50 values for DPPH, Nitric Oxide and Reducing Power of plant extracts

Sample	**DPPH**		**Nitric Oxide**		**Reducing Power**	
	IC_{50}	**R^2**	**IC_{50}**	**R^2**	**IC_{50}**	**R^2**
A. nyassana	2.32	0.52	7.47	0.97	9.56	0.94
E. whyteana	1.99	0.69	5.45	0.87	2.43	0.77
P. amarus	2.26	0.51	4.27	0.89	0.43	0.29
R. acuminatissinia	3.11	0.59	8.90	0.92	0.17	0.94
E. milanjiana	1.89	0.75	9.74	0.85	2.42	0.77
Ascorbic acid	0.64	0.39	11.47	0.94	1,82	0.52

IC_{50} values are half maximal inhibitory concentration which is a measure of the potency of a substance in inhibiting a specific biological or biochemical function while R^2 is a statistical measure of how close the data are to the fitted regression line. The IC_{50} values and R^2 were calculated for DPPH, Nitric oxide and Reducing power assays for all the plants understand

The results showed that the five plants' extracts had moderate scavenging activity as compared to ascorbic acid at all concentration in all the experiments. Results are means of 3 replicates.

In Table 4.3.1, the results showed that the IC_{50} values for DPPH free radical scavenging activity were within the range of 0.64 – 3.11 µg/ml, with Ascorbic acid, *E. milanjiana,* and *E. whyteana* showing a significantly marked IC_{50} values of 0.64, 1.89 and 1.99 µg/mL respectively. The IC_{50} values of the DPPH tested samples were in the order: Ascorbic acid < *E. milanjiana* < *E. whyteana* < *P. amarus* < *A. nyassana* < *R. acuminatissinia.*

For nitric oxide scavenging activity, the IC_{50} values were within the range of 5.45 –11.47 µg/mL with *R. acuminatissinia* and *E. milanjiana* significantly showing the IC_{50} values of 8.90 and 9.74 µg/mL respectively as compared to 11.47 for Ascorbic acid. The IC_{50} values of the nitric oxide scavenging activity tested samples were in the order: *P. amarus* < *E. whyteana* < *A. nyassana* < *R. acuminatissinia* < *E. milanjiana* < Ascorbic acid.

For the reducing power assay, the IC_{50} values were within the range of 0.43 – 9.56 µg/mL with *A. nyassana, E. whyteana,* and *E. milanjiana* showing IC_{50} values of 9.56, 2.43 and 2.4 respectively above the reference ascorbic acid. The IC_{50} values of the reducing power assay tested samples were in the order: *R. acuminatissinia* < *P. amarus* < Ascorbic acid < *E. milanjiana* < *E. whyteana* < *A. nyassana.*

Table 4.3.2: High-Performance Thin-Layer Chromatography (HPTLC) separation of the extracts

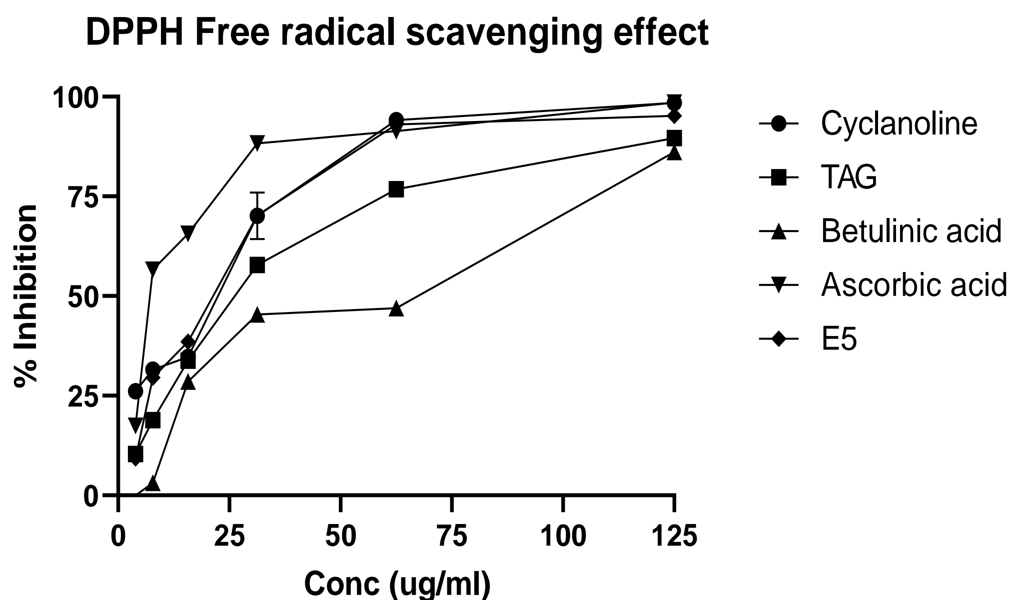
Plant	**Rf value**	**Number of spots**	**Colour Observation**	
			methanol/ sulphuric acid	**Folin-Ciocalteu**
A. nyassana	0.97	1	Pale-yellow	Blue colour
E. whyteana	0.75, 0.88, 0.94, 0.97	4	Pale-yellow	-
P. amarus	0.56, 0.67, 0.97	3	Pale-yellow	Blue colour
R. acuminatissinia	0.38, 0.94, 0.97	3	Yellow	Blue colour
E. milanjiana	0.69, 0.75, 0.88, 0.94, 0.97	5	Pale-yellow	Blue colour

Chromatographic separation of the extracts was developed using ethyl acetate/methanol/water (10:1.35:1), the profiles of separated spots sprayed with 90:10 methanol/sulphuric acid reagent after visualization under UV (366 nm) and visible light respectively. For phenolic compounds, a separate HPTLC run was done in which plates were stained with Folin-Ciocalteu reagent to assess the phenolic compounds.

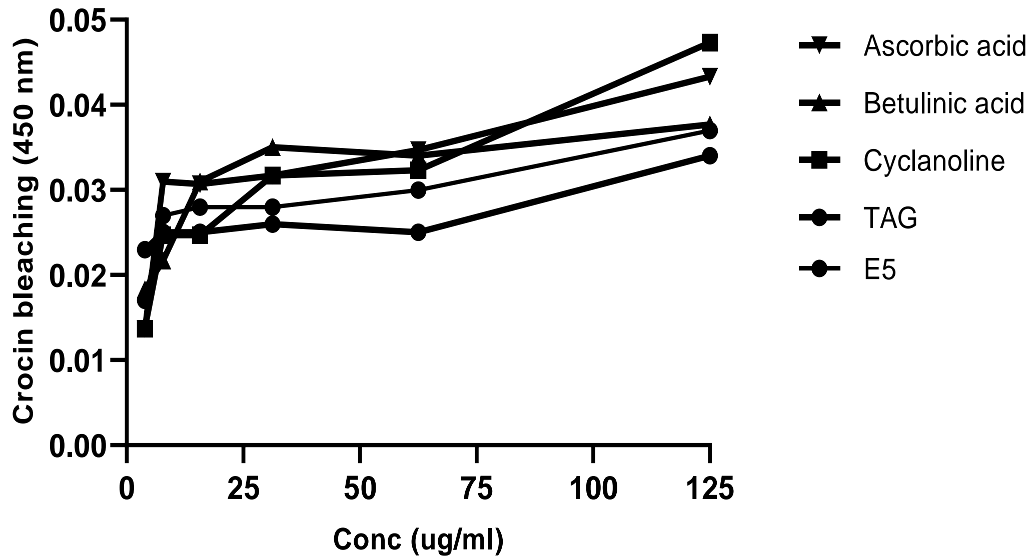
As shown in Table 4.3.2, the presence of antioxidant substances was demonstrated by a change in colour to pale-yellow colour spots on purple background and these changes were observed in *A. nyassana*, *E. whyteana, P. amarus, R. acuminatissima* and *E. milanjiana* plants. While in a

separate HPTLC run of plates stained with Folin-Ciocalteu reagent, the presence of phenolic compounds was demonstrated by change to blue colour of spots with yellow background. The changes were observed in *E. milanjiana* (0.69, 0.75, 0.88, 0.94, 0.97), *E. whyteana* (0.75, 0.88, 0.94, 0.97), *P. amarus* (0.56, 0.67, 0.97), *R. acuminatissima* (0.38, 0.94, 0.97) and *A. nyassana* (0.97).

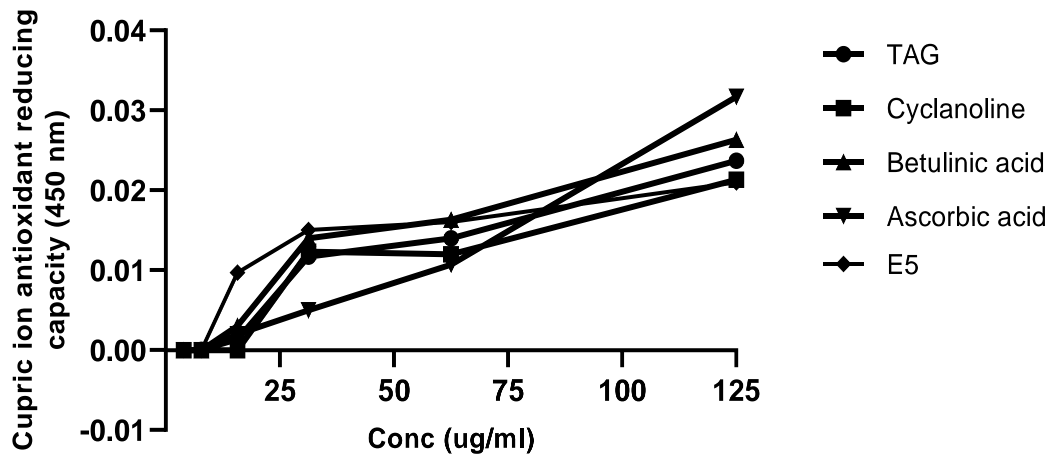
4.4 Antioxidant and Anti-Inflammatory potential of E5, TAG, Cyclanoline and Betulinic acid compounds



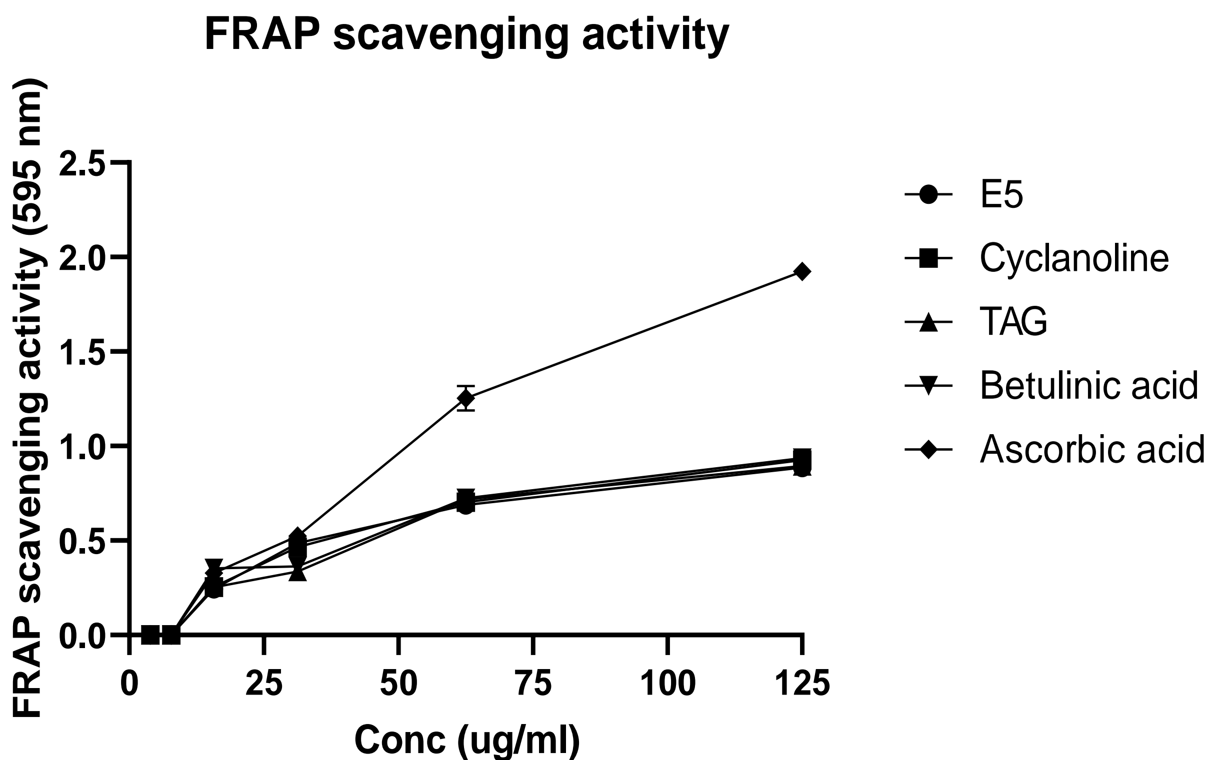
(a)



(b)



(c)



(d)
Figure 4.4.1: Antioxidant effect of Cyclanoline and Betulinic acid compounds[2]

In this study, It can be observed that all the four compounds showed moderate DPPH radical scavenging activity. Figure 4.4.1 (a) shows a steady increase in the percentage inhibition with increasing concentration. The maximum DPPH radical scavenging activity was observed in Ascorbic acid 98.54 ± 0.08 % while the lowest was observed in Betulinic acid 0.08 ± 3.97 %. The lowest IC50 value was observed in TAG 15.59 ug/ml while the highest was observed in Betulinic acid 51.68 ug/ml. The DPPH assay is normally used to evaluate the ability of antioxidants to scavenge free radicals which are known to be a major factor in biological damages caused by oxidative stress. The DPPH assay is known to give reliable information concerning the antioxidant

[2] The DPPH free radical scavenging, crocin bleaching effect, cupric ion reducing activity, and the FRAP are shown in a, b, c and d respectively. The DPPH data is shown as percentage inhibition.

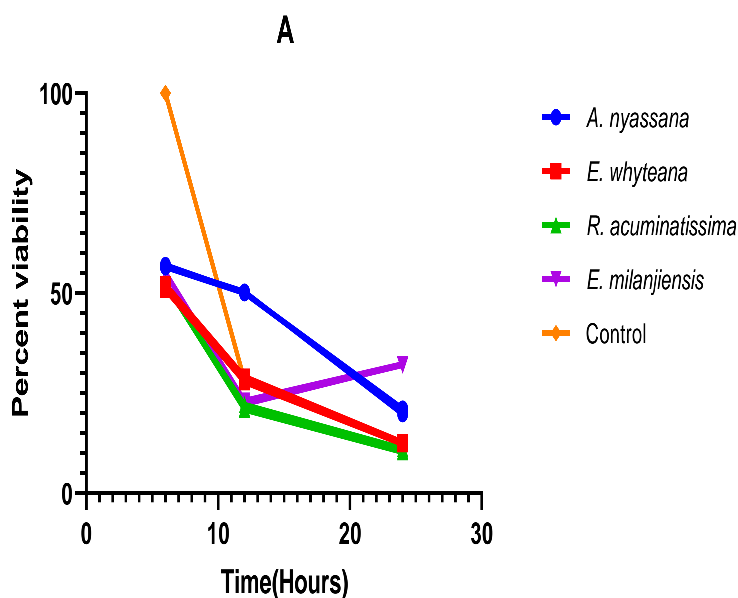
ability of the tested compounds (160). DPPH radical involves a hydrogen atom transfer process (119). In this assay, it can be noted that moderate-high antioxidant activity on DPPH radicals of Betulinic acid can be attributed to a direct role in trapping free radicals by donating a hydrogen atom.

The ferric reducing antioxidant power (FRAP) mechanism is based on the ability of pH to reduce Fe^{3+} to Fe^{2+} in electron transfer rather than hydrogen atom transfer (161). Ferrozine can quantitatively form complexes with $Fe^{2+,}$ however, in the presence of chelating agents, the complex formation is disrupted with the result that the red colour of the complex is decreased. Consequently, measurement of colour decrease, allows the assessment of the chelating activity of the coexisting chelator (162). In this study, the result of the ferrous ion chelating assay is presented in Figure 4.4.1(d). It can be observed that all the compounds understudy showed ferrous ion chelating activity in a dose-dependent manner. The highest IC_{50} value of 93.3 was observed in TAG while the lowest IC_{50} value of 3.83 was observed in Ascorbic acid. The ferrous ion chelating activity was found to be lower for E5 and moderately higher in TAG. Studies have shown that iron chelators mobilize tissue iron by forming soluble, stable complexes, this is then excreted in the faeces and/or urine. Chelation therapy reduces iron-related complications and thereby improves the quality of life and overall survival (182). The accumulation of toxic quantities of iron causes tissue damage and leads to various complications in human. Consequently, E5 can be observed as a potent ferrous-chelating source worthy of further investigation.

A variant of the FRAP assay using Cu instead of Fe is known as CUPRAC (copper reduction assay) in Figure 4.4.1 (c), and this is based on the reduction of Cu^{2+} to Cu^{+} by the combined action

of reducing agents in the sample. Neocuproine (2,9-dimethyl-1,10-phenanthroline) is used to form chromophores with Cu^{+} that is absorbed at 490 nm or 450 nm, respectively. Studies have shown that CUPRAC values are generally comparable to TEAC values for pH. The low redox potential of copper both in the free and complexed form makes it more selective in reactions than iron and can also indicate the potential pro-oxidant activity of compounds.

4.5 Investigation of the Cytotoxicity, Anti-oxidant, and Anti-inflammatory Effects of extracts and compounds on Human Peripheral Lymphocytes



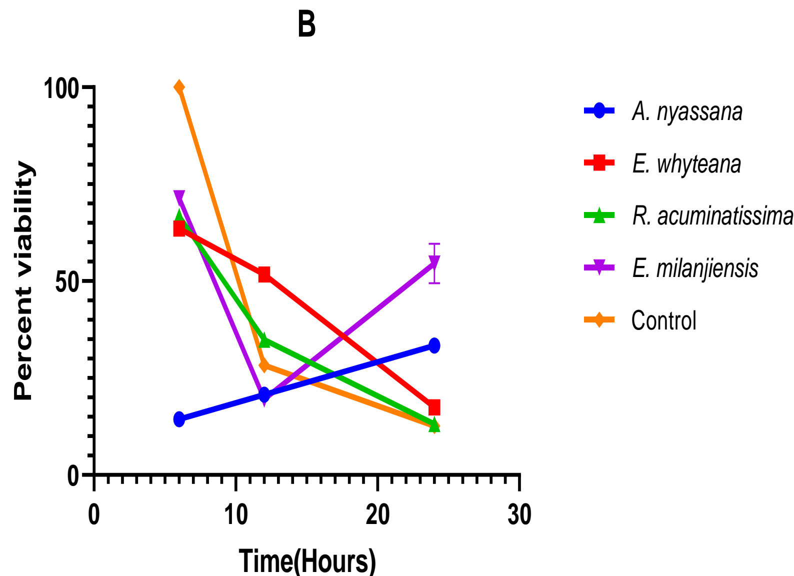


Figure 4.5.1: Cell viability studies were conducted using the trypan blue dye exclusion assay. Lymphocytes viability was evaluated at 250 (**A**) and 500ug/ml (**B**) against plant extracts. The samples were analysed in triplicate presented as mean ± standard deviation (SD)

It can be observed from the results that after 6, 12 and 24 hrs of incubation, percent cell viability (lowest cytotoxic and apoptotic activity) of both control (250 and 500 ug/ml) and treated cells exhibited some dose dependent response as shown in Figure 4.5.1. The highest percent viability (lowest cytotoxic and apoptotic activity) was observed in the control (100 %) while the lowest percent viability was observed in *E. whyteana* (51.6 %) after 6 hrs of incubation, at 250 ug/ml. There was a steady decrease in percent viability with the highest percent viability being observed in *A. nyassana* (50.0 %) while the lowest percent viability was observed in *R. acuminatissima* (21.2 %) between 6-12 hrs of incubation. *E. milanjiana* showed a steady increase in percent viability (22.7 to 32.0 %) while *A. nyassana*, *E. whyteana* and *R. acuminatissima* showed a significant decrease in percent viability with the lowest being observed in *R. acuminatissima* (10.5 %) between 12-24 hrs of incubation. The highest percent viability (lowest cytotoxic and apoptotic activity) was also observed in the control (100 %) while the lowest percent viability was observed in *A. nyassana* (14.3 %) after 6 hrs of incubation, at 500 ug/ml. It can be observed from the results,

all the plants showed a steady decrease in percent viability except for A. *nyassana* which showed significant increase in percent viability (from 14.3 to 20.7 %). Between the period of 12-24 hrs, *E. milanjiana* and A. *nyassana* showed a steady increase in percent viability of 19.4 to 51.9 % and 20.7 to 33.3 % respectively with the highest being observed in *E. milanjiana* while all the other plants showed a decrease in percent viability with the lowest being observed in *R. acuminatissima* (13.0 %). Literature have shown that measurement of cytotoxicity is usually determined by the delay in the proliferation of target cells either through destruction of the genetic material or by blocking the nutrient supply. Consequently, this has a direct relationship with the efficacy of the therapeutic compound being determined (163).

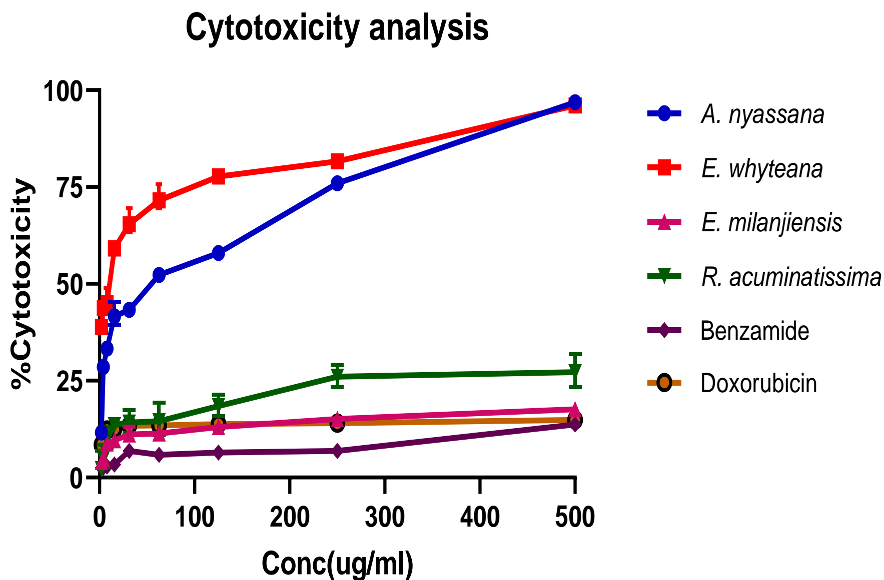


Figure 4.5.2: Dose response curves of *A. nyassana, E. whyteana, E. milanjiana, R. acuminatissima* extracts, Benzamide and Doxorubicin effects on PBMCs. The curves were constructed by Graph Pad Prism software

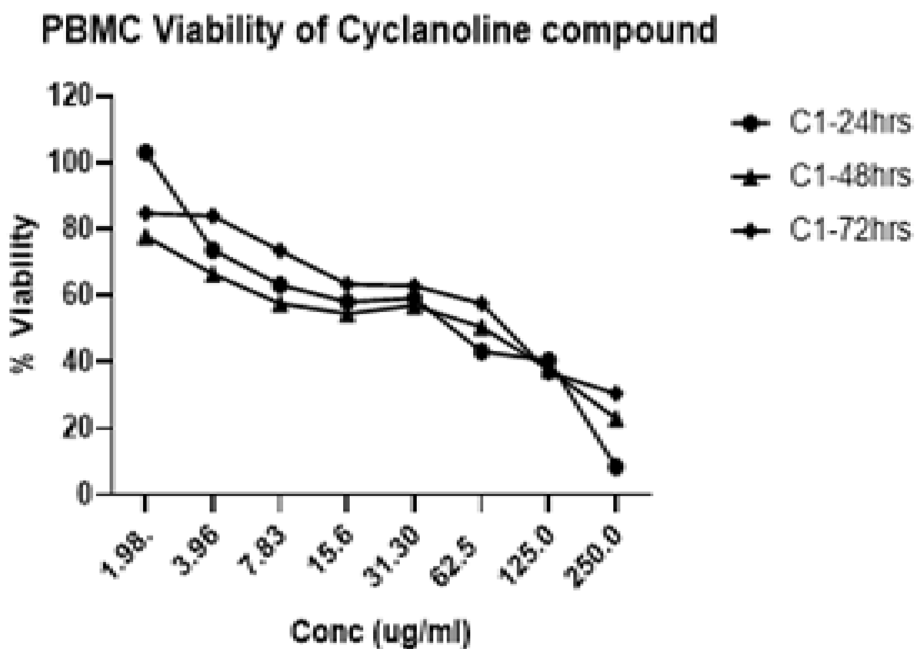
Table 4.5.1: Inhibitory concentration of Benzamide, Doxorubicin and 4 medicinal plants

Test compound	**IC_{50} (ug/ml)**	**R^2**
A. nyassana	4.818	0.938
E. whyteana	6.944	0.944
E. milanjiana	20.238	0.964
R. acuminatissima	7.101	0.944
Benzamide	32.849	0.726
Doxorubicin	6.213	0.679

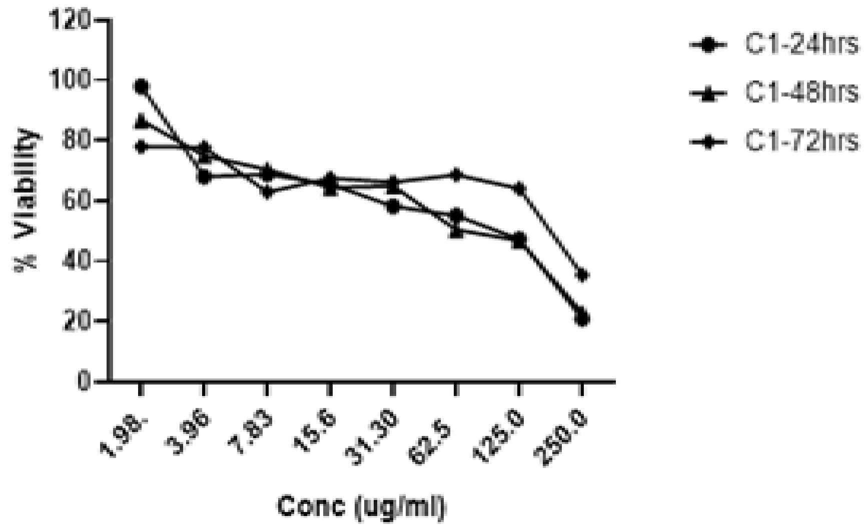
All values were determined by GraphPad Prism nonlinear regression analysis. Log (inhibitor) vs. response - Variable slope (four parameters) equation was the best fit model for all the extracts, Benzamide and Doxorubicin curves when compared by the software to the other dose response inhibition models. The bottom of all curves was constrained to zero value. R^2 values and 95 % confidence intervals for each IC_{50} are also indicated.

The cytotoxicity of plant extracts, benzamide (used for treatment of acute lymphoblastic leukaemia) and standard drug, doxorubicin (used to treat different types of cancers that affect the breast, bladder, kidneys, ovaries, thyroid, stomach, lungs, bones, nerve tissues, joints, and soft tissues. Doxorubicin is also used to treat Hodgkin lymphoma, non-Hodgkin lymphoma, and certain types of leukemia) were investigated at concentrations of 1.98 – 500 µg/ml on PBMCs. Highest percent cytotoxicity was observed in *A. nyassana* (96.05 %) followed by *E. whyteana* (95.72 %) while the lowest percent cytotoxicity was observed in standard drug, doxorubicin (13.85 %) and Benzamide (13.68 %) respectively at 500 ug/ml. Similarly, at concentration 1.98 µg/ml, the lowest percent cytotoxicity was observed in Benzamide (1.89 %) and *R. acuminatissima* (1.69 %) respectively as compared to standard drug doxorubicin (7.85 %) as shown in Figure 4.5.2. However, all the extracts showed increased cytotoxicity that is likely to have apoptotic or necrotic activity. It can be observed that *A. nyassana* was found to be more potent cytotoxic (IC_{50} value 4.818 µg/ml) than the standard drug, doxorubicin (IC_{50} value 6.213 µg/ml). In addition, *E. whyteana* and *R. acuminatissima* showed moderate activity against PBMC as shown in Table 4.5.1.

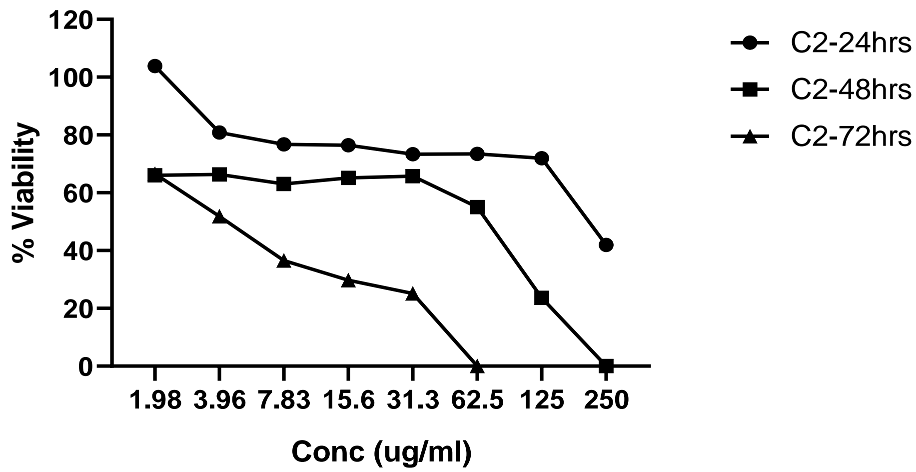
A. nyassana and *E. whyteana* displayed relatively high cytotoxicity on PBMCs as compared to *E. milanjiana*, *R. acuminatissima* and the compounds understudy. It can be observed that crude extracts contain many unpurified and unspecified materials which may exert non-specific inhibition against the already difficult to grow PBMCs. However, the compounds in plants that possess antimicrobial activities might not necessarily be the same compounds that exhibit non-specific inhibition against the normal cells. It can also be noted from the results that at relatively low concentrations, the extracts were able to inhibit normal cells proliferation and this inhibition was in a dose-dependent manner.



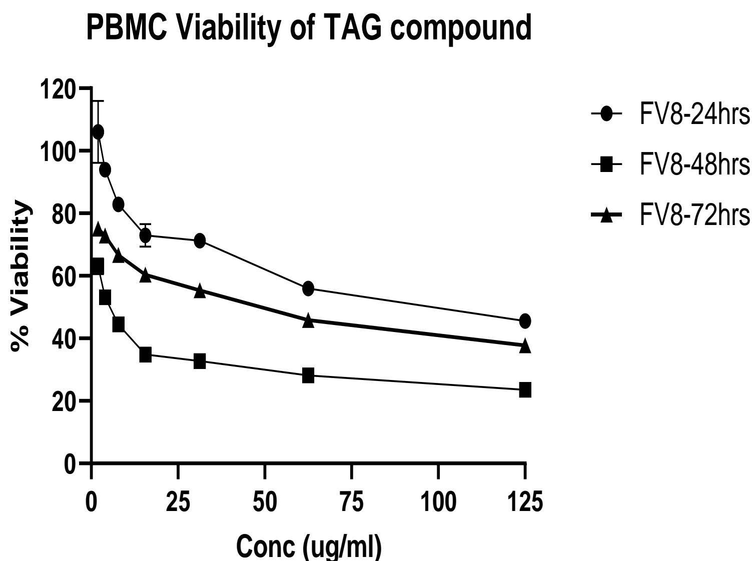
(a) Cell viability of cyclanoline



(b) Cell viability of Betulinic acid



(c) Cell viability of E5



(d) Cell viability of TAG

Figure 4.5.3: PBMC viability of E5, TAG, Cyclanoline and Betulinic acid compounds.

In this study as shown in Figure 4.5.3, compounds E5, TAG, Cyclanoline and Betulinic acid showed a dose dependent response at different concentrations. Compound, E5 showed a steady decrease in viability from 24 to 72 hours and IC_{50} of 11.13 ug/ml was observed after 72 hours. A small stimulative effect was observed in TAG between 24 to 72 hrs with IC_{50} of 14.41, 4.57 and 34.55 ug/ml respectively. However, this study was aimed at assessing whether the four compounds understudy were deleterious for the viability of PBMC at different concentrations. Studies have shown that PBMC cells are a critical component in the immune system to fight infection and adapt to intruders. They also play significant roles in neurodegenerative diseases and ageing (164). It has been reported in literature that long-term proliferation and survival of in vitro lymphocytes is dependent on IL-2 growth factor and that the repeated addition of IL-2 into the media is essential to maintain cell proliferation (161). Toxic compounds destroy live cells including lymphocytes when they are exposed to them and they either undergo accidental cell death or programmed cell death and this may compromise the immune system (163).

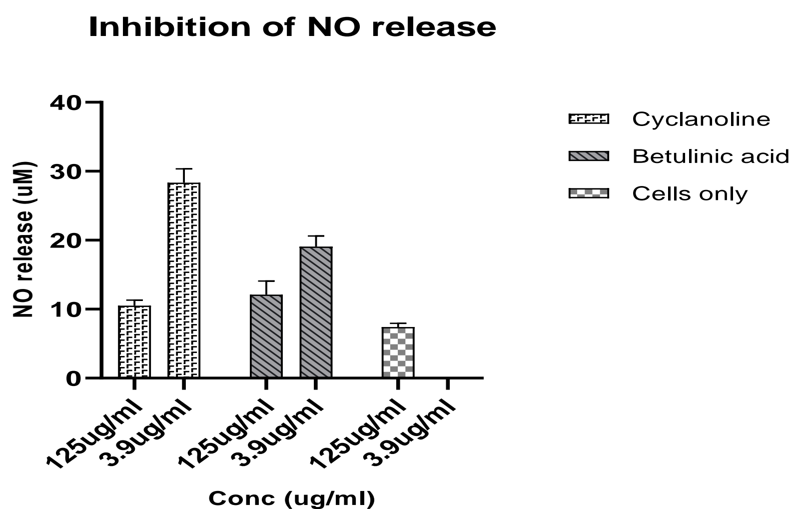


Figure 4.5.4: Inhibition of Nitric oxide release Cyclanoline and Betulinic acid compounds.

In this study, it can be observed from the results that Cyclanoline and Betulinic acid are able to reduce the production of nitric oxide in the PBMC supernatant. At higher concentration of 125 ug/ml nitrite accumulation was reduced to 10 and 12 uM while at lower concentration of 3.8 ug/ml, it was at 28 and 21 uM for Cyclanoline and Betulinic acid respectively while in control cells was at 7 uM as shown in Figure 4.5.4. Consequently, it can be concluded that the compounds down-regulate the release of NO production by human PBMC. The inhibitory effect of these compounds on PBMC proliferation as well as NO production suggests that these compounds might have therapeutical potential. NO is an important chemical mediator generated by the endothelial. Furthermore, excess concentration or production of NO is generally associated with several diseases.

4.5.1 Proposed model of molecular mechanism of induced apoptosis and down-regulation of NFkB activation in cells

It can be observed from this study, that compounds E5, Cyclanoline and TAG have shown considerable in vitro antioxidant, free radical scavenging capacity activities in a dose-dependent manner when compared with the standard antioxidant in scavenging and ferrous ion chelating assay. These findings show that these compounds may possess antioxidant activity with different mechanisms of actions towards the free radicals tested. Therefore, E5, Cyclanoline and TAG can be possible candidates for use as an antioxidant agent. To understand the molecular mechanisms of action, this study recommends that future studies should focus on whether E5, Cyclanoline and TAG can suppress pro-inflammatory transcription factors such as transcription factor nuclear factor-kB (NF-kB) and NF-IL-6 or activating protein-1 (AP-1). The transcription factor NFkB regulates the expression of a wide range of genes involved in immune response, inflammation, and acute phase response, as well as several viral genes. Recent evidence also suggests that NFkB is involved in apoptosis (165). Moreover, various kinds of antioxidants have been reported to down-regulate NFkB activation in a wide range of cell types (164). Furthermore, as shown in Figure 4.5.5, E5, Cyclanoline and TAG treatment may up-regulate Bax, down-regulate Bcl-2 expression, promote Bax translocation to mitochondria, activate mitochondria-mediated apoptotic pathway, which in turn causes the release of caspase-3, and promote cell apoptosis.

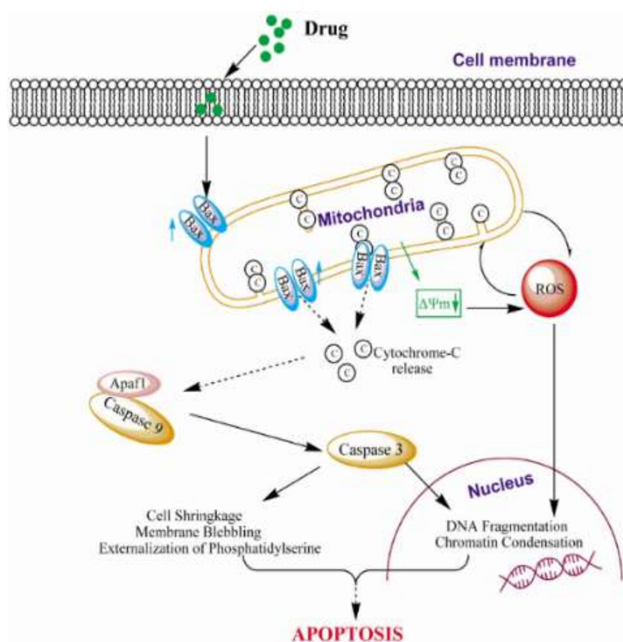


Figure 4.5.5: Proposed model of molecular mechanism of E5, Cyclanoline and TAG induced apoptosis and down-regulation of NFkB activation in cells

4.6 In vitro anti-mycobacterial and cytotoxic activities of five Malawian medicinal plants

Results of the mammalian macrophage cytotoxicity assay showed a dose-dependent response for the extracts and fractions understudy with a considerable good response observed in *F. virosa* and *E. milanjiana* extracts as shown in Figure 4.6.1. *A. nyassana, E. whyteana, F. virosa* and *E. milanjiana* extracts showed median cytotoxic concentration (CC50) of >512, 320.1, 282.2 and 105.91 respectively while *A. nyassana* ANX, *A. nyassana* A9, *E. whyteana* W1 and *E. whyteana* WX fractions showed median cytotoxic concentration (CC_{50}) of >256, 252.25 and 289.4 respectively. Moderate cytotoxic levels were observed in *E. cooperi* extract and *E. milanjiana* EM21 at CC_{50} value of 43.88 and 69.07 respectively as shown in Table 4.6.1. The results obtained from antimycobacterial analysis of *M. smegmatis* showed *F. virosa* and *E. milanjiana* crude extracts had minimum inhibition concentration (MIC) of 128 ug/ml and 512 ug/ml respectively

while *A. nyassana* ANX fraction had MIC of 256 ug/ml. *E. cooperi, A. nyassana* and *E. whyteana* extracts showed MIC of >512 ug/ml while fractions *E. milanjiana* EM21, *A. nyassana* A9, *E. whyteana* W1 and *E. whyteana* WX showed MIC of >252 ug/ml as shown in Table 4.6.2. The results obtained from antimycobacterial analysis of *M. ulcerans* showed *E. cooperi, E. milanjiana* and *E. whyteana* crude extracts had MIC of 512 ug/ml respectively while *F. virosa and A. nyassana* had MIC of >512 ug/ml. For fractions, *E. whyteana* W1 had MIC of 252 ug/ml while *A. nyassana* ANX, *E. milanjiana* EM21, *A. nyassana* A9 and *E. whyteana* WX had MIC of >252 ug/ml respectively as shown in Table 4.6.2. The results from selectivity index analysis ranged from <0.082 – 2.20 and *F. virosa. E. whyteana* extracts and *E. whyteana* W1 fraction had a considerably good Selective Index of 2.20, 0.625 and 0.985 respectively as shown in Table 4.6.2.

Table 4.6.1: Determination of median cytotoxic concentration of 5 crude extracts and 5 fractions

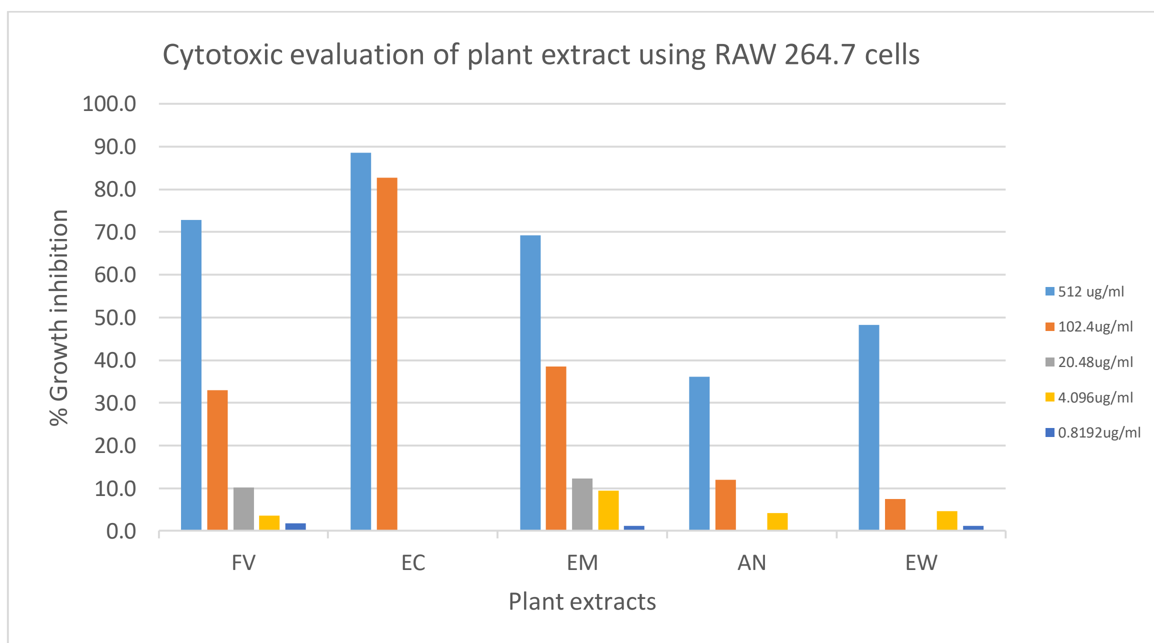
	EXTRACT	**CC50**	**SD**
Crude	*F. virosa*	282.2	14.79
	E. cooperi	43.88	8.97
	E. milanjiana	105.91	36.26
	A. nyassana	>512	
	E. whyteana	320.1	26.38
Fractions	*A. nyassana* ANX	>256	
	E. milanjiana EM21	69.06	3.74
	A. nyassana A9	>256	
	E. whyteana W1	252.25	5.30
	E. whyteana WX	289.4	39.29

All values were determined by GraphPad Prism nonlinear regression analysis. Log (inhibitor) vs. response – Variable slope (four parameters) equation was the best fit model for all the extracts

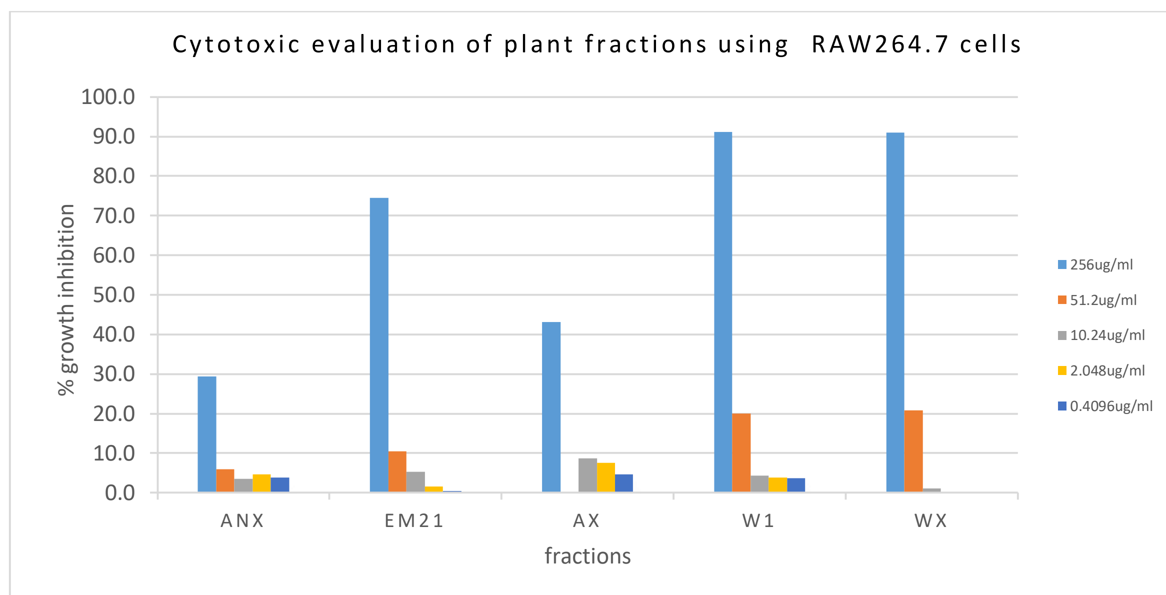
Table 4.6.2: Selective index (SI) and Minimum inhibitory concentration (MIC) of 5 crude extracts and 5 fractions against *M. ulcerans* and *M. smegmatis*

Plant	**extract**	***M. ulcerans***		***M. smegmatis***	
		SI	**MIC (ug/ml)**	**SI**	**MIC (ug/ml)**
Crude	*F. virosa*		>512	2.20	128
	E. cooperi	0.086	512		>512
	E. milanjiana	0.206	512	0.206	512
	A. nyassana		>512		> 512
	E. whyteana	0.625	512		> 512
Fractions	*A. nyassana* ANX		>256		256
	E. milanjiana EM21		>256		> 256
	A. nyassana A9		>256		> 256
	E. whyteana W1	0.985	256		> 256
	E. whyteana WX		>256		> 256
Compound	EM25		128		
	Betulinic acid		>128		
	TAG		128		
	E5		64		
	Cyclanoline		64		
	FV9		>128		

Selectivity index is calculated as a ratio of CC_{50} (ug/ml) to MIC (ug/ml). *MIC values presented are averages of three replicates.*



(a) Cytotoxic evaluation of crude extracts



(b) Cytotoxic evaluation of fractions

Figure 4.6.1: Cytotoxic evaluation of Plant extracts and fractions using macrophage RAW 264.7 cells

The samples *FV: F. virosa, EC: E. cooperi, EM: E. milanjiana, AN: A. nyassana EW: E. whyteana, A. nyassana ANX, E. milanjiana* EM21, *A. nyassana* A9, *E. whyteana* W1 and *E. whyteana* WX *were analysed in duplicate presented as mean ± standard deviation (SD)*

According to Molina-Salinas et al. (2007), extracts or compounds that displays MIC value of 125 ug/ml or below are considered to be active against mycobacteria. Consequently, it can be noted from the results that *F. virosa* crude extract displayed good activity at 128 ug/ml, *E. milanjiana* showed considered noteworthy antimycobacterial activity at 512 ug/ml and *A. nyassana ANX* fraction displayed moderate antimycobacterial activity at 256 ug/ml against *M. smegmatis.* Moderate activities against *M. ulcerans* were observed in *E. cooperi, E. milanjiana* and *E. whyteana* extracts and *E. whyteana* W1 fraction at 512 ug/ml and 256 ug/ml respectively. Compounds E5, Cyclanoline, TAG and EM25 showed moderately good anti-mycobacterial activity against *M. ulcerans* at 64 and 128 ug/ml respectively. Consequently, the results from this study showed that *E. cooperi, E. milanjiana* and *E. whyteana* have moderately anti-mycobacterial activity against *M. ulcerans,* the mycobacterium that cause Buruli ulcers. Selective Index values measures potential efficacy of the extract and fraction against adverse effects therefore, a good

selectivity index is an indication of a large safety margin between the concentration of the extract or fraction that is able to kill and the concentration that is toxic. Therefore, the higher the selectivity index the more likely it is that the activity is not due to a general metabolic toxic [Cho-Ngwa et al., 2010]. It can be observed from the results that *F. virosa* crude extract showed higher SI values of 2.20 as compared to 0.26 reported by Dzoyem et al., 2014. However, the difference between the two studies is that macrophage RAW 264.7 cells were used as opposed to Vero cells and also methanol solvent was used instead of acetone solvents for extraction of plant materials. Methanol is known to extract more active components from plant material as compared to acetone.

This is a major public health concern, therefore, there is need to promote research on natural products especially on those compounds that are showing promising results to find an all-oral and less toxic treatment as encouraged by WHO.

4.6.1 Proposed model of molecular mechanism of induced DNA damage by oxidative stress

In this study, compounds E5 and Cyclanoline have shown considerable antimycobacterial activity against *M. ulcerans.* Currently, an all-oral and less toxic treatment regimen of Buruli ulcer is been sought after and encouraged by (167). This is because most of the newly developed treatments are based on rifampin although it is well known for its interactions with many drugs, including anti-retroviral agents. Recently, rifampin-resistance have been reported in *M. ulcerans* isolates from patients and infected animals (167).

Therefore, E5 and Cyclanoline can be possible candidates for use as an antimycobacterial agent. To understand the molecular mechanisms of action, we recommend future studies should focus on whether E5 and Cyclanoline induced DNA damage by oxidative stress.

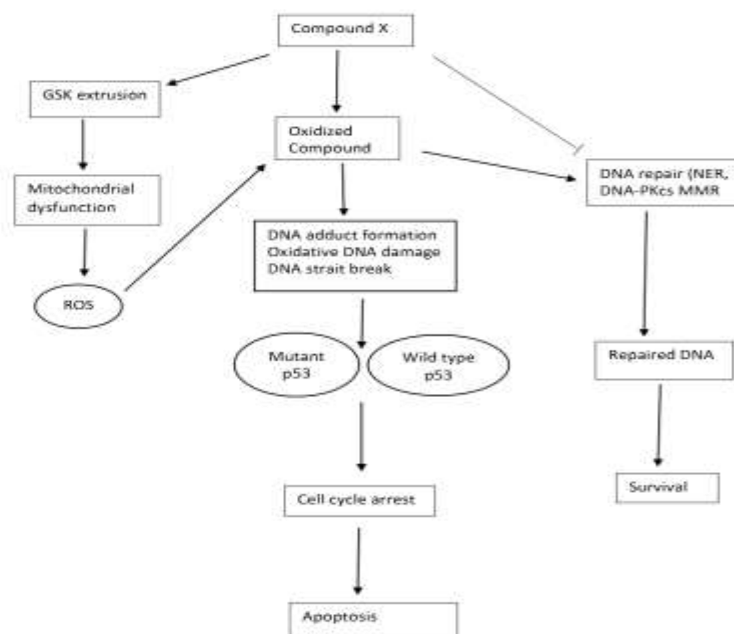


Figure 4.6.2: Proposed model of molecular mechanism of E5, Cyclanoline and TAG induced DNA damage by oxidative stress

4.7 In vitro synergistic activity between herbal compounds and SIRE drugs in combination against Multidrug Resistant Mycobacterium tuberculosis

Table 4.7.1: Summary results of synergistic activity of compounds and SIRE drugs

Sample	**Reference Drugs**									
	Control		**Strep (1ug/ml)**		**Inh (0.1ug/ml)**		**Rif (1ug/ml)**		**Eth (5ug/ml)**	
	GU	**Status**	**GU**	**Status**	**GU**	**Status**	**GU**	**Status**	**GU**	**Status**
Growth control	400	D	0	S	400	R	0	S	0	S
A2	400	D	0	S	400	R	0	S	0	S
C5	400	D	0	S	276	R	0	S	0	S
EM9	400	D	0	S	400	R	32	S	400	R
FV8	400	D	0	S	17	S	0	S	0	S
E1	400	D	0	S	400	R	0	S	400	R
EM8	400	D	15	S	1	S	0	S	0	S

GC: Growth control; S: Susceptible; R: Resistance; A2: Compound 5; E1: Compound 3; D: Drug control; EM9: Compound 7; EM8: Betulinic acid; C5: Cyclanoline; FV8: 1,2,3,6 – tetra(3,4,5-trihydroxyphenyl) acetyl ester - β-D-glucopyranose

BACTEC MGIT 320

Unloaded AST Set Report

Instrument Number	Current Date/Time	Temperature A	Software Version	Page Number
1	05/05/20 11.09	35.7°C	V6.01B	1

Em9

Sequence No: 439550128876 **TIP:** 10;0 **SOP:** 04/05/20 16.42 **Removed Date:** 05/05/20

Tube Position	Growth Unit	Status	Concentration	Drug Name
A/J15	400	D 0110		Growth Control
A/J16	0	S 0010	1.00 ug/mL	Streptomycin
A/J17	400	R 0010	0.10 ug/mL	Isoniazid
A/J18	32	S 0010	1.00 ug/mL	Rifampin
A/J19	400	R 0010	5.00 ug/mL	Ethambutol

Em9

FV8

Sequence No: 439550139080 **TIP:** 10;4 **SOP:** 04/05/20 16.43 **Removed Date:** 05/05/20

Tube Position	Growth Unit	Status	Concentration	Drug Name
A/K01	400	D 0110		Growth Control
A/K02	0	S 0010	1.00 ug/mL	Streptomycin
A/K03	17	S 0010	0.10 ug/mL	Isoniazid
A/K04	0	S 0010	1.00 ug/mL	Rifampin
A/K05	0	S 0010	5.00 ug/mL	Ethambutol

FV8

E1

Sequence No: 439550128875 **TIP:** 9;15 **SOP:** 04/05/20 16.43 **Removed Date:** 05/05/20

Tube Position	Growth Unit	Status	Concentration	Drug Name
A/K06	400	D 0110		Growth Control
A/K07	0	S 0010	1.00 ug/mL	Streptomycin
A/K08	400	R 0010	0.10 ug/mL	Isoniazid
A/K09	0	S 0010	1.00 ug/mL	Rifampin
A/K10	400	R 0010	5.00 ug/mL	Ethambutol

E1

Em8

Sequence No: 439550128874 **TIP:** 10;5 **SOP:** 04/05/20 16.43 **Removed Date:** 05/05/20

Tube Position	Growth Unit	Status	Concentration	Drug Name
A/K11	400	D 0110		Growth Control
A/K12	15	S 0010	1.00 ug/mL	Streptomycin
A/K13	1	S 0010	0.10 ug/mL	Isoniazid
A/K14	0	S 0010	1.00 ug/mL	Rifampin
A/K15	0	S 0010	5.00 ug/mL	Ethambutol

Em8

(a)

BACTEC MGIT 320

Unloaded AST Set Report

Instrument Number	Current Date/Time	Temperature A	Software Version	Page Number
1	05/05/20 11.09	35.7°C	V6.01D	2

C5

Sequence No: 439550129096 TIP: 10;8 SOP: 04/05/20 16.43 Removed Date: 05/05/20

Tube Position	Growth Unit	Status	Concentration	Drug Name
A/K16	400	D 0110		Growth Control
A/K17	0	S 0010	1.00 ug/mL	Streptomycin
A/K18	276	R 0010	0.10 ug/mL	Isoniazid
A/K19	0	S 0010	1.00 ug/mL	Rifampin
A/K20	0	S 0010	5.00 ug/mL	Ethambutol

C5

Sequence No: 439550139081 TIP: 9:1 SOP: 04/05/20 16.44 Removed Date: 05/05/20

A2

Tube Position	Growth Unit	Status	Concentration	Drug Name
A/L01	400	D 0110		Growth Control
A/L02	0	S 0010	1.00 ug/mL	Streptomycin
A/L03	400	R 0010	0.10 ug/mL	Isoniazid
A/L04	0	S 0010	1.00 ug/mL	Rifampin
A/L05	0	S 0010	5.00 ug/mL	Ethambutol

A2

Sequence No: 439550128894 TIP: 9;23 SOP: 04/05/20 16.44 Removed Date: 05/05/20

Tube Position	Growth Unit	Status	Concentration	Drug Name
A/L06	400	D 0110		Growth Control
A/L07	0	S 0010	1.00 ug/mL	Streptomycin
A/L08	400	R 0010	0.10 ug/mL	Isoniazid
A/L09	0	S 0010	1.00 ug/mL	Rifampin
A/L10	0	S 0010	5.00 ug/mL	Ethambutol

Set Control

Sample Control

END OF AST SETS

(b)

BACTEC MGIT 320
Unloaded Positives Report

Instrument Number	Current Date/Time	Temperature A	Software Version	Page Number
1	04/02/20 12.34	37.1°C	V6.03B	1

Tube Position	Accession Number	Sequence Number	Growth Unit	Tube Status	TTD	Date Positive	Protocol Length	Start of Protocol
A/J14	002 Testing	430195919888	1729	+	5:2	03/31/20	42	03/26/20 16.34

(c)

Figure 4.7.1: BACTEC MGIT 320 unloaded AST Set and positives report

The combination drug action showed that only Betulinic acid (EM8), 1,2,3,6 – tetra(3,4,5-trihydroxyphenyl) acetyl ester - *β*-D- glucopyranose (FV8) and Cyclanoline (C5) exhibited synergistic antimycobacterial activity at a final concentration of 18 ug/ml as shown in the table 4.7.1. The best synergistic activity (susceptibility) were observed in combined EM8+INH and FV8+INH while a moderate synergistic activity was observed in C5 indicating an additive effect of the combination. However, reduced synergistic activity (resistance) was observed in combined EM9+ETH and E1+ETH as shown in Figure 4.7.1.

The mechanism of action of isoniazid (INH), Ethambutol (EMB), Streptomycin (STR), and rifampicin (RIF) are well known. The mode of action of rifampicin in *M. tuberculosis* is by binding to the β-subunit of the RNA polymerase, inhibiting the elongation of messenger RNA [Blanchard, 1996]. The majority of rifampicin-resistant clinical isolates of *M. tuberculosis* harbour mutations in the *rpoB* gene that codes for the β-subunit of the RNA polymerase. As a result of this, conformational changes occur that decrease the affinity for the drug and results in the development of resistance (164). In about 96% of *M. tuberculosis* isolates resistant to rifampicin, there are mutations in the so-called "hot-spot region" of 81-bp spanning codons 507–533 of the *rpoB* gene. This region is also known as the rifampicin resistance-determining region (164). Unlike rifampicin, isoniazid is only active against metabolically-active replicating bacilli. Also known as isonicotinic acid hydrazide, isoniazid is a pro-drug that requires activation by the catalase/peroxidase enzyme KatG, encoded by the *katG* gene, to exert its effect (164). Isoniazid acts by inhibiting the synthesis of mycolic acids through the NADH-dependent enoyl-acyl carrier protein (ACP)-reductase, encoded by *inhA* (168). Although simple in its structure, resistance to this drug has been associated with mutations in several genes, such as *katG*, *inhA*, *ahpC*, *kasA* and

NDH. The two main molecular mechanisms of isoniazid resistance are associated with gene mutations in *katG* and *inhA* or its promoter region. Indeed, numerous studies have found mutations in these two genes as the most commonly associated with isoniazid resistance (169). Several studies have found single nucleotide polymorphisms in other genes in isoniazid-resistant clinical isolates of *M. tuberculosis*, including *kasA* and the *oxyR-ahpC* and *furA-katG* intergenic regions (170). However, their direct role as a cause of isoniazid resistance has not been fully demonstrated. On the other hand, co-resistance to isoniazid and ethionamide has been demonstrated to be caused by mutations in ndh in *M. smegmatis* and *M. bovis* BCG, by altering the NADH/NAD ratios inside the cell, leading to competitive inhibition of the INH-NAD adduct (171). A recent study has also found that a silent mutation in mabA conferred isoniazid resistance through upregulation of *inhA* in *M. tuberculosis* (170). Ethambutol is bacteriostatic against multiplying bacilli interfering with the biosynthesis of arabinogalactan in the cell wall (172). In *M. tuberculosis*, the genes *embCAB*, organized as an operon, code for arabinosyl transferase, which is involved in the synthesis of arabinogalactan, producing the accumulation of the intermediate D-arabinofuranosyl-P-decaprenol (173).The recognized mechanism of resistance to ethambutol has been linked to mutations in the gene *embB* with mutations at position *embB*306 as the most prevalent in most of the studies performed (174). Some studies have also found mutations in *embB*306 in ethambutol susceptible isolates (175). Streptomycin is an aminocyclitol glycoside active against actively growing bacilli and its mode of action is by inhibiting the initiation of the translation in the protein synthesis (176). More specifically, streptomycin acts at the level of the 30S subunit of the ribosome at the ribosomal protein S12 and the 16S rRNA coded by the genes *rpsL* and *rrs*, respectively (177).

4.7.1 Proposed model of molecular mechanism of efflux pump inhibitor

In this study, compounds E5, Cyclanoline and TAG have shown considerable antimycobacterial activity against Resistant *Mycobacterium tuberculosis.* The antimycobacterial mechanism of action of EM and FV8 has to be investigated to confirm this supposition as a different mechanism of action to INH could hold positive implications on preventing drug resistance as well as targeting strains already resistant to INH with the use of a drug therapy that combines EM or FV8 with INH. Therefore, E5, Cyclanoline and TAG can be possible candidates for use as an antimycobacterial agent. To understand the molecular mechanisms of action, we recommend future studies should focus on whether E5, Cyclanoline and TAG inhibit the efflux pump. Efflux pumps are bacterial transport proteins which are involved in the extrusion of substrates from the cellular interior to the external environment. These substrates are often antibiotics, imparting the efflux pump expressing bacteria antibiotic-resistant phenotype (179). The efflux pumps are broadly classified into two categories based on the mechanism by which they derive energy. As shown in Figure 4.7.2, the primary efflux pumps draw energy from active hydrolysis of ATP, whereas the secondary efflux pumps draw energy from chemical gradients formed by either protons or ions such as sodium (179). Abolishment the efflux could be achieved by different ways namely, (*i*) downregulating the expression of efflux pump genes by interfering in genetic regulation, (*ii*) redesigning antibiotics that are no longer recognized as substrates, (*iii*) inhibiting the assembly of functional efflux pumps, (*iv*) blocking the pump to avoid substrate binding to the active site, and (*v*) collapsing the energy mechanism responsible for energizing these pumps (178). However, for the compounds understudy, there is need to investigate last two categories that inhibit the efflux pumps using chemical entities called efflux pump inhibitors (EPIs). EPIs are the molecules that inhibit efflux pumps by one or more mechanisms, leading to inactive drug transport. Since this could eventually

lead to successful build-up of an antibiotic inside the cell, these EPIs can be used as adjuncts in combination with antibiotics to enhance their activity against bacteria expressing efflux pumps (179).

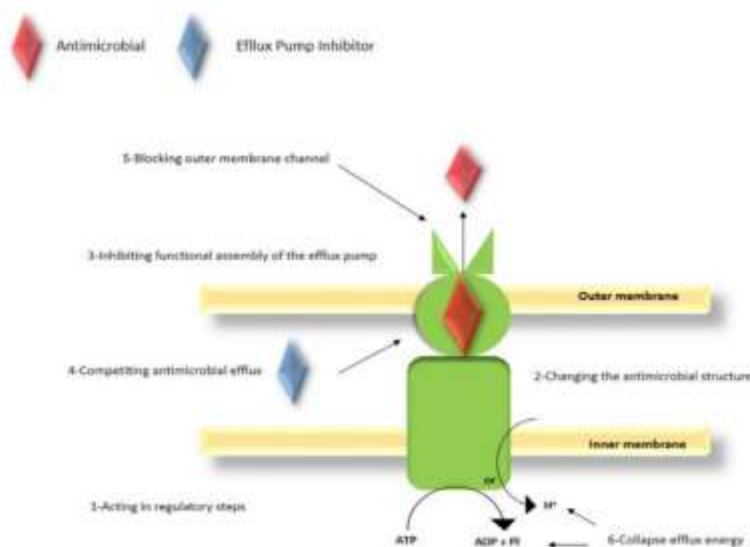


Figure 4.7.2: Proposed model of efflux pump inhibitor molecular mechanism of Cyclanoline and TAG

4.8 Contribution to knowledge and practice

Previous studies showed that Betulinic acid has anticancer; Antibacterial; AntiHIV activity; Antimalarial and other biological activities (180,181). The findings in this study collaborate with these findings and extend them as they revealed that Betulinic acid can also be used synergistically with the standard drugs of resistant tuberculosis and for treatment of Buruli ulcers.

This study is also the first to report on isolation and activities of novel compounds 1,2,3,6 – tetra(3,4,5-trihydroxyphenyl) acetyl ester - β-D- glucopyranose and semi-synthesized cyclanoline and E5 from the medicinal plants under study.

CHAPTER 5: CONCLUSION AND RECOMMENDATIONS

5.1 Conclusion

The study aimed to investigate some of the medicinal plants used by traditional healers to treat viral and mycobacterial infections in Malawi. The study achieved this aim as it revealed that some plants used in traditional medicine in Malawi contain phytochemical compounds with antiviral, antimycobacterial and pharmacological properties including antimicrobial, anti-inflammatory, antioxidant and immune-stimulant activities. The compounds also demonstrated synergistic properties with the first-line tuberculosis drugs, rifampicin; isoniazid ethambutol and streptomycin. This study further investigated the levels of heavy metals present in the plants under study and the cytotoxic effect of the plant extracts and compounds on peripheral blood mononuclear cells and macrophage RAW 265.7 cells. The heavy metal analysis showed a least levels of toxic metals concentration in order of Cadmium< Chromium<Lead. This study is the first to report on isolation and activities of novel compounds 1,2,3,6 – tetra(3,4,5-trihydroxyphenyl) acetyl ester - β-D- glucopyranose and semi-synthesized cyclanoline and E5 from the plants under study. This explains the interest Malawians have in medicinal plants and substantiates some of claims that Malawian traditional healers have made that when AIDS and TB patients use the medicinal plants, their health can improve. Specifically, the study showed that *Euphorbia whyteana and Flueggea virosa,* have antimycobacterium properties. These findings confirm what was known about the plants from previous studies.

The study further revealed that although the medicinal plants are a promising therapy for the HIV pandemic, their potential toxicity and that of their end products on several organs remains non-negligible. Based on these findings we propose further studies on drug combination of INH with

1,2,3,6 – tetra(3,4,5-trihydroxyphenyl) acetyl ester - β-D- glucopyranose and cyclanoline has the potential to prevent and combat-resistant strains.

5.2 Recommendations

This study recommends that:

(i) Information about the presence of phytochemical constituents responsible for antimicrobial activities in the herbal plants understudy should be provided to the communities and traditional healers.

(ii) Even though the compounds have been found to inhibit the growth of Resistant *Mycobacterium tuberculosis* H37Rv and *Mycobacterium ulcerans* at certain concentrations, the minimum bactericidal concentrations (MBC) should be performed for Resistant *Mycobacterium tuberculosis* H37Rv to ascertain whether it is bactericidal or bacteriostatic

(iii) Further study should include other local medicinal plants to determine their potency and activity against multi-drug resistance tuberculosis to come out with a new drug for its treatment and management

Further research studies should be instituted to:

(i) Build on the findings of this study and determine the mechanism of action of compounds in order to unravel its exact potential to inhibit several pathogenic microbes

(ii) Further evaluate the cytotoxicity effect of the phytochemical compounds and how this affect safety.

(iii) Determine the possible synthetic pathway of the extracted phytochemical compounds for the possible development of potential chemotherapeutical agents.

(iv) In vivo analysis should be conducted to determine whether its true pharmacological activities exist.

(v) Assess the potential cultivation of the medicinal plants that were investigated in this study

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APPENDICES

Appendex 1: In vitro antioxidant activities and HPTLC fingerprint analysis of five Malawian medicinal plants

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Full Length Research

In Vitro Antioxidant Activities and HPTLC Fingerprint Analysis of five Malawian Medicinal Plants.

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Antioxidants, as natural compounds have been documented to possess scavenging abilities which helps in fighting body infections. Therefore, the aim of this study was to analyse the phytochemical constituents and antioxidant potential of some medicinal plants reported to possess anti-viral activity in Malawi. The antioxidant potentials of *Aeschynomene nyassana*, *Phyllanthus amarus*, *Euphorbia whyteana*, *Rhus acuminatissima* and *Ericae milanjiensis* plants were evaluated using DPPH, FRAP, reducing power, Nitric oxide scavenging activity and HPTLC while phytochemical analysis was done using spectrophotometric techniques. The phytochemical analysis showed the presence of saponins, flavonoids, alkaloids, phenolic in the plant extracts. The results showed that ascorbic acid had significantly higher DPPH scavenging activity as compared to the plant extracts at all levels.NO scavenging activity demonstrated a significant dose dependent decrease except for *R. acuminatissinia. A. nyassana* revealed reducing power ability that was significantly greater as compared to Ascorbic acid. In HPTLC analysis, DPPH pale-yellow coloured spots were observed

while the phenolic active blue colour spots were observed only in *A. nyassana*, *P. amarus, R. acuminatissinia* and *E. milanjiensis*. Therefore, this study recommends further research on the 5 plants should be undertaken in order to inform science of traditional use in Malawi.

Keywords: Antioxidant, phytochemical compounds, reactive oxygen species, medicinal plants, High performance thin-layer chromatography, viral infections.

1. INTRODUCTION

The World Health Organisation (WHO) estimates that about 80% of the population living in the developing countries especially in Asia and Africa rely almost exclusively on traditional medicine for their primary healthcare needs due to limited availability of health facilities. In almost all the traditional medical systems in these countries, the medicinal plants play a major role and constitute their backbone [1].

Viral infections are a major cause of high morbidity and mortality rates in most of these developing countries. The emergence of viral resistance to drugs, as well as the serious adverse effect induced by antiviral drugs, has caused serious medical problems in many countries, particularly when administered in combination over prolonged treatment periods [2]. Traditionally, medicinal herbs are known to relieve the symptoms of different human diseases, including infectious diseases, and they are acknowledged to have been used for over thousands of years [3].

Many plants contain large amount of antioxidants which plays an important role in absorbing and neutralizing free radicals, quenching singlets and triplet's oxygen or decomposing peroxidase. Antioxidant substances block the action of free radicals which have been implicated on the pathogenesis of many infectious diseases. Radical scavenging antioxidants are particularly important in anti-oxidative defense in protecting cells from injury of free radicals especially common in immunosuppressed individuals [4].

Therefore, the aim of this study was to analyse the phytochemical constituents and antioxidant potential of some medicinal plants reported to possess anti-viral activity in Malawi. *Aeschynomene nyassana* is a shrub that can grow up to 120cm, has reddish-brown viscid stem that arises from woody rootstock and it belongs to the family, *Fabaceae*. Leaves are 2.5cm long, pinnate in shape with 15-20 pairs of oblong and obtuse leaflets [5]. *Phyllanthus amarus* is an annual herb found in shady places among other common weeds and it belongs to the family, *Euphorbiaceae*. It can grow up to 30-60 cm in height and blooms with yellow flowers. All parts of the plant are used as medicines because of their medicinal properties [6]. *Euphorbia whyteana* is a hairless perennial herb with annual stems that can grow up to 30 cm high from a woody rootstock and it belongs to the family *Euphorbiaceae* which have over 2000 species. Leaves are normally 30 mm long, rounded and apiculate at the apex, they are numerous, sessile, spreading to reflexed and linear-lanceolated. *Rhus acuminatissima* is a small tree often straggling and can grow up to 9metres in length and it belongs to the family, *Anacardiaceae*. The leaves are 3-foliolate while the leaflets are broadly elliptic, obtuse. The flowers are in loose panicle and small yellowish green in colour. While *Ericae milanjiensis* belongs to the family, *Ericaceae* which have over 3000 species consists mainly of shrubs or climbers. It can grow up to 6metresin length and has greyish brown bark. Leaves are crowded linear, small and dark green in colour [7].

2. MATERIALS AND METHODS

2.1 Chemicals

Trichloroacetic acid (TCA), 1,1-diphenyl-2-picryl hydrazyl (DPPH), Diethyl ether, Ammonium hydroxide, Acetic acid, Ethanol, Methanol, Sodium nitroprusside, Sodium bicarbonate, Potassium ferricyanide, Aluminum chloride, Gallic acid, Quercetin, Griess reagent, Phosphate buffered saline, Folin-Ciocalteu reagent and n-butanol were purchased from Sigma Chemicals Co. (USA). N-(1-naphthyl) ethylenediamine dihydrochloride, EDTA, Ferric chloride and Ascorbic acid were purchased from Merck (Germany). All other chemicals were of analytical grade.

2.2 Preparation of plant materials and extraction

Aeschynomene nyassana, *Phyllanthus amarus*, *Euphorbia whyteana, Rhus acuminatissima* and

Ericae milanjiensis medicinal plants were collected and identified by Mr. Edwin Kathumba from Herbarium and Botanical Gardens of Malawi and voucher specimens deposited in the same herbarium. The plants were air- dried at room temperature and then grounded into fine powder. The powdered material was further dissolved twice in methanol for 48hours. Filtration was conducted using a vacuum system and the filtrates concentrated using Rota vapour (Buchi B-100) to evaporate the solvents from the plant material. The extracts were air- dried at room temperature, weighed and stored for further use.

2.3 Phytochemical analysis

2.3.1 Determination of flavonoids contents

Aluminum chloride method was used to determine flavonoid contents according to standard procedures [8]. About 0.5ml of 1mg/ml methanol extract was mixed with 0.5ml of 2% aluminum chloride, then it was allowed to stand at room temperature for 60 minutes. Absorbance was then measured at 420nm using the PerkinElmer Victor X3 Multimode plate reader. The total flavonoid content was evaluated as quercetin equivalents (mg/g) using the following equation based on the calibration curve $y = 0.3812x + 0.1257$, $R^2 = 0.9583$, where y was the absorbance and x was the concentration.

2.3.2 Determination of total phenolic

Total phenolic contents were evaluated with Folin-Ciocalteu's phenol reagent according to standard procedures [9]. Five millilitres of the extract solution was mixed with 5 ml Folin-Ciocalteu reagent previously diluted with water (1:9 v/v). The mixture was allowed to stand for 5 minutes, then 4 ml of 7% Na_2CO_3 solution was added. The tubes were vortexed for 15 seconds and allowed to stand for 30 min at 40°C for the development of color. Absorbance was then measured at 765 nm using the PerkinElmer Victor X3 Multimode plate reader. The extracts were evaluated at a final concentration of 0.1 mg/ml. Total phenolic content was expressed as mg/g gallic acid equivalent (GAE) using the following equation based on the calibration curve: $y = 0.3947x - 0.0423$, $R^2 = 0.981$, where y was the absorbance x was the concentration.

2.3.3 Saponins determination

The saponins content in the plant extracts was determined according to standard procedures [10]. Ten grams of the powdered plant sample was placed in 200 ml of 20% ethanol. The suspension collected was heated for 4 hours in a water bath at 55°C while continuously being stirred. The mixture was filtered and the residue was re-extracted twice as above. The resultant combined extracts were reduced in a water bath at 90°C to about 40 ml. The final concentrate was added to 20 ml diethyl ether in a 250 ml separator funnel and shaken vigorously. The layer of ether solution was discarded, while the purification process was repeated.

60 ml of n-butanol was added and the combined n-butanol extracts was washed twice with 10ml of 5% aqueous sodium chloride. The solution that remained was heated in a water bath to evaporate the solvents and then the sample was dried in the oven to a constant weight. The saponins content was determine according to the equation:

Amount of saponins (mg/g) = weight of residue/weight of sample.

2.3.4 Alkaloids determination

The alkaloids content in the plant extracts was determined according to standard procedures [11]. Five grams of the powdered plant sample was weighed into 200 ml of 20% acetic acid in ethanol and allowed to stand for 5 hours. The extracts were filtered and concentrated using a water bath at 55°C to approximately one-quarter of the original volume. Then dropwise, concentrated ammonium hydroxide solution was added into the resultant extract until precipitation was complete. The precipitate collected after allowing the solution to settle was washed with dilute ammonium hydroxide solution and then filtered. The residue of the crude alkaloid was weighed and calculated according to the equation:

Amount of alkaloid (mg/g) = weight of precipitate/weight of sample.

2.4 Antioxidant assays

2.4.1 Assay of DPPH scavenging activity

The DPPH radical-scavenging activity of the methanol extracts was determined according to standard procedures [12]. DPPH free radical scavenging assay was performed using 96-micro-well flat plates. Stock solutions of the extracts were prepared as 1 mg/ml in methanol. Each well was filled in with 200 µl extract of different concentrations (3.13 - 25.0 µg/ml). Then, 5 µl of the DPPH solution (2.5 mg/ml in methanol) was added to each well. Ascorbic acid was used as standard control while a blank was prepared by mixing DPPH and methanol. Three replicates were made for each test sample. After 30 minutes of incubation at room temperature in the dark, the optical density of each well was read using PerkinElmer Victor X3 Multimode plate reader at wavelength 517 nm, and results were expressed as percentage antioxidant activity using the following equation:

100 – [(Sample Optical density (OD) – Sample background OD)/ (DPPH only OD) × 100].

The IC_{50} values were calculated by plotting a linear regression, where the abscissa represented the concentration of the tested plant extracts and the ordinate represented the average percent of scavenging capacity from three replicates.

2.4.2 Assay of nitric oxide-scavenging activity

The nitric oxide radical scavenging assay was determined according to standard procedures [13]. The extracts were prepared from a 1mg/mL stock solution of methanol and serial diluted to make concentrations (1.56−50μg/mL). Griess reagent was prepared by mixing equal amounts of 1% sulphanilamide in 2.5% phosphoric acid and 0.1% naphthylethylene diamine dihydrochloride in 2.5% phosphoric acid immediately before use. A volume of 0.5mL of 10mM sodium nitroprusside in phosphate buffered saline was mixed with 1mL of the different concentrations of the methanol extracts (1.56−50μg/mL) and incubated at 25°C for 180mins. The freshly prepared Griess reagent was mixed with equal volume of the plant extracts. Control samples were prepared by mixing equal volume of buffer prepared in a similar manner to the test samples but without the extracts. The colour tubes containing methanol extracts at the same concentrations with no sodium nitroprusside was also prepared. Then, 150μL of the reaction mixture was transferred to a 96-well plate and absorbance was measured at 546nm using a PerkinElmer Victor X3 Multimode plate reader. Ascorbic acid was used as the positive control. The percentage nitric oxide scavenging activity of the methanol extracts and ascorbic acid were calculated using the following formula:

Nitric Oxide Scavenging activity (%) = A control - A test/A control x 100.

2.4.3 Reducing power assay

The reducing power of the plant extracts was determined according to standard procedures [14]. Different amounts of plant extracts (3.125 - 50 μg/ml) in methanol were prepared and mixed with 2.5 ml of 0.2 M phosphate buffer (pH 6.6) and 2.5 ml potassium ferricyanide (1% w/v). The mixture was incubated at 50°C for 20 minutes, followed by the addition of 2.5 ml of trichloroacetic acid (10% w/v) and the mixture centrifuged at 3000 rpm for 10 minutes. About 2.5 ml of the supernatant was mixed with an equal volume of distilled water and 0.5 ml of $FeCl_3$ (0.1% w/v) and the absorbance was measured at 700 nm. Ascorbic acid was used as positive controls.

2.4.4 Ferric-Reducing Antioxidant Power Assay (FRAP)

The FRAP assay was evaluated according to standard procedures [15]. The FRAP reagent was prepared by mixing acetate buffer (25 mL, 300 mmol/L, pH 3.6), TPTZ solution (2.5 mL, 10 mmol/L) in 40 mmol/L HCl and $FeCl_3$ solution (2.5 mL, 20 mmol/L) in proportions of 10:1:1 (v/v), respectively. The freshly prepared FRAP reagent was warmed at 37°C in a water bath prior to use. Then 150μlof the sample was added to the 4.5 mL of FRAP reagent. The absorbance of the reaction mixture was measured at 595nm. The standard curve for analysis was constructed using $FeSO_4$ solution (0.5-10 mg/mL). The results obtained were expressed as μmol Fe (II)/g dry weight of plant material. Ascorbic acid was used as a control.

2.4.5 High Performance Thin Layer Chromatography (HPTLC) study of Phenol and DPPH

High performance thin-layer chromatography (HPTLC) was performed on a silica gel glass plate (20 × 20 cm, Silica gel 60 F254, Merck) according to standard procedures [16]. The extracts were dissolved in ethyl acetate and were directly deposited on glass silica gel. TLC plates were developed in a sandwich TLC chamber with Ethyl acetate/Methanol/Water (10:1.35:1) solvent mixture as mobile solvent. The profiles of the separated spots were sprayed with 90:10 methanol/sulphuric acid reagent after visualization under UV (366 nm) and visible light respectively. The plates were further examined for DPPH active spots. After 25 min, the pale-yellow spots on purple background indicated spots antioxidant activity. A separate run of the plates were stained with Folin-Ciocalteu's reagent and heated at 80°C/10min. The plates were further examined for phenolic active spots. After 25 min, the blue colour spots indicated presence of phenolic compounds.

2.5 Statistical Analysis

All experiments analysis were done in triplicates and where applicable, the data were subjected to one-way analysis of variance (ANOVA). P Values < 0.05 were regarded as significant.

3 RESULTS AND DISCUSSIONS

As shown in Figure 1, significant amounts of saponins ($P < 0.05$) were quantitatively indicated from *P. amarus*(514.24mg/g) and *R. acuminatissinia* (509.90 mg/g) as compared to 209.9, 370.82 and 167.60 mg/g indicated from *A. nyassana*. *E. whyteana* and *E. milanjiensis* respectively. Saponins are known to have characteristics that includes

bitterness, formation of foams in aqueous solutions, hemolytic activity, antifungal and cholesterol binding properties [19]. Significant amounts of flavonoids ($P < 0.05$) were also indicated from *E. whyteana* (579.46 mg/g) as compared to 127.51 mg/g indicated from *A. nyassana*. Literature indicates that flavonoids decrease free radicals by chelating radical intermediate compounds and through quenching, up-regulating or protecting antioxidant defences [17]. There were more alkaloids compounds ($P < 0.05$) indicated from *E. whyteana*(766.90mg/g) and *E. milanjiensis (*698.48 mg/g) respectively as compared to the other plants. Alkaloids have been shown to exhibit marked physiological activity when administered to animals and also they are reported to have analgesic activities [18]. Significant amounts of phenolic compounds ($P < 0.05$) were quantitatively indicated from all the 5 plants understudy. Literature indicates that phenolic compounds can readily donate hydrogen atom to the radical and this could be responsible for the DPPH radical scavenging activity reported in the study [13]. In Figure 2A, the results of DPPH scavenging activity demonstrated an inhibition percent of above 80 (87.7, 99.6, 95.7, 81.1, and 94.3) at a higher concentration of 500ug/ml, and also showed considerably higher inhibition percent of above 50 (70.5, 65.8, 58.3, 62.6 and 67.0) at lower concentration of 7.8ug/ml for *A. nyassana, E. whyteana, E. milanjeinsis,*
R. acuminatissinia and *P. amarus* respectively. However, it can be observed that Ascorbic acid had stronger activity as compared to the plants understudy and this might be attributed to the additive or synergistic effects of polyphenols making the antioxidant activity of the extracts weaker than that of the isolated bioactive compounds [21].

In Figure 2B, the results of FRAP scavenging activity showed increased absorbance with increased concentration trend for all the plants. The absorbance increased from 0.1 to 0.8, 0.1 to 0.3, 0.2 to 0.5, -0.03 to 0.4, 0.07 to 0.8 and 0.2 to 1.9 for A*. nyassana, E. whyteana, E. milanjeinsis, R. acuminatissinia, P. amarus* and Ascorbic acid respectively at a concentration range of 0.16-0.5ug/ml. Literature indicates that compounds with iron chelating ability can act as powerful antioxidants in most of the times. The iron ions are

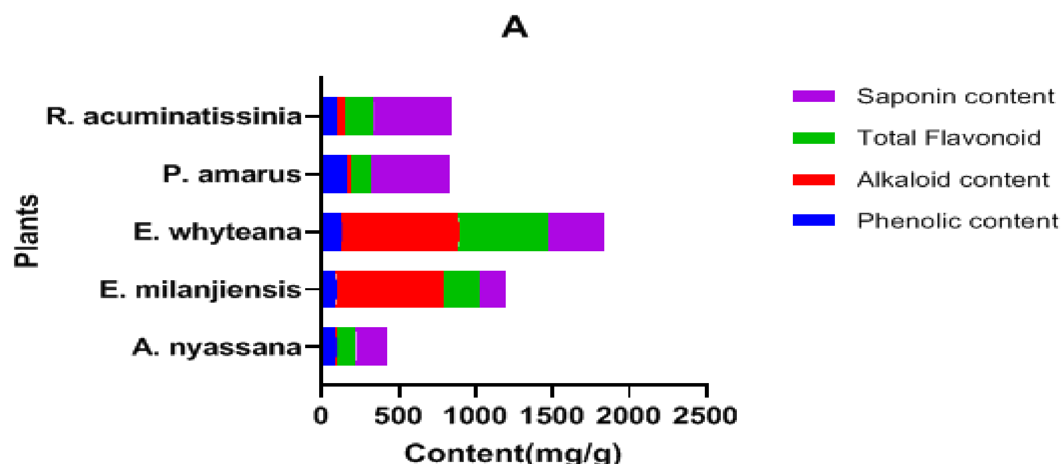

Figure.1: Phytochemical contents for the five medicinal plants were evaluated. Aluminium chloride method was used to assess the flavonoids contents, Folin-Ciocalteu reagents method was used to evaluate the total phenolic contents, Harborne method was used to measure the alkaloids content and also saponins contents were also evaluated. The samples were analysed in triplicate presented as mean ± standard deviation (SD)

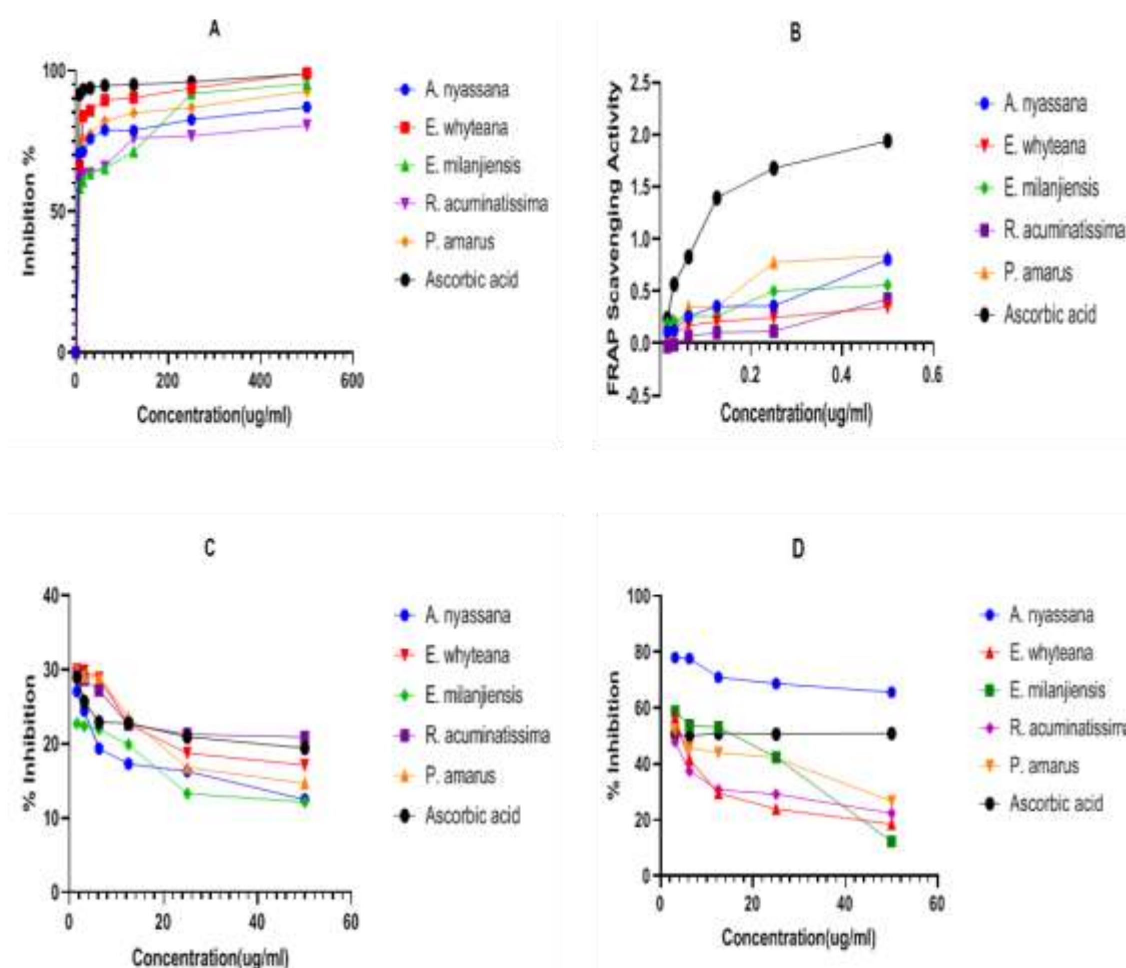

Figure 2: The analysis of 1,1-diphenyl-2-picryl hydrazyl (DPPH) free radical scavenging, the ferric ion reducing capacity (FRAP), Nitric oxide scavenging and Reducing power activities are shown in Figure 2 A, B, C, and D respectively. The results showed that the five plants extracts had moderate scavenging activity as compared to ascorbic acid at all concentration in all the experiments. Results are means of 3 replicates

known to catalyse the conversion of less reactive species such as lipid peroxides to more reactive species such as hydroxyl, peroxyl/alkoxyl radicals. And release of iron by cellular damage can accelerate oxidative damage to tissues [23].

In Figure 2C, the nitric oxide scavenging activity for the plant extracts and ascorbic acid demonstrated the scavenging activity of less than 50% inhibition for all the plants understudy. *P. amarus, R. acuminatissinia, E. whyteana*, and Ascorbic acid showed lower percentage inhibition of 29.8, 29.4, 29.2 and 28.9 respectively at a concentration of 50 µg/ml. A dose-dependent decrease in NO scavenging activity was observed for all the extracts except *R. acuminatissinia* at all the tested concentrations. In the human body, nitric oxide is generated from the amino acid, L-arginine, by vascular endothelial cells, phagocytes and certain cells of the brain. Nitric acid is classified as free radical, because of its unpaired electron and normally displays important reactivity with certain types of proteins and other free radicals. It becomes adverse when it reacts with superoxide radical, forming a highly reactive peroxynitrite anion hence being toxic [20].

In Figure 2D, the results for reducing power activity displayed an inverse relationship between concentration and percentage inhibition of the plant extracts. *A. nyassana, E. milanjiensis, E. whyteana*, and ascorbic acid showed reducing power of 78.0, 58.8, 56.5 and 50.8% respectively at 50ug/ml concentration. Increased absorbance of the reaction mixture indicates an increased reducing power of the plant extracts. The reducing properties of plant extracts has been shown in literature to exert antioxidant action that breaks the free radical chain through donation of a hydrogen atom to the compound [22],

In Table 1, the results showed that the IC_{50} values for DPPH free radical scavenging activity were within the range of 0.64–3.11 µg/ml, with Ascorbic acid, *E. milanjiensis,* and *E. whyteana* showing a significantly marked IC_{50} values of 0.64, 1.89 and 1.99 µg/mL respectively. The IC_{50} values of the DPPH tested samples were in the order: Ascorbic acid<*E. milanjiensis*< *E. whyteana*< *P. amarus*< *A. nyassana*< *R. acuminatissinia*

Table 1: The IC_{50} values for DPPH, Nitric Oxide and Reducing Power of plant extracts

Sample	DPPH		Nitric Oxide		Reducing Power	
	IC_{50}	R^2	IC_{50}	R^2	IC_{50}	R^2
A. nyassana	2.32	0.52	7.47	0.97	9.56	0.94
E. whyteana	1.99	0.69	5.45	0.87	2.43	0.77
P. amarus	2.26	0.51	4.27	0.89	0.43	0.29
R. acuminatissinia	3.11	0.59	8.90	0.92	0.17	0.94
E. milanjiensis	1.89	0.75	9.74	0.85	2.42	0.77
Ascorbic acid	0.64	0.39	11.47	0.94	1,82	0.52

IC_{50} values are half maximal inhibitory concentration which is a measure of the potency of a substance in inhibiting a specific biological or biochemical function while R^2 is a statistical measure of how close the data are to the fitted regression line. The IC_{50} values and R^2 were calculated for DPPH, Nitric oxide and Reducing power assays for all the plants understand

For nitric oxide scavenging activity, the IC_{50} values were within the range of 5.45 –11.47 µg/mL with *R. acuminatissinia* and *E. milanjiensis* significantly showing the IC_{50} values of 8.90 and 9.74 µg/mL respectively as compared to 11.47 for Ascorbic acid. The IC_{50} values of the nitric oxide scavenging activity tested samples were in the order: *P. amarus*<*E. whyteana*< *A. nyassana*< *R. acuminatissinia*< *E. milanjiensis*< Ascorbic acid

For the reducing power assay, the IC_{50} values were within the range of 0.43 –9.56 µg/mL with *A. nyassana, E. whyteana,* and *E. milanjiensis* showing IC_{50} values of 9.56, 2.43 and 2.4 respectively above the reference ascorbic acid. The IC_{50} values of the reducing power assay tested samples were in the order: *R. acuminatissinia*<*P. amarus*< Ascorbic acid< *E. milanjiensis*< *E. whyteana*< *A. nyassana.*

As shown in Table 2, the presence of antioxidant substances were demonstrated by a change in colour to pale-yellow colour spots on purple background and these changes were observed in *A. nyassana*, *E. whyteana, P. amarus, R. acuminatissima* and *E. milanjiana* plants. While in a separate HPTLC run of plates stained with Folin-Ciocalteu reagent, the presence of phenolic compounds were demonstrated by change to blue colour of spots with yellow background. The changes were observed in *E. milanjiensis* (0.69, 0.75, 0.88, 0.94, 0.97), *E. whyteana* (0.75, 0.88, 0.94, 0.97), *P. amarus* (0.56, 0.67, 0.97), *R. acuminatissima* (0.38, 0.94, 0.97) and *A. nyassana* (0.97).

Table 2: High-Performance Thin-Layer Chromatography(HPTLC) separation of the extracts

Plant	Rf value	Number of spots	Colour Observation	
			methanol/ sulphuric acid	Folin-Ciocalteu
A. nyassana	0.97	1	Pale-yellow	Blue colour
E. whyteana	0.75, 0.88, 0.94, 0.97	4	Pale-yellow	-
P. amarus	0.56, 0.67, 0.97	3	Pale-yellow	Blue colour
R. acuminatissinia	0.38, 0.94, 0.97	3	Yellow	Blue colour
E. milanjiensis	0.69, 0.75, 0.88, 0.94, 0.97	5	Pale-yellow	Blue colour

Chromatographic separation of the extracts was developed using ethyl acetate/methanol/water (10:1.35:1), the profiles of separated spots sprayed with 90:10 methanol/sulphuric acid reagent after visualization under UV (366 nm) and visible light respectively. For phenolic compounds, a separate HPTLC run was done in which plates were stained with Folin- Ciocalteu reagent to assess the phenolic compounds.

4 CONCLUSIONS

The presence of phytochemical compounds such as phenolic, alkaloids, flavonoids, saponins and the inhibitory effect of *A. nyassana, E. milanjiensis, E. whyteana, P. amarus* and *R. acuminatissinia* extracts on the free radicals provides some scientific evidence on the traditional usage in the management of opportunistic infections. These phytochemical compounds provide scavenging abilities which assist the body to fight infections. However, further studies on these plants are recommended to be undertaken in order to inform science.

Conflicts of interest

The authors have no conflicting of any interest.

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Appendix 2: Levels of heavy metals in four Malawian medicinal plants used for treatment of infectious diseases

Levels of heavy metals in four Malawian medicinal plants used for treatment of infectious diseases

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- **Article In Press Abstract**

Medicinal plants could potentially be contaminated with heavy metals during growing in the field, processing and/or handling. These heavy metals may be toxic to humans and cause damage to organs such as liver, kidneys and lungs. Currently, there is no data available on the levels of heavy metals in medicinal plants traditionally used for management of infectious diseases in Malawi. The aim of this study was to determine the levels of heavy metals; lead, chromium, cadmium, zinc, manganese, iron and copper in selected medicinal plants in Malawi. Four plants namely, Aeschynomene nyassana Taub., Euphorbia whyteana Baker f., Rhus acuminatissima R. Fern. & A. Fern. and Ericae milanjiana Bolus medicinal plants were collected from Southern parts of Malawi and identified at National Herberium and Botanical Gardens of Malawi. The leaves and roots were shade-dried at room temperature and grounded into fine powder. Two grams of each sample was transferred into silica crucible and mixed with 5 ml HCl. The mixture was subjected to ashing for 3 hours at 450 °C in a furnace. The ash residues were dissolved in HNO3 and made up to 50 ml in a conical flask. The digested material was analysed in triplicates for presence of metals using Atomic Emission Spectrophotometer. The results showed that heavy metals were present in all the four selected plants. The levels of metals were within the WHO permissible ranges of lead, chromium, cadmium, zinc, manganese, iron and copper. The percentage recovery of heavy metals from each sample was within the acceptable range of 88 to 103 %. Lead was shown to be the most abundant metal while cadmium was the least abundant in all the plants understudy. A statistical analysis of variance at 95 % confidence level showed significant differences in the levels of metals. This study has shown that selected medicinal plants could be contaminated with heavy metals. While, the metal concentrations are low in the present study, plants growing in heavily polluted environments could accumulate very high levels of metals. This can cause adverse health effects on people consuming these plants. Therefore, this study recommends screening of medicinal plants for heavy metals prior to usage.

Keywords: Levels of heavy metals, Medicinal plants, Microwave Plasma-Atomic Emission Spectrophotometer

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Appendix 3: Certificate of Ethics Approval

CERTIFICATE OF ETHICS APPROVAL

This is to certify that the College of Medicine Research and Ethics Committee (COMREC) has reviewed and approved a study entitled:

P.11/17/2309 - NATURAL TREATMENT OF INFECTIOUS DISEASES: IN VITRO TESTING OF ANTI-HIV AND ANTI-MYCOBACTERIAL ACTIVITY OF SOME MALAWIAN MEDICINAL PLANTS by Frank Ngonda

On 20-Dec-17

[illegible]

20-Dec-17

[illegible]