BIOASSAY-GUIDED EVALUATION OF ANTI-TUBERCULOSIS AND PHYTOCONSTITUENTS IN EXTRACTS OF *Guiera senegalensis* (J.F. GMEL), *Scoparia dulcis* (LINN) AND *Pulicaria crispa* (FORSSK)

By

Adedayo Babafemi ADEBIYI

DEPARTMENT OF CHEMISTRY
FACULTY OF SCIENCE
AHMADU BELLO UNIVERSITY, ZARIA
NIGERIA

FEBUARY, 2016

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By

Adedayo Babafemi ADEBIYI, B.Sc Chemistry (UNILAG) 2008

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FEBUARY, 2016

DECLARATION

I declare that the work in this dissertation entitled "**BIOASSAY-GUIDED EVALUATION OF ANTI-TUBERCULOSIS AND PHYTOCONSTITUENTS IN EXTRACTS OF *Guiera senegalensis* (J.F. GMEL), *Scoparia dulcis* (LINN) AND *Pulicaria crispa* (FORSSK)**" has been carried out by me in the Department of Chemistry under the supervision of Prof. R.G. Ayo and Dr I.A. Bello. The information derived from the literature has been duly acknowledged in the text and a list of references provided. No part of this thesis was previously presented for another degree or Diploma at any University.

Adedayo Babafemi ADEBIYI

Name of Student	Signature	Date

CERTIFICATION

This thesis entitled "**BIOASSAY-GUIDED EVALUATION OF ANTI-TUBERCULOSIS AND PHYTOCONSTITUENTS IN EXTRACTS OF *Guiera senegalensis* (J.F. GMEL), *Scoparia dulcis* (LINN) AND *Pulicaria crispa* (FORSSK)**" by Adedayo Babafemi Adebiyi meets the regulations governing the award of the degree of Master of Science Degree in (Organic) Chemistry of the Ahmadu Bello University, Zaria, and is approved for its contribution to knowledge and literary presentation.

Prof. R.G. Ayo Chairman, Supervisory Committee	Signature	Date
Dr I.A. Bello. Member, Supervisory Committee	Signature	Date
Prof. V. O Ajibola Head of Department	Signature	Date
Prof. K. Bala Dean, School of Postgraduate Studies	Signature	Date

DEDICATION

This work is dedicated to the Almighty God who makes all impossibilities possible.

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ABSTRACT

Guiera senegalensis, Scoparia dulcis and *Pulicaria crispa* are plants known in Nigeria for the treatment of various infectious diseases. These plants were subjected to phytochemical, antimicrobial and anti-TB screening. Phytochemical screening of the plants revealed the presence of saponin in all the hexane fractions, ethyl acetate fraction of *Scoparia dulcis* and methanolic fraction of *Guiera senegalensis*. Cardenolides, phlobatanins, volatile oil and resins were absent in all the three solvent extracts. Flavonoids and volatile oils were present in only the ethyl acetate extract and methanolic extract of *Pulicaria crispa* . Cardiac glycosides, glycosides, balsams and phenols were only present in the methanolic extract of *Scoparia dulcis*. Steroids were found in the ethyl acetate extracts. Flavonoids, cardiac glycosides and glycosides were also found in the methanolic extract of *Guiera senegalensis* and *Scoparia dulcis*. The antimicrobial activity of the plant fractions tested against Gram positive bacteria (*Staphylococus aureus, Bacillus subtilis, Streptococcus pyogenes*) and Gram negative bacteria (*Shigella dysenteriae, Salmonella typhii, Escherichia coli*) and fungus (*Candida albicans, Candida krusei*) using agar in-well diffusion method have their activities ranging between 16 mm to 35 mm respectively as against the control (ciproflaxin and fluconazole ranging between 31mm to 41mm respectively) while the zone of inhibition of the test microbes against the three plant extracts were found to have high zone of inhibition in both ethyl acetate fraction and hexane fraction of *Guiera senegalensis*, at a concentration of 3.12 µg/ml as against *S. aureus,* also in ethyl acetate fraction of *Pulicaria crispa* at a concentration of 12.5 µg/ml as against *P. mirabilis,* also in all fractions o *G. Senegalensis* at a concentration 3.12 µg/ml as against *C.albican*, followed by the fractions of *Pulicaria crispa* at concentration of 3.12 µg/ml as against *S. pyogenes* and *N.gonorrhea*, the inhibitory effect of various extracts were compared with standard drug ciprofloxacin and fluconazole. Minimum inhibitory concentration and Minimum bactericidal and fungicidal concentration for both extracts were also determined and ranged between 12.25 to 25 µg/ml using broth dilution method. The anti-mycobacteria study showed that activity were found in methanol extract of *Pulicaria crispa* at concentration of 8.01±1.70 µg/ml, extracts of ethyl acetate of *Scoparia dulcis* at concentration of 12.03±0.86 µg/ml and methanol extract of *Guiera senegalensis* at concentration of 2.80±1.40 µg/ml, however low activity was observed for the hexane extract of *Scoparia dulcis* at concentration of 20.40±0.24 µg/ml and ethyl acetate extract of *Guiera senegalensis* at concentration of 40.01±1.20 µg/ml, hence when compared with the control drug rifampicin at concentration of 0.38±1.40 µg/ml, methanol fraction for

Guiera senegalensis showed the highest anti-mycobaterial activity. Extensive chromatographic separation of the methanol extract of *Guiera senegalensis* led to the isolation of compound Dy1 (oleanolic acid). The structure of the compound were established by spectral analysis including 1D NMR (^{1}H, ^{13}C and DEPT), 2D NMR (COSY, HSQC, HMBC and NOESY) hence showing 8 quatenary carbons, 5 methine carbons, 10 methylene carbon and 7 methyl carbons, hence the total of thirty carbons. The high anti-TB activity on the methanolic fraction of *Guiera sengalensis* is been reported for the first time from this plant. The use of the plants for the treatment of infectious disease is therefore justified.

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LIST OF ABBREVIATION

TB: Tuberculosis

M. TB: Mycobacterium Tuberculosis

DNA: Deribonucleic Acid

HIV/AIDS: Human Immuno-deficiency Virus/Acquired Immune Deficiency Syndrome

RFP: Rifampicin

INH: Isoniazid

PZA: Pyrazinamide

EMB: Ethambutol

CDC: Centre for Disease Control

DOTS: Direct Observation Treatment Short course

COSY: Correlation Spectroscopy

DEPT: Distortionless Enhancement Polarization Transfer

HMBC: Heteronuclear Multiple Bond Correlation

HSQC: Heterenuclear Single Quantum Correlation

MBC: Minimum Bactericidal Concentration

MHA: Muller Hinton Agar

MIC: Minimum Inhibitory Concentration

NMR: Nuclear Magnetic Resonance

BDH: British Drug Home

WHO: World Health Organization

MDRTB: Multidrug Resistant Tuberculosis

XDRTB: Extensively Drug Resistant Tuberculosis

MABA: Micro-plate Alamar Blue Assay

CHAPTER ONE

1.0 INTRODUCTION

1.1 Historical background and importance of medicinal plants.

Plants have been used for thousands of years to flavour and conserve food, to treat health disorders and to prevent diseases including epidemics. The knowledge of their healing properties has been transmitted over the centuries within and among human communities. The use of plants for treating diseases is as old as the humans (Balunas and Kinghorn, 2005). Plant materials have been used for the treatment of various diseases throughout the world before the advent of modern clinical drugs. The use of medicinal plants still plays an important role to cover the basic health needs in the developing countries and the industrialized societies. The extraction and development of several drugs have been traced from these plants as well as from traditionally used folk medicines (Shrikumar and Ravi, 2007). Popular observations on the use and efficacy of medicinal plants significantly contribute to the disclosure of their therapeutic properties, so that they are frequently prescribed, even if their chemical constituents are not always completely known. All over the globe, the use of medicinal plants has significantly supported primary health care (Maciel *et al*., 2002). From 250 to 500 thousand plant species are estimated to exist on the planet, and only between 1 and 10 % are used as food by humans and other animals (Cowan, 1999). About 80 % of the world population uses only 37 % of the commercially available drugs and depend exclusively on medicines of natural origin (Funari and Ferro, 2005). Thus, phytotherapeutics entered the market promising a shorter and cheaper production, since basic requirements to use medicinal plants do not involve strict quality control regarding safety and efficacy compared to the other types of drugs (Niero, 2010).

Infectious diseases represent an important cause of morbidity and mortality among the general population, particularly in developing countries. Therefore, pharmaceutical companies have been motivated to develop new antimicrobial drugs in recent years, especially due to the constant emergence of microorganisms resistant to conventional antimicrobials. Apparently, bacterial species present the genetic ability to acquire and transmit resistance against currently available antibacterial since there are frequent reports on the isolation of bacteria that are known to be sensitive to routinely used drugs and became multi-resistant to other medications available on the market (Nascimento *et al.,* 2000; Sakagami and Kajimura, 2002). Consequently, common strategies adopted by pharmaceutical companies to supply the market with new antimicrobial drugs include changing the molecular structure of the existing medicines in order to make them more efficient or recover the activity lost due to bacterial resistance mechanisms (Chartone-Souza, 1998).

1.2 Ethnobotany and pharmacology of *Guiera senegalensis* (J.F. Gmel), *Scoparia dulcis* (Linn) and *Pulicaria crispa* (Forssk)

The plant selected for study in this project were root part of *Guiera senegalensis* (J.F. Gmel), whole plant part of *Scoparia dulcis* (Linn) and whole plant part of *Pulicaria crispa* (Forssk).These species were mentioned by traditional healers in Nigeria to be used in the treatment of tuberculosis symptoms and chest related infections according to an ethno-botanical survey that was done (Gautam, *et al*., 2007)

1.2.1. *Guiera senegalensis* (J.F. Gmel)

Guiera senegalensis is a flowering plant in the genus *Guiera* of the family of combretaceae and it is a native of tropical regions of Africa. The plant produces the Tannin 3, 4, 5- tri-o-galloylguinic acid and several alkaloids (Gmel *et al.,* 2006). It is popularly named in Hausa as kululu or saabaraa in the northern parts of Nigeria. It is used to treat a variety of microbial infections, can be used as dyes. It is also used for the following purposes; as an essential oil,

exudates, fibre meals, fuel plant, medicinal plant, timber and vegetables. They are soft on both surfaces, with scattered black glands underneath (Hutchison and Dalziel, 1965). The leaves are bitter-tasting and have widespread acknowledgement in African medicine as a "cure-all" in herbal concoctions (Hiermann and Bucar, 1994). The usual form of preparation for internal use is in decoctions or mixed with food preparations *G. senegalensis* leaves are widely administered for pulmonary and respiratory complaints, for coughs, as a febrifuge, colic and diarrhea, syphilis, beriberi, leprosy, impotence, rheumatism, diuresis and expurgation (Hutchinson and Dalziel, 1965; Zeljan *et al*., 1998). In Northern Nigeria powdered leaves are mixed with food as a general tonic and blood restoratives (Koumare *et al*., 1968). In Nigeria and other West African Countries, the leaves are used to treat dysentry and fever due to malaria (Abbiw, 1990). Earlier findings by Etkin (1997) indicated *Guiera* leaf extracts markedly oxidized glutathione and also generated high levels of methaemoglobin *in vitro*, both conditions being unfavourable for the survival of *Plasmodium* in red cells. These reported activity and the application of the plant in herbal medicine for analgesic and antimalarial properties necessitated *in vivo* screening with parasitized animals. Leaf extracts of the plant were tested for antiplasmodial, analgesic and anti-inflammatory effects *in vivo* by Jigam *et. al*., 2009, his results indicated the safe dose of extracts as 600 mg/kg body weight of mice with LD50 of 1100 mg/kg body weight. Only the methanol fraction had antiplasmodial effect while ethyl acetate and hexane fractions were ineffective. Furthermore the methanol extract produced a significant ($p<0.05$) suppression of up to 67.52% levels. The extracts were found to have no prophylactic effect or high parasitaemia, rather it gave 44.83% analgesic effect which was devoid of anti-inflammatory activity.

1.2.2 *Scoparia dulcis* (Linn)

Scoparia dulcis (Linn) belongs to the family Scrophulariaceae. It is an herbal plant found almost around the globe: In European, African, American and Asian countries. Synonyms are *Scoparia grandiflora, Scoparia ternata, Capraria dulcis* and *Gratiola micrantha.* In Nigeria the plant is known by such names as *roma fada* (Hausa), *aiya* (Igbo), *bibiimbelemo* (Ijaw), *mesenmesèn gogoro* (Yoruba), *ndiyang* (Efik), and *ungungbuhi* (Gwari).

The plant is an erect, shrubby herb that grows up to about 50 cm high (Orhue *et al*, 2006). It usually has many auxiliary shoots and reproduces from seeds. The stem is more or less woody, ribbed, mainly branched and glabrous. The leaves are opposite or three at a node, oval or narrowly oblanceolate; about 2.5 cm to 5.0 cm long and 1.5 cm wide. The Brazilian folkloric use it to treat bronchitis, gastric disorders, hemorrhoids, insect bites and skin wounds. Asian medicine uses the herb to treat hypertension Latha *et. al.*, 2004. The herb also has antiulcer properties Cragg *et. al.*, 2008. In tropical and subtropical regions, the fresh or dried plant of *Scoparia dulcis* has traditionally been used as one of remedies for stomach troubles (Satyanarayana, 1969), hypertension (Chow *et al.*, 1974), diabetes (Perry, 1980), bronchitis (Freire *et al.*, 1996), and as an analgesic and antipyretic (Gonzales, 1986). Three antidiabetic compounds have been isolated from *Scoparia dulcis*; amellin, scoparic acid D and diasulin. Amellin, an antidiabetic compound is a glycoside which has been reported in the leaf and stem of fresh green plants. An infusion of the leaf is used in fever, cough and bronchitis and as gargle for toothache. A hot infusion is a diuretic. An infusion of roots leaves and tops are useful in diarrhea and dysentery. The diterpenoid, scoparic acid A, isolated from the plant has been reported to be a potent α-glucoronidase inhibitor (Hayashi *et al*, 1992). The constituents, scopadulciol, scopadulcic acid-B and diacetylscopadiol, have been shown to be responsible for the inhibitory activity of the plant on gastric H+-K+ ATPase enzyme (Asano *et al.*, 1990). All parts of the plant are useful as emetic. However, very limited work has been

carried out on this specie toward documenting its ethno medicinal uses and establishing its phytochemical and anti-TB finger prints.

1.2.3. *Pulicaria crispa*(Forssk)

Pulicaria crispa (forssk) is an annual herb or sometimes a perennial sub shrub belonging to the family Asteraceae producing small bright yellow flowers and Genus Pulicaria is represented in the flora of Iran by five species (Nematollahi *et al*., 2006). *P. undulata* is an example of the species which is used as a medicinal plant by people of Southern Egypt to treat inflammation. This species are distributed in Iran, Saudi Arabia, Kuwait, Iraq, Egypt, Afghanistan, Pakistan, India and parts of North and West tropical Africa (Al-Rawi, 1987; Boulos, 2002).

Pulicaria crispa is used as an insect repellent (Stavri *et al*., 2007) and also as an herbal tea (Ross *et al*., 1997). These plants have been reported in the past to have medicinal values just as reviewed above, they possess potentials for inhibiting TB in the human body and hence justifying the research.

1.3 Aim and Objectives of the study

1.3.1 Aim

The aim of this work is to justify or otherwise the ethnomedicinal claims on the root of *Guiera senegalensis,* whole plant of *Scoparia dulcis,* and *Pulicaria crispa* as medicinal plants in the treatment of tuberculosis and any other microbial infectious diseases.

1.3.2 Objectives

The objectives of this study are:

- Collection, proper botanical identification and drying of the plants.

- Extraction of the ground plant material using different solvents, from non-polar to polar ones.
- Phytochemical screening of the crude extracts.
- Antimicrobial screening of the extracts
- To determine the anti-mycobacterial activity of the plant extractsChromatographic purification of the extracts
- Spectroscopic Structural elucidation and characterization of the isolated compounds using available spectral techniques
- To determine the anti-mycobacterial activity of the isolated compound.

1.4 Justification of the study

Trends in the incidence of tuberculosis together with the development of multi-drug and extensively drug resistant strains of tuberculosis raises the need to intensify the search for more efficient drugs to combat this disease (Corbett *et al*., 2003; CDC Report, 2005). There are widespread claims by some traditional healers that TB can be treated using herbs. However these claims have no scientific justification mainly because, Nigeria has one of the least published literatures on plants screened for anti-Tubercular activity (Mann *et al*., 2007). The results of this study will go a long way to authenticate the claims by 5 traditional healers and will as well enrich the databases on plants with anti-mycobacterial activity that can be used in drug discovery.

Among infectious diseases, tuberculosis is one of the leading killers of adults in the world today. The incidence of tuberculosis is exacerbated by the emergence of drug-resistant strains multi-drug resistant and extensive drug resistant (MDR and XDR) and HIV co-infection (Furin, 2007). Available treatment regimens are lengthy and complex, leading to problems of

non-adherence and inadequate response. In the case of MDRTB, second line drugs used are more toxic and expensive while XDRTB is virtually untreatable. HIV/AIDS patients presenting with tuberculosis stand a risk of drug adverse reactions as a result of possible drug-drug interactions. On the other hand a number of traditional medicinal plants have been reported to treat tuberculosis, however their efficacy and safety remains unknown. This study was conducted to determine the efficacy and safety of the plants *(Guiera senegalensis, Scoparia dulcis,* and *Pulicaria crispa)* that have been suggested in treatment of TB symptoms by traditional healers.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Natural products in drug discovery and development

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

The plant kingdom can be looked at as an important source of new drugs for the treatment of tuberculosis because of its enormous chemical diversity (Gautam *et al*., 2007). In addition to the antimicrobial action of plant extracts and essential oils, a synergism between conventional antimicrobial drugs and products obtained from medicinal plants has also been reported. Possible interactions among medications are frequently observed, which has motivated researchers to test such possibilities. However, it must be emphasized that interactions between synthetic and natural drugs depend on several factors including pharmacokinetics and employed doses, since combinations confirmed *in vitro* may not have the same effect on humans (Szalek *et al*., 2006). Nevertheless, numerous studies on this particular theme can be found in the literature. Synergism assays between terpenes and penicillin against MRSA and *E. coli* revealed a synergistic effect produced by the combination of carvone and penicillin whereas an antagonistic effect between thymol and penicillin was detected against MRSA strains. (Maciel *et al*., 2002).

The World Health Organization (WHO) recommended that standard drug-susceptibility testing be performed at the same time that the experimental MTB/RFP assay is performed to confirm rifampicin resistance and the susceptibility of the *M. tuberculosis* isolate to other drugs (WHO 2009). The search for an improved and better alternative to rifampicin, leads us to investigate plants for anti-TB activity. Some medicinal properties of these plants have been known for a while, even though plants have been known to serve as a source of potent anti-microbial agents in the form of secondary metabolites. There have been some claims that these plants have some anti-TB activity, this research hence seeks to justify that claim and at the same time characterize the phyto-constituents responsible for this activity.

2.2 The global burden of Tuberculosis

Tuberculosis (TB) has been part of human history for thousands of years and signs of this disease have been found ever before the identification of mycobacterial deoxyribonucleic acid (DNA) in ancient Egyptian mummies dated between 3000 and 2400 years B.C. (Crubezy *et al.*, 1998; Rothschild *et al.*, 2001; Donoghue *et al.*, 2004). In ancient Greece, Hippocrates described TB as phthisis or consumption, a term by which this disease was known until the early 20th century. At the beginning of the 17th century and for the next 200 years, the TB epidemic (known as the Great White Plague) started in Europe and became the major cause of mortality (Bates and Stead, 1993). However, despite the long co-existence of TB and mankind, *Mycobacterium tuberculosis*, the agent responsible for this infection in Man, was described only in 1882 by Robert Koch (Koch, 1882).

In the early 20th century, the general improvement in public health in Europe and in the United States of America (U.S.A) helped to reduce the burden of TB. The establishment of TB control programmes, along with the introduction of a successful anti-TB treatment, resulted in an evident decrease of infection and mortality (WHO, 2007). In the middle of the

20th century, the fact that the disease was considered so close to elimination caused a decreased interest of the industrialized countries and, consequently, the abandonment of the TB control programmes (WHO, 2004). In fact, it was considered that the recent developments in anti-mycobacterium chemotherapy had determined the disappearance of the disease as a public health problem. However, despite the decrease of TB cases, the disease never disappeared completely and around 1985, TB began to increase again in industrialized countries (Brudney and Dobkin, 1991; Daniel, 2006). The other major factor that contributed to this resurgence, along with the decline of the TB control programmes, was the advent of the human immunodeficiency virus/acquired immunodeficiency syndrome (HIV/AIDS) (Brudney and Dobkin, 1991). TB has once more gained the attention of the international community and is still without doubt one of the major threats to public health. In 1993, the World Health Organization (WHO) declared TB a global health emergency and in 1998, together with the International Union against Tuberculosis and Lung Disease (IUATLD) and other international partners, formed the Stop TB Initiative, a new global strategy to control TB that has recently evolved into a global partnership, the Stop TB Partnership (WHO, 2006). However, despite these global efforts, TB is still a major threat. In fact, the WHO estimates that one third of the world population (approximately two billion people) is infected with *M. tuberculosis*, with eight million new cases of TB every year and two million deaths from TB each year (WHO, 2009).

The higher rates of TB ($\geq$ 300/100 000 inhabitants/year) occur in Sub-Saharan Africa, If effective TB control programmes are not created, WHO estimates that by 2020 *M. tuberculosis* will infect approximately 1000 million people, 150 million will develop the disease and 36 million will die of TB (WHO, 2009).

The global resurgence of TB is not only related to the higher rates of co-infection with HIV, but also with the emergence of *M. tuberculosis* strains resistant to anti-TB drugs. An example was the extensive outbreak of TB in New York City in the early 1990's. This form of TB was resistant to the most effective anti-TB drugs and occurred mainly in patients co-infected with HIV. In fact, one in three new cases were found resistant to one drug and one in five to more than one drug (Frieden *et al.*, 1995). WHO defined multidrug resistant TB (MDRTB) as TB cases caused by *M. tuberculosis* strains resistant, simultaneously, to isoniazid (INH) and rifampicin (RFP), two of the firstline drugs used to treat TB. It is estimated that 50 million people are infected with *M.tuberculosis* strains resistant to anti-TB drugs, with 300 000 new cases of MDRTB each year (WHO, 2008). Recently, a new form of TB has been reported that presents resistance not only to first-line drugs, but also to second-line drugs. The WHO has defined this form of TB as extensively drug resistant TB (XDRTB) and refers to *M. tuberculosis* strains that present resistance not only to INH and RFP (MDRTB), but also to any fluoroquinolone and at least to one of the three injectable second-line drugs kanamycin, amikacin and capreomycin (WHO, 2006). XDRTB can develop when these second-line drugs are misused or mismanaged and, consequently, become ineffective (Migliori *et al.*, 2007).

2.3 Etiology of Tuberculosis

Tuberculosis (TB) is caused by different tubercle bacilli from the genus Mycobacterium (Sensi and Grassi, 2003). The genus contains over 50 species including *Mycobacterium tuberculosis,* the most common pathogen for humans, *Mycobacterium africanum* common in West Africa and *Mycobacterium. bovis* that causes infection in animals but can also infect humans (Adeniyi *et al.*, 2004; Gautam *et al.*,2007). Other members of Mycobacteria tuberculosis complex include *Mycobacterium microti* and *Mycobacterium pinnipedii.*

Furthermore *Mycobacterium. avium, Mycobacterium scrofulaceum* and *Mycobacterium intracellurae* are potentially pathogenic species that have been reported to cause opportunistic infections in HIV/AIDS patients. Other species, *Mycobacterium chelonae, Mycobacterium vaccae* and *Mycobacterium fortuitumare* are important causes of cutaneous, pulmonary and nosocomial infections (Newton *et al*., 2000; Adeniyi *et al*., 2004).

Mycobacteria are non-motile rod shaped obligate aerobes (Bruton *et al*., 2007), that are characterized as acid fast bacteria because of their fast action on dyes treated with acidified organic material (Madison, 2001). One of the major characteristics of mycobacteria is that it is slow growing and may remain inactive in the host for a long period. This characteristic contributes to its virulence nature (Tripathi *et al*., 2004). Secondly, the cell wall of mycobacteria is 60% lipophilic, containing lipids like mycolic acids; this hinders antibacterial agents from penetrating the cell (Tripathi *et al*., 2004). The complete sequencing of the *Mycobacterium tuberculosis* H37Rv genome has provided more information concerning the characteristics of this organism, such as its slow growth, cell-wall complexity and adaptation to environmental conditions (Cole *et al*., 1998). From the genome sequence it is clear that *Mycobacterium tuberculosis* has the potential to switch from one metabolic route to another, including aerobic (*e.g.* oxidative phosphorylation) and anaerobic respiration (*e.g.* nitrate reduction). This flexibility is useful for survival within the human host environment that can range from high oxygen tension in the lung alveolus to micro aerophilic/anaerobic conditions within the tuberculous granuloma (Cole *et al*., 1998).

2.4 Tuberculosis in Nigeria

Tuberculosis is one of the leading causes of illness and mortality worldwide; it has a mortality rate and incidence rate of approximately 2 million deaths per year and 9.2 million new cases respectively (CDC, 2005; WHO, 2007). Nigeria ranks 10th among the 22 high-

burden TB countries in the world. WHO estimates that 210,`000 new cases of all forms of TB occurred in the country in 2010, equivalent to 133/100,000 population. There were an estimated 320,000 prevalent cases of TB in 2010, equivalent to 199/100,000 cases. There were 90,447 TB cases notified in 2010 with 41, 416 (58 %) cases as new smear positives, and a case detection rate of 40 %. 83 % of cases notified in 2009 were successfully treated. The main goal of Nigeria's TB program is to halve the TB prevalence and death rates by 2015 (WHO, 2007).

According to Adeniyi *et al.*, (2004), 95 % of the world's TB burden is in developing countries. Sub-Saharan Africa has the highest incidence rate of 29 % of the world's TB burden and 34 % of the world's total death from TB (Chaisson and Martinson, 2008). The TB burden is compounded by a high prevalence of HIV in the country which stands at about 4.1 % in general population and the prevalence of HIV among TB patients increased from 2.2% in 1991 to 19.1 % in 2001 and 25 % in 2010. This indicates that the TB situation in the country is HIV-driven.

The proportion of TB patients tested for HIV was 79 % in 2010, with a 25 % TB-HIV co-infection rate, 59 % of these patients were started on cotrimoxazole (CPT) prophylaxis and 1.8 % provided with isoniazid (INH) prophylaxis. This high incidence rate, morbidity and mortality of TB in Africa has led WHO to declare TB an emergency in the continent. HIV/AIDS, resistance of TB to first line drugs, poverty and overcrowding are some of the reasons that explain the increase in Africa (Tripathi *et al.*, 2004). The U.S response in the control of TB in Nigeria was relatively limited until late 2002, when USAID began its program in Nigeria in 2003 with the establishment of TB DOTS centers in the 17 supported states in Northern Nigeria and Lagos. Over time, the USG effort to address TB has expanded to cover the entire country in all thematic areas of the STOP TB partnership (CDC, 2005).

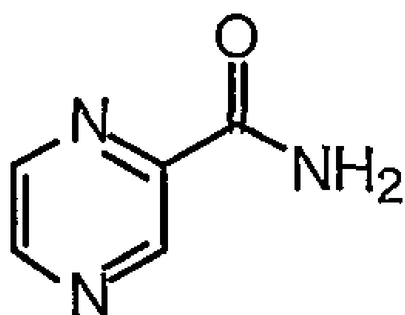
2.5 Treatment of Tuberculosis

Treatment of TB is used not only to cure the disease but also to interrupt the transmission and to prevent relapse (most relapses occur within 6-12 months after the end of therapy) (Böttger and Springer, 2008). In 1994, the WHO introduced the Directly Observed Treatment Short Course (DOTS), a strategy for the detection and treatment of TB, in which patients are observed to take each dose of anti-TB medication, until the end of therapy (WHO, 2004). Monthly sputum specimens are then taken until 2 consecutive specimens are negative. However, the effectiveness of DOTS is facing new challenges due to the increase of MDRTB and the emergence of XDRTB. This lead to a new strategy called DOTS-plus, a comprehensive management initiative built upon the DOTS strategy with the goal of preventing further development and spread of MDRTB (WHO, 2007; WHO, 2008). Effective treatment of TB involves targeting the multiple populations of bacteria that reside in the host (Bryrne *et al*., 2007).

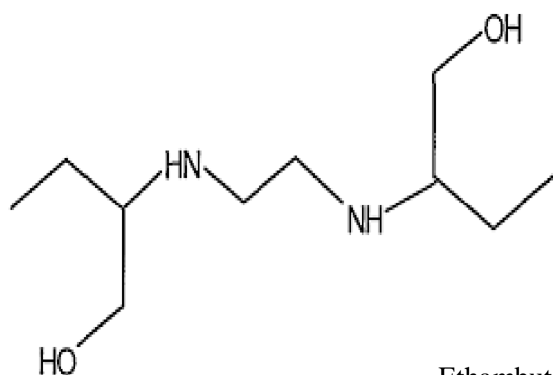
Treatment can be divided into first-line and second-line drugs: the first-line drugs used are isoniazid (INH), rifampicin (RFP), pyrazinamide (PZA), ethambutol (EMB) and streptomycin (STR) and the second-line drugs include fluoroquinolones, aminoglycosides such as kanamycin and amikacin, cyclic peptides like capreomycin, D-cycloserine, ethionamide, and D-amino salicylic acid. Each treatment regimen for pulmonary TB caused by susceptible organisms has an initial 2 months intensive phase with INH, RFP, PZA and EMB, followed by a continuation phase with INH and RFP for 4 to 7 months (CDC, 2006). Treatment of patients with MDR-TB is much more difficult and relies extensively on second-line drugs that include fluoroquinolones, ethionamide, the aminoglycosides capreomycin, cycloserine, para-aminosalicylic acid, and clofazimine. These agents have poorer activity than the first-line drugs and greater tendency to cause adverse reactions (CDC, 2006).

Fluoroquinolones such as moxifloxacin and levofloxacin have considerable activity against *M. tuberculosis* and are preferred in the treatment of all MDR-TB cases, unless resistance to this class is also demonstrated as the case is in Extensively Drug Resistant TB strains (XDR). XDR-TB is that which is resistant to any fluoroquinolone, and at least one of three injectable second-line drugs (capreomycin, kanamycin, and amikacin), in addition to MDR-TB (Furin, 2007).

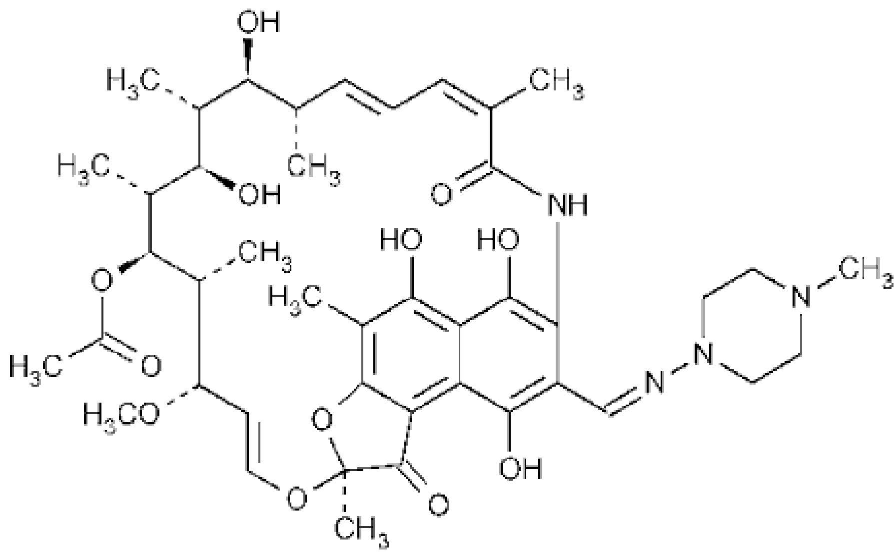
Previously discovered compunds in the treatment of TB include; thiolactoycin and tryptanthin which have demonstrated significant activity against MDR TB. However lack of *in vivo* toxicity profile has greatly hindered their use in humans (Tripathi *et al*., 2004). Structures of anti-TB drugs are shown below.



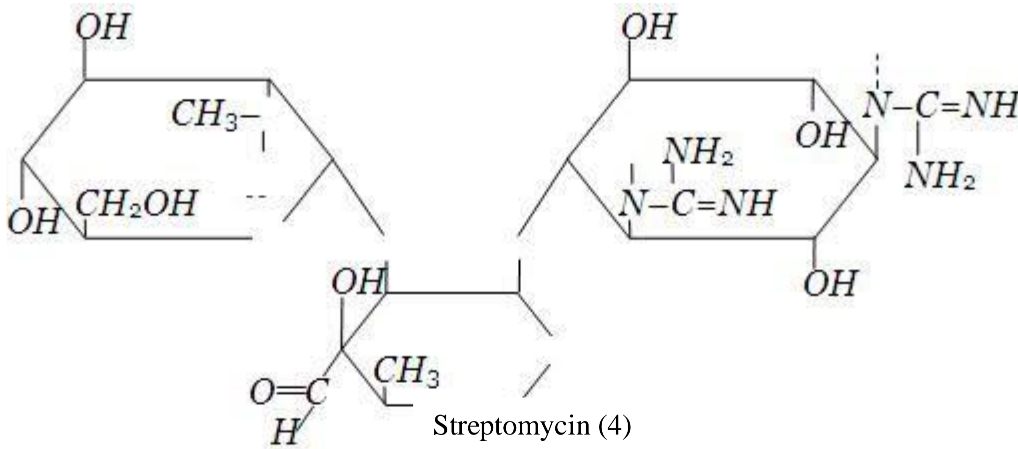
Pyrazinamide (1)



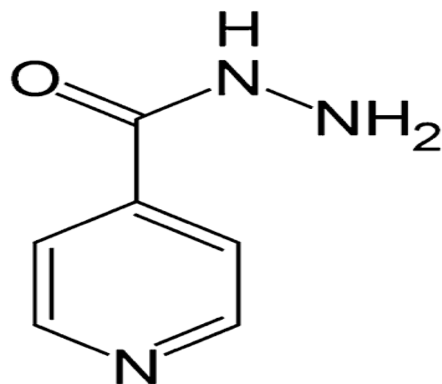
Ethambutol (2)



Rifampicin (3)



Streptomycin (4)



Isoniazid (5)

2.6 Problems with Controlling TB

One of the major challenges in TB control is the development of resistance that is Multi Drug Resistant TB (MDR TB) and Extensively Drug Resistant TB (XDRTB). MDRTB is TB that is resistant to at least rifampicin and isoniazid while XDR TB is TB that is resistant to all the first line drugs, any flouroquinolone and one of the injectable second line drugs (Fattorini *et al*., 2007). In addition, therapy should be based on individual drug susceptibility testing including residual first line and second line drugs (Fattorini *et al*., 2007). If MDRTB is not well managed, XDRTB will eventually develop which poses a much more powerful hindrance for management of TB since it is virtually untreatable (CDC, 2006).

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

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

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

In the macro and micro dilution method known concentrations of the extracts are tested on the bacteria in agar media (Pauli *et al.*, 2005). The method allows for quantification and determination of minimum inhibitory concentration (Gautam *et al.*, 2007). The medium is supplemented with oleic acid, albumin, dextrose and catalase (OADC) (Pauli *et al.*, 2005). The major disadvantage with the method is that it requires at least 18 days for visibly detection of colonies growth (Gautam *et al.*, 2007). Micro broth dilution method is used in the determination of minimum inhibitory concentration. In this method, serial dilutions of the extract are placed in different test tubes containing inoculated broth (Suffredin *et al.*, 2004). Quantification is done by visualizing turbidity in the test tube. This poses a disadvantage of misinterpretation due to the tendency of mycobacteria to clump and also crude extracts may impart some turbidity to the medium (Gautam *et al.*, 2007).

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

2.8 Drug discovery from natural products

Natural products continue to be a source for innovation in drug discovery by playing a significant role in the discovery and understanding of cellular pathways that are an essential component in the drug discovery process. In many cases, natural products provide compounds as clinical/marketed drugs, or as biochemical tools that demonstrate the role of specific pathways in disease and the potential of finding drugs. Numerous reviews have been written that describe the importance of compounds derived from microbes, plants and animal

sources to treat human diseases (Newman *et al*., 2003; Butler, 2004; Butler, 2005; Koehn and Carter, 2005).

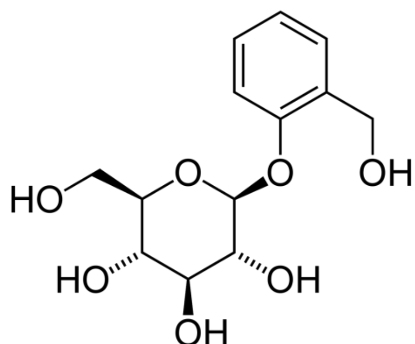
The ability of plants to synthesize aromatic substances and secondary metabolites has been of importance in drug development (Cowan, 1999). Some of the most important compounds of plants with medicinal uses are alkaloids, tannins, flavonoids, sterols and phenols (Edeoga *et al.,* 2005).

These compounds can be extracted from plants by use of different solvents ranging from alcohols, chloroform, ether, hexane and water. Alcohols and aqueous solvents extract the polar components of the plant while hexane and ether extract the non-polar compounds (Cowan, 1999). Prescription drugs in 1980 were estimated to be plant-based (Principe, 1985). An authoritative review concluded that 61% of all the new drugs introduced worldwide can be traced to or were inspired by natural products (Principe, 1985).

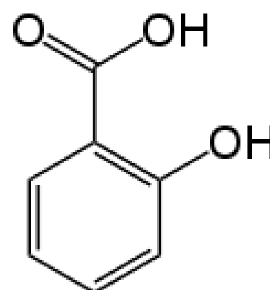
This “drug-like” nature of natural products has been demonstrated both by statistical analyses (Principe, 1985; Newman *et al*., 2003) and by various analyses of the importance of natural products as pharmaceuticals. A comparison between natural products and drugs, and shows that there is more similarity between natural products and drugs in several areas (LogP, number of chiral centres, number of nitrogen atoms, number of oxygen atoms, percent of aromatic rings) than between synthetic compounds and drugs. (Principe, 1985)

The treatment of disease with pure pharmaceutical agent is a relatively modern phenomenon. Among the earliest pure compounds discovered was salicin **(I)** isolated from the bark of the white willow, *Salix alba*, in 1825-26. It was subsequently converted to salicylic acid **(II)** via hydrolysis and oxidation. The use of salicylic acid, however often led to severe gastro intestinal toxicity. This was overcome when Felix Hoffmann of Bayer Company converted

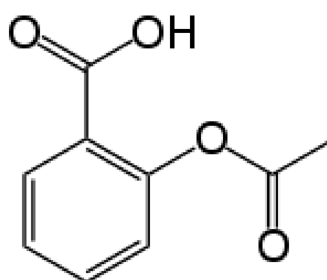
salicylic acid into acetylsalicylic acid (ASA) **(III)** via acetylation. Bayer then began marketing ASA under the trade name aspirin in 1899. Today, aspirin is still the most widely used analgesic and antipyretic drug in the world (Newman *et al.*, 2003).



Salicin **(I)**

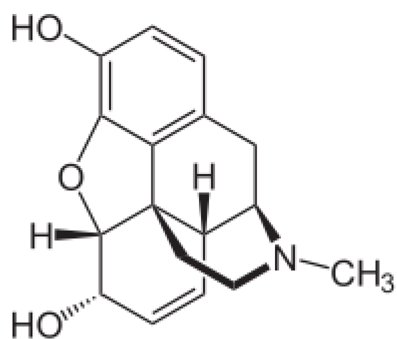


Salicyclic acid **(II)**

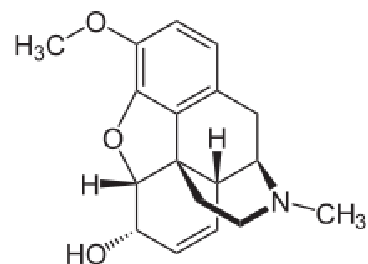


Acetylsalicyclic acid **(III)**

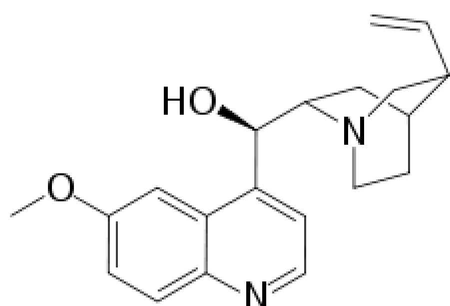
Plants-derived products have always been a rich source of lead compounds for potent pain killers such as morphine **(IV)** and codeine **(V)**, isolated from the *Opium poppy* (Newman *et al.*, 2003). The antimalarial agent quinine **(VI)** was isolated from cinchona bark, and based on the structure of quinine, many classes of antimalarial agents were synthesized including the 4-aminoquinoline (chloroquine) **(VII)** and 8-aminoquinoline (primaquine) **(VIII)** (Newman *et al.*, 2003).



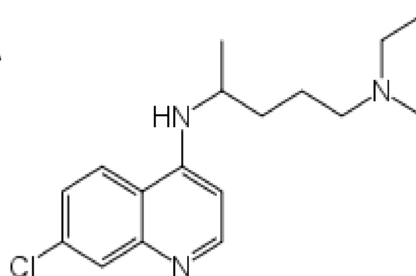
Morphine **(IV)**



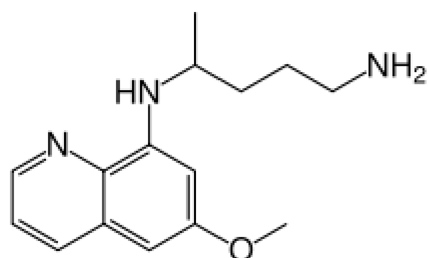
Codeine **(V)**



Quinine **(VI)**

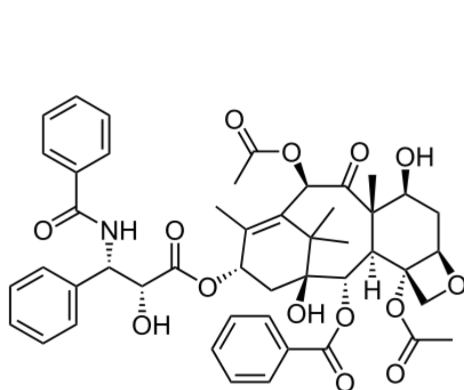


Chloroquine **(VII)**

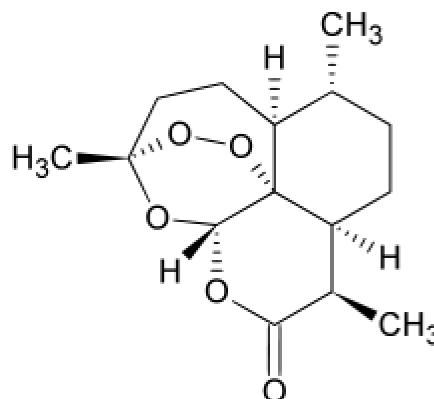


Primaquine **(VIII)**

Clinically useful drugs which have been recently isolated from plants include the anticancer agent paclitaxel (Taxol) **(IX)**, a natural product derived from the pacific yew tree (Shu, 1998) and the antimalarial agent artemisinin **(X)** from *Artemisia annua*.

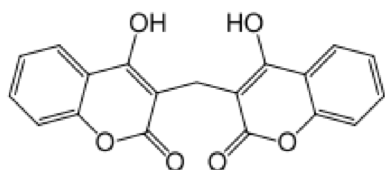


Taxol **(IX)**

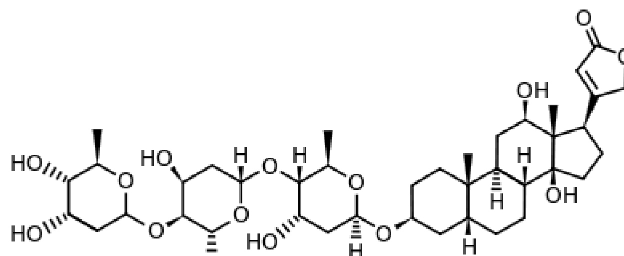


Artemisinin **(X)**

Natural products have been and still are an inexhaustible source of drug leads as well as drugs. Compounds isolated from natural products can be used directly as drugs e.g. the anticoagulant, dicoumarol **(XI)**, isolated from the sweet clover hay, *Melilotus officinalis*. Nature still supplies the best drug for the treatment of heart failure e.g. Digoxin **(XII)**, as till date there is no synthetic drug capable of replacing it in heart failure (Newman *et al*., 2003).

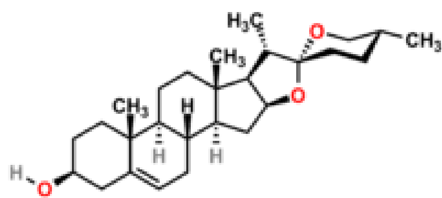


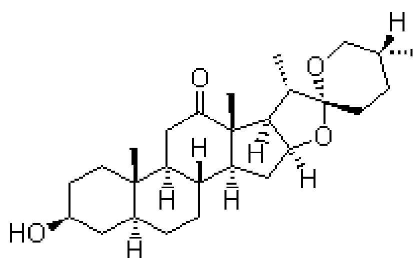
Dicoumarol **(XI)**



Digoxin **(XII)**

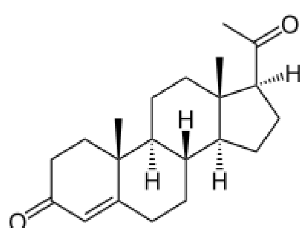
Drugs can also be semi synthesized from natural products leads e.g. the plants steroids diosgenin **(XIII)** and hecogenin **(XIV)** remain key precursors for the synthesis of many modern contraceptive drugs such as progesterone **(XV)** and its derivatives norethindrone **(XVI)**.

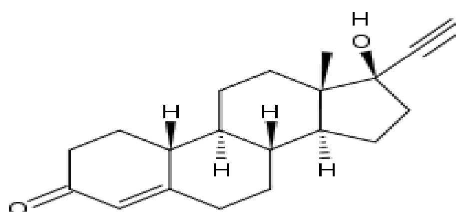




Diosgenin **(XIII)**

Hecogenin **(XIV)**





Progesterone **(XV)**

Norethindrone **(XVI)**

Natural products were significant from the perspective of a major pharmaceutical company (Principe, 1985), and are still significant for some companies. Thus, Butler, writing from the perspective of a small pharmaceutical company that is heavily invested in natural products, states "Another misconception has been that natural product research has failed to deliver many new compounds that have undergone clinical evaluation over the last few years. However, in reality, 15 natural product derived drugs have been launched in the key markets of the United States, Europe, and Japan over the last three years." (Principe, 1985).

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

environments for a long time (Tribuddharat and Fennewald, 1999). Likewise the plant kingdom continues to provide new and important leads against various pharmacological targets (Balunas and Kinghorn, 2005). Several drugs have been derived from medicinal plants. These include quinine from cinchona tree, codeine and morphine from *Papaver somniferum* and artemether, artemisinin, an antimalarial agent isolated from *Artemisia annua* (Chin *et al*., 2006). Owing to the plant kingdom's enormous chemical diversity, it can be looked at as an important source of new TB agents (Gautam, *et al*., 2007). A number of anti-mycobacterial compounds have been isolated from plants used in traditional medicine though they have not so far yielded comparable potency to those from microorganisms (Newton *et al*., 2000). According to Gautam *et al*., (2007), 70% of 365 plant species in India have shown anti-mycobacterial activity and chemical derivatives like Indicanine B and C, have been isolated from *Erythrina variegate* and *E. indica* (Waffo, *et al*., 2000).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Materials

3.1.1 Reagents

The solvents used were all of analytical grade and were all distilled before use. The solvents were of the BDH brand. They are Hexane, Ethyl acetate and Methanol.

3.1.2 Equipment

(i) NMR spectra were obtained on a Bruker AVANCE spectrometer (400 MHz for ^{1}H and 100 MHz for ^{13}C), using the TMS peaks as reference

(ii). FTIR Spectroscopy (Shimadzu FTIR-8400S Fourier Transform Infra-Red Spectrophotometry).

(iii). the melting point of the compound was determined using Ernst Leitz Wetzlar micro melting point apparatus.

3.1.3 Collection and identification of plant materials

The root part of *Guiera senegalensis,* whole plant of *Scoparia dulcis* and whole plant of *Pulicaria crispa* were collected at Samaru, Basawa and Kufena regions of Zaria, Kaduna state, Nigeria respectively. The plants were identified at the Herbarium of Department of Biological Sciences, Faculty of Science, Ahmadu Bello University, Zaria, Kaduna State, Nigeria. With voucher specimen numbers of 4446, 1064 and 2583 respectively. The plant material in each case was air-dried, powdered with the use of a mortar and pestle and then kept in an airtight container until required for further laboratory analysis.

3.2 Methods

3.2.1 Extraction

The pulverized plant materials (1.0 kg) each were exhaustively extracted using maceration method (Harborne, 1984). Starting with n-Hexane, ethyl acetate and methanol in batches. The extracts were concentrated *in vacuo* at 40˚C using a rotatory evaporator and later air-dried to give dried crude extracts. The extracts were weighed and their weights recorded.

3.2.2 Phytochemical screening

Phytochemical screening was carried out on the hexane, ethyl acetate and methanolic extracts for the qualitative determination of major chemical constituents such as alkaloids, glycosides, flavonoids, phenol, tannins, saponin glycosides and volatile oils etc. using methods previously described (Harborne, 1984; Sofowora, 1993; Evans 2009).

3.2.2.1 Test for carbohydrates

Molisch's test

The extracts (1.0 g respectively) were dissolved in about 5 ml of distilled water and heated in a water bath. The solution was filtered. To the filtrate, four drops of Molisch's reagent was added. Concentrated sulphuric acid (3 ml) was carefully added to the mixture from the side of the test tube to form a lower layer. A purple colour appeared at the interface on all the extracts.

Fehling’s test

Fehling’s solution A (aqueous solution of $CuSO_4$) 1.0 ml and 1.0 ml of Fehling solution B (potassium tartrate), were added to 2.0 ml of sugar solution mixed and boiled. A red precipitate was formed which indicated the presence of carbohydrates.

3.2.2.2 Test for tannins

Ferric chloride test

A small quantity of the extract was boiled with water and filtered. Two drops of ferric chloride was added to the filtrate. Formation of a blue-black, or dark green precipitate was taken as evidence for the presence of tannins (Evans, 2009).

Lead sub-acetate test

To a small quantity of the extract, three drops of lead sub-acetate was added. The solution was observed for the presence of green precipitate (Evans, 2009).

3.2.2.3 Tests for flavonoids

Shinoda Test

A small quantity of the extract was dissolved in methanol. Some pieces of magnesium chips were added followed by five drops of concentrated hydrochloric acid. It was observed for the appearance of pink, orange or red to purple colour which indicates the presence of Flavonoids (Evans, 2009).

Sodium hydroxide test

The extract (2.0 ml) was dissolved in 10% aqueous sodium hydroxide solution. The solution was observed for the presence of a yellow colour, a change in colour from yellow to colourless on addition of dilute hydrochloric acid indicates the presence of flavonoids (Evans, 2009).

Ferric chloride test

A drop of ferric chloride was added to a small quantity of the extract. The solution was observed for the formation of a green precipitate (Evans, 2009).

3.2.2.4 Test for cardiac glycoside

Kella-Killani test

The extracts (0.5g) were respectively dissolved in 5 ml glacial acetic acid containing traces of ferric chloride in a test tube was held at an angle of 45° and 1 ml of concentrated sulphuric acid was added carefully down the side, a purple ring colour indicates its presences.

3.2.2.5 Test for steroids and triterpenes

Leiberman-Burchard test

A small portion of the extract was dissolved in chloroform. Equal volume of acetic anhydride was added, and concentrated sulphuric acid was added down the test tube. The solution was observed for the presence of a brown ring at interphase which indicates its presence (Evans, 2009).

Salkowski test

A small quantity of the extract was dissolved in 1.0 ml chloroform and to it 1.0 ml of concentrated sulphuric acid was added down the test tube. Formation of red or yellow colouration was taken as an indication for the presence of sterols (Sofowora, 1993).

3.2.2.6 Test for alkaloids

The extract 0.5 g was stirred with 5 ml of 1% aqueous hydrochloric acid on a water bath and filtered. 3.0 ml of the filtrate was divided into three. To the first 1.0 ml few drops of freshly prepared Dragendoff reagent was added and observed for formation of orange to brownish precipitate. To the second, one drop of Meyer reagent was added observed for formation of white to yellowish or cream colour precipitate. To the third, 1.0 ml of Wagner reagent was added to give a brown or reddish or reddish-brown precipitate (Evans, 2009).

3.2.2.7 Test for Saponins

Test solution 2.0 ml in a test tube was shaken vigorously for few minutes. Frothing which persists on warming was taken as an evidence of the presence of saponins.

3.2.2.8 Test for Glycosides

H_2SO_4 of 5.0 ml was added to each of the test extracts in a separate tubes. The mixture was heated in boiling water for 15minutes. Fehling solutions were then added and the resulting mixture was heated to boiling. A brick red precipitate indicates the presence of glycosides.

3.2.2.9 Test for Phlobatannins

Aqueous extract 10.0 ml of each plant sample was boiled with 1% HCl acid in a test tube or conical flask, a deposition of a red precipitate indicates the presence of phlobatannins.

3.2.2.10 Test for phenols

The extract was first extracted with ethyl acetate and then filtered with Whatman filter paper No. 1. The development of blue-black or brown colouration on the addition of ferric chloride reagent to the filtrate indicates the presence of phenols.

3.2.2.11 Test for terpenoid

The extracts 0.5g each was added 2.0 ml of chloroform. Concentrated H_2SO_4 (1 ml) was carefully added to form a layer. A reddish brown colouration of the interface indicates the presence of terpenoids.

3.2.2.12 Test for resins

Copper acetate solution (5 ml) was added to 5ml of the methanolic extract. The resulting solution was shaken vigorously and allowed to separate. A green coloured solution is an evidence of the presence of resins.

3.2.3 Antimicrobial Screening

Antimicrobial activities of the hexane, ethyl acetate and methanolic extracts were determined using some selected pathogens; Gram positive bacteria (*Staphylococcus aureus, Streptococus pyogenes, and Vancomycin resistant Enterococci*), Gram negative bacteria (*Escherichia coli, Pseudomonas aeruginosa, Salmonella typhi,* and *Shigella dysenteriae*), fungi (*Candida albicans, Candida krusei, Candida stellatoidea,* and *Candida tropicalis*).

3.2.3.1 Preparation of microbial culture

All the media were purchased from Oxoid, England and were prepared in accordance with manufacturer instructions. The bacterial isolates were collected from the Medical Microbiology Department, Specialist Teaching Hospital, and University of Abuja Federal Capital Territory Nigeria on a slant Nutrient agar. The isolates were restored on Nutrient broth and confirmed using standard biochemical tests procedure (Cheesbrough, 2002). While the collected fungal isolates were identified using fungi chrome test kits in Specialist Teaching Hospital, University of Abuja, Federal Capital Territory Nigeria. The isolates were collected on a Potato dextrose agar slant and restored in Potato dextrose broth. Agar diffusion method was adopted.

3.2.3.2 Antimicrobial screening test

The crude extracts were screened for antimicrobial activity using agar in-well diffusion technique with little modification. Clinical isolates were tested to determine the antibacterial activity of the ethyl acetate, methanol and n-hexane extracts. The media Mueller Hinton agar (Sigma Aldrich) was prepared based on the manufacture's instruction. The agar plates were sterilized at 37°C. The sterilized Mueller Hinton agar plates were inoculated with the test culture by surface spreading using sterile cotton bud and each bacterium evenly spread on the entire surface of the plate to obtain uniformity of the inoculum. The wells were made in each

culture plates using a sterile 6 mm cork borer. Ciproflaxin and Fluconazole at 30 µg/ml respectively were used as control references. Approximately, 0.1 ml of the crude extract (50, 25, 12.5, 6.25 3.12 µg/ml) respectively was dispensed in each well and incubated for 24 h at 37°C for the bacteria and 48 h at 37°C for fungi . The plates were examined for the presence of bacterial inhibition zones around each well. The zones of inhibition were measured using a rule and the results were reported in millimetres (mm).

3.2.3.3 Determination of minimum inhibitory concentrations (MIC) of the extracts

The minimum inhibitory concentration (MIC) was determined on the test organisms that were sensitive to the extracts and was done by broth dilution method (Mann *et al.,* 2007). Mueller Hinton broth was prepared, dispersed into test tubes and the broth was sterilized at 121°C for 15 min, the broth was allowed to cool. Normal saline was prepared, 10 ml was dispersed into sterile test tube and the test microbes were inoculated and incubated at 39°C for 6 hrs. Dilution of the test microbes was done in the normal saline until the turbidity marched that of the McFarland's standard scale by visual comparison at this point, this test microbes has a concentration of about 1.5 x 10^{8} CFU/ml.

Two fold serial dilution of the extract in sterilized broth was made to obtain the concentration of 50 µg/ml, 25 µg/ml, 12.5 µg/ml, 6.25 µg/ml and 3.2 µg/ml. The initial concentration was obtained by dissolving 5.0 mg of the extract in 10 ml of sterile broth. Having obtained the different concentration of the extracts in the sterile broth, was observed for turbidity (growth), the lowest concentration of the extract in the broth which showed no turbidity was recorded as the MIC

3.2.3.4 Minimum Bactericidal Concentration/ Minimum Fungicidal Concentration MBC/MFC

Method was carried out to determine whether the test microbes were bactericidal or only their growth was inhibited (bacteriostatic), Mueller Hinton was prepared following the manufacturer's instructions by sterilizing at 121°C for 12 min poured into sterile petri-dishes was allowed to cool and solidify. The content of the MIC in the serial dilution were then sub-cultured into the prepared medium, incubation was made at 37°C for 24 h (bacteria) and 37°C for 48 h (fungi) after which the plates of the medium were observed for colony growth, the MBC/MFC were the plates with lowest concentration of the extract without colony growth.

3.2.4. Anti-Mycobacteria Screening

3.2.4.1 Microplate Alamar Blue Assay (MABA)

Stock solutions of the individual plant extracts were prepared in 0.05% DMSO (Dimethyl sulphoxide) diluted to the final concentration of 20 and 40 µg/ml in sterile distilled water as part of experimental standardization.

The sensitivity of *Mycobacterium tuberculosis* strain to the various extracts was demonstrated by agar diffusion method (Chaturvedi *et al*., 2007). A sterile cork borer of 7 mm diameter was used to bore holes into the inoculums seeded solidified nutrient agar. A 50 µl volume of each 20 and 40 µg/ml of the plant extracts was loaded into the labelled well in the prepared media plate using sterile pipette. The test was performed in triplicates and the plates were kept in a refrigerator for pre-diffusion of the sample and incubated at 37°C and 48h. Growth of *Mycobacterium tuberculosis* was observed after the incubation of 48 h and the diameter of inhibition zone was measured subtracting the well size.

3.2.5 Chromatographic Purification of Extracts

3.2.5.1 Thin layer chromatography (TLC)

Purification was done based on the extract with the most anti-TB activity. This test was carried out using pre-coated silica gel analytical TLC plates prepared by MERCK. The extracts were dissolved in minimum volume of their respective extracting solvent. The extracts were spotted on TLC using capillary tube, hence plates were allowed to dry. The plates were developed in a development tank and were visualized under UV light (254-366 nm) and spraying with 10 % sulphuric acid, followed by heating at 110°C for 5-10 mi**n.**

3.2.5.2. Purification and Isolation of compounds

A small portion of the methanol fraction of cold extraction was dissolved in methanol and the solution was spotted on TLC plates. Then the TLC plates were run by solvent systems ranging from non-polar hexane to polar ethyl acetate and were viewed individually under UV light. Through several pilot experiments, it was found that the constituent of methanol fraction were separated by the solvent system of hexane and ethyl acetate in the proportion of 3:7. The methanol fraction, 10 g, was packed on a column with silica gel (60-120 mesh) as stationary phase with gradient elution using n-hexane: ethyl acetate 1:9 and finally with 100 % methanol. Two fractions were found homogeneous on TLC plate by using n-hexane: ethyl acetate (1:9), n-hexane: ethyl acetate (3:7) solvent systems.

3.2.5.6. Preparative thin layer chromatography

The purified sample from the above column was subjected to preparative TLC. The different layers were scraped out, dissolved in chloroform, filtered and recovered using rotary evaporator. The result gave rise to a component coded Dy1, the second isolate with 1:9

Hexane: Ethyl acetate solvent system was named Dy2, hence both isolates were weighed and recorded.

3.2.6. Spectral Analysis

3.2.6.1. UV- Visible Spectrometer

The UV-Visible spectroscopic analyses was carried out on UV 752 PEC medical USA and the scanning wavelength was set between 200-600 nm at the Department of Chemistry, ABU, Zaria.

3.2.6.2. Infra-Red (IR)

The Infra-Red (IR) spectroscopic determination was carried out using Shimadzu FTIR-8400S Fourier Transform Infra-Red Spectroscopy at the Department of Chemistry, ABU, Zaria.

3.2.6.3. Nuclear Magnetic Resonance (NMR)

The ^{1}H NMR and ^{13}C NMR spectroscopic analysis were obtained and was carried out on Brucker AVANCE FT-NMR (600 MHz) at the school of Chemistry and Physics, University of Kwazulu-Natal, Durban, South Africa. Chemical shift values were reported in parts per million (ppm) relative to TMS standard and coupling constant are given in Hz. The NMR solvent used for these measurements is deuterated methanol.

CHAPTER FOUR

4.0 RESULTS

4.1 Result of Extraction of *Guiera senegalensis* (J.F. GMEL), *Scoporia dulcis* (*LINN*) and *Pulicaria crispa* (FORSSK)

Table 4.1 Extraction of Plant Extracts

	Weight of Extracts (g)			**Recovery yield (%)**		
Solvent	***Guiera senegalensis***	***Scoporia dulcis***	***Pulicaria crispa***	***Guiera senegalensis***	***Scoporia dulcis***	***Pulicaria crispa***
n-Hexane	15.00	16.00	22.00	1.50	1.60	2.20
Ethyl acetate	23.60	24.00	28.60	2.36	2.40	2.86
Methanol	56.00	25.70	31.00	5.60	2.57	3.10

4.2 Result of phytochemical screening

The results of the phytochemical screening are presented in Tables 4.2 below: Which show the phytochemical screening of all hexane fractions, ethyl acetate fraction of *Scoparia dulcis* and methanolic fraction of *Guiera senegalensis* to have the presence of saponin. Cardenolides, phlobatanins, volatile oils and resins were absent in all the three solvent extracts. Flavoniods was present in only the ethyl acetate extract and methanolic extract of *Pulicaria crispa*. Both the ethyl acetate and methanol extract were found to show high percentage of triterpeniods in extract of *Guiera senegalensis*. Cardiac glycosides, glycosides, balsams and phenols were only present in the methanolic extract of *Scoparia dulcis.*

The extracts of *Pulicaria crispa* was found to show a high percentage of terpeniods and moderate presence of balsams in the methanolic extract only, while tannin was absent in the n-hexane extract (Table 4.2). Steroids were found in the ethyl acetate extract, flavonoids, cardiac glycosides and glycosides were also found in the methanolic extract, hence confirming their solubility in relatively more polar solvents. Alkaloid was found to be present in the ethyl acetate extract and present in the methanolic and hexane extracts.

Table 4.2 gives the details of phytochemical screening of *Guiera senegalensis* root extract, the extracts contained some phytoconstituents. Trace amounts of alkaloids, terpeniods, tannin and flavonoids were found in the ethyl acetate extract. Steroids, saponins, cardenolides, terpeniods and phlobatanins were also found in trace amounts in the methanol extract as shown in Table 4.2. A very strong positive presence of tannins and alkaloid were observed in the methanolic extract, while the ethyl acetate extract showed positive result for steroids. Cardenolides and phlobatanins are very polar compounds and hence were only found in the in methanol extract (polar solvent), while flavonoids which are considered as compounds of intermediate polarity were found in the ethyl acetate.

4.3 Result of Antimicrobial Screening of Extract

The anti-microbial screening of *Guiera senegalensis*, *Scoparia dulcis* and *Pulicaria crispa*, and their zones of inhibition are presented in Table 4.3 and Table 4.4 below. The n-hexane, ethyl acetate and methanol extracts of the plants were found to inhibit the growth of the test microbes; *Methicillin resistant Staphyloccocus aureus, Vancomycin resistant Enterococci, Shigella dysenteriae, Salmonella typhi, Escherichia coli, Proteus mirabilis, Candida albicans, Candida krusei and Candida tropicalis*, and in Table 4.5 below, their MIC values were found to be within the range of 3.12-12.5 μg/ml, while in Table 4.6 below, the MBC/MFC values were found in the range of 12.5-25 μg/ml. The extracts of Pulicaria *crispa* were found to inhibit some microbes in addition to those inhibited by *Scoparia dulcis* extracts. These includes: *Staphylococcus aureus, Streptococus pyogenes, Neisseria gonorrhea, Pseudomonas aeruginosa*.

Table 4.2: Phytochemical screening of the hexane, ethyl acetate and methanol fractions of G. *senegalensis, Scoporia dulcis* and *Pulicaria crispa*

Phytoconstituents	N-hexane extract			Ethyl acetate extract			Methanolic extract		
	Guiera senegalensis (J.F. GMEL)	*Scoporia dulcis (LINN)*	*Pulicaria crispa (Forssk)*	*Guiera senegalensis (J.F. GMEL)*	*Scoporia dulcis (LINN)*	*Pulicaria crispa (Forssk)*	*Guiera senegalensis (J.F. GMEL)*	*Scoporia dulcis (LINN)*	*Pulicaria crispa (Forssk)*
Steroid	–	-	-	++	+	+	+	+	-
Triterpenoid	–	-	-	++	-	-	++	-	-
Glycoside	–	-	-	–	-	-	–	+	+
Saponins	+	+	+	–	++	-	+	-	-
Phenols	–	-	-	–	-	-	–	+	++
Alkaloid	–	-	-	+	+	++	+++	+	+
Cardenolides	–	-	-	–	-	-	+	-	-
Terpeniods	–	-	-	+	+	-	+	-	+++
Cardiac glycoside	–	-	-	–	-	-	–	+	+
phlobatanin	–	-	-	–	-	-	+	-	-
Resins	–	-	-	–	-	-	–	-	-
Balsam	–	-	-	–	-	-	–	+	++
Volatile oil	–	-	-	–	-	-	–	-	-
Tannin	+	+	-	+	-	+	+++	+	+
Flavonoid	–	-	-	+	+++	-	–	-	+

KEY: (+++) Very strong positive, (++) positive, (+) Trace, (-) Negative

Table 4.3: RESULTS OF THE ANTIMICROBIAL SCREENING

S/No.	Test microbes	GSN	GSE	GSM	SDN	SDE	SDM	PCN	PCE	PCM	Ciproflaxin	Fluconazole
1	Methicillin Resistant *Staphylococcus aureus*	S	S	S	S	S	S	S	S	S	S	R
2	Vancomycin Resistant *Enterococci*	R	R	R	S	S	S	S	S	S	R	R
3	*Staphylococcus aureus*	S	S	S	R	R	R	S	S	S	S	R
4	*Corynebacterium ulcerans*	R	R	R	R	R	R	R	R	R	S	R
5	*Shigella dysenteriae*	S	S	S	S	S	S	R	R	R	S	R
6	*Salmonella typhi*	R	R	R	S	S	S	R	R	R	S	R
7	*Streptococus pyogenes*	R	R	R	R	R	R	S	S	S	S	R
8	*Neisseria gonorrhea*	S	S	S	R	R	R	S	S	S	R	R
9	*Escherichia coli*	S	S	S	S	S	S	R	R	R	S	R
10	*Pseudomonas aeruginosa*	R	R	R	S	S	S	S	S	S	R	R
11	*Proteus mirabilis*	S	S	S	S	S	S	S	S	S	S	R
12	*Candida albicans*	S	S	S	S	S	S	S	S	S	R	S
13	*Candida krusei*	S	S	S	S	S	S	S	S	S	R	S
14	*Candida stellatoidea*	R	R	R	R	R	R	S	S	S	R	S
15	*Candida tropicalis*	S	S	S	S	S	S	S	S	S	R	S

GSN - *Guiera senegalensis* n-Hexane extract
GSE - *Guiera senegalensis* Ethyl acetate extract
GSM - *Guiera senegalensis* methanol extract
*SD*N - *Scoparia dulcis* n-Hexane extract
SDE - *Scoparia dulcis* Ethyl acetate extract
SDM - *Scoparia dulcis*methanol extract
PCN - *Pulicaria crispa* n-Hexane extract
PCE - *Pulicaria crispa* Ethyl acetate extract
PCM - *Pulicaria crispa* methanol extract
R - Resistant
S - Sensitive

Table 4.4: RESULTS OF THE ANTIMICROBIAL SCREENING
Zone of Inhibition (mm)

S/No.	Test microbes	GSN	GSE	GSM	SDN	SDE	SDM	PCN	PCE	PCM	Ciproflaxin	Fluconazole
1	Methicillin Resistant *Staphylococcus aureus*	22	27	20	16	20	20	29	26	24	35	-
2	Vancomycin Resistant *Enterococci*	-	-	-	31	30	34	29	29	31	-	-
3	*Staphylococcus aureus*	34	35	33	-	-	-	29	32	30	37	-
4	*Corynebacterium ulcerans*	-	-	-	-	-	-	-	-	-	34	-
5	*Shigella dysenteriae*	35	30	32	34	32	30	-	-	-	40	-
6	*Salmonella typhi*	-	-	-	27	26	24	-	-	-	41	-
7	*Streptococus pyogenes*	-	-	-	-	-	-	27	29	24	35	-
8	*Neisseria gonorrhea*	26	29	24	-	-	-	29	30	32	-	-
9	*Escherichia coli*	27	27	25	24	26	28	-	-	-	39	-
10	*Pseudomonas aeruginosa*	-	-	-	26	27	27	32	32	30	-	-
11	*Proteus mirabilis*	27	26	25	32	35	30	31	34	30	35	-
12	*Candida albicans*	35	34	30	30	32	33	26	26	22	-	35
13	*Candida krusei*	30	32	34	22	20	24	22	24	25	-	34
14	*Candida stellatoidea*	-	-	-	-	-	-	16	18	21	-	37
15	*Candida tropicalis*	26	24	22	20	22	20	24	25	27	-	31

GSN - *Guiera senegalensis* n-Hexane extract
GSE - *Guiera senegalensis* Ethyl acetate extract
GSM - *Guiera senegalensis* methanol extract
*SD*N - *Scoparia dulcis* n-Hexane extract
SDE - *Scoparia dulcis* Ethyl acetate extract
SDM - *Scoparia dulcis*methanol extract
PCN - *Pulicaria crispa* n-Hexane extract
PCE - *Pulicaria crispa* Ethyl acetate extract
PCM - *Pulicaria crispa* methanol extract

Table 4.5: Results of Minimum Inhibitory Concentration (MIC) of Plantextracts against test microbes (μg/ml)

	Guiera senegalensis						*Scoparia dulcis*						*Pulicaria crispa*					
	n-H		E.A		Me		n-H		E.A		Me		n-H		E.A		Me	
Test Microbes																		
Methicillin Resistant *Staphylococcus aureus*	-	3.12	-	3.12	-	3.12	-	3.12	-	3.12	-	3.12	-	3.12	-	3.12	-	3.12
Vancomycin Resistant *Enterococci*	-	3.12	-	3.12	-	3.12	-	3.12	-	3.12	-	3.12	12.5	+	12.5	+	12.5	+
Staphylococcus aureus	-	3.12	-	3.12	-	3.12	-	3.12	-	3.12	-	3.12	-	3.12	-	3.12	12.5	+
Corynebacterium ulcerans	-	3.12	-	3.12	-	3.12	-	3.12	-	3.12	-	3.12	-	3.12	-	3.12	12.5	+
Shigella dysenteriae	-	3.12	-	3.12	12.5	+	-	3.12	-	3.12	-	3.12	-	3.12	-	3.12	12.5	+
Salmonella typhi	12.5	+	12.5	+	12.5	+	-	3.12	-	3.12	-	3.12	-	3.12	-	3.12	12.5	+
Streptococus pyogenes	12.5	+	12.5	+	12.5	+	-	3.12	-	3.12	-	3.12	-	3.12	-	3.12	-	3.12
Neisseria gonorrhea	12.5	+	12.5	+	12.5	+	-	3.12	-	3.12	-	3.12	-	3.12	-	3.12	-	3.12
Escherichia coli	-	3.12	-	3.12	-	3.12	-	3.12	-	3.12	-	3.12	12.5	+	12.5	+	12.5	+
Pseudomonas aeruginosa	-	3.12	-	3.12	-	3.12	12.5	+	12.5	+	12.5	+	12.5	+	12.5	+	12.5	+
Proteus mirabilis	-	3.12	-	3.12	-	3.12	12.5	+	12.5	+	12.5	+	-	3.12	-	3.12	-	3.12
Candida albicans	12.5	+	12.5	+	12.5	+	12.5	+	12.5	+	12.5	+	-	3.12	-	3.12	-	3.12
Candida krusei	-	3.12	-	3.12	-	3.12	-	3.12	-	3.12	-	3.12	-	3.12	-	3.12	-	3.12
Candida stellatoidea	12.5	+	12.5	+	12.5	+	12.5	+	12.5	+	12.5	+	12.5	++	12.5	+	12.5	+
Candida tropicalis	12.5	+	12.5	+	12.5	+	12.5	+	12.5	+	12.5	+	-	3.12	-	3.12	-	3.12

KEY: (+) Turbid (Light growth) (++) Moderate turbidity (-) No growth

Table 4.6: Results of Minimum Bactericidal/Fungicidal Concentration (MBC/MBF) of Plant extracts against test microbes (µg/ml)

	Guiera senegalensis						*Scoparia dulcis*						*Pulicaria crispa*					
	n-H		E.A		Me		n-H		E.A		Me		n-H		E.A		Me	
Test Microbes																		
Methicillin Resistant *Staphylococcus aureus*	-	12.5	25	+	-	12.5	-	12.5	-	12.5	-	12.5	-	12.5	-	12.5	-	12.5
Vancomycin Resistant *Enterococci*	-	12.5	25	+	-	12.5	-	12.5	-	12.5	-	12.5	-	12.5	25	+	25	+
Staphylococcus aureus	-	12.5	-	12.5	-	12.5	-	12.5	-	12.5	-	12.5	25	+	-	12.5	25	+
Corynebacteriumulcerans	-	12.5	-	12.5	-	12.5	-	12.5	-	12.5	-	12.5	-	12.5	-	12.5	25	+
Shigella dysenteriae	-	12.5	-	12.5	-	12.5	-	12.5	-	12.5	-	12.5	-	12.5	-	12.5	25	+
Salmonella typhi	25	+	-	12.5	-	12.5	-	12.5	-	12.5	-	12.5	-	12.5	-	12.5	25	+
Streptococus pyogenes	25	+	-	12.5	-	12.5	-	12.5	-	12.5	-	12.5	-	12.5	-	12.5	25	+
Neisseria gonorrhea	25	+	-	12.5	-	12.5	-	12.5	-	12.5	-	12.5	-	12.5	-	12.5	-	12.5
Escherichia coli	-	12.5	25	+	-	12.5	-	12.5	-	12.5	-	12.5	-	12.5	25	+	-	12.5
Pseudomonas aeruginosa	-	12.5	25	+	-	12.5	25	+	25	+	25	+	25	+	25	+	25	+
Proteus mirabilis	-	12.5	25	+	-	12.5	25	+	25	+	25	+	25	+	-	12.5	25	+
Candida albicans	25	+	25	+	25	+	25	+	25	+	25	+	-	12.5	-	12.5	-	12.5
Candida krusei	-	12.5	-	12.5	25	+	-	12.5	-	12.5	-	12.5	-	12.5	-	12.5	-	12.5
Candida stellatoidea	25	+	25	+	25	+	25	+	25	+	25	+	25	+	25	+	25	+
Candida tropicalis	25	+	25	+	-	+	25	+	25	+	25	+	-	12.5	-	12.5	-	12.5

KEY: (+) Turbid (Light growth) (-) No growth

4.4 Result of Anti-mycobateria Tuberculosis Screening of the Extracts

The results of the anti-TB assay from Table 4.7 below, the hexane extract of *Guiera senegalensis*, methanol extract of S*coparia dulcis* and ethyl acetate of *Pulicaria crispa* showed no reaction under experimental conditions. All other extracts showed activity to the mycobacterium strain based on its inhibitory concentrations, which was determined at 20 and 40 µg/ml respectively. High inhibitory concentrations were observed for methanol fractions of *Guiera senegalensis* at 31.24µg/ml and 48.44 µg/ml for hexane fraction of *Pulicaria crispa* which were comparable and higher than the reference drug rifampicin. Moderate activity were observed in methanol extract of *Pulicaria crispa* and ethyl acetate extract of *Scoparia dulcis,* while low activity was observed for the hexane extract of *Scoparia dulcis* and ethyl acetate extract of *Guiera senegalensis*. When compared to reference drug rifampicin, the methanol extract of *Guiera senegalensis* showed MIC of 2.80 µg/ml which is comparable to the MIC observed at 0.38 µg/ml for the standard drug.

Based on these results, the methanol extract of *Guiera senegalensis* was chosen for the purification, isolation and characterization study.

4.5 Physical and Chemical test on Dy1

Dy1 was obtained as a white crystalline solid with melting point range of 280^{0C}, which tested positive to the Liebermann-Buchard for triterpenes.

Table 4.7: Result of Anti-TB Activity of Guiera *Senegalensis, Scoporia dulcis* and *Pulicaria crispa*

Plants used	Solvent extract	Dilutions		MIC(μg/ml)
		20(μg/ml)	40(μg/ml)	
Guiera senegalensis	N-hexane extract	NR	NR	NR
	Ethyl acetate extract	7.23 ± 1.35	12.69 ± 0.5	40.01 ± 1.20
	Methanol extract	22.71± 0.47	31.24 ± 0.52	2.80 ± 1.40
Scoparia dulcis	N-hexane extract	8.62 ± 0.07	12.14 ± 0.02	20.40 ± 0.24
	Ethyl acetate extract	12.56 ± 0.60	17.00 ± 0.91	12.03 ± 0.86
	Methanol extract	NR	NR	NR
Pulicaria crispa	N-hexane extract	15.17 0.42	48.44 ± 0.75	10.03 ±1.33
	Ethyl acetate extract	NR	NR	NR
	Methanol extract	20.86 1.24	24.10 ± 1.35	8.01 ±1.70
	RFP (10μg/ml)	33.70 0.64		0.38 ±1.40

RFP = Rifampicin
NR = No reaction under experimental conditions
The results presented here are the mean values of three independent experiment

4.6 Column chromatography

Table 4.8 Result of Column Chromatography of Methanol Extract of *Guiera senegalensis*

Fraction	Eluting Solvent	No. of collection	No. of clear spots.
F0	Hex ; 100%	1-10	nil
F1	Hex : EtOAc ; 95%-5%	11-26	2
F2	Hex: EtOAc; 97.5%-2.5%	27-36	2
F3	Hex: EtOAc ; 92.5%-7.5%	37-46	3
F4	Hex: EtOAc ; 90%-10%	47-52	2
F5	Hex: EtOAc ; 80%-20%	53-56	3
F6	Hex: EtOAc ; 70%-30%	57-63	4
F7	Hex: EtOAc ; 60%-40%	64-74	4
F8	Hex: EtOAc ; 50%-50%	75-90	3
F9	EtOAc ; 100%	91-96	nil

96 collections,100 ml each was collected and pull together based on TLC profile to give 9 major fractions

4.7 Thin layer chromatography of the methanol extract of *Guiera senegalensis*

Table 4.9: TLC profile of ethyl acetate extract in hexane: ethyl acetate (3:7)

Spot	R_f Value	Colour in 10% H_2SO_4
1	0.82	Red
2	0.74	Purple
3	0.56	Yellow
4	0.17	Brown

Table 4.10: Result of TLC Analysis of Dy1

Solvent system	No. of spot	Colour of spot on heating	Rf
EtOAc: N-Hex ; 70%-30%	1	Brown	0.54

4.8 Spectral Analysis

The structure of Dy1 was determined using ^{1}HNMR Fig 4.1, ^{13}CNMR Fig 4.2, Correlation Spectroscopy (COSY) Fig 4.3, Distortionless Enhancement Polarization Transfer (DEPT) Fig 4.4, Heteronuclear Multiple Bond Correlation (HMBC) Fig 4.5, Heteronuclear Single Quantum Correlation (HSQC) Fig 4.6 and Nuclear Overhauser Enhancement Spectroscopy (NOESY) Fig 4.7.

4.8.1 Result of the ^{1}H NMR spectra of Dy1

The proton NMR spectrum of Dy1 (Fig 4.1) shows th presence methyl group at δ 1.02 and methylene protons at δ 1.83 and δ 0.95. It also showed five methyl singlets at δ 0.82, δ 1.00, δ 1.24, δ 1.27 and one singlet of secondary hydroxyl group at δ 3.45. and the following multiplicities:^{1}H-NMR of Dy1(400 MHz, CD_3OD) (δ-1.02 t, 2H, H-1), (δ-1.82 q, 2H, H-2), (δ-3.45 t, 1H, H-3), (δ-0.82 t, 1H, H-5), (δ-1.29 q, 2H, H-6) (δ-1.55 t, 2H, H-7), (δ-1.50t, 1H, H-9), (δ-1.96 t, 2H, H-11), (δ-5.50 t, 1H, H-12), (δ-1.30 t, 2H, H-15), (δ-2.22 t, 2H, H-16), (δ-3.20 t, 1H, H-18), (δ-1.83 d, 2H, H-19), (δ-1.51 t, 2H, H-21), (δ-1.62 t, 2H, H-22).

4.8.2Result of the^{13}C NMR spectra of Dy1

The ^{13}C NMR spectrum (Fig 4.2) revealed thirty signals ranging from 15.6 ppm to 179.3 ppm, implying that there were thirty carbon atoms in the compound. The signals at 23.7 and

145.6 ppm are typical of olefinic carbons and the signals at 179.6 ppm suggested the presence of a carboxylic acid carbon. These signals are also characteristic of a triterpene (Ghias *et al.,* 2011, Fauziah *et al.,* 2013).

4.8.3. DEPT

The DEPT experiment (Fig 4.4) , revealed the presence of eight quaternary carbons signals at 31.0, 37.4, 39.4, 39.8, 42.2, 46.7, 145.6 and 179.3 ppm, five methine carbon signals at 42.0, 48.2, 58.8, 78.1, and 122.6 ppm, ten methylenes carbon signals at 18.8, 23.7, 23.8, 28.2, 28.4, 33.3, 33.3, 34.3 39.0, and 46.5 ppm while seven methyl carbon signals were seen at 15.6, 16.6, 17.5, 23.8, 26.5, 28.8 and 33.3 ppm respectively. This shows that the compound contains 7 methyls, 10 methylenes, 5 methines and 8 quatenary carbons atoms.

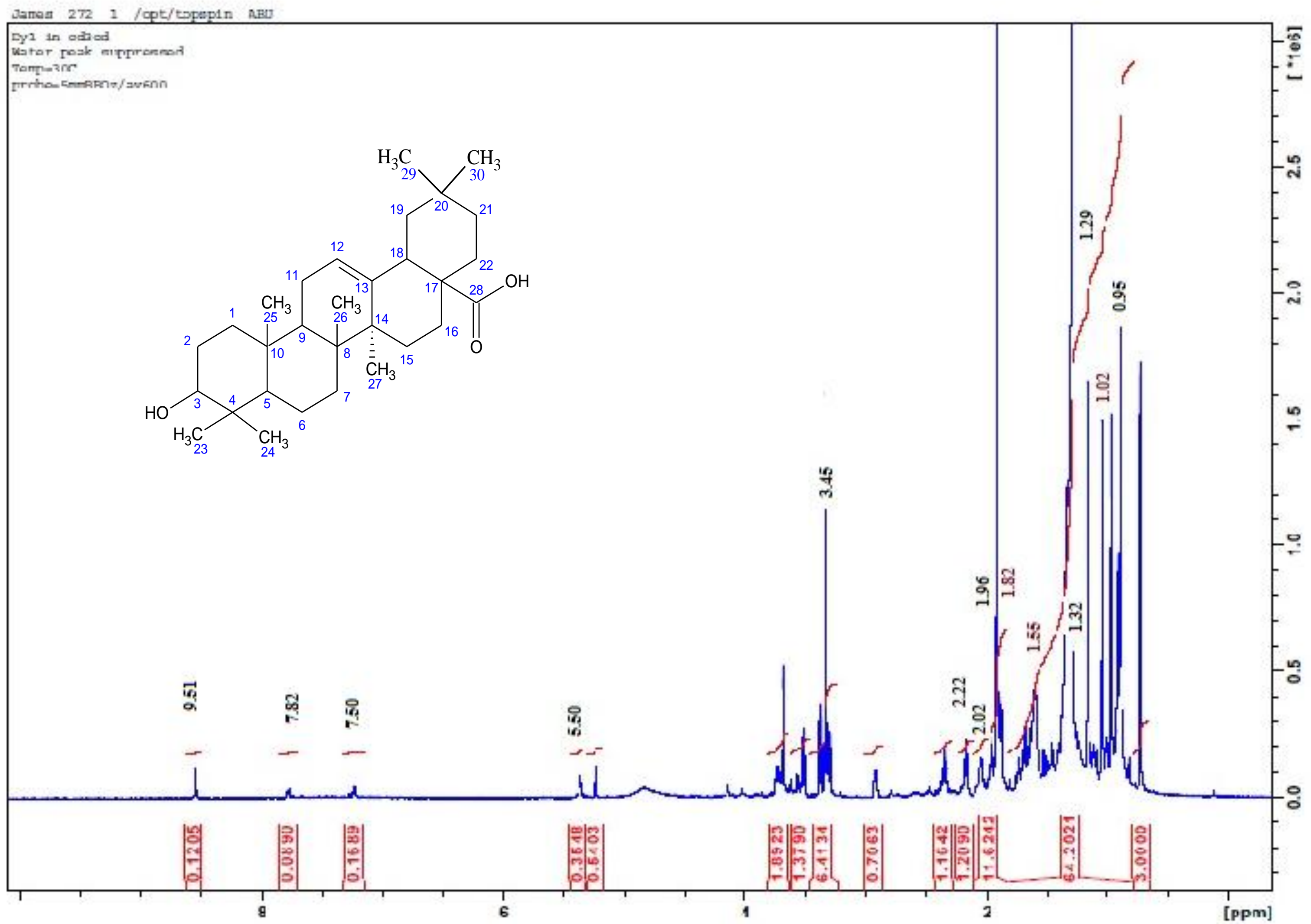


Figure 4.1 ^{1}H NMR Spec trum of DY1

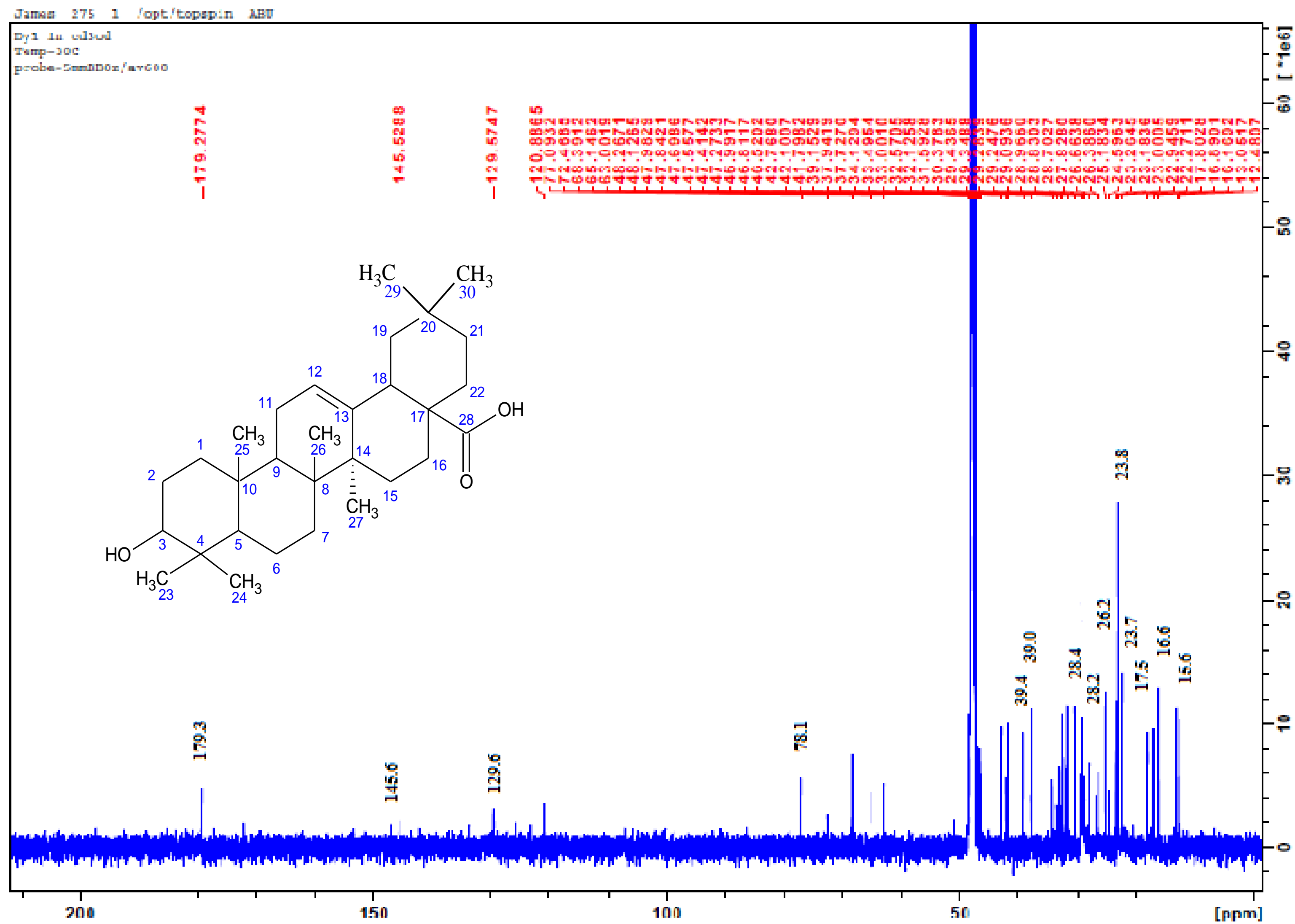


Figure 4.2: ^{13}C NMR Spectrum of DY1

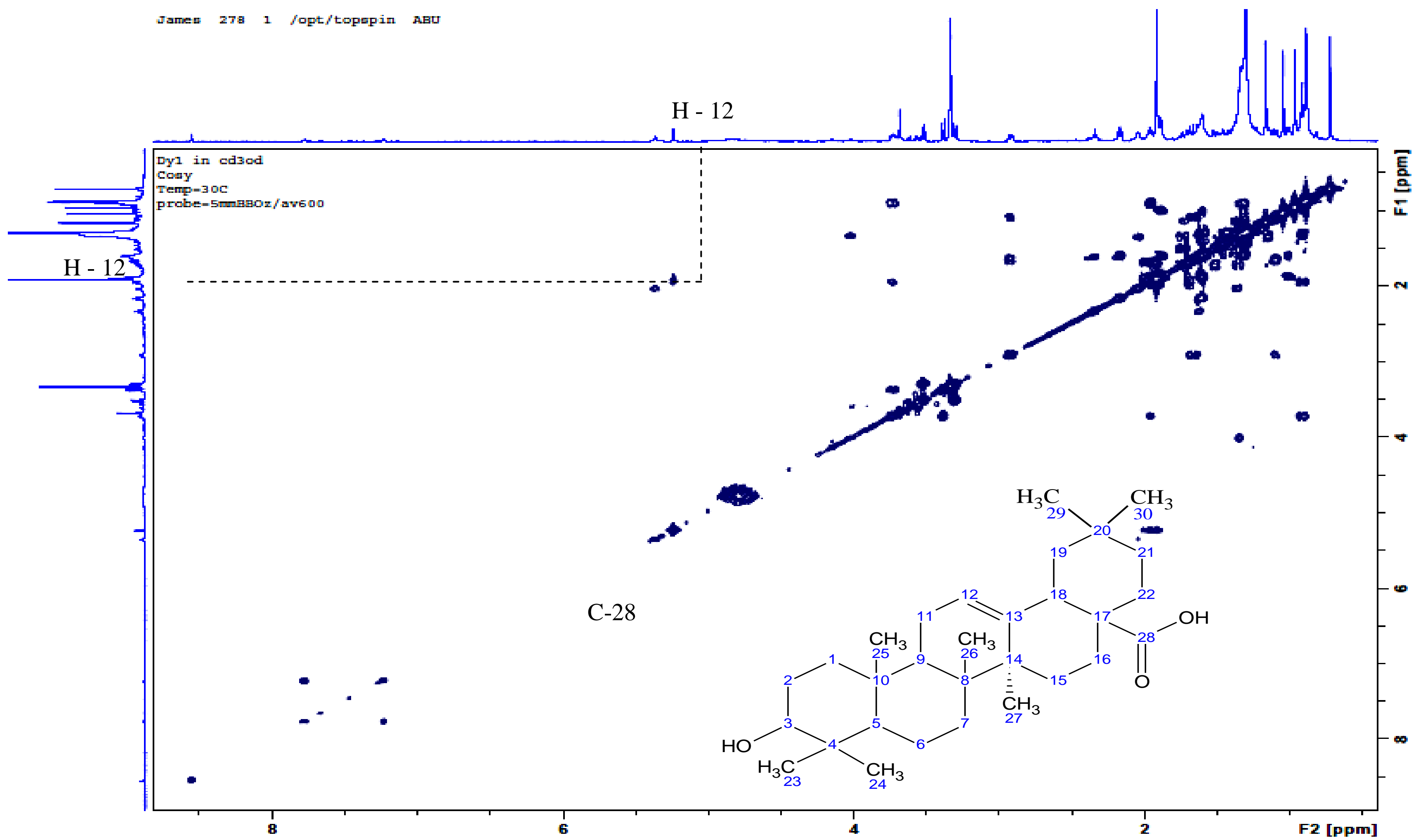


Figure 4.1: COSY Spectrum of DY1

James 275 1 /opt/topspin ABU

Figure 4.2: DEPT Spectrum of DY1

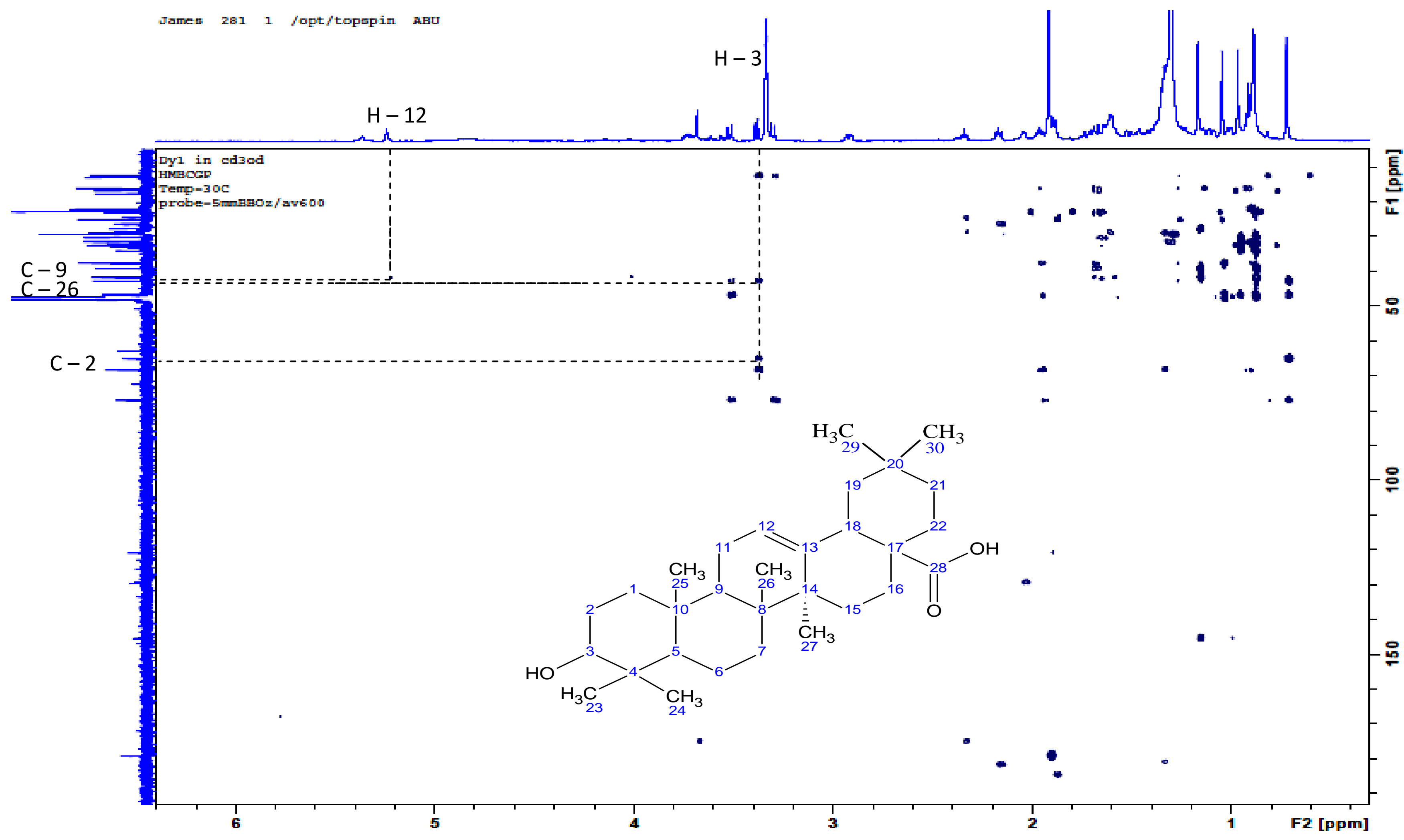


Figure 4.3: **HMBC spectrum of DY1**

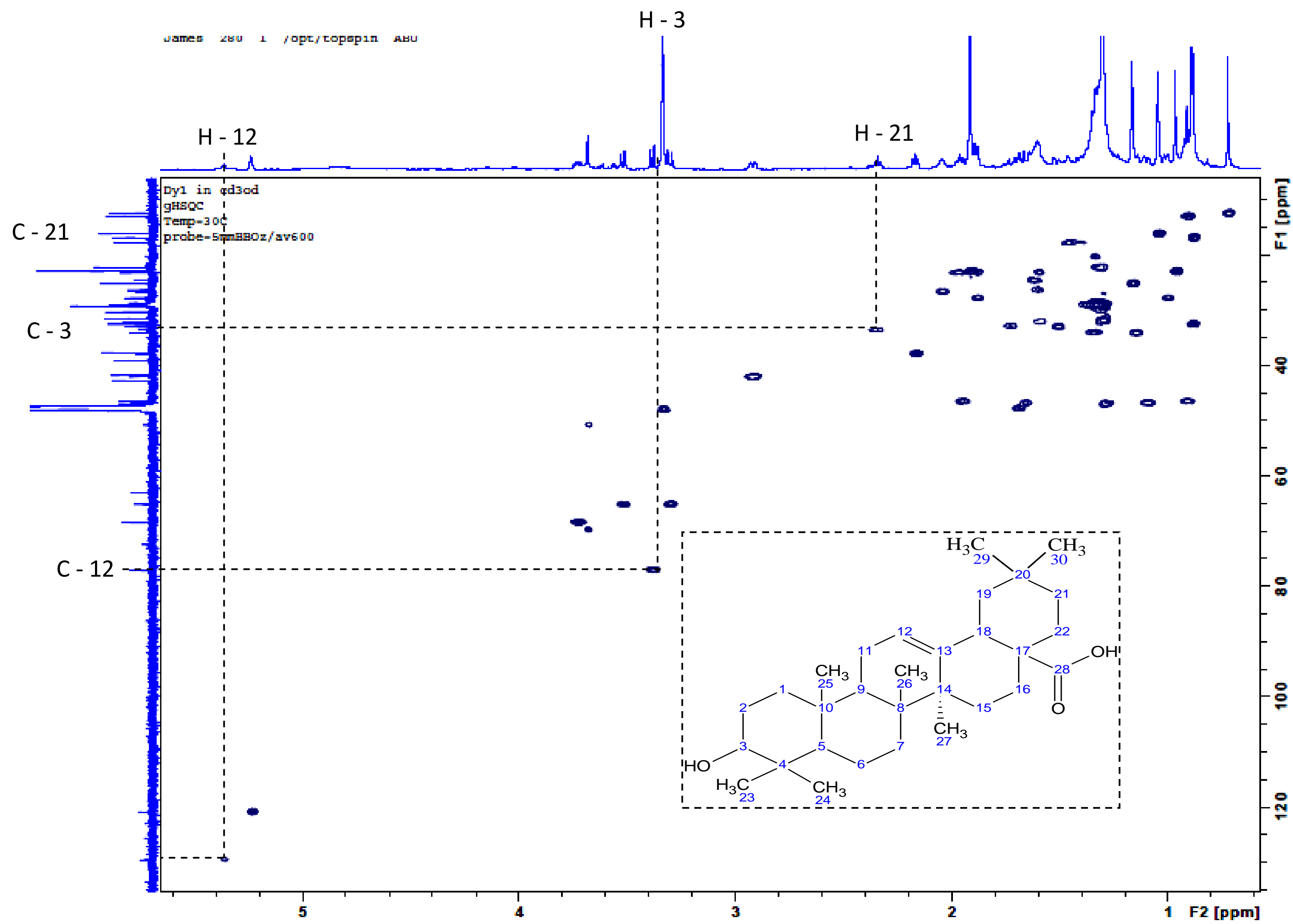


Figure 4.4: **HSQC spectrum of DY1**

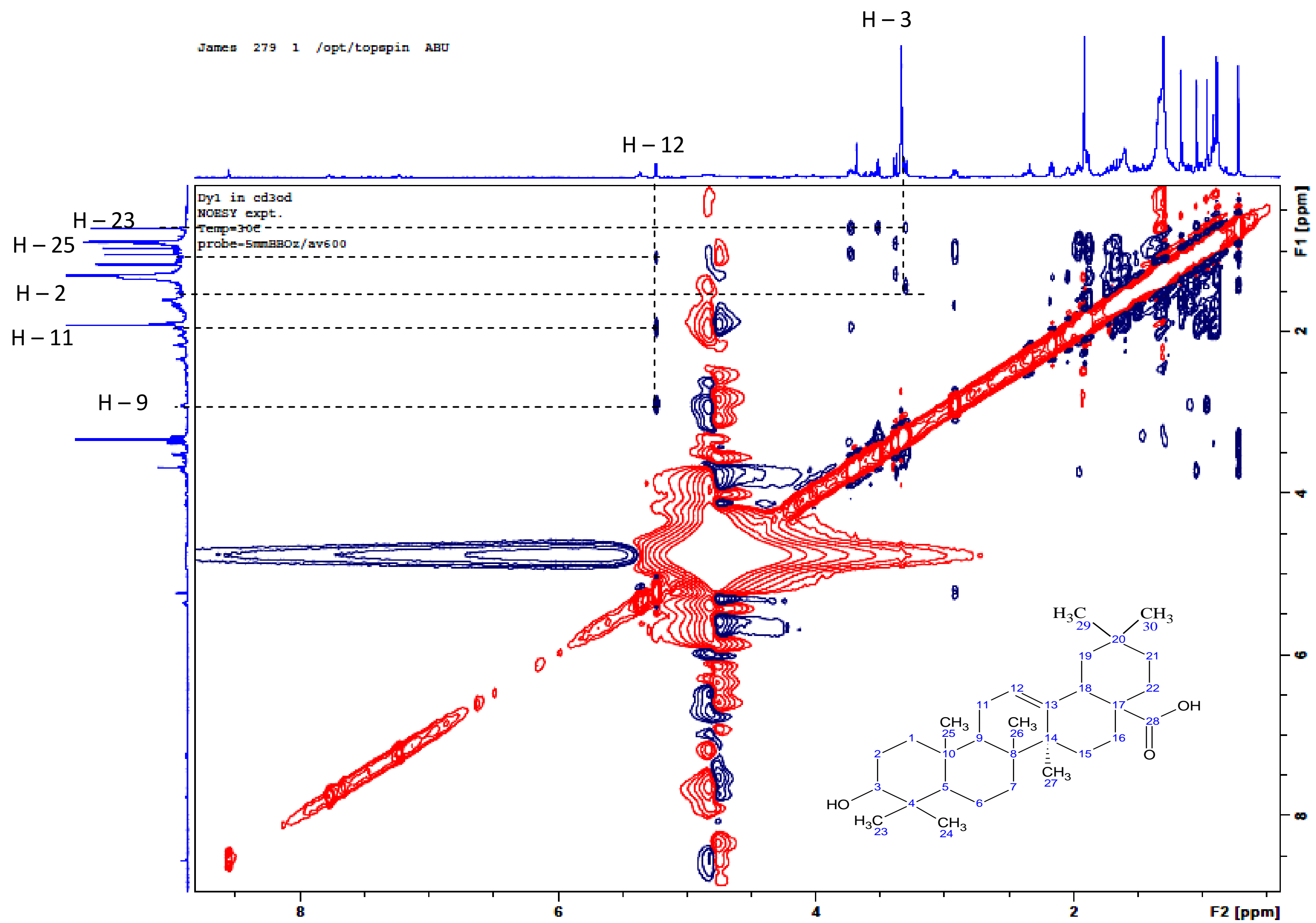


Figure 4.5: **NOESY spectrum of DY1**

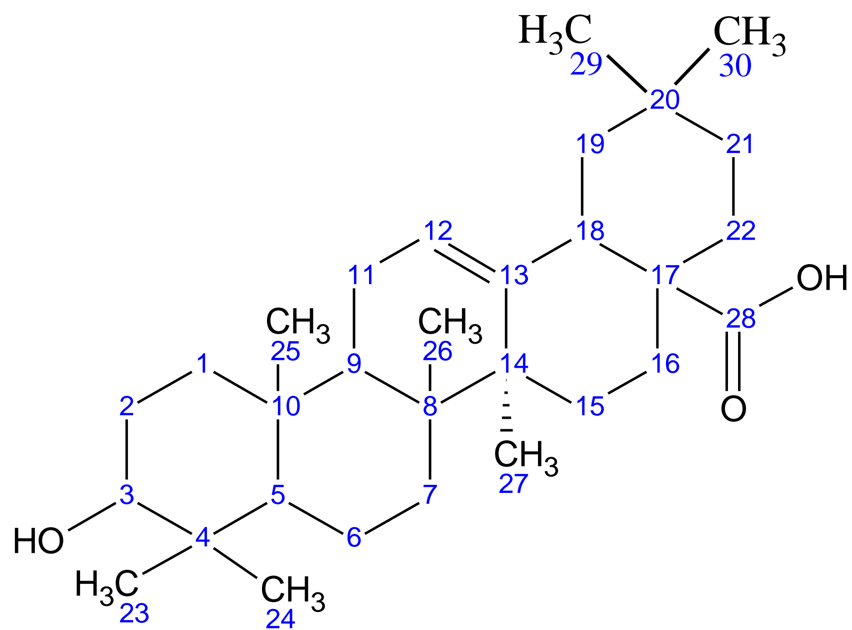


Figure 4.8: **DY1- (Oleanolic acid)**

4.8.4. 2 D Spectrum of Dy1

The **COSY** spectrum of Dy1 shows the correlations between adjacent protons, The heteronuclear multiple bond correlation **HMBC** of Dy1, The homonuclear single quantum correlation spectrum **HSQC** of Dy1 shows the correlation between carbons and protons were all shown in (Fig 4.3), (Fig 4.5) and (Fig 4.6) respectively, while **NOESY** spectrum of Dy1is shown in figure 4.7 showing coupling correlation in space.

4.8.5 Summary of assignment (spectral data) comparism with literature.

As shown in Table 4.11, comparism of Dy1 with reference showed that isolated product was well purified, and results were comparable with that of literature.

Table 4.11: Comparing the ^{13}CNMR and ^{1}HNMR spectral data of DY1 with that obtained from the literature (Werner Seebacher *et al*., 2003)

Position	δ_c (DY1)	δ_H(DY1)	δ_c (Reference)	δ_H (Reference)	
1	39.0	1.02	39.0	1.02	CH_2
		1.55		1.57	
2	28.2	1.82	28.1	1.82	CH_2
3	78.1	3.45 dd	78.2	3.44 dd	CH
4	39.4	-	39.4	-	C
5	58.8	0.82 d	55.9	0.88 d	CH
6	18.8	1.55	18.8	1.58	CH_2
		1.29		1.39	
7	33.3	1.55	33.4	1.53	CH_2
		1.27		1.36	
8	39.8	-	39.8	-	C
9	48.2	1.51	48.2	1.71	CH
10	37.4	-	37.4	-	C
11	23.7	1.96	23.8	1.96	CH_2
12	129.6	5.50 s	122.6	5.49 s	CH
13	145.6	-	144.8	-	C
14	42.2	-	42.2	-	C
15	28.4	1.30	28.4	1.22	CH_2
		2.20		2.19	
16	23.8	2.22	23.8	2.12	CH_2
		1.96		1.96	
17	46.7	-	46.7	-	C
18	42.0	3.20 dd	42.1	3.30 dd	CH
19	46.5	1.83	46.6	1.83	CH_2
		1.32		1.32	
20	31.0	-	31.0	-	C
21	34.3	1.51	34.3	1.46	CH_2
		2.24		2.04	
22	33.3	1.62	33.2	1.82	CH_2
		2.04		2.04	
23	28.8	1.24s	28.8	1.24s	CH_3
24	16.6	1.00s	16.5	1.02s	CH_3
25	15.6	0.82s	15.6	0.93s	CH_3
26	17.5	1.01s	17.5	1.04s	CH_3
27	26.2	1.27s	26.2	1.30s	CH_3
28	179.3	-	180.2	-	C
29	33.3	0.95s	33.4	0.97s	CH_3
30	33.8	1.02s	23.8	1.02s	

CHAPTER FIVE

5.0 DISCUSSION

5.1 Phytochemical screening

The phytochemical study of the n-hexane, ethyl acetate and methanol extracts of *Guiera senegalensis, Scoparia dulcis* and *Pulicaria crispa*, revealed that more phyto-constituents were present in the methanolic extracts followed by the ethyl acetate extract and then the hexane extract respectively as shown in Table 4.2. Both the ethyl acetate and methanol extracts showed the presence of triterpenes in the phytochemical screening presence of saponin in all the hexane fractions, ethyl acetate fraction of *Scoparia dulcis* and methanolic fraction of *Guiera senegalensis*. Cardenolides, phlobatanins, volatile oil and resins were absent in all the three solvent extracts. Flavonoids and volatile oils were present in only the ethyl acetate extract and methanolic extract of *Pulicaria crispa*. Cardiac glycosides, glycosides, balsams and phenols were only present in the methanolic extract of *Scoparia dulcis*. Steroids were found in the ethyl acetate extracts. Flavonoids, cardiac glycosides and glycosides were also found in the methanolic extract of *Guiera senegalensis* and *Scoparia dulcis*. Cardiac glycoside, resins and balsams were not detected in any of the fractions. These phyto-constituents possess various medicinal or herbal importance which further explains its anti-microbial activities (Tsechesche, 1971), flavonoids have anti-fungal, antibacterial and anti-inflammatory properties (Iwu *et al*., 1999; Hassan *et al*., 2004; Usman *et al*., 2007).

5.2 Antimicrobial activity

The antimicrobial activity (Table 4.6) of the plant fractions tested against Gram positive bacteria (*Staphylococus aureus, Bacillus subtilis, Streptococcus pyogenes*) and Gram negative bacteria (*Shigella dysenteriae, Salmonella typhii, Escherichia coli*) and fungus (*Candida albicans, Candida krusei*) using agar in-well diffusion method have their activities ranging between 16 mm to 35 mm respectively as against the control (ciproflaxin and

fluconazole ranging between 31 mm to 41 mm respectively) while the zone of inhibition of the test microbes against the three plant extracts were found to have high zone of inhibition in both ethyl acetate fraction and hexane fraction of *Guiera senegalensis*, at a concentration of 3.12 µg/ml as against *S. aureus,* also in ethyl acetate fraction of *Pulicaria crispa* at a concentration of 12.5 µg/ml as against *P. mirabilis,* also in all fractions of *G. Senegalensis* at a concentration 3.12 µg/ml as against *C.albican*, followed by the fractions of *Pulicaria crispa* at concentration of 3.12 µg/ml as against *S. pyogenes* and *N.gonorrhea*, the inhibitory effect of various extracts were compared with standard drug ciprofloxacin and fluconazole. Minimum inhibitory concentration and Minimum bactericidal and fungicidal concentration for the extracts were also determined and ranged between 12.25 to 25 µg/ml using agar in-well diffusion method. The zone of inhibition of the extracts was highest for the ethyl acetate fractions of *Guiera senegalensis* and *Pulicaria crispa* followed by the hexane and methanolic extracts of *Pulicaria crispa.* With the least observed for hexane extract of *Scoparia dulcis.* In general, the accumulation and concentration of secondary metabolites are responsible for antibacterial activity and varies according to the plant extracts base on their polarity (Sakagami and Kajimura, 2002). The sensitivity of *E. coli*, *S. aureus* and S. dysenteriae to some of the extracts implies that chemical compounds in the extracts can be further developed to fight against these microorganisms and the use of the plant for the treatment of diarrhea, stomach pain and skin itching is justified since this bacteria are responsible for such illness (Ramanathan *et al*., 2013; Uttu *et al*., 2015). The extracts also showed activities against *S. dysenteriae*, the bacteria responsible for bacillary dysentery (Uttu *et al*., 2015). Therefore all the extracts could serve as source of compounds that may be effective in the management of the ailments.

5.3 Anti-mycobacterial activity

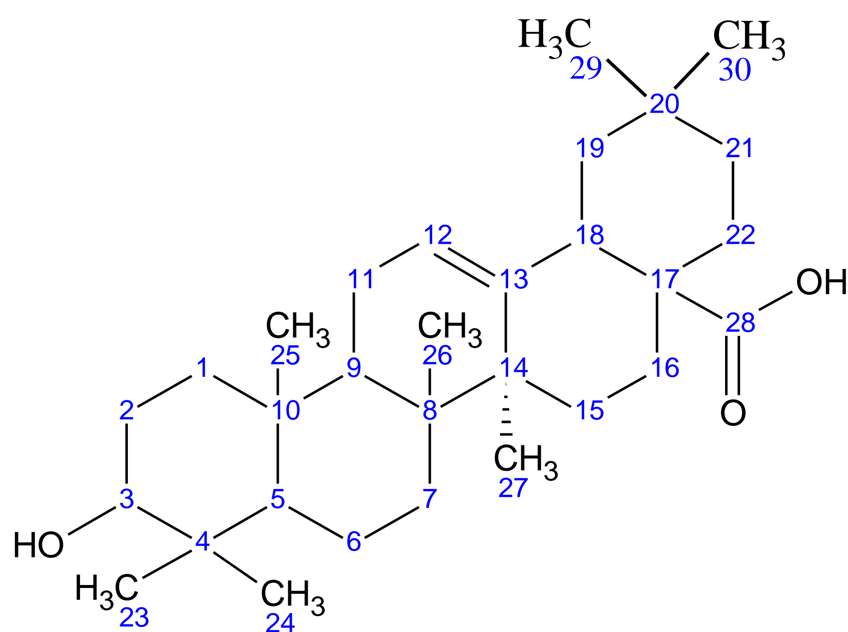
The study showed that the plant extracts had anti-mycobacterial tuberculosis (Anti-TB) activity at various levels. The anti-mycobacteria study showed that activity were found in methanol extract of *Pulicaria crispa* at concentration of 8.01±1.70 µg/ml, extracts of ethyl acetate of *Scoparia dulcis* at concentration of 12.03±0.86 µg/ml and methanol extract of Guiera senegalensis at concentration of 2.80±1.40 µg/ml. However low activity was observed for the hexane extract of *Scoparia dulcis* at concentration of 20.40±0.24 µg/ml and ethyl acetate extract of *Guiera senegalensis* at concentration of 40.01±1.20 µg/ml. Hence when compared with the control drug rifampicin at concentration of 0.38±1.40 µg/ml, methanol fraction for *Guiera senegalensis* showed the highest anti-mycobaterial activity. These activities were observed in ethyl-acetate extract and methanol extracts of *Guiera Senegalensis,* hexane and ethyl-acetate extract of *Scoparia dulcis* and hexane and methanol extract of *Pulicaria crispa*. All the above extracts were active against the rifampicin resistant strain of *Mycobacterium tuberculosis* however the methanol extracts of *Guiera senegalensis* was more active on the rifampicin resistant strain of *Mycobacterium tuberculosis*. This is important because rifampicin resistance is a good indication of multi drug resistant TB and so the extracts may also be active on multi drug resistant tuberculosis. (Tripathi, 2004)

5.4 Chromatographic and Spectral Analysis.

Chromatographic separation and purification of the methanol extract of *Guiera senegalensis* led to isolation of a compound Dy1, a white amorphous powder re-crystallised from MeOH which showed positive to triterpenes test (Liebermann-Buchard test)

The ^{1}H NMR spectrum revealed the presences of methyl group at δ 1.02 and methylene protons at δ 1.83 and δ 0.95. It also showed five methyl singlets at δ 0.82, δ 1.00, δ 1.24, δ

1.27 and one singlet of secondary hydroxyl group at δ 3.45. The following signals show the various multiplicities in ^{1}H NMR spectrum: (δ-1.02 t, 2H, H-1), (δ-1.82 q, 2H, H-2), (δ-3.45 t, 1H, H-3), (δ-0.82 t, 1H, H-5), (δ-1.29 q, 2H, H-6) (δ-1.55 t, 2H, H-7), (δ-1.50t, 1H, H-9), (δ-1.96 t, 2H, H-11), (δ-5.50 t, 1H, H-12), (δ-1.30 t, 2H, H-15), (δ-2.22 t, 2H, H-16), (δ-3.20 t, 1H, H-18), (δ-1.83 d, 2H, H-19), (δ-1.51 t, 2H, H-21), (δ-1.62 t, 2H, H-22). The proton decoupled ^{13}C NMR spectrum and showed 30 carbon atoms (table 4.11). The Distortionless Enhancement polarization Transfer (DEPT 90, Fig. 4.4) indicated five methine carbon while (DEPT 135, Fig. 4.4) suggested ten methylenes carbon (CH_2, negative), seven methyl carbon (CH_3, positive) groups. Quartenary carbon don't contain attached protons hence do not appear in the DEPT spectrum. They may be identified as the signals which further appear in the proton decoupled ^{13}C NMR spectra, hence eight quaternary carbons were identified. Heteronuclear Multiple Bond Coherance (HMBC) correlates ^{13}C- ^{1}H shifts through both two and three bonds and Heteronuclear Single Quantum Correlation correlates the ^{13}C in 1D with the proton shift one bond CH coupling. NOESY correlates couple protons in space and were all used in elucidating the structure DY1. spectra revealed thirty signals ranging from 15.6 to 179.3 ppm. The signals between 26.2 to 58.8 ppm correspond to overlapping methine (-CH), methylene (-CH_2) and methyl (-CH_3).. Based on the analysis above and after comparison with data in literature (Ghias *et al.*, 2011; Fauziah *et al.*, 2013), the structure of Dy1 was found to be oleanolic acid (Figure 4.8)



Compound Dy1- (Oleanolic acid)

Lupine, oleanane, ursane and oleanolic acid triterpenes display various pharmacological effects while being devoid of prominent toxicity. These triterpenes are promising leading compounds for the development of new multi-targeting bioactive agents. Depending on the plant material, betulin, betulinic acid, oleanolic acid and ursolic acid are the common pentacylcic triterpenes. They are isolated in high concentration of their active principles and therefore can be used for development of phytopharmaceutical formulations (Sebastian *et al.*, 2009).

It is well known that triterpenes exhibit moderate to high *in vitro* antimycobacterial activity against *Microbacterium tuberculosis*. (Okunade, *et. al*., 2004; Copp and Pearce, 2007). The modification of oleanolic acid a natural triterpene through introduction of cinnamoyl frames at the C-3 position has been reported (Tanachatchairatana *et al*., 2008) and Oleanolic acid (Phillips *et al.*, 2012) isolated from *L. hispida* was also reported to have an MIC of 25 µg/mL against *Mycobacterium tuberculosis* (H37RV).

CHAPTER SIX

6.0 Conclusion

The findings in this study have hence provided scientific support for the ethnomedicinal antiTB use of the root of *Guiera senegalensis*. The methanolic extract was found to have a high activity when compared to the standard drug (Rifampicin). The phytochemical analyses of the plants showed that the methanolic extracts contained more active secondary metabolites. Steroids, terpenoid, alkaloids and tannins were high in the methanol extract, hence promoting the antimicrobial activity of all the extracts. Isolation of the active compound were compared with that of literature and confirmed to be oleanolic acid, which is a known compound that has very good medicinal potentials.

This research finally justifies the claim that the plants have anti-TB activity based upon the attributed findings from both the phytochemical screening and anti-microbial screening. Hence the secondary metabolites anti-microbial strength may be responsible for the good anti-tuberculosis activity found in the methanolic extract of *Guiera senegalensis.*

6.1 Recommendation

More compounds should be isolated from other extracts and subjected to biological and pharmacological studies to investigate the medicinal potential of *Guiera senegalensis Scoparia dulcis* and *Pulicaria crispa.*

Derivatives of Dy1 (Oleanolic acid) should be synthesized to improve on its activity on the TB drug-resistant strains.

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