PHYTOCHEMICAL SCREENING AND ANTIMYCOBACTERIAL POTENTIALS OF *Syzygium guineense* WILD DC. AND *Mimosa pigra* LINN

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BY

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A Thesis in the Department of Chemistry,
Submitted to the Faculty of Science
in partial fulfillment of the requirements for the Degree of

DOCTOR OF PHILOSOPHY

of the

UNIVERSITY OF IBADAN

SEPTEMBER, 2014

ABSTRACT

The prevalence of multi-drug resistant tuberculosis is an increasing health challenge. Attention has therefore been shifted to the use of ethno-medicines in combating this disease. Traditionally, *Syzygium guineense* (stem-bark) and *Mimosa pigra* (aerial parts) are used in the treatment of respiratory tract infections with no scientific justification. This study was designed to identify the constituents of *Syzygium guineense* and *Mimosa pigra* that may be active against *Mycobacterium tuberculosis.*

The *S. guineense* (stem-bark) and *M. pigra* (aerial parts) were obtained from farmlands in Abuja and authenticated at the herbarium of National Institute for Pharmaceutical Research and Development, Abuja. The *S. guineense* (1.0 kg) was extracted successively with chloroform and methanol. The extracts were separately fractionated with n-hexane followed by acetone. *Mimosa pigra* (0.5 kg) was extracted with methanol and partitioned with diethylether and n-butanol. The fractions were purified using chromatographic techniques. The extracts, fractions and isolated compounds were subjected to anti-mycobacterial assay with *Mycobacterium tuberculosis* (ZMC 050 and 303) strains using the Lowenstein-Jensen and mycobacterium growth indicator tube methods. Streptomycin, isoniazid, rifampicin, and ethambutol were used as standard drugs and dimethylsulphoxide as negative control. The structures of the isolated compounds were elucidated using infra-red, ultra-violet/visible, nuclear magnetic resonance and mass spectroscopic techniques.

The chloroform extract of *S. guineense* yielded 4.1 g n-hexane fraction and 5.2 g acetone fraction A while the methanol extract gave 5.2 g acetone fraction B and 98.0 g residue. Crude methanol extract of *M. pigra* gave 40.1, 4.6 and 12.3 g of diethylether, n-butanol and residue fractions respectively. Two new triterpenoids were isolated from *S. guineense* namely: 12-hydroxy-27-demethylfriedelan-3-one and betulinic acid methylenediol ester, in addition to the two known triterpenoids: betulinic acid, and friedelan-3-one. One new flavonoid, 8-hydroxy-3-phenyl-4-benzopyrone rhamnoside was isolated from *M. pigra*. The two plants' methanol extracts, n-butanol fraction, acetone fraction A, and isolated compounds: betulinic acid, betulinic acid methylenediol ester and 8-hydroxy-3-phenyl-4-benzopyrone rhamnoside were active against ZMC 050 strain with Minimum Inhibitory Concentrations (MIC) of 5.0, 5.0, 2.0, 0.6, 0.6, 0.15 and 0.5 mg/mL respectively. The ZMC 303 strain also gave similar MIC values as ZMC 050 strain. Spectroscopic analysis of 12-hydroxy-27-demethylfriedelan-3-one provided evidence for δ_H signals: 0.8-1.2

($7CH_3$), 1.2-1.8 ($10CH_2$), and 2.2-2.4 (5CH), δ_C signals: 29.9-41.7 (5C), 72.8 (1CHOH) and 213.4 (1C=O) with a molecular ion of 428 corresponding to $C_{29}H_{48}O_2$. The betulinic acid methylenediol ester showed δ_H and δ_C signals similar to betulinic acid except for the downfield methylenedioxy carbinol (OCH_2O) δ_C signal at 79.0 and molecular ion of 486 correspond to $C_{31}H_{50}O_4$. Betulinic acid and friedelan-3-one signals are similar to published spectra. The 8-hydroxy-3-phenyl-4-benzopyrone rhamnoside showed δ_H signals: 0.9 (3Hd, J=5.8 Hz), 3.1-3.8 (overlapping glycone multiplet) and 5.2 (anomeric) typical of a rhamnose moiety, with δ_H 6.2 (1H), 6.4 (1H), 6.9 (5H) and δ_C 178.0 (1C=O) of the flavonoid ring.

The bioactive compounds obtained from *Syzygium guineense* and *Mimosa pigra* exhibited anti-mycobacterial activities. They have potentials for the development of drugs for the management of tuberculosis. The new triterpenoids and flavonoids are addition to the library of compounds.

Keywords: *Syzygium guineense*, *Mimosa pigra*, Antimycobacterial activity, Triterpenoids
Word count: 484

ACKNOWLEDGEMENTS

I am grateful to the Department of Chemistry for giving me the opportunity to obtain this degree. The Head of Department, Prof. A. A. Adesomoju and the Head of Organic Chemistry unit, Prof. O. Ekundayo are well acknowledged for their tutelage. I wish to appreciate my supervisors; Dr. Olapeju O. Aiyelaagbe and Dr. 1. A. Oladosu for their doggedness in, and meticulous approach to the supervision of the work.

I cherish the cordial relationship maintained with my friends: Tayo Ogundajo, Abdulrazaq Ogunmoye, Toyin Babatunde, Adeniran Oluremi, Nkop Josiah and Sunday Ajibade. Others are Dr. O. Balogun, Dr. J. Nvau and Dr. Olaoluwa Olaolu. I appreciate the technical support rendered by Mallam Mua' azam Ibrahim and Hajia Jemilat Ibrahim during the ethnobotanical survey.

With a deep sense of gratitude, I acknowledge the Royal Society of Chemistry, United Kingdom for the conference travel bursaries which afforded me the opportunity to use spectroscopic instruments in laboratories at the Sao Paulo State University, Brazil, and Rhodes University, Grahmstown, South Africa. I also wish to acknowledge Professors Ian Castro-Gamboa and Rui Krause of the Sao Paulo State University, Brazil and Rhodes University, South Africa for making their NMR facilities available and accessible to me for the two dimensional spectroscopic analysis of compounds isolated in this research. I am also expressing my gratitude to Mrs. Dorcas Okoroafor for proof-reading this work. Also worthy of gratitude are Dr. L. Lawson and Mr. N. Emenyonu for allowing me free access to the facilities at the Tuberculosis Research laboratories of the Zankli Medical Centre, Abuja, Nigeria.

My acknowledgement would not be complete without appreciating my uncle Dr. G.O. Ohore and family for always being there for me whenever I am in Ibadan during the course of this study. To my siblings (Engr. and Mrs. Erovie-oghene Afieroho, Otho-oghene Afieroho, Engr. and Mrs. Eze Afieroho, Ewomazino Afieroho, Onorieva-oghene Afieroho, Edooghogho Afieroho and Evawere Afieroho), wife (Mercy Afieroho), and parents (Mr. and Late Mrs. S.A. Afieroho), I remain indebted for their support and love.

Above all, I owe much gratitude to the Lord Jesus Christ my saviour and redeemer for His grace that saw me through from the beginning to the end of this programme.

AFIEROHO, O.E.

September, 2014.

DEDICATION

This work is dedicated to all who use science as a tool to solve human health challenges.

CERTIFICATION

We certify that this work was carried out by Mr. O. E. Afieroho in the Department of Chemistry, University of Ibadan.

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CHAPTER ONE

1.0 INTRODUCTION

1.1 An overview of the global tuberculosis burden and traditional medicine practice

Tuberculosis (TB) is a common and often deadly infectious disease caused by *Mycobacterium tuberculosis*. The World Health Organization (WHO) reported 8.6 million new cases of TB in 2012 (WHO, 2013). Less developed countries, mostly in Africa and Asia accounted for the highest percentage. An increasing challenge in the control of TB infections is the emergence of multi-drug and extensive drug resistant tuberculosis (MDR-TB and XDR-TB respectively). Deaths due to MDR-TB is currently put at 170, 000 worldwide (WHO, 2013). The increasing prevalence of MDR-TB and XDR-TB coupled with the expensive cost of treatment-especially in the third world countries, has made people living in these countries to resort to various traditional medications as alternatives to orthodox drugs.

Traditional medicine is defined as ‘‘the sum total of all the knowledge whether explic- able or not, used in diagnosis, prevention and elimination of physical, mental or social imbalance and relying exclusively on practical experience and observation handed down from generation to generation whether verbally or in writing” (WHO, 1978). From time immemorial, plants and plant-derived drugs have been used as traditional remedies for the treatment of diseases (Daziel and Hutchison, 1937; Kokwaro, 1976; Oliver-Bever, 1986). Over two-third of the world population still depend on herbal medicine for its healthcare needs (Farnsworth *et al.*, 1985). This number is still growing daily due to the high cost of orthodox therapy especially in the third world countries. The United Nations Educational, Scientific and Cultural Organisation (UNESCO) have described traditional medicines as one of the surest means to achieve total health coverage of the world’s population (UNESCO, 1994).

In view of the paradigm shift towards plant based alternative medications, interest in research aimed at the scientific validation of these ethno-medicinal claims is on the rise. Some of such researches have resulted in the isolation and characterisation of the active secondary metabolites in these medicinal plants which have served as useful leads for the development and synthesis of several orthodox drugs (Olaniyi, 2000). Notable among such orthodox drugs derived from bioactive secondary metabolites in plants are: aspirin (antipyretic and pain relieving drug), codeine (an antitussive drug), digoxin (a cardiotonic agent), quinine (an antimalarial drug) and vincristine (an anticancer drug).

Several plants have been used traditionally, in the treatment of diseases. This practice commonly known as ethno-medicine have been passed down from one generation to another. Some of such medicinal plants have been documented and include: *Azadirachta indica* (used for the treatment of malaria), *Abrus precatorius* (used for the treatment of tuberculosis), *Euphorbia hirta L* (a remedy for cough, chronic bronchitis and other pulmonary disorders), the root bark of *Moringa oleifera* Lam (used as a diuretic, stomachic and an arbortoficient), stinging nettles, *Urtica dioca* (used in the treatment of inflammatory conditions like; osteoarthritis, gouts, rheumatism, and kidney stone), *Senna alata* (used as a laxative), *Aloe vera* (used as an immune booster), *Ricinus communis* (used traditionally to enhance the circulation of blood), *Momordica charantia* (used in the treatment of diabetes), *Carica papaya* (used in the treatment of jaundice, peptic ulcer, cancer, worm infestation, and male impotency) and *Ocimum gratissium* (used in the treatment of chronic diarrhoea) among others (Daziel and Hutchison, 1937; Kokwaro, 1976). These plants are usually prepared or formulated in the form of: decoctions, infusions, concentrates, essential balms, inhalations, tablets and capsules, syrups, tinctures, tea, compresses and poultices.

1.2 Justification of present study

In the traditional treatment of respiratory tracts infections, there are documented reports on the use of *Syzygium guineense* (Tsakala *et al*., 1996; Djoukeng *et al.*, 2005; Nvau, 2011) and *Mimosa pigra* (Sonibare and Gbile, 2008, and Genest *et al.*, 2008). However there is no previous scientific work on the antimycobacterial activity of the stem bark of *Syzygium guineense* and *Mimosa pigra*. Based on this, these two

medicinal plants were selected for antimycobacterial study with the view of isolating bioactive constituent(s) that could serve as leads for the development of drugs for the treatment of tuberculosis and related mycobacterial infections.

1.3 Research aim

This research is aimed at identifying the constituents of *Syzygium guineense* and *Mimosa pigra* that may be active against *Mycobacterium tuberculosis.*

1.4 Research objectives

1. To collect, identify and prepare extracts of *Syzygium guineense* (stem bark) and *Mimosa pigra* (aerial part) using standard phytochemical methods.
2. To determine the antimycobacterial activities of the plants' extracts using the Lowenstein Jensen and mycobacterium growth indicator tube methods.
3. To isolate the chemical constituents of the plants' extracts using chromatographic methods, and characterised them using spectroscopic techniques.
4. To determine the antimycobacterial activity of the isolated compounds using the mycobacterium growth indicator tube method.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 The Syzygium genus

The genus Syzygium is a member of the Myrtaceae family of plants. The Myrtaceae are a family of dicotyledonous plants with over 5000 species distributed among 130-150 genera. Plants species like: Myrtle, clove, guava, feijoa, allspice, and eucalyptus belong to this family. The syzygium genus comprises about 1200 species of evergreen trees and shrubs. Several species of syzygium are grown as ornamental plants for their attractive glossy foliage, and a few among them produce edible fruits that are eaten fresh or used in jams and jellies. The most economically important species is *Syzygium aromaticum* (the clove plant), of which the unopened flower buds are important spice for household use. Some of the edible species of *Syzygium* are planted throughout the tropics (Johnson and Briggs, 1984; Wilson *et al.*, 2001; Wilson *et al.*, 2005; Govaerts *et al*., 2008).

2.2 The medicinal uses of *Syzygium* genus

Syzygium cumini (synonym: *Eugenia jambolama*), is used in Indian folk medicine for the treatment of diabetes (Prince *et al.*, 1998). Similar use has been reported on the infusion of the leaves of *S. jambos* (Teixeira *et al.*, 1997).The potent antibacterial and antidiarheal effect of *S. aromaticum* (Dorman *et al.*, 2000) and *S. guineense* (Tsakala *et al.*, 1996) are documented. The methanol leaf extracts of *S. aromaticum* have also shown antibacterial activity against Gram-negative anaerobes (Cai and Wu, 1996). Extracts from the leaves of *S. claviflorum* inhibited the replication of the Human Immuno-deficiency Virus (HIV) (Fujioka *et al.*, 1994; Kashiwada *et al.*, 1998). The *in vitro* growth inhibition of *Mycobacterium tuberculosis* by extracts from *S. jambos* has been reported (Frame *et al.*, 1998). The anti- inflammatory (Lee *et al.*, 1995), larvicidal (Pushpalata *et al.*, 1995), weight reduction (Palit *et al.*, 1999) and diuretic (Silva-Netto, 1986) activities of *S. jambolanum* leaves extracts are documented. Inhibition of the aggregation of the human blood platelets by the oil of cloves (*S. aromaticum*) (Srivastava *et al.*, 1991) and the anti-anaphylaxis effects by the flower bud aqueous extract of the same plant in rats (Kim *et al.*,1998) have also been reported. Anti-fertility and spermatogenesis activities of

triterpenoid from the flowers of *S. cumuni* in male rats, without changes in body weight or reproductive organ's weight have been documented (Rajasekaran *et al.*, 1988). Castor oil induced diarrhea was reduced by the bark extracts of *S. cumini* (Mukherjee *et al.*, 1998). Bark decoction of *S. guineense* is used traditionally in the treatment of diarrhea (Abebe *et al.*, 2003). The twigs and aerial roots were used for different stomach ailments (Kassu, 2002). The aqueous extracts of the leaves of *S. guineense* were reported to have *in vitro* antibacterial effect against diarrhea causing bacteria (Tsakala *et al*., 1996).

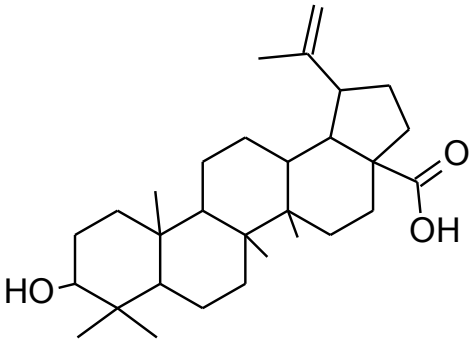
2.3 Morphological description of Syzygium *guineense*

Syzygium guineense (Figure 2.1) is a dense leafy forest tree species. It is about 30 m tall and belongs to the botanical family Myrtaceae. It is commonly known as water berry in English (Agwu and Okeke, 1996), "Málmóó'' in Hausa, and "Adere" in Yoruba. Morphologically, it has a broad fluted trunk with the crown rounded and heavy. The bark is smooth when young, but becomes rough and black with age. The branches are dropping while the stems are thick and angular. The leaves are purple-red in color when young, but as they mature their colour becomes dark green. The flowers of *S. guineense* have white, showy stamens, in dense branched heads 10 cm across, yielding a honey-sweet smell that attracts many insects. The fruits are ellipsoid drupe, purplish in colour (Bekele, 1993; Abebe *et al.*, 2003). They are edible fruits, with high nutritional value (Saka and Msonthi, 1994; Nievergelt *et al.*, 1998).

2.4 Isolated compounds from Syzygium genus

Betulinic [2.1], dihydrobetulinic [2.2], platanic [2.3] and oleanolic [2.4] acids have been isolated from the leaves of *S. claviflorum* (Fujioka *et al.*, 1994; Kashiwada *et al.*, 1998). Oleanolic [2.4], 2-hydroxyoleanolic [2.5], 2-hydroxyursolic [2.6], arjunolic [2.7], asiatic [2.8], terminolic [2.9] and 6-hydroxyasiatic [2.10] acids were isolated from the leaf of *S. guineense* (Djoukeng *et al.*, 2005). Acetyl eugenol [2.11] has been isolated from the volatile oils of *S. aromaticum* (Srivastava *et al.*, 1991). Caryophyllene oxide [2.12], d-cadinene [2.13] , viridiflorol [2.14], α-cadinol [2.15] and α-humulene [2.16] are present in the essential oils from the leaves of *S. guineense* (Noudogbessi1 and Yédomonhan 2008).

Figure 2.1: Matured tree of *Syzygium guineense* with the twig containing drupe (insert)



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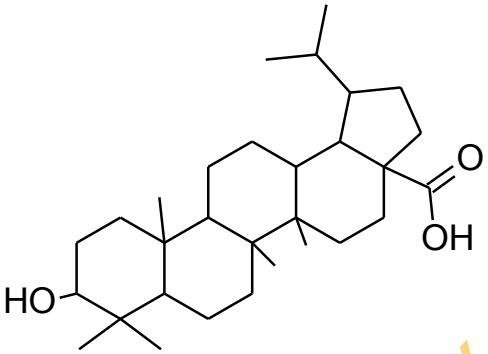

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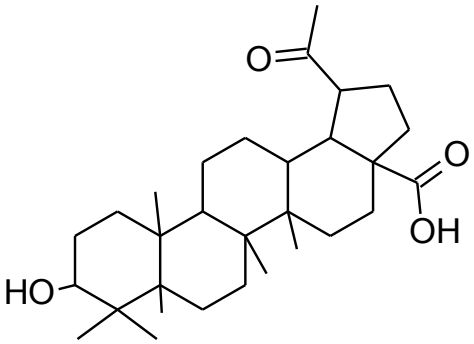

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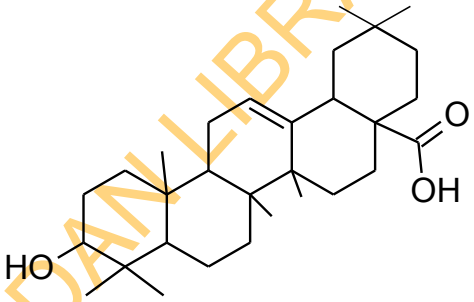

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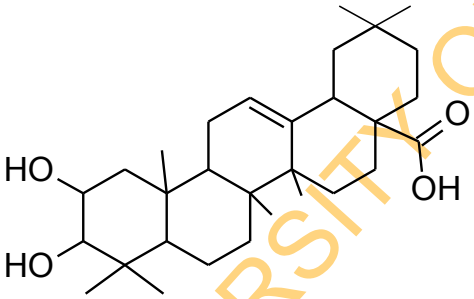

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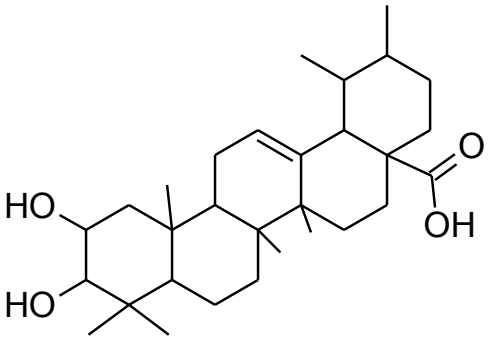

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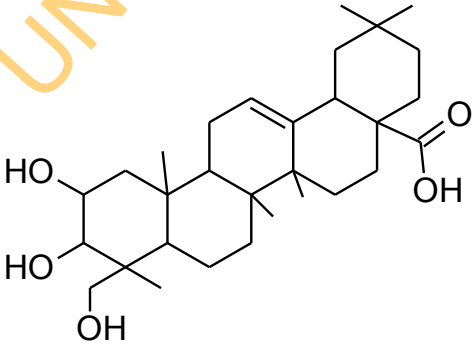

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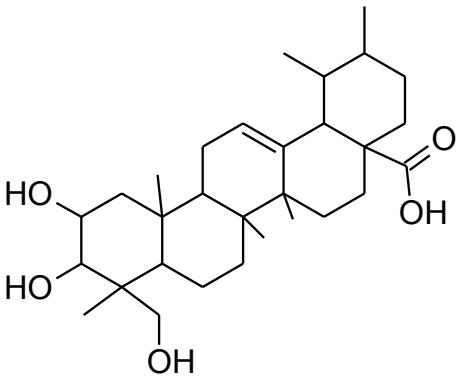

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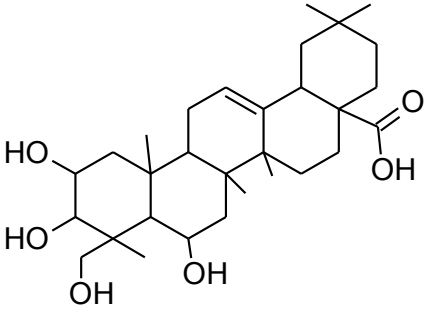

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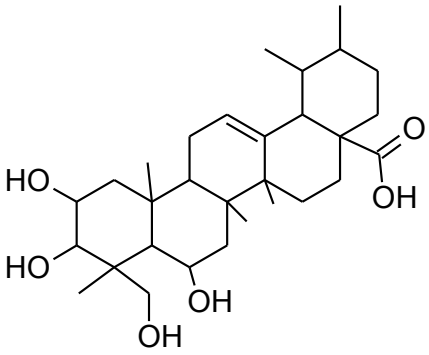

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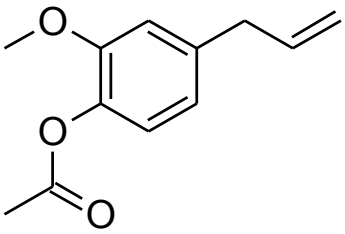

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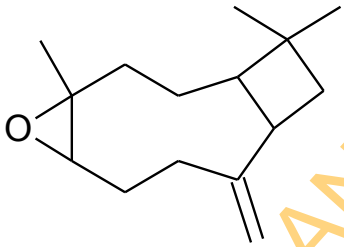

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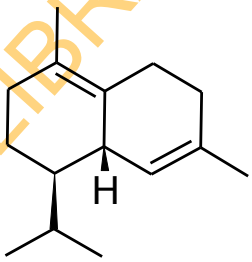

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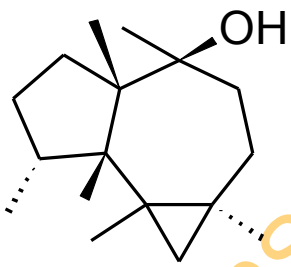

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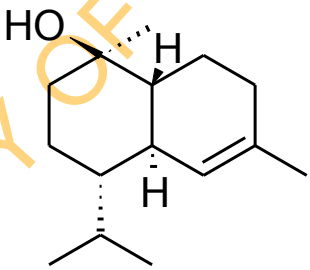

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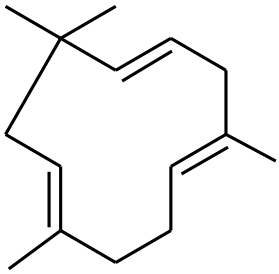


2.16

2.5 Pharmacological properties of some compounds isolated from *Syzygium spp*

The potent anti-HIV activity of betulinic [2.1], dihydrobetulinic [2.2], platanic [2.3] and oleanolic [2.4] acids isolated from the leaves of *S. claviflorum* have been reported (Fujioka *et al.*, 1994; Kashiwada *et al.*, 1998). The anti-fertility and spermatogenesis inhibition activities of oleanolic acid [2.4] from the flowers of *S. cumuni* in male rats have also been documented (Rajasekaran *et al.*, 1988). Acetyl eugenol [2.5] isolated from the essential oil of *S. aromaticum* has been reported to have human blood platelets aggregation inhibitory activity (Srivastava *et al.*, 1991). The 2-hydroxyursolic acid [2.7], arjunolic [2.8] and

asiatic acid [2.9], isolated from the aqueous leaves extracts of *S. guineense* were reported to have potent *in vitro* antibacterial effects against diarrhea causing bacteria (Tsakala *et al.*, 1996).

2.6 Morphological description of *Mimosa pigra*

Mimosa pigra (Fig. 2.2) is a species of the genus Mimosa. The members of the mimosa genus comprising of about 400 species of herbs and shrubs, belong to the legume family Fabaceae. *Mimosa pigra* is a shrub reaching up to 6 m in height. It is called "giant sensitive plant, bashful plant, catclaw mimosa, or black mimosa" in English, "Kai dafi" in Hausa, "Ganjanje" in Fulani and "Enwa agogo" in Yoruba. The stem which is armed with broad-based prickles up to 7 mm long, is greenish in young plants but becomes woody as the plant matures (Lonsdale *et al.*, 1995). The leaves are bright green and bipinnate, consisting of a central pricky rachis 20 to 25 cm long with up to 16 pairs of pinnae 5 cm long, each divided into pairs of leaflets 3 to 8 mm long. Leaves are sensitive and fold up when touched and at nightfall. Flowers are mauve or pink, born in tight sub-globose pedunculate heads 1 cm in diameter each containing approximately 100 flowers. Each flower head produces a cluster of 10 to 20 seedpods which then matures and breaks into segments. Each segment contains an oblong shaped seed. Hairs on the segments allow them to float on water and stick to hair or clothing thereby aiding their dispersal. Ripe seeds vary in colour from light brown to brown or olive green. Mimosa is hard seeded, surviving at least 23 years on sandy soils, with viability decreasing more rapidly on clay soils. *Mimosa pigra* can germinate year-round if the soil is moist but not flooded though optimum at the start and end of the wet season. Growth in seedling is rapid and flowering occurs between 4 and 12 months after germination. The process from the emergence of flower bud to ripe seed takes about five weeks.

Figure 2.2: *Mimosa pigra* plant

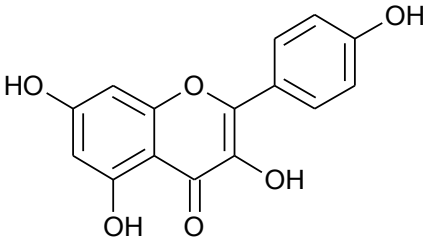
Mimosa pigra can be distinguished from its closely related species *Mimosa pudica (*commonly called sensitive plant) by its large size, large pods (6 to 8 cm long as opposed to 2.5 cm long), and leaves which have 6 to 16 pairs of pinnae as opposed to 1 to 2 pairs in *Mimosa pudica* leaves (Lonsdale *et al.*, 1995).

2.7 The medicinal uses of species of *Mimosa* genus.

The use of *Mimosa pigra* in the traditional treatment of asthma and other respiratory diseases has been reported (Sonibare and Gbile, 2008). Among the Bawku in Upper East Ghana, it is used in the treatment of diarrhea, typhoid fever and genitourinary tract infections (Mbatchou *et al.*, 2011). Its anti-hypertensive (Rakotomalala, *et.al.*, 2013) and anti-oxidant (Lee *et al.*, 2004) activities have also been reported. *Mimosa pudica* the closely related creeping species identified as 'Lajjalu' in Ayurveda has been found to have anti-asthmatic, aphrodisiac, analgesic and anti-depressant activities (Gandhiraja *et al.*, 2009). Also reported is its antimicrobial activity (Ojalaa *et al*., 1999). It is also used for the treatment of snakebite in Africa (Irvine 1961). In Sumatra, roasted and ground mimosa leaves are made into an infusion or a decoction, which is drunk to treat a weak heart or weak pulse (Grosvenor *et al.*, 1995). In Mexico, an infusion of mimosa was traditionally used in Mayan medicine for the treatment of diarrhoea (Rosado-Vallado *et al.*, 2000).

2.8 Isolated compounds from Mimosa genus

The flavonoids; Kaempferol [2.17], quercetin [2.18] and myricetin [2.19] and their glycosides have been isolated from the leaves of *Mimosa pigra* (Lee *et al.*, 2004; Sulaiman *et al.*, 1997). Genest *et al.*, (2008) reported on the antioxidant and antibacterial activities of flavonoids isolated from *Mimosa pigra.* Mimosine [2.20] an alkaloid with antiproliferative and apoptotic effects, has been isolated from *Mimosa pudica* (Restivo *et al.*, 2005) while the psychedelic indole alkaloid dimethyltryptamine [2.21] was isolated from *Mimosa tenuiflora* (Carmargo-Ricalde, 2000; Ott, 1998).



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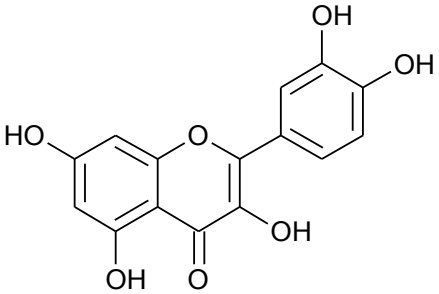

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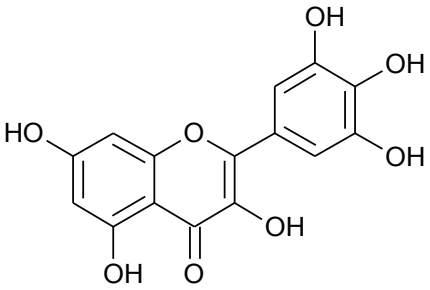

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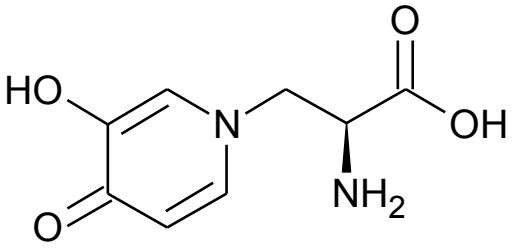

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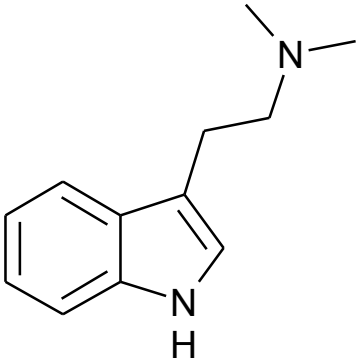

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2.9 Secondary plant metabolites

Secondary metabolites are molecules that are products of secondary metabolism in plants. Their functions in plants include; the regulation of growth and reproduction, herbivore deterrence due to astringency or acting as phytoalexins that kill bacteria that the plant recognizes as a threat. Secondary metabolites are often involved in key interactions between plants and their abiotic and biotic environments (Facchini *et al*., 2000). Plants produce secondary metabolites as a defence mechanism against fungi, bacteria, insects and viruses. They also produce them as colourful pigments to attract insects for pollination. Depending on their biosynthetic origin, secondary plant metabolites, could be classified as; terpenoids (monoterpenoids, sesquiterpenoids, diterepenoids, triterpenoids and tertraterpenopoids), phenolics (pigments, flavonoids, tannins, phenylpropanoids and related compounds) and alkaloids (a group of nitrogen containing compounds derived from amino acids) (Edwards and Gatehouse, 1999).

In this section, the chemistry and pharmacology of terpenoids and phenolics compounds will be discussed because of their relevance to this research.

2.9.1 Terpenoids

Terpenoids are secondary metabolites consisting of one or more isoprene units linked together in a head to tail, or rarely tail to tail manner. They are believed to have their biosynthetic origin from the mevalonic acid pathway (Figure 2.3) (Finar, 2000; Rohdich *et al.*, 2001). They exist in the free form or as esters, glycosides or ethers. Based on the number of isoprene units, the various classes of terpenoids are outlined in Table 2.1 below.

The triterpenoids which consist of six isoprene units are believed to have their biosynthetic origin from the mevalonic acid pathway through the cyclisation of squalene (Figure 2.3). The cyclisation is usually in a manner that makes most naturally occurring triterepnoids to be hydroxylated at C_{-3} position of the pentacyclic ring. Pentacyclic triterpenoids are classified structurally into twenty-two series (Mahato *et al.*, 1992; Mahato and Kundu., 1994). These include: Bauerane [2.28], Bicadinane [2.29], Fernane [2.30], Friedelane [2.31], Friedomadeirane [2.32], Friedoursane [2.33], Gammacerane [2.34], Glutinane [2.35], Hancokinane [2.36], Hopane [2.37], Kairatane [2.38], Lupane [2.39], Mimusopane [2.40], Oleanane [2.41], Onocerane[2.42], Serratane [2.43], Sorghumane [2.44], Spirosupinane [2.45], Stictane [2.46], Swertane [2.47], Taraxarane [2.48], Taraxastane [2.49] and Ursane [2.50]. Triterpenes are normally hydroxylated at C_{-3} and certain methyl groups are frequently oxidised to hydroxymethyl, aldehyde or carboxylic acid functionalities. When a sugar moiety is linked to a triterpene, the resulting compound is refered to as saponins in which case the non sugar triterpene moiety or aglycone is specifically called the sapogenin (Hostettmann and Marston, 1995). The sugar moieties of saponins are generally oligosaccharide, linear or branched, attached to a hydroxyl or a carboxyl group or both. The site of attachment may be one (monodesmoside), two (bidesmoside) or three (tridesmoside) (Mahato *et al.*, 1992; Mahato and Kundu, 1994). Triterpenoids and their saponins are widely distributed in the plant kingdom.

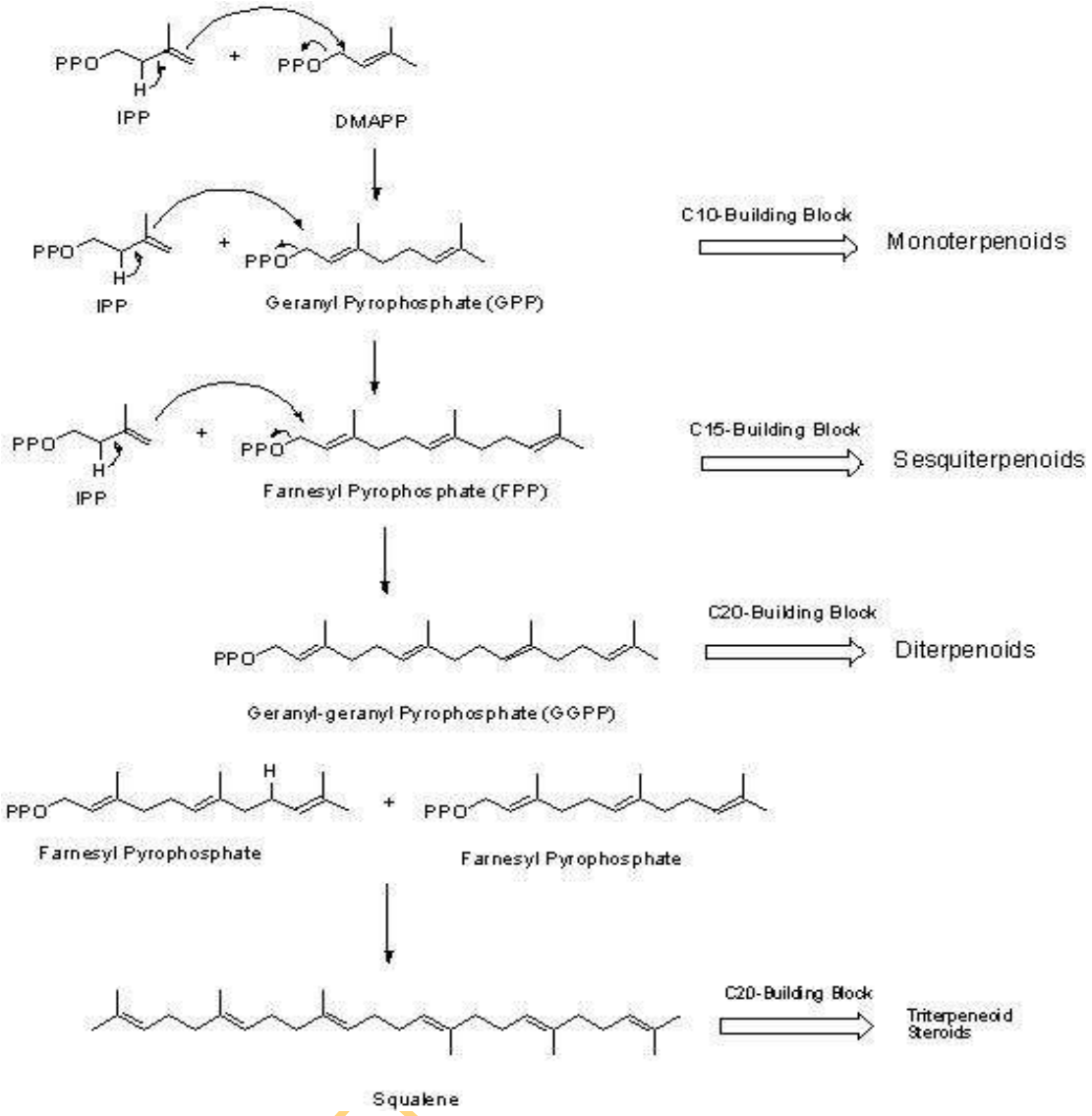
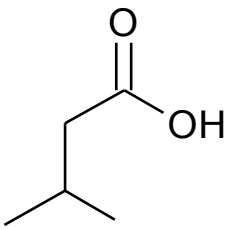


Figure 2.3: The mevalonic acid pathway (Rohdich *et al.*, 2001)

Table 2.1: Classes and examples of some naturally occurring terpenoids

Classes of Terpenoids	Number of Isoprene units	Examples
Hemitepenes	1	Isovaleric acid[2.22]
Monoterpenoids	2	Eucalyptol [2.23]
Sesquiterpenoids	3	Artemisinin [2.24]
Diterpenoids	4	Pleuremutillin[2.25]
Triterpenoids	6	Ursolic acid[2.26]
Tetraterpenoids	8	Lycopene [2.27]



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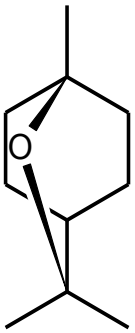

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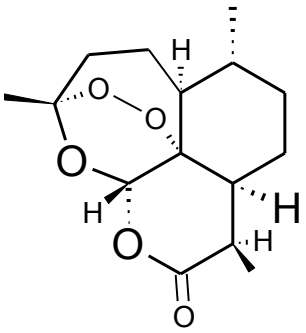

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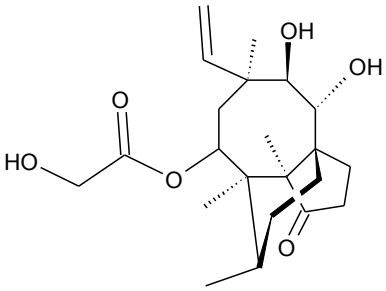

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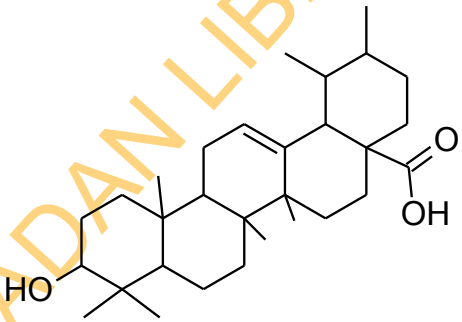

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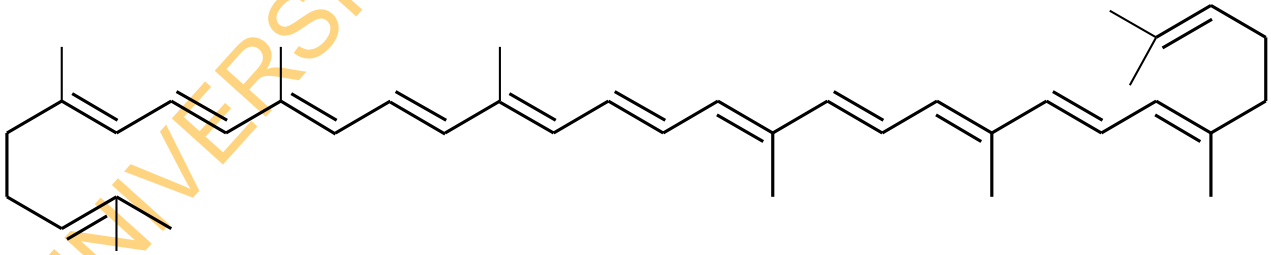

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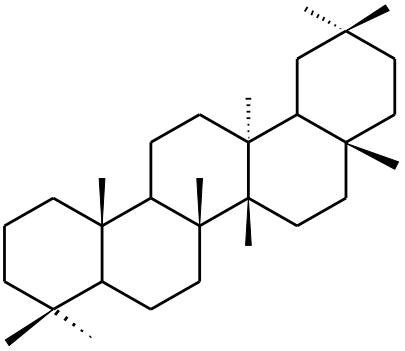

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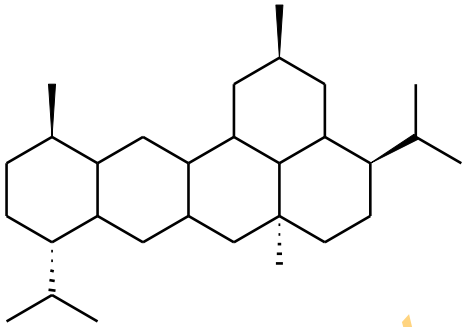

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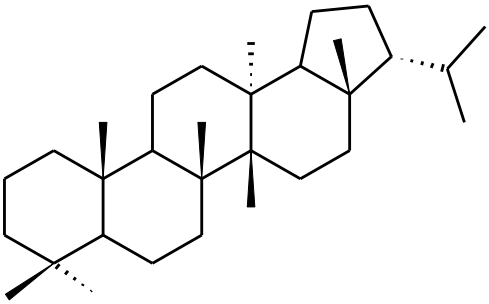

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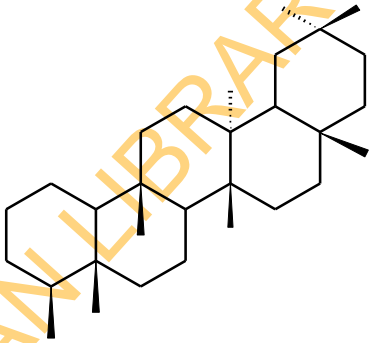

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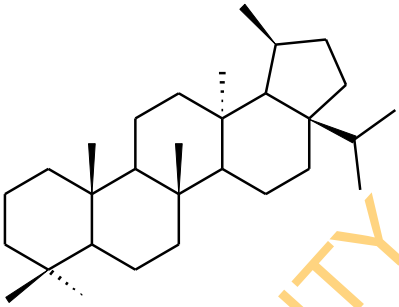

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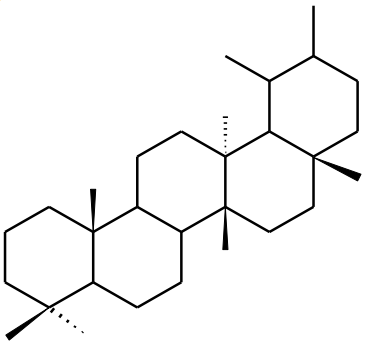

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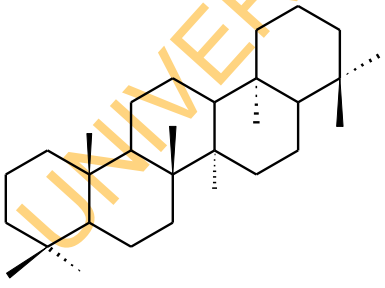

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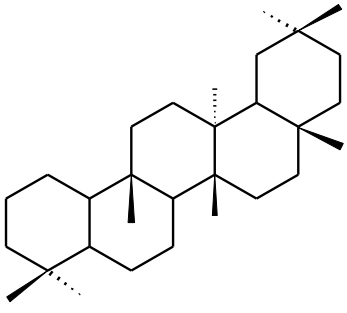

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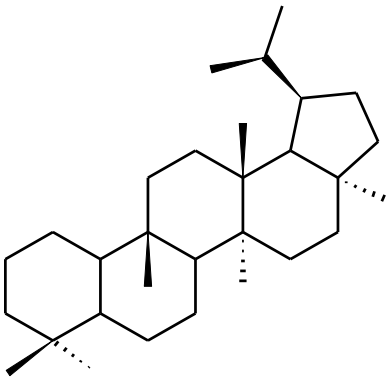

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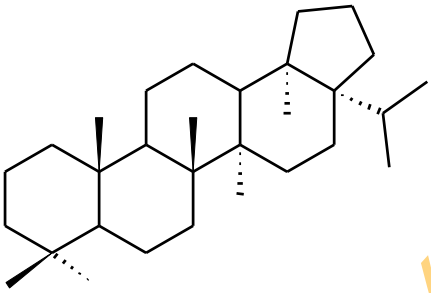

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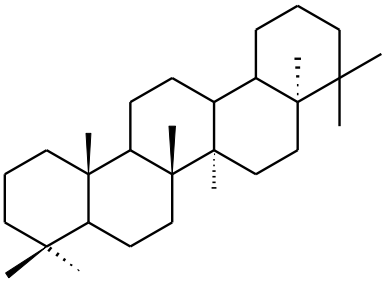

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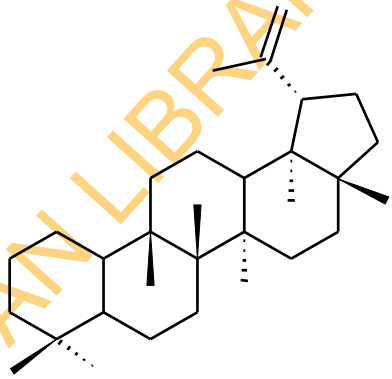

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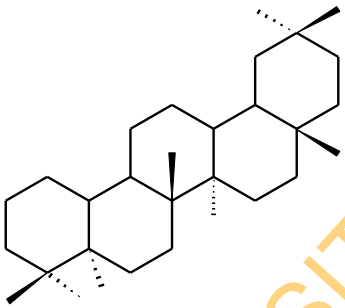

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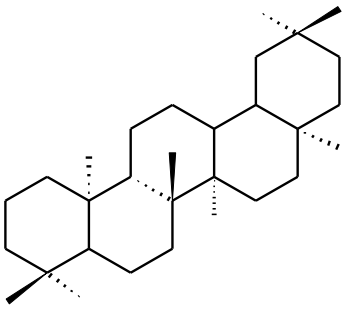

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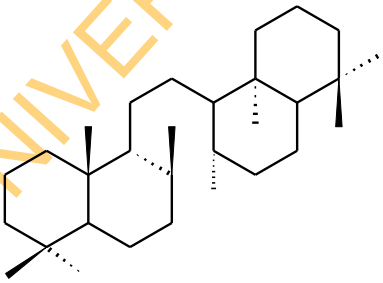

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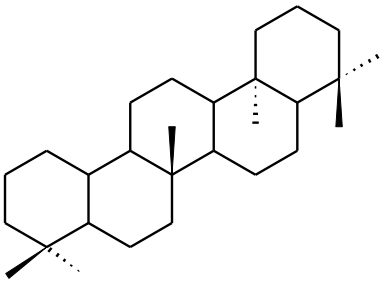

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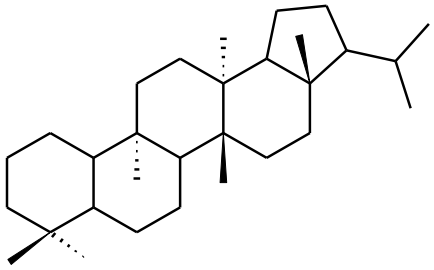

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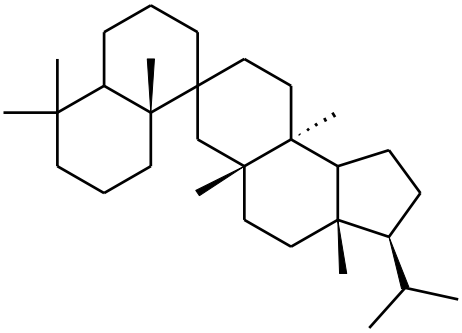

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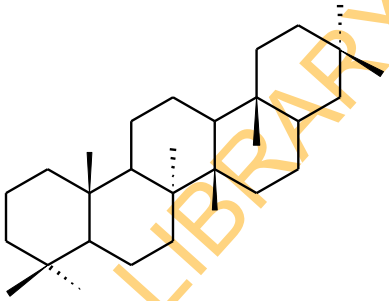

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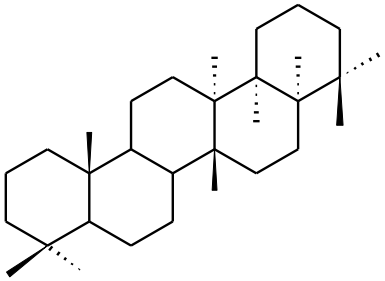

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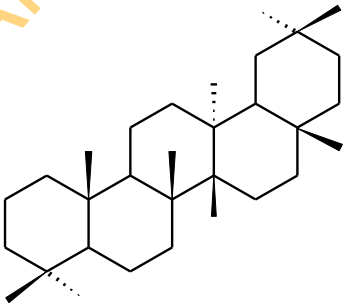

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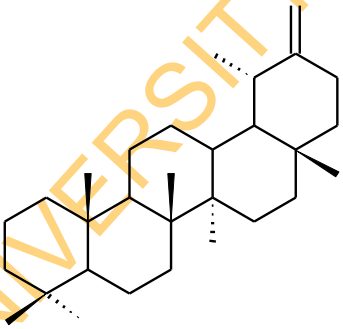

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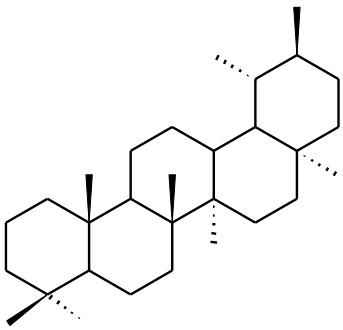

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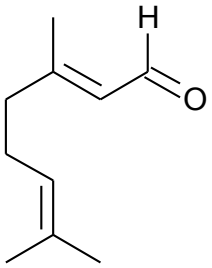
Some plants contain large quantities of triterpenes in their latex and resins where, in addition to their physiological functions, they act as a chemical defence against pathogens and herbivores (Brown, 1998).

2.9.2 Pharmacological relevance of terpenoids

Terpenoids are used extensively for their aromatic qualities. They play a role in traditional herbal remedies and are under investigation for antibacterial, antineoplastic, and other pharmaceutical functions. Terpenoids contribute to the scent of eucalyptus, the flavours of cinnamon, cloves, and ginger, and the colour of yellow flowers. Well-known terpenoids include; citral [2.51], and menthol [2.52], in the plant *Salvia divinorum*. Isovaleric acid [2.22] has a strong pungent cheesy or sweaty smell, but its volatile esters have pleasing scents and are used widely in perfumery. It has been suggested that it is the anticonvulsant agent in valerian. Eucalyptol [2.23] is an ingredient in many brands of mouthwash and cough suppressant. It controls airway mucus hypersecretion and asthma via anti-inflammatory cytokine inhibition. Although eucalyptol is used as an insecticide and insect repellent, it is one of many compounds that are attractive to males of various species of orchid bees, who apparently gather the chemical to synthesize pheromones. Artemisinin [2.24] is another important sesquiterpene lactone with highly potent antimalarial activity. Pleuremutillin [2.25] a diterpenoid from some species of mushroom (Novak and Shlaes 2010, and Sreedhar *et al.*, 2009) and 12- demethylmulticauline [2.53] also a diterpenoid from *Salvia multicaulis* (Ulubelen *et al.*, 1997) have been reported to have antimycobacterial activity. Several triterpenoids have been found to be of pharmacological relevance. Friedelin [2.54] is reported to exhibit antifeedant, anti-cancer, antiinflammatory, anticonvulsant, antidysentery and antiulcer activities (Gunatilaka, 1986). Ursolic [2.26] and oleanolic [2.4] acids and their derivatives have been reported to exhibit antitumour (Tokuda *et al.*, 1986), gastroprotective (Astudillo *et al.*, 2001), and antimicrobial activities (Woldemichael *et al.*,2003) with the 28- ester derivatives exhibiting weak antimicrobial effect (Weimann *et al.*,2002). The antimycobacterial activity of oleanolic acid [2.4] and its naturally occurring derivatives have been reported (Jim´enez-Arellanes *et al.*, 2003). Betulinic [2.1], ursolic [2.26] and oleanolic [2.4] acids and derivatives have been found to inhibit viral replication (Ma *et al.*, 2002; and Kashiwada *et al.*, 1998). Lanostane–type [2.55] triterpenes have been isolated from several species of mushrooms such as *Astraeus pteridis*. The compound has good inhibitory activity against *M. tuberculosis* (Stanikunaite *et al.*, 2008).



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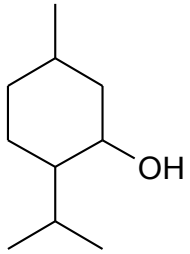

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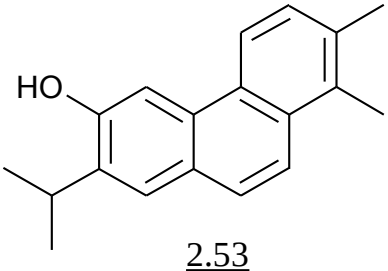

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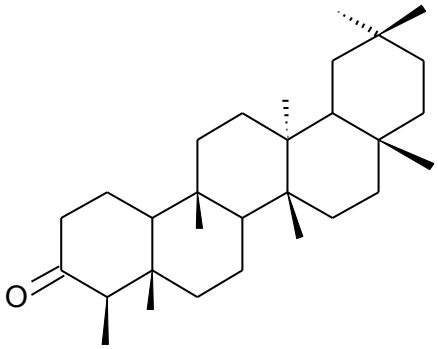

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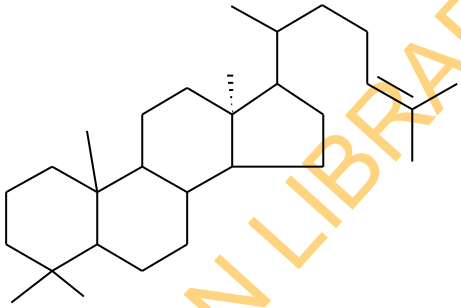

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2.9.3 Phenolic compounds

Phenolic compounds include all secondary plant metabolites that possess an aromatic ring bearing one or more hydroxyl groups and include phenols and free phenolic acids, phenylpropanoids, flavonoids, tannins, and quinine pigments. Their biosynthetic origin is through the shikimate pathway (Figure 2.4) (Finar, 2000; Petrussa *et al.*, 2013).

2.9.3.1 Flavonoids

Flavonoids are naturally occurring phytophenolic compounds. Like every other phytophenol, they possess an aromatic ring bearing one or more hydroxyl groups. They possess a 15- carbon atom skeleton commonly refered to as the C_6-C_3-C_6 system. Flavonoids are highly ubiquitous in higher plants. They are preponderant in flower pigments of most angiosperms. They also occur in other parts of the plants like seeds, and barks. In most naturally occurring flavonoids, the sugar moiety is linked to the flavone aglycone nucleus at position C_{-3}. Generally, the 15-carbon skeleton of most flavonoids consist of a "chromane" ring and an aromatic benzene ring as a substituent at position 2, 3, or 4. This chromane-benzene C_6-C_3-C_6 nucleus is known as the "flavone skeleton" (Deans, 1963). In some few and often rare cases, the six- membered heterocyclic epoxide ring C could exist as an open 3-carbon chain as in chalcones or as a five-membered furanoid ring

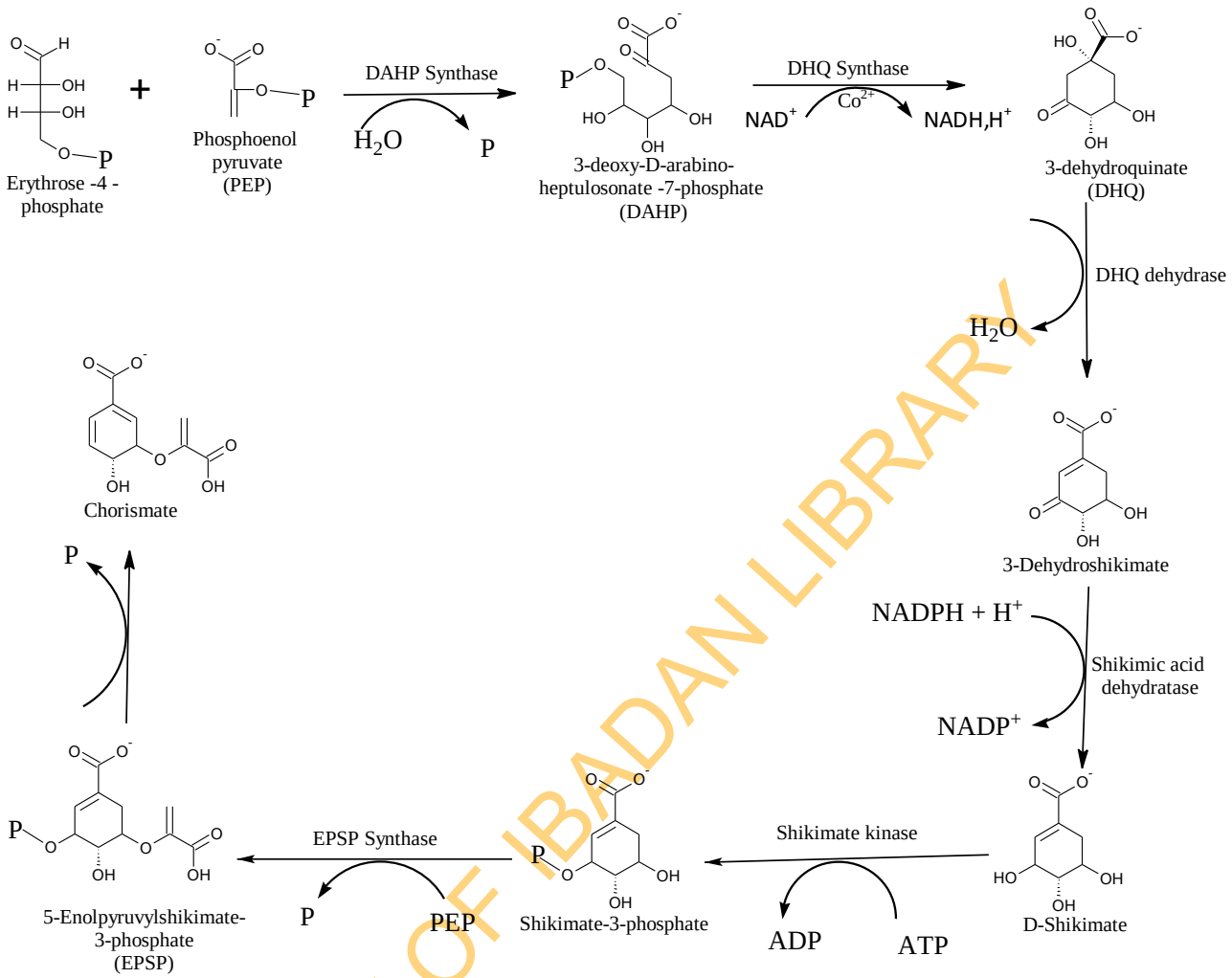
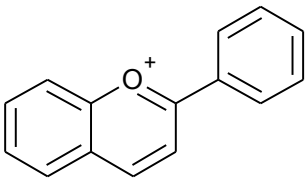


Figure 2.4: The Shikimate pathway (Petrussa *et al.*, 2013)

as in aurones. These structural variations in the heterocyclic C-ring of flavonoids and the interlinkage between benzopyran and benzene groups are the basis for the classification of flavonoids into anthocyanidins [2.56], flavones [2.57], flavonols [2.58], chalcones [2.59], aurones [2.60], isoflavonoids [2.61], flavanones [2.62], neoflavonoids [2.63] and related coumarines[2.64**]** (Deans, 1963; Harbourne, 1998; Finar, 2000). The anthocyanins, flavones, and flavonols, are the common naturally occurring flavonoids. They are therefore referred to as the "major flavonoids" (Deans, 1963; Harbourne, 1998; Finar, 2000). The others are of rare occurrence and are referred to as the minor flavonoids. Of the minor flavonoids, the isoflavonoids and the neoflavonoids, in which the aromatic ring B is attached to the ring C at positions C_3 and C_4 respectively, are regarded as the "abnormal flavonoids"(Deans, 1963; Harbourne 1998; Finar, 2000).



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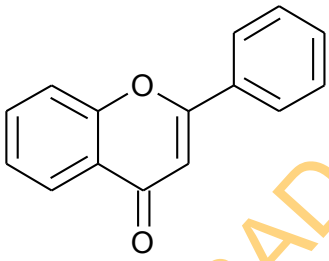

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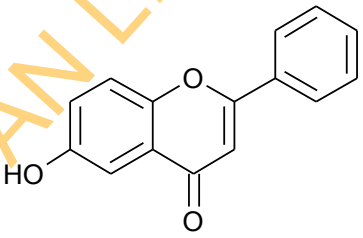

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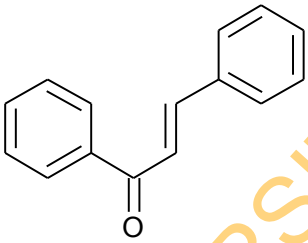

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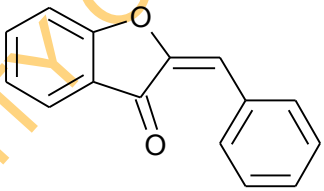

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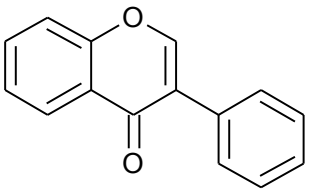

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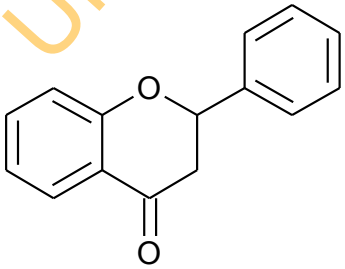

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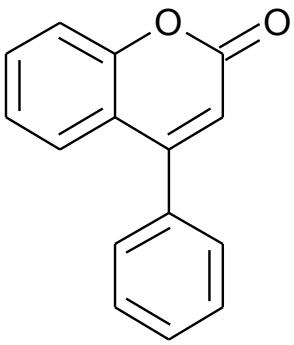

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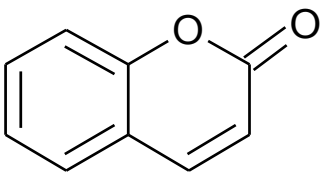

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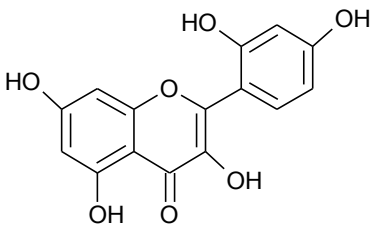
2.9.3.2 Pharmaological relevance of flavonoids

Flavonoids are of immense pharmacological importance. Claus *et al.* (1970) reported on the capillary vaso-constriction, and permeability increasing activities of flavonoid glycosides. Quercetin [2.18], an ubiquitous flavonoid, has been identified as the anti-diarrrhoea principle in *Euphorbia hirta* L (Galvez *et al.*, 1993). The flavonoids, quercetin [2.18], morin [2.65], gossypterin [2.66], chrysin [2.67], myricetin [2.19], and catechin [2.68] have been shown to exhibit *in vitro* inhibition of the oxidation of low density lipoprotein (LDL) cholesterol. This inhibition has been adduced to their anti-oxidant properties (Frankel, 1993; Knekt, 1996; Wiseman, 1996). The insecticidal, fish poisoning, anaesthetic, and anti-inflammatory activities of rotenone [2.69] -an isoflavonoids, have been reported (Dean, 1963). The antiviral (Musci, 1987) and antibacterial (Cushnie *et al.*, 2003) activities of flavonoids have also been reported.



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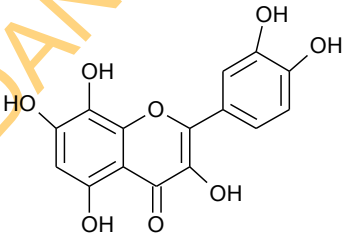

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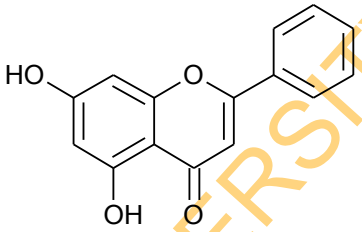

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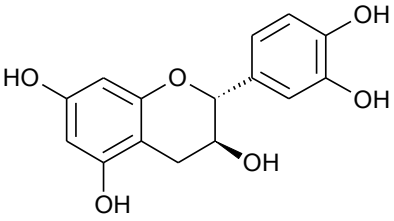

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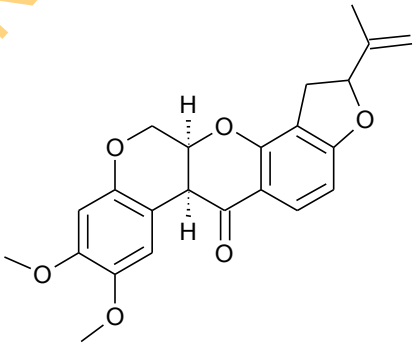

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2.10 Structural elucidation of isolated secondary plant metabolites

The structure of a pure secondary metabolite isolated from plants could be elucidated using combination of spectroscopic techniques like; ultraviolet/visible, fourier transform infra-red, mass and nuclear magnetic resonance spectroscopic techniques. In this section, the application of these spectroscopic techniques in the elucidation of triterpenoids and flavonoids will be reviewed considering their relevance to this study.

2.10.1 Ultraviolet and visible spectroscopy

Ultraviolet and visible spectroscopy is of relevance in the elucidation of the structure of pure compounds. It gives information about the chromophores and conjugation present in the structure of the compound. The distinct nature of the aromatic chromophore in flavonoids makes their UV spectra a useful tool in the determination of their basic structure, number and position of hydroxyl group as well as sugar groups in the case of glycosidic flavonoids (Agrawall, 1989). Absorption bands of the various categories of flavonoid compounds are known and have been reported. Flavones have two strong absorption bands at 250-270 nm (band I due to ring A absorption) and 300-380 nm (band II due to ring B absorption) Flavonols have absorption band at 350-390 nm and 250-270 nm. The anthocyanins show two absorption bands at 470-560 nm (Visible region) and 275-280 nm (UV region). Because flavonoids behave like enols, their absorption maxima can be shifted by certain reagents, particularly chelating bases or Lewis acid (Hendrickson, 1965). The use of shift reagents has led to the detection of flavonoids with a free hydroxyl group at C_{-5}. Also, lack of 3-methoxy substitution or presence of 3, 4-dihydroxy groups can be detected with shift reagents as in flavones (Harbourne, 1998).

Triterpenoids of the pentacyclic class have a characteristic absorption maximum at 310 nm in sulphuric acid due to the formation of a carbocation. The substituents on the ring do not normally affect this maximum (Ponomarev *et al.*, 1973).

2.10.2 Infra-red spectroscopy

Infrared (IR) spectroscopy is based on the principle that functional groups have vibrational frequencies, characteristic of the functional group (Dudley and Fleming, 1980). This spectroscopic method provides information on functional groups of various types. Peaks representing functional groups are expected in the I.R spectra of flavonoids and triterpenoids. Flavonoids and related naturally occurring compounds are benzopyrones. As

such their IR spectra have the characteristic pyrone carbonyl(C=O) stretching frequency in the region of 1700-1750 cm^{-1}, strong absorption bands at 1600-1660 cm^{-1} due to the C=C skeletal vibrations, the aromatic ring band at 1500 cm^{-1}, OH stretching frequency around 3400 cm^{-1}, and C-O stretching at 1050- 1200 cm^{-1}. Triterpenoids exist in the free saturated or unsaturated form, or as their respective oxo, alcohol, esters, glycosides or ethers derivatives. Based on these structural variations, their IR spectra show vibrational frequencies corresponding to hydroxyl group giving a broad peak at 3400 cm^{-1}, carbonyl (C=O) stretching frequency in the region 1680-1750 cm^{-1}, C-H aliphatic stretching arbsorption at 2800 -2980 cm^{-1} and C-O stretching at 1050- 1200 cm^{-1} for compounds containing them (Finar, 2000).

2.10.3 Mass spectroscopy

When a molecule is bombarded with electrons, it is converted to a highly energetic positively charged ion known as the molecular ion. This breaks up into smaller fragment ions that are separated by deflection in a variable magnetic field depending on their mass and charge ratio. This is the basis of mass spectroscopy in structural elucidation of organic molecules. Organic chemists use mass spectrometry in three principal ways:

1. to measure relative molecular masses with very high accuracy thereby deducing the exact molecular formulae.
2. to detect within a molecule the places at which it prefers to fragment. From this, the presence of recognizable groupings within the molecules can be deduced.
3. as a method for identifying analytes by comparison of their mass spectra with libraries of mass spectra of known compounds

A typical mass spectrometer consists of three operational components. These are the ionisation source, mass analysers and detector. Based on the ionisation mode, mass spectroscopic techniques are classified to include Chemical Ionisation (CI), Atmospheric Pressure CI (APCI), Electron Impact (EI), Electro-Spray Ionization (ESI), Fast Atom Bombardment (FAB), Field Desorption/Field Ionisation (FD/FI), Matrix Assisted Laser Desorption Ionisation (MALDI) and Thermospray Ionisation (TI).

Electron Impact Ionisation (EI): A beam of electrons passes through a gas-phase sample and collides with neutral analyte molcules to produce a positively charged ion or a

fragment ion. Generally, electrons with energies of 70 eV are used. This method is applicable to all volatile compounds (>103 Da) and gives reproducible mass spectra with fragmentation to provide structural information (Rose and Johnstone, 2001).

Chemical Ionisation (CI): In this method, a reagent gas is first ionized by electron impact and then subsequently reacts with analyte molecules to produce analyte ions. This method gives molecular weight information and reduced fragmentation in comparison to EI.

Fast Atom Bombardment (FAB): Ions are produced by using a high current of bombarding particles. This is a soft ionisation technique and is suitable for analysis of low volatility species. It produces large peaks for the pseudo-molecular ion species $[M+H]^+$ and $[M-H]^-$ along with other fragment ions and some higher mass cluster ions and dimers (Kalsi, 2004).

Electro-Spray Ionization (ESI): A solution is nebulized under atmospheric pressure and exposed to a high electrical field which creates a charge on the surface of the droplet. The production of multiple charged ions makes electrospray extremely useful for accurate mass measurement, particularly for thermally labile, high molecular mass substances (ie. proteins, oligonucleotides, synthetic polymers, etc.) (Hoffman and Stroobank, 2002)

Matrix-Assisted Laser Desorption/Ionization (MALDI): This is a soft ionization technique suitable for the analysis of molecules which tend to be fragile and fragment when ionized by more conventional ionization methods such as biomolecules (biopolymers e.g DNA, proteins, peptides and sugars) and large organic molecules (such as polymers, dendrimers and other macromolecules). It is similar in character to electrospray ionization both in relative softness and the ions produced (although it causes many fewer multiple charged ions). MALDI is also more tolerant of salts and complex mixture analysis than ESI (Rose and Johnstone, 2001).

The mass analyzer sorts the ions by their masses by applying electromagnetic fields. Examples include: Time-of-Flight (TOF), magnetic sectors, Fourier transform and quadrupole ion traps.

The high resolution mass spectrometry (HR-MS) is applied for the establishment of the exact molecular formula since it gives information about the elemental composition through exact mass measurements.

Pentacyclic triterpenoids exhibit diagnostic fragmentation pattern which depends on the nature of unsaturation or sort of functional groups contained in the ring. One of the important features of the EI-MS of terpenoids is the Retro-Diels-Alder (RDA) cleavage of the molecule at the ring containing a double bond. This provides useful information as regards the substituents in the ring system (Karliner and Djerassi, 1966; Budzikiewicz *et al.*, 1963; Hostettmann and Marston, 1995). A C_{-5} unsaturated triterpenoid with no substitution on rings C, D and E as in fornane, oleanane and lupane type skeletons have a base peak at m/z 274 or 259. However, with substitution, the base peak shifts to higher m/z values. Triterpenoids with unsaturation (double bond) at C_{-7}, or C_{-8} or C_{-9}, C_{-11}, fragment in either of two ways depending on which skeleton bears the double bond (Levin *et. al.* 1988). Strong peaks at m/z M^+ - 167 and M^+ - 15 occur in arborane and fernane skeletons that have no substitution at C_{-12} (Obafemi *et al.*,1979) The diagnostic peak of oleanene or ursane skeletons with double bond at C_{-12} and no substitution in rings D and E is at m/z 218, while a peak at m/z 203 is formed where there are functional groups in rings D and E in addition to the mass of such functional groups (Obafemi *et al.*,1979; Mahato *et al.*, 1992; Ogunkoya, 1981). As a result of RDA cleavage at ring C, the unsaturated bond of compounds with a 12-unsaturated oleanene or ursene skeleton give diagnostic m/z ion peaks at 203, 189 and 133 (due to the upper right of the molecule) and 207 (3-OH), 205(3-oxo) or 191 for the left part of the molecule (Budzikiewicz *et al.*, 1963). The introduction of a ketone group at postion C-12 as in saturated oleananes and ursanes leads to an abundant fragment at m/z 234 due to the RDA decomposition of the enol form (Budzikiewicz *et al.*, 1963; Karliner and Djerassi, 1966). Peaks at m/z 189 and 205 are diagnostic of saturated triterpenoids of lupane and hopane skeleton (Kumar and Seshadri, 1976).Lupanes can be distinguished from 12-oleanenes as the upper part yield fragmentation m/z ions at 191 or 189 for saturated and unsaturated side chains unlike m/z 203 and 189 for 12-oleanenes. The loss of a sugar moiety m/z 180, 164, for hexose and 150 and/or 134 for pentose is indicative of triterpene glycosides. Flavonoids glycosides exhibit similar loss of sugar moiety but with characteristic loss of 28 mass units equivalent to carbon monoxide, and 75, 78, 91 and 106 mass units due to their charactistic aryl unit.

Mass fragmentation pattern of related phenolic compounds like furano- and pyranocoumarins follows in a similar fashion.

2.10.4 Nuclear Magnetic Resonance (NMR) spectroscopy

Nuclear Magnetic Resonance (NMR) is a form of absorption spectrometry based on the principle that under appropriate conditions in magnetic field, a sample can absorb electromagnetic radiation in the radio frequency region at frequencies governed by the characteristics of the sample. Proton (^{1}H-) and carbon-13 (^{13}C-) NMR spectroscopy is used in the elucidation of the structure of pure forms of isolated secondary metabolites using various techniques of signal assignments like the Attached Proton Test (APT), Distortionless Enhancement Polarisation Transfer (DEPT) and two dimensional 2D-NMR spectroscopy (Das and Mahato, 1983; Mahato *et al.*,1992.).

In the NMR spectra of flavonoids and related compounds the H_{-3} and H_{-4} protons exhibit characteristic chemical shifts which enable the substituted coumarins to distinguish from one another. In the case of the glycoside derivatives, the vast majority of proton resonances of the carbohydrate moiety appear as overlapping signals in a very small spectra width of 3.0-4.2 ppm with the carbinol carbon signals resonating in the region of 68 – 80 ppm (Table 2.2), (Agrawall and Jain, 1992; Hostettmann and Marston, 1995).The upfield shift of about 0.17 ppm of H_{-3} proton in 7-oxygenated coumarins as compared with the parent compound coumarin, is due to the electron released resulting in the electron density at C_{-3}. In contrast, an oxygen or alkyl substituent at C_{-5} shifts the resonance of H_{-4} downfield by 0-3 ppm due to the peri effect.

For triterpenoids, their ^{1}H-NMR spectra are usually complex and tedious to interprete, the methyl peaks are readily resolved as sharp singlet or doublet absorption peaks in the upfield region of 0.5-2.0 ppm (Kojima and Ogura, 1989, and Ageta and Arai, 1983). Valuable information from the analysis of the ^{1}H-NMR signal peaks of the protons on oxygen-bearing carbon atoms could make it possible for unambiguous assignment of the configuration of the hydroxyl groups at C_{-2}, C_{-3} C_{-23}, and C_{-24} of oleanane and ursane triterpenes (Kojima and Ogura, 1989). For example, the distances ($\Delta\delta_{2-3}$) between the chemical shifts of H_{-2} and H_{-3} are 0.88 ppm in 2β,3β-$(OH)_2$ and 0.7 ppm in 2β,3β-$(OAc)_2$ > 0.69 ppm in 2α,3β-$(OH)_2$ and 0.35 ppm in 2α,3β-$(OAc)_2$ > 0.57 ppm in 2α,3α-$(OH)_2$ and

0.27 ppm 2α,3α-$(OAc)_2$> 0.12 ppm in 2β,3α-$(OH)_2$ and –0.08 ppm in 2β,3α-$(OAc)_2$. The values for the dihydroxy compounds are larger (ca 0.2 to 0.3 ppm) than those of the corresponding diacetates because of the lower downfield shift of the H_{-3} on acetylation (Kojima and Ogura, 1989).

^{13}C-NMR spectroscopy is an improvement on ^{1}H-NMR technique. It has provided for easier elucidation of structures particularly complex ones. The limitations of the ^{1}H- NMR spectroscopy in the resolution of complexities in structural elucidation of complex compounds are effectively taken care of by ^{13}C-NMR spectroscopy. Although it requires larger amount of sample for analysis, it is more informative compared to ^{1}H-NMR in the chemistry of triterpenoids. Several techniques such as the APT, DEPT and Insensitive Nuclei Enhanced by Polarisation Transfer (INEPT) are now used to distinguish between carbon types (i.e quaternary, tertiary, secondary and primary). Like ^{1}H-NMR, introduction of substituents on the pentacyclic carbon skeleton could result in the shielding or deshielding effect. This is observed on introducing a hydroxyl group into the pentacyclic system which results in downfield shifts of 34- 50 ppm for α–carbons and 2-10 ppm for β–carbons and upfield shifts of 0-9 ppm for γ– carbons (Mahato and Kundu, 1994). This phenomenon is commonly referred to as the hydroxyl substituent effect. An added advantage of NMR spectroscopy in unambiguous assignment of the configuration of pentacyclic triterpenoids could be linked to the use of 2D-spectrocopy that incorporates second order effects in assigning all peaks in the complex spectrum of a triterpenoid. This reveals nuclei that are directly or indirectly bonded. This can be between two protons (^{1}H-^{1}H-COSY) or a proton and a carbon (^{1}H-^{13}C-COSY) (Gonzalez–sirra *et al.*, 1989).

Table 2.2: ^{13}C chemical shift assignment for some sugars commonly found in glycosides.

C-position	β-D-Glc	α-D-Glc	β-D-Gal	α-D-Gal	β-D-Man	α-D-Man	β-L-Rha	α-L-Rha	β-L-Fuc	α-L-Fuc
C-1	104.0	100.0	104.5	100.1	102.3	102.2	102.4	102.1	97.2	93.1
C-2	74.1	72.2	71.7	69.2	71.7	71.4	71.8	71.2	72.7	69.1
C-3	76.8	74.1	73.8	70.5	74.5	72.1	74.1	71.5	73.9	70.3
C-4	70.6	70.6	69.7	70.2	68.4	68.3	73.4	73.3	72.4	72.8
C-5	76.8	72.5	76.0	71.6	77.6	73.9	73.4	69.5	71.6	67.1
C-6	61.8	61.6	62.0	62.2	62.6	62.5	17.9	17.9	16.3	16.3

Key: Glc : Glucose, Gal: Galactose, Man.: Mannose, Rha: Rhamnose, Fuc: Fucose

(Agrawall and Jain, 1992; Hostettmann and Marston, 1995)

2.11. Tuberculosis

2.11.1 Causes and Epidemiology

In humans, *Mycobacterium tuberculosis* is the primary causative bacterium of tuberculosis (TB) although other mycobacteria such as *M. bovis, M. africanum, M. canetti, and M. microti* could be responsible. Mycobacteria are aerobic and non-motile rod-like bacilli (Figure 2.5) that are characteristically acid-alcohol fast (Ryan and Ray, 2004). Mycobacteria do not seem to fit the Gram-positive category from an empirical standpoint (i.e. they do not retain the crystal violet stain), they are classified as an acid-fast Gram-positive bacterium due to their lack of an outer cell membrane. All *Mycobacterium* species share a characteristic cell wall, thicker than the one in many other bacteria, which is hydrophobic, waxy, and rich in mycolic acid [2.70] derivatives. The cell wall consists of the hydrophobic mycolate layer and a peptidoglycan layer held together by polysaccharide residue. This explains the hardiness of this genus. The biosynthetic pathways of cell wall components are potential targets for new drugs for tuberculosis (Bhamidi *et al.*, 2009).

Based on the World Health Organization (WHO) report, about 2 billion people-equivalent to one third of the world's population, have been exposed to the tuberculosis pathogen. However, on annual basis, 8 million people become ill with tuberculosis, and 2 million people die from the disease worldwide. The annual incidence rate varies from 356 per 100,000 in Africa to 41 per 100,000 in the Americas. Tuberculosis is the world's greatest infectious killer of women of reproductive age and the leading cause of death among people with HIV/AIDS (WHO, 2006; CDC, 2003; Cox, 2004). The rise in HIV infections and the neglect of tuberculosis control programmes have enabled a resurgence of tuberculosis. The emergence of drug-resistant strains has also contributed to this new epidemic. The rate at which new TB cases occur varies widely, even in neighbouring countries, apparently because of the differences in health care system. Generally, overcrowding, poverty and severe malnutrition with its damaging effects on the immune system which are common in parts of the developing world increase the risk of developing active tuberculosis (Schaible and Kaufmann, 2007; and Lonnroth and Raviglione, 2008).

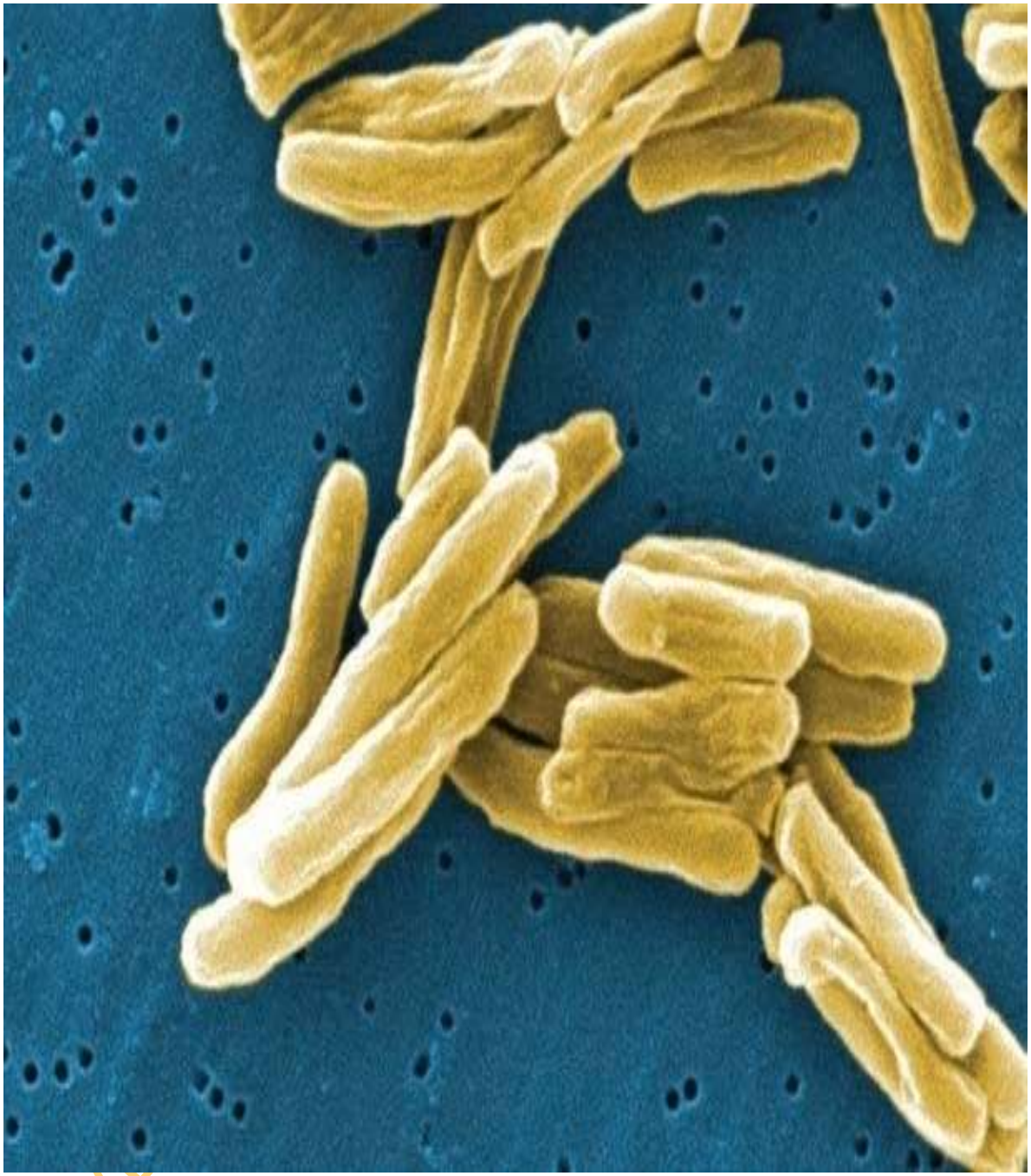
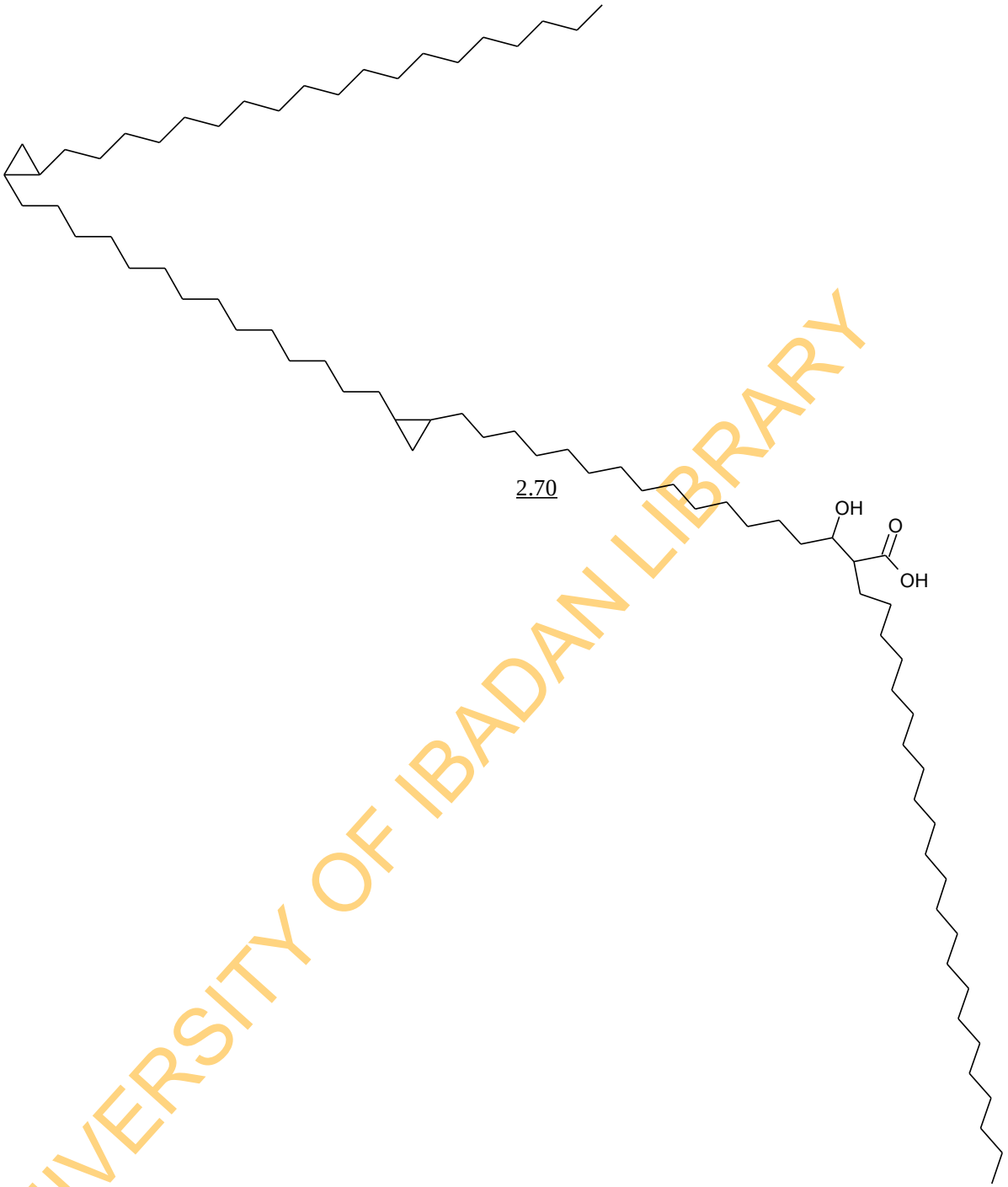


Figure 2.5: Rod- like bacilli of *M. tuberculosis*



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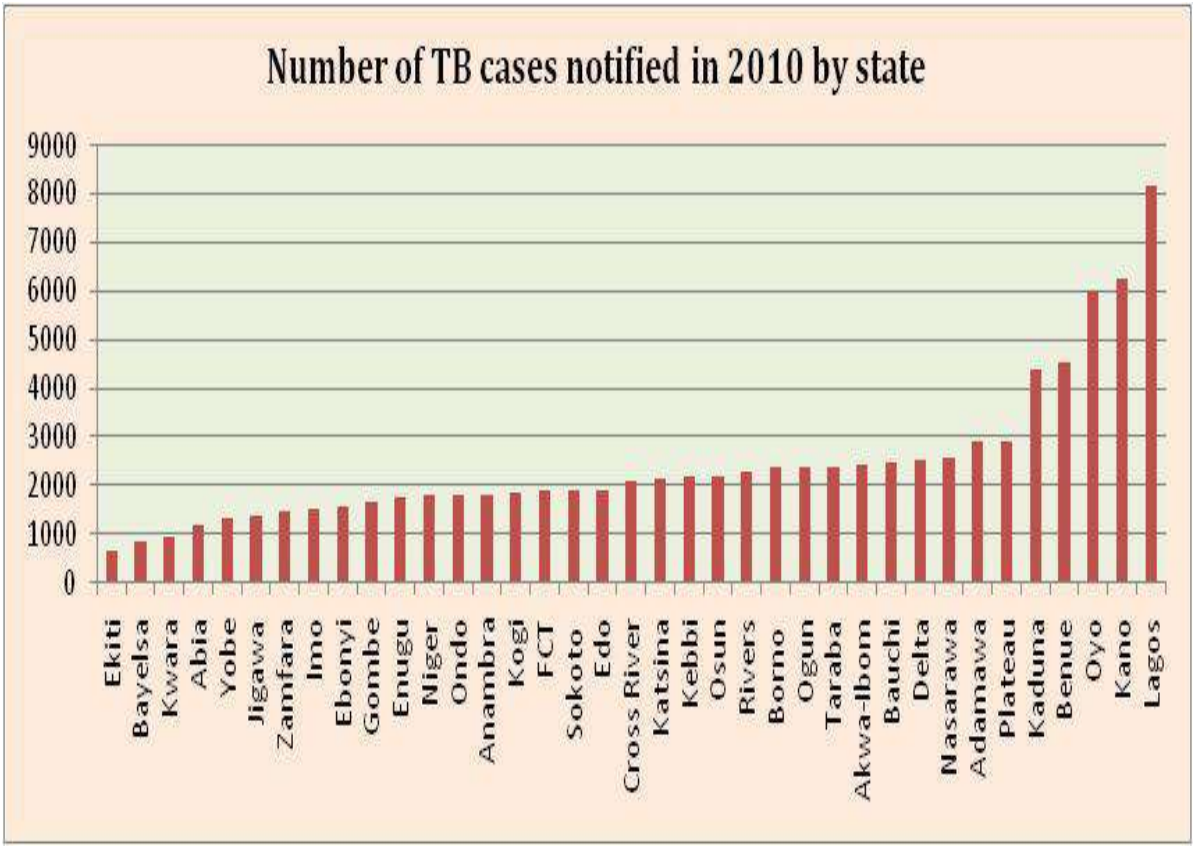
In the developed countries, tuberculosis is less common and is mainly an urban disease. According to the WHO's report, Nigeria had about 320,000 cases of all forms of TB with yearly prevalence of 199 cases per 100,000 population and 210,000 new cases representing an incidence rate of 133 per 100,000 populations (WHO, 2013). The United States economic report (2012), shows densely populated states like Lagos, Kano, and Oyo in Nigeria to have the highest cases of TB infections (Figure 2.6). In the United Kingdom, TB incidence ranges from 40 per 100,000 in London to less than 5 per 100,000 in the rural South West of England; the national average is 13 per 100,000. The highest rates in Western Europe are in Portugal (31.1 per 100,000 in 2005) and Spain (20 per 100,000). These rates are lower when compared with 113 per 100,000 in China and 64 per 100,000 in Brazil. The incidence of TB also varies with age. In Africa, TB primarily affects adolescents and young adults. However, in countries such as the United States, where the incidence of TB has gone from high to low, TB is mainly a disease of older people, or of the immuno-compromised. Co-infection with HIV is a particular problem in Sub-Saharan Africa. Smoking more than 20 sticks of cigarettes a day also increases the risk of TB by 200 -400%. *Diabetes mellitus* is also an important risk factor that is growing in importance in developing countries. Other disease states that increase the risk of developing tuberculosis are *Hodgkin lymphoma*, end-stage renal disease, chronic lung disease, and malnutrition (Kumar *et al.*, 2007; Sobero and Peabody, 2006; WHO, 2007 and CDC, 2005). Generally, overcrowding occassioned by poverty and severe malnutrition, with its damaging effects on the immune system, which are common in parts of the developing world increase the risk of developing active tuberculosis (Schaible and Kaufmann, 2007; and Lonnroth and Raviglione, 2008).

2.11.2 Transmission and Pathogenesis

Tuberculosis is spread through the air when infected patients cough, sneeze or spit (Figure 2.7). The TB infection in humans could result in latent infection and about 10% of latent infections will progress into active disease. Active TB, if left untreated, kills over 50% of its sufferers. The primary site of TB infection in the lungs is called the Ghon focus, and it is generally located in either the upper part of the lower lobe, or the lower part of the upper lobe (Houben *et al.*, 2006). Bacteria are picked up by dendritic cells, which do not allow replication, although these cells can transport the bacilli to local (mediastinal) lymph nodes. Further spread is through the bloodstream



United States economic report (2012).

Figure 2.6: Epidemiological trend of tuberculosis in Nigeria.

to other tissues and organs where secondary TB lesions can develop in other parts of the lung (particularly the apex of the upper lobes), peripheral lymph nodes, kidneys, brain, and bone. All parts of the body can be affected by the disease, though it rarely affects the heart, skeletal muscles, pancreas and thyroid.

Tuberculosis is classified as one of the granulomatous inflammatory conditions. Macrophages, T lymphocytes, B lymphocytes and fibroblasts are among the cells that aggregate to form a granuloma, with lymphocytes surrounding the infected macrophages. The granuloma functions not only to prevent dissemination of the mycobacteria, but also provides a local environment for communication among cells of the immune system. Within the granuloma, T lymphocytes secrete cytokines such as interferon gamma, which activates macrophages to destroy the bacteria with which they are infected. Cytotoxic T cells can also directly kill infected cells, by secreting perforin and granulysin (Houben *et al.,* 2006). Importantly, bacteria are not always eliminated within the granuloma, but can become dormant, resulting in a latent infection. Another feature of the granulomas of human tuberculosis is the development of cell death, also called necrosis, in the center of tubercles. To the ordinary eye this has the texture of soft white cheese and has been termed caseous necrosis (Grosset, 2003). If TB bacteria gain entry into the bloodstream from an area of damaged tissue they spread through the body and set up many foci of infection- all appearing as tiny white tubercles in the tissues. This severe form of TB disease is most common in infants and the elderly and it is called miliary tuberculosis. Patients with this disseminated TB are reported to have an approximate fatality rate of 20%, even with intensive treatment (Kim *et al.,* 2003). In many patients, the infection waxes and wanes. Tissue destruction and necrosis are balanced by healing and fibrosis. Affected tissue is replaced by scars and cavities filled with cheese-like white necrotic material. During active disease, some of these cavities are joined to the bronchi and could be coughed up. This cough contains living bacteria and can therefore pass on infection. If untreated, infection with *Mycobacterium tuberculosis* can become lobar pneumonia. (Sambandamurthy *et al.,* 2002).

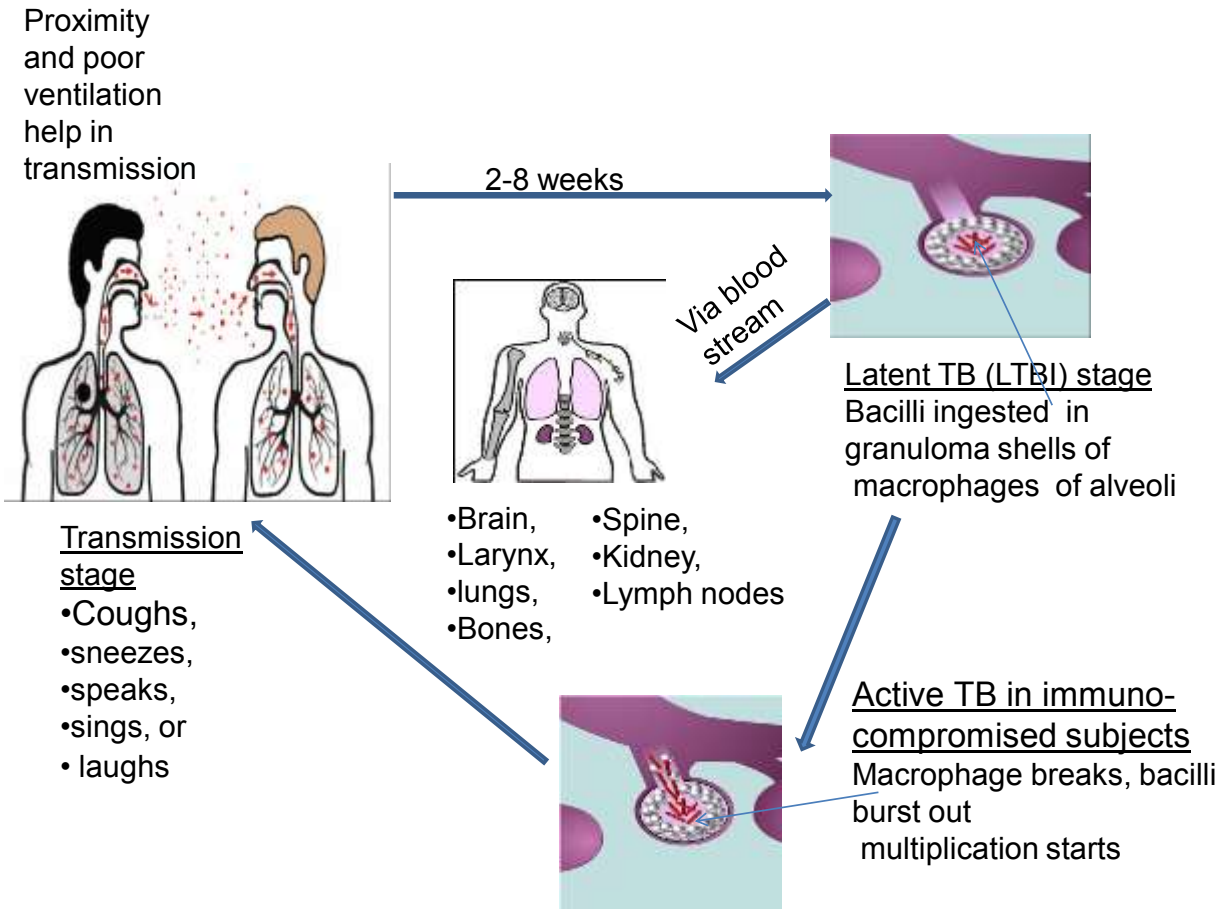


Figure 2.7: Transmission cycle of tuberculosis

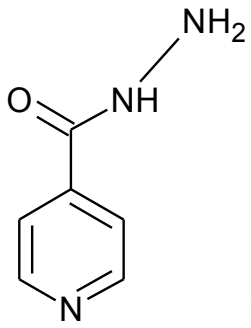
2.11.3. Treatment and Prevention

Treatment with appropriate antibiotics kills bacteria and allows healing to take place. Upon cure, affected areas are eventually replaced by scar tissue (Sambandamurthy, *et al*, 2002). TB prevention and control takes two parallel approaches. In the first, people with TB and their contacts are identified and then treated. Identification of infections often involves testing high-risk groups for TB. In the second approach, children are vaccinated to protect them from TB. Unfortunately, no vaccine is available that provides reliable protection for adults. However, in tropical areas where the levels of other species of mycobacteria are high, exposure to non-tuberculous mycobacteria gives some protection against TB. The World Health Organization (WHO) declared TB as a global health emergency in 1993, and the Stop TB Partnership developed a global plan to stop tuberculosis that aims to save 14 million lives between 2006 and 2015. Since humans are the only host of *Mycobacterium tuberculosis*, eradication would be possible: a goal that would be helped greatly by an effective vaccine. Many countries use the Bacillus Calmette-Guérin (BCG) vaccine as part of their TB control programmes, especially for infants. According to the WHO, 85% of infants in 172 countries were immunized with the BCG vaccine in 1993. This makes it to be the most often used vaccine worldwide. The Directly Observed Treatment Short-course (DOTS) strategy of tuberculosis treatment recommended by the WHO was based on clinical trials done in the 1970s by the Tuberculosis Research centre, Chennai, India. The two commonly used antibiotics in the DOTS are isoniazid [2.71] and rifampicin [2.72]. However, instead of the short course of antibiotics typically used to cure other bacterial infections, TB requires much longer periods of treatment (around 6 to 24 months) to entirely eliminate mycobacteria from the body (CDC, 2003). Latent TB treatment usually uses a single antibiotic while active TB disease is best treated with a combination of several antibiotics to reduce the risk of the bacteria developing antibiotic resistance (Parish and Stoker, 1999). People with latent infections are treated to prevent them from progressing to active TB disease later in life. Drug resistance tuberculosis is transmitted in the same way as regular TB. Primary resistance occurs in persons who are infected with a resistant strain of TB. A patient with drug susceptible TB will develop secondary resistance (acquired resistance) during TB therapy if there is inadequate treatment or if he did not take the prescribed regimen appropriately (Parish and Stoker, 1999). Drug resistant TB is a public health issue in many developing countries as treatment is longer and requires more

expensive drugs. Multi Drug Resistance Tuberculosis (MDR-TB) is defined as resistance to the two most effective first line TB drugs: isoniazid [71] and rifampicin [72]. Extensively drug resistant TB (XDR-TB) is also resistant to three or more of the six classes of second line drugs.

2.2.11.4 Some orthodox drugs used in the treatment of tuberculosis

2.11.4.1 Isoniazid



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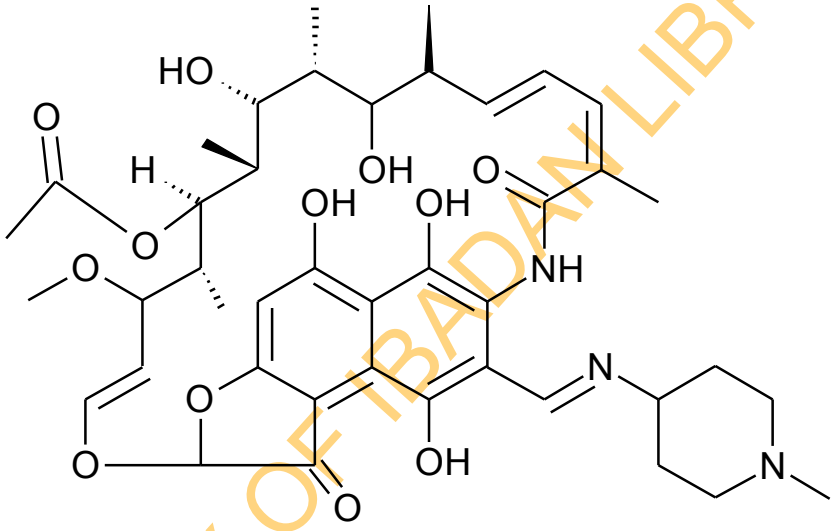
Isoniazid [2.71] systemically known as isonicotinylhydrazine (INH), is the first-line antituberculosis medication. It was first discovered in 1912, and later in 1951 it was found to be effective against tuberculosis by inhibiting its mycolic acid [2.70] synthesis. It is a pro-drug activated by KatG, a bacterial catalase-peroxidase enzyme in *M. tuberculosis* (Suarez *et al.*, 2009). The KatG couples the isonicotinic acyl with NADH to form isonicotinic acyl-NADH complex. This complex binds tightly to the enoyl-acyl carrier protein reductase known as InhA, thereby blocking the natural enoyl-AcpM substrate and the action of fatty acid synthase (Suarez *et al.*, 2009). This process inhibits the synthesis of mycolic acid, required for the mycobacterial cell wall. Isoniazid is bactericidal to rapidly-dividing mycobacteria but is bacteriostatic if the mycobacterium is slow-growing. It is never used on its own to treat active tuberculosis because resistance to it quickly develops.

2.11.4.2. Rifampicin

Rifampicin [2.72] is used in the treatment of a number of bacterial infections. However, it is best known for activity against *Mycobacterium* strains. It can be used as a monotherapy in prophylaxis for a few days, against meningitis, but resistance

develops quickly during long treatment of active infections, so the drug is always used against active infections in combination with other antibiotics. It inhibits deoxyribonucleic acid (DNA)-dependent ribonucleic acid (RNA) polymerase in bacterial cells by binding to its beta-subunit, thus preventing transcription to RNA and subsequent translation to proteins (Coulson, 1994; Hardman *et al.,* 2001; O'Sullivan *et al.,* 2005). Its lipophilic nature makes it a good candidate for treating the meningitis form of tuberculosis, which requires distribution to the central nervous system and penetration through the blood-brain barrier.



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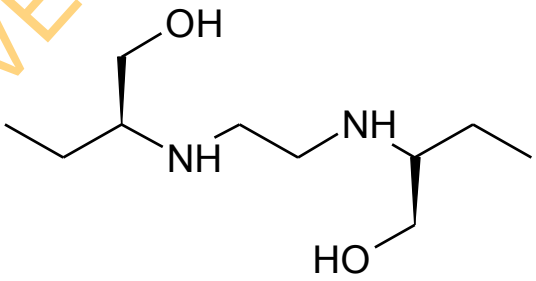
2.11.4.3. Ethambutol



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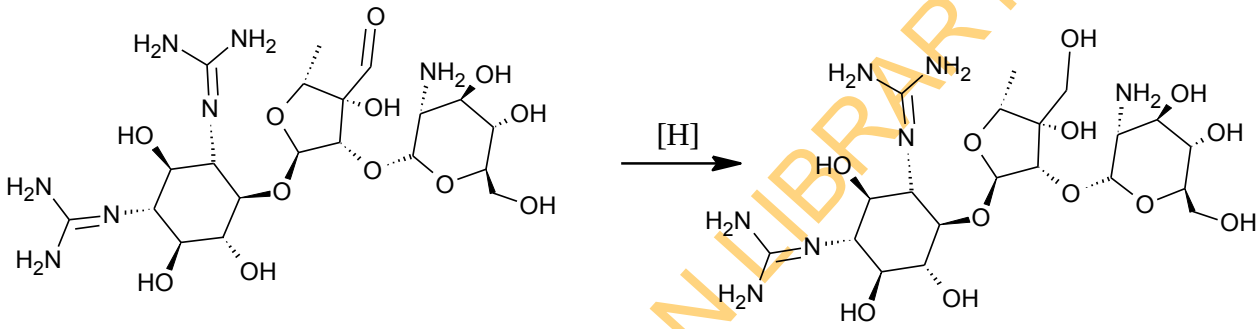
Ethambutol [2.73] systemically known as (2*S*, 2′*S*)-2, 2′-(ethane-1, 2-diyldiimino) dibutan-1-ol is a bacteriostatic antimycobacterial drug prescribed to treat tuberculosis

(Yendapally and Lee, 2008). It is usually given in combination with other tuberculosis drugs, such as isoniazid [2.71], rifampicin [2.72] and pyrazinamide [2.76]. It works by obstructing the formation of cell wall mycolic acids [2.70]. This disrupts the formation of cell wall peptidoglycan.This leads to increased permeability of the cell wall.

2.11.4.4. Dihydrostreptomycin



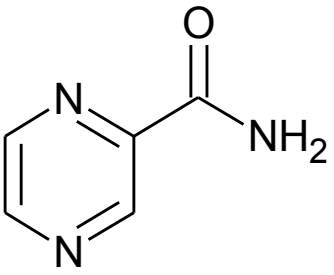
2.74 2.75

Dihydrostreptomycin [2.75] is one of the derivatives of streptomycin [2.74] class of aminoglycoside antibiotic derived from *Streptomyces griseus*. The streptomycins [2.74] are protein synthesis inhibitor. They binds to the small 16S rRNA of the 30S subunit of the bacterial ribosome, interfering with the binding of formyl-methionyl-tRNA to the 30S subunit (Sharma *et al.,* 2007; Voet and Voet, 2004). This leads to codon misreading, eventual inhibition of protein synthesis and ultimately death of microbial cells.

2.11.4.5 Pyrazinamide

Pyrazinamide [2.76] is a drug used to treat tuberculosis. The drug is largely bacteriostatic, but can be bactericidal on actively replicating tuberculosis bacteria. It is a prodrug that stops the growth of *Mycobacterium tuberculosis*. It is converted to the active form, pyrazinoic acid [2.77] by the enzyme pyrazinamidase (Zhang and Mitchison, 2003). Pyrazinoic acid [2.77] is thought to inhibit the enzyme fatty acid synthase (FAS) I, which is required by the bacterium to synthesize fatty acids. Pyrazinoic acid [2.77] binds to the ribosomal protein S1 (RpsA) and inhibits trans-translation. This may explain the ability of the drug to kill dormant mycobacteria (Zimhony *et al.,* 2000., Boshoff *et al.,* 2002., Zimhony *et al.,* 2007., and Ngo and

Zimhony, 2007). Mutations in the *pncA* and *rpsA* genes are linked to pyrazinamide resistance in *M. tuberculosis* (Shi *et al.*, 2011; and Scorpio and Zhang, 1996).



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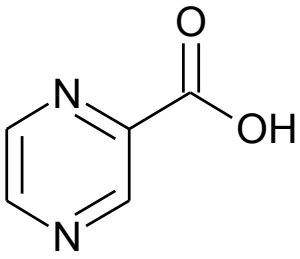

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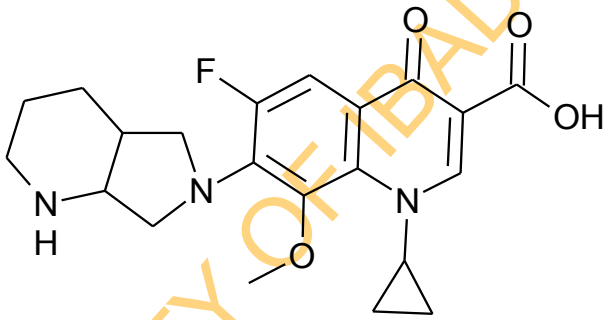
2.11.4.6. Moxifloxacin

Moxifloxacin [2.78] is a fluoroquinolone and as such acts by the inhibition of DNA replication and transcription. The flouroquinolone derivatives are the second line anti-TB drugs used in the treatment of multi-drug resistant TB.



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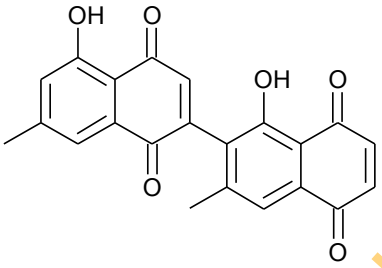
2.11.5 Some natural products isolates with antimycobacterial activity

In recent times, there has been an increase in the literature on bioactive compounds isolated from plants with antituberculosis activity. Although no bench-to-market plant- derived drug for the treatment of TB has been reported, some of the bioactive isolates have been identified as useful lead compounds for the development of antituberculosis drugs. Diospyrin [2.79] a naphthoquinone isolate from the roots of *Euclea natalensis* was found to inhibit the growth of drug resistant and drug sensitive strains of *Mycobacterium tuberculosis* (Lall and Meyer, 2001). Newton *et al.*, (2002) reported the antituberculosis activity (MIC 29.0 ug/ml) of Chelerylthrine [2.80] a benzophenanthridine alkaloid isolated from the root of *Sanguinaria canadensis*

against *M. smegmatis.* 12-demethylmulticauline [2.53] a norditerpenoids isolate from the roots of *Salvia multicaulis* was found to be more active than ethambutol [2.73] and nearly as active as rifampicin [2.72] in an *in vitro* study (Ulubelen *et al.,* 1997). Berberine [2.81], and phaseollidin [2.82] from *Phaseollus vulgaris* and related species, erythrabyssin II [2.83] a benzofuran neoflavonoid from *Erythrina spp*, and tryptanthrin [2.84] have been reported as useful antitubercolosis lead compounds from plants. Pleuremutillin [2.25] a novel protein synthesis inhibitor with antituberculosis activity has been reportedly isolated from some species of mushrooms. Calanolide A [2.85] a naturally occurring pyranocoumarin from *Calophyllum* seed oil have been reported to exhibit good *in vitro* activity towards *M. tuberculosis* (McKee *et al.,* 1998; Currens *et al.,*1996; Dharmaratne *et al.,* 1998; Galinis *et al.,* 1996; Spino *et al.,* 1998).



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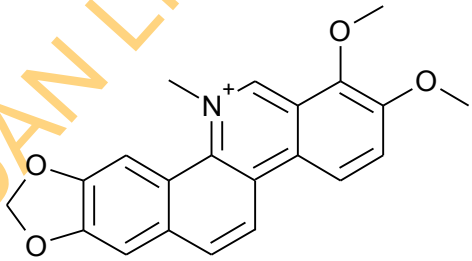

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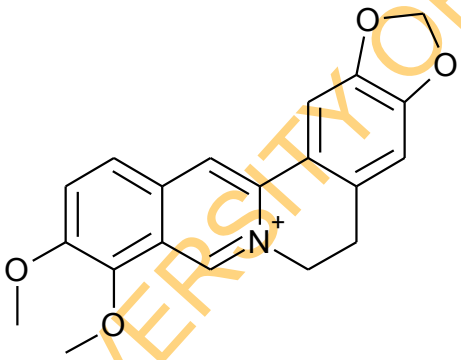

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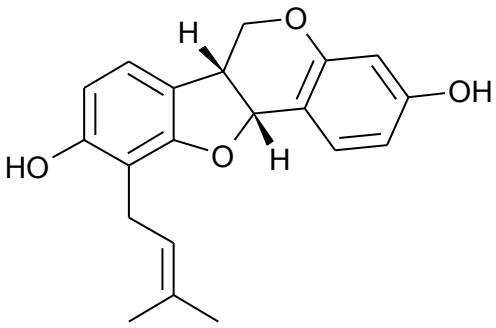

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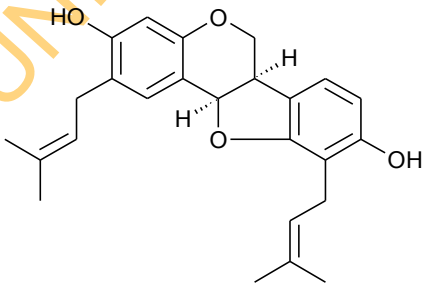

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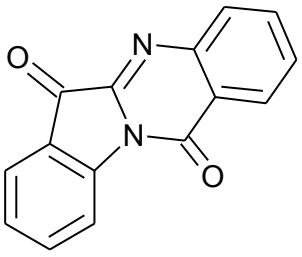

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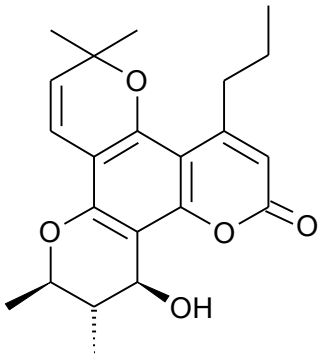

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2.11.6 Some antimycobacterial bioassay methods

Several methods have been established for the screening of drugs and drug lead substances for anti-mycobacterial activity. These includes: agar diffusion, agar dilution, microbroth dilution, radiometric, Mycobacterium Growth Indicator Tube (MGIT), the microplate Alamar Blue assay, the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide-based assay, and the luciferase assay methods among others (Canetti *et al.*,1963; Selvakumar, 2010).

Agar diffusion: This involves placing filter paper disks (soaked with the test substance) on agar plate surfaces that have been previously inoculated with the mycobacteria strain. The test substance then diffuses into the agar and inhibits the growth of the mycobacteria (Hadacek and Greger, 2000). After incubation, the diameter of the zones of growth inhibition is recorded. Generally, diffusion assays are recommended more for polar rather than non-polar molecules or mixture of compounds such as essential oils (Lahlou, 2004). With mycobacteria, there are additional reasons to avoid diffusion assays. This is because the mycobacteria cell wall is often more susceptible to less-polar compounds. Non-polar compounds will diffuse more slowly than polar compounds in the aqueous agar medium thereby giving the erroneous impression of weak activity (Pauli *et al.,* 2009). Furthermore, agar-diffusion method is difficult to run on high capacity screening platforms (Cos *et al.*, 2006).

Agar dilution: This entails the preparation of serial concentrations of the test substance either in a solid base (Lowenstein-Jensen (LJ)) medium usually made into slants (Canetti *et al.,* 1963; Van-Klingeren *et al.,* 2001). The bacterial cultures are

then inoculated on the slants or well plates. After incubation, the inhibition of growth by the test substance (at the different concentrations) is read by counting the Colony Forming Units (CFU). The agar dilution test is suitable for the determination of the Minimum Inhibition Concentration (MIC). Its major disadvantage is the high amount of test compounds (20 mg/plate to test 1.000 mg/mL) needed. This restricted its use to easily available test materials (Canetti *et al.*, 1963; Pauli *et al.*, 2009). It also has the disadvantage of not being rapid as it takes 4 – 6 weeks for the results to be available. This is because of the slow growth rate of the mycobacteria.

Micro broth dilution: This is like the agar dilution method but makes use of a liquid broth medium in microtitre well plates. Because of the use of microtitre well plates, a significant reduction in the amount of test substance is achieved compared to using the LJ medium. It also has the added advantage of enormous high-throughput when used in combination with spectrophotometric or fluorometric plate readers (Hadacek and Greger, 2000; Pauli *et al.*, 2009). However, since the growth of the mycobacteria is quantitated by turbidity in a liquid medium, the tendency of the mycobacteria to clump makes this a difficult test. Also, test samples that are not fully soluble may interfere with turbidity readings, making interpretation difficult. This disadvantage could be de- emphasized by incorporating a negative sterility control (extract dissolved in blank medium without microorganisms) (Cos *et al.*, 2006; Pauli *et al.*, 2009). An important alternative is the use of oxidation/reduction indicator dyes such as Alamar Blue (Franzblau *et al.*, 1998; Jimenez-Arellanes *et al.*, 2003; 2007), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Abate *et al.*, 1998; De-Logu *et al.*, 2001), 2,3,5-triphenyltetrazolium chloride (TTC) (Mohammadzadeh *et al.*, 2006), and 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino) carbonyl]-2H-tetrazolium- hydroxide (XTT) (De-Logu *et al.*, 2003). In these cases, the growth/inhibition can be read visually; and the reduced form of these dyes can also be quantified by using a colorimeter at 530 nm (excitation) or 590 nm (emission). The latter mode is more sensitive.

Radiometric method: This is a semi-automated radiometric drug susceptibility testing method. It is done in the BACTEC 460® instrument. The principle is based on the measurement of the $^{14}CO_2$ produced by metabolic breakdown of (1-^{14}C) palmitic acid in a liquid Middlebrook 7H12 medium (Laszlo *et al.*, 1983; Collin and

Franzblau, 1997). With this method, multiple concentrations can be tested and an MIC calculated. Although this method is rapid with the results available in 5 days, it is costly and prone to the generation of radioactive wastes.

Mycobacteria Growth Indicator Tube (MGIT) method. This makes use of the BACTEC 960® instrument. It is a liquid base method. Each MGIT contain about 7 mL of Middlebrook 7H9 broth. In addition, the MGITs contain a fluorescent sensor in the base of the tube which binds with O_2 and quenches fluorescence. Antimycobacterial assays using this method takes 4-13 days (Selvakumar, 2010). The test is based on growth of the *M. tuberculosis* strain in a drug containing tube compared to a drug free tube (Growth control). The instrument continuously monitors the tubes for increase in fluorescence. Analysis of fluorescence in the drug containing tube compared to the fluorescence of the growth control tube is used by the instrument to determine susceptibility results. Results are interpreted automatically and reported as susceptible or resistant. Unlike the radiometric method, radioactive wastes are not generated.

Flow cytometry: In this method, Fluorescein diacetate (FDA) stain is used in a flow cytometer (Norden *et al.*, 1995). FDA is a non-fluorescent diacetyl fluorescein ester that becomes fluorescent upon hydrolysis by cytoplasmic esterases. It is a rapid method but has high cost of equipment as a limitation.

Reporter gene assays: This makes use of Genes encoding luciferase enzymes that are cloned from several species of firefly, beetle, crustacean, bacteria and the sea pansy (New *et al.*, 2003). Other fluorescent proteins such as the red fluorescent protein (RFP) and green fluorescent protein (GFP) could be used. These are introduced in mycobacteria on plasmids. The determination of bacterial viability is by measuring the expression of an introduced fluorescent or luminescent protein (Arain *et al.*, 1996; Collins *et al.*, 1997*;* Pauli *et al.*, 2009). Patent restrictions limit the use of this method (New *et al.*, 2003).

High-performance liquid chromatography (HPLC) analysis of mycolic acid: This method is based on the quantitative analysis of mycolic acid using HPLC. p-bromophenacyl bromide derivatizing reagent is used for UV detection (Butler and

Gulhertz, 2001; Viader-Salvado *et al.*, 2006). The Total Area under the Mycolic Acid (TAMA) chromatographic peaks of a culture of *M. tuberculosis* and log CFU per mL serve as the basis for the estimation of the growth of the mycobacteria (Gaz-Gonzalez, 1997; Viader-Salvado, 2006). However the cost of the HPLC equipment limits the use of this method.

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Materials

Syzygium guineense stem bark and *Mimosa pigra* aerial part used for this study were selected based on ethnomedicinal information and collected from farmlands in Chaza village, Suleja, Niger State, Nigeria in the month of November 2010, by Mallam Muazam and authenticated at the herbarium unit of the National Institute for Pharmaceutical Research and Development, Idu, Abuja, Nigeria. Voucher specimens; NIPRD/H/6418 for *S. guineense* and NIPRD/H/6405 for *M. pigra* have been deposited in the herbarium of the Institute. The plant materials were sorted out to remove humus, washed and dried under ambient condition. The dried plant materials were then pulverised into fine powder and kept in air-tight polythene bags prior to extraction.

Most solvents used were of analytical grade product of Sigma Aldrich and purchased from Zayo Chemicals Nigeria Ltd, the Sigma Aldrich accredited agent in Nigeria. Other solvents used were distilled before use. Column chromatography was performed on silica gel (Merck 60-230 mesh) under gravity. Thin layer chromatography (TLC) was performed on plates pre-coated with silica gel 60 HF_{254} (Merck, TLC grade, with gypsum binder). The TLC bands were visualized under ultraviolet light (at 254 nm and 365 nm), by exposure to iodine and by spraying with concentrated H_2SO_4, and 10% $FeCl_3$ in aqueous ethanol using Sigma gun spray. All glassware were washed with chromic acid solution, rinsed with water several times, oven dried overnight at 125 °C and allowed to cool prior to use. Solvents were removed under reduced pressure using a Buchi rotary evaporator at pump pressure of 0.1 mmHg.

The ^{1}H and ^{13}C-NMR spectra were recorded at 200 and 400/600 MHz [50/100/150 MHz for ^{13}C-NMR analysis] on a JEOL GSX–200 and Brucker Avance III spectrometer respectively in deuterated solvents: $CDCl_3$, or DMSO-d6 as appropriate. Chemical shifts are expressed in parts per million (ppm) downfield of Trimethylsilane (TMS) as internal reference for ^{1}H resonances, and referenced to the central peak of the appropriate

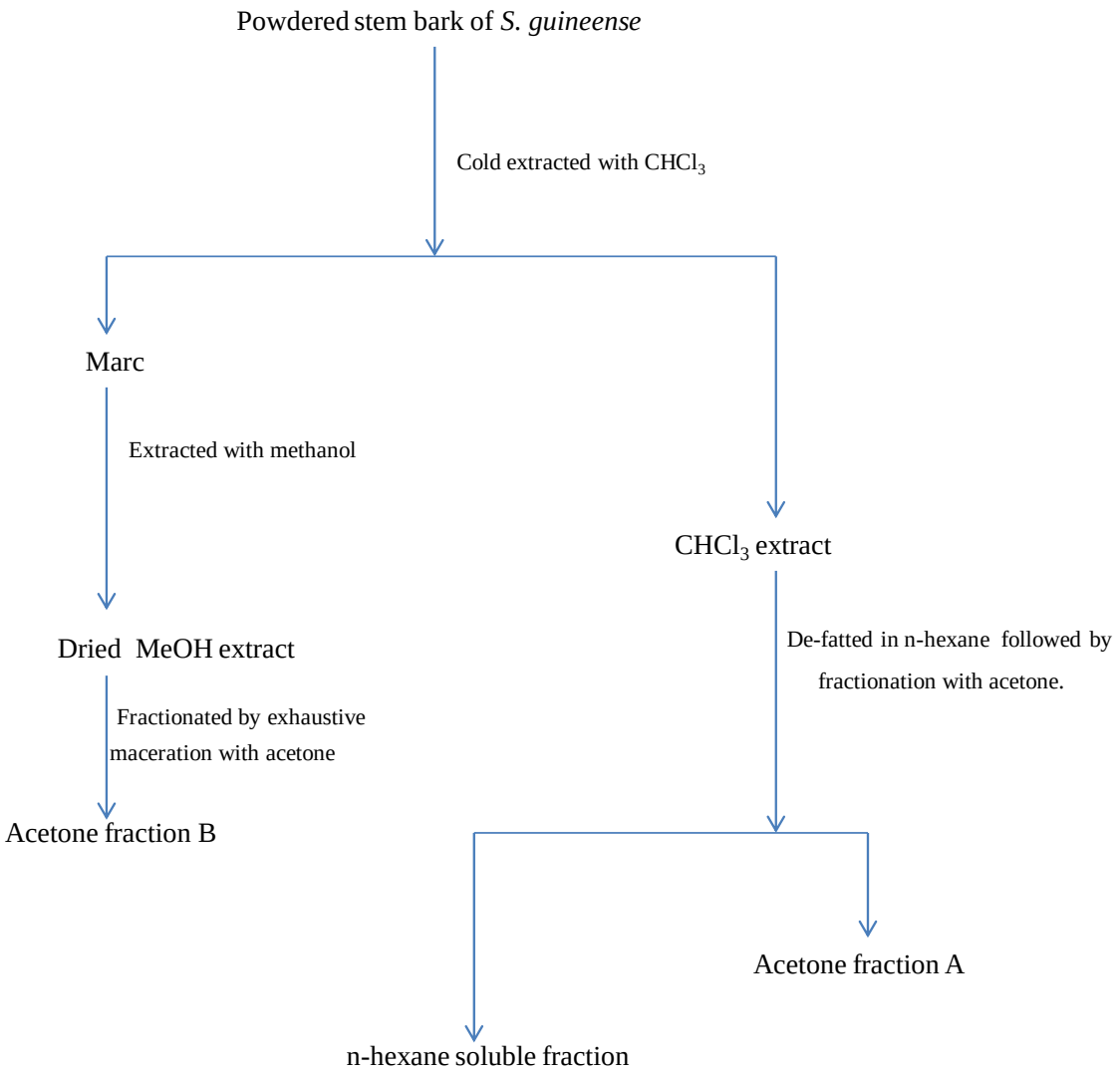
deuteriated solvent's resonances. Infra red (IR) spectra were recorded on 1600 ATI Matson Genesis series FTIR^{TM} spectrometer. Mass spectra were recorded on a FINNIGAN MAT 12 spectrometer. The Ultra-violet (UV)/visible spectra were obtained using a CECIL UV –Visible spectrophotometer. Melting points were recorded on an Electrothermal melting point apparatus and are uncorrected.

The TB Research Laboratory facilities at The Zankli Medical Centre, Abuja, Nigeria were used for the antimycobacterial assay. De-contaminated clinical strains: ZMC 303 (Rifampicin susceptible) and ZMC 050 (Rifampicin resistant) of *Mycobacterium tuberculosis* obtained from the same laboratory were used for the investigation.

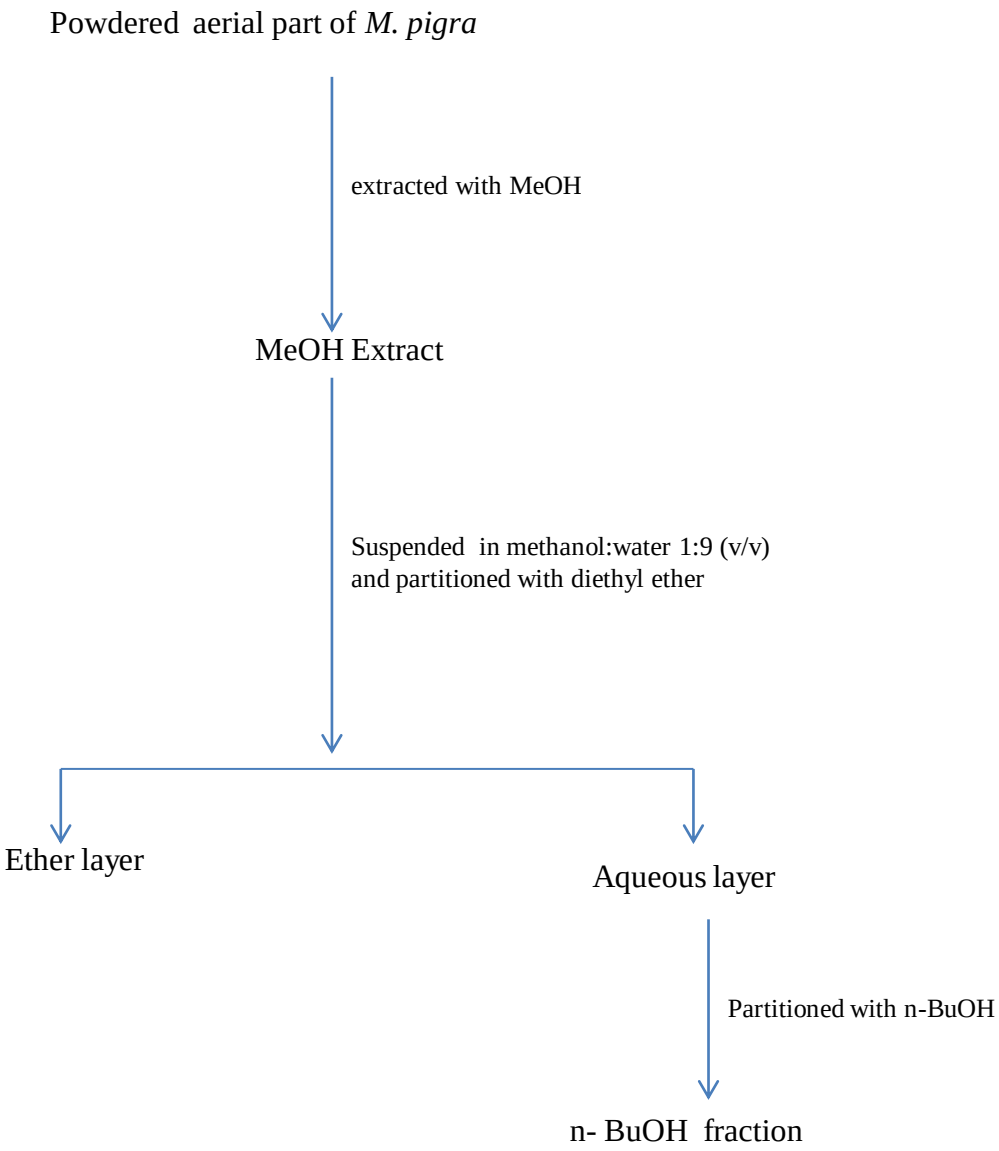
3.2 Methods.

3.2.1 Extraction and fractionation procedures

The dried powdered stem bark of *S. guineense* (1000 g) was extracted by cold maceration with frequent agitation for 72 hours in succession with chloroform (4 x 2.5 L) and methanol (4 x 2.5 L) to afford the chloroform and methanol extracts respectively. The vacuum dried chloroform extract was defatted with n-hexane and fractionated with acetone. The vacuum dried methanol extract was also macerated separately with acetone. The extraction and fractionation protocol is shown in Scheme 3.1. Also, the powdered dried aerial part of *M. pigra* (500 g) was extracted by cold marceration with intermintent agitation for 72 hours in absolute methanol (3 x 2.5 L). The vacuum dried methanol extract was suspended in methanol: water (1:9 v/v) and partitioned with diethyl ether and n-butanol in succession according to Scheme 3.2.



Scheme 3.1: Fractionation flow chart for *Syzygium guineense* stem bark



Scheme 3.2: Fractionation flow chart for *Mimosa pigra* aerial part

3.2.2 Antimycobacterial assay procedures

The solid based agar dilution (using the egg enriched Lowenstein Jensen (LJ) medium), and the liquid based Mycobacterium Growth Indicator Tube (MGIT) methods were used. Colony counting was done as reported by the International Union against Tuberculosis LJ Medium (IUTM) (Selvakumar, 2010) as outlined below:

- ➔ No growth at the end of incubation period at 37 ^{o}C
+ ➔ 1-19 colonies growth units at the end of incubation period at 37 ^{o}C
1+ ➔ 20-100 colonies growth units at the end of incubation period at 37 ^{o}C
2+ ➔ 100-200 colonies growth units at the end of incubation period at 37 ^{o}C
3+ ➔ 200-400 colonies growth units at the end of incubation period at 37 ^{o}C

Where a test substance showed inhibition with greater than 100 colonies it is taken as not being active. Where a no colony growth was observed, the test substance is taken as bactericidal.

3.2.2.1 Antimycobacterial assay using the solid based LJ medium method.

This was done using the method reported by Selvakumar (2010). Freshly laid eggs (not more than 7 days old) were scrubbed with a brush in warm water and plain alkaline soap. The washed eggs were then soaked in 70% aqueous ethanol for 15 mins. These were thereafter cracked aseptically and the content poured into a sterile blender and homogenized. The LJ stock solution was prepared by aseptically mixing 1 L of the homogenised eggs with 20 mL of aqueous malachite green solution (2% w/v). To this resulting mixture was added 600 mL of mineral salt solution (a solution containing 6 g of L-Aspargine, 4 g of anhydrous dibasic potassium phosphate, 1.0 g of magnesium citrate, 0.4 g of magnesium sulphate heptahydrate, and 20 mL of glycerol in 1000 mL of sterile disilled water). The freshly prepared LJ medium was then mixed with the test samples as shown in Table 3.1. About 7-8 mL of the re-constituted test sample-LJ mixture was then dispensed into sterile bijou tubes and inspissated for 45 min at 85 ^{o}C in a slanting position. The LJ medium (7 mL) without the test samples or standard drugs but re-constituted with DMSO was also separately dispensed into sterile bijou tubes and inspissated for 45 min at 85 ^{o}C in a slanting position as negative control.The de-contaminated clinical isolates of *Mycobacterium tuberculosis* (MTB) was diluted in sterile distilled water to 10^{-2} and 10^{-4}. This corresponds to 1.0 and 0.5 McFarland respectively. Using a sterile micropipette, 10 µL each of the 10^{-2} and 10^{-4} innoculum concentrations were inoculated on separate standard

drugs, phytodrugs, and negative control re-constitued LJ media slant and incubated for six weeks at 37 ^{o}C. The LJ media slant containing neither the standard control drugs, phytodrugs nor MTB inoculums was also incubated along side for six weeks at 37 ^{o}C. Inoculated media were checked after 3 days for contamination. Each sample analysis was done in duplicate. The results are presented in Tables 4.2 and 4.3 for *S. guineense* and *M. Pigra* respectively.

3.2.2.2 Antimycobacterial assay using the liquid based MGIT method

This was done using the method reported by Selvakumar (2010). To the MGIT containing 7 mL of middlebrook 7H9 broth was added 0.8 mL of Oleic acid-Albumin- Dextrose-Catalase -Polymyxin B, Amphotericin B, Nalidixic acid, Trimethoprim and Azlocillin (OADC-PANTA) antibiotics mixture to inhibit the growth of non-tuberculosis micro-organism contaminants. Using a sterile micro-pipette, 0.5 mL of 1.0 and 0.5 Mcfarland decontaminated clinical strains of the *Mycobacterium tuberculosis* was added to the MGIT. The re-constituted MGITs were then labelled corresponding to the test samples. Using a sterile pipette, 0.5 mL of 10 mg/mL solution of each of the test samples from *S. guineense* were separately added to their respective re-constituted MGIT while 0.1 mL of each of dihydrostreptomycin (84.0 µg/mL), isoniazid (8.4 µg/mL), rifampicn (84.0 µg/mL), and ethambutol (420.0 µg/mL) were separately used as standard control drugs. The MGIT tubes containing the test samples were placed inside the BACTEC 960 SIRES system programmed as per manufacturer's instruction and incubated for 14 days alongside separate MGIT re-constituted with dimethylsulphoxide (DMSO) as negative control. Innoculation was done in a Lamiplus 7 innoculation chamber. Each sample analysis was done in duplicate. The results are presented in Table 4.2.

Table 3.1 Preparation of Standard drugs and Phytodrug containing LJ slopes

Drugs	Stock concentration (mg/mL)	Volume of stock solution added to LJ media (mL)	Final volume of dilution with LJ media (mL)	Final test concentration of drug (mg/mL)
Isoniazid	0.02	1	100	0.0002
Dihydrostreptomycin	0.80	1	100	0.0080
Ethambutol	0.20	1	100	0.0020
Rifampicin	8.00	1	100	0.0800
Phytodrug	500.00	1	100	5.0000
	200.00	1	100	2.0000

3.2.3 Phytochemical Methods

Preliminary phytochemical tests were carried out on the extracts and fractions. The methods were based on standard procedures reported in literature (Harbourne, 1998; Houghton and Raman, 1999; Trease and Evans, 1985; Sofowora, 1982).

3.2.3.1 Tests for flavonoids

$FeCl_3$ solution test

Two drops of freshly prepared 10% $FeCl_3$ solution were added to 2 mL of aqueous filtrate of the test sample. Formation of dark blue or blue-green precipitate is a positive test for the presence of a phenolic nucleus.

NaOH solution test

Two to three drops of 10% NaOH (aq) were added to 2 mL of the aqueous filtrate of the test extract/fraction. A yellow colour which disappears on addition of dilute HCl indicates the presence of flavonoids.

Ammonia solution test

Two to three drops of 10% aqueous ammonia were added to 2 mL of the aqueous filtrate of the test extract/fraction. An intense yellow colour indicates the presence of flavonoids.

$AlCl_3$ solution test

Two to three drops of 10% alcoholic $AlCl_3$ solution were added to 2 mL of the aqueous filtrate of the test extract/fraction. An intense yellow colour indicates the presence of flavonoids.

Shinoda test

A small strip of magnessium filings was added to 2 mL of the aqueous filtrate of the test extract/fraction followed by the addition of 1 mL of concentrated HCl using a dropping pipette. An intense pink colouration indicates the presence of flavonoids

3.2.3.2 Tests for tannins

$FeCl_3$ solution test

Two drops of freshly prepared 10% $FeCl_3$ solution were added to 2 mL of aqueous filtrate of the test sample. Formation of dark blue or blue-green precipitate is a positive test for the presence of a phenolic nucleus.

Gelatin test

A 1 mL aliquot of the aqueous filtrate of the test extract/fraction was added to 5 mL of 10% w/v gelatin in normal saline solution. Precipitation of gelatin from solution indicates the presence of tannins

3.2.3.3 Test for saponins

The test extract/fraction (0.5 g) was boiled in 5 mL of distilled water and filtered into a test tube. About 20 mL of distilled water was added to the aqueous filtrate and the test-tube was closed with a stopper, shaken vigorously for about 1minute and then allowed to stand for about 30 mins. A persistent honey comb froth indicates the presence of saponins.

3.2.3.4 Tests for alkaloids

About 0.5 g test extract was stirred with 5 mL of 1% dilute hydrochloric acid on a water bath. The resulting solution was then filtered and the filtrate was divided into three equal portions in separate test tubes labelled D, M and H. To the test tube labeled D was added 3 drops of Dragendorff's reagent. Formation of reddish-brown precipitates is positive for alkaloids. To the test tube labelled M was added 3 drops of Mayer's reagent. Formation of a creamy white precipitate on adding the Mayer's reagent is positive for alkaloids. To the third test tube labelled H was added 3 drops of Hager's reagent. Formation of a yellow precipitate on addition of the Hager's reagent indicates the presence of alkaloids.

3.2.3.5. Test for Tritepenoids/ Steroids.

Liebermann – Buchard test for terpenoids.

The chloroform filtrate (2 mL) of the test extract/fraction was transferred into a clean dry test tube and mixed with 1 mL of acetic anhydride. Using a dropping pipette 1mL of conc. H_2SO_4 was then poured carefully down the wall of the test tube to form a layer underneath.

A reddish-violet colour at the junction of the two liquids, and a green to violet to purple colour in the chloroform layer indicate the presence of triterpenoids

Salkowski test for steroidal nucleus.

The chloroform filtrate (2 mL) of the test extract/fraction was transferred into a clean dry test tube. Using a dropping pipette, 1 mL of conc. H_2SO_4 was then poured carefully down the wall of the test tube to form two layers. A reddish- brown ring at the interface of the two liquids indicates the presence of a steroidal ring.

3.2.3.6. Tests for carbohydrates, reducing sugars and glycosides

Molisch test

Two drops of the Molisch reagent (a solution of ∝-napthol in 95% ethanol) was added to 2 mL of a test solution in a test tube. The solution is then poured slowly into a test tube containing 2 mL of concentrated sulphuric acid to give two layers. The formation of a purple interface of the layer indicates the presence of carbohydrates.

Fehling's test for reducing sugars

The test extract/fraction (0.5 g) was boiled with 10 mL of distilled water and filtered. The filtrate was divided into two equal portions in separate test tubes labelled A and B. To the test tube labelled A was added 2 mL of 1:1v/v mixture of Fehling solutions A and B and boiled in a water bath for about 3 mins. Formation of a brick red precipitate indicates the presence of free reducing sugars.

To the second portion of the aqueous filtrate in the test tube labelled B was added 3 mL of dilute 10% $HCl_{(aq)}$ and boiled for 15minutes. The mixture was filtered cooled and neutralised with 10% $NaOH_{(aq)}$. The Fehling test was then repeated. Formation of brick red precipitate that is about 2 or 3 times that obtained in test tube 'A' indicates the presence of glycosides or combined reducing sugars.

3.2.4 Column chromatography of the n-hexane soluble fraction of *S. guineense* stem bark

The n-hexane soluble fraction coded SGHS (1.0 g) was dissolved in chloroform and pre-adsorbed on silica gel (1.0 g) by mixing to form a homogenous paste which was allowed to

air-dry in a fume cupboard. The mixture was then loaded on a chromatography column (Length = 30 cm, internal diameter = 2 cm and weight of silica gel (mesh 60 -230) = 100 g). The column was eluted with the following mobile phase gradient in increasing order of polarity: n-hexane (100%, 100 mL), n-hexane: $CHCl_3$ (3:1, 100 mL); (1:1, 100 mL); and 1:3, 100 mL), and $CHCl_3$ (100%, 150 mL). A total of 20 fractions (20 mL portions) were collected and pooled to 3 sub-fractions SGHS-1-3 based on TLC analysis. The SGHS-2 eluted with n-hexane: $CHCl_3$ (1:1) yielded a white crystal coded HSB after washing with acetone to remove coloured pigments while the SGHS-3 eluted with n-hexane : $CHCl_3$ (1:3 to 0:4) gave a creamy to off white solid coded HSD which was further subjected to column chromatography.

3.2.4.1 Characterisation of HSB

Appearance: white crystal

Melting point: 249-250 ^{o}C

Phytochemistry: positive to Liebermann and Salkowski test

Molecular formula (HREIMS): $C_{30}H_{50}O$

Molecular Mass (HREIMS): 426.3903 g/mol.

Molecular Mass (calculated): 426.7174 g/mol.

Major EIMS m/z fragmentation peaks m/z (relative abundance): 426 (60) [M+], 411(35) (M-CH_3), 395(5) [M-2CH_3], 341 (17), 302(42), 273(76), 231(54), 218(54), 205(78), 163(57), 137(54), 125(100), 109(80), 95(80).

^{13}C-NMR (150 MHz, $CDCl_3$, δ ppm): 22.3 (t, C_{-1}), 41.5 (t, C_{-2}), 212.3 (s, C_{-3}), 58.2 (d, C_{-4}), 42.2 (s, C_{-5}), 41.3 (t, C_{-6}), 18.2 (t, C_{-7}), 53.1 (d, C_{-8}), 37.5 (s, C_{-9}), 59.5 (d, C_{-10}), 35.6 (t, C_{-11}), 30.5 (t, C_{-12}), 39.7 (s, C_{-13}), 38.3 (s, C_{-14}) 32.4 (t, C_{-15}), 36.0 (t, C_{-16}), 30.0 (s, C_{-17}), 42.8 (d, C_{-18}), 35.4 (t, C_{-19}), 28.2 (s, C_{-20}), 32.8 (t, C_{-21}), 39.3 (t, C_{-22}), 6.8 (q, C_{-23}), 14.7 (q, C_{-24}), 18.0 (q, C_{-25}), 20.3 (q, C_{-26}), 18.7 (q, C_{-27}), 32.1 (q, C_{-28}), 35.0 (q, C_{-29}), 31.8 (q, C_{-30}). The results are presented in Table 4.7.

^{1}H-NMR (600 MHz, $CDCl_3$, δ ppm) : 1.72/1.98 (2H, m, $H_{-1a/b}$), 2.31/ 2.41(2H, m, $H_{-2a/b}$), 2.26(1H, m, H_{-4}), 1.30 (2H, m, H_{-6}), 1.51 (2H, m, H-$_{7}$), 1.40 (1H, m, H_{-8}), 1.54 (1H, m, H_{-10}), 1.38/1.46 (,2H, m, $H_{-11a/b}$), 1.36 (2H, m, H_{-12}), 1.30 (2H, m, H_{-15}), 1.57 (2H, m, H_{-16}), 1.56 (1H,m, H_{-18}), 1.23,(2H, m, H_{-19}), 1.48 (2H, m, H_{-21}), 0.95/1.50 (2H, m, H_{-22}) , 0.91 (H, d, H_{-23}), 0.75(3H, s, H_{-24}), (0.89, 3Hs, H_{-25}), 1.03 (3H, s, H_{-26}), 1.07, 3H, s, H_{-27}), 1.20 (3H, s, H_{-28}), 0.97, 3H, s, H_{-29}),1.02 (3H, s, H_{-30}). The results are presented in Table 4.7.

FTIR spectrum (V_{max} cm^{-1}, KBr): C-H (2917), C=O (1734).

3.2.4.2 Purification of HSD

The impure HSD (100 mg) was further purified by chromatography on a finger column packed with silica gel. Elution was done with 25% stepwise gradient of n-hexane and chloroform in the order of n-hexane: $CHCl_3$ (4:0, 50 mL; 3:1, 50 mL; 1:1, 50 mL; 1:3, 50 mL and 0:4, 50 mL). The fractions were collected in 5 mL portions to give forty-nine fractions which were pooled together into four sub-fractions HSD-1-4. The sub-fraction HSD-2 eluted with n-hexane: $CHCl_3$ (1:1) afforded a white crystal coded SGB (5 mg) while the sub-fraction HSD-4 eluted with n-hexane: $CHCl_3$ 0:4 yielded a white solid GHII (24 mg).

3.2.4.2.1 Characterisation of compound SGB

Appearance: white crystal

Melting point: 249-250 ^{o}C

Phytochemistry: positive to Liebermann and Salkowski test

Molecular formula (HREIMS): $C_{30}H_{50}O$

Molecular Mass (EIMS): 426.4 g/mol.

Molecular Mass (calculated): 426.7174 g/mol.

Major EIMS m/z fragmentation peaks m/z (relative abundance): 426 (60) [M+], 411(35) (M-CH_3), 395(5) [M-2CH_3], 341 (17), 302(42), 273(76), 231(54), 218(54), 205(78), 163(57), 137(54), 125(100), 109(80), 95(80)

^{13}C-NMR (50 MHz, $CDCl_3$, δ ppm): 22.3 (t, C_{-1}), 41.5 (t, C_{-2}), 213 (s, C=O, C_{-3}), 58.2 (d, C_{-4}), 42.1 (s,C_{-5}), 41.3 (t, C_{-6}), 18.2 (t, C_{-7}), 53.1 (d,C_{-8}), 37.4 (s,C_{-9}), 59.5 (d, C_{-10}), 35.6 (t, C_{-11}), 30.5 (t, C_{-12}), 39.7 (s, C_{-13}), 38.3 (s, C_{-14}) 32.4 (t, C_{-15}), 36.0 (t, C_{-16}), 30.0 (s, C_{-17}), 42.8 (d, C_{-18}), 35.3 (t, C_{-19}), 28.2 (s, C_{-20}), 32.8 (t, C_{-21}), 39.3 (t, C_{-22}), 6.8 (q, C_{-23}), 14.7 (q, C_{-24}), 17.9 (q, C_{-25}), 20.3 (q, C_{-26}), 18.7 (q, C_{-27}), 32.1 (q, C_{-28}), 35.0 (q, C_{-29}), 31.8 (q, C_{-30}). The results are presented in Table 4.9.

^{1}H-NMR (200 MHz, $CDCl_3$, δ ppm) 0.73 (3H, s, H_{-24}), 0.87, (3H, s, H_{-25}), 0.90 (3H, d, H_{-23}), 0.96 (3H, s, H_{-29}), 1.05 (3H,s, H_{-27}), 1.18 (3H, s, H_{-28}), 1.00(6H, s, $H_{-26/30}$). Overlapping complex methine (1H) and methylene (2H) multiplets at 1.8 – 2.4 (H_{-1}, H_{-2}, H_{-6} , H_{-7}, H_{-8}, H_{-10}, H_{-18}, H_{-11}, H_{-12}, H_{-15}, H_{-16}, H_{-19}, H_{-21}, H_{-22}). The results are presented in Table 4.9.

FTIR spectrum (V_{max} cm^{-1}, KBr): C-H (2917), C=O (1734).

3.2.4.2.2 Characterisation of compound GHII

Appearance: white solid

Melting point: 95-98 ^{o}C

Phytochemistry: positive to Liebermann and Salkowski test

Molecular formula (EIMS): $C_{29}H_{48}O_2$

Molecular Mass (EIMS): 428.4 g/mol.

Molecular Mass (calculated): 428.6902 g/mol.

EI-MS (m/z (relative abundance): 428.4(23) [M+], 413(49) [M-15], 410(4) [M-18], 395(9) [M-15-18], 275(59) [M- H_20 - 153 (ring A loss)], 259(19), 234(45) [M-194(ring A/B loss)], 220(54), 205(58), 165(94), 137 (45) [M-H_2O-275(ring C/D/E loss)], 125(100), 109(91), 96(94).

^{13}C-NMR (100 MHz, $CD_3OD/CDCl_3$, δ ppm): 22.3 (t, C_{-1}), 35.0 (t, C_{-2}), 212.3 (s, C=O, C_{-3}), 58.5 (d, C_{-4}), 40.8(d, C_{-5}), 40.9 (t, C_{-6}), 32.0 (t, C_{-7}), 48.5 (d,C_{-8}), 37.3 (s, C_{-9}), 60.6 (d, C_{-10}), 34.0 (t, C_{-11}), 70.8 (d, CHOH, C_{-12}), 52.0 (s, C_{-13}), 38.7 (s, C_{-14}), 29.5 (t, C_{-15}), 36.5 (t, C_{-16}), 37.1 (s, C_{-17}), 42.0 (d, C_{-18}), 34.8 (t, C_{-19}), 27.4 (s, C_{-20}), 15.0 (t, C_{-21}), 39.6 (t, C_{-22}), 11.0 (q, C_{-23}), 14.0 (q, C_{-24}), 18.0 (q, C_{-25}) 15.6 (q, C_{-26}),

-- (C_{-27}), 19.2 (q, C_{-28}), 34.0 (q, C_{-29}), 31.2 (q, C_{-30}). The results are presented in Table 4.10.

^{1}H-NMR (400 MHz, $CD_3OD/CDCl_3$, δ ppm) 0.71 (3H, s, H_{-24}), 0.87, (3H, s, H_{-25}), 0.87 (3H, d, H_{-23}), 0.96 (3H, s, H_{-29}), 1.05 (3H,s, H_{-26}), 1.18 (3H, s, H_{-28}), 1.00(3H, s, H_{-30}). Overlapping complex methine (1H) and methylene (2H) multiplets at 1.8 – 2.4 (H_{-1}, H_{-2}, H_{-6} , H_{-7}, H_{-8}, H_{-10}, H_{-18}, H_{-11}, H_{-12}, H_{-15}, H_{-16}, H_{-19}, H_{-21}, H_{-22}). The results are presented in Table 4.10.

FTIR spectrum (V_{max} cm^{-1}, $CHCl_3$): O-H (3444), C-H (2919) , C=O (1714) and C-O (1024)

3.2.5 Column chromatography of the acetone fraction A of *S. guineense* stem bark

The acetone fraction A (1.12 g) was dissolved in acetone and pre-adsorbed on silica gel (1.12 g) to form a homogenous paste which was allowed to air dry in a fume cupboard. The mixture was loaded on a chromatography column (silica gel; mesh 60 -230) . The column (Length = 30 cm, internal diameter = 2 cm and weight of silica gel (mesh 60 -230) = 100 g) was eluted with the following mobile phase gradient in increasing order of polarity: n-hexane (100%, 100 mL), n-hexane: ethyl acetate ((3:1, 100 mL); (1:1, 100 mL); and (1:3, 100 mL)), ethyl acetate (100%, 150 mL) and ethyl acetate: methanol (3:1, 150

mL). A total of 28 fractions (20 mL portions) were collected and pooled to 3 sub-fractions SGHI-1-3 based on TLC analysis. The SGHI-2 eluted with n-hexane: ethyl acetate (1:1 to 1:3) yielded a white solid coded SGD (50 mg) after washing with n-hexane and re-crystalising in acetone. The SGHI-3 eluted with ethyl acetate 100% to ethyl acetate: methanol (3:1) gave a creamy to off white solid coded SGE (145 mg) after washing with n-hexane and re-crystalising in acetone.

3.2.5.1 Characterisation of SGD

Appearance: white solid

Melting point: 225-230 ^{o}C

Solubility: freely soluble in chloroform, dichloromethane

Phytochemistry: positive to Liebermann and Salkowski test

Molecular formula (EIMS): $C_{31}H_{50}O_4$

Molecular mass (EIMS): 486.6 g/mol.

Molecular Mass (calculated): 486.7263 g/mol.

Major EI-MS m/z fragmentation peaks (relative abundance): 486 (4) [M+], 470 (4) [M+16], 456(8) [M-30], 439(8) [M-OCH_2OH], 411(9) [M-$COOCH_2OH$], 248(100) and 207(96), 189(56).

^{13}C-NMR (150 MHz, $CDCl_3$, δppm): 38.7(t, C_{-1}), 27.4 (t, C_{-2}), 79.0 (d, CHOH, C_{-3}), 38.9 (s, C_{-4}), 55.4 (d, C_{-5}), 18.3 (t, C_{-6}) 34.3 (t, C_{-7}), 40.7 (s, C_{-8}), 50.5 (d, C_{-9}), 37.0 (C_{-10}), 20.9 (t, C_{-11}), 25.5 (t, C_{-12}), 38.4 (d, C_{-13}), 42.5 (s, C_{-14}), 32.5 (t, C_{-15}), 30.6 (t, C_{-16}), 55.9 (s, C_{-17}), 49.8 (d, C_{-18}), 47.5 (d, C_{-19}), 150.4 (s, C_{-20}), 29.7 (t, C_{-21}), 37.2 (t, C_{-22}), 28.0 (q, C_{-23}), 15.3 (q, C_{-24}), 16.1(q, C_{-25}), 16.1 (q, C_{-26}), 14.7 (q, C_{-27}), 181 (s, C=O, C_{-28}), 109.7 (t, C_{-29}), 19.4 (q, C_{-30}), 79.0 (t, OCH_2O, C_{-31}),. The results are presented in Table 4.12.

^{1}H-NMR (600 MHz, $CDCl_3$, δppm): 0.90/1.68 (2H, m, $H_{-1a/1b}$), 1.60 (2H, m, H_{-2}), 3.25 (1H, m, H_{-3}), 2.12(1OH, 3β-OH), 0.69 (1H, m, H_{-5}), 1.40/1.54 (2H, m, $H_{-6a/6b}$), 1.40 (2H, m, H_{-7}), 1.25 (2H, m, H_{-9}), 1.27/1.41 (2H, m, $H_{-11a/11b}$), 1.05/1.75 (2H, m, $H_{-12a/12b}$), 2.19 (1H, m, H_{-13}), 1.43/1.98 (2H, m, $H_{-15a/15b}$), 1.43/2.25 (2H, m, $H_{-16a/16b}$), 1.63 (1H, m, H_{-18}), 3.00 (1H, m, H_{-19}), 1.27 (2H, m, H_{-21}), 1.50/1.98 (2H, m, $H_{-22a/22b}$), 1.00 (3H, s, H_{-23}), 0.77 (3H, s, H_{-24}), 0.84 (3H, s, H_{-25}), 0.95 (3H, s, H_{-26}), 0.98 (3H, s, H_{-27}), 4.63/ 4.76 ($J_{Ha/Hb}$ 25.8 Hz, geminal coupled exomethylene protons H_{-29a}/ H_{-29b}), 1.71 (3H, s, H_{-30}), and 3.26 (2H, s, H_{-31}),. The results are presented in Table 4.12.

3.2.5.2 Characterisation of SGE

Appearance: creamy to off white solid

Melting point: 250-252 ^{o}C

Solubility: freely soluble in chloroform, dichloromethane

Phytochemistry: positive to Liebermann and Salkowski test

Molecular formula (HREIMS): $C_{30}H_{48}O_3$

Molecular mass (HREIMS): 456.3561 g/mol

Molecular mass (calculated): 456.7003 g/mol.

Major EI-MS m/z fragmentation peaks (relative abundance): 457 (12) [M+H], 456(30) [M+], 438(15) [M-18], 411(9) [M-COOH]), fragmentation ions due to ring C cleavage: 248(69) and 207(60), base peak 189(100).

^{13}C-NMR (150 MHz, $CDCl_3$, δppm): 38.7(t, C_{-1}), 27.4 (t, C_{-2}), 79.0 (d, CHOH, C_{-3}), 38.9 (s, C_{-4}), 55.4 (d, C_{-5}), 18.3 (t, C_{-6}) 34.3 (t, C_{-7}), 40.7 (s, C_{-8}), 50.5 (d, C_{-9}), 37.0 (C_{-10}), 20.9 (t, C_{-11}), 25.5 (t, C_{-12}), 38.4 (d, C_{-13}), 42.5 (s, C_{-14}), 32.5 (t, C_{-15}), 30.6 (t, C_{-16}), 56.6 (s, C_{-17}), 49.8 (d, C_{-18}), 47.5 (d, C_{-19}), 150.4 (s, C_{-20}), 29.7 (t, C_{-21}), 37.2 (t, C_{-22}), 28.0 (q, C_{-23}), 15.3 (q, C_{-24}), 16.1(q, C_{-25}), 16.1 (q, C_{-26}), 14.7 (q, C_{-27}), 180.0 (s, C=O, C_{-28}), 109.7 (t, C_{-29}) and 19.4 (q, C_{-30})**.** The results are presented in Table 4.13.

^{1}H-NMR (600 MHz, $CDCl_3$, δppm): 0.90/1.68 (2H, m, $H_{-1a/1b}$), 1.60 (2H, m, H_{-2}), 3.25 (1H, m, H_{-3}), 2.12(1OH, 3β-OH), 0.69 (1H, m, H_{-5}), 1.40/1.54 (2H, m, $H_{-6a/6b}$), 1.40 (2H, m, H_{-7}), 1.25 (2H, m, H_{-9}), 1.27/1.41 (2H, m, $H_{-11a/11b}$), 1.05/1.75 (2H, m, $H_{-12a/12b}$), 2.19 (1H, m, H_{-13}), 1.43/1.98 (2H, m, $H_{-15a/15b}$), 1.43/2.25 (2H, m, $H_{-16a/16b}$), 1.63 (1H, m, H_{-18}), 3.00 (1H, m, H_{-19}), 1.27 (2H, m, H_{-21}), 1.50/1.98 (2H, m, $H_{-22a/22b}$), 0.98 (3H, s, H_{-23}), 0.98 (3H, s, H_{-23}), 0.77 (3H, s, H_{-24}), 0.84 (3H, s, H_{-25}), 0.95 (3H, s, H_{-26}), 0.99 (3H, s, H_{-27}), 4.63/ 4.76 ($J_{Ha/Hb}$ 25.8 Hz, geminal coupled exomethylene protons H_{-29a}/ H_{-29b}), 1.71 (3H, s, H_{-30}). The results are presented in Table 4.13.

FTIR spectrum (V_{max} cm^{-1}, $CHCl_3$): O-H (3424), C-H (2928), C=O (1681) and C-O (1029).

3.2.6 Column chromatography of the n-butanol fraction of *M. pigra* (aerial part)

The n-butanol fraction (0.9 g) from the crude methanol extract of *M. pigra* aerial part was dissolved in methanol and pre-adsorbed on silica gel (0.9 g) by mixing to form a homogenous paste which was allowed to air dry in a fume cupboard. The mixture was separated using column chromatography ((Length = 30 cm, internal diameter = 2 cm and

weight of silica gel (mesh 60 -230) = 100 g). The column was eluted with the following gradient of mobile phase: ethyl acetate: methanol (10:0, 150 mL), ethyl acetate: methanol (9:1, 100 mL), and ethyl acetate: methanol (8:2, 100 mL). A total of 15 fractions (20 mL portions using pre-calibrated glass sample bottles) were collected and pooled to three sub-fractions BSMP-1-3 based on TLC analysis. The sub-fraction BSMP-2 eluted with ethyl acetate: methanol (10:0 (4^{th} – 5^{th} eluate), to 9:1(6th – 10^{th} eluate)) afforded a light yellow solid coded MPL after washing with diethyl ether and re-crystalisation in acetone.

3.2.6.1. Characterisation of MPL

Appearance: light yellow solid

Melting point: 148 -152^{o}C

Phytochemistry: positive to $FeCl_3$ and Molisch tests

Molecular mass (calculated): 400.3787 g/mol.

EI-Mass spectrum: (m/z): 398[M-2H], 318(25) [M-ring B], 302 (55) [M-15-ring B], 286(64) , 258(5), m/z 184 [due to ring C cleavage], and base peak at 153(100).

^{13}C-NMR (50 MHz, DMSO-d6 δ ppm): Rhamnose moiety : 102.6 (d, $C_{-1''}$), 70.7 (d, $C_{-2''}$),71.1 (d, $C_{-3''}$), 71.3 (d, $C_{-4''}$), 72.0 (d, $C_{-5''}$)and 19.0 (q, $C_{-6''}$), chromone ring : 94.7 (d, C_{-2}), 137.2 (s, C_{-3}), 178.5 (s, C=O, C_{-4}), 99.4 (d, C_{-5}), 164.9 (s, C_{-6}), 108.6 (d, C_{-7}), 161.9 (s, C_{-8}), 120.3 (s, C_{-9}), and 157.1 (s, C_{-10}), and aryl ring B: 145.6 (s, $C_{-1'}$) and 116.0 (d, $C_{-2'}$-$C_{-6'}$ of monosubstituted aromatic ring B,). The results are presented in Table 4.16.

^{1}H-NMR (200 MHz, DMSO-d6, δ ppm): Rhamnose moiety : 5.2 (1H, d, $H_{-1''}$ due to anomeric proton), 3.1-3.9 (4 x 1H, m, $H_{-2''}$- $H_{-5''}$) and 0.9 (3H, d, J=5.8 Hz, $H_{-6''}$), chromone ring: 7.3 (1H, H_{-2}), and two meta coupled aromatic protons 6.2 (1H, H_{-5}) and 6.4(1H, H_{-7}), aryl ring B :, 6.9 ppm (5H, mono-substituted aromatic ring B, $H_{-2'}$-$H_{-6'}$). The results are presented in Table 4.16.

FTIR (V_{max} cm^{-1}, Nujol): 3280 (OH), 2920, 2850 (CH), 1657 (C=O), 1576, (Aromatic C=C), 969, 827.31, 717(Aromatic C=C deformation) and 1167 (C-O).

UV/Visible spectrum (λ_{max} nm, methanol): 217, 233, 335.

3.2.7 Antimycobacterial assay of column sub-fractions from the bioactive n-butanol fraction of *M. Pigra* using the liquid based MGIT method

This was done using the liquid based MGIT method (Selvakumar, 2010). To 7 mL of middlebrook 7H9 broth was added 0.8 mL of Oleic acid-Albumin- Dextrose-Catalase-Polymyxin B, Amphotericin B, Nalidixic acid, Trimethoprim and Azlocillin (OADC-

PANTA) antibiotics mixture to inhibit the growth of non-tuberculosis micro-organism contaminants. Using a sterile pipette, 0.1 mL of 30 mg/ mL solution of sample was added followed by the addition of 0.5 mL of 1.0 and 0.5 Mcfarland decontaminated clinical strains of the *Mycobacterium tuberculosis* The MGIT tubes containing the test samples were placed inside the BACTEC 960 SIRES system programmed as per manufacturer's instruction and incubated for 14 days alongside separate MGIT re-constituted with dimethylsulphoxide (DMSO) as negative control. Innoculation was done in a Lamiplus 7 innoculation chamber. Each sample analysis was done in duplicate. The results are presented in Tables 4.17.

3.2.8 Minimum Inhibition Concentration (MIC) determination of isolated compounds from bioactive fractions of *S. guineense* and *M. pigra*

This liquid base MGIT method (Selvakumar, 2010) was used to determine the MIC following the two fold serial dilution approach in the Middlebrook 7H9 broth as shown in Table 3.2. Only compounds separated from the acetone fraction A of *S. guineense*, and the column fraction BSMP-2 of *M. pigra* were evaluated for their MIC. This is because these fractions showed promising antimycobacterial activity. Three MGIT containing 7 mL of Middlebrook 7H9 were labelled A-C separately for each compound. From each of the MGIT, 2.8 mL of Middlebrook 7H9 was withdrawn using a sterile pipette such that the content of each of the MGIT A-C will be 4.2 mL of Middlebrook 7H9. The SGD (10 mg), SGE (10 mg) and MPL (8 mg) were dissolved separetely in 4.2 mL of DMSO in sterile vials. This is to give a stock solution of 2.381, 2.381, and 1.905 mg/mL respectively. The content of the SGD stock solution was completely transferred aseptically into the MGIT that has been labelled A, covered with the cork and mixed by vortexing to obtain test sample solution A. This is to re-constitute the SGD in the Middlebrook 7H9. From the resulting test sample solution A, 4.2 mL of the mixture was withdrawn using a sterile micro-pipette and transferred into the MGIT labelled B, covered with the cork and mixed by vortexing to obtain test sample solution B. From the resulting test sample solution B, 4.2 mL of the mixture was withdrawn and transferred into the MGIT labelled B, covered with the cork and mixed by vortexing to obtain test sample solution C. Using a sterile pipette a 4.2 mL aliquot of the content of the resulting mixture in test sample solution C was withdrawn and discarded. This is to ensure that the final volume in each of the three test sample solutions A-C (now re-constituted in the Middlebrook 7H9) is 4.2 mL. To each

of the re-constituted test sample solutions A-C was added in succession 2.9 mL of fresh middlebrook 7H9, 0.7 mL of OADC-PANTA supplement, and 0.5 mL of the decontaminated MTB inoculums. This adjusted the final volume in each of the MGIT labelled A-C to 8.4 mL. The SDE and MPL stock solution were re-constituted in the Middlebrook 7H9 and innoclated following the same procedure as described for SGD. This give a final concentrations (after inoculation) of: 0.5953, 0.2977 and 0.1488 mg/mL separately for compound SGD and SGE isolated from *S. guineense* stem bark, and 0.476, 0.238 and 0.119 mg/mL for MPL isolated from *M. pigra* aerial part (Table 3.2). After inoculation, the various test samples A-C for each of SGD, SGE and MPL, were separately transferred respectively to the BACTEC 960 SIRES and incubated for 14 days. The results are presented in Table 4.18.

Table 3.2: Re-constitution of isolated compounds in Middlebrook 7H9 broth for MIC determination

Isolated compounds	Conc. of test compound in stock solution (mg/mL)	Test solution A		Test solution B		Test solution C	
		Conc. of test sample in MGIT –A before innoculation (mg/mL)	Final conc.. of test sample in MGIT–A after innoculation (mg/mL)	Conc. of test sample in MGIT–B before innoculation (mg/mL)	Final conc. of test sample in MGIT–B after innoculation (mg/mL)	Conc. of test sample in MGIT–C before innoculation (mg/mL)	Final conc. of test sample in MGIT–C after innoculation (mg/mL)
SGD	2.3810	1.1905	0.5953	0.5953	0.2976	0.2976	0.1488
SGE	2.3810	1.1905	0.5953	0.5953	0.2976	0.2976	0.1488
MPL	1.9048	0.9524	0.4762	0.4762	0.2381	0.2381	0.119

Key: SGD and SGE are compounds isolated from the bioactive acetone fraction A of *S. guineese.*

MPL: compound isolated from the bioactive n-butanol fraction of *M. pigra*

CHAPTER FOUR

4.0 RESULTS AND DISCUSSION

4.1 Extraction and fractionation

The *S. guineense* (stem bark) was cold macerated with chloroform and methanol in succession while the *M. pigra* (aerial part) was cold macerated with absolute methanol. The cold extraction method was used in order to avoid the possibility of thermal degradation of heat labile constituents that could occur while using the soxhlet or other thermal processes. The respective extracts were concentrated to dryness *in vacuo*. The *S. guineense* stem bark chloroform extract was defatted with n-hexane followed by fractionation with acetone. Its methanol extract was similarly fractionated with acetone to obtain the respective fractions (Scheme 3.1). The *M. pigra* (aerial part) methanol extract was fractionated by partitioning with diethyl ether and n-butanol in succession (Scheme 3.2). The yields of the extracts and the fractions are presented in Table 4.1.

4.2 Antimycobacterial assay

The two medicinal plants; *S. guineense* (stem bark) and *M. pigra* (aerial part) were selected for antimycobacterial investigation based on information obtained from ethnomedicinal survey. The liquid based MGIT and solid based LJ methods were used (were applicable). This is because of their availability aside their high degree of reproducibility. The bio-guided fractionation approach was used to investigate the extracts and their respective fractions for antimycobacterial activity with the view of isolating the antimycobacterial constituent(s). Only the MGIT method was used for the evaluation of the isolated constituents for antimycobacterial activity. This is because it is rapid, requires lesser amount of test substance and time, as well as being less prone to the likelihood of uneven mixing of test drug within the media. It also has the additional advantage of the non-involvement of heat compared to the solid based LJ method. This is to ensure quicker detection of activity and the avoidance of thermal decomposition of isolated compounds. The stem bark methanol extract of *S. guineense* (5.0 mg/mL) < aerial part crude methanol extract of *M. pigra* (5.0 mg/mL) < aerial part n-butanol fraction of *M. pigra* (2.0 mg/mL) < stem bark acetone fraction A of *S. guineense* (0.6 mg/mL) inhibited the growth of the *M.*

tuberculosis at the stated test concentrations (Tables 4.2 and 4.3). The activity of the stem bark methanol extract of *S. guineense* was not regarded as promising due to the observed moderate Colony Forming Unit (CFU) of '+' at the relatively higher test concentration of 5 mg/mL. Whereas the acetone fraction B (0.6 mg/mL, CFU = +) from the stem bark methanol extract of *S. guineense* was bacteriostatic, the n-hexane soluble fraction (0.6 mg/mL, CFU = 3+) from the chloroform extract of the same plant did not inhibit the growth of the *M. tuberculosis* compared to the observed complete growth inhibition by the acetone fraction A (0.6 mg/mL, CFU = -) from the chloroform extract of the same plant. This is an indication that the antimycobacterial constituent(s) in the stem bark of *S. guineense* are in the acetone fraction A of the chloroform extract of the plant. The complete inhibition of the *M. tuberculosis* by the acetone fraction A (0.6 mg/mL) from the stem bark of *S. guineense* (Table 4.2), and the n-butanol fraction (2 mg/mL) from the aerial part of *M. pigra* (Table 4.3) are comparable (in colony growth unit) with the standard drug (isoniazids, rifampicin and dihydrostreptomycin) and were taken as promising for further investigation with the view of isolating the antimycobacterial drug lead compounds using the bio-activity guided chromatographic separation approach. Generally, crude plant extracts with antimicrobial activity at a test concentration of 2 mg/mL or lower are regarded as having promise for the isolation of drug lead compounds (Nvau *et. al*, 2011). Based on the MIC method, bacterial growth is defined as the presence of 20 or more colonies (Selvakumar, 2010). However, a substance could be regarded as being antibacterial if it showed a bacterial growth inhibition of 100 or lower colonies (Selvakumar, 2010).

Table 4.1: The yields of extracts and fractions from *S. guineense* stem bark (1000 g) and *M. pigra* aerial part (500 g)

Extracts/Fractions:	SGCE	SGHS	Acetone fraction A	SGME	Acetone fraction B	Residue B	MEMP	DEMP	BSMP	BIMP
Weight (g)	10.3	4.1	5.2	105.0	5.2	98.0	57.0	40.1	4.6	12.3
% w/w yield	1.03	0.41	0.52	1.50	0.52	9.8	11.4	8.0	0.9	2.5

Key:

SGCE: The chloroform extract, of *S. guineense* stem bark.

SGHS: The n-hexane soluble fraction of SGCE.

Acetone fraction A: The acetone soluble fraction of SGCE.

SGME: The methanol extract of *S. guineense* stem bark.

Acetone fraction B: The acetone soluble fraction of SGME

Residue B: The acetone insoluble fraction SGME

MEMP: The crude methanol extract of *M. pigra* (aerial part)

DEMP: The diethyl ether soluble fraction of *M. pigra* (aerial part)

BSMP: The n-butanol soluble fraction of *M. pigra* (aerial part)

Table 4.2: Antimycobacterial activity of extracts and fractions from *S. guineense* (stem bark)

Test samples	Description	Test concentration (mg/ mL)	Assay method used	CFU at 0.5 McFarland Inoculums	CFU at 1.0 McFarland Inoculums
n-hexane fraction	Fraction from $CHCl_3$ extract of *S. guineense*	0.5682	MGIT	3+	3+
Acetone fraction A	Fraction from $CHCl_3$ extract of *S. guineense*	0.5682	MGIT	-	-
Methanol extract	Extract of *S. guineense*	5.0000 2.0000	LJ*	+ 2+	+ 2+
Acetone fraction B	Fraction from methanol extract of *S. guineense*	0.5682	MGIT	+	+
INH	Reference drug	0.0001	MGIT	-	-
DHS	Reference drug	0.0010	MGIT	-	-
INH	Reference drug	0.0002	LJ	-	-
DHS	Reference drug	0.0080	LJ	-	-
Growth control	Control	NA	Both	3+	3+
Sterility control	Control	NA	Both	-	-

Key to Table 4.2 : NA => Not applicable, nd➔ not determined; *MGIT not used due to colour/ppt reaction, - ➔ No growth after 2 weeks incubation at 37°C, + ➔ 1-19 colonies growth after 2 weeks incubation at 37°C, 1+ ➔ 20-100 colonies growth after 2 weeks incubation at 37°C, 2+ ➔ 100-200 colonies growth after 2 weeks incubation, 3+ ➔ 200-400 colonies growth after 2 weeks incubation at 37°C, 4+ ➔ > 400 colonies growth after 2 weeks. DHS- Dihydrostreptomycin, INH- Isoniazid, CFU- Colony Forming unit,

Table 4.3: Antimycobacterial activity of extracts and fractions from *M. pigra* (aerial part)

Test samples	Description	Test concentration (mg/ mL)	Assay method used	Colony Forming Units (CFU)			
				ZMC 303 at 0.5 McFarland Inoculums	ZMC 303 at 1.0 McFarland Inoculums	ZMC 050 at 0.5 McFarland Inoculums	ZMC 050 at 1.0 Mcfarland inoculums
MPME	Crude Extract	5.0000 2.0000	LJ	- 3+	- 3+	nd nd	nd nd
BSMP	Fraction	2.0000	LJ	-	-	-	-
INH	Reference drug	0.0002	LJ	-	-	-	-
DHS	Reference drug	0.0080	LJ	-	-	-	-
EMB	Reference drug	0.0020	LJ	-	-	-	-
RIF	Reference drug	0.0800	LJ	-	-	1+	3+
Growth control	Control	NA	LJ	3+	3+	3+	3+
Sterility control	Control	NA	LJ	-	-	-	-

Key to table 4.3 : NA => Not applicable, nd→ not determined; *MGIT not used due to colour/ppt reaction, - → No growth after 2 weeks incubation at 37°C, + → 1-19 colonies growth after 2 weeks incubation at 37°C, 1+ → 20-100 colonies growth after 2 weeks incubation at 37°C, 2+ → 100-200 colonies growth after 2 weeks incubation, 3+ → 200-400 colonies growth after 2 weeks incubation at 37°C, 4+ → > 400 colonies growth after 2 weeks.DHS- Dihydrostreptomycin, INH- Isoniazid, RIF- Rifampicin, EMB- Ethambutol, CFU-colony Forming unit, ZMC 303 is INH/RIF.DHS/EMB susceptible clinical isolates of *M.tuberculosi*., MPME→ The crude methanol extract of *M. pigra* (aerial part), BSMP→ The n-butanol soluble fraction of *M. pigra* (aerial part),

4.3 Phytochemical Analyses

The extracts and fractions from *S. guineense* (stem bark) and *M. pigra* (aerial part) were separately screened for secondary metabolites. The results of the phytochemical investigation of the various extracts/fractions (Table 4.4) showed the presence of phenolics and saponins in the methanol extracts of both plants. Only tritepenoids were present in the n-hexane soluble (SGHS) and acetone fraction A of *S. guineense* (stem bark). Alkaloids were absent in all extracts and fractions of both plants. The n-butanol fraction of *M. pigra* (aerial part) and the acetone fraction B of *S. guineense* (stem bark) gave a positive test for phenolic constituents. The presence of triterpenoids in the bioactive acetone fraction A of *S. guineense* supported earlier report on bioactive triterpenoids of the lupane [2.39], oleanane [2.41] and ursane [2.50] series among *Syzygium spp* (Fujioka *et al.*, 1994; Kashiwada *et al.*, 1998, Djoukeng *et al.*, 2005; Noudogbessi1, and Yédomonhan 2008). This could reasonably explain the observed antimycobacterial activity of the acetone fraction A of *S. guineense* while the inactivity observed for the other triterpenoids containing fractions could be attributed to structural modification. In the same vein, phenolic compounds have been shown to exhibit antibacterial activities (Cushnie, *et al.*, 2003). Calanolide A [2.94] a naturally occurring pyranocoumarin from *Calophyllum spp* seed oil has been reported to exhibit good *in vitro* activity towards *M. tuberculosis* (McKee *et al.*,1998; Currens *et al.*,1996; Dharmaratne *et al.*, 1998; Galinis *et al.*, 1996; Spino *et al.*, 1998). The presence of phenolic compounds in the BSMP fraction of *M. pigra* could as well be linked in part to its observed activity.

Table 4.4: Phytochemical screening of extracts and fractions from *S. guineense* (stem bark) and *M. pigra* (aerial part)

Secondary metabolites	SGHS	Acetone fraction A	SGME	Acetone fraction B	MEMP	BSMP
Alkaloids	-	-	-	-	-	-
Saponins	-	-	+	-	+	+
Phenolics	-	-	+	+	+	+
Flavonoids	-	-	+	+	+	+
Tannins	-	-	+	+	+	+
Triterpenoids/steroids	+	+	-	-	+	-
Free reducing	-	-	+	+	+	+
Carbohydrate/glycosides	-	-	+	+	+	+

Key :

(+) = present

(–) = absent.

SGCE: The chloroform extract, of *S. guineense* stem bark.

SGHS: The n-hexane soluble fraction of SGCE.

Acetone fraction A: The acetone soluble fraction of SGCE.

SGME: The methanol extract of *S. guineense* stem bark.

MEMP: The crude methanol extract of *M. pigra* (aerial part)

BSMP: The n-butanol soluble fraction of *M. pigra* (aerial part)

4.4: Column chromatography of the n-hexane soluble fraction of *S. guineense* stem bark

The chromatographic separation of the n-hexane soluble fraction coded SGHS afforded twenty chromatography fractions which were pooled by TLC analysis to three sub-fractions labelled SGHS-1-3 (Table 4.5). The sub-fraction SGHS-2 yielded a white crystal coded HSB after washing with acetone to remove coloured pigments. After washing the sub-fraction SGHS-3 with acetone to remove pigments, a creamy to off-white solid coded HSD was obtained.

4.4.1 Characterisation of HSB

The HSB (m.pt 249-250 ^{o}C) was obtained from the pooled column sub-fraction SGHS-2 (Table 4.5) as a white crystal (15 mg). Using pre-coated silica gel HF_{254} plates for TLC examination, a single spot with R_f value of $0.83_{(Chloroform)}$ was observed. The HSB gave a positive test reaction with Liebermann-Buchard and Salkowski's reagents a characteristic of triterpenoid. The molecular ion peak at m/z 426 (Scheme 4.1, Table 4.6 and Figure 4.2) calculated for a molecular formula of $C_{30}H_{50}O$. It showed fragmentation peaks at m/z : 426 [M+], 411 [M-CH_3], and the base peak at m/z 125 due to cleavages at ring B. Subsequent loss of a methyl group from the base peak produced the fragmentation peak at m/z 109.

The ^{13}C-NMR (150 MHz, $CDCl_3$, δppm) spectrum in Figures 4.3 to 4.5 showed a total of thirty carbon signals: 22.3 (t, C_{-1}), 41.5 (t, C_{-2}), 212.3 (s, C_{-3}), 58.2 (d, C_{-4}), 42.2 (s, C_{-5}), 41.3 (t, C_{-6}), 18.2 (t, C_{-7}), 53.1 (d, C_{-8}), 37.5 (s, C_{-9}), 59.5 (d, C_{-10}), 35.6 (t, C_{-11}), 30.5 (t, C_{-12}), 39.7 (s, C_{-13}), 38.3 (s, C_{-14}) 32.4 (t, C_{-15}), 36.0 (t, C_{-16}), 30.0 (s, C_{-17}), 42.8 (d, C_{-18}), 35.4 (t, C_{-19}), 28.2 (s, C_{-20}), 32.8 (t, C_{-21}), 39.3 (t, C_{-22}), 6.8 (q, C_{-23}), 14.7 (q, C_{-24}), 18.0 (q, C_{-25}), 20.3 (q, C_{-26}), 18.7 (q, C_{-27}), 32.1 (q, C_{-28}), 35.0 (q, C_{-29}), 31.8 (q, C_{-30}). These were rationalised on the basis of the DEPT-135 experiment (Figure 4.5) to be: eight methyl, four methine, eleven methylene, five quarternary and one carbonyl (213 ppm) carbon signals due to the ketone group. This is suggestive of a triterpene ketone derivative. The presence of this ketone carbon signal corroborated the proposed 3-oxo configuration in line with biosynthetic origin of triterpenoids. The absence of the characteristic sp^2 carbon signal in the range 100-160 ppm typical of olefinic carbon signals is an indication that HSB is a saturated triterpene ketone.

The ^{1}H-NMR (600 MHz, $CDCl_{3,}$ δppm) spectrum in Figure 4.6 showed the presence of eight angular methyl groups (one doublet and seven singlets)at δ_H ppm; 0.75 (3H, s),, 0.89 (3H, d), 0.89 (3H, s), 0.97(3H, s), 1.02(3H, s), 1.03 (3H, s) and 1.2 (3H, s). The absence of olefinic proton signal in the region 3-5 ppm corroborated similar trend in the ^{13}C-NMR spectrum. This supported the proposed saturated skeleton (Figure 4.1). Other ^{1}H signals and their corresponding carbon signals were rationalised unambiguously from the HSQC spectrum (Figure 4.7) as indicated in Table 4.7. The angular methyl carbon signals: 6.8 (q, C_{-23}), 14.7 (q, C_{-24}), 17.9 (q, C_{-25}), 20.3 (q, C_{-26}), 18.7 (q, C_{-27}), 32.1 (q, C_{-28}), 35.0 (q, C_{-29}), and 31.8 (q, C_{-30}) with their corresponding proton signals: 0.91 (H, d, H_{-23}), 0.75(3H, s, H_{-24}), (0.89, 3Hs, H_{-25}), 1.03 (3H, s, H_{-26}), 1.07, 3H, s, H_{-27}), 1.20 (3H, s, H_{-28}), 0.97, 3H, s, H_{-29}) and 1.02 (3H, s, H_{-30}) respectively from the HSQC spectrum (Figure 4.7), showed a trend characteristic of triterpenoids of the friedelane series (Mahato and Kundu, 1994; Igoli and Alexander, 2008). Comparison of ^{1}H and ^{13}C-NMR spectroscopic data of HSB was found to be consistent with that published for Friedelan-3-one (Mahato *et al.*, 1992; Mahato and Kundu, 1994; Igoli and Alexander, 2008). In view of this, HSB was characterised to be friedelan-3-one (Figure 4.1).

Friedelan-3-one commonly known as friedelin is reported to exhibit antifeedant, anticancer, antiinflammatory, anticonvulsant, antidysentery and antiulcer activities (Gunatilaka, 1986). Its presence therefore in the stem bark of *S. guineese* aside justifying some of the reported ethnomedicinal uses of this plant, could as well be a natural mechanism for preventing attack by insect pests.

Table 4.5: Column chromatography of the n-hexane soluble fraction of *S. guineense* stem bark

.

Column Eluents	Pooled Fraction	Pool No
n-hexane (100 %,100 mL),	1-4	SGHS-1
n-hexane: $CHCl_3$ (3:1 v/v, 100 mL),	5-8	
n-hexane: $CHCl_3$ (1:1 v/v, 100 mL)	9-12	SGHS-2
n-hexane:$CHCl_3$ (1:3 v/v, 100 mL),	13-16	SGHS-3
Chloroform (0:4 v/v, 100 mL)	17-20	

Key:

SGHS-1 - Chromatography sub-fraction eluted with n-hexane (100%) – n-hexane: $CHCl_3$ (3:1 v/v) from the n-hexane soluble fraction of *S. guineense*

SGHS-2 - Chromatography sub-fraction eluted with n-hexane: $CHCl_3$ (1:1 v/v) from the n-hexane soluble fraction of *S. guineense*

SGHS-3- Chromatography sub-fraction *eluted* with n-hexane: $CHCl_3$ (1:3 to 0:4 v/v) from the n-hexane soluble fraction of *S. guineense*

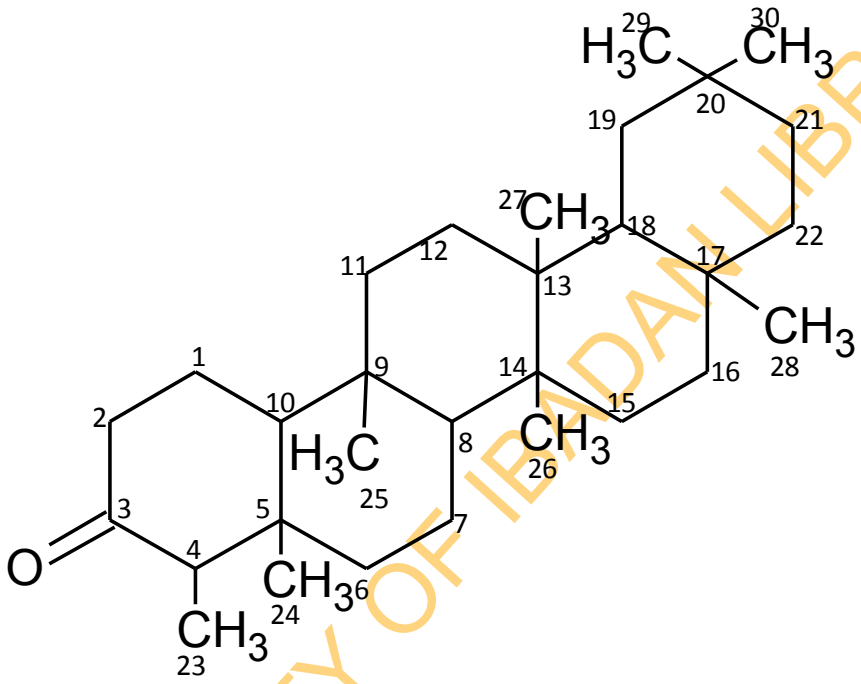
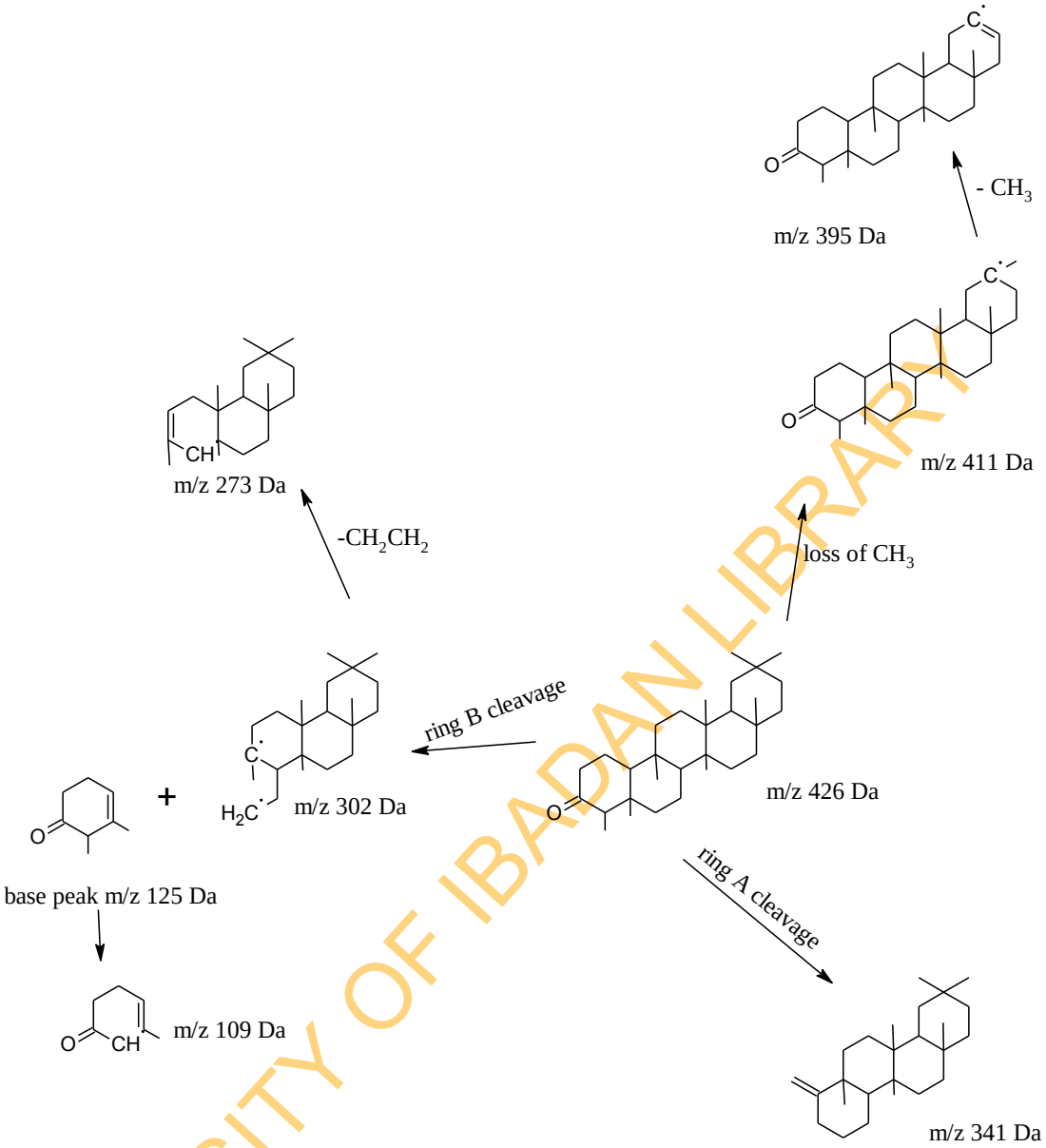


Figure 4.1: Compound HSB (Friedelan-3-one)



Scheme 4.1: Rationalised fragmentation scheme for compound HSB/SGB isolated from *S. guineense* stem bark

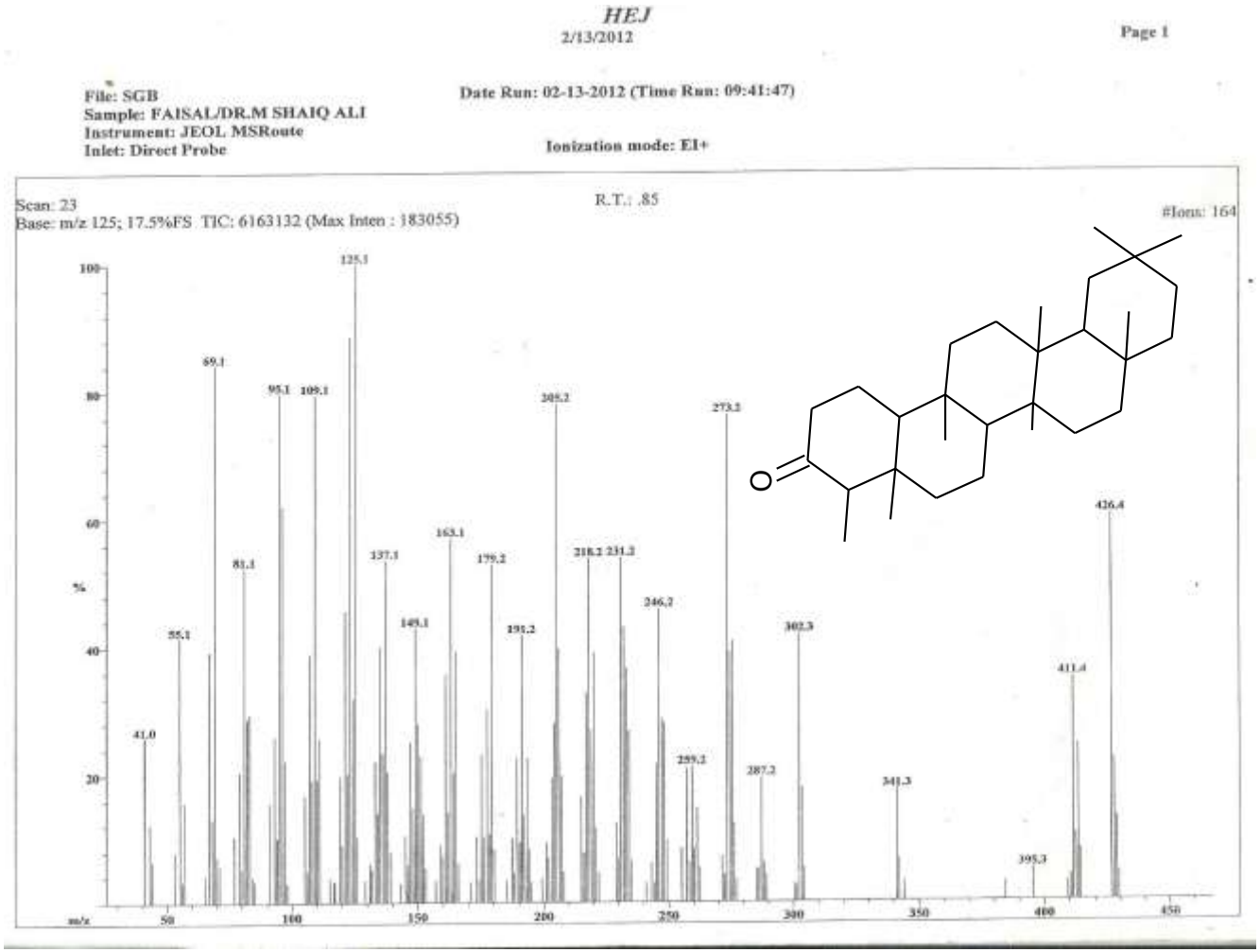


Figure 4.2: EI-mass spectrum of compound HSB

Table 4.6: High Resolution EIMS data of HSB

File : D:\Xcalibur\data\sgb(03-03-12)-c6.RAW
Full ms [39.500 - 600.500] - Range: 39.500 - 600.500
Scan No. 1 of 1

Mass	Relative Intensity	Theoretical Mass	Delta [ppm]	Delta [mmu]	RDB	Composition
218.2030	8.8732	218.2035	-2.1	-0.4	4.0	$C_{16}H_{26}$
218.9856	7.2907					
219.1723	1.4006	219.1749	-11.9	-2.6	4.5	$C_{15}H_{23}O_1$
219.2090	2.8350	219.2113	-10.2	-2.2	3.5	$C_{16}H_{27}$
220.1813	2.4040	220.1827	-6.4	-1.4	4.0	$C_{15}H_{24}O_1$
220.2187	1.4689	220.2191	-1.9	-0.4	3.0	$C_{16}H_{28}$
221.1866	0.9079	221.1905	-17.8	-3.9	3.5	$C_{15}H_{25}O_1$
227.1791	1.0739	227.1800	-3.8	-0.9	6.5	$C_{17}H_{23}$
229.1951	3.2040	229.1956	-2.1	-0.5	5.5	$C_{17}H_{25}$
230.2009	1.0926	230.2035	-11.0	-2.5	5.0	$C_{17}H_{26}$
230.9856	9.2622					
231.1752	6.1005	231.1749	1.5	0.4	5.5	$C_{16}H_{23}O_1$
231.2076	2.1732	231.2113	-16.1	-3.7	4.5	$C_{17}H_{27}$
232.1828	4.4386	232.1827	0.3	0.1	5.0	$C_{16}H_{24}O_1$
233.1926	2.3572	233.1905	8.8	2.0	4.5	$C_{16}H_{25}O_1$
234.2336	1.5082	234.2348	-4.9	-1.2	3.0	$C_{17}H_{30}$
241.1906	1.1054	241.1956	-20.9	-5.0	6.5	$C_{18}H_{25}$
242.9856	5.1489					
243.2111	1.1519	243.2113	-0.7	-0.2	5.5	$C_{18}H_{27}$
245.1892	2.2121	245.1905	-5.3	-1.3	5.5	$C_{17}H_{25}O_1$
245.2278	1.6326	245.2269	3.5	0.9	4.5	$C_{18}H_{29}$
246.1984	4.1708	246.1984	0.2	0.1	5.0	$C_{17}H_{26}O_1$
247.2038	2.2030	247.2062	-9.8	-2.4	4.5	$C_{17}H_{27}O_1$
248.2101	1.6742	248.2140	-15.8	-3.9	4.0	$C_{17}H_{28}O_1$
254.9856	1.7103					
255.2064	2.3340	255.2113	-19.1	-4.9	6.5	$C_{19}H_{27}$
256.2147	0.7308	256.2191	-17.2	-4.4	6.0	$C_{19}H_{28}$
257.2235	3.7619	257.2269	-13.5	-3.5	5.5	$C_{19}H_{29}$
258.2277	0.9943					
259.2046	2.1478	259.2062	-6.2	-1.6	5.5	$C_{18}H_{27}O_1$
259.2406	3.2127	259.2426	-7.8	-2.0	4.5	$C_{19}H_{31}$
260.2445	0.7853	260.2504	-22.6	-5.9	4.0	$C_{19}H_{32}$
261.2564	0.8578	261.2582	-7.1	-1.8	3.5	$C_{19}H_{33}$
266.9840	1.3074					
268.9824	2.8622	268.9875	-18.8	-5.1	17.5	$C_{17}H_1O_4$
269.2225	1.1014	269.2269	-16.3	-4.4	6.5	$C_{20}H_{29}$
271.2417	1.3570	271.2426	-3.1	-0.8	5.5	$C_{20}H_{31}$
272.2487	0.8599	272.2504	-6.2	-1.7	5.0	$C_{20}H_{32}$
273.2188	8.0388	273.2218	-11.2	-3.1	5.5	$C_{19}H_{29}O_1$
274.2314	3.9488	274.2297	6.4	1.8	5.0	$C_{19}H_{30}O_1$
275.2365	1.9836	275.2375	-3.5	-1.0	4.5	$C_{19}H_{31}O_1$
280.9824	5.5508	280.9875	-18.0	-5.1	18.5	$C_{18}H_1O_4$
287.2335	2.2169	287.2375	-14.0	-4.0	5.5	$C_{21}H_{31}O_1$
288.2419	0.8314	288.2453	-11.7	-3.4	5.0	$C_{21}H_{32}O_1$
292.9824	4.2895	292.9875	-17.2	-5.1	19.5	$C_{19}H_1O_4$
302.2602	3.4593	302.2610	-2.6	-0.8	5.0	$C_{22}H_{34}O_1$
303.2644	1.2777	303.2688	-14.6	-4.4	4.5	$C_{22}H_{35}O_1$
304.9824	1.2359	304.9875	-16.6	-5.1	20.5	$C_{20}H_1O_4$
316.9747	0.8140	316.9722	7.9	2.5	17.5	$C_{17}H_1O_7$
318.9792	1.2633					
330.9792	4.2629					
341.3180	1.6405	341.3208	-8.2	-2.8	5.5	$C_{25}H_{41}$
342.3281	0.8451	342.3287	-1.8	-0.6	5.0	$C_{25}H_{42}$
342.9792	2.2481					
354.9792	0.8822					
380.9760	2.4306					
392.9760	1.2897					
393.3457	0.8167					
395.3585	1.4715	395.3525	15.1	6.0	2.5	$C_{25}H_{47}O_3^-$
404.9760	0.7339					
409.3490	1.1826	409.3470	4.8	2.0	7.5	$C_{29}H_{45}O_1$
410.3789	1.0435	410.3760	7.1	2.9	2.0	$C_{28}H_{50}O_3$
411.3653	2.5399	411.3627	6.4	2.6	6.5	$C_{29}H_{47}O_1$
412.3627	0.8735					
416.9760	0.7289					
426.3903	3.3818	426.3862	9.7	4.1	6.0	$C_{30}H_{50}O_1$
427.3939	0.8266	427.3940	-0.2	-0.1	5.5	$C_{30}H_{51}O_1$

Table 4.7: ^{1}H and ^{13}C NMR (600 and 150 MHz, $CDCl_3$) spectra data of HSB

Assign-ment	δ_C ppm	published[a-c] δ_C ppm	DEPT	HSQC (δ_H ppm)	Published[a-c] δ_H ppm	^{1}H-multiplicity
1	22.3	22.3	CH_2	1.72,1.98	1.65,1.90	m
2	41.5	41.5	CH_2	2.31, 2.41	2.22, 2.32	m
3	212.3	212.3	>C=O	-	-	
4	58.2	58.2	CH	2.26	2.18	q
5	42.2	42.1	C	-	-	
6	41.3	41.3	CH_2	1.30	1.21,1.66	m
7	18.2	18.2	CH_2	1.51	1.35, 1.45	m
8	53.1	53.1	CH	1.40	1.35	s
9	37.5	37.4	C	-	-	
10	59.5	59.4	CH	1.54	1.48	m
11	35.6	35.6	CH_2	1.38,1.46	1.19, 1.38	m
12	30.5	30.5	CH_2	1.36	1.24, 1.31	m
13	39.7	39.7	C	-	-	
14	38.3	38.3	C	-	-	
15	32.4	32.4	CH_2	1.30	1.27, 1.49	m
16	36.0	36.0	CH_2	1.57	1.28, 1.50	m
17	30.0	30.0	C	-	-	
18	42.8	42.8	CH	1.56	1.51	m
19	35.4	35.3	CH_2	1.23	1.14, 1.31	m
20	28.2	28.1	C	-	-	-
21	32.8	32.7	CH_2	1.48	1.37, 1.42	m
22	39.3	39.2	CH_2	0.95,1.50	0.90, 1.41	m
23	6.8	6.8	CH_3	0.91	0.88	d
24	14.7	14.6	CH_3	0.75	0.73	s
25	18.0	17.9	CH_3	0.89	0.88	s
26	20.3	20.2	CH_3	1.03	1.01	s
27	18.7	18.6	CH_3	1.07	1.05	s
28	32.1	32.1	CH_3	1.20	1.18	s
29	35.0	35.0	CH_3	0.97	0.97	s
30	31.8	31.8	CH_3	1.02	1.01	s

Key: m = overlapping proton multiplet, s = singlet proton d = doublet proton t = triplet proton q = quartet protons, [a] Mahato and Kundu, 1994 ; [b]Mahato *et al.*, 1992; [c]Igoli and Alexander, 2008.

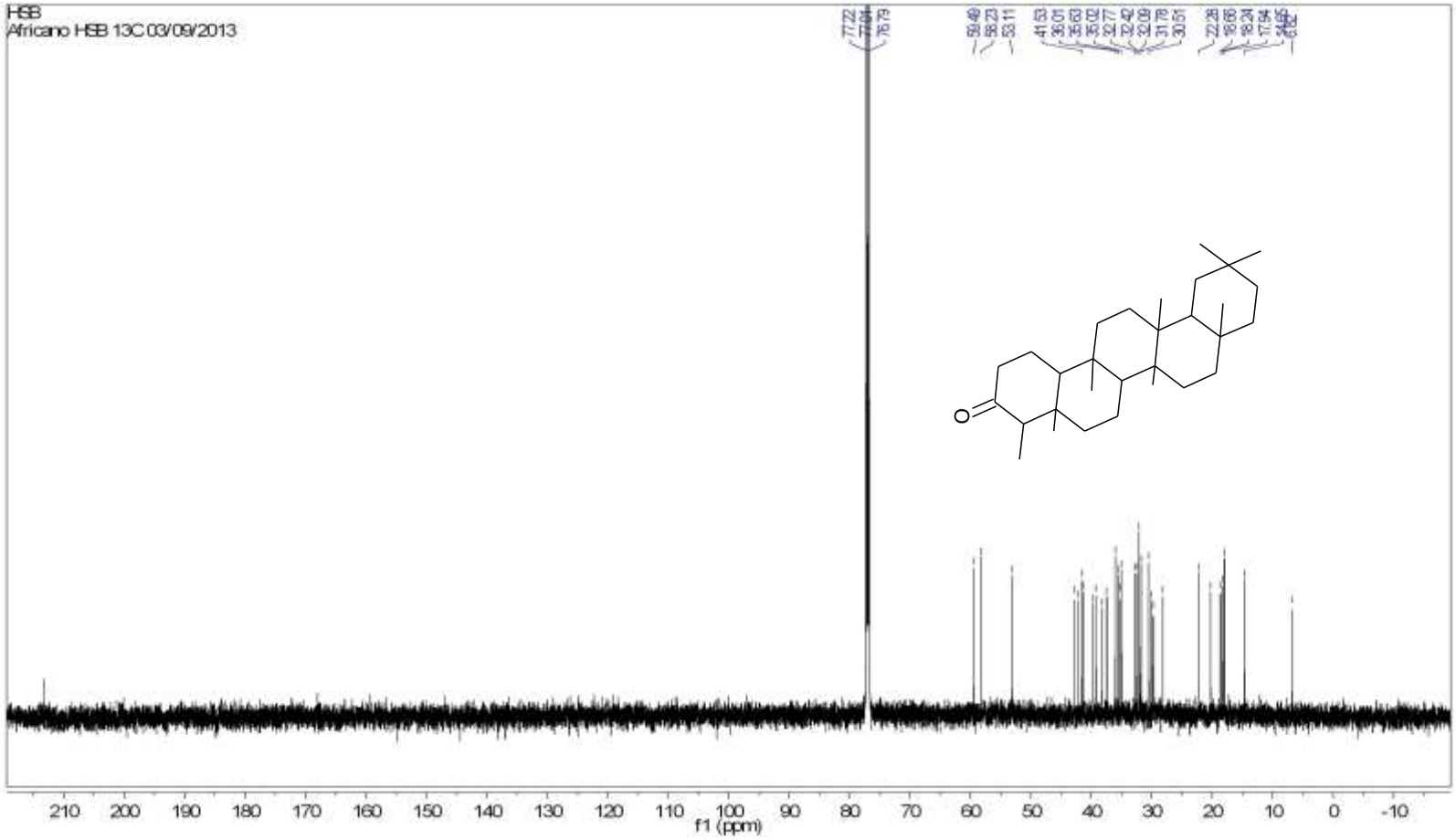


Figure 4.3: ^{13}C-NMR (150 MHz, $CDCl_3$) spectrum of HSB from *S. guineense* stem bark

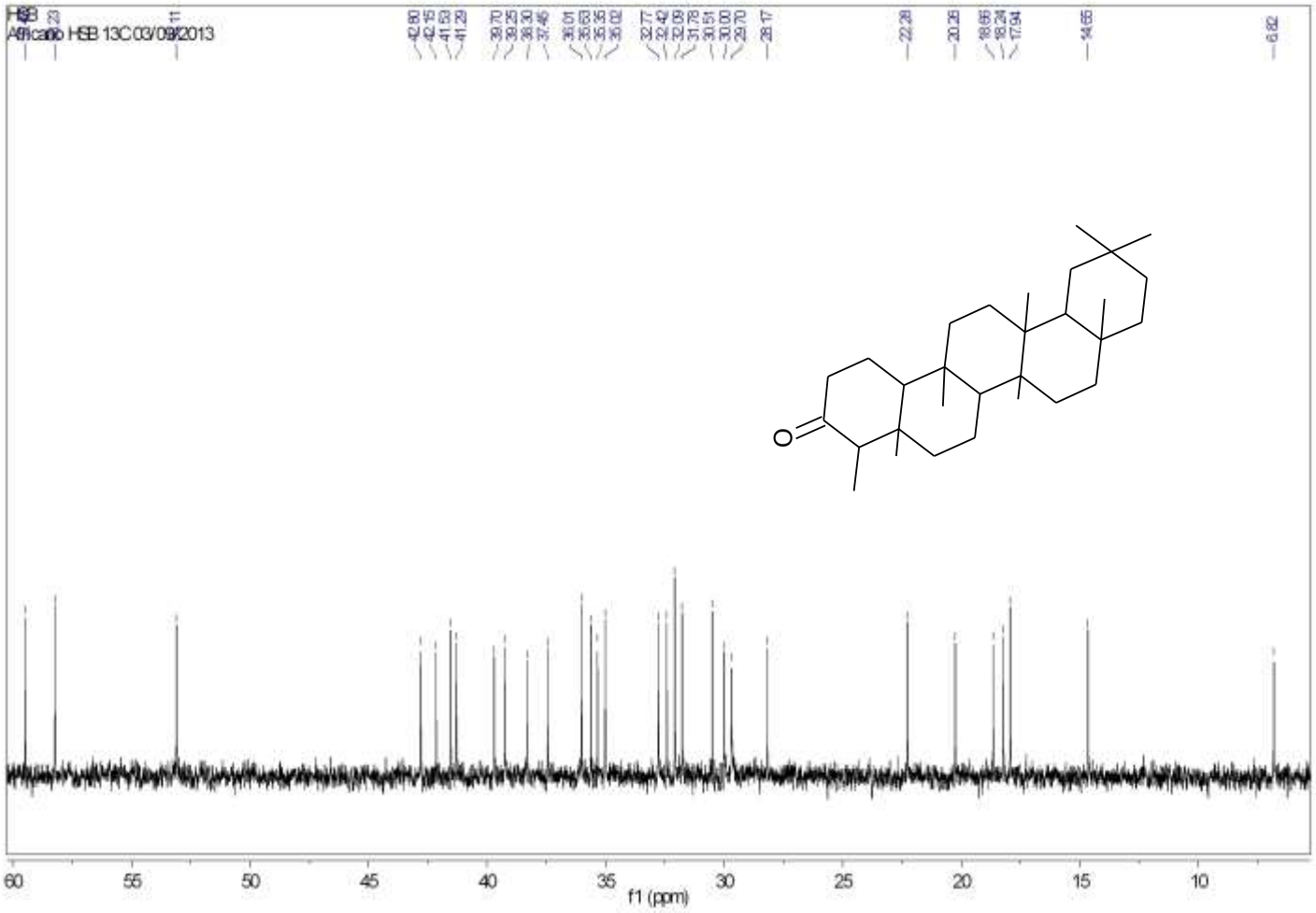


Figure 4..4: Expanded ^{13}C-NMR (150 MHz, $CDCl_3$) spectrum of HSB from *S. guineense* stem bark

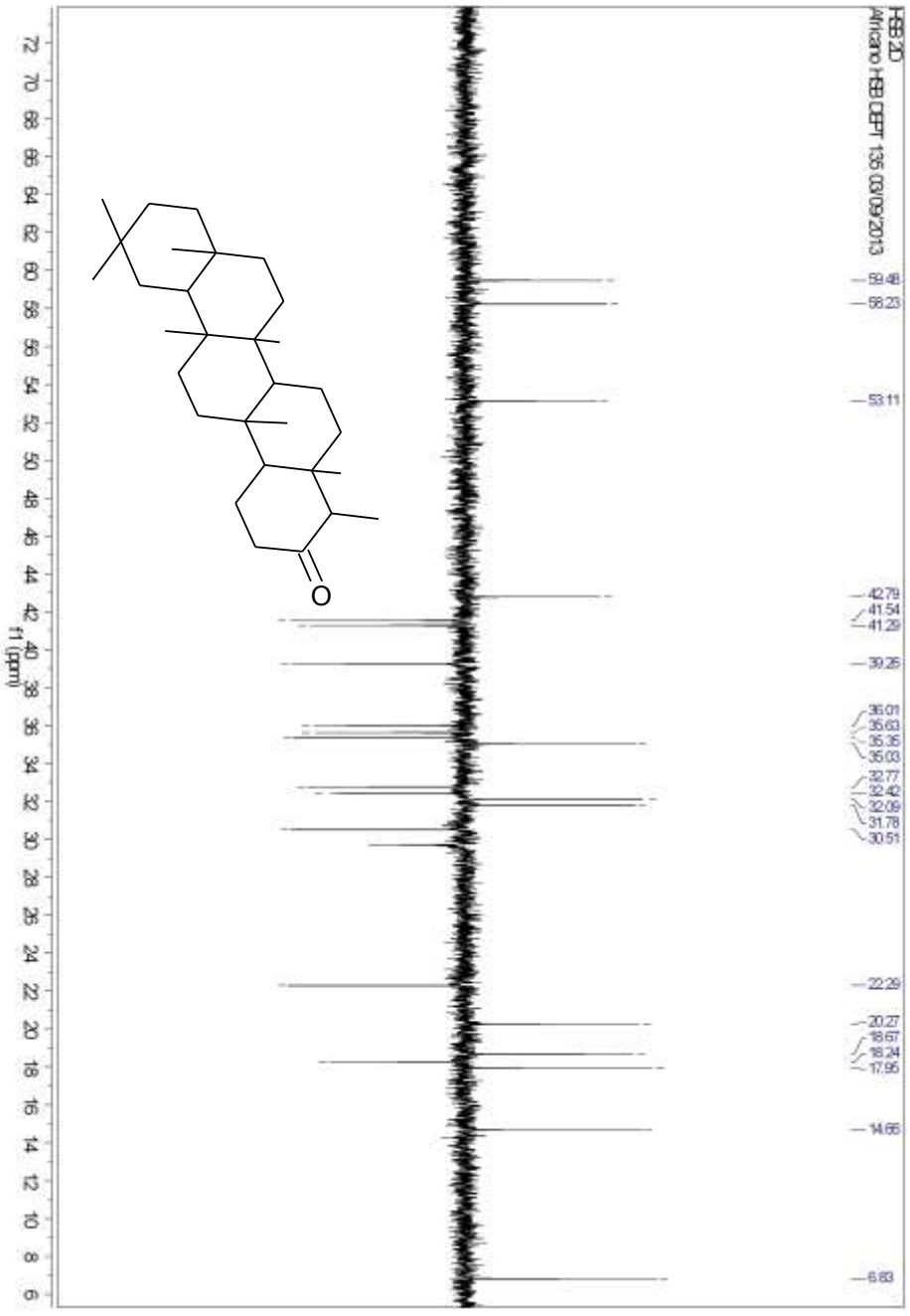


Figure 4.5: DEPT-135 (150 MHz, $CDCl_3$) spectrum of HSB from *S. guineense* stem bark

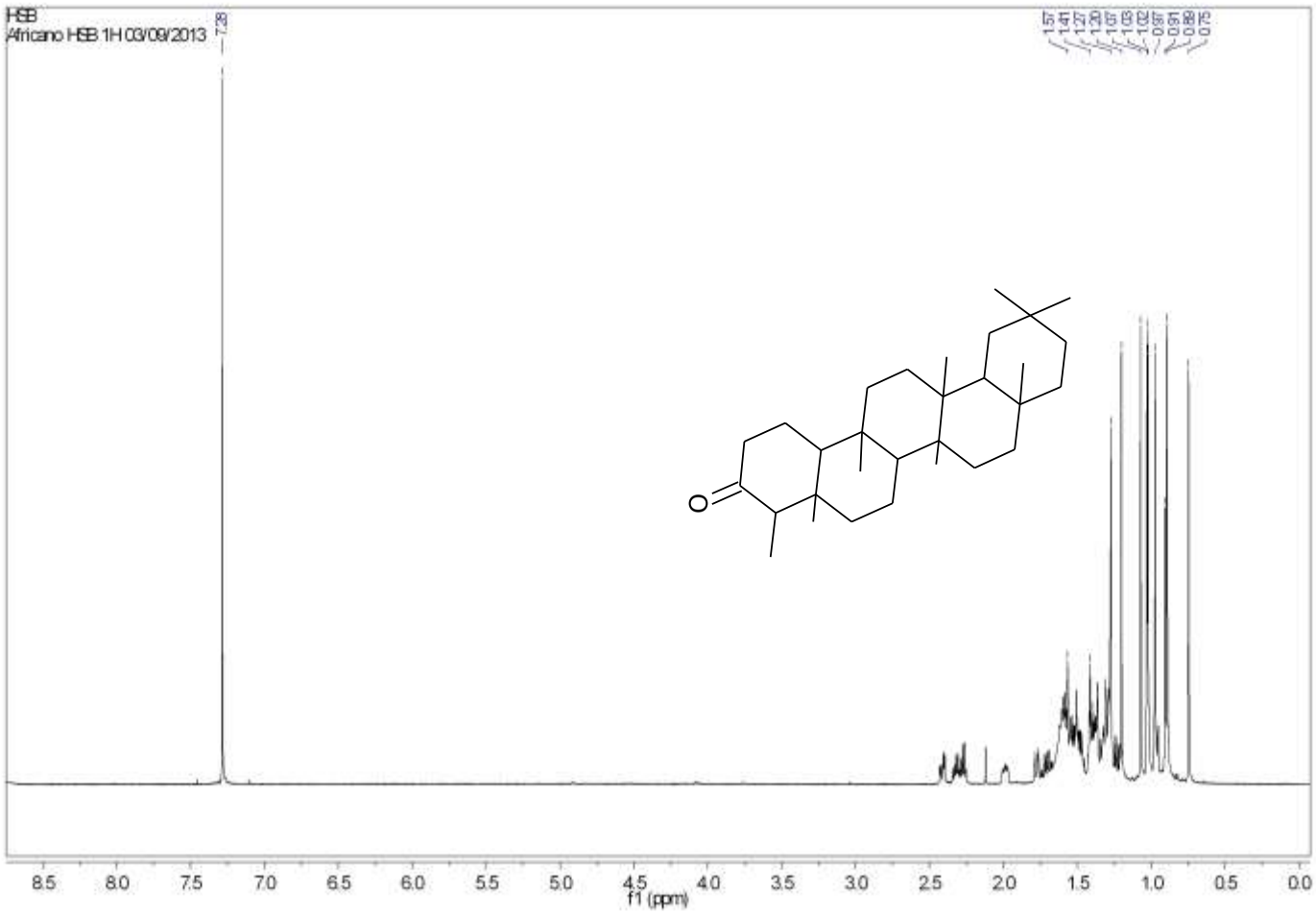


Figure 4.6: ^{1}H-NMR (600 MHz, $CDCl_3$) spectrum of HSB from *S. guineense* stems bark

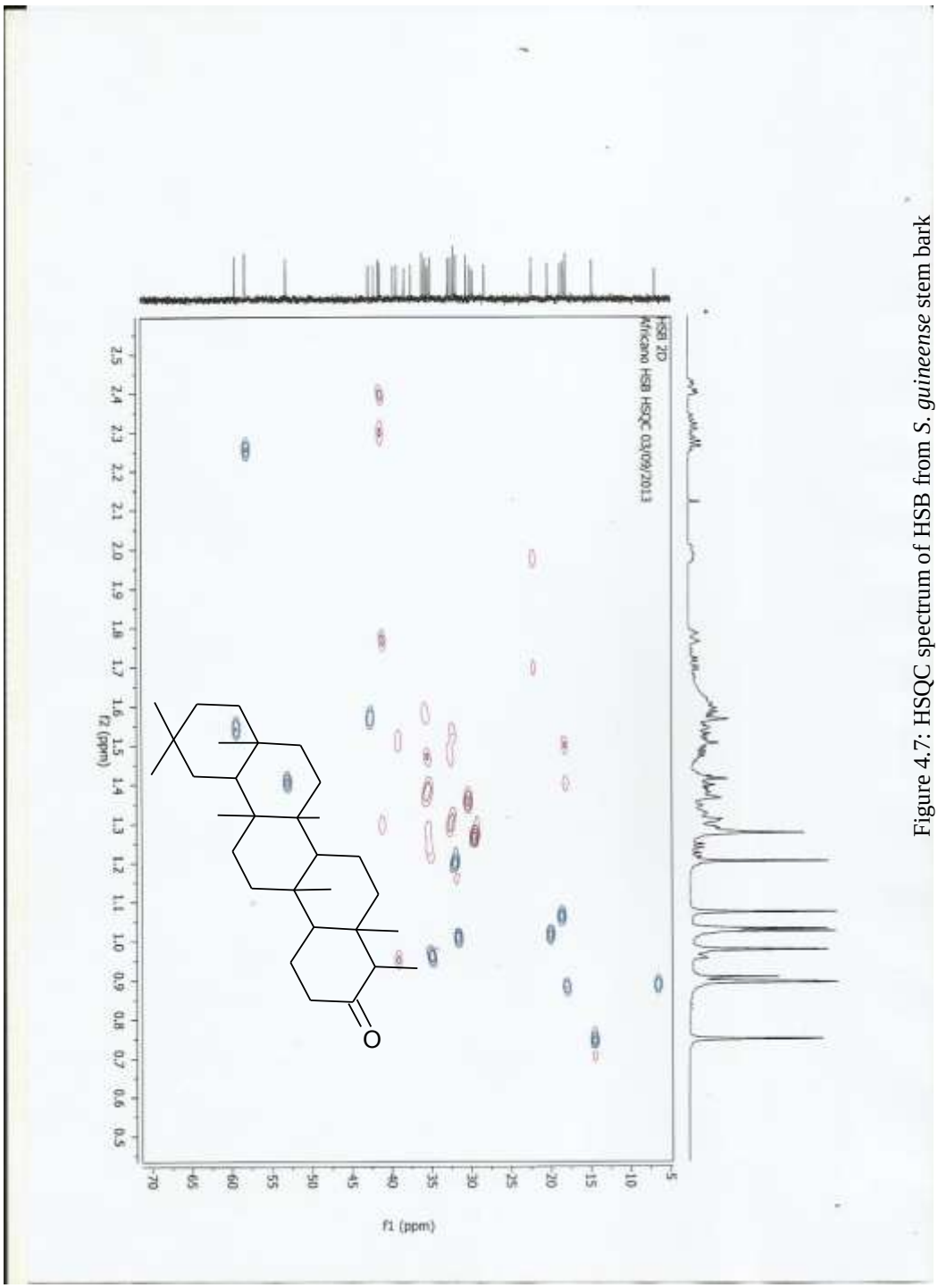


Figure 4.7: HSQC spectrum of HSB from *S. guineense* stem bark

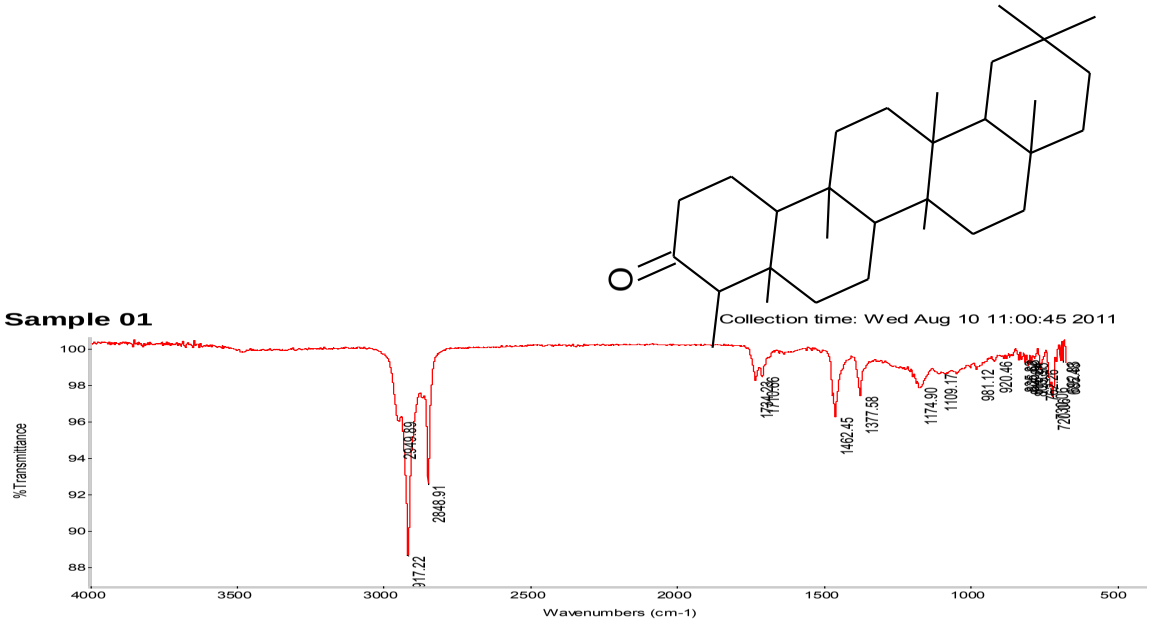


Figure 4.8: FTIR spectrum (KBr) of HSB from *S. guineense* stems bark

4.4.2. Chromatographic purification of HSD

The HSD was further purified by chromatography. A total of forty-nine fractions collected in 5 mL portions were obtained (Table 4.8) which were pooled into four sub-fractions HSD-1-4 based on TLC analysis. The sub-fraction HSD-2 afforded a white crystal coded SGB (5 mg) while the sub-fraction HSD-4 yielded a white solid GHII (24 mg).

4.4.2.1 Characterisation of SGB

The compound SGB (5 mg) was obtained from chromatography sub-fraction HSD-2 eluted with n-hexane: $CHCl_3$ (1:1) as a white crystal. Its physical, TLC profile and NMR spectroscopic data (Figures 4.9-4.11, and Table 4.9) were similar to that obtained for HSB earlier isolated from the same n-hexane soluble fraction. It was elucidated as Friedelan-3-one.

4.4.2.2 Characterisation of GHII

The compound GHII was obtained as a white solid from the sub-fraction HSD-4 eluted with n-hexane: $CHCl_3$ (0:4). The TLC examination using silica gel HF_{254} plate had a single spot with an R_f value of 0.78 in chloroform. Compound GHII (Figure 4.12) gave a positive test to Liebermann-Buchard and Salkowski's reagents. This is typical of a triterpenoidal nucleus. The Attached Proton Test experiment (APT) (100 MHz, $CD_3OD/CDCl_3$, δ ppm) in Figure 4.14 did not show the carbon signals in the region above 100 ppm where the sp^2 and carbonyl carbon signal resonate. However its FTIR spectrum (Figure 4.19) showed strong stretching vibrational frequencies for carbonyl functional group at 1714.52 cm^{-1} in addition to the OH stretching frequency at 3444 cm^{-1}. A total of twenty-nine carbon signals (one carbonyl, one secondary carbinol at 71 ppm, five methine, seven methyl, ten methylene and five quaternary) were rationalised suggestive of a demethyl- or nor-triterpenod derivative. The trend in the region for angular methyl δc signals at 11.0, 14.0, 15.6, 18.0, 19.2, 31.2 and 34.0 ppm (Figure 4.14), is characteristic of triterpenoids of the friedolenane series (Mahato and Kundu 1994). The ^{1}H-NMR (400 MHz, $CD_3OD/CDCl_3$, δ ppm) in Figure 4.15 showed the angular methyl protons doublet signal at δ_H 0.87 ppm typical of friedelin-type triterpenoids. Also observed are the overlapping proton multiplets signals in the region 1.330-1.544 and 1.726-2.301 typical of ring methylene and methine protons signal of triterpenoids. These trends in addition to the broad proton signal at 3.7 ppm

due to the 12-OH confirmed GHII to be a keto-triterpene alcohol. The unambiguous chemical shift assignment is as rationalised in Table 4.10 after 2D NMR $^{1,2,3}J_{(CH)}$ connectivities (Figure 4.16 – 4.18) spectroscopic analyses of its mixture HSD with the proposed structure in Figure 4.12 for 12-hydroxy-27-demethylfriedelanan-3-one or 12-hydroxy-27-norfriedelanan-3-one. This is further confirmed from the EI^{+}-mass spectrum (Figure 4.13) having a molecular ion peaks at m/z 428 and the rationalised fragmentation pattern in Scheme 4.2.

Table 4.8: Column chromatography purification of HSD

Column Eluents	Pooled Fraction	Pool No
n-hexane (100 %, 50 mL),	1-9	HSD-1
n-hexane: $CHCl_3$ (3:1 v/v, 50 mL),	10-20	
n-hexane: $CHCl_3$ (1:1 v/v,50 mL)	21-30	HSD-2
n-hexane:$CHCl_3$ (1:3 v/v, 50 mL),	31-39	HSD-3
Chloroform (100 %, 50 mL)	40-50	HSD-4

Key:

HSD- Creamy to off-white solid obtained from chromatography sub-fraction SGHS-3 in Table 4.5

HSD-1 - Chromatography sub-fraction eluted with n-hexane (100%) – n-hexane: $CHCl_3$ (3:1 v/v) from HSD

HSD-2 - Chromatography sub-fraction eluted with n-hexane: $CHCl_3$ (1:1 v/v) from HSD

HSD-3- Chromatography sub-fraction eluted with n-hexane: $CHCl_3$ (1:3) from HSD

HSD-4- Chromatography sub-fraction eluted with $CHCl_3$ (100 %) from HSD

Table 4.9: ^{13}C NMR (50 MHz, $CDCl_3$) of SGB compared with the ^{1}H and ^{13}C NMR (600 and 150 MHz, $CDCl_3$) spectra data of HSB

Assignment	δ_C ppm for HSB	δ_C ppm for SGB	APT	HSQC* (δ_H ppm)	^{1}H-multipli-city	^{1}H-^{1}H-COSY#	^{2}J, $^{3}J_{(H-C)}$ HMBC# (δ_C ppm)
1	22.3	22.3	CH_2	1.72,1.98	m	H_{-1b}, H_{-1a}	
2	41.5	41.5	CH_2	2.31, 2.41	m	H_{-2b}, H_{-2a}	C_{-1}, $C_{-3,}$, C_{-4}
3	212.3	212.3	>C=O	-			
4	58.2	58.2	CH	2.26	q	H_{-23}	C_{-5}, C_{-23}, C_{-24}
5	42.2	42.1	C	-			
6	41.3	41.3	CH_2	1.30	m		
7	18.2	18.2	CH_2	1.51	m		
8	53.1	53.1	CH	1.40	s		
9	37.5	37.4	C	-			
10	59.5	59.4	CH	1.54			
11	35.6	35.6	CH_2	1.38,1.46			
12	30.5	30.5	CH_2	1.36			
13	39.7	39.7	C	-			
14	38.3	38.3	C	-			
15	32.4	32.4	CH_2	1.30			
16	36.0	36.0	CH_2	1.57			
17	30.0	30.0	C				
18	42.8	42.8	CH	1.56			C_{-30}
19	35.4	35.3	CH_2	1.23			C_{-21}
20	28.2	28.1	C	-	-		
21	32.8	32.7	CH_2	1.48	m		
22	39.3	39.2	CH_2	0.95, 1.50	m		
23	6.8	6.8	CH_3	0.91	d	H_{-4}	
24	14.7	14.6	CH_3	0.75	s		C_{-4}
25	18.0	17.9	CH_3	0.89	s		C_{-11}
26	20.3	20.2	CH_3	1.03	s		C_{-13}
27	18.7	18.6	CH_3	1.07	s		C_{-18}, C_{-19} ,C_{-12}
28	32.1	32.1	CH_3	1.20	s		
29	35.0	35.0	CH_3	0.97	s		
30	31.8	3.8	CH_3	1.02	s		

Key: * unambiguous assignment from HSQC spectrum of HSB,
# extrapolated from 2D correlation data of HSD in Figure 4.16-4.18
m = overlapping proton multiplet,
s = singlet proton
d = doublet proton
t = triplet proton
q = quartet protons

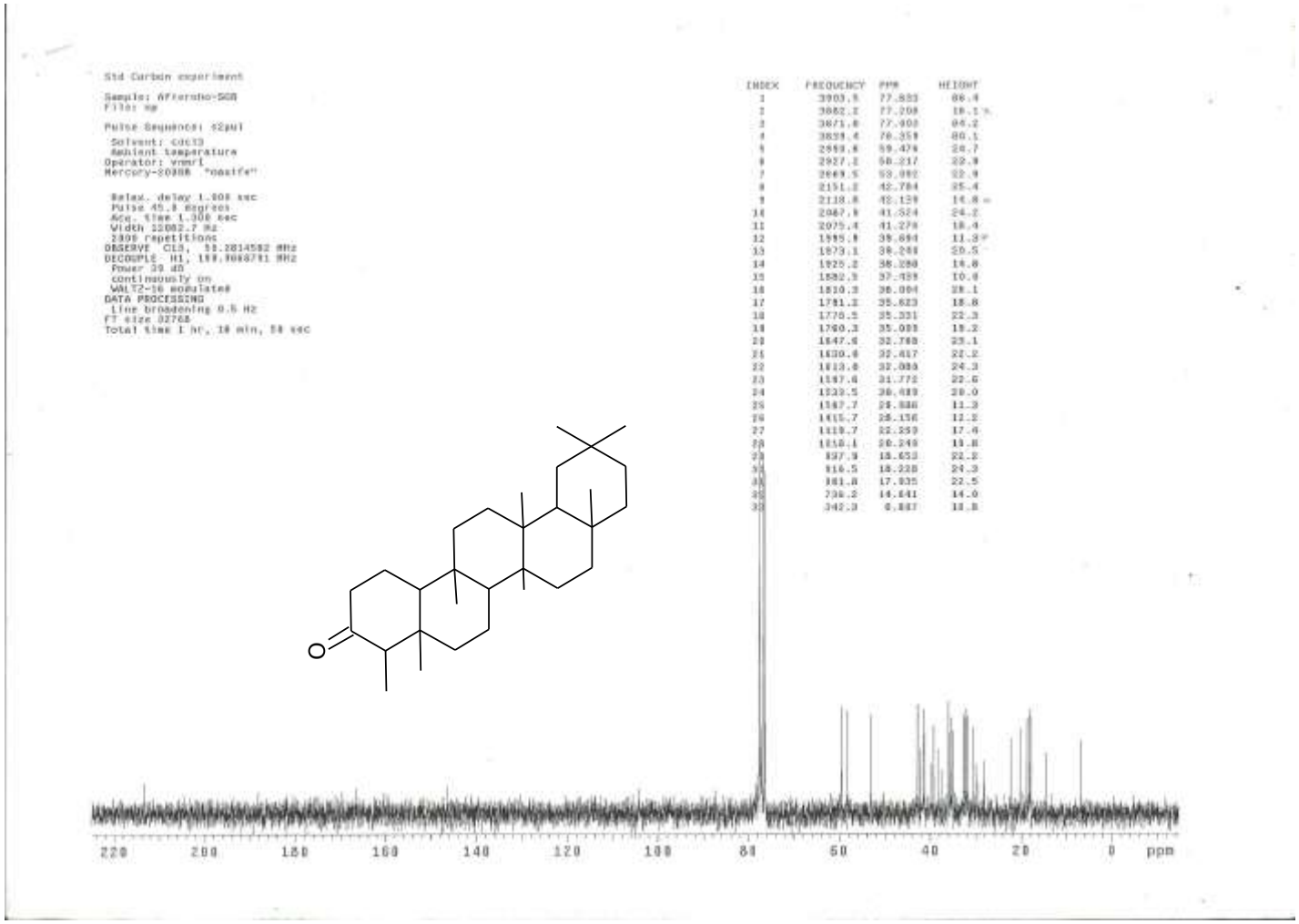


Figure 4.9 ^{13}C-NMR(50 MHz, $CDCl_3$)Spectrum of SGB

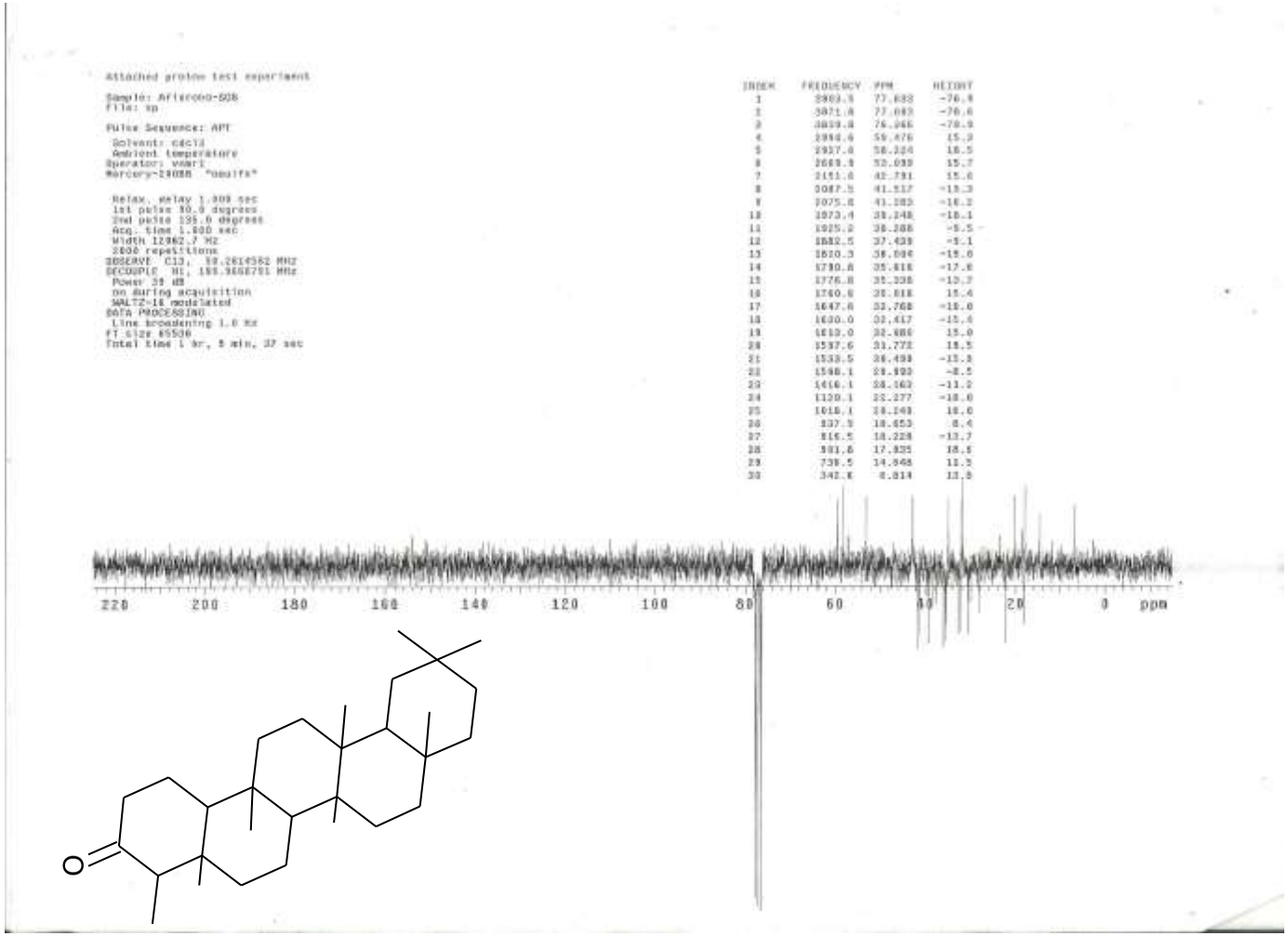


Figure 4.10: APT (50 MHz, $CDCl_3$) spectrum of SGB

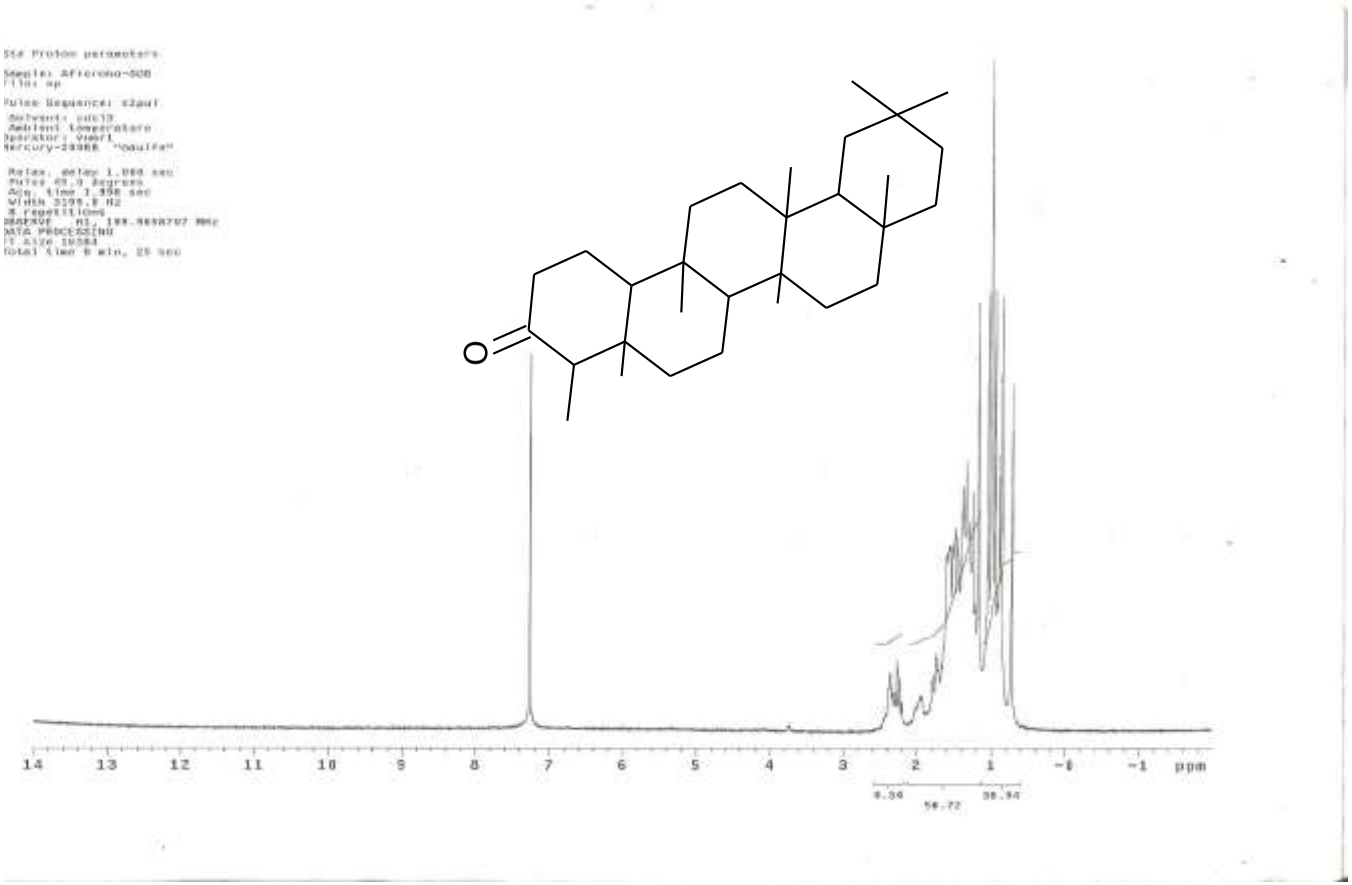


Figure 4.11: ^{1}H-NMR (200 MHz, $CDCl_3$) spectrum of SGB

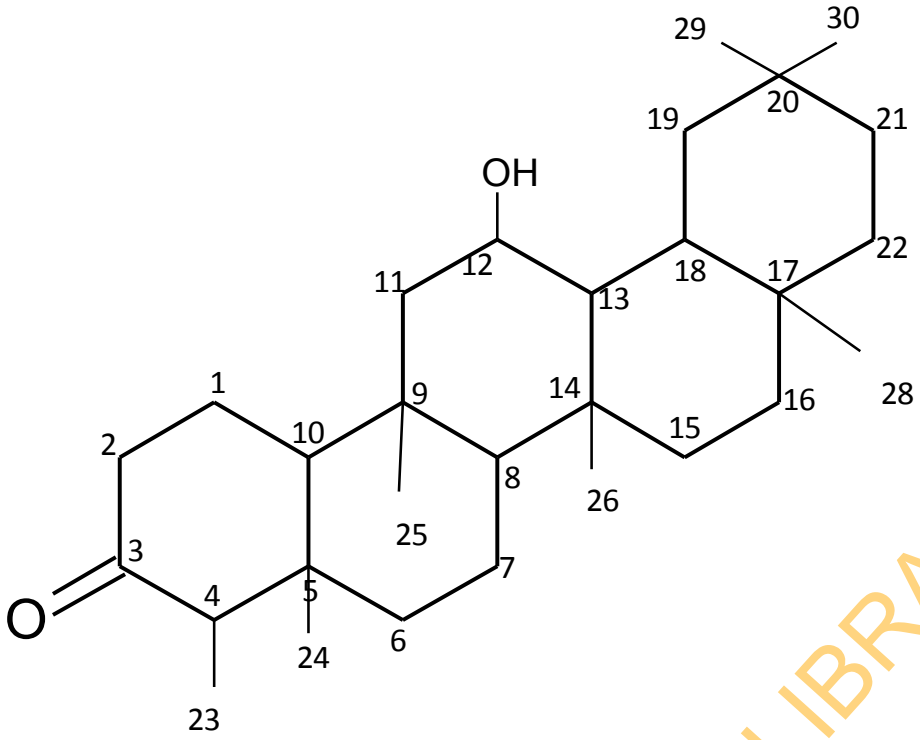
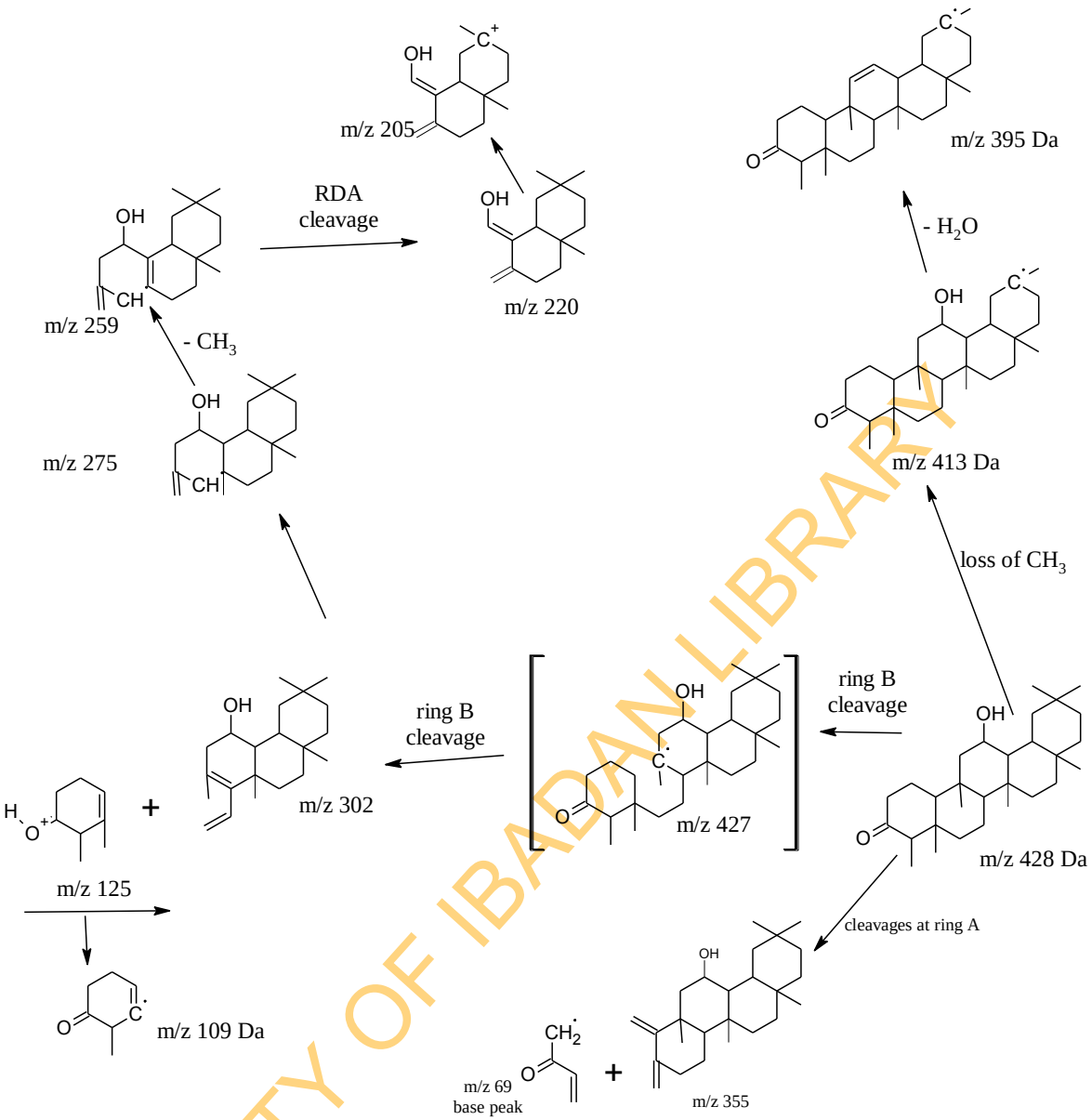


Figure 4.12: Compound GHII (12-hydroxy-27-demethylfriedoleanan-3-one or 12-hydroxy-27-norfriedoleanan-3-one)



Scheme 4.2: EI-Mass fragmentation pattern of compound GHII

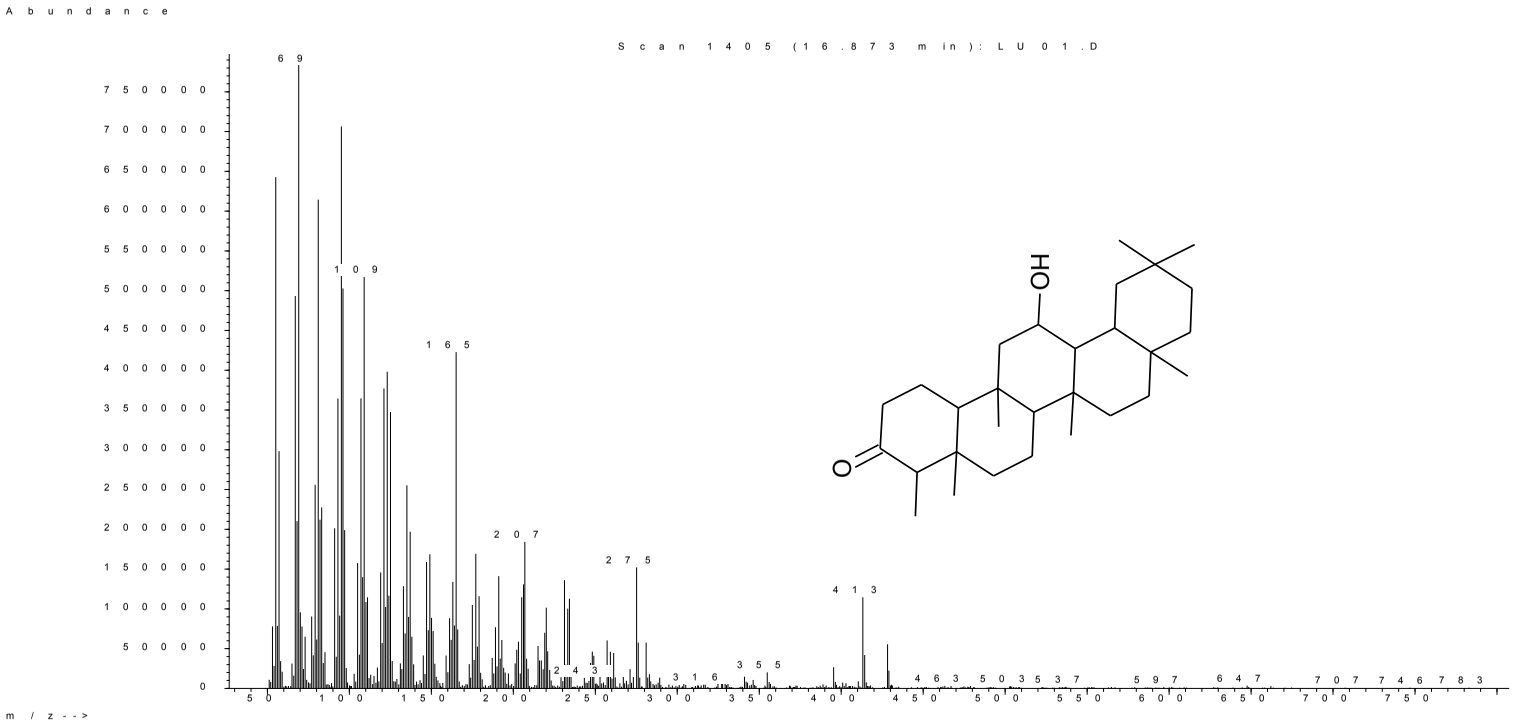


Figure 4.13: EI-Mass spectrum of compound GHII

Table 4.10: ^{1}H and ^{13}C NMR chemical shifts signals of GHII .

			^{1}H- (600 MHz) and ^{13}C- (150 MHz) NMR and selected 2D correlation data from its mixture in HSD in Figure 4.16-4.18			
Assign-ments	δ_C ppm	APT	HSQC (δ_H ppm)	^{1}H-multi-plicity	^{1}H-^{1}H-COSY	^{2}J, $^{3}J_{(H-C)}$ HMBC (δ_C ppm)
1	22.3	CH_2	1.98, 1.7	m	H_{-1b}, H_{-1a}	
2	35.0	CH_2	2.3	m	H_{-2b}, H_{-2a}	C_{-1}, C_{-3}
3	212.3ca	>C=O	-			
4	58.5	CH	2.26	q	H_{-23}	C_{-5}, C_{-23}, C_{-24}
5	40.8	C	-			
6	40.9	CH_2	0.98	m		C_{-8}, C_{-10}
7	32.0	CH_2	1.19	m	H_{-8}	
8	4.5	CH	1.26	m	H_{-7}	C_{-15}
9	37.3	C	-			
10	60.6	CH	0.91			C_{-4}, C_{-7}, C_{-25}
11	34.0	CH_2	1.91	m		
12	70.8	CHOH	3.76		H_{-11}, H_{-13}	
13	52.0	CH	1.28			
14	38.7	C	-			
15	29.5	CH_2	1.27	m		
16	36.5	CH_2	1.15			
17	37.1	C				
18	42.0	CH	1.56		H_{-13}	C_{-30}
19	34.8	CH_2	1.23			C_{-21}
20	27.4	C	-	-		
21	15.0	CH_2	1.48	m		
22	39.6	CH_2	1.50	m		
23	6.1	CH_3	0.88	d	H_{-4}	C_{-4}, C_{-7},C_{-10},C_{-25}
24	14.0	CH_3	0.75	s		
25	18.0	CH_3	1.01	s		
26	19.2	CH_3	0.97	s		C_{-7}, C_{-8},C_{-14},C_{-12}
27	-					
28	15.6	CH_3	1.20	s		
29	34.0	CH_3	0.94 ca/0.95	s		
30	31.2	CH_3	1.02	s		

Key: m = overlapping proton multiplet,
s = singlet proton
d = doublet proton
t = triplet proton
q = quartet protons

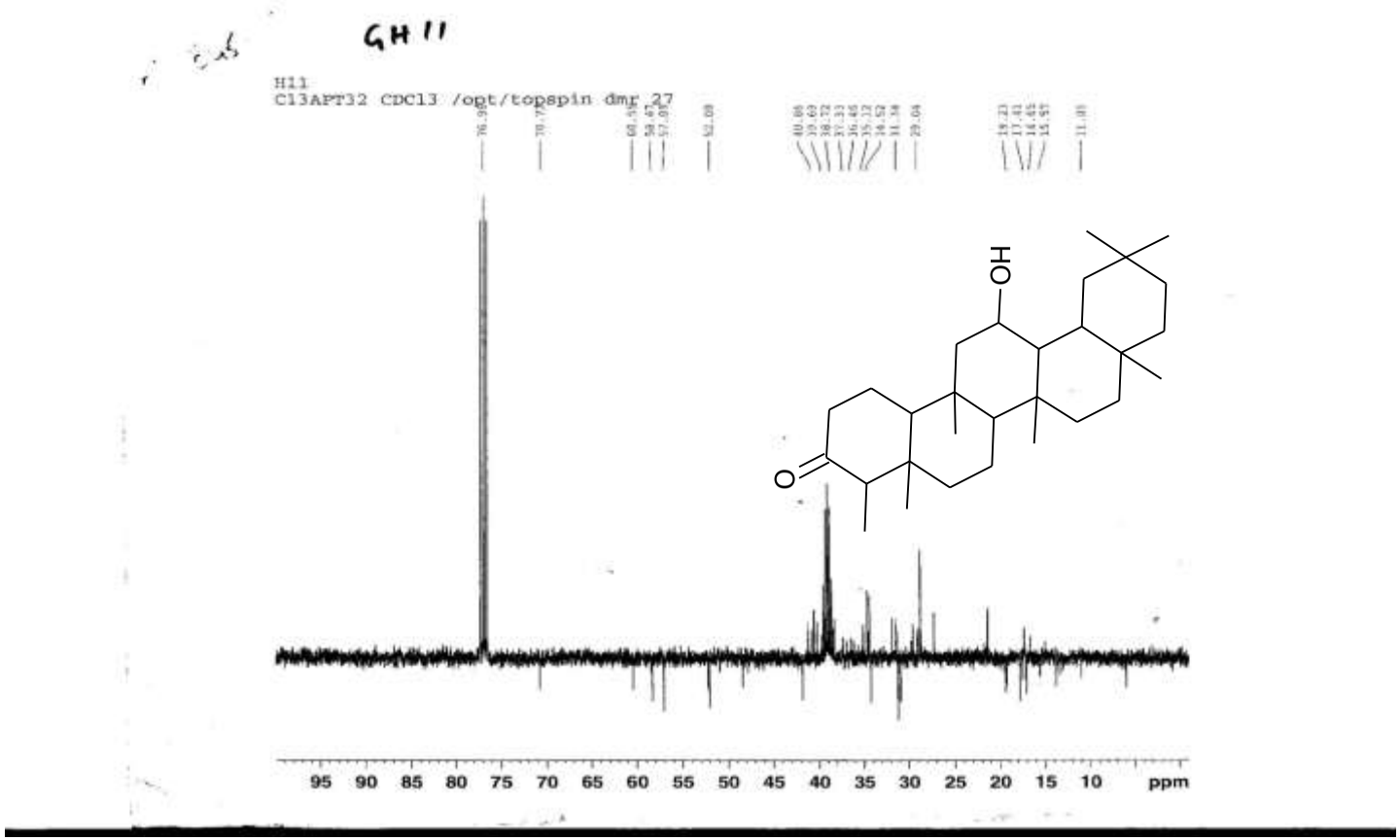


Figure 4.14: APT (100 MHz, $CD_3OD/CDCl_3$) spectrum of pure GHII

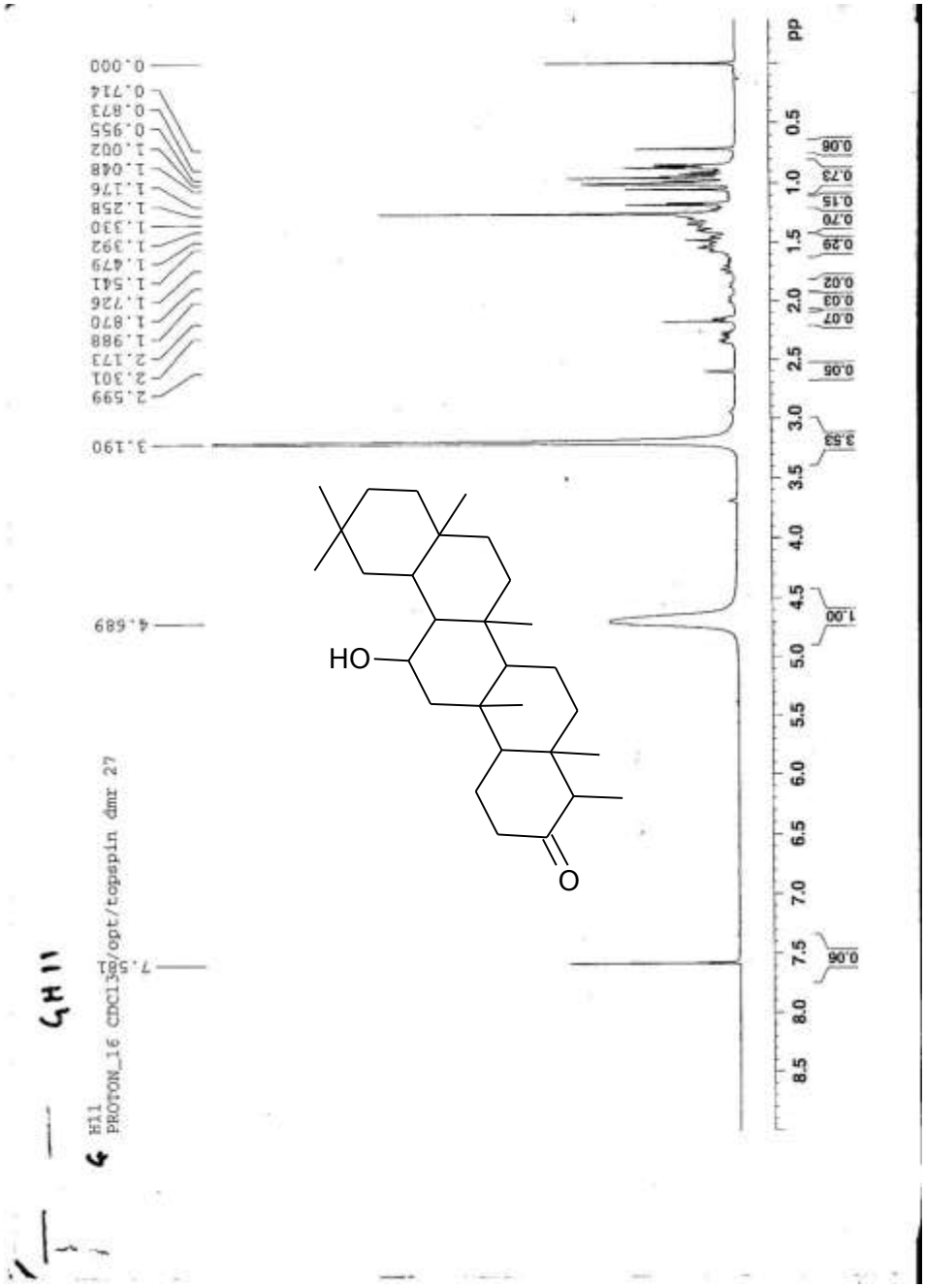


Figure 4.15: ^{1}H-NMR (400 MHz, $CD_3OD/CDCl_3$) of compound GHII

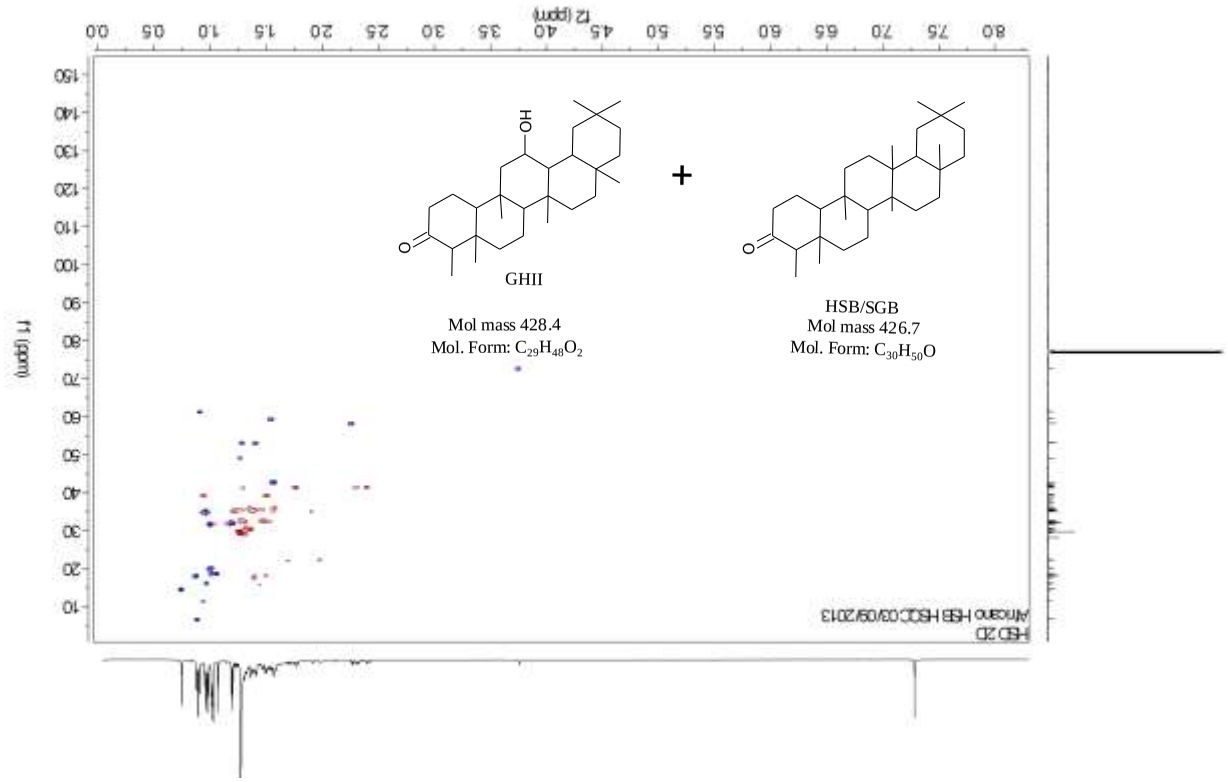


Figure 4.16: HSQC (600 MHz, $CDCl_3$) spectrum of HSD

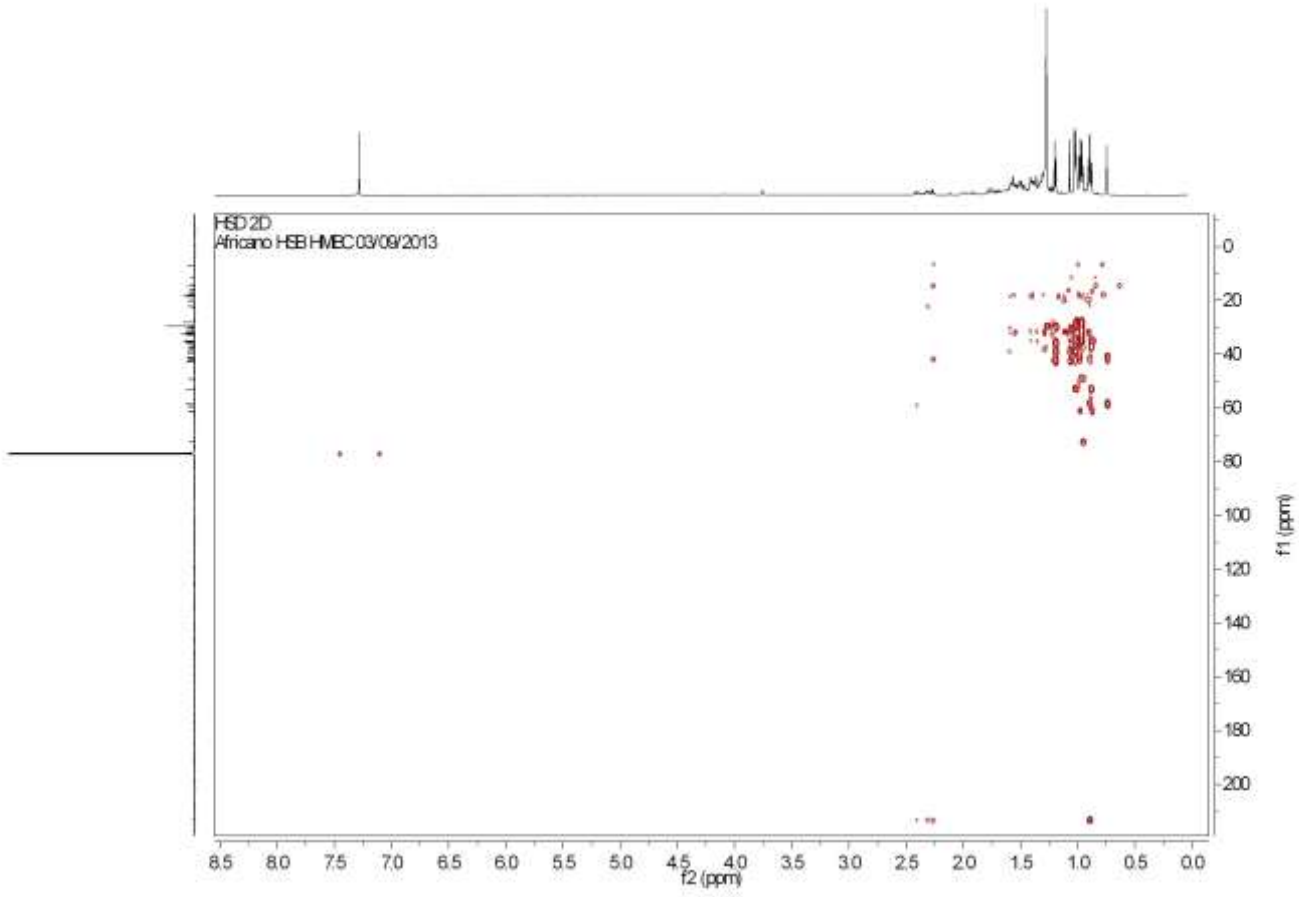


Figure 4.17. HMBC (600 MHz, $CDCl_3$) spectrum of HSD

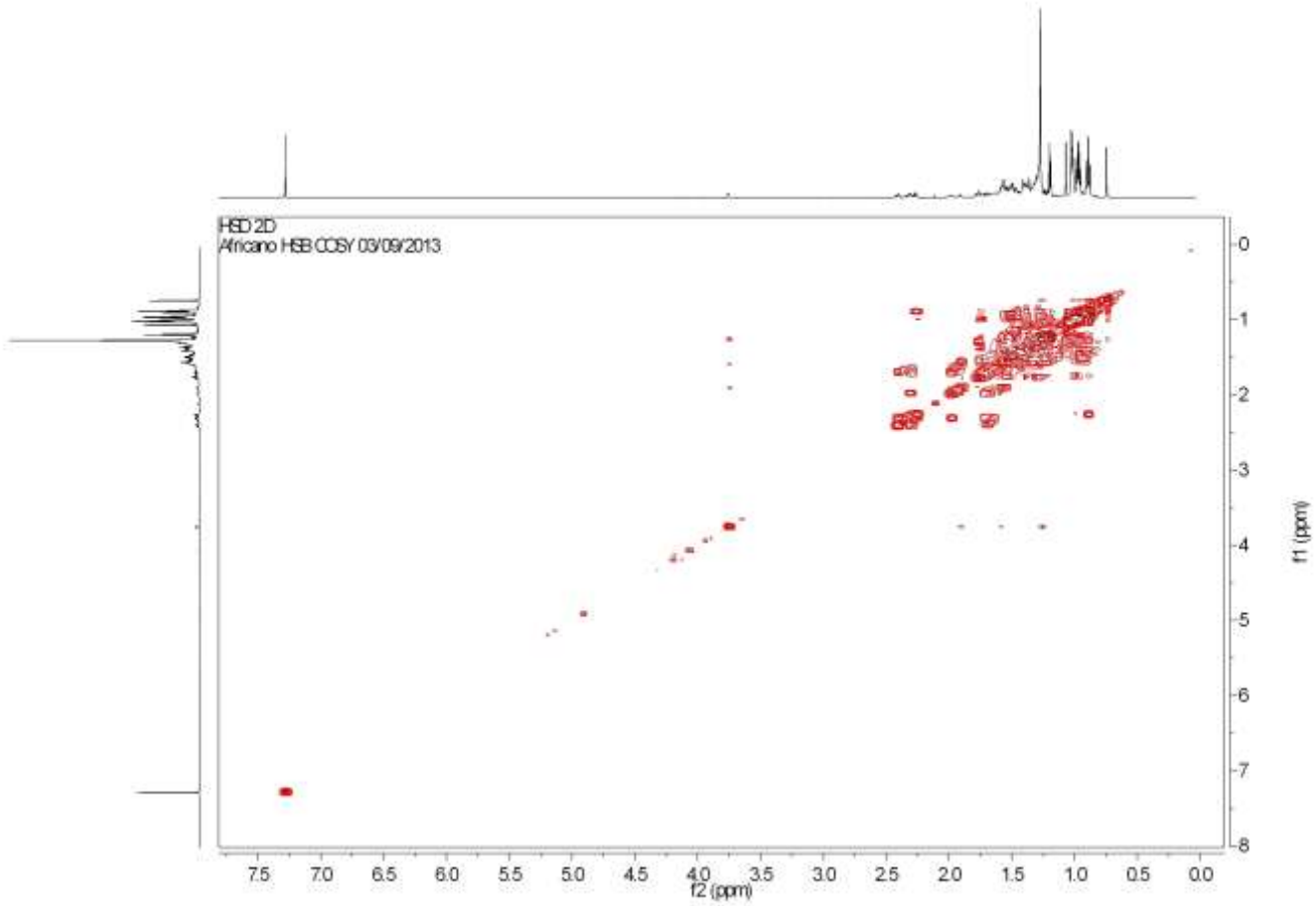


Figure 4.18. ^{1}H-^{1}H-COSY spectrum of HSD

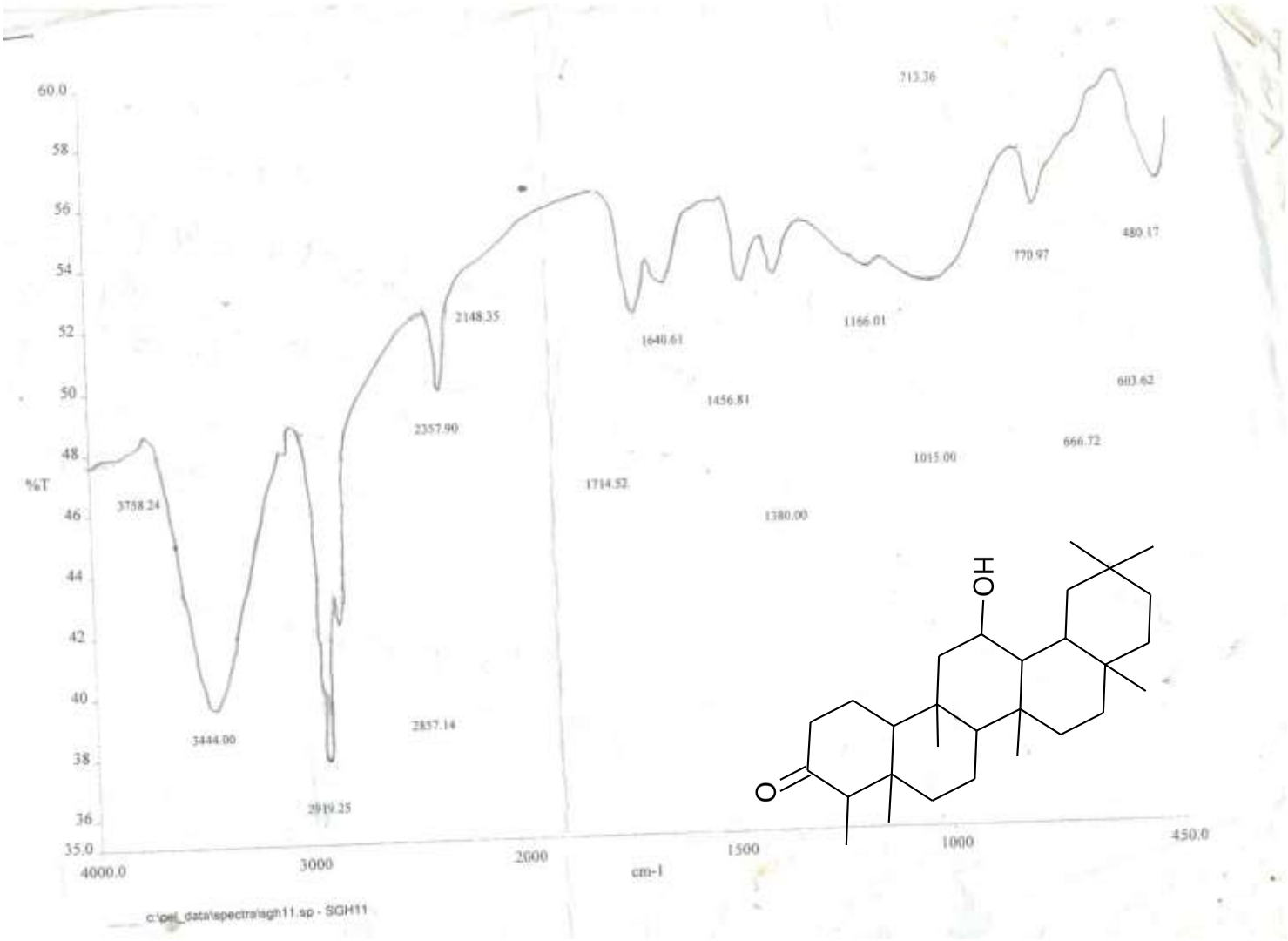


Figure 4.19: FTIR spectrum of pure GHII isolated from *S. guineense*

4.5 Column chromatography of the acetone fraction A of *S. guineense* stem bark

The chromatographic separation of the acetone fraction A gave twenty-eight chromatography fractions which were collected and pooled by TLC analysis to afford a total of three sub-fractions (Table 4.11) labelled SGHI-1-3. The chromatography fraction SGHI-2 yielded a white solid coded SGD (50.0 mg) after washing with n-hexane and re-crystalising in acetone while the chromatography fraction SGHI-3 gave a creamy to off white solid coded SGE (145 mg) after washing with n-hexane and re-crystalising in acetone.

4.5.1. Characterisation of compound SGD

Compound SGD (Figure 4.20) was obtained as a white solid with a melting point of 225-230 ^{o}C. It gave positive Liebermann and Salkowski test confirming its triterpenoidal nucleus. The EI-MS showed a molecular ion peak at m/z 486 corresponding to the molecular formula $C_{31}H_{50}O_4$ (Figure 4.21). The loss of -CH_2O gave the m/z peak at 456 which undergoes furher cleavages at ring C to produced the peaks at m/z 207 (due to ring A and B) and 248 (due to ring D and E). Subsequent loss of COOH unit from the peak at m/z 248 formed the base peak at m/z 189. The presence of these two fragment ion peaks at m/z 189 and 207, which resulted from cleavages at C_{-11} and C_{-14} of ring C, are typical of lupane-type triterpenes (Budzikiewicz *et al.*, 1963; Ryu *et al.,* 1992; Macias *et al.,* 1998). Other major m/z fragmentation peaks at 470 [M-16], 456 [M-CH_2O] and 411 [M- $COOCH_2OH$] were also observed (Scheme 4.3). The ^{1}H and ^{13}C-NMR (600 and 150 MHz, $CDCl_3$, δppm) chemical shift signals of SGD (see Table 4.12 and Figures 4.22–4.27) showed similar pattern to the ones reported for betulinic acid (Mahato and Kundu, 1994) except for the presence of a downfield methylenedioxy carbinol carbon signal at 79 ppm having its corresponding proton signal at δ_H 3.26 ppm (Figure 4.25). This additional signal validated the observed molecular ion peak at m/z 486. The molecular ion peak is an increment of 30 m/z units when compared to that of betulinic acid (m/z 456).

Its ^{1}H-NMR (600 MHz, $CDCl_3$, δ ppm) spectrum in Figure 4.24 showed a total of six methyl protons singlets (five angular methyl at δ: 0.98, 0.77, 0.84, 0.95 and 0.99 due to H_{-23}-H_{-27} respectively, and the downfield H_{-30} methyl signal at δ 1.71). In addition,

the two downfield geminally coupled ^{1}H singlets at δ 4.63(H_{-29a}) and δ 4.76 (H_{-29b}) of an exomethylene group were seen. The ^{13}C-NMR spectrum (Figure 4.22 and 4.24) is evident for the exomethylene δ_C signal at 109.7 ppm (C_{-29}) which was unambiguously assigned from the HSQC experiment (Figure 4.25). Also observed is the quaternary sp^2 δ_C signal at 150.4 ppm due to C_{-20} of the isopropenyl side chain. These trend in spectra data are characteristic of 20(29)-lupene derivatives (Adnyana *et al.*, 2000; Yogeeswari and Sriram, 2005). The 3-OH δ_C carbon signal at 79.0 ppm is an attestation to a 3β-hydroxylupene stereochemistry and not a 3α-hydroxy derivative which should resonate slightly upfield at about < 75 ppm (Mahato and Kundu, 1994). The $^{2}J_{CH}$ and $^{3}J_{CH}$ HMBC analysis spectrum in Figure 4.26 confirmed the connectivity between H_{-30} and its neighbouring carbons: C_{-20} ($^{2}J_{CH}$), C-19 ($^{3}J_{CH}$) and C_{-20} ($^{3}J_{CH}$). The ester carbonyl δ_C signal at 180 ppm, demonstrated $^{3}J_{CH}$ connectivities with H_{-18}, H_{-16} and H_{-22} respectively. The ^{1}H-^{1}H-COSY experiment (Figure 4.27) showed the H_{-3} and H_{-2} protons to be vicinally coupled. In view of these spectra data and after comparison with those reported for its parent compound betulinic acid (Mahato *et al.*, 1992*;* Mahato and Kundu, 1994) compound SGD (Figure 4.20) was named betulinic acid methylenediol ester. It is a new compound reported for the first time from this family of plants.

Table 4.11: Column chromatography separation of acetone fraction A of *S. guineense* stem bark

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Column eluent	Pooled Fraction	Pool No
n-hexane (100 ml),	1-4	SGHI-1
n-hexane:ethyl acetate (3:1 v/v, 100 mL)	5-8	
n-hexane:ethyl acetate (1:1 v/v, 100 mL)	9 -12	SGHI-2
n-hexane:ethyl acetate (1:3 v/v, 100 mL)	13-16	
Ethyl acetate (100 % 150 mL),	17-20	SGHI-3
Ethyl acetate: methanol (3 :1 v/v , 150 mL)	21-28	

Key:

SGHI-1 - Chromatography sub-fraction eluted with n-hexane (100%) – n-hexane: ethyl acetate (3:1 v/v) from the acetone fraction A of *S. guineense*

SGHI-2 - Chromatography sub-fraction eluted with n-hexane: ethyl acetate (1:1 to 1:3) from the acetone fraction A of *S. guineense*

SGHI-3 - Chromatography sub-fraction eluted with ethyl acetate (100%) to ethyl acetate: methanol (3:1) from the acetone fraction A of *S. guineense*

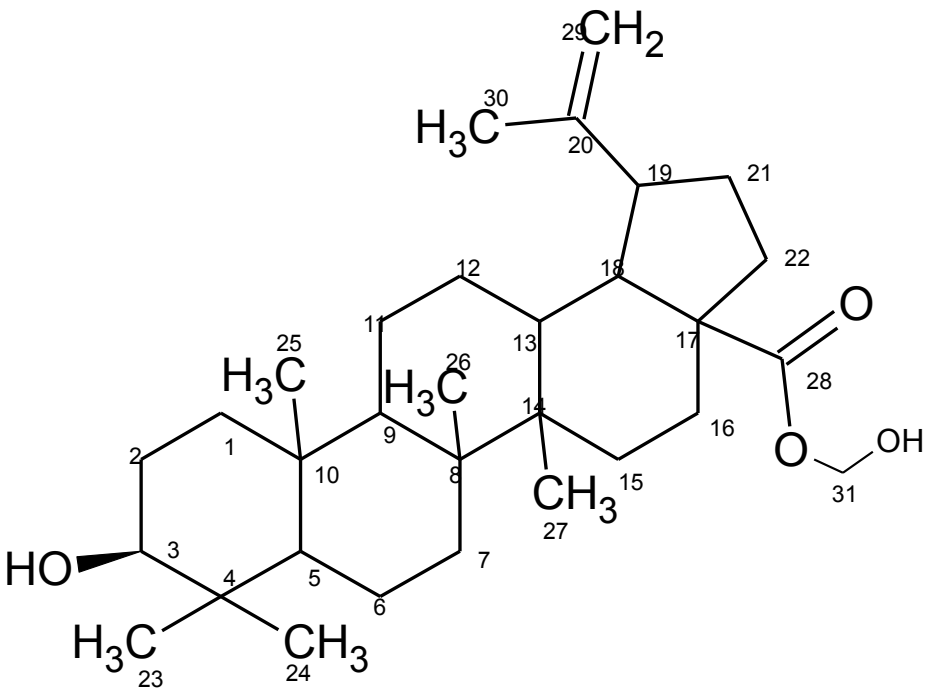

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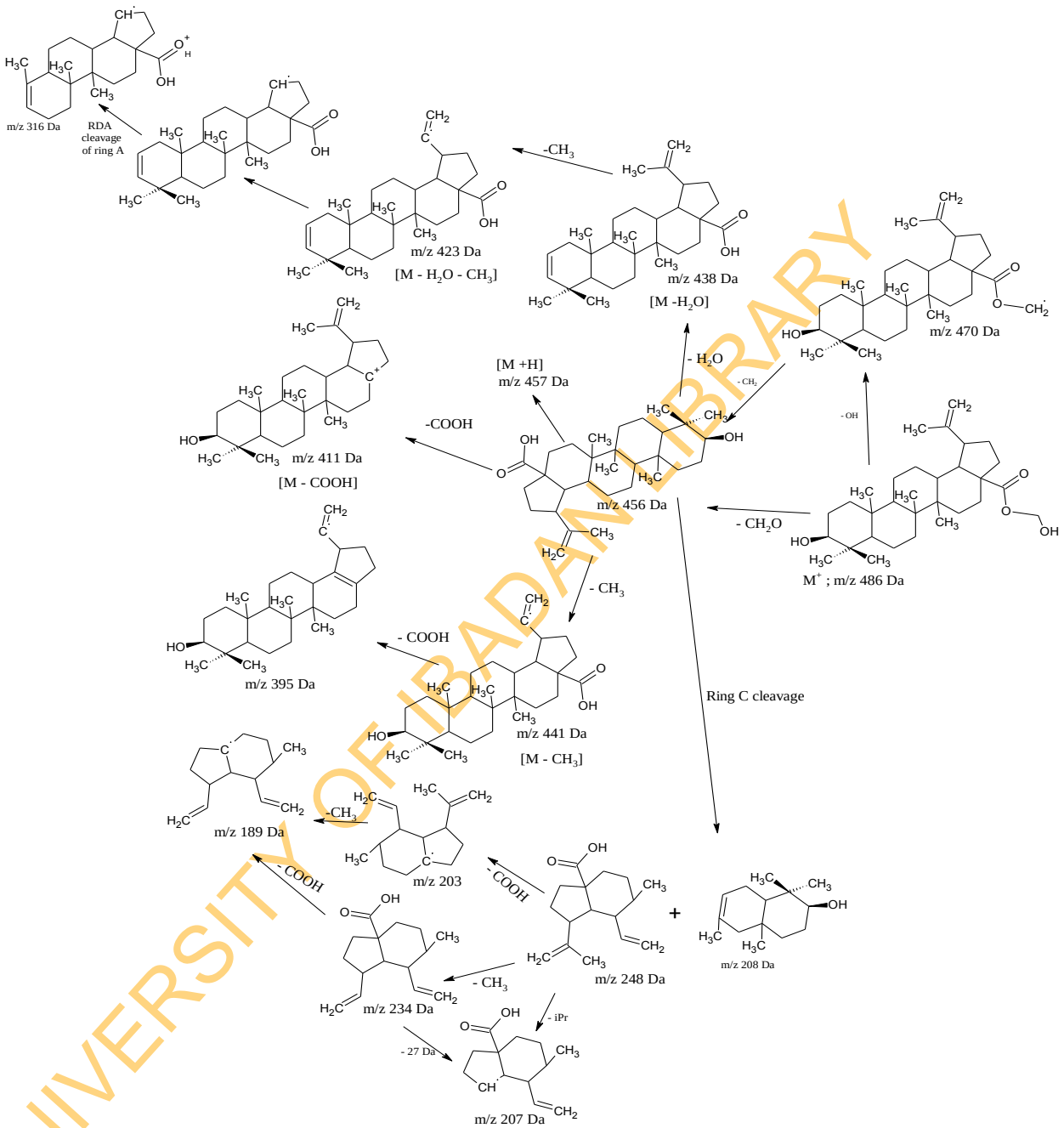
Figure 4.20: Compound SGD (Betulinic acid methylenediol ester)

Systemic name: (3β-hydroxylup -20(29) -en - 28 -oic acid methylenediol ester

Table 4.12: 1D and 2 D ^{1}H-NMR(600 MHz, $CDCl_3$) and ^{13}C-NMR (150 MHz, $CDCl_3$) spectra data of SGD

Assign-ment	$\delta_{C\ ppm}$	Pub-lished[a]	DEPT	HSQC (δ_H ppm)	^{1}H-multiplicity	^{1}H-^{1}H-COSY	^{2}J, ^{3}J$_{(CH)}$HMBC (δ_C ppm)
1	38.7	38.5	CH_2	0.90,1.68	m	H_{-2}	
2	27.4	28.2	CH_2	1.60	m	H_{-3} , H_{1a}	
3	79.0	78.1	CHOH	3.25	m	H_{-2}	$C_{-1,}$ $C_{-23,}$ C_{-24}
3-OH	-		OH	2.12	s(br)		
4	38.9	39.4	C	-	-		
5	55.4	55.9	CH	0.69	m	H_{-6a}	
6	18.3	18.7	CH_2	1.40, 1.54	m	H_{-5}	
7	34.3	34.7	CH_2	1.40	m		
8	40.7	41.0	C	-	-		
9	50.5	50.5	CH	1.29	m		
10	37.0	37.5	C	-	-		
11	20.9	21.1	CH_2	1.27, 1.41	m		
12	25.5	26.0	CH_2	1.05, 1.75	m		
13	38.4	39.2	CH	2.19	m		
14	42.5	42.8	C	-	-		
15	32.5	30.2	CH_2	1.43, 2.25	m	H_{-15b} , H_{-15a}	
16	30.6	32.8	CH_2	1.42 , 1.98	m	H_{-16a} ,H_{-16b}	$C_{-20,}$ C_{-28}
17	55.9	56.6	C	-	-		
18	49.8	49.7	CH	1.63	m	H_{-19}	$C_{-28,}$ C_{-30}
19	47.5	47.7	CH	3.00	m	H_{-18} ,H_{-21b}	C_{-20}, $C_{-29,}$ $C_{-18,}$ $C_{-16,}$ C_{-30}
20	150.4	150.4	>C=	-	-		
21	29.7	31.1	CH_2	1.27	m		
22	37.2	37.4	CH_2	1.50,1.98	m		
23	28.0	28.5	CH_3	0.98	s		
24	15.3	16.2	CH_3	0.77	s		
25	16.1	16.3	CH_3	0.84	s		
26	16.1	16.2	CH_3	0.95	s		
27	14.7	14.8	CH_3	0.99	s		
28	180.0	178.0	>C=O	-	-		
29	109.7	109.5	= CH_2	4.63 (J=4.6 MHz) , 4.76(J=4.6 MHz)	sl, sl	H_{29a} , H_{29b} ,	C_{-19} , C_{-30}
30	19.4	19.4	CH_3	1.71	s		C_{-20} , C_{-29}
31	79.0		O-CH_2OH	3.26			

Key: m = overlapping proton multiplet, s = singlet proton d = doublet proton t = triplet proton q = quartet protons, [a] Mahato and Kundu (1994)



Scheme 4.3: Mass fragmentation scheme of SGD

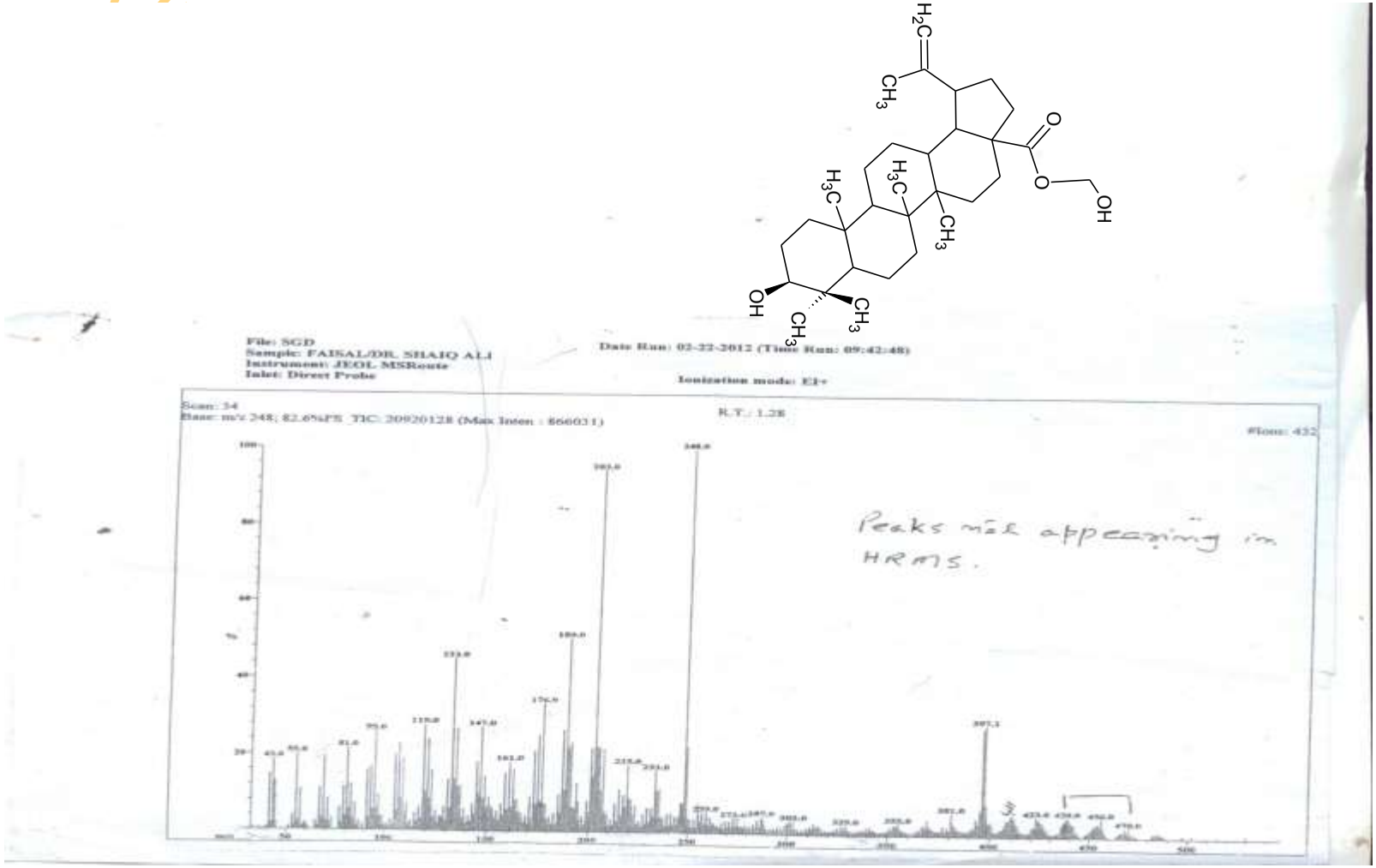


Figure 4.21: EI-mass spectrum of SGD

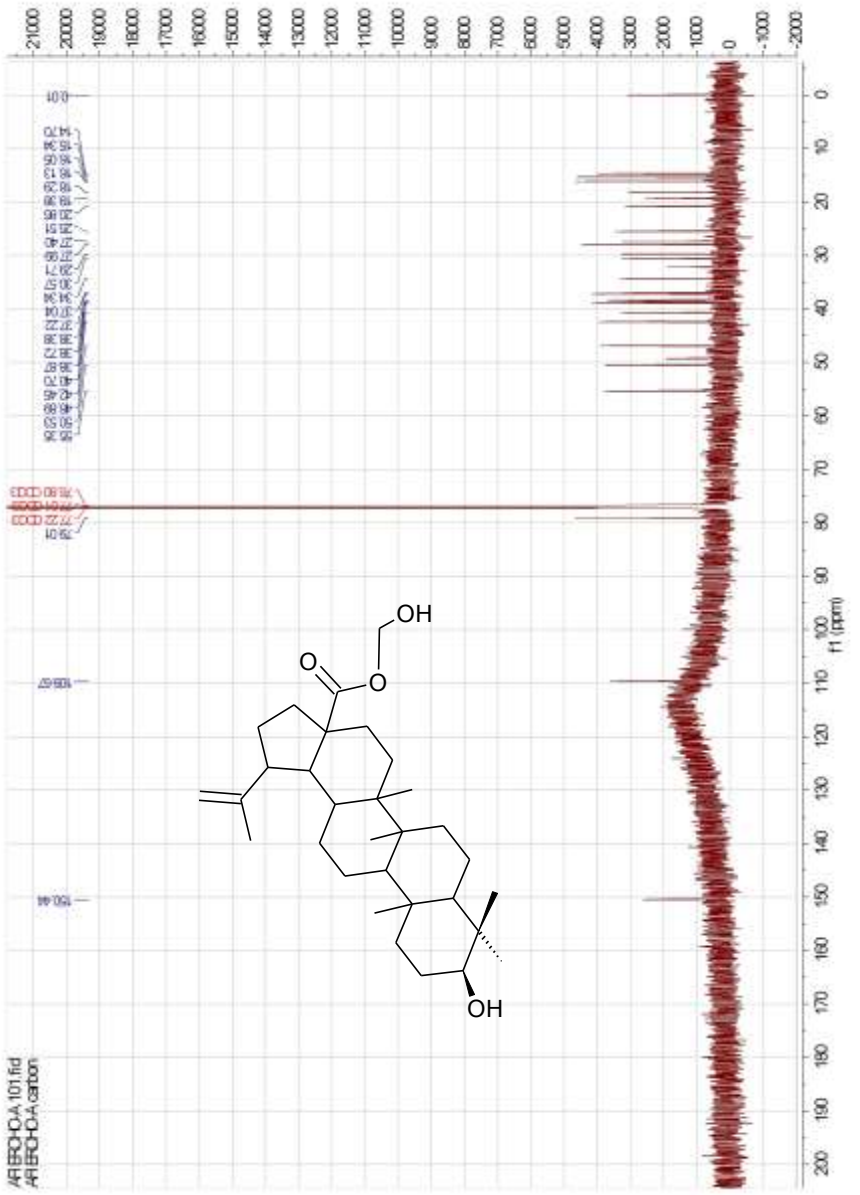


Figure 4.22: ^{13}C NMR(150 MHz. $CDCl_3$) spectrum of SGD

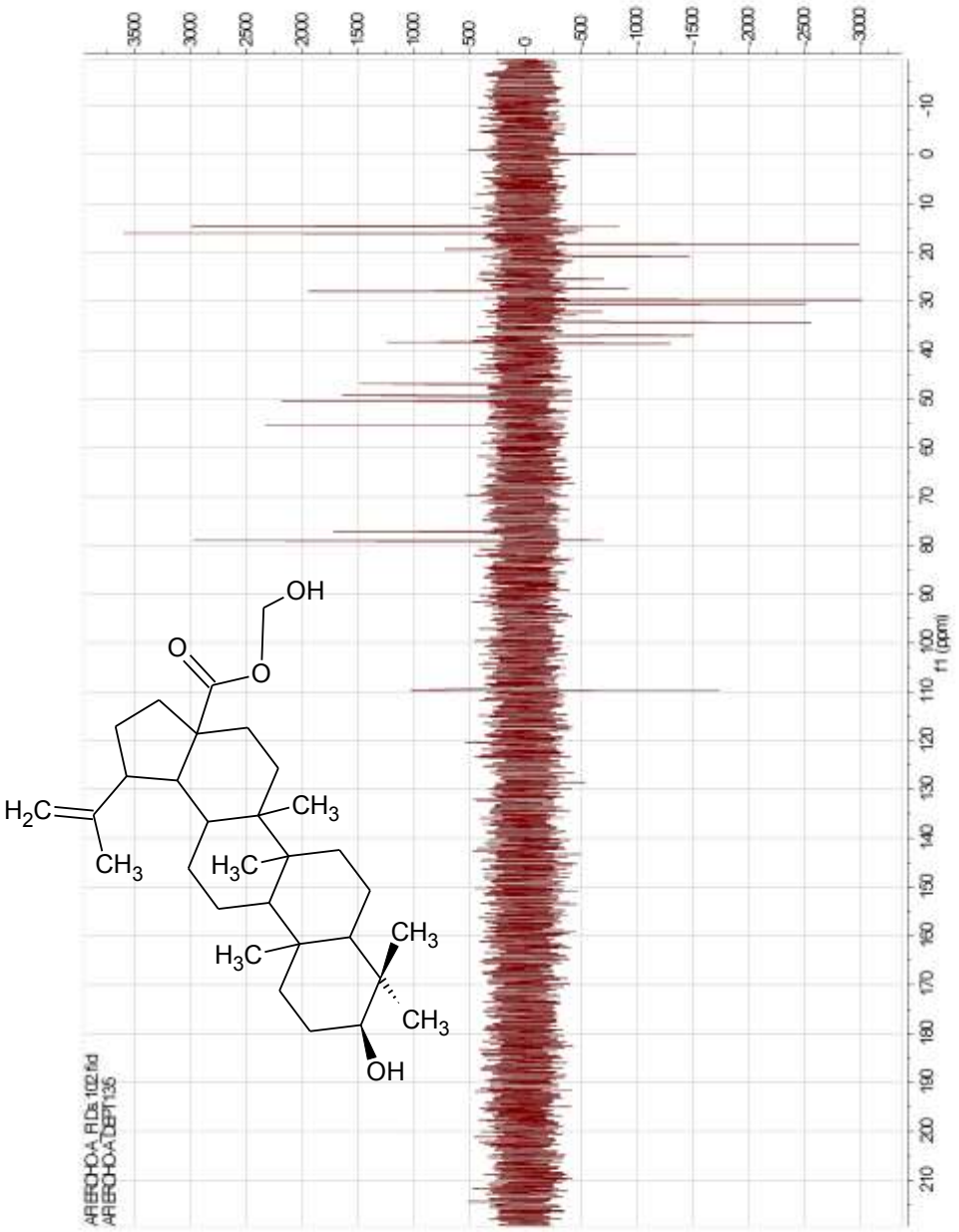


Figure 4.23: DEPT- 135 (150 MHz. $CDCl_3$) spectrum of SGD

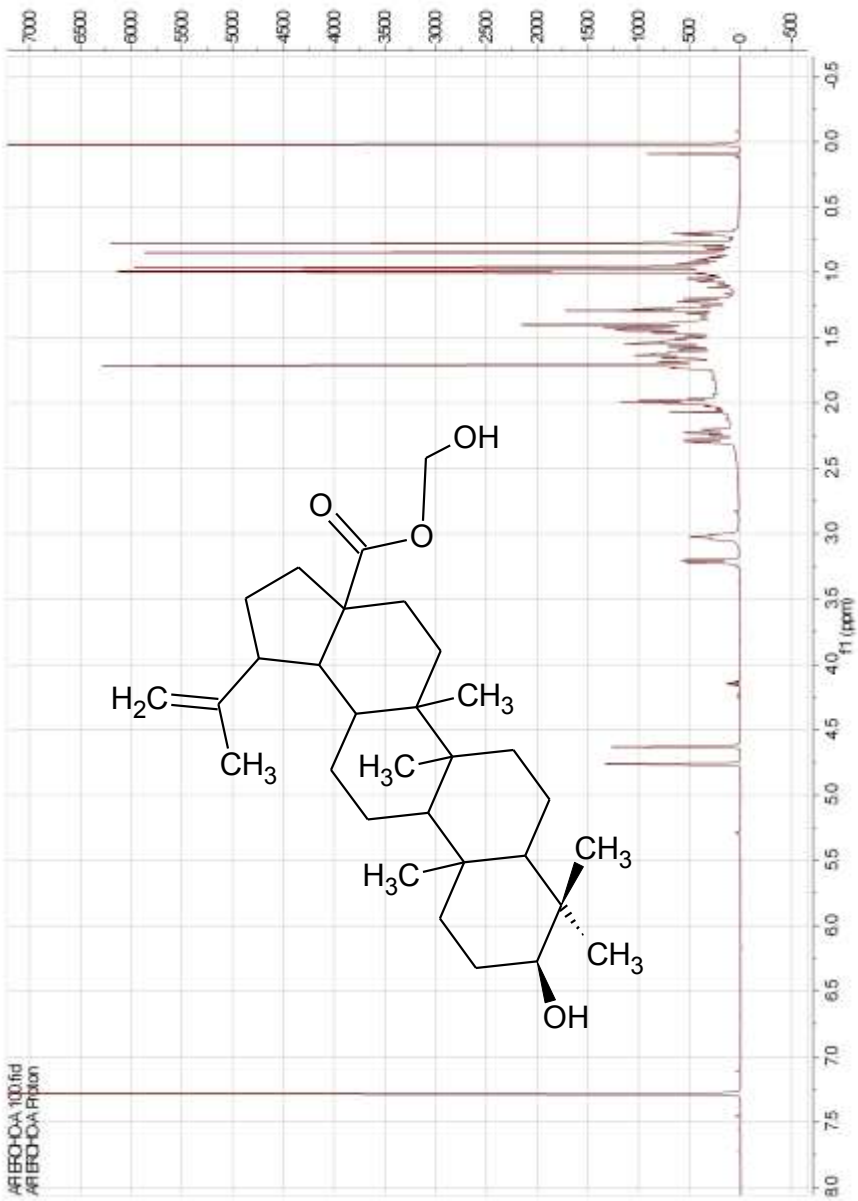


Figure 4.24: ^{1}H- NMR (600 MHz. $CDCl_3$) spectrum of SGD

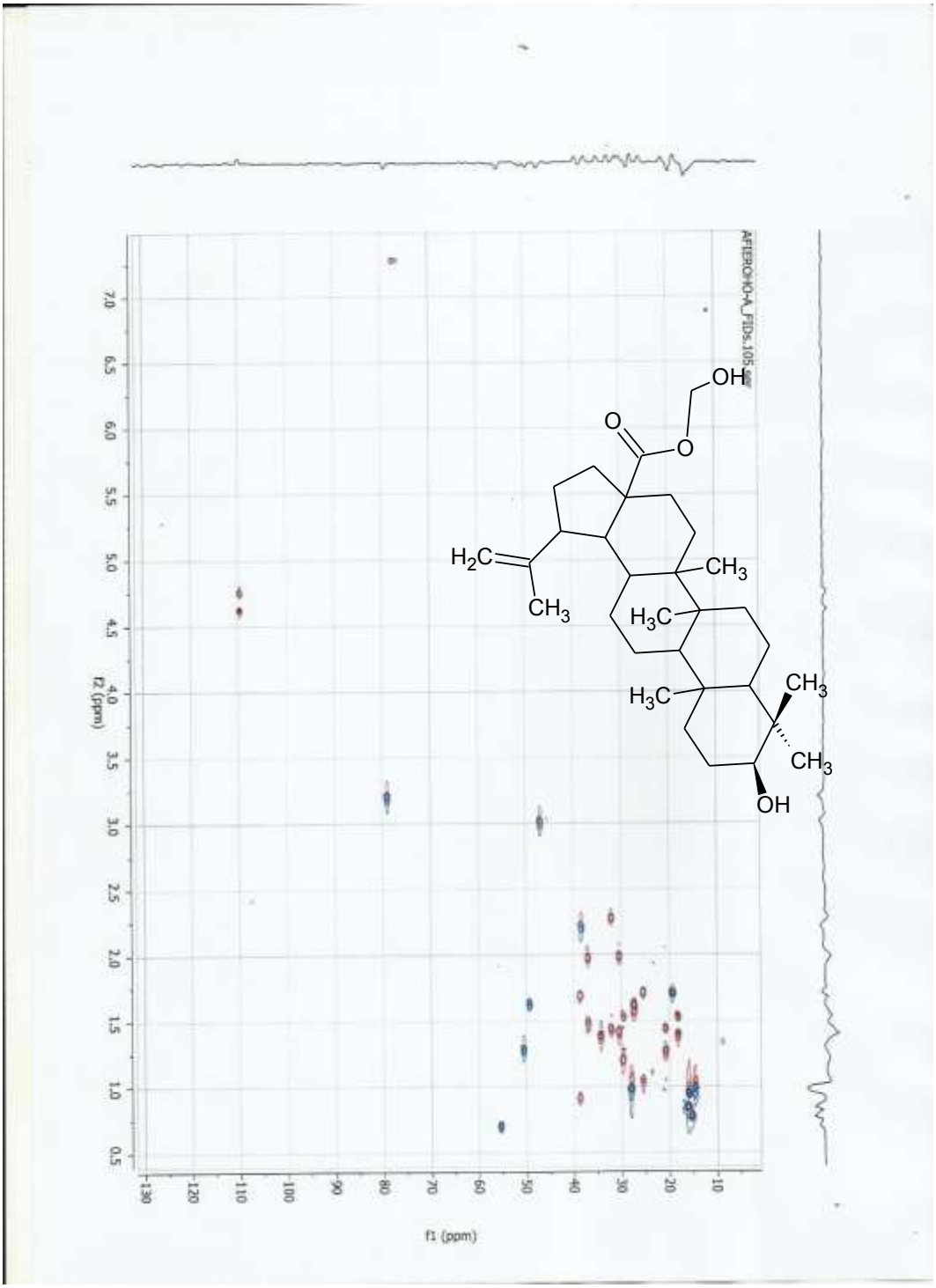


Figure 4.25: HSQC spectrum of SGD

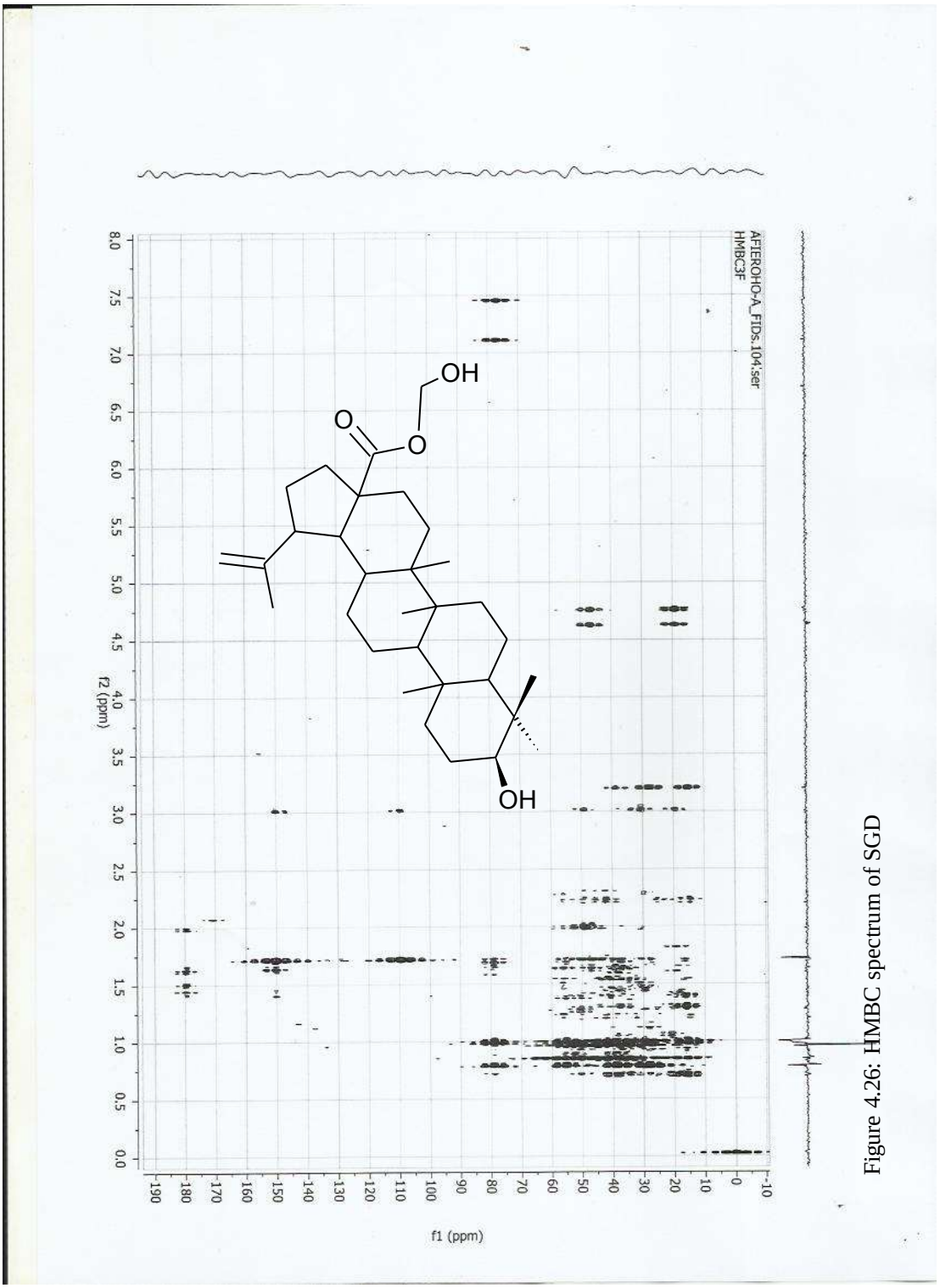


Figure 4.26: HMBC spectrum of SGD

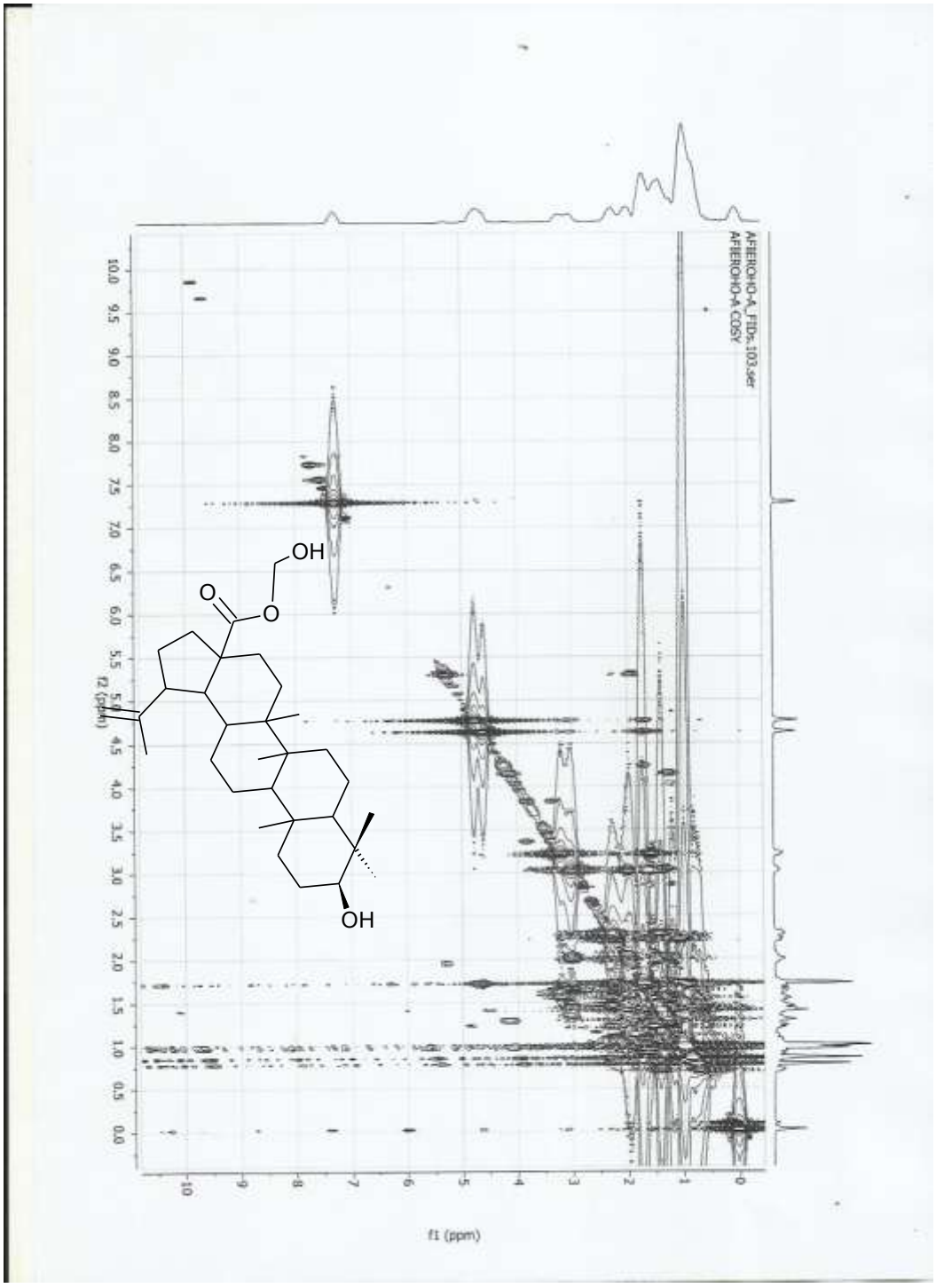
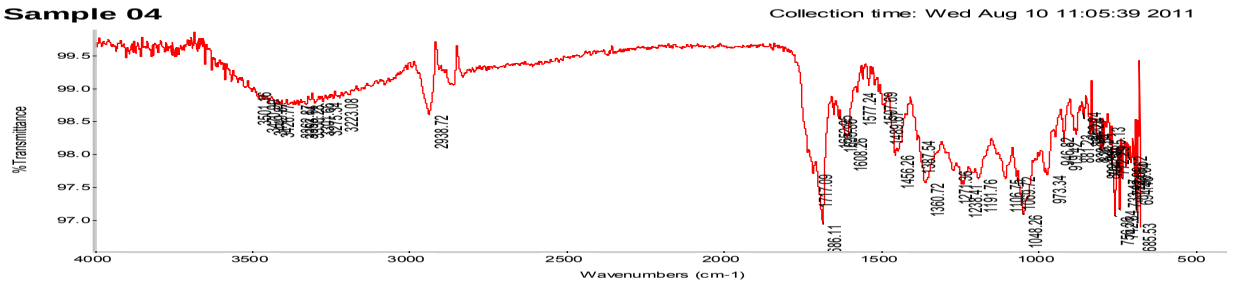
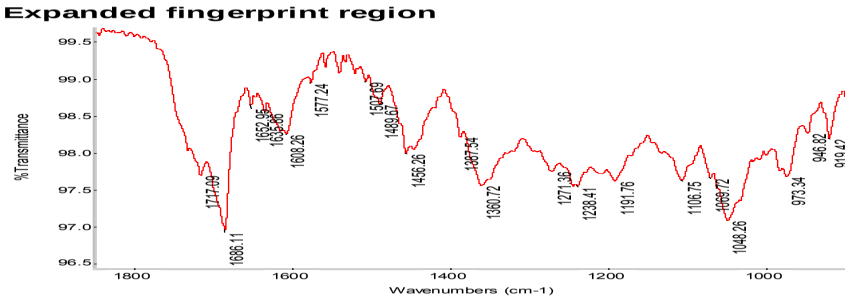


Figure 4.27: ^{1}H-^{1}H COSY spectrum of SGD





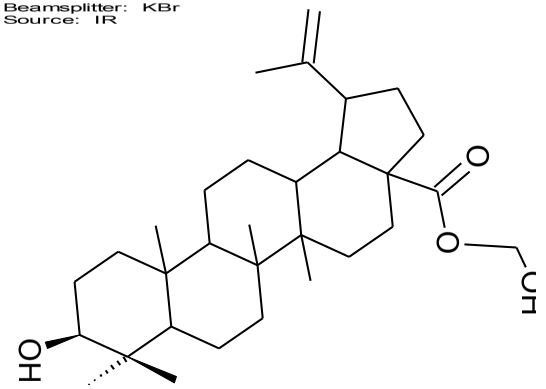


Figure 4.28: FTIR spectrum of SGD

4.5.2 Characterization of compound SGE

Compound SGE (melting point 250-252^{o}C.) was obtained from the column sub-fraction SGHI-3 (Table 4.11) after washing with n-hexane followed by re-crystalisation of the resulting residue in acetone to yield a creamy to off white solid. The SGE (Figure 4.29) gave positive Liebermann and Salkowski test confirming it to be a triterpenoid. The molecular ion m/z peak at 456.7 (Figure 4.30 and Table 4.14) was obtained which correspond to the molecular formula of $C_{30}H_{48}O_3$. Other major fragmentation pattern are observed at m/z: 457 [M+H], 456 [M+], 411 [M-COOH], 438 [M-H_2O], 207 [due to ring A and B cleavages], 248 [due to ring D and E cleavages] and the base peak at 189 (Scheme 4.4 and Figure 4.30). These established SGE to be a 3-hydroxylupenoic acid derivative typical of betulinic acid (Budzikiewicz *et al.*, 1963; Ryu *et. al.*, 1992; Macias *et al.*, 1998).

The ^{1}H- (Figures 4.31 to 4.33), HSQC (Figures 4.34 to 4.36), HMBC (Figures 4.37 to 4.39) and ^{1}H-^{1}H –COSY (Figures 4.40 to 4.41) NMR spectra of SGE are evident for betulinic acid as reported in literature (Mahato and Kundu 1994). A total of thirty carbon signals seven quaternary, eleven methylene, six methine carbon signals and six angular methyl carbon signals were seen. Their corresponding proton signals were unambiguously assigned from the HSQC experiment (Figures 4.34 to 4.36) as rationalised in Table 4.13. The downfield methyl proton signal at 1.71 ppm (H_{-30}) showed $^{2}J_{CH}$ and $^{3}J_{CH}$ connectivities with C_{-19} ($^{3}J_{CH}$), C_{-20}($^{2}J_{CH}$) and C_{-29} ($^{3}J_{CH}$), as seen in the HMBC spectrum (Figures 4.37 to 4.39). The two geminally coupled protons signals at δ_H ppm: 4.63(H_{-29a}) and 4.76(H_{-29b}), were unmistakably assigned to the C_{-29} carbon signal at 109 ppm from the HSQC experiment (Figure 4.36). The C_{-20} is a quaternery olefinic carbon resonating at δ_C 150 ppm. These spectra data confirmed the presence of an isopropenyl unit at position 19 of the triterpene skeleton which typical of 20(29)- lupene derivatives (Adnyana *et al.*, 2000; Yogeeswari and Sriram, 2005). The C_{-3} secondary carbinol signal at 79.02 ppm is typical of 3β-hydroxy derivatives (Mahato and Kundu, 1994), while the carbonyl δ_C signal at 180 ppm due to the 28-COOH. Their respective positions were unambiguously assigned based on the ^{1}H-^{1}H-COSY, HMBC and HSQC experiments. This additional spectra data for SGE is supportive of the 3β-hydroxy-20(29)- lupen-28-oic acid nucleus (Adnyana *et al.*, 2000; Yogeeswari and Sriram, 2005). In view of these spectra data which are similar to the ones reported for betulinic acid (Mahato *et al.*, 1992; Mahato and Kundu,

1994), the SGE isolated from the stem bark of *S. guineense* stem bark was characterised to be betulinic acid (systemic name: 3β-hydroxylup -20(29) -en - 28 -oic acid) as proposed in Figure 4.29. The FTIR spectrum (V_{max}, cm^{-1}) in Figure 4.42 showing the presence of O-H stretching of hydroxyl group (3000 - 3424), C-H stretching of methyl groups (2928) and C=O (1681) and C-O (1029) stretching of carboxylic acid, is a proved support. The observed melting point of 250-2 ^{o}C is also similar to that reported for betulinic acid (Menezes and Kaplan, 2005).

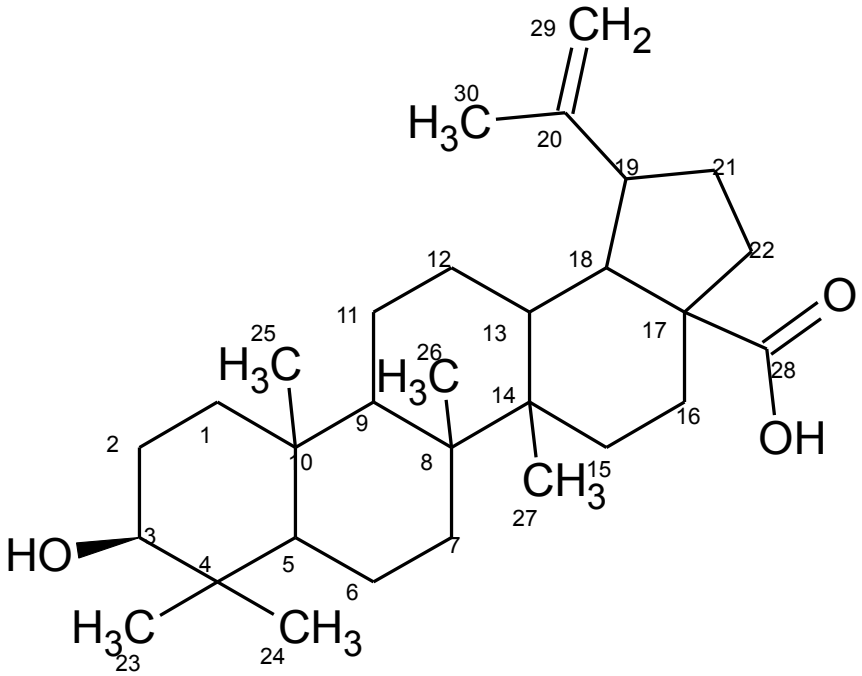


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Figure 4.29: Compound SGE (3β-hydroxylup -20(29) -en - 28 -oic acid)

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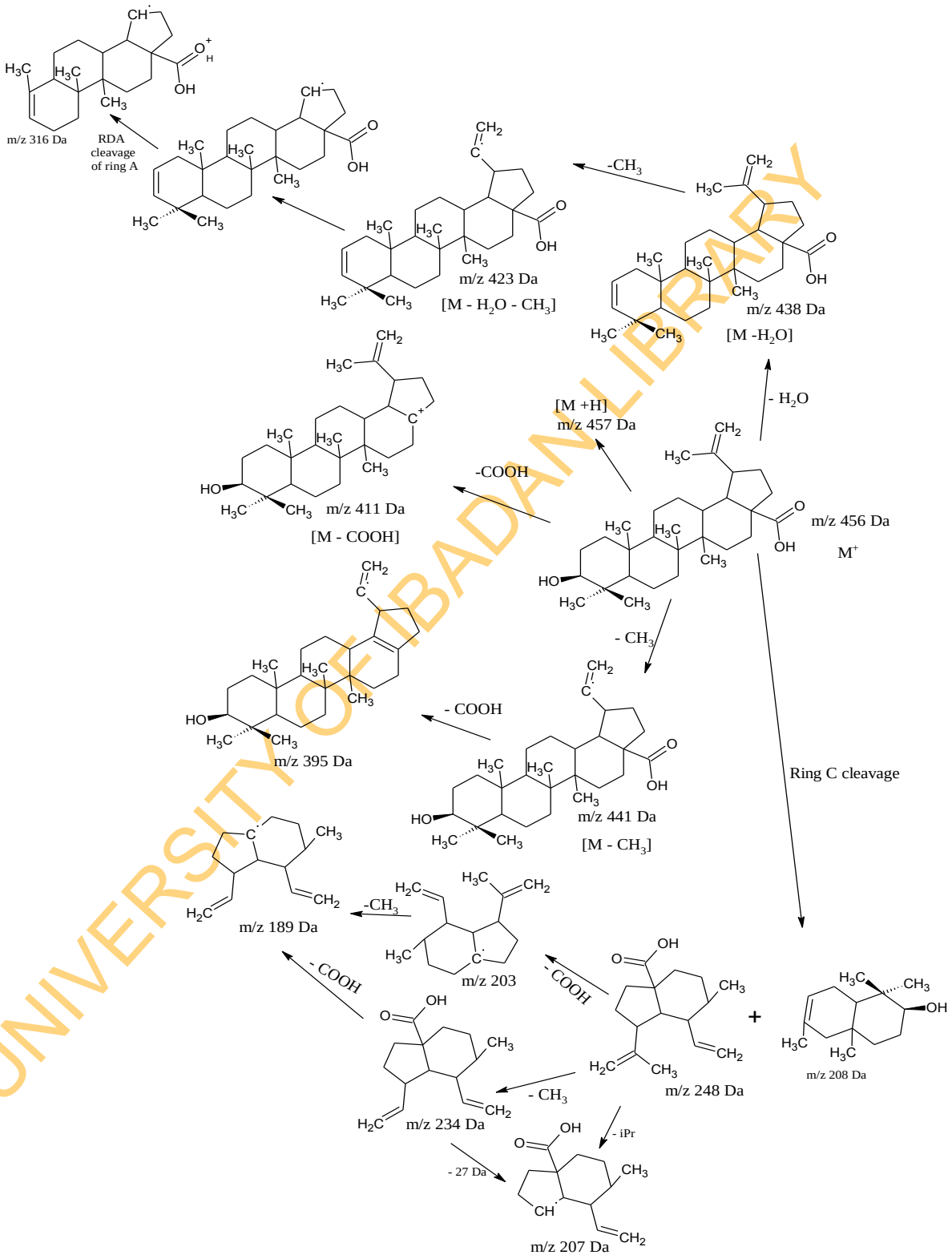
Table 4.13: 1D and 2 D ^{1}H-NMR (600 MHz, $CDCl_3$) and ^{13}C-NMR (150 MHz, $CDCl_3$) spectra data of SGE

S/No	$\delta_{C\ ppm}$	published[a]	APT	HSQC (δ_H ppm)	^{1}H-multiplicity	^{1}H-^{1}H-COSY	^{2}J, ^{3} $J_{(CH)}$HMBC (δ_C ppm)
1	38.7	38.5	CH_2	0.90,1.68	m	H_{-2}	
2	27.4	28.2	CH_2	1.60	m	H_{-3} , H_{1a}	
3	79.0	78.1	CHOH	3.20	m	H_{-2}	
3-OH	-		OH	2.12	s (br)		
4	38.9	39.4	C	-	-		
5	55.4	55.9	CH	0.69	m	H_{-6a}	
6	18.3	18.7	CH_2	1.40, 1.54	m	H_{-5}	
7	34.3	34.7	CH_2	1.40	m		
8	40.7	41.0	C	-	-		
9	50.5	50.5	CH	1.29	m		
10	37.0	37.5	C	-	-		
11	20.9	21.1	CH_2	1.27, 1.41	m		
12	25.5	26.0	CH_2	1.05, 1.75	m		
13	38.4	39.2	CH	2.19	m		C_{-27}
14	42.5	42.8	C	-	-		
15	32.5	32.8	CH_2	1.42 , 1.98	m	H_{-16a} ,H_{-16b}	
16	30.6	30.22	CH_2	1.43, 2.25	m	H_{-15b} , H_{-15a}	
17	56.5	56.6	C	-	-		
18	49.8	49.7	CH	1.63	m	H_{-19}	C_{-28}
19	47.5	47.7	CH	3.00	m	H_{-18} ,H_{-21b}	
20	150.4	150.4	>C=	-	-		
21	37.2	37.4	CH_2	1.27	m		
22	29.7	31.1	CH_2	1.50,1.98	m	H_{-19}	
23	28.0	28.5	CH_3	0.98	s		C_{-3} , C_{-5} , C_{-24}
24	15.3	16.2	CH_3	0.77	s		C_{-3} , C_{-4} , C_{-5} , C_{-23}
25	16.1	16.3	CH_3	0.84	s		C_{-5} , C_{-9} , C_{-10}
26	16.1	16.2	CH_3	0.95	s		C_{-7} , C_{-8} , C_{-9} , C_{-29}
27	14.7	14.8	CH_3	0.99	s		C_{-8} , C_{-14} , C_{-15}
28	180.0	178	>C=O	-	-		
29	109.7	109.5	= CH_2	4.63 (J=4.6) , 4.76(J=4.6)	s, s	H_{29a} , H_{29b} ,	C_{-19} , C_{-30}
30	19.4	19.4	CH_3	1.71	s		C_{-19} , C_{-20} , C_{-29}

Key: m = overlapping proton multiplet, s = singlet proton d = doublet proton t = triplet proton q = quartet protons, [a] Mahato and Kundu (1994)



Scheme 4.4: EI-mass fragmentation pattern of SGE

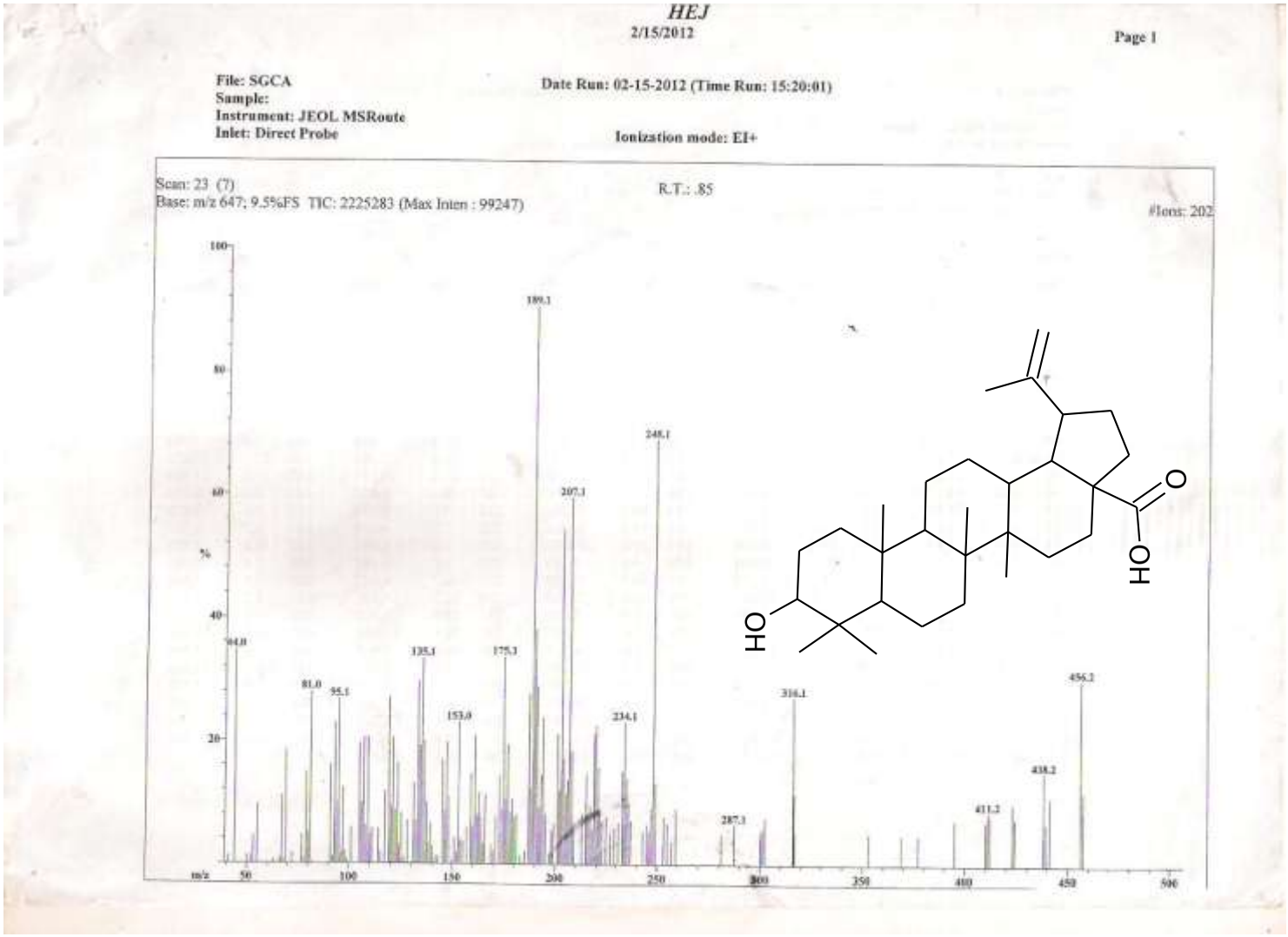


Figure 4.30: EI-mass spectrum of SGE from *S. guineense* stems bark

Table 4.14: High Resolution EIMS data of SGE

File : D:\Xcalibur\data\sgca(05-03-12)-c1.RAW
Full ms [39.500 - 600.500] - Range: 50.000 - 600.500
Scan No. 1 of 1

Mass	Relative Intensity	Theoretical Mass	Delta [ppm]	Delta [mmu]	RDB	Composition
380.3249	0.4579	380.3290	-10.9	-4.2	3.0	$C_{24}H_{44}O_3$
380.9760	1.0146					
381.2801	0.6737	381.2794	1.8	0.7	8.5	$C_{24}H_{37}O_2$
382.2821	1.2825	382.2872	-13.2	-5.0	8.0	$C_{26}H_{38}O_2$
383.2879	0.7678					
386.2955	0.3913	386.2974	-4.9	-1.9	11.0	$C_{29}H_{38}$
388.3110	0.7644	388.3130	-5.3	-2.1	10.0	$C_{29}H_{40}$
390.3303	2.2415	390.3287	4.2	1.6	9.0	$C_{29}H_{42}$
		390.3345	-10.8	-4.2	0.0	$C_{22}H_{46}O_5$
391.3273	1.4058					
392.3414	8.7432	392.3443	-7.4	-2.9	8.0	$C_{25}H_{44}$
392.9760	0.6646					
393.3454	4.7584					
394.3505	1.6391	394.3447	14.6	5.8	3.0	$C_{25}H_{46}O_3$
395.2951	8.9083	395.2950	0.1	0.1	8.5	$C_{27}H_{39}O_2$
396.2959	3.0646					
397.3027	0.8814					
404.9842	0.4444	404.9824	4.6	1.8	27.5	$C_{27}H_{1}O_5$
405.3108	0.4109	405.3157	-12.1	-4.9	9.5	$C_{29}H_{41}O_1$
406.3165	0.4483					
407.3350	0.4939	407.3314	8.9	3.6	8.5	$C_{29}H_{43}O_1$
408.3310	1.8702					
409.3287	0.8276	409.3318	-7.6	-3.1	3.5	$C_{29}H_{45}O_4$
410.3516	3.1132	410.3549	-8.1	-3.3	7.0	$C_{29}H_{46}O_1$
411.3556	1.9070					
412.3628	0.6420					
421.3084	0.9556	421.3107	-5.3	-2.2	9.5	$C_{29}H_{41}O_2$
422.3313	0.5211					
423.3258	6.8336	423.3263	-1.2	-0.5	8.5	$C_{29}H_{43}O_2$
424.3305	2.5142	424.3341	-8.6	-3.7	8.0	$C_{29}H_{44}O_2$
425.3275	0.6025	425.3267	1.9	0.8	3.5	$C_{25}H_{45}O_5$
436.3372	1.1677	436.3400	-6.4	-2.8	0.0	$C_{23}H_{48}O_7$
		436.3341	7.1	3.1	9.0	$C_{30}H_{44}O_2$
437.3362	0.8089	437.3420	-13.2	-5.8	8.5	$C_{30}H_{45}O_2$
438.3530	8.7384	438.3498	7.3	3.2	8.0	$C_{30}H_{46}O_2$
439.3528	3.1742	439.3576	-11.0	-4.8	7.5	$C_{30}H_{47}O_2$
440.3492	1.3024	440.3502	-2.2	-1.0	3.0	$C_{26}H_{48}O_5$
		440.3443	11.1	4.9	12.0	$C_{33}H_{44}$
441.3396	1.3288	441.3369	6.1	2.7	7.5	$C_{28}H_{45}O_3$
456.3561	4.0760	456.3603	-9.4	-4.3	7.0	$C_{30}H_{48}O_3$

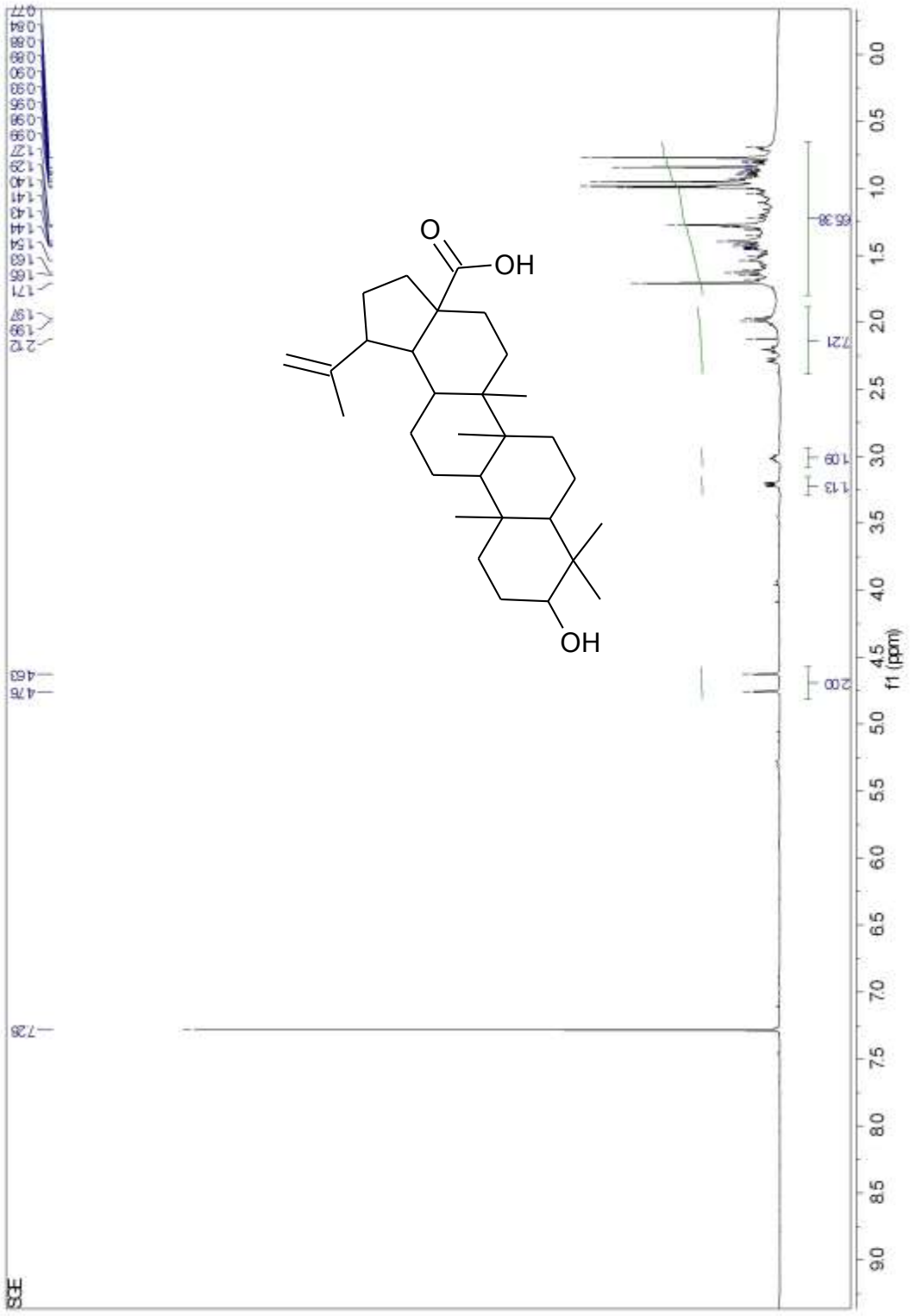


Figure 4.31: ^{1}H-NMR (600 MHz, $CDCl_3$) spectrum of compound SGE from *S. guineense* stems bark

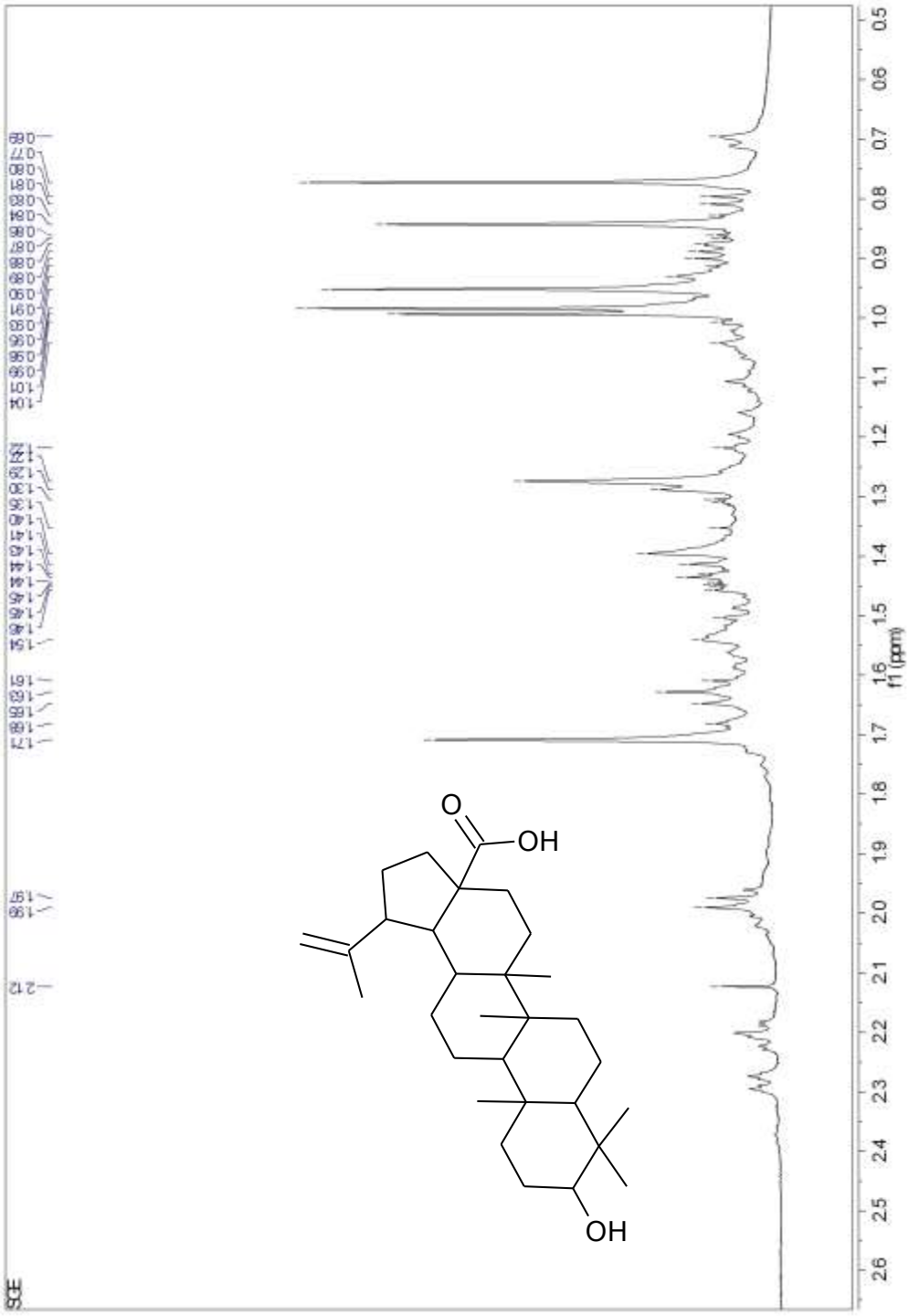


Figure 4.32: Expanded-1 ^{1}H-NMR (600 MHz, $CDCl_3$) spectrum of SGE from *S. guineense* stems bark

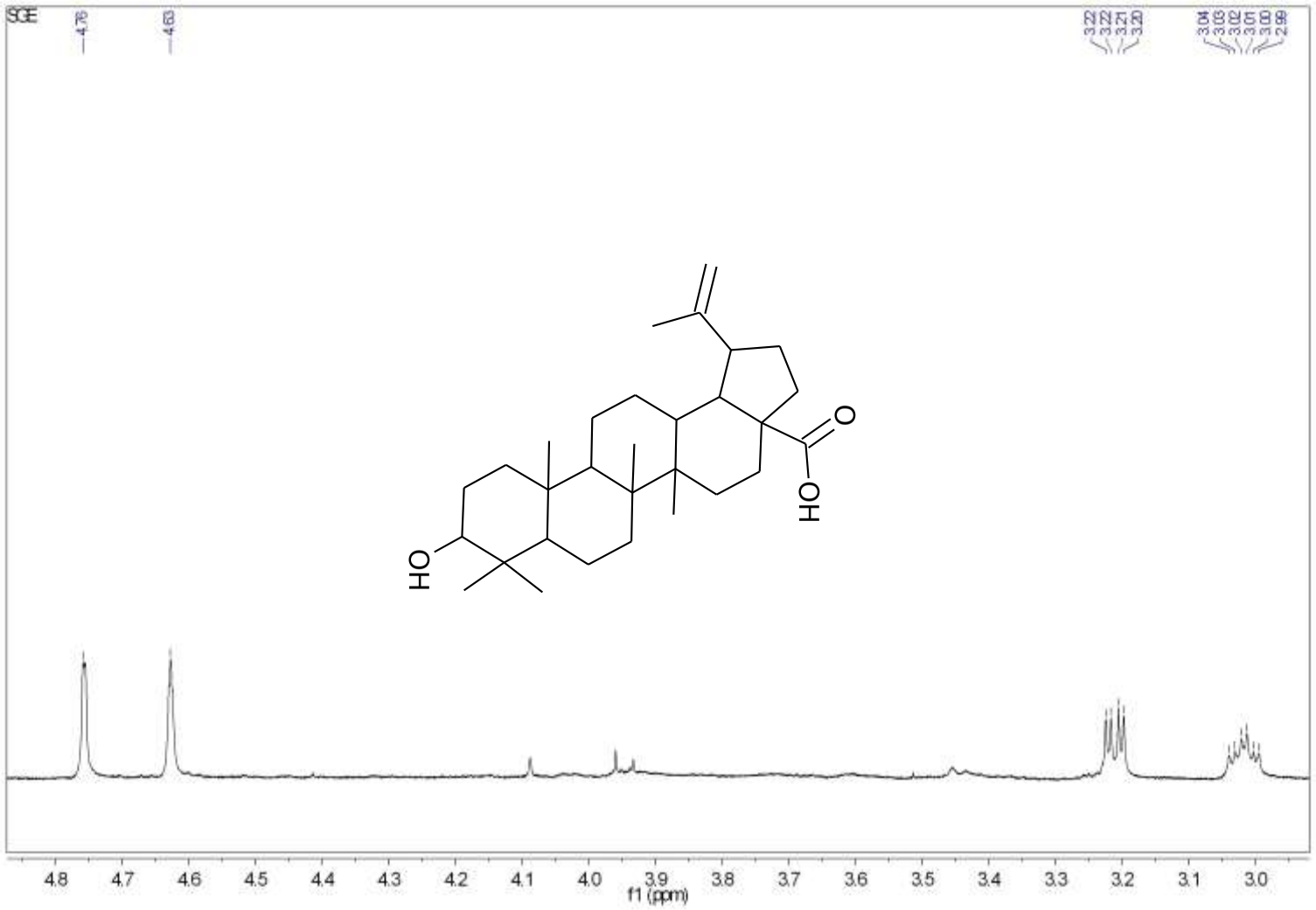


Figure 4.33: Expanded-2 ^{1}H- NMR (600 MHz, $CDCl_3$) spectrum of SGE from *S. guineense* stems bark

Figure 4.34: HSQC spectrum of SGE from *S. guineense*

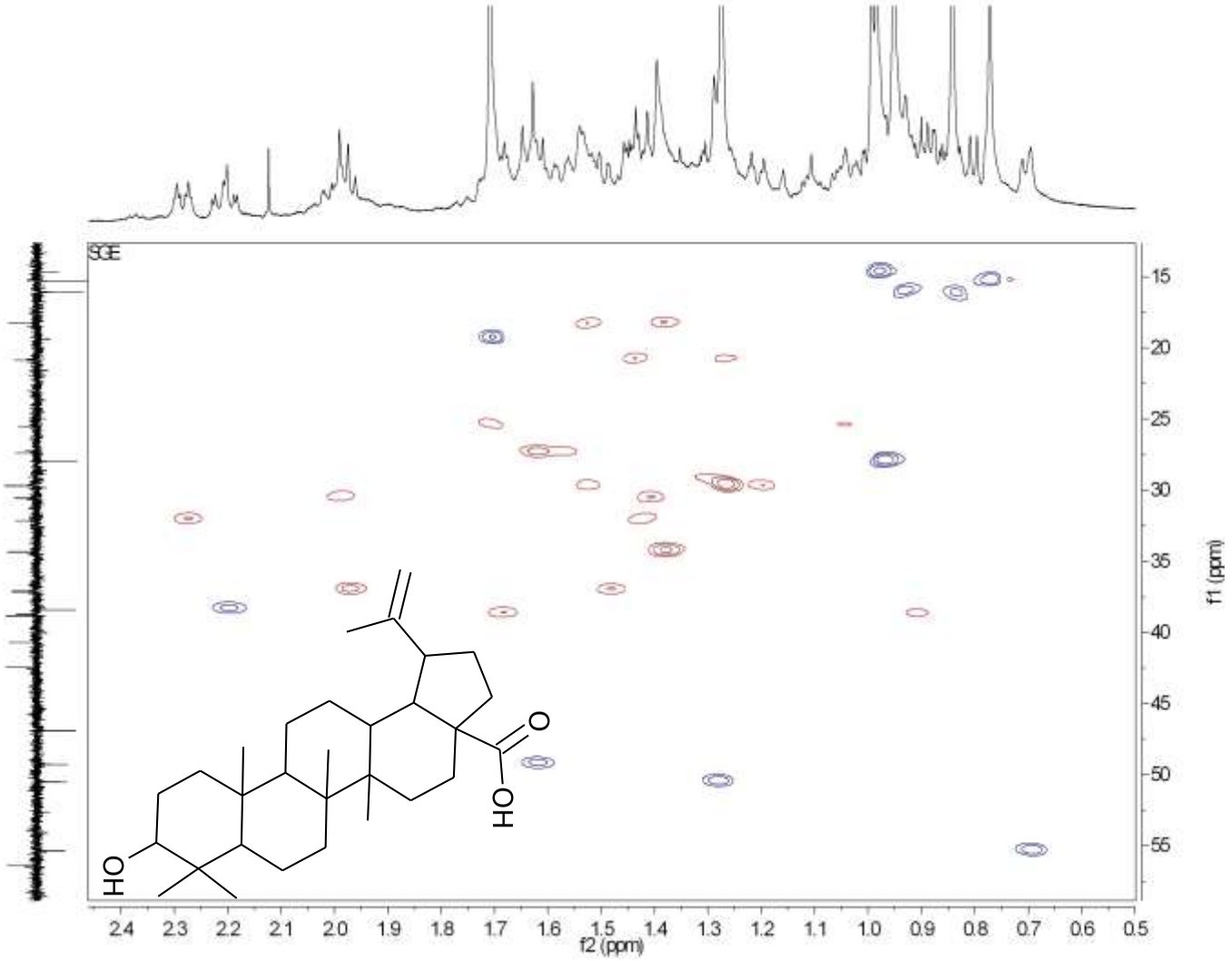


Figure 4.35: Expanded-1 HSQC spectrum of SGE from *S. guineense*

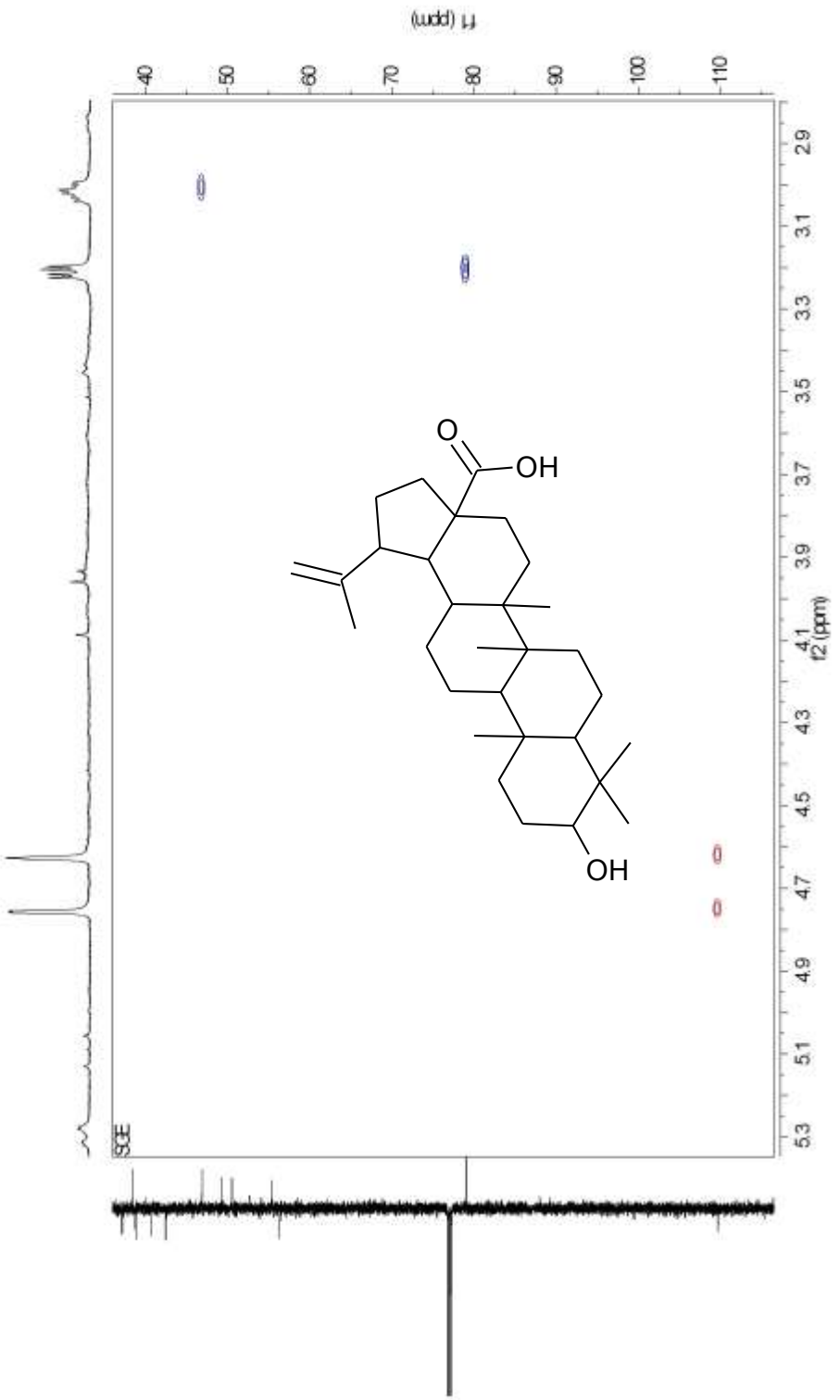


Figure 4.36: Expanded-2 HSQC spectrum of SGE from *S. guineense*

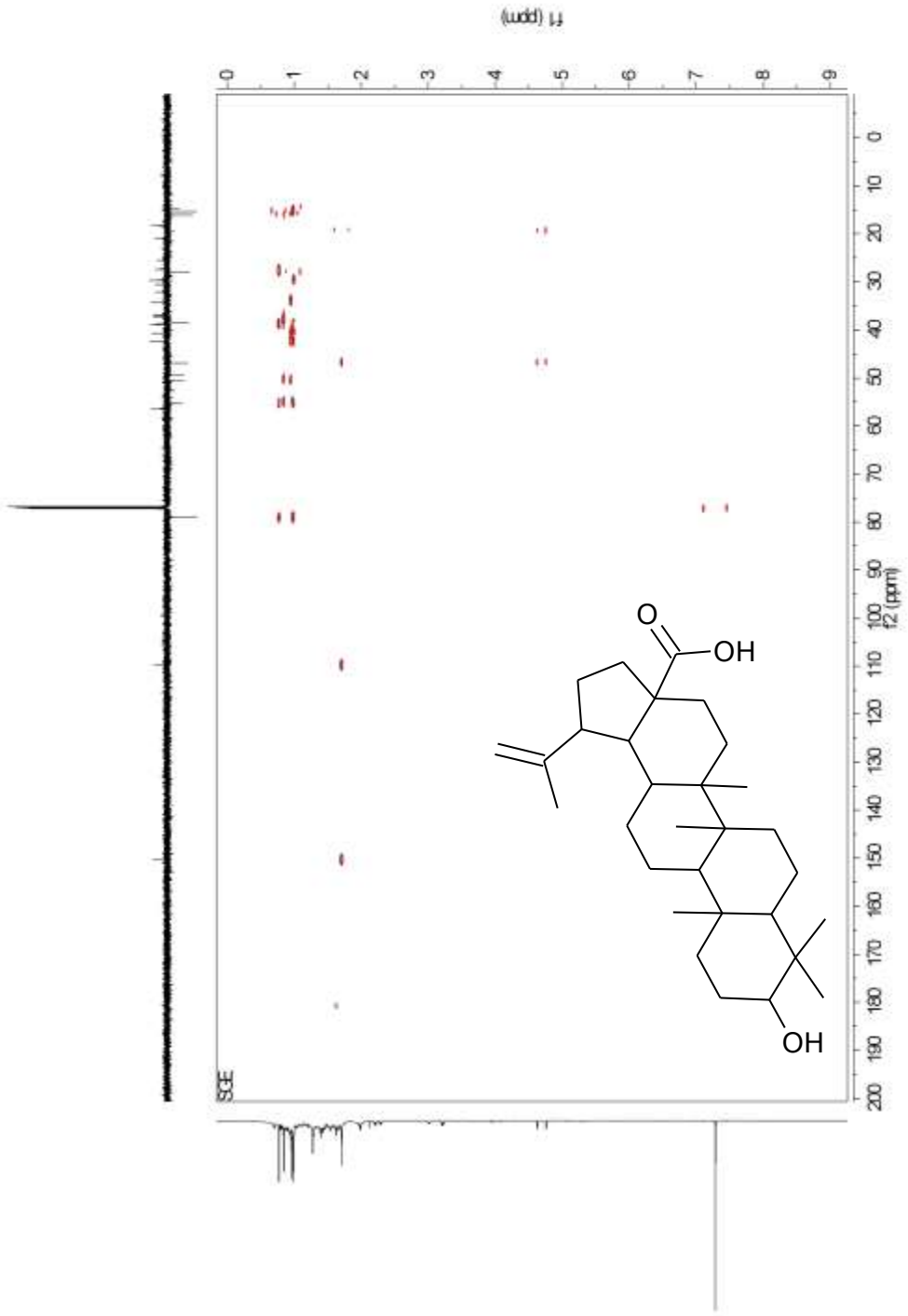


Figure 4.37: HMBC spectrum of SGE from *S. guineense*

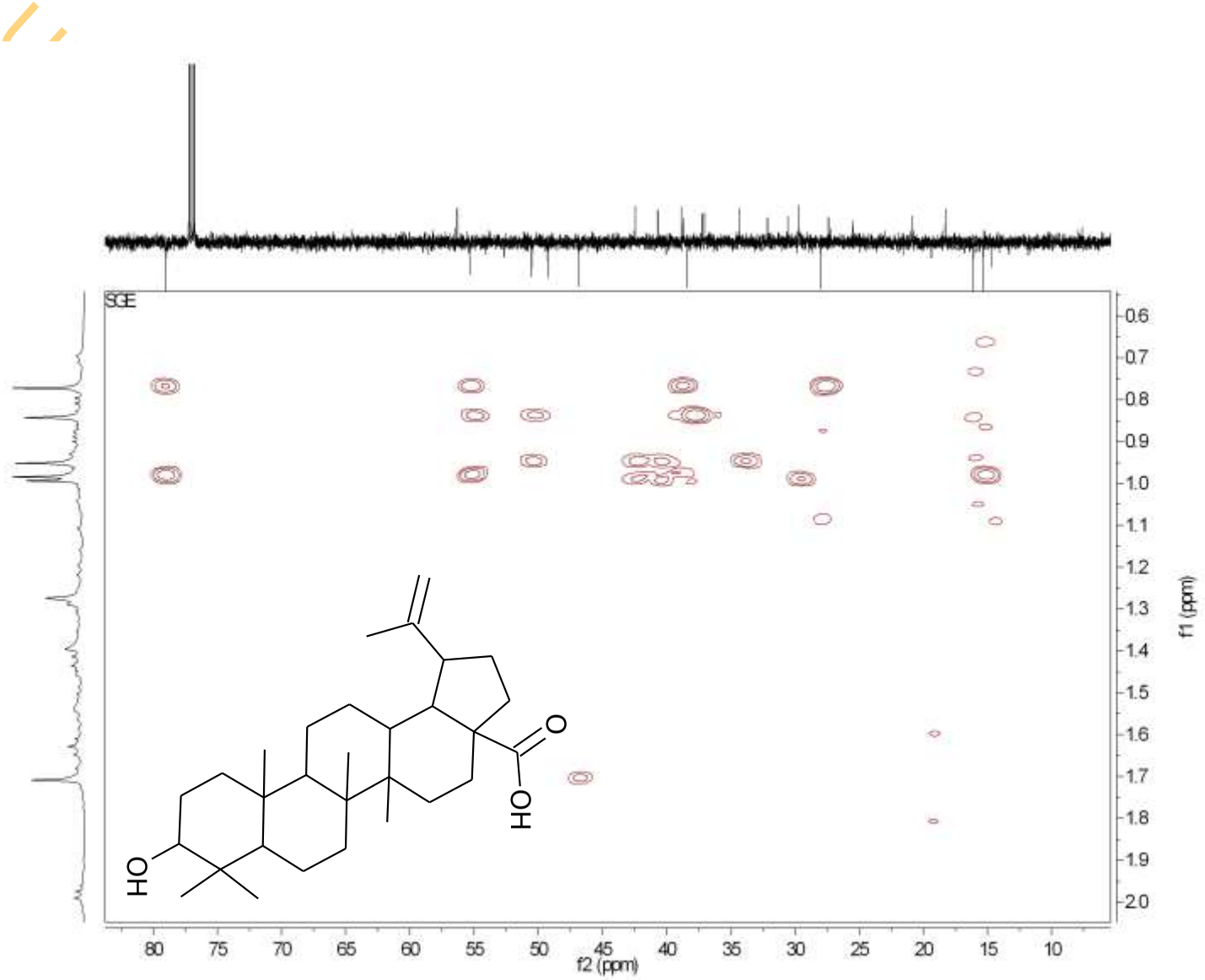


Figure 4.38: Expanded-1 HMBC spectrum of SGE from *S. guineense*

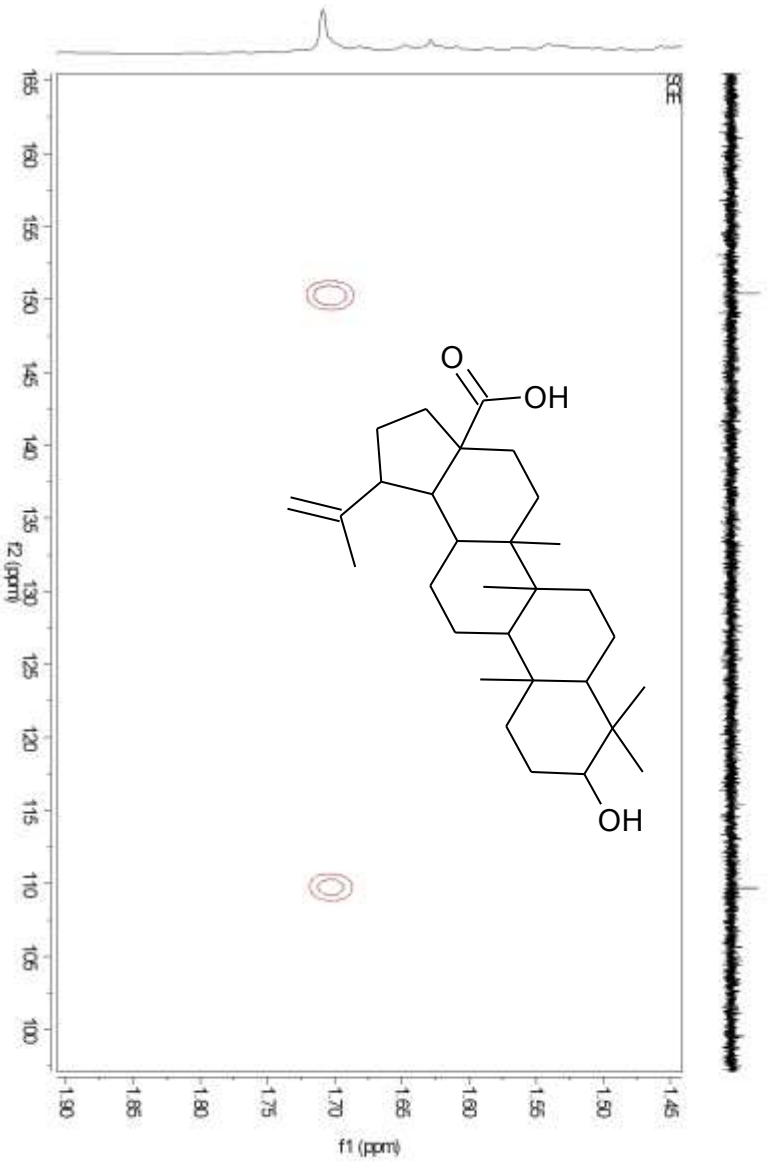


Figure 4.39 : Expanded-2 HMBC spectrum of compound SGE from *S. guineense*

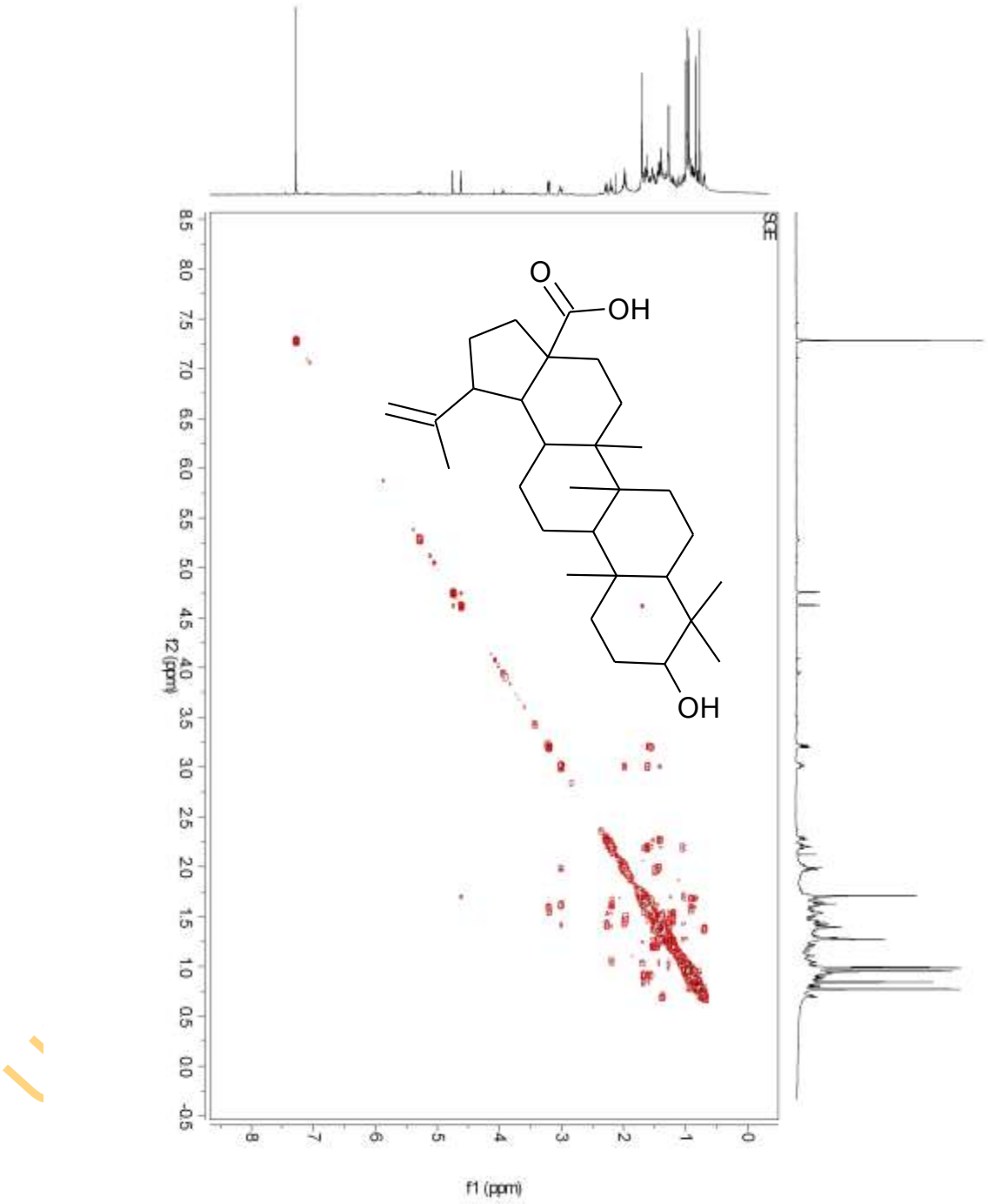


Figure 4.40: ^{1}H-^{1}H- COSY spectrum of SGE from *S. guineense*

Figure 4.41: Expanded ^{1}H-^{1}H-COSY spectrum of SGE from *S. guineense*

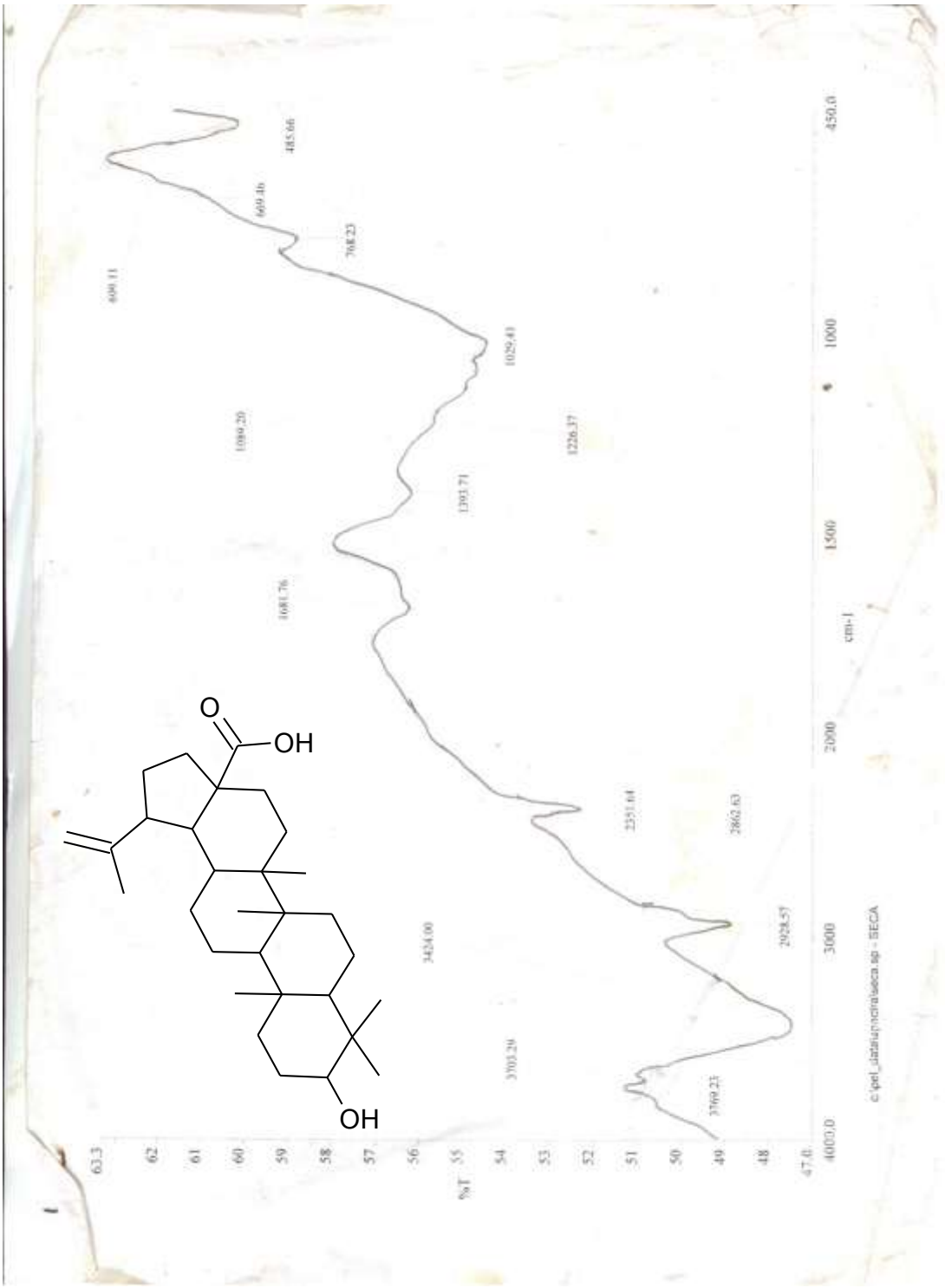


Figure 4.42: FTIR spectrum of SGE from *S. guineense*

4.6. Column chromatography of the n-butanol soluble fraction of *M. pigra* (aerial part)

A total of fifteen chromatography fractions were collected after chromatographic separation of the n-butanol soluble fraction. Based on TLC analysis, the fractions were pooled to afford a total of three sub-fractions (Table 4.15) labelled BSMP-1-3. The sub-fraction BSMP-2 afforded a light yellow solid coded MPL (80 mg) after washing with diethyl ether and re-crystalisation in acetone.

4.6.1 Characterisation of MPL

Compound MPL (melting point: 148 - 152^{o}C) gave a single spot (R_f = 0.65) on TLC examination with $CHCl_3$: MeOH (3:1) as mobile phase. The MPL (Figure 4.43) gave a positive test observation with 10% alcoholic $FeCl_3$ reagents. This is an indication that it phenolic compound.

The EI-MS (Figure 4.44) showed the base peak at m/z 153. This is typical of flavonoids with hydroxylation at ring A (Abbas *et al.*, 2007). It is due to cleavages at ring C, in addition to loss of the sugar residue (Scheme 4.5).

The ^{13}C-NMR (50 MHz, DMSO-D6, δ_C ppm) in Figures 4.45 and 4.46 indicated a total of seventeen δ_C carbon signals which on the basis of the Attached Proton Test (APT) analysis reflected for; one methyl, nine methine (one of which is due to the five equivalent methine of the mono-substituted aryl ring B), six quaternary and one carbonyl. The trend in δ_C (ppm) signals: 102.62 (d, OCHO, $C_{-1''}$), 70.70 (d, CHOH, $C_{-2''}$), 71.1 (d, CHOH, $C_{-3''}$), 71.3 (d, CHOH, $C_{-4''}$), 72.0 (d, CHOH, $C_{-5''}$) and 19.0 (q, CH_3, $C_{-6''}$) is a characteristic of a rhamnose residue (Agrawall and Jain, 1992; Hostettmann and Marston, 1995). This supported MPL to be a rhamnoside (Figure 4.43). In addition the δ_C (ppm) signals: 94.7 (d, C_{-2}), 137.2 (s, C_{-3}), 178.5 (s, C=O, C_{-4}), 99.4 (d, C_{-5}), 164.9 (s, C_{-6}), 108.6 (d, C_{-7}), 161.9 (s, C_{-8}), 120.3 (s, C_{-9}), and 157.1 (s, C_{-10}), 145.6 (s, $C_{-1'}$) and 116.0 (d, $C_{-2'}$-$C_{-6'}$ of monosubstituted aromatic ring B,) are evident for an aryl benzopyrone (or aryl chromone) nucleus typical of the C_{-6}-C_{-3} biosynthetic origin of flavonoids and related phenolics secondary metabolites like the coumarines and phenylpropanoids. The quaternary carbon signal at 178.5 ppm correspondings to C_{-4} is indicative of the C=O functionality of the chromone.

The ^{1}H-NMR (200 MHz, DMSO-d6) spectrum in Figure 4.47 showed the overlapping proton multiplet peaks in the region 3.1-3.9 ppm typical of sugar residue and the corresponding anomeric proton doublet signal at δ_H : 5.21 ppm (1Hd, J =5.2 MHz), one upfield methyl doublet proton 0.9 ppm (3H, d J=5.8 MHz due to CH_3 of rhamnose unit). This corroborated similar observation in the ^{13}C-NMR spectrum. Rhamnose is a methyl deoxy monosaccharide which has resonance for the C_{-6} methyl protons as a doublet upfield in addition to four ring secondary carbinol and one anomeric proton signal (Delazar *et al.*, 2006). The δ_H (ppm) resonance for the two meta coupled aromatic protons 6.18 (H_{-5}) and 6.38 (H_{-7}) in addition to that at 6.9 (5H, $H_{-2'}$-$H_{-6'}$) due to the mono-substituted phenyl ring B of the flavonoid nucleus were also seen. After comparing the spectra data with that simulated using ACD software, the chemical shift assignment for MPL (Figure 4.43), was done as shown in Table 4.16.

The FTIR $_{nujol}$ spectrum (Figure 4.48): 3280.34 cm^{-1}(OH), 2919.71 cm^{-1}, 2850.29 cm^{-1} (CH), 1656.56 cm^{-1} (C=O), 1575.56 cm^{-1} (Aromatic C=C), 968.49 cm^{-1}, 827.31 cm^{-1}, 717.39 cm^{-1} (Aromatic fingerprinting), and 1166.72 cm^{-1} (C-O) is indicative of a phenol with cyclic carbonyl moiety. UV-Visible λmax: 217, 233, 335 nm are indication of the conjugated chromane or benzopyran system typical of flavonoids and related naturally occurring compounds (Ma *et al.*, 2002).

4.6.2: Antimycobacterial activity of column sub-fractions from the bioactive n-butanol fraction of *M. pigra* (aerial part)

The three sub-fractions BSMP-1-3 obtained after coloumn chromatography separation of the bioactive n-butanol fraction of *M. pigra*, were investigated for antimycobacterial activity using the Liquid based MGIT method. This is to identify the sub-fraction(s) that contain the bioactive compound(s). The results are presented in Table 4.17. None of the sub-fractions was active as a colony growth unit of '3+' was observed. The MPL isolated from BSMP-2 however showed only bacteriostatic effect at 0.476 mg/mL (Table 4.18) as the observed colony growth unit was '2+'.

Table 4.15: Column chromatography of the n-butanol soluble fraction of *M. pigra* (aerial part).

Column Eluents	Fraction	Pool No
ethyl acetate (100 %, 100 mL),	1-3	BSMP-1
ethyl acetate (100 %, 50 mL),	4-5	BSMP-2
ethyl acetate: MeOH (9:1 v/v, 100 mL),	6-10	
ethyl acetate : MeOH (8:2 v/v, 100 mL),	11-15	BSMP-3

Key:

BSMP-1 - Chromatography sub-fraction eluted with ethyl acetate (100%, first 3 x 20 mL eluate) from the n-butanol fraction of *M. pigra*

BSMP-2 - Chromatography sub-fraction eluted with ethyl acetate (100%, last 2 x 20 mL eluate) to ethyl acetate:methanol (8:1 v/v) from the n-butanol fraction of *M. pigra*

BSMP-3 - Chromatography sub-fraction eluted with ethyl acetate:methanol (89:1 v/v) from the n-butanol fraction of *M. pigra*

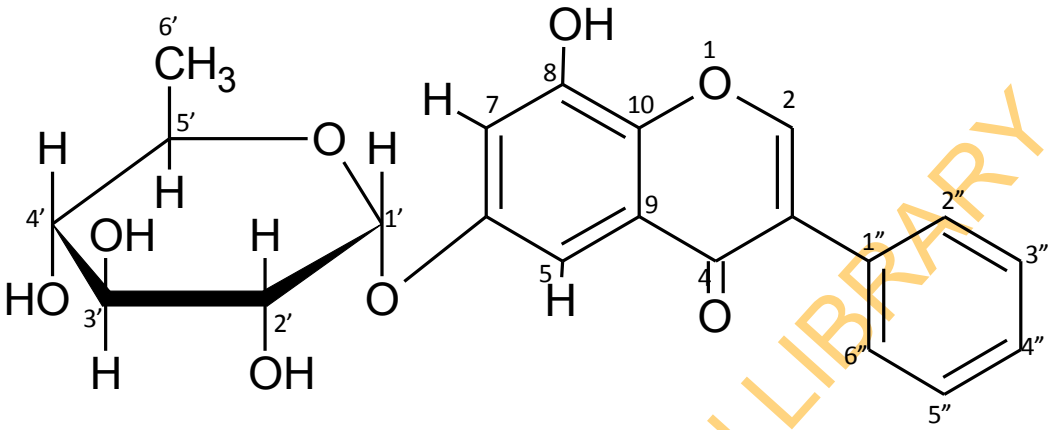
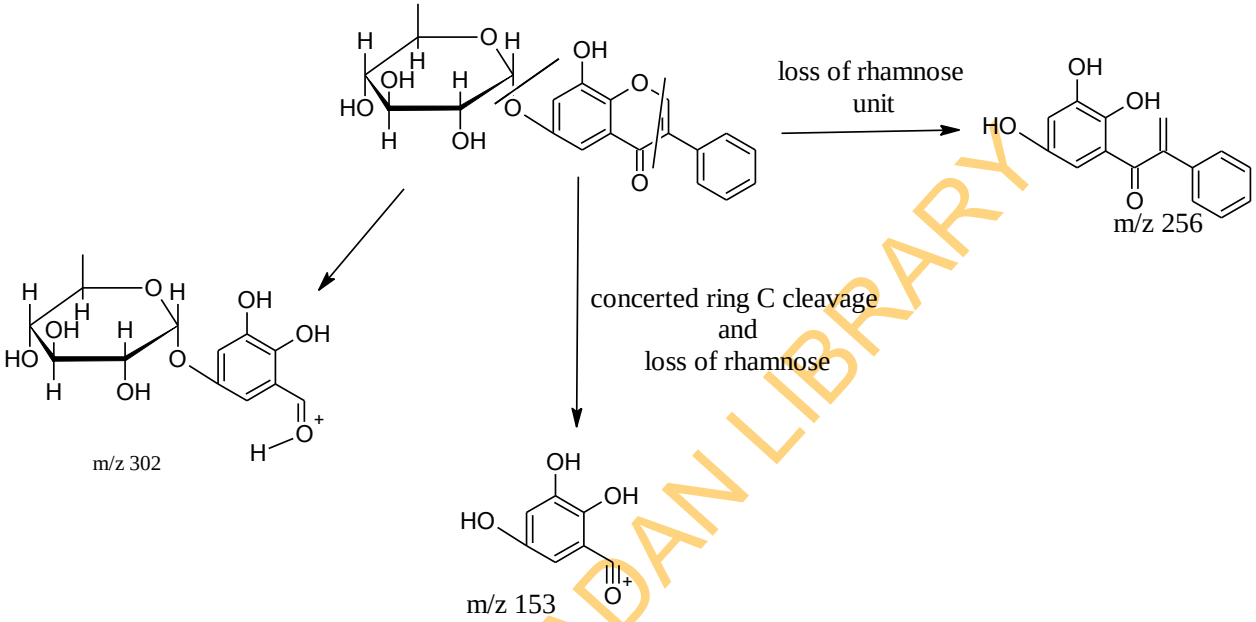


Figure 4.43: Compound MPL (8-hydroxy-3-(phenyl)-4-benzopyrone-6-O-rhamnoside)

Table 4.16: Rationalised ^{1}H and ^{13}C NMR ($CDCl_3$) spectra data of compound MPL.

S/No	δ_C ppm	APT	δ_H ppm
1	-	-	-
2	94.7	CH	7.3
3	137.2	C	-
4	178.5	C=O	-
5	99.4	CH	6.2
6	164.9	C	-
7	108.6	CH	6.4
8	161.9	-	-
9	120.3	-	-
10	157.1	-	-
1’	145.6	-	-
2’	116.0	CH	6.9
3’	116.0	CH	6.9
4’	116.0	CH	6.9
5’	116.0	CH	6.9
6’	116.0	CH	6.9
1’’	102.6	CH	5.2 (1H,d, J= 5.2 MHz)
2’’	71.1	CH	3.1-3.9
3’’	71.3	CH	3.1-3.9
4’’	72.0	CH	3.1-3.9
5’’	70.7	CH	3.1-3.9
6’’	19.0	CH_3	0.9 (3H, d, J= 5.8 MHz)

Key: om = overlapping multiplet, s = singlet proton or C, d = doublet proton or CH, t = triplet proton or CH_2, q = quartet proton or CH_3



Scheme 4.5: EI-MS fragmentation scheme for MPL from *M. pigra* aerial part

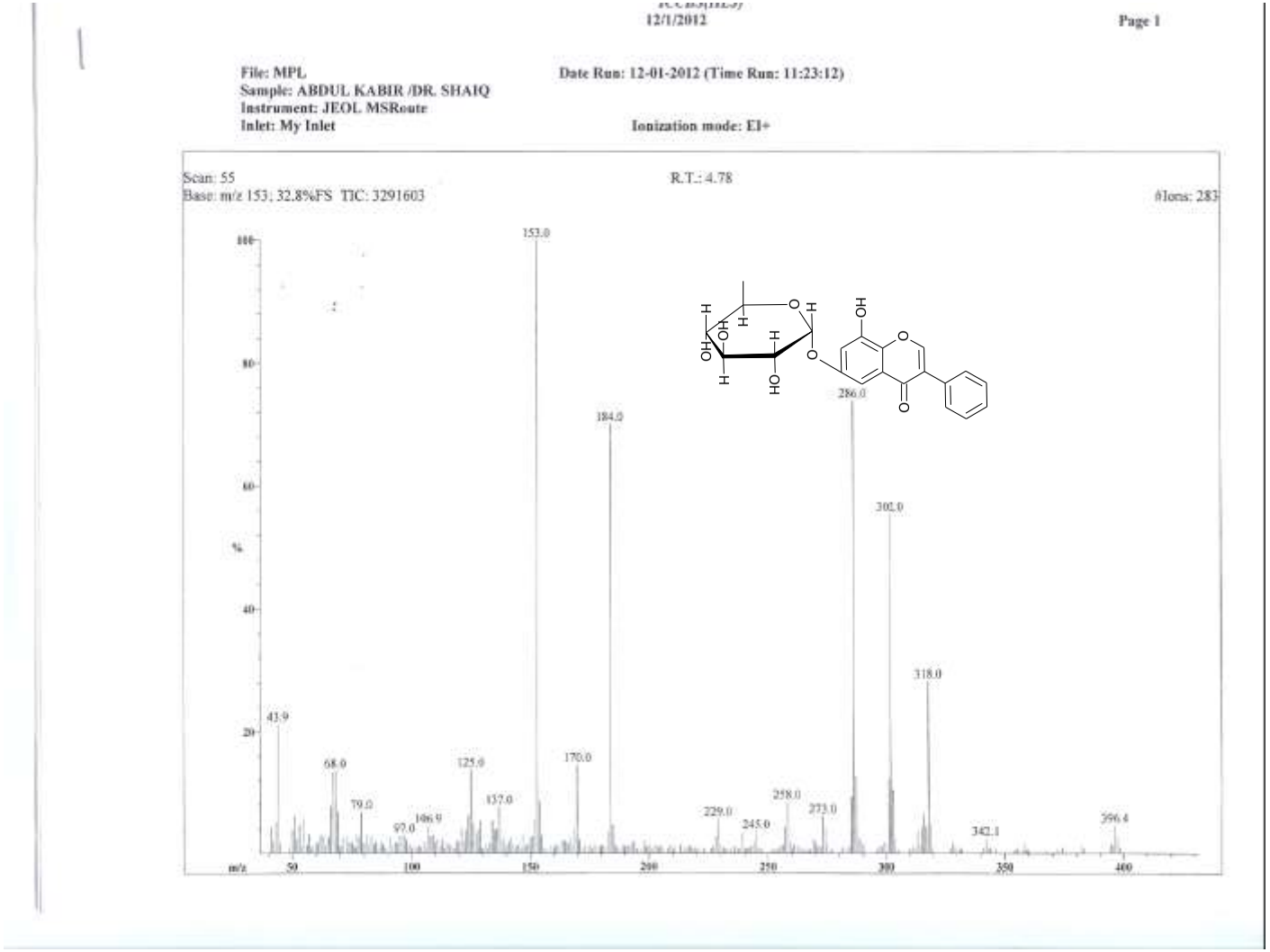


Figure 4.44: EI-mass spectrum of MPL isolated from *M. pigra* aerial part

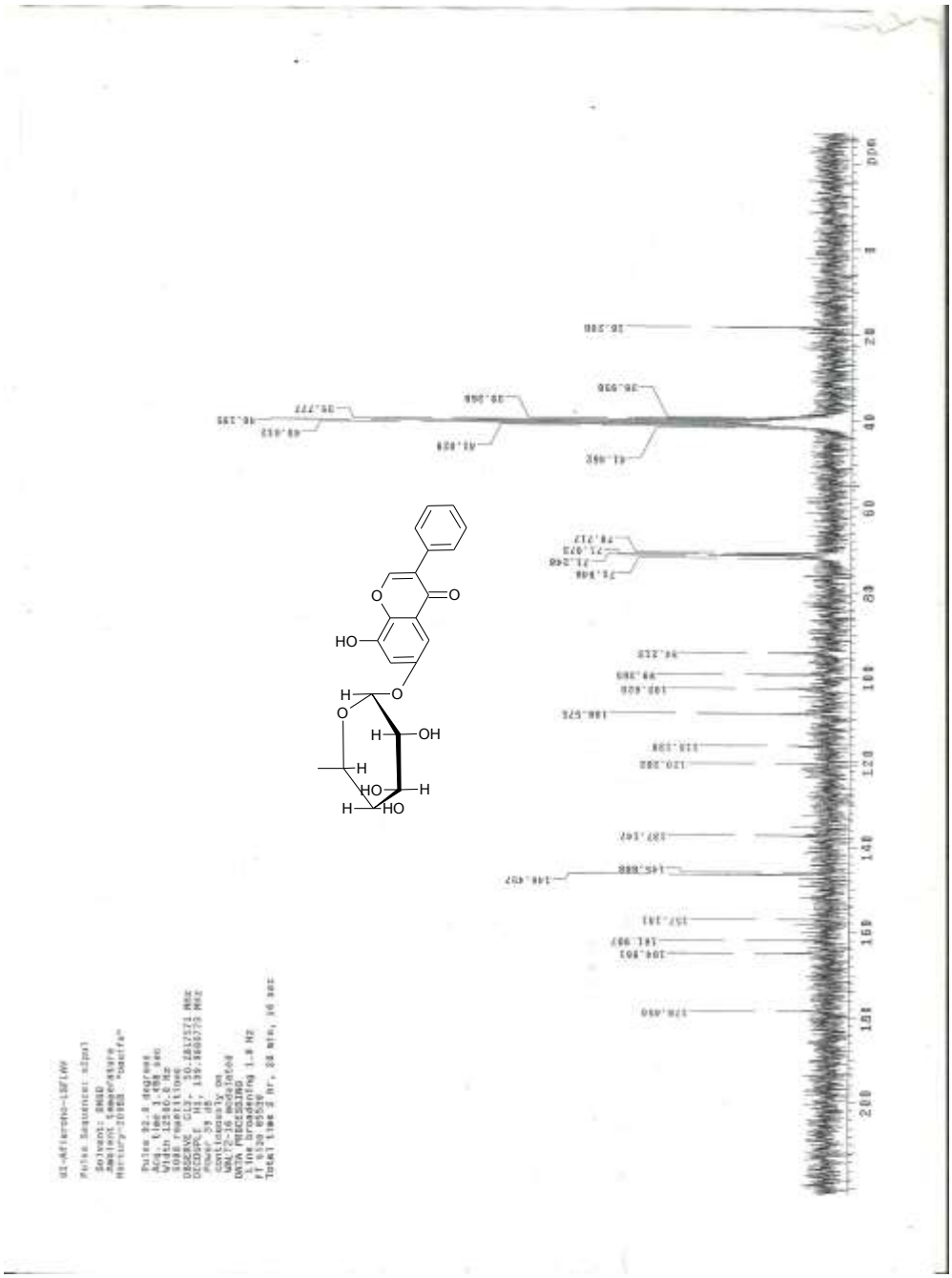


Figure 4.45: ^{13}C-NMR spectrum (50MHz, DMSO-d6) of MPL from *M. pigra* aerial part

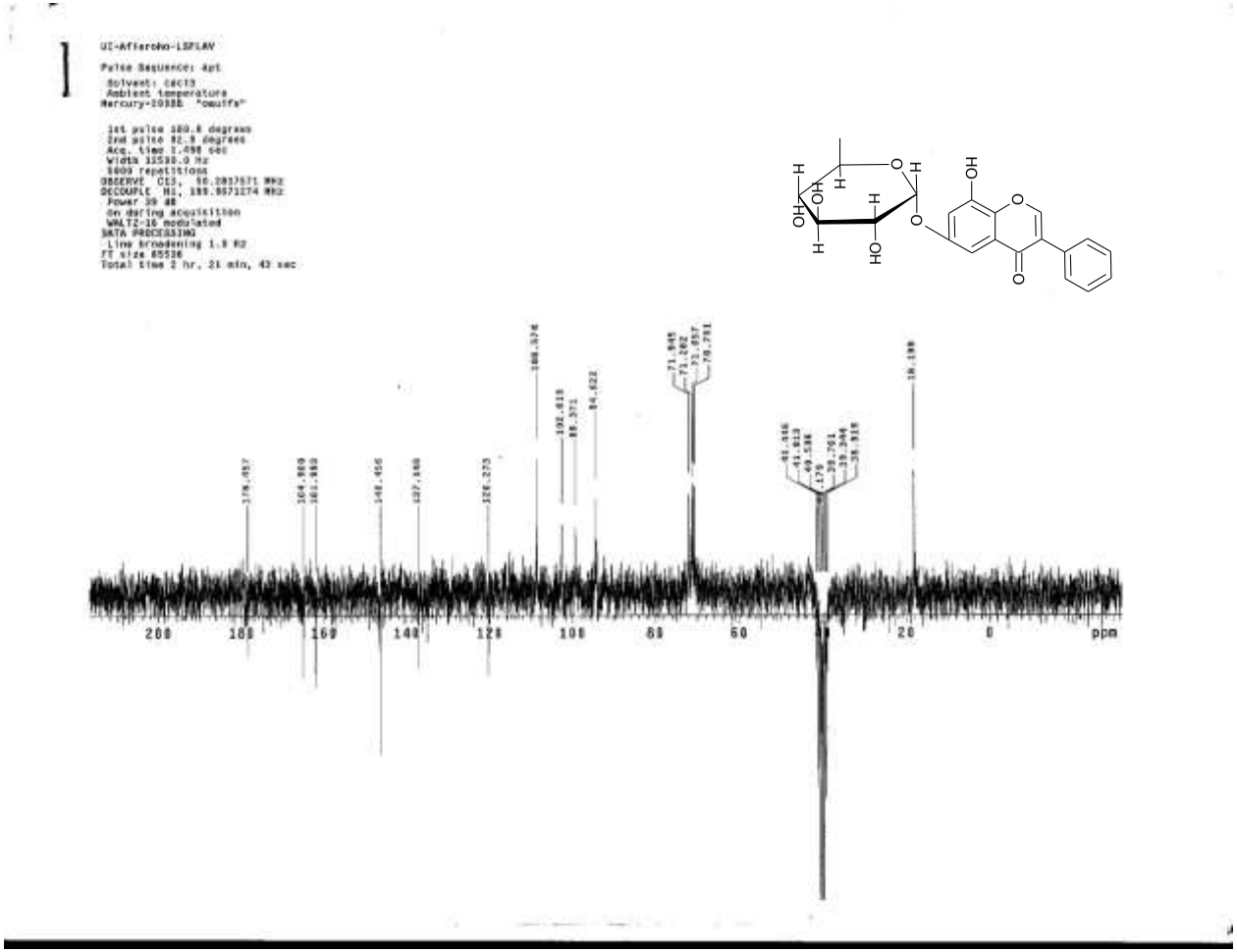


Figure 4.46: APT spectrum (50MHz, DMSO-d6) of MPL from *M. pigra* aerial part

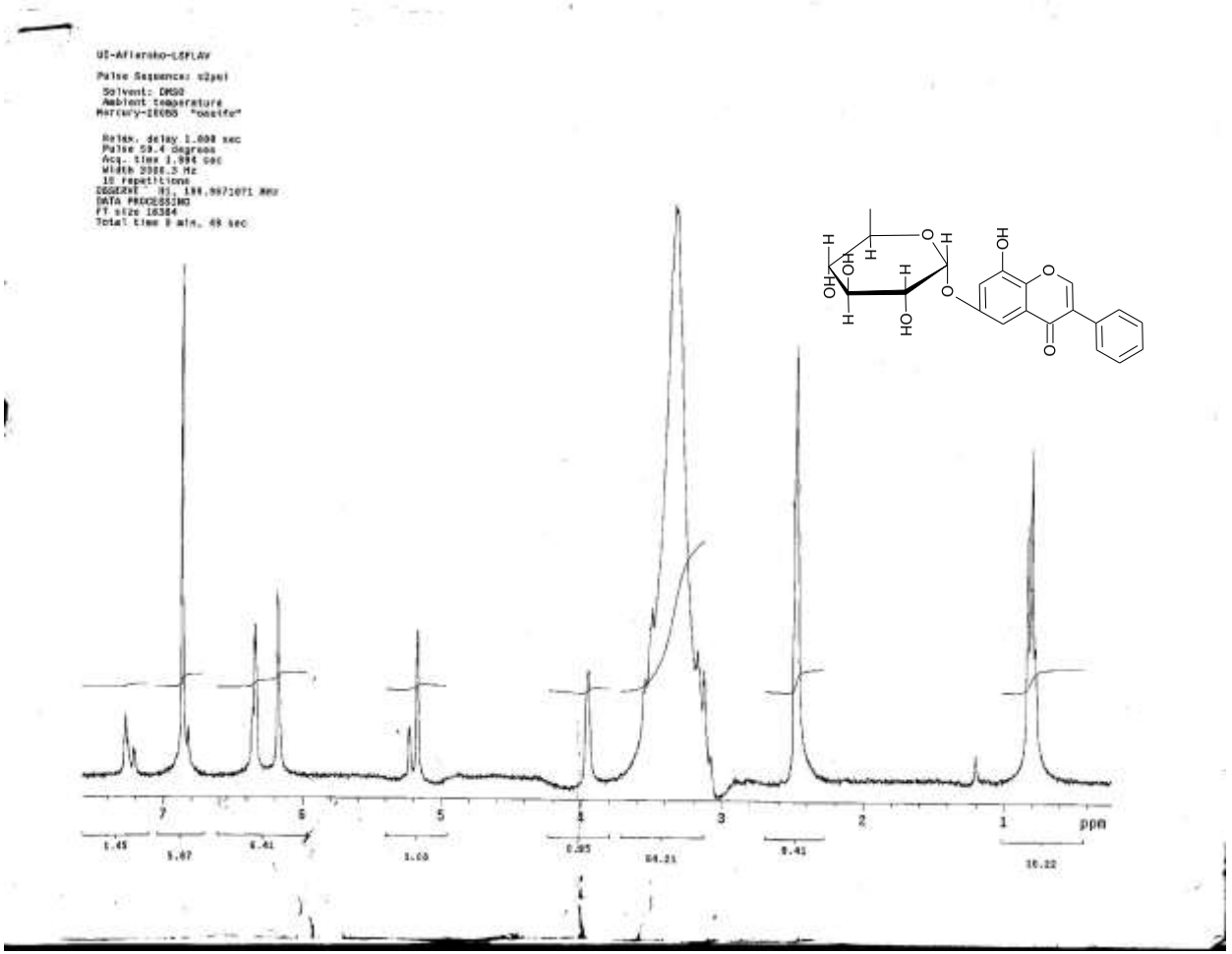


Figure 4.47: ^{1}H-NMR spectrum (200MHz, DMSO-d6) of MPL isolated from *M. pigra* aerial part

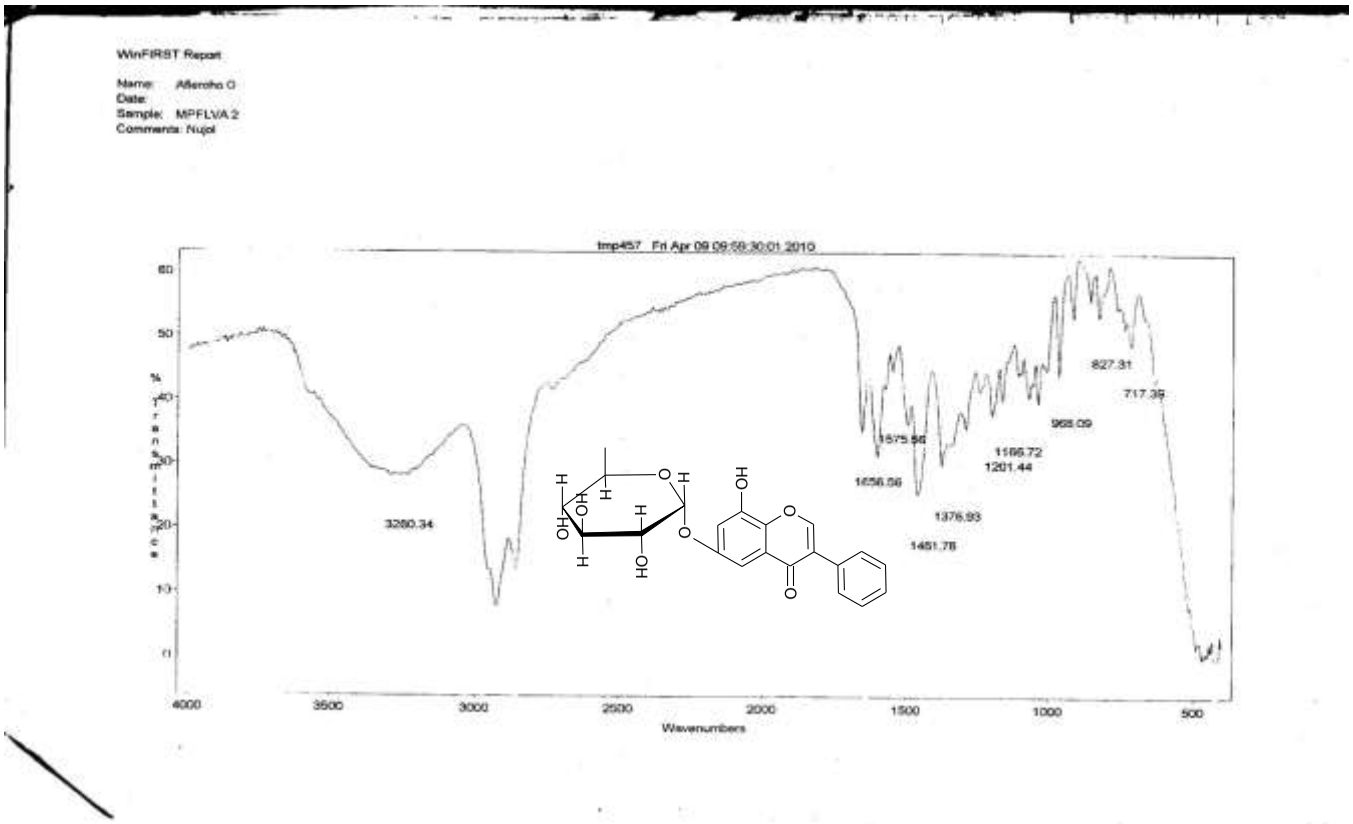


Figure 4.48: FTIR spectrum of MPL isolated from *M. pigra* aerial part

Table 4.17: Antimycobacterial activity of column sub-fractions from the bioactive n-butanol fraction of *M. pigra* (aerial part)

Test samples	Description	Test concentration (mg/ mL)	Assay method used	Colony Forming Units (CFU)	
				ZMC 303 at 0.5 McFarland Inoculums	ZMC 303 at 1.0 McFarland Inoculums
BSMP-1	Column sub-fraction from n-butanol fraction of *M. pigra*	0.357	MGIT	3+	3+
BSMP-2	Column sub-fraction from n-butanol fraction of *M. pigra*	0.357	MGIT	3+	3+
BSMP-3	Column sub-fraction from n-butanol fraction of *M. pigra*	0.357	MGIT	3+	3+
INH	Reference drug	0.0001	MGIT	-	-
DHS	Reference drug	0.0010	MGIT	-	-
EMB	Reference drug	0.0050	MGIT	-	-
RIF	Reference drug	0.0010	MGIT	-	-
Growth control	Control	NA	MGIT	3+	3+
Sterility control	control	NA	MGIT	-	-

Key to Table 4.17 : NA => Not applicable, nd→ not determined; - ➔ No growth after 2 weeks incubation at 37^{o}C, + ➔ 1-19 colonies growth after 2 weeks incubation at 37^{o}C, 1+ ➔ 20-100 colonies growth after 2 weeks incubation at 37^{o}C, 2+ ➔ 100-200 colonies growth after 2 weeks incubation, 3+ ➔ 200-400 colonies growth after 2 weeks incubation at 37^{o}C, 4+ ➔ > 400 colonies growth after 2 weeks incubation. DHS- Dihydrostreptomycin, INH- Isoniazid, RIF- Rifampicin, EMB- Ethambutol, CGU-colony growth unit, ZMC 303 is INH/RIF.DHS/EMB susceptible clinical isolates of *M.tuberculosis*, BSMP-1-3 are column sub-fractions obtain after chromatography separation of the n-butanol fraction of *M. pigra*.

4.7 Minimum Inhibiton Concentration (MIC) of isolated constituents from the bioactive fractions of *S. guineense* (Stem bark) and *M. pigra* (aerial part)

The constituents isolated from the acetone fraction A of *S.* guineense¸ and n-butanol fraction *M .pigra* were evaluated for their MIC. This is because these two fractions showed promising anti-mycobacterial activity (Tables 4.2 and 4.3). The results of the MIC determination are as shown in Table 4.18. The MPL from *M. pigra* (MIC > 0.476 mg/mL) was not as active as SGD and SGE from *S. guine*ense. Compound SGD characterised as betulinic acid methylenediol ester inhibited the growth of the clinical isolate of *Mycobacterium tuberculosis* with an MIC of 0.149 mg/mL which is better than that of compound SGE (betulinic acid, 0.30 < MIC < 0.60 mg/mL). This pointed to the presence of 3β-OH, 28 -carboxylate, and 20(29) unsaturation as pharmacophores responsible for antimycobacterial activity among lupane tritepenes. Betulinic acid and its derivative are common among members of the Myrtaceae family of plants of which *S. guineense* is a species. The antimycobacterial activity of betulinic acid isolated from some Argentine plants have been reported (Warhter *et al.*, 1999). It has also been reported to exhibit anti-cancer (Pisha *et al.*, 1995*;* Fulda *et al.*, 1999; Selzer *et al.*, 2000), anti-HIV (Fujioka *et al.*, 1994), antimalarial (Steele *et al.*, 1999), and anti-bacterial (Schuhly *et al.*, 1999; Nick *et al.*, 1995; Jeong *et al.*, 1999*;* Warhter *et al.*, 1999) activities among others. In view of this observed and reported activities, the presence of betulinic acid (SGE) and its methylene diol ester derivative (SGD) in *S. guineese* could further explain the ethnomedicinal uses of this plant in the treatment of tuberculosis and opportunistic infections of the respiratory tracts.

Table 4.18: Minimum Inhibiton Concentration (MIC) of isolated constituents from the bioactive fractions of *S. guineense* (Stem bark) and *M. pigra* (aerial part)

Test samples	Description	Test concentration (mg/ mL)	CFU	MIC (mg/mL)
SGD	Isolate from acetone fraction A	0.595 0.298 0.149	- - -	0.15
SGE	Isolate from acetone fraction A	0.595 0.298 0.149	- 1+ 1+	0.30 < MIC < 0.60
MPL	Isolate from n-butanol fraction of *M. pigra*	0.476 0.238 0.119	2+ 3+ 3+	>0.48
INH	Reference drug	0.1	-	-
DHS	Reference drug	1.0	-	-
Growth control	Control	NA	3+	3+
Sterility control	Control	NA	-	-

Key to table 4.18:
NA ➔ Not applicable
nd ➔ not determined
- ➔ No growth after 2 weeks incubation at 37°C
+ ➔ 1-19 colonies growth after 2 weeks incubation at 37°C
1+ ➔ 20-100 colonies growth after 2 weeks incubation at 37°C
2+ ➔ 100-200 colonies growth after 2 weeks incubation
3+ ➔ 200-400 colonies growth after 2 weeks incubation at 37°C
4+ ➔ > 400 colonies growth after 2 weeks incubation at 37°C
DHS- Dihydrostreptomycin
INH- Isoniazid
CFU-Colony Forming Unit
SGD and SGE -> compounds isolated from *S. guineense*,
MPL-> compound isolated from *M. pigra*

CHAPTER FIVE

5.0 CONCLUSION AND RECOMMENDATION

Both *Syzygium guineense* stem bark and *Mimosa pigra* aerial part have antimycobacterial activities. The *Syzygium guineense* stem bark showed better activity than the *Mimosa pigra* aerial part. This is a validation of the ethno-medicinal uses of *Syzygium guineense* and *Mimosa pigra.* The isolation and characterisation of the lupane-type triterpenoids, betulinic acid, and the new methylene diol ester of betulinic acid from *S. guineense*, and a benzopyrone rhamnoside from *Mimosa pigra* were identified for the first time from these plants as the antimycobacterial substances. They could serve as leads for the development of drugs for the management of tuberculosis. The isolation and characterisation of the triterpenoid, 12-hydroxy-norfriedoleanan-3-one and the known compound Friedelan-3-one from *S. guineense* is also being reported for the first time for this plant. The pharmacokinetic, structure activity relationship and toxicological profile of these isolated bioactive compounds could be carried out. This is to explore the possibility of translating them to prescription drugs for orthodox therapies.

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