ISOLATION AND CHARACTERISATION OF BIOACTIVE COMPOUND FROM THE ROOT BARK OF *Ficus sycomorus* (LINN).

By

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NOVEMBER, 2018

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DEPARTMENT OF CHEMISTRY,
FACULTY OF PHYSICAL SCIENCES,
AHMADU BELLO UNIVERSITY, ZARIA
NIGERIA

NOVEMBER, 2018

Declaration

I declare that the work in this dissertation entitled "Isolation and Characterisation of Bioactive Compound from the Root Bark of *Ficus sycomorus* L.*"* has been performed by me in the Department of Chemistry, Ahmadu Bello University, Zaria, Nigeria, under the supervision of Dr. I. A. Bello and Prof. M. S. Sallau. The Information derived from literature has been duly acknowledged in the text and a list of references provided. No part of this thesis was previously presented for another degree or diploma in any institution.

Bello MUKTAR		
Name of Student	Signature	Date

Certification

This dissertation entitled "Isolation and Characterisation of Bioactive Compound from the Root Bark of *Ficus sycomorus* L.)" by Bello MUKTAR, meets the regulations governing the award of the Degree of Master of Science in Organic Chemistry of the Ahmadu Bello University, Zaria, and is approved for its contribution to knowledge and literary presentation.

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Dedication

This research work is dedicated to my beloved family, the Muktars', and to the family of late Alh. Muhammad Yakubu Wanka (Kanawa).

Acknowledgement

Firstly, I would like to sincerely thank my respected supervisors Dr. I. A. Bello and Prof. M. S. Sallau for their support and the time they devoted towards my progress and final completion of my program. Their encouragement and patience were enormous. Their expertise in the area of natural products is very invaluable and I thank them for letting me tap into their wealth of knowledge.

I wish to appreciate the Head of Department of Chemistry, Prof. A. O. Oyewale, all professors and other respected academic and non-academic staff of the Department for their contribution in one way or the other to my success.

In this research work, there are a number of Institutions/Departments that generously rendered support to my work, which I would like to appreciate for their facilities and expert staffs. These include;

1. Department of Biological Sciences, Ahmadu Bello University Zaria-Nigeria, for the botanical identification of the plant.
2. Department of Microbiology, Ahmadu Bello University, Zaria, Nigeria, for performing the *in vitro* antimicrobial activity assay.
3. Department of Pure and Applied Chemistry, University of Strathclyde, Scotland-UK, for NMR analyses.

Lastly, but not the least, my deepest gratitude and appreciation go to my beloved parents, my siblings and my friends for their support financially and morally.

Abstract

Microorganisms have developed and are still developing resistance to most antimicrobial agents in use. Thus there is a need to search for new drugs that are of organic origin from medicinal plants, because they are relatively safer than synthetic alternatives, offering profound therapeutic benefits at affordable costs, and serve as a primary source of new medicine and lead compounds for the development of new drugs against various diseases. The percentage recovery of 3.67 % for n-butanol extract (MBS) from the microwave-assisted extraction was recorded whereas the extracts obtained from the target extraction; MBC1, MBC2 and MBC3 were found to have the percentage recovery of 0.15%, 0.28% and 0.20 %, respectively. Phytochemical investigation of n-butanol extract from the root bark of *Ficus* sycomorus showed the occurrence of steroids, triterpenes, flavonoids, alkaloids, and tannins. Detailed chromatographic techniques performed on the n-butanol extract led to the successful isolation of two triterpenoid compounds; lupeol (MB01) and lupeol acetate (MB03). Compound (MB01) was isolated as a white crystalline solid with melting point of 213 - 215°C and compound (MB03) was isolated as an off-white powder with melting point of 216 - 218°C. Antimicrobial activity study of the isolated compounds using agar well diffusion method, against *Escherichia coli, Samonella typhi, Bacillus subtilis* and *Staphylococcus aureus,* with inhibition zones ranging from 11-18 mm as compared with ciprofloxacin (standard drug) with inhibition zones ranging from 26-31 mm, showed that the compounds are potential source of antibacterial agents.

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List of Abbreviations

PPM	Part per million
NMR	Nuclear magnetic resonance
TLC	Thin layer chromatography
MIC	Minimum Inhibitory Concentration
MBC	Minimum Bactericidal Concentration
^{1}H NMR	Proton Nuclear Magnetic Resonance
^{13}C NMR	Carbon Nuclear Magnetic Resonance
δ	Delta, for mical shift in ppm
WHO	World Health Organization
IZTECH	Izmir Institute of Technology
R_f	Retardation factor
s	Singlet
t	Triplet
m	Multiplet
MeOH	Methanol
EtOH	Ethanol
BuOH	Butanol
ZI	Zone of inhibition
MAE	Microwave-Assisted Extraction
Conc.	Concentrated

CHAPTER ONE

1.0 INTRODUCTION

Natural products, for ages, have been used as medicines and today, they continue to be a reservoir of potential drugs (Lamottke *et al*., 2011). Medicinal plants are essential and resorted to by more than 70 % of the world's population that do not have access to Western medicine (WHO, 2008). The primary benefits of using plant-derived medicine are that they are relatively safer than synthetic alternatives and offer profound therapeutic benefits at affordable costs (Iwu, *et al*., 1999). There is an increasing awareness towards the use of herbal drugs and medicinal plant in the world. Germany, for example, is an active country among developed countries on herbal drug research, and France, where herbal extracts are sold as prescription drugs (Subramoniam, 2014).

On the other hand, natural products provide new medicine and lead compounds for the development of new drugs against various ailments. Out of the 1,135 new drugs approved from 1981 to 2010, 50 % were of natural product origin (natural, derivatives and analogues) (Cragg 2007; Newman and Cragg 2012). A more recent example is the development of artesunate (antimalarial and profound anticancer agent) a derivative of artemisinin from the Chinese medicinal plant *Artemisia annua* L. (Krusche *et al*., 2013) and the widely used breast cancer drug, paclitaxel (Taxol®), isolated from the bark of the Pacific Yew, *Taxus brevifolia* (Dewick, 2009).

The presence of secondary metabolites in natural products accounts for their contribution towards drug development. There is wide consensus that the potential for new natural products is not exhausted, therefore natural products remain an important source for the lead in drug discovery (Chin *et al*., 2006; Zaku *et al*., 2009).

The extraction method is one of the most important steps of any medicinal plant study in the processing of the bioactive constituents from the plant materials (Azwanida, 2015). The complete phytochemical profile of a given plant species can be investigated by fractionating the crude extract in order to separate the main classes of constituent from each other, known as target extraction, prior to chromatographic analysis.

The microwave-assisted extraction (MAE) is considered a selective method that favours polar molecules and solvents with high dielectric constants, for extracting bioactive constituents from the plant materials using microwave energy (Pare *et al*., 1994).

1.1 Statement of Research Problem

Plant materials have been the primary sources of medicine since early civilization (Subramoniam, 2014) and they continue to provide new medicine and lead compounds for the development of new drugs against various ailments (Parekh and Chanda, 2007). However, since these traditional herbal medicines were commonly prepared from crude materials, there are many questions concerning their specific medicinal effects and reproducibility, mechanism of action, and the identity of the active ingredients (Kim *et al*., 2010). Therefore, current some researches have focused on the specific components of an active herb rather than on the herb in its entirety. Several bioactive chemical constituents such as alkaloids, tannins, flavonoids and phenolic compounds have been reported in *Ficus sycomorus* (Sandabe *et al.*, 2006; Mudi *et a*l., 2015) and it is an important medicinal plant used traditionally for the treatment of different conditions including gastrointestinal, respiratory, cardiovascular disorders and inflammation (Hedberg and Staugard, 1989; Arnold and Gulumian, 2002; Sandabe *et al*., 2006; Hassan *et al*., 2007). However, research on this plant is scanty (Galil and Esiskowitch,

1974; Sandabe *et al*., 2006) and to the best of our knowledge, no phytochemicals have been characterized from the root bark of this plant before now.

1.2 Justification

Most of the targeted microorganisms have developed resistance to most of the antimicrobial agents in use, thus there is need for search for new ones that are of organic origin (Larhsini *et al.*, 2001). There are many advantages of using antimicrobial compounds from medicinal plants such as being safer and cheaper compared to the synthetic alternatives (Iwu, *et al*., 1999), acceptance due to long history of use, and being renewable in nature (Gur *et al*., 2006) and also higher plants represent a potential source of novel antibiotic prototypes (Parekh and Chanda, 2007). To the best of our knowledge, there is no reported work on the establishment of the antimicrobial study based on the individual classes of phytochemical constituents of the plant, *Ficus Sycomorus,* as well as characterization of bioactive compounds from the part of the plant under study.

1.3 Aim

The aim of this research is to carryout MAE, isolate, characterize and investigate the biological activity of phytochemical components from the root bark of *Ficus sycomorus.*

1.4 Objectives of the Study

The aim will be achieved through the following objectives:

i. MAE and Target extraction on the plant material.

ii. Preliminary Phytochemical screening of the extracts.

iii. Separation, purification and isolation of the bioactive constituents using chromatographic techniques.

iv. Characterization and structural elucidation of the isolated compound(s) using spectroscopic techniques such as:

- Infrared spectroscopy (IR);
- Nuclear Magnetic Resonance (^{1}H NMR, ^{13}C NMR and 2D NMR)

v. Determination of the antimicrobial activity of the plant extracts and the isolated compound.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Extraction

Medicinal plants are in considerable significance view due to their special attributes as a primary source of new medicine and lead compounds for the development of new drugs against various diseases (Parekh and Chanda, 2007) in addition to their ethno pharmacology.

Pre-extraction and the extraction procedures are the first and important steps of any medicinal plant study in the processing of the bioactive constituents from the plant materials (Azwanida, 2015). The extract thus obtained may be ready for use as a medicinal agent, it may be further processed to be incorporated in any dosage form such as tablets or capsules, or it may be fractionated to isolate individual chemical entities as modern drugs (Handa *et al.,* 2008). Various solvents have been used to extract different phyto-constituents and alcohol among other solvents, in any case, remains a good all-purpose solvent for preliminary extraction (Harborne, 1998). El-Sayed and his co-workers reported that n-butanol (BuOH) fraction of *Ficus sycomorus* leaves has strong antioxidant activity and the compounds isolated from it were found as major components and principally responsible for the antioxidant activity of *F. sycomorus* (El-Sayed *et al.,* 2010).

Medicinal plants are biosynthetic laboratories, not only for chemical compounds but for phytochemicals, which exert physiological and therapeutic effects (Barthel and Reuter, 1968). Due to the presence of many bioactive compounds in natural product extracts, synergistic effects or antagonistic interactions are likely to occur and as such; natural product extracts are claimed to have better pharmacological activity compared to single drug component. However, such claims are difficult to prove experimentally.

Synergistic effects, can result in the following: (i) the constituents of a natural product extract affects different targets (ii) they can interact with one another to improve the solubility and thereby enhancing the bioavailability of one or several substances of a natural product extract and (iii) compounds may also have their efficacy enhanced with agents that antagonize mechanisms of resistance (Wagner and Ulrich-Merzenich, 2009).

The analysis of the discrimination between antagonistic interactions or real synergism, of different classes of phytochemicals, can be carried out by investigating the complete phytochemical profile of a given plant species and fractionating the crude extract to obtain different classes of phytochemicals. This is known as target extraction, prior to chromatographic analysis. Target extraction, on an alkaloid-containing plant might be employed, that is based on varying polarity and basicity. However, modification is possible when investigating labile substances (Harborne, 1998).

Maceration and Soxhlet extraction are conventional extraction methods commonly used in laboratories. The microwave-assisted extraction (MAE) is a method for extracting bioactive constituents from the plant materials using microwave energy (Pare *et al*., 1994) and it offers some combinations of the following advantages over conventional extraction methods: purity of crude extracts, improved stability of marker compounds, reduced processing costs, increased recovery and purity of marker compounds, very fast extraction rates, reduced energy and solvent usage (Patil and Shettigar, 2010). Microwave-assisted extraction (MAE), can also be considered a selective method that favours polar molecules and solvents with high dielectric constants: in MAE, microwave radiation interacts with dipoles of polar and polarizable materials (e.g. solvents and sample) causes heating near the surface of the materials and heat is transferred by conduction (Azwanida, 2015). In non-polar solvents, poor

heating occurs as the energy is transferred by dielectric absorption (soakage) only (Handa, 2008). The extraction mechanism of microwave assisted extraction is supposed to involve three sequential steps: first, separation of solutes from active sites of sample matrix under increased temperature and pressure; second, diffusion of solvent across sample matrix; third, release of solutes from sample matrix to solvent (Alupului *et al*., 2012).

2.2 The Genus *Ficus*

The genus *Ficus* L. belongs to the family Moraceae. It is one of the most populous species in number of all plant genera (Lansky and Paavilainen, 2011). They are flowering plants and are often called the mulberry or fig family, which comprises about 40 genera and over 1000 species. Most of the species are widespread in tropical and subtropical regions (Noort *et al*., 2007). *Ficus*, the largest genus in the mulberry family, contains *Ficus sycomorus*, commonly known as fig Mulbrry. They are characterized by latex that exudes from the bark, branches, leaves and fruits on injury. The leaves are mostly entire and some are rarely lobed with irregular margins. The other characteristic feature of the *genus*, which runs in the whole family Moraceae is the fruit termed the syconium (Bwalya, 2014). They are up to 10 m in height. Their fruits, roots and leaves have been traditionally used by different ethnic communities worldwide for treatment of different diseases (Fowler, 2007).

2.2.1 Ethnomedical uses of the genus *Ficus*

Plant traditional medicines are still in use for the treatment of a variety of diseases caused by different pathogens. The genus *Ficus* is no exception as it has worldwide use, because members of this genus are one of the earliest sources of cultivated medicines and food by both humans and animals (Ipulet, 2007; Lansky and Paavilainen, 2011).

Many reports come from the use of *Ficus* species as food, some of which are the fruits and leaves of *F. dicranostyla* Mildbr., *F. sur* Forssk. (Synonym: Thunb.), *F. natalensis* Hochst., *F. sycomorus* L. and *F. wakefieldii* Hutch. Edible fruits are chewed for dyspepsia, while leaves (also edible) or bark and root infusions are used in the treatment of infectious diseases, abdominal pains and diarrhoea (Kuete *et al*., 2011)

There are many *Ficus* species reportedly used as folk medicines throughout the continent of Africa and a few have been reported as being used for domestic purposes (Burrows and Burrows, 2003; Ipulet, 2007). As folk medicines, they have been used as astringents, laxatives, antihelmintics (De Amorin *et al*., 1999), for skin inflammations and warts (Bwalya, 2014). In Ghana, the bark infusion of *F. asperifolia* Miq. was reportedly used for washing sores and ulcers and applied to circumcision wounds, while the rough leaves are used for scraping patches of ringworm before further treatment (Annan and Houghton, 2008). Decoctions of the bark of *F. virgata* are used in treating various skin diseases and ulcers (Abdel-Hameed *et al*., 2008). It is also said to be effective in the treatment of piles, asthma, gonorrhea, hemoptysis, and urinary diseases. Decoctions of some *Ficus* fruits (*F. carica L.* and *F. natalensis*), the stem barks (*F. trichopoda* Baker, *F. asperifolia*, *F. craterostoma* Warb. ex Mildbr. and Burret., *F. exasperata* Vahl, *F. thonningii* Blume (synonym: *F. iteophylla* Miq.)), leaves (*F. sagittifolia* Warb. ex Mildbr. and Burret and *F. populifolia* Vahl), roots (*F. sur* Forssk. (synonym: *F. capensis*)) and the milky latex of *F. sycomorus* are reportedly used against sore throats and coughs (Burrows and Burrows, 2003 and Neuwinger, 2000). In Senegal, the maceration of the leaves of *Ficus dekdekena* are used to treat tuberculosis (TB), while in Cameroon the 1:1 decoction of *Ficus chlamydocarpa* and *Ficus cordata* is also used traditionally and indiscriminately in the treatment of filariasis, diarrhoeal infections and tuberculosis (Kuete *et al.*, 2008).

2.2.2 Biological activities found in the genus *Ficus*

Medicinal plants are biosynthetic laboratories, not only for chemical compounds but for phytochemicals that exert physiological and therapeutic effects (Barthel and Reuter, 1968). Phytoconstituents employed by plants to protect themselves against pathogenic insects, bacteria, fungi or protozoa have found applications in human medicine (Nascimento *et al*., 2000). A number of scientific research has been carried out to validate and document the pharmacological properties that the plant exhibits in addition to all the acclaimed traditional uses of the genus *Ficus*. The genus has been well documented for its biological activities like antioxidant activity (Li *et al*., 2005), anticancer activity (Mradu *et al*., 2012), antidiarrhoeal activity (*F. lutea, F. cordata* Thunb. subsp. *salicifolia* (Vahl) C.C. Berg. (syn: *F. religiosa* Forssk.) *F. sycomorus*), antibacterial activity *(F. ingens*), antifungal activity (Kuete *et al*., 2009), antiplasmodial activity (F. sycomorus, *F. polita*), antiulcer activity, gastroprotective activity and wound healing activity in experimental animals (Thakare *et al*., 2010; Kuete *et al*., 2009; Lansky *et al*., 2008).

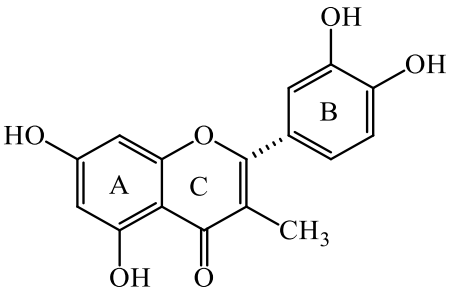
2.2.3 Phytochemistry of the genus *Ficus*

Most members of genus *Ficus* have been investigated pharmacologically and phytochemically because of their medicinal uses. A phytochemical review shows that members of this genus are rich sources of several secondary metabolites such as prenylated flavanoids and isoflavanoids, lignans, terpenoids, alkaloids, coumarins, chromones, phenylpropanoids and tannins (Chen, *et al.*, 2010 and Kuete, *et al*., 2008). Other classes reported include steroids, triterpenoids, fatty acids, saponins and anthocyanins (Chawla *et al*., 2012).

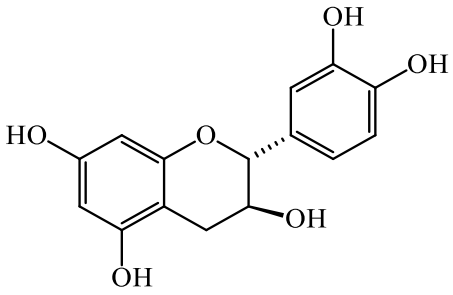
2.2.3.1 Phenolic compounds

Phenolic compounds are chemically diverse secondary metabolites, which are characterized by aromatic rings with one or more hydroxyl functional groups. Phenolic compounds can be classified into different groups such as flavanoids, flavanones, flavones, anthraquinones and tannins.

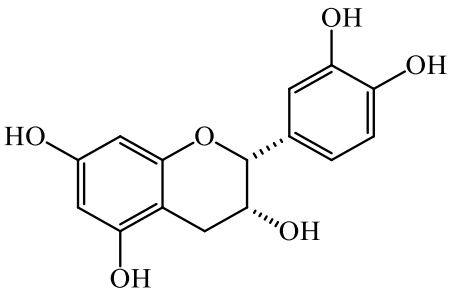
Flavonoids such as catechins, epicatechins and epiafzelechins isolated from *F. ovata* are common to the genus (Kuete *et al*., 2009). Furthermore, the following compounds were also isolated from the *Ficus gnaphalocarpa:* 3-methoxyquercetin (**I**), catechin (**II**), epicatechin (**III**), quercetin (**IV**), and quercitrin (**V**) (Hubert et *al*., 2011) (Figure 2.1).



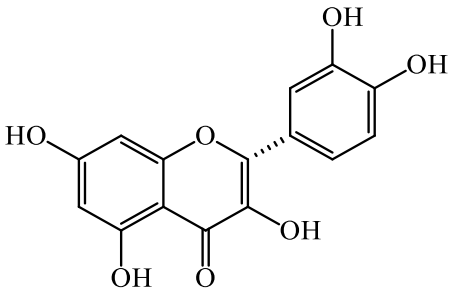
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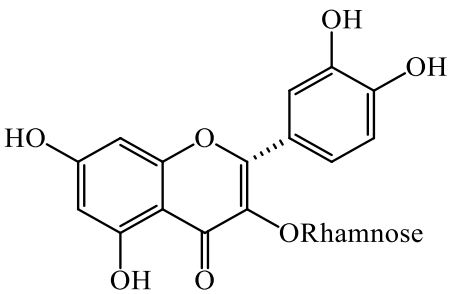
II



III



IV



V

Figure 2.1: Some Phenolic compounds isolated from *Ficus* species.

2.2.3.2 Terpenoids

Terpenes are among the most widespread and chemically diverse groups of natural products. Thus, terpenes are all based on the hydrocarbons of plant origin known as isoprene molecule (5 carbon atom unit), **CH_2=C-(CH_3)-CH=CH_2**, and their carbon skeletons are built up from the union of two or more of these five carbon atoms units. They are classified according to whether they contain two (10 carbon atoms), three (15 carbon atoms), four (20 carbon atoms), six (30 carbon atoms) or eight (40 carbon atoms) such units. They are ranged from the essential oil components, the volatile mono-and sesquiterpenes (10 and 15 carbon atoms) through the less volatile diterpenes (20 carbon atoms) to the non-volatile triterpenes and triterpenoids (30 carbon atoms) and carotenoid pigments (40 carbon atoms). Each of these various classes of terpenes is of significance in either plant growth, metabolism or ecology. The main types and occurrence of plant triterpenes include sterols (e.g. sitosterol), triterpenoids (e.g. β-amyrin), saponins (e.g. yamogenin) and cardiac glycosides (e.g digitoxin). (Firn, 2010; Harborne, 1998).

Terpenoids such as betulinic acid is a common bioactive compound that has been isolated from the genus, and is known to have antiprotozoal properties (Hubert *et al*., 2011). The following Chemical compounds were also reported to have been isolated from the roots of *F. polita*. lupeol **(VI)**; betulinic acid **(VII)**; taraxar14-ene **(VIII)** (Kuete *et al*., 2011) (Figure 2.2).

2.2.3.3 Steroids

Another interesting class of bioactive compounds found in the genus *Ficus* are steroids. These include *ß*-sitosterol **(IX)**; isolated from *F. polita* and *ß*-sitosterol-D-glucoside **(X)** isolated from *F. glomerata* and *F. cordata* stem bark. Potent hypoglycaemic and

antibacterial properties of ß-sitosterol-D-glucoside have been reported (Channabasavaraj *et al.*, 2008). (Figure 2.2).

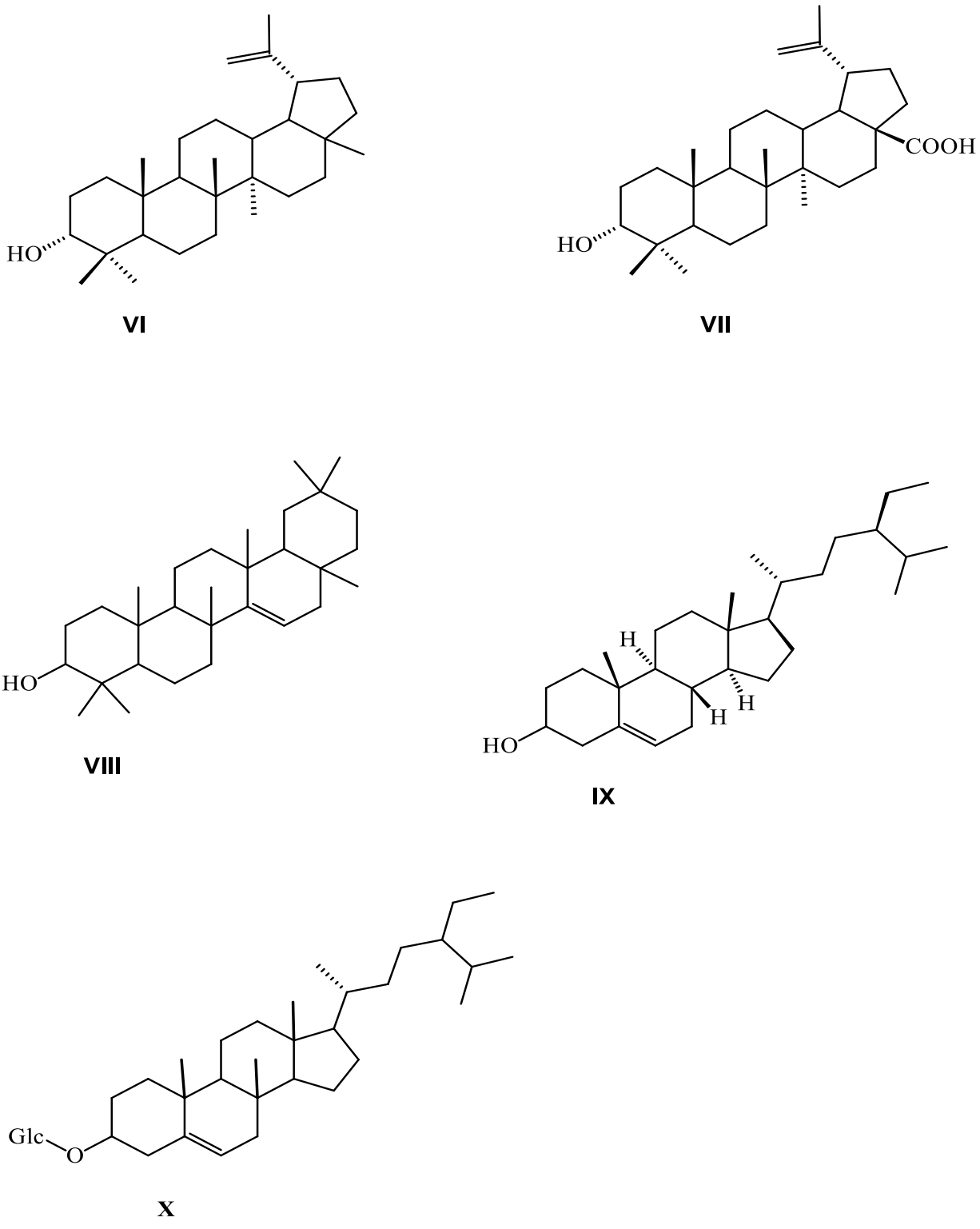


Figure 2.2: Some terpenoids and steroids isolated from *Ficus* species.

2.2.3.4 Terpenoid biosynthesis in plants

The pathways of biosynthesis are responsible for the occurrence of both primary and secondary metabolites (Herbert, 1989; Nicolaou and Chen, 2011). Terpenoids are polymeric isoprene derivatives and synthesized from acetate via the mevalonic acid pathway. During their formation, the isoprene units are linked in head to tail fashion. The number of units incorporated into a particular terpene serves as a basis for their classification. Although terpenoids are derived biogenetically from isoprene, which does occur as a natural product, this substance is not the *in vivo* precursor. Instead, the compound actually involved is isopentenyl pyrophosphate, $CH_2=C(CH_3)CH_2CH_2OPP$, which is formed itself from acetate *via* mevalonic acid, $CH_2OH\text{-}CH_2C(OH,CH_3)CH_2CO_2H$. Isopentenyl pyrophosphate (IPP) exists in living cells in equilibrium with the isomeric dimethylallyl pyrophosphate (DMAPP), $(CH_3)_2C=CHCH_2OPP$. In biosynthesis, a molecule of isopentenyl pyrophosphate and one of dimethylallyl pyrophosphate are linked together to give geranyl pyrophosphate (GPP). The key intermediate in monoterpene formation; geranyl pyrophosphate and isopentenyl pyrophosphate are, in turn, linked to give farnesyl pyrophosphate (C_{15}), the key intermediate of sesquiterpene synthesis. Different combinations of these C_5, C_{10} and C_{15} units are then involved in the synthesis of the higher terpenoids, triterpenoids being formed from two farnesyl units and carotenoids from the condensation of two geranyl-geranyl units (Fig. 2.3). Most natural 'terpenoids' have cyclic structures with one or more functional groups (hydroxyl, carbonyl, etc.) so that the final steps in synthesis involve cyclization and oxidation or other structural modification (Harborne, 1998; Justin *et al*., 2014).

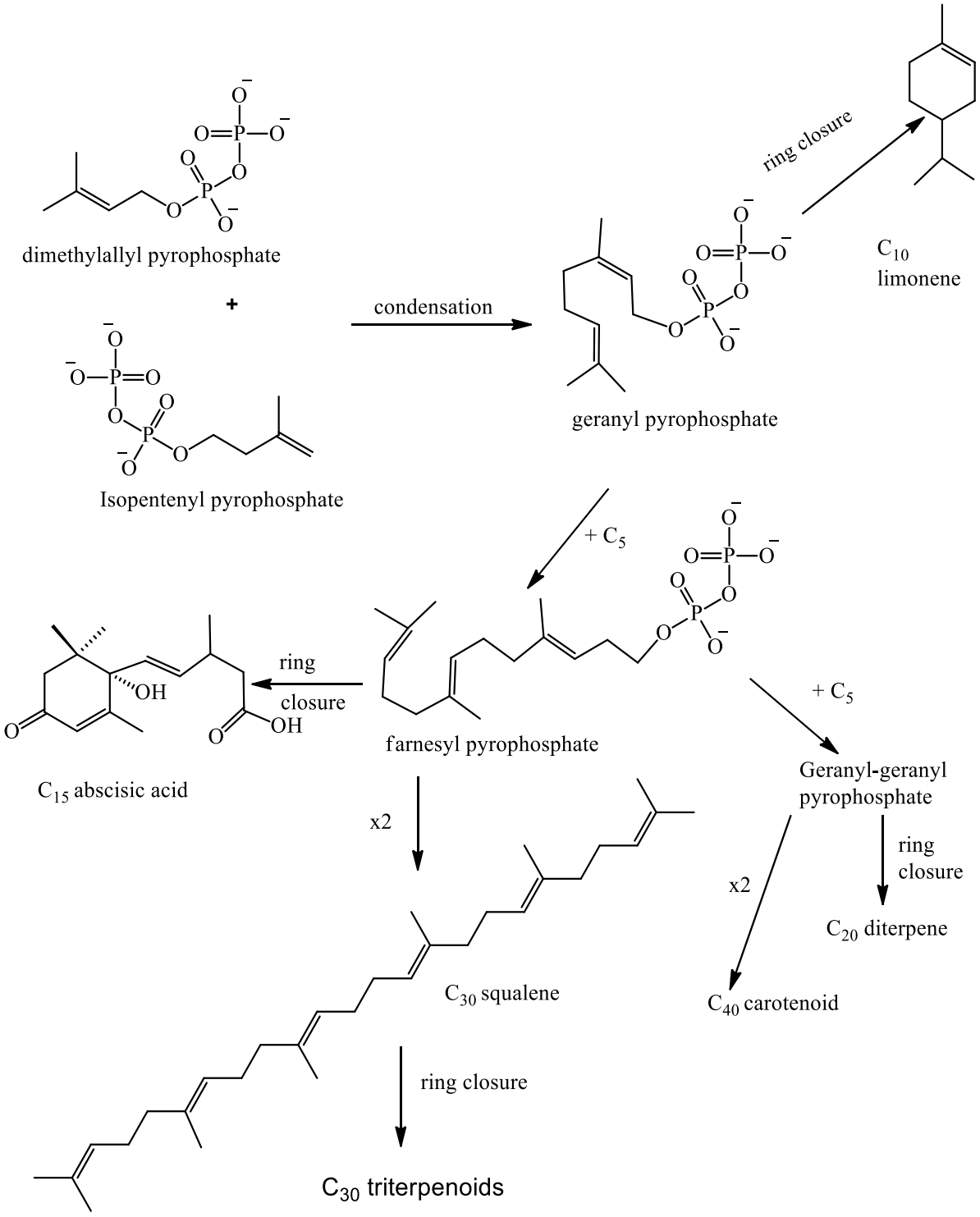


Figure 2.3: The path of terpenoid biosynthesis in plants.

2.3 *Ficus sycomorus*

Ficus sycomorus, subspecies *sycomorus* is commonly known as the sycamore fig or mulberry fig. It is commonly known among Yoruba people as epin (Zumbes *et al*, 2015), as Ji-ewu in Igbo language (Ogbuewu *et al*., 2015) and Hausa people of Northern Nigeria as Farin Baure. It is a large, spreading tree up to about 30 m tall and 30 m wide (canopy covering), deciduous or evergreen with a typically short, thick trunk extending to 3.5 m in diameter. Old trees develop wide-spreading branches and buttresses without root suckers and aerial roots (Bwalya, 2014) (plate I and II).

2.3.1 Ethnomedicinal uses of *Ficus sycomorus*

Ficus sycomorus, in particular, is also known for its antimicrobial role in treatment of Chest complaints, coughs, dysentery, diarrhoea, swollen glands, inflammation, tonsillitis and sore throat, sores and skin rashes, ulcers and TB (swollen lymph nodes) (Fowler, 2007). In Nigeria, the root bark of this plant is traditionally used for the treatment of epilepsy, diarrhoea, dysentery, painful urination and vaginal infections (Abubakar *et al*., 2015), and pathologic haemorrhoid, M. L. Abdullah (personal communication, March 26, 2017).

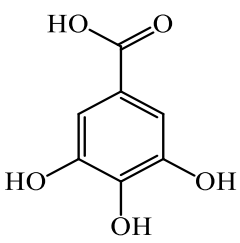
2.3.2 Pharmacological activities of *Ficus sycomorus*

Treatment of various diseases using plant material has always been practiced worldwide (Heinrich *et al*., 2004). Preliminary *in vivo* toxicity study was carried out on the plant and the result showed that the plant is safe for use as a pharmaceutical ingredient (Njagi *et al*, 2012) and as such, different parts of the plant were subjected to biological activity screenings which included both *in vitro* and *in vivo* assays and the results have been reported: antibacterial activities of the fruits of the plant (Mousa *et al*. 1994), antioxidant activity and antibacterial activity of the leaves of the plant (El-Sayed *et al*., 2010; Saleh *et al*, 2015), activities on sperm cell production, pH of homogenates of

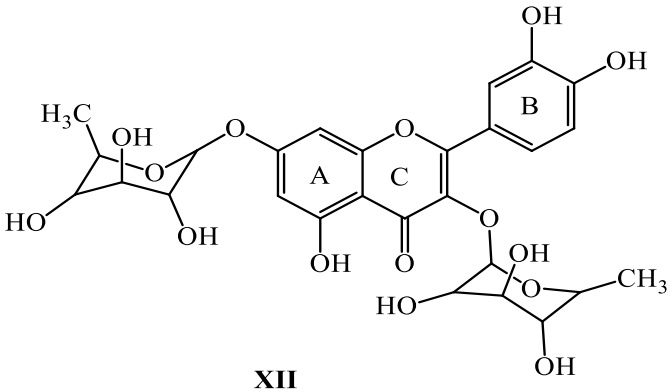
testes and epididymes of albino rats, anti-diabetic and antibacterial of the aqueous stem-bark extract of the plant (Igbokwe *et al*, 2009; Njagi *et al*, 2012; Saleh *et al*, 2015). Furthermore, the root bark of the plant has also been reported to have pharmacological effect on hepatoprotective, *petit mal* epilepsy, muscular relaxation, anaesthetic and sleeping time on laboratory animals (Garba *et al*, 2007; Abubakar *et al*., 2017; Zaku *et al*., 2009).

2.3.3 Phytochemistry of *Ficus sycomorus*

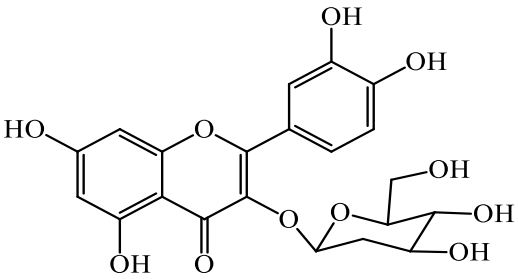
Chemically diverse secondary metabolites such as tannins, flavanoids, steroids and terpenoids were isolated and characterized from the different parts of the plant. El-Sayed and his co-workers reported (2010) the isolation of gallic acid (3,4,5-trihydtxybenzoic acid) **(XI)**, a tannin and the following flavonoids from the leaves of the plant: quercetin 3, 7-*O*-α-L-dirhamnoside **(XII)**, quercetin-3-*O*-β-D-glucopyranoside (isoquercitrin) **(XIII),** quercetin-3-*O*-α-L-rhamnopyronosyl (1→6) β-D-glucopyranoside (rutin) **(XIV)**, quercetin **(XV)**, and quercetin-3-*O*-β-D-galactopyranosyl (1→6) glucopyranoside **(XVI)** (El-Sayed et *al*., 2010) (Figure 2.3).



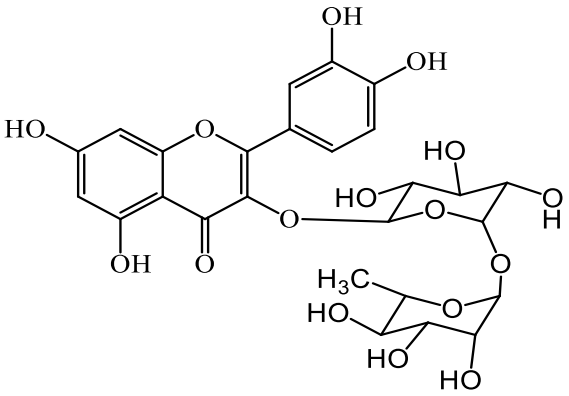
XI



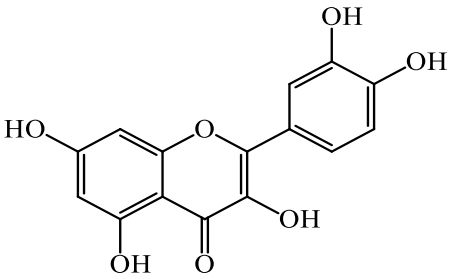
XII



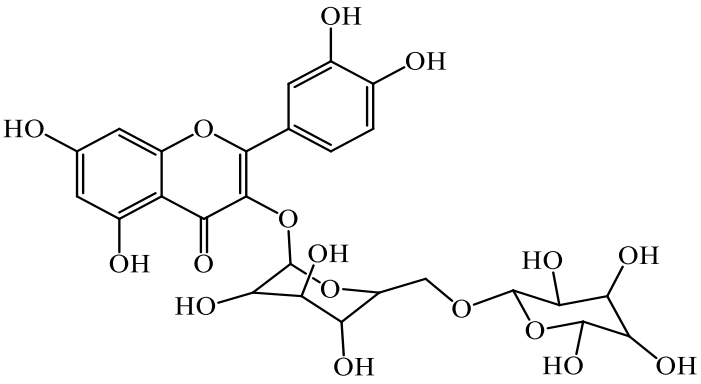
XIII



XIV



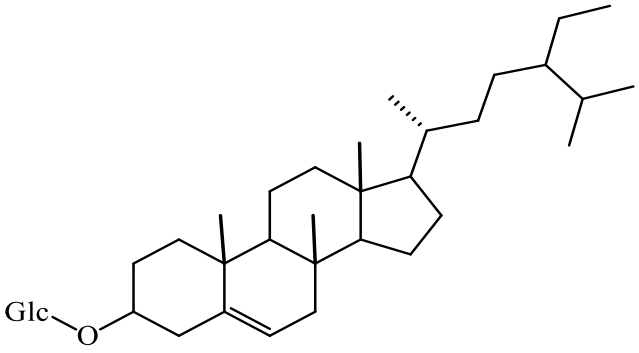
XV



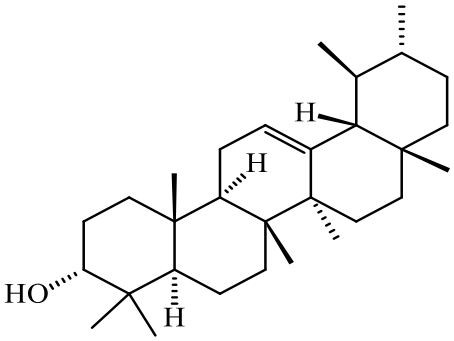
XVI

Figure 2.3: Some Phenolic compounds isolated from *Ficus sycomorus*.

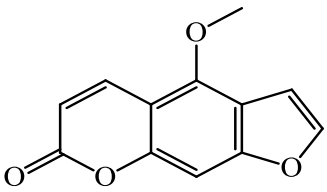
Bioactive steroids and terpenoids found in the the plant include ß-sitosterol-3-*O*-β-D-glucopyranoside **(XVII)** that has been isolated from the leaves (El-Sayed et *al*., 2010), α-amyrin **(XVIII)**, Bergapten **(XIX)**, and Xanthotoxin **(XX)** isolated from the latex of the plant (Lansky and Paavilainen, 2011) (Figure 2.4)



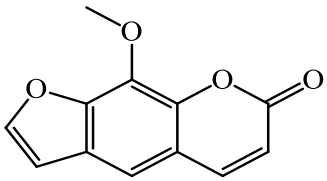
XVII



XVIII



XIX



XX

Figure 2.4: Some terpenoids and steroids isolated from *Ficus sycomorus*.

2.3.4 Taxonomic description

Kingdom: *Plantae*

Phylum: *Magnoliophyta*

Class: *Magnoliopsida*

Order: *Rosales*

Family: *Mulberry (Moraceae)*

Genus: *Ficus L.*

Species: *Sycomorus*



Plate I: *Ficus sycomorus* tree.

[image]. (2017). Retrieved from http://www.prota4u.org/database/protav8.asp?g=psk&p=ficus+syncomorus+L



Plate II: Leaves and fruit of *Ficus sycomorus*.

[image]. (2017). Retrieved from http://www.figweb.org/Figs_and_fig_wasps/index.htm and www. Plantzafrica.com

2.4 Microorganisms and their Association with Human Host

Bacteria are microorganisms live in close association with human and other organisms. The association may be beneficial to the host on no detrimental effect to the host where they colonize the skin, existing on the skin and mucous membranes without causing any harm. However, when they gain entry into the deeper tissue and the immune system of the host fails to fight off the invasion, the bacteria causes infection and establishes a disease condition such as skin infections, respiratory illnesses, cerebral and food-borne illnesses. Bacteria are broadly classified according to the staining properties of their cell walls. Thus, they can be classified as gram-negative bacteria which have less murein or peptidoglycan in their cell walls compared to gram-positive bacteria (Bwalya, 2014). The following are some of the common pathogenic bacteria:

2.4.1 *Escherichia coli*

Escherichia coli are Gram negative microorganisms and classified by the characteristics of their virulence properties and each group causes disease by a different mechanism (Atlas, 2010). The majority of community acquired urinary tract infections are caused by uropathogenic *E. coli* and that accounts for approximately 90% of first urinary tract infections in young women (Atlas, 2010; Ad Dhhan *et al*., 2005; Schilling and Hultgren, 2002). Most uropathogenic *E. coli* strains produce hemolysin, which initiates tissue invasion and makes iron available for infecting pathogens (Huges, 1996). It causes diarrhoea and is common worldwide.

2.4.2 *Salmonellae*

Salmonellae are Gram negative microorganisms that are often pathogens for humans or animals when acquired by the oral route. They are transmitted from animals and animal products to humans, where they cause enteritis, systemic infections and enteric fever.

Salmonellae vary in length and grow on simple media, the following are some of their classes: *S. typhi, S. cholerasuis, S. paratyphi A, S. paratyphi B.* They produce diseases such as typhoid fever, headache, vomiting and nausea. The most important is *Salmonella typhi* which reaches the small intestine after ingestion of *Salmonellae*, from where they enter the lymphatic system and then the blood stream. It can be treated with antibiotics (Podschun and Ullman, 1998).

2.4.3 *Staphylococci*

Staphylococci are Gram positive spherical cells, usually arranged in grape like irregular clusters. They grow readily on many types of media and are active metabolically, fermenting carbohydrates and producing pigments that vary from white to deep yellow. The genus *Staphylococcus* has at least 40 species. The three frequently encountered species of clinical importance are S*taphylococcus aureus, Staphylococcus epidermidis* and *Staphylococcus saprophyticus. S. aureus* is a major pathogen for humans and causes nosocomial infection such as skin infection, gastrointestinal and urinary tract infections, and pneumonia (Bronner, 2004 and Al-Dahmoshi *et al*., 2013). Almost every person will have some type of *S. aureus* infection during their life time, ranging from food poisoning or minor skin infections to severe life-threatening infections. *Staphylococci* are non-motile and do not form spores. Under the influence of drugs like penicillin, *Staphylococci* are lysed. Staphylococci are easily cultured on most bacteriological media under aerobic or microaerophilic conditions. *Staphylococci* can cause disease both through their ability to multiply and spread widely in tissues and through their production of many extracellular substances. Some of these substances are enzymes. However, it is difficult to eradicate pathogenic staphylococci from infected persons, because the organisms rapidly develop resistance to many antimicrobial drugs

and the drugs cannot act in the central necrotic part of a suppurative lesion. It is difficult to eradicate the *S. aureus* carrier state (Bronner, 2004).

2.4.4 *Bacillaceae*

The genus *Bacillus* belongs to the family bacillaceae and it is the largest in the order. The genus contains Gram-positive, endospore-forming and chemo-heterotrophic rods that are usually motile with peritrichous flagella. Occasionally, *B. cereus, B. subtilis,* and some other species cause genuine infections, including infections of the eye, soft tissues, and lungs. Infection is usually associated with immunosuppression, trauma, an indwelling catheter, or contamination of complex equipment such as an artificial kidney. The relative resistance of *Bacillus* spores to disinfectants aids their survival in medical devices that cannot be heat sterilized. *Bacillus subtilis* produces the proteolytic enzyme subtilisin and its spores can survive the extreme heating that is often used to cook food. (Ryan and Ray, 2004)

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Materials

3.1.1 Solvents/Reagents and Chromatographic Materials

Solvents used, which include methanol, ethanol, n-butanol, ethyl acetate, chloroform and n-hexane were of general purpose grade and were distilled before use. Chromatographic materials include: aluminium TLC plate pre-coated with silica gel 60 PF_{254}, Shandon chromatographic tank (developing jar), Glass columns, Ultraviolet lamp (254 and 366 nm), Silica gel (Qualikens 60-120 mesh), 10 % H_2SO_4, conc. H_2SO_4 and conc. NaOH.

3.1.2 Equipment

Conventional microwave oven (MATSUI M180TC), digital pH meter (Hanna 4221), and the melting points apparatus (Suart SMP40 automatic melting point apparatus) were obtained from the Department of Chemistry, Ahmadu Bello University Zaria. Infra-red Spectroscopy (Shimadzu FTIR 400 Fourier Transform Infra-Red Spectroscopy) from the Multi user Laboratory, Department of Chemistry, Ahmadu Bello University Zaria. NMR analyses were run on a 600 MHz Bruker AVANCE spectrometer at the Department of Pure and Applied Chemistry, University of Strathclyde, Scotland-UK.

3.1.3 Test organisms for antimicrobial study

The microorganisms tested include two Gram negative bacteria (*Escherichia coli* and *Samonella typhi*) and two Gram positive bacteria (*Bacillus subtilis* and *Staphylococcus aureus*). Clinical isolates of the organisms were obtained from the Department of Microbiology, Ahmadu Bello University, Zaria, Nigeria.

3.2 Methodology

3.2.1 Collection of Plant Material.

The plant sample, root bark of *Ficus sycomorus* was collected around Tsauni Basawa, Samaru-Zaria, in Kaduna State, Nigeria in the month of March, 2017. The plant sample was collected by digging down to the root and peeling the root bark without cutting the root. The plant was identified by Namadi Sunusi of the Herbarium unit, Department of Biological Sciences, Ahmadu Bello University Zaria-Nigeria with the voucher specimen number, 1466. The plant sample was air-dried, pulverized using a wooden pestle and mortar. The pulverized plant material was then stored in an air-tight polythene bag ready for analysis.

3.2.2 Extraction

The Pulverized plant material was subjected to extraction using two different procedures as follows:

3.2.2.1 Microwave-Assisted Extraction (MAE)

The Pulverized plant material (600 g) was divided into five different portions and macerated in n-butanol and allowed to stand overnight. Afterward they were placed in a microwave under the lowest power (10 ^{O}C) and microwaved 5 times at 3 minutes pulses with intermittent cooling between the pulses. The microwaved plant material was then washed exhaustively with the same solvent. The extract was concentrated by allowing the solvent to evaporate at room temperature to obtain a gummy dark red product referred to as crude n-butanol extract (MBS). The crude extract was dissolved in chloroform-ethanol (4:1) and decanted to remove the gummy part before phytochemical profiling. The decant was concentrated and 10 g out of the 15 g of the decant was loaded on the column for further purification.

3.2.2.2 Microwave-Assisted polarity based extraction

Partitioning of phytochemicals into different classes based on polarity and pH was carried out based on Harbone's method (1998) using ethanol (Figure 3.1). Three fractions were obtained; the chloroform fraction (MBC1), Chloroform-ethanol fraction (MBC2) and ethanol fraction (MBC3).

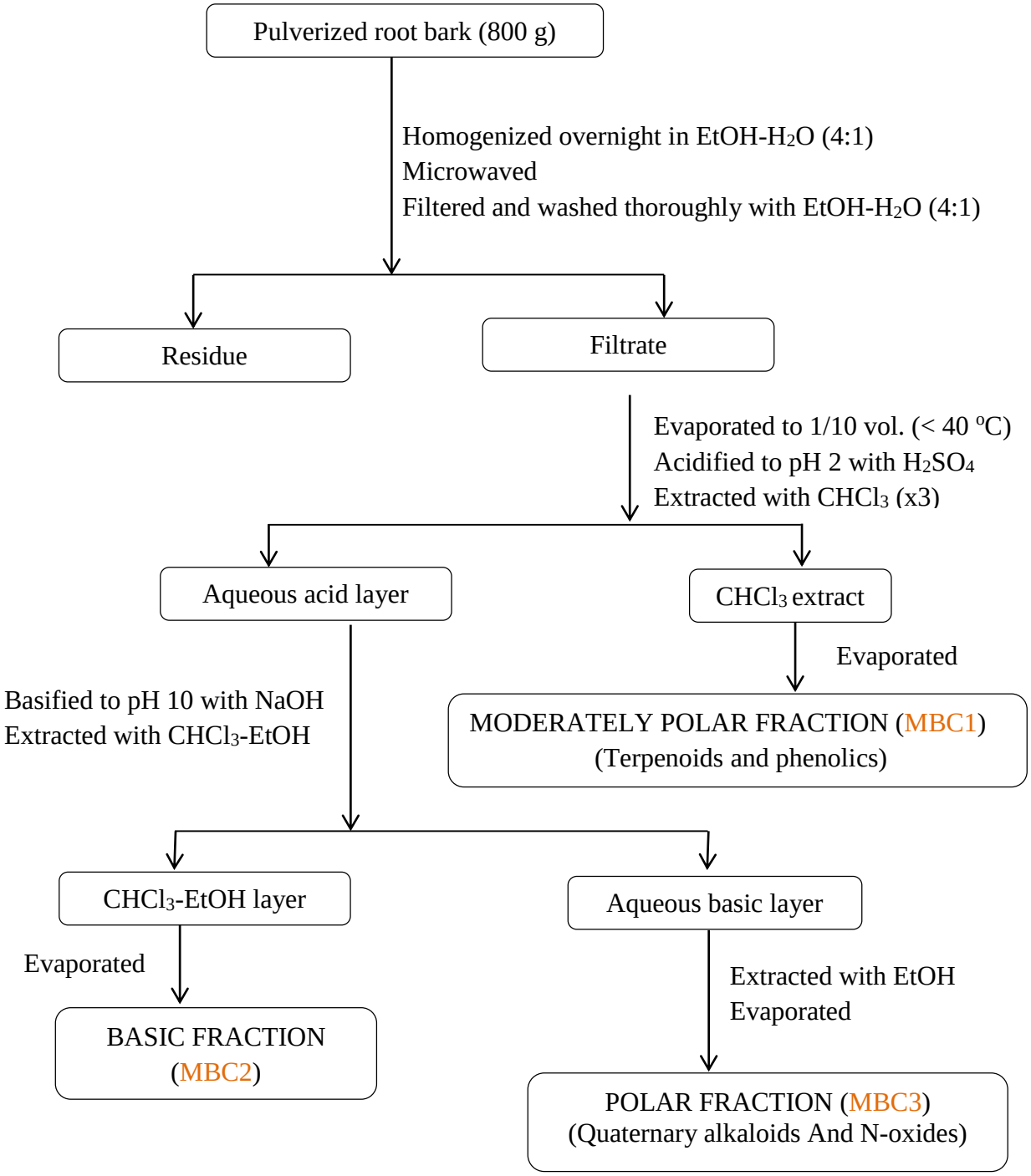


Figure 3.1: General Procedure for microwave assisted extraction (MAE) of root bark of *Ficus Sycomorus* and fractionating the phytochemicals into different classes according to polarity and pH (Harborne, 1998).

3.2.3 Preliminary Phytochemical Screening of the Plant Extract

The crude n-butanol extract (MBS), chloroform (MBC1), Chloroform-Ethanol (MBC2) and ethanol (MBC3) fractions of the root bark of *Ficus Sycomorus* were subjected to preliminary phytochemical screening for the presence of secondary metabolites using standard methods (Evans, 2002; Silva *et al.*, 1998; Sofowora, 1993).

3.2.3.1 Liebermann-Buchard test for steroids/terpenes

A portion of the extract/fractions (100 mg) was dissolved in water-ethanol mixture, acetic anhydride was added and the mixture was carefully mixed. Concentrated sulphuric acid (1 mL) was added down the side of the test tube to form a lower layer and the resulting solution was observed. Green colouration indicated the presence of steroids/terpenes (Silva *et al.,* 1998).

3.2.3.2 Sodium hydroxide test for flavonoids

Few drops of 10 % sodium hydroxide solution were added to the extract/fractions. Yellow colouration indicated the presence of flavonoids. (Silva *et al*., 1998)

3.2.3.3 Ferric chloride test for phenols

Few drops of ferric chloride solution were added to a solution of the extract/fractions; green colouration indicated the presence of phenolic hydroxyl groups (Evans, 2002).

3.2.3.4 Dragendoff's test for alkaloids

To an ethanolic solution of the extract/fractions, a few drops of Dragendoff's reagent were added. A reddish brown precipitate indicated the presence of alkaloids (Evans, 2002).

3.2.3.5 Wagner's test for alkaloids

To an ethanolic solution of the extract/fractions, a few drops of Wagner's reagent was added. A white precipitate indicated the presence of alkaloids (Evans, 2002).

3.2.3.6 Ferric chloride test for tannins

To an ethanolic solution of the extract/fractions 3 - 5 drops of ferric chloride solution was added. A brownish-blue precipitate indicated the presence of hydrolysable tannins (Evans, 2002).

3.2.3.7 Frothing test for saponins

Water (10 mL) was added to the extract/fractions and was shaken vigorously for 30 seconds. The tube was allowed to stand in a vertical position and was observed for 30 minutes. A honey comb froth that persists for 10 -15 minutes indicated the presence of saponins. (Silva *et al.*, 1998)

3.2.4 Methods of isolation/purification of bioactive compound

Thin-Layer chromatography (TLC), column chromatography and Preparative Thin-Layer chromatographic techniques were used for the isolation and purification processes of the bioactive compound.

3.2.4.1 Thin layer chromatography (TLC)

Thin layer chromatography was carried out on aluminium TLC sheets pre-coated with silica gel 60 PF_{254}, layer thickness of 0.2 mm.

Technique: Spots were applied manually using capillary tube; plates were allowed to dry and developed at room temperature in a chromatographic tank.

Visualization of Spots: Spots on TLC plates were visualized under UV light (254 and 366 nm) and also by spraying with 10 % sulphuric acid, followed by heating at 110 ^{o}C for 5-10 min.

3.2.4.2 Column chromatography (CC)

The following column conditions were employed in running the column chromatography.

a) Technique - Gradient elution.

b) Column - A glass columns (350, 200 and 100 mL).

c) Stationary phase - Silica gel, 60-120 mesh size.

d) Column packing - both dry and wet slurry method were applied.

e) Sample loading - the n-butanol extract (10 g) was first loaded by the dry loading method; the extract was dissolved in minimum amount of chloroform, mixed with a small quantity of silica gel, dried, triturated and then loaded on top of the previously packed column (wet slurry method). The same procedure was applied on the subsequent columns, for further purification of some fractions from the first column, on dried silica packed column (Appendix V).

f) Solvent System/Elution - Various solvent systems comprising 100 % Hexane, hexane/ethyl acetate mixtures and 100 % ethyl acetate were used in eluting the column by gradient elution.

g) the summary of the collections on the column chromatography and preparative TLC of the n-butanol extract form the root bark of *Ficus sycomorus* is presented below:

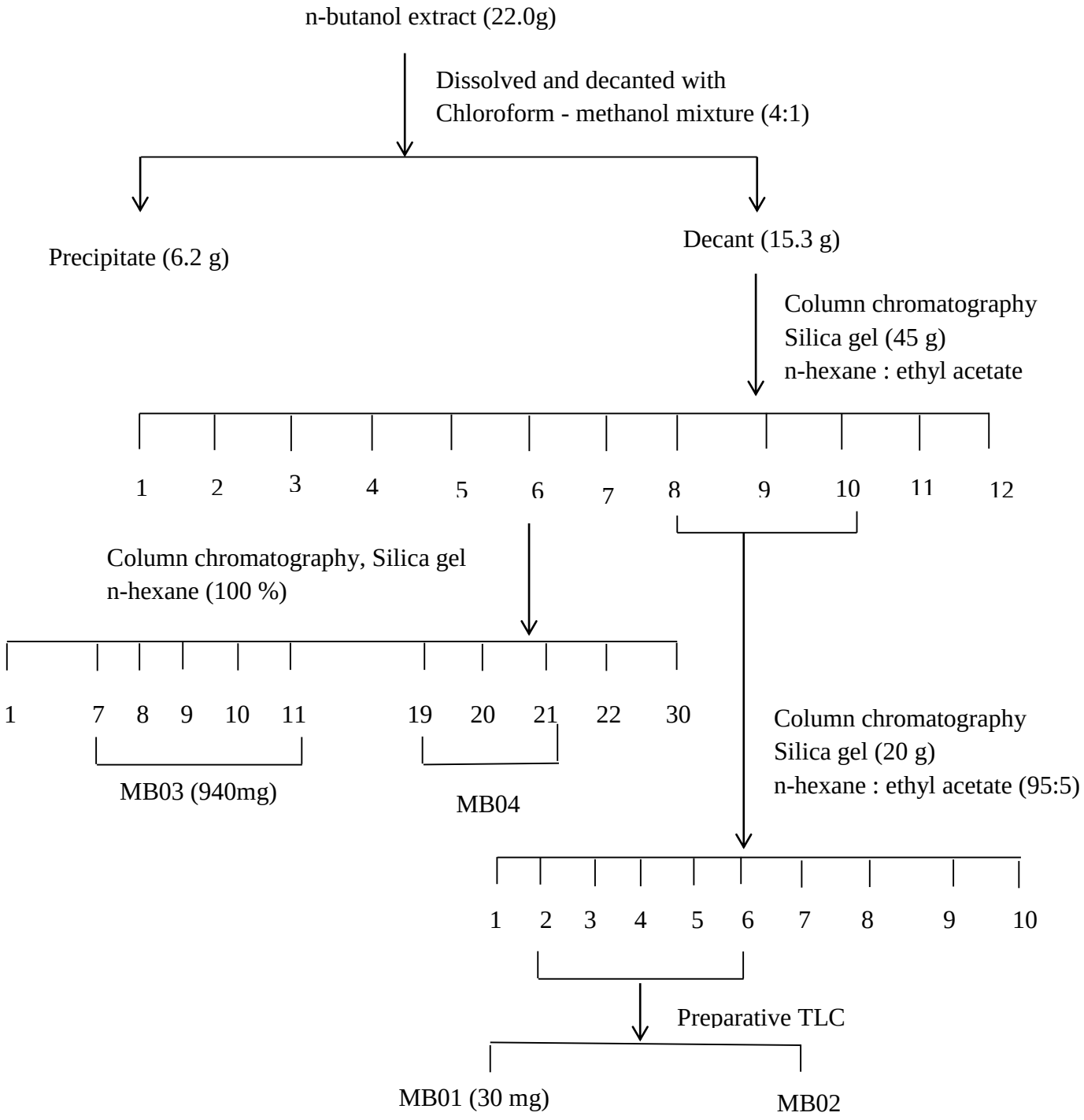


Figure 3.2: Fractionation scheme of the n-butanol extract (MBS).

3.2.5 Determination of Antimicrobial Activity of the plant extract and the isolated compound

3.2.5.1 Culture media

Antimicrobial activity was measured using agar well diffusion method according to the Clinical and Laboratory Standard Institute (CLSI, 2017). The culture media used for the analysis include: Mueller Hinton Agar (MHA), Mueller Hinton Broth (MHB) and Nutrient agar (NA). They were used for the sensitivity test, determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the extract/fractions and the isolated compounds. All the media were prepared and sterilized by autoclaving at 121^{o}C for 15 minutes in an Astral Scientific autoclave.

3.2.5.2 Preparations of different concentrations of the extracts/fractions and the isolated compounds; Various concentrations ranging from 100 to 3.125 mg/mL and 100 to 3.125 µg/mL of the extract/fractions and the isolated compounds, respectively, were prepared by first dissolving one gram of each extract or one milligram of the isolated compound in sterile distilled water (10 mL) to obtain 100 mg/mL and 100 µg/mL, respectively. A 10-5 serial dilutions of the extract/fractions and the isolated compounds were carried out to obtain concentrations of 50, 25, 12.5, 6.25 and 3.125 mg/mL and µg/mL of the extract/fractions and the isolated compounds, respectively.

3.2.5.3 Preparation of standard inoculums of the test organisms

The inoculum of the test organisms were prepared by first streaking the organisms on the prepared nutrient agar plates to obtain discrete colonies of the bacteria. A colony was picked and subcultured unto sterile nutrient broth and incubated at 37 ^{o}C for 24 hours. A loopful of each bacterial suspension, from the broth culture, was transferred into bottles containing sterile distilled water and standardized to obtain a bacterial

density of 1.5 $\times 10^8$ cfu/mL as determined by the McFarland scale No 1 (Vollekova *et al.,* 2001).

3.2.5.4 Determination of zone of inhibitory activity (sensitivity test) of the extracts and the isolated compound.

The standardized inocula of the bacterial isolates were sterilized on Mueller Hinton Agar plates with the aid of sterile swab sticks. Four wells were notched on each inoculated agar plate with a sterile cork borer. The wells were properly labeled according to different concentrations of the extract/fractions and the isolated compounds prepared which were 100, 50, 25, 12.5, 6.25, 3.125 mg/mL and µg/mL of the extract/fractions and the isolated compounds, respectively. Each well was filled up with approximately 0.2 mL of the different extract/fractions or the isolated compounds concentrations, respectively. The inoculated plates with the extract/fractions or the isolated compounds were allowed to stay on the bench for about an hour; this is to enable the extract to diffuse into the agar. The plates were then incubated at 37 ^oC for 24 hours. At the end of the incubation period, the plates were observed for any evidence of inhibition which appeared as clear zone that was completely devoid of growth around the wells. The diameters of the zones were measured using a transparent and calibrated ruler.

3.2.5.5 Determination of Minimum Inhibitory Concentration (MIC)

The minimum inhibitory concentration of the extract/fractions and the isolated compounds were determined using tube dilution method with the Mueller Hinton Broth. The lowest concentration of the extract/fractions and the isolated compounds showing inhibition for each organism when the extract/fractions and the isolated compounds were tested during sensitivity test was serially diluted in the test tube containing Mueller

Hinton Broth. A loopful of the organism suspensions were inoculated into each tube containing the broth and the extract/fractions or the isolated compounds. The inoculated tubes were then incubated at 37 ^{o}C for 24 hours. At the end of the incubation period, the tubes were examined for the presence or absence of growth using turbidity as a criterion, the lowest concentration in the series without visible sign of growth (turbidity) was considered to be the Minimum Inhibitory Concentration (MIC).

3.2.5.6 Determination of Minimum Bactericidal Concentration (MBC)

The result from the Minimum Inhibitory Concentration (MIC) was used to determine the Minimum Bactericidal Concentration (MBC) of the extract/fractions or the isolated compounds. A sterilized wire loop was dropped into the test tubes that did not show turbidity (clear) in the MIC test and the loopful was taken and streaked on a sterile nutrient agar plates. The plates were incubated at 37 ^{o}C for 24 hours. At the end of the incubation period, the plates were observed for the presence or absence of growth. Growth indicated bacteriostatic activity while no growth indicated bacteriocidal activity of the extract/fractions or the isolated compounds.

CHAPTER FOUR

4.0 RESULT

Table 4.1 shows the percentage recovery of n-butano extact (3.67 %), chloroform fraction (0.15 %), chloroform-ethanol fraction (0.28 %) and ethanol fraction (0.20 %) from the root bark of *Ficus sycomorus.*

Table 4.2 shows the Phytochemical constituents present in the extract/fractions from the root bark of *Ficus sycomorus.*

Table 4.3 shows the solvent ratio of the eluting solvent, number of spots and their Rf value for each of the collections from first column chromatography of n-butanol extract.

Plate III shows the Thin-Layer Chromatography profile of the isolated compounds.

Table 4.4 shows the Rf value of each of the isolated compound at solvent system 9:1 (n-hexane : ethyl acetate)

Table 4.5 shows the result of the chemical test carried out on the isolated compounds.

Table 4.6 shows the melting point, physical state and the weight of the isolated compounds

Figure 4.3.1 shows the ^{1}H NMR spectrum depicting the characteristic peaks of lupeol (MB01); seven methyl signals at δH 1.53, 1.04, 0.98, 0.94, 0.83, 0.78 and 0.76 ppm. A doublet of doublets at δH 3.21 ppm for C-3. Doublets for geminal protons at δH 4.69 and 4.57 ppm at C-29, along with the methyl signal at δH 1.53 ppm for C30.

Figure 4.3.2 shows the ^{13}C NMR spectrum depicting the characteristic peaks of lupeol (MB01); pair of sp2 hybridized carbon atoms at δ 151.14 and 109.47 ppm for C20 and C29, respectively, and oxygenated carbon shift at δ 79.15 ppm for C3.

4.1 Result of Extraction of Plant Material

Table 4.1: Percentage recovery of the various solvent extract/fractions

Method of Extraction	extract/fractions	Weight (g)	(%) Recovery
Microwave-Assisted Extraction (MAE) (600g)	n-butanol (MBS)	22.00	3.67
Microwave-Assisted Polarity-Based Extraction (800g)	Chloroform (MBC1)	1.20	0.15
	Chloroform-Ethanol (MBC2)	2.20	0.28
	Ethanolic (MBC3)	1.60	0.20

4.2 Result of Phytochemical Screening of the Extract/fractions

Table 4.2**:** Phytochemical constituents of the root bark of *Ficus sycomorus*.

Phytochemical constituent	Test	extract/fractions			
		MBS	MBC1	MBC2	MBC3
Flavonoids	Sodium hydroxide	+	+	–	–
Alkaloids	Dragendorff's	+	–	+	–
	Wagner's	+	–	+	–
Saponins	Frothing	–	–	–	–
Terpenes/Steroids	Lieberman	+	+	+	+
Tannins	Ferric chloride	+	+	+	–

MBS = n-butanol extract, MBC1= Chloroform fraction, MBC2= Chloroform-Ethanol fraction and MBC3= Ethanol fraction.
Key: + = Present, – = Absent.

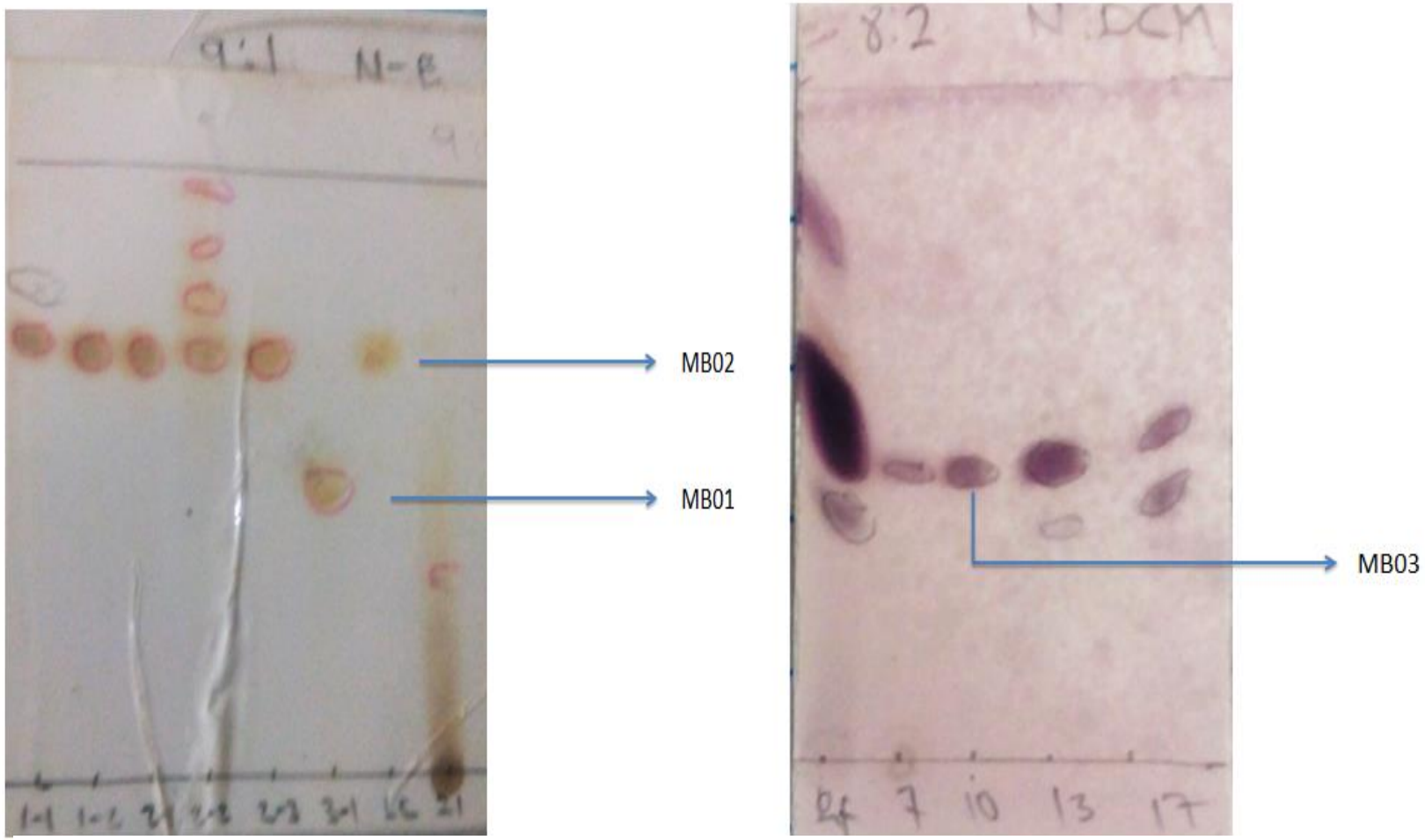
4.3 Result of Chromatographic Separation

4.3.1 TLC Analysis of the column fractions and the isolated compounds

Table 4.3: Fractions from first column chromatography of n-butanol extract of *Ficus sycomorus* (350 mL column)

Fraction	Eluting solvent (H: EA)	Number of major spots	TLC	
			R_f value	solvent system (H: EA)
1	100:00%	No spot	-	-
2	100:00%	No spot	-	-
3	95:05%	No spot	-	-
4	95:05%	3	0.74, 0.85, 0.94	8:2
5	90:10%	3	0.74, 0.85, 0.94	8:2
6	90:10%	2	0.85, 0.94	8:2
7	85:15%	2	0.85, 0.94	8:2
8	85:15%	2	0.85, 0.94	8:2
9	80:20%	2	0.50, 0.55	9:1
10	80:20%	2	0.26, 0.50	9:1
11and12	75:25%	4	0.26,0.50,0.55, 0.87	9:1

Key: H = n-hexane, EA = ethyl acetate.



(a) **(b)**

Plate III (a and b): TLC profile of the isolated compounds

Figure 4.3.3 shows the FTIR spectrum depicting the characteristic bands of lupeol (MB01); broad band at 3421 cm^{-1}, intense band at 1192 cm^{-1} for hydroxyl group (O-H), weak band at 1662 cm^{-1} for C=C vibrations .

Figure 4.3.4 shows the ^{1}H NMR spectrum depicting the characteristic peaks of lupeol acetate (MB03); eight methyl signals at δH 2.02, 1.66, 1.05, 0.96, 0.85, 0.85, 0.83, and 0.78 ppm. Doublets for geminal protons at δH 4.66 and 4.54 ppm for C-29, methyl signal at δH 1.66 ppm for C30, A multiplet at δH 4.48 ppm, for C-3 and singlet methyl α-oriented proton at δH 2.02ppm for 2'.

Figure 4.3.5 shows the ^{13}C NMR spectrum depicting the characteristic peaks of lupeol acetate (MB03); carbonyl peak at 171.02 ppm for 1'.

Figure 4.3.6 shows the FTIR spectrum depicting the characteristic bands of lupeol acetate (MB03); a very sharp carbonyl peak at 1733 cm^{-1} weak band at 1640 cm^{-1} for C=C vibrations .

Table 4.7 shows the comparison of the spectral data for the isolated compound (MB01) with that of the literature data.

Table 4.8 shows the comparison of the spectral data for the isolated compound (MB03) with that of the literature data.

Table 4.9 shows the sensitivity test result of the extract/fractions from the root bark of *Ficus sycomorus* against the selected microorganisms ranging from 12 - 26 mm.

Table 4.10 shows the result of Minimum Inhibitory Concentration (MIC) ranging from 6.25 -12.5 mg/mL and MBC ranging from 12.5 -25 mg/mL, of the extract/fractions from the root bark of *Ficus sycomorus* against the selected microorganisms.

Table 4.11 shows the result of inhibition zones of the isolated compounds ranging from 11-18 mm as compared with ciprofloxacin (standard drug) with inhibition zones ranging from 26-31 mm.

Table 4.4: TLC profiles of the isolated compounds.

Isolated Compound	R_f value	Solvent system
MB01	0.36	9:1 (n-hexane: ethyl acetate)
MB03	0.94	9:1 (n-hexane: ethyl acetate)

4.3.2: Chemical test on the Isolated Compound

Table 4.5: Chemical test on the isolated compounds.

Isolated compounds	Test	Result	Inference
MB01	Ferric Chloride test	–	Non –phenolic
	Liebermann test	+	Terpenoid/steroid
MB03	Ferric Chloride test	–	Non –phenolic
	Liebermann test	+	Terpenoid/steroid

Key: + = positive, – = negative

4.3.3: Physical properties of the Isolated Compounds

Table 4.6: Physical properties of the isolated compounds

Isolated compounds	Melting point (^oC)	Physical State	Weight (mg)
MB01	213 - 215	White crystalline solid	30
MB03	216 - 218	Off White powder	940

4.3.4 Spectroscopic Analysis of the Isolated Compounds

The following spectra show the result obtained from the ^{1}H NMR, ^{13}C NMR and FTIR spectroscopic analysis

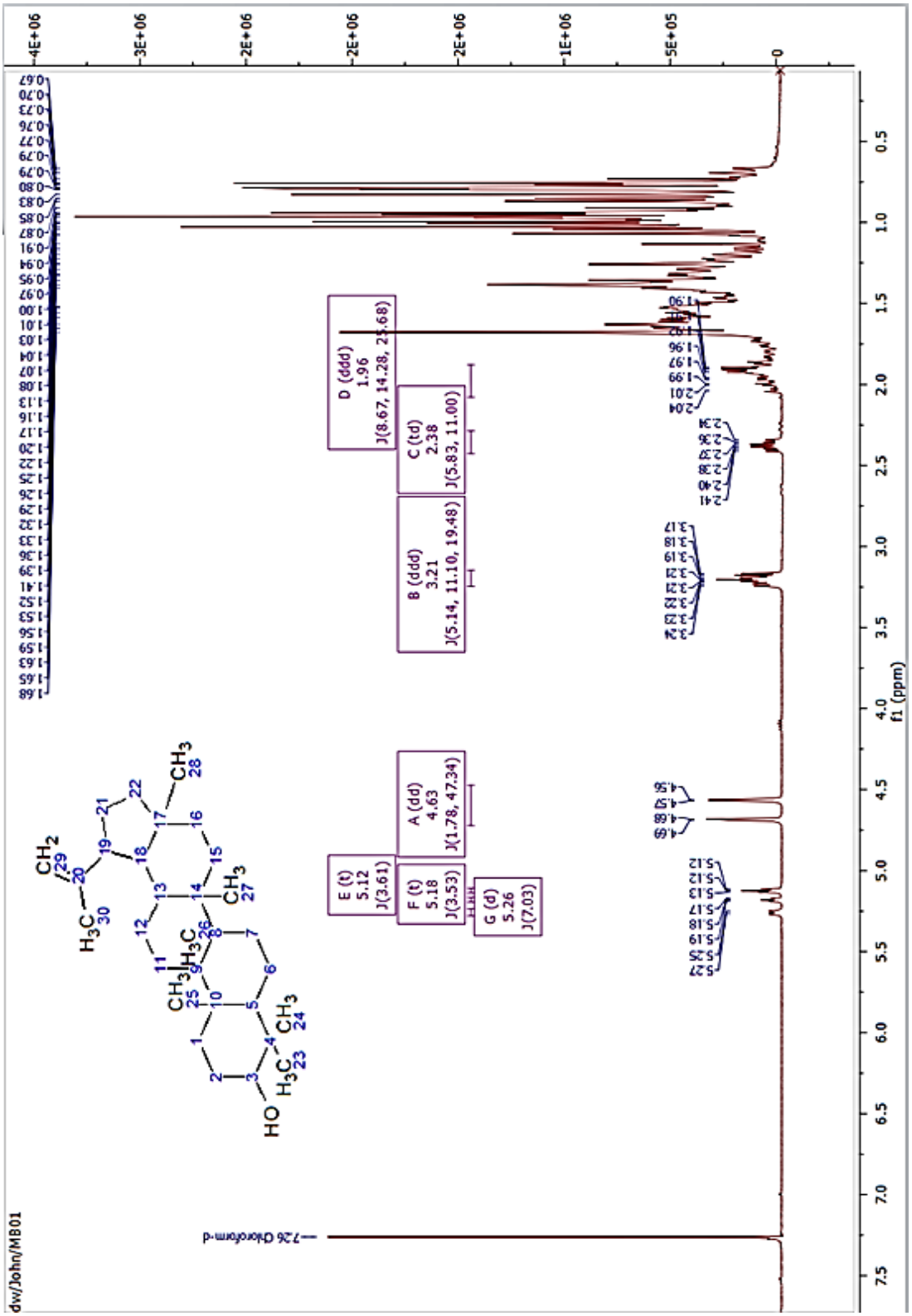


Figure 4.3.1: ^{1}H NMR spectrum of lupeol (MB01)

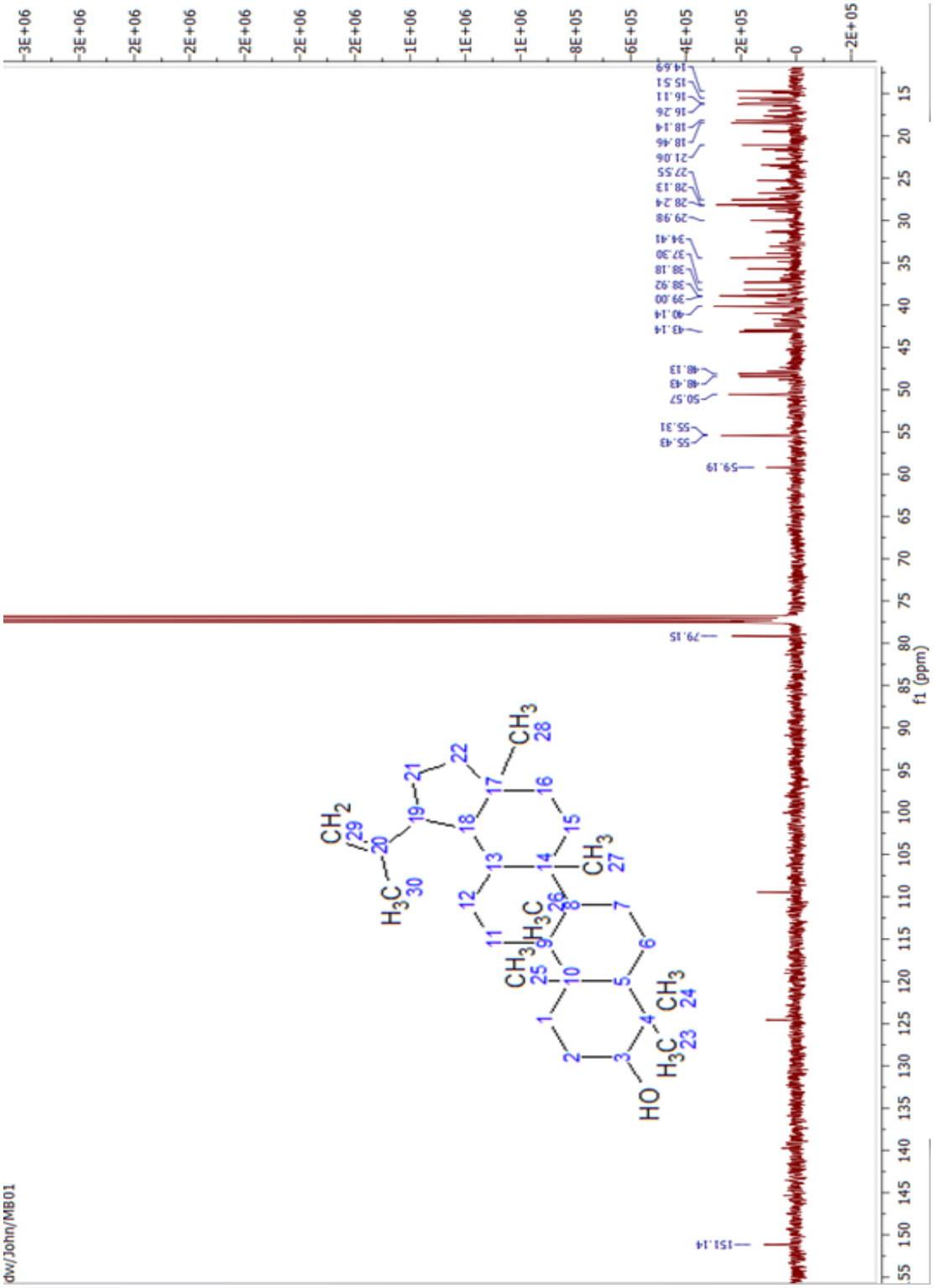


Figure 4.3.2: ^{13}C NMR spectrum of lupeol (MB01)

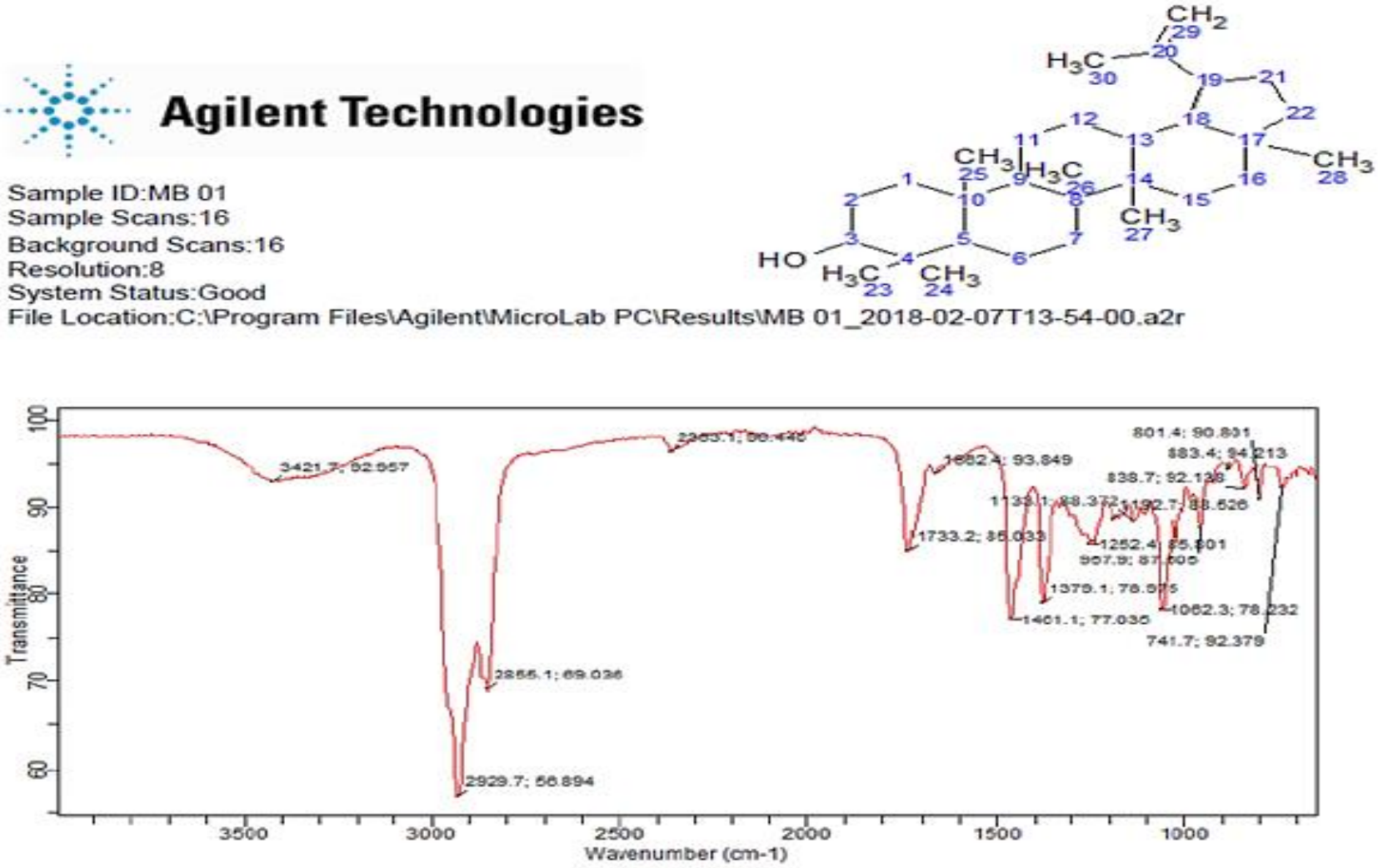


Figure 4.3.3: FTIR spectrum of lupeol (MB01)

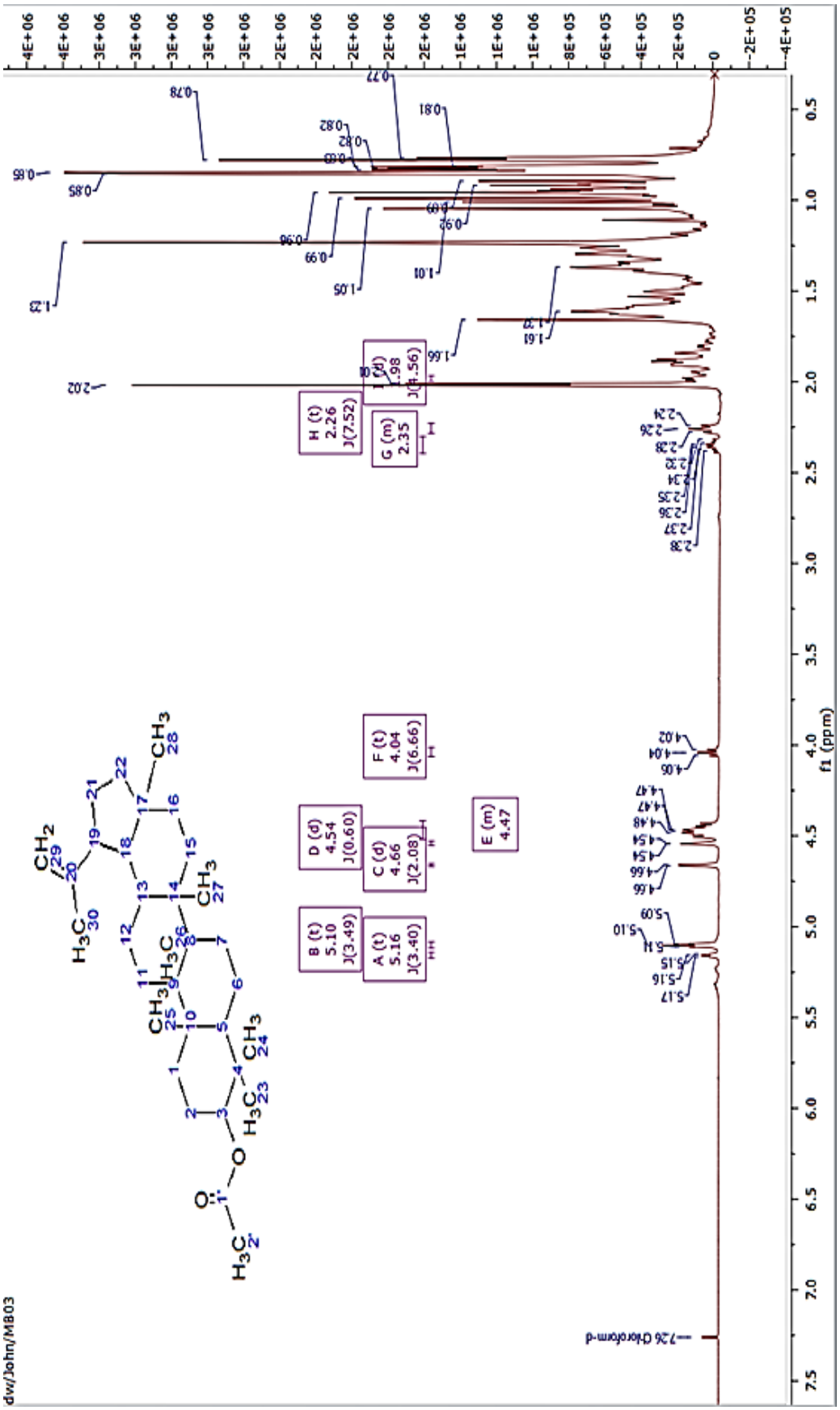


Figure 4.3.4: ^{1}H NMR spectrum of lupeol acetate (MB03)

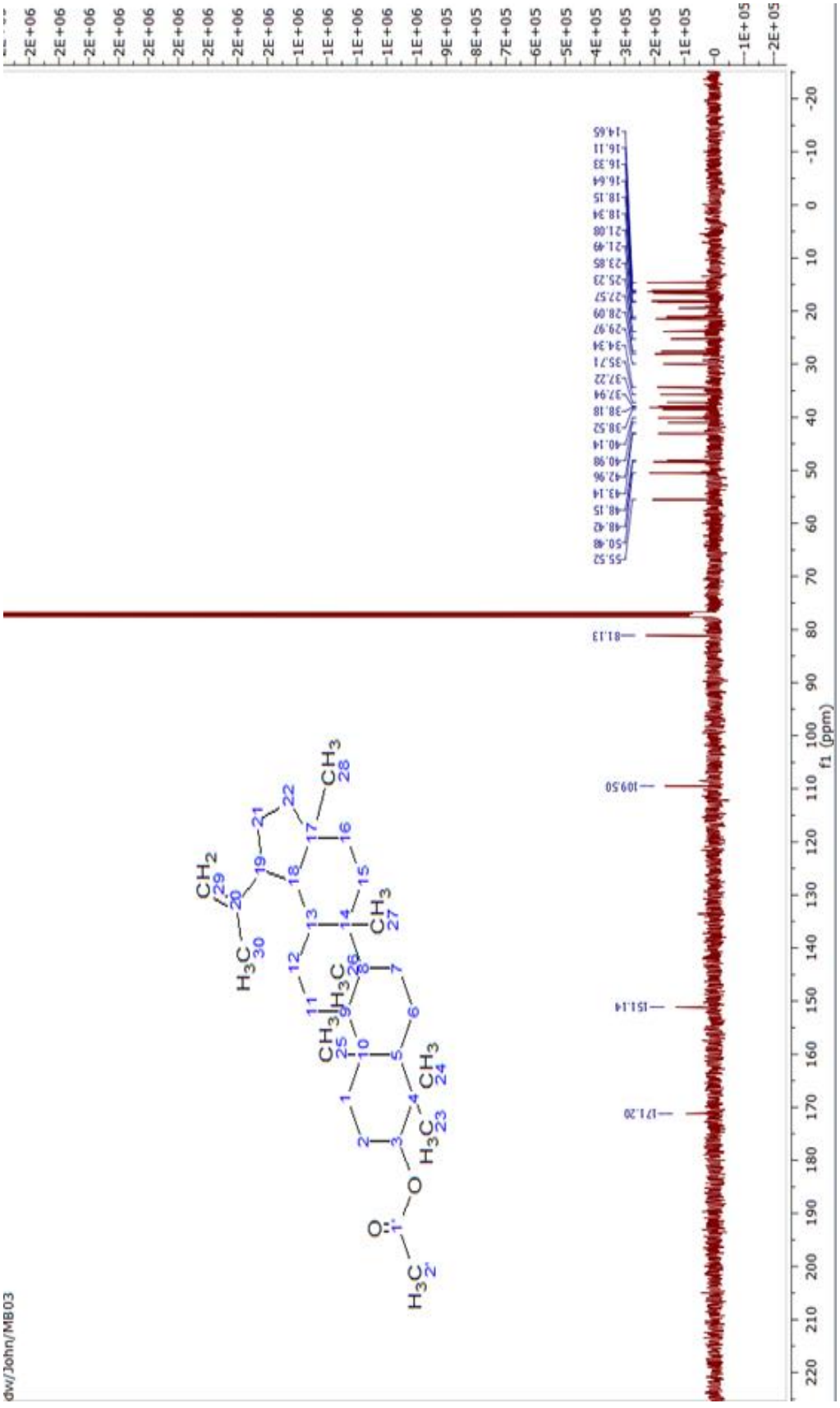


Figure 4.3.5: ^{13}C NMR spectrum of lupeol acetate (MB03)

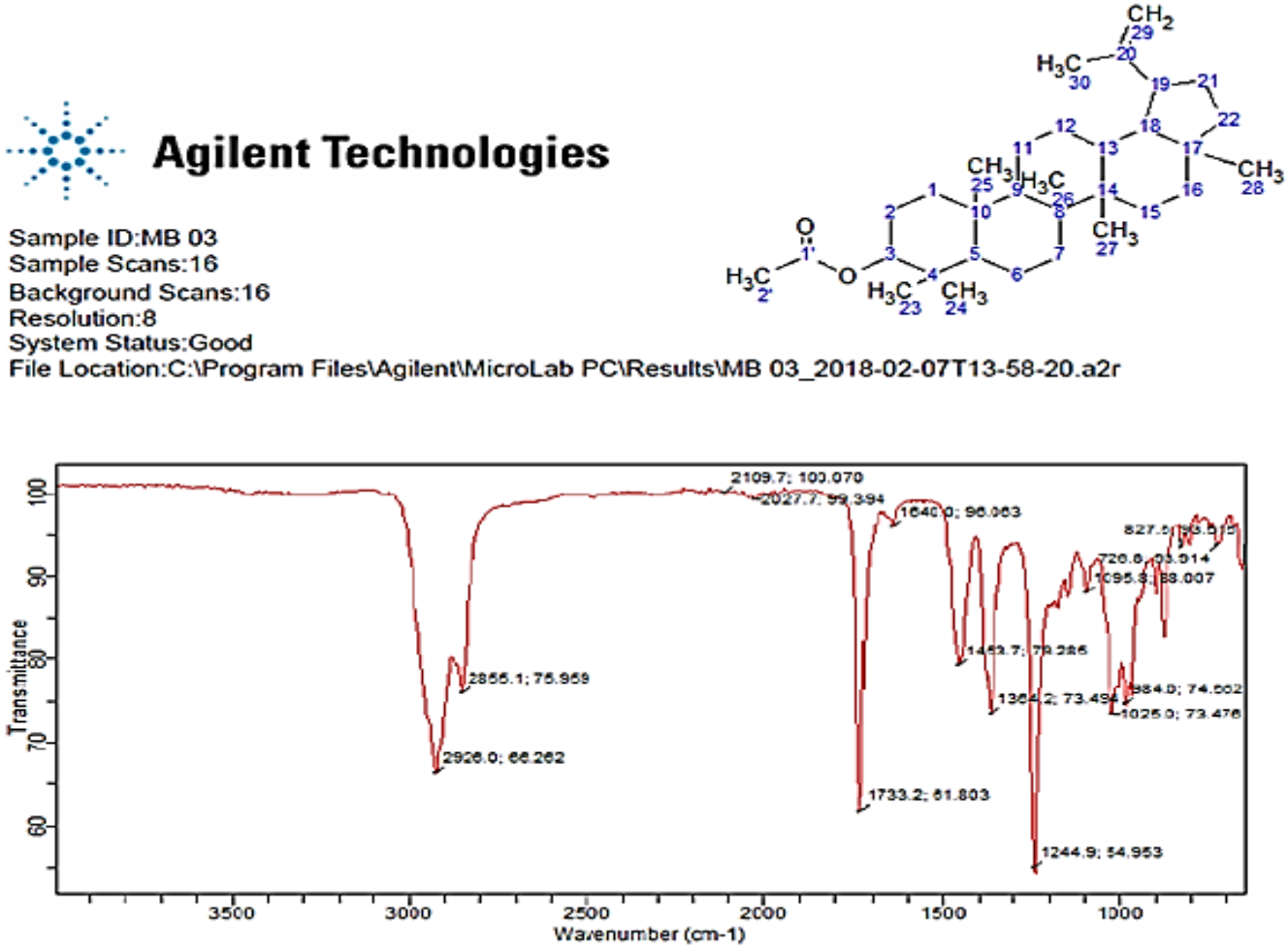


Figure 4.3.6: FTIR spectrum of lupeol acetate (MB03)

4.3.5: Summary of Spectral Data for the Isolated Compounds and Comparison with Literature Data

Table 4.7: Summary of spectral data for MB01 and Comparison with literature data

C	Compound MB01 (CD_3OD)		Jamal *et al*., 2008 (CD_3OD)		
position	$\delta^{13}C$ (ppm)	$\delta^{1}H$(ppm), J (Hz)	$\delta^{13}C$(ppm)	$\delta^{1}H$(ppm)	CH_n
1	38.18		38.2		CH_2
2	25.30		25.3		CH_2
3	79.15	3.21 (dd, *J = 8.00, 12.00* Hz)	79.1	3. 16 (1H, *dd*, *J* = 4.76, 11.00 Hz)	CH
4	38.92		38.9	-	C
5	55.43		55.5		CH
6	18.46		18.5		CH_2
7	34.41		34.5		CH_2
8	41.00		41.0	-	C
9	50.57		50.6		CH
10	37.30		37.3	-	C
11	21.06		21.1		CH_2
12	27.50		27.5		CH_2
13	39.00		39.0		CH
14	43.14		43.0	-	C
15	27.55		27.6		CH_2
16	35.80		35.8		CH_2
17	43.20		43.2	-	C
18	48.43		48.5		CH
19	48.13	2.41 (m)	48.1		CH
20	151.40		151.1	-	C
21	29.98		30.0		CH_2
22	40.14		40.2		CH_2
23	28.24	0.95 (s)	28.2	0.95 (s)	CH_3
24	15.51	0.76(s)	15.6	0.75(s)	CH_3
25	16.26	0.83(s)	16.3	0.82(s)	CH_3
26	16.11	1.04(s)	16.2	1.02(s)	CH_3
27	14.69	0.94(s)	14.7	0.93(s)	CH_3
28	18.14	0.78(s)	18.2	0.78(s)	CH_3
29	109.47	4.57, 4.69 (dd, *J=1.78, 8.58*)	109.5	4.56(s), 4.68(s)	CH_2
30	19.40	1.53(s)	19.5	1.25(s)	CH_3

Table 4.8: Summary of spectral data for MB03 and Comparison with literature data

C position	Compound MB03 (CD_3OD)		Rasoanaivo *et al*., 2014 (CD_3OD)		
	$\delta^{13}C$ (ppm)	$\delta^{1}H$(ppm), J (Hz)	$\delta^{13}C$(ppm)	$\delta^{1}H$(ppm)	CH_n
1	38.18		38.50		CH_2
2	23.85		23.86		CH_2
3	81.13	4.48(m)	81.22	4. 48 (m)	CH
4	38.58		38.86	-	C
5	55.52		55.40		CH
6	18.34		18.39		CH_2
7	34.34		34.26		CH_2
8	41.98		40.96	-	C
9	50.48		50.49		CH
10	37.22		37.23	-	C
11	21.08		21.07		CH_2
12	25.23		25.01		CH_2
13	37.94		38.10		CH
14	42.96		42.97	-	C
15	27.57		27.58		CH_2
16	35.71		35.72		CH_2
17	43.14		43.17	-	C
18	48.15		48. 00		CH
19	48.42	2.38(m)	48.43		CH
20	151.14		151.19	-	C
21	29.97		29.86		CH_2
22	40.14		40.20		CH_2
23	16.64	0.83 (s)	16.55	0.83 (s)	CH_3
24	28.09	0.84(s)	28.09	0.84(s)	CH_3
25	16.33	0.85(s)	16.33	0.85(s)	CH_3
26	16.11	1.05(s)	16.04	1.03(s)	CH_3
27	14.65	0.96(s)	14.50	0.94(s)	CH_3
28	18.15	0.78(s)	18.05	0.78(s)	CH_3
29	109.50	4.54, 4.66 (dd, *,J=0.60,2.08*)	109.78	4.56(m), 4.68(m)	CH_2
30	19.40	1.66(s)	1947	1.69(s)	CH_3
1^{1}	171.20	-	171.02	-	C
2^{1}	21.49	2.02	21.42	2.03	CH_3

4.4 Result of Antimicrobial Activity of the Plant Extract/Fractions and the Isolated Compounds

4.4.1 Result of zones of Inhibition of the extract/fractions

Table 4.9: Result of zones of Inhibition (mm) of the extract/fractions

Plant Extract		**Zone of Inhibition (mm)**			
Code	Conc.(mg/mL)	*S. aureus*	*B. subtilis*	*E. coli*	*S. typhi*
MBS	100	0	15	0	0
	50	0	14	0	0
	25	0	12	0	0
	12.5	0	0	0	0
MBC1	100	17	26	24	19
	50	14	18	18	17
	25	12	14	14	13
	12.5	0	0	0	0
MBC2	100	21	0	20	15
	50	16	0	18	14
	25	14	0	14	13
	12.5	12	0	13	12
MBC3	100	0	0	0	0
	50	0	0	0	0
	25	0	0	0	0
	12.5	0	0	0	0

MBS = n-butanol extract, MBC1= Chloroform fraction, MBC2= Chloroform-Ethanol fraction and MBC3= Ethanol fraction.

4.4.2 Result of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of the extract/fractions

Table 4.10: Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of the extract/fractions in mg/mL

Analysis	Plant Extract	Microorganism			
		S. aureus	*B. subtilis*	*E. coli*	*S. typhi*
MIC	MBS	ND	12.5	ND	ND
	MBC1	6.25	12.5	6.25	6.25
	MBC2	6.25	ND	6.25	6.25
MBC	MBS	ND	25	ND	ND
	MBC1	12.5	25	12.5	12.5
	MBC2	12.5	ND	12.5	12.5

MBS = n-butanol extract, MBC1= Chloroform fraction and MBC2= Chloroform-Ethanol fraction.

Key: (**#)** = no inhibition observed or bacteriostatic, ND = not determined

4.4.3 Result of zones of Inhibition of the Isolated Compound

Table 4.11**:** Determination of Zones of Inhibition of the Isolated Compounds

Isolated compounds		**zone of inhibition (mm)**			
Code	Conc.(µg/mL)	*S. aureus*	*B. subtilis*	*E. coli*	*S. typhi*
MB01	100	0	18	16	0
	50	0	16	14	0
	25	0	14	12	0
	12.5	0	0	0	0
MB03	100	18	16	0	16
	50	16	14	0	14
	25	14	12	0	11
	12.5	0	0	0	0
Ciprofloxacin	30	31	26	27	28

MB01= Lupeol, MB03= Lupeol acetate

Table 4.12 shows the Minimum Inhibitory Concentration (MIC) of 12.5 μg/ml and Minimum Bactericidal Concentration (MBC) of 25 μg/ml of the Isolated Compounds from n-butanol extract of the root bark of *Ficus sycomorus* against the tested microorganisms.

4.4.4: Result of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of the isolated compound

T able 4.12: Minimum Inhibitory Concentration (μg/ml) and Minimum Bactericidal Concentration (μg/ml) of the Isolated Compounds

Analysis	Isolated Compound	Microorganism			
		S. aureus	*B. subtilis*	*E. coli*	*S. typhi*
MIC	MB01	ND	12.5	12.5	ND
	MB03	12.5	12.5	ND	12.5
MBC	MB01	ND	25	25	ND
	MB03	25	25	ND	25

MB01= Lupeol, MB03= Lupeol acetate

Key: (#) = no inhibition observed or bacteriostatic, ND = not determined.

CHAPTER FIVE

5.0 DISCUSSION

5.1 Plant Extraction

The result of the percentage yield of the extract/fractions from the root bark of *Ficus sycomorus* reported in Table 4.1 shows that the n-butanol extract from MAE had the highest recovery of 3.66 % followed by the fractions from Microwave-Assisted polarity based extraction; moderately polar chloroform-ethanol (0.28 %), more polar ethanol (0.20 %) and less polar chloroform (0.15 %).

5.2 Phytochemical Profiling

The result of the phytochemical analysis of n-butanol revealed the presence of flavonoids, alkaloids, steroids/triterpenes and tannins. The analysis also revealed the presence of flavonoids, steroids/triterpenes and tannins in the less polar $CHCl_3$ fraction (MBC1), the moderately polar $CHCl_3$-EtOH fraction (MBC2) had alkaloids, steroids/triterpenes and tannins and lastly the polar EtOH (MBC3) fractions had steroids/triterpenes (Table 4.2). These plant constituents were also reported from the parts of the plant extracts (El-Sayed *et al*., 2010 and Garba *et al.*, 2007).

5.3 Isolation, Purification, Characterization and Biological Activity of the Isolated Compounds

Compound MB01 was isolated as a white crystalline solid with melting point of 213 – 215 °C. The ^{1}H NMR spectrum showed seven methyl signals at δH 1.53, 1.04, 0.98, 0.94, 0.83, 0.78 and 0.76 ppm. A doublet of doublets at δH 3.21 ppm characteristic of an α-oriented proton at C-3. Doublets for geminal protons at δH 4.69 and 4.57 ppm at C-29, along with the methyl signal at δH 1.53 ppm for C30, suggested that the

compound MB01 was a lupane-type triterpenoid. The ^{13}C NMR spectrum further suggested compound MB01 as a lupane-type triterpene derivative. A total of 30 carbon signals were observed from the spectrum. The characteristic pair of sp2 hybridized carbon atoms comprising the double bond of lupeol was observed at δ 151.14 and 109.47 ppm. Oxygenated carbon shift was observed at δ 79.15 ppm for C3. Consequently, after comparing these NMR data with data in the literature (Jamal *et al*., 2008), the compound was identified to be (3β)-Lup-20(29)-en-3-ol, more commonly known as lupeol ($C_{30}H_{50}O$). The FTIR spectrum complemented the assignment; a very intensely broad band at 3421 cm^{-1} and moderately intense band at 1192 cm^{-1} indicates the characteristic hydroxyl group (O-H). The corresponding C=C vibrations was shown around 1662 cm^{-1} as a weak band. The stretching and bending vibrations of sp^2 C-H part were noticed by the intense band 2929 cm^{-1} and medium intensity band at 1461 cm^{-1}, respectively. The stretching vibration of the sp^3 C-H part was shown by the band at 2855 cm^{-1}. The IR absorbance values are in concordance with Silverstein *et al.* (2014).

Compound (MB03) was isolated as an off white powder with melting point of 216 - 218°C. The ^{1}H NMR spectrum showed eight methyl signals at δH 2.02, 1.66, 1.05, 0.96, 0.85, 0.85, 0.83, and 0.78 ppm. Doublets for geminal protons at δH 4.66 and 4.54 ppm at C-29, along with the methyl signal at δH 1.66 ppm at C30, suggested that compound MB03 was a lupane-type triterpenoid. A multiplet at δH 4.48 ppm, characteristic of an α-oriented proton at C-3 and the characteristic singlet methyl α-oriented proton at δH 2.02 for C2' suggested that compound MB03 was a ester derivative of lupeol-type triterpenoid. The ^{13}C NMR spectrum further suggested compound MB03 was an ester derivative of lupeol-type triterpenoid. A total of 32 carbon signals were observed from the spectrum. There was a carbonyl peak at 171.02 ppm in addition to the characteristic peaks of lupeol. Consequently, after comparing these NMR data with data in the

literature (Rasoanaivo *et al*., 2014), the compound was identified to be (3β)-Lup-20(29)-en-3-yl acetate, more commonly known as lupeol actate (**$C_{32}H_{52}O_2$**). The FTIR spectrum complemented the assignment; a very sharp carbonyl peak was observed at 1733 cm^{-1} in addition to the assigned absorbance of lupeol, except the intensely broad band at 3421 cm^{-1} of the characteristic hydroxyl group (O-H) had disappeared. The IR absorbance values are in concordance with Silverstein *et al.* (2014).

5.4 Antimicrobial Screening of the Extract/Fraction and the Isolated Compounds

The dried crude extracts and fractions were screened *in vitro* for antimicrobial activities. The antimicrobial activities included sensitivity test, determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) against *S. aureus, B. subtilis, E. coli* and *S. typhi*. Overall, the fractions (MBC1 and MBC2) from the Microwave-Assisted polarity based extraction had very significant antibacterial activity with wider inhibition diameters in contrast to the n-butanol extract (MBS) that had an activity against *B. subtilis* only. Among the fractions from the Microwave-Assisted polarity based extraction, MBC1 that had steroids/triterpenoids and flavonoids, was more active than MBC2 and MBS that had more phytochemical. This could be as a result of a mixture of numerous chemical compounds with different functional groups and properties in MBC2 and MBS. These active metabolites could be antagonistic to each other. The active metabolites could also be in too small concentration to have a very significant effect against the tested organisms. The flavonoid could also be more active than the alkaloid against the tested organisms at the given concentrations and the experimental conditions. The polar solvents used to extract polar phenolic compounds, of which flavonoids and alkaloids are some of the chemical classes reported to exhibit antibacterial activity (Kuete *et al.*, 2008). These results are quite encouraging in that, plant extracts used in traditional herbal preparations are

usually extracted with water in which case the polar metabolites are extracted. Hence, the moderate antimicrobial activity observed at the tested concentrations and the experimental conditions indicate that higher concentrations of the extracts maybe used in traditional preparations. The presence of these secondary metabolites in the root bark of the plant could be linked to the observed antimicrobial properties in the plants. This is because these secondary metabolites have been scientifically proven to act as antioxidants, anti-inflammatory, anticancer and antimicrobial agents (Shah *et al*., 2008; Stapleton *et al.*, 2004 and Singh *et al.*, 2002).

The tested microorganisms were sensitive to the isolated compounds and showed zones of inhibition which ranged from 11-18 mm. Both MB01 (lupeol) and MB03 (lupeol acetate) were active against *Bacillus subtilis* with MIC of 12.5 µg/mL respectively. The sensitivity of *B. subtilis* to the isolated compounds implies that the compounds are potential sources of antibacterial agents that can treat infections caused by *B. subtilis*, including infections of the eye, soft tissues, and lung (Ryan and Ray, 2004). MB01 (lupeol) also had activity against *Escherichia coli* with MIC of 12.5 µg/ml. This implies that the compound is a potential source of antibacterial agents that can treat urinary tract infections and diarrhoea (Atlas, 2010). MB03 (lupeol acetate) was also active against S*taphylococcus aureus* and *Salmonella typhi* with MIC of 12.5 µg/mL (Table 4.12 - Table 4.14 and Appendix III-IV). The sensitivity of these microorganisms implies that MB03 is a potential source of antibacterial agent that can treat systemic infections and enteric fever (typhoid fever) and even pathogenic diseases that are difficult to eradicate (Podschun and Ullman, 1998 and Al-Dahmoshi, 2013). Zone of inhibition of the isolated compounds against the microorganisms which ranged from 11-18 mm as compared to the standard drugs used as positive control (ciprofloxacin) with zone of

inhibition of 26 - 31 mm indicated that the chemical compounds can be synthetically modified for the better fight against these microorganisms.

The compounds isolated from the n-butanol extract of the root bark of the plants are triterpenes. Triterpenes are a class of chemical compounds with the least functional groups that are composed of three terpenes or six isoprene units with the molecular formula $C_{30}H_{48}$. Triterpenes are widely distributed in edible and medicinal plants and are an integral part of the human diet. They have been evaluated for use in drugs, cosmetics and healthcare products. Screening plant material has identified plants as promising and highly available sources of triterpenes (Szakiel *et al*., 2012). The presence of lupeol (MB01) could be linked to the observed antibacterial properties in the root bark of the plant, because lupeol have been reported to have antimicrobial, antioxidants, immunomodulatory, anti-inflammatory, anticancer and antitubercular activities (Sutomo *et al*., 2013; Suryati *et al.*, 2011; Saleem, 2009 and Wachter *et al.*, 1999). The presence of lupeol acetate could also be the linked to the observed antimicrobial properties in the root bark of the plant, because lupeol acetate was reported to have antimicrobial, anti-inflammatory, antimalarial and antituberculosis activity (Tahany *et al*., 2010; Prachayasittikul *et al.*, 2010; Lucetti *et al.*, 2010 and Wachter *et al.*, 1999).

CHAPTER SIX

6.0 CONCLUSION AND RECOMMENDATIONS

6.1 Conclusion

Microwave-assisted extraction (MAE) and Microwave-assisted polarity based extraction (target extraction) were carried out on the pulverized root bark of *Ficus sycomorus*. The fractions from the Microwave-assisted polarity based extraction were concentrated under reduced pressure while the n-butanol extract from MAE was evaporated and dried at room temperature. The highest percentage recovery was recorded on the n-butanol extract. The results of the phytochemical analysis of the extract/fractions revealed the presence of alkaloids, flavonoids, steroids/triterpenes and tannins, which are in agreement with the previous work (Garba *et al*, 2007) except for saponins, which were absent in all the extract/fractions of this work.

Silica gel column purifications followed by preparative thin layer chromatography led to the successful isolation of two compounds which were identified to be lupeol (30 mg) and lupeol acetate (940 mg) using ^{1}H NMR and ^{13}C NMR and by comparing with literature data. The isolated compounds demonstrated good activity against the tested microorganism, which implies that the compounds are potential sources of antimicrobial agents against various ailments.

6.2 Recommendations

1. MAE with more polar solvent such as water or methanol should be carried out on the root bark of the plant with a view to isolating more potent bioactive compounds.
2. While *in vitro* assays can be sensitive, quick and inexpensive, the results that are obtained may not necessarily predict *in vivo* activity as observed by Wright

(2010), therefore, there will be a need to further screen the extracts and the isolated compounds using suitable *in vivo* assays.

3. The isolated compounds are potent bioactive compounds with characteristic sides of reaction, therefor, they can be synthetically modified with a view to improving their antibacterial activity.

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Appendices

Appendix I: Result of Minimum Inhibitory Concentration (MIC)

Table 4.3.2: Minimum inhibitory concentration (MIC) of the extracts in mg/mL

Plant Extract		**Microorganism**			
Code	Conc.(mg/mL)	*S. aureus*	*B. subtilis*	*E. coli*	*S. typhi*
MBS	25	ND	-	ND	ND
	12.5	ND	-	ND	ND
	6.25	ND	+	ND	ND
	3.125	ND	+	ND	ND
MBC1	25	-	-	-	-
	12.5	-	-	-	-
	6.25	-	+	-	-
	3.125	+	+	+	+
MBC2	12.5	-	ND	-	-
	6.25	-	ND	-	-
	3.13	+	ND	+	+
	1.56	+	ND	+	+

MBS = n-butanol extract, MBC1= Chloroform fraction and MBC2 = Chloroform-Ethanol fraction

Key: (**-**) = inhibition observed, (**+**) = no inhibition observed, ND = not determined.

Appendix II: Result of Minimum Bactericidal Concentration (MBC)

Table 4.3.3: Minimum bactericidal concentration (MBC) of the extracts in (mg/mL)

Plant Extract		**Microorganism**			
Code	Conc.(mg/mL)	*S. aureus*	*B. subtilis*	*E. coli*	*S. typhi*
MBS	25	ND	-	ND	ND
	12.5	ND	+	ND	ND
	6.25	ND	+	ND	ND
	3.125	ND	+	ND	ND
MBC1	25	-	-	-	-
	12.5	-	+	-	-
	6.25	+	+	+	+
	3.125	+	+	+	+
MBC2	12.5	-	ND	-	-
	6.25	+	ND	+	+
	3.13	+	ND	+	+
	1.56	+	ND	+	+

MBS = n-butanol extract, MBC1= Chloroform fraction and MBC2 = Chloroform-Ethanol fraction

Key: **(-)** = bacteriocidal, (+) = bacteriostatic, ND = not determined.

Appendix III: Result of Minimum Inhibitory Concentration (MIC)

Table 4.10.2: Minimum Inhibitory Concentration (µg/ml) of the Isolated Compounds

Isolated compounds		**Microorganism**			
Code	Conc.(µg /mL)	S. aureus	B. subtilis	E. coli	S. typhi
MB01	25	ND	-	-	ND
	12.5	ND	-	-	ND
	6.25	ND	+	+	ND
	3.125	ND	+	+	ND
MB03	25	-	-	ND	-
	12.5	-	-	ND	-
	6.25	+	+	ND	+
	3.125	+	+	ND	+

MB01= Lupeol, MB03= Lupeol acetate

Key: (-) = inhibition observed, (+) = no inhibition observed, ND = not determined.

Appendix IV: Result of Minimum Bactericidal Concentration (MBC)

Table 4.10.3: Minimum Bactericidal Concentration (µg /ml) of the Isolated Compounds in comparison with the crude extract

Isolated compounds		**Microorganism**			
Code	Conc.(µg /mL)	*S. aureus*	*B. subtilis*	*E. coli*	*S. typhi*
MB01	25	ND	-	-	ND
	12.5	ND	+	+	ND
	6.25	ND	+	+	ND
	3.125	ND	+	+	ND
MB03	25	-	-	ND	-
	12.5	+	+	ND	+
	6.25	+	+	ND	+
	3.125	+	+	ND	+

MB01= Lupeol, MB03= Lupeol acetate

Key: (-) = bacteriocidal, (+) = bacteriostatic, ND = not determined.

Appendix V: Column chromatography





Appendix VI: Antimicrobial analysis





