PHYTOCHEMICAL AND ANTIMICROBIAL STUDIES OF THE ETHANOL EXTRACT OF THE LEAVES OF *CUSSONIA BARTERI* SEEMANN (ARALIACEAE)

CHINYERE ELUWA

DEPARTMENT OF PHARMACEUTICAL AND MEDICINAL CHEMISTRY, FACULTY OF PHARMACEUTICAL SCIENCES, AHMADU BELLO UNIVERSITY, ZARIA.

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ChinyereELUWA

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A THESIS SUBMITTED TO THE SCHOOL OF POST GRADUATE STUDIES, AHMADU BELLO UNIVERSITY, ZARIA, IN PARTIAL FULFILLMENT OF THE REQUIREMENT FOR THE AWARD OF MASTERS OF SCIENCE (M.Sc.) DEGREE IN PHARMACEUTICAL AND MEDICINAL CHEMISTRY.

DEPARTMENT OF PHARMACEUTICAL AND MEDICINAL CHEMISTRY, AHMADU BELLO UNIVERSITY, ZARIA.

NOVEMBER, 2014

DECLARATION

I declare that the work in this thesis entitled: ***"Phytochemical and Antimicrobial Studies of the Ethanol extract of the Leaves of CussoniabarteriSeemann (Araliaceae)"*** has been carried out by me in the Department of Pharmaceutical and Medicinal Chemistry of Ahmadu Bello University, Zaria under the supervision of Prof. M. Ilyas and Dr. U. U.Pateh. The information derived from the literature has been duly acknowledged in the text and a list of references provided. No part of this thesis was previously presented for the award of another degree and/or diploma at this or any other Institution.

Chinyere Mary Eluwa

……	……..	…….
Name of Student	Signature	Date

CERTIFICATION

This thesis entitled: "PHYTOCHEMICAL AND ANTIMICROBIAL STUDIES OF THE ETHANOL EXTRACT OF THE LEAVES OF *CUSSONIA BARTERI* SEEMANN (ARALIACEAE)" by Chinyere Mary Eluwa meets the regulations governing the award of the degree of Master of Science in Pharmaceutical and Medicinal Chemistry of the Ahmadu Bello University, Zaria, and is approved for its contribution to knowledge and literary presentation.

Prof M. Ilyas

……. …… ……

Chairman, Supervisory Committee Signature Date

Prof. U. U. Pateh

…… ……. ……..

Member, Supervisory Committee Signature Date

Dr.A. M. Musa

……. ……..

Head of Department Signature Date

Prof. A. A. Joshua

……. ……..

Dean, School of Post Graduate StudiesSignature Date

DEDICATION

This research work is dedicated to the Almighty God. For in HIM I live, and move and have my being.

AKNOWLEDGEMENT

Iam forever grateful to God who has given me the grace and strength to carry out this research work for without Him I can do nothing. May all Glory, Honor, Respect and Adoration be ascribed to His Holy name, amen.

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Abstract

*Cussoniabarteri*a plant used in traditional medicine in the treatment of infectious diseases such as conjunctivitis, cataracts, diarrhoea anddysentery was subjected to phytochemical and antimicrobial studies.

The powdered leaves of *Cussoniabarteri*were extracted with ethanol using maceration method and the resulting crude ethanol extract (EE) 140g was fractioned into hexane (HE)21.31g and ethyl acetate (EA) 8.51g extracts. The preliminary phytochemical screening was carried out on the extract and the fractions, while silica gel column chromatographic separation was carried out on the hexane fractions. The phytochemical screening revealed the presence of carbohydrates, flavonoids, saponins, tannins, cardiac glycosides, steroids and triterpenes while the column chromatographic separation followed by preparative TLC resulted in the isolation of β sitosterol ester (8 mg) and structure determination was achieved using chemical tests and spectroscopic techniques(UV, IR, 1D and 2D NMR). The extract was tested for antimicrobial activities againstMRSA, *Staphylococcus aureus, Streptococcus pyogenes, Corynebacteriumulcerans, Proteus vulgaris, Pseudomonas aeruginosa, Escherichia coli, Klebsiellapneumoniae, Candida albicans*and *Candidatropicalis*using agar diffusion method.The result of the antimicrobial studies showed zones of inhibition ranging from 16-31mm. The strong activity of the isolated compound against *S. aureus, C. albicans* and *E. coli* shows that it may be effective against infectious diseases which these microorganisms cause. The minimum inhibitory concentration (MIC) showed a range between 2.5µg/ml-15mg/ml, the MIC of the isolated compound against *Staphylococcus aureus, Escherichia coli*and*Klebsiella pneumonia* was 2.5µg/ml while *Corynebacteriumulcerans*and *Candida albicans*had 5µg/ml as their MIC. The minimum bactericidal/ fungicidal (MBC/MFC) concentration showed a range between 5µg/ml-30mg/ml, the MBC of the isolated compound against *Staphylococcus aureus, Escherichia coli*and *Klebsiella pneumonia* was 5µg/ml while *Corynebacteriumulcerans*and *Candida albicans*had 10µg/ml as their MBC/MFC.

The ethanol extract of the leaves of *Cussoniabarteri*contain phytochemical constituent with antimicrobial activity.

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LIST OF ABBREVIATIONS

cm	centimeter
ml	millilitre
UV	ultra violet
TLC	thin layer chromatography
%	percentage
R_f	retardation factor
HMBC	heteronuclear multiple bond correlation
HSQC	hetero nuclear single quantum coherence
COSY	correlation spectroscopy
DEPT	distortionless enhancement by polarization transfer
rDNA	recombinant deoxyribonucleic acid
DNA	deoxyribonucleic acid
CAM	complementary and alternative medicine
WHO	World Health Organization
MIC	minimum inhibitory concentration
MBC	minimum bactericidal concentration
MFC	minimum fungicidal concentration
FTIR	fourier transform infrared spectroscopy
NMR	nuclear magnetic resonance
1D/2D	one dimensional/ two dimensional
H_2SO_4	sulphuric acid
Conc. HCl	concentrated hydrochloric acid
MRSA	methicillin-resistant *Staphylococcus aureus*
DMSO	dimethyl sulfoxide
EE	ethanol extract
EA	ethyl acetate extract
HE	hexane extract

CHAPTER ONE

1.0 INTRODUCTION

1.1 Medicinal Plants

Medicinal plants are plants that have at least one of their parts (leaves, stem, barks or roots used for therapeutic purposes (Bruneton,1993). Recently, medicinal plants have become important for the treatment of different disease conditions, such as diabetes, malaria andanaemia (Fola, 1993). The availability and relatively cheaper cost of medicinal plants in sub-Saharan Africa, makes them more attractive as therapeutic agents when compared to 'modern' medicines (Agbor and Ngogang, 2005; Agbor*et al*., 2005). Indeed, the market and public demand has been so great that there is a great risk that many **medicinal plant**s today, face either extinction or loss of **genetic diversity** (Misra, 2009).The history of plants being used for medicinal purpose is probably as old as the history of mankind(Shrikumar and Ravi, 2007).

Medicinal plants constitute the main source of new pharmaceuticals and healthcare products (Ivanova*et al*., 2005).The use of **medicinal plant**s in the industrialised societies has been traced to the extraction and development of several drugs from these plants as well as from traditionally used folk medicine (Shrikumar and Ravi, 2007). The use of traditional medicine is widespread in India (Jeyachandran and Mahesh, 2007). A growing body of evidence indicates that secondary plant metabolites play critical roles in human health and may be nutritionally important (Herto*get al*., 1993). It is believed that crude extract from **medicinal plant**s are more biologically active than isolated compounds due to their synergistic effects (Jana and Shekhawat, 2010).Phytochemical screening of plants has revealed the presence of numerous chemicals including alkaloids, tannins, flavonoids, steroids, glycosides and

saponins etc. Secondary metabolites of plants serve as defense mechanisms against predation by many microorganisms, insects and herbivores (Cowan, 1999).

The use of plants for healing purposes predates human history and forms the origin of much modern medicine (Vickers and Zollman, 1999).

The knowledge of their healing properties has been transmitted over the centuries within and among human communities. Currently, data on the antimicrobial activity of numerous plants, so far considered empirical, have been scientifically confirmed, concomitantly with the increasing number of reports on pathogenic microorganisms resistant to antimicrobials. Products derived from plants may potentially control microbial growth in diverse situations and in the specific case of disease treatment, numerous studies have aimed to describe the chemical composition of these plant antimicrobials and the mechanisms involved in microbial growth inhibition, either separately or associated with conventional antimicrobials (Silvaand Fernandes, 2010).

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

structure of the existing medicines in order to make them more efficient or recover the activity lost due to bacterial resistance mechanisms (Chartone-Souza, 1998).

1.2 Sources of Drugs

Drugs are substances administered to humans and animals for diagnosis or treatment of diseases (Somnath, 2010). There are four sources of drug:

1. Natural sources
2. Semi synthetic sources
3. Synthetic sources
4. Biosynthetic sources (genetically engineered drug)

1.2.1 Natural Sources

Drugs can be obtained from plants, animals, minerals and microorganisms (Somnath, 2010):

A) PLANTS:

Natural products once served human kind as the source of all drugs, and higher plants provided most of these therapeutic agents. Today natural products (and their derivatives and analogs) still represent over 50% of all drugs in clinical use, with higher plant-derived natural products representing 25% of the total, (Balandrin*et al.*, 1993). The World Health Organization estimates that 80% of the people in developing countries of the world rely on traditional medicine for their primary health care, and about 85% of traditional medicine involves the use of plant extracts. This means that about 3.5 to 4 billion people in the world rely on plants as sources of drugs, (Farnsworth, 1988).

In the United States plant-derived drugs represent about 25% of the prescription drugs market, and in 1991 this equated to a retail value of approximately $15.5 billion, (Pezzuto,

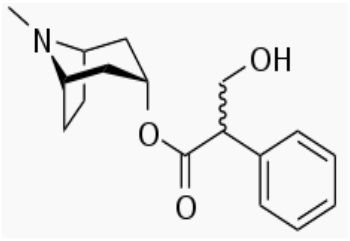
1997).The following phytochemical constituents are derived from roots, leaves or barks of plants:

a) Alkaloids:

These are nitrogenous heterocyclic bases, which are pharmacologically active principles of plants.They are composed of carbon, hydrogen, nitrogen and oxygen.They are bitter in taste and are often poisonous. These are, therefore, used in small doses.They are insoluble in water. However, they form salts with acids which are soluble in water.Some examples of alkaloids and their sources are listed in Table 1.1, (Somnath, 2010):

Table 1.1 Examples of alkaloids and their sources

DRUG	SOURCE
Atropine (i)	*Atropa belladonna*
Quinine (ii)	Cinchona bark
Morphine (iii)	*Papaverumsemniferum*
Reserpine (iv)	*Rauwolfiaserpentina*
Nicotine (v)	Tobacco
Digoxin (vi)	*Digitalis lanata*
Caffeine (vii)	Coffee, tea, cocoa



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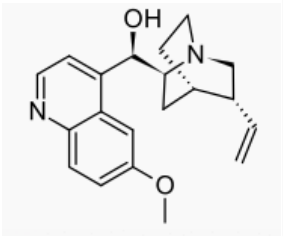

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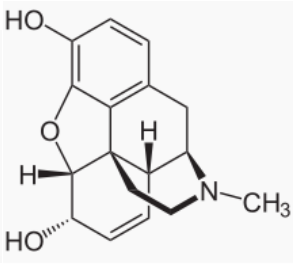

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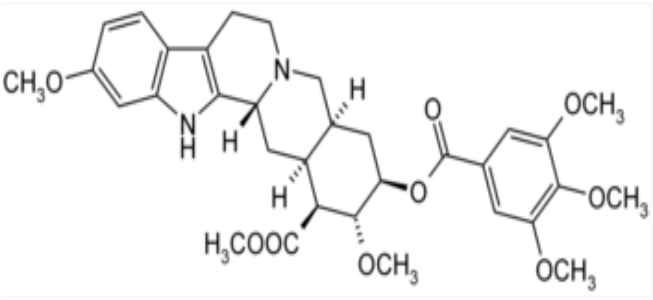

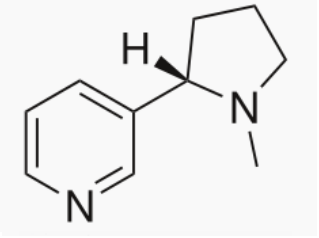

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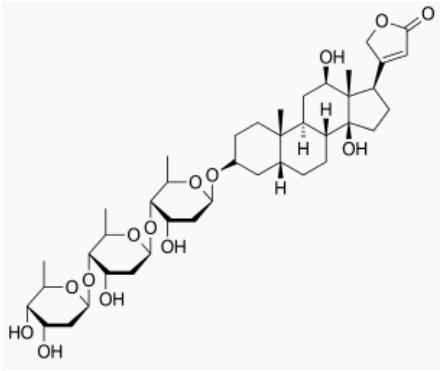
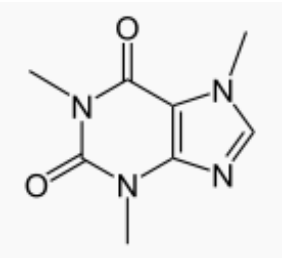
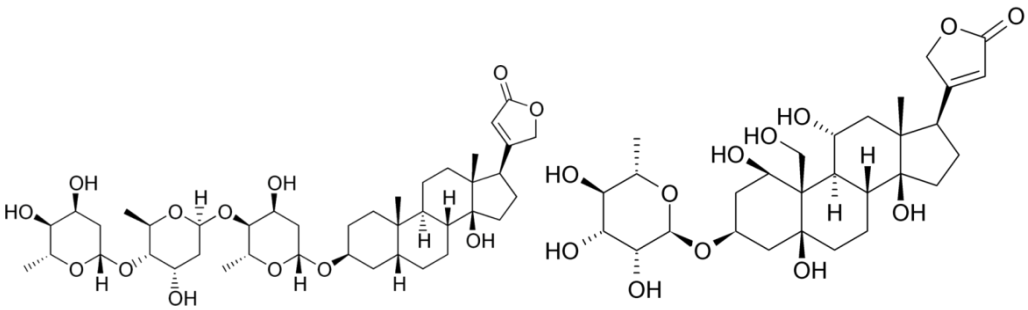




(vi) (vii)

b) Glycosides:

They are ether-like combination of sugar moiety with non-sugar moiety.Sugar moiety is not essential for the pharmacological activity but it governs the pharmacokinetic properties of the glycoside. In the body it may be removed to liberate aglycone. Pharmacological activity resides in the non-sugar moiety that is called aglycon (or genin). Some examples are digitoxin (viii), digoxin and ouabain (ix), (Somnath, 2010).

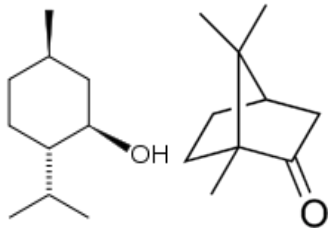


(viii) (ix)

c) Oils:

Oils are of three types and are used for various medicinal purposes.

i) Essential oils (or volatile oils): Essential oils are obtained from leaves or flower petals, by steam distillation and have an aroma. They have no caloric or food value, they do not form soaps with alkalis; they do not leave greasy stain after evaporation. On prolonged stay, they do not become rancid (foul smell). They are frequently used as carminatives and astringents in mouth wash (Somnath, 2010).Some of these oils are sold at room temperature and sublime on heating, e.g. menthol (x) and camphor (xi). Other examples are clove oil, peppermint oil, eucalyptus oil and ginger oil.



(x) (xi)

ii) Fixed oils are glycerides of stearic, oleic and palmitic acid. They are obtained from the seeds that are present within the cells as crystals or droplets. They are non-volatile and leave greasy stains on evaporation. They have caloric or food value, they form soaps with alkalis, on prolonged stay they become rancid. They do not have marked pharmacological activity and have little pharmacological use except castor oil (purgative) or arachis oil (demulcent). They may be of vegetable origin e.g. olive oil, castor oil, croton oil and peanut oil or of animal origin e.g. cod liver oil, shark liver oil and lard (Somnath, 2010).

iii) Mineral Oils: They are obtained by dry distillation of wood.E.g liquid paraffin (hydrocarbon derived from petroleum). They are used as lubricant or laxative to promote defecation, (Vijay, 2013).

d) Resins:

They are used for inhalation in common cold and also used in the treatment of cough, (Vijay, 2013).

e) Gums:

They are secretary products of plants.They are dispersible in water and form adhesive mucilaginous colloids e.g. Gum acacia and are used as emulsifying or suspending agents.

f) Tannins:

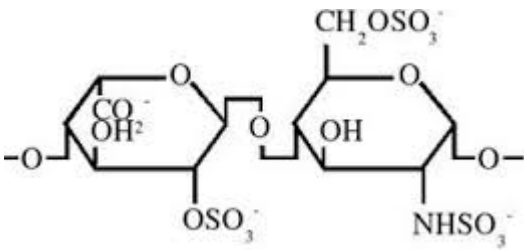
They are non-nitrogenous phenolic derivatives from plants and are soluble in water e.g. Astringents (precipitate surface proteins), (Vijay, 2013).

B) ANIMALS:

Some drugs are obtained from animals, like Cow and Pig as listed in Table 1.2(Vijay, 2013).

Table 1.2 Examples of drugs from animal sources

Drug	Animal
Heparin(xii)	Cow, Pig
Insulin	Pig
Thyroxin(xiii)	Thyroid gland
Vitamin B_{12}	Liver extract
Cod liver oil	Fish
Pepsin	Cow



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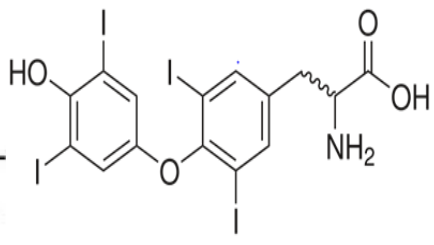


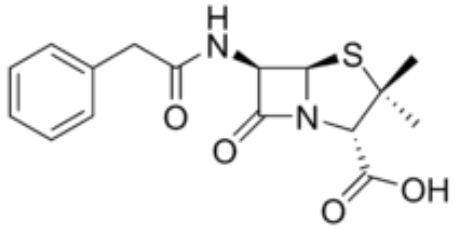
(xiii)

C) MICROORGANISM:

They have served as important source of life saving drug e.g. bacteria and fungi(Vijay, 2013). Some drugs obtained from them are listed in table 1.3:

Table 1.3 Examples of drugs obtained from microorganisms

Drug	Microorganism
Benzylpenicillin (xiv)	*Peniciliumnotatum*
Chloramphenicol (xv)	*Streptomyces venezuelace*
Griseofluvin	*Penicillin grisofullivum*
Streptomycin	*Streptomyces griseus*
Neomycin	*Streptomyces fradiae*



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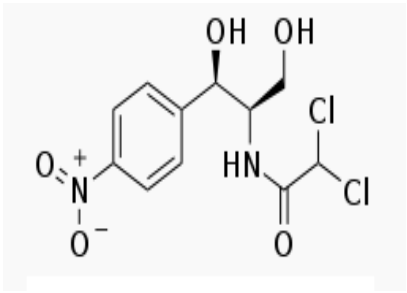


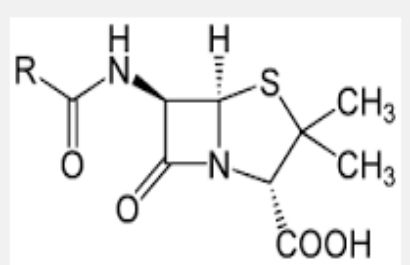
(xv)

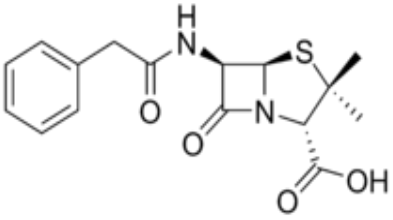
D) MINERAL:

They are used in pharmacotherapy, examples are; Ferrous sulphate ($FeSO_4$), Magnesium sulphate ($MgSO_4$), Sodium bicarbonate ($NaHCO_3$), Aluminium Hydroxide (Vijay, 2013).

1.2.2 Semi Synthetic Sources

These are mainly obtained by changing the chemical structure of drugs obtained from natural sources, example, atropine bromide, penicillin (xvi),benzylpenicillin (xvii), vinblastine, vincristine (xviii) (by changing –R side chain), (Vijay, 2013).





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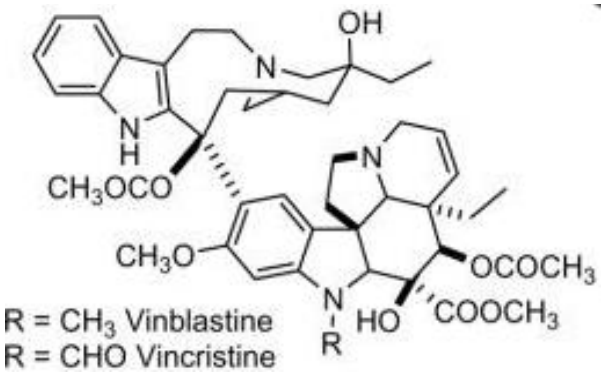


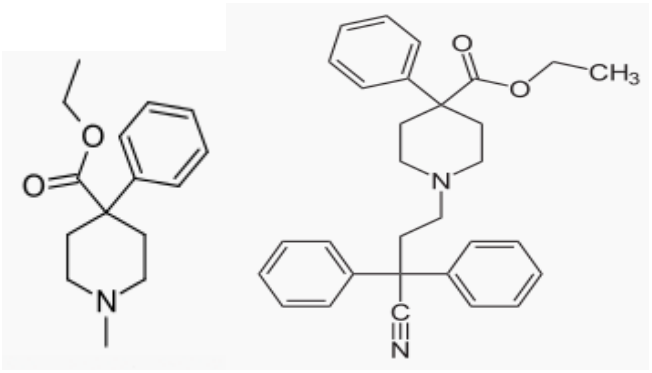
(xviii)

1.2.3 Synthetic Sources

Presently, majority of drugs are obtained synthetically. Some of the drugs which are earlier obtained from plants today are synthesized in the lab, (Vijay, 2013). The advantages of drugs from synthetic sources include; controlled quality, easy and cheap process, large scale production and they are safer and more potent.

Table 1.4 Examples of drug from synthetic sources

Example	Trade name	Classification
Meperidin (xix)	Demerol	Analgesic
Diphenoxylate (xx)	Lomotil	Anti-diarrhoea



(xix) (xx)

1.2.4 Biosynthetic Sources (Genetically engineered drugs)

This is a relatively new method and involves the blending of discoveries from molecular biology, rDNA technology, DNA alteration, immuno pharmacology. Example: Insulin (Human insulin of rDNA techniques), (Vijay, 2013).

1.3 STATEMENT OFRESEARCH PROBLEM

Infectious diseases are a significant cause of morbidity and mortality worldwide which accounts for approximately 50% of all deaths in tropical countries and as much as 20% of deaths in America. Despite the significant progress made in microbiology and the control of microorganisms, sporadic incidents of epidemics due to drug resistant microorganisms and hitherto unknown disease-causing microbes pose an enormous threat to public health. These negative health trends call for a global initiative for the development of new strategies for the prevention and treatment of infectious disease (Mahady, 2005).

Also, Pharmaceutical scientists are experiencing difficulty in identifying new lead structures, templates and scaffolds in the finite world of chemical diversity. A number of synthetic drugs have adverse and unacceptable side effects (Dahanukar,*et al*., 2000).

In an attempt to solve the problem caused by infectious diseases, search for medicinal plants containing novel compounds with fewer side effects and more efficacies is being made.

1.4 JUSTIFICATION OF THE STUDY

Active compounds produced during secondary metabolism are usually responsible for the biological properties of some plant species used throughout the globe for various purposes, including treatment of infectious diseases(Silva and Fernandes, 2010).

Cussoniabarteri has been reported to be used in the treatment of infectious diseases. Previous studies carried out on the leaves of *Cussoniabarteri* did not give detailed report on the active ingredient responsible for the treatment of infections. This has led to the investigation of the active ingredients present in the leaves of *Cussoniabarteri* that are responsible for the treatment of infectious diseases as claimed by traditionalists.

1.5 AIM AND OBJECTIVES

1.5.1 Aim

The aim of this research is to carry out phytochemical studies on the leaf of *Cussoniabarteri*and to justify the ethno medicinal claims on the plant.

1.5.2 Objectives of the Study

The Objectives of this study is to:

1. Carry out preliminary phytochemical screening on the leaves of *Cussoniabarteri* using standard procedures.
2. Isolate some of the bioactive compounds present in the leaves of *Cussoniabarteri*
3. Elucidate the structure of the isolated compound.

4. Validate the ethno medicinalclaim for the use of the leaves of the plantin the treatment of infectious diseases.
5. To test the antimicrobial properties of the isolated compound.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 The family Araliaceae

Araliaceaefamily is composed of 55 genera and 700 species. They are shrubs or trees found mainly in the tropical regions of the world, particularly South East Asia and America. However, there are representatives in Tasmania and in the cool temperate rainforests of South America and New Zealand. In Australia there are 12 genera and 32 species of which only 2 tree species of Polyscias are in New South Wales rainforests, (Floyd, 1982).They are also found in Eurasia, Africa, New Caledonia and Pacific islands (Plunkett *et al*, 1997).

2.2 Taxonomy/Nomenclature of the plant

According to the International Plant Names Index IPNI, the plant *Cussoniabarteri*was classified under the following:

Kingdom: Plantae

Family: Araliaceae

Species: *Cussoniabarteri*

2.2.1 Common names

The plant *Cussoniabarteri* is locally known in Hausa as gwabsa (JMD) ha'nnu'mku'tu'ruu' = leper's hand; a nickname (JMD) takanda'rgii'waa' = elephant's sugarcane, Yoruba -ako-sigo (JRA) sigo, Tiv -ityovor

There are other species of Cussonia some of them are: *Cussoniaarborea*Hochst, *Cussoniadjalonensis*A. Chev.and*Cussonialongissima*Hutch. &Dalz.

2.3 Botanical description and habitat

It is a tree that is up to 10m high and 40cm in diameter, with a spreading, open crown. Habitat recognizable by verticillate branches bearing short, thick, whorled stems, like stumps. The Bark is thick, corky, fistured, brown-grey to blackish, slash pale brown. The Stems are corky, thick (1-2cm in diameter), pale brown. Leaves are compound digitate, alternateand usually grouped at the end of stems, 20-80cm long. Petiole is with more or less sheathing base, 10-20(-50) cm long,Nervesare pinnate, slightly prominent. TheInflorescenceis a terminal fascicle of spikes, 15-50cm long. Flowers are greenish-yellow, sessile with a short triangular bract at base, (4-5) petals, flowering occurs in the dry season before the first leaves appear. Fruit is a globose sessile berry, green or more or less whitish when ripe, fruiting occurs at the end of the dry season or beginning of the rainy season (Michel, 2005).

Its habitat isSudano-Guinean to Guinean savannahs, on well-drained light soils. Resistant to fire, thanks to its thick, fireproof bark. It is distributed in Tropical Africa, from Senegal to Central African Republic. Irregularly but widely distributed, locally common (Michel, 2005).

In the dry season, the tree becomes completely defoliated and the thick stumpy branches resembling amputated and deformed limbs sticking up into the sky which gives the hausa name in Northern Nigeria as ‘leper’s hand’. The plant does not appear to have any marked physiological activity (Burkill, 1985).

2.4 Ethno medicinal uses

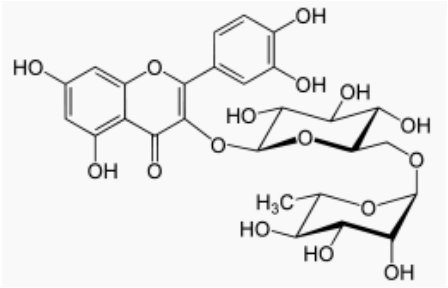
The root extract is used as an emetic for poisoning and measles prevention, painful menstruation and gonorrhea. The leave extract are used for the treatment of conjunctivitis, cataracts and epilepsy. The shoot extract is used to treat dysentery and diarrhea while the timber extract is used for the treatment of leprosy, trypanosomiasis and oedema (Michel, 2005, Burkill, 1985).

*Cussonia*species are used in African traditional medicine mainly against pain, inflammation, gastrointestinal problems, malaria and sexually transmitted diseases, (De Villiers*et al.*, 2010).

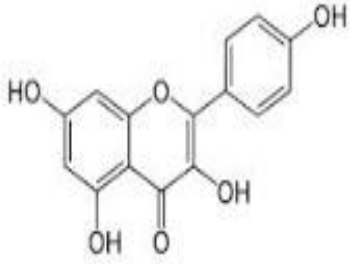
2.5Chemical Constituent

Previous phytochemical screening of the bark of*Cussoniabarteri*revealed the presence of alkaloids, tannins, saponins, flavonoids and glycosides (Prosper-Cabral*et al.*, 2007).

Also, previous phytochemical study of the leaves of *Cussoniabarteri* revealed the presence ofcatechinic and gallic tannins, flavonoids and saponins. The most abundant materials in the leaves are rutosides (xxi), kaempferol(xxii) and isoquercetin (xxiii) which are the major flavonoids. The most predominant sapogenins found in *Cussoniabarteri*which distinguishes it from other species of the genus are oleanolic acid (xxiv) and hederagenol (xxv) (Trotin*et al.*, 1973).



(xxi)



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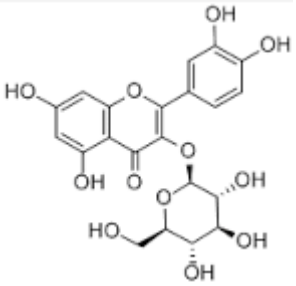

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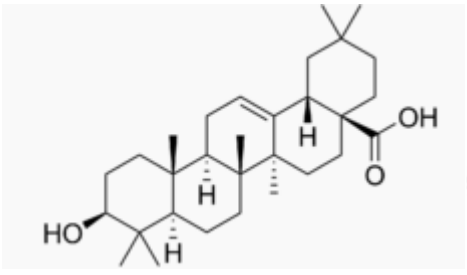

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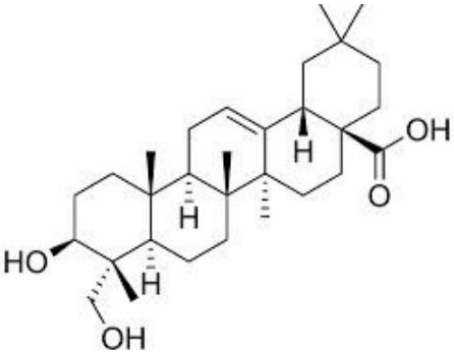
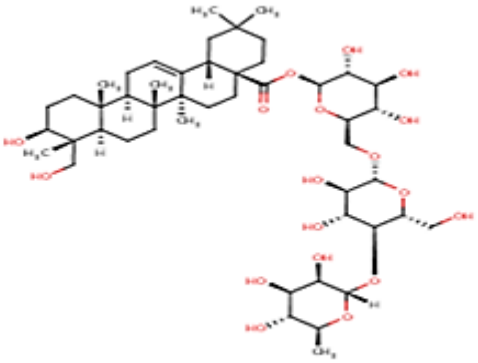


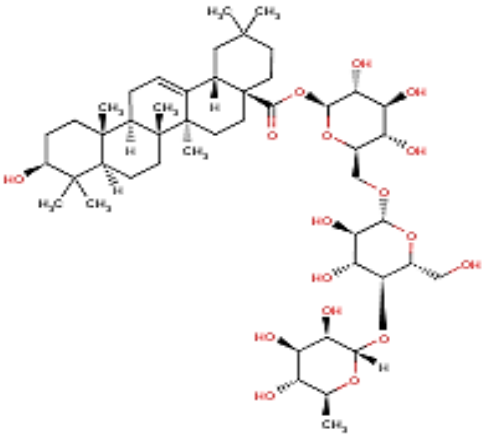
(xxv)

A new hederogenin 28-D-(α-L-rhamnopyranosyl(1-4)-β-D-glucopyranosyl(1-6)-β-D-glucopyranosyl ester (Cussonoside A) (xxvi) and the corresponding oleanolic acid

analogue(Cussonoside B)(xxvii) were the two major saponins isolated from the bark of *Cussoniabarteri* (Dubois *et al*., 1985).



(xxvi)



(xxvii)

2.6 Antimicrobial activity of *Cussonia* species

The antimicrobial activity of the leaves of thirteen Cussonia species has been reported using the minimum inhibitory concentration (MIC) method. They were tested against pathogens associated with diarrhoea (*Enterococcus faecalis* and *Escherichia coli*), sexually transmitted infections (*Neisseria gonorrhoeae* and *Trichomonasvaginalis*) and general infectious diseases (*Staphylococcus aureus* and *Pseudomonas aeruginosa*). Methanolic extracts were active against *Pseudomonas aeruginosa* (MIC of 1.0–1.5 mg/mL), *Trichomonasvaginalis* (MIC of 0.8–1.3 mg/mL) and *Staphylococcus aureus* (*Cussoniaarborea*, 1.8 mg/mL). All samples were active against *Neisseria gonorrhoeae* (MIC of 0.02–0.7 mg/mL) (De Villier*set al*., 2010).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Materials

3.1.1 Materials, Chemicals and Reagents

Solvents used were of analytical grade and they include methanol, ethyl acetate, chloroform and hexane. Reagents used were those for phytochemical screening such as Molisch's reagent, Salkowski's reagent, Shinoda's reagent, Dragendorf's reagent and Borntrager's reagent. Chromatographic materials used are TLC plates (aluminium), silica gel of 60-120µm mesh size (Qualikems), chromatographic tanks and columns.

3.1.2 Equipment

Thermoelectron UV machine obtained from the Department of Pharmaceutical and Medicinal Chemistry, Ahmadu Bello University, Zaria.

Shimadzu FTIR-8400S spectrophotometer obtained from the National Research Institute for Chemical Technology, Zaria.

Bruker AVANCE III NMR spectrometer at the School of Chemistry and Physics, University of Kwa-Zulu Natal-Durban, South Africa.

3.1.3 Plant Collection and Identification

The leaves of *Cussoniabarteri* were collected in November, 2012 from Kakeyi village of Zaria local government area of Kaduna State. It was confirmed and authenticated by U.S. Gallah of the Herbarium Section of the Department of Biological Sciences, Ahmadu Bello University, Zaria by comparing it with a standard specimen with voucher number 193. The collected leaves of the plant were air dried at room temperature, powdered and weighed.

3.2 Methods

3.2.1 Extraction and Partitioning

The powdered leaves (1000g) were extracted with 7000ml of ethanol using maceration method for 10days. The extract was filtered using Whatman No. 1 filter paper. The solvent was removedusing rotatory evaporator to yield 140g subsequently referred to as the crude ethanol extract and partitioned with n-hexane and ethylacetate to obtain hexane fraction (21.31g) and ethylacetate fraction (8.51g). The n-hexane fraction was used for the study.

3.2.2 Extraction of Alkaloids

To a portion of the ethanol extract 20ml of 5% hydrogen chloride was added and shaken frequently for 5 minutes using a glass funnel and a filter paper. 25ml of chloroform was used to further extract the mixture. Two layers were formed, the acid layer which was carefully separated and made alkaline with ammonium hydroxide solution until it was basic. This layer was shaken with 30ml of chloroform and allowed to separate. The chloroform layer was collected and washed with water.

Anhydrous sodium sulphatewas added to chloroform portion for 24 hours to remove any residual water. The extract was then concentrated on a hot water bath and labeled as A which was used for preliminary alkaloidal chemical test. The second half of the ethanol extract was labeled B which was used for antimicrobial screening.

3.3 Phytochemical Screening

Different chemical tests were carried out on the ethanol extract (EE), hexane fraction (HF) and ethylacetate fraction (EF) for the presence of various chemical constituents such as

carbohydrates, alkaloids, flavonoids, saponins, tannins, anthraquinones, cardiac glycosides and steroids/triterpenes.

3.3.1*Tests for Carbohydrates*

a) Molisch test: to a small portion of the extract distilled water was added and mixed with a few drops of Molisch reagent, this was then followed by addition of 1ml of conc. H_2SO_4 by the side of the test tube. Formation of a reddish colored ring at the interfaceindicates the presence of carbohydrates (Silva *et al*., 1998).

b) Fehling's test: About 0.5g of the extract was dissolved in distilled water and filtered. The filtrate was heated with 5ml of equal volumes of Fehling's solution A and B. Formation of a red precipitate of cuprous oxide was an indication of the presence of reducing sugars, (Sofowora, 1993).

3.3.2*Tests for Alkaloids*

a) Meyer's test: To a small portion of the extract in a test tube, few drops of Meyers reagent is added, a cream precipitate indicates the presence of alkaloids (Evans, 1983).

b)Dragendoff's test: few drops of Dragendoff's reagent are added to a portion of the extract, rose red precipitate indicates the presence of alkaloids (Evans, 1983).

c) Wagner's test: to a small portion of the extract in a test tube, few drops of Wagner's reagent is added, formation of brown/ reddish precipitate indicates the presence of alkaloids (Sofowora, 1993).

3.3.3 *Tests for Flavonoids*

a)Shinoda's test: About 0.5g of the extract was dissolved in ethanol, warmed and then filtered. Three pieces of Magnesium chips were then added to the filtrate followed by few

drops of conc. HCl. A pink, orange, or red to purple coloration indicates the presence of flavonoids, (Evans, 2002).

b)Sodium Hydroxide test: Few quantity of the extract was dissolved in water and filtered; to this 2ml of the 10% aqueous sodium hydroxide was added to produce a yellow coloration. A change in color from yellow to colorless on addition of dilute hydrochloric acid was an indication for the presence of flavonoids, (Evans, 2002).

3.3.4*Test for Saponins*

A small quantity of the extract was boiled with 5ml of distilled water, filtered. To the filtrate, about 3ml of distilled water was further added and shaken vigorously for about 5minutes. Frothing which persisted on warming was taken as an evidence for the presence of saponins, (Sofowora, 1993).

3.3.5 *Tests for Tannins*

a)Ferric Chloride test: About 0.5g of the extract was stirred with about 10ml of distilled water and then filtered. Few drops of 1% ferric chloride solution were added to 2ml of the filtrate, occurrence of a blue-black, green or blue-green precipitate indicates the presence of tannins, (Evans, 2002).

b)Lead Sub-acetate Test: To a small quantity of the extract, distilled water was added. 3-5 drops of lead acetate solution was added, a cream colored precipitate indicates the presence of tannins(Silva *et al*., 1998).

3.3.6 *Test for Anthraquinones*

a)Borntrager's test: About 0.2g of each portion to be tested was shaken with 10ml of benzene and then filtered. 5ml of the 10% ammonia solution was then added to the filtrate

and therafter shaken. Appearance of a pink, red or violet color in the ammoniacal (lower) phase was taken as the presence of free anthraquinones, (Sofowora, 1993).

b) Modified Borntrager's test: About a small quantity of each portion in a test tube was boiled with 5ml of 10% hydrochloric acid for 2-3mins this would hydrolyse the glycosides to yeild aglycones, which are soluble in hot water. This was then filtered and the filtrate was cooled and extracted with 5ml of benzene. The benzene layer was pipetted off and shaken gently in a test tube with half of its volume of 10% ammonium hydroxide. If the lower ammonium layer becomes rose pink to cherry red, it shows the presence of anthraquinones derivative (free or in combined state), (Evans, 1996).

3.3.7 *Test for cardiac Glycosides*

a) Kellerkiliani test:0.5g of extract will be dissolved in 2ml of glacial acetic acid containing one drop of ferric chloride solution. This will then be underlayed with 1ml of concentrated sulphuric acid.

A brown ring obtained at the interface will indicate the presence of a deoxy sugar characteristic of cardenolides. A violet ring may appear below the brown ring while, in the acetic acid layer a greenish ring may form just above the brown ring and gradually spread throughout this layer (Evans 2002).

b) Kedde's test:to a portion of the extract, 1ml of 2% solution of 3,5-Dinitrobenzoic acid in 95% alcohol. The solution was made alkaline with 5% sodium hydroxide, appearance of purple-blue color, indicates the presence of cardenolides, (Evans, 1996).

3.3.8 *Test for Steroids/Terpenes*

a) Liebermann-Burchard test:To a portion of the extract, equal volume of acetic acid anhydride was added and mixed gently. 1ml of concentrated sulphuric acid was added down the side of the test tube to form two layers. Color changes were observed immediately and

over a period of one hour. Blue to blue-green color in the upper layer and a reddish, pink or purple color at the junction of the two layers indicate the presence of triterpene, (Evans, 1996).

b) Salkowski test : a small portion of the extract was dissolved in 1ml of chloroform, 2-3 drops of concentrated sulphuric acid was added at the side of the test tube. The appearance of red colour indicates the presence of steroids (Sofowora, 1993).

3.4 Chromatographic Procedures

3.4.1 Thin Layer Chromatography (TLC)

Thin layer chromatography was carried out using pre-coated TLC plates by the one way ascending technique. Spotting was done manually using capillary tubes and chromatograms were developed in an air tight chromatographic tank at room temperature using different solvent systems. Developed chromatograms were sprayed using 10% sulphuric acid followed by heating in an oven at 150^{o}C for 5 minutes.

3.4.2 Column Chromatography of n-Hexane Fraction

The column was packed using the wet slurry method. 7g of the n-hexane fraction was dissolved in n-hexane and absorbed over silica gel (60-120 mesh size) was added to form slurry which was allowed to dry. The column was prepared by placing cotton wool at the bottom end of the column; estimated quantity of silica gel was poured into a minimum amount of n-hexane, stirred vigorously and poured into the column while stirring and was allowed to settle. The adsorbed extract was introduced on to the packed column and was gradiently eluted starting from n-hexane (100%), n-hexane: ethyl acetate (97.5%:2.5%) to n-hexane: ethyl acetate (75%:25%). A total of 62 column fractions were collected and combined based on their TLC profiles to give 8 major fractions labeled F1-F8. The column

was then washed with ethanol. The fractions collected after washing with ethanol (A-D) were monitored with TLC.

3.4.3 Column Chromatography of Fraction B

Repeated silica gel column chromatography of fraction B was gradiently eluted starting from chloroform (100%), chloroform: ethyl acetate (9:1) to chloroform: ethyl acetate (6:4). A total of 70 column fractions were collected from 1-70, 66 and 67 were combined based on their TLC profiles, coded B^{+}.

3.4.4 Preparative Thin Layer Chromatography of B^{+}

B^{+} was dissolved in ethyl acetate and subjected to preparative thin layer chromatography using pre-coated TLC plates. Spotting was done manually using capillary tubes and then allowed to dry.The dried plates were developed in a chromatographic tank using ethyl acetate: chloroform (4:1) as solvent system.

The region containing the compound of interest was marked, scraped off, dissolved in ethyl acetate and filtered.The solvent was removed to afford a white crystalline compound coded B^{1}.

3.4.5 Melting point Determination

The melting point of compound B^{1} was determined using the melting point apparatus.

3.4.6 Spectroscopic Analysis of Isolated Compound

B^{1} was subjected to UV, FTIR and NMR spectroscopy.

3.4.7 Chemical Test

Compound B^1 was subjected to Liebermann Burchard test and Salkowski's test for steroids and triterpenes.

3.5 Antimicrobial Studies

3.5.1 Test Organisms

The test organism used for the study are*MRSA, Staphylococcus aureus, Streptococcus pyogenes, Corynebacteriumulcerans, Proteus vulgaris, Pseudomonas aeruginosa, Escherichia coli, Klebsiellapneumoniae, Candida albicans and Candidatropicalis*. The microbes were obtained from the Department of Medical Microbiology, A.B.U Teaching Hospital, Zaria.

3.5.2 Preparation of Sample Extract for Microbiological Assay

0.002mg of the isolated compound and 0.3g of the crude ethanol extract, n-hexane extract and ethyl acetate extract was dissolved in 10mls of Dimethyl Sulphur Oxide (DMSO) to obtain a concentration of 20µg/ml and 30mg/ml of the extracts respectively.

3.5.3 Preparation of Culture Media

The medium (Mueller Hinton agar) was prepared according to the manufacturer's specification, sterilized at 121^0C for 15minutes, cooled to 40-45^0C and poured into sterile petri dishes.

3.5.4 Antimicrobial Profile (Susceptibility Test)

The antibacterial screening was carried out using the agar diffusion method. The sterilized medium was seeded with standard inoculums (0.1ml)of the test microorganism. The inoculum was spread evenly over the surface of the medium by the use of a sterile swab; the

seeded plates were allowed to dry at 37^0C for 30 mins inside the incubator. By the use of a standard cork borer (6mm in diameter), a well was cut at the centre of each inoculated medium and the solution of the extracts (0.1ml) was then introduced into the well on the inoculated medium. The medium was incubated at 37^0C for 24 hours after which the plate of the medium was observed for the zones of inhibition. The zones of inhibition were measured in diameterwith a transparent ruler and the results recorded.

3.5.5 Determination of Minimum Inhibitory Concentration (MIC)

The minimum inhibitory concentration of the extract was determined using the broth dilution method (Vollekova*et al.*, 2001; Usman *et al.*, 2007). Mueller Hinton broth was prepared. In this method, 10ml nutrient broth was prepared, 10mls was dispensed into test tubes and was sterilized at 121^0C for 15mins, and the broth was allowed to cool. McFarland's turbidity standard scale number 0.5 was prepared to give a turbid solution.

Normal saline was prepared, 10mls was dispensed into sterile test tube and the test microorganism was inoculated and incubated at 37^0C for 6hours. Dilution of the test microorganism was done using normal saline until the turbidity matched that of the McFarland standard, number 0.5 by visual comparison; at this point the test microbe has a concentration of about 1.5×10^8cfu/ml.

Two-fold serial dilution of the extracts was done using sterile broth was made to obtain the concentrations of 20µg/ml, 10µg/ml, 5µg/ml, 2.5µg/ml and 1.25µg/ml for $B^1$and 30mg/ml, 15mg/ml, 7.5mg/ml, 3.75mg/ml and 1.875mg/ml for the crude ethanol extract, n-hexane and ethyl acetate fractions. 0.1ml of the test microorganism in the normal saline was then inoculated into the different concentration. Incubation was made at 37^0C for 24hrs after which each of the test tubes was observed for turbidity (growth). The lowest concentration of

the extract in the sterile broth which shows no turbidity was recorded as the minimum inhibitory concentration.

3.5.6 Determination of Minimum Bactericidal/Fungicidal Concentration (MBC/MFC)

The MBC/MFC was determined according to the method described by Vollekova*et al.,* 2001 and Usman *et al.,* 2007. The tubes that showed no growth from the determination of MIC above were sub cultured on freshly prepared Mueller-Hinton agar plates and incubated at 37^0C for 24 hours. The lowest concentration of the extract which showed no bacteria growth was noted and recorded as the MBC/MFC.

CHAPTER FOUR

4.0 RESULTS

4.1 Extraction And Partitioning Yield

1000g of the powdered leaves of *Cussoniabarteri*gave the following yields;Crude Ethanol extract – 140g, N-Hexane fraction – 21.31g, Ethyl acetate fraction – 8.51g.

4.2 Phytochemical Constituents

Phytochemical analysis of the crude ethanol extract and ethyl acetate extract revealed the presence of carbohydrates, flavonoids, saponins, tannins and cardiac glycosides, while the hexane extract revealed the presence of flavonoids, steroids and triterpenes (Table 4.1).

Table 4.1 Results of Phytochemical Constituents of the ethanol, hexane and ethyl acetate extracts of the leaves of *Cussoniabarteri*

Constituents	Test	Observation	Inference		
			EE	EA	HE
Carbohydrates	a. Molish test	Purple to violet colour at the interface	+	-	-
	b. Fehlins's A & B test	Brick red precipitate	+	+	-
Alkaloids	a. Mayers test	No colour change observed	-	-	-
	b. Dragendoff's test	No colour change observed	-	-	-
	c. Wagners test	No colour change observed	-	-	-
Flavonoids	a. Shinoda test	Red and Orange coloration	+	+	-
	b. NaOH test	colourless	+	+	+
	c. Ferric Chloride test	Blue black coloration	+	+	-
Saponins	a. Frothing test	Honey comb formed	+	+	-
Tannins	a. Lead Sub acetate test	Cream precipitate	+	-	-
	b. Ferric Chloride test	Blue black coloration	+	+	-
Anthraquinones derivatives					
Free Anthraquinones	a. Borntrager's test	No colour change observed	-	-	-
Combined Anthracene	b. Modified Borntrager's test	No colour change	-	-	-
Cardiac Glycosides	a. Keller kiliani test	Purple colored ring	+	+	-
	b. Kedde test	Purple blue coloration	+	+	+
	c. Salkowski test	Reddish coloration	+	-	-
Steroids &Triterpenes	a. Liebermann Burchard test	Purple coloration	+	+	+
	b.Salkowski's test	Red coloration	+	+	+

Key: += Present, - = Absent

EE= ethanol extract, EA= ethyl acetate extract, HE= hexane extract

4.3Result of Chromatographic Analysis

4.3.1 Result of Thin layer Chromatography of crude ethanol extract

The thin layer chromatographicanalysis of the crude ethanol extract using ethyl acetate: n-hexane (7:3) as solvent system revealed 6 spots (Plate I).

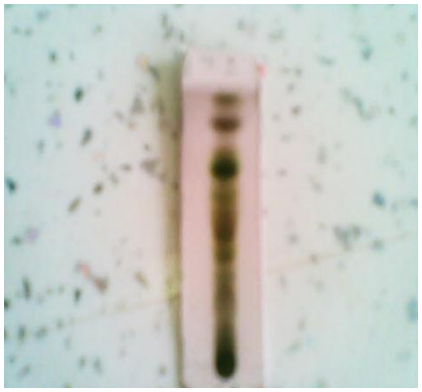


Plate I: TLC profile of crude ethanol extract

4.3.2 Result of Thin layer Chromatography of n-Hexane and Ethyl acetate Fractions

The thin layer chromatographic analysis of the n-hexane and ethyl acetate fractions using n-hexane: ethyl acetate (8:2) as solvent system gave 3 major spots when sprayed with 10% sulphuric acid (Plate II).

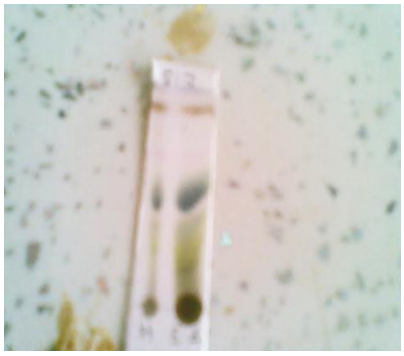


Plate II: TLC profile of n-Hexane and ethyl acetate fractions

4.3.3 Column Chromatographic separation of n-Hexane fraction

Result of the TLCs of 62 column fractions obtained from the column chromatographic separation of the n-hexane fraction using n-hexane: ethyl acetate (8:2) as solvent system revealed various spots as shown in Plate III (1-50 column fractions pooled together to afford 6 major fractions F1-F6) and Plate IV (51-62 column fractions) respectively.

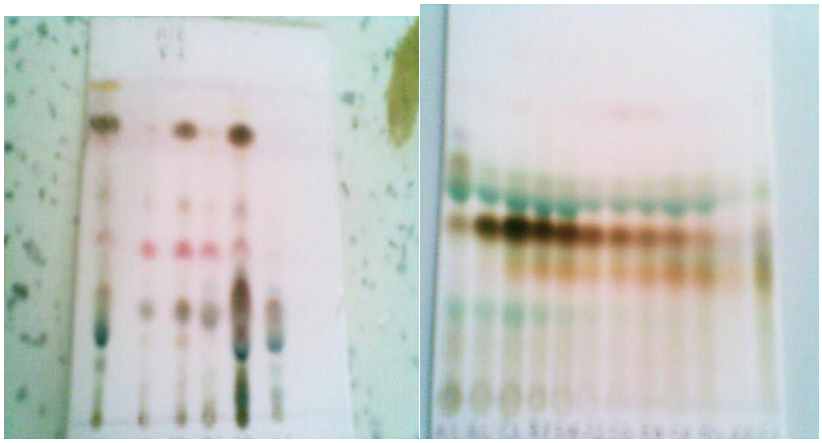


Plate III (F1-F6)–H: EA (8:2) **Plate IV (F7-F8) 51-62 column fractions**– H: EA (8:2)

Plate III and IV: TLC profile of column chromatographic separation of n-Hexane fraction

4.3.4 Result of Thin layer Chromatography of ethanol washing of the Column

The result of the TLC's of A-D fractions collected after washing the column with ethanol revealed 3 spots using n-hexane: ethyl acetate (1:1) as solvent system, (Plate V).

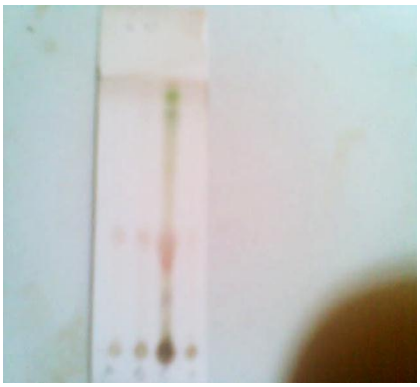


Plate V: TLC profile of ethanol washing of the column

4.3.5 Result of Thin layer Chromatography of B^+

Result of the TLC of fraction B^+ using chloroform: ethyl acetate (1:1) revealed 1 major spot and is represented in Plate VI.

Plate VI – TLC Profile of B^+

4.3.6 Thin layer Chromatographic analysis of B^1

TLC analysis of B^1using ethyl acetate: chloroform (4:1) as solvent system when sprayed with 10% sulphuric acid revealed a pink colouredsingle spot with R_f value 0.6(Plate VII).

Plate VII: TLC Profile of B^1

4.4 Melting Point Determination of B^1

The melting point of the isolated compound was found to be between 204^0C-206^0C.

4.5 Chemical test on B^1

B^1 gave positive test to LiebermannBurchard and Salkowski's test.

4.6 Spectral Analysis of B^1

4.6.1 Ultra violet (UV) Spectroscopy of B^1

The UV spectroscopy of B^1 showed maximum absorption at 253nm (Figure 4.1).

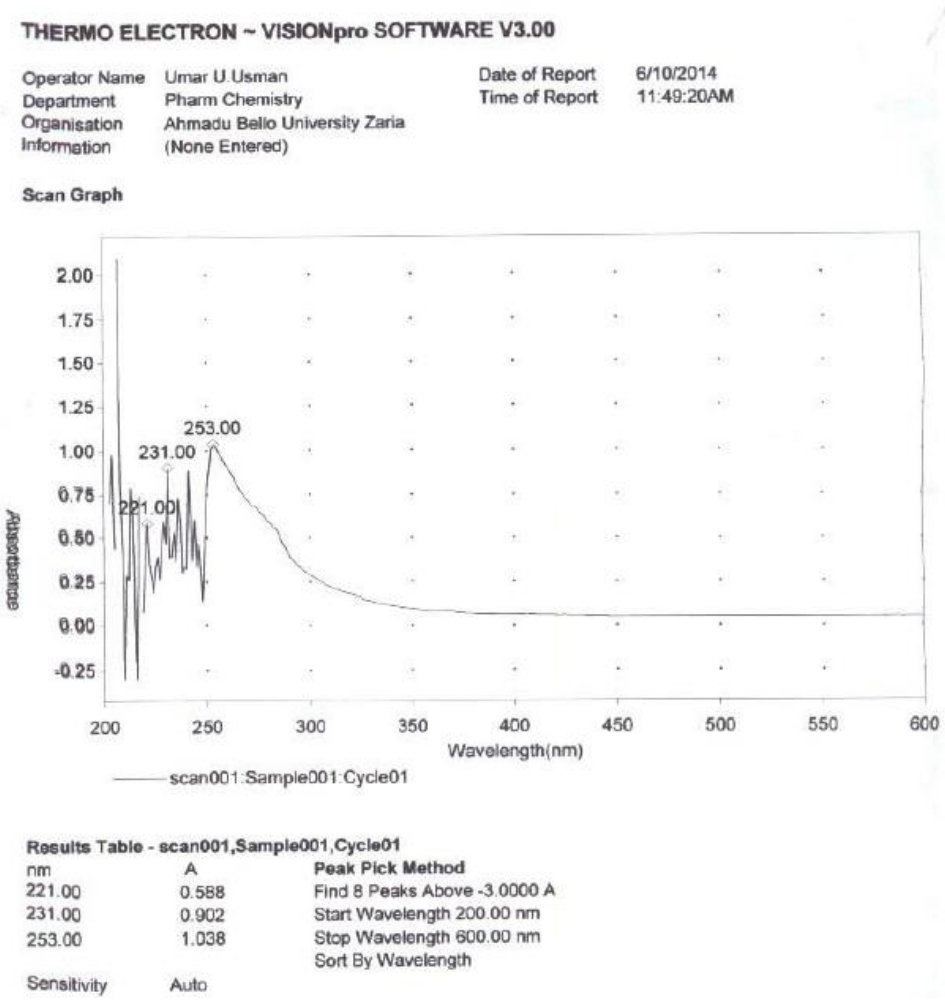
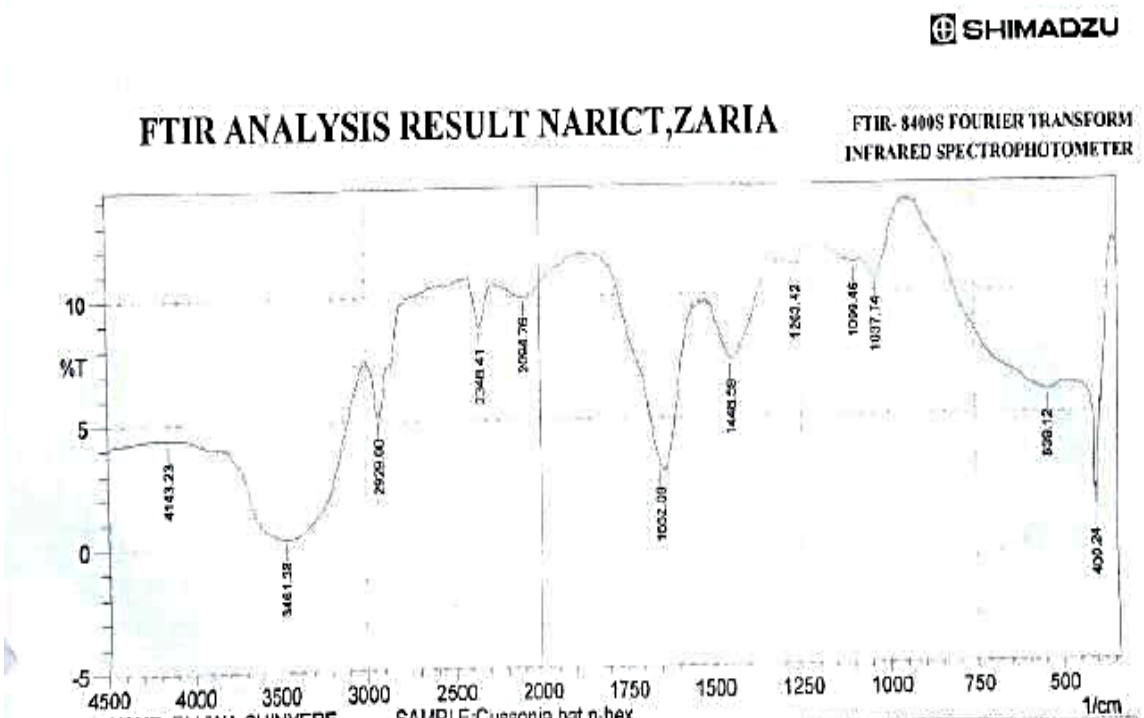


Figure 4.1: UV Spectroscopy of B^1

4.6.2 Infrared (IR) Spectroscopy of B^1

The IR spectrum of B^1 revealed major bands at: 3461.38cm^{-1} (O-H stretch),2929cm^{-1} (C-H aliphatic stretch), 2348.41cm^{-1}, 1652.09cm^{-1}(C=C stretch), 1448.59cm-1 (C-H scissoring and bending) and 400.24cm^{-1} (Figure 4.2).



NAME: ELUWA CHINYERE, SAMPLE:Cussonia bat n-hex

	Peak	Intensity	Corr. Intensity	Base (H)	Base (L)	Area	Corr. Area
1	400.24	1.074	1.714	403.14	388.67	22.719	1.492
2	539.12	5.896	0.049	567.09	531.41	43.736	0.088
3	1037.74	10.61	1.415	1077.28	948.04	119.647	2.66
4	1099.46	11.191	0.031	1199.76	1096.57	96.709	0.322
5	1263.42	11.141	0.011	1276.92	1261.49	14.688	0.002
6	1448.59	7.327	0.062	1452.45	1444.73	8.738	0.018
7	1652.09	3.054	0.061	1796.68	1651.12	169.998	-11.782
8	2094.76	9.971	0.014	2112.12	2088.98	23.168	0.01
9	2348.41	8.759	1.914	2414.96	2276.08	140.525	5.529
10	2929	5.163	2.136	2999.41	2872.1	152.327	7.854
11	3461.38	0.333	0.006	3579.04	3469.45	281.591	3.546
12	4143.23	4.376	0.004	4148.06	4135.52	17.036	0.003

Figure 4.2: IR Spectroscopy of B^1

4.6.3 Proton Nuclear Magnetic Resonance Spectral Analysis of B^1

The ^{1}H-NMR spectra (in *DMSO-D6*) of B^1 revealed signals at δ 5.19, 1H, d; 3.52, 1H, d; 3.19, 2H, d; 2.78, 2.55, 2.53, 2.32, 1.98, 1.85, 1.63, 1.62, 1.52, 1.5, 1.45, 1.38, 1.36, 1.29, 1.17, 1.03, 0.98, 0.94, 0.91, 0.90, 0.85, 0.79 (Figure 4.3- 4.4).

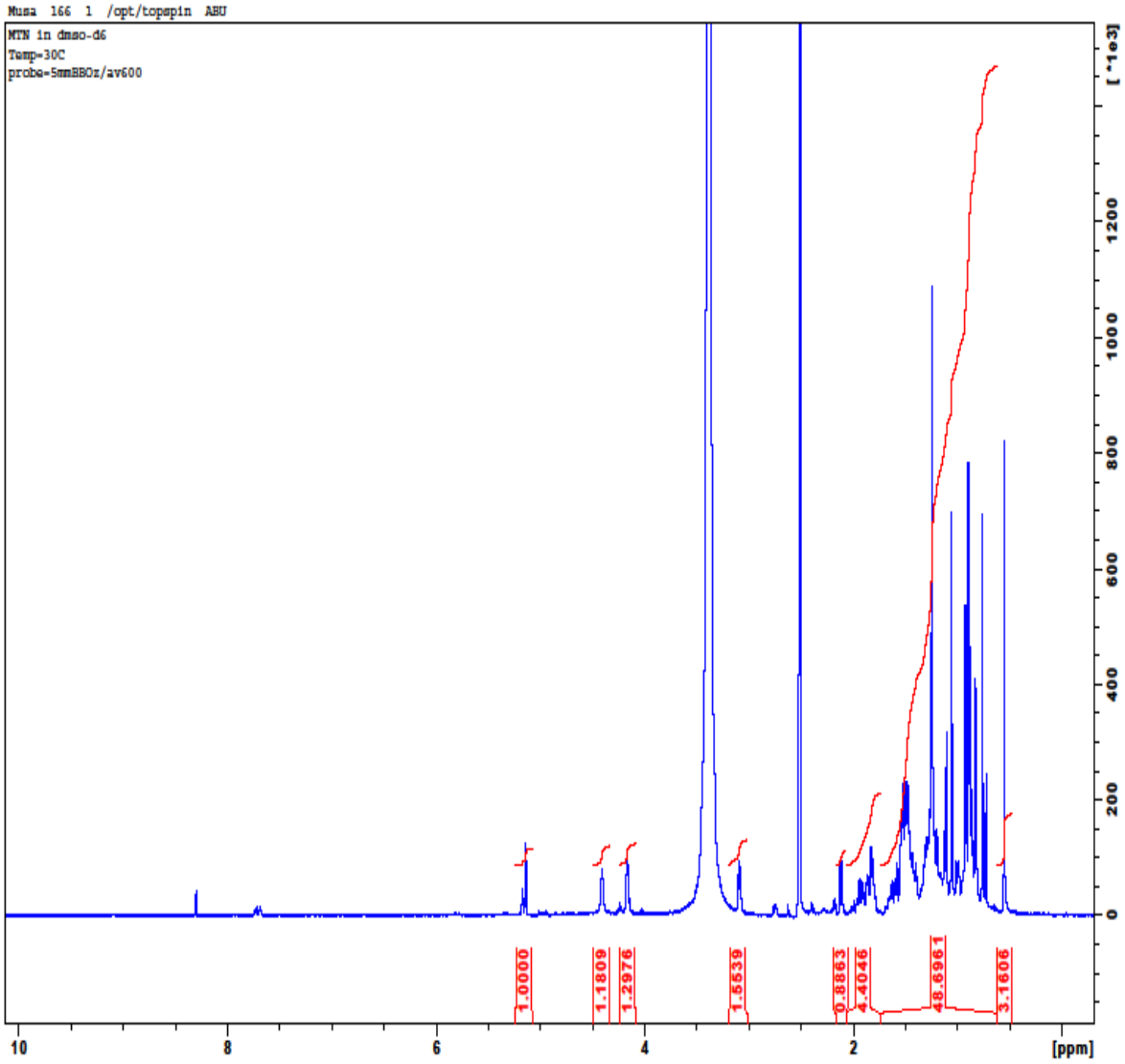


Figure 4.3: ^{1}H-NMR spectrum of B^1 in DMSO-D6

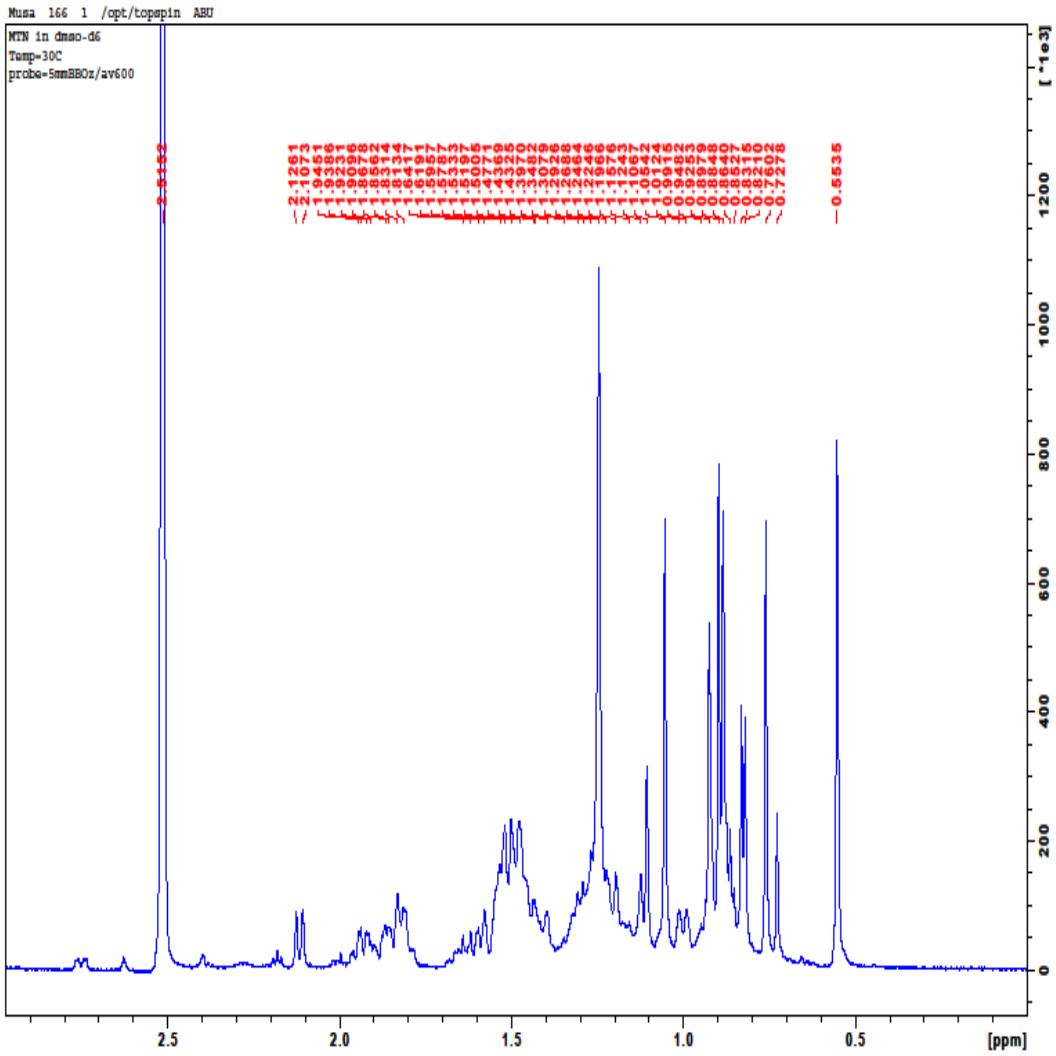


Figure 4.4: ^{1}H-NMR spectrum of B^{1} in DMSO-D6 (expanded)

4.6.4 ^{13}C NMR Spectral Analysis of B^1

The ^{13}C NMR (δ ppm, 125MHz, DMSO- D6) and DEPT experiments indicated presence of 29 carbon atoms; 178.77 (q), 138.71 (q), 125.09 (CH), 70.92 (CH), 65.08 (CH_2), 52.89 (CH), 47.58 (CH), 47.51 (CH), 47.32-45.94 (solvent), 42.31 (CH_2), 42.17 (q), 40.25 (CH), 40.11 (CH_2), 39.31 (CH_2), 38.58 (CH_2), 36.80 (CH_2), 36.70 (CH_2), 33.81 (q), 32.59 (CH), 32.48 (CH_2), 31.73 (CH), 30.85 (CH_2), 28.02 (CH_2), 24.29 (CH_2), 23.10 (CH_3), 22.53 (CH_2), 21.53 (CH_3), 17.49 (CH_3), 17.96 (CH_3), 16.07 (CH_3) (Figure 4.5-4.6).

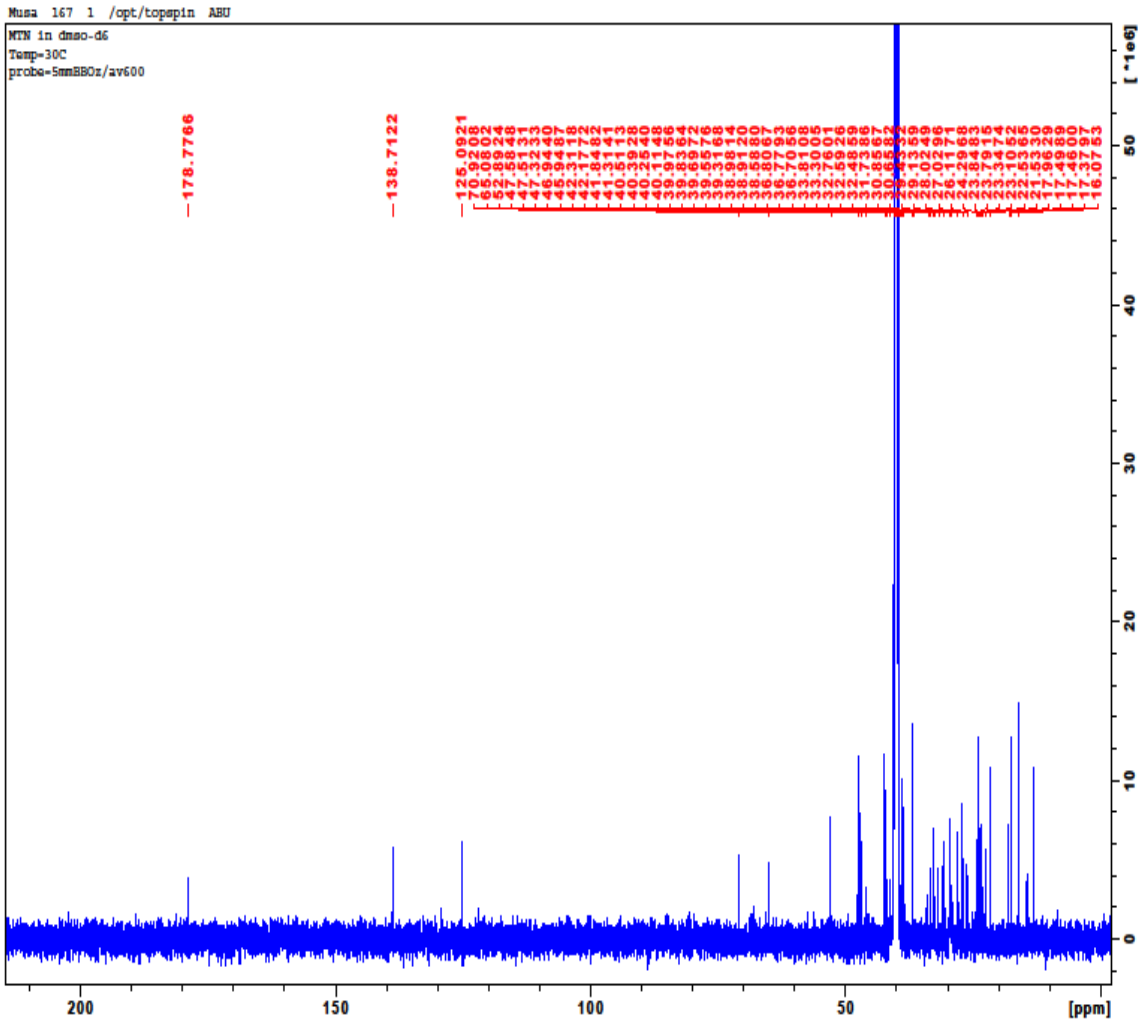


Figure 4.5: ^{13}C-NMR spectrum of B^1 in DMSO-D6

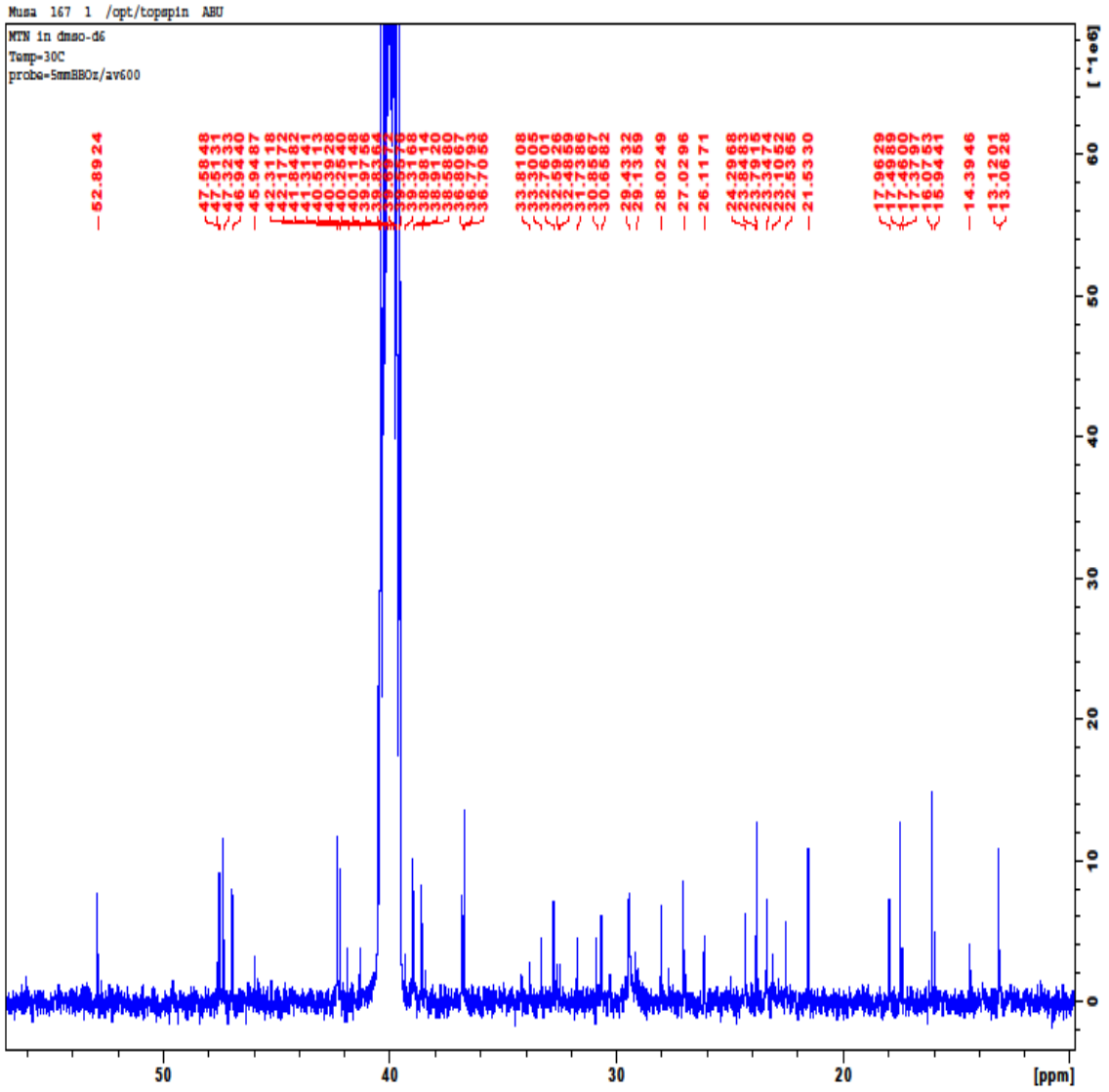


Figure 4.6: ^{13}C-NMR spectrum of B^{1} in DMSO-D6 (expanded)

4.6.5 ^{13}C-DEPT NMR Spectral Analysis of B^{1}

The DEPT further revealed the following carbons and their multiplicity: five methyl, twelve methylene, eight methine and four quaternary carbons. (Figure 4.7).

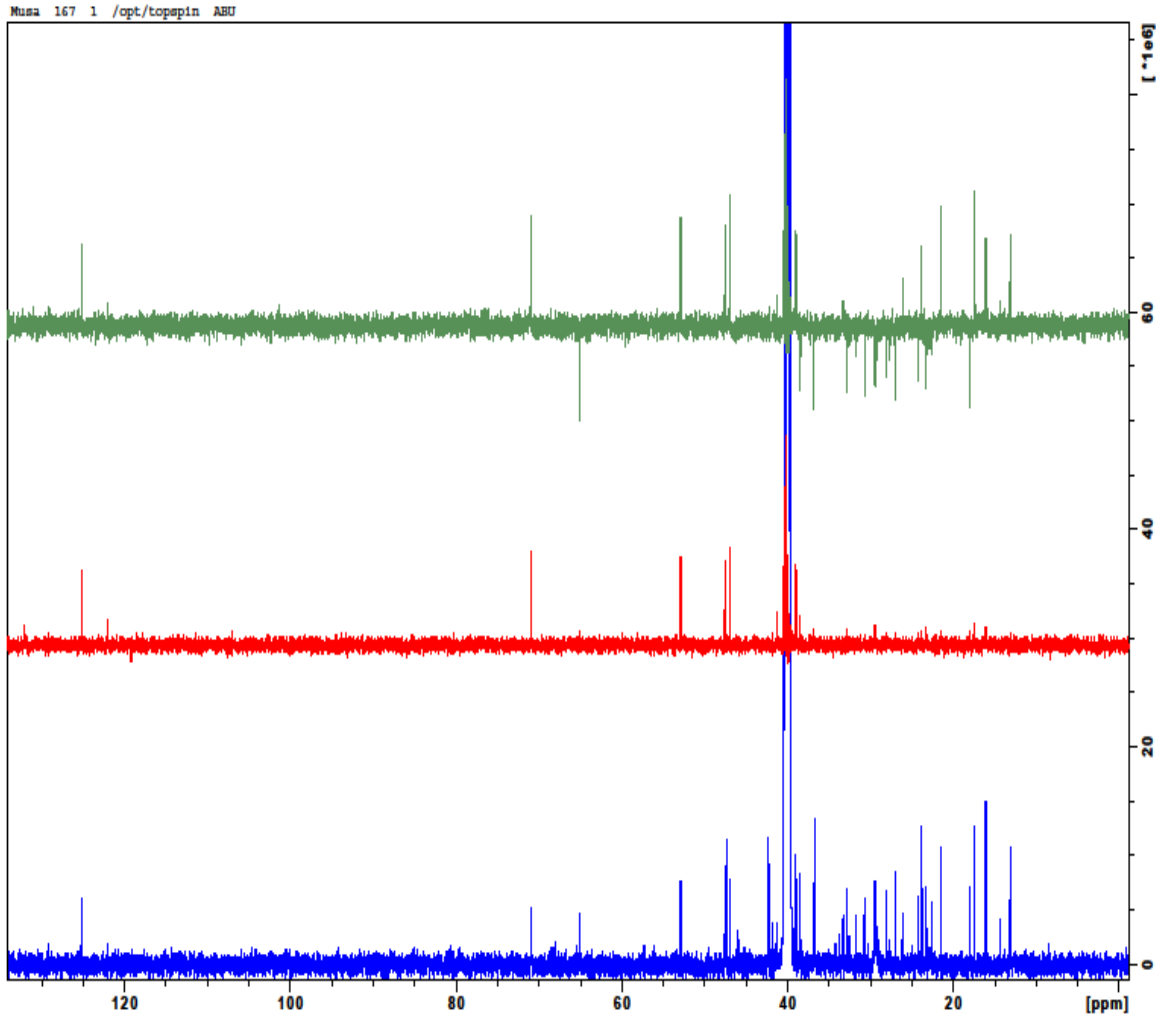


Figure 4.7: ^{13}C-DEPT spectrum of B^{1} in DMSO-D6

4.6.6 ^{1}H-^{1}H COSY NMR Analysis of B^{1}

The ^{1}H-^{1}H COSY spectra of B^{1} indicates the protons that are present in the same environment. Prominent ones are: H6(5.19) # H7(1.82), H8(0.9) # H9(1.50). (Figure 4.8-4.9).

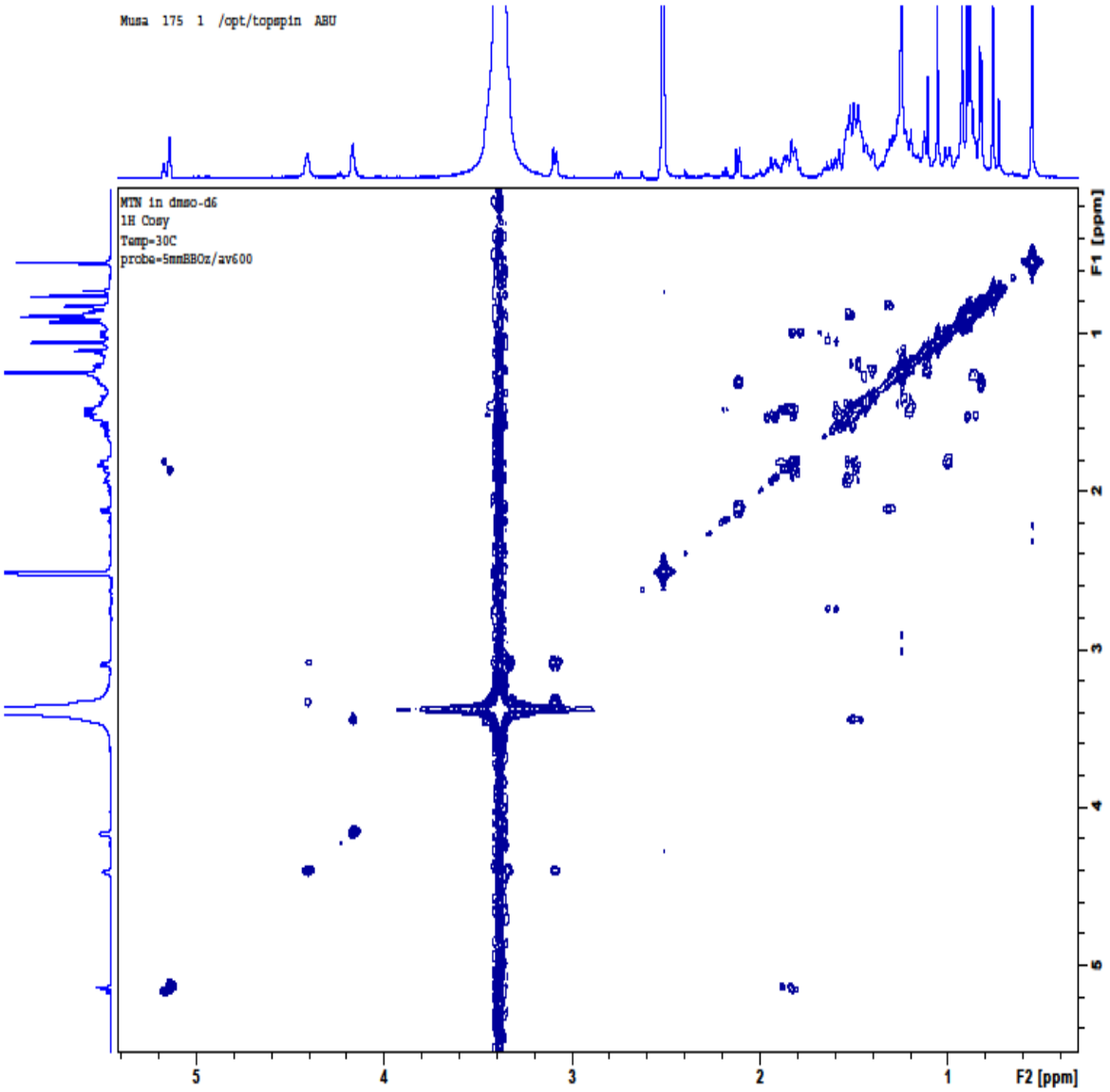


Figure 4.8: ^{1}H-^{1}H COSY spectrum of B^{1} in DMSO-D6

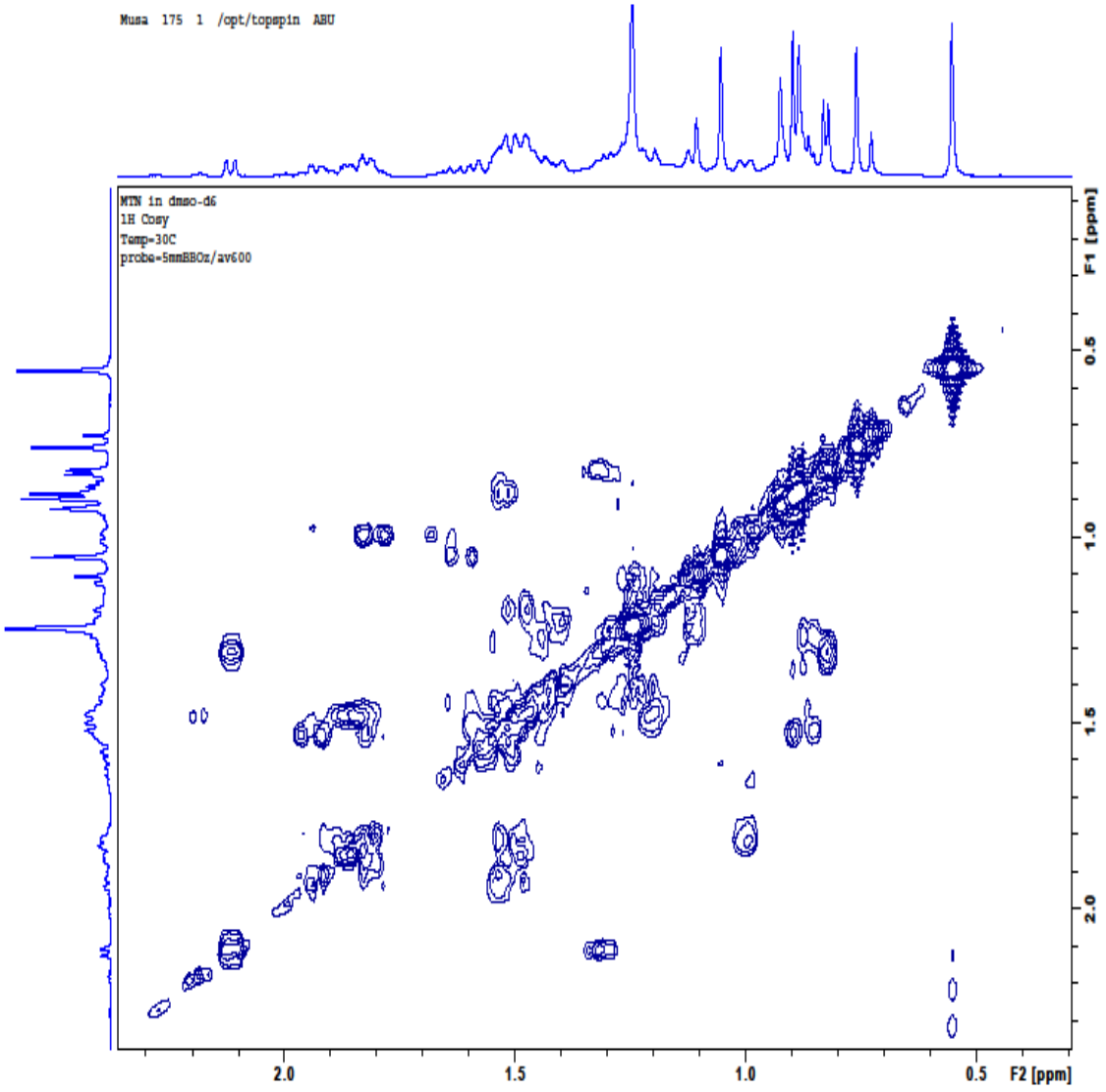


Figure 4.9: ^{1}H-^{1}H COSY spectrum of B^{1} in DMSO-D6 (expanded)

4.6.7 HSQC Spectra Analysis of B^1

The HSQC spectrum of B^1(Figure 4.10 – 4.11) was used to assign protons to their respective carbons as shown in Table 4.6.

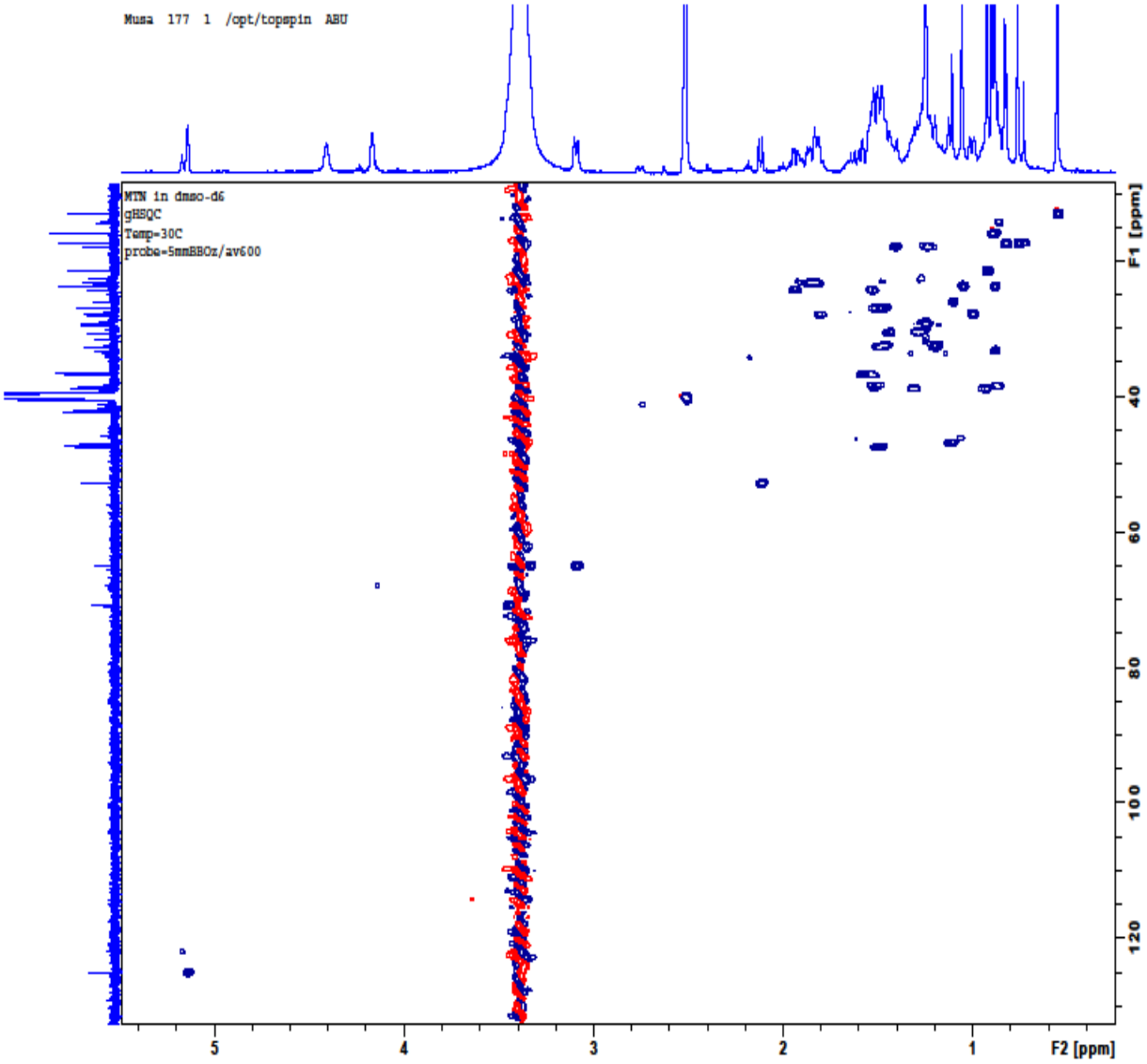


Figure 4.10: HSQC spectrum of B^1 in DMSO-D6

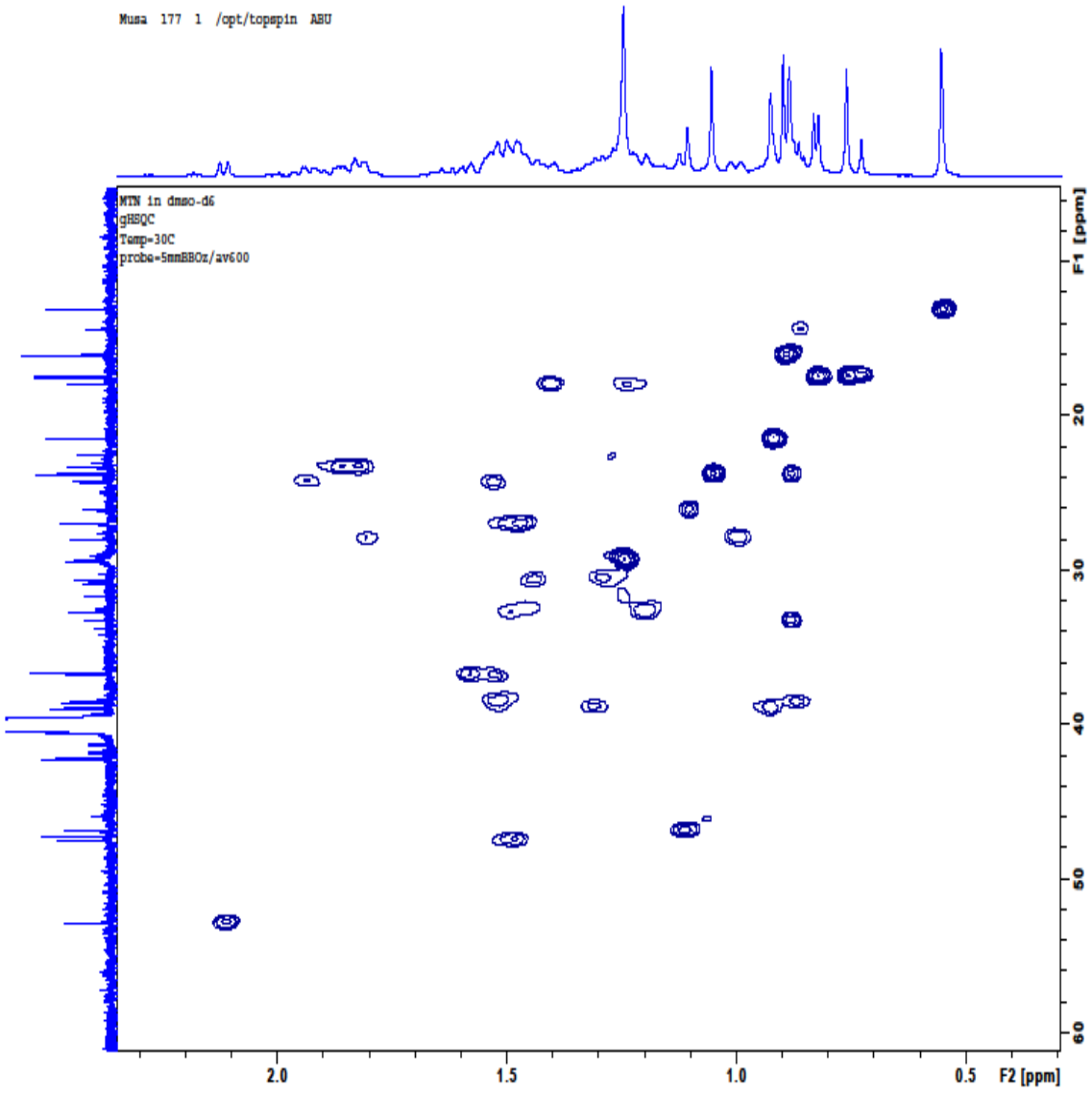


Figure 4.11: HSQC spectrum of B^1 in DMSO-D6 (expanded)

4.6.8 HMBCSpectra Analysis of B^1

The HMBC spectra are used to assign connectivity between various fragments. (Figure 4.12-4.13).

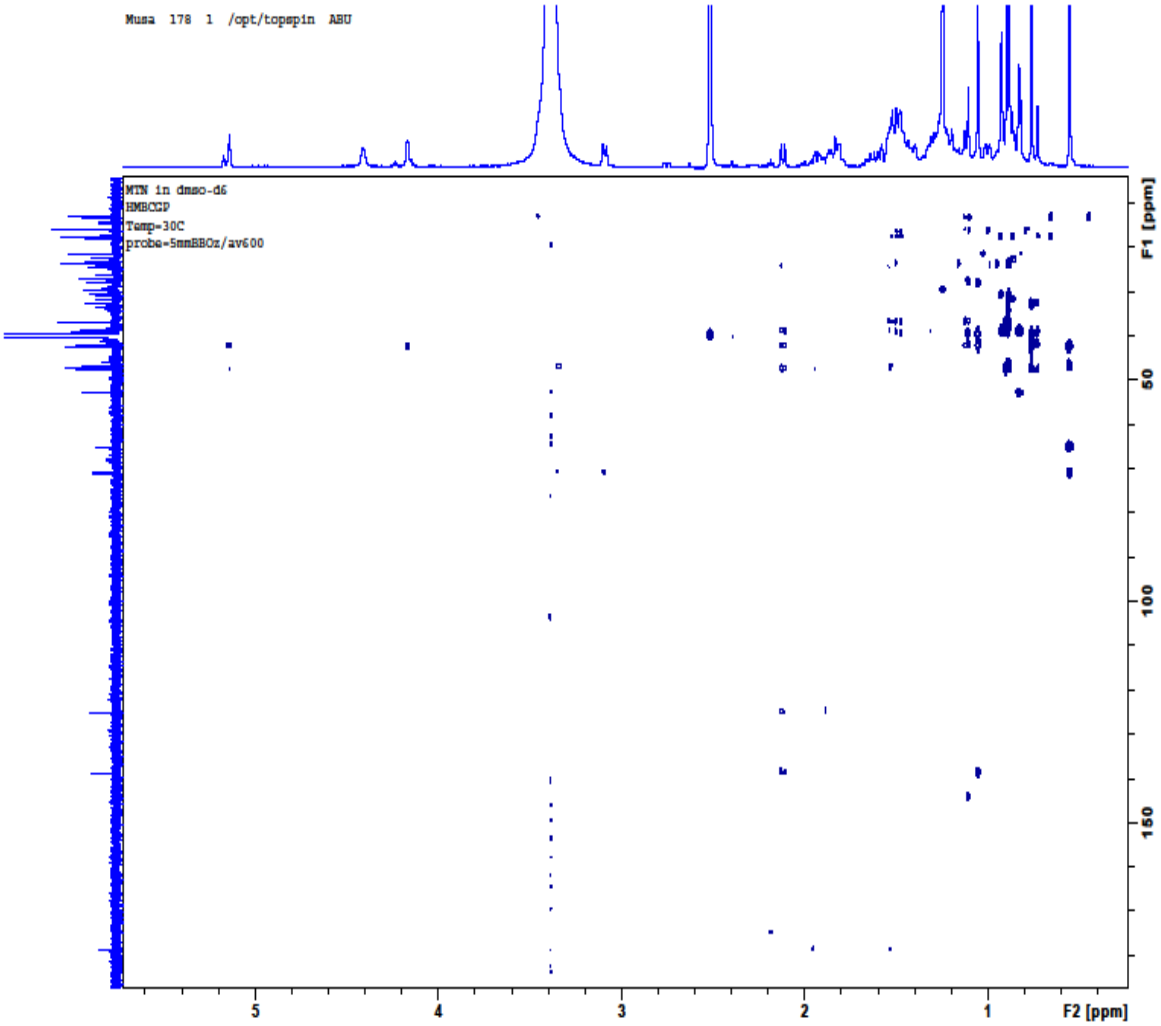


Figure 4:12: HMBC spectrum of B^1 in DMSO-D6

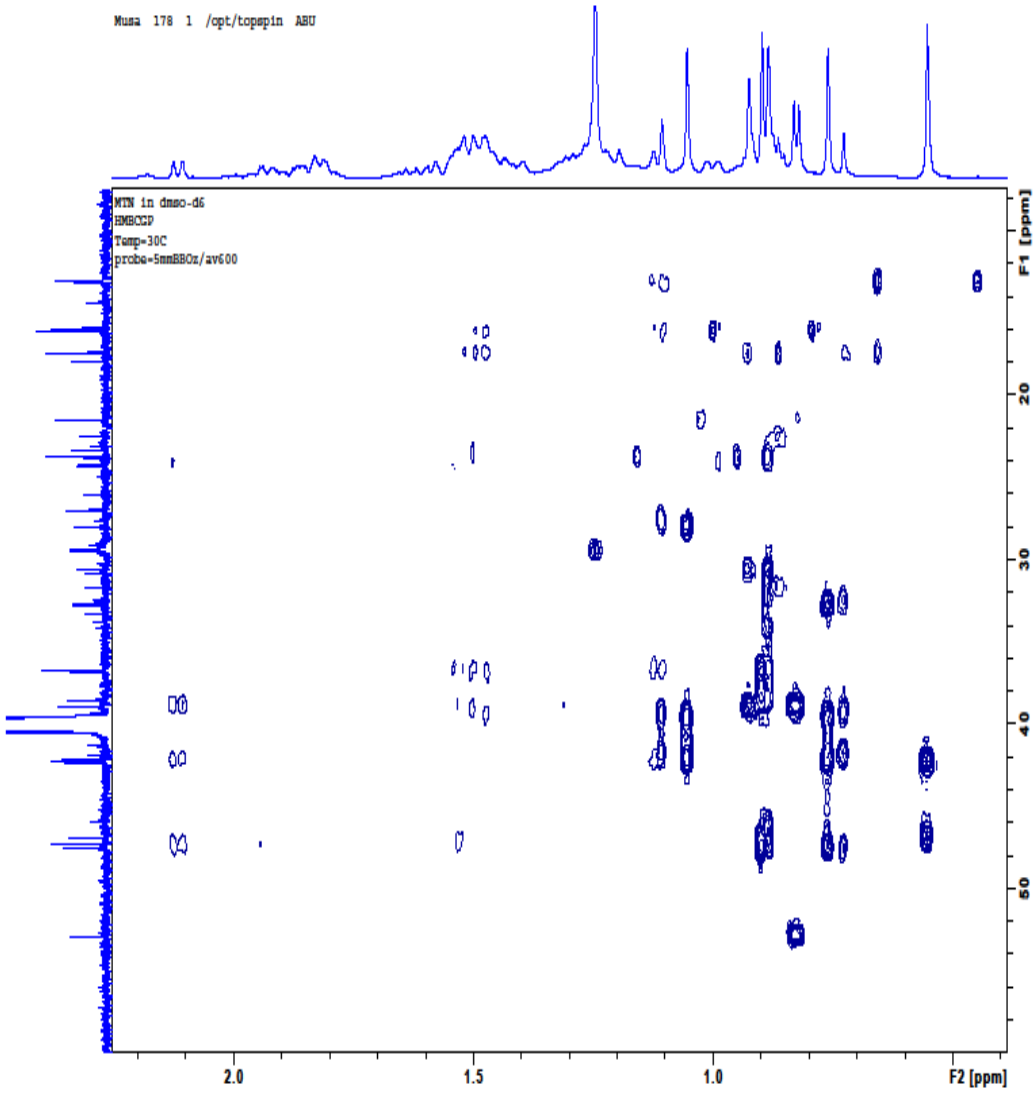


Figure 4.13: HMBC spectrum of B^1 in DMSO-D6 (expanded)

4.6.9 NOESYSpectra Analysis of B^1

The NOESY spectrum of B^1 in DMSO-D6 reveals correlations between protons in space (Figure 4.14).

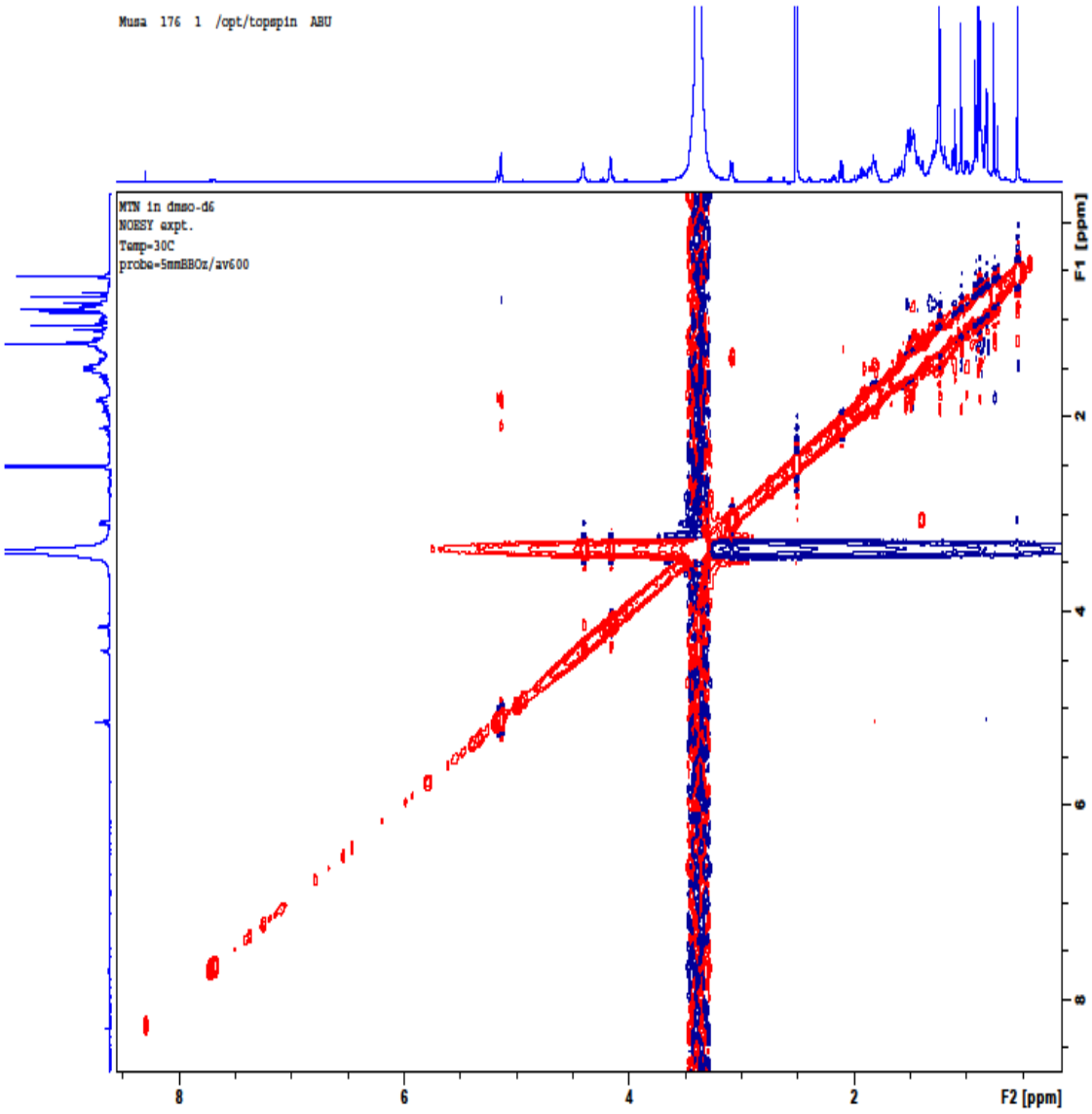


Figure 4.14: NOESY of B^1 in DMSO-D6

Table 4.2: $^{1}H,^{13}C$ NMR and DEPT data of B[1]

Position	^{13}C (ppm)	^{1}H (ppm)	DEPT
1	36.8	1.62	CH_2
2	30.85	1.29	CH_2
3	70.92	3.52	CH
4	42.31	2.78	CH_2
5	138.71	-	C
6	125.09	5.19	CH
7	32.48	1.52	CH_2
8	32.59	0.9	CH
9	47.58	1.5	CH
10	33.81	-	C
11	22.53	1.36	CH_2
12	39.31	0.98	CH_2
13	42.17	-	C
14	52.89	2.32	CH
15	24.29	1.98	CH_2
16	28.02	1.03	CH_2
17	47.51	1.17	CH
18	16.07	0.91	CH_3
19	17.96	0.85	CH_3
20	40.25	2.53	CH
21	17.49	0.79	CH_3
22	36.7	1.63	CH_2
23	38.58	1.38	CH_2
24	40.11	2.55	CH_2
25	31.73	1.45	CH
26	65.08	3.19	CH_2
27	23.1	1.85	CH_3
28	178.77	-	C
29	21.5	0.94	CH_3

4.7 Result of Antimicrobial Screening

The results of the antimicrobial screening expressed in terms of minimum inhibitory concentration and minimum bactericidal/fungicidal concentration are shown in the tables below.

Table 4.3: Zone of Inhibition (diameter) of the Extracts and Standard drugs against the Test Microorganism (mm)

Test Organism	IS	EE	HE	EA	Ciprofloxacin	Fluconazole
MRSA	27	0	0	0	0	0
Staphylococus aureus	29	24	20	28	35	0
Streptococcus pyogenes	0	26	21	29	37	0
Corynebacteriumulcerans	24	22	18	28	37	0
Proteus vulgaris	27	0	0	0	35	0
Psudomonas aeruginosa	0	0	0	0	0	0
Escherichia coli	30	25	18	30	37	0
Klebsiellapneumoniae	31	27	20	29	39	0
Candida albicans	25	23	18	25	0	35
Candida tropicalis	0	25	16	28	0	37

Drug Concentration: Ciprofloxacin-5µg/disc, Fluconazole-5µg/disc

Table 4.4: Minimum Inhibition Concentration of the Extracts against the Test Microorganism

Test Organism	IS (µg/ml)	EE (mg/ml)	HE (mg/ml)	EA (mg/ml)
MRSA	2.5	0	0	0
Staphylococus aureus	2.5	7.5	7.5	3.25
Streptococcus pyogenes	0	7.5	7.5	3.25
Corynebacteriumulcerans	5	7.5	15	3.25
Proteus vulgaris	2.5	0	0	0
Psudomonas aeruginosa	0	0	0	0
Escherichia coli	2.5	7.5	15	3.25
Klebsiellapneumoniae	2.5	3.25	7.5	3.25
Candida albicans	5	7.5	15	7.5
Candida tropicalis	0	7.5	15	3.25

Key: MRSA- Methicillin Resistant *Staphylococcus aureus*

IS- Isolated compound, EE- ethanol extract, HE- hexane extract, EA- ethyl acetate extract.

Table 4.5 Minimum Bactericidal/Fungicidal Concentration of the Extracts against the Test Microorganism

Test Organism	IS(µg/ml)	EE(mg/ml)	HE(mg/ml)	EA(mg/ml)
MRSA	10	0	0	0
Staphylococus aureus	5	15	30	7.5
Streptococcus pyogenes	0	15	30	7.5
Corynebacteriumulcerans	10	30	30	7.5
Proteus vulgaris	10	0	0	0
Psudomonas aeruginosa	0	0	0	0
Escherichia coli	5	15	30	7.5
Klebsiellapneumoniae	5	15	30	7.5
Candida albicans	10	30	30	15
Candida tropicalis	0	15	30	7.5

Key: MRSA- Methicillin Resistant *Staphylococcus aureus*

IS- Isolated compound, EE- ethanol extract, HE- hexane extract, EA- ethyl acetate extract.

CHAPTER FIVE

5.0 DISCUSSION

5.1 Phytochemical Studies

The preliminary phytochemical screening of the ethanol extract of *Cussoniabarteri* revealed the presence of flavonoids, cardiac glycosides, steroids and triterpenesin the n-hexane fraction while carbohydrates, alkaloids, saponins, tannins and anthraquinones were absent. Many naturally occurring compounds found in plants have been shown to have antimicrobial properties (Yusha'u*et al*, 2008, Aliyu *et al*., 2009, Kawo*et al*., 2009). The presence of flavonoids in the plant extract for instance shows that the plant has antibacterial or antifungal property (Evans, 1996).

The column chromatography of the n-hexane fraction afforded the fraction B^+ while the preparative TLC of B^+ resulted in the isolation of a white-crystalline solid which revealed a pink coloured single spot using ethyl acetate: chloroform (4:1) as solvent system when sprayed with 10% sulphuric acid, coded as B^1 (8mg). The compound was crystallized and the melting point of 204^0C-206^0C was recorded. The positive chemical tests displayed by B^1 when subjected to Liebermann Burchard and Salkowski's test indicates that it has a steroidal or triterpenenucleus (Evans, 1996).

The appearance of maximum absorption at 253nm in the (UV) spectroscopy of B^1 shows evidence of conjugationin the structure (Jerry, 1985). The IR spectrum of B^1 revealed major bands at: 3461.38cm^{-1} which is characteristic of O-H stretch, 2929$cm^{-1}$is due to C-H aliphatic stretch, 1652.09$cm^{-1}$is due to C=C olefinic stretch and 1448.59cm^{-1}characteristic of C-H scissoring and bending (Bellamy, 1961; Nakanishi, 1962; Patehet *al*., 2009).

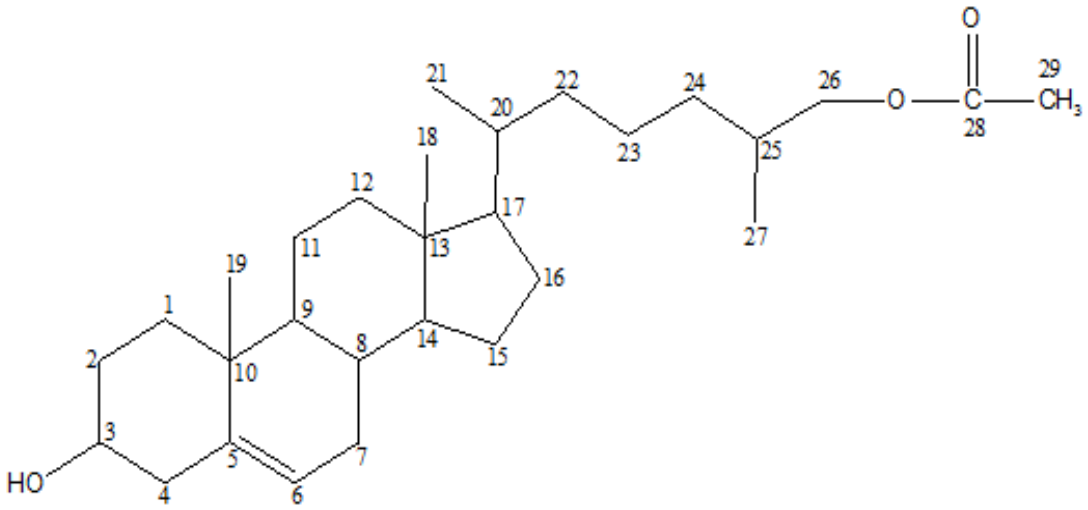
The ^{1}H-NMR spectrum of B^{1}gave signals at δ3.52 which indicates the presence of a proton on an oxygenated carbon, signals at δ5.19 signifying olefinic proton while the others are region of overlapping CH_3, CH_2, and CH signals in the upfield.

The ^{13}C-NMR and DEPT spectral showed the structure of the compound to consist of an unsaturated aliphatic molecule with 29 carbon atoms which comprises of 5 methyl (CH_3) groups, 12 methylene (CH_2) groups, 8 methine (CH), group and 4 quartenary (C) carbons further indicating the steroidal nature of the compound (Agrawal *et al*., 1985;Patehe*t al*., 2009). The downfield resonances at δ 138.71(C-5) and δ 125.09(C-6) indicate the presence of unsaturation, the resonance at δ 70.92 indicates the presence of a hydroxyl group at C-3 position, resonances at δ 16.07 and δ 17.96 corresponds to angular methyl carbon atoms at C_{18} and C_{19} respectively while resonances at δ 36.70 and δ 38.58 corresponds to a single bond at C_{22} and C_{23} respectively which suggest the compound as a β-sitosterol(Pretsche*t* al., 2000;Patehe*t al*., 2009). The value for C_{18} is lower due to γ-gauche interaction that increases the screening of the C_{18} hence lower chemical shift. However the loss of H in C_6 results in decrease in screening of the C_{19} leading to increase in ^{13}C chemicals shift to higher frequency (Smith, 1978).The downfield shift of C_{26}atδ 65.08 and C_{28}atδ 178.77 indicates the presence of acetyl group at C_{26}(Shuhuae*t al*., 2004).

The results of the ^{1}H-^{1}H COSY correlation and the HSQC spectroscopy confirmed the relationship between the various protons and carbons in the molecule. Some prominent correlations between protons established by the ^{1}H-^{1}H COSY spectra include: H6(5.19) # H7(1.82), H8(0.9) # H9(1.5), H11(0.98) # H12(1.36), H3(3.52) # H1(1.62), H3(3.52) # H4(2.78), H3(3.52) # H2(1.29). The assignment of the protons, carbons and their various linkages in the molecule was confirmed through the cross peaks detected on the HMBC spectroscopy. Some of the major correlations observed between protons and carbons include:

proton H3(3.52) showed correlation with C5, C26, C19; proton H9(1.5) correlated with C28, C17, C12, C15; proton H15(1.98) correlated with C28.

On the basis of the above UV, IR, ^{1}H NMR and ^{13}C NMR data compound B^{1} was identified asβ-sitosterol ester.



Acetic acid 6-(3-hydroxy-10,13-dimethyl-2,3,4,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-1H-cyclopenta[a]phenanthren-17-yl)-2-methyl-heptyl ester

Figure 5.1: β-sitosterol ester

5.2 Antimicrobial Studies

The results show that the isolated compound has various inhibitory effects against the test organisms except*Streptococuspyogenes, Pseudomonasaeruginosa* and *Candida tropicalis*.

The zone of inhibition ranges from 16-31mm. The isolated compound showed higher activity than the other fractions. The best activity was shown by the isolated compound against *K. pneumonia*, with zone of inhibition (31mm), then the isolated compound and ethyl acetate fraction also showed a good activity against *E. coli* with 30mm inhibition. The fractions showed moderate activity against *C. ulceran*sand *C. albicans*.

*Staphylococcus aureus*is known to play a significant role in treatment of skin diseases (Srinivesan*et al*., 2001); the strong activity of the isolated compound against *Staphylococcus aureus*indicates that it may be effective against skin infections and diarrhea. It is important to note the activity of the isolated compound against *Candida albicans*which indicates that the isolated compound can be used as an anti-fungal especially for the treatment of fungal diseases such as vaginitis and other generalized infections including endocarditis caused by *Candidaalbicans*(Thomas, 1979).

Also, the isolated compound demonstrated an interesting activity against *Escherichia coli*, this microorganism causes infections that are difficult to combat due to multi drug resistance (Salie*etal*., 1996, Afolayan and Aleiro, 2006).The results obtained indicated the existence of antimicrobial compound in the various extracts and showed a good correlation between the reported uses of this plant in traditional medicine against infectious diseases. For example the inhibition of *E. coli* by the extracts justifies their use in the treatment of diarrhea and dysentery in traditional medicine.

As low as MIC value of 2.5µg/ml and MBC/MFC value of 5µg/ml of the isolated compound had activity against *Staphylococcus aureus*, *Escherichia coli* and *Klebsiella pneumonia*, this shows the strength of activity of the isolated compound against the tested microorganisms.

CHAPTER SIX

6.0 SUMMARY, CONCLUSION AND RECOMMENDATIONS

6.1 Summary

The preliminary phytochemical screening of the ethanol extract of the leaves of *Cussoniabarteri* revealed the presence of carbohydrates, flavonoids, saponins, tannins, cardiac glycosides, steroids and triterpenes. Flavonoids,cardiac glycosides, steroids and triterpenes were found to be present in all the extracts while alkaloids and anthraquinones were absent in all the extracts.

The chromatographic studies of the n-hexane fraction resulted in the isolation of the compound B^1 which is a β- sitosterol ester.

The isolated compound showed significant activity against *Staphylococcus aureus*, *Escherichia coli*, *Klebsiella pneumonia*. A low MIC value of 2.5µg/ml and MBC/MFC value of 5µg/ml showed the strength of activity of the isolated compound against the tested microorganisms. This results support the traditional use of the leaves of this plant as a remedy for the treatment of infectious diseases.

6.2 Conclusion

βsitosterol ester has been isolated from the leaves of *Cussoniabarteri*. The plant has demonstrated a strong activity against some organisms responsible for infections rationalizing its ethno medicinal use in the treatment of infectious diseases and the presence of some secondary metabolites revealed during the phytochemical studies could be responsible for the activity.

6.3 Recommendation

Based on the work carried out on the leaf of *Cussoniabarteri*the following recommendations have been made:

1. Other parts of the plant such as the roots and bark should be worked on so as to validate their ethno medicinal uses.
2. Other biological activities such as pharmacological activities should be carried out so as to validate other ethno medicinal uses of the leaf of the plant.
3. Other fractions of the leaf extract should be worked on to isolate more compounds and also validate its ethno medicinal uses.

REFERENCE

Afolayan , A. J. andAliero, A. A. (2006). Antimicrobial activity of *Solanumtomentosum.AfricanJournal of Biotechnology*, 5 (4): 369-372.

Agbor A. G. andNgogang Y. J. (2005).*Toxicity of herbal preparations. Complementary and alternative medicine, Journal of Ethnobotany, U.S.* ; 1:23–28.

Agbor A. G.Oben J. E. and Ngogang J. Y. (2005).*Haematinic activity ofHibiscus cannabinus.African Journal Biotechnology, U.S*; 4(8):833–837

Agrawal, P.K., Jain, D.C, Gupta, R.K, and Thakur, R.S. (1985) "Carbon -13 NMR spectroscopy of steroidal sapogenins and steroidal saponins" *Phytochemistry.*China. 24(11): 2476-2496.

Aliyu, M. S., Hanwa, U. A., Tijjani, M. B., Aliyu, A. B. and Yaú, B. (**2009**). Phytochemical and Antibacterial properties of leaf extract of *Stereospermumkunthianum* (Bignoniaceae). *Nigerian Journal of Basic and Applied Sciences* 17 (2): 235-239.

Balandrin N. F, Kinghorn A. D, and Farnsworth N. R. (**1993**).In: Human medicinal agents from
plants. Kinghorn A. D, Balandrin M. F, editors. ACS Symposium Series 534. Washington DC, USA: American Chemical Society Books. p. 2-12.

Bellamy, L. J.(**1961**). "The Infrared Spectra of Complex Molecules", 2nd edition, John Wiley & Sons, New York, U.S.A. PP. 299

Bruneton J. (1993).PlantesMédicinales: Phytochimie, Pharmacognosie. 2 éme. New York, U.S.A: Lavoisier; p. 914.

Burkill, H. M. (**1985**). *The useful plants of west tropical Africa*, Vol 1, London, U.K: Royal BotanicalGardens, Kew, Richmond Surrey, U. K. pp. 443-444

Chartone-Souza, E. (**1998**).Bactérias ultra-resistentes: umaguerraquaseperdida. CiencHoje. ; 23(138):27-35.

Cowan, M.M., (**1999**).*Plant Products as Antimicrobial Agents.* Clinical. Microbiology, U. S.Review. 12: 564-582.

Dahanukar, S. A., Kulkarni, R. A. and Rege, N. N. (**2000**). *Pharmacology of Medicinal Plants*
and Natural products. *Indian Journal of Pharmacology*, India. **32**, S81–S118.

De Villiers, B. J, Van Vuuren, S.F, Van Zyl R. L and Van Wyk B. E (**2010**). "*Antimicrobial and*
*Antimalarial activity of Cussonia species (Araliceae)"*Journal of Ethnopharmacology,
U.S, Vol. 129, Issue 2, pp, 189-196

Dubois , M. A., Ilyas, M. and Wagner, H. (**1986**). Cussonoside A and B, two triterpene-saponins
from *Cussoniabarteri*, Planta Medica 2, France. pp. 80-83

Evans, W. C. (**1983**). Trease and EvansPharmacognosy, 14th edition W. B.
Saunders Company Limited. 24-28 Oval Road London NWI TDX, U.K. pp. 13-53, 117-139, 293-334, 471-511

Evans, W. C.(**1996**).*Trease and Evans' Pharmacognosy*. 13th edition W.B. Sauders, London, U.K. 612, 832-833 p.

Evans, W. C. (**2002**). Trease and Evans Pharmacognosy15th Edition. London, U.K: Saunders Publishers.pp. 42-44. 214-314, 221-229, 246-249, 304-306, 331-332, 391-393

Farnsworth, N. R. (**1988**). In *Biodiversity.*Wilson, E. O. Editor, National Academy Press Washington DC, U.S.A.pp. 83 -97

Floyd, A. G. (1982). N. S. W. Rainforest Trees Part XI. Forestry Commission of N.S.W; Sydney. Australia.pp. 5.

Fola, A. (1993).Local Medicinal Plants and the Health of the Consumers. A paper delivered at the PSN/CF PCON organization workshop: in Clinical Pharmacy and Herbal Medicine.NewYork, U.S.A.9:28–31

Hertog, M.G.L., Feskens E. J. M., Kromhout D. Hertog, M. G. L., Hollman, M. C.H.,Hertog M. G. L and Katan, M. B. (**1993**).Dietary Antioxidant Flavonoids and Risk of Coronary
Heart disease. The Zutphen elderly study. Lancet, Netherland. 342: 1007-1011.

Ivanova, D.,Gerova, D., Chervenkov T. and Yankova, T. (**2005**).*Polyphenols and Antioxidant Capacity of Bulgarian Medicinal Plants.Journal of Ethnopharmacology*. Bulgaria. 96: 145-150.

Jana, S. and Shekhawat, G. S. (**2010**).Phytochemical Analysis and Antibacterial Screening of *in*
vivo and *in vitro* extracts of Indian medicinal herb: *Anethumgraveolens*. *Research Journal of Medicinal Plant*, India.4: 206-212.

Jerry, M. (**1985**).Advanced Organic Chemistry reactions, mechanisms and structure.3rd edition. New York, U.S.A. pp. 1-5

Jeyachandran, R. and Mahesh A, (**2007**).Enumeration of Antidiabetic Herbal Flora of Tamil Nadu *Research Journal of Medicinal Plant*, India. 1: 144-148.

Kawo, A. H., Mustapha, A., Abdullah, B. A., Rogo, L. D., Gaiya, Z. A and Kumurya, A. S. (**2009**). Phytochemical properties and Antibacterial activities of the leaf and latex extracts of *Calotropisprocera* (AIT. F.). *Bayero Journal of Pure and Applied Sciences***2 (1)**: 34-40.

Mahady, G. B. (**2005**). Medicinal plants for the prevention and treatment of Bacterial Infections.
WHO collaborating centre for Traditional Medicine, University of Illinois, Chicago,
U.S.A. 11(19): 2405-27.

Michel A.(**2005**).*Trees Shrubs and Lianas of West African dry zones*: Cambridge University Press, U.S.A. pp 172.

Misra, A.(**2009**).Studies on biochemical and physiological aspects in relation to phyto-medicinal qualities and efficacy of the active ingredients during the handling, cultivation and harvesting of the medicinal plants. *Journal of Medicinal Plants Research,*India. 3: 1140-1146.

Nascimento G. G. F, Locatelli J, Freitas P. C, Silva G. L.(**2000**).Antibacterial activity of plant extracts and phytochemicals on antibiotic-resistant bacteria.*Brazilian Journal of Microbiology,*Brasil.*31*:247-256.

Nakanishi, K. (**1962**)."Infrared Absorption spectroscopy - Practical", Holden-Day, Inc., San Francisco, U.S.A. pp. 233.

Pateh , U.U., Haruna, A.K., Garba, M., Iliya, I., Sule, I.M., Abubakar, M.S., and Ambi, A.A.(**2009**). Isolation of Stigmasterol, β-Sitosterol and 2-hydroxyhexadecanoic acid methyl Ester from the Rhizomes of *Stylochitonlancifolius*Pyer and Kotchy (Araceae).*Nigerian Journal of Pharmaceutical sciences*. Nigeria. 8(1):19-25

Pezzuto, J. M. (**1997**). *Biochemical Pharmacology*, Chicago, U.S.A.*53*, 121-133

Plunkett, G. M., Soltis, D. E. &Soltis, P. S. (**1997**). Clarification of the relationship between Apiaceace and Araliaceae based on MATK and RBCL sequence data. *AmericanJournal of Botany* 84: 565-580.

Pretsch, E. B. and Affolter, A. (2000).“Structure Determination of Organic Compounds – Tableof spectral data” springer-verlag Berlin Heidelberg, Germany. Pp. 71-150.

Prosper-Cabral N. B., Agbor G. A., Oben J. E., and NgogangJ. Y. (**2007**),
Phytochemical Studies and Antioxidant Properties of Four Medicinal Plants Used in Cameroon.*African Journal of Traditional, Complimentary and Alternative Medicines,*
*Vol.4,*Yaounde, Cameroun. No. 4, pg 495-500

Sakagami Y.and Kajimura K. (**2002**).*Bactericidal activities of disinfectants against*

vancomycin-resistantenterococci. *Journal of Hospital Infection.*Japan;50(2):140-4

Salie F., Eagles P.F.K, and Leng H.M.J (**1996**). Preliminary Antimicrobial screening of four South African Asteraceae species.*Journal of Ethnopharmacology*, 76: 347-354.

Shrikumar, S. and Ravi, T. K., (**2007**).*Approaches towards development and promotion of herbaldrugs*.PharmacognosyReview, India. 1: 180-184.

Shuhua, Q., Zhang, S., Jianshi H, Zhihui, X., Jun, W., and Qingxin, L. (**2004**). Complete 1H and 13C NMR assignments of four new steroidal glycosides from a gorgonian coral *JunceellaJuncea*. In: Magnetic resonance in Chemistry, 43:266-268.

Silva, G.L., Lee I., and Douglas K.A. (**1998**). Special problems with extraction of plants. In: Cannell J.P.R (eds). *Natural Products Isolation, Human Publishers*, New Jersey, USA, pp 251-293.

Silva, N. C. andFernandes J. A. (**2010**), Biological Properties of Medicinal Plants: A review oftheir antimicrobial activity. In: *Journal of Venomous Animals and Toxins IncludingTropical Diseases*,Brazil. vol. 16 no. 3, pp 402-413

Smith, W.B. (**1978**), "Carbon-13 NMR Spectroscopy of steroids" **in**: Webb G.A (Ed.) Annual

Reportson NMR spectroscopy" vol. 8, Academic press incorporated London, U.K. Pp199-226

Somnath, M. (**2010**). Sources of drugs. NSHM College of Pharmaceutical Technology,NSHMKnowledge Campus, Kolkata, India.

Sofowora A. (**1993**). Medicinal Plants and Traditional Medicine in Africa. 2nd Edition. SunshineHouse, Ibadan, Nigeria: Spectrum books Limited. Screening Plants for Bioactive agents;pp 134-156

Thomas, C. G. (**1979**). *Medical Microbiology*. 4th edition. Texas, U.S.A. Pp. 356-358

Trotin, F., Bezanger-Beanquesna, L., Pinkas, M. and Robelet, A. (**1973**). Research concerning two plants of Malagasy folk medicine *Cussoniabarteri* and *D. madagascariensis*Ann Pharmacy pp. 30(7-8), 555-60

Usman, H, Abdulrahman, F. I. and Ladan, A. H. (**2007**). Phytochemical and Antimicrobial Evaluation of Tribulusterresris L. (Zygophylaceae) growing in Nigeria. Journal of Biological Science, Medwell journals, 2 (3): 244-247

Vickers A. and Zollman C. (**1999**). ABC of Complementary Medicine: *Herbal medicine*. Wiley-Blackwell, *British Medical Journal*,Bristol, U.K. 319, 1050-3.

Vijay, P. (**2013**). Source of drugs. PDVVPF Medical College,ViladGhatMaharashatra, India. Pp. 2-38

Vollekova, A., KostalovaD. and Sochorova, R. (**2001**).Isoquinoline alkaloids from *Mahonia aquifolium* stem bark as active against *Malassezia Species*. Folia Microbiology, 46: 107-111.

Yushaú, M., Bukar, A. and Balarabe, A. I. (**2008**). Prevalence and Sensitivity of enterobacterial

Isolates from patients with urinary tract infections to *Acaliphawilkisenia* extracts. *Biological and Environmental Sciences Journal for the Tropics*. 5(3):72-76.