CHARACTERISATION OF THE COMPOUNDS OF THE ETHYLACETATE EXTRACT OF THE LEAVES OF *ALSTONIA BOONEI* DE WILD AND THEIR ANTIOXIDANT AND ANTIMICROBIAL POTENTIALS

OKOYE, NKEOMA NKASI

PG/Ph.D/08/50321

DEPARTMENT OF PURE AND INDUSTRIAL CHEMISTRY

FACULTY OF PHYSICAL SCIENCES

UNIVERSITY OF NIGERIA NSUKKA

SEPTEMBER, 2014

CHARACTERISATION OF THE COMPOUNDS OF THE ETHYLACETATE EXTRACT OF THE LEAVES OF *ALSTONIA BOONEI* DE WILD AND THEIR ANTIOXIDANT AND ANTIMICROBIAL POTENTIALS

OKOYE, NKEOMA NKASI

PG/Ph.D/08/50321

A THESIS SUBMITTED TO THE DEPARTMENT OF PURE AND INDUSTRIAL CHEMISTRY, FACULTY OF PHYSICAL SCIENCES
UNIVERSITY OF NIGERIA NSUKKA, IN FULFILMENT OF THE REQUIREMENTS FOR THE AWARD OF THE DEGREE OF DOCTOR OF PHYLOSOPHY (Ph. D) IN ANALYTICAL CHEMISTRY.

CERTIFICATION

Okoye, Nkeoma Nkasi, a postgraduate student with registration number: **PG/Ph.D/08/50321** has satisfactorily completed the requirements for the award of the degree of Doctor of Philosophy (Ph.D) of the Department of Pure and Industrial Chemistry, Faculty of Physical Sciences, University of Nigeria, Nsukka.

The research embodied in this thesis is original and has not been submitted in part or in full for the award of any other diploma or degree of this or any other University.

---------- ----------

Prof. C.O.B. Okoye
(Supervisor)

Dr. A.E. Ochonogor
(Head of Department)

.....

External Examiner

DEDICATION

This work is dedicated to all women, who recognise God's strength in their weaknesses and seek to allow Him manifest it.

ACKNOWLEDGEMENT

The laboratory stage as well as the writing up of this work has been filled with series of seemingly endless challenges. Coming to the stage of writing this acknowledgement, it has become obvious to me that it has actually been brought to a close. Now I can join the Germans to say, "Endlichmal"! (meaning, 'At long last'!).

Sincerely, I want to appreciate the Almighty God who made all these possible. He made me surprise myself. It scares me when I hear people say, "You are really trying in this research" because I know my usual abilities and limitation. Obviously, it's all about Him. And so I want to return all the glory to Him.

This work, however would not have been accomplished without the help of a wonderful set of people God made available to me through this period. I want to appreciate my supervisor, Prof. C. O. B. Okoye, who guided me in this work. Even when the on-going construction work in the University led to the demolition of his office block, he was not deterred but continued to raise a lot of useful contributions, questions and advice. The result is the beauty of this work.

I want to appreciate the Head of the Department of Pure and Industrial Chemistry, Prof. P. O. Ukoha and his wife, my friend, too, I appreciate, see you at the top! Dr. M.O. Agbo of the Department of Pharmaceutical and Medicinal Chemistry was also very encouraging and challenging to me.

My appreciation still go to my parents and my mother-in-law who were ready to stand in for me any time I had clashes between my role as a Ph.D student and as a mother. They regularly encouraged me to keep it up and finish the programme I had begun. My siblings, too, Ihedimma, Ada, and Ebuka have continued to see me as the woman I hope to become and this energised me to push on. I won't forget my husband's siblings, you all have been great in your own peculiar ways.

I will never forget the Alexander von Humboldt Foundation (AVH) who gave my husband the post doctoral experience, sponsored my whole family to Germany where most of the laboratory work was carried out. I also appreciate Prof. Proksch of the Institute of Pharmaceutical Biology and Biotechnology, Heinrich-Heine University, Düsseldorf, Germany who generously made every equipment, solvent and reagent in his lab freely available for this work.

My thanks also goes to Dr. Peters of the Institute of Organic Chemistry, Heinrich-Heine University, Düsseldorf, Germany who recorded the NMR of the compounds.

My regards to the Pastor, his wife, and all the members of the New Life Church, Düsseldorf, Germany. You all made our stay great. You were a family away from home.

I also appreciate so much the Society for Medicinal Plant and Natural Product Research (GA) for the Travel Grant Award they granted me back to Germany (September 1-5, 2013) in order to enable me attend the 61st International Congress and Annual Meeting in Münster, Germany. There I presented this work.

My daughters Solu, Favour and Marvy have been wonderful. With time, they learnt to give mummy time to sleep during the day, after sleepless nights.

I would want to finally appreciate my husband, friend and mentor, Dr. F. B. C. Okoye. All through this period of research, he has sponsored, encouraged, advised, taught, corrected, prayed, etc. for me. He has often ended up babysitting or assisting me in my domestic work when I found it difficult to cope.

My prayer for you all is that you will all find help in your time of need. God bless you all real good!

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF SCHEMES

LIST OF TABLES

ABSTRACT

Dried and pulverised leaves of *A. boonei* De Wild (Apocynacea) were extracted using methanol for 48 h. The methanol extract obtained was defatted using n-hexane and fractionated using ethylacetate. The ethyl acetate fraction of the extract was subjected to vacuum liquid chromatography (VLC) in silica gel using gradients of hexane-ethyl acetate. The VLC fractions were further separated on Sephadex LH-20. A total of 10 compounds were successfully isolated and purified using the reverse phase semi preparative HPLC (L-7100, Merck/Hitachi). The structures of these compounds were determined using UV, HPLC-MS, one-dimensional: ^{1}H, ^{13}C and DEPT NMR, and two-dimensional: $^{1}H^{1}H$ COSY, HMQC, and HMBC NMR. The antioxidant properties were assessed using the 1, 1-Diphenyl-2-picrylhydrazyl (DPPH) radical scavenging test. The isolated compounds were also subjected to anti-microbial studies using Agar well diffusion technique against the organisms: *Staphylococcus aureus*, *Escherichia coli, Pseudomonas aeruginosa* and *Candida albicans*. Based on the spectral data, the isolated compounds were identified as: quercetin-3-O- [Ŭ-L-rhamnopyranosyl(1Ÿ 6) ɓ-D-glucopyranoside] (**1**); quercetin-3-O- [Ŭ-L-rhamnopyranosyl(1Ÿ 6) ɓ-D-galactopyranoside] (**2**); kaempferol-3-O-[Ŭ-L-rhamnopyranosyl(1Ÿ 6) ɓ-D-glucopyranoside] (**3**); kaempferol-3-O-[Ŭ-L-rhamnopyranosyl(1Ÿ 6) ɓ-D-galactopyranoside] (**4**); quercetin-3-O-[Ŭ -L-rhamnopyranosyl(1Ÿ 4) ɓ-D-glucoctopyranoside] (**5**); kaempferol-3-O-[Ŭ -L-rhamnopyranosyl(1Ÿ 4) ɓ-D-glucopyranoside] (**6**); quercetin-3-O-[Ŭ-L-rhamnopyranosyl(1Ÿ 2) ɓ-D-glucopyranoside] (**7**); quercetin-3-O-[Ŭ-L-rhamnopyranosyl(1Ÿ 2) ɓ-D-galactopyranoside] (**8**); chlorogenic acid (**9**) and 4,5-dicaffeoylcinnamic acid (**10**). Compounds **1**, **2**, **5**, **7, 8** (derivatives of quercetin) and **9**, a caffeic acid derivative, showed a dose dependent antioxidant activity on DPPH free radical scavenging model with IC_{50} values of 52, 48, 36, 66, 56 and 22 ε g/mL respectively. The three kaempferol derivatives (**3**, **4** and **6**) showed poor anti-oxidant activity (IC_{50} >100 ε g/mL). This suggests that the presence of at least two *ortho* coupled OH groups in RING B of the flavonoid nucleus is necessary for a good antioxidant activity. Compounds **7** and **8** were active against *Escherichia coli* with MIC values of 1.77 ε g/mL and 1.92 ε g/mL respectively. The profound antioxidant activity of the isolated quercetin derivatives and chlorogenic acid may explain the ethnomedicinal use of the leaf extract in the management of inflammatory disorders. These groups of compounds isolated could be very useful for SAR studies as well as other studies on the effects of glycosyl substitution patterns on chemical shifts of the ring carbons of flavonoid nuclei.

CHAPTER ONE

1.0 INTRODUCTION

1.1 Medicinal Plants

Over the centuries humans have relied on plants for basic needs such as food, clothing, and shelter, all produced or manufactured from plant matrices (leaves, woods, fibers) and storage parts (fruits, tubers). Plants have also been utilized for additional purposes, namely: arrow and dart poisons for hunting, poisons for murder, hallucinogens used for ritualistic purposes, stimulants for endurance, and hunger suppression, as well as inebriants and medicines (Salim *et al*., 2008).They have also formed the basis of sophisticated Traditional Medicine (TM) systems that have been in existence for thousands of years and continue to provide mankind with new remedies (Barboza *et al*., 2009). They provide a large bank of rich, complex and highly varied structures which are unlikely to be synthesized in laboratories (Newman, 2008).

Some of the oldest known medicinal systems of the world such as Ayurveda of the Indus civilization, Arabian medicine of Mesopotamia, Chinese and Tibetan medicine of the Yellow River civilization of China and Kempo of the Japanese are based mostly on plants. These ancient cultures are known for their systematic collection of information on herbs and their rich and well-defined herbal pharmacopoeias. Although some of the therapeutic properties attributed to plants have proven to be erroneous, medicinal plant therapy is based on the empirical findings of hundreds and thousands of years (Gurib Fakim, 2006).

A medicinal plant is therefore defined as (1) any plant used in order to relieve, prevent or cure a disease or to alter physiological and pathological process, or (2) any plant employed as a source of drugs or their precursors (Arias, 1999). A *phytopharmaceutical preparation* or *herbal medicine* is any manufactured medicine obtained exclusively from

plants (aerial and non-aerial parts, juices, resins and oil), either in the crude state or as a pharmaceutical formulation (Rates, 2001). Phytotherapy is referred to as the study of the use of plant extracts from natural origin as medicines or health-promoting agents. The main difference between phytotherapy medicines and the medicines containing the herbal elements lies in the methods of plants processing. The preparation of medicines containing herbal elements involves the extraction of the chemically clean active substances, while in the case of phytotherapy medicines all complex active substances of plant are incorporated in the crude natural form. Phytotherapeutic medicines do not include drugs from medicinal plants made for homeopathy, anthroposophic medicine, as well as non-standardized mixture of plant and synthetic bioactive substances or isolated in a pure form from natural bioactive substances.

There is ample archaeological evidence indicating that medicinal plants were regularly employed by people in prehistoric times. In several ancient cultures botanical products were ingested for biomedically curative and psychotherapeutic purposes (Halsberstein, 2005). However, medicinal plants are constrained by procedures such as classification, identification, and characterization. Nearly 50,000 species of higher plants have been used for medicinal purposes. In systems of traditional healing, major pharmaceutical drugs have been either derived from or patterned after compounds from biological diversity (Bisset, 1994).

Plants have the ability to synthesize a wide variety of chemical compounds that are used to perform important biological functions, and to defend themselves against attack from predators such as insects, fungi and herbivorous mammals. Many of these phytochemicals have beneficial effects on long-term health when consumed by humans,

and can be used to effectively treat human diseases. The beneficial medicinal effects of these plant materials typically result from the combinations of secondary products present in the plant making the medicinal actions of plants unique to particular plant species or groups. As a result, the combinations of secondary products in a particular plant are often taxonomically distinct (Kaufman *et al*., 1999).

Phytochemicals are actually divided into (1) primary metabolites/products such as sugars and fats, which are found in all plants; and (2) secondary metabolites/products ï compounds which are found in a smaller range of plants, serving a more specific function (Meskin and Mark, 2002). For example, some secondary metabolites are toxins used to deter predation and others are pheromones used to attract insects for pollination. It is these secondary metabolites and pigments that can have therapeutic actions in humans and which can be refined to produce drugs - examples are inulin, a naturally occurring form of fruit sugar, extracted from dahlia root tubers (Williams, 1895), quinine from the cinchona, morphine and codeine from the poppy, and digoxin from the foxglove (Meskin and Mark, 2002). Chemical compounds in plants mediate their effects on the human body through processes identical to those already well understood for the chemical compounds in conventional drugs; thus herbal medicines do not differ greatly from conventional drugs in terms of how they work. This enables herbal medicines to be as effective as conventional medicines, but also gives them the same potential to cause harmful side effects (Briskin, 2000; Lai and Roy 2004; Tapsell *et al.* 2006). In contrast to synthetic pharmaceuticals based upon single chemicals, many phytomedicines exert their beneficial effects through the additive or synergistic action of several chemical compounds acting at single or multiple target sites associated with a physiological process. This synergistic or additive

pharmacological effect can be beneficial by eliminating the problematic side effects associated with the predominance of a single xenobiotic compound in the body (Tyler, 1999).

The development of ethnomedicine is usually based on information transferred from generation to generation among peoples in rural societies. This knowledge is often acquired through trial and error methods, and is majorly based on speculation and superstition. For instance, it is common knowledge that plants are more likely to survive if they contain potent compounds which deter animals from eating them. This helps the plants to survive in the midst of an adverse environmental condition, stress and competition or to overcome a prevalent infection destroying other plants around. The pharmaceutical and biotechnological industries are much interested in using this knowledge for the discovery, development and application, within biodiversity, of new active products on health and new genes with properties for food improvement (Heinrich & Gibbons, 2001). Chemical analyses and biological assays have begun to play an important role in ethnobotanical studies and there are now numerous examples where scientific analyses have provided objective evidence to validate traditional plant use, for example *Homalanthus nutans* (G. Forst.) Guill. (Euphorbiaceae), used by Samoan healers against the viral disease yellow fever; extracts have been found to exhibit potent antiviral activity, particularly against the human immunodeficiency virus HIV-1 (Balick & Cox, 1996). This however does not rule out that a lot of claims in ethnomedicine are baseless. Tyler listed some common fallacies including claims that there is a conspiracy to suppress safe and effective herbs, herbs cannot cause harm, that whole herbs are more effective than molecules isolated from the plants, herbs are superior to drugs, the doctrine of signatures (the belief that the shape of the plant indicates its function) is valid, dilution of substances increases their potency (a doctrine of the pseudoscience of homeopathy), astrological alignments are significant, animal testing is not

appropriate to indicate human effects, and that anecdotal evidence is an effective means of proving a substance works. Tyler believes that none of these beliefs have any basis (Tyler and Robbers, 1999; Tyler, 2012).

As new uses of medicinal plants are discovered and popularized, the concern for sustainability is being increasingly addressed; concern over the growth in biopiracy also combines with the critical need for the conservation of species and their habitat (Science Reference Services, 2008). A 2008 report from the Botanic Gardens Conservation International (BGCI) (representing botanic gardens in 120 countries) warned that "cures for things such as cancer and HIV may become 'extinct before they are ever found'." They identified 400 medicinal plants at risk of extinction from over-collection and deforestation, threatening the discovery of future cures for disease. This means that complementary medicinal plant/tree-planting exercises of these endangered plant species are necessary in order to preserve them.

The search for new molecules, nowadays, has taken a slightly different route where the science of ethnobotany and ethnopharmacognosy are being used as guide to lead the chemist towards different sources and classes of compounds (Gurib-Fakim, 2006). Plant derived natural products hold great promise for discovery and development of new pharmaceuticals (McChesney *et al*., 2007). Since ancient times, medicinal plants have been harvested from the wild (Mshigeni *et al*., 1991; Balick & Cox, 1996; Sheldon *et al*., 1997; Dhillion & Ampornpan, 2000; Singh & Padmalatha, 2004). In many rural communities, traditional medicine (TM) is still viewed as the mainstay of primary health care systems (Bannerman *et al*., 1983; Manandhar, 1994; Svarstad & Dhillion, 2000; Manandhar, 2002) due to its

effectiveness, cultural preference or absence of modern alternatives (Plotkin & Famolare, 1992; Taylor *et al.*, 1995; Balick *et al.*, 1996; Tabuti *et al.*, 2003).

1.2 Phytochemistry in Drug Developement

Despite the recent interest in drug discovery by molecular modelling, combinatorial chemistry, and other synthetic chemistry methods, natural-product-derived compounds are still proving to be an invaluable source of medicines for humans (Salim *et al.*, 2008). Plants remain rich sources of lead compounds (e.g. alkaloids such as, morphine, cocaine, digitalis, quinine, tubocurarine, nicotine, and muscarine). Many of these lead compounds are useful drugs in themselves (e.g. the alkaloids, morphine and quinine), and others have been the basis for synthetic drugs (e.g. local anaesthetics developed from cocaine). In 1805, morphine became the first pharmacologically active compound to be isolated in pure form from a plant, although its structure was not elucidated until 1923 (Sneader , 2005). The 19th century marked the isolation of numerous alkaloids from plants used as drugs, for instance, atropine (*Atropa belladonna*), caffeine (*Coffea arabica*), cocaine (*Erythroxylum coca*), ephedrine (*Ephedra* species), morphine and codeine (*Papaver somniferum*), pilocarpine (*Pilocarpus jaborandi* Holmes), physostigmine (*Physostigma venenosum*), quinine (*Cinchona cordifolia* Mutis ex Humb.), salicin (*Salix* species), theobromine (*Theobroma cacao*), theophylline (*Camellia sinensis*), and (+)-tubocurarine (*Chondodendron tomentosum* Ruiz & Pav.) (Sneader, 2005). Following these discoveries, bioactive secondary metabolites from plants were later utilized more widely as medicines, both in their original and modified forms (Sneader, 1996; Samuelsson, 2004).

Although relatively few plant-derived drugs have been launched into the market over the last few years, many plant-derived compounds are currently undergoing clinical trials for the

potential treatment of various diseases. The majority of such drugs under clinical development are in the oncological area, including new analogs of known anticancer drugs based on the camptothecin-, taxane-, podophyllotoxin-, or vinblastine-type skeletons (Butler, 2005).

Plants as sources or starting points of drugs is associated with some advantages, for instance, the selection of a candidate species for investigations can be done on the basis of a long-term use by humans (ethnomedicine). This approach is based on an assumption that the active compounds isolated from such plants are likely to be safer than those derived from plant species with no history of human use. On the other hand, more often than not, drug discovery and eventual commercialization would pressurize the resource substantially and might lead to undesirable environmental concerns. While synthesis of active molecule could be an option, not every molecule is amenable for complete synthesis. Hence, certain degree of dependence on the lead resource would continue. For instance, anticancer molecules like etoposide, paclitaxel, docetaxel, topotecan, and irinotecan continue to depend upon highly vulnerable plant resources for obtaining the starting material since a complete synthesis is not possible (Katiyar *et al*., 2012). One major challenge in drug development from plants remains that it is time consuming and very costly. The process of identifying the structures of active compounds from an extract could take weeks, months, or even years, depending on the complexity of the problem. Nowadays, the speed of bioassay-guided fractionation has been improved significantly by improvements in instrumentation such as high-performance liquid chromatography (HPLC) coupled to mass spectrometry (MS)/MS (liquid chromatography, LC-MS), higher magnetic field-strength nuclear magnetic resonance (NMR) instruments, and robotics to automate high-throughput bioassays (Salim *et al*., 2008). Screening of plant extract libraries can be problematic due to the presence of compounds that may either

autofluoresce or have UV absorptions that interfere with the screen readout, but fractionation of extracts can be used to alleviate some of these types of problems. Also, most high-throughput screening assay methods have been developed with computational filtering methods to identify and remove potentially problematic compounds that can give false-positive results (Walters and Namchuk, 2003).

1.3 Folkloric Uses of *Alstonia boonei*

Fresh leaves, stem bark and root bark extracts of *Alstonia boonei* de Wild have been used for centuries now in various parts of the northern tropical Africa and some parts of Asia for the treatment of various ailments ranging from malaria to inflammatory diseases as well as hypertension. Plants with such a wide range of validated folkloric use are expected to contain compounds which show good antioxidant properties. *Alstonia boonei* , especially the leaves has, however not been fully chemically investigated in order to identify the bioactive compounds.

1.4 Statement of Problem

Plants have provided humans with many of their essential needs, including life-saving pharmaceutical agents. In the last few years, some new plant-derived drugs have been launched onto the market, and many more are currently undergoing clinical trials. As a vast proportion of the available higher plant species have not yet been screened for biologically active compounds, drug discovery from plants should remain an essential component in the search for new medicines, particularly with the development of highly sensitive and versatile analytical methods (Salim *et al*., 2008).

1.5 Aims and Objectives of the Study

The aim of the study is to isolate and characterise the principles/constituents of the ethyl acetate fraction of *Alstonia boonei* de Wild leaves and trace the principles to some known pharmacological effects especially those of folkloric origin. This will enhance further studies and drug development on them. To achieve this, the following specific objectives will be pursued:

I. Extraction and fractionation to obtain the ethyl acetate fraction of the leaves of *Alstonia boonei* de Wild.

II. Isolation of the constituents by vacuum column chromatography, separation and purification using HPLC.

III. Structure elucidation of the isolated constituents by a combination of UV, HPLC-EIMS, 1D ^{1}H-NMR, COSY, HMBC, HMQC, ^{13}C-NMR, and DEPT analyses.

IV. Screening of the isolated constituents for antimicrobial and antioxidant effects using established *in vitro* models.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Common phytochemicals in plants

In order to understand the biological activity of a plant, be it medicinal, poisonous, or nutritive, it is necessary to know its chemical constituents. Thise phytochemicals include the primary and secondary metabolites (e.g. alkaloids, terpenoids, phenolics, gums, mucilages, carbohydrates, amino acids, proteins, fatty acids, glycolipids, etc.) that organize medicinal plants (Croteau *et al*., 2000). The medicinal properties of plants are mainly due to the presence of the secondary metabolites which the plants need in their natural environments.

Some of the commonly reported phytochemicals in plants are:

I. Alkaloid
II. Triterpenoids
III. Saponin
IV. Tannin
V. Steroids
VI. Flavonoids
VII. Cardiac glycosides

2.1.1 Alkaloids. These are a large group of pharmacologically active nitrogen containing organic compounds of plants, microbial or animal origin. In most alkaloids, the nitrogen atom is a part of the ring. They are biosynthetically derived from amino acids. The name alkaloid is derived from the word ólalkalineô, which means a water soluble base. A number of natural alkaloids and their derivatives have been developed as drugs to treat various diseases e.g. morphine, reserpine, nicotine and taxol. Alkaloids are usually insoluble in water but soluble

in ethanol, ether, chloroform and other organic solvents. They are basic in nature, forming water soluble salts. The degree of basicity varies considerably depending on the structure of the molecule, and presence and location of the functional groups. Most alkaloids are bitter in taste. They contain oxygen and are usually colourless crystals at ambient conditions. Oxygen free alkaloids for instance nicotine or conine are typically volatile, colourless, oily liquids. There are however some alkaloids that are coloured eg berberine (yellow) and sanguinarine (orange). Alkaloids precipitate with heavy metal iodides (except caffeine). In plants, alkaloids may exist in the free state, as salts or as N-oxides.

Many of the earliest isolated pure compounds with biological activity were alkaloids. Naturally occurring alkaloids constitute the pharmacogenically active basic principles of flowering plants. Alkaloids have been divided into three major classes depending on the precursors and the final structure. They can also be classified in terms of their biological activity, chemical structure (the nucleus must contain nitrogen) or their biosynthetic pathway. Common alkaloid ring structures include the pyridines, pyrroles, indoles, pyrrolidines, isoquinolines, and piperidines. Several plants of the *Alstonia* species have been reported to contain the monoterpene indole group of alkaloids.

2.1.2 Triterpenoids/Terpenes: Terpene is a generic name of a group of natural products structurally based on the isoprene (Isopentenyl) units. They are one of the largest and most diverse groups of plant secondary metabolites. They include sterols and triterpenes, complex compounds that are formed by the cyclization of 2,3-oxidosqualene. Sterols and triterpenes can accumulate as glycoside conjugates in substantial quantities in plants (Ncube *et al*., 2008).

Classifications of Terpenes:

Terpenes are normally grouped based on the number of isoprene units from which they are biogenetically derived. The higher terpenes are further subdivided into several subgroups based on the type of skeletons they posses.

(a) Hemiterpenes: These are terpenes made up of just one isoprene unit. e. g Isoprene.

(b) Monoterpenes: This class of terpenes contains two isoprene units. They are found mostly in essential oils. They are useful in perfumery and flavour industries. Essential oils are reported to possess some biological activities including antibacterial, antiviral, antifungal and antiinflammatory effects (Ncube *et al*., 2008).

(c) Sesquiterpenes: These contain three isoprene units. They are found mainly in higher plants.

(d) Diterpenes: They contain four isoprene units.

(e) Triterpenes: They contain thirty carbon atoms made from six isoprene units. They are believed to be derived from squalene which is formed from a head to head coupling of two sesquiterpenoid units. Triterpenes may be tetracyclic or pentacyclic, and best classified biogenetically.

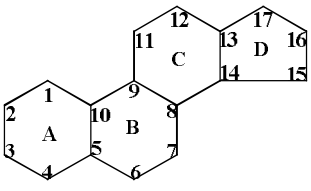
(f) Tetrapenes: These are compounds based on eight isoprene units. Important among these are the C-40 carotenoids.

(g) Terpenoids often refer to oxygen derivatives of terpenes. They are compounds derived from a combination of two or more isoprene units. Terpenoids encompass a large and diverse group of naturally occurring 30-carbon compounds derived from squalene. A number of triterpenoids are bioactive and are useful in medicine.

2.1.3 Saponins. Saponins are glycosides with a distinctive foaming characteristic. They are found in many plants especially certain desert plants but get their names from the soap worth plant (Saponaria, Caryophyllacae), the root of which was used historically as soap. Saponins consist of polycyclic aglycones attached to one or more sugar side chain. The aglycone part which is also called sapogenin is either a steroid (C27) or a triterpene (C30). The foaming ability of saponins is caused by the combination of a hydrophobic sapogenin and a hydro phyllic sugar part. They generally have bitter taste. Some saponins are toxic and are known as sapotoxins.

A range of pharmacological effects have been reported for saponins among which are anti inflammatory effects, anti neoplastic effects, cholesterol lowering effect hemolytic, expectorative,. They demonstrate antimicrobial properties particularly against fungi and additionally against bacteria and protozoa. They also build the immune system (immune-stimulating activity) as well as promote wound healing.

2.1.4 Steroids. These are chemical messengers known as hormones. They are lipids characterised by a common basic ring structure: three fused cyclohexane rings (phenanthrene part) fused to a cyclopentane ring system. This is referred to as the cyclopentaphenanthrene ring (**1**)



1 (Cyclopentaphenanthrene Ring Structure)

Many steroids have methyl groups at C-10 and C-13 positions. These are called the angular methyl groups. Many steroids have an alcoholic hydroxyl attached to the ring system and are known as sterols. The most common sterol is the cholesterol which occurs in most animal tissues. There are many different steroids hormones, and cholesterol is the precursor for all of them. Cholesterol is also the precursor of vitamin D.

Types of steroids

(a) Anabolic steroids or anabolic androgenic steroids: These are a class of natural and synthetic steroids that interact with androgen receptors to promote cell growth and division, resulting in growth of several types of tissues especially muscles and bones. There are natural and synthetic anabolic steroids eg. testosterone, nandrolone and methandrotenolone.

(b) Corticosteroids (glucocorticoids and mineralocorticoids): Glucocorticoids are a class of steroidal hormones characterised by the ability to bind with the cortisol receptor and trigger similar effects. They regulate many aspects of metabolism and immune functions, and are often prescribed as a remedy for inflammatory conditions such as asthma and arthritis e.g. Cortisol. Mineralcorticoids are corticosteroids that help maintain blood volume and control renal excretion of electrolytes e.g. Aldosterone

(c) Sex steroids or gonadal steroids: These are a subset of sex hormones that interact with vertebrate androgen or oestrogen receptors to produce sex differences (primary and secondary sex characters) and support reproduction. E.g. androgens, oestrogens, and progestagens.

(d) Phytosterols or plant sterols: They are steroid alcohols that occur naturally in plants. E.g. ɓ-sitosterol.

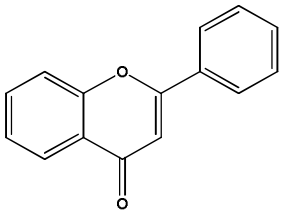
(e) Ergosterols: These are steroids that occur in fungi. They include some vitamin D supplements.

2.1.5 Flavonoids. Flavonoids generally occur in plants as glycosylated derivatives and they contribute to the brilliant shades of blue, scarlet and orange in leaves, flowers and fruits. They are also found in seeds, nuts, grain, spices and different medicinal plants as well as in beverages such as wine (particularly red wine), tea and at low levels, beer (Kuhnau, 1976)

Flavonoids are a class of water soluble plant pigments. Though they are not considered essential nutrient, they are a class of plant secondary metabolites originally referred to as vitamin P, likely due to the effect they had on the permeability of vascular capillaries. The original "flavonoid" research apparently began in 1936, when Hungarian scientist Albert Szent-Gyorgi was uncovering a synergy between pure vitamin C and as yet unidentified co-factors from the peels of lemons, which he first called "citrin," and, later, "vitamin P" This term is rarely used now (Murray, 1998). The name flavonoid (biflavonoid) comes from the Latin word flavus meaning yellow, which is the natural colour of flavonoids.

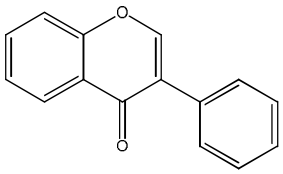
According to the IUPAC nomenclature, flavonoids can be classified into:

(a) flavonoids, derived from 2-phenyl chromen-4-one (2-phenyl-1,4-benzopyrone) structure (**2**). e.g. Quercetin)



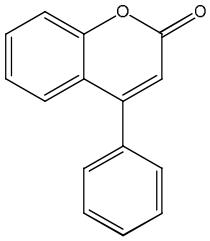
2 [2-phenyl chromen-4-one (2-phenyl-1,4-benzopyrone]

(b) isoflavonoids, derived from 3-phenylchromen-4-one (3-phenyl-1,4-benzopyrone) structure (**3**).



3 [3-phenylchromen-4-one (3-phenyl-1,4-benzopyrone)]

(c) neoflavanoids, derived from 4-phenylcoumarine (4-phenyl-1,2-benzopyrone) structure (**4**).



4 [4-phenylcoumarine (4-phenyl-1,2-benzopyrone)].

Flavonoids have been referred to as natureôs óbiological response modifiersô because of strong experimental evidence of their inherent ability to modify the bodyôs reaction to allergens, viruses and carcinogens (Yamamoto and Gaynor, 2001). They play different roles in the ecology of plants. Because of their astringency, cathechins and other flavonols can represent a defense system against insects harmful to the plant (Mazza and Miniati 1993). Cyanidin-3-O-glucoside has been reported to inhibit the growth of larvae of tobacco budworm *Heliothis virescen*, an important pest of cotton and other crops. Anthocyanins serve as antifungal agents in red coloured tropical young leaves as well as assist in heavy metal tolerance of plants.

Parasitic plants often use chemicals released by their host plant to stimulate seed germination, to locate the host, or for development. Many different compounds are involved, including

strigolactones, quinines, coumarins, flavonoids, and other phenolics. Flavonoids contribute to signalling in some species (Andersen and Markham, 2006).

Flavonoids give protection against UV radiation. UV light is a potential hazard to plants because it can damage DNA and impair various physiological processes. Plants often respond to UV light by the action of flavonoid biosynthetic genes with favorable UV absorbing properties. Flavonoids therefore protect plants from UV (especially UV-B) radiation of the sun and scavenge UV-generated reactive oxygen species (ROS) (Shirly, 1996; Andersen and Markham, 2006).

Anthocyanins, flavonols, or chalcones which often have attractive colours accumulate in both male and female sex organs in a flower, including the pollen grains (Farrant 2000). Insects can detect light from the UV to orange waveband (Primack, 1982) and may act as visual signals for pollinating insects.

Flavonoids act as catalysts in the light phase of photosynthesis/or as regulators of iron channels involved in phosphorelation (Pietta and Simonetti 1999). They can also function as stress protectants in plant cell by scavenging reactive oxygen species produced by the photosynthetic electron transport system (Harbourne 1986).

Flavonoids have been more often used than any other group of plant substances in chemotaxonomic studies. Flavonoids have traditionally been thought of as waste products and their functions largely remain obscure. Many act as flower pigments while some have been implicated in the control of Indole Acetic Acid (IAA) activity. For example, flavonols, such as quercitin, with two hydroxyls in the ɗ ring (forming a catechol group) inhibit IAA oxidase while flavonols with one hydroxyl (e.g. kaempferol) promote the enzyme's activity. Other flavonoids, especially certain of the isoflavonoids, serve as phytoalexins.

By the year 2000, more than 7000 flavonoids had been identified in plants with the list constantly increasing (Harbourne and Williams 2000). This is because of the occurrence of numerous substitution patterns in which primary substituents themselves can be substituented sometimes yielding highly complex structures (DôArchivio *et al.,* 2007).

In vitro studies have shown that some flavonoids have anti allergenic, anti inflammatory (Yamamoto and Gaynor 2001), anti microbial (Cushnie and Lamb 2006), anticancer (Chahar *et al*., 2011) as well as anti diarrheal (Yao *et al*., 2011) activities. Some flavonoids show anti histaminic and antiviral, antioxidant and anti mutagenic properties. Others such as anthocyanins from bilberry, purple cabbage and grapes may protect the lens of the eye from cataracts. Studies using animal models suggest that naringenin found in grapefruit, may have anticancer activity (So *et al*., 1996).

Flavonoids belong to a group of phytochemicals known as the phenolics. Phenolics belong to a large group of phenolic secondary metabolite compounds of plant origin. They have a wide range of functions in plants including defence against herbivores, pathogens aggression or other sources of injury; structural components of cell walls; protection from ultraviolet radiation; pigments and signaling molecule (Bravo, 1998). They are attracting increasing attention due to their reputed beneficial effects on human health protection. They are reported to play a role in the prevention of cardiovascular diseases, cancer, diabetes mellitus and neurodegenerative disease (Scalbert *et al*., 2005). Particularly for diseases associated with oxidative stress, polyphenols inhibit the oxidation of cholesterol and formation of low density lipo-proteins (LDP) particle that are involved in atherosclerosis (Marrugat *et al*., 2004). The main groups of polyphenols are: flavonoids, tannins, phenolic acids, stilbenes and lignans.

2.1.6 Glycosides: These are molecules in which a sugar is bound to a non carbohydrate moiety, usually a small organic molecule. They are composed of two moieties:

the sugar portion (glycone) and the non-sugar portion (aglycone or genin). They yield one or more sugars upon hydrolysis. Glycosides play numerous important roles in living organisms. Most plants store chemicals in the form of inactive glycosides. These can be activated by enzyme hydrolysis which causes the sugar part to be broken off, making the chemical available for use. Many such plant glycosides are used as medications. In animals and humans, poisons are often bound to sugar molecules as part of their elimination from the body.

Several glycosides are formed from polyphenols, phenols, steroidal and terpenoidal alcohols through glycosidic linkages to sugars. Among the sugars found in natural glycoside, D-glucose is the most prevalent one but L-rhamnose, D- and L- fructose and L- arabinose also occur quite frequently. Of the pentoses, L-arabinose is more common than D-xylose (Sarker and Nahar, 2007).

Glycosides can also be referred to as any molecule in which a sugar group is bonded through its anomeric carbon to another group via a glycosidic bond . Glycosides can be linked by an O- (an *O*-glycoside), N- (a glycosylamine), S-(a thioglycoside), or C- (a *C*-glycoside) glycosidic bond.

The anomeric proton

Cyclisation of sugars leading to the formation of a cyclic hemiacetal or hemiketal occurs due to intramolecular nucleophillic addition reaction between ï OH and a C=O group. This produces a new chiral centre at C-1 in the cyclic form. This carbon is called the anomeric carbon. On the anomeric carbon, is attached, the anomeric proton and the ï OH group. The anomeric proton could be axial (ɓ-configuration) or equatorial (Ŭ-configuration) (**5**).

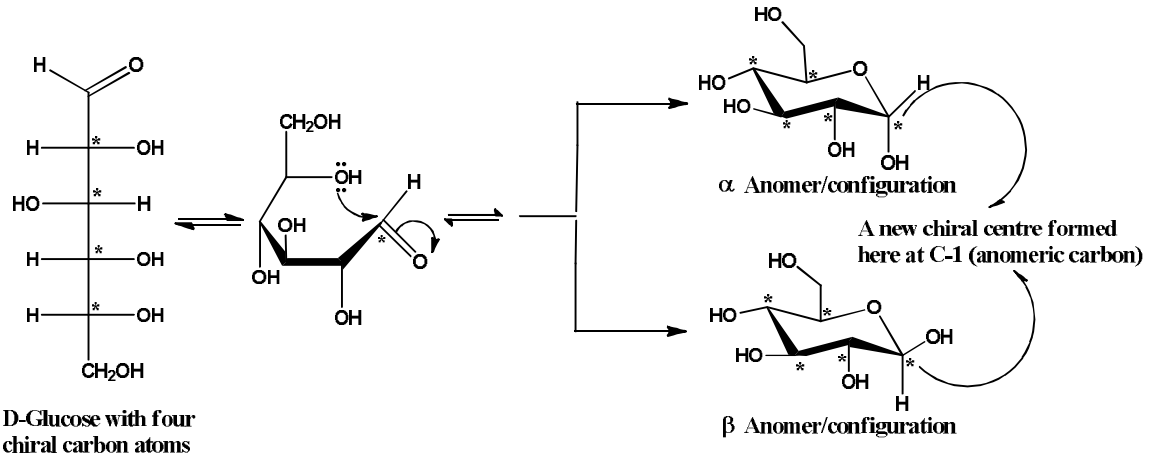


Figure 2.1 (Cyclisation of Glucose)

2.1.7 Tannins. They are a heterogeneous group of natural products widely distributed in the plant kingdom. They are often present in unripe fruits but disappear during ripening.

There are two distinct types of tannins: condensed tannins whose fundamental structural unit is the phenolic flavan-3-ol (cathechin) and the hydrolysable tannins which are polymers composed of a monosaccharide core (most often glucose) with several catechin derivatives attached. The two types of tannins have most properties in common, but hydrolysable tannins are less stable and have greater potential to cause toxicity.

The water solubility is restricted and decrease in general with the size of the tannin molecule. Tannins indiscriminately bind to proteins and larger tannins are used as astringents in cases of diarrhoea, skin bleedings and transudates. Examples of plant families associated with presence of tannins are Fagaceae (beech family) and Polygonaceae (knotweed family).

The name tannin is derived from the ability to tan leather and is not based on any class of compounds with a common basic structure (Finar, 1997). They precipitate proteins and many alkaloids from their solutions. Many human physiological activities, such as stimulation of

phagocytic cells, host-mediated tumor activity and a wide range of anti-infective actions have been associated with tannins (Ncube *et al.*, 2008). Tannins have been traditionally used for the protection of inflamed surfaces of the mouth and treatment of catarrh, wounds, hemorrhoids and diarrhea (Ogunleye and Ibitoye, 2003; Okwu and Okwu 2004). As a group, Tanins have been found to stimulate macrophages, which could have an indirect negative effect on infections (Cowan, 1999).

2.2 The Plant *Alstonia boonei* De Wild

Alstonia is a wide spread genus of evergreen trees and shrubs from the Dogbane family (Apocynaecae). Apocynaecae family consists of about 50 species widely distributed in Africa, Asia and America. *Alstonia* was named by Robert Brown in 1811 after Charles Alston (1685-1760), a Professor of Botany at Edinburgh from 1716-1760.

2.2.1 Taxonomy

Kingdom:	Plantae
Division:	Sympetalae
Order:	Gentianales
Class:	Asterids
Family:	Apocynaceae
Subfamily:	Plumerioideae
Tribe:	*Alstoninae*
Genus:	*Alstonia*
Species:	*Alstonia boonei*

(Trease and Evans, 2002)

2.2.2 Ethnobotanical uses of *Alstonia boonei*

Alstonia boonei De Wild (Apocynaecae) is a medicinal plant that is widely used across Africa for various ailments. A cold infusion of the stem bark in palm wine with fruits of *Capsicum frutensis* or a decoction of stem bark of *A. boonei*, *Khaya grandifoliola*, *Cleistopholia patens* and some quantity of red small pepper (*Capsicum frutensis*) in palm wine is a remedy for malaria. An infusion of the fresh stem bark is used as an effective antidote against snake, rat or scorpion poison. It is also used in treating painful micturition and rheumatic conditions (Ojewole, 1984; Asuzu and Anaga, 1991). An infusion of stem bark of *A. boonei* and a bunch of *Piper guineensis* fruit in local gin is drunk once daily to treat impotence. Dosage never exceeds 1 tablespoonful per day. An infusion of the stem bark in cold water is drunk or used for bathing as a remedy for dizziness. Breast pains are treated with native soap and bathing the affected part with extracts from young leaves of *A. boonei* in water. An infusion of stem bark is drunk as a cure for round worms, thread worms and other intestinal parasites in children. An infusion of root and stem bark and leaves is drunk as a remedy for asthma. The bark of *Alstonia* tree is one of the effective analgesic herbs available in nature (Abbiw, 1990). A decoction could be sweetened with pure honey and be taken up to 4 times daily as an effective painkiller. The leaves and latex of *A. boonei* are used topically to reduce swellings, for the treatment of rheumatic pains, muscular pains and hypertension (Iwu, 1993). Fresh decoction of the stem bark of *A. boonei* is administered for the management tooth ache, painful menstruation (dysmenorrhoea), when associated with uterine fibroid or ovarian cysts in women; lower abdominal and pelvic congestion associated with gynaecological problems such as pelvic inflammatory diseases (Adotey *et al*., 2012). It is also used to relieve the painful urethritis common with gonococcus or other microbial infections in men. *Alstonia boonei* decoction also exerts a mild antibacterial effect in this case. A cold infusion made

from the fresh or dried bark of *Alstonia boonei* taken orally two to three times daily exerts a mild hypoglycaemic effect on diabetic patients.

A. boonei has been listed in the African Pharmacopoeia as an antimalarial drug. It is also useful in expelling retained products of conception and afterbirth (like the placenta) (Orwa *et al*., 2009). Asthma can also be treated with a drink prepared from parts of *Trema orientalis* and decoction of the bark of *Alstonia boonei* mixed with the roots and bark of cola and fruits of *Xylopia parviflora* with hard potash (Akinmoldun *et al*., 2007). The bark decoction of *Alstonia boonei* is used with other preparations in the treatment of fractures or dislocation jaundice, and for inducing breast milk. Its latex is taken as a purgative. The hardened latex is used for the treatment of yaws (Abbiw, 1990). *Alstonia boonei* De Wild is regarded as one of few herbs with potential anti-HIV indicators (Orwa *et al*., 2009).

The triterpenes present in *A. boonei* however have been reported to show varying degrees of toxicity to the liver, kidney or spleen which may limit their benefits in antiarthritic therapy (Kweifo-Okai and Caroll, 1992). In Ghana, the root barks are often used in combination with *Elias guineensis* nuts to reduce toxicity and *Rauvolfia vomitoria* to provide sedative cover during antiarithmatic therapy (Kweifio-Okai, 1991a). The basis for the reduction of toxicity when taken with *E. guineensis* is not known except for the knowledge that esterification normally reduces toxicity of theurapeutic agents and that an esterified triterpene, Ŭ-amyrin palmitate, was identified in a crude extract of the herbal plants shown to reduce carageenin pedal oedema and adjuvant arthritis in rats (Kweifio-Okai, 1991a, b). It was suggested that the demonstration of antiarthritic effect for Ŭ-amyrin palmitate with reduced toxicity is a new approach for therapeutic intervention in rheumatoid arthritis based on triterpene compounds.

Fresh leaves and stem bark of *A.boonei* show good insecticidal activity against *Sesamia calamistis* making it an option as a crop protectant against the insect and possibly other pest

species (Oigiangbe *et al*., 2007). In some African countries *Alstonia boonei* is considered a sacred tree and worshiped in the forest and hence human beings in those countries do not eat its parts. Other folkloric uses of *Alstonia boonei* include in the treatment of oedema, sores, ulcers, cancer, hypertension, insomnia, arrow poison and chronic diarrhoea (Olajide *et al.,* 2000).

2.2.3 Ecology and Distribution

Alstonia boonei is a light demanding species of *Alstonia* (Voorhoeve, 1965), widely distributed in the northern tropical Africa (Senegal, Gambia, Guinea, Ivory Coast, Ghana, Nigeria, Cameroon, Sudan, Uganda) but may extend into drier types, including gentle, to even, steep, rocky hill sites in Liberia. It is most commonly found scattered or in small groups in wet or marshy places that are occasionally inundated.

2.2.4 Description of *Alstonia boonei*

Alstonia boonei De Wild is one of the two indigenous species of *Alstonia* in Africa (Palla, 2005). In Nigeria, it is commonly called Egbu, Egbu-Ora (Igbo), Awun (Yoruba), Ukhu (Bini), Okugbe (Itsekiri, Ukpukuhu (Urhobo), Ndondo (Ijaw), Ukpo (Efik). In other African countries: Osen-nuri, Onyame-dua (Ashanti, Twi :-Ghana);, Etiap (Ekoi), Bokuk (Boki),Ouguie, Sinduru (Ivory Coast), Tsonguti, Bokuka, Otando (Zaire), Myna, Mujwe (Uganda), Kaiwi (Sierra Leone), Ekouk, Kanja (Guinea), Kinje, Kaika (Gabon) Nfomba, Ubangi, Moguga (Angola). Emien (Trade name); Yung (Gio), It is also referred to as cheesewood, stoolwood, patternwood (Palla, 2005).

Fig 2.2: ***Alstonia boonei*** **De Wild (Apocynacea)**

Alstonia boonei is a large deciduous tree up to 45 m tall and 1.2 m in diameter. Its bole could be branchless up to 25 m. Its bark is greyish-green or grey, smooth or rough, exuding copious milky latex. It branches in whorls. The leaves are simple, subsessile to petiolate, stipules are absent; petiole is 2-10 (-15) mm long, and stout; blade is oblanceolate to obovate, rarely elliptic, 7-26 cm long, 3-9. 3 cm wide, apex is acute to rounded or sometimes emarginate, base narrowly cuneate, margins are entire, subcoriaceous to coriaceous, dark shiny green above, light green below, midrib more prominent below, lateral veins 25-50, more-or-less at right angles to midrib, parallel, anastomising and very close to the margin, occurs in whorls of 4-8. The flowers are bisexual, regular, fragrant and hermaphrodite. The fruits are composed of two linear follicles with many oblong flat seeds (Palla, 2005). Flowering occurs from October to March and fruiting between December and May.

2.2.5 Pharmacological Properties of *Alstonia boonei* already Reported

Alstonia boonei stem bark has been reported to possess anti pyretic, analgesic and anti inflammatory properties (Olajide *et al*., 2000, Osadebe, 2002). The stem bark is also reported to have potent neuroleptic and anxiolytic properties in mice (Elizabetsky and Costa-Campos,

2006). Independent studies by Odugbemi and Akinsulire (2007) and Olajide *et al*., (2000) confirmed indigenous medicinal usefulness of *Alstonia boonei* for malaria therapeutic usage in south-western part of Nigeria. Echitamine, an alkaloid largely reported to be present in *Alstonia boonei* has been validated to be cytotoxic *in vivo* and *in vitro* against fibrosarcoma in rats and sarcoma-180 in mice (Kamarajan *et al*., 1991, Saraswathi *et al.,* 1998a). It has also been shown to increase the cytotoxic effect of doxorubicin, cyclophosphamide and berberine *in vivo* (Saraswathi *et al.,* 1997). In order to test the anticancer activity of echitamine in various systems, establishing its efficacy, another research team (Jagetia *et al*., 2005) evaluated the anticancer activity of echitamine in various cultured neoplastic cancer cells of human origin and in mice bearing Ehrlich ascities carcinoma. Their results further validated the *in vitro* and *in vivo* antitumor activity echitamine. *Alstonia boonei* triterpenes also show antiarthritic (Kwefio-Okai and Caroll, 1992), anti proteolytic (Chaturvedi *et al*., 1974) as well as varying degrees of toxicity to the liver, kidney or spleen which may limit their benefits in antiarthritic therapy (Kweifio-Okai, 1991a). Other pharmacological activities of *Alstonia boonei* include: immuno stimulant antihelminthic (Wesche *et al*., 1990) hypotensive (Akinloye *et al*., 2013, Iwu, 1993, Sabu and Kuttan, 2002) aphrodisiac and antidiabetic (Adotey *et al.*, 2012) properties. The wide range of medicinal activity could be traceable to its antioxidant activity. Antioxidant substances block the action of free radicals which have been implicated in the pathogenesis of many diseases. In addition to all these, the insecticidal activity of *A. boonei* has been validated by Oigiangbe and his colleagues (Oigiangbe *et al.,* 2007) against *Sesamia calamistis* Hampson.

2.2.6 Other Uses of *Alstonia boonei*

The wood of *Alstonia boonei*, called alstonia in international trade, is used for light construction, light carpentry, open boats, moulding, furniture, interior joinery, implements,

boxes, crates, matches, pencils, sculptures, and for veneer and plywood. It is locally popular for the production of household implements because of its good working properties and stability. In Ghana it is used for the famous Asante stool and in Nigeria for sound boxes of musical instruments of the Yoruba people. The latex has been used as birdlime and as an inferior alternative to rubber.

2.2.7 Review of some Secondary Metabolites Isolated from *Alstonia* species

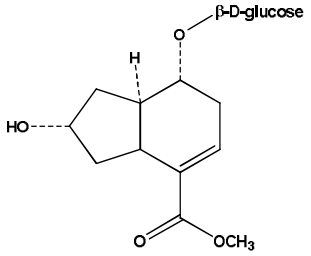
Alstonia which has over 40 species, grow widely in the tropical regions of Africa and Asia. Hundreds of compounds from *Alstonia* species have been isolated and characterised. Most of the compounds identified so far belong to the monoterpene indole alkaloid and quinolone alkaloids and are thought to originate from the condensation of tryptophan with secologanin. *Alstonia* species are rich in alkaloids, steroids, terpenoids and flavonoids (Arulmohzhi *et al*., 2007, Nadkarni, 1976).

It has been suggested that the existence of the monoterpene indole alkaloids is related to the plantôs location. For instance, Macebeo *et al.,* (2005) reported that the picrine type indole alkaloids are generally found in *Alstonia* plants from continental countries such as India, Pakistan and Thailand while alkaloids bearing the angustilobane skeleton exist predominantly in those in Indonesia and the Phillipines.

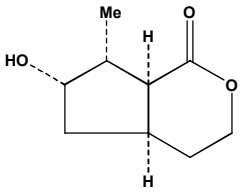
Chromatography of bark extracts of *Alstonia boonei* on silica gel plates with the solvent system AcOEt-MeOH-H_2O (150ززز26ززز19) produced 6 separate spots with alkaloid reactions and the alkaloids isolated from the plant include echitamine and echitamidine, voacangine and akuammidine, Nα-formylechitamidine and Nα-formyl-12-methoxyechitamidine (Ayiku, 1992),(Kucera *et al.,* 1972), Croquelois *et al.,* 1972, Oguakwa, 1984, Shang *et al*., 2010). Echitamine, which is also isolated from the bark of *Alstonia scholaris*, *Alstonia cogenesis*,

and *Alstonia neriifolia*, has been assigned the nomenclature [(3*β*, 16R)-3,17-Dihydroxy-16-(methoxycarbbonyl)-4-methyl-2-4(14)-cyclo-3, 4-secoakuammilinium. Echitamidine, on the other hand, has a molecular formula of $C_{20}H_{22}O_3N_2$, with a melting point of 244°C and molecular weight of 338 g/mol.

These alkaloids, especially echitamine, possess a battery of pharmacological and autonomic activities (Ojewole, 1984, 1983) including anticancer activities (Chandrasekaran and Nagarajan, 1983; Saraswathi *et al.,* 1998, Saraswathi *et al.,* 1999). Alkaloids and related compounds isolated from other *Alstonia* species include the glycosides of venoterpene (Biswas and Saharia, 1978), nareline, (Kam *et al.,* 1997; Morita *et al*, 1977) lagunamine, (Yamauchi 1990) 19-epischolaricine (Yamauchi, 1990) butamine, dobutamine (Yamauchi, 1990; Boonchuay and Court 1976), alschomine (Abe *et al* 1989), N*α*-methylbutylamine, isoaslchomine (Abe *et al*., 1989), picrinine (Ghosh *et al.,* 1988), strictamine, (Banerji and Chakrabarti 1984, Bhattacharya,1979), rhazimanine (Rahman and Alvi, 1987, vallesamine (Rahman *et al.,* 1987), (20S)-19,20 dihydrocondylocarpine (Rahman 1986), scholarine (Banerji and Siddhanta, 1981), pseudoakuammigine (Banerji and Banerji, 1977), tetrahydroalstonine (Dutta, 1976), akuammicine (Boonchuay and Court 1976), picralinal (Rastogi,1970, rhazine,1969) scorlarine-N(4)- oxide, scholaricine (Rahman 1985, Banerji, *et al*., 1984), and flavone glycosides (Chauhan, 1985, Desoky, 1999). Other compounds isolated from *A. boonei* include: loganin (**5**), an iridoid is a key intermediate in the biosynthesis of indole alkaloids. Boonein, a C-9 monoterpenoid *α*-lactone has also been isolated from *A. boonei* (**6**). It is a possible precursor in the indole alkaloid biogenesis (Marini-Bettolo *et al.,* 1983).



5 (Loganin)

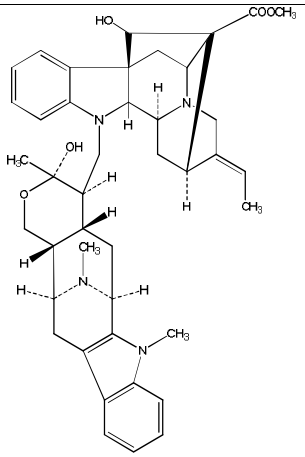
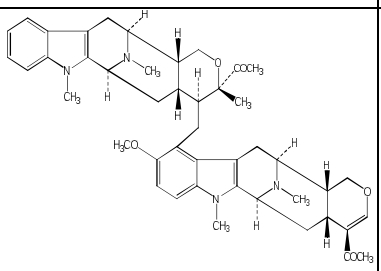
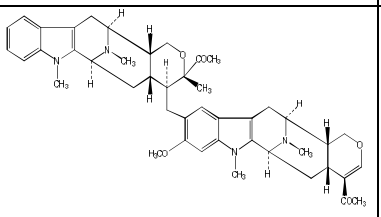
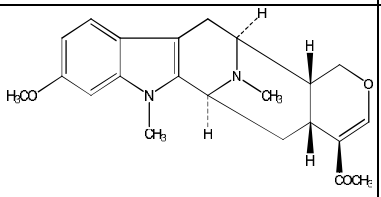
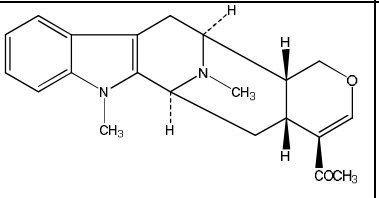


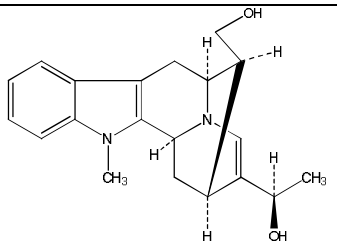
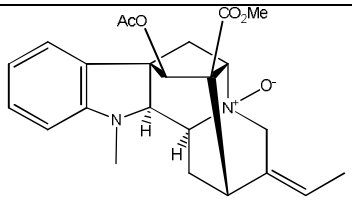
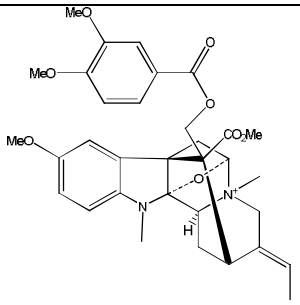
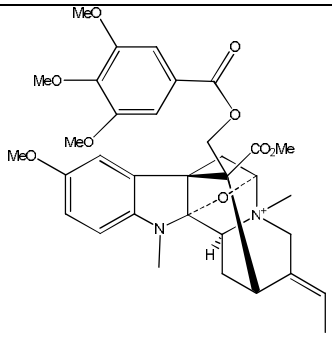
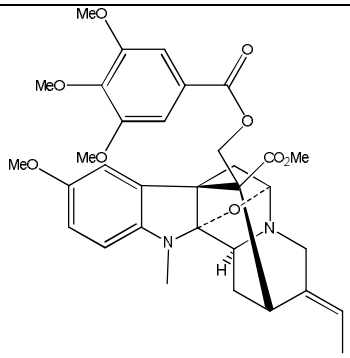
6 (Boonein)

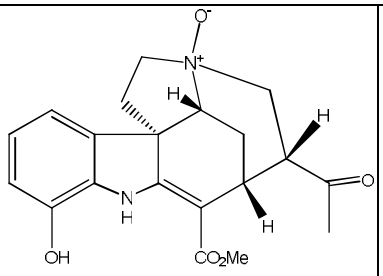
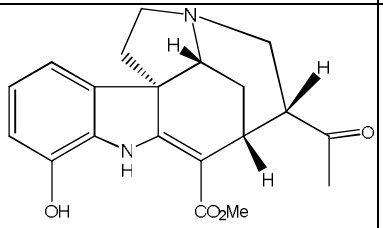
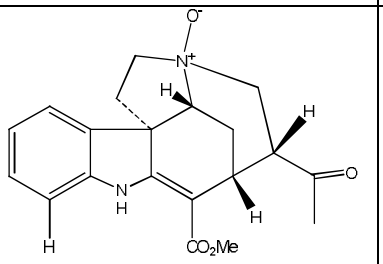
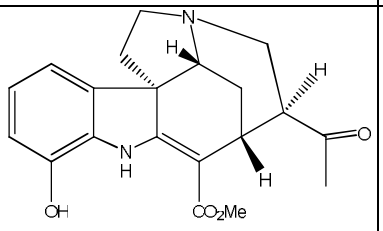
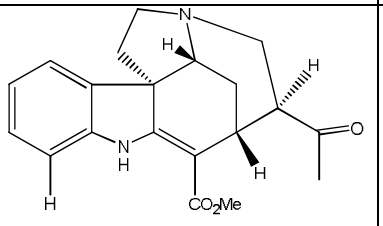
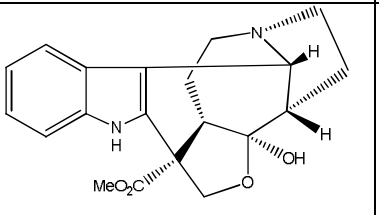
2.2.7.1 Alkaloids from *Alstonia* species

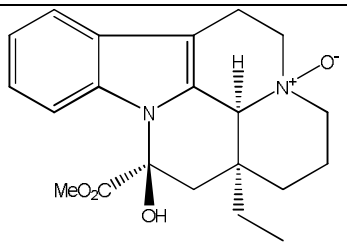
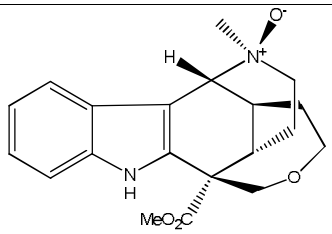
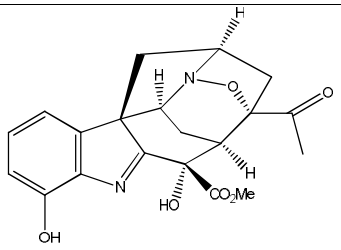
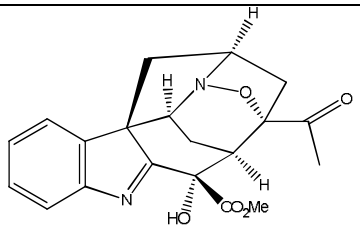
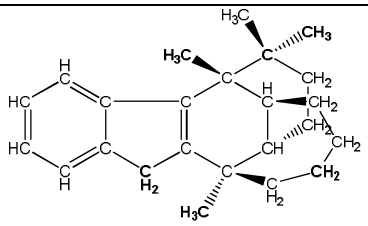
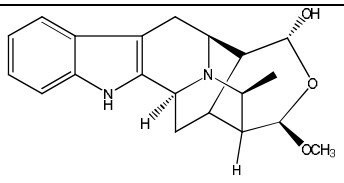
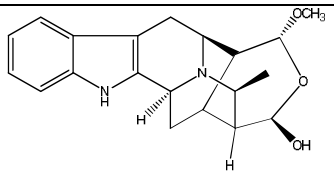
Some of the alkaloids, their sources and structures are summarised in Table 2.1.

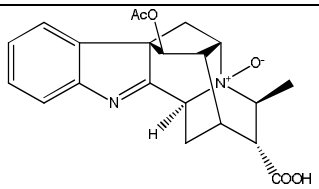
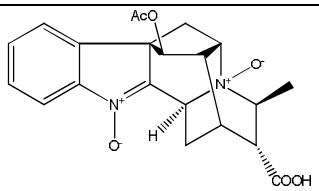
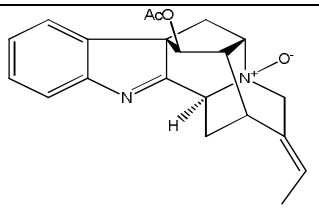
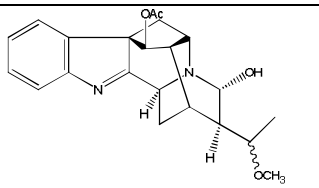
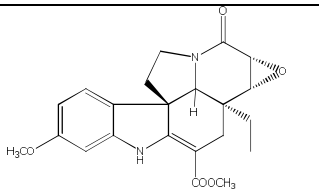
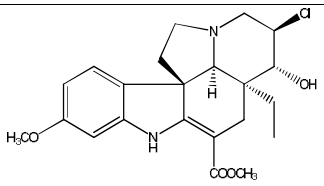
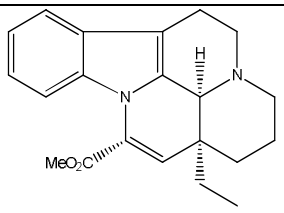
Table 2.1: Chemical Structures of some Alkaloids Isolated from *Alstonia* spp.

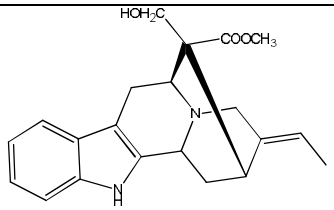
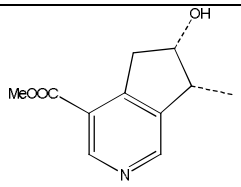
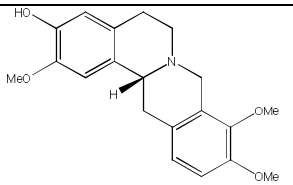
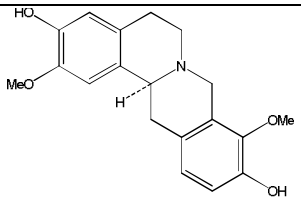
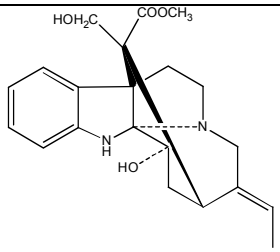
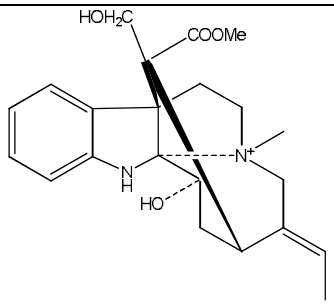
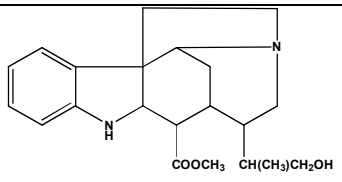
Compound	Source	Structure	Reference
---	---		Keawpradub *et al.*, 1999
Alstomacrophylline	*A.macrophylla*		Keawpradub *et al.*, 1999
O-Acetylmacralstonine	*A.macrophylla*		Keawpradub *et al* .,1999
Alstophylline	*A.macrophylla*		Keawpradub *et al.*, 1999
Alstonerine	*A.macrophylla*		Keawpradub *et al.*, 1999

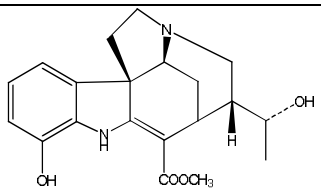
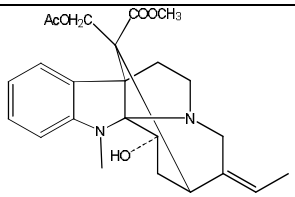
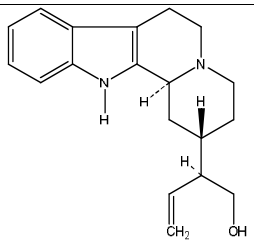
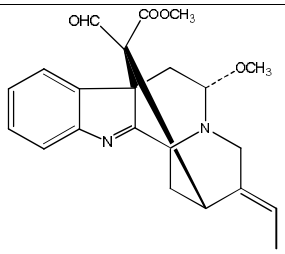
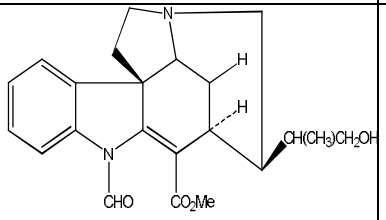
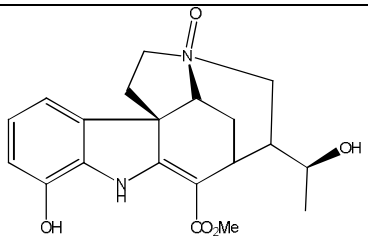
Alstoumerine	*A.macrophylla*		Keawpradub *et al.,* 1999
Alstiphyllanine A	*A.macrophylla*		Hirasawa *et al* .,2009
Alstiphyllanine B	*A.macrophylla*		Hirasawa *et al* .,2009
Alstiphyllanine C	*A.macrophylla*		Hirasawa *et al* .,2009
Alstiphyllanine D	*A.macrophylla*		Hirasawa *et al* .,2009

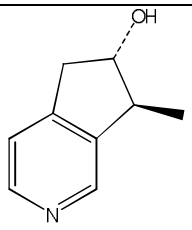
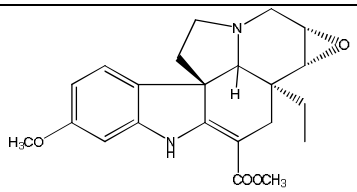
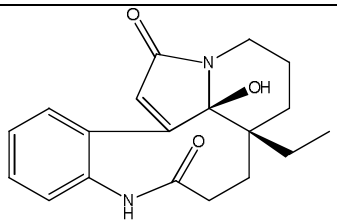
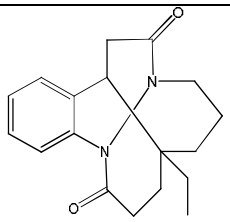
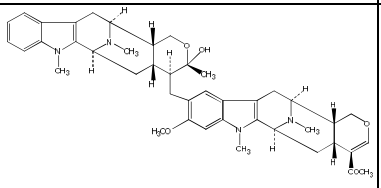
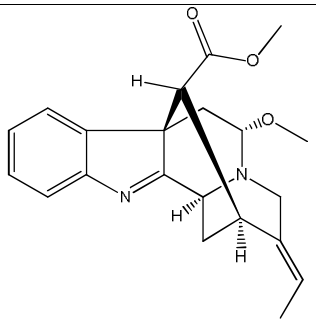
Alpneumine A	*A.pneumatophora*		Koyama *et al.,* 2010 A
Alpneumine B	*A.pneumatophora*		Koyama *et al.,* 2010 A
Alpneumine C	*A.pneumatophora*		Koyama *et al.,* 2010 A
Alpneumine D	*A.pneumatophora*		Koyama *et al.,* 2010 A
Alpneumine E	*A.pneumatophora*		Koyama *et al.,* 2010 A
Alpneumine F	*A.pneumatophora*		Koyama *et al.,* 2010 A

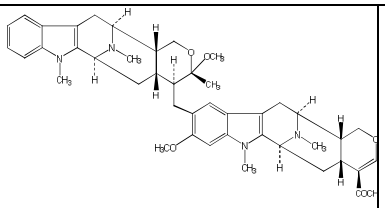
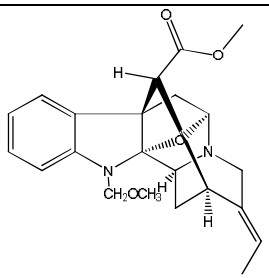
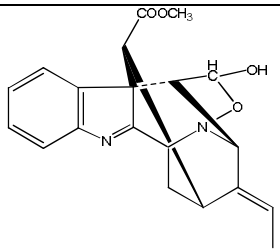
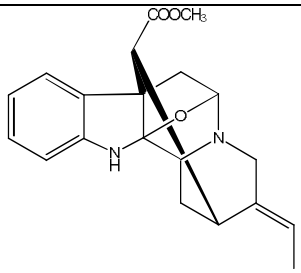
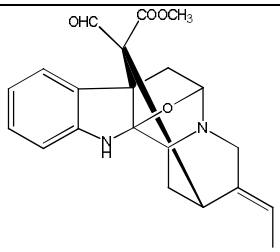
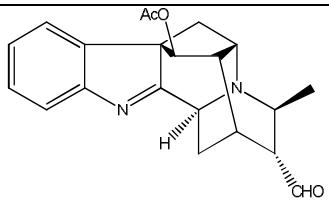
Alpneumine G	*A.pneumatophora*		Koyama *et al.,* 2010 A
Alpneumine H	*A.pneumatophora*		Koyama *et al.,* 2010 A
Alsmaphorazine A	*A.pneumatophora*		Koyama *et al* .,2010 B
Alsmaphorazine B	*A.pneumatophora*		Koyama *et al* .,2010 B
Alstilobanine B	*A. pneumatophora*		Koyama *et al* .,2010 B
Alstoyunine A	*A.yunnanensis*		Feng *et al* .,2009
Alstoyunine B	*A.yunnanensis*		Feng *et al* .,2009

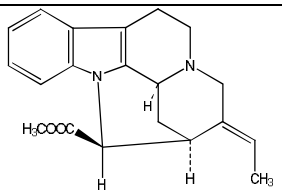
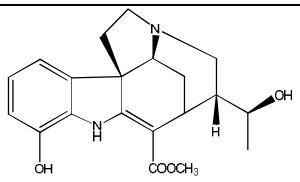
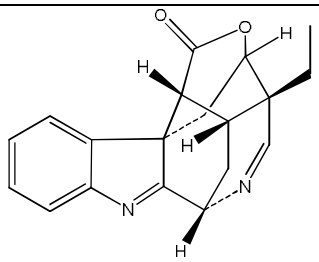
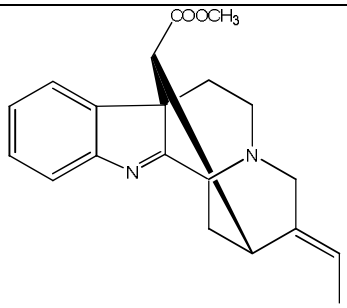
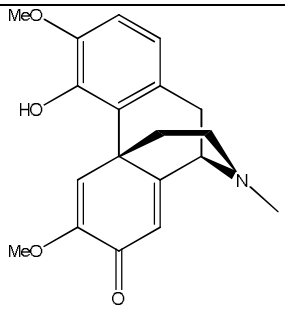
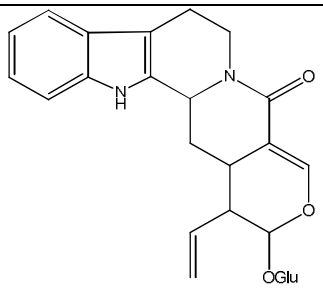
Alstoyunine C	*A.yunnanensis*		Feng *et al* .,2009
Alstoyunine D	*A.yunnanensis*		Feng *et al* .,2009
Alstoyunine E	*A.yunnanensis*		Feng *et al* .,2009
Alstoyunine F	*A.yunnanensis*		Feng *et al* .,2009
Alstoyunine G	*A.yunnanensis*		Feng *et al* .,2009
Alstoyunine H	*A.yunnanensis*		Feng *et al* .,2009
Apovincamine	*A.scholaris*		Koyama *et al* .,2010 A

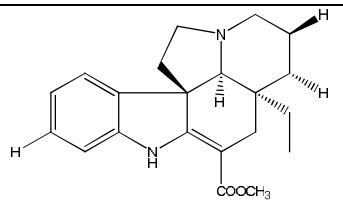
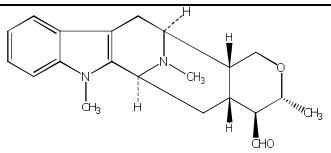
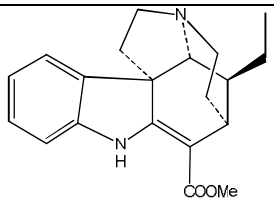
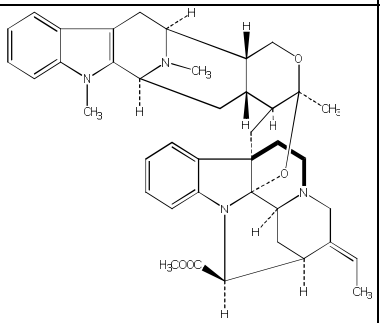
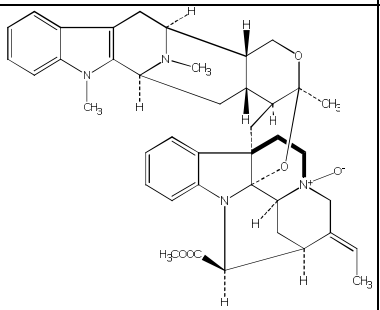
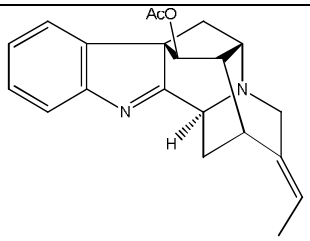
Akuamidine	*A.scholaris*, *A.boonei*		Shang *et al.,* 2010
cCantleyine	*A.scholaris*		Shang *et al.,* 2010
Corypalmine	*A.scholaris*		Shang *et al.,* 2010
Discretamine	*A.scholaris*		Shang *et al.,* 2010
N(4)-Demetylechitamine	,,		Shang *et al.,* 2010
Echitamine	*A.scholaris*		Saraswatti *et al.,* 1997,Shang *et al.,* 2010
Echitamidine	*A.pneumatophora*		Koyama *et al.,* 2010 A

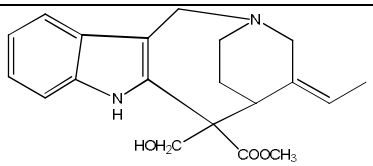
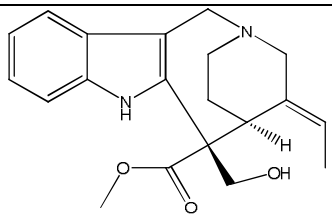
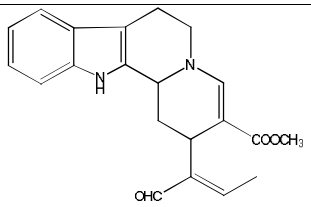
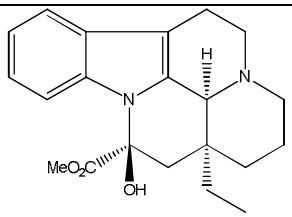
19-Epi-scholaricine	*A.scholaris*		Shang *et al.,* 2010
3-Epi-dihydrocorymine-17-acetate	*A.scholaris*		Shang *et al.,* 2010
20-Epi-antirhine	*A.macrophylla*		Keawpradub *et al.,* 1999
16-Formyl-5Ŭ-methoxystrictamine	*A.scholaris*		Shang *et al.,* 2010
N-formylechitamidine	*A.boonei*		Oguakwa 1984, Kucera *et al.,* 1972, Ayiku 1992
12-Hydroxy-echitamidine Nb-oxide	*A.scholaris*		Shang *et al.,* 2010

Isogentialutine	*Ascholaris*		Shang *et al.,* 2010
Lochnerinine	*A.yunnanensis*		Feng *et al.,* 2009
Leuconolam	*A.scholaris*		Wang *et al.,* 2009
Leuconoxine	*A.scholaris*		Shang *et al* .,2010
Macralstonine	*A.macrophylla*		Keawpradub *et al* .,1999
5Ŭ-Methoxystrictamine	*A.scholaris*		Wang *et al.,* 2009.

O-methylmacralstonine	*A.macrophylla*		Keawpradub *et al.,* 1999
N'-methoxymethyl picrnine	*A.scholaris*		Wang *et al.,*2009
Nareline	*A.scholaris*		Shang *et al.,* 2010
Picrinine	*A.scholaris*,*A.yunnanensis*		Abe *et al.,* 1989,Wang *et al.,* 2009, Shang *et al.,* 2010, Feng *et al.,* 2009
Picralinal	*A.scholaris*		Wang *et al.,* 2009, Abe *et al.,* 1989, Shang *et al.,*2010
Perakine	*A.yunnanensis*		Feng *et al.,* 2009

Pleiocarpamine	*A.macrophylla*		Keawpradub *et al.,* 1999
Scholaricine	*A.scholaris*		Shang *et al.,* 2010
Scholarisine A	*A.scholaris*		Cai *et al* .,2008
Strictamine	*A.scholaris*		Shang *et al*., 2010
Salutaridine	*A.scholaris*		Shang *et al*., 2010
Strictosamine	*A.scholaris*		Shang *et al.,* 2010

Tabersonine	*A. yunnanaensis*		Feng *et al.,* 2009
Talcarpine	*A.macrophylla*		Keawpradub *et al.,* 1999
Tubotaiwine	*A.scholaris*		Shang *et al.,* 2010
Villalstonine	*A.macrophylla*		Keawpradub 1999
Villalstonine N_b Oxide	*A.macrophylla*		Keawpradub 1999
Vinorine	*A.yunnanensis*		Feng *et al.,* 2009

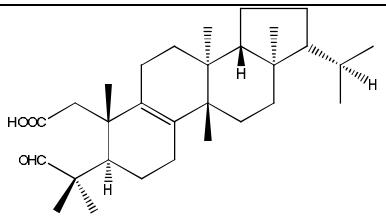
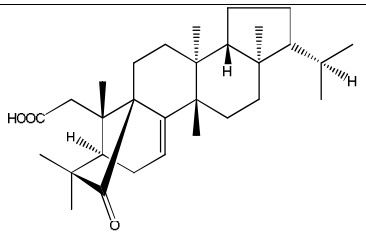
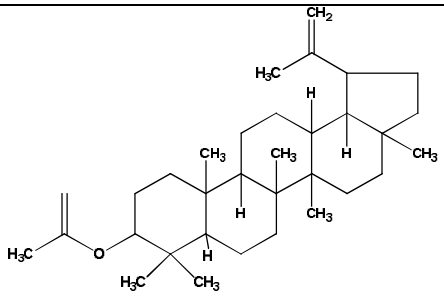
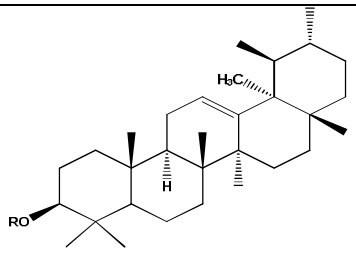
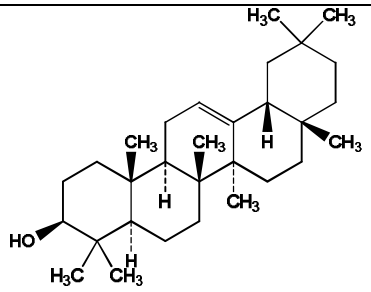
Vallesamine	*A.scholaris*		Shang *et al.,* 2010
19,20(-E-)Vallesamine	*A.scholaris*		Wang *et al.,* 2009
Vallesiachotamine	*A.scholaris*		Shang *et al.,* 2010
Vincamine	*A.pneumatophora*		Koyama *et al*., 2010 [A]

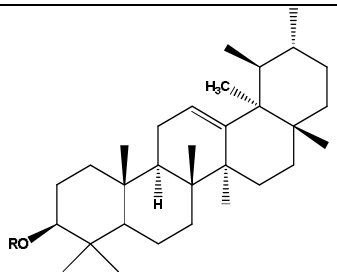
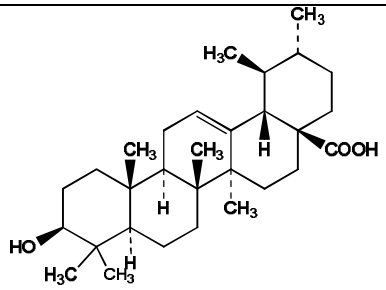
2.2.7.2 Triterpenoids from *Alstonia* species

The triterpenoids isolated from *Alstonia boonei* include lupeol (Gosse *et al* 1990) also known as (3*β*)-Lup-20(29)-en-3-ol, monogynol B, *β*-viscol, or fagarasterol, ursolic acid (Abbiw 1990), also known as urson, prunol micromerol, or malol. Its systematic name is (3*β*)-3-hydroxyurs-12-en-28-oic acid and *β*-amyrin (Hadi and Bremner 2001).

Some of the triterpenoids, their sources and structure are summarised in the Table 2.2:

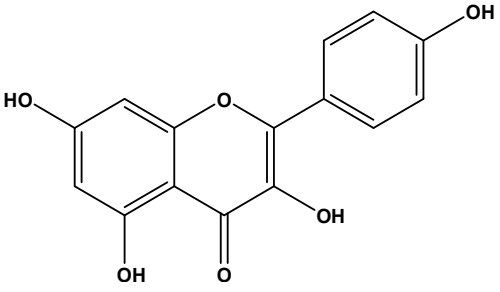
Table 2.2: Chemical Structures of some Triterpenoids Isolated from *Alstonia* spp

Compound	Source	Structure	Reference
Alstonic acid A	*A.scholaris*		Wang *et al.,* 2009
Alstonic acid B	*A.scholaris*		Wang *et al*., 2009
Lupeol acetate	*A. scholaris, A. boonei*		Kweifio-Okai and Caroll, 1992
Ŭ-Amyrin	*A. scholaris, A. boonei*		Kweifio-Okai and Caroll, 1992
ɓ- Amyrin	*A. boonei*		Kweifio-Okai and Caroll, 1992

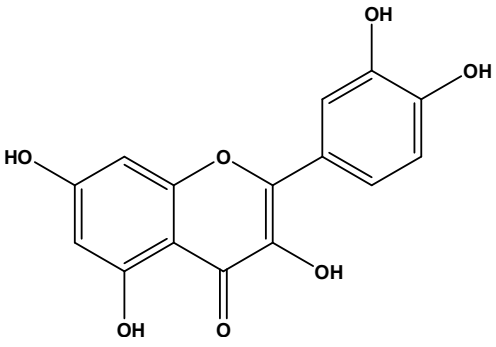
Ŭ-Amyrin Palmitate	*A. boonei*		Adotey, 2012
Ursolic acid	*A. boonei*		Abbiw 1990

2.2.7.3 Flavonoids from *Alstonia* species

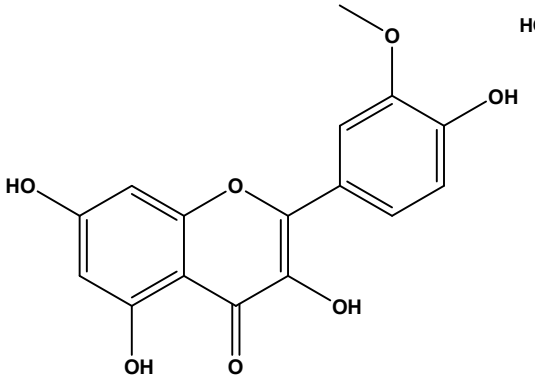
Eight flavonoids have been reportedly isolated from *Alstonia scholaris* leaves and identified as kaempferol (**7**), quercetin (**8**), isorhamnetin (**9**), kaempferol-3-O-beta-D-galactopyranoside (**10**), quercetin-3-O-beta-D-galactopyranoside (**11**), isorhamnetin-3-O-beta-D-galactopyranoside (**12**), kaempferol-3-O-beta-D-xylopyranosyl-(2-1)-)O-beta-D-galactopyranoside (**13**) ,quercetin-3-O-beta-D-xylopyranosyl-(2-1)-O-beta-D-galactopyranoside (**14**) (Hui *et al.,* 2009).



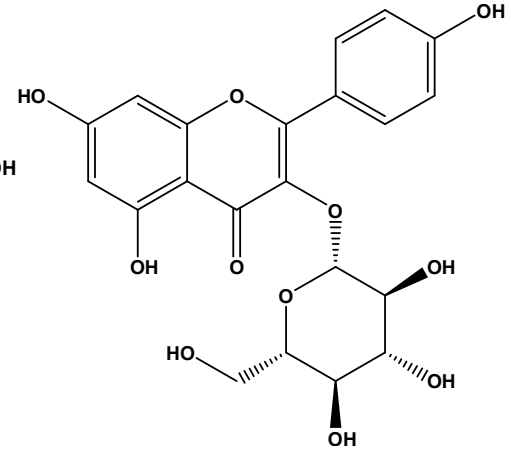
7



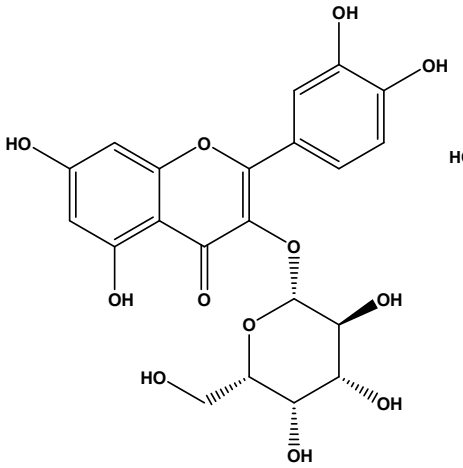
8



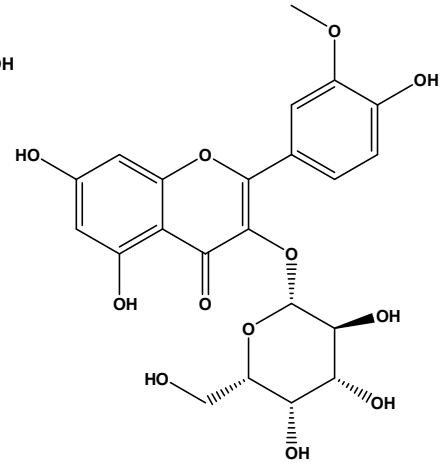
9



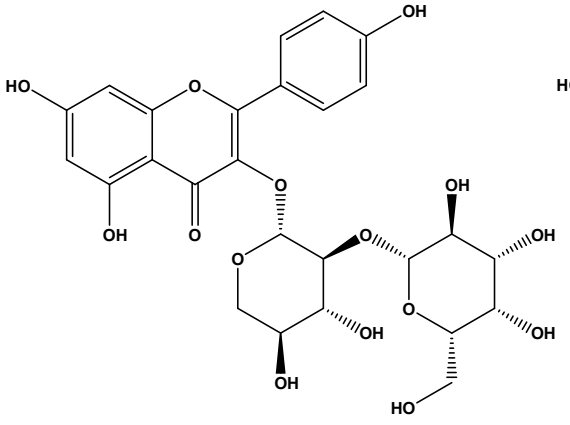
10



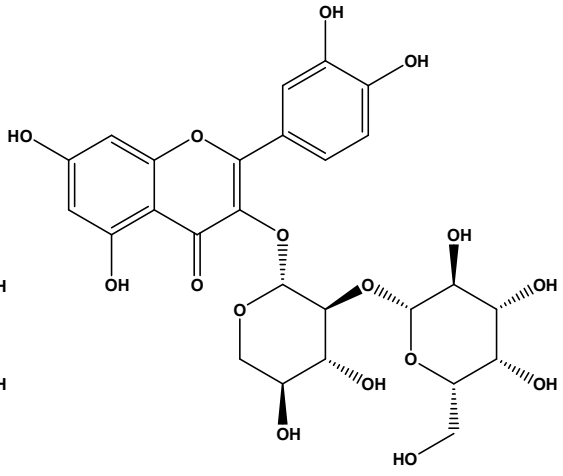
11



12



13



14

2.3 Plant Derived Antioxidants

An increased intake of fruits and vegetables has been associated with a reduction in morbidity and mortality from a number of degenerative diseases including heart attacks, diabetes, cancer, etc (Stevenson and Lowe, 2009,Rimm *et al*.,1996). As a result, a huge number of them have so far been studied for their potentials as lead molecules with antioxidant potentials. Their industrial-by-products, like peel and seed, are even proven to have much higher antioxidant levels than the flesh and other parts of the fruit with the presence of high polyphenol content. This is true for *Viburnum opulus* seed (Cam *et al*., 2007), peach peel (Chang *et al*., 2000), apple peel (He & Liu, 2007), mangosteen peel (Moongkarndi *et al*., 2004) and grape seed and skin (Rockenbach *et al*., 2011).

So far, it is not known which specific dietary constituents (prevalent in fruits and vegetables) are responsible for this association, but antioxidants are assumed to be the major compounds that play the important role (Gey *et al*., 1991). In vitro studies have shown them to contain groups of compounds which possess a high antioxidant capacity such as Oxygen Radical Absorbance Capacity (ORAC) and Ferric Reducing Antioxidant Power (FRAP) (Clifford, 2004). Most of these free radical scavenging molecules are a wide variety of polyphenols, dietary glutathione, vitamins and endogenous metabolites (Ling and Palanisamy, 2012).

The term ñphenolicò is used to define substances that possess one or more hydroxyl groups (OH) substituents bonded to an aromatic benzene ring while compounds with several phenolic hydroxyl substituents are often referred to as polyphenols. The antioxidant activity of phenolic acids and phenoxides is mainly due to their redox properties, which allow them to act as reducing agents, hydrogen donators, and singlet oxygen quenchers. In addition, some phenolic acids have a metal chelation potential (Maestri *et al*., 2006; Waterman and Mole, 1994).

Generally, natural antioxidant phenolics can be classified into:

(a) lipophilic group mainly tocopherols, and

(b) hydrophilic group, including

(i) simple phenolics,

(ii) phenolic acids,

(iii) anthocyanins,

(iv) flavonoids and

(v) tannins.

(a) Vitamin 'E' which is a collective name of tocopherols and tocotrienols (Sen *et al*., 2007). Naturally occurring Vitamin 'E' contains different proportion of these compounds but the synthetic is primarily Ŭ- tocopherols. They are thought to prevent lipids from peroxidation (Herrera and Barbas 2001).

(b) Phenolic acids include gallic acid, a natural product arising from tannin hydrolysis. Others are monohydroxylated phenolic acids, such as vanillic, caffeic, sinapic, siringic, ferulic and coumaric acids whose antioxidant capacities have been demonstrated in several ñ*in vitro*ò and ñ*in vivo*ò studies (Taruscio, *et al*., 2004; Baldioli *et al*., 1996; Anderson *et al*., 2001) as well as hydroxycinnamic acids such as *p*-coumaric acid, an effective radical inhibitor *in vitro* (Rice-Evans *et al*., 1997) but contains only moderate inhibitory properties against lipid peroxidation (Kikuzaki, et al., 2002).

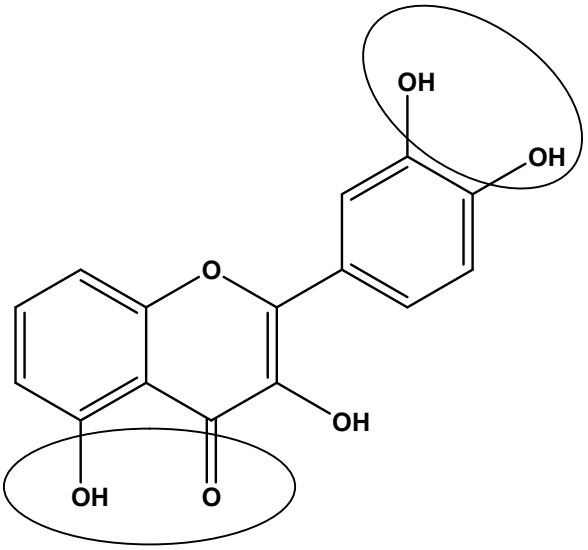
Polyphenolics which include anthocyanins, flavonoids and tannins are moderately hydrophobic polyhydroxy aromatic compounds, a class made up of thousands of different structures thought to be made by plants for many purposes including feeding deterrence against mammals and insects and shielding from damaging solar UV radiations. These

polyphenolics have also been shown to contribute to the health benefits of fruits and vegetables (Gould and Lister, 2006, Stevenson and Hurst, 2007). Prominent among the polyphenols are the flavonoids. Flavonoids are natural polyhydroxylated aromatic compounds widely distributed in the plant kingdom, including fruits and vegetables. About two-third of the polyphenols obtained in human diet are flavonoids. It is estimated that humans consume on the average 1 g / day of flavonoids (Das and Ramanathan, 1992). Flavonoids display pronounced biological effects. Various investigations have established a relationship between the structure of different flavonoids and their relative efficiencies as antioxidants (Bors *et al*., 1992; Masteri *et al*., 2006) This effect is commonly associated with their ability to scavenge reactive free radicals, including hydroxyl, peroxyl and superoxide radicals (Roginsky *et al*., 1996) and/or to deactivate catalytic metals due to complexation (Afanasèv *et al*., 2000). Flavonoids can also inhibit lypoxygenase and cyclooxygenase enzymes (Hsieh *et al*., 1988). Studies have also found both an increased antioxidant capacity of human blood plasma following ingestion of flavonoids, and a decreased level of Low density lipoprotein (LDL) and increased cholesterol oxidation (Serafini 1998).

One factor that seems to influence the antioxidant capacity of a flavonoid is the degree of hydroxylation on the "A" and ñBò ring. Bors *et al*. (1992) concluded that for maximal radical scavenging activity a flavonoid molecule needs to meet the following characteristics:

a) 3ô,4ô-dihydroxy structure (**15**) on the ñBò ring,

b) 2,3-double bond in conjunction with a 4-oxo group in the ñCò ring and a 5-hydroxyl group on the ñAò ring (**15**). The flavonoids luteolin, quercetin, myricetin, and their glycosides meet these structural requirements (Bors *et al*.,1992 ; Maestri D.M. *et al*.,2006).



15

Other major groups of antioxidants in plants

Compounds such as the carotenoids, and ascorbic acids (vitamin C) have demonstrated to have antioxidant and synergistic activity despite their non-phenolic structure. Carotenoids are highly lipophyllic terpenoids (usually highly coloured yellow, orange, red), with no known specific biological function in human metabolism (Humphrey and Beale, 2006). The most common and most used carotenoid in plants as supplements is ɓ-carotene. Some carotenoids can be converted into vitamin A, an important component of visual system. Vitamin C is the only major antioxidant with a known specific biological function in humans: it is an electron donor for eight different enzymes (Stevenson and Lowe 2009).

Several other micronutrients such as selenium, copper and zinc are essential for humans to maintain an effective antioxidant system. Selenium is an essential cofactor for glutathione peroxidase (an important antioxidant enzyme) and deficiency is associated with poor health with studies associating low levels with cardiovascular disease (CVD). Copper and zinc are

essential cofactors for superoxide dismutase, another antioxidant enzyme (Stevenson and Lowe 2009).

2.4 Antioxidant Activity

Oxidation is the transfer of electrons from one atom to another and represents an essential part of aerobic life called metabolism. Oxygen is the ultimate electron acceptor in the electron flow system that produces energy in the form of ATP (Davies, 1995). However problems may arise when the electrons become uncoupled (transfer of unpaired single electrons) generating free radicals with chain reactions, usually produced in the mitochondrial respiratory chain, liver mixed function oxidases, by bacterial leucocytes, through xanthine oxidase activity, atmospheric pollutants, from transitional metal catalysts, drugs and xenobiotics. In addition, chemical mobilization of fat stored under various conditions such as lactation, exercise, fever, infection and even fasting, can result in increased radical activity and damage, in particular, to the immune and nervous systems, while the stress hormones (adrenalin and noradrenalin) secreted by the adrenal glands under conditions of continuing and excessive emotional stress, are metabolised into simpler, albeit, free radical molecules (Atawodi, 2005).

These Reactive Oxygen Species (ROS) actually play different roles *in vivo*. Some are positive and are related to their involvement in energy production, phagocytosis, regulation of cell growth and intercellular signaling and synthesis of biologically important compounds (Halliwell, 1997). Free radicals or oxidative injury may however be very damaging and now appears to be the fundamental mechanism underlying a number of human neurologic and other disorders. This is because they attack lipids (peroxidation) in cell membranes, proteins in tissue or enzymes(inactivation), carbohydrates and DNA to induce oxidations which cause membrane damage, protein modifications (including enzymes) and DNA damage. This

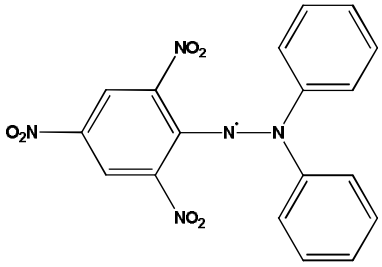
oxidative damage is considered to play a causative role in ageing and several degenerative diseases associated with it such as heart disease, cataracts, cognitive disfunction and cancer (Harman, 1956; Blake and Winyard, 1995; Halliwell and Gutteridge, 1998; Sabu and Kuttan, 2002). Similarly, in carcinogenesis, reactive oxygen species are responsible for initiating the multistage carcinogenesis process starting with DNA damage and accumulation of genetic events in one or few cell lines which lead to progressively dysplastic cellular appearance, deregulated cell growth, and finally carcinoma (Tsao *et al*., 2004). Owing to the incomplete efficiency of the bodyôs defense system and the existence of physiopathological situations (cigarette smoke, air pollutants, UV radiation, high poly unsaturated fatty acid diet, inflammation, ischemia/reperfusion etc) in which ROS are produced in excess and at the wrong time and place, dietary antioxidants are needed for diminishing the cumulative effects of oxidative damage over the life span (Wayner *et al*., 1987; Halliwell, 1994).

Vitamins C, E, A, and carotenoids are well established antioxidants derived from diets. Plant polyphenols generally are an important class of antioxidants (Pier-Giorgio, 2000) which are found virtually in all plants and often at high level.

2.4.1 Review of Assay Methods for Investigation of Antioxidant Activity

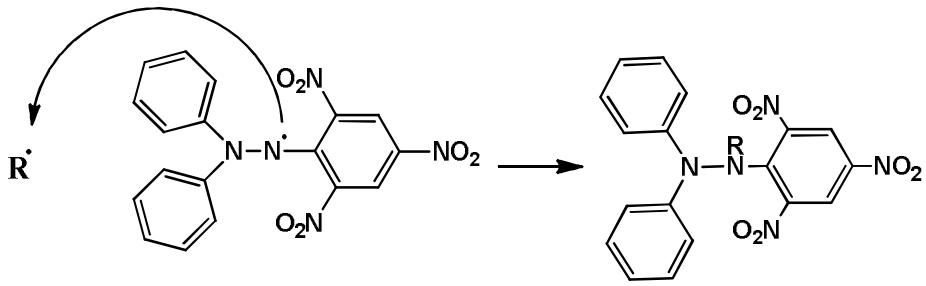
2.4.1.1 Radical Scavenging-Activity (RSA) Assay

Free radical scavenging activity of plant materials can be obtained using 1,1-Diphenyl-2-picrylhydrazyl radical ($DPPH^{\cdot}$) (**16**), a stable organic nitrogen free radical commercially available.



16 (1,1-Diphenyl-2-picrylhydrazyl) ($DPPH^{\cdot}$)

The methanol solution of this compound (radical state) is purple and absorbs light at wavelength of 540 nm). When it reacts with an antioxidant (e.g, flavonoid) it is reduced to the molecular form (DPPHR) (**17**) which is yellow and absorbs at 517 nm.



16 **17**

This change in absorbance can be a measure of the radical scavenging power of the test sample (Amic *et al.,* 2003). Trolox® (6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid, a vitamin E derivative) or ascorbic acid, known for their potent radical scavenging activity are usually used as positive control or assay calibrator.

The RSA assay measures the reducing capacity of antioxidant. This DPPH test is simple and quick. It has a wide spread use in antioxidant capacity screening, probably due to the simplicity of the equipment required. The DPPH method remains unaffected by certain side reactions eg metal chelation and enzyme inhibition which is similar to laboratory-generated free radicals such as the hydroxyl radical and the superoxyl anion (Amarowicz *et al*., 2004).

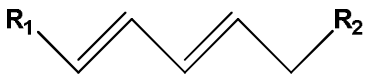
The interpretation of result can be complicated when the test compounds have spectra that overlap DPPH e.g. carotenoids (Nomura *et al*., 1997). Due to the stability and stearic inaccessibility of DPPH, many antioxidants that react quickly with peroxide radicals may react slowly with or may even be inert to DPPH (Prior *et al*., 2005). It has also been reported that the reaction of DPPH with eugenol was reversible (Bondet *et al*., 1997), which can lead to falsely low readings for antioxidant capacities of samples containing eugenol and other phenols with similar structures eg o-methoxyphenol.

2.4.1.2 Reducing Power Assay

In the reducing power assay, the reductants, (in this case the antioxidants) in the test compounds reduce the Fe^{3+}/ferricyanide complex [$FeCl_3/K_3Fe(CN)_6$] to the ferrous Fe^{2+} form (Chung *et al*., 2002). Therefore, depending on the reducing power of the test compounds, the yellow colour of the test solution changes to various shades of green or blue (Amarowicz *et al*., 2004) or the presence of Fe^{2+} results in formation of Perl's Prussian blue, which can be measured spectrophotometrically at 700 nm (Yen and Chen, 1995). The reducing power assay is considered to be a sensitive method for the semiquantitative determination of dilute concentrations of polyphenols, which participate in the redox reaction (Amarowicz *et al*., 2004).

2.4.1.3 Bulk Stripped Oil Model System

The term conjugated diene (CD) implies two double bonds separated by a single bond, which is an unusual structure for polyunsaturated fatty acids (PUFA).



Conjugated Diene

The presence of CDs in a PUFA can be interpreted, therefore as an indication of autoxidation of fatty acid moieties (Corngui and Banni, 1994). Conjugation of the pentadiene structure is a result of the formation of hydroperoxides from PUFA (Gordon, 2001). This structure causes absorption of UV radiation at 233-234 nm. Hence the bulk stripped oil model system represents a simple and rapid method of monitoring the oxidative deterioration of marine oils or vegetable oils (Decker *et al*., 2005). Oils need to be stripped to prevent any possible interaction of minor old constituents before adding test compounds having antioxidant capability. Therefore oil samples containing antioxidants exhibit a lower number of CDs, compared to oil samples without antioxidant after a specific period.

2.5 Antimicrobial Activity

Infectious diseases are those caused by pathogenic microorganisms like bacteria, viruses, fungi, protozoa and multicellular parasites. They are also referred to as communicable or transmissible diseases since they can be transmitted from one person to another through a vector or a replicating agent. Infectious diseases account for about half of the deaths in tropical countries (Khosravi and Behzadi, 2006). An antimicrobial is an agent that kills microorganisms or inhibits their growth (Webster online Dictionary). They are among the most commonly used drugs. They are subdivided according to the microorganisms they primarily act against. These include:

(a) Antibacterials or antibiotics: These are used to treat pathogenic bacterial infections.

A vast majority of bacteria are non pathogenic and therefore are not harmful to human health. Some bacteria are helpful and necessary for good health. Normally, millions of bacteria are found in the human intestine, skin and the genitalia. Bacterial diseases only occur when the harmful bacteria multiply excessively and attack the bodyôs defensive mechanism (Solanki, 2010).

Pathogenic bacteria can invade the body through inhalation into nose and lungs, ingestion in food or through sexual contact. On enterance into the system, the immune system of the body recognizes the bacteria as foreign and tries to kill or stop them from multiplying. However, even a healthy immune system is not always able to stop the bacteria from reproducing and spreading. As a result bacteria thrive in the body and emit toxins which damage cells and tissues that consequently results in the symptoms of bacterial disease.

Commonly occurring pathogenic bacteria are *Neisseria meningitidis*, which can cause meningitis, *Streptococcus pneumoniae*, which can cause pneumonia, *Helicobacter pylori,* which can cause gastric ulcers, *Escherichia coli* which can cause food poisoning, *Salmonella typhi*, which can cause typhoid, and *Staphylococcus aureus,* which can cause skin infections.

(b) Antifungals: Antifungals are used to kill or prevent further growth of fungi. In medicine, they are used as a treatment for infections such as athlete's foot, ringworm, thrush etc. and work by exploiting differences between mammalian and fungal cells. They kill off the fungal organism without dangerous effects on the host.

(c) Antivirals: Antiviral drugs are a class of medicaments used specifically for treating viral infections like cold sores, AIDS, genital herpes, viral hepatitis, influenza, etc. Like antibiotics, specific antivirals are used for specific viruses. They are relatively harmless to the host and therefore can be used to treat infections. They are distinguishable from viricides, which actively deactivate virus particles outside the body.

(d) Antiparasitics: Antiparasitics are a class of medications indicated for the treatment of infection by parasites, such as nematodes, infectious protozoa, and amoebae.

Plants are a rich and unique source of antimicrobials. Different parts of plants (e.g leaves, stem bark, root bark, flowers etc.) have long been used in traditional medicine to treat

infectious diseases. Many of these plants have been investigated scientifically for antimicrobial activity, and a large number of plant products have been shown to inhibit the growth of pathogenic microorganisms.

The increasing prevalence of multidrug resistant strains of bacteria and the recent appearance of strains with reduced susceptibility to antibiotics raises the specter of untreatable bacterial infections and adds urgency to the search for new infection-fighting strategies (Sierdaski *et al.,* 1999). This multidrug resistance is traceable to an indiscriminate use of commercial antimicrobial drugs commonly employed in the treatment of infectious diseases (Iwu *et al.,* 1999). The search is on-going and has proved fruitful since a number of these antimicrobial agents from plants appear to have structures and modes of action that are distinct from those of the antibiotics in current use, suggesting that cross resistance with agents already in use may be minimal.(Wikipedia). This explains the claim by several traditional medicine practitioners that herbs are more potent than conventional drugs. Moreover, contrary to the synthetic drugs, antimicrobials of plant origin are not associated with many side effects and have enormous therapeutic potentials to heal many infectious diseases (Iwu *et al.*, 1999).

2.5.1 Review of Assay Methods for Investigation of Antimicrobial Activity

2.5.1.1 The agar diffusion Test

Agar diffusion refers to the movement of molecules through the matrix that is formed by the gelling of agar. The agar diffusion test, or the Kirby-Bauer disk-diffusion method, is a means of measuring the effect of an antimicrobial agent against bacteria grown in culture.

When the seaweed extract known as agar is allowed to harden, the resulting material is not impermeable. Rather, there are spaces present between the myriad of strands of agar that

comprise the hardened polymer. Small molecules such as antibiotics are able to diffuse through the agar. When performed under controlled conditions, the extent of the molecular movement can be related to the concentration of the molecule. This phenomenon forms the basis of the agar diffusion assay that is used to determine the susceptibility or resistance of a bacterial strain to an antibacterial agent, (including antibiotics).

The sample of the bacteria in question is swabbed uniformly across a culture plate. A filter-paper disk, impregnated with the compound to be tested, is then placed on the surface of the agar. The compound diffuses from the filter paper into the agar. The concentration of the compound will be highest at the disk, and will decrease as distance from the disk increases. If the compound is effective against bacteria at a certain concentration, no colonies will grow where the concentration in the agar is greater than or equal to the effective concentration. This is the zone of inhibition. Thus, the size of the zone of inhibition is a measure of the compound's effectiveness: the larger the clear area around the filter disk, the more effective the compound.

The agar diffusion assay allows bacteria to be screened in a routine, economical and easy way for the detection of resistance. More detailed analysis to ascertain the nature of the resistance can then follow.

2.5.1.2 The Broth Microdilution Method

The micro-titre plate or broth microdilution method has provided a potentially useful technique for determining Minimum Inhibitory Concentration (MIC) of large numbers of test samples. Its advantages over diffusion techniques include increased sensitivity for small

quantities of extract which is important if the antimicrobial is scarce as is the case for many natural products; ability to distinguish between bacteriostatic and bactericidal effects; and quantitative determination of the MIC (Langfield *et al*., 2004). This method can also be used for a wide variety of microorganisms, it is not expensive and it produces reproducible results.

In the micro-titre plate method, a stock solution of the extract is first obtained in solvent, usually the solvent used for extraction (Grierson and Afolayan, 1999) or in DMSO (Salie *et al*., 1996; Nostro *et al*., 2000; Baris *et al*., 2006). Methanol and acetone are sometimes chosen as solvents because, in addition to dissolving the extracts completely, they show no inhibition of the microorganisms even at 2 % final concentration (Meyer and Afolayan, 1995; Afolayan and Meyer, 1997; Mathekga *et al*., 2000). Mueller Hinton Broth or water is often used as diluents in the wells of the microtitre plate before transferring an equal volume of stock solution to the plate. Two fold serial dilutions are then made from the first well to obtain a concentration range. An equal volume of a fixed bacterial culture is added to the wells and incubated at 37 ^{o}C for 24 h (Lourens *et al*., 2004).The inoculum size for the microtitre plate procedure is usually 1×106 cfu/ml (Lourens *et al*., 2004; Basri and Fan, 2005). Plates are then examined for changes in turbidity as an indicator of growth. The first well that appears clear is taken to be the MIC of the extract. Indicators could be used (Umeh *et al*., 2005) or spectrophotometry to determine presence of growth in microtitre plates (Devienne and Raddi, 2002, Matsumoto *et al*., 2001). Indicators (usually tetrazolium salts or resazurin dye) are added after the incubation period and left for about 6 h and changes in color or absence of color, depending on the indicator, is used to detect the MIC breakpoint.. When the spectrophotometric method is used the absorbance, usually at 620 nm with the negative control as a blank, is used to detect the breakpoint (Salie *et al*., 1996). The concentration at which there is a sharp decline in the absorbance value (Devienne and Raddi, 2002), or the

lowest concentration which gives a zero absorbance reading (Salie *et al*., 1996) is deemed to be the MIC.

The minimum bactericidal concentration (MBC) is determined by subculturing the preparations that would have shown no evidence of growth in the MIC determination assay. These subcultures are made either in broth or in agar plates. In broth, the MBC is regarded as the lowest concentration of extract which does not produce an absorbance reading at 620 nm relative to the negative control (Salie *et al*., 1996, Ncube *et al*., 2008). On agar the lowest concentration showing lack of growth represents the MBC.

2.6 Biological Activities of Flavonoids

2.6.1 Flavanoids as antioxidants

Flavonoids might induce mechanisms that affect cancer cells and inhibit tumour invasion (News release issued by Oregon State University. URL accessed). *In vitro* studies have shown flavonoids to be effective scavengers of free radicals. In preliminary studies, UCLA (University of California Los Angeles), Cancer researchers proposed that smokers who ate foods containing certain flavonoids such as flavan-3-ols (cathechins) found in strawberries and green and black teas, kaempferol from brussel sprouts and apples and quercetin from beans, onions and apples have reduced risk of obtaining lung cancer (UCLA news, 2008).

Antioxidants are compounds that protect cells against the damaging effects of oxygen-centred free radicals known as reactive oxygen species (ROS) such as singlet oxygen $^1O_2$, superoxide $O_2^{\bullet -}$, peroxyl radicals $ROO^{\bullet}$, hydroxyl radicals $HO^{\bullet}$, alkyl radicals $RO^{\bullet}$ and nitric oxide $NO^{\bullet}$. The hydroxyl and alkoxy free radicals are very reactive and rapidly attack the molecules in nearby cells. Probably the damage caused by them is unavoidable and is dealt with by repair

process. On the other hand, the superoxide anion, lipid hydroperoxide and nitro peroxide are less reactive (Ames *et al*., 1993). An imbalance between antioxidants and reactive oxygen species, results in oxidative stress, leading to cellular damage.

Mechanisms of antioxidant action of flavonoids include (1) suppressing reactive oxygen species formation either by inhibition of enzymes or chelating trace elements involved in free radical production, (2) scavenging reactive oxygen species, (3) up regulating or protecting antioxidant defences (Halliwell and Gutteridge 1998).

This ability of flavonoids to chelate metal ions such as copper and iron which can catalyze the production of free radicals may contribute to their antioxidant activity. The proposed binding sites for metals to flavonoids are the catechol moiety in ring B, the 3-hydroxyl, 4-oxo groups in the heterocyclic ring, and the 4-oxo, 5-hydroxyl groups between the heterocyclic and the A rings. However, the major contribution to metal chelation is due to the catechol moiety, as exemplified by the more pronounced bathochromic shift produced by chelation of level of kaempferol (which differs from quercetin because it has a lone hydroxyl group in ring B). These data further confirmed that the catechol structure in the B ring is the major determinant for radical-scavenging capacity of the flavonoids. In the case of isoflavones, the location of ring B at the 3-position of the heterocyclic ring greatly affects the radical scavenging capacity, as determined by the TEAC assay. Thus, genistein is 2 times more potent than its flavone relative apigenin. For the other flavonoid classes, glycosylation negatively influences the radical-scavenging capacity.

2.6.2 Flavonoids as antimicrobials

For centuries, preparations that contain flavonoids as the principal physiologically active constituents have been used by physicians and lay healers in attempts to treat human diseases (Havsteen, 1983). For instance, owing to the widespread ability of flavonoids to inhibit spore

germination of plant pathogens, they have been proposed for use against fungal pathogens of man (Harbourne, 2000). Several flavonoids with antimicrobial properties have been isolated and reported. A prenylated flavanone was isolated from the shrub *Eysenhardtia texana* identified as 5,7,4-trihydroxy-8-methyl-6-(3-methyl-[2-butenyl])-(2*S*)-flavanone and shown to possess activity against the opportunistic pathogen *Candida albicans* (Wachter *et al*., 1999). The flavonoid 7-hydroxy-3-,4-(methylenedioxy) flavan, isolated from *Terminalia bellerica* fruit rind, has also been shown to possess activity against *C.albicans* (Valsaraj *et al.,* 1997). Two more flavones from *Artemisia giraldi*, identified as 6,7,4-trihydroxy-3-,5-dimethoxyflavone and 5,5-dihydroxy-8,2-,4-trimethoxyflavone, together with 5,7,4-trihydroxy-3-,5-dimethoxyflavone have been reported to exhibit activity against *Aspergillus flavus (*Zheng *et al.,* 1996), a species of fungi that causes invasive disease in immunosuppressed patients (Prescott *et al*., 1999). The activity of propolis against dermatophytes and *Candida* spp. has been partially attributed to its high flavonoid content (Cafarchia *et al*., 1990). Galangin, a flavonol commonly found in propolis samples (Fearnley, 2001) has been shown to have inhibitory activity against *Aspergillus tamarii*, *A. flavus*, *Cladosporium sphaerospermum*, *Penicillium digitatum* and *Penicillium italicum (*Afolayan and Meyer, 1997).

An area of research that is of particular interest is the apparent inhibitory activity of some flavonoids against human immunodeficiency virus (HIV). Most, if not all investigations have involved work with the pandemic HIV-1 strain and its enzymes. *In vitro* studies have shown that baicalin inhibits HIV-1 infection and replication. In a study by Hu and colleagues, chrysin was reported to have the highest therapeutic index of 21 natural and 13 synthetic flavonoids against HIV-1(Hu *et al.,* 1994; Cushnie and Lamb, 2005). Several research groups have investigated the relationship between flavonoid structure and inhibitory activity against

HIV-1 and its enzymes [Lin *et al.,* 1997, Brinkworth *et al*., 1992, Fesen *et al*., 1994, Ono *et al*., 1990, Hu *et al*., 1994].

Flavonoids also have inhibitory activity against a variety of other viruses. For example, Selway (1986) reported that quercetin, morin, rutin, dihydroquercetin, dihydrofisetin, leucocyanidin, pelargonidin chloride and catechin possess activity against up to seven types of virusus, including herpes simplex virus (HSV), respiratory syncytial virus, poliovirus and Sindbis virus (Middleton and Chitan, 1993). Proposed antiviral mechanisms of action include inhibition of viral polymerase and binding of viral nucleic acid or viral capsid proteins (Selway, 1986). In addition to the flavonoids mentioned above, three proanthocyanidins from *Pavetta owariensis* (with structural similarity to proanthocyanidin A2 and cinnamtannin B1 and B2) have been shown to have activity against HSV and coxsackie B virus (Yamada, 1991, Balde *et al*., 1990). It has also been demonstrated that two of the flavonoids found in propolis, chrysin and kaempferol, inhibit viral replication of HSV, human coronavirus and rotavirus (Cheng and Wong, 1996). More recently, the flavonol galangin has been reported to have significant antiviral activity against HSV and coxsackie B virus (Meyer *et al*., 1997). Although naturally occurring flavonoids with antiviral activity have been recognised since the 1940s, it is only in the last 45 years that attempts have been made to synthetically modify flavonoids for improved antiviral activity. One such synthesized compound is 6,4-dichloroflavan. However, despite showing strong *in vitro* activity, this compound proved unsuccessful in clinical trials (Middleton and Chitan, 1993). Synergism has been demonstrated between various combinations of flavones and flavonols. For example, kaempferol and luteolin show synergy against HSV. It has been suggested that this is why propolis is more active than its individual component compounds (Amoros *et al*., 1992). Synergism has also been reported between flavonoids and other antiviral agents. Quercetin,

for example, potentiates the effects of 5-ethyl-2-dioxyuridine (Middleton and Chitan 1993) and acyclovir (Musci *et al*., 1992) against HSV and pseudorabies infection. Apigenin also enhances the antiviral activity of acyclovir against these viruses (Musci *et al*., 1992).

Many flavonoids with antibacterial activities have been isolated, however, a more careful review of the antibacterial studies of flavonoids show conflicting results (Cushnie and Lamb, 2005). Such discrepancies, according to Cushnie and Lamb report, could be attributed to the different assays employed. Even when the same methods are used, differences in bacterial innoculum could lead to such discrepancies. Other factors which lead to such conflicting results when using the same methods are used include the volume of broth or agar (Sakanaka *et al*., 1989, Li and Yoshikawa 1998) type of broth or agar (Ng *et al*., 1996) size of wells (Khanna *et al*., 1980,Rauha *et al*., 2000), size of paper disc (Al-saleh *et al*., 1997, Khanna *et al*., 1980), strains of a particular bacterial species used (Bashir *et al*., 1994, Basile *et al*., 2000) as well as inoculation period. Other variables worth noting include whether the test flavonoids are obtained from a commercial or natural source (Afolayan and Meyer, 1997, Nishino *et al*., 1987) and which companies (Nishino *et al*., 1987, Cushnie *et al*., 2003) natural products (Basile *et al*., 1999, Basile *et al*., 2000) the compounds are from.

Some researchers have reported synergy between naturally occurring flavonoids and other antibacterial agents against resistant strains of some bacteria. Examples of these include epicatechin gallate (Hamilton-Miller and Shah, 2000; Shiota *et al*., 1999; Yam *et al*., 1998; Stapleton *et al*., 2004) and sophora flavanone G (Sakagami *et al*., 1998, Sato *et al*., 1995). Arima and colleagues demonstrated synergy between flavonoids with antibacterial activity (Arima *et al*., 2002). Wang and colleagues have complexed 5-hydroxy-7,4-dimethoxyflavone with a number of transition metals and shown that this process increases antibacterial activity

(Wang *et al*., 1992). Another group reported increased antibacterial activity of 3-methyleneflavanones when the B ring contained bromine or chlorine substituents (Ward *et al*., 1981).

2.7 An Overview of some Analytical Methods

2.7.1 Liquid–liquid Extraction, also known as **solvent extraction** and **partitioning**, is a method used in separating compounds based on their relative solubilities in two different immiscible liquids, usually water and an organic solvent. It is an extraction of a substance from one liquid phase into another liquid phase. Liquidï liquid extraction is a basic technique in the extraction of compounds of plants.

2.7.2 Thin Layer Chromatography (TLC)

Chromatography refers to any of the various methods of separating and identifying the components of a complex mixture by differential movement through a two phase system in which the movement is effected by a flow of a liquid or a gas (mobile phase which percolates through an adsorbent (stationary phase) or a second liquid phase. It is performed on a sheet of glass, plastic, or aluminum foil, which is coated with a thin layer of adsorbent material, usually silica gel, aluminium oxide, or cellulose (blotting paper). This layer of adsorbent is known as the stationary phase. After the sample has been applied on the plate, a solvent or solvent mixture (known as the mobile phase) is drawn up the plate via capillary action. Because different analytes ascend the TLC plate at different rates, separation is achieved .

Thin layer chromatography can be used to monitor the progress of a reaction, identify compounds present in a given mixture, and determine the purity of a substance.

2.7.3 Column chromatography

In column chromatography, the stationary phase, a solid adsorbent, is placed in a vertical glass (usually) column. The mobile phase, a liquid, is added to the top and flows down through the column by either gravity or external pressure. Column chromatography is generally used as a purification technique: it isolates desired compounds from a mixture.

The mixture to be analyzed by column chromatography is placed inside the top of the column. The liquid solvent (the eluent) is passed through the column by gravity or by the application of air pressure. Equilibrium is established between the solute adsorbed on the adsorbent and the eluting solvent flowing down through the column. Because the different components in the mixture have different interactions with the stationary and mobile phases, they will be carried along with the mobile phase in varying degrees and a separation will be achieved. The eluates, are collected as the solvent drips from the tap of the column.

Column chromatography is separated into two categories, depending on how the solvent flows down the column. If the solvent is allowed to flow down the column by gravity, or percolation, it is called **gravity column chromatography**. If the solvent is forced down the column by positive air pressure, it is called **flash chromatography**, a "state of the art" method currently used in organic chemistry research laboratories. The term "flash chromatography" was coined by Professor W. Clark Still because it can be done in a flash.

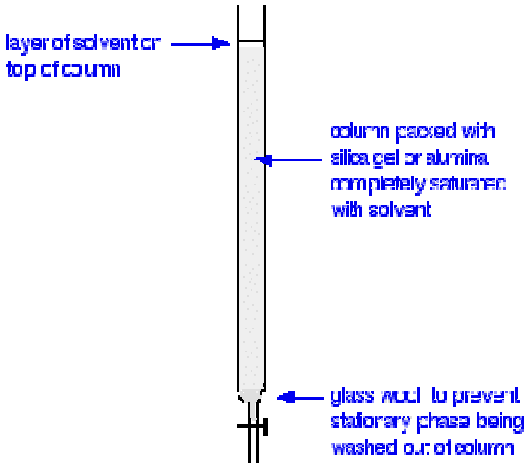


Fig 2.3: Simple Column Chromatography

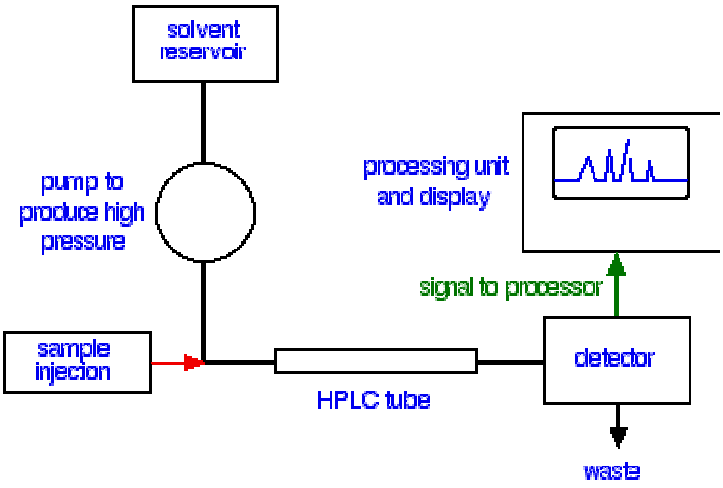
Silica gel (SiO_2) and alumina (Al_2O_3) are two adsorbents commonly used for column chromatography. The polarity of the solvent which is passed through the column affects the relative rates at which compounds move through the column. Proper choice of an eluting solvent is thus crucial to the successful application of column chromatography as a separation technique. Thin-Layer Chromatography (TLC) is generally used to determine the system for a column chromatography separation.

Often a series of increasingly polar solvent systems are used to elute a column. A less-polar solvent is first used to elute a less-polar compound. Once the less-polar compound is off the column, a more-polar solvent is added to the column to elute the more-polar compound.

During the entire chromatography process the eluate is collected in a series of fractions. The composition of the eluent flow can be monitored and each fraction is analyzed for dissolved compounds, e.g. by analytical chromatography like TLC, HPLC; UV absorption, or fluorescence. Coloured compounds (or fluorescent compounds with the aid of an UV lamp) can be seen through the glass wall as moving bands.

2.7.4 High pressure Liquid Chromatography (HPLC)

HPLC is a form of liquid chromatography that utilizes small size columns (typically 250 mm or shorter and 4.6 mm i.d. or smaller; packed with smaller particles).



Scheme 2.1: A Flow Scheme for HPLC

Components in a mixture are separated on a column packed with silica-based particles (stationary phase) by pumping a solvent (mobile phase) through the column. Depending on the unique affinity of each analyte between the mobile phase and the stationary phase, each analyte migrates along the column at different speeds and emerges from the column at different times, thus establishing a separation of the mixture. Analytes with higher affinity for the mobile phase migrate faster down the column, whereas those with higher affinity for the stationary phase migrate slower. This migration time (retention time) is unique for each analyte and can be used in its identification. With the appropriate use of a detection method after the column, each analyte can also be quantified for analysis. Smaller column particle size can improve chromatographic resolution, but increased solvent delivery pressure is

needed. Further reduction of column particle size can allow for higher solvent flow rates, reducing analysis time without sacrificing resolution.

2.7.5 Liquid Chromatography–Mass Spectrometry (**LC-MS**, or alternatively **HPLC-MS**) is an analytical technique that combines the physical separation capabilities of liquid chromatography (or HPLC) with the mass analysis capabilities of mass spectrometry. LC-MS is a powerful technique used for many applications which has very high sensitivity and selectivity. Generally its application is oriented towards the general detection and potential identification of chemicals in the presence of other chemicals (in a complex mixture).

Coupled HPLCï MS is one of the most important techniques of the last decade of the 20th century. The combination offers the possibility of taking advantage of chromatography as a separation method and MS as an identification tool. The amazing number of applications and the rapid drop in price (and size) of MS instruments has meant that the use of LCï MS is now extremely widespread. MS is one of the most sensitive methods of molecular analysis. Due to its high power of mass separation, very good selectivities can be obtained.

2.7.5.1 HPLC-MS of Flavonoid Glycosides

Flavonoid glycosides are thermally labile compounds and the evaporation without decomposition of the analyte is impossible, even in the ion source of a MS, where vacuum exists. In this situation soft ionisation methods need to be applied for the analysis of this group of compounds and the analyte molecules are ionised without evaporation in high vacuum (FAB or LSIMS, MALDI) or under atmospheric pressure (ESI,APCI). From normal mass spectra, information can be obtained about the molecular weight of the whole conjugate, the size of the sugar moieties attached to the aglycone and the molecular weight of

the aglycone (Stobiecki, 2000, Cuyckens and Claeys, 2004) as well as and to establish the distribution of substituents between A- and B-rings.

A careful study of the fragmentation pattern is useful in the determination of the nature and the site of attachments of the sugars in O- and C- glycosides. For flavonoid aglycones and glycosides with a limited number of sugar units (not more than three) TSP LC-MS analysis leads to a soft ionisation providing only intense $[M+H]^+$ ions for the aglycones and weak $[M+H]^+$ ions of glycosides (mono or disaccharide), together with an intense fragment ions due to the loss of the saccharide units leading to the aglycone moity $[A+H]^+$.

The O-glycosides of flavonoids give positive ion mass spectra containing intense $[M+H]^-$ ions as well as fragment ions created after the cleavage of glycosidic bonds between sugar moieties or sugar and aglycone in this case Y_n^+ type ions (for the different situation is observed in the negative ion mass spectra mode where much lower fragmentation of the deprotonated molecule ions $[M-H]^-$ occurs. In this case of O-glycosyl-glycosides, the arrangement of sugars may take place during the fragmentation process and the sequence of sugar losses does not correspond to the sugar moieties in the intact molecule (Cuyckens *et al.,* 2001)

In general, HPLC coupled with diode array and mass spectrometric detection provides an efficient method of rapid identification of flavonoids in nature.

2.7.6 Nuclear Magnetic Resonance

Nuclear magnetic resonance (NMR) is a phenomenon which occurs when the nuclei of certain atoms are immersed in a static magnetic field and exposed to a second oscillating magnetic field. Some nuclei experience this phenomenon, while some do not, depending upon whether they possess a property called spin. When an atom is placed in a magnetic

field, its electrons circulate about the direction of the applied magnetic field. This circulation causes a small magnetic field at the nucleus which opposes the externally applied field (Harris 1997).

In some cases, such as the benzene molecule, the circulation of the electrons in the aromatic ´ orbitals creates a magnetic field at the hydrogen nuclei which enhances the field. This phenomenon is called deshielding.

Generally, if a nucleus experiences a smaller total magnetic field, it is said to be shielded leading to an upfield shift in NMR frequency (low chemical shift) and vice versa. The electron density around each nucleus in a molecule varies according to the types of nuclei and bonds in the molecule. The opposing field and therefore the effective field at each nucleus will vary. This is called the chemical shift phenomenon. The chemical shift is a very precise measure of the chemical environment around a nucleus. For example, the hydrogen chemical shift of a CH_2 hydrogen next to a Cl will be different from that of a CH_3 next to the same Cl. Nuclei experiencing the same chemical environment or chemical shift are called equivalent. Those nuclei experiencing different environment or having different chemical shifts are nonequivalent. Nuclei which are close to one another exert an influence on each other's effective magnetic field. This effect shows up in the NMR spectrum when the nuclei are nonequivalent. If the distance between non-equivalent nuclei is less than or equal to three bond lengths, this effect is observable. This effect is called spin-spin coupling or J coupling. Chemical shift is influenced by:

(a) Electronegativity/Inductive effect.

(b) Shielding/deshielding effect

(c) Anisotropy of chemical bonds

(d) Hydrogen bonding.

With respect to electronegativity/inductive effect, an atom of high electronegativity in a molecule draws electron density towards itself. This causes a decrease in the electron density leading to deshielding of the nucleus. Thus, with increasing electronegativity ŭ values will become high or go downfield.

´-bonded carbons (double, triple or aromatic systems) usually generate a weak, local magnetic field leading to a deshielding of the protons attached on them. This is called anisotropy.

In the case of H-bonding, it leads to the H-atom being shared by two electronegative atoms. This makes it highly deshielded. The resonance occurs at low field which corresponds to a high ŭ value being observed. All alcohols, amines and thiols show this.

The family of peaks centred at a particular chemical shift is referred to as an absorption peak. For example, if there is a triplet of peaks at a specific chemical shift, the number is the sum of the area of the three. The rule is that peak area is proportional to the number of a given type of spins in the molecule and in the sample. For example:

For the methyl ethyl ketone ($CH_3CH_2(C=O)CH_3$) molecule and its hydrogen NMR spectrum.

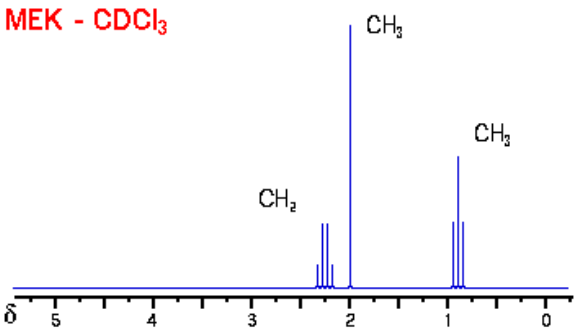


Fig 2.4: NMR Spectrum of methylethylketone

When the -CH_2- (δ = 2.25 ppm), -CH_3 (δ = 2.0 ppm), and CH_3- (δ = 0.9 ppm) peaks are integrated the following spectrum is obtained.

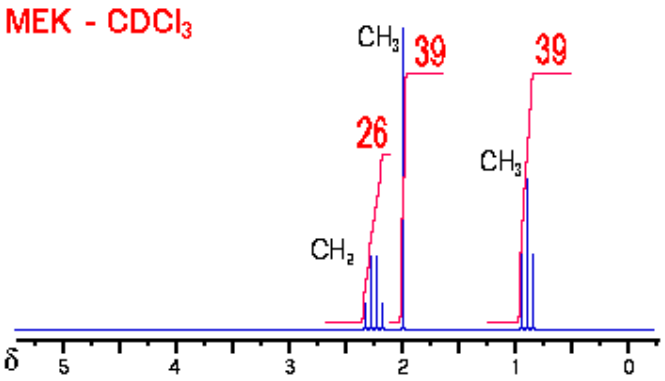


Fig 2.5: NMR Intrgrated Spectrum of Methyl ethyl ketone

The areas under the three types of peaks on this spectrometer are 26:39:39. Dividing each number by 13, we obtain a 2:3:3 ratio which is proportional to the number of -CH_2- to -CH_3 to CH_3- hydrogens .

The distance (in *Hz*) between peaks in a multiplet is called the coupling constant, *J*.

NMR can be studied in two dimensions:

2.7.6.1 The One Dimensional NMR Technique

(i) Proton NMR (^{1}H-NMR)

Proton NMR is a plot of signals arising from absorption of radio frequency during an NMR experiment by different protons in a compound under study as a function of frequency. A ^{1}H NMR spectrum gives three sets of information: the integrals, the coupling pattern and the chemical shifts. The integrals define the number of protons represented by each signal or group of signals. Coupling (splitting) patterns reflect the mutual arrangement of the coupling

protons. It provides information about the number of neighbouring (vicinal or geminal) protons. In aromatic compounds, the coupling constants are 7-9 *Hz* between ortho positions,, 1-3 *Hz* between meta positions and <1 *Hz* between para positions. The para coupling however is usually not resolved but causes broadning of the signal only, which may in turn obscure the meta coupling. The position of the signals (chemical shift) reveals information regarding the chemical and electronic environment of the protons and the splitting patterns (Derome, 1987; Abraham *et al.*, 1988; Sanders and Hunter, 1993)

(ii) Carbon-13 NMR (^{13}Carbon NMR)

This is similar to the proton NMR. It is a plot of the signals arising from different carbons as a function of frequency. Its signal appears as singlets as a result of the decoupling of the attached protons. Different techniques of recording of the 1 D carbon NMR has been developed such that it is possible to differentiate between the various types of carbon such as primary, secondary, tertiary quaternary from the 1D ^{13} Carbon NMR plot.

2.7.6.2 Two dimensional NMR Techniques

There are now several NMR pulse sequences that offer structural information from NMR studies. They include (i) COSY

(ii) HMQC

(iii) HMBC

(iv) NOE, NOESY and ROESY

(i) COSY. There are two types of COSY (COrrelation SpectroscopY) used extensively: The homonuclear (^{1}H^{1}H) COSY and the heteronuclear (^{1}H^{13}C) COSY.

With the $^{1}H^{1}H$ COSY, the one dimensional spectrum is displayed along each axis with a contour projection of the 1D spectrum along the diagonal axis. Off diagonal peaks represent the H-H shift correlations (couplings). One can see which protons couple with one another and which are therefore attached to neighbouring carbons. $^{1}H^{1}H$ COSY gives information regarding three-bond couplings (from a proton to its carbon, to the adjacent carbon, then to that carbon's proton).

$^{1}H^{13}C$ COSY spectra, on the other hand, show the ^{13}C NMR spectrum along one axis and the ^{1}H NMR on spectrum along the other, H-C shift correlations being evidenced by óspotsô in the 2D display.

$^{1}H^{1}H$ COSY identifies which protons are coupled to which while $^{1}H^{13}C$ COSY experiments identify which protons are coupled to which carbons. $^{1}H^{13}C$ COSY is used to assign proton resonances when the carbon resonances for the same rates are known and vice versa.

(ii) ^{1}H-^{1}H TOCSY (Total COrrelation SpectroscopY also known as HOHAHA - HOmonuclear HArtmann HAhn) is useful for dividing the proton signals into groups or coupling networks, especially when the multiplets overlap (have very similar chemical shifts) or there is extensive second order coupling. It is usually used in large molecules with many separated coupling networks such as peptides, proteins, oligosaccharides and polysaccharides. If an indication of the number of bonds connecting the protons is required, for example in order to determine the order in which they are connected, a COSY spectrum becomes preferable.

(iii) HMQC (Heteronuclear Multiple Quantum Correlation or Heteronuclear Multiple Quantum Coherence) or Proton-detected H-C COSY experiment provides direct H-C shift correlations from data related to the connectivity of the proton to its directly bound carbon.

The basic idea behind this experiment is related to the echo difference technique which is used to eliminate proton signals not coupled to the heteronuclei. From this experiment, important information regarding the number and chemical shifts between methyl, methylene and methine groups can be extracted (Ernst *et al.,* 1987, Kessler *et al.*, 1988).

(iv) HMBC (Heteronuclear Multiple Bond Correlation) The HMBC experiment detects long range coupling between proton and carbon (two or three bonds away) with great sensitivity. This enables the spanning of hetero atoms and quaternary carbons, which, because they lack attached protons, would otherwise block the sequence of interactions along the chain from one protonated carbon to the next.

(iv) NOE (Nuclear Overhauser Effect), NOESY (Nuclear Overhauser Effect SpectroscopY) and ROESY (Rotating-frame Overhauser enhancement Spectroscopy also known as the CAMEL SPIN).

NOE is the enhancement produced in the signal of a nearby proton when the nucleus of its neighbour in (space) is irradiated.

2D NOESY is a homonuclear correlation via dipolar coupling which may be due to NOE or chemical change. The magnitude of the enhancement is related to the distance between the nuclei. As in COSY, it is possible to see which protons are nearer to each other in space by drawing a straight line from any of the dark spots to each axis of the plot.

ROESY is a spin-locked NOE method, which is particularly used for intermediate sized molecules with molecular weights 800-2000. This more recent technique takes care of the earlier encountered problem with NOE: NOE interactions are commonly near zero in the important mid-range of molecular weights of about 800-2000. This means that with NOESY, NOE, useful results are often only obtained with either small or large molecules.

2.7.6.3 NMR of the Glycosyl Moiety

The need to derivatize to obtain sufficiently well resolved and interpretable H- NMR spectra of the saccharide moiety has diminished owing to the wide availability of high field NMR spectrometers and the development of COSY and DQF techniques. An important signal to look out for is the anomeric proton signal. This usually occurs as a singlet peak (per glycosyl moiety) appearing around 5 ppm. This alone can provide much useful information regarding the glycosyl moiety. For example, the H-1/H-2 coupling constant can at times indicate which signal relate to which sugar in a poly glycoside and more frequently is also indicative of the Ŭ-or ɓ- linkage of the glycosidic bonds. ɑb-linked glucopyranosides, which exhibit H-1/H-2 coupling constants of 7-8 *Hz,* are readily distinguishable from the Ŭ-linked glucopyranosides with 3-4 *Hz* couplings. The same is true for many other glycosides, as is evident from the coupling constant data for a range of pyranosides and furanosides. A selection from their data is as follows:

1. Pyranosides
   (a) ɓ-D-glucose, galactose and xylose, 7-8 *Hz*
   (b) Ŭ-D-glucose, galactose, xylose, 3-4 *Hz*
   (c) ɓ-L-rhamnose, 1 Hz, Ŭ-L-2 *Hz*
   (d) ɓ-L-arabinose, 2-5 *Hz*

Some other information that can be derived from the chemical shift data of glycosides includes the following:

(i) The H-1 signal of a sugar attached to another sugar (referred to as ósecondaryô, óterminalô as the case may be), can usually be distinguished from that of a sugar directly attached to a flavonoid (primary) in that it normally resonates upfield relative to the latter. Exceptions to

this are evident when the primary glycoside is a C-glycoside (C-glycosides commonly exhibit higher field anomeric proton signals than O-glycosides), or when the terminal sugar is apiofuranoside (which exhibits a relatively low field H-1 signal).

(ii) The 3- and 7-O-glycosides of flavonoids other than anthocyanidines, involving the same sugars can be genarally distinguished eg. 3-O-glucosides (5.35-5.56ppm), 7-O-glucosides (4.9-5.25; 3-O-rhamnosides (4.96-5.36ppm, 7-O-rhamnosides (5.22-5.75ppm); and 3-O-glucuronides (5.48ppm), 7-O-glucuronides (5.1-5.18ppm)

(iii) The presence of glycosylation or acylation on the C-2 hydroxyl of a primary sugar in both O- and C-glycosides is generally evidenced by a 0.1-0.4ppm downfield shift in the H-1 signal of the primary sugar. Acylation seems to have the greater effect.

(iv) Rhamnosyl-(1Ÿ 6)-glucosides (rutinosides) can readily be distinguished from rhamnosyl-1Ÿ 2)-glucosides (neohesperidosides) in DMSO-d_6 solutions by the chemical shift of the the rhamnose H-1. For example, in rutinosides, the glucose and rhamnose H-1 signals appear as *ca* 5.3 and 4.4 ppm respectively, whereas in the 3-neohesperidosides they are found at *ca* 5.7 and 5 ppm respectively. Similar differences are evident for the 7-linked neohesperidosides and rutinosides and for the (1Ÿ 2)- and (1Ÿ 6) linked rhamnogalactosides.

(v) Although the glucosylation site on an anthocyanin is generally not evident from the chemical shift of the glucose H-1 signal, it appears that the 5-O-glucoside (with H-1 at 5.1-5.16 ppm) may be distinguishable from the 3-,7-,3'- or 5'-O-glucosides, which exhibit their H-1 signals in the range 5.21-5.44 ppm.

CHAPTER THREE

3.0 Materials and Methods

3.1 Materials

3.1.1 General laboratory Chemicals

Acetic acid	Merck
Anisaldehyde (4-methoxybenzaldehyde)	Merck
(-)-2-butanol	Merck
Hydrochloric acid	Merck
Potassium hydroxide	Merck
ortho-phosphoric acid 85% (p.a.)	Merck
Sulphuric acid, conc.	Merck
Trifloroacetic acid (TFA)	Merck

3.1.2 General solvents:

Acetone

Acetonitrile

Dichloromethane

Ethanol

Ethyl acetate

Hexane

Methanol

The solvents were obtained from the Institute of Chemistry, University of Duesseldorf, Germany. They were distilled before using and special grade were used for spectroscopic measurements.

3.1.2.1 Solvents for HPLC:

Methanol LiChroSolv HPLC (Merck),

nano-pure water (distilled and heavy metals free water) obtained by passing distilled water through nano- and ionexchange filter cells (Barnstead, France).

3.1.2.2 Solvents for optical rotation:

Methanol spectral grade	Sigma
Chloroform special grade	Sigma

3.1.2.3 Solvents for NMR:

Deuterated methanol	Uvasol, Merck
Water	Uvasol, Merck

3.1.3 Materials for Chromatography

Pre-coated TLC plates	Merck
(Aluminium, Silica Gel 60 F254, layer thickness 0.2mm)	
Silica Gel 60, 40ī 63 μm mesh size	Merck
Pre-coated TLC plates	
(Aluminium, RP-18, F254 S, layer thickness 0.25mm)	Merck
RP-18, 40-63 μm mesh size	Merck
Amberlit XAD. Diaion PH 20, Dowex MCI Gel	Merck
Sephadex LH 20, 25ī 100 μm mesh size	Merck

3.1.4 Laboratory instruments

3.1.4.1 General instruments

Analytical balances MC-1	Sartorious
Half-micro and analytical balance MC-1	Sartorious
pH-meter inoLab, pH-Electrode Sen Tix 21	WTW
Desiccator	Glaswerk Werthein
Hot plate and magnetic stirrer: IKA-Combimag RCH	Janke & Kunkel KG
Glass ware	Schott Duran
Drying Oven ET6130	Heraeus
Ultra sonicator RK 510H	Bandelin
UV-Lamp (254 and 366 nm)	Camag
Rotary evaporator	Büchi Rotavapor R-200
Vacuum pump CVC 2000	Vacuubrand
Centrifuge Pico	Heraeus
Nitrogen generator UHPN 3001	Nitrox
Air generator ZA 20	WGA
Fraction collector Retriever II	ISCO
Lyvac GT2 (Freeze dryer)	Steris
Vacuum pump Trivag D10E (Freeze dryer)	Leybold
SPD 111V (Speedvac)	Savant
Cooling trap RVT 400 (Speedvac)	Savant
Vacuum pump VLP 80 (Speedvac)	Savant
Syringe	Hamilton 1701 RSN
Mill	Molinex 354
Magnetic stirrer	Variomag Multipoint HP

Behrotest PH 10-Set

3.1.4.2 Semipreparative HPLC

Pump: L-7100	Merck/Hitachi
Detector: UV-L7400 (Photodiode array detector)	Merck/Hitachi
Printer: Chromato-Intergartor D-2000	Merck/Hitachi
Column: Eurospher 100-C18, [10 μm; 300 mm × 8 mm]	Knauer
Pre-column: Eurospher 100-C18, [10 μm; 30 mm × 8 mm]	Knauer

3.1.4.3 Analytical HPLC

Pump: P 580A LPG	Dionex
Autosampler: ASI-100T (injection volume = 20 μL)	Dionex
Detector: UVD 340S (Photodiode array detector)	Dionex
Column oven: STH 585	Dionex
Column: Eurospher 100-C18, [5 μm; 125 mm × 4 mm]	Knauer
Pre-column: Vertex column, Eurospher 100-5 C18 [5-4 mm]	Knauer

Software: Chromeleon (V. 6.30)

3.1.4.4 HPLC-MS

Analytical HPLC: Agilent 1100 series	Agilent
(Photodiode array detector)	
MS: Finigan LCQ-DECA	Thermoquest
Ionizer: ESI and APCI	Thermoquest
Vacuum pump: Edwards 30	BOC
Column: Eurospher 100-C18, [5 μm; 227 mm × 2 mm]	Knauer

Pre-column: Vertex column, Eurospher 100-5 C18 [5ī 4 mm] Knauer

3.1.4.5 NMR

DRX-500 Bruker

ARX-400 Bruker

3.1.5 Materials for Antioxidant Assay

DPPH

methanol

Ascorbic acid

NaCl

KCl

Na_2HPO_4

HCl

H_2O_2.

3.1.6 Materials for Antimicrobiological Assay

The following materials were used for antimicrobial studies: Sterile nutrient agar, sterile petri dishes, sterile cork borer, sterile forceps, filter paper strips, innoculating loops, wax pencil, tween 20, tween 65, Sabouraud Dextrose Agar (SDA), Culture of laboratory strains of *Staphylococcus aureus*, *Pseudomonas aeruginosa, Escherichia coli,* and *Candida albicans.*

3.2 Methods

3.2.1 Collection and preparation of *A. boonei* de Wild leaves

The leaves of *Alstonia boonei* De Wild were collected in December 2011 in Nsukka Enugu State, Nigeria and identified by Mr. Ozioko, a taxonomist at the Centre for Ethnomedicine

and Drugs Development, a subsidiary of the Bioresources, Development and Conservation Program (BDCP), Nsukka, Enugu State, Nigeria. A voucher specimen of the leaf with Herbarium number INTERCEDD/024 was deposited at the Herbarium section of the BDCP. The clean leaves were dried under room temperature to a constant weight and pulverised into a fine powder using a mechanical grinder. The powdered materials were stored at room temperature (25-27^{0}C), protected from sunlight until required for extraction and analysis.

3.2.2 Extraction

About 500 g of the pulverised leaves were extracted for 48 hours by cold maceration in 2.5 L methanol with continuous stirring using a magnetic stirrer. The solvent was changed after every 24 hours for maximum extraction. The combined methanol extract was filtered through Whatman No 1 filter paper and evaporated at 40 °C under reduced pressure to obtain the methanol extract (ME).

3.2.3 Liquid-liquid fractionation

The methanol extract (20 g) was dispersed in 200 ml of 10 % methanol in distilled water and sonicated for 10 min. The dispersion was transferred into a 1 L separating funnel and defatted by partitioning with n-hexane (500 mL X 3). The defatted extract was partitioned in ethyl acetate (500 mL X 3) to obtain ethyl acetate fraction (EF).

3.2.4 Chromatographic Separation

The general features of the molecule of a compound which are helpful in this isolation process include differences in solubility, acid-base properties, stability, and molecular size.

3.2.4.1 Vacuum Liquid Chromatography (VLC)

Vacuum liquid chromatography (VLC) is a useful method and an initial isolation step for large amounts of sample using silica gel. Silica Gel is regarded as a typical polar sorbent and

has a weakly acidic surface. Polar compounds containing carboxylic or hydroxyl group are strongly absorbed on silica gel.

VLC is a liquid column chromatography under a negative pressure. A 5-Litre column was packed with silica gel 40-63 μm to a 10 cm bed size. The EF was mixed in about 20 g silica gel and introduced into the column. The column was covered with sea sand up to 1 cm and then glass wool. The column was eluted in succession with 500 mL each of gradient mixture of hexane and ethyl acetate (9:1, to 0:10) and dichloromethane (DCM): methanol (9:1,to 0:10) as shown in Tables 3.1 and 3.1.

Table 3.1: Standard Gradient (Ratio) for Hexane : Ethylacetate used in VLC

Hexane	Ethyl acetate
9	1
8	2
7	3
6	4
5	5
4	6
3	7
2	8
1	9

Table 3.2: Standard Gradient (Ratio) for Dichloromethane (DCM) : Methanol used in VLC

DCM	Methanol
9	1
7	3
5	5
3	7
1	9
0	10

Altogether 15 fractions (EF1 to EF15) were collected.

3.2.4.2 Thin layer chromatography (TLC)

Analytical TLC was applied in order to detect and monitor compounds through the separation process. It was used to optimize solvent systems for column chromatography. TLC was performed on pre-coated TLC plates with silica gel 60 F254 (layer thickness 0.2 mm, E. Merck, Darmstadt, Germany) with dichloromethane : methanol (4:1) as mobile phase. The band separation on the TLC describing the separation of compounds was detected under UV absorbance at 254 and 366 nm (fluorescence) respectively.

3.2.4.3 Column chromatography (CC)

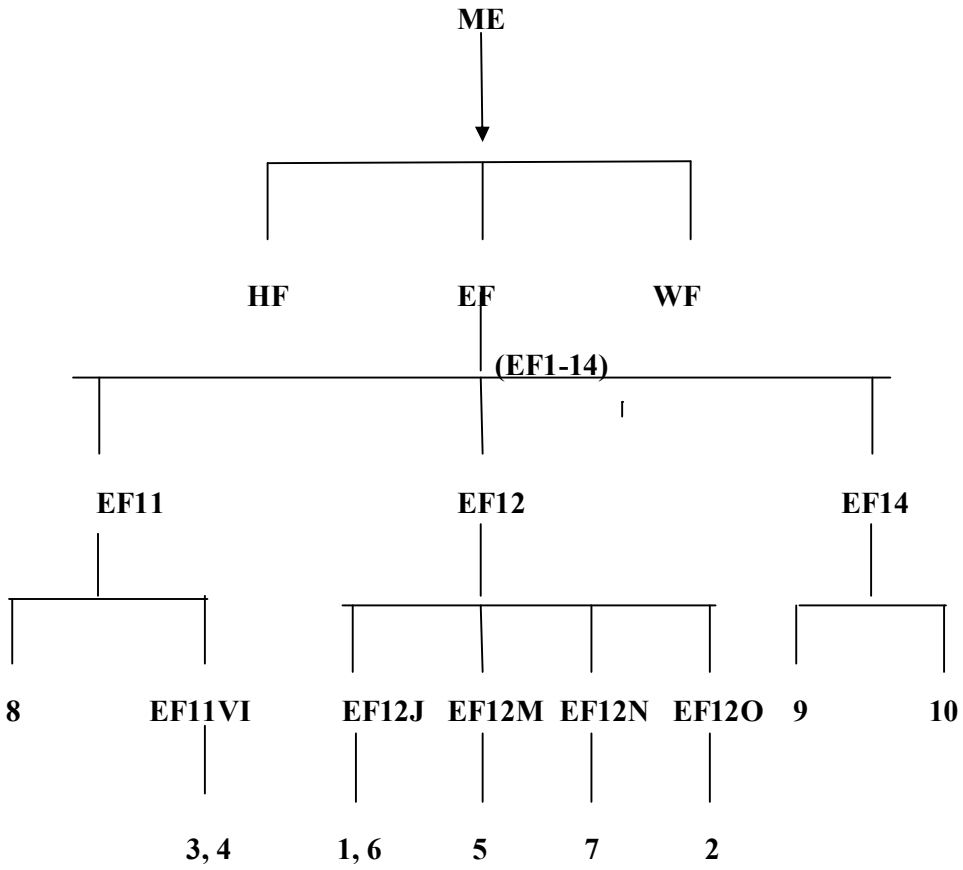
Sephadex LH-20 (200 g) was sonicated with 300 mL of DCM: MeOH (1:1) for about 30 min and the gel gradually transferred into column (3x60 cm) with the tap open. The solvent was allowed to drain until the gel was firmly formed. The sample to be separated was dissolved in 2 mL of DCM: MeOH (1:1) sonicated for 10 min and then centrifuged. The supernatant was transferred into the column and allowed to permeate the gel, after which fresh DCM: MeOH (1:1) was added with the help of a dropping funnel and the whole set up connected to the Fraction collector (Retriever II ISCO, Germany) and adjusted to a flow rate of 0.2 mL/ min. The VLC fraction (EF11) was separated on a Sephadex LH-20 column (3x 60 cm) eluted with dichloromethane: MeOH (1:1) to afford 7 pooled fractions EF11A to EFG, while the VLC fraction (EF12) was separated on a Sephadex LH-20 column (3x 60) eluted with 100 % MeOH to afford 20 pooled fractions EF12A to EF12T.

3.2.4.4. Semi-Preparative High-Performance Liquid Chromatography (HPLC)

Semi-preparative HPLC was used for isolation of pure compounds from fractions previously cleaned up using conventional column chromatography. The reversed-phase C18 chromatography was used as the exclusive stationary phase of HPLC in this study. The

separation column (125 x 4 mm, i.d.) was prefilled with Eurospher C-18 (Knauer, Berlin, Germany). The amount of each injection is dependent on the column size. For a smaller column, about 30 mg/ml sample was prepared in starting gradient of solvent system and alequotes of 100 εl used for each injection. With a big column, samples reaching 20 mg can be injected at once. The gradient solvent system was a mixture of methanol and nanopure water and the flow rate of 5 mL/min. The paper speed of the recorder was 5 mm/min. The eluted substances were collected after detection by manual work based on the records of a UV-VIS diode array detector.

The Sephadex fraction EF11E was purified by semipreparative HPLC to obtain compounds **3**, **4** and **8** respectively. EF12J was similarly purified to obtain compounds **1** and **6** while the purification of EF12M, EF12N and EF12O gave compounds **5**, **7**, and **2** respectively. The VLC fraction (EF14) was directly processed with semipreparative HPLC to obtain compounds **9** and **10**. The extraction scheme is shown in Schem 3.1



HF = Hexane Fraction; EF = Ethyl acetate fraction; WF = Water Fraction

Scheme 3.1: Procedure for Isolation of the Secondary Metabolites from Ethyl Acetate Fraction of *A. boonei* Leaves

3.2.4.5. Analytical High-Performance Liquid Chromatography (DAD-HPLC)

Measurements were done at Institute of Pharmaceutical Biology and Biotechnology, Heinrich-Heine Universität, Düsseldorf, Germany.

Analytical HPLC was used to identify peaks from raw fractions, and to evaluate the purity of isolated compounds. The different components in the mixture passed through the column at

different rates and separation was achieved due to differences in their partitioning behaviour between the mobile liquid phase and the stationary phase.

The solvent gradient used started with 10:90 (MeOH: nanopure water (adjusted to pH 2 with 0.1 % formic acid) increasing to 100 % MeOH in 45 minutes. The compounds were detected by an UV-VIS diode array detector which helps to analyze individual HPLC peaks, and to obtain complete UV spectrum of individual components. The HPLC retention time and the UV spectrum for any component (HPLC peak) is characteristic of compounds. The whole system was run by sophisticated software (Chromeleon® Version 6.30) that allows building up of spectral libraries for reference compounds and automated compound search.

The conditions used for the analytical HPLC are:

Flow rate: 1 mL/min

Injection volume: 20 μL

Sample concentration: ca. 0.1 mg/mL

Column temperature: 20 ^{0}C

UV detection wavelengths: 235, 254, 280, and 340 nm.

For a standard method, the gradient eluant was composed by 0.1% formic acid in nanopure water (pH 2.0) and methanol, which was showed in the table below.

Table 3.3 Standard Gradient for Analytical HPLC

Time (min)	Acidic water (%)	Methanol (%)
0	90	10
5	90	10
35	0	100
45	0	100
50	90	10
60	90	10

All the fractions and subfractions were analysed by HPLC to ascertain the composition while the purity of the isolated compounds were assessed by HPLC

3.2.5. HPLC-MS

Measurements were done at Institute of Pharmaceutical Biology and Biotechnology, Heinrich-Heine Universität, Düsseldorf, Germany.

HPLC/ESI-MS was carried out using a ThermoFinningan LCQ-Deca mass spectrometry connected to an UV detector. The samples were dissolved in MeOH and injected to the HPLC/ESI-MS set up. A solution of the sample is then sprayed at atmospheric pressure through a 2-5 kV potential. HPLC was run on a Eurospher C-18 (6 x 2 mm, i.d.) reversed phase column. The mobile phase was H_2O 0.1% Formic acid (A), to which MeOH (B) was added by a linear gradient: initial, 0% of B; 45 min, 80% of B; 55 min, 100% of B. The flow rate was at 400 εL/min and the absorbance detected at 254 nm. ESI (electrospray ionization) was performed at a capillary temperature of 200^0C and drift voltage of 20eV. The molecular ion peak is the most abundant ion in ESI spectra. All the isolated compounds were analysed by ESI-MS for the purpose of obtaining the molecular masses. MS/MS analyses of the

molecular ions indicated the fragmentation patterns which were used to deduce the type of aglycones present, types of sugar, the number of sugars and also the possible sugar connections.

3.2.6. Nuclear Magnetic Resonance Spectroscopy (NMR)

NMR measurements were recorded by Dr. Peters at the Institute of Organic Chemistry, Heinrich-Heine-Univeristy Düsseldorf, Germany. The ^{1}H NMR and ^{13}C NMR spectra were recorded at 300^{0}K on Bruker ARX 500 NMR spectrometer. All 1D and 2D spectra were obtained using the standard Bruker software.

The pure compounds were each dissolved in 0.75 ml of deuterated methanol (i.e. CD_3OD) (the choice of which is dependent on the solubility of the samples) sonicated for 5 minutes and thereafter centrifuged (10000 rpm) for 10 minutes. The clear supernatant was each carefully transferred into respective NMR tubes to a hight of 5 cm and each tube submitted to the NMR technician for measurement. TMS was used as internal standard reference signal. The observed chemical shifts (ŭ) were recorded in ppm and the coupling constants (*J*) were recorded in Hz. Several other modern NMR techniques were performed in order to elucidate the structure of isolated compounds, such as; **DEPT** (**D**istortionless **E**nhancement by **P**olarization **T**ransfer) which distinguishes and classifies the carbons as doublets (CH), triplets (CH_2), or quartets (CH_3); **HMQC** (**H**etero **M**ultinuclear **Q**uantum **C**oherence) was used to directly correlate proton and carbon nuclei through one bond and the **HMBC** (**H**etero **M**ultinuclear **B**ond **C**oherence) was utilized to obtain long range correlations of proton and carbon nuclei through two, three, or four bonds.

3.2.7. Optical Activity

The rotation of plane-polarized light is known as optical activity. Optical rotation was determined on a Perkin-Elmer-241 MC polarimeter by measuring the angle of rotation at the wavelength of 589 nm of mercury vapour lamp at room temperature (20^0C) in a 0.5 mL cuvette with 0.1 dm length. The specific optical rotation was calculated using the formula:

$$[\alpha]_{\lambda}^{T} = \frac{100\alpha}{lc}$$

where $[\alpha]_{\lambda}^{T}$ = the specific rotation at a given temperature and wavelength and a temperature.

Ŭ measured angle of rotation in degrees

T temperature in degrees Celcius

 wavelength (Sodium D-line 589 nm)

l length in dm of polarity tube

c concentration of substance g/100 ml of solution

Rotation to the right is given a positive value, rotation to the left a negative one. Many stereoisomers do not differ significantly in their electronic or fluorescence spectra so the optical activity of the species is often a useful analytical parameter (Havsteen, 2002).

3.2.8 Biological Assays

3.2.8.1 Antioxidant Activity

3.2.8.1.1 Radical-Scavenging Activity by DPPH

Preparation of Standard Stock Solution of Ascorbic Acid

Ascorbic acid used as standard for the study and its stock solution was prepared in the concentration of 1000 ε g/ml in water. It was freshly prepared and used immediately for the

study. From the stock solution, different concentrations viz 10, 20, 40, 60, 80 and 100 εg/ml were prepared in water and used for antioxidant studies.

Each of the isolated compounds and standard ascorbic acid solution (25 εl) of different concentrations viz 10, 20, 40, 60, 80 and 100 εg/ml was added to 25 εl of a 0.02 % methanol solution of DPPH.

An equal amount of methanol and DPPH served as control. After 30 minutes incubation in the dark, absorbance was recorded at 517 nm and 490 nm in UV spectrophotometer and ELISA machine respectively. The ELISA is a high through-put equipment containing a 96 well plate having the ability to capture UV absorbances of a maximum of 96 samples at the same time. The advantage is that all samples are given the same time of incubation before readings were taken since no time was lost between the interval when the first and the subsequent samples were read, thereby providing more reliable and accurate readings.

The percentage inhibition activity was calculated from the formula below:

$$\frac{A_0 - A_1}{A_0} X\ 100$$

Where: A_0 is the absorbance of the control,

A_1 is the absorbance of the compound/standard.

The antioxidant activity of the extract was expressed as IC_{50}

The IC_{50} value was defined as the concentration (in εg/ml) of compounds that inhibits the formation of DPPH radicals by 50 %. All the tests were performed in triplicate and the graph was plotted with an average of three observations (Kumaran, 2007). The results obtained are as recorded in Table 4.12

3.2.9.2 Antimicrobial Screening of the Isolated Compounds

The antimicrobial activity of the compounds against *Staphylococcus aureus, Escherichia coli, Pseudomona aerigunosa* and *Candida albicans* were determined by agar well diffusion technique.

3.2.9.2.1 Preparation of Medium

All culture media used were prepared according to the manufacturerôs specification.

3.2.9.2.2 Turbidity Standard for Inoculum Preparation

To standardize the inoculum density for a susceptibility test, $BaSO_4$ turbidity standard, equivalent to a 0.5 McFarland standards was used. A 0.5 McFarland standard was prepared as described in NCCLS (NCCLS, 2003). One percent V/V solution of sulphuric acid was prepared by adding 1 ml of concentrated sulphuric acid to 99 ml of water and mixed well. A 1.175% W/V solution of barium chloride was prepared by dissolving 2.35 g of dehydrated barium chloride ($BaCl_2.H_2O$) in 200 ml of distilled water. To make the turbidity standard, 0.5 ml of the barium chloride solution was added to 1% 99.5 ml sulphuric acid solution and mixed well. A small volume of these turbid solutions was transferred to a screw-capped tube of the same type as used for preparing the control inocula and stored in the dark at room temperature.

Broth culture (0.1 mL) containing approximately 1×10^7 cfu/mL of the selected bacteria and fungi was introduced into a sterile Petri dish and 20 ml of molten nutrient agar (for bacteria) or 20 mL Sabouraud dextrose agar (for fungi). The dish was conventionally rotated to ensure even distribution of the organism and then left to solidify.

The dishes were each divided into 4 segments with permanent markers. Cups were bored in each segment of the seeded agar using sterile cork borer.

A 1000 µl of each of the isolated compounds were diluted separately, five-fold serially in 1ml of DMSO to obtain different concentrations (500 µl/ml, 250 µl/ml, 125µl/ml and 65.5µl/ml). A total of 4 wells were made for the different concentrations using a sterile cork borer of 6 mm. Approximately 0.1 ml of the isolated compounds were introduced into each of the well respectively by means of a sterile pipette. The dishes were left to stand at room temperature for about 30 minutes to allow for prediffusion. Thereafter, the plates were incubated at 37 ^{0}C for 24 hours for bacteria and at 25 ^{0}C for 48 hours for fungi.

The inhibition zone diameters (IZD) were determined and MIC (minimum inhibitory concentration) of each of the test compounds was calculated from the graph of log (concentration) and square of IZD.

CHAPTER FOUR

4.0 RESULTS

The chemical investigation of the ethyl acetate fraction of the leaves of *Alstonia boonei* De Wild led to the isolation of ten phenolic compounds. This was achieved using a combination of UV, Mass Spectroscopy, 1 D and 2 D NMR Spectroscopy. Compound **1**-**8** were identified as flavonoids while compounds **9** and **10** were caffeic acid derivatives. The details of the results are shown as follows**:**

4.1 Compound 1:

Compound **1** had the following characteristics:

Yellow amorphous solid

Optical rotation: $[\alpha]_D^{20} = +88.00 \pm 1.76$

HPLC retention time (t_R): 20.183 min

UV maxima: λ_{max} 256.0 and 356.0 nm

HPLC-ESIMS Major Peaks: *m/z* 611.0 $[M+H]^+$

m/z 612.0 $[M+2H]^+$

*m/z 609.0*1 $[M-H]^-$

m/z 465.0 $(M-147)^+$

m/z 303.3 $[M-147-163]^+$

NMR spectral data are shown in Table 4.1

Table 4.1: ^{1}H, ^{13}C and HMBC Assignments for Compound 1 (500 MHz in Methanol d-4)

Position	δ_H (J in Hz)	δ_C (J in Hz)	HMBC
2	---	159.47	
3	---	135.92	
4	---	179.48	
5	---	163.07	
6	6.20 (d, *J*=2.0)	100.17	5, 7, 8, 10
7	---	166.41	
8	6.39 (d, *J*=2.0)	95.11	6, 7, 9, 10
9	---	158.75	
10	---	105.74	
1'	---	123.32	
2'	7.67 (d, *J*=2.1)	117.82	2, 1', 3', 4'
3'	---	149.82	
4'	---	145.95	
5'	6.87 (d, *J*=8.4)	116.18	1'
6'	7.63 (dd, *J*=2.1,8.4)	123.70	2,1'
1''	5.11 (d, *J*=7.6)	104.89	2'',
2''	3.4		
3''	3.4		
4''	3.26		
5''	3.32		
6''	6"A 3.81 (d, *J*=10.9) 6"B 3.28 (dd, *J*=4.2,8.9)	68.69	1'''
1'''	4.52 (s,1H)	104.89	2''',6''
2'''	2.64 (s)		
3'''	3.54 (dd, *J*=3.4, 9.5)		
4'''	3.28 (dd, *J*=4.2, 8.9)		
5'''	3.4		
6'''	1.12 (d, *J*=6.2, 3H)	18.03	5''', 4'''

4.2 Compound 2

Compound **2** showed the following characteristics: Yellow amorphous solid

Optical rotation: $[\alpha]_D^{20} = +38.17 \pm 0.76$

HPLC retention time (t_R): 19.867 min

UV absorption maxima peaks λ_{max}: 256.0 and 354.0 nm

HPLC-ESIMS Major Peaks: *m/z* 633.2 $[M+Na]^+$

m/z 611.1 $[M+1]^+$

m/z 612.1 $[M+2]^+$

m/z 609.4 $[M-1]^-$

m/z 303[querc+1] $^+$

m/z 465.1 [M-rh+1] $^+$

m/z 301.3 $[querc-1]^-$

NMR spectra data are shown in Table 4.2

4.3 Compound 3

Compound **3** was found to have the following characteristics: Yellow powdery solid

HPLC retention time (t_R): 21.680 min

Optical rotation: $[\alpha]_D^{20} = -34.17 \pm 1.80$

UV absorption maxima peaks λ_{max}: 265.5 nm and 348.7 nm

HPLC-ESIMS Major peaks: *m/z* 595.0 $[M+1]^+$

m/z 596.0$[M+2]^+$

m/z 617.3 $[M+Na]^+$

m/z 593.2 $[M-1]^-$

m/z 449.1 $[M+1-146]^+$

m/z 287.3 $[M+1-146-162]^+$

NMR spectral data are shown in Table 4.3

4.4 Compound 4

Compound **4** showed the following characteristicss: Yellow powdery solid with:

HPLC retention time (t_R): 21.287 min

Optical rotation: $[\alpha]_D^{20}$ = -50.47 ±0.76.

UV spectrum absorption maxima peaks λ_{max}: 265.6 and 348.7 nm

HPLC-ESIMS Major Peaks *m/z* 595.1 $[M+1]^+$

m/z 596.1[M+2]

m/z 617.3 $[M+Na]^+$

m/z 593.4 $[M-1]^-$

m/z 449.0 [M+1-146]

m/z 287.3 [M+1-146-162].

NMR spectral data is shown in Table 4.4

Table 4.2: ^{1}H NMR Assignments for Compound 2 (500 MHz in Methanol d-4)

Position	δ_H (J in Hz)
2	---
3	---
4	---
5	---
6	6.20 (s)
7	---
8	6.40 (s)
9	---
10	---
1'	---
2'	7.87(s)
3'	---
4'	---
5'	6.87 (d, *J*=8.5)
6'	7.60 (d, *J*=8.5)
1''	5.07 (d, *J*=7.8)
2''	3.84 (d, *J*=9.5)
3''	3.54 (d, *J*=9.9)
4''	3.81 (s)
5''	3.64 (d, *J*=6.3)
6''A 6''B	3.75 (dd, *J*=5.8, 10.1) 3.41
1'''	4.53 (s)
2'''	3.58 (s)
3'''	3.50 (dd, *J*=3.4, 9.9)
4'''	3.27
5'''	3.54 (d, *J*=9.9)
6'''	1.19 (d, *J*=6.2)

Table 4.3: ^{1}H, ^{13}C and HMBC Assignment for Compound 3 (500 MHz in methanol d-4)

Position	δ_H ppm, (J in Hz)	δ_C ppm (J in Hz)	HMBC
2	-----	159.58	
3	-----	135.65	
4	-----	179.52	
5	-----	163.22	
6	6.20 (d, J=2.1)	100.37	5, 7, 8, 10,
7	-----	166.75	
8	6.39 (d, J=2.0)	95.50	6, 7, 9, 10,
9	-----	158.79	
10	-----	105.72	
1'	-----	123.04	
2'	8.06 (d, J=8.9)	132.52	6', 1'
3'	6.89 (d, J=8.9)	116.28	5'
4'	-----	161.72	
5'	6.89 (d, J=8.9)	116.28	3'
6'	8.06 (d, J=8.9)	132.52	1'
1''	5.12 (d, J=7.4)	105.08	
2''	3.44	78.29	1'', 3''
3''			
4''			
5''			
6''	6"A 3.81 (d, J=10.6, 1H) 6"B 3.39 (d, J=6.7, 1H)	68.73	1'''
1'''	4.52 (s, 1H)	102.84	2''', 3''', 6''
2'''	3.64 (s, 1H)	72.22	
3'''		72.45	1''', 2'''
4'''	3.27	74.03	
5'''	3.44	69.87	4'''
6'''	1.13 (d, J=6.2)	18.06	5''', 4'''

Table 4.4: ^{1}H NMR Assignment for Compound 4 (500 MHz in methanol d-4)

Position	δ_H (J in Hz)
2	---
3	---
4	---
5	---
6	6.18 (J=1.19)
7	---
8	6.36 (s)
9	---
10	---
1'	---
2'	8.09 (d, J=8.8)
3'	6.88 (d, J=8.8)
4'	---
5'	6.88 (d, J=8.8)
6'	8.09 (d, J=8.8)
1''	5.01 (d, J=7.8)
2''	3.77 (d, J=7.3)
3''	3.54 (d, J=3.4)
4''	
5''	3.61 (s)
6''A 6''B	3.73 (dd, J=5.7, 10.1) 3.39 (s)
1'''	4.52 (s)
2'''	3.59 (s)
3'''	3.52 (d, J=6.0)
4'''	3.27 (s)
5'''	3.52 (d, J=6.0)
6'''	1.19 (d, J=6.2)

4.5 Compound 5

Compound **5** was isolated as a yellow amorphous solid with the following characteristics:

Optical rotation: $[\alpha]_D^{20}$ = -23.83±0.76

HPLC retention time (t_R): 19.713 min

UV absorption maxima λ_{max}: 256 and 355.6 nm

NMR data is shown in Table 4.5

4.6 Compound 6

Compound **6** was observed as a yellow amorphous solid with:

Optical rotation: $[\alpha]_D^{20}$ = -28.29 ±1.17

HPLC retention time (t_R): 21.797 min

UV absorption peaks λ_{max}: 266.0 nm and 348.0 nm

HPLC-ESIMS Major peaks: *m/z* 595.0 $[M+1]^+$

m/z 596.0 $[M+2]^+$

m/z 617.2 $[M+Na]^+$

m/z 593.2 $[M-1]^-$

m/z 449.0 $[M+1-146]^+$

m/z 284.2 $[M-2-146-162]^-$

m/z 285.3 $[M-1-146-162]^-$

NMR data are shown in Table 4.6

Table 4.5: ^{1}H NMR Assignments for Compound 5 (500 MHz in Methanol d-4)

Position (δ_H)	δ_H (J in Hz)
2	---
3	---
4	---
5	---
6	6.21 (s)
7	---
8	6.40 (s)
9	---
10	---
1'	---
2'	7.67 (s)
3'	---
4'	---
5'	6.87 (d, J=8.4)
6'	7.64 (d, J=8.4)
1''	5.11 (d, J=7.6)
2''	3.47
3''	---
4''	3.63
5''	---
6''A 6''B	3.80 (d, J=10.9) 3.38 (d, J=10.5)
1'''	4.52 (s)
2'''	3.63 (s)
3'''	3.54
4'''	3.27 (d, J=6.3)
5'''	3.42 (d, J=9.8)
6'''	1.12 (d, J=6.2)

Table 4.6: ^{1}H NMR Assignments for Compound 6 (500 MHz in Methanol d-4)

Position	δ_H, J in Hz
2	---
3	---
4	---
5	---
6	6.21, *J*=2.0
7	---
8	6.40, *J*=2.0
9	---
10	---
1'	----
2'	8.09, *J*=8.8
3'	6.91, *J*=8.8
4'	---
5'	6.91, *J*=8.8
6'	8.09, *J*=8.8
1''	5.13, *J*=7.3
2''	3.45
3''	
4''	3.37
5''	3.33
6''A 6''B	3.80 3.40
1'''	4.54, s
2'''	
3'''	
4'''	
5'''	3.50
6'''	1.15 *J*=6.2

4.7 Compound 7

Compound **7** appeared as a yellow isomeric solid mixture with **8** having:

Optical rotation: $[\alpha]_D^{20}$ = -18.17 ±1.05

HPLC Retention Time (t_R) = 20. 247 min

UV λ_{max}: 256.0 and 356.0 nm

HPLC-ESIMS spectrum: *m/z* 611.0 $[M+1]^+$

m/z 612.1 $[M+2]^+$

m/z 633.2 $[M+Na]^+$

m/z 609.4 $[M-1]^-$

m/z 303.3 $[quercetin+1]^+$

m/z 465.1 $[M-rhamnose+1]^+$

m/z 301.4 $[quercetin-1]^-$

NMR spectra data are shown in Table 4.7

4.8 Compound 8

Compound **8** was isolated as a yellow amorphous compound in isomeric mixture with compound **7** and with:

Optical rotation: $[\alpha]_D^{20}$ = +48.00 ±0.29

HPLC retention time: 20.520 min

Two major UV peaks λ_{max}: 256.6 nm and 356.0 nm

Diagnostic HPLC-ESIMS fragment peaks: *m/z* 611.0 $[M+1]^+$

m/z 612.0 $[M+2]^+$

m/z 633.2 $[M+Na]^+$

m/z 609.3 $[M-1]^-$

m/z 301.1 [quercetin-1]$^-$

NMR data are shown in Table 4.8

Table 4.7: ^{1}H NMR Assignments for Compound 7 (500 MHz in Methanol d-4)

Position	δ_H (J=Hz)
2	---
3	---
4	---
5	---
6	6.21
7	---
8	6.40
9	---
10	---
1'	---
2'	7.67
3'	---
4'	---
5'	6.87
6'	7.63.
1''	5.11(d, 7.7)
2''	3.48
3''	3.63
4''	
5''	3.32
6''A 6''B	3.8 3.4
1'''	4.52
2'''	3.53
3'''	
4'''	
5'''	3.48 (d, 7.7)
6'''	1.13 (d, 6.2)

Table 4.8: ^{1}H NMR Assignments for Compound 8 (500 MHz in Methanol d-4)

Position	δ_H (J=Hz)
2	---
3	---
4	---
5	---
6	6.21 (d, 2.0)
7	---
8	6.41 (d, 2.0)
9	---
10	---
1'	---
2'	7.88 (d, 2.1)
3'	---
4'	---
5'	6.87 (d, 8.5)
6'	7.61 (d, 7.5)
1''	5.07 (d, 7.8)
2''	3.81
3''	3.42
4''	
5''	
6''	
1'''	4.53 (br s)
2'''	
3'''	
4'''	
5'''	
6'''	1.19 (d, 6.2)

4.9 Compound 9

Compound **9** was isolated as a brown amorphous solid with the following parameters:

UV absorption λ_{max}: 218 nm, 242 nm, and 326 nm

HPLC-ESIMS important peaks in the spectrum: *m/z* 355 $[M + H]^+$

m/z 353 $[M - H]^-$

NMR data are shown in Table 4.9

4.10 Compound 10

Compound **10** was a yellowish brown amorphous solid with following parameters:

UV absorption maxima peak at 219, 244 and 329 nm.

HPLC-ESIMS spectrum revealing peaks: 516.9 nm $[M+1]^+$

517.9 nm$[M+2]^+$

539.0 nm $[M+Na]^+$

515.3 nm $[M-1]^-$

NMR data are shown in Table 4.10

Table 4.9 ^{1}H NMR Assignments for Compound 9 (500 MHz in Methanol d-4)

Position	δ_H **(*J*=Hz)**
1	---
2A 2B	1.94 (d, *J*=14.5) 2.15 (d, *J*=14.7)
3	4.11
4	3.67
5	5.38
6A 6B	2.00 (d, *J*=11.9) 2.10 (d, *J*=12.6)
7	---
1'	---
2'	7.05 (d, *J* = 1.9)
3'	---
4'	---
5'	6.77 (d, *J* = 8.2)
6'	6.95 (d, *J* = 1.9, 8.2)
7'	7.57 (d, *J*=15.9)
8'	6.29 (d, *J*=15.9)
9'	---

Table 4.10 ^{1}H NMR Assignments for Compound 10 (500 MHz in Methanol d-4)

Position	δ_H (*J*= Hz)
1	--
2A 2B	2.18 2.28 A
3	4.44 m
4	5.14, d
5	5.71
6A 6B	2.28 A 2.37
7	---
1'	---
2'	7.00
3'	---
4'	---
5'	6.73
6'	6.86 A
7'	7.58
8'	6.27
9'	---
1''	---
2''	6.96
3''	---
4''	---
5''	6.70
6''	6.86 A
7''	7.49
8''	6.18
9''	---

4.11 Antimicrobial Assay Results

The sensitivity of each of the test microorganisms to the compounds is shown in Table 4.11. Only compounds **7** and **8** showed activity against *Escherichia coli* with zones of inhibition of 17 and 13 mm respectively, at a concentration of 62.5 µg/ml. The MIC values as calculated from the graph of log of concentration vs square of IZD are 1.77 µg/mL and 1.92 µg/mL respectively. None of the compounds showed activity against the other tested microorganisms.

4.12 Antioxidant Assay Results

Compounds **1- 9** were evaluated for their antioxidant potentials using the DPPH radical scavenging model and the inhibition concentrations obtained are shown in Figure 4.1 and Table 4.12. Compounds **1**, **2**, **5**, **7** and **8** which are derivatives of quercetin and **9**, a caffeic acid derivative, showed a dose-dependent antioxidant activity on DPPH free radical scavenging model.

Figure 4.2 below shows a plot of the IC_{50} values of compounds **1, 2, 3, 5, 7** and **9,** which showed antioxidant activity, each corresponding to 52, 48, 36, 66, 56 and 22 ε g/mL respectively. The three kaempferol derivatives **3**, **4** and **6** did not show any antioxidant activity. Compounds **5** and **9** showed better antioxidant activity than the standard drug, Vitamin C (IC_{50}= 49 ε g/mL)

Table 4.11: Zones of Inhibition of the Compounds against *Escherichia coli*

Compounds	Concentrations µg/ml			
	500	250	125	62.5
1	---	---	---	---
2	---	---	---	---
3	15 mm	---	---	---
4	---	---	---	---
5	---	---	---	---
6	---	---	---	---
7	14 mm	---	13 mm	17 mm
8	15 mm	14 mm	13 mm	13 mm

Table 4.12: Inhibition Concentrations of the Compounds using the DPPH Model

Conc (µg/mL)	Percent Inhibition (%) of the Compounds							
	1	**2**	**3**	**5**	**7**	**8**	**9**	**Vitamin C**
100	62.14	64.85	-4.17	62.68	61.69	62.50	85.1	66.85
80	62.32	62.50	-3.08	64.13	62.31	61.78	84.0	66.30
40	44.75	47.30	-14.38	53.53	27.71	42.03	60.0	45.11
20	18.21	24.46	-14.68	37.51	12.5	26.63	29.8	14.67
10	11.41	7.07	-18.75	24.19	3.44	14.95	25.3	7.61

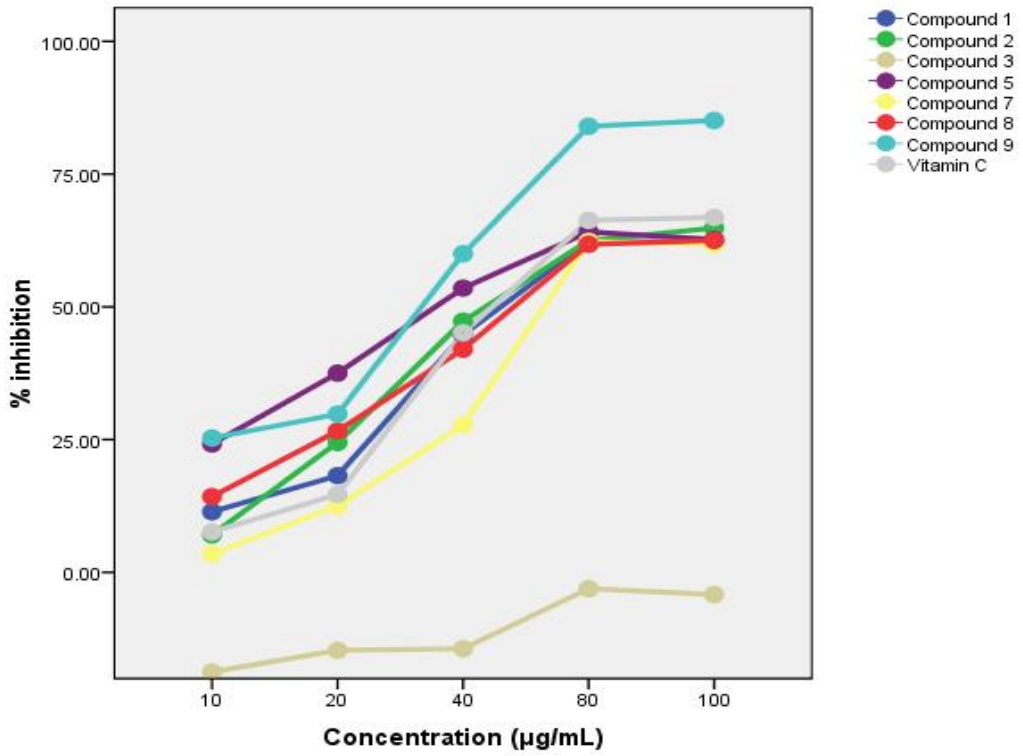


Fig 4.1 **Graph of percent inhibition against concentration for compounds 1 to 9**

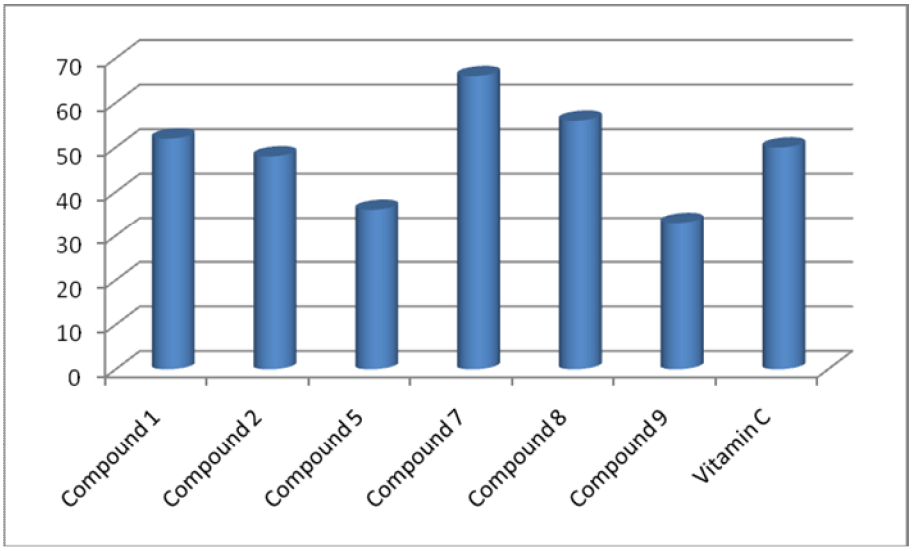


Fig 4.2 **IC_{50} values of the compounds**

CHAPTER FIVE

5.0 Discussion

The ten compounds isolated in this study from the ethyl acetate fraction of the leaves of *Alstonia boonei* de Wild were phenolic and had not been reported in the leaves of *Alstonia* species. Eight of them were flavonoid glycosides and two were caffeic acid derivatives.

5.1 Compound 1

Compound **1** was obtained as a yellow amorphous solid with an optical rotation of +88.00 ±1.76. It has HPLC retention time (t_R) of 20.183 min and UV maxima at λ_{max} 256.0 and 356.0 nm (Appendix 1 and 2) which is typical of quercetin flavonoids.

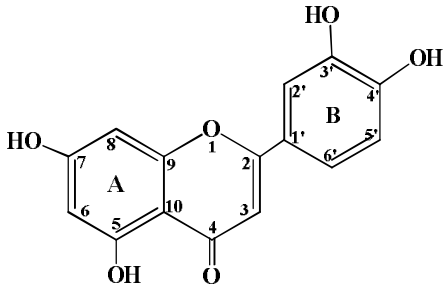
Its LC-ESI-MS (Appendix 3) exhibited strong peaks at *m/z* 611.0 $[M+H]^+$ and 612.0 $[M+2H]^+$ in the positive mode and at *m/z 609.0*1 $[M-H]^-$ in the negative mode, indicating a molar mass of 610 g/mol and a molecular formula of $C_{27}H_{30}O_{16}$. ESI-MS/MS of **1** showed fragments corresponding to loss of one desoxyhexose *m/z* 465.0 $(M-147)^+$ and subsequent loss of a hexose *m/z* 303.3 $[M-147-163]^+$. The fragment 303.3 is diagnostic of quercetin or morin aglycone. Compound **1** is likely a diglycoside derivative of quercetin or morin. Monosaccharides known to occur in association with flavones and flavonols include: the pentoses (D-apiose, L-Arabinose, L-rhamnose, D-xylose); the hexoses (D-allose, D-galactose, D-glucose, D-mannose); the desoxyhexose (rhamnose) as well as the uronic acids (D-galacturonic acid and D-glucuronic acid) (Harbourne, 1993). The most abundant hexoses occurring in plants are glucose and galactose, while rhamnose is the commonest desoxyhexose. Based on the MS fragmentation pattern of compound **1** the glycoside component consists of rhamnose attached either to a glucose or a galactose moiety. The aglycone and the glycoside components can, however, be assigned using NMR spectroscopy.

In any ^{1}H NMR spectrum, three sets of information can be deduced: the integrals, the coupling pattern and the chemical shifts. The integral defines the number of protons represented by each signal or group of signals. Coupling pattern reflects the mutual arrangement of the coupling protons.

In aromatic compounds the coupling constants of 7-9 Hz designate *ortho* coupled protons, while coupling constants of 1-3 Hz between designate *meta* coupled protons. Coupling constants <1 Hz are usually shown by *para* protons. The *para* coupling, however, is usually not resolved but causes broadening of the signal only, which may in turn obscure a *meta* coupling. The chemical shift of a proton indicates its chemical environment, i.e. its position relative to any other part of the molecule (Harbourne, 1993).

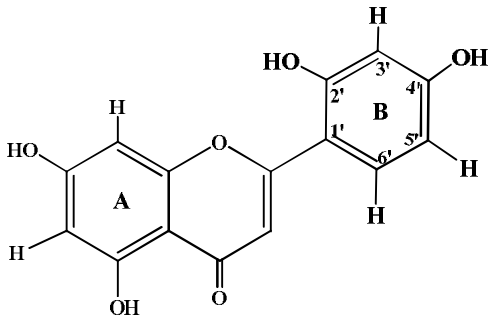
Appendix 4 shows the proton NMR spectrum of compound **1** with five proton peaks in the aromatic region. A closer look at it discloses an ABX aromatic spin system denoted by resonances at δ_H 6.87(d, *J*=8.4, 1H) 7.63(dd, *J*=2.1, 8.4, 1H) 7.67(d, *J*=2.1, 1H) which is attributable to the protons of Ring 'B' of the quercetin/morin nucleus:

The signal at δ_H 7.67 (with a weak coupling constant denoting *meta* coupling) was assigned to H-2' of the quercetin nucleus, while the signal at δ_H 7.63 (split by two protons: δ_H 7.67 and an *ortho* coupled proton at δ_H 6.87) corresponds to H-6' of the quercetin/morin nucleus. The signal at δ_H 6.87 was similarly assigned to H-5'.



The Quercetin Nucleus

This flavonoid nucleus is confirmed to be a quercetin nucleus, not morin as evidenced by the presence of a proton at position 2'. HMBC results (Appendix 7) also show correlations between H-2' and C-2 as well as C-4'. No proton signal is observed on position 3. This is in consistency with the 3', 4' dihydroxy system of quercetin unlike Morin which has a 2', 4' dihydroxy system.



The Morin Nucleus

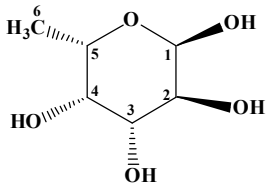
The remaining two meta coupled protons found in the aromatic region, δ_H 6.20 (d, *J*=2.0, 1H) and 6.39(d, *J*=2.0, 1H) are assignable to H-6 and H-8, respectively of the 'Ring A' of quercetin nucleus.

The next pair of protons in the ^{1}H NMR spectrum of compound **1** (Appendix 4) are two anomeric proton signals at δ_H 5.12 ppm (d *J*=7.6 Hz, 1H) and 4.52 ppm (br s, 1H). This further confirms the diglycoside nature of the compound. These signals are assignable to ß-D- glucosyl/galactosyl and Ŭ-L- rhamnosyl units respectively. Anomeric protons of ß-linked glucopyranosides exhibit H-1/H-2 coupling constants of 7-8 Hz. This is readily distinguishable from the Ŭ-linked glucopyranoside with 3-4 Hz coupling (Harbourne 1993). On the other hand, anomeric protons belonging to Ŭ-L-rhamnose have a coupling constant between 1 and 2 (ie. very low) and sometimes appear as a broad singlet. This is because its anomeric proton has a *cis*-conformation (Ŭ-linkage) with its H-2 (Harbourne 1993). An Ŭ-configuration of the rhamnosyl moiety was thus adopted on the basis of this negligible

coupling constant of the anomeric rhamnosyl proton (4.52 ppm, s, 1H). Structural elucidation of flavonoid glycosides with two, three or even more sugar moieties is not easy to achieve due to the complexity of NMR spectra region. This difficulty in complete assignment concerns both the position and configuration of the inter glycosyl linkage (Viet and Pauli, 1999; Halabalaki *et al* 2011).

Each of the carbon atoms was however accounted for; their positions traced using C-13 NMR and DEPT-135(Appendix 8 and 9 respectively).

The chemical shift of the anomeric proton of rhamnose occurring at 4.52 ppm is typical of terminal anomeric protons. This is because the chemical shift signal of a sugar attached to another sugar (in this case referred to as 'terminal') normally resonates a bit upfield relative to the latter (except for C-glycosides or when the terminal sugar is apiofuranoside).



L-rhamnose

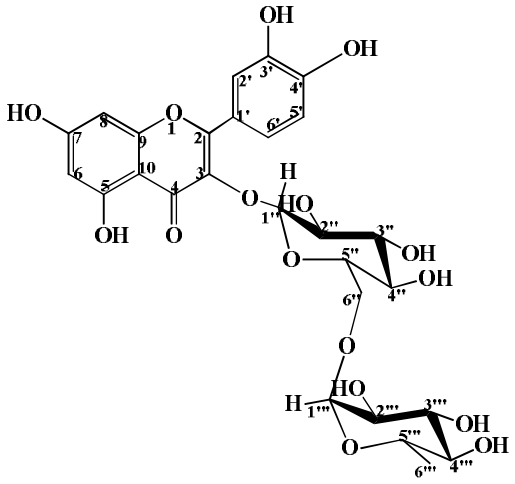
Each of these two anomeric protons is directly linked to its anomeric carbons located at 105.74 and 104.89 ppm (Appendix 8) respectively while the remaining carbons resonate below 90 ppm. COSY (Appendix 5) reveals these two anomeric protons to be linked to other protons located in the sugar region (3-4 ppm). There also appears no direct correlation between them and any of the aromatic protons or carbons.

Furthermore, a rhamnosyl methyl doublet signal at δ_H 1.12(d, J=6.2, 3H) (Appendix 4) was observed, (Bello *et al.,* 2011) showing COSY correlations with H-5''' and HMBC correlations with C-5''' as well as a long range correlation with H-4''' (Hasan *et al*., 1996). The β-glucose

moiety was evident from the ^{1}H NMR resonances of oxymethine protons, together with a pair of oxymethylene protons at δ_H 3.81 and 3.28 ppm. The remaining sugar protons resonate in the range of 3.2-3.5 ppm but can each be accounted for as in the COSY spectrum (Table 4.1)

A 1Ÿ 6 interglycosidic conformation was adopted for compound **1** based on the HMBC correlation observed between the anomeric proton of the rhamnosyl unit (δ_H 4.52) and C-6 of the glucosyl unit. In the same way, a HMBC link (Appendix 7) between the anomeric proton (δ_H 5.11) of the glucosyl moiety and C-3 (δ_C 135.92) (**Table 4.1**)of the quercetin nucleus implies a substitution at C-3 of the quercetin, leading to a downfield shift of C-2 and C-3 (δ_C 159.47 and 135.92, respectively) (Halabalaki *et al.,* 2011). Each of the remaining carbons were accounted for using C-13 NMR as well as DEPT-135 (Appendix 8 and 9).

Compound **1** was thus identified as Quercetin-3-O-[Ŭ-L-rhamnopyranosyl(1Ÿ 6) ɓ-D-glucopyranoside]. Trivial name is Quercetin-3-O-rutinoside or Rutin and the chemical structure is as shown below.



Compound **1**

5.2 Compound 2

Compound **2** is a yellow amorphous solid compound with an optical rotation of +38.17 ±0.76 with a HPLC retention time (t_R) of 19.867 min. It exhibited UV absorption maxima peaks of 256.0 and 354.0 nm, (Appendix10, 11) typical of the quercetin flavonoids. A molecular mass of 610.15 was deduced on the basis of its pseudomolecular ion peak at *m/z* 633.2 $[M+Na]^+$, 611.1 $[M+1]^+$, and 612.1 $[M+2]^+$ on the positive ion mode and 609.4 $[M-1]^-$ on the negative ion mode (Appendix 12). Other informative peak fragments observed include: *m/z* 303[querc+1] $^+$, 465.1 [M-rh+1] $^+$ on the positive ion mode and 301.3 $[querc-1]^-$ on the negative ion mode. It can be inferred that this is a quercetin based glycoside having a terminal sugar in addition to hexose directly attached to its nucleus. Compound **2** shows very similar UV, mass and NMR spectral data with Compound **1** however, a careful comparison reveals some significant differences:

1. An earlier retention time than Compound **1** was observed, confirming that they are different.

2. The anomeric proton of the directly attached sugar of Compound **1** appear a bit downfield (δ_H 5.11) compared to that of Compound **2** (δ_H 5.07)(Appendices 4 and 13).

3. The methyl protons of rhamnose attached to Compound **1** show a slight downfield shift (δ_H 1.12, qpå0.07) when compared to Compound **2** (δ_H 1.19).

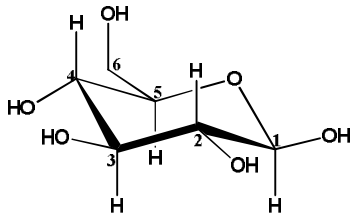
4. The coupling constant of H-4 of the directly attached sugar of Compound **2** appears as a broad singlet unlike that of Compound **1** with a coupling constant of 3.28.

5. H-2 of the directly attached sugar of Compound **2** resonates further downfield δ_H 3.81 than that of Compound **1** δ_H 3.64.

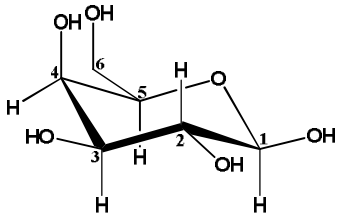
As earlier shown, the mass spectra show the compound to contain a hexose. Coupling constants of anomeric protons are characteristic of sugar moieties. Among the hexoses which occur in flavonoids, the anomeric protons of ɓ-D-glucose, and galactose show coupling constants between 7 and 8 Hz (Harbourne, 1994). The anomeric proton of Compound **2** has a coupling constant of 7.8 while that of Compound **1** is 7.6. This rules out the possibility of the presence of other hexose moieties.

Thus the difference between the two compounds lies in their sugar region: One of the compounds is a glucoside while the other is a galactoside.

The structural difference between glucose and galactose is found in their H-4. Glucose has an axial H-4 while galactose has an equatorial H-4. This results in a diaxial (*trans*)-conformation between H-4 and each of the neighbouring H-5 and H-3 of glucose as well as a diequitorial (*cis*)-conformation between that of galactose as shown below.



β-D-Glucose



β-D-Galactcose

β-D Glucose and Galactose

Trans-conformations lead to higher *J* values than *cis*-conformations. The coupling constant of H-4 of galactose is usually 1 Hz and in most cases the proton appear as a broad singlet while that of glucose is expected to be much higher (8 to 9 Hz). From the results obtained, Compound **2** is proposed to be a galactoside (H-4" δ_H 3.81 brs) while Compound **1** is a glucoside (H-4" δ_H 3.26 dd *J*=4.2, 8.9). This is supported by the following:

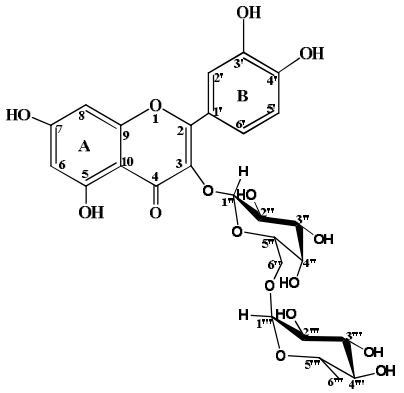
1. The anomeric protons of glucosides resonate slightly downfield compared to those of galactosides linked to the same nucleus (Harbourne 1994). δ_H 5.11 vs 5.07 was observed for Compound **1** and Compound **2** respectively.

2. The methyl protons of a rhamnosyl unit attached to a glucosyl unit show a slight upfield shift (ca 0.06) compared to that of galactose attached at the same position. Compound **1** δ_H 1.12 vs Compound **2** δ_H 1.19.

3. H-2 of galactose attached to position 3 of flavonoids resonates a bit downfield compared to that of glucose attached at the same position. The H-2 of Compound **2** (δ_H 3.84) vs the H-2 of Compound **1** (δ_H 3.44).

4. Axial hydrogens appear upfield with respect to equatorial hydrogens (Finar, 1997). The chemical shift of H-4 of Compound **1**, a glucoside (having a diaxial conformation), is δ_H =3.26 while that of Compound **2**, a galactoside (having a diequatorial conformation), is δ_H =3.81.

Compound **2** was therefore identified as Quercetin-3-O-[α-L-rhamnopyranosyl(1→ 6) β-D-galactopyranoside]. The trivial name is Quercetin-3-O-robinobioside and the chemical structure is as shown below.

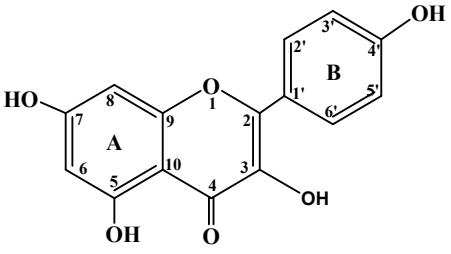


Compound 2

5.3 Compound 3.

Compound **3** was obtained as a yellow powdery solid with HPLC retention time (t_R) of 21.680 min (Appendix 15) and had an optical rotation of -34.17 ±1.80. Its UV absorption maxima peaks were observed at 265.5 nm and 348.7 nm (Appendix 16) which is typical of kaempferol flavonoids. An ESIMS of the compound (Appendix 17) exhibited strong peaks at *m/z* 595.0 $[M+1]^+$, 596.0$[M+2]^+$ and 617.3 $[M+Na]^+$ in the positive mode and at *m/z* 593.2 $[M-1]^-$ in the negative mode. These are consistent with a molar mass of 594 g/mol and a molecular formula of $C_{27}H_{30}O_{15}$. Its ESI-MS/MS showed fragments from subsequent loss of one desoxyhexose *m/z* 449.1 [M+1-146], loss of another hexose at *m/z* 287.3 [M+1-146-162]. The fragment ion at *m/z* 287.3 together with the observed UV maxima confirms that the compound is a kaempferol derivative. This observation is in agreement with earlier reports (Hasan *et al*., 1996, Gohar *et al*., 2000). The fragmention pattern shows the presence of a desoxyhexose (a rhamnose) and a hexose (glucose/galactose) and a kaempferol aglycone.

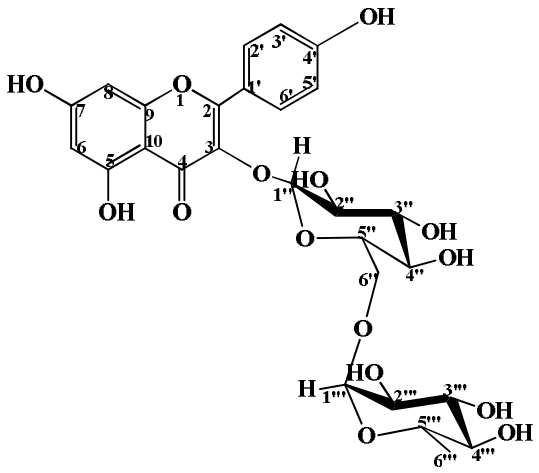
^{1}H and ^{1}H-^{1}H COSY NMR spectra of Compound **3** showed characteristic aromatic spin systems of the rings A and B of kaempferol. (Appendix 18 and 19 respectively). There were signals of an AA'BB' system at δ_H 8.06 (d, *J*=8.9) and δ_H 6.89 (d, *J*=8.9 Hz), each integrated to two protons, assigned to H-2'/H-6' and H-3'/H-5', respectively. In addition, *meta*-coupled protons at δ_H 6.20 (d, *J*=2.1 Hz) and 6.39 (s, *J*=2.0 Hz) were observed and assigned to H-6 and H-8, respectively (Hasan *et al*., 1996, Fang *et al.,* 2005, Muzitano *et al* 2006).



The kaempferol nucleus

The compound was confirmed to be a diglycoside by the presence of two anomeric proton signals which resonate at δ_H 5.12(d, *J*=7.4, 1H) shown earlier to be a ɓ-linkage and 4.52(s, 1H), an Ŭ-linkage, the latter being assigned to a terminal L-rhamnose. Coupling constants between 7 and 8 is traceable to glucose/galactose among the hexoses as reported earlier. The presence of rhamnose sugar was confirmed by three methyl protons observed resonating upfield (δ_H 1.13 d, *J*=6.2), corresponding to an earlier report (Hasan *et al*., 1996). These methyl protons were observed (through COSY) to be linked to H-5"' found in the sugar region. A slight upfield shift of these rhamnosyl methyl protons(δ_H 1.13), a slight downfield shift of the anomeric proton of the glycosyl unit (δ_H 5.12) as well as a higher coupling constant of H-4" show the compound to be a glucoside.

COSY showed the rhamnosyl moiety to be linked to the glycosyl unit which, in turn is linked to the aglycone (Appendix 19). This was earlier shown to be so by the mass spectrum. A 1Ÿ 6 link was established between the anomeric proton of the rhamnosyl moiety (δ_H 4.52) and C-6 of the glycosyl moiety by HMBC (Appendix 21) correlations observed. A correlation was also seen between the anomeric proton (δ_H 5.12) of the glucosyl moiety and C-3 (δ_C 135.65) of the kaempferol nucleus. Furthermore, the chemical shifts of C-2 and C-3 (δ_C 159.58 and 135.65, respectively) indicated that the glycosylation occurred at C-3 of the nucleus (Appendix 22). Compound **3** was identified as Kaempferol-3-O-[Ŭ-L-rhamnopyranosyl(1Ÿ 6) ɓ-D-glucopyranoside]. Trivial name is Kaempferol-3-O-rutinoside or Nicotiflorin and the chemical structure is as shown below.



Compound 3

5.4 Compound 4

Compound **4**, was a yellow powdery solid, and had a HPLC retention time (t_R) of 21.287 min (Appendix 24) with an optical rotation of -50.47 ±0.76. Its UV spectrum had absorption maxima peaks of 265.6 and 348.7 nm (Appendix 25), suggesting it to be a kaempferol flavonoid. ESIMS of compound **4** (Appendix 26) gave strong psuedomolecular ion peaks at *m/z* 595.1 $[M+1]^+$, 596.1[M+2] and 617.3 $[M+Na]^+$ in the positive mode and at *m/z* 593.4 $[M-1]^-$ in the negative mode, confirming a molar mass of 594 g/mol and molecular formula of $C_{27}H_{30}O_{15}$. Its ESI-MS/MS showed fragments at *m/z* 449.0 [M+1-146], possibly due to loss of a desoxyhexose moiety and at *m/z* 287.3 [M+1-146-162], due to a subsequent loss of a hexose moiety . The fragment ion at *m/z* 287.3 together with the observed UV maxima confirms that it is a kaempferol derivative. This observation is consistent with the reports of Hasan *et al.,* (1996) and Gohar *et al.,* (2000). The fragmention pattern shows the presence of two hexoses (a rhamnose and a glucose/galactose) and a kaempferol aglycone.

Compound **4** also showed UV, mass and NMR spectral data similar to those of Compound **3**. However, the following differences were observed:

i. A difference in retention time on HPLC, indicating that the two compounds are different in polarity.

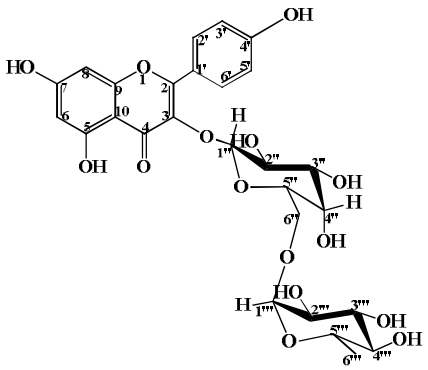
ii. A down field shift in the anomeric proton of the directly attached sugar of compound **3** (δ_H 5.12) when compared to compound **4** (δ_H 5.01).

iii. The rhamnosyl methyl protons of Compound **3** were found to resonate slightly upfield (δ_H 1.13) as against 1.19 of Compound **4**

iv. The H-2 chemical shift of the directly attached sugar of Compound **3** appears at δ_H 3.44 while that of Compound **4** was found downfield (δ_H 3.77)

v. H-4 of the directly attached sugar unit of Compound **3** resonates at 3.53 as against that of Compound **4** (δ_H 3.61).

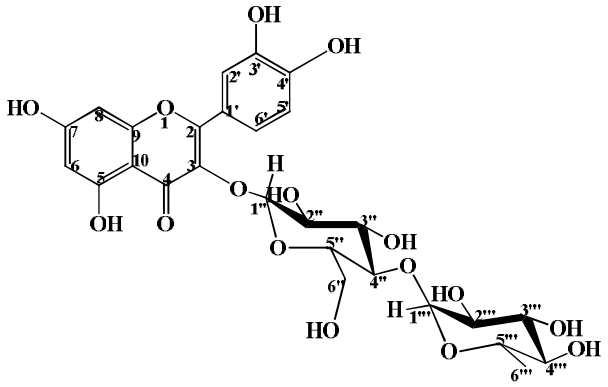
From these results obtained, and comparing with Compounds **1** and **2** earlier discussed, Compound **4** is observed to be a galactoside (H-4 δ_H 3.61 brs) with a *cis* (diequitorial) conformation on H-4" while Compound **3** is a glucoside (H-4 δ_H 3.53 dd *J*=3.4, 9.5) with a *trans* (diaxial) conformation on H-4". Compound **4** was identified as Kaempferol-3-O-[Ŭ-L-rhamnopyranosyl(1Ÿ 6) ɓ-D-galactopyranoside]. Trivial name is Kaempferol-3-O-robinobioside and the chemical structure is as shown below.



Compound **4**

5.5 Compound 5

Compound **5** was a yellow amorphous solid and was eluted by HPLC at a retention time of 19.713 min (Appendix 29). It showed identical mass and UV spectra with compound **1** but had an optical rotation of -23.83±0.76 as against +88.00±1.76 of compound **1**. 1 D H-NMR spectra of compound **5** is shown in (Appendix 31). Studies of the 1 D H-NMR spectra of both compounds showed a downfield shift of the H-5" of compound **5** relative to compound **1**, which is an indication that there is a substitution on its H-4'. Based on this, Compound **5** was identified as Quercetin-3-O-[Ŭ-L-rhamnopyranosyl(1Ÿ 4) ɓ-D-glucopyranoside]. The chemical structure is as shown below.

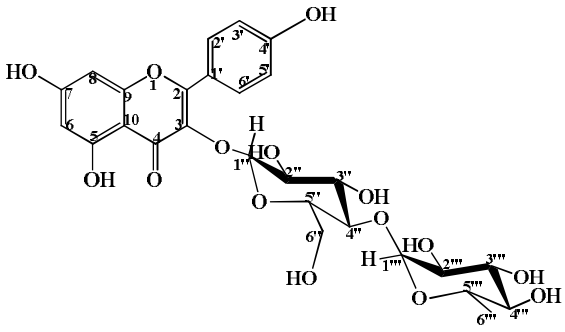


Compound 5

5.6 Compound 6

At a HPLC retention time (t_R) of 21.797 min (Appendix 33), compound **6,** a yellow amorphous solid was eluted. Its optical rotation was found to be -28.29 ±1.17. Compound **6** showed UV absorption peaks at 266.0 nm and 348.0 nm (Appendix 34) typical of kaempferols. This was supported by a set of mass and NMR spectra very similar to compound **3**, for instance *m/z* 595.0 $[M+1]^+$, *m/z* 596.0 $[M+2]^+$, *m/z* 617.2 $[M+Na]^+$, *m/z* 593.2 $[M-1]^-$, *m/z* 449.0 $[M+1-146]^+$, *m/z* 284.2 $[M-2-146-162]^-$, *m/z* 285.3 $[M-1-146-162]^-$

(Appendix 35) and Table 4.6 respectively. A few peak differences were however seen in the sugar region: a slight upfield shift of H-5" indicating a substitution on H-4. A 1Ÿ 4 substitution was therefore proposed for compound **6**. The compound was therefore identified as Kaempferol-3-O-[Ŭ-L-rhamnopyranosyl (1Ÿ 4) ɓ-D-glucopyranoside]. The chemical structure is as shown below.



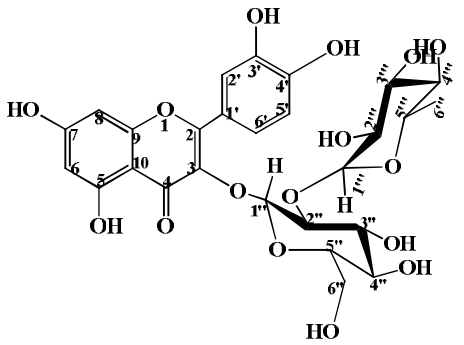
Compound 6

5.7 Compound 7

Compound **7** was isolated as a yellow solid in isomeric mixture with **8**. A HPLC retention time of 20.247 (Appendix 38) was observed with an optical rotation of -18.17 ±1.05. It has UV λ_{max} at 256.0 and 356.0 nm (Appendix 39), which is typical of the quercetin flavonoids.

On the positive ion mode of the ESI-MS spectrum, were 611.0 $[M+1]^+$, 612.1 $[M+2]^+$ and 633.2 $[M+Na]^+$ peaks, while on the negative ion mode 609.4 $[M-1]^-$was observed. From these, a molecular mass of 610 g/mol was deduced. Three other useful peaks were namely: 303.3 $[quercetin+1]^+$, 465.1 $[M-rhamnose+1]^+$ as well as 301.4 $[quercetin-1]^-$ (Appendix 40), From these, one can deduce that the compound is a quercetin based flavonoid diglycoside containing rhamnose as one of the sugars. The rhamnose glycone was considered to be terminal.

The quercetin nucleus of Compound **7** was confirmed by the presence of the ABX aromatic spin system observed in its proton NMR spectra. Compound **7** and **8** are very closely related as was observed in their proton (Appendices 41 and 46) and COSY spectra (Appendices 42 and 47). HPLC retention time (Appendices 38 and 43), while some shifts in their sugar region (Appendices 41 and 46) showed them to be different. The difference once again lies in the sugar region as was observed from the differences in some of their chemical shifts and coupling constants. Compound **7** is a glucoside as observed in the upfield shift in its rhamnosyl protons (ŭ$_H$=1.13) and the downfield shift of the anomeric proton of the directly attached hexose. Furthermore, a downfield shift of proton 3" (ŭ$_H$=3.63) supported the substitution on carbon 2. Compare. Compound **7** is therefore confirmed to be Quercetin-3-O-[Ŭ-L-rhamnopyranosyl(1Ÿ 2) ƃ-D-glucopyranoside]. The chemical structure is given below.

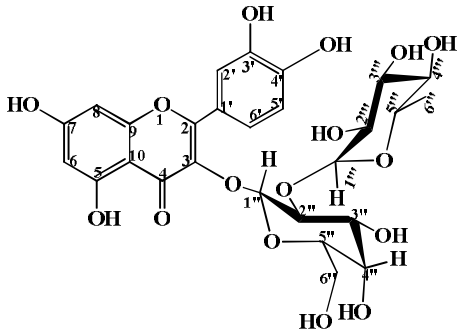


Compound 7

5.8 Compound 8

This is a yellow amorphous compound with an optical rotation of +48.00 ±0.29 and HPLC retention time of 20.520 min (Appendix 43) and was isolated in an isomeric mixture with **7**. Two major UV peaks at 256.6 nm and 356.0 nm (Appendix 44) were observed, typical of the quercetin flavonoids. A molecular mass of 610 was deduced, considering the following diagnostic HPLC-ESI-MS fragment peaks: 611.0, 612.0, 633.2, and 609.3 corresponding to

$[M+1]^+$, $[M+2]^+$, $[M+Na]^+$ and $[M-1]^-$ fragments respectively (Appendix 45). Peak 301.1 was also observed, resulting from [quercetin-1]$^-$. The presence of quercetin nucleus was further confirmed by an ABX aromatic spin system observed at δ_H 6.87(d, *J*=8.5, 1H), 7.61(dd *J*=8.5, 2.1, 1H) and 7.88 (d, *J*=2.1 1H) (Appendix 46). These were assignable to H-5', H-6' and H-2' respectively. The remaining aromatic peaks were identical to that obtained in the quercetin nucleus earlier discussed. Compound **8** was thus identified as Quercetin-3-O-[Ŭ-L-rhamnopyranosyl(1Ÿ 2) ɓ-D-galactopyranoside]. The structure is as shown below.

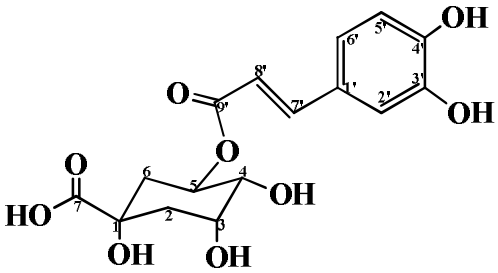


Compound 8

5.9 Compound 9

Compound **9** had a HPLC retention time of 11.537 min (Appendix 48) and was isolated as a brown amorphous solid. It showed UV absorption maxima at 218 nm, 242 nm, and 326 nm (Appendix 49), which is typical of caffeic acid derivatives. Its molecular mass was determined as 354 by ion peaks found in ESIMS spectrum at *m/z* 355 $[M + H]^+$ and *m/z* 353 $[M \bar{\imath} H]^{\bar{\imath}}$ (Appendix 50). Its aliphatic region of the ^{1}H NMR spectrum displayed three oxymethine protons at δ_H 5.38, 4.11 and 3.67 (H-5, H-3 and H-4) together with two pairs of sp^3 methylene protons at δ_H 2.15/1.94 and 2.10/2.00 for H_2-2 and H_2-6, respectively (Appendix 51). These belong to the quinic acid unit of the molecule. This is comparable to earlier reports. Its aromatic region showed resonances for an ABX system at δ_H 7.05 (d, *J* = 1.9 Hz), 6.77 (d, *J* = 8.2 Hz) and 6.95 (d, *J* = 8.2 Hz), corresponding to H-2', H-5' and 6'

respectively. A pair of doublet at δ_H 7.57 and 6.29 with coupling constants of 15.9 Hz were observed. These belong to the two trans olefinic protons found in hydroxy cinnamic acids: H-7' and H-8' respectively. Along with the analysis of mass and UV spectra, a caffeic acid unit was indicated to be present. Compound **9** was identified to be chlorogenic acid (5-caffeoylquinic acid) by spectral comparison of the ^{1}H NMR and MS data with those of already reported data [Pauli *et al.*, 1998]. The structure is as shown below.

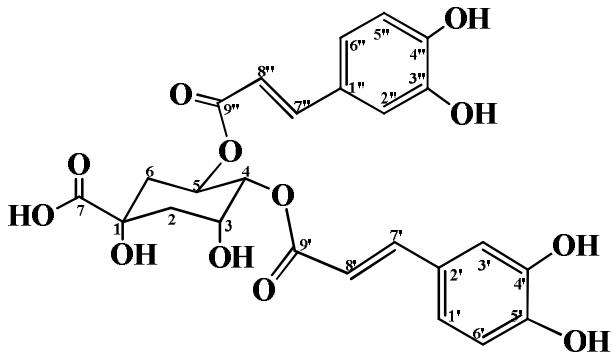


Compound 9

5.10 Compound 10

This compound was obtained as a yellowish brown amorphous solid with UV absorption maxima peaks at 219, 244 and 329 nm. Its molecular mass, 516 nm was deduced from the ESIMS spectrum which had peaks at *m/z* 516.9, 517.9, 539.0 and 515.3, corresponding to $[M+1]^+$, $[M+2]^+$ $[M+Na]^+$and $[M-1]^-$ (Appendix 55). The MS spectrum showed other supporting peaks at 354.9 due to the loss of a caffeic acid unit. The ^{1}H NMR spectrum (Appendix 56) of compound **10** exhibited one quinic acid and two caffeoyl moieties. In the aromatic region, resonances for two ABX systems δ_H 7.05 (d, J =1.9 Hz), 6.77 (d, J = 8.2 Hz) and 6.95 (dd, J = 8.2 Hz); and δ_H 7.09 (d, J = 2.2 Hz), 6.80 (d, J = 8.2 Hz) and 6.95 (d, J = 8.2 Hz)] were observed, which were assignable to two 1,3,4-trisubstituted phenyl units. The assignments and configuration of the quinic acid moiety were determined by the analysis of ^{1}H-^{1}H COSY. The ^{1}H NMR signals at δ_H 5.14 and 5.71 were assignable to H-4 and H-5

respectively. When comparing the ^{1}H NMR of compound **10** with those of chlorogenic acid, a downfield shift of H-4 by 1.47 ppm and for H-5 by 0.33 ppm were observed. This can be due to the attachment of the second caffeoyl unit at C-5. From these data obtained and comparing with earlier reported data, Compound **10** was identified as 4,5-dicaffeoylquinic acid. The structure is as shown below.



Compound 10

The hexoses, glucose/galactose and rhamnose was found to be common to all the flavonoid compounds isolated. They also show a common substitution pattern of 3-O-rhamnopyranosyl glycosylation. These groups of compounds isolated could be very useful for Structure Activity Relationship (SAR) studies as well as other studies on the effects of glycosyl substitution patterns on chemical shifts of the ring carbons of flavonoid nuclei.

5.11 Antimicrobial Activity of the Compounds

The use of antibiotics which is often accompanied by side effects, and the development of resistant strains (Beale 2004) has led to a continuous search for antibiotic compounds among the flavonoids which are non toxic or have low toxicity (Narayana *et al.*, 2001). So far, numerous flavonoids have been reported to show antibacterial activity against the Gram positive and Gram negative bacteria. Compounds that contain hydroxyl groups in 'Ring B' have been reported to show

activity against Gram positive bacteria (e.g. *S. aureus*) (Bylka *et al*., 2004). Mori *et al* observed a relationship between the structure of the flavonoids and their activity against P. *vulgaris* and S. *aureus.* Most of the activity were related to the presence of hydroxyl groups 3', 4' 5' in Ring B and at C-3 (Mori *et al.,* 1987). Glycosylation, however appeared to reduce the antibacterial activity of the compounds.

The sensitivity of the test microorganisms to the compounds is shown in Table 4.11. Only compounds **7** and **8** showed activity against *Escherichia coli* with zones of inhibition of 17 and 13 mm at a concentration of 62.5 εg/ml. The MIC values as calculated from the graph of log of concentration vs square of IZD are 1.77 µg/mL and 1.92 µg/mL respectively. None of the compounds showed activity against the other tested microorganisms.

A previous report on the antimicrobial screening of using the broth dilution method showed some antimicrobial activity against *Pseudomonas aeruginosa* although at a much higher concentration of 6.25 mg/mL with corresponding MBC was at 12.5 mg/mL(Bello *et al*., 2011). However, activity against *Pseudomonas aeruginosa* was not observed at lower concentrations of the compound (below 500 εg/ml) which was employed in the present study.

5.12 Antioxidant Study of the Compounds

The DPPH free radical scavenging assay measures the reducing capacity of antioxidants towards DPPH. Upon reduction the colour of DPPH fades. Consequently compounds with high antioxidant activity lead to a rapid decline in the absorbance of the DPPH solution (Amarowicz *et al*., 2004). Compounds **1- 9** were evaluated for their antioxidant potentials using the DPPH model and the results are shown in **Figure 4.1** and **Table 4.12**. Compounds **1**, **2**, **5**, **7** and **8** which are derivatives of quercetin and **9**, a caffeic acid derivative, showed a dose-dependent antioxidant activities on DPPH free radical scavenging model (**Figure 4.1**)

with IC_{50} values of 52, 48, 36, 66, 56 and 22 εg/mL respectively (**Figure 5.7**). The three kaempferol derivatives **3**, **4** and **6**) did not show any antioxidant activity, (**Figure 5.6b**). This is not surprising since it appears that flavonoids require at least two OH groups in 'Ring B' in order to show good antioxidant activity. In an earlier report De Martino *et al*., (2012) showed that an ortho-dihydroxyl functional group at the B-ring of flavonoids is highly effective for scavenging free radicals (De Martino *et al.,* 2012). Bors *et al* (1992) added that a 2,3-double bond in conjunction with a 4-oxo group in the ñCò ring and a 5-hydroxyl group on the ñAò ring are also required. Compounds **5** and **9** showed better antioxidant activity (**Figure 5.6b,d**) than the standard drug, Vitamin C (IC_{50}= 49 εg/mL) The antioxidant activity of compounds **1**, **2**, **5**, **7**, **8** and **9** may be attributed to their proton donating ability. The above report is consistent with an independent report by Hou *et al* (2005). In the report, quercetin-3-O-rutinoside showed good antioxidant activity while kaempferol-3-O-rutinoside and kaempferol-3-O-robinobioside had very poor IC_{50} results (Hou *et al*., 2005). Quercetins and caffeic acid derivatives are known to show good antioxidant activity unlike the kaempferols (Hou *et al*., 2005). The antioxidant efficiency of chlorogenic acid (5-caffeoyl quinic acid) was reported to be weaker than those of the dicaffeoylquinic acids predicting that compound **10** is expected to show an even stronger antioxidant activity.

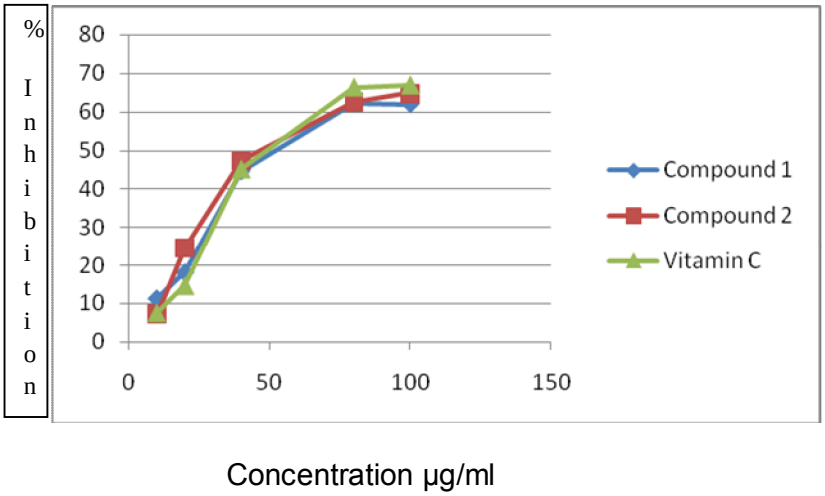


Figure 5.6a: Percent Inhibition against Concentration for Compounds 1, 2 and Vit C.

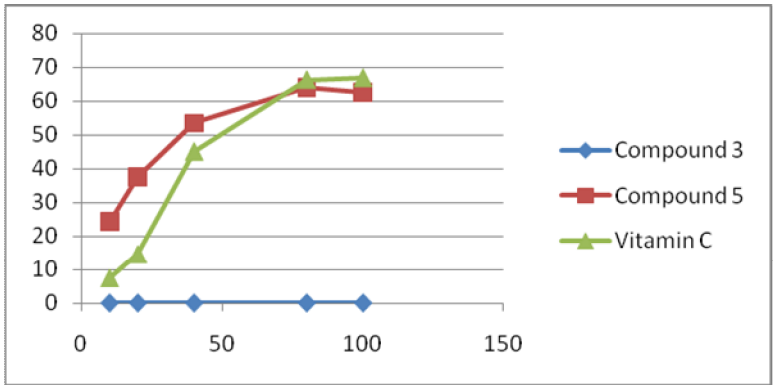


Figure 5.6b: Percent Inhibition against Concentration for Compounds 3, 5 and Vit C.

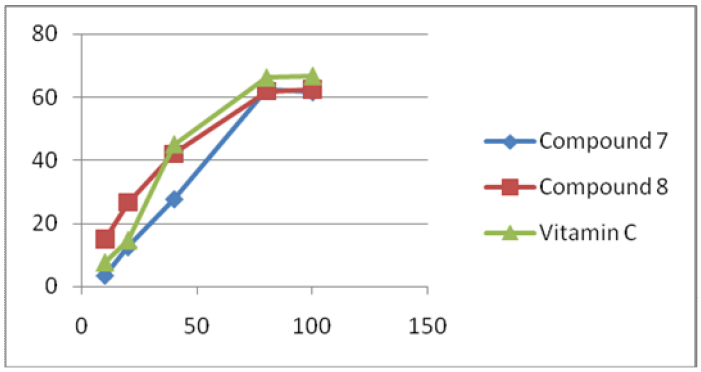


Figure 5.6c: Percent Inhibition against Concentration for Compounds 7, 8 and Vit C.

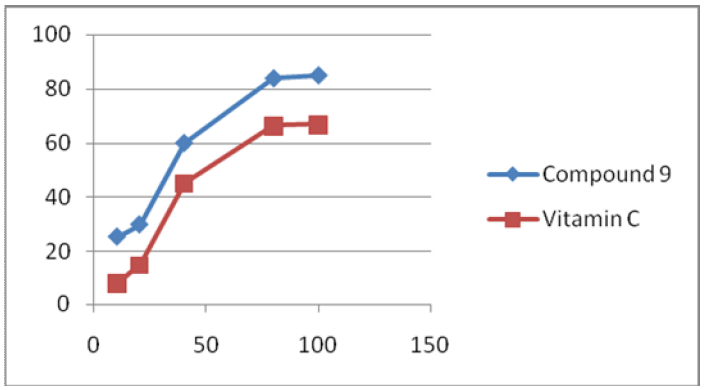


Figure 5.6d: Percent Inhibition against Concentration for Compounds 9 and Vit C.

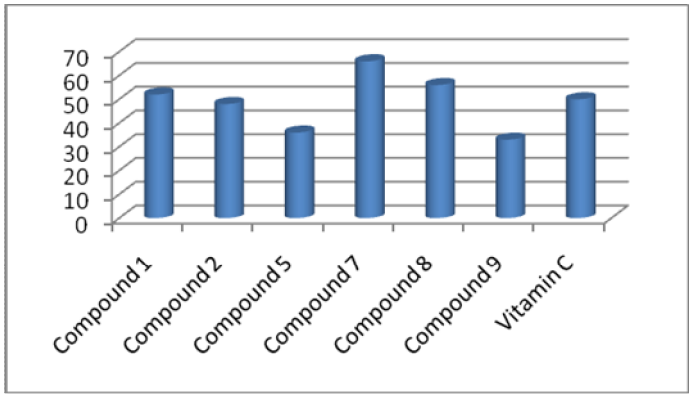


Fig. 5.7: IC_{50} values of the Compounds against Standard Vitamin C

The antioxidant capacity of a flavonoid is linked to its three structural groups.

i. The ortho-dihydroxy (catechol) structure in the B-ring, which confers greater stability to aroxyl radicals, possibly through hydrogen bonding, and which participates in electron dislocation.

ii. The 2,3-double bond, in conjugation with a 4-oxo function, responsible for electron dislocation from the B-ring.

iii. The presence of both 3-and 5-hydroxyl groups on 3ô,4ô-catechol structure in B-ring strongly enhances lipid peroxide inhibition and this arrangement is an important characteristic of most potent scavengers of peroxyl, superoxide and peroxynitrite radicals (Heim *et al.,* 2002) and its absence decreases antioxidant activity.

CHAPTER SIX

6.1 SUMMARY

Natural products, which include secondary metabolites from plants, have been the major sources of chemical substances which have become useful starting materials for drug discovery over the past century. Several of the important secondary metabolites are derived from plants with known ethnomedicinal uses.

Flavonoids are a group of phenolic molecules found in fruits, leaves, and certain beverages. They are derived from phenyl- and malonyl-coenzyme A and are generally classified into several groups, e.g., flavones, flavonols, chalcones, flavanones, isoflavones, anthocyanidins and others, according to the chemical features of rings A and B, as well as the oxidation of the C-ring (De Martino *et al.,* 2012). Flavonoids are reputed to have anti inflammatory, anti-allergic, antitumor, antimicrobial and antioxidant effects. Owing to these reported rich potential beneficial effects on human health, they have been studied widely, leading to the isolation of thousands of them (Harbourne and Williams 2000). The occurrence of numerous substitution patterns in which primary substituents themselves can be substituted sometimes yielding highly complex structures has contributed to this large variety of flavonoids (DôArchivio *et al.,* 2007).

Flavonoids are UV-absorbing compounds and are thought to provide a means of protection against UV damage and subsequent cell death by protecting DNA from dimerization and breakage (Dixon and Paiva, 1995). This may justify the high content of flavonoids in leaves and fruits of plants in response to high light irradiation in order to attenuate the amount of light reaching the photosynthetic cells. It was reported that UV irradiation induces the synthesis of flavonoids particularly kaempferol derivatives [Dixon and Paiva, 1995].

The major functions of flavonoids in plants are to protect them from predators and infectious agents, shield plants from UV-B radiation, act as signaling molecules in plant-bacterium symbioses and are the primary pigments that attract pollinators and seed dispersers (Mol *et al.,* 1998).

Dixon and Paiva suggested that chlorogenic acid production in plants may be induced in response to wounding or to feeding by herbivores, and then act directly as defence compounds or may serve as precursor for the wound-induced polyphenolic barriers [Dixon and Paiva, 1995].

Several herbal mixtures and supplements obtained from leaves of plants contain flavonoids. *Alstonia* is a wide spread genus of evergreen trees and shrubs from the dogbane family (Apocynaecae). Apocynaecae family consists of about 50 species widely distributed in Africa, Asia and America.

Alstonia boonei is a medicinal plant that is widely used across Africa for various ailments. Its ethomedicinal uses include the efficacy of its leaves and latex when applied topically to reduce swellings for the treatment of rheumatic pains, muscular pains, hypertension and malaria, as an antidote against snake, rat or scorpion poison. *A. boonei* has been listed in the African Pharmacopoeia as an antimalarial drug. It is also useful in expelling retained products of conception and afterbirth (like the placenta) when given to women.

In this study, ten phenolic compounds including eight flavonoids as well as two caffeic acid derivatives were, to the best of our knowlegde, isolated for the first time from the ethyl acetate fraction of the methanol leaf extract of the plant *Alstonia boonei*. The flavonoid glycosides isolated were very closely related. Within each aglycone group, differences were observed arising from presence of either glucosyl or galactosyl moieties. Subtle differences arising as a result of differing positions of sugar linkages were also observed.

Isolation of this group of closely related compounds was made possible by various modern chromatographic techniques used for separation and purification of the compounds from the crude extract. Their structures were also elucidated by the use of modern one-dimensional (H, C, DEPT-135) and two dimensional (H-H COSY, HMQC, HMBC) NMR techniques as well as Mass Spectroscopy.

The compounds that have been isolated and structurally elucidated from the ethyl acetate fraction of the leaves of *A. boonei* are:

quercetin-3-O- [Ŭ-L-rhamnopyranosyl(1Ÿ 6) ɓ-D-glucopyranoside] (**1**)

quercetin-3-O- [Ŭ-L-rhamnopyranosyl(1Ÿ 6) ɓ-D-galactopyranoside] (**2**)

kaempferol-3-O-[Ŭ-L-rhamnopyranosyl(1Ÿ 6) ɓ-D-glucopyranoside] (**3**)

kaempferol-3-O-[Ŭ-L-rhamnopyranosyl(1Ÿ 6) ɓ-D-galactopyranoside] (**4**)

quercetin-3-O-[Ŭ-L-rhamnopyranosyl(1Ÿ 4) ɓ-D-glucoctopyranoside] (**5**)

kaempferol-3-O-[Ŭ-L-rhamnopyranosyl(1Ÿ 4) ɓ-D-glucopyranoside] (**6**)

quercetin-3-O-[Ŭ-L-rhamnopyranosyl(1Ÿ 2) ɓ-D-glucopyranoside] (**7**)

quercetin-3-O-[Ŭ-L-rhamnopyranosyl(1Ÿ 2) ɓ-D-galactopyranoside] (**8**).

The two caffeic acid derivatives are chlorogenic acid (**9**) and 4,5-dicaffeoylcinnamic acid (**10**)

The quercetin and caffeic acid derivatives isolated showed very promising and dose dependent antioxidant activity in the DPPH free radical model. Only two of the compounds (compounds **7** and **8**) showed promising antimicrobial effect with MIC values in very low microgram per ml range.

6.2 CONCLUSION

The quercetin and caffeic acid derivatives (compounds **1**, **2**, **5**, **7, 8** and **9**) showed very good antioxidant activity in the DPPH free radical scavenging model unlike the three kaempferol derivatives (**3**, **4** and **6**). This observed antioxidant activity can be correlated with the chemical structures of the isolated compounds. This agrees with the works of De Martino, *et al*., (2012) whereby the authors observed that the presnece of least two *ortho* coupled OH groups in RING B of the flavonoid nucleus or in the aromatic rings of the caffeic acid derivatives is necessary for a good antioxidant activity.

The two compounds which showed promising antimicrobial effect against *Escherichia coli* at a concentration of 62.5 ε g/ml are derivatives of quercetin and they both have a 1Ÿ 2 linkage between the terminal rhamnose and the directly attached sugar. This study is a typical case where nature provides unique and complex molecules for structure activity relationship studies. These compounds can be considered for further detailed pharmacological studies like anti inflammatory and anti cancer activities. The profound antioxidant activity of some of the isolated quercetin derivatives and chlorogenic acid compounds justify the efficacy of the *Alstonia boonei* De Wild (Apocynacea) leaf extracts in management of disorders like inflammation and cancer, which are associated with oxidative stress. These groups of molecules could be very useful for Structure Activity Relationship (SAR) studies as well as for the studies on the effects of glycosyl substitution patterns on NMR chemical shifts of the aromatic ring protons and carbons in flavonoids.

6.3 CONTRIBUTIONS TO KNOWLEDGE

Hitherto, the chemistry of *Alstonia boonei* leaves has been poorly investigated. Most of the works reported in the literature are limited to pharmacological investigations of crude

methanol extracts of *A boonei* leaves, with little or no attempt on the characterization of the bioactive compounds from this plant species.

The present work is thus the first attempt on the systematic isolation, purification and structure elucidation of bioactive compounds from the leaves of *A. boonei*. We report in this thesis the isolation and structure elucidation of ten (10) phenolic compounds, which to the best of our knowledge, have not been previously isolated from the leaves of *Alstonia boonei*. This work is also the first attempt at validating the antioxidant and antimicrobial activities of *Alstonia boonei* leaves and associating these activities to specific chemical compounds isolated from the plant.

The compounds isolated from this plants also provided a basis for further investigation on Structure Activity Relationship (SAR) studies of plant polyphenols.

REFERENCES

Abbiw, D. (1990) Useful Plants of Ghana: West African Uses of wild and cultivated plants, Intermediate Technology Publications, Royal Botanical Garden, Kew, London.

Abe, F., Chen, R. F., Yamauchi, T. *et al*., (1989) Alkaloids from the leaves of *Alstonia scholaris,* 31,Fukuoka University, Fukuoka, Japan.

Abe, F.; Chen, R. F., Yamauchi, T., Marubayashi, N. and Ueda I., (1989) ñStudies on the constituents of Alstonia scholaris. Part I. Alschomine and isoalschomine, new alkaloids from the leaves of *Alstonia scholaris*,ò Chemical & Pharmaceutical Bulletin; 37(4) 887ï 890.

Abraham, R. J., Fisher, J. and Loftus, P. (1988) Introduction to NMR Spectroscopy John Wiley & Sons Chichester

Adotey, J. P. K, Adukpo, G. E., Boahen, Y. O. and Armah, F. A.; (2012) A Review of the Ethnobotany and Pharmacological Importance of *Alstonia boonei* De Wild (Apocynaceae) ISRN Pharmacol. Vol 2012.

Afanasèv, I.B., Dorozhko, A.I., Brodskii, A.V., Kostyuk, V.A., and Patapovitch, A.I. (2000), Biochem. Pharmacol., 38:1763.

Afolayan, A. J. and Meyer, J. J. (1997) The antimicrobial activity of 3,5,7-trihydroxyflavone isolated from the shoots of *Helichrysum aureonitens*. J Ethnopharmacol; 57:177ï 181.

Akinloye, O.A., Oshilaja, R.T., Okelanfa O.A., Akinloye, D.I. and Idowu, O.M.O (2013) Hypoglyceamic activity of *Alstonia boonei* stem bark extract in mice Agric. Biol. J. N. Am. 4(1): 1-5

Akinmoladun, A.C., Ibukun, E.O., Afor, E., Akinrinlola, B.L., Onibon, T.R., Akinboye, A.O., Obuotor, E.M., Farombi, E.O. (2007). Chemical constituents and antioxidant activity of *Alstonia boonei.* African Journal of Biotechnology 6: 1197 ï 1201.

Al-Saleh, F. S., Gamal, El-Din, A. Y., Abbas, J. A. and Saeed, N. A. (1997) Phytochemical and biological studies of medicinal plants in Bahrain: family Chenopodiaceae. Part 2. Int J Pharmacogn; 35: 38ï 42.

Amarowicz , R., Pegg, R. P., Rahimi-Moghaddam,P., Barl, B., and Weil, J. A. (2004) Free radical scavenging capacity and antioxidant activity of selected plant species from the Canadian prairies, Food Chem; 84:551-562

Ames, B. N.; Sjigenaga, N. K. and Hagen, T. M. (1993) Proc 1 Atl Acad Sci USA; 90:7915-7922

Amic, D. et al. (2003). "Structure-Radical Scavenging Activity Relationships of Flavonoids. "Croatica Chemica Acta; 76 (1) 55-61.

Amoros, M., Simoes, C. M., Girre, L., Sauvager, F. and Cormier, M. (1992) Synergistic effect of flavones and flavonols against herpes simplex virus type 1 in cell culture. Comparison with the antiviral activity of propolis. J Nat Prod; 55:1732ï 1740.

Anderson, K., Teuber, S.S., Gobeille, A., Cremin, P., Waterhouse, A.L., and Steinberg, F.M. (2001) J. Nutr., 131, 2837.

Anderson, Ø. M. and Markham, K. R. (Editors) (2006) Flavonoids, Chemistry Biochemistry and Applications CRC Press.

"Antimicrobial - Definition from the Merriam-Webster Online Dictionary"

Arias T. D. (1999). Glosario de Medicamentos: desarrollo, evaluación y uso. Organización Panamericana de la Salud. OMS, Washington D. C.

Arima, H.; Ashida, H. and Danno, G. (2002) Rutin-enhanced antibacterial activities of flavonoids against Bacillus cereus and *Salmonella enteritidis*. Biosci Biotechnol Biochem; 66:1009ï 1014.

Arulmozhi, S. Mitra-Mazumder, P., Ashok, P, et al., (2007) Pharmacological activities of *Alstonia Scholaris* Linn (Apocynaceae) : a review. Phcog Rev.; 1: 163-170

Asuzu, I. U. and Anaga, A. O. (1991) ñPharmacological screening of the aqueous extract of *Alstonia boonei* bark,ò Fitoterapia; 63(5) 411ï 417.

Atawodi, S.E. (2005) Antioxidant potential of African medicinal plants African Journal of Biotechnology; 4 (2) 128-133,

Ayiku, M.N.B (1992) Ghana Herbal Pharmacopoeia, Technology Transfer Centre.

Balde, A. M; Van Hoof, L.; Pieters, L. A.; Vanden-Berghe, D. A and Vlietinck, A. J. (1990) Plant antiviral agents. VII. Antiviral and antibacterial proanthocyanidins from the bark of *Pavetta owariensis*. Phytother Res; 4:182ï 188.

Baldioli, M., Servili, M. Perretti, G., and Montedoro, G.F. (1996). JAOCS, 73, 1589.

Balick, M. J. and Cox, P.A. (1996). Plants, people and culture: the science of ethnobotany. The Scientific American Library, New York.

Balick, M. J., Elisabetsky, E. and Laird, S.A. (1996). Medicinal resources of the Tropical Forest: Biodiversity and its importance to Human Health. Columbia University Press, New York.

Banerji, A. and Banerji, J. (1977) ñIsolation of pseudoakuammigine from the leaves of *Alstonia scholaris* R. Br.,ò Indian Journal of Chemistry B; 15 (4) 390ï 391.

Banerji, A. and Siddhanta, A. K. (1981) ñScholarine: an indole alkaloid of *Alstonia scholaris*,ò Phytochemistry; 20 (3) 540ï 542.

Banerji, J. and Chakrabarti, R. (1984) ñConstituents of *Alstonia schorlaris* R.Br:-conversion of picrinine into strictamine and to a pyrrolidinoindolenine system,ò Indian Journal of Chemistry B; 23(5) 453ï 454.

Banerji, J.; Mustafi, R. and Roy, D. J. (1984) ñ(-)-Scholarine and (+)-lochneridineð constituents of *Alstonia scholaris* R.Br. (Apocynaceae),ò Indian Journal of Chemistry B; 23(5) 455.

Bannerman, R. H., Burton, J. and Wen-Chieh. Ch (1983). TM and health care coverage: a reader for health administrators and practitioners. World Health Organization 1983: 318-27. Geneva, Switzerland

Barboza, G. E., Cantero,J.J, Núñez,C., Pacciaroni, A and Ariza Espinar, L. (2009). Medicinal Plants: A general review and a phytochemical and ethnopharmacological screening of the native Argentine Flora. Kurtziana; 34 (1-2): 7-365.

Baris, O., Gulluce, M., Sahin, F., Ozer, H., Kilic, H., Ozkan, H., Sokmen, M. and Ozbek, T. (2006) Biological activities of the essential oil and methanol extract of *Achillea Biebersteinii* Afan. (Asteraceae). Turk. J. Biol.; 30: 65-73.

Bashir, A. K., Abdalla, A.A., Wasfi, I. A., Hassan, E. S., Amiri, M. H. and Crabb, T. A. (1994) Flavonoids of *Limonium axillare*. Int J Pharmacogn; 32:366ï 372.

Basile, A., Giordano, S., Lopez-Saez, J. A. and Cobianchi, R. C. (1999) Antibacterial activity of pure flavonoids isolated from mosses. Phytochemistry; 52:1479ï 1482.

Basile, A., Sorbo. S., Giordano, S. et al. (2000) Antibacterial and allelopathic activity of extract from *Castanea sativa* leaves. Fitoterapia; 71:110ï 116.

Basri, D .F. and Fan, S. H. (2005). The potential of aqueous and acetone extracts of galls of Quercus infectoria as antibacterial agents. Indian J. Pharmacol. 37(1):26-29.

Bauer, A. W, Kirby, W .M. M.; Sherris, J. C. and Turk, M. (1966) Antibiotic susceptibility testing by a standardized single disc method. Am J Clin Pathol; 45:493ï 496

Bello, I.A, Ndukwe, G.I, Audu, O.T and Habila, J.D. (2011) A bioactive flavonoid from *Pavetta crassipes* K. Schum, Organic and Medicinal Chemistry Letters, 1:14

Bhattacharya, S. K., Bose, R., Dutta, S. C., Ray, A. B. and S. R. Guha, S. R. (1979) ñNeuropharmacological studies on strictamine isolated from *Alstonia scholaris*,ò Indian Journal of Experimental Biology; 17(6)598ï 600.

Bisset, N. (ed.). (1994). Herbal drugs and phytopharmaceuticals. A handbook for practice on a scientific basis. Ed. Medpharm. Sc. Publishers, Stutgarts-CRC Press, Boca Raton.

Biswas, G. K. and Saharia, G. S. (1978) ñA new alkaloidal glycoside from *Alstonia scholaris,*ò Indian Journal of Chemistry; 1(1) 66ï 67.

Blake, D. and Winyard P. G. (1995) Immunopharmacology of free radical species Academic press San Diego

Bondet, V., Brand-Williams, W. and Berset, C. Kinetics and Mechanisms of Antioxidant Activity using the DPPHÅFree Radical Method ©1997 Academic Press Limited

Boonchuay, W. and Court, W. E. (1976) ñAlkaloids of Alstonia scholaris from Thailand,ò Planta Medica; 29(4) 380ï 390.

Boonchuay, W. and Court, W. E. (1976) ñMinor alkaloids of *Alstonia scholaris* root,ò Phytochemistry; 15(5) 821.

Bors, W., Heller, W., Muchel, C., and Saran, M. (1992) Structural Principles of Falvonoid Antioxidants, in Free Radical and the Liver, ed. by G. Cosmós and J. Fehér, Springer, Berlin, 77.

Boynes J.W. (1991). Role of oxidative stress in the development of complication in diabetes. Diabetes; 40:405 ï 411.

Bravo, L. (1998) Polyphenols: Chemistry, dietary sources, metabolism and nutritional significance. Nutr Rev; 56(11) 317-333

Brinkworth, R. I., Stoermer, M. J. and Fairlie, D. P. (1992) Flavones are inhibitors of HIV-1 proteinase. Biochem Biophys Res Commun; 188:631ï 637.

Briskin, D. (2000). Medicinal plants and phytomedicines. Linking plant biochemistry and physiology to human health. Pl. Physiol.; 124: 507-514.

Butler, M. S (2005) Nat Prod Rep; 22:162

Cafarchia, C., De Laurentis, N., Milillo, M. A, Losacco, V. Puccini, V. (1990) Antifungal activity of Apulia region propolis. Parassitologia; 41:587ï 590.

Cai, X-H., Tan, Q-G., Liu, Y-P., Feng, T., Du, Z-Z., Li, W-Q and Luo, X-D. (2008). A Cage-Monoterpene Indol Alkaloids from *Alstonia scholaris*. Organic Letters; 10(4): 577-580.

Cam, M., Hisil, Y. and Kuscu, A. (2007). Organic Acid, Phenolic Content, and Antioxidant Capacity of Fruit Flesh and Seed of *Viburnum Opulus*. *Chemistry of Natural Compounds.* 43(4):460-461.

Chahar, M. K., Sharma, N., Dobhal, M. P. and Joshi Y. C. (2011) Flavonoids: A Versatile Source of Anticancer Drugs. Pharmacogn. Rev.; 5(9): 1-12

Chandrakant, K., Arun, G. Satyajyoti, K. and Shefali K. (2012) Drug discovery from plant sources: An integrated approach Ayu.; 33(1): 10ï 19.

Chandrasekaran, B. and Nagarajan, B. (1983)ñGrowth inhibitory effect of echitamine and plumbagin on fibrosarcoma in rats,ò Arogya; 9(1) 60ï 63.

Chang, S., Tan, C., Frankel, E. N. and Barrett, D. M. (2000). Low-Density Lipoprotein Antioxidant Activity of Phenolic Compounds and Polyphenol Oxidase Activity in Selected Clingstone Peach Cultivars. *Journal of Agricultural and Food Chemistry,* Vol. 48.; (2):147-151.

Chatterjee, A., Mukherjee, B., Ghosal, S. and Banerjee, P. K (1969) ñOccurrence of rhazine in Alstonia scholaris: biogenetic and chemotaxonomic significance of the co-occurrence of several indole alkaloids having a common structural pattern,ò Journal of the Indian Chemical Society; 46 (7) 635ï 638.

Chaturverdi, A. K., Parmar, S. S. Bhatnagar, S. C., Misra, G. And Nigam, S. K. (1974) Anticonvuslant and antiinflammatory activity of natural plant coumarins and triterpenoids. Research Communications in Chemical Pathology and Pharmacology; 9:11-22

Chauhan, J. S., Chaturvedi, R. and S. Kumar, S. (1985) ñIsookanin-7-O-a-L-rhamnopyranoside, a new flavanone glycoside from the roots of *Alstonia scholaris* R. Br.,ò Indian Journal of Chemistry B; 24(2) 219.

Cheng, P.C and Wong,G. (1996) Honey bee propolis: prospects in medicine. Bee World; 77:8ï 15.

Chung, Y., Chang, C., Chao, W., Lin C. and Chou, S. (2002) Antioxidative activity and safety of the 50 ethanolic extract from the red bean fermented by Bacillus subtilis IMR-NKI J. Agric food chem.; 5: 2454-2458

Clifford, M. N. (2004) Diet-Derived Phenols in Plasma and Tissues and Their Implications for Health. Planta Medica.; 70:1103-1114

Collier A., Wilson R., Bradley H., Thomson J.A. and Small M. (1990). Free radical activity in type 2 diabetes. Diabetes; 7:27 ï 30.

Corongui, F. P. and Banni, S. (1994) Detection of conjugated dienes by second derivative ultra violet spectrophotometry; 233:303-310

Cottiglia, F., Loy, G., Garau, D. *et al.* (2001) Antimicrobial evaluation of coumarins and flavonoids from the stems of Daphne gnidium L. Phytomedicine; 8:302ï 305.

Cowan, M. M. (1999). Plant products as antimicrobial agents. Clin. Microbiol. Rev.; 12(4): 564-582.

Croquelois, G, Kunesch, N, Debray, M and Poisson, J (1972) ñ*Alstonia boonei* alkaloids,ò Medicinal Plants and Phytotherapy; 6 (2) 122ï 127.

Croteau R., Kutchan, T. M. and Lewis, N. G. (2000). Natural products (secondary metabolites), in B. Buchanan, W. Gruissem & R. Jones (eds.). Biochemistry and Molecular Biology of Plants; 1250-1318. American Society of Plant Physiologists, Rockville, MD, USA.

Cushnie, T. P. T. and Lamb, A. J. (2005) Antimicrobial activity of þavonoids International Journal of Antimicrobial Agents; 26: 343-356.

Cushnie, T. P. T., Hamilton, V. E. S. and Lamb, A. J. (2003) Assessment of the antibacterial activity of selected flavonoids and consideration of discrepancies between previous reports. Microbiol Res; 158: 281ï 289.

Cuyckens, F and Claeys, M. (2004) Mass spectrometry in structural analysis of flavonoids. J mass Spectrom; 39:1-15.

Cuyckens, F., Rozenberg, R., de Hoffman, E. and Claeys, M. (2001) Structure characterisation of flavonoid O-glycosides by positive and negative nano-electrospray ion trap mass spectrometry. J Mass spectrum; 36: 1203-1210.

DôArchivo,M., Filesi,C., Di Bendetto, R., Guarguilo,R., Giovannini,C. and Masella, R. (2007) Polyphenols, dietry sources and bioactivity. Ann !st Super Sanita; 43(4), 348-361

Das, N.P., and Ramanathan, L. (1992) Studies on Flavonoids and related compounds as antioxidants in food. In Lipid-Soluble Antioxidants: Biochemistry and Clinical Applications (ASH Ong. and L. Packer Eds.), Birkhäuser Verlag, Basel, 295.

Davies, K. J.(1995) Biochem Soc Symp. (61) 1-31

De Martino, L., Mencherini, T., Mancini, E., Aquino, R.P, De Almeida, L.F.R and De Feo, V (2012) In Vitro Phytotoxicity and Antioxidant Activity of Selected Flavonoids Int J Mol Sci; 13(5): 5406ï 5419

Decker, E. A., Warner, M. M., Richards, M. P. and Shahidi, F. (2005) Measuring antioxidant effectiveness in food J Agric Food chem.; 53: 4303-4312

Delanty, N. and Dichter, M. (2000). Antioxidant therapy in neurologic diseases. Arch. Neurol.; 57(9):1265-1270.

Derome, A.E. (1987) Modern NMR Techniques for Chemistry Research Pergamon Press, Oxford

Desoky, E. K. (1999) ñFlavonoidal contents of *Alstonia scholaris* R.Br,ò Bulletin of Pharmaceutical Sciences; 22 (2) 117ï 121.

Devienne, K. F. and Raddi, M. S. G. (2002). Screening for antimicrobial activity of natural products using a microplate photometer. Braz. J. Microbiol.; 33(2): 97-105.

Dhillion S. S. & Ampornpan, L. (2000). Bioprospecting and phytomedicines in Thailand: conservation, benefit sharing and regulations, in H. Svarstad & S. S. Dhillion (eds.). Responding to Bioprospecting: from biodiversity in the south to medicines in the north. Spartacus Forlag AS, Oslo

Dutta, S. C., Bhattacharya, S. K. and Ray, A. B. (1976) ñFlower alkaloids of *Alstonia scholaris*,ò Planta Medica; 30 (1) 86ï 89.

Elisabetsky, E. and Costa-Campos, L. (2006). The alkaloid alstonine: a review of its pharmacological properties. Evidence-Based Complementary and Alternative Medicine; 3(1): 39-48.

Ernst R. R., Bodenhausen G. and Wokaun A. (1987) Principles of nuclear magnetic resonance in one and two dimensions. Claredon Press, Oxford

Fang, S.; Rao, K.Y and Tzeng, Y (2005) Inhibitory effects of flavonol glycosides from *Cinnamomum osmophloeum* on inflammatory mediators in LPS/IFN-ɔ-activated murine macrophages Bioorg. Med Chem.; 13 2381-2388

Farrant, J. M., (2000) A Comparison of Mechanisms of Desiccation Tolerance among Three Angiosperm Resurrection Plants. Plant Ecol; 151,(29)

Fearnley, J. (2001) Bee propolis. London, UK: Souvenir Press Ltd.

Feng, T., Li, Y., Cai, X-H., Gong, X., Liu, Y-P., Zhang, R-T., Zang, X-Y., Tan, Q-G and Luo, X-D. (2009). Monoterpene Indole Alkaloids from *Alstonia yamanensis*. J. Nat. Prod. 72: 1836-1841.

Fesen, M. R, Pommier, Y.; Leteurtre, F.; Hiroguchi, S.; Yung J. and Kohn, K. W. (1994) Inhibition of HIV-1 integrase by flavones, caffeic acid phenethyl ester (CAPE) and related compounds. Biochem Pharmacol; 48:595ï 608.

Finar, I. L., (1997) Organic Chemistry. Vol 2: Stereochemistry and the Chemistry of Natural Products. Longman

Gey, K. F., Puska, P., Jordan, P. and Moser, U. K. (1991). Inverse Correlation between Plasma Vitamin E and Mortality from Ischemic Heart Disease in Cross-Cultural Epidemiology. *The American Journal of Clinical Nutrition.;* 53(1):326S-334S.

Ghosh, R., Roychowdhury, P., Chattopadhyay, D. and Iitaka, Y, (1988) ñThe structure of an alkaloid, picrinine, from *Alstonia scholaris*,ò Acta Crystallographica C; 44(12) 2151ï 2154

Gohar, A. A., Maatooq, G. T. and Niwa, M. (2000) Two flavonoid glycosides from *Chenopodium murale* Phytochemistry; 53:299-303

Gordon, M. H. (2001) The development of oxidative rancidity on foods In: Porkony J., Yanishlieva N., Gordon M editors. Antioxidants in food: Practical application. Cambridge England. Woodhead publishing limited 21

Gosse, B. K.; Bryson, T. A. and Gokou, T. (1999) ñStudy of triterpenoids from *Alstonia boonei*,ò Société Ouest-Africaine de Chimie; 5 (8) 123ï 128.

Gould, K.S and Lister, C. (2006) Flavonoid function in Plants In: Anderson, O. M. and Markham K. R. (Eds) Flavonoids, Chemistry, Biochemistry and Applications. CRC Press, Boca Raton FL 397-442

Grierson, D. S. and Afolayan, A. J. (1999) Antibacterial activity of some indigenous plants used for the treatment of wounds in the Eastern Cape, South Africa. J. Ethnopharmacol. 66: 103-106.

Gupta, M. B., Bhalla, T. N., Tangri, K. K. and Bhargava, K. P. (1971) Biochemical study of anti-inflammatory activity of Ŭ- and ɓ-amyrin acetate. Biochemical pharmacology; 20: 401-405.

Gurib-Fakim A. & Brendler, T.; (2004). Medicinal and Aromatic Plants of Indian Ocean Islands. Medpharm GmbH, Scientific Publishers, Stuttgart, Germany.

Gurib-Fakim A. (2006). Traditions of yesterday and drugs of tomorrow: Molecular Aspects of Medicine. Special Edition. Elsevier Publications, UK

Hadi, S. and Bremner, J. B. (2001) ñInitial studies on alkaloids from Lombok medicinal plants,ò Molecules; 6 (2) 117ï 129.

Halabalaki, M., Urbain, A., Paschali, A., Mitakou, S., Tillequin, F. and Skaltsounis, A. (2011) Quercetin and Kaempferol 3-O-[Ŭ-L-Rhamnopyranosyl-(1Ÿ 2)-Ŭ-L-rabinapyranoside]-7O-Ŭ-L-rhamnopyranosides from *Anthylllis hermanniae*: Structure Determination and Conformational Studies J. Nat. Prod; 74, 1939-1945

Halberstein R. (2005). Medicinal Plants: historical and cross-cultural usage patterns. Ann. Epidemiol. 15: 686-699

Halliwell, B. (1994) Lancet; 344: 721-724.

Halliwell, B. (1997) I Utr Rev (55) 44-52.

Halliwell, B. and Gutteridge, J. M. C. (1998) In Free radicals in Biology and Medicine Oxford University press Oxford.

Hamilton-Miller, J. M. T. and Shah, S. (2000). Activity of the tea component epicatechin gallate and analogues against methicillin-resistant *Staphylococcus aureus*. J Antimicrob Chemother; 46:852ï 853.

Harborne, J. B. (1986) In: Plant flavonoids in Biology and Medicine E; Cody V, Middleton Harbourne, J. B. Eds: Alan R Liss New York; 15-24.

Harborne, J. B. (1994) In Flavonoids: Advances in research since 1986 Harborne JB Ed Chapman and Hall London; 589-618.

Harbourne, J. B and Williams,C. A (2000) Advances in Flavonoid research since 1992, Phytochemistry; 55(6), 481-504

Harman, D. J (1956) Gerontology; (11) 289-300.

Harris, R. K. (1997) Nuclear Magnetic Resonance Spectroscopy Pitman, London)

Hasan A.; Ahmad, I.; Khan, M. A. and Chudhary (1996) Two flavonol triglycosides from the leaves of *Indigofera Hebepetala* Phytochemistry; 43 (5) 1115-1118

Havsteen, B. (1983) Flavonoids, a class of natural products of high pharmacological potency. Biochem Pharmacol; 32: 1141ï 1148.

Havsteen, B. H. (2002) The biochemistry and medical significance of the flavonoids. Pharmacology and Therapeutics; 96: 67-202.

He, X.. and Liu, R. H. (2007). Triterpenoids Isolated from Apple Peels Have Potent Antiproliferative Activity and May Be Partially Responsible for Apple's Anticancer Activity. *Journal of Agricultural and Food Chemistry.;* 55(11):4366-4370.

Heim, K. E.; Tagliaferro, A. R. and Bobliya, D. J. (2002) Flavonoids antioxidants: Chemistry, metabolism and structure-activity relationships. The Journal of Nutritional Biochemistry; 13: 572-584.

Heinrich M. and S. Gibbons, S.; (2001). Ethnopharmacology in drug discovery: an analysis of its role and potential contributions. J. Pharm. & Pharmacol.; 53: 425-432.

Herrera, E. and Barbas, C. (2001) Vitamin E: Action, Metabolism and Perspectives. Journal of Physiology and Biochemistry. 57: 43-56

Hirasawa, Y., Arai, H., Zaima, K., Oktarina, R., Rahman, A., Ekasari, W., Widyawarunyanti, A., Indrayanto, G., Yaini, N.C and Morita, H. (2009). Alstiphyllanines A-D, Indole Alkaloids from *Alstonia macrophylla*. J. Nat. Prod. 72: 304-307.

Hou, W., Lin, R., Lee, T., Huang, Y., Hsu, F. and Lee, M. (2005) The phenolic constituents and free radical scavenging activities of *Gynura formosana* Kiamnra J Sci Food Agric; 85: 615ï 621

Hsieh, R.J., German, J.B., and Kinsella J.E. (1988), Lipids, 23:322.

Hu, C. Q; Chen, K.; Shi, Q.; Kilkuskie, R. E.; Cheng, Y. C. and Lee, K. H. (1994) Anti-AIDS agents, 10. Acacetin-7-O-beta-D-galactopyranoside, an anti-HIV principle from *Chrysanthemum morifolium* and a structureï activity correlation with some related flavonoids. J Nat Prod; 57:42ï 51.

Hui, T., Zhu, L,. Guo, W. and Rao G. (2009) Flavonoids in leaves of *Alstonia scholaris* China journal of Chinese materia medica; 34(9): 1111-1113.

Humphrey, A. J. and Beale, M. H. (2006) Terpenes: In Crozia, A., Clifford, M. N. and Ashihara, H. (Eds) *Plant Secondary Metabolites*, Blackwell, Oxford. 47-65

Hutchinson, C. R. (1981), Tetrahedron; 37: 1047-1065

Idowu, O.A., Soniran, O.T., Ajana, O and Aworinde, D.O. (2010). Ethnobotanical survey of antimalarial plants used in Ogun State, Southwest, Nigeria. Afr. J. Phar. Pharmacol.; 4: 55-60.

Iwu, M. W., Duncan A.R. and Okunji C.O. (1999) New Antimicrobials of Plant Origin. In: Janick J. (ed.): Perspectives on New Crops and New Uses. ASHS Press, Alexandria, VA: 457ï 462.

Iwu, M. M. (1993) Handbook of African Medicinal Plants, CRC Press, London, UK,.

Jagetia, G. C., Baliga, M. S, Venkatesh, P., Ulloor, J. N., Mantena, K. S., Genebriera, J. and Mathuram, V. (2005) Evaluation of the cytotoxic effect of the monoterpene indole alkaloid echitamine in-vitro and in tumour bearing mice. Journal of Pharmacy and Pharmacology; 57: 1213-1219

Kam, T. S., Nyeoh, K. T., Sim, K. M. and Yoganathan, K. (1997) ñAlkaloids from Alstonia scholaris,ò Phytochemistry; 45(6) 1303ï 1305.

Kamarajan, P., Sekar, N., Mathuram, V. and Govindasamy, S. (1991) ñAntitumor effect of echitamine chloride on methylcholonthrene induced fibrosarcoma in rats,ò Biochemistry International; 25 (3) 491ï 498.

Kaufman P. B., Cseke ,L. J., Warber, S., Duke J. A. and Brielmann,H. L. (1999). Natural Products from Plants. CRC Press, Boca Raton, FL.

Keawpradub, N., Houghton, P. J., Eno-Amooquaye, E. and Burke, P.J. (1997) ñActivity of extracts and alkaloids of Thai *Alstonia* species against human lung cancer cell lines,ò Planta Medica; 63 (2) 97ï 101.

Keawpradub, N., Eno-Amooquaye, E., Burke, P.J. and Houghton, P. J. (1999). Cytotoxic Activity of Indole Alkaloids from *Alstonia macrophylla*. Planta Medica; 65: 311-315.

Kessler, H., Gehrke M. and Griesinger C. (1988) Two dimensional NMR Spectroscopy, principles and survey of experiments. Ang. Chem.; 100(4) 507-54

Khanna, P., Sharma, O. P., Sehgal, M. et al. (1980) Antimicrobial principles from tissue culture of some plant species. Indian J Pharm Sci; 42:113ï 7.

Khosravi, A. and Behzadi, A. (2006) Evaluation of the antibacterial activity of the seed hull of *Quercus barantii* on some gram-negative bacteria. Pak. J. Med. Sci.; 22:429-432.

Kim, H. J., Woo, E. R., Shin, C. G and Park, H. (1998) A new flavonol glycoside gallate ester from *Acer okamotoanum* and its inhibitory activity against human immunodeficiency virus-1 (HIV-1) integrase. J Nat Prod; 61:145ï 148.

Koyama, K., Hirasawa, Y., Hosaya, T., Hoe, T.C., Chan, K., Morita, H. (2010). Alpneumines A-H, new anti-melanogenic Indole Alkaloids from *Alstonia pneumatophora*. Bioorg. Med. Chem; 18: 4415-4421.

Koyama, K., Hirasawa, Y., Nugroho A.E., Hosaya, T., Hoe, T.C., Chan, K., Morita, H. (2010). Alsmaphorazines A and B, Novel Indole Alkaloids from *Alstonia pneumatophora*. Org. Lett.; 12 (18): 4188-4191.

Kucera, M., Marquis,V.O and Kucerova, H (1972)ñNigerian medicinal plants. I. [TLC thin-layer chromatographic] separation and quantitative evaluation of *Alstonia boonei* alkaloids,ò Planta Medica; 21 (4) 343ï 346.

Kuhnau, J. (1976) World Rev I utr Diet; 24, 117-191

Kumar, B., Sandhar, H. K. ;Prasher, S., Tiwari, P., Salhan, M. and Sharma, P. (2011) A Review of Phytochemistry and Pharmacology of Flavonoids. Internationale Pharmaceutica Sciencia; 1(1)

Kweifio-Okai, G. 1991. ñAntiinflammatory activity of a Ghanaian antiarthritic herbal preparation: II,ò Journal of Ethnopharmacology; 33 (2) 129ï 133.

Kweifio-Okai, G. 1991. ñAntiinflammatory activity of a Ghanaian antiarthritic herbal preparation: I,ò Journal of Ethnopharmacology; 33 (3) 263ï 267,

Kweifio-Okai. G and Carroll, G. (1992). Antiarthritic effect of *Alstonia boonei* triterpenes. In: P. Gopalakrishnakone and C.K. Tan (eds). Recent Advances in Toxinology Research; TRG Publishers: Singapore pp 19ï 28.

Lai P. K; Roy J (2004). "Antimicrobial and chemopreventive properties of herbs and spices". Curr. Med. Chem. 11 (11): 1451ï 60.

Langfield, R. D., Scarano, F. J., Heitzman, M. E., Kondo, M., Hammond, G. B. and Neto, C. C. (2004). Use of a modified microplate bioassay method to investigate antibacterial activity in the Peruvian medicinal plant *Peperomia galiodes*. J. Ethnopharmacol; 94(2-3): 279-281.

Laszczyk, M. N. (2009). "Pentacyclic Triterpenes of the Lupane, Oleanane and Ursane Group as Tools in Cancer Therapy". Planta Medica 75 (15): 1549ï 1560.

Li, W.; Asada, Y. and Yoshikawa, T. (1998) Antimicrobial flavonoids from *Glycyrrhiza glabra* hairy root cultures. Planta Med; 64:746ï 747.

Lin, Y. M.; Anderson, H, Flavin, M. T.; et al. (1997) In vitro anti-HIV activity of biflavonoids isolated from *Rhus succedanea* and *Garcinia multiflora*. J Nat Prod; 60:884ï 888.

Ling, L. T. and Palanisamy, U. D. (2012). Review: Potential Antioxidants from Tropical Plants, Food Industrial Processes - Methods and Equipment, Dr. Benjamin Valdez (Ed.)
López-Lázaro, M. (2002) Curr Med Chem Anticancer Agents; Nov 2(6):691-714.

Lourens, A. C. U., Reddy, D., Baser, K. H. C., Viljoen, A. M. and Van Vuuren, S. F. (2004). In vitro biological activity and essential oil composition of four indigenous South African *Helichrysum species*. J. Ethnopharmacol. 9: 253-258.

Macabeo, A. P. C., Krohn, K., Gehle, D., Read, R. W., Brophy, J. J. Cordell G. A., Franzblau, S. G. and Aguinaldo, A. M. (2005) Indole alkaloida from the leaves of Phillipines *Alstonia Scholaris*. Phytochemistry; 66:1158-1162

Maestri D.M., Nepote V., Lamarque A. L. and Zygadlo J.A. (2006) Phytochemistry: Advances in Research: 105-135
Manandhar, N. P. (1994). An ethnobotanical survey of herbal drugs of Kaski District, Nepal. Fitoterapia; 65: 7-13

Manandhar, N. P. (2002). Plants and People of Nepal. Timber Press, Inc., Portland, U.S.A

Marco, B (2007). Synthesis and Characterization of Glycosides. Springer.

Marini-Bettolo, G. B., Nicoletti, M., Messana, I., et al., (1983) ñResearch on African medicinal plants-IV1 1 Part III: see ref. 1. Boonein, a new C-9 terpenoid lactone from *Alstonia boonei*: a possible precursor in the indole alkaloid biogenesis,ò Tetrahedron; 39(2) 323ï 329.

Marrugat, J., Covas, M. I., Fito, M., Schroder, H., Miro-Casas, E., Gimeno, E., Lopez-Sabater M. C.; de la Torre, R. and Farre, M. (2004) Effects of diferring phenolic content in dietary olive oils on lipids and LDL oxidationð a randomised controlled trial Eur J Nutr; 43(3), 140-147

Mathekga, A. D. M., Meyer, J. J. M., Horn, M. M. and Drewes, S. E. (2000). An acylated phloroglucinol with antimicrobial properties from *Helichrysum caesptitium*. Phytochemistry; 53: 93-96.

Matsumoto, M., Ishida, K., Konagai, A., Maebashi, K. and Asaoka, T. (2001). Strong antifungal activity of SS750, a New Triazole Derivative, Is based on its Selective Binding Affinity to Cytochrome P450 of fungi. Antimicrob. Agents Chemother.; 46(2): 308-314.

Mazza, G. and Miniati, E. (1993) Anthocyanins in Fruits, Vegetable and Grains CRC Press Boca Raton FL

McChesney J., Venkataraman S. and Henri, J. (2007). Plant natural products: Back to the future or into extinction? Phytochemistry 68: 2015-2022.

Meskin, M. S. (2002). Phytochemicals in Nutrition and Health. CRC Press. 123.

Meyer, J. J. M. and Afolayan, A. J. (1995). Antibacterial activity of *Helichrysum aureonitens* (Asteraceae). J. Ethnopharmacol. 47: 109-111.

Meyer, J. J., Afolayan, A. J., Taylor, M. and Erasmus, D. Antiviral activity of galangin isolated from the aerial parts of *Helichrysum aureonitens*. J Ethnopharmacol 1997; 56:165ï 169.

Middleton Jr, E. and Chithan, K. (1993). The impact of plant flavonoids on mammalian biology: implications for immunity, inflammation and cancer. In: Harborne JB, editor. The flavonoids: advances in research since 1986. London, UK: Chapman and Hall.

Mohana, K., Purushothaman, K. K. and Susan, T. (1985) Drug Potential of echitamine chloride in cancer chemotherapy. Bulletin Medico-Ethnobotanical Res.; 6: 124-132

Mol, J. , Grotewold, E. and Koes, R. (1998) How genes paint the flowers and seeds. Trends Plant Sci.; 3:212ï 218.

Moongkarndi, P., Kosem, N., Kaslungka, S., Luanratana, O., Pongpan, N. and Neungton, N. (2004). Antiproliferation, Antioxidation and Induction of Apoptosis by *Garcinia Mangostana* (Mangosteen) on Skbr3 Human Breast Cancer Cell Line. *Journal of Ethnopharmacology.;* 90(1):161-166.

Moore, P. S. and Pizza, C. (1992) Observations on the inhibition of HIV-1 reverse transcriptase by catechins. Biochem J; 288:717ï 719.

Morita, Y., Hesse, M. and Schmid, H. (1977) ñ*Alstonia scholaris*: the structure of the indole alkaloid nareline,ò Helvetica Chimica Acta; 60 (4) 1419ï 1434.

Mshigeni, K. E., Nkunya M. H. H., Fupi, V., Mahunnah, R. L. A. and Mshiu, E. N. (eds.). (1991). Proceedings of an International Conference of Experts from Developing Countries on Traditional Medicinal Plants, University Press, Tanzania.

Mucsi, I., Gyulai, Z. and Beladi, I. (1992) Combined effects of flavonoids and acyclovir against herpesviruses in cell cultures. Acta Microbiol Hung; 39:137ï 147.

Murray, M. T. (1998) Quercetin: Natureôs antihistamine. Better Nutrition.

Muzitano, F. M., Tinoco, L.W., Guette, C., Kaiser, C. R., Rossi-Bergmann B. and Costa, S. S. (2006) The antileishmanial activity assessment of unusual flavonoids from *Kalanchoe pinnata*. Phytochemistry; (67) 2071-2077

Nadkarni, A. K. (1976) Indian Material Medica. 3rd edition, Popular Press Ltd, Mumbai, India.

Ncube, N. S., Afolayan, A. J. and Okoh, A. I. (2008) Assessment techniques of antimicrobial properties of natural compounds of plant origin: current methods and future trends. African Journal of Biotechnology; 7 (12) 1797-1806

Newman, D.J.(2008). Natural products as leads to potential drugs: An old process or the new hope for drug discovery? J. Med. Chem; 51:2589ï 2599

Ng, T. B., Ling, J. M, Wang, Z. T., Cai, J. N. and Xu, G. J. (1996) Examination of coumarins, flavonoids and polysaccharopeptide for antibacterial activity. Pharmacol; 27:1237ï 1240.

Nishino, C., Enoki, N., Tawata, S., Mori, A., Kobayashi, K. and Fukushima, M. (1987) Antibacterial activity of flavonoids against *Staphylococcus epidermidis*, a skin bacterium. Agric Biol Chem; 51:139ï 143.

Nomura, T., Kikuchi, M., Kubodera, A., Kawakami, Y. (1997) Proton-donative antioxidant activity of fucoxanthin with 1,1-diphenyl-2-picrylhydrazine (DPPH) Biochem Mol Biol Int; 42: 361-370

Nostro, A., Germano, M. P., DôAngelo, V., Marino, A. and Cannatelli, M. A. (2000). Extraction methods and bioautography for evaluation of medicinal plant antimicrobial activity. Lett. Microbiol; 30(1): 379-384.

Odugbemi, T.O. and Akinsulire, O.R. (2007). Medicinal plants useful for malaria therapy in Okeigbo, Ondo State, Southwest, Nigeria. Afr. J. Trad. CAM.; 4(2): 191-198.

Oguakwa, J.U (1984) ñNŬ-formylechitamidine, an alkaloid from *Alstonia boonei*,ò Phytochemistry; 23 (11) 2708ï 2709.

Ogunleye, D. S. and Ibitoye, S. F. (2003). Studies of antimicrobial activity and chemical constituents of *Ximenia americana*. Trop. J. Pharm. Res. 2(2): 239-241.

Oigiangbe, O.N., Igbinosa, I.B. and Tamo, M. (2010). Insecticial properties of an alkaloid from *Alstonia boonei* De Wild. J. Biopesticides; 3(1): 265-270.

Ojewole, J. A. O. (1983) ñAutonomic pharmacology of echitamine, an alkaloid from *Alstonia boonei* De Wild,ò Fitoterapia; 54 (3) 99ï 113.

Ojewole, J. A. O. (1984) ñStudies on the pharmacology of echitamine, an alkaloid from the stem bark of *Alstonia boonei* L. (Apocynaceae),ò International Journal of Crude Drug Research; 22(3) 121ï 143.

Okwu, D. E., Okwu, M. E. (2004). Chemical composition of *Spondias mombin* Linn plant parts. J. Sustain. Agric. Environ; 6: 140-147.

Omoya, F., Oladipupo, K., Abe, A. and Udensi, O. (2012), "Bioactivity, Qualitative and Quantitative Components of *Alstonia boonei* Leaf Extracts on Anopheles Mosquito Larvae in Nigeria", Journal of Medical and Bioengineering; 1 (1) 39-41,

Ono, K., Nakane, H., Fukushima, M., Chermann, J. C. and Barre-Sinoussi F. (1990) Differential inhibitory effects of various flavonoids on the activities of reverse transcriptase and cellular DNA and RNA polymerases. Eur J Biochem; 190:469ï 76.

Orwa C, Mutua A, Kindt R, Jamnadass R, Simons A. 2009. Agroforestfree Database: a tree reference and section guide version 4.0 (http// www.worldagroforestry.org/af/treedb/).

Osadebe, P.O. (2002). Anti inflammatory properties of the root bark of *Alstonia boonei*. *Nig. J. Nat. Prdt. Med*.; 6: 39-41.

Palla, F., 2005. *Alstonia boonei* De Wild. [Internet] Record from Protabase. Louppe, D., Oteng-Amoako, A.A. & Brink, M. (Editors). PROTA (Plant Resources of Tropical Africa / Ressources végétales de lôAfrique tropicale), Wageningen, Netherlands.

Patel, A., Patel, A., Patel, A., and Patel, N.M. (2010) Determination of polyphenols and free radical scavenging activity of *Tephrosia purpur* Linn. Leaves (Leguminosae). *Pharmacogn Res* 2: 152-158.

Pauli, G. F., Poetsch, F. and Nahrstedt, A. (1998). Structure assignment of natural quinic acid derivatives using proton nuclear magnetic resonance techniques. Phytochemical Analysis; 9(4), 177-185.

Pier-Giorgio Pietta (2000) J. I. Nat Prod 63, 1035-1042

Pietta, P. G. (1998) In Flavonoids in Health and disease; Rice Evans C. A. Packer L. Marcel Dekker New York 61-110.

Pietta, P. G. and Simoneti, P. (1999) In Antioxidant food supplements in Human Health Parker L Hiramatsu M Yoshikawa T. Eds; Academic press San Diego; 283-308.

Plotkin, M. J. and Famolare, L. (1992). Conclusions and recommendations, en M. J. Plotkin & L. Famolare (eds.). Sustainable Harvest and Marketing of Rain Forest Products, Island Press, Washington, DC; 310-313.

Prescott, L. M., Harley, J. P. and Klein, D. A. (1999) Microbiology. London, UK: WCB/McGraw-Hill.

Primack, R. B., (1982) Ultraviolet Patterns in Flowers, or Flowers as Viewed by Insects.

Prior, R., Wu, X. and Schaich, K. (2005) Standardised methods for the determination of antioxidant capacity and phenolics in foods and dietary supplements J Agric Food Chem; 53(10): 4290-4302

Rahman, A. and Alvi, K. A. (1987) ñIndole alkaloids from *Alstonia scholaris*,ò Phytochemistry; 26 (7) 2139ï 2142.

Rahman, A., Alvi, K. A. and Muzaffar, A. (1986) ñIsolation and 1H/13C-NMR studies on 19,20-dihydrocondylocarpine: an alkaloid from the leaves of *Ervatamia coronaria* and *Alstonia scholaris*, òPlanta Medica; 52(4) 325ï 326.

Rahman, A., Alvi, K. A., Abbas, S. A. and Voelter, W. (1987)ñIsolation of 19,20-Z-vallesamine and 19,20-E-vallesamine from *Alstonia scholaris*,ò Heterocycles; 26 (2) 413ï 419.

Rahman, A., Asif, M., Ghazala, M., Fatima, J. and Alvi, K. A. (1985)ñScholaricine, an alkaloid from *Alstonia scholaris*,ò Phytochemistry; 24 (11) 2771ï 2773.

Ramirez, A. and Garcia-Rubio, S. (2003) Current progress in the chemistry and pharmacology of akuamiline alkaloids. Curr. Med. Chem. (10) 1891-1915.

Rastogi, R. C., Kapil, R. S. and Popli, S. P. (1970) ñPicralinalð A key alkaloid of picralima group from *Alstonia scholaris*,ò Experientia; 26 (10) 1056.

Rates, S. M. K. (2001). Plants as source of drugs. Toxicon; 39: 603-613.

Rauha, J. P., Remes, S., Heinonen, M. et al. (2000) Antimicrobial effects of Finnish plant extracts containing flavonoids and other phenolic compounds. Int J Food Microbiol; 56:3ï 12.

Rockenbach, I. I., Gonzaga, L. V., Rizelio, V. M., Goncalves, A. E. d. S. S., Genovese, M. I. and Fett, R. (2011). Phenolic Compounds and Antioxidant Activity of Seed and Skin Extracts of Red Grape (*Vitis Vinifera* and *Vitis Labrusca*) Pomace from Brazilian Winemaking. *Food Research International,* Vol. 44.; (4):897-901

Roginsky, V.A., Barsukova, T.K., Remorova, A.A., and Bors, W. (1996), JAOCS, 73:777.

Sabu M.C., Kuttan R. (2002). Antidiabetic activity of medicinal plants and its relationship with their antioxidant property. J. Ethnopharmacol; 81:155 ï 160.

Sakagami, Y., Mimura, M., Kajimura, K. et al. (1998) Anti-MRSA activity of sophoraflavanone G and synergism with other antibacterial agents. Lett Appl Microbiol; 27:98ï 100.

Sakanaka, S., Kim, M., Taniguchi, M. and Yamamoto, T. (1989) Antibacterial substances in Japanese green tea extract against Streptococcus mutans, a cariogenic bacterium. Agric Biol Chem; 53:2307ï 11.

Salie, F., Eagles, P. F. K. and Lens, H. M. J. (1996). Preliminary antimicrobial screening of four South African Asteraceae species. J. Ethnopharmacol.; 52(1): 27-33.

Salim, A.A., Chin, Y. W. and Kinghorn, A.D., (2008) In: Bioactive Molecules and Medicinal Plants Ramawat KG, Merillon JM (eds.), Springer

Samuelsson, G. (2004) Drugs of Natural Origin, 5th edn, Apotekarsocieteten, Stockholm

Sanders, J. K. M and Hunter, B.K. (1993), Modern NMR Spectroscopy 2nd Edition Oxford University Press, Oxford.

Saraswathi, V., Mathuram, V., Subramanian, S. and Govindasamy, S. (1999)ñModulation of the impaired drug metabolism in sarcoma-180 bearing mice by echitamine chloride,ò Cancer Biochemistry Biophysics; 17 (2) 79ï 88.

Saraswathi, V., Ramamoorthy, N., Subramaniam, S., Mathuram, V., Gunasekaran, P. and Govindasamy, S. (1998)ñInhibition of glycolysis and respiration of sarcoma-180 cells by echitamine chloride,ò Chemotherapy; 44 (3) 198ï 205.

Saraswathi, V., Shyamala, A. C., Subramanian, S. and Govindasamy, S. (1998) ñStudies on the effects of echitamine chloride on serum glycoproteins and lysosomal hydrolases of sarcoma-180 induced mice,ò Fitoterapia; 69 (1) 73ï 75.

Saraswathi, V., Subramanian, S., Ramamoorthy, N., Mathuram,V. and Govindasamy, S. (1997) ñIn vitro cytotoxicity of echitamine chloride and adriamycin on Ehrlich ascites carcinoma cell cultures,ò Medical Science Research; 25 (3) 167ï 170. Sarker, S. D and Nahar, L. (2007) General, Organic and Natural Product Chemistry - TiERA Chemistry for Pharmacy Students. ©John Wiley & Sons, Ltd.

Sarker, S. D. and Nahar, L. (2007) Chemistry for Pharmacy Students John Wiley and Sons Ltd.

Sato, M., Tsuchiya, H., Takase, I., Kureshiro, H., Tanigaki, S. and Iinuma, M. (1995) Antibacterial activity of flavanone isolated from *Sophora exigua* against methicillin-resistant *Staphylococcus aureus* and its combination with antibiotics. Phytother Res; 9:509ï 12.

Scalbert, A., Manach, C., Morand, C., Remesy, C. and Jimenez, L. (2005) Dietry polyphenol and the prevention of diseaes. Crit Rev Food Sci Nutr.; 45(4), 287-306

Schuier, M., Sies, H., Illek, B. and Fischer, H. (2005) Cocoa-related flavonoids inhibit CFTR-mediated chloride transport across T84 human colon epithelia. Journal of Nutrition; 135(10), 2320ï 2325.

Science Reference Services. (2008). Library of Congress. http://www.loc.gov/rr/scitech/

Selway, J. W. T. (1986) Antiviral activity of flavones and flavans. In: Cody V, Middleton E, Harborne JB, editors. Plant flavonoids in biology and medicine: biochemical, pharmacological, and structureï activity relationships. New York, NY: Alan R. Liss, Inc.

Sen, C. K. Khanna,S. and Roy,S. (2007) Tocotrienols in Health and Diseases:The Other Half of the Natural Vitamin E Family. Molecular Aspects of Medicine.; 28:692-728

Serafini, M., Maiani, G., and Ferro-Luzzi, A. (1998), J. Nutr., 128:1003.

Shang, J., Cai, X., Feng, T., Zhao, Y., Wang, J., Zhag, L.,Yan, M. and Luo, X. (2010) Pharmacological evaluation of *Alstonia Scholaris*: Anti inflammatory and analgesic effects. Journal of Ethnopharmacology; 129: 174-181

Sheldon, J. W., Balick, M. J. and Laird, S. A. (1997). Medicinal plants: can utilization and conservation coexist? Advances Econ. Bot. 12: 1-104.

Shiota, S., Shimizu, M., Mizushima, T. *et al*. (1999) Marked reduction in the minimum inhibitory concentration (MIC) of beta-lactams in methicillin-resistant *Staphylococcus aureus* produced by epicatechin gallate, an ingredient of green tea (*Camellia sinensis*). Biol Pharm Bull; 22:1388ï 1390.

Shirly, B. W, (1996) Trends plant Sci.; (31) 377-382.

Sieradski, K., Roberts, R.B., Haber, S.W. and Tomasz, A. (1999): The development of vancomycin resistance in a patient with methicillin-resistant *Staphylococcus aureus* infection. N. Engl. J. Med.; 340: 517ï 523.

Singh, R. and Padmalatha, C. (2004). Ethno-entomological practices in Tirunelveli district, Tamil Nadu. Indian J. Traditional Knowledge; 3: 442-446

Sneader, W. (1996) Drug Prototypes and their Exploitation, Wiley, Chichester, UK

Sneader, W. (2005) Drug Discovery: a History, Wiley, Chichester, UK

So, F. V., Guthrie, N., Chambers, A. F., Moussa, M. , Carroll, K. K. (1996) Inhibition of human breast cancer cell proliferation and delay of mammary tumorigenesis by flavonoids and citrus juices. Nutr Cancer. ; 26(2):167-181.

Solanki, R. (2010) Some medicinal plants with antibacterial activity. Pharmacie Globale (IJCP); 4 (10)

Stapleton, P. D., Shah, S., Anderson, J. C., Hara, Y., Hamilton-Miller, J. M. T. and Taylor, P. W. (2004) Modulation of beta-lactam resistance in *Staphylococcus aureus* by catechins and gallates. Int J Antimicrob Agents; 23:462ï 467.

Stevenson, D. E, and Hurst, R. D. (2007) Polyphenolic phytochemicals-Just antioxidants or much more? Cellular and Molecular Life Sciences; (64) 2900-2916

Stevenson, D. E. and Lowe, T. (2009) Plant-Derived Compounds as Antioxidants for Health-Are They all Really Antioxidants? Functional Plant Science and Biotechnology. Global Science Books 3 (Special Issue1) 1-12

Stobiecki, M. (2000). Review-Application of mass spectrometry for identification and structural studies of flavonoid glycosides Phytochem; 54:237-256

Svarstad H. and Dhillion, S. S. (2000). Responding to Bioprospecting: Rejection or regulation?, in Svarstad H. & Dhillion S. S. (eds.). Responding to Bioprospecting: from biodiversity in the south to medicines in the north. Spartacus Forlag AS, Oslo

Tabuti, J. R. S., Dhillion S. S. and Lye K. A.. (2003). TM in Bulamogi county, Uganda: its practitioners, users and viability. J. Ethnopharmacol.; 85: 119-129

Tapsell L. C., Hemphill I. and Cobiac L. (August 2006). "Health benefits of herbs and spices: the past, the present, the future". Med. J. Aust.; 185 (4): 4ï 24.

Taruscio, T.G., Barney, D.L., and Exon, J. 2004, J. Agric. Food. Chem., 52, 3169.

Taylor, R. S., Manandhar, N.P and G. H. Towers. (1995). Screening of selected medicinal plants of Nepal for antimicrobial activities. J. Ethnopharmacol.; 46: 153-159.

Trease, G. E and Evans, W. C. (2002) Text book of Pharmacognosy, 15th Edition. London: W. B Saunders Company Ltd. 20, 21, 32

Tsao A.S., Kim E.S. and Hong W.K (2004). Chemoprevention of Cancer. CA Cancer J. Clin.; 54:150 ï 180.

Tyler, V. E (2012). "False Tenets of Paraherbalism". Quack watch.

Tyler, V. E. (1999). Phytomedicines: back to the future. J. Nat. Prod.; 62: 1589-1592.

Tyler, V. E. and Robbers J. E. (1999). Tyler's Herbs of Choice: The Therapeutic Use of Phytomedicinals. Routeledge; 6-8.

Umeh, E. U., Oluma, H. O. A., Igoli, O. (2005). Antibacterial screening of four local plants using an indicator-based microdilution technique. Afr. J. Tradit. Complement. Alternat. Med.; 2(3): 238-243.

Valsaraj, R., Pushpangadan, P., Smitt, U. W., et al. (1997) New anti-HIV-1, antimalarial, and antifungal compounds from *Terminalia bellerica*. J Nat Prod; 60:739ï 742.

Ververidis, F., Trantas, E., Doughlas C., Vollmer G., Krezschmer G. and Panopoulus, N.; (2007) Biotechnology of flavonoids and other phenylpropanoid-derived natural products part 1:Chemical diversity ,impacts on plant biology and human health, Biotechnology J; 2(10), 1214-1234

Viet, M; and Pauli, G. F, J. Nat. Prod. (1999); 62, 1301-1303

Viswanathan, S., Ramamurthy, N., Subramanian, S., Mathuram, V. and Govindasamy, S. (1997) ñEnhancement of the cytotoxic effects of Echitamine chloride by vitamin A: an in vitro study on Ehrlich ascites carcinoma cell culture,ò Indian Journal of Pharmacology; 29 (4) 244ï 249.

Wachter, G. A., Hoffmann, J. J, Furbacher, T., Blake, M, E. and Timmermann, B.N. (1999) Antibacterial and antifungal flavanones from *Eysenhardtia texana.* Phytochemistry; 52:1469ï 1471.

Walters, W. P and Namchuk M (2003) Nature Rev Drug Discov 2:259

Wang, S. X., Zhang, F. J, Feng, Q. P. and Li, Y. L. (1992) Synthesis, characterization, and antibacterial activity of transition metal complexes with 5- hydroxy-7,4-dimethoxyflavone. J Inorg Biochem; 46:251ï 257.

Wang, F., Ren F-C and Liu J-K. (2009). Alstonic Acids A and b, unusual 2,3-secofernane triterpenoids from *Alstonia scholaris*. Phytochemistry; 70: 650-654.

Ward, F. E., Garling, D. L., Buckler, R. T., Lawler, D. M. and Cummings, D.P. (1981) Antimicrobial 3-methyleneflavanones. J Med Chem; 24: 1073ï 1077.

Waterman, P. G., and Mole, S. 1994, Analysis of Phenolic Plant Metabolites. Blackwell Scientific Publications, Oxford, UK.

Wayner, D. D. M., Burton, G. W., Ingold, K. U., Barclay, L. C. R. and Locke, S. J. (1987) Biochem Biophys Act; (924) 408-419.

Webster Online Dictionary

Wesche, D., Black, D. and Milhous, W. K. (1990) Poster No. 138, Book of Abstracts, 39th Annual Meeting of the American Society of Tropical Medicine and Hygiene, New Orleans.

Wikipedia 2011

Wikipedia 2013

Williams, F. M., (1895) Report on Therapeutics, On the effect of giving levulose and inulin to patients suffering diabetes mellitus, Boston medical and surgical journal, Massachusetts Medical Society, New England Surgical Society; 133(2) 37.

Yam, T. S., Hamilton-Miller, J. M. T. and Shah, S. (1998) The effect of a component of tea (*Camellia sinensis*) on methicillin resistance, PBP2_ synthesis, and beta-lactamase production in Staphylococcus aureus. J Antimicrob Chemother; 42:211ï 216.

Yamada, H. (1991) Natural products of commercial potential as medicines. Curr Opin Biotechnol; 2:203ï 210.

Yamamoto, Y. and Gaynor, R.B. (2001) Therapeutic potential of inhibition of the NF kappa B pathway in the treatment of inflammation and Cancer. J Clin Invest; 107(2), 135-142

Yamauchi, T. and Abe, F. (1998) ñRegional differences of indole alkaloids in *Alstonia scholaris* and *Alstonia macrophylla*,ò International Congress Series; 1157: 51ï 58.

Yamauchi, T., Abe, F., Chen, R. F., Nonaka, G. I, Santisuk, T. and Padolina, W. G. (1990) ñAlkaloids from the leaves of *Alstonia scholaris* in Taiwan, Thailand, Indonesia and the Philippines,ò Phytochemistry; 29, (11) 3547ï 3552.

Yamauchi, T., Abe, F., Padolina, W.G. and Dayrit, F. M. (1990) ñAlkaloids from leaves and bark of *Alstonia scholaris* in the Philippines,ò Phytochemistry; 29 (10) 3321ï 3325.

Yao, W. R., Wang, H. Y., Wang, S. T., Sun, S. L., Zhou, J. and Luan, Y. Y. (2011) Assessment of the Antibacterial Activity and the Antidiarrheal Function of Flavonoids from Bayberry Fruit. J. Agric. Food Chem.; 59(10):5312-5317

Yee, Y. K. and Koo, M. W. (2000) Anti-Helicobacter pylori activity of Chinese tea: in vitro study. Aliment Pharmacol Ther; 14:635ï 638.

Yen, G. H. and Chen, H. Y. (1995) Antioxidant activity of various tea extracts in relation to their antimutagenicity J. Agric Food Chem; 43:27-32

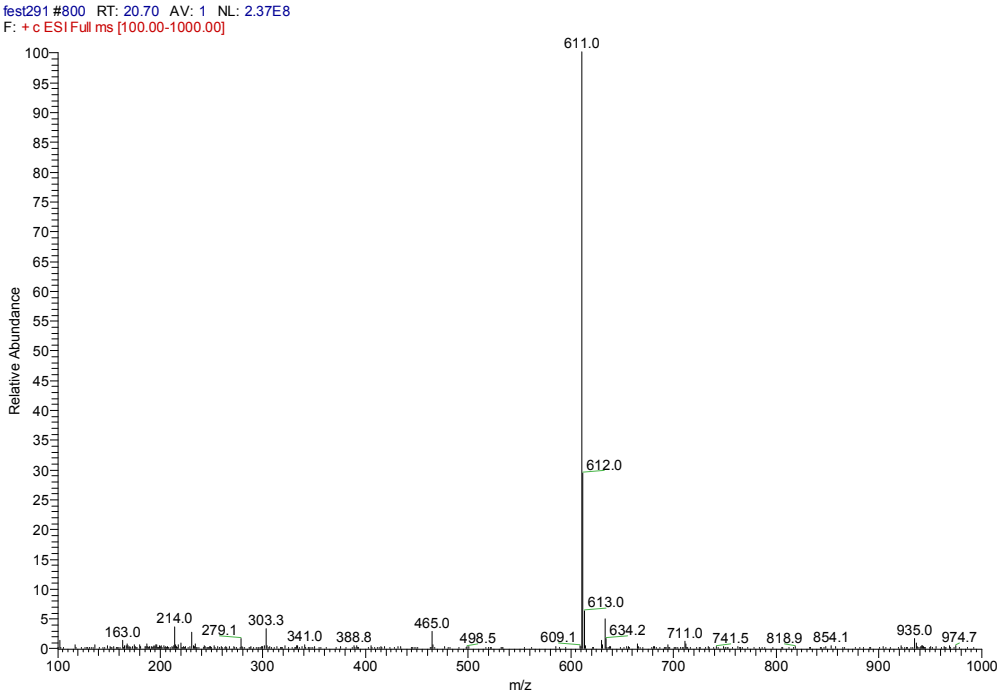
Zheng, W.F., Tan, R. X., Yang, L. and Liu, Z. L. (1996) Two flavones from *Artemisia giraldii* and their antimicrobial activity. Planta Med; 62:160ï 162.

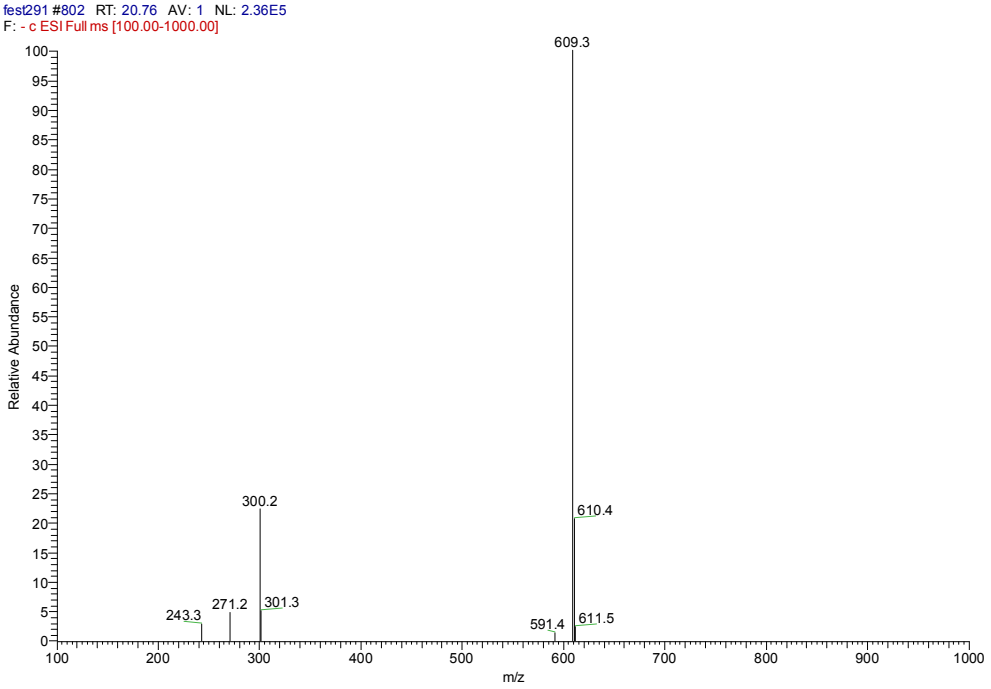
APPENDICES

1 HPLC Spectrum of Compound **1**

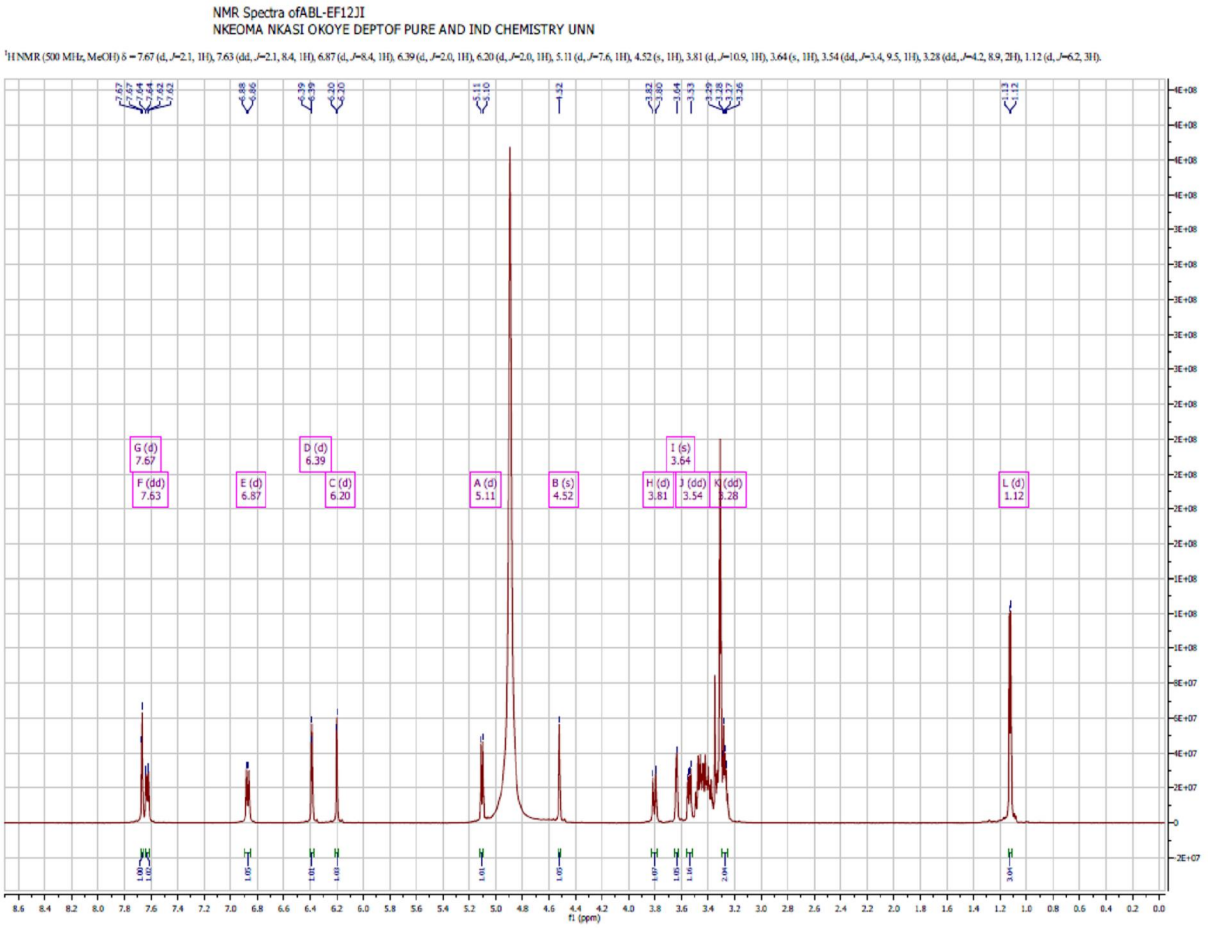
2. UV Spectrum of Compound **1**

3. LC-MS Spectra of Compound **1**

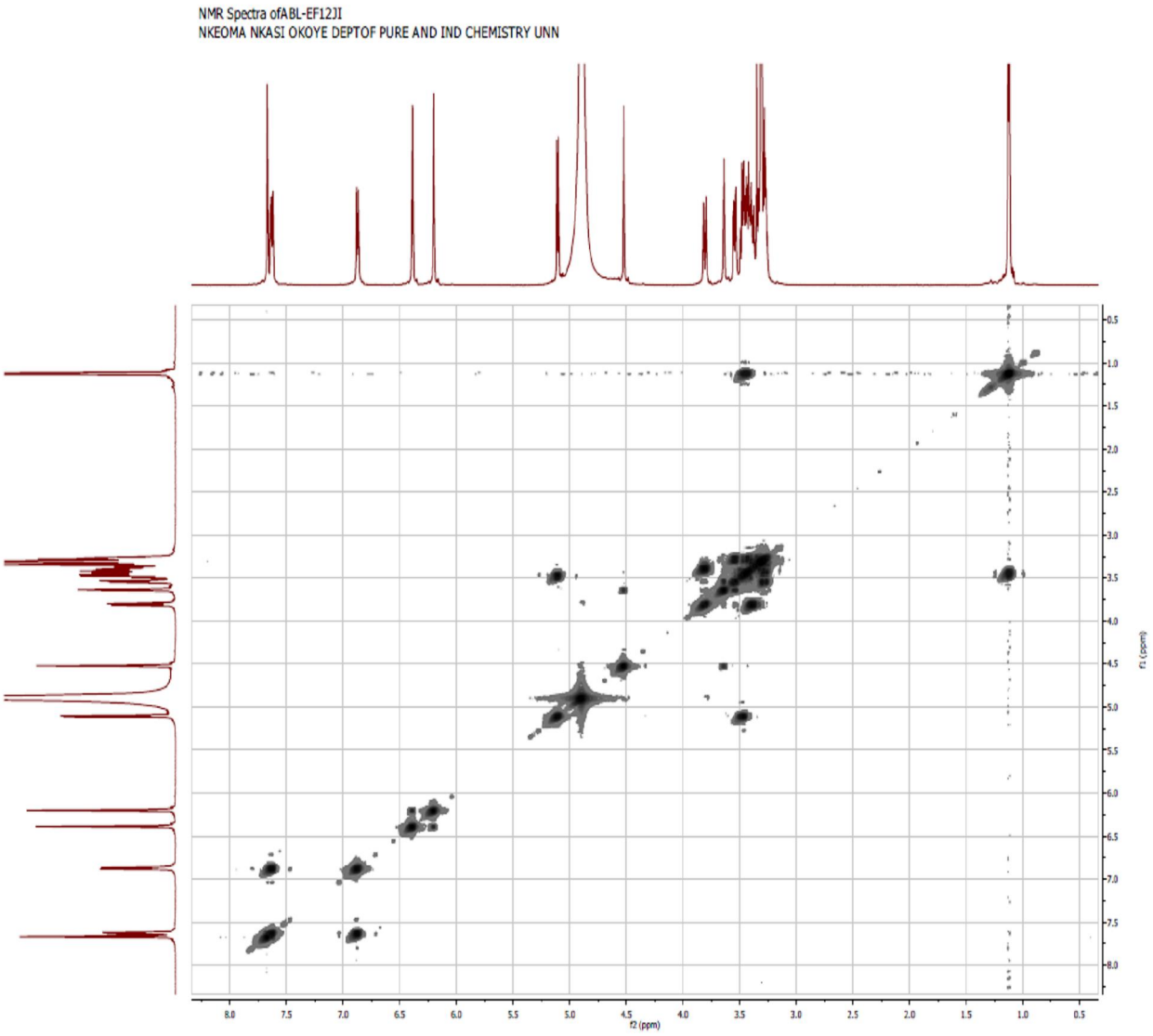




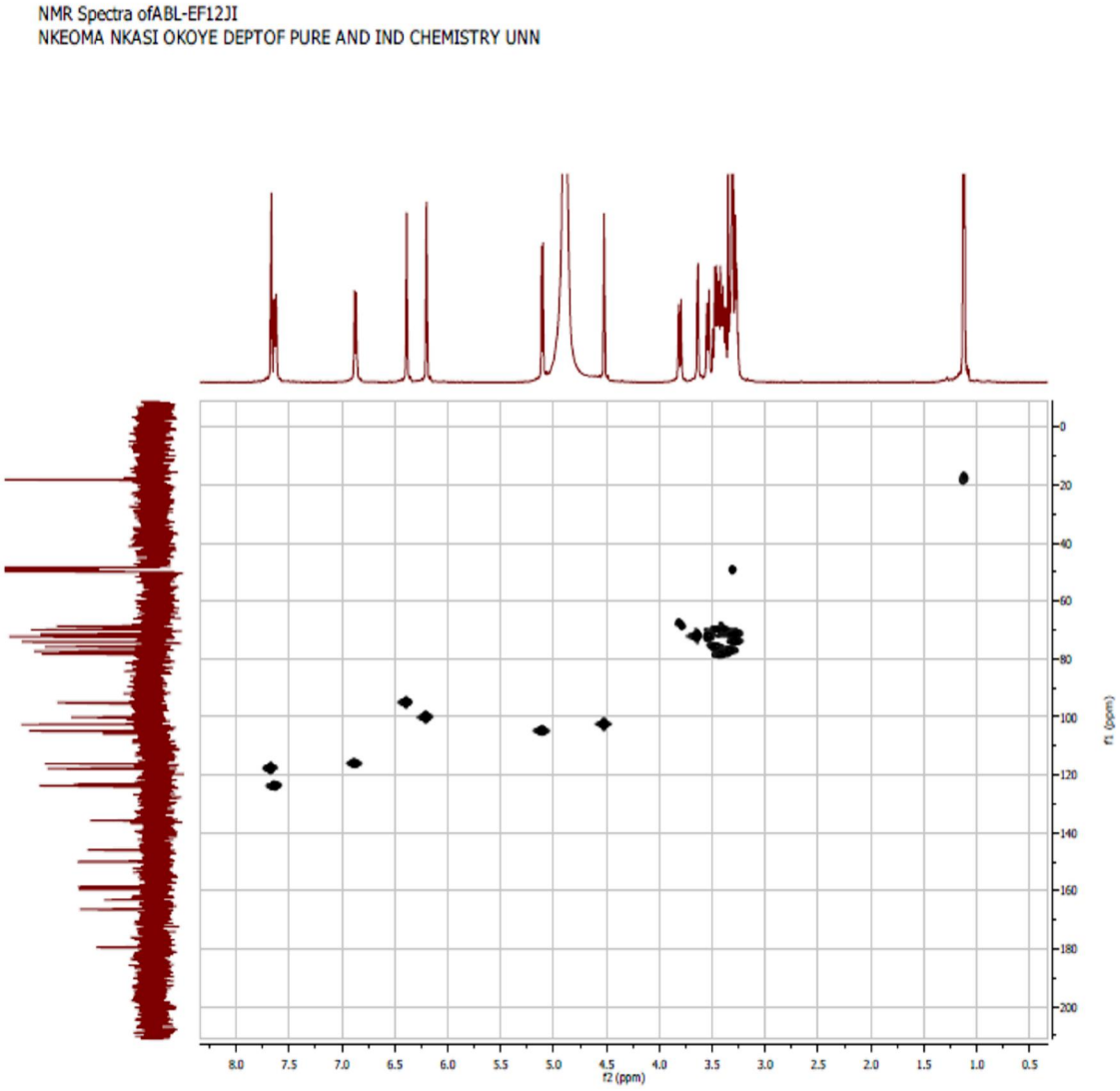
4. Proton NMR spectrum of compound **1**



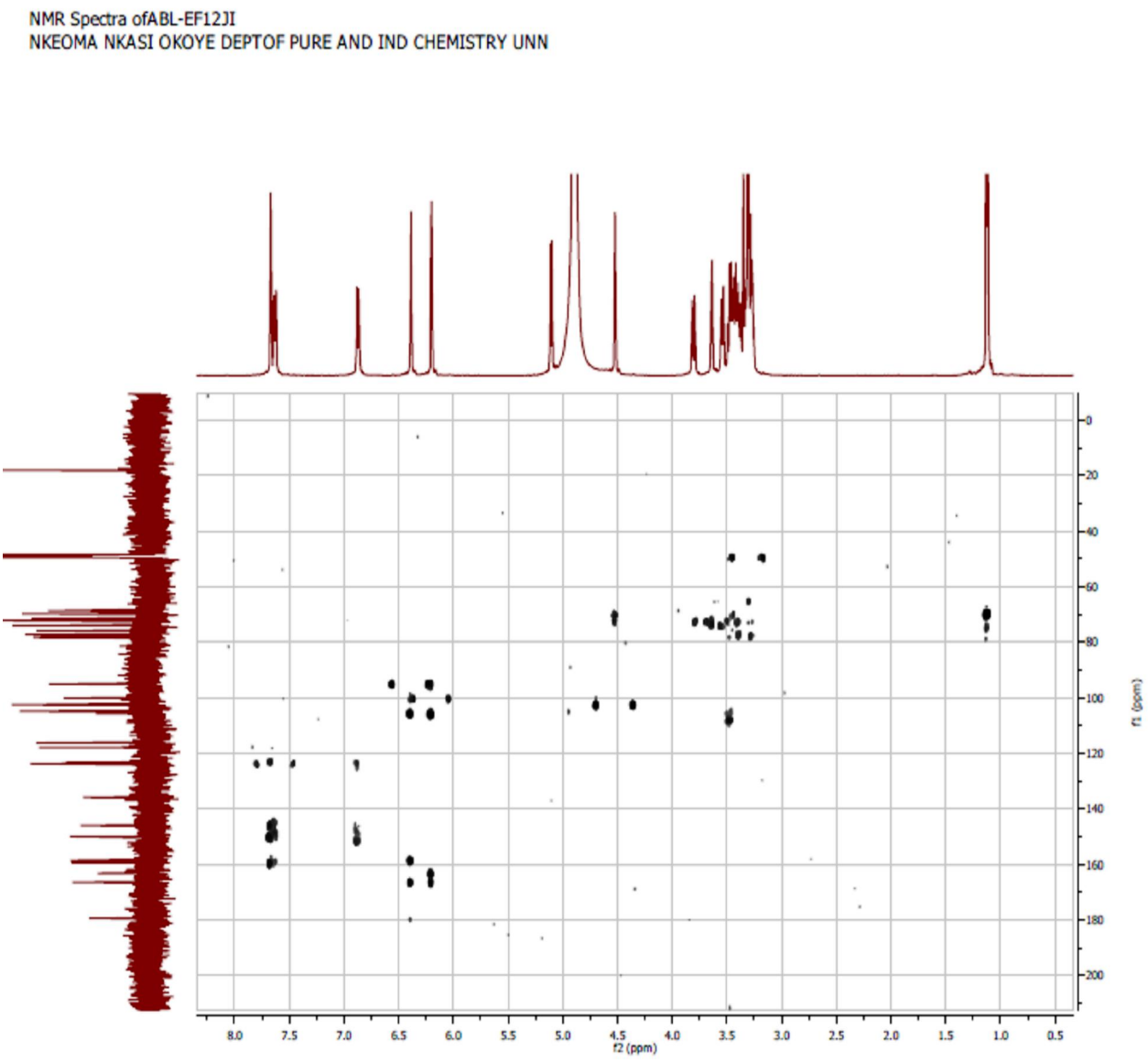
5. (HH-COSY) proton NMR spectrum of compound **1**



6. HMQC Spectrum of Compound **1**

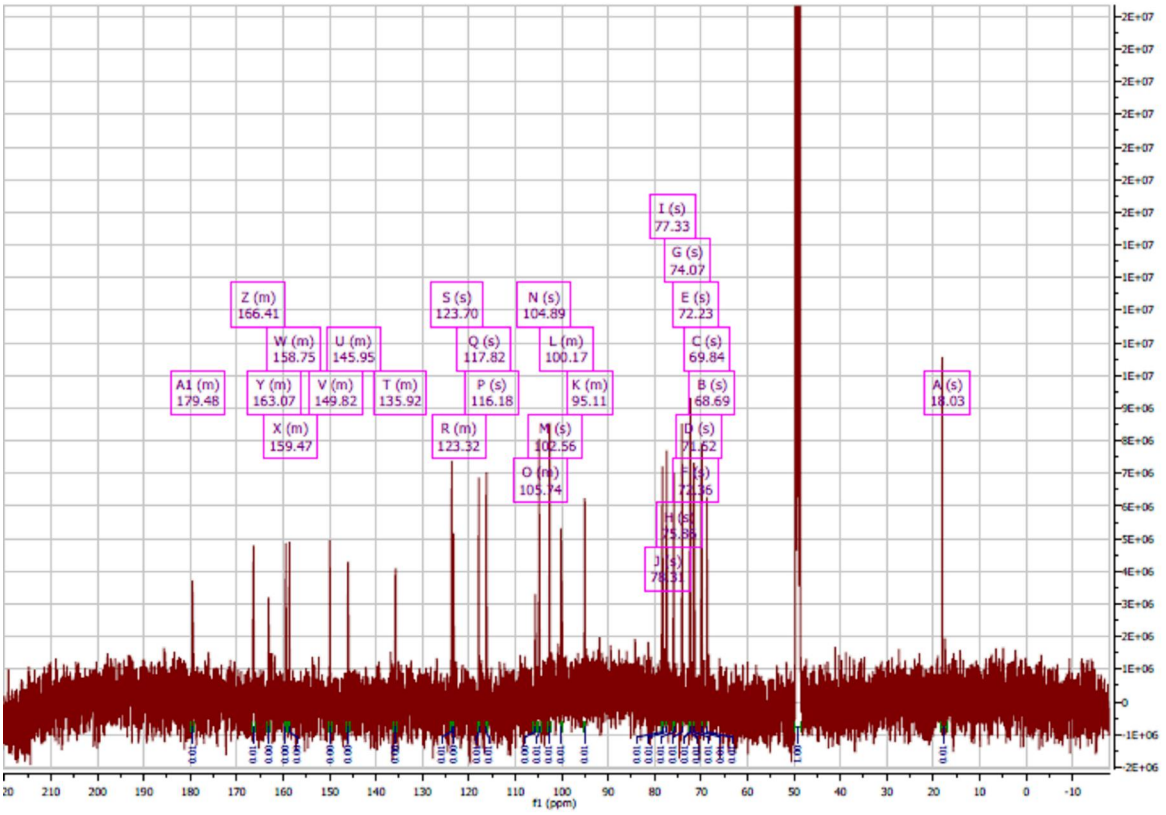


7. HMBC Spectrum of Compound **1**

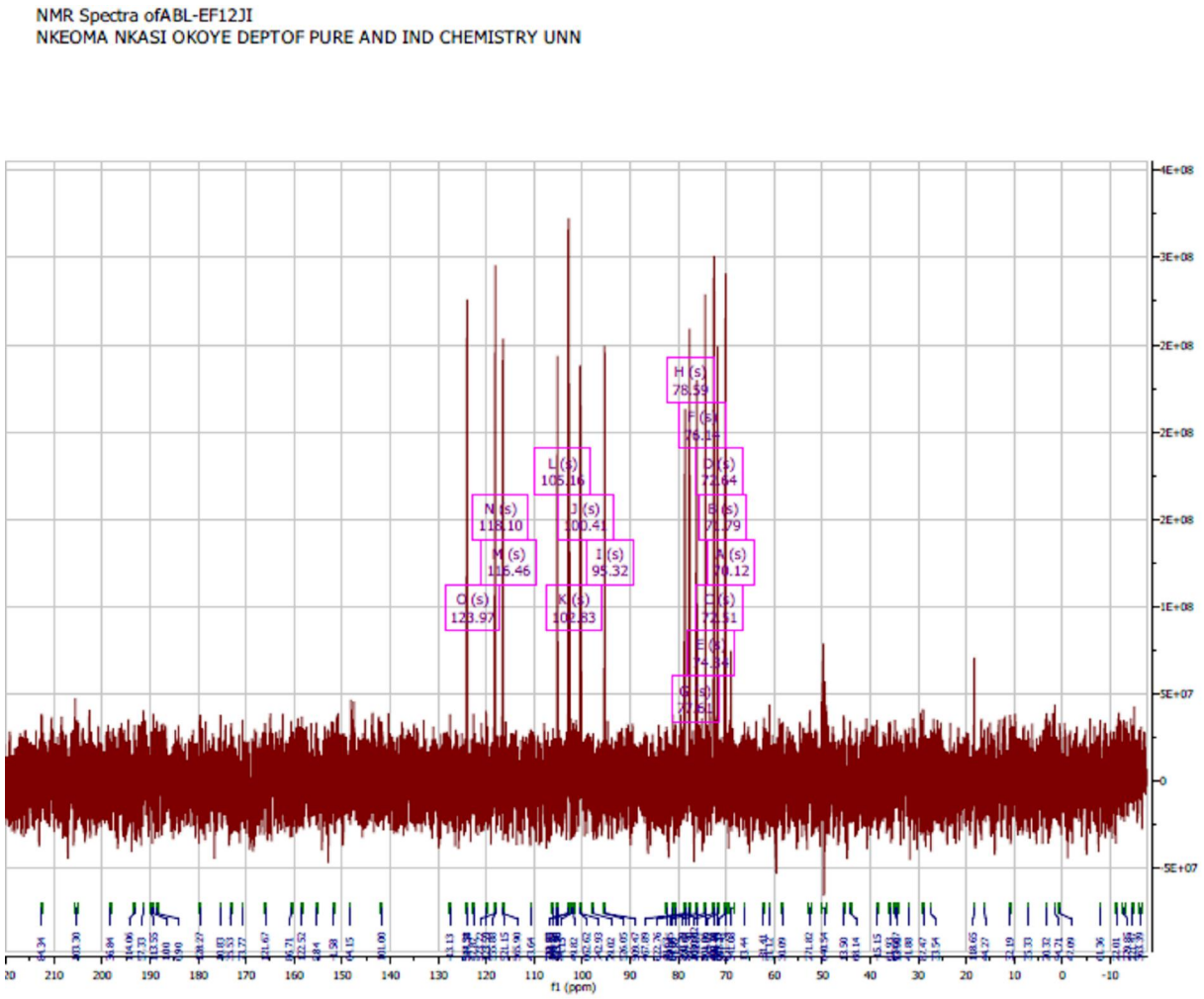


8. C-13 NMR of Compound **1**

NMR Spectra ofABL-EF12JI
NKEOMA NKASI OKOYE DEPTOF PURE AND IND CHEMISTRY UNN



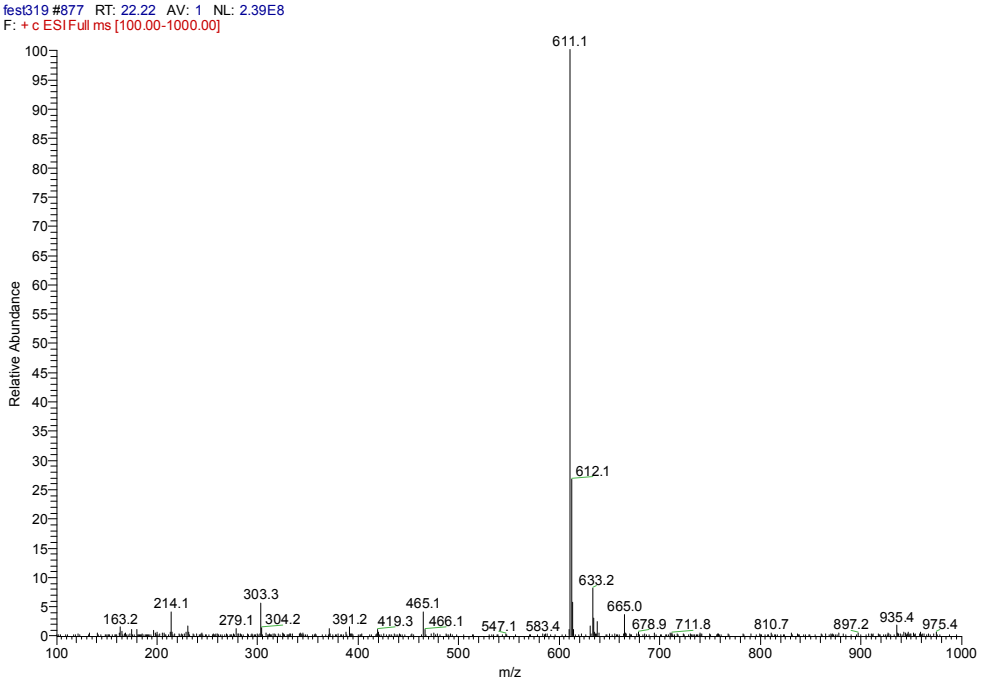
9. C-13 (DEPT-135) Spectrum of Compound **1**

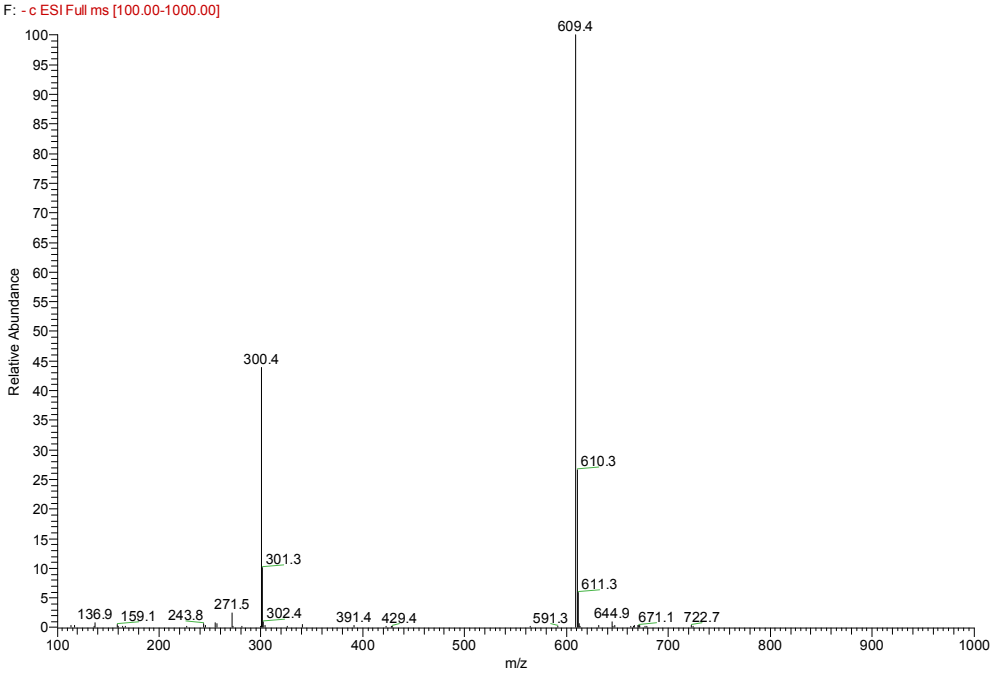


10. HPLC Spectrum of Compound **2**

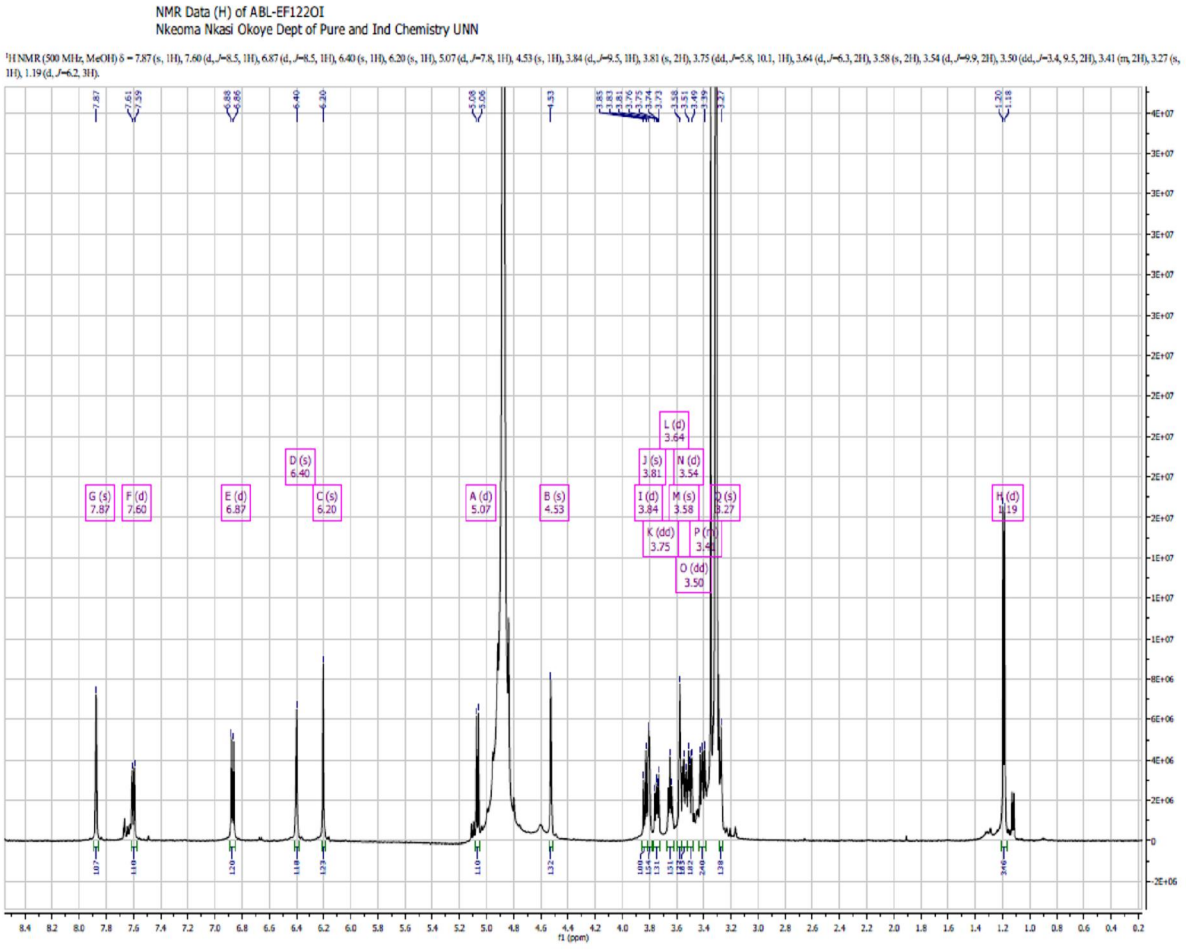
11. UV Spectrum of Compound **2**

12. LC-MS Spectrum of Compound **2**

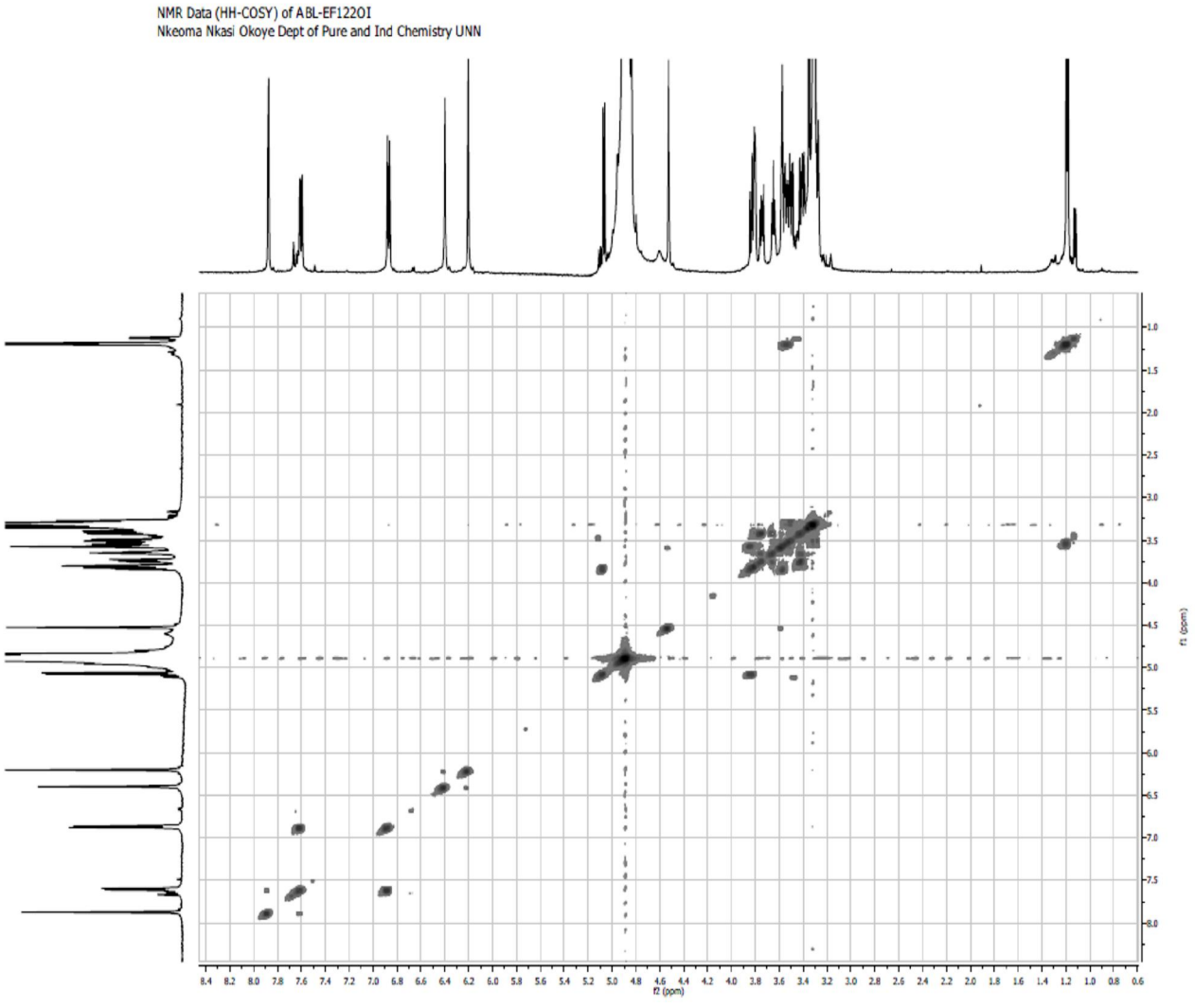




13. Proton NMR Spectrum of Compound **2**



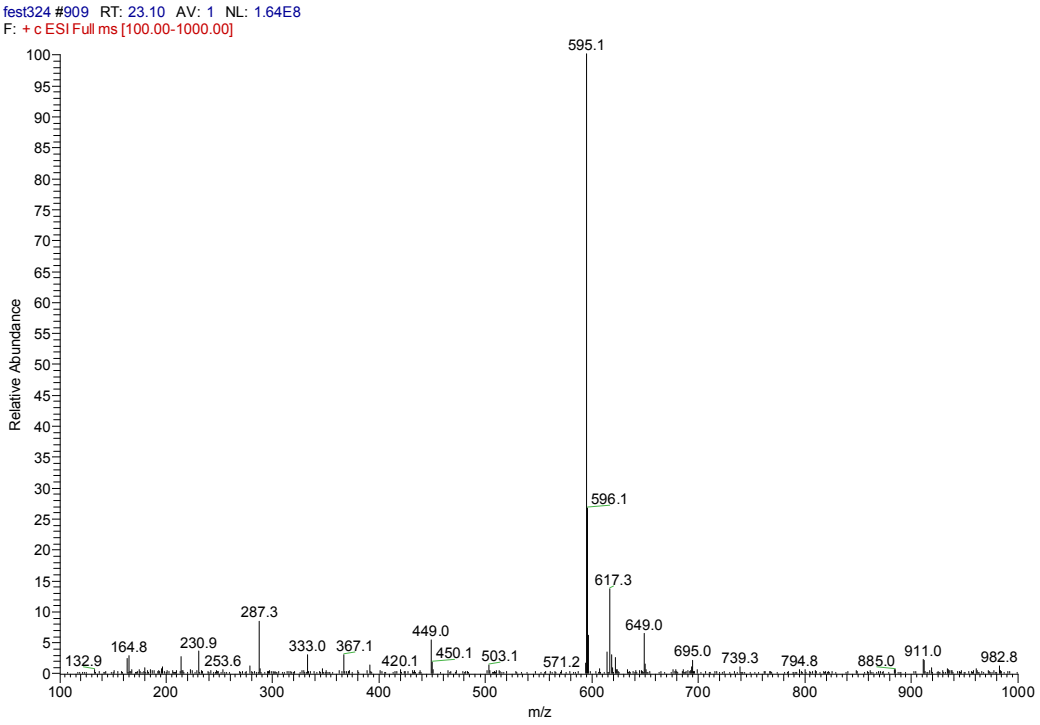
14. (H-H COSY) Proton NMR Spectrum of Compound **2**

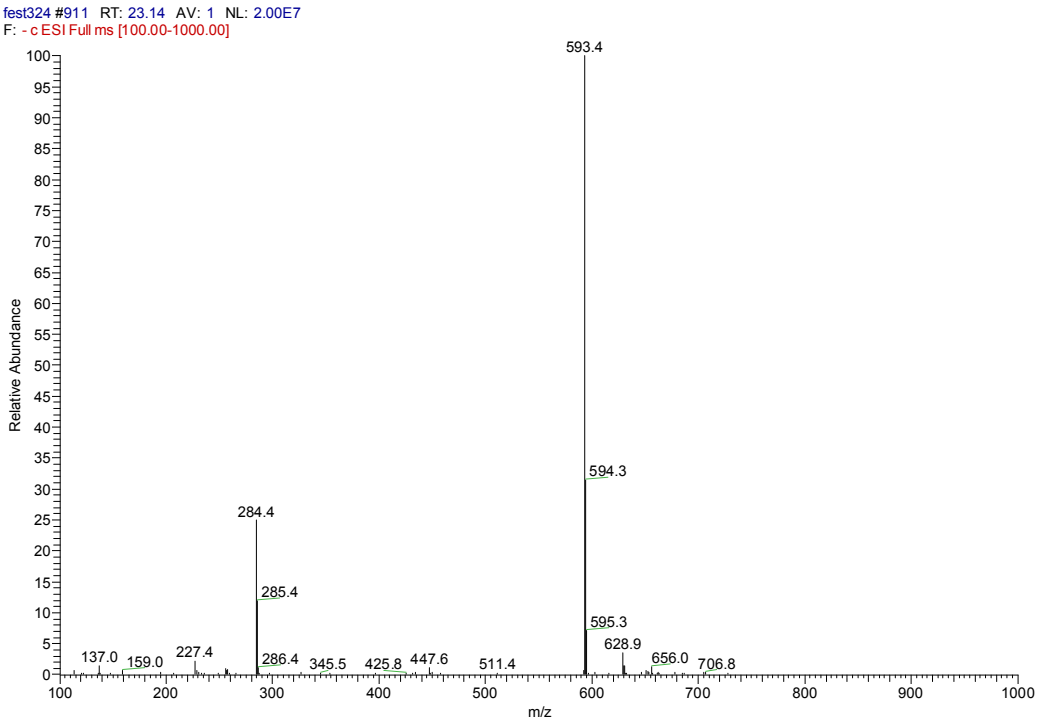


15. HPLC Spectrum of Compound **3**

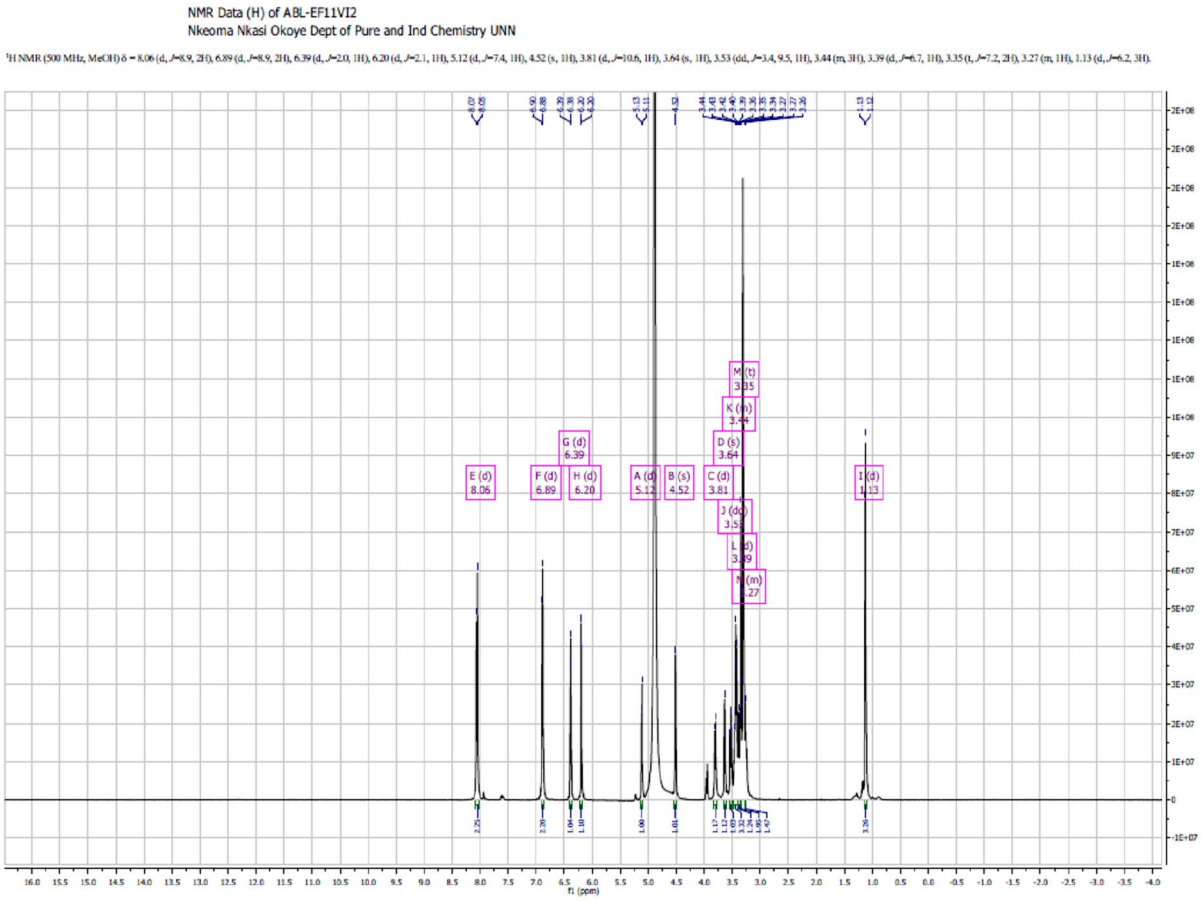
16. UV Spectrum of Compound **3**

17. LC-MS Spectra of Compound **3**

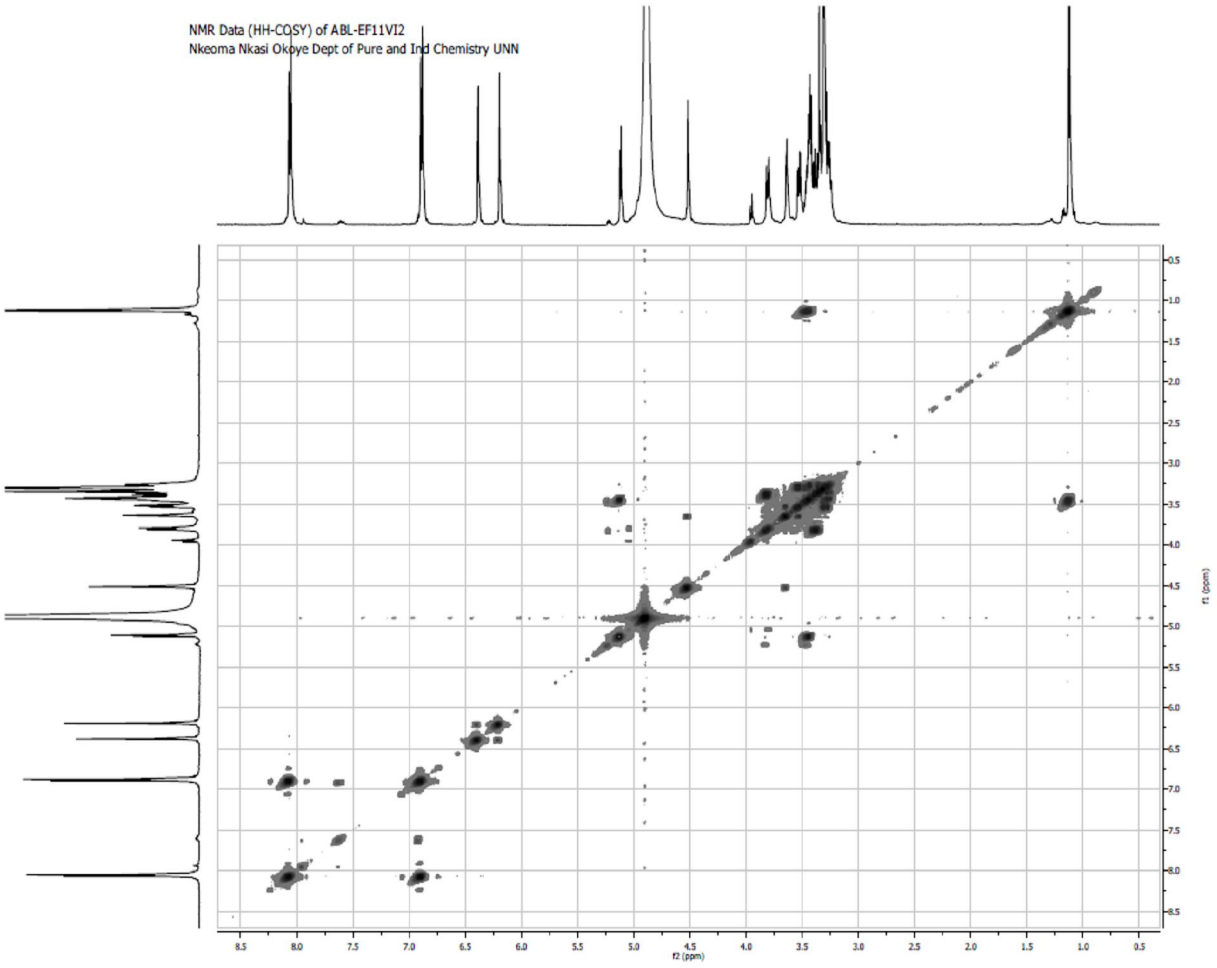




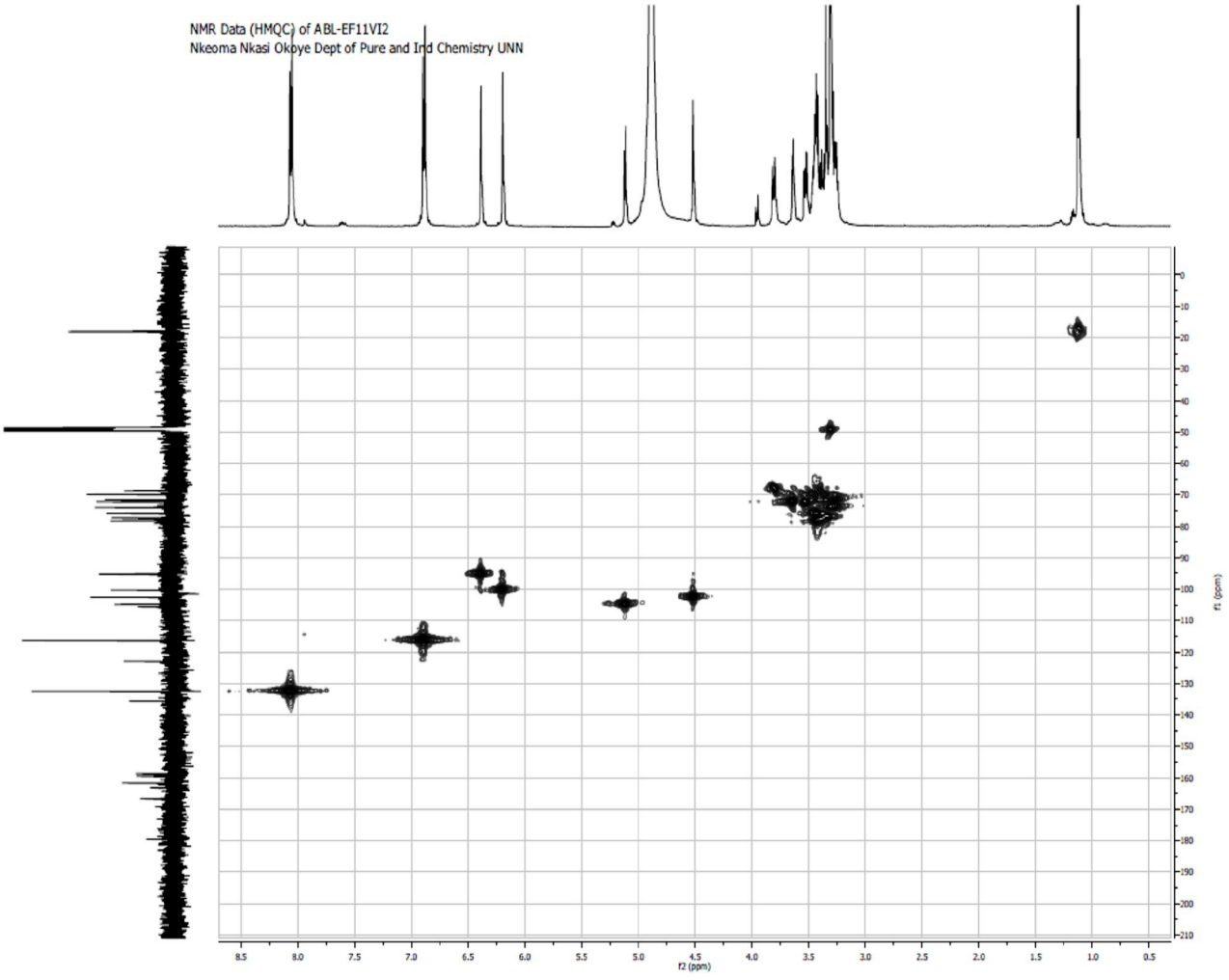
18. Proton NMR Spectrum of Compound **3**



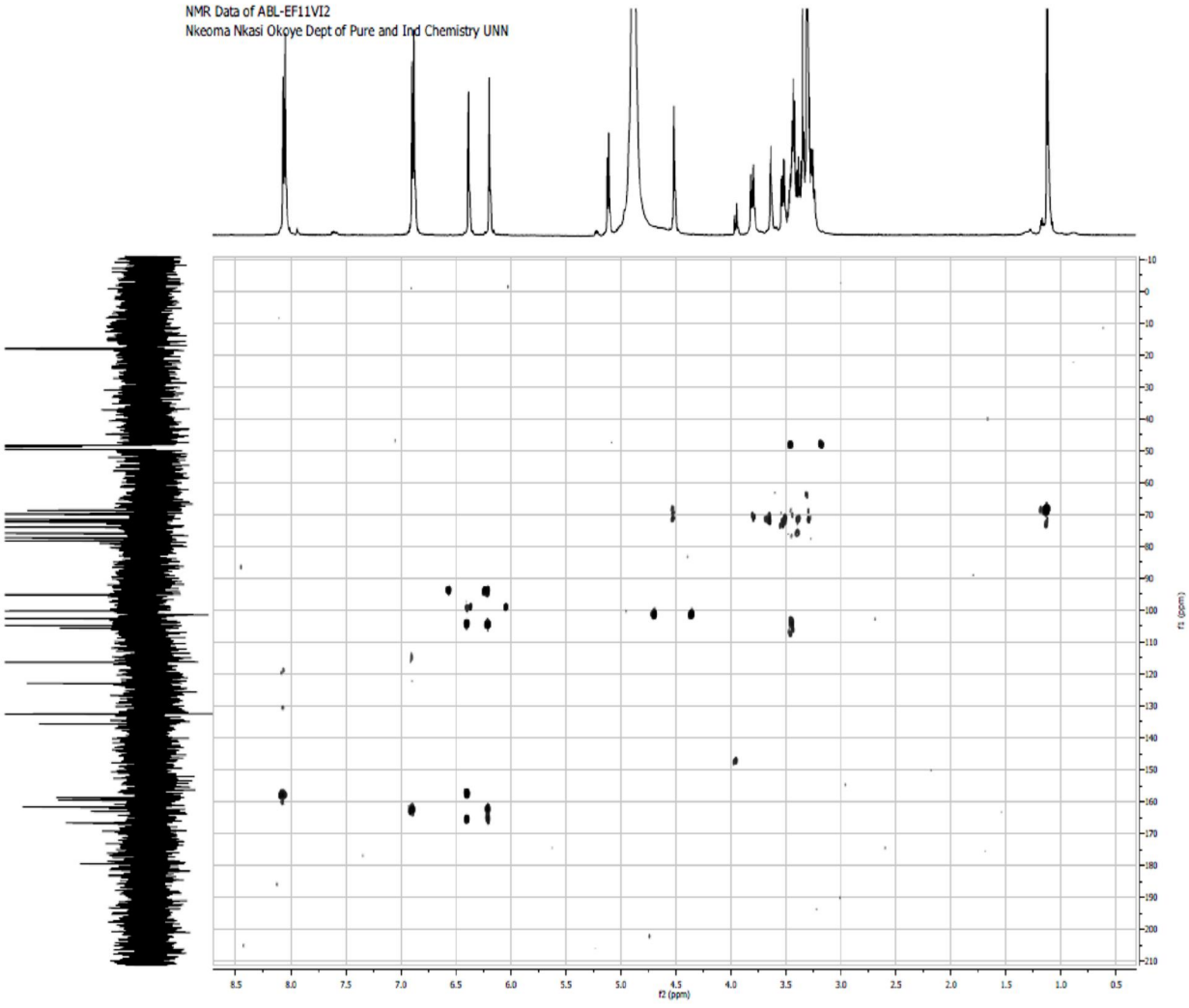
19. (H-H COSY) Proton NMR Spectrum of Compound **3**



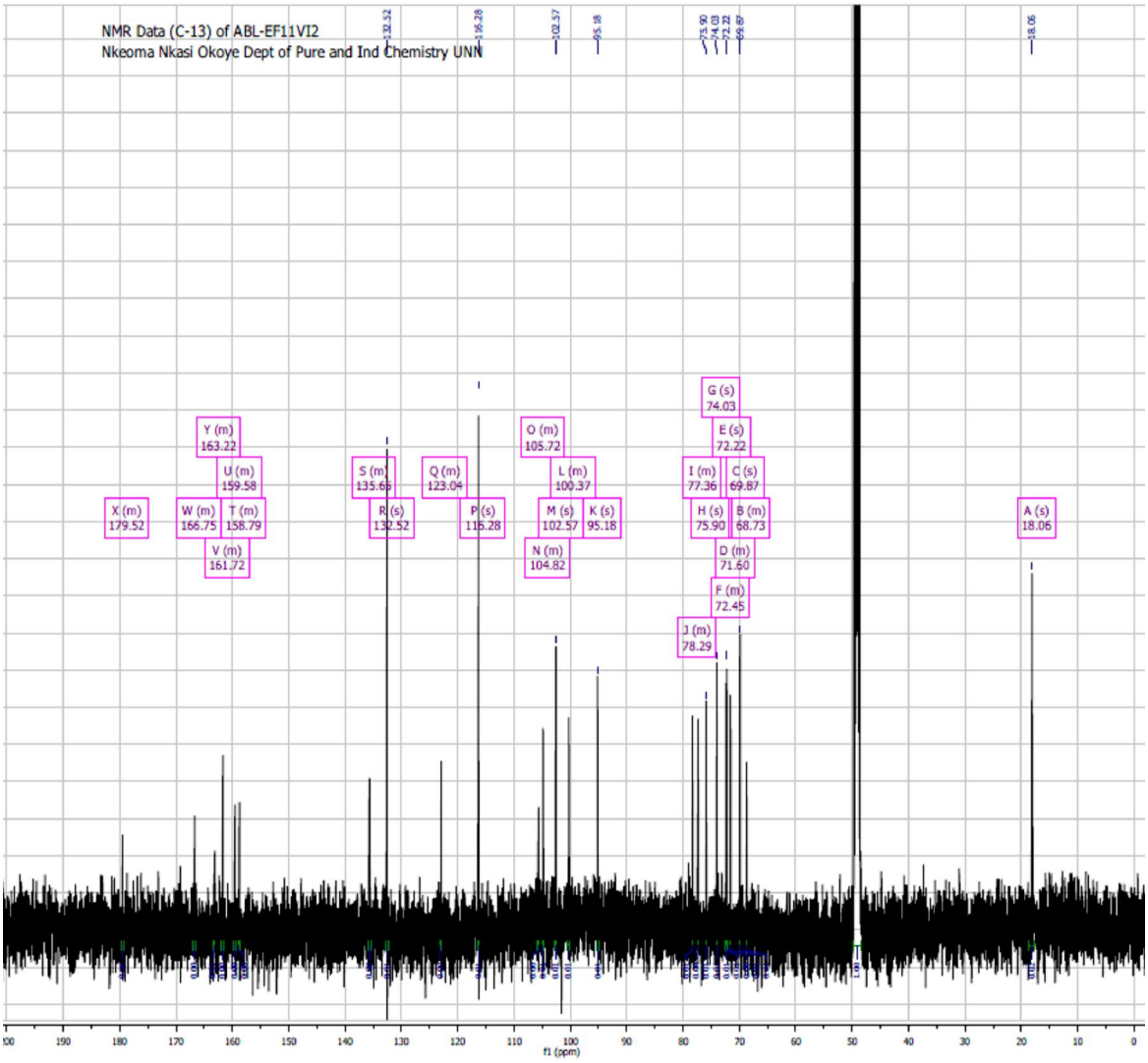
20. HMQC Spectrum of Compound **3**



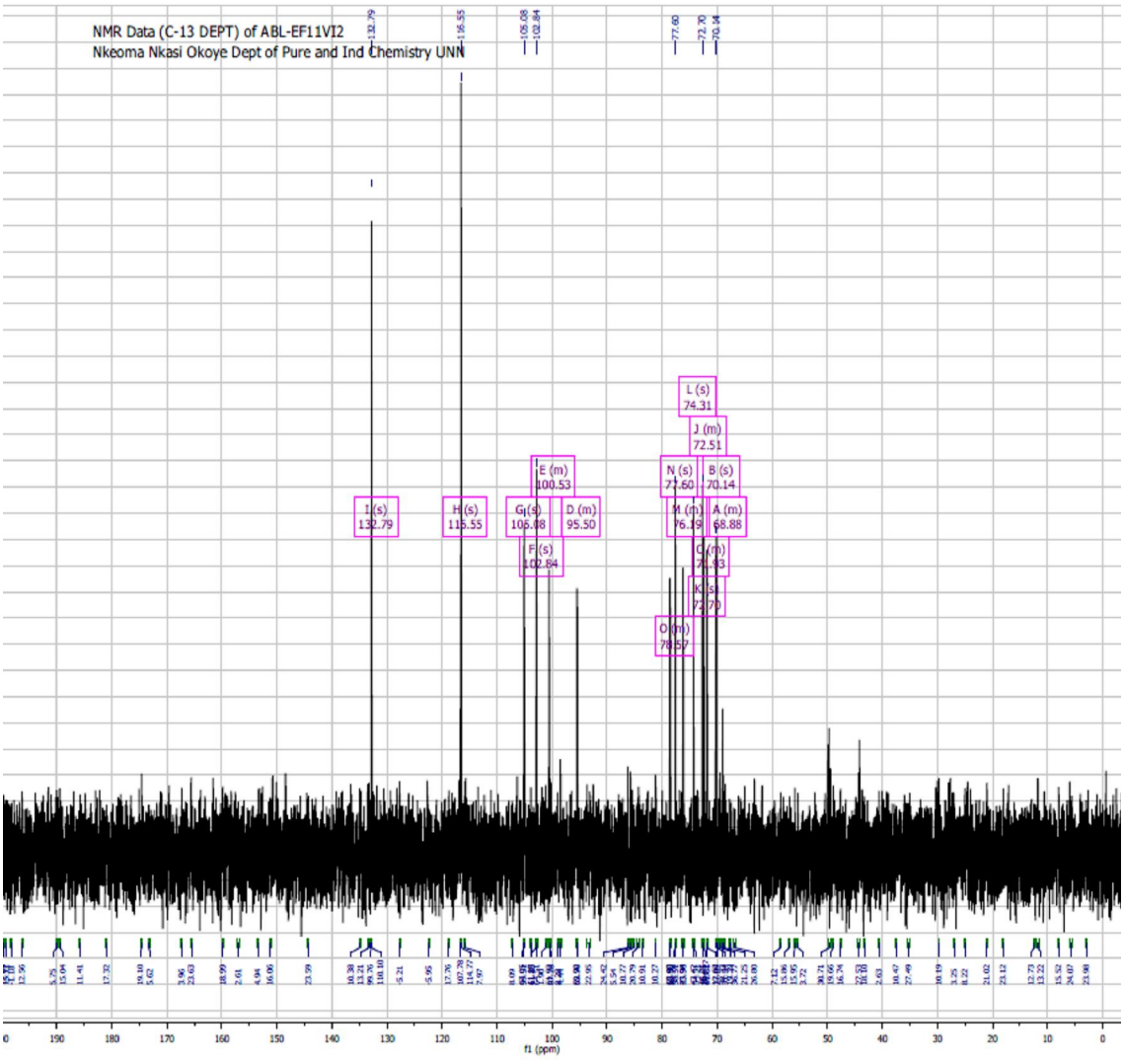
21. HMBC Spectrum of Compound **3**



22. C-13 NMR Spectrum of Compound **3**



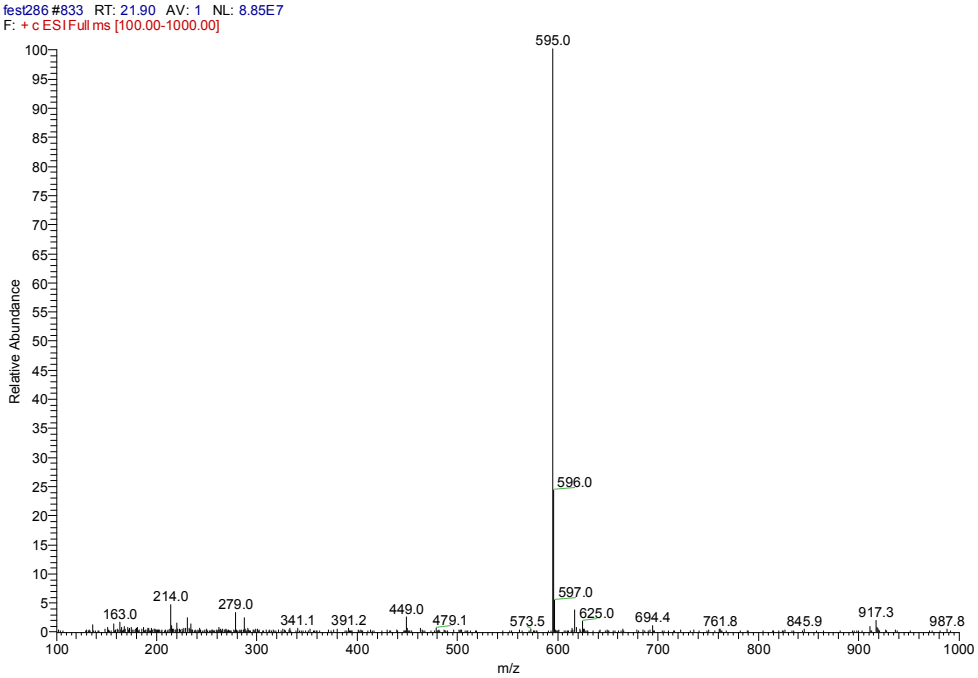
23. C-13 NMR (DEPT-135) of Compound **3**

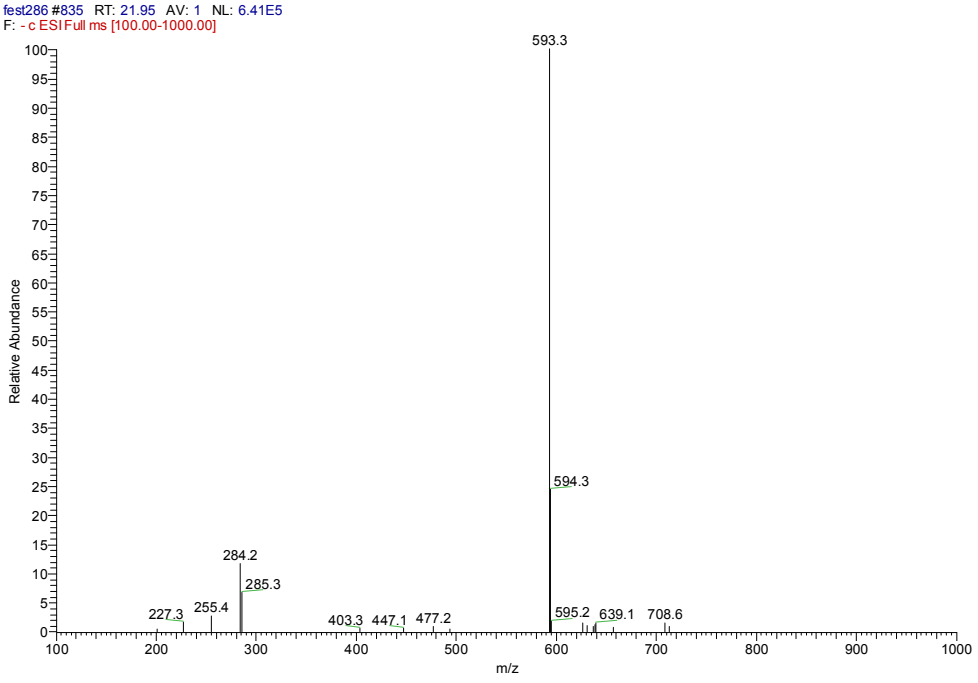


24. HPLC Spectrum of Compound **4**

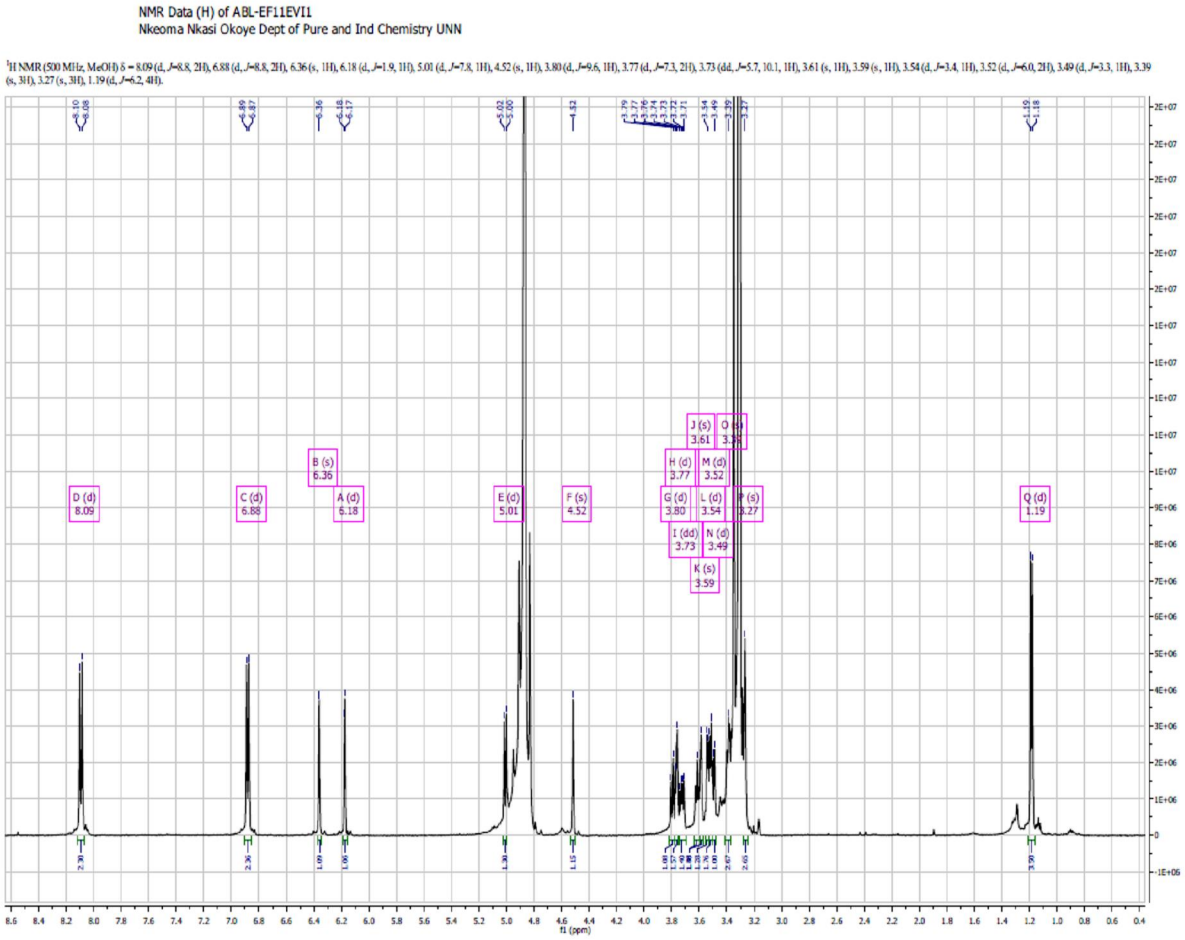
25. UV Spectrum of Compound **4**

26. LC-MS Spectra of Compound **4**

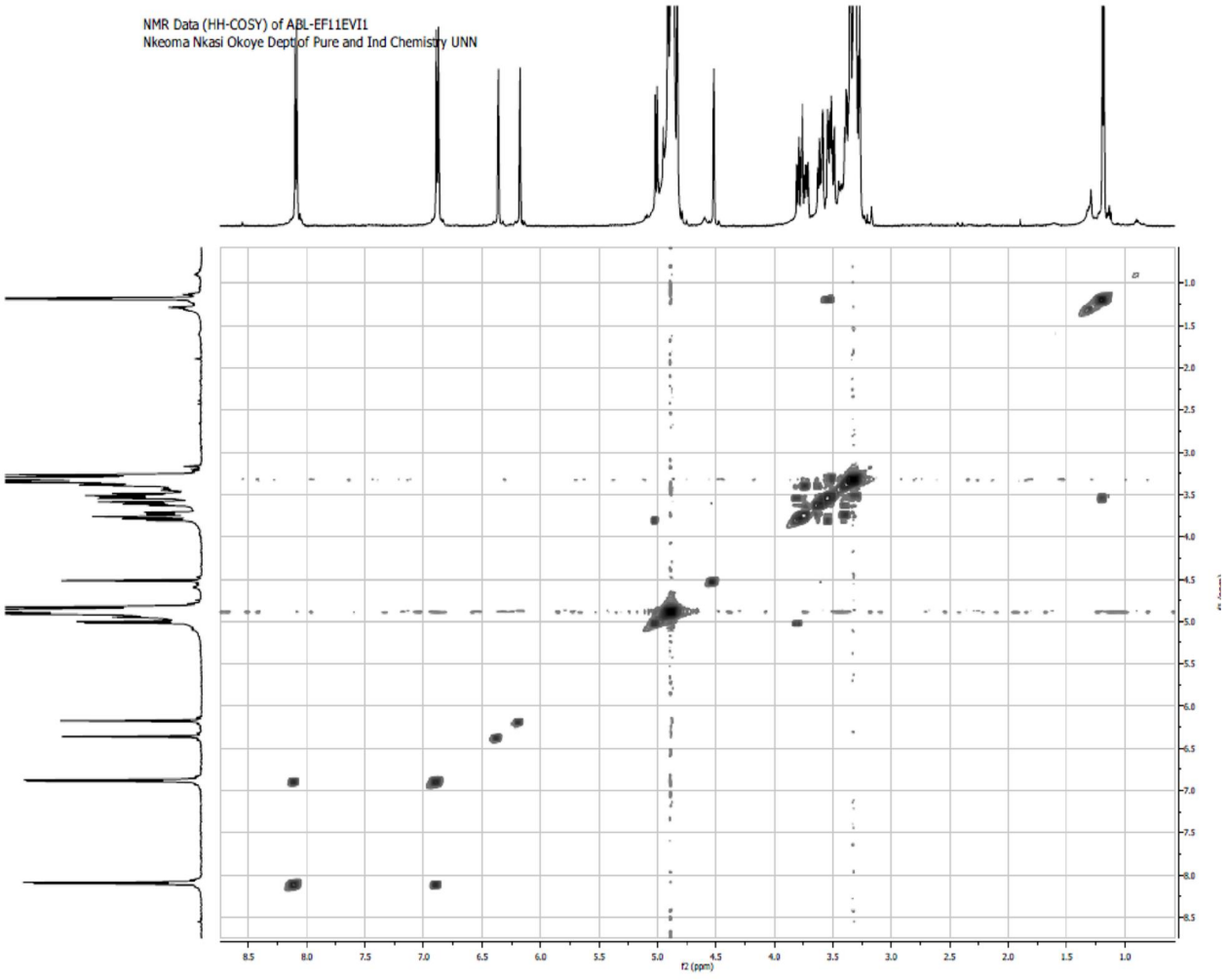




27. Proton NMR Spectrum of Compound **4**



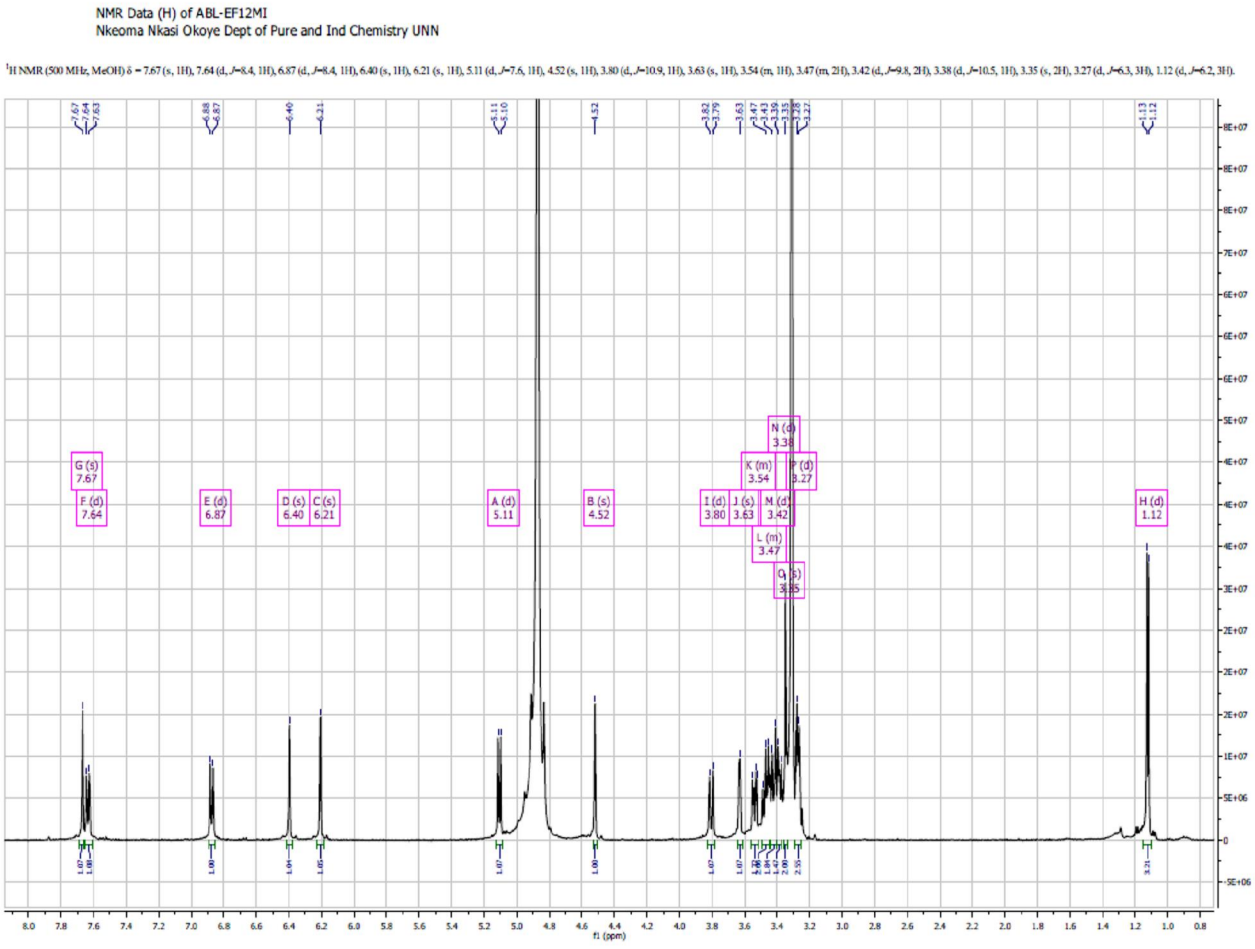
28. (H-H COSY) Proton NMR Spectrum of Compound **4**



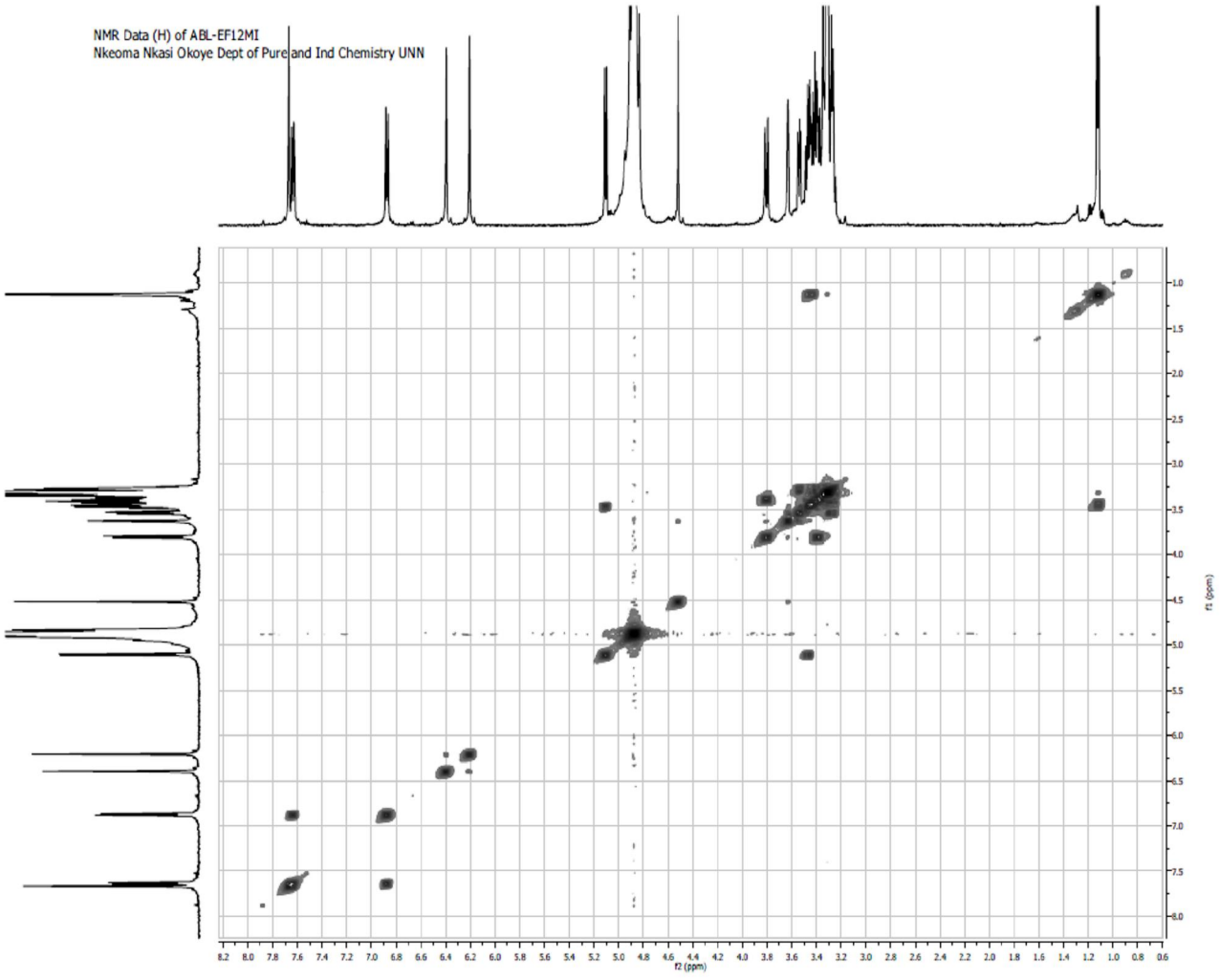
29. HPLC Spectrum of Compound **5**

30. UV Spectra of Compound **5**

31. Proton NMR Spectra of compound **5**



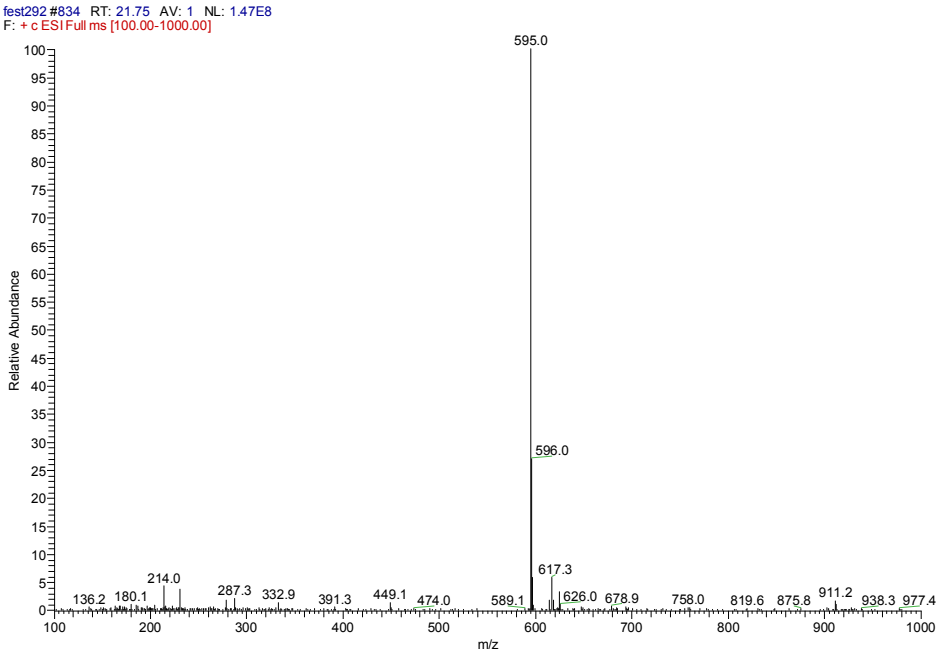
32. (H-H COSY) Proton NMR Spectrum of Compound **5**

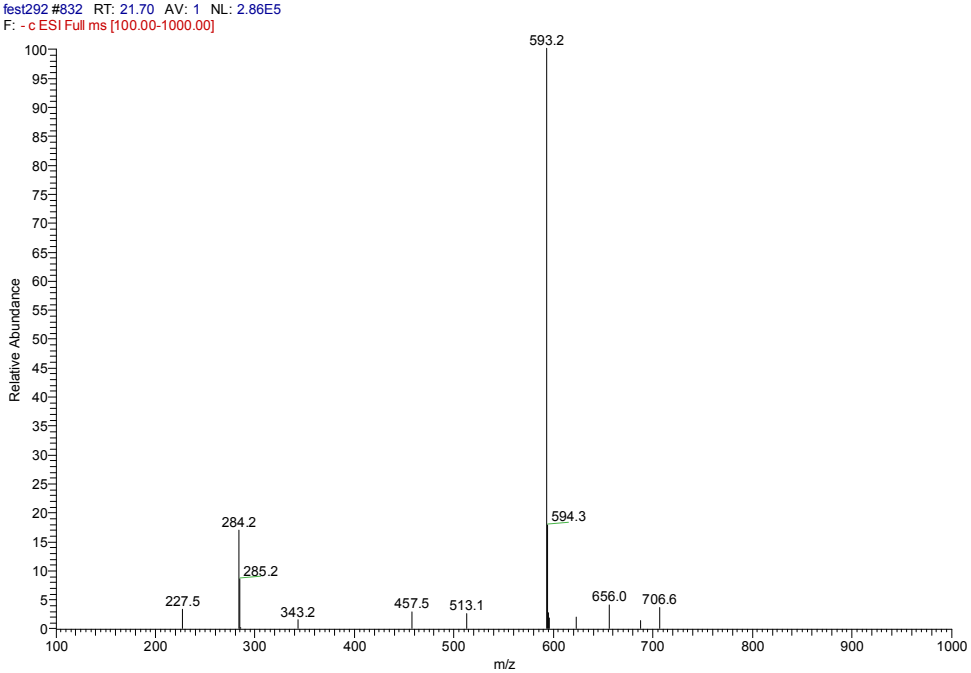


33. HPLC Spectrum of Compound **6**

34. UV Spectrum of Compound **6**

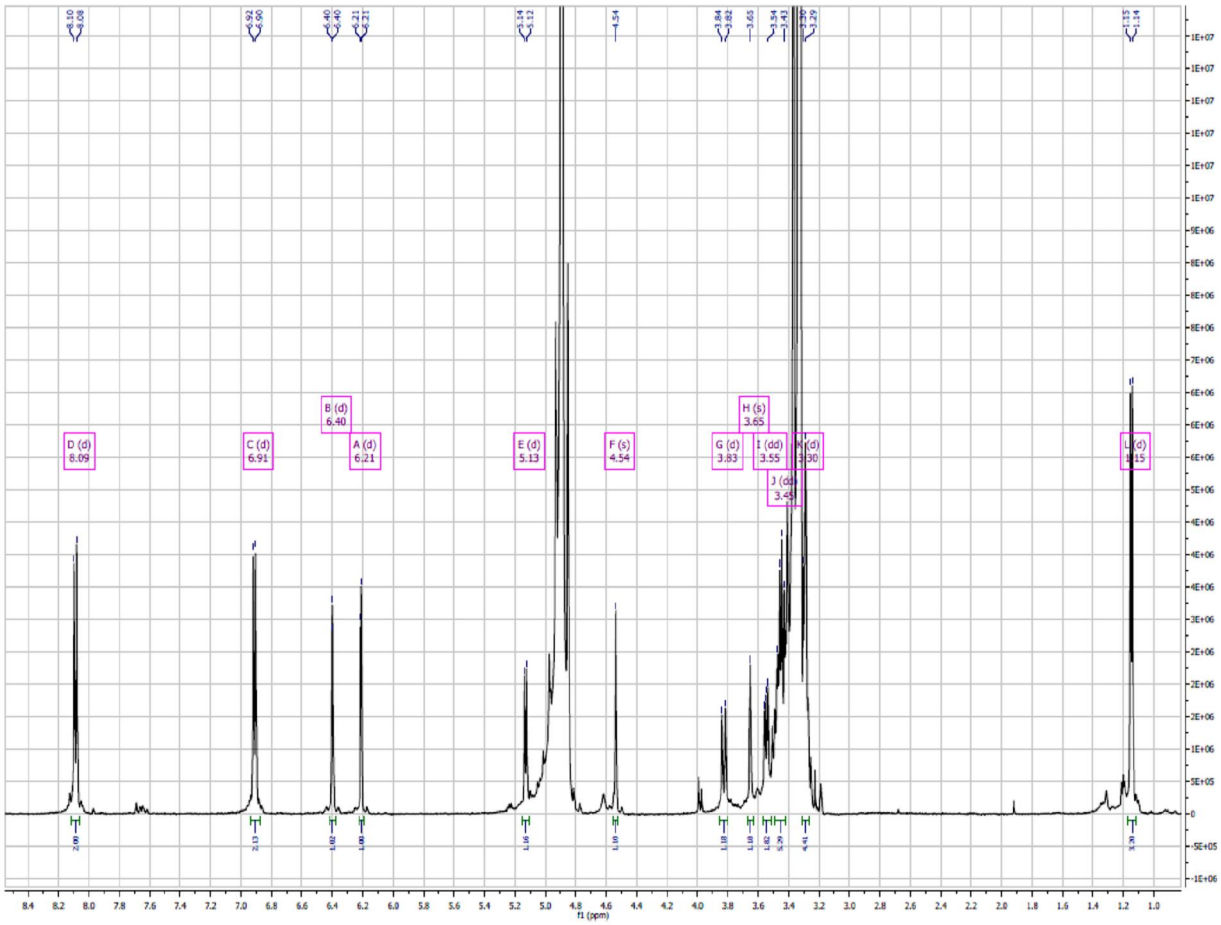
35. LC-MS Spectrum of Compound **6**



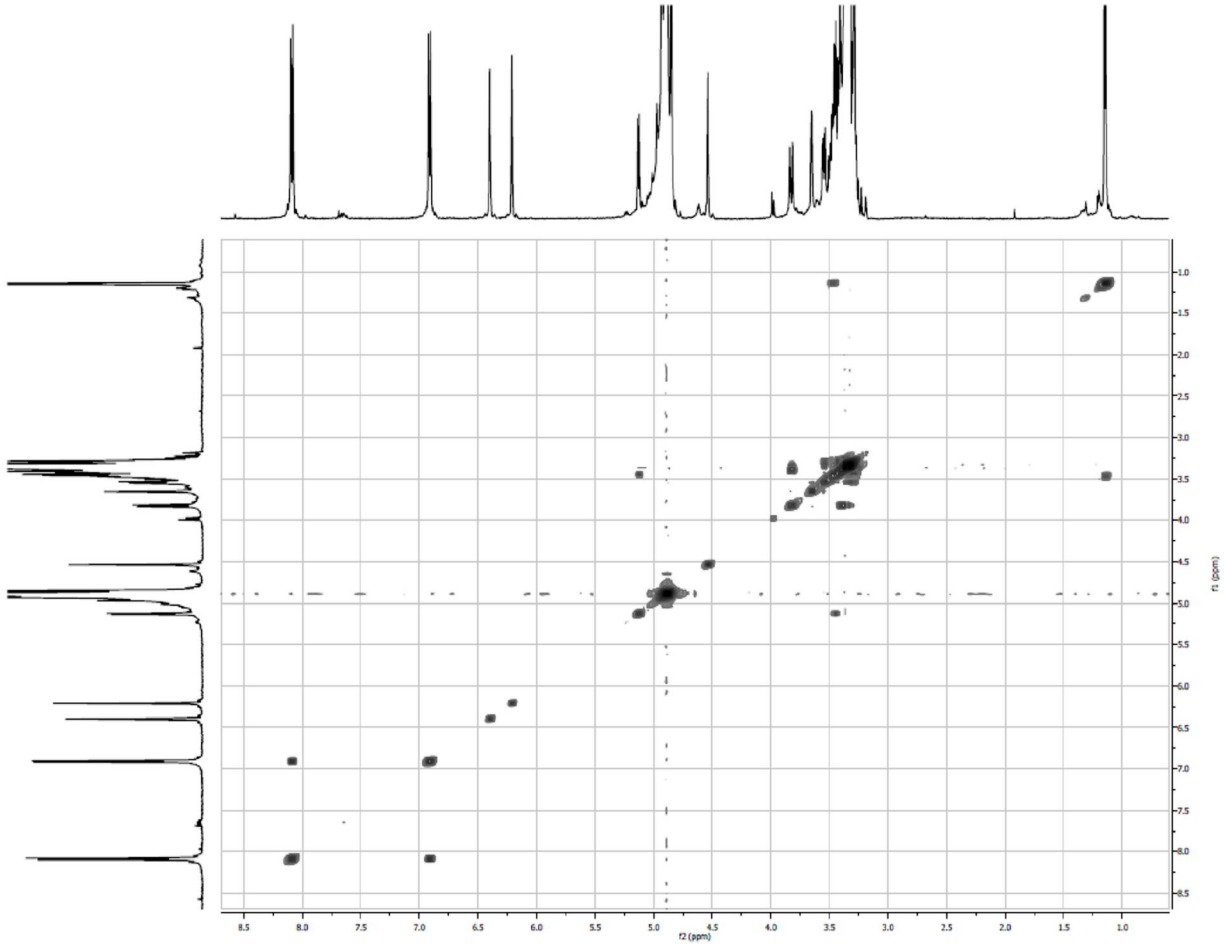


36. Proton NMR Spectrum of Compound **6**

^{1}H NMR (500 MHz, MeOH) δ = 8.09 (d, *J*=8.8, 2H), 6.91 (d, *J*=8.8, 2H), 6.40 (d, *J*=2.0, 1H), 6.21 (d, *J*=2.0, 1H), 5.13 (d, *J*=7.3, 1H), 4.54 (s, 1H), 3.83 (d, *J*=11.0, 1H), 3.65 (s, 1H), 3.55 (dd, *J*=3.4, 9.5, 2H), 3.45 (dd, *J*=7.1, 14.7, 5H), 3.30 (d, *J*=6.6, 4H), 1.15 (d, *J*=6.2, 3H).



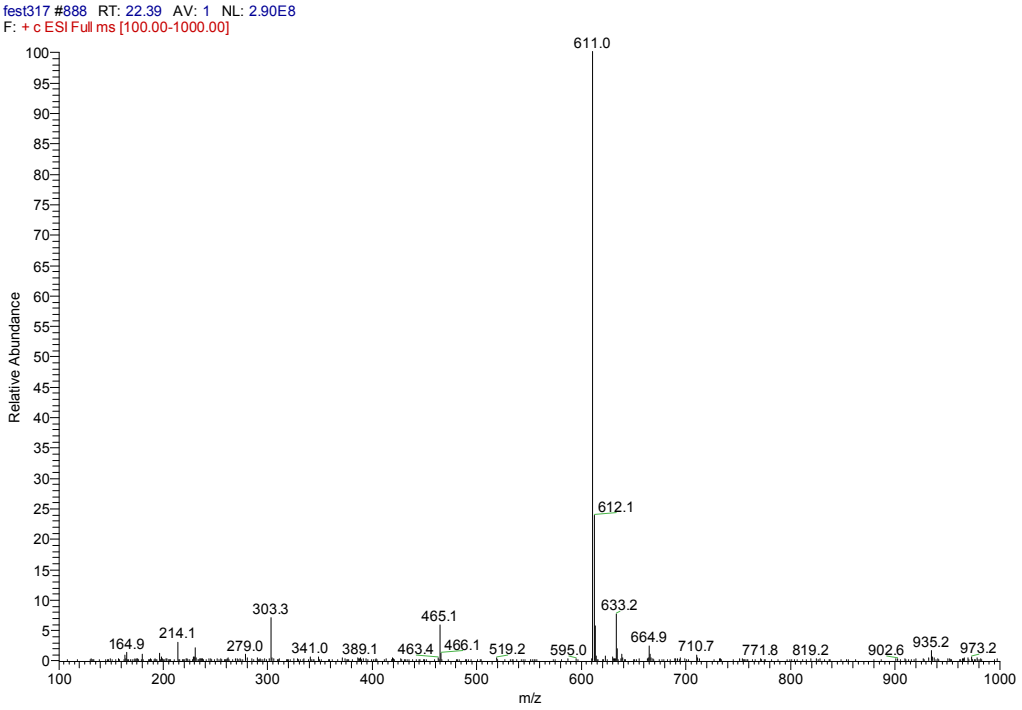
37. (H-H COSY) Proton NMR Spectrum of Compound **6**

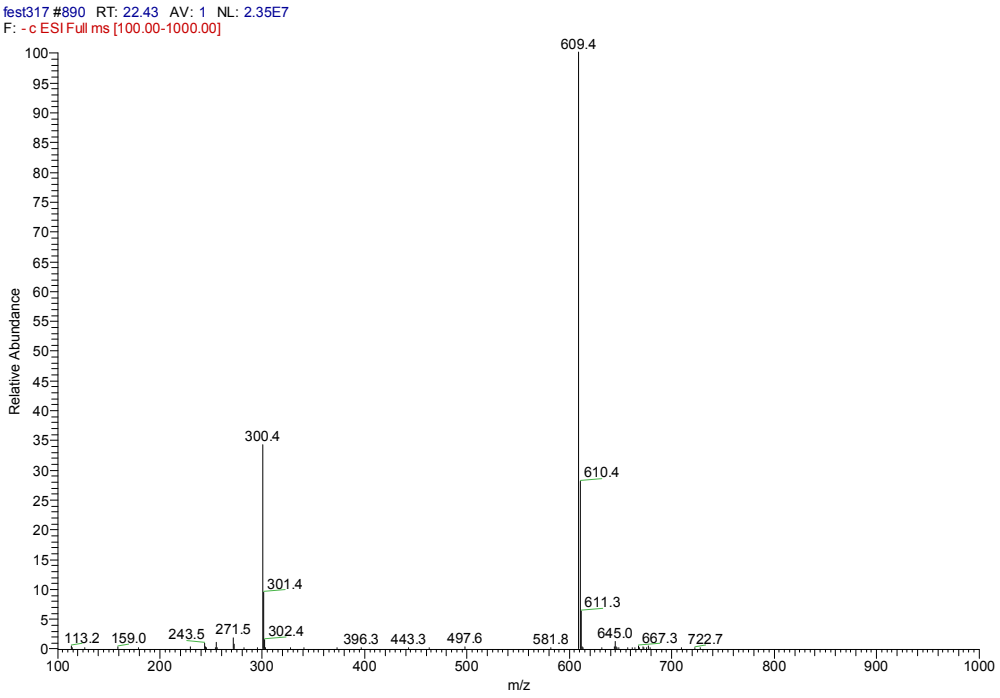


38. HPLC Spectrum of Compound **7**

39. UV Spectrum of Compound **7**

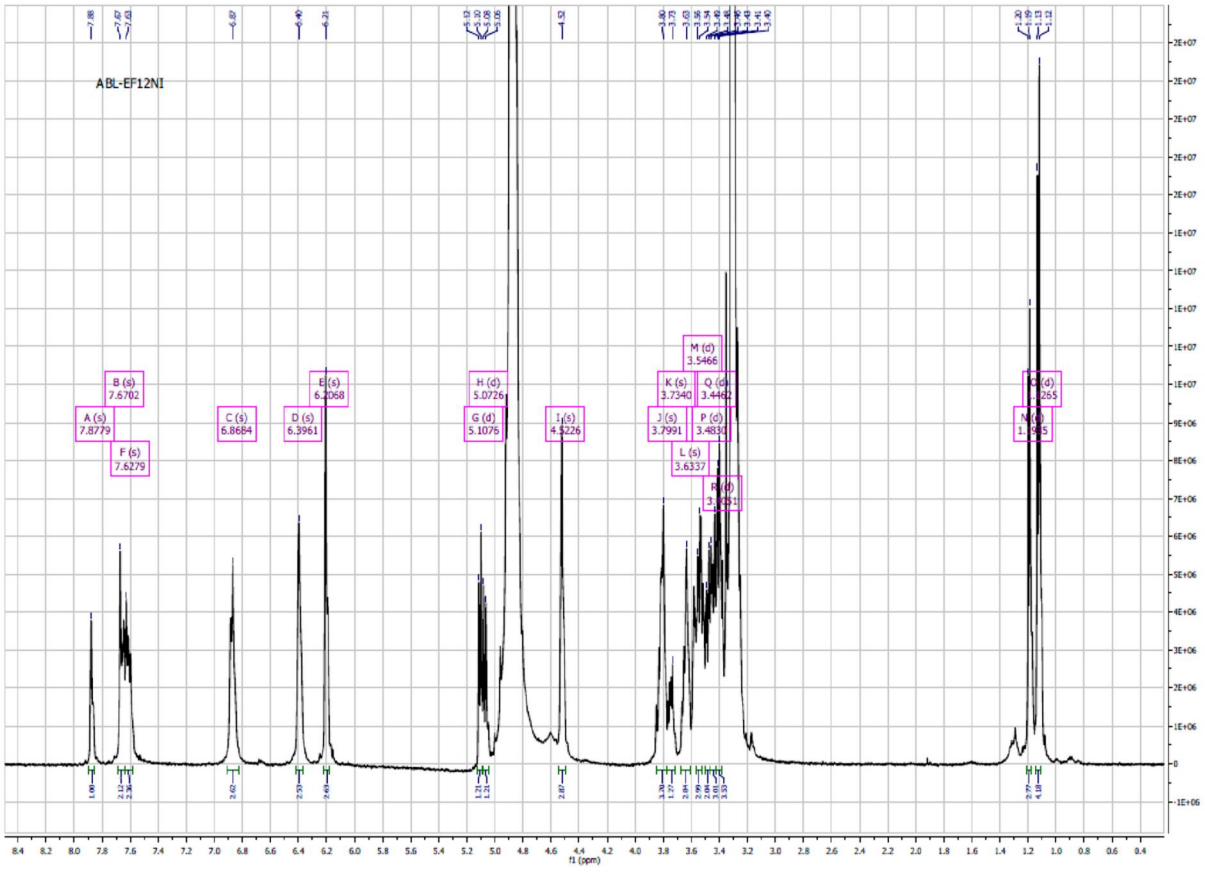
40. LC-MS Spectra of Compound **7**



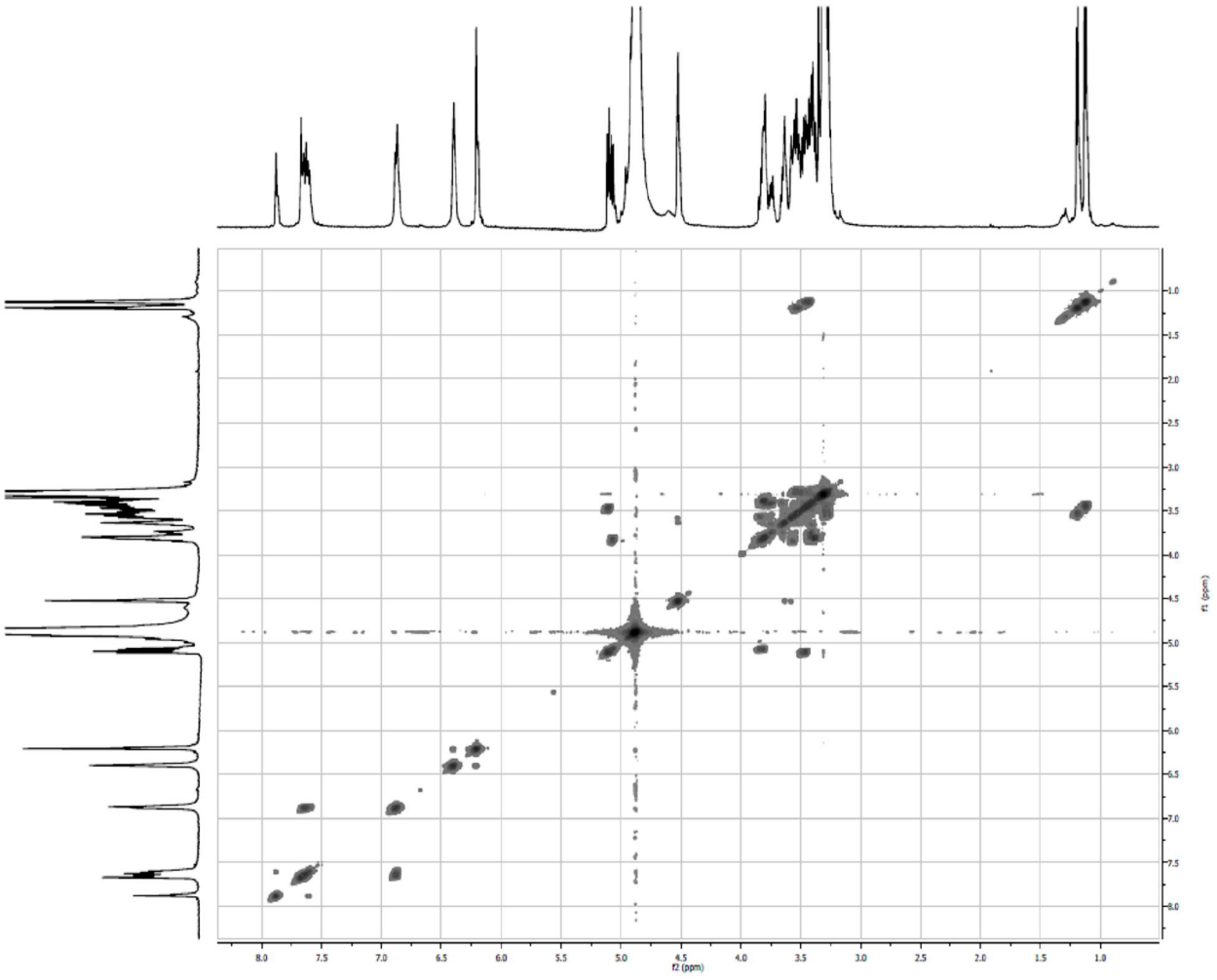


41. Proton NMR Spectrum of Compound 7

^{1}H NMR (500 MHz, MeOH) δ = 7.88 (s, 1H), 7.67 (s, 2H), 7.63 (s, 2H), 6.87 (s, 3H), 6.40 (s, 3H), 6.21 (s, 3H), 5.11 (d, *J*=7.7, 1H), 5.07 (d, *J*=7.9, 1H), 4.52 (s, 3H), 3.80 (s, 4H), 3.73 (s, 1H), 3.63 (s, 3H), 3.55 (d, *J*=9.4, 3H), 3.48 (d, *J*=7.7, 2H), 3.45 (d, *J*=13.2, 3H), 3.41 (d, *J*=6.8, 4H), 1.19 (d, *J*=6.2, 3H), 1.13 (d, *J*=6.2, 4H).



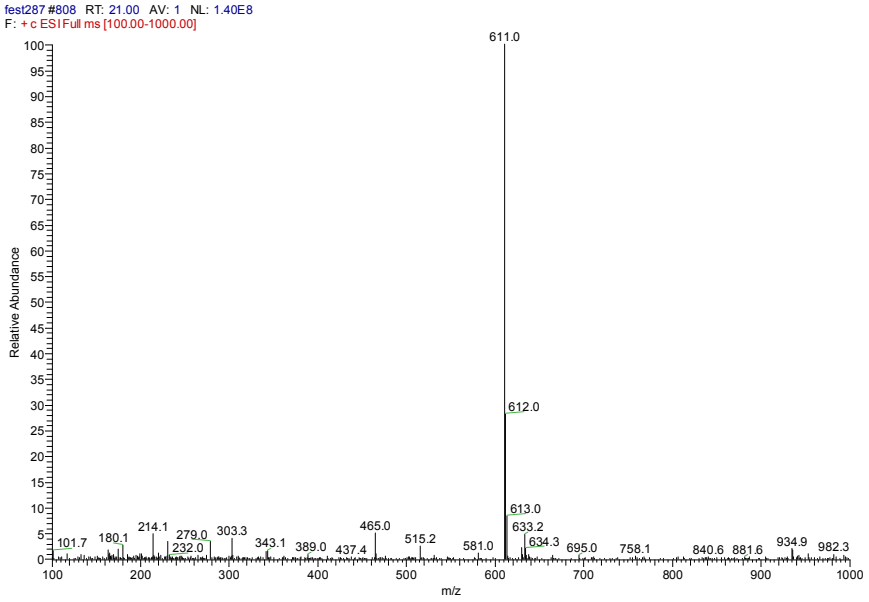
42. (H-H COSY) Proton NMR Spectrum of Compound 7

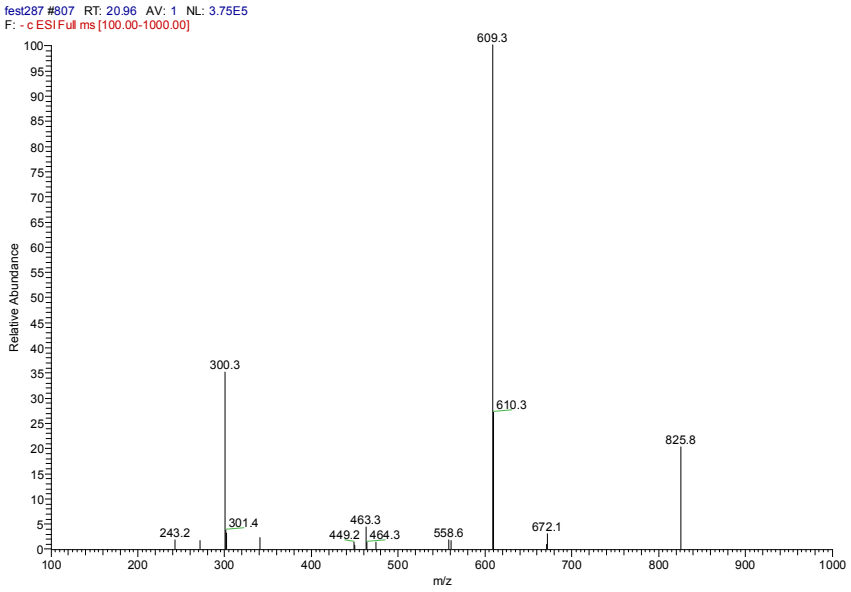


43. HPLC Spectrum of Compound **8**

44. UV Spectrum of Compound **8**

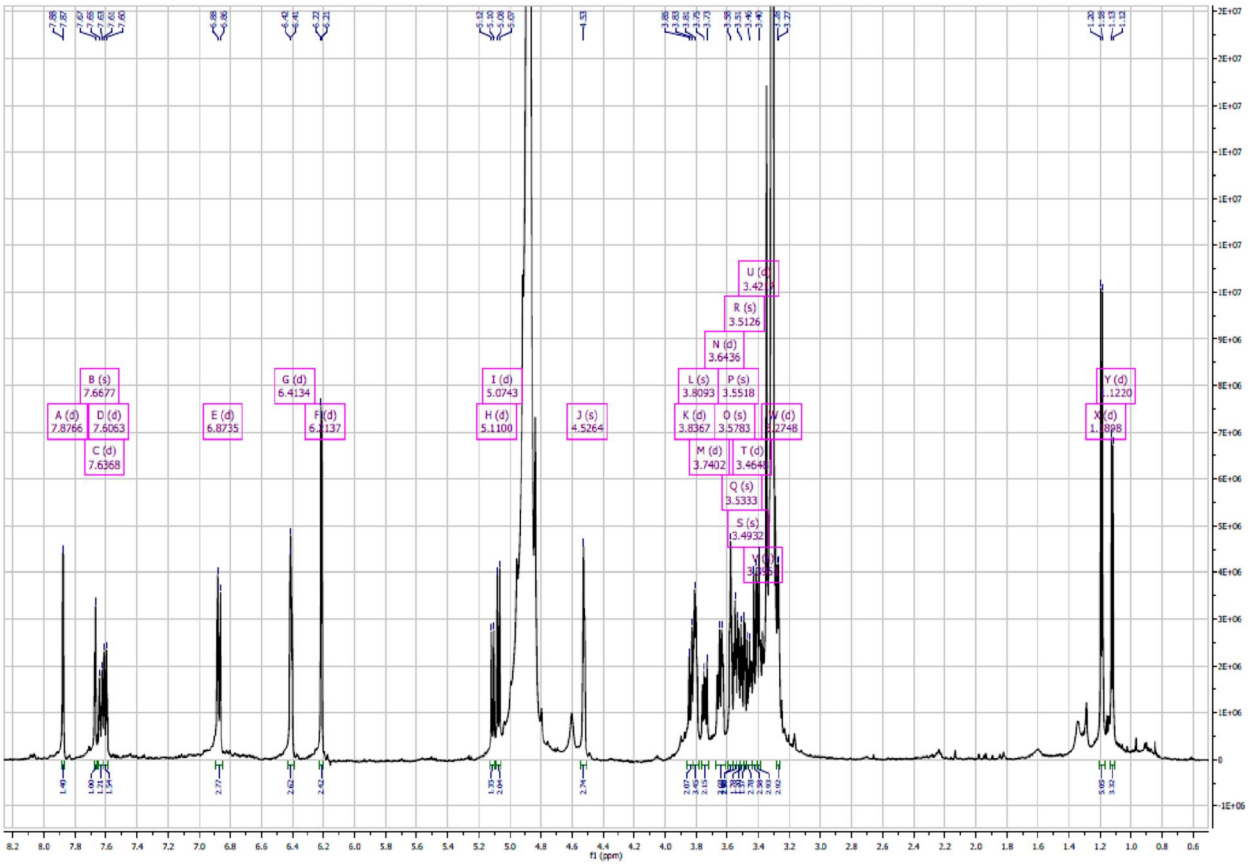
45. LC-MS Spectra of Compound **8**



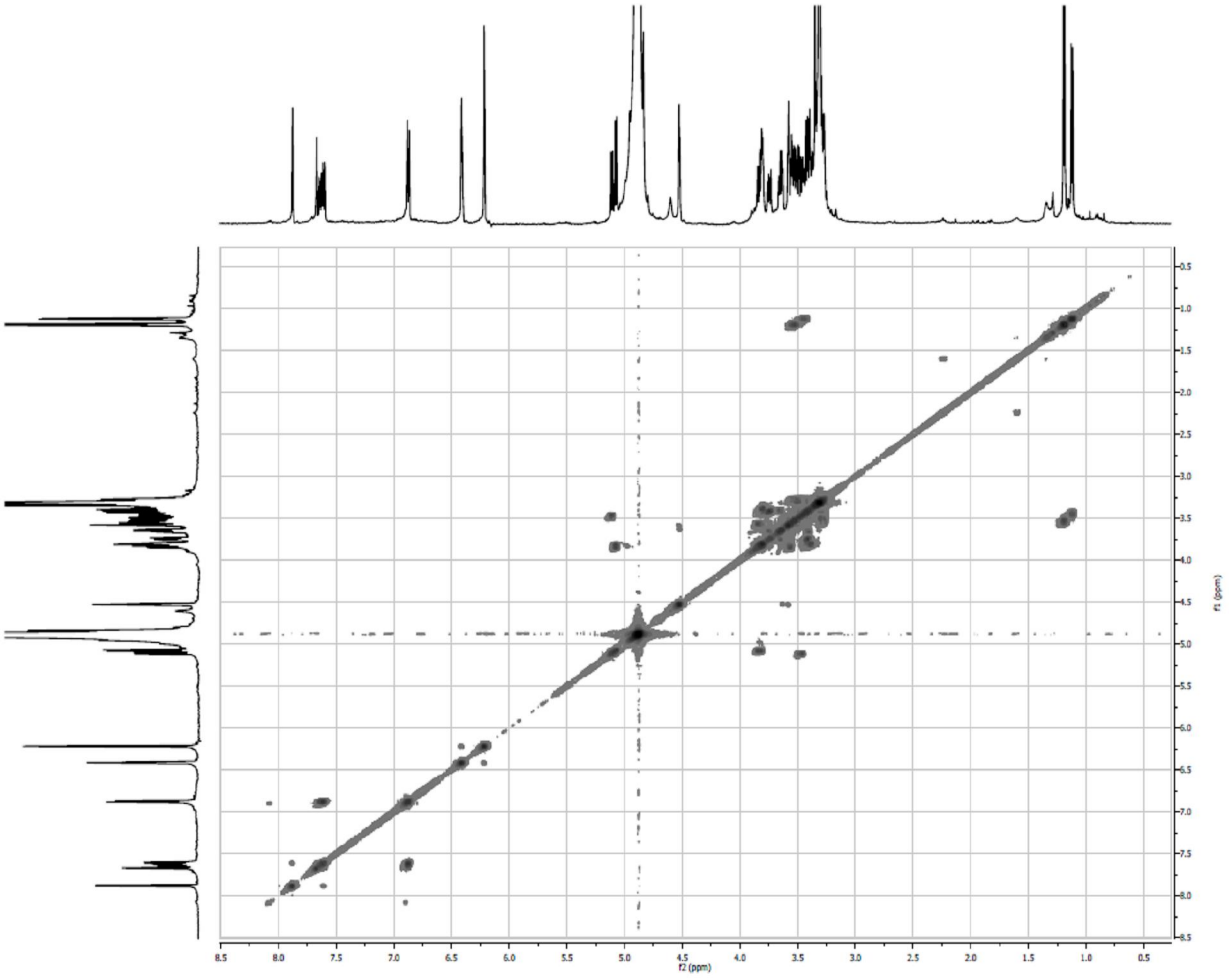


46. Proton NMR Spectrum of Compound **8**

^{1}H NMR (500 MHz, MeOH) δ = 7.88 (d, J=2.1, 1H), 7.67 (s, 1H), 7.64 (d, J=8.4, 1H), 7.61 (d, J=8.5, 2H), 6.87 (d, J=8.5, 3H), 6.41 (d, J=2.0, 3H), 6.21 (d, J=2.0, 2H), 5.11 (d, J=7.7, 1H), 5.07 (d, J=7.8, 2H), 4.53 (s, 3H), 3.84 (d, J=9.6, 2H), 3.81 (s, 3H), 3.74 (d, J=10.1, 2H), 3.64 (d, J=6.7, 4H), 3.58 (s, 3H), 3.55 (s, 2H), 3.53 (s, 2H), 3.51 (s, 2H), 3.49 (s, 1H), 3.46 (d, J=7.8, 3H), 3.42 (d, J=8.1, 3H), 3.40 (s, 3H), 3.27 (d, J=4.1, 3H), 1.19 (d, J=6.2, 5H), 1.12 (d, J=6.2, 3H).



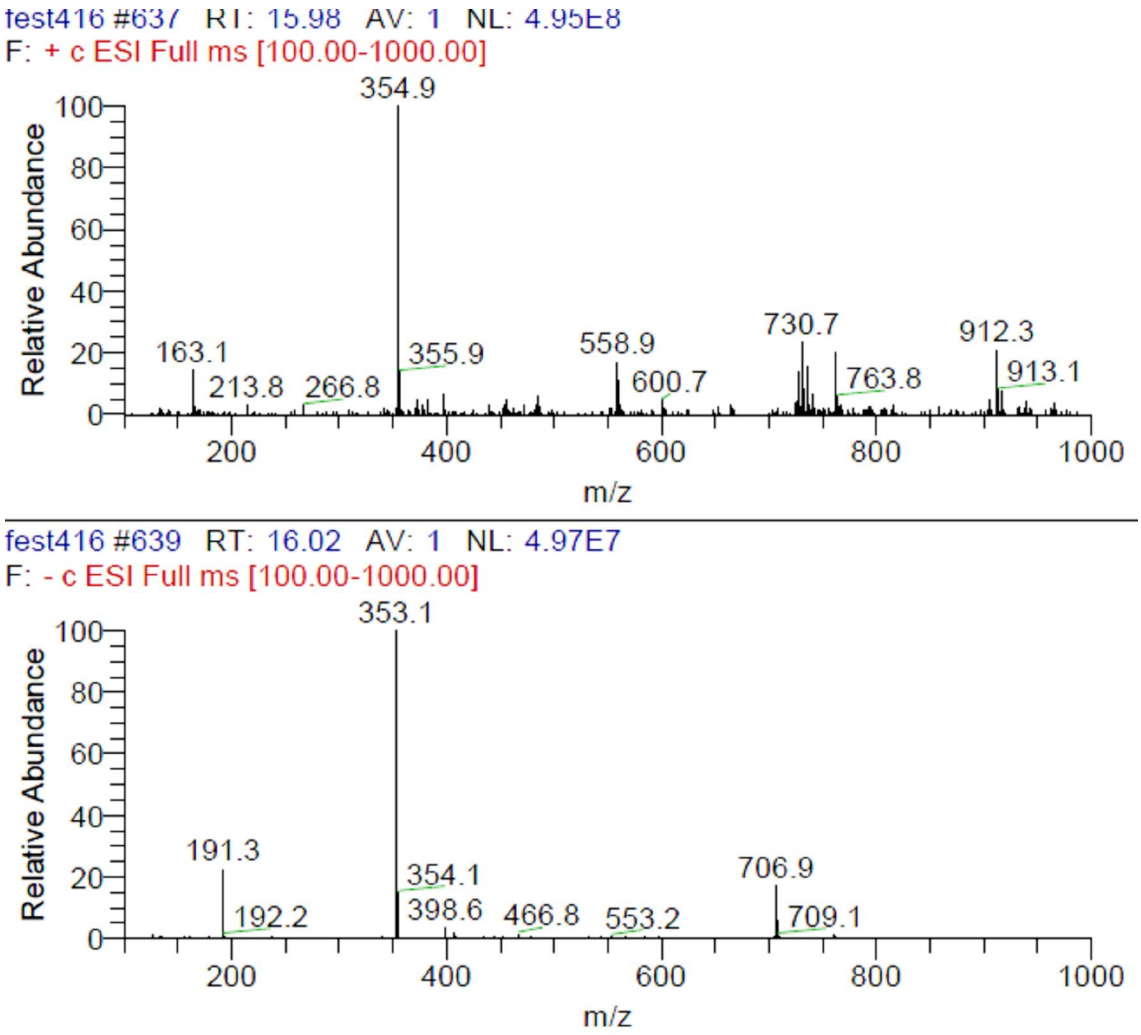
47. (H-H COSY) Proton NMR Spectrum of Compound **8**



48. HPLC Spectrum of Compound **9**

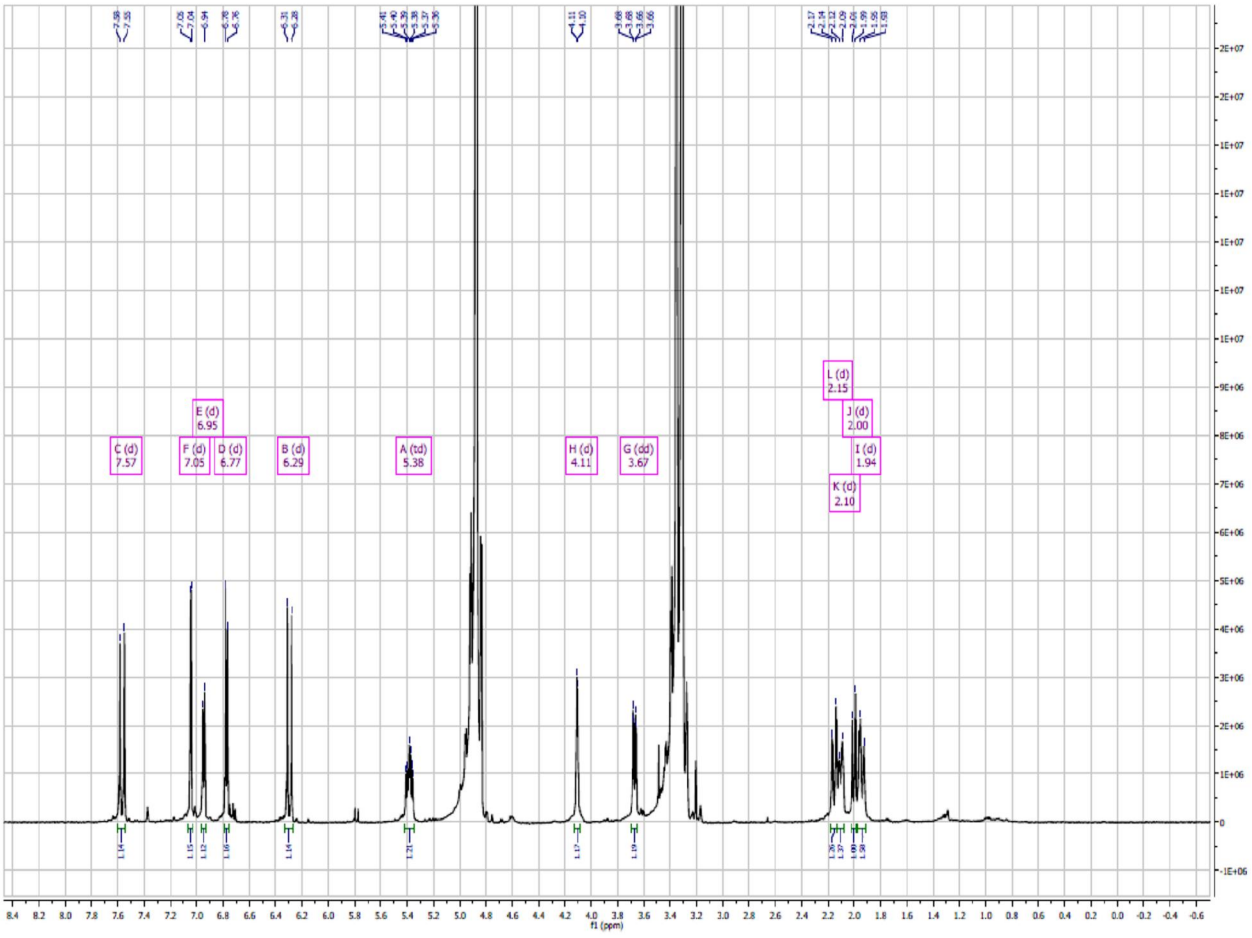
49. UV Spectrum of Compound **9**

50 LC-MS Spectra of Compound **9**

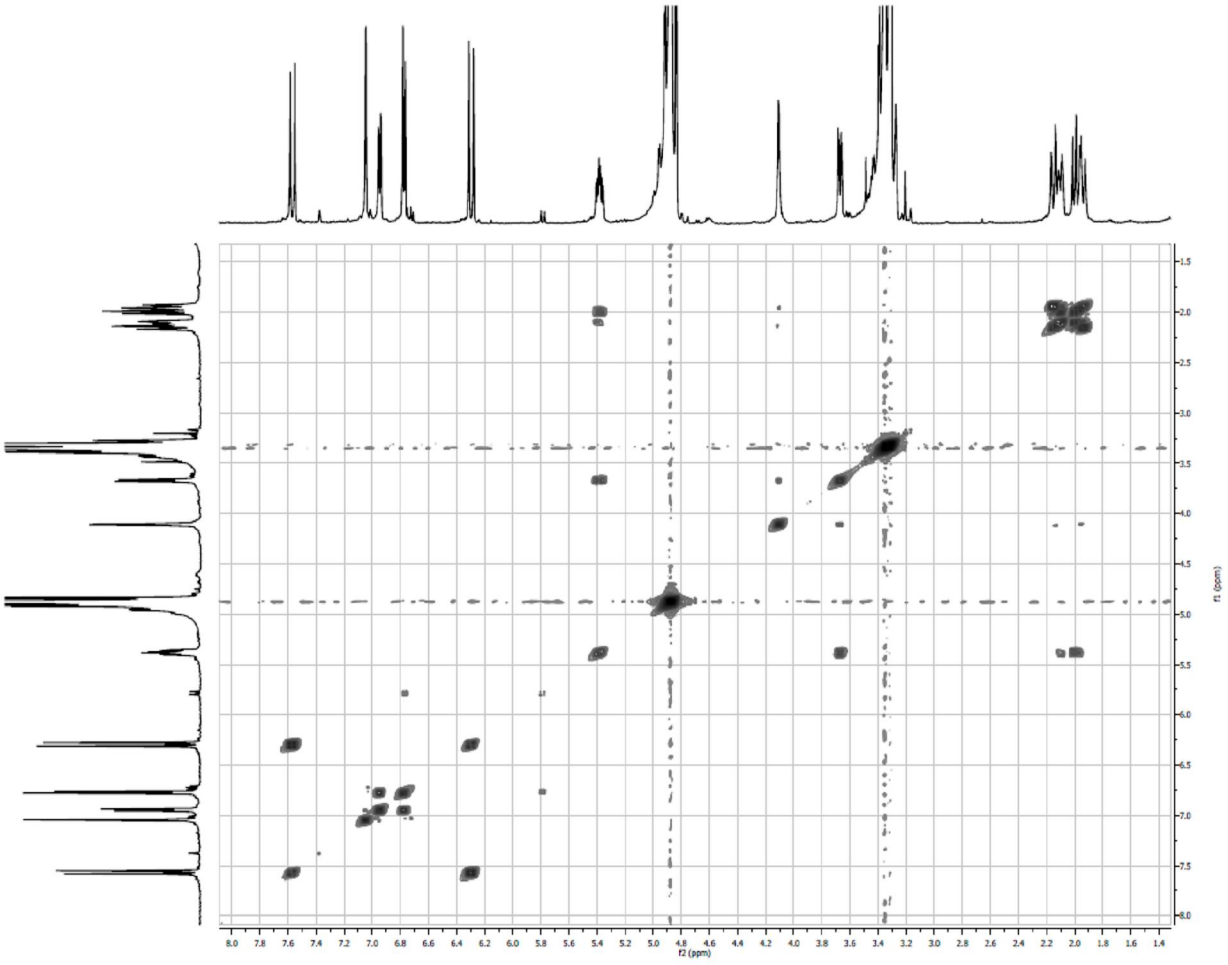


51. Proton NMR Spectrum of Compound **9**

^{1}H NMR (500 MHz, MeOH) δ = 7.57 (d, *J*=15.9, 1H), 7.05 (d, *J*=1.9, 1H), 6.95 (d, *J*=8.2, 1H), 6.77 (d, *J*=8.2, 1H), 6.29 (d, *J*=15.9, 1H), 5.38 (td, *J*=4.9, 11.2, 1H), 4.11 (d, *J*=3.1, 1H), 3.67 (dd, *J*=3.2, 9.9, 1H), 2.15 (d, *J*=14.7, 1H), 2.10 (d, *J*=12.6, 1H), 2.00 (d, *J*=11.9, 1H), 1.94 (d, *J*=14.5, 2H).



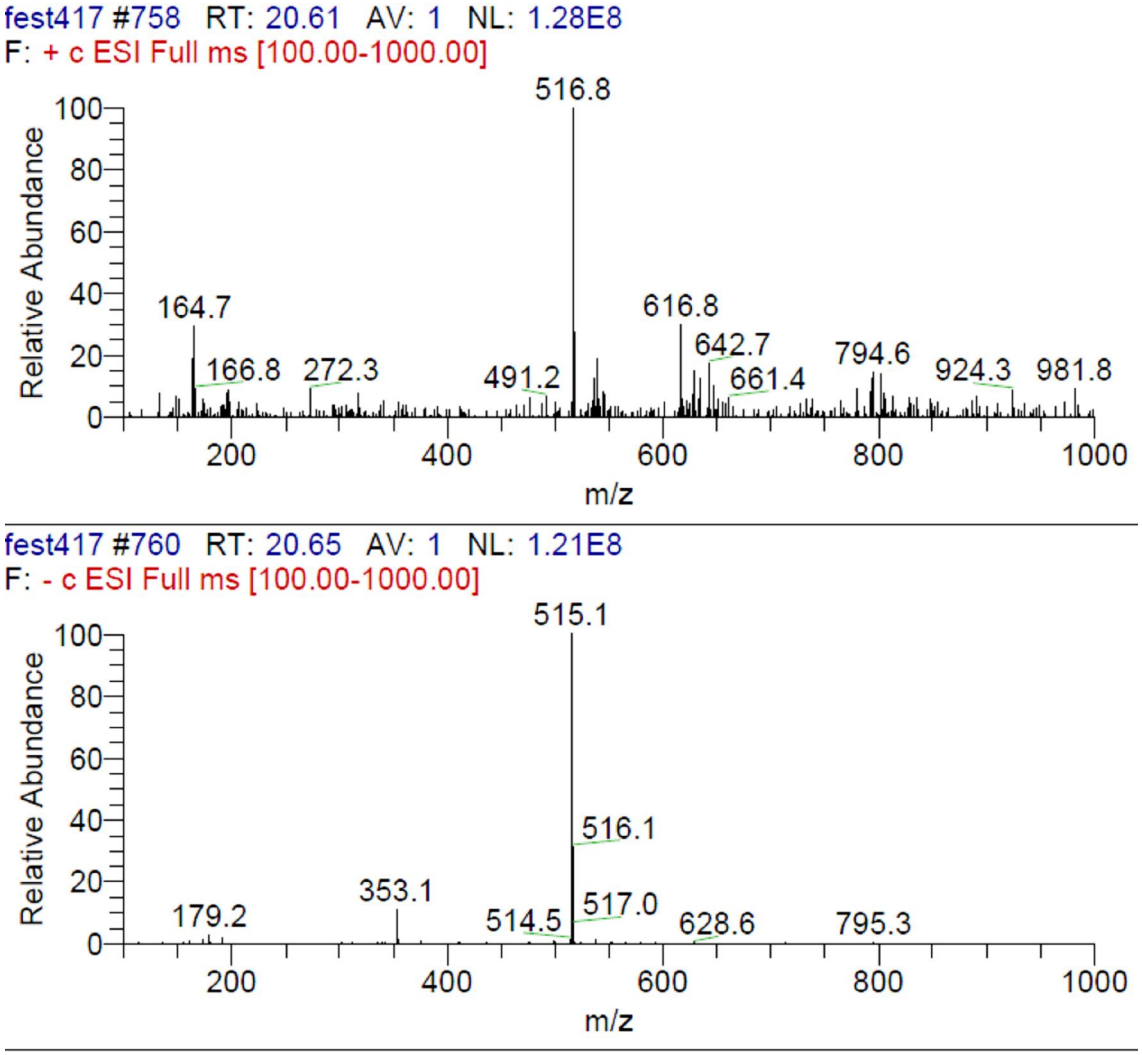
52. (COSY) Proton NMR Spectrum of Compound **9**



53. HPLC Spectrum of Compound **10**

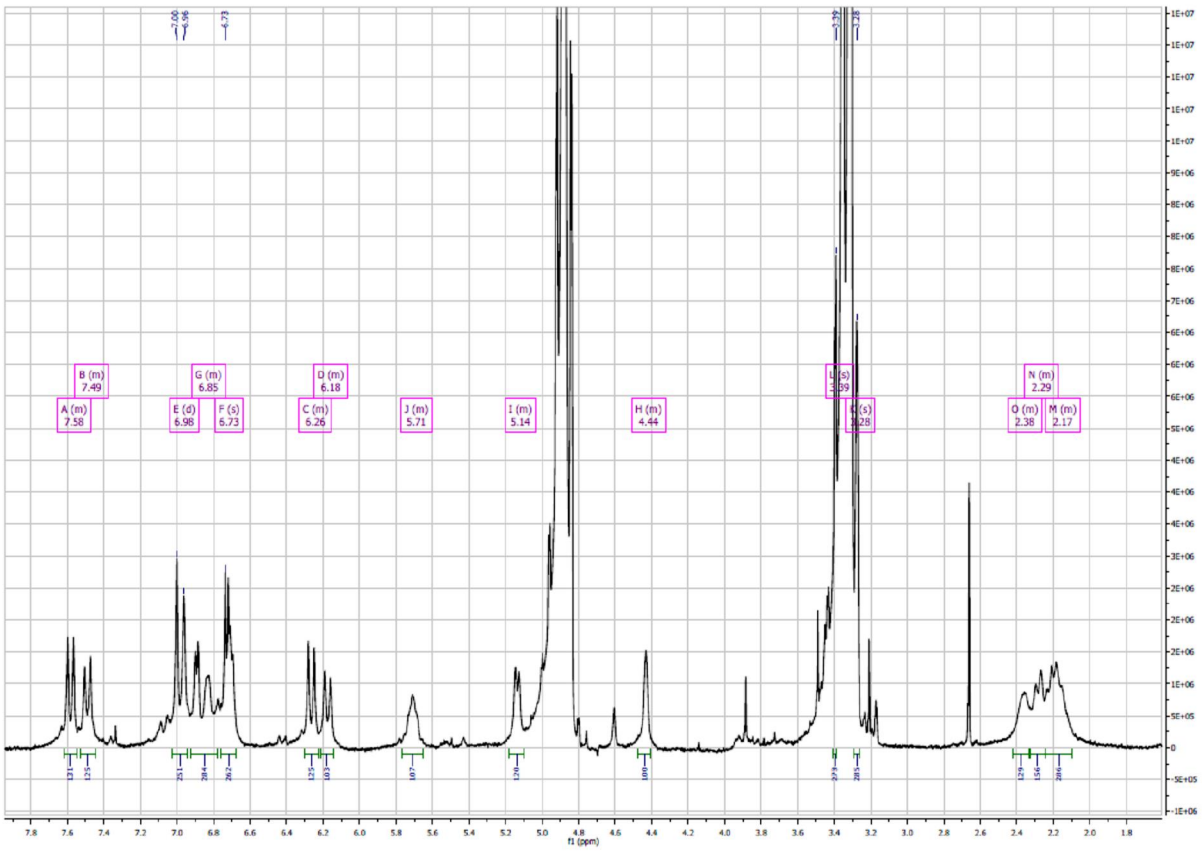
54. UV Spectrum of Compound **10**

55 LC-MS Spectra of Compound **10**



56. Proton NMR Spectrum of Compound **10**

^{1}H NMR (500 MHz, MeOH) δ = 7.58 (m, 1H), 7.49 (m, 1H), 6.98 (d, *J*=19.5, 3H), 6.85 (m, 3H), 6.73 (s, 3H), 6.26 (m, 1H), 6.18 (m, 1H), 5.71 (m, 1H), 5.14 (m, 1H), 4.44 (m, 1H), 3.39 (s, 3H), 3.28 (s, 3H), 2.38 (m, 1H), 2.29 (m, 2H), 2.17 (m, 3H).



57. (COSY) Proton NMR Spectrum of Compound **10**