Chapter one

Introduction

1.1. Background of Study

Plants over thousands of years have been utilized as important element of traditional medicine (Samuelsson, 2004). WHO has also confirmed its crucial role in the primary health care of the greater percentage of people living in the world (Wang, 2009). There is already more than 119 bioactive compounds isolated from about 90 plant species being used as drugs, where 74 % of them were discovered by chemical studies through the isolation of the bioactive compounds present in the plants (Newman *et al.*, 2000). Early nineteenth century, isolation of the bioactive compounds from extracts has been from medicinal plant materials (Kinghorn, 2001). Since 1930s, series of compounds isolated from plants have been used as clinical agents and still in use up till today (Kong *et al.*, 2003). Recently, isolation and characterization of the bioactive compounds from these medicinal plants for discovery of more drugs have become more efficient through the use of new technologies. More interests are been shown for these investigation due to some well-known pharmaceutical and research institutions involved in some research.

More than 50% of the 25 best-selling drugs worldwide are related directly to natural products. In 1990's, 61% of anticancer agents approved were natural products and their derivatives (Wang, 2009). In the same period, among the projects performed by the top twenty international pharmaceutical companies, more than 80% of the compounds involved were from microorganisms and natural products. Moreover, approximately 40% of the projects at the clinical test and pre-clinical test stage were correlated to natural products (Liu, 2000).

Biological organisms produce mainly two types of chemical products. The first being primary metabolites, which consists of compounds such as sugars and proteins. These compounds are

common to most organisms and essential for functional metabolism. The second being secondary metabolites, and, are chemicals unique to a single species or related group of organisms. Before 1990s, scientists did not fully realize that these secondary metabolites are more than mere leftovers from an organism's metabolic processes. The secondary metabolites play important roles such as: its function as communications tools, defense mechanisms, or sensory devices. Although the biological activity of these secondary metabolites is beneficial to the organism that produces them, many are harmful to other species, especially humans due to its toxicity. And this can have an effect on the normal functions of the entire human body or a specific biological process, such as cancer cells growth. Because of its toxicity, some secondary metabolites can act as drugs when administered at a minimal concentration. The use of natural product compounds as a source of remedies dates back to ancient empires in Mesopotamia, Egypt, China, Greece, and Rome. Today scientists in the world are turning to plants, microbes, and marine organisms as a potential storehouse of drugs waiting to be discovered (da Rocha *et al.*, 2001).

Drugs from natural sources may either be isolated from biological organisms, or modified versions of natural bioactive compounds, or completely synthetic, yet based upon models of natural origin (Yoder, 2005). Today, natural products are responsible for about half of the approved drugs that are currently available (Vlietinck and Apers, 2001). The percentage is even higher for treatment of infection or cancer, as natural products for those illnesses account for approximately 60% of the drugs either in use or awaiting FDA approval between 1989 and 1995. For example, 18 of the 42 new drugs discovered in 1992 are either natural products or synthetic analogs of natural products. Hence, the role of the chemist has become more crucial in the isolation, structure determination, and synthesis of bioactive compounds (Yoder, 2005).

1.2. Statement of problem

Today approximately 80% of the world's population relies on traditional plant based medicines for primary health care. The remaining 20% of the world's population also depends on plant products for health care. About 25% of prescription drugs dispensed in the United States contain plant extracts or active ingredients derived from plants. Out of a total of 520 new drugs approved for commercial use between 1983 and 1994, 30 were new natural products and 127 were chemically modified natural products.

Despite the great successes already achieved in natural products chemistry and drug development, we have barely begun to tap the potential of our molecular diversity. Only an estimated 5% to 15% of the 250,000 species of higher terrestrial plants in existence have been chemically and pharmacologically investigated in systematic fashion (Myint, 2006). Now the chemists have to solve the puzzle of which biological species produce these compounds, what is the structure of the bioactive compounds, and how potent are they as therapeutic agents (Yoder, 2005)?

The plant *Millettia aboencis* has many biological activities which include: antibacterial activity, antioxidant activity, hepatoprotective activity, anti-inflammatory activity, etc, but there is little or no information on the isolation and structural elucidation of the bioactive principles. There is need to isolate, identify (or structurally elucidate) the bioactive principles present in the extracts of these plant parts which could lead to its potential exploitation for pharmaceutical use.

1.3.Aim of study

The aim of this study is to isolate and identify bioactive compounds via HPLC-UV, NMR and MS.

1.4. Justification of the study

The genus Millettia (family Leguminosae, subfamily Papilionoideae) with approximately 260 species is widespread in Africa (139 species) and Asia (121 species) (Marco *et al.,* 2017). Out of the West African countries, one of the species (*Millettia aboencis*) is indigenous to Nigeria.

The genus is a rich source of secondary metabolites such as chalcones, isoflavones, rotenoids, isoflavans, flavanones, coumarins and pterocarpans. Some of these metabolites inhibit nitric oxide formation or possess larvicidal, pesticidal, cytotoxic, anti-inflammatory, antimicrobial and cancer chemopreventive activities (Marco *et al.,* 2017).

Millettia aboensis being a plant that all its parts has medicinal properties; reports have been given about its pharmacological activities or screening, but there is scanty information on the isolation and structural elucidation of the bioactive compounds. Hence the present study is on the isolation and identification of the bioactive principles from the plant parts of *Millettia aboensis*.

1.5. Research objectives

- ✓ To isolate the phytochemicals present in the plant using various chromatographic techniques.
- ✓ HPLC-DAD and determination of the molecular weight and fragmentation patterns using ESI-MS of the isolated compounds.
- ✓ To elucidate the structures of the active pure compounds using one dimensional NMR techniques.
- ✓ To evaluate the cytotoxicity of the leaf, stem, root and pod of the plant.
- ✓ To evaluate the antioxidant activity of the isolated compounds.

1.6. Research Hypothesis

- **H_O:** *Millettia aboensis* plant parts have no novel bioactive compounds.

- **H_1:** *Millettia aboensis* plant parts have novel bioactive compounds.

1.7. Scope of study

This study was basically aimed at the isolation, purification, identification, antioxidant screening of the promising bioactive compounds from the plant parts of *Millettia aboensis* and also the cytotoxicity screening of the crude extracts of *Millettia aboensis*.

1.8. Significance of study

This study revealed the isolation, purification and identification of bioactive compounds which ten of them are new compounds and the remaining seven compounds are already known compounds. This is the first time of the report of the isolation, purification and identification of these bioactive compounds from the plant parts *Millettia aboensis*. These compounds will serve as therapeutic compounds and biomarkers.

Chapter two

Literature review

2.1. Sources of Compounds: Microbes, Marine Organisms and Plants

Many of the drugs form natural products come from plant materials, although some have been gotten from micro-organisms and marine sources. From plant materials or sources, anticancer agents (e.g. Taxol), and analgesics (e.g. salicyclic acid, codeine, morphine), anti-malarial drugs (e.g. quinine), pupil dilators (e.g. atropine), and cardiac glycosides (e.g. digitalis) have been produced. Many of these natural products have been associated with extracts from the stem, root and leaf of plants, but yet natural products is not limited to plant sources alone. Some active principles such as antibiotics (penicillin and tetracycline) have been derived from micro-organisms. The accidental discovery of penicillin by Alexander Fleming in 1928 is still one of the most important developments in the history of pharmaceutical chemistry. As an inhibitor of the growth of gram-positive bacteria, it became the first natural product to demonstrate that microorganisms, specifically fungi, are a source of medically useful secondary metabolites (Grabley and Thiericke, 1999). The inhibition occurs because penicillin can inhibit a key step in the biosynthesis of the bacterial cell wall (Newman *et al.*, 2000). Today, penicillins are a class of over a dozen compounds that can be natural, synthesized, or semi-synthesized (Yoder, 2005). One of the most well-known penicillin molecules is penicillin G (also called benzylpenicillin), which contains the characteristic β-lactam-thiazolidine structure (Figure 2.1).

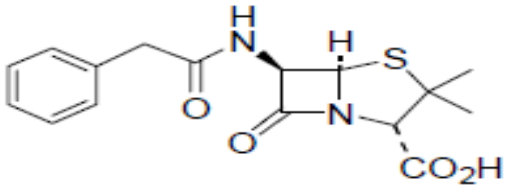


Figure 2.1: Structure of Pencillin G

Tetracyclines are another class of natural (from *Streptomyces sp.*) and semisynthetic antibiotics that are composed of a polyketide fused tetracyclic structure (Yoder, 2005). After initial discovery almost 50 years ago, many semi-synthetic analogs have been successfully created from the parent molecule (Newman *et al.,* 2000). One specific tetracycline that has found use outside of its traditional application as an antibiotic is doxycycline (Figure 2.2), and it is used to aid in the treatment of malaria, often in combination with an alkaloid like quinine (Brogger and Kharazmi, 2001). Both penicillin and tetracycline are the results of 1960s programs that sought to structurally modify and compare bioactivities of several antibiotic natural products (Grafe, 1999).

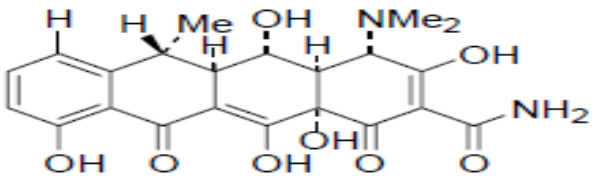


Figure 2.2: Structure of doxycycline

One type of drug that is often taken for granted and viewed as slightly less glamorous than its pharmaceutical counterparts are medicines that prevent the rejection of organs following surgery to transplant organs. These immunosuppressant compounds are administered not to treat an illness, but to stop the human body from performing a normal function for which one is usually grateful. Cyclosporin A is one such immunosuppressant drug. This molecule was first isolated from its parent fungus, *Tolypocladium inflatum*, in Switzerland**,** but a variety of advanced studies were necessary before the efficiency of the drug was fully realized and the medicine was introduced into the market in 1983 (Yoder, 2005).

It was the first of its kind, and current worldwide use still places it on the list of the 25 top overall drugs and number one among all immunosuppressive drugs (Grabley and Thiericke, 1999). Even

after popular and effective drugs are synthesized in the laboratory, the creation of structurally unique analogues remains a top priority. However, midway through the 1990s, combinatorial analogues of cyclosporin A (Figure 2.3) were still unproduced (Yoder, 2005).

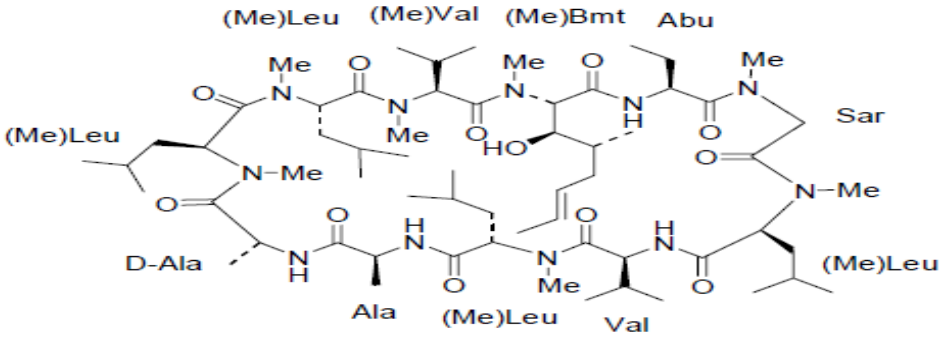


Figure 2.3: Structure of cyclosporin A

Bleomycins are microbial compounds from *Streptomyces verticillus* that interfere with the replication of DNA by cleaving both single and double strands of genetic material (Yoder, 2005). The two glycopeptides that compose the bleomycin family are bleomycin A2 (Figure 2.4), which accounts for 55-70% of that isolated, and bleomycin B2, which accounts for the remaining 30% (Newman *et al.*, 2000; Yoder, 2005). The enzymes involved in the biosynthesis of these anticancer agents are amide synthases, which create highly complex molecules that contain amino acid, sugar, pyrimidine ring, and dithiazole ring components (Sattler *et al.*, 1999; Yoder, 2005). The bleomycins have found clinical use as treatment for squamous cell cancers of the head, neck, cervix, and lymphomas, all without greatly affecting the patient (da Rocha *et al.*, 2001; Yoder, 2005)

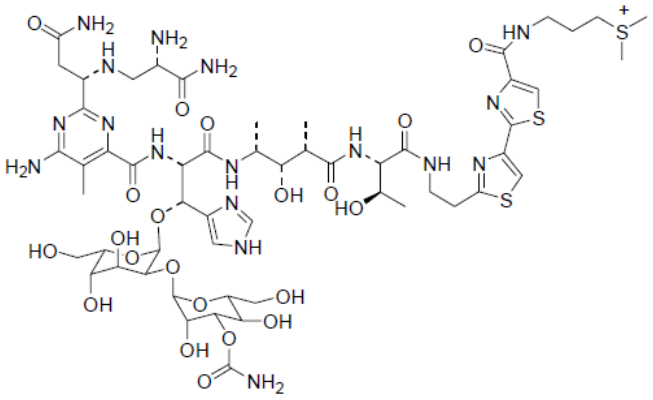


Figure 2.4: Structure of bleomycin A2

The prospecting for biologically active molecules in marine organisms is a relatively new and rapidly expanding branch of natural products chemistry. Over the past 30 years alone, the ocean depths have produced some 3000 novel natural products (da Rocha *et al.,* 2001; Yoder, 2005).

The search includes the examination of sponges, molluscs, corals, and sea-dwelling microorganisms as potential sources of potent drugs. Manoalide (Figure 2.5) is a 25- carbon marine natural product with anti-inflammatory activity towards the cyclooxygenase (COX)-2 enzyme. The COX-2 enzyme has been identified as a catalytic source of prostaglandins (and thus, unwanted inflammation), so drugs that can regulate the enzyme have the ability to control the pain that results from inflammation (Perera *et al.,* 2001).

Manoalide is isolated from a sponge, *Luffariella variabilis*, but there exist a number of synthetic analogues that may function through irreversible deactivation of phospholipase A2 (Yoder, 2005).

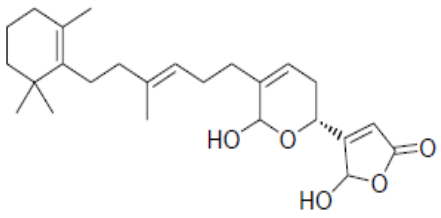


Figure 2.5: Structure of manoalide

There are quite a few cytotoxic marine natural products that have been isolated and identified in the past two decades, as methods of collection in these remote environments have improved (Pomponi, 1999; da Rocha *et al.,* 2001). A great majority of these potential medicines are still under investigation in the early stages of clinical trials, but one drug, citarabine, is showing promise as an inhibitor of DNA synthesis in leukemia and lymphomas (da Rocha *et al.,* 2001). Other experimental anticancer medicines include aplidine, which halts the progression of the cell-cycle; bryostatin 1, which was found through the use of a leukemia cell line bioassay; dolastatin 10, a microtubule inhibitor; and ecteinascidin 743, which alkylates specific amino acid components in the minor groove of DNA (da Rocha *et al.,* 2001).

2.2. Medicinal Plant-Derived Compounds

The realm of drugs obtained from plants is vast, wider than any other source of natural products. They are the basis for the traditional medicine philosophies and practices in China, India, and isolated tribal peoples (Newman *et al.,* 2000). It is known that nearly 120 compounds from 90 different plant species were being used around the world as drugs in 1985, and the numbers have certainly grown since then (Yoder, 2005). Approximately 25% of the prescriptions that were filled in the U.S. between 1959 and 1980 are directly tied to extracts of higher plants (Newman *et al.,* 2000). Although anticancer agents are the focal point of this review and research occurring in this laboratory, plants provide a multitude of medicines for all types of ailments and diseases.

Taxol (paclitaxel), a cytotoxic diterpene alkaloid, was first isolated from the bark of the Pacific yew tree *Taxus brevifolia* in the late 1960s. The discovery process involved the screening for anti-cancer bioactivity of over 110,000 compounds from 35,000 different plants by the National Cancer Institute over a 22-year period (Yoder, 2005). *Taxus brevifolia* became one of a number of plant species that was developed into very effective anti-cancer drugs. The anti-tumor activity was

originally tested against leukemia cells, but paclitaxel (Figure 2.6) proved to be most effective against breast and refractory ovarian types of cancer. However, it has also been used to treat melanoma and certain types of lung cancers (da Rocha *et al.*, 2001; Yoder, 2005).

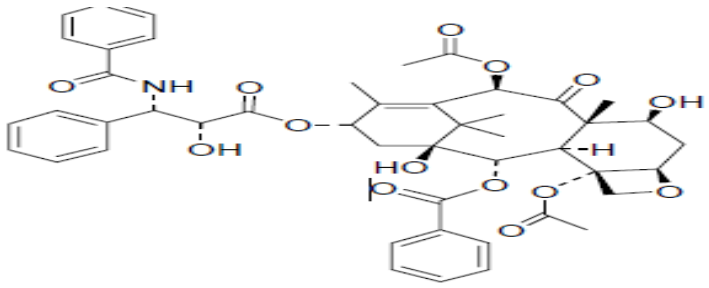


Figure 2.6: Structure of taxol

Paclitaxel functions by inhibiting the cancer cells ability to divide (mitotic arrest), and that inability leads to cell death. The drug binds to and stabilizes the microtubules of a cell, preventing the breakdown of tubulin, which was a very surprising mechanism of action when it was first determined in 1979 (Yoder, 2005). Paclitaxel is currently marketed commercially as Taxol by Bristol-Myers Squibb, which manufactured the compound semi synthetically until 2004, and now produces it by plant tissue culture. In 1998, total worldwide sales of the drug topped the billion-dollar mark (Grabley and Thiericke, 1999; Goodman and Wash, 2001). It goes without saying that this drug is perhaps the most important anticancer development of the past decade (Kingston, 2001).

After the publication of its structure in 1971, paclitaxel was also isolated from the leaves of *Taxus baccata* (a renewable source of the compound), and bioactive taxoid structures can now be found in a wide variety of other plant parts throughout the *Taxus* genus (Yoder, 2005). There are many analogs of the taxoid structure which are bioactive, including baccatin III (Figure 2.7) and docetaxel (Figure 2.8) (Jaziri and Vanhaelen, 2001). The latter analog can actually be synthesized in the chemistry laboratory from the former. However, the direct conversion of paclitaxel to

docetaxel (by selective debenzoylation) and other analogs is still a process that hails interest and demands attention (Jagtap and Kingston, 1999). Other semi-synthetic structures can be created by modifying a part of the compound, such as the substitution of an oxygen atom for a sulfur or selenium atom within a ring, even though the resulting molecule may be less biologically active than the original model (Gunatilaka *et al.*, 1999).

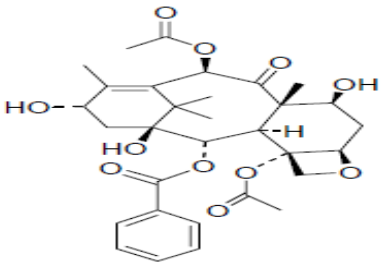


Figure 2.7: Structure of Baccatin III

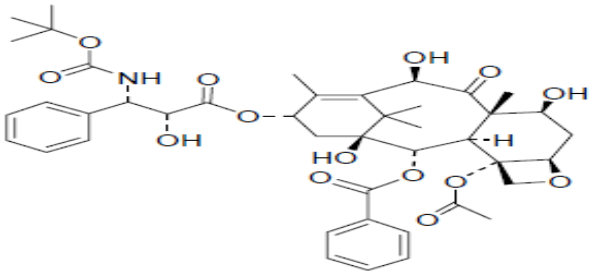


Figure 2.8: Structure of Docetaxel

Aspirin is a powerful synthetic drug that is used to treat a wide variety of ailments. Since its synthesis and initial use in the 1800s, it has come to be known mostly as an anti-inflammatory drug and pain reliever. The natural product that provides the basis for aspirin is salicylic acid, which is isolated from the bark of the willow tree (Swerdlow, 2000). Use of the willow tree for medicinal purposes dates back nearly 2500 years to the time of the ancient Mediterranean empires. One of the side effects of salicylic acid is gastric discomfort and irritation, so the acetyl derivative of salicylic acid (acetylsalicylic acid or aspirin) is used clinically to partially reduce the side effects (Grabley and Thiericke, 1999). Aspirin (Figure 2.9) functions by inhibiting the COX-1 and COX-2 enzymes and, therefore, the synthesis of human hormones called prostaglandins (Swerdlow, 2000). It is the production of COX-2 that induces pain within the human body, so inhibition of the enzyme is a biochemical form of pain management. Aspirin also functions as an important

preventative treatment against heart disease because of its inhibition of prostaglandins, which affect the clotting of blood (Yoder, 2005).

Prostaglandins are vital to many normal biological processes within the human body, so aspirin unfortunately produces many of its own unwanted side effects. Ulcers and other conditions resulting from the loss of stomach lining are due to the unwanted inhibition of COX-1.

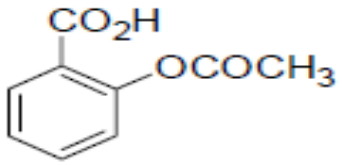


Figure 2.9: Structure of aspirin

Codeine and morphine are two other well-known and often prescribed analgesics. Both of these similarly structured alkaloids come from unripened seedpods of the opium poppy plant (Swerdlow, 2000). In fact, the two compounds are so alike that the codeine molecule can be partially synthesized in the laboratory from morphine, which is the more abundant natural product (Yoder, 2005). Use of morphine (Figure 2.10) as a drug dates back many centuries to a time when monks saw the anaesthetic and pain-relieving properties of *Papaver somniferum*, even though morphine was not isolated until 1806 and it was commercially manufactured 20 years later (Grabley and Thiericke, 1999). While codeine (Figure 2.10) is not nearly as effective in its pain-relieving abilities as morphine, it also can be used as a cough suppressant, and it is a considerably less addictive drug, producing fewer effects of euphoria as compared to its narcotic cousin. Each compound includes constipation among its list of side effects, but only morphine leads to additional mental/emotional ailments as well as physical symptoms (Yoder, 2005).

In spite of side effects and the possibility of addiction, morphine remains one of the most powerful and effective medicines for intense pain in clinical situations, an advantage that cannot be matched by any human-made compound (Harry and Brielmann, 1999).

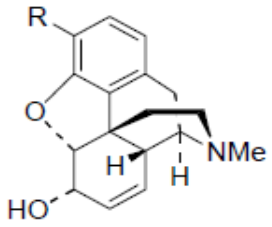


Figure 2.10: Structure of morphine (R=OH) and codeine (R=OMe)

Quinine is one of the oldest of a number of anti-malarial drugs that are currently available. Only the bark from the *Cinchona* genus of trees, located mainly in South America, is known to be the source of this compound (Brogger and Kharazmi, 2001). The first pure form of the active drug (isolated in 1820, nearly 150 years before its structure was determined) is the 10 precursor of a variety of synthetic analogs that were developed during World War II when the natural supply became too difficult to obtain (Yoder, 2005). Throughout its history, quinine (Figure 2.11) has perhaps saved more lives than any other drug (Brogger and Kharazmi, 2001). The mechanism of action of this alkaloid is believed to involve the inhibition of heme polymerization, although debate exists over exactly how the drug operates (Yoder, 2005). Heme is the part of hemoglobin that is left over after the protein part has been digested (Brogger and Kharazmi, 2001). Although other quinoline drugs used to treat malaria are known to have few side effects, medical treatment with quinine produces a large number of dangerous side effects, including toxicity to the heart and various sensory and nervous system disorders (Yoder, 2005). With an IC_{50} value of around 100-440 nM, the difference between the toxic and therapeutic doses is very small and difficult to manage in a health care environment. However, a greater concern with quinine (and other anti-malarials) might be the resistance that has developed towards the drug in certain parts of the globe

where it is administered. Brazil and Africa have been most affected by the resistance of the malaria parasite *Plasmodium falciparum* to the drug (Yoder, 2005).

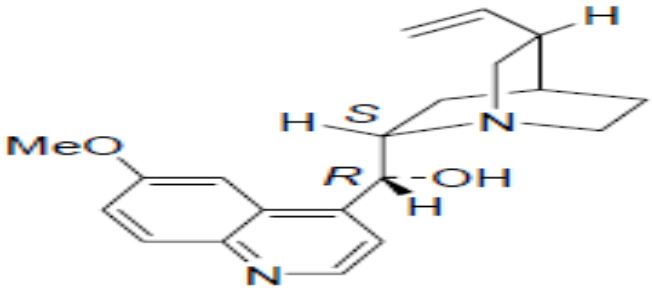


Figure 2.11: Structure of quinine

Other natural products which are well-known from plants are the tropane alkaloid atropine gotten from *Atropa belladonna* and digitalis from *Digitalis purpurea*. Atropine is being used as an antitoxin and muscle relaxant, but mostly known as a mydriatic (pupil dilator).

The use of the *belladonna* fruit juice for such a purpose originated from the Italian women who would brighten the eyes of their young females through a practice that Louisa May Alcott details in her book *An Old Fashioned Girl* (Swerdlow, 2000). Belladonna which really means beautiful woman or beautiful lady. Modern doctors use the drug to prepare patients for eye examinations or surgery because it acts as a local pain reliever by halting the passage of nerve impulses and decreasing sensitivity in the parasympathetic endings (Swerdlow, 2000). The drug binds to the muscarinic receptor site that is normally occupied by acetylcholine (Yoder, 2005).

Atropine is actually a racemic mixture of two compounds, (+)-hyoscyamine (Figure 2.12) and (-)-hyoscyamine (Figure 2.13), although the natural, (-)-enantiomer is considerably more bioactive than the (+)-enantiomer. These tropane alkaloids can be quite addicting, causing dry mouth, sedation (it was historically used during childbirth), or even death (Yoder, 2005). Indeed, the ancient Romans found use for *belladonna* as a poison because there is such a fine line between the dose that is therapeutic and the dose that swiftly kills (Swerdlow, 2000).

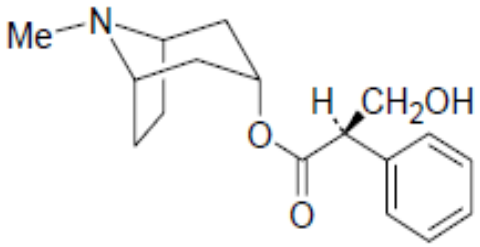


Figure 2.12: Structure of (+)-Hyoscyamine

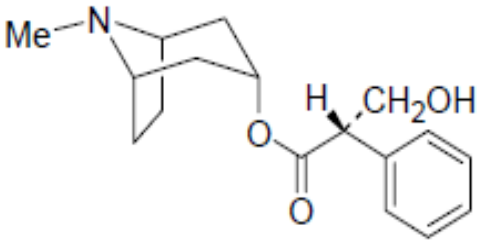


Figure 2.13: Structure of (-)-Hyoscyamine

Digitalis comes from *Digitalis purpurea*, a large flowering herb native to Great Britain. The leaves of the plant produce digitoxin (another name for digitalis), a glycoside prescribed for heart failure and irregular heart rhythm, as well as digoxin, a kidney diuretic, both of which are toxic at high concentrations. The ability of digitoxin (Figure 2.14) to strengthen the muscle contractions of the heart and slow the heart rate has made it a popular natural treatment since its discovery by William Withering in the 18th century (Swerdlow, 2000). One aspect of this drug that makes it unique in the pharmaceutical industry is that it is still isolated from plants today because the cost of synthesizing the drug in the laboratory is so high (Swerdlow, 2000). *D. purpurea* has also shown potential as an anticancer agent because of its ability to inhibit protein kinase C in certain yeast bioassays. Many other important compounds have been isolated from this plant, including two cardiac glycosides (gitoxin and gitaloxin), anthraquinones, phenylethanoids, and flavonoid glycosides (Yoder, 2005).

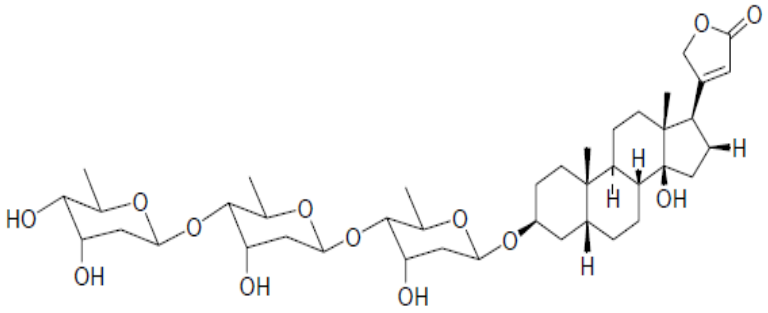


Figure 2.14: Structure of digitoxin

2.3. Anticancer Drugs Isolated from Plants

While there are a plethora of novel, bioactive natural products to examine, further discussion will be limited to those that are isolated from botanical sources, specifically phytochemicals with anticancer activity. As previously discussed, Taxol is currently the World's best-selling anticancer drug available for chemotherapy, and it is one of the most famous of the plant-derived medicines. However, other natural drugs (often alkaloids) play a role in the expanding realm of cancer treatment options. A handful of such compounds are camptothecin, flavopiridol, homoharringtonine, podophyllotoxin, and the *Vinca* alkaloids, vincristine and vinblastine. Camptothecin comes from the wood and bark of a Chinese tree, *Camptotheca acuminata*, which is a tree well known for its anticancer metabolites. It is a pyrrolo[3,4-*b*]-quinoline alkaloid that was extracted using ethanol from the stem-wood of the plant (Yoder, 2005). Although it was initially discovered in 1966 by Wani and Wall, it is now known that the drug binds to topoisomerase I, making it unique in that most other drugs that interact with topoisomerase do so with topoisomerase II. Cells are unable to replicate when the drug is bound to a complex of topoisomerase I and DNA that has been stabilized (Yoder, 2005). Early chemical studies on camptothecin were performed by a National Cooperative Drug Discovery Group (NCDDG) under the guidance of the National Cancer Institute (Newman *et al.*, 2000). Many chemical modifications

have been attempted on camptothecin (Figure 2.15), but most have resulted in a loss of efficiency and biological activity. However, the substitution of various functional groups for hydrogen atoms at select locations has led to an increase in the water solubility of the compound and the creation of two useful analogues, topotecan and irinotecan (Brogger and Kharazmi, 2001). These two drugs show bioactivity towards ovarian cancer (topotecan, Figure 2.16) and colorectal cancer (irinotecan, Figure 2.17) (Yoder, 2005).

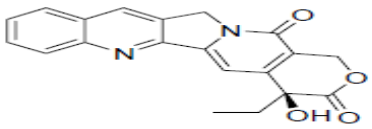


Figure 2.15: Structure of camptothecin

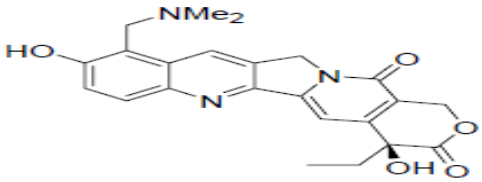


Figure 2.16: Structure of topotecan

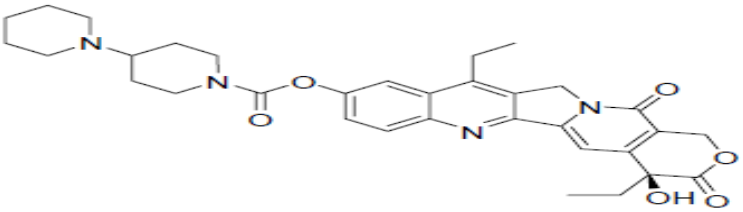


Figure 2.16: Structure of Irinotecan

Flavopiridol (Figure 2.18) is a flavone inhibitor of the cyclin-dependent kinase (CDK) family that was semi-synthesized from rohitukine, a plant natural product (Yoder, 2005). It appears to be non-selective towards any particular CDK. The drug is in the early stages of clinical trials, but it is creating excitement because of its interesting mechanism of action (Newman *et al.,* 2000). The progression of the cell cycle is blocked during stages of growth after the compound interferes with

the kinase phosphorylation step (Kelland, 2000). The only toxic side effect realized to date is diarrhea (Yoder, 2005).

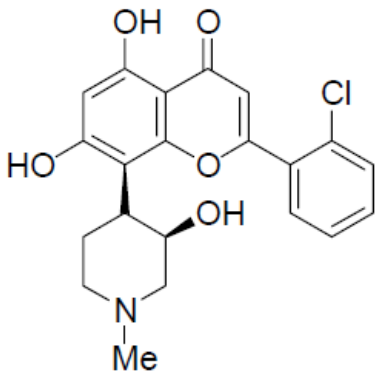


Figure 2.18: Structure of flavopiridol

Homoharringtonine comes from the seeds of a Chinese evergreen (*Cephalotaxus harringtonia)* widely used in China for traditional medicine and known for efficiency as a cytotoxic anti-leukemia drug (Warber, 1999; Yoder, 2005). As in the case of Taxol, this drug was a product of discovery through an extensive research program carried out by the National Cancer Institute in the 1960s, and in 1993, it was classified as one of the NCI's investigational new drugs (Warber, 1999; Yoder, 2005). Homoharringtonine (Figure 2.19) is thought to function during the cell cycle when proteins are being elongated by peptidyl transferase (Warber, 1999). This interruption of protein synthesis leads to apoptosis and differentiation of cancer cells because of the loss of cell-cycle progression (Warber, 1999; Yoder, 2005).

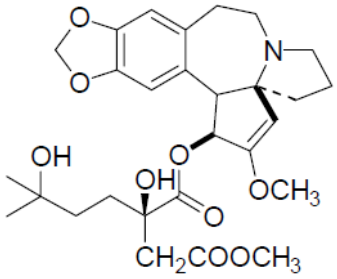


Figure 2.19: Structure of homoharringtonine

A non-alkaloid bioactive compound from a higher plant that deserves some attention is podophyllotoxin (Figure 2.20). It is isolated from the roots of two different plant species (one from the genus *Podophyllum* and one from the genus *Juniperus)* and identified as an antitumor dimeric lignan in 1880 (Kuo and King, 2001). The epimer of podophyllotoxin is epipodophyllotoxin, giving rise to two semi-synthetic compounds with high activites and clinical applications, etoposide and teniposide (Newman *et al.*, 2000). These drugs are much less toxic than their grandparent compound (podophyllotoxin). The former is used to battle lung carcinomas and, along with the bleomycins, as a treatment for testicular cancer (da Rocha *et al.*, 2001; Yoder, 2005).

Like many anticancer drugs, etoposide functions by inhibiting topoisomerase II, during mitosis, which leads to DNA cleavage (Yoder, 2005). Podophyllotoxin, however, causes cells to arrest during metaphase after microtubule assembly interference has occurred (Kuo and King, 2001).

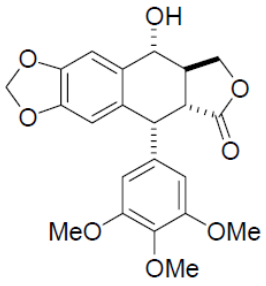


Figure 2.20: Structure of podophyllotoxin

Vincristine and vinblastine are known as the *Vinca* alkaloids. Both come from *Catharanthus roseus,* a type of periwinkle from the rain forests of Madagscar, and like Taxol, they target the formation of microtubules to stop the process of cell division at metaphase (Warber, 1999; Yoder, 2005). However, with vincristine and vinblastine, it is the *disassembly* of the microtubules, formed by the polymerization of free tubulin dimers, which halts the formation of spindles and asters necessary for mitosis (Warber, 1999). Depolymerization begins at metaphase after a dimer of tubulin and one of the *Vinca* alkaloids has bonded to the microtubule (Yoder, 2005).

Vincristine (Figure 2.21) has traditionally been used for acute childhood leukemia and Hodgkin's disease, while vinblastine (Figure 2.21) is a common treatment for lymphoma types of cancer (Warber, 1999; Yoder, 2005). The side effects most commonly seen with vincristine and vinblastine are peripheral neuropathy and depression of bone marrow, respectively (Yoder, 2005). The periwinkle source of these drugs continues to be of great interest to Eli Lilly, the pharmaceutical company that grows it in Texas, and others involved in the search for antitumor compounds. Eli Lilly managed to discover these anticancer agents on the 40th attempt in their program to screen plants with possible antineoplastic activity (Yoder, 2005).

Currently, over 500 interesting alkaloids from this plant have been examined and documented (Yoder, 2005).

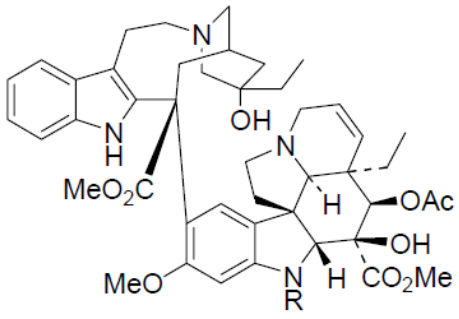


Figure 2.21: Structure of vincristine (R=CHO and vinblastine (R=Me)

2.4. Recent Discoveries of Cytotoxic Phytochemicals

All of the natural products discussed to this point are well known, commercially available drugs that have been used clinically for many years, decades, or even centuries. However, in the past few years, scientists have isolated many phytochemicals that show promise as potential anticancer drugs, but they are awaiting further investigation by pharmaceutical companies (Yoder, 2005).

One plant species that has produced a number of interesting alkaloid structures is *Menispermum dauricum*, a species native to China. Past studies have indicated as many as nine useful alkaloids found in the plant and roots, but two isolated oxoisoaporphine alkaloids have recently shown activity against a human breast cancer cell line (Yu *et al.*, 2001). The compounds that have been isolated, daurioxoisophines A and B, are shown in Figure 2.22 and Figure 2.23, respectively.

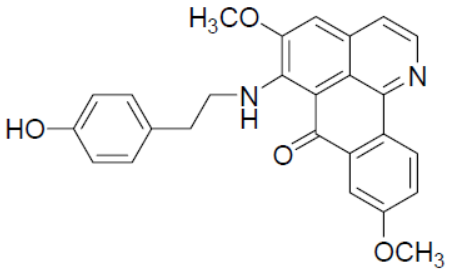


Figure 2.22: Structure of daurioxoisophine A

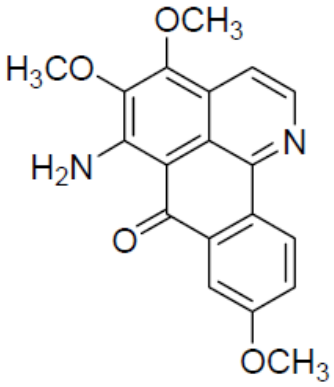


Figure 2.23: Structure of daurioxoisophine B

Another plant that was recently identified as a source of interesting alkaloids is *Cananga odorata*, from Taiwan. Although this evergreen tree had been traditionally known for its anti-malarial properties and treatment of fever and infection, two novel compounds demonstrated activity against hepatocarcinoma cell lines. Both alkaloids (cananodine, Figure 2.24, and crytpomeridiol 11-α-L-rhamnoside, Figure 2.25) were isolated from the fruit of the plant and bear structural resemblance to other sesquiterpenes that had been previously obtained from the species (Hsieh *et al.*, 2001).

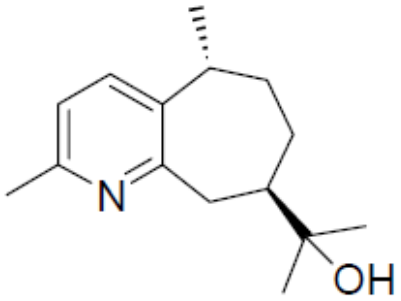


Figure 2.24: Structure of cananodine

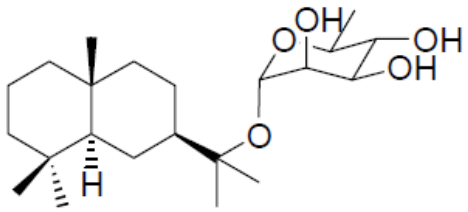


Figure 2.25: Structure of crytpomeridiol 11-α-L-rhamnoside

Lippsidoquinone (Figure 2.26) is a new naphthoquinone that has been located in *Lippia sidoides* (Costa *et al.*, 2001). The compound, extracted with ethanol, is a dimer and has shown activity against a pair of human leukemia cell lines. The plant itself grows in the northeastern part of the

country and the oil from its leaves has previously demonstrated antiseptic bioactivity (Yoder, 2005).

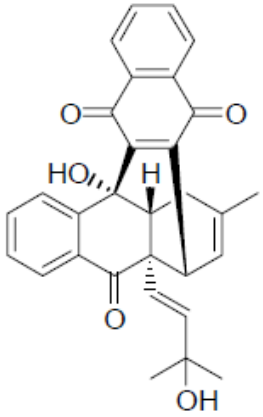


Figure 2.26: Structure of lippsidoquinone

Another novel molecule that was recently isolated is solavetivone (Figure 2.27). It is produced by the root of a Chinese plant that is popular in Taiwan (*Solanum indicum*). Traditionally used to treat breast cancer, the plant has also been accepted as an anti-inflammatory and anti-toxin source. Cytotoxicity testing of the new compound gives an IC_{50} of 0.1 mM on the OVCAR-3 cell line (Syu *et al.,* 2001).

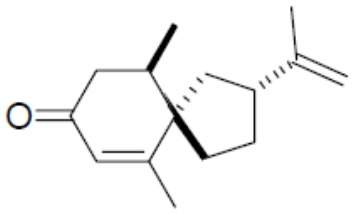


Figure 2.27: Structure of solavetivone

Yet another shrub from Taiwan that is used in Chinese traditional medicine is *Ventilago leiocarpa*. Its folk uses include treatment for pain and rheumatism, but stem extracts have also shown cytotoxicity towards various cancer cell lines. One of the newest quinones from the dried stems of this plant is 2-hydroxyemodin 1-methyl ether (Figure 2.28). The activity against so many different

cell lines is assumed to be partly due to the trihydroxy nature of the anthraquinone (Lin *et al.*, 2001).

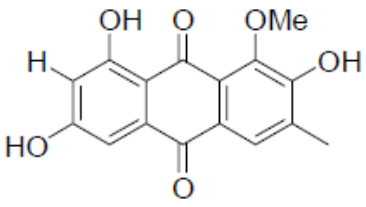


Figure 2.28: Structure of 2-hydroxyemodin 1-methyl ether

Not all phytochemical compounds of current interest are new discoveries. One molecule that is back in the news is hypericin, an anthraquinone from St. John.s wort (Peebles *et al.*, 2001). *Hypericum perforatum* is an extremely popular over-the-counter remedy for depression, but the active ingredient in this plant is now being examined as an inhibitor of the topoisomerase IIα enzyme in humans. Topoisomerase IIα is an isoform of DNA topoisomerase II enzyme that is regulated by the cell cycle and selectively cleaved during apoptosis of human epithermoid carcinoma cells. Hypericin (Figure 2.29) has shown *in vitro* activity against various leukemia cell lines, making it a potentially interesting drug for cancer patients who also experience depression because of their illness.

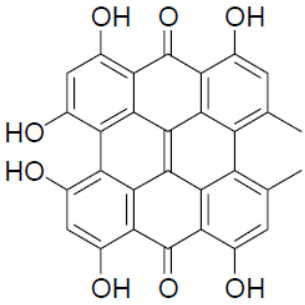


Figure 2.29: Structure of Hypericin

Another Asian plant of interest in traditional medicine, and now cytotoxicity studies, is *Clerodendrum cyrtophyllum*. Folklore has labeled this plant from Taiwan as a form of treatment for a number of illnesses including syphilis and typhoid fever (Cheng *et al.*, 2001). The most biologically active constituent in terms of cytotoxicity is a methyl ester of a compound found in the related *Clerodendrum calamitosum*. It is a known compound, but it had never been isolated as a plant natural product before this discovery. This structure (Figure 2.30) appears to be potent towards a number of cancer cell lines and has ED_{50} values as low as 0.27 μg/mL (Cheng *et al.*, 2001).

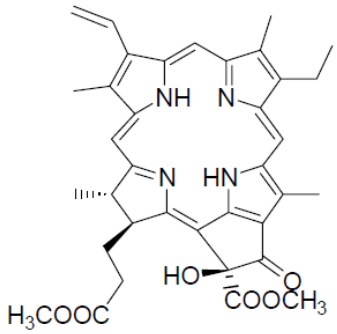


Figure 2.30: Structure of methyl ester from *Clerodendrum calamitosum*

A chemical class that has not been explored yet in this discussion, but is a viable source of cytotoxic compounds, is that of the acetogenins, which are long molecules with aliphatic chains. One such source of acetogenins is *Annona cherimolia*, a tropical tree from Peru used traditionally to kill insects and parasites (Kim *et al.*, 2001). The seeds from this plant have yielded two antitumor compounds from an ethanol extract: annomolin (Figure 2.31) and annocherimolin (Figure 2.32). The former has shown activity against a prostate cancer cell line, while the latter has shown activity against both breast and colon cancer cell lines. Both compounds appear to be 104 times as potent as Adriamycin (Kim *et al.*, 2001).

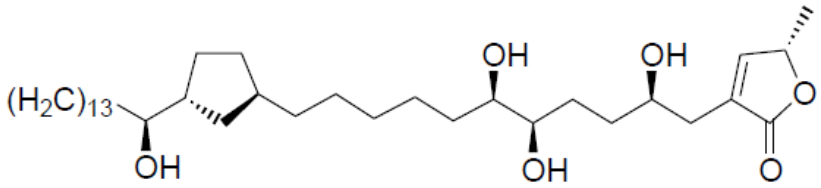


Figure 2.31: Structure of annomolin

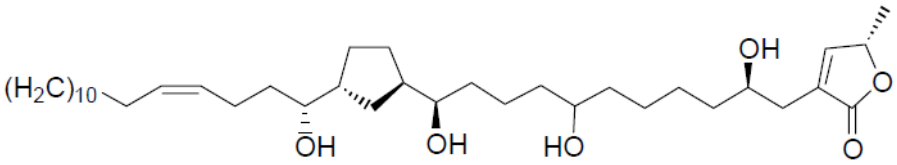


Figure 2.32: Structure of annocherimolin

Another series of long-chain hydrocarbon compounds with cytotoxic activity are a collection of alkynes from *Ochanostachys amentacea*. The tree which is native to South Pacific islands like Malaysia and Indonesia, and from its small twigs were isolated two new potent polyacetylenes (Ito *et al.*, 2001). (*S*)-17,18-hydroxy-9,11,13,15-octadecatetraynoic acid (Figure 2.33) which has indicated activity against oral epidermoid cancer and (*S*)-17-hydroxy-15*E*-octadecen-9,11,13-triynoic acid (Figure 2.34); this can be a potential treatment for ovarian and hormone-dependent prostate cancers (Yoder, 2005).

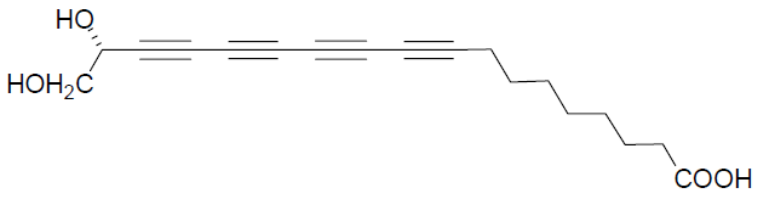


Figure 2.33: Structure of (S)-17,18-hydroxy-9,11,13,15-octadecatetraynoic acid

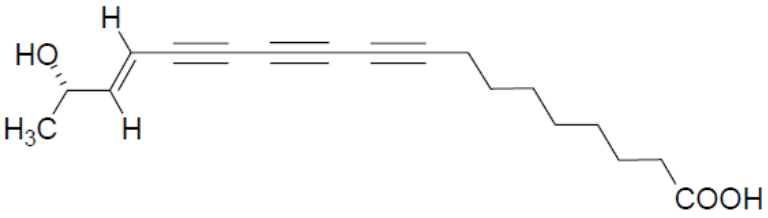


Figure 2.34: Structure of (S)-17-hydroxy-15E-octadecen-9,11,13-triynoic acid

Coumarin structures which are familiar compounds in the pharmaceutical industry. The plant *Calophyllum dispar* has been recently identified as a source of eleven coumarin compounds, eight of which are new (Guilet *et al.,* 2001). These new molecules are 4-phenylfuranocoumarins and are extracted from the fruits and bark of the species. Nearly all of the compounds show cytotoxic activity with IC_{50} values as low as 5 µg/mL. An example of one of these coumarins is provided in Figure 2.35.

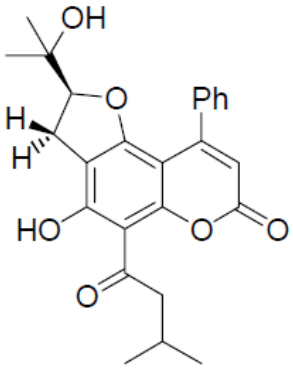


Figure 2.35: Structure of coumarin from *Calophyllum dispar*

One new natural product, a benzoquinone, 2-methoxy-6-heptyl-1,4-benzoquinone (Figure 2.36), was isolated from *Miconia lepidota*, a species native to the rainforests of South America and West Africa (Gunatilaka *et al.*, 2001). The compound discovery was made through fractionation of an ethyl acetate extract from Suriname, and cytotoxicity testing gave an IC_{50} value of 7.9 µg/mL in the A2780 ovarian cancer cell line. This moderate reading indicated the true potency of the

molecule, but it was not enough to warrant further examination as an anticancer drug (Gunatilaka *et al.,* 2001).

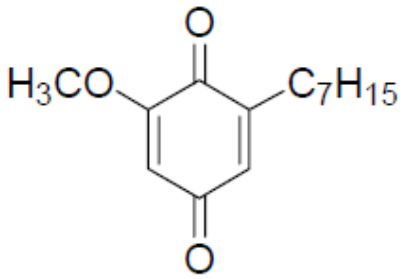


Figure 2.36: Structure of 2-methoxy-6-heptyl-1,4-benzoquinone

2.5. Isoflavones

Isoflavones are a class of phytochemicals found in soybeans, chickpeas, and other legumes. Soybeans have the highest concentration of isoflavones, as well as the highest concentration of the individual isoflavones thought to contain medicinal properties – genistein and daidzein. Isoflavones have antioxidant properties that protect the cardiovascular system from LDL oxidation. Isoflavones are also a type of phytoestrogen that have been studied for their role in the prevention of osteoporosis and symptoms of menopause, as well as breast and prostate cancer (Alt. Med. Monographs, 2002).

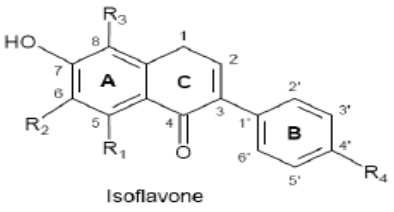
Traditionally, breast cancer, prostate cancer, and diabetes were less common diseases in Asian population than in Western populations and it has been suggested that soy foods may contribute to the prevention of these hormone-related diseases (Kim, 2008; Kang et al., 2012; Kwang-Pil, 2014). Soybean products contain organic compounds related to the isoflavone which act as phytoestrogen. Phytoestrogens are called as diphenolic non-steroidal estrogens because those are structurally similar to 17-β-estradiol and thus physiologically functioned as weak estrogens.

Endogenous estrogens are circulated and bound with biologically active unconjugated form, while dietary isoflavones are nearly conjugated in circulation. There are a few unconjugated isoflavones in circulation (1-5% of the total isoflavones) (Barnes, 2010; Kwang-Pil, 2014). Moreover, compared to endogeneous estrogens, isoflavones have approximately 100 times weaker affinities of estrogen receptor (Kuiper et al., 1997; Kwang-Pil, 2014). So isoflavones have estrogenic or anti-estrogenic effects according to estrogen hormone level in body.

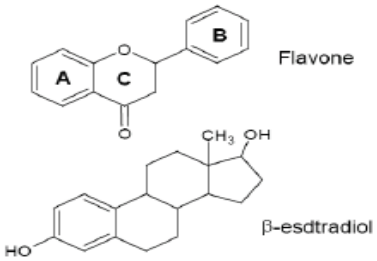
Although recently many researchers have shifted their attention to beneficial effects of soybean products on health, yet the health benefits of soy for many diseases are small and inconsistent in epidemiologic studies.

Isoflavones in Foods and Natural Plants

Isoflavones are naturally-occurring plant compounds. More than 300 kinds of plants, in particular roots and seeds, include isoflavones (Klejdus et al., 2005; Kwang-Pil, 2014). The sources are the Fabaceae/Leguminosae family, including various legumes such as kudzu, lupine, broccoli, cauliflower, barley, fava beans, and soy, are the major source of isoflavones (Prasad et al., 2010; Kwang-Pil, 2014).



Isoflavone



Compound	R1	R2	R3	R4	Substitution
Daidzein	H	H	H	OH	7,4'-$(OH)_2$
Genistein	OH	H	H	OH	5,7,4'-$(OH)_3$
Glycitein	H	OCH_3	H	OH	7,4'-$(OH)_2$, 6-OCH_3
Formononetin	H	H	H	OCH_3	7-OH, 4'-OCH_3
Biochanin A	OH	H	H	OCH_3	5,7-$(OH)_2$, 4'-OCH_3

Also red wine extracts contain small amounts of isoflavones and natural plants such as red clover, germs of alfalfa, and linseed contain isoflavones (Pilsakova et al., 2010; Kwang-Pil, 2014). Of these sources, soybeans and their products are one of the richest sources of isoflavones in human. As other source in food is soy oil and soy protein isolates. Textured soy protein is a kind of soy protein isolates. Vegetarians like to eat food made textured soy protein instead of meat and cheese. Textured soy protein usually containing 50-70% soy protein is commonly used as a meat substitute in hotdogs, hamburgers, sausages and other meat products. Another soy protein isolate containing 90% soy protein is used to enrich energy bars, sports drinks, infant formula, cereals, imitation dairy products, ice cream, cheese and even doughnuts (Rakosky, 1975; Thomas and Lutz, 2001; Kwang-Pil, 2014). Soy protein isolate is popularly added in many canned food and bakery products to improve the appearance of the food or to control dietary intake because it is a cholesterol-free, vegetable protein rich in complex carbohydrates and unsaturated fats, high fiber, and free of lactose (Patisaul and Jefferson, 2010; Kwang-Pil, 2014). For those reasons, soy is used to make low fat soy mild and tofu, and fermented food in the school breakfast and lunch programs (Thomas and Lutz, 2001; Kwang-Pil, 2014).

Dry soybeans contain 1.2-4.2 mg/g isoflavones. Their exact concentration depends on many environmental factors and cooking methods, including the soil in which they are grown, climate, and stage of their maturity or processing (Pilsakova et al., 2010; Kwang-Pil, 2014). High processed foods contain lower isoflavone contents than the amount found in unprocessed soybeans. For example, the second generation of soybean such as tofu contains only 6-20% (5.1-64 mg/100g) of the isoflavone amount in unprocessed soybeans (Duncan et al., 2003; Kwang-Pil, 2014). Miso, doenjang, and natto contain a large amount of isoflavones in foods (20-126 mg/100g) and tofu and tempeh contain a moderate amount of isoflavones (5-64 mg/100g) (Liggins et al., 2000; Kwang-

Pil, 2014). New generation soy foods such as soy milk, yogurts and cheeses have a small amount (1-33 mg/100g) (Wiseman et al., 2002; Kwang-Pil, 2014). Canned foods including tuna or meatless chili, meat products like sausages and bakery products like bread and muffins have a very small amount (usually 2 mg/100g) (Pillow et al., 1999; Horn-Ross et al., 2000; Kwang-Pil, 2014). However, nutritional supplements such as infant formulas contain high concentrations of isoflavones (up to 31 mg/100g instant product). In natural plants, red clover contains moderate levels of isoflavones (nearly 21 mg/100g), however other plants such as green tea, flaxseeds, and raw broccoli have a very small amount (0.02-0.07 mg/100g) (Mortensen et al., 2009; Kwang-Pil, 2014). The isoflavone content of an array of foods is now published in numerous on-line databases the most accessible of which for consumers is maintained by the US Departments of Agriculture (Kwang-Pil, 2014).

There is a big difference of isoflavone intake according to race. Intakes of isoflavones are lower in Western populations than in Asian populations since soybean is rarely consumed food in Western diets. In western countries, soymilk is frequent soybean containing products. In contrast, in Asia, tofu, natto, soymilk and fermented foods such as miso, doenjang (fermented soybean paste in Korea), soy sauce, tempeh are frequent sources of isoflavone intake in food.

Usually mean daily isoflavone intake in Asians is about 8-50 mg (expressed as aglycone equivalents). Blood genistein levels are generally in the range of 25 ng/mL for Asian women, slightly less for vegetarian women, and under 2 ng/ml for US women (Verkasalo et al., 2001; Kwang-Pil, 2014). Generally, isoflavone contents in food are measured as sum of daidzein, genistein, and glycitein in aglycone equivalents (Mortensen et al., 2009; Kwang-Pil, 2014).

2.5.1. Chemistry

Isoflavones are the secondary metabolite formed by symbiotic relationship with the rhizobial bacteria and the defense responses of leguminous plant (Yu et al., 2000; Kwang-Pil, 2014). Isoflavones are synthesized as part of the phenylpropanoid pathway, the same biosynthetic pathway of flavonoid biosynthesis (Barnes, 2010). Phenylalanine converts 4-hydroxycinnamoyl CoA by reaction with malonyl CoA. Chalcone synthase catalyzes the reaction of this intermediate to convert to 4,2',4',6'-tetrahydrozychalcon (naringenin chalcone) and the combined enzyme reaction of chalcon synthase and chalcone reductase convert this intermediate to 4,2',4'-trihydrozychalcone (isoliquiritigenin). And then, chalcone isomerase catalyzes the ring closure of the heterocyclic ring to form 7, 4'-dihydrozyflavone (liquirintigenin) and 5, 7, 4'-trihydroxyflavone (naringenin). The B-ring is moved from the 2-position to 3-position by isoflavone synthase. Isoflavone dehydratase removed water to generate the 2, 3 double bond in the heterocyclinc ring. The products generated by this reaction were daidzein (7, 4'-dihydroxyisoflavone) and genistein (5, 7, 4'-trihydroxyisoflavone) (Fig. 2.37)

2.5.2. Antioxidant function

An antioxidant is a molecule capable of inhibiting the oxidation of other molecules. Oxidation is a chemical reaction that transfers electrons or hydrogen from a substance to an oxidizing agent. Oxidation reactions can produce free radicals. In turn, these radicals can start chain reactions. When the chain reaction occurs in a cell, it can cause damage or death to the cell. Antioxidants terminate these chain reactions by removing free radical intermediates, and inhibit other oxidation reactions. They do this by being oxidized themselves, so antioxidants are often reducing agents such as thiols, ascorbic acid, or polyphenols (Sies, 1997).

A paradox in metabolism is that, while the vast majority of complex life on earth requires oxygen for its existence, oxygen is a highly reactive molecule that damages living organisms by producing reactive oxygen species (Davies, 1995). Consequently, organisms contain a complex network of antioxidant metabolites and enzymes that work together to prevent oxidative damage to cellular components such as DNA, proteins and lipids (Sies, 1997; Vertuani *et al*., 2004). In general, antioxidant systems either prevent these reactive species from being formed, or remove them before they can damage vital components of the cell (Davies, 1995; Sies, 1997). However, reactive oxygen species also have useful cellular functions, such as redox signaling. Thus, the function of antioxidant systems is not to remove oxidants entirely, but instead to keep them at an optimum level (Rhee, 2006).

The reactive oxygen species produced in cells include hydrogen peroxide (H_2O_2), hypochlorous acid (HClO), and free radicals such as the hydroxyl radical (·OH) and the superoxide anion (O_2^-) (Valko *et al*., 2007). The hydroxyl radical is particularly unstable and will react rapidly and non-specifically with most biological molecules. This species is produced from hydrogen peroxide in metal-catalyzed redox reactions such as the Fenton reaction. (Stohs, 1995). These oxidants can damage cells by starting chemical chain reactions such as lipid peroxidation, or by oxidizing DNA or proteins (Sies, 1997).

Damage to DNA can cause mutations and possibly cancer, if not reversed by DNA repair mechanisms,(Valko, 2004; Nakabeppu, 2006) while damage to proteins causes enzyme inhibition, denaturation and protein degradation (Stadtman, 1992).

The use of oxygen as part of the process for generating metabolic energy produces reactive oxygen species (Raha *et al*., 2000). In this process, the superoxide anion is produced as a by-product of

several steps in the electron transport chain (Lenaz, 2001). Particularly important is the reduction of coenzyme Q in complex III, since a highly reactive free radical is formed as an intermediate ($Q^{\cdot -}$). This unstable intermediate can lead to electron "leakage", when electrons jump directly to oxygen and form the superoxide anion, instead of moving through the normal series of well-controlled reactions of the electron transport chain (Finkel, 2000). Peroxide is also produced from the oxidation of reduced flavoproteins, such as complex I (Hirst *et al*., 2008). However, although these enzymes can produce oxidants, the relative importance of the electron transfer chain to other processes that generate peroxide is unclear (Imlay, 2003; Seaver *et al*., 2004). In plants, algae, and cyanobacteria, reactive oxygen species are also produced during photosynthesis (Demmig-Adams, 2002), particularly under conditions of high light intensity (Krieger-Liszkay, 2004). This effect is partly offset by the involvement of carotenoids in photo inhibition, which involves these antioxidants reacting with over-reduced forms of the photosynthetic reaction centres to prevent the production of reactive oxygen species (Kerfeld, 2004; Szabó *et al*., 2005).

Genistein has antioxidant properties as scavenger of radicals and chelaters of metals. This function occurs by affecting gene expression of enzymes that react with antioxidants such as catalase and superoxide dismutase, and inhibiting with secondary oxidant production such as hydrogen peroxide or hypochlorous acid (Mortensen et al., 2009). Genistein is more active as antioxidant than daidzein due to having its third hydroxyl group in the C-5 position. Equol is another compound with better antioxidant actions than its precursor compounds due to the absence of the 2,3-double bond in conjunction with a loss of the 4-oxo group which is enhancing antioxidant properties (Arora et al., 1998).

Isoflavone metabolism pathway in intestine and liver affect the antioxidant properties of isoflavones and the metabolites. Although genistein significantly reacts with superoxide

dismutase, catalase, and glutathione peroxidase, isoflavone metabolites such as equol, 8-OH-daidzein, O-desmethylangolensin (O-DMA), and 1,3,5-trihydroxybenzene have also played a role as potent scavengers. 8-hydroxy-daidzein is the most potent scavenger for hydroxyl and superoxide anion radicals. These isoflavone metabolites are highly chelating with ferrous compound relative to genistein and daidzein. However sulfated isoflavones can decrease antioxidant activity. Isoflavones can reduce low-density lipoprotein oxidation and lipid peroxidation by reacting with lipid radicals (Tikkanen et al., 1998).

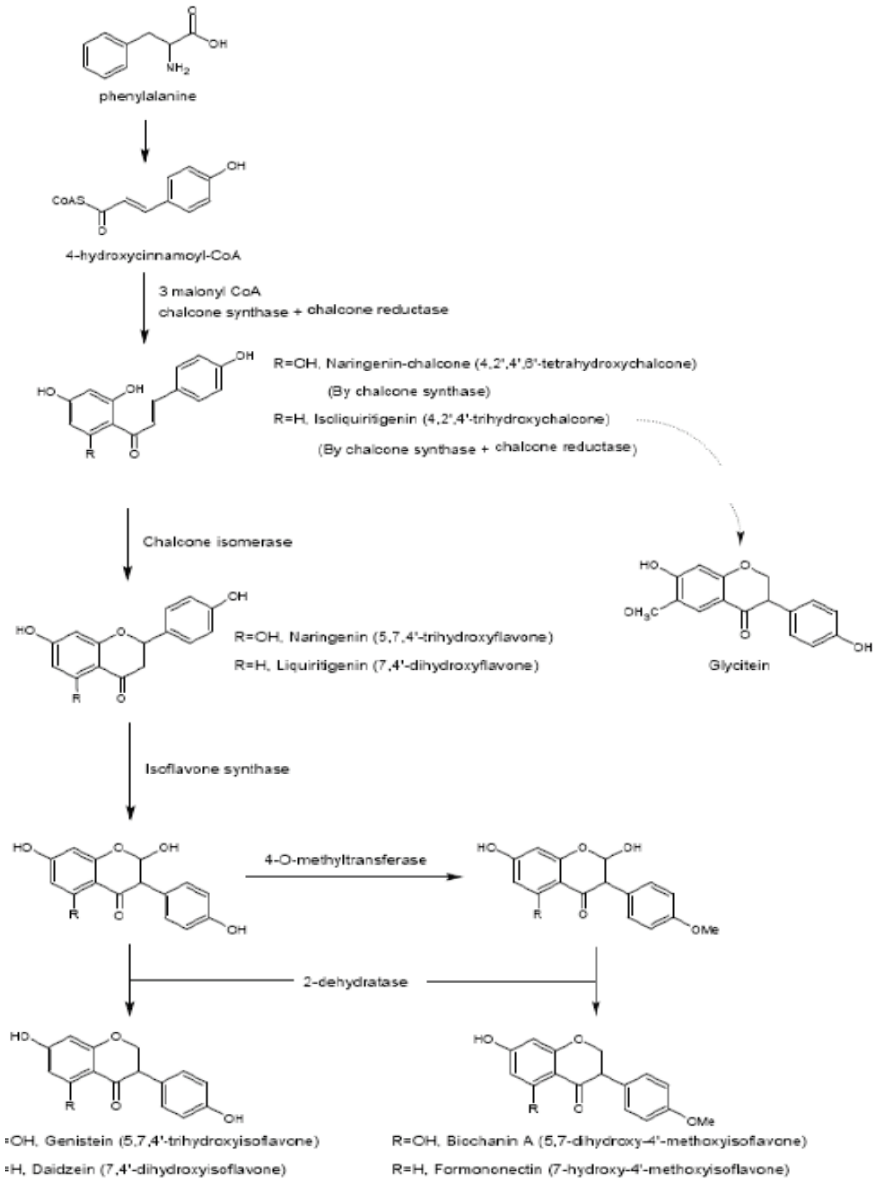


Figure 2.37: Biosynthesis pathway of Isoflavones (Barnes, 2010)

2.6. Chromatography

2.6.1. Introduction

Chromatography encompasses a diverse and important group of methods that permit the scientist to separate closely related components of complex mixtures; many of these separations are impossible by other means. In all chromatographic separations the sample is transported in a mobile phase, which may be a gas, a liquid, or a supercritical fluid. This mobile phase is then forced through an immiscible stationary phase, which is fixed in place in a column or on a solid surface. The two phases are chosen so that the components of the sample distribute themselves between the mobile and stationary phase to varying degrees. Some components of the mixture are strongly retained to stationary phase than others and as a consequence of these differences in mobility; sample components are separated (Cooke and Poole, 2000; Meyer, 2004; Ajaegbu, 2013).

Chromatographic methods can be classified according to the type of mobile phase such as liquid, gas, and super critical fluid chromatography (Ajaegbu, 2013). The most important one used in this research is liquid chromatography.

2.6.2. Liquid Chromatography

In liquid chromatography, it implies that the mobile phase used in chromatographic process is liquid and the stationary phase can be either liquid, solid, bonded phase or ion exchange (Ajaegbu, 2013).

2.6.2.1. Liquid Chromatographic Separation Modes

2.6.2.2. Adsorption Chromatography

The principle of adsorption chromatography is known from classical open column and thin-layer chromatography. A relatively polar material with a high specific surface area is used as the stationary phase, (silica gel and alumina are the most popular). The mobile phase is relatively non-polar (heptane to tetrahydrofuran). The different extents to which the various types of molecules in the mixture are adsorbed on the stationary phase provide the separation effect. Polar compounds are eluted later than non-polar compounds separated (Cooke and Poole, 2000; Meyer, 2004; Ajaegbu, 2013).

Silica gel is the most frequently used solid-phase in adsorption chromatography. The weakly acidic properties of its surface may be the reason for the chemisorption of alkaloids, especially when neutral nonpolar solvents are used such as chloroform and dichloromethane (Cooke and Poole, 2000).

2.6.2.3. Partition Chromatography

Partition chromatography can be subdivide d into liquid-liquid and -bonded-phase chromatography. The difference in these techniques lies in the method by which the stationary phase is held on the support particles of the packing. With liquid-liquid, a liquid stationary phase is retained on the surface of the packing by physical adsorption (e.g. viscous liquid of hydrocarbons chain) (Ajaegbu, 2013).

With bonded-phase stationary phase is covalently bonded to its support by chemical reaction. A large number of stationary phases can be produced by careful choice of suitable reaction partners.

The most important special case of chemically bonded-phase chromatography is the reverse-phase method, where the stationary phase is very non-polar and the mobile phase is relatively polar separated (Cooke and Poole, 2000; Meyer, 2004; Ajaegbu, 2013).

2.6.3. Column Chromatography

Column chromatography is the most widely and the oldest technique used for separation of organic compounds with the aim of isolating and purifying compounds from 1-100 grams. It is based upon the stationary phase (silica gel is the most popular) held in narrow tube and the sample injected through which the mobile phase is eluted under the force of gravity. The mobile phase is eluted with gradient increase in its polarity. Polar compounds are eluted later than non-polar compounds separated (Cooke and Poole, 2000; Meyer, 2004; Ajaegbu, 2013).

2.6.4. Size-exclusion chromatography

Size-exclusion chromatography (SEC) is a chromatographic method in which molecules in solution are separated by their size, and in some cases molecular weight (Paul-Dauphin *et al*., 2007). It is usually applied to large molecules or macromolecular complexes such as proteins and industrial polymers. Typically, when an aqueous solution is used to transport the sample through the column, the technique is known as gel-filtration chromatography, versus the name Gel permeation chromatography, which is used when an organic solvent is used as a mobile phase. SEC is a widely used polymer characterization method because of its ability to provide good molar mass distribution (Mw) results for polymers

2.6.4.1. Gel filtration chromatography

Gel filtration separates molecules according to difference in size as they pass through a gel filtration medium packed in a column. Unlike ion exchange or affinity chromatography, molecules do not bind to the chromatography medium so buffer composition does not directly affect resolution (the degree of separation between peaks). Consequently, a significant advantage of gel filtration is that conditions can be varied to suit the type of sample or the requirements for further purification, analysis or storage without altering the separation.

Gel filtration is well suited for biomolecules that may be sensitive to changes in pH, concentration of metal ions or co-factors and harsh environmental conditions. Separations can be performed in the presence of essential ions or co-factors, detergents, urea, guanidine hydrochloride, at high or low ionic strength, at 37^0C or in the cold room according to the requirements of the experiment. Purified proteins can be collected in any chosen buffer (Amersham Biosciences AB, 2002).

2.6.4.2. Gel permeation chromatography

Gel permeation chromatography (GPC) is a type of size exclusion chromatography (SEC) that separates analytes on the basis of size. The technique is often used for the analysis of polymers. As a technique, SEC was first developed in 1955 by Lathe and Ruthven (Lathe, and Ruthven, 1956). The term gel permeation chromatography can be traced back to J.C. Moore of the Dow Chemical Company who investigated the technique in 1964 and the proprietary column technology was licensed to Waters, who subsequently commercialized this technology in 1964 (Moore, 1964). It is often necessary to separate polymers, both to analyze them as well as to purify the desired product. When characterizing polymers, it is important to consider the polydispersity index (PDI)

as well the molecular weight. Polymers can be characterized by a variety of definitions for molecular weight including the number average molecular weight (M_n), the weight average molecular weight (M_w) (see molar mass distribution), the size average molecular weight (M_z), or the viscosity molecular weight (M_v). GPC allows for the determination of PDI as well as M_v and based on other data, the M_n, M_w, and M_z can be determined (Ajaegbu, 2013).

2.6.5. High Performance Liquid Chromatography

High-performance liquid chromatography (HPLC) is a form of liquid chromatography used to separate compounds that are dissolved in solution. HPLC instruments consist of a reservoir of mobile phase, a pump, an injector, a separation column, and a detector. Compounds are separated by injecting a plug of the sample mixture onto the column. The different components in the mixture pass through the column at different rates due to differences in their distribute behavior between the mobile liquid phase and the stationary phase (Meyer, 2004; Ajaegbu, 2013).

2.6.6. Analytical vs. Semi-Preparative HPLC

Analytical HPLC is a very efficient instrument used quantitatively for accurate determination of the amount of analyte in a sample. Few micrograms of the analyte could be detected accurately. The method used for analysis must be improved to reach a good separation between analyte peak and sample matrix peaks. The retention time and area under the peaks, comparing with standard, are used for quantitative determination of the analyte (Meyer, 2004).

Semi-preparative and preparative HPLC equipment are used to speed up the efficiency of separation and purification of compounds in amounts varying from 3 mg up to several grams.

Special preparative HPLC column, high pressure pump and large sample volume injector are used for efficient separation (Meyer, 2004).

2.6.7. Analytical Thin-Layer Chromatography

Thin-layer chromatography is a technique in which a solute undergoes distribution between two phases, a stationary phase acting through adsorption and a mobile phase in the form of a liquid. The adsorbent is a relatively thin, uniform layer of dry finely powdered material applied to a glass, plastic or metal sheet or plate. Glass plates are most commonly used. Separation may also be achieved on the basis of partition or a combination of partition and adsorption, depending on the particular type of support, its preparation and its use with different solvent (Ajaegbu, 2013). Identification can be effected by observation of spots of identical R_f value and about equal magnitude obtained, respectively, with an unknown and a reference sample chromatographed on the same plate. A visual comparison of the size and intensity of the spots usually serves for semi-quantitative estimation (Ajaegbu, 2013).

2.6.8. Preparative Thin-Layer (Planar) Chromatography

Preparative planar (thin-layer) chromatography (PPC) is a liquid chromatographic technique performed with the aim of isolating and purifying compounds, in amounts of 10-100 mg, for structure elucidation and for evaluation of biological activity. PPC is a valuable simple method of sample purification for preparative purposes and isolation. The scope of modifying operating parameters such as the vapor space, development mode and for offline sample application is enormous in planar chromatography. One of the most important experimental variables in PPC is the vapor space, because the separation process occurs in a three-phase system of stationary,

mobile, and vapors phases, all of which interact until equilibrium is reached. In classical PPC the mobile phase migrates by capillary action (Cooke and Poole, 2000; Meyer, 2004; Ajaegbu, 2013).

2.7. Nuclear Magnetic Resonance Spectroscopy (NMR)

Nuclear magnetic resonance spectroscopy (NMR) has been the single most important physical method for the determination of molecular structures for more than 30 years. The power of the technique lies in that it not only defines the numbers and types of nuclei present in an organic molecule, but it also describes their individual chemical environments and, more importantly, the way they are interconnected. Driven by its potential to determine the structures of organic compounds, NMR spectroscopy has seen substantial development in the four and a half decades since the first experiments. In particular, the implementation of the pulsed Fourier transforms method and, subsequently, the concept of two-dimensional experiments provided the seeds for vibrant growth. There are currently hundreds of multipulse experiments available to the NMR spectroscopist. The most important experiments are correlation spectroscopy (COSY), heteronuclear multiple bond connectivity (HMBC), heteronuclear multiple quantum coherence (HMQC), nuclear overhauser effect spectroscopy (NOESY), rotating frame overhauser enhancement spectroscopy (ROESY), HSQC and distortionless enhancement by polarization transfer (DEPT) experiments (Lambert and Mazzola, 2000; Hallas, 2004).

2.8.Mass Spectrometry

Mass spectrometry is a fairly routine tool for molecular weight determination and a specialty tool for complex structure identifications. The principle of mass spectrometry (MS) is the separation of ions in a vacuum, using an electrical or magnetic field or a combination of both. The ions may

be formed through a variety of ionization methods; fast atom bombardment (FAB), desorption chemical ionization (DCI), chemical ionization (CI), electrospray ionization (ESI), atmospheric pressure chemical ionization (APCI), and electron impact ionization (EI). Once a molecule is ionized, the mass spectrometer provided separation of ions according to mass to charge ratio. The chemical structure of the parent ion may be determined from the molecular ion mass and the pattern of the fragment ions recorded. Experienced mass spectrometrists can recognize typical fragment ion patterns (Cooke and Poole, 2000; Lee, 2002).

Ion sources classified as being hard sources or soft sources. Hard sources (e.g. EI) impart sufficient energy to analyte molecules so that they are left in highly excited energy state. Relaxation then involves rupture of bonds, producing fragment ions that have mass-to-charge (m/z) ratios less than that of the molecular ion. Soft sources (e.g. CI, ESI, and APCI) cause little fragmentation and then the spectrum consists of the molecular ion peak and only a few, if any other peaks (Cooke and Poole, 2000; Ajaegbu, 2013; Lee, 2002).

2.9. ESI-MS of Flavonoids and Phenolics

MS is a very sensitive analytical method used to identify flavonoid conjugates or to perform partial structural characterization using microgram amounts of sample (Cuyckens and Claeys, 2004). Indeed, significant structural data can be obtained from less than 1 mg of the analyzed compound when different MS techniques are used in combination with chemical derivatization of the characterized compounds (Franski et al., 1999, 2002, 2003). 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 high vacuum exists. In this situation, soft ionization methods need to be applied for the analysis of this group of compounds, and the analyte molecules are ionized

without evaporation in high vacuum (FAB or LSIMS (liquid secondary ion mass spectrometry), MALDI (matrix-assisted laser desorption ionization)) 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). 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 ion different situation is observed in the negative ion mass spectra mode, where much lower fragmentation of the deprotonated molecule ions $[M-H]^-$ occurs. In the case of flavonoid O-glycosyl-glycosides, the rearrangement of sugars may take place during the fragmentation process, and the sequence of sugar losses does not correspond to the sequence of the sugar moieties in the intact molecule (Cuyckens *et al*., 2001, 2002).

2.10. Millettia aboensis

Figure 2.38: The Plant *Millettia aboensis* (IITA Forest Center)

Millettia is a genus of legume in the Leguminosae family. It consists of about 150 species, which are distributed in the tropical and subtropical regions of the world (https://en.wikipedia.org/wiki/Millettia).

The Specie *Millettia aboensis* is of the plant kingdom and the phylum is Magnoliophyta (flowering plants). It is classified as a Magnoiiphisida and of the order Fabales (legumes, milkworts, and snakeroot) and belongs to the family known as Fabaceae (Pea family) and the Genius Millettia. They are mostly shrubs that have streaked dark reddish or chocolate coloured wood (Figure 1). The leaves are usually alternate and compound; they have margins that are seriated. They have stipule which can be leaf-like (example, pisum), thorn-like (example, robinia) or inconspicuous. Leaf margins are entire or occasionally, seriate. Both the leaves and the leaflets often have wrinkled pulvini to permit mastic movements. Most species have leaves with structures which attract ants that protect the plant from attack by herbivore insects. The plants have indeterminate inflorescences which are sometimes reduced to a single flower. They have short hypanthium and a single purple-coloured carpel with short gynophores and after fertilization produces fruits that are legumes and the common name for this type of fruit is a ‘pod’. Most of the fabaceae host bacteria in their root nodules which have the ability to take up nitrogen from air for nitrogen fixation. The family name fabaceae was coined from Latin word ‘Faba’ which means ‘bean’. Leguminosae is an older name still considered valid (Onyegeme-Okerenta *et al*., 2013).

In Nigeria, the leaves are chewed and rubbed on the painful spots in case of respiratory difficulties. The sap of the leaves is drunk to treat the constipation of the children. A decoction of the twigs is given to the constipated babies and to children as laxative. Leaves and fruits are used in case of colds or headaches.

In Nigeria, the roots are macerated in alcohol to treat the hemias, mixed with egg, oil and slices of plantain, banana; they are used to treat jaundice, 3 days for a man and 4 days for a woman. Its twigs are used as chewing sticks.

Local names in Nigeria: awo (Edo), erurumesi, ukperurumwesi (Edo), odudu (Efik), ndu ezi, mkpukpu manya, ogba odolo (Igbo), ofoni (Ijo-izon) (Banzouzi *et al.*, 2008)

2.10.1. Pharmacognostic Profile

Kingdom: Plantae

Phylum: Angiosperms

Unranked: Eudicots

Unranked: Rosids

Order: Fabales

Family: Fabaceae

Subfamily: Faboideae

Tribe: Millettieae

Genus: *Millettia*

Specie: *aboensis*

2.10.2. Biological actions of the plant

Ethanol leaf extract of *Millettia aboensis* and reference drug (commercial Geniclox 500) were tested in vitro against four clinical isolates, namely, *Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa,* and *Staphylococcus aureus* for antibacterial activity. The ethanol leaf extract of *M. aboensis* inhibited the growth of *K. pneumonia*e, *P. aeruginosa*, and *S. aureus* but did not inhibit the growth of *E. coli* while the reference drug inhibited the growth of all the isolates.

Interaction with local traditional healers on their knowledge and usage of *Millettia aboensis* reveals that the leaf extract is useful in the management of sexual transmitted disease; it is also abortifacients if taken in combination with other herbal plant in excess by pregnant women. The stem extract is also used as a laxative in both children and Adult. In addition, *Millettia aboensis* is used in management of oedema in pregnant women, fever, and malaria locally (Onyegeme-Okerenta and Okafor, 2014).

Onyegeme-Okerenta et al., (2013) also reported on the effect of ethanol leaf extract of *Millettia aboensis* on selected haematological indices of Wistar albino rats.

Almost all the parts of Millettia aboensis have medicinal properties. The leaf is used by traditional herbalist for general healing including ulcer healing and laxatives while the root is used in treating gastro-intestinal disturbances and hepatic disease. Also the leaf, stem and roots mixed with other plant materials are used to cure veneral diseases such as syphilis and Chlamydia trachomatic infection. The aqueous extract and alcoholic extracts of *Millettia aboensis* root has shown promising hepatoprotective activity on paracetamol- hepatotoxicity in rats at the administered dose of 215 mg/kg and 431 mg/kg body weight, orally. Both extracts showed dose dependant activity (Ugwueze and Adonu, 2013). Anti-inflammatory activity was reported by Ajaghaku et al., (2013).

2.10.3. Chemical composition of *Millettia aboensis*

Recent qualitative analysis by Onyegeme-Okerenta *et al.,* (2013) revealed that leaves of *Millettia aboensis* contains tannins, saponins, alkaloids, flavonoids, phlobatanins, reducing sugars, and cardiac glycosides; and the absence of cyanogenic glycoside and anthraquinone. The ethanolic extract of leaves of *Millettia aboensis* was found to contain two flavonoid glycosides: quercetin-3-O-rutinoside and quercetin-3-O-robinobioside (Ajaghaku *et al*., 2018).

Chapter three

Materials and methods

3.1. Materials

3.1.1. ANALYTICAL INSTRUMENTS

3.1.1.1. Analytical HPLC

Pump	P580A LPG, Dionex
Detector	UVD 340S (photodiode array detector), Dionex.
Autosampler	ASI-100 (standard injection volume 0 20µL), Dionex
Column oven	STH 585, Dionex.
Column	Eurosphere 100-C18 (5 µM; 125 x 4 mm), with integrated pre-column
Software	Chromeleon 6.30
Manufacturer	Dionex

3.1.1.2. Semi preparative HPLC

Pump	L-7100 and Chromaster 5110, Merck/Hitachi
Detector	UV-L7400 and Chromaster 5410 (photodiode array detector), Merck/Hitachi
Column	Eurosphere 100-C18 (10 µm; 300 x 8 mm), Knauer

Precolumn	Eurosphere 100-C18 (10 μm; 30 x 8 mm), Knauer
Printer	Chromato-Integrator D-2000, Merck/Hitachi
Manufacturer	Hitachi

3.1.1.3. ESI-MS

MS spectrometer	Finnigan LCQDeca, Thermoquest
Vacuum pump	Edwards 30, BOC
Column	Eurosphere 100-C18 (5 μM; 227 x 2 mm), Knauer
Software	Xcalibur, version 1.3.
Manufacturer	Knauer

3.1.1.4. High resolution ESI-MS Qtof 2, Micromass.

3.1.1.5. NMR 1H and 13C spectra were recorded at 300º K on Bruker DPX 300, ARX 400, 500 or AVANCE DMX 600 NMR spectrometers. All 1D and 2D spectra were obtained using the standard Bruker software.

3.1.1.6. Polarimeter 241 MC, Perkin-Elmer.

3.1.2. GENERAL INSTRUMENTS

1. Freeze dryer: Lyovac GT2, Steris; pump Trivac D10E, Savant
2. Speedvac: SPD 111V with cooling trap RVT 400, Savant
3. Ultra Turrax®: T18 basic, IKA

4. Vacuum pump: 4EKF56CX-4, Greiffenberger Antriebstechnik, Germany
5. Balance: BL1500, Sartorius
6. Analytical balance: MC-1, Sartorius
7. Desiccator: Glaswerk Wertheim
8. Hot plate and magnetic stirrer: IKA-Combimag RCH, Janke & Kunkel KG
9. Drying oven: ET6130, Heraeus
10. Ultra sonicator: RK 510H, Bandelin Sonorex
11. Rotary evaporator: RV 05-ST, Vaccubrand
12. Membrane vacuum pump: Vaccubrand
13. UV lamp: Camag (wave length 254 and 366 nm)
14. Nitrogen generator: UHPN 3001, Nitrox (All made in Germany).

3.1.3. General solvents

1. Acetone p.a.: Merck
2. n-Butanol p. a.: Merck
3. Methanol, ethyl acetate, dichloromethane and n-hexane were purchased as technical solvents and distilled before using.

3.1.4. HPLC solvents

1. Methanol: LiChroSolv HPLC grade, Merck
2. Acetonitrile: LiChroSolv HPLC grade, Merck
3. Distilled and heavy metal free water was obtained by passing through nano- and ion-exchange filter cells (Barnstead) to yield nanopure water.

3.1.5. Solvents for optical rotation

1. Chloroform: Spectroscopic grade, Sigma

2. Methanol: Spectroscopic grade, Sigma

3.1.6. Solvents for NMR

1. Chloroform-d: Uvasol, Merck
2. Methanol-d4: Uvasol, Merck

3.1.7. GENERAL LABORATORY CHEMICALS/REAGENTS

Other chemicals/reagents include: acetic acid, formic acid, hydrochloric acid, potassium hydroxide, sodium hydroxide, sulphuric acid, chloramphenicol, ammonium chloride, peptone, yeast extract, corn steep liquor, rhamnose, glucose, sucrose, sodium chloride, sodium iodide, sodium bromide, etc.

3.1.8. Plant material

The plant parts of *Milletia aboensis* were collected from their natural habitat in Amudo-Awka, Anambra State of Nigeria, in the month of January 2015. The plants were identified by Mr. Alfred Ozieko of Bioresources, Development, and Conservation Program (BDCP), Nsukka. Voucher specimens have been deposited in the herbarium section of the Department of Pharmacognosy and Traditional Medicine, Faculty of Pharmaceutical Sciences, Nnamdi Azikiwe University, Awka, with specimen number PCG/474/A/021. The plant parts were dried under the shade for some days. The dried plant parts were grounded into a fine powder using a mechanical grinder. Powdered materials were maintained at room temperature (25-27°C), protected from light until required for extraction and analysis.

3.2. Methods

3.2.1. Extraction and fractionation

The powdered plant materials of leaf (750 g), stem (900 g) and root (800 g) of *M. aboensis* were extracted for 2 days using cold maceration method in methanol (500 ml) in intermittent shaking. The maceration process was then repeated once in methanol (300 ml) for maximal extraction. The methanol extract was then collected and concentrated almost to dryness under vacuum at 40ºC using rotary evaporator. The concentrated methanol leaf (90 g) extract were reconstituted with 200 ml of methanol: water (2:8) and fractionated successively with hexane (500 ml x 10), ethyl acetate (500 ml x 7) and butanol (100 ml) to give the hexane (HF), ethyl acetate (EF), butanol (BF) and aqueous (AF) fractions.

The concentrated methanol stem (18.5 g) extract were reconstituted with 200 ml of methanol: water (2:8) and fractionated successively with hexane (500 ml x 4), ethyl acetate (500 ml x 5) and butanol (100 ml) to give the hexane (HF), ethyl acetate (EF), butanol (BF) and aqueous (AF) fractions.

The concentrated methanol root (19.8 g) extract were reconstituted with 200 ml of methanol: water (2:8) and fractionated successively with hexane (500 ml x 2), ethyl acetate (500 ml x 6) and butanol (100 ml) to give the hexane (HF), ethyl acetate (EF), butanol (BF) and aqueous (AF) fractions.

3.2.2. Chromatographic separation of the fractions

3.2.2.1.Thin layer chromatography (TLC)

The fractions or isolated compounds (50 µl) in each test tube were applied on the entire length of aluminum pre-coated silica gel TLC plates as stationary phase of size 6.5 x 10 cm with the help

of a fine bore glass capillary tube,. The solvent system, dichloromethane: methanol (5:5) as the mobile phase was used for the development of chromatograms of the respective fractions. The plates were examined under UV at 254 (fluorescence absorption) and 365 nm. The TLC was also used to ascertain the identity and purity of the isolated compounds.

3.2.2.2.Vacuum Liquid Chromatography

The ethyl acetate fractions of the leaf [EF (3.385 g), stem [EF (3.1066 g), and root [EF (4.7829 g) were dissolved in methanol, silica gel (200 – 400 mesh size) were added to the mixture to make slurry to get uniform mixing.

The glass column (diameter 6 cm × 20 cm height) was packed with silica gel (200 – 400 mesh size) using hexane as solvent. To the top of the column, admixture was loaded and column was eluted gradually with increasing order of polarity of solvents from non- polar to highly polar solvents (Appendix A, B and C) using Vacuum Liquid Chromatography (VLC) method with gradient elution. The fractions were collected with the help of a round bottom flask to give a total of 35 fraction. The fractions were concentrated under vacuum at 40ºC using rotary evaporator and completely dried using the oven

3.2.2.3. Sephadex LH-20 Separation of ethyl acetate fraction

3.2.2.3.1. Sephadex LH-20 Separation of ethyl acetate fractions of the plant parts

100 g of Sephadex LH-20 was dispersed in either 200 mL of methanol or dichloromethane: methanol (50:50) and the mixture sonicated for 30 mins. The slurry was transferred into a column (50 × 2 cm, length × internal diameter) and the gel allowed to stabilize for about 5 hours before use.

The ethyl acetate fractions of the plant parts were reconstituted in either 2 mL of methanol or dichloromethane: methanol (50:50) and the solution sonicated for 5 minutes and then centrifuged × 10000 for 10 mins to remove undissolved solid particles. The supernatant was pipetted and used for the separation studies.

The supernatant was introduced into the sephadex LH-20 column adjusted to the flow rate of approximately 0.2 mL/min. About 100 eluates of 2 mL each were collected with the aid of fraction collector and monitored with TLC on silica gel G_{254} developed with dichloromethane: methanol, 5:5. Similar eluates with same R_f were combined and concentrated with rotary evaporator to obtain the Sephadex fractions.

3.2.3. Isolation and purification of chromatographic fractions

Fraction 5 (120 mg) was separated using Sephadex LH-20 column with DCM: MeOH (50:50) as eluent to yield three sub-fractions (5-1 to 5-3). Sub-fraction 5-1 (51.5 mg) was further purified using semi-preparative HPLC (gradient MeOH-H_2O, 25:85 to 100:0) to yield compound **1** (1 mg).

Fraction 7 (200 mg) was separated using Sephadex LH-20 column with DCM: MeOH (50:50) as eluent to yield five sub-fractions (7-1 to 7-5). Sub-fraction 7-2 (51.6 mg) was further purified using semi-preparative HPLC (gradient MeOH-H_2O, 20:80 to 100:0) to yield compound **2** (10.1 mg).

Fraction 8 (1.2 g) was separated using Sephadex LH-20 column with DCM: MeOH (50:50) as eluent to yield four sub-fractions (8-1 to 8-4). Sub-fraction 8-2 (965.1 mg) was further subjected to Sephadex LH-20 column using methanol as mobile phase followed by further purification using semi-preparative HPLC (gradient MeOH-H_2O, 20:80 to 100:0) to yield compound **3** (9.6 mg).

Fraction 9 (350 mg) was separated using Sephadex LH-20 column with MeOH as eluent to yield three sub-fractions (9-1 to 9-3). Sub-fraction 9-2 (145.7 mg) was further purified using semi-preparative HPLC (gradient MeOH-H_2O, 10:90 to 100:0) to yield compound **4** (2 mg) and **5** (1 mg).

Fraction 14 was separated using Sephadex LH-20 column with DCM: MeOH (50:50) to yield four sub-fractions (3-1 to 3-4). Sub-fraction 3-2 and 3-3 were combined together, diisolved in methanol and was further purified using semi-preparative HPLC (gradient MeOH-H_2O, 15:85 to 100:0) to yield compound **16** (1.6 mg), and **17** (1.6 mg)

Fraction 24 (570 mg) was separated using Sephadex LH-20 column with DCM: MeOH (50:50) to yield four sub-fractions (3-1 to 3-4). Sub-fraction 3-2 (117 mg) was further purified using semi-preparative HPLC (gradient MeOH-H_2O, 15:85 to 100:0) to yield compound **6** (2.3 mg), **7** (1.5 mg). Sub-fraction 3-3 (163 mg) was further purified using semi-preparative HPLC (gradient MeOH-H_2O, 15:85 to 100:0) to yield compound **8** (1.2 mg) and **9** (1.1 mg). Sub-fraction 3-4 (97 mg) was further purified using semi-preparative HPLC (gradient MeOH-H_2O, 45:55 to 100:0) to yield compound **10** (1.4 mg), **11** (1.3 mg), and **12** (1.4 mg).

Fraction 25 (650 mg) was separated using Sephadex LH-20 column with DCM: MeOH (50:50) to yield four sub-fractions (3-1 to 3-4). Sub-fraction 4-2 (330 mg) was further purified using semi-preparative HPLC (gradient MeOH-H_2O, 45:55 to 100:0) to yield compound **13** (2.3 mg), and **14** (1 mg).

The pod of the plant was extracted with methanol and the concentrated methanol extract was reconstituted with 200 ml of methanol: water (2:8) and fractionated successively with hexane,

ethyl acetate and butanol to give the hexane (HF), ethyl acetate (EF), butanol (BF) and aqueous (AF) fractions. The ethyl acetate fraction was fractionated using Sephadex LH-20 column with MeOH to give compound **15** (1 mg).

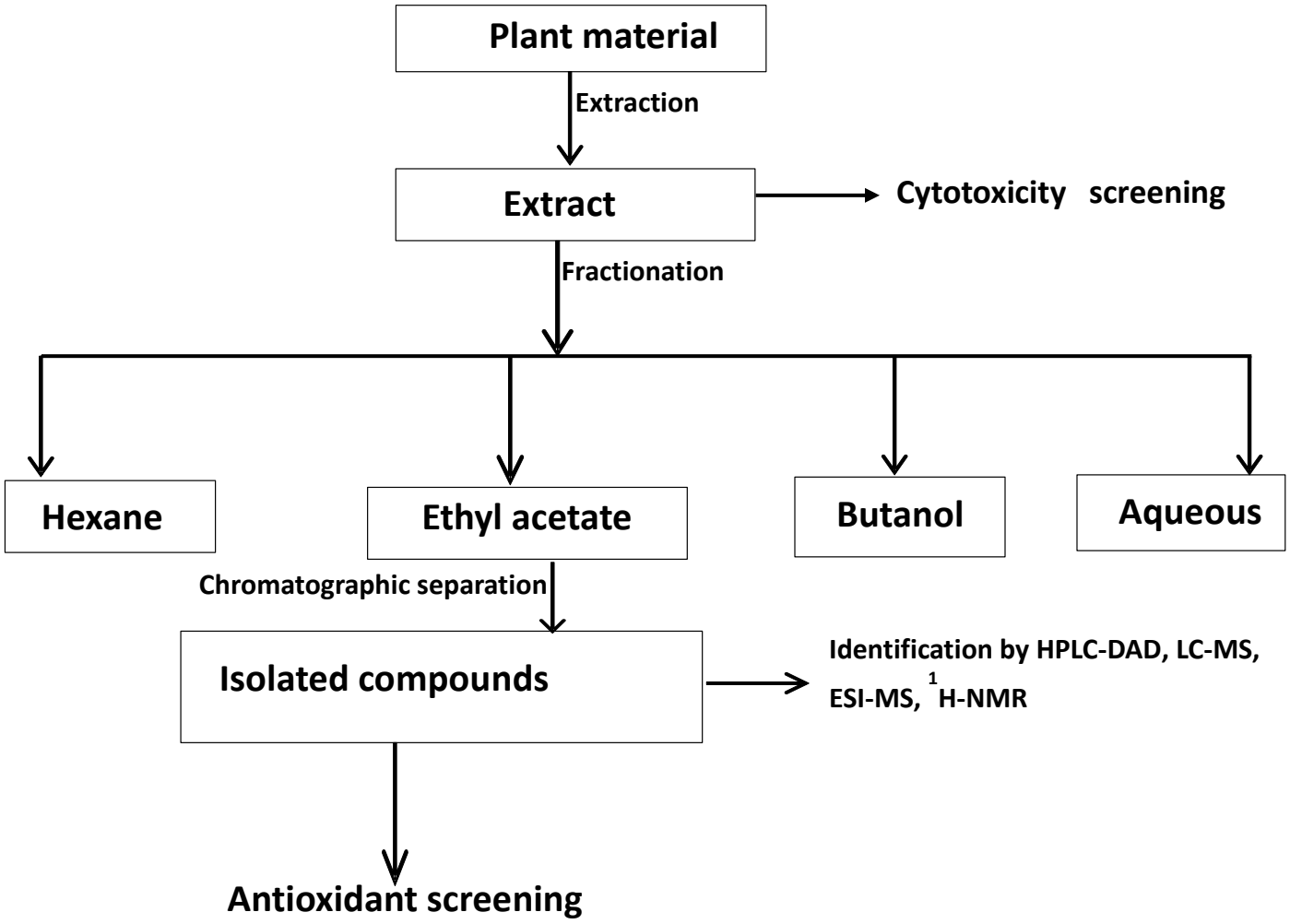


Figure 3.1. Methodology chart of the plant *Millettia aboensis*

3.2.3.1.Analytical HPLC

Each of the dried fractions was reconstituted with 2 mL of HPLC grade methanol. 100 µL of the dissolved samples were each transferred into HPLC vials containing 500 µL of HPLC grade methanol. HPLC analysis was carried on the samples with a Dionex P580 HPLC system coupled to a photodiode array detector (UVD340S, Dionex Softron GmbH, Germering, Germany). Detection was at 235, 254, 280 and 340 nm. The separation column (125 cm × 4 mm; length × internal diameter) was prefilled with Eurospher-10 C18 (Knauer, Germany), and a linear gradient of nanopure water (adjusted to pH 2 by addition of formic acid) and methanol was used as eluent. The solvent gradient used started with 10:90 (MeOH: nanopure water (adjusted to pH 2 with formic acid) increasing to 100 % MeOH in 45 minutes. The compounds were detected by an UV-VIS diode array detector

3.2.3.2. Semi-preparative HPLC

Semi-preparative HPLC was used for the isolation and purification of the compounds from complex and nearly pure fractions. Each injection was concentrated to 3 mg/ml and the maximum injection volume was 100 µl. The flow rate was set to 5 ml/min.

3.2.4. Spectroscopic methods

3.2.4.1. Electron Spray Ionization Mass Spectrometry (ESI-MS)

ESI-MS is a gentle ionization method where the sample is passed through a high voltage metal capillary. At the end of this capillary it is sprayed by a flow of nitrogen gas at atmospheric pressure to form an aerosol. Together with heating, the nitrogen evaporates the emerging droplets forcing the ions in each droplet together until repulsion causes them to eject from the surface. The ions are

extracted into the vacuum of the mass analyser where they are detected. Additionally to the molecular ion peaks [M+H]+ or [M-H]- fragments of these compounds can be detected.

3.2.4.2. Nuclear Magnetic Resonance Spectroscopy (NMR)

The proton NMR spectrum of the isolated compounds was acquired by varying the magnetic field that is applied to the sample dissolved in a deuterated solvent over a small range while observing the resonance signal from the sample.

All proton NMR spectra were measured at the Institut für Anorganische Chemie und Strukturchemie, Heinrich-Heine Universität, Düsseldorf with a Bruker ARX-300, and 600, by Dr. Peter Tommes and co-workers.

3.2.5 Optical activity

Optical rotation was determined on a Perkin-Elmer-241 MC polarimeter by measuring the angle of rotation at the wavelength of 546 and 579 nm of mercury vapour lamp at room temperature αthe formula:

$$[\alpha]_D = \frac{3.199 * [\alpha]_{579}}{4.199 - \frac{[\alpha]_{579}}{[\alpha]_{546}}}$$

where $[\alpha]_D$ is the specific rotation at the wavelength of the sodium D-line at 589 nm and a temperature of 20ºC. $[\alpha]_{579}$ and $[\alpha]_{546}$ are the optical rotation at wavelengths 579 and 546 nm, respectively, calculated using the formula:

$$[\alpha]_\lambda = \frac{100 * c}{I * c}$$

Where α is the measured angle of rotation in degrees, I is the length of the polarimeter tube in dm, c is the concentration of the substance expressed in g/100 ml.

3.2.6. Biological Assays

3.2.6.1. Cytotoxicity Assay (MTT/Cell Proliferation Assay)

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was performed following the method described by Carmichael *et al*. (1987), and % cell viability was determined by spectrophotometric determination of accumulated formazan derivative in treated cells at 560 nm in comparison to control cells (Ashour *et al*. 2006).

L5178Y mouse lymphoma cells were grown in Eagle's minimal essential medium supplement with 10% horse serum in roller tube culture. The medium contained 100 units/mL penicillin and 100 units/mL streptomycin. The cells were maintained in a humidified atmosphere at 37°C with 5% CO_2. Of the test samples to be analyzed in the bioassay, stock solutions of crude extracts (10 μg/mL) in ethanol (96% v/v) were prepared. Exponentially growing cells were harvested, counted and diluted appropriately. Of the cell suspension, 50 μL containing 3750 cells were pipetted in 96-well microtiter plates. Subsequently, 50 μL of a solution of the test samples containing the appropriate concentration was added to each well.

The test plates were incubated at 37°C with 5% CO_2 for 72 h. A solution of MTT was prepared at a concentration of 5 μg/mL in phosphate buffered saline (PBS; 1.5 mM KH_2PO_4; 6.5 mM Na_2HPO_4; 137 mM NaCl; 2.7 mM KCl; pH 7.4), and from this solution, 20 μL was pipetted into each well.

The yellow MTT penetrated the cells and in the presence of mitochondrial dehydrogenases MTT was transformed to its blue formazan complex.

After an incubation period of 3 h 45 min at 37°C in a humidified incubator with 5% CO_2, the medium was centrifuged (15 min at 210 x g) with 200 µL DMSO and the cells were lysed to liberate the formazan product.

After thorough mixing, the absorbance was measured at 520 nm using Microplate reader. The color intensity could be correlated with the number of healthy living cells and cell survival was calculated using the formula:

$$Survival\ \% = \frac{100}{1} * \frac{absorbance\ of\ untreated\ cells - absorbance\ of\ culture\ medium}{absorbance\ of\ treated\ cells - absorbance\ of\ culture\ medium}$$

All experiments were carried out in triplicate.

As negative controls, media with 0.1% (v/v) ethanol were included in all experiment.

3.2.6.2. Free radical scavenging activity: DPPH test

The free radical scavenging activity of the isolated compounds was evaluated using 1,1-diphenyl-2-picrylhydrazyl using the method of Sajjad and Patel (Sajid *et al.*, 2006; Patel *et al.*, 2010) with little modification. DPPH solution (0.6 mMol) was freshly prepared using methanol as solvent; 0.5 ml of this solution was mixed with 0.5 ml of different dilutions of the fractions. The volume of the solution was adjusted with methanol to a final volume of 5 ml. After incubation in the dark for 30 minutes at room temperature, the absorbances of the mixtures were measured at 520 nm against methanol as blank using UV-spectrophotometer. Ascorbic acid was used as standard. Absorbance measurements were done in triplicate.

The antioxidant activities of the isolated compounds were evaluated by comparing their absorbances with that of the control (containing 0.5 ml of DPPH solution and 4.5 ml of methanol). The free radical scavenging activity of the isolated compounds of different concentrations – 10,

20, 40, 80 and 100 µg/ml were obtained using the relationship shown below. The concentrations that produce 50% inhibition (IC_{50}) of free radical were also deduced from dose response curve.

DPPH scavenging activity = 100 * [(AC – AS)/ AC]

AC = Absorbance of control

AS = Absorbance of Sample

3.2.7. Statistical analysis

Results were analysed by SPSS Version 16.0 using Student's T-tests and expressed as mean ± standard error of mean (SEM). Differences between means were considered significant at $P<0.05$.

Chapter four

Results and Discussion

4.1. Plant material

4.1.1. Extraction

The yields of the extract and fractions of the extraction process of *M. aboensis* leaf are shown in Table 4.1

Table 4.1: Extract, fractions and yields of extraction process of *M. aboensis* leaf

Extracts	Yield (% w/w)
MLE[a]	12.72
HLF[b]	20.57
ELF[b]	6.65
BLF[b]	4.19
WLF[b]	30.38

MLE = Methanol leaf extract

HLF = Hexane leaf fraction

ELF = Ethyl acetate leaf fraction

BLF = Butanol leaf fraction

WLF = Water leaf fraction

a = yield calculated from 50 g of plant material

b = yield calculated from 90 g of methanol leaf extract

The yields of the extract and fractions of the extraction process of *M. aboensis* root are shown in Table 4.2

Table 4.2: Extract, fractions and yields of extraction process of *M. aboensis* root

Extracts	Yield (% w/w)
MRE[a]	2.48
HRF[b]	5.56
ERF[b]	24.10
BRF[b]	6.63
WRF[b]	51.11

MRE = Methanol root extract

HRF = Hexane root fraction

ERF = Ethyl acetate root fraction

BRF = Butanol root fraction

WRF = Water root fraction

a = yield calculated from 800 g of plant material

b = yield calculated from 19.84 g of methanol root extract

The yields of the extract and fractions of the extraction process of *M. aboensis* stem are shown in Table 4.3

Table 4.3: Extract, fractions and yields of extraction process of *M. aboensis* stem

Extracts	Yield (% w/w)
MSE[a]	2.94
HSF[b]	9.48
ESF[b]	16.79
BSF[b]	12.17
WSF[b]	57.73

MSE = Methanol stem extract

HSF = Hexane stem fraction

ESF = Ethyl acetate stem fraction

BSF = Butanol stem fraction

WSF = Water stem fraction

a = yield calculated from 900 g of plant material

b = yield calculated from 18.5 g of methanol root extract

The yields of the extract and fractions of the extraction process of *M. aboensis* pod are shown in Table 4.4

Table 4.4: Extract, fractions and yields of extraction process of *M. aboensis* pod

Extracts	Yield (% w/w)
MPE[a]	5.44
HPF[b]	43.48
EPF[b]	3.43
BPF[b]	8.60
WPF[b]	9.19

MPE = Methanol stem extract

HPF = Hexane stem fraction

EPF = Ethyl acetate stem fraction

BPF = Butanol stem fraction

WPF = Water stem fraction

a = yield calculated from 171 g of plant material

b = yield calculated from 9 g of methanol pod extract

4.2. Compounds isolated from the leaf of *Millettia aboensis*

4.2.1 (z)-2-decylpent-2-enedioic acid (compound 1, known)

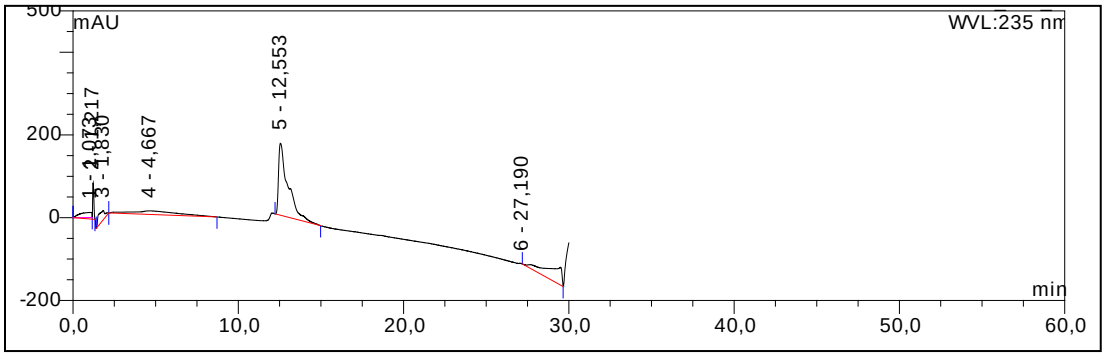


Figure 4.1: HPLC Chromatogram of Compound **1**

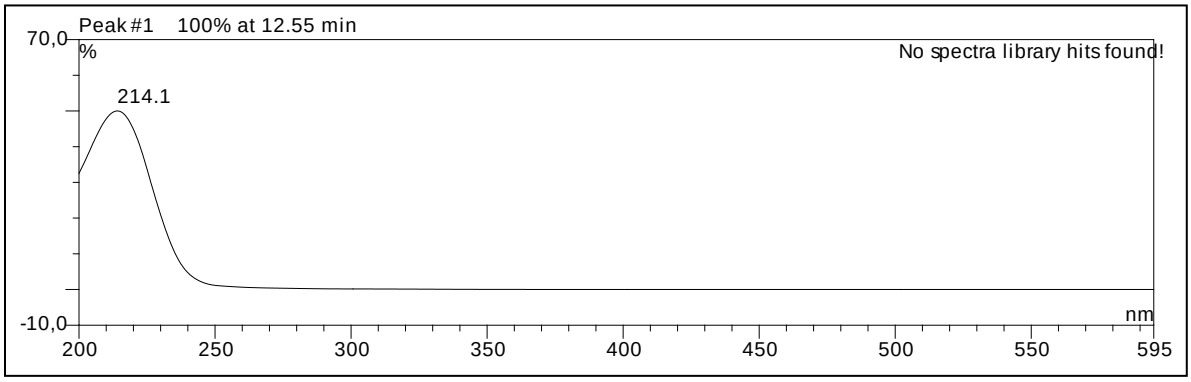


Figure 4.2: Uv spectrum of Compound **1**

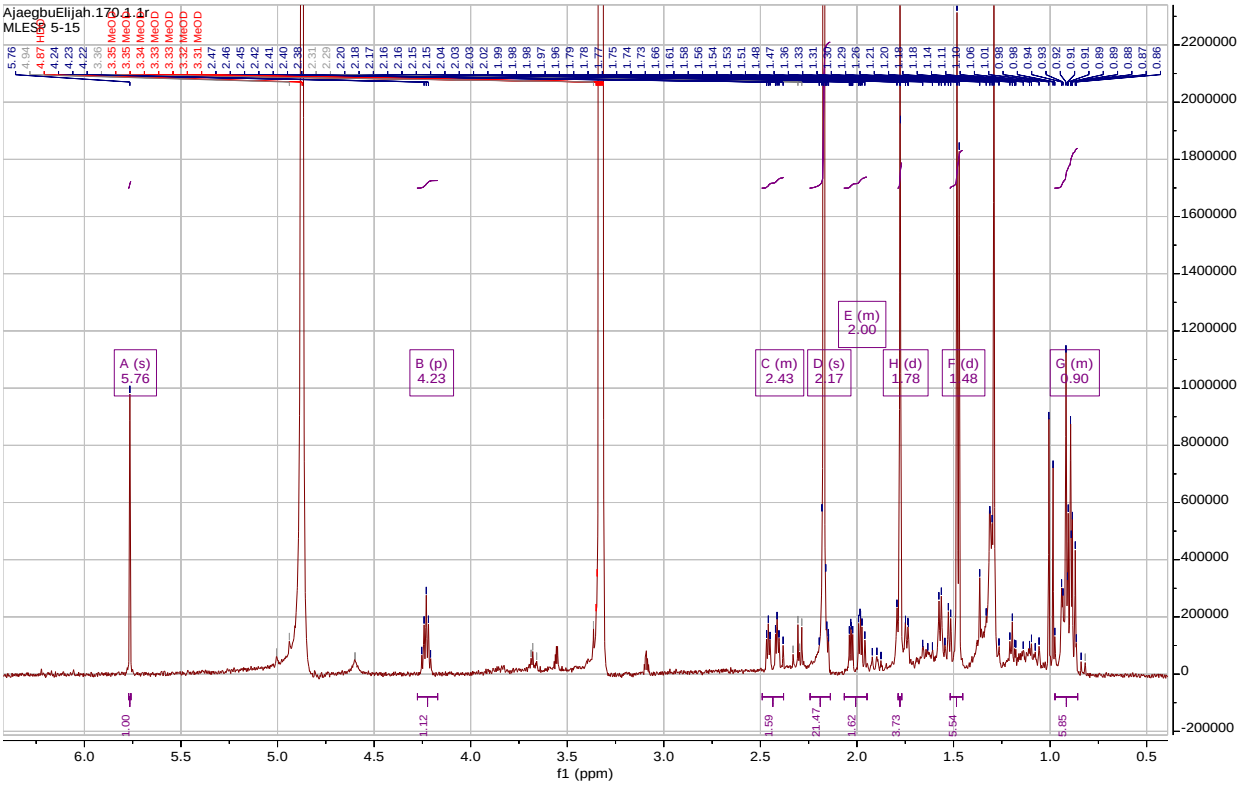


Figure 4.3: [1]HNMR spectrum of Compound **1**

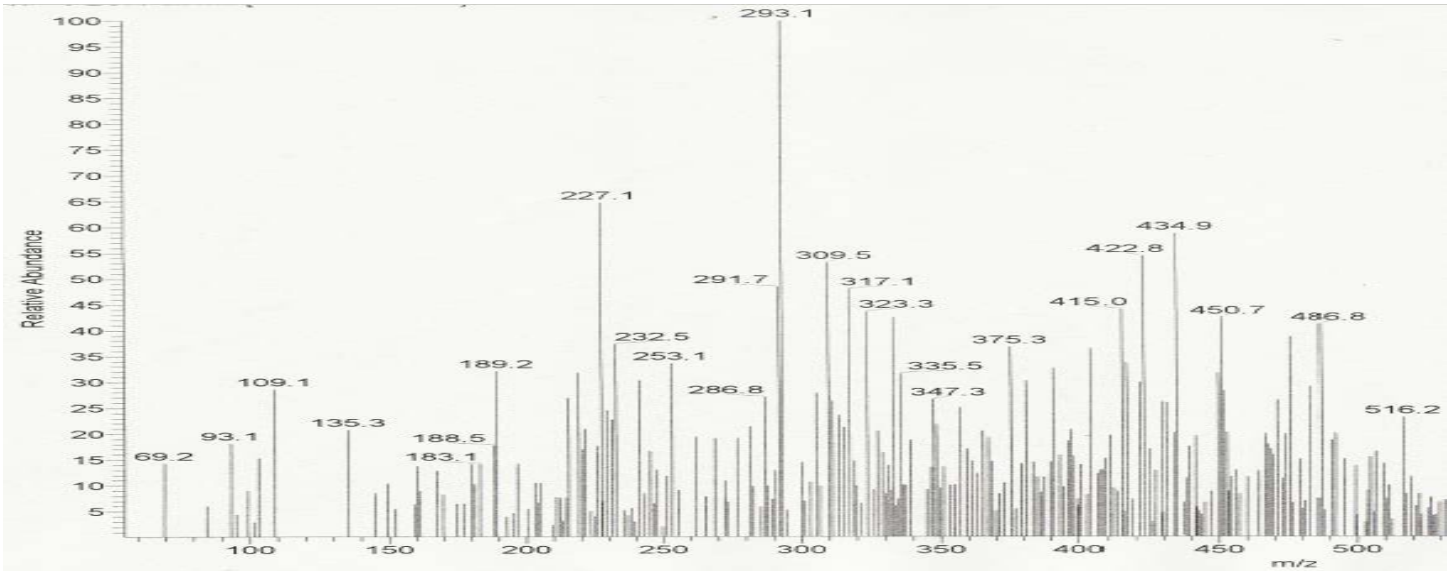
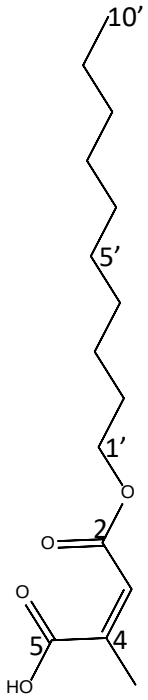


Figure 4.4: ESI-MS spectrum of Compound **1**

4.2.1.1 Structure of (z)-2-decylpent-2-enedioic acid (compound 1, known)



Molecular formula	$C_{15}H_{26}O_4$
Molecular weight	270.37
Amount	1 mg

Compound **1** was isolated from the leaf of *Millettia aboensis* as a colorless oil. It showed UV (MeOH) λ_{max} 214.1 (see Fig. 4.2); the Uv is suggestive of a pentenedioic acid nucleus and $[\alpha]^{20}_{D}$ -15.4 (c, 0.4, MeOH). The ESI-MS showed the pseudomolecular ion peak at *m/z* 293.1 [M+Na^+] (see Fig. 4.4). The fragment ions peak occurred at *m/z* 227.1 [M±H^+-CO_2^-], indicating presence of carboxylation; at *m/z* 286.8 [M+OH^-], which shows the presence of hydroxylation and also at *m/z* 309.5 [M+Na^++CH_3], indicating the presence of sodium and methylation. ^{1}H NMR measured in methanol shows a proton singlet at δ 5.76 (s, H-3), characteristic of an olefinic proton or one trisubstituted double bond as singlets, and a methyl singlet at δ 2.17 (s, H-6) . It also displayed one methylene group at δ 4.23 (p, J=3.4 Hz, H-1'), together with nine aliphatic methylene groups at δ

2.43 (m, H-2', H-3'), 2.00 (m, H-4', H-5'), 1.78 (d, J=0.9 Hz, H-6', H-7'), 1.48 (d, J=3.4 Hz, H-8'), and 0.90 (m, H-9', H-10'). In comparison with literature data (Endo *et al.,* 1985 and Akone *et al.*, 2014), compound **1** was established as (z)-2-decylpent-2-enedioic acid and which was previously isolated from a soil-derived fungus *G. butleri* and this is the first report of its isolation from plant.

Table 4.5: Proton NMR spectroscopic data of compound **1** (CD_3OD, 300 MHz)

Position	**δ_H (J in Hz)**
2	-
3	5.76 (s)
4	-
5	-
6	2.17 (s)
1’	4.25 (p, 3.4)
2’	2.43 m
3’	2.43 m
4’	2.00 m
5’	2.00 m
6’	1.78 (d, 0.9)
7’	1.78 (d, 0.9)
8’	1.48 (d, 3.4)
9’	0.90 (m)
10’	0.90 (m)

4.2.2. Genistein-4'-O-arabinofuranoside (compound 2, new)

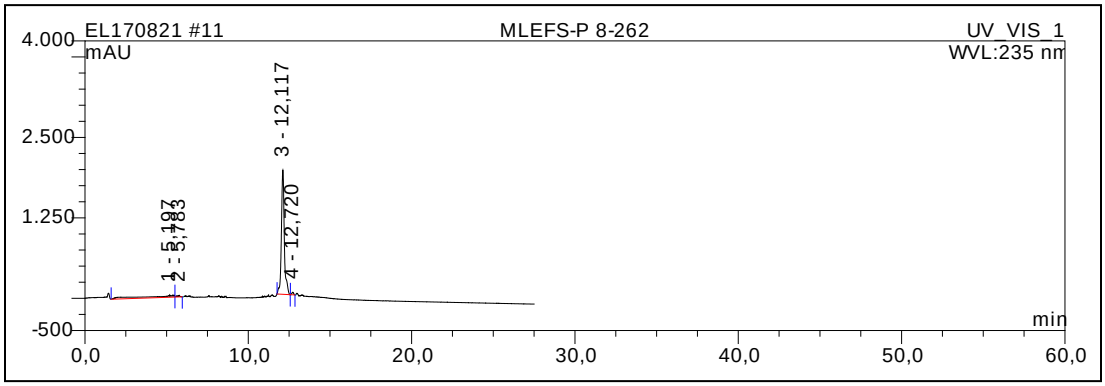


Figure 4.5: HPLC Chromatogram of Compound **2**

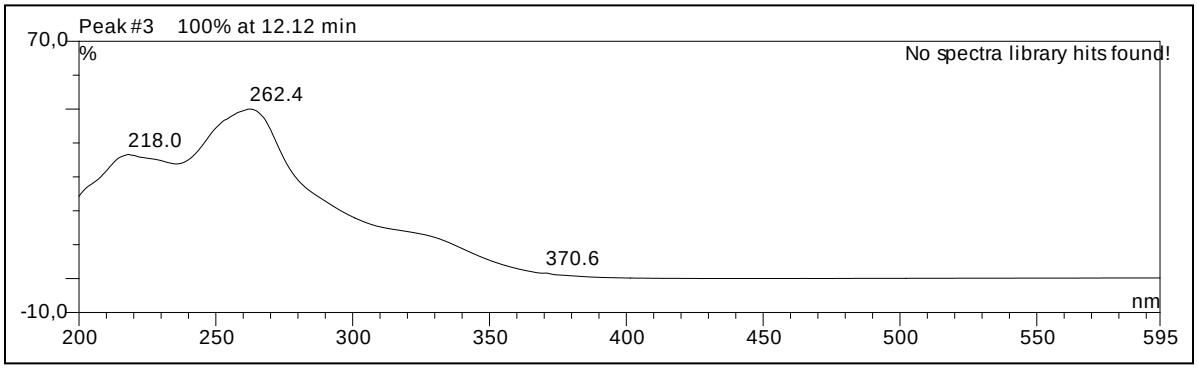


Figure 4.6: Uv spectrum of Compound **2**

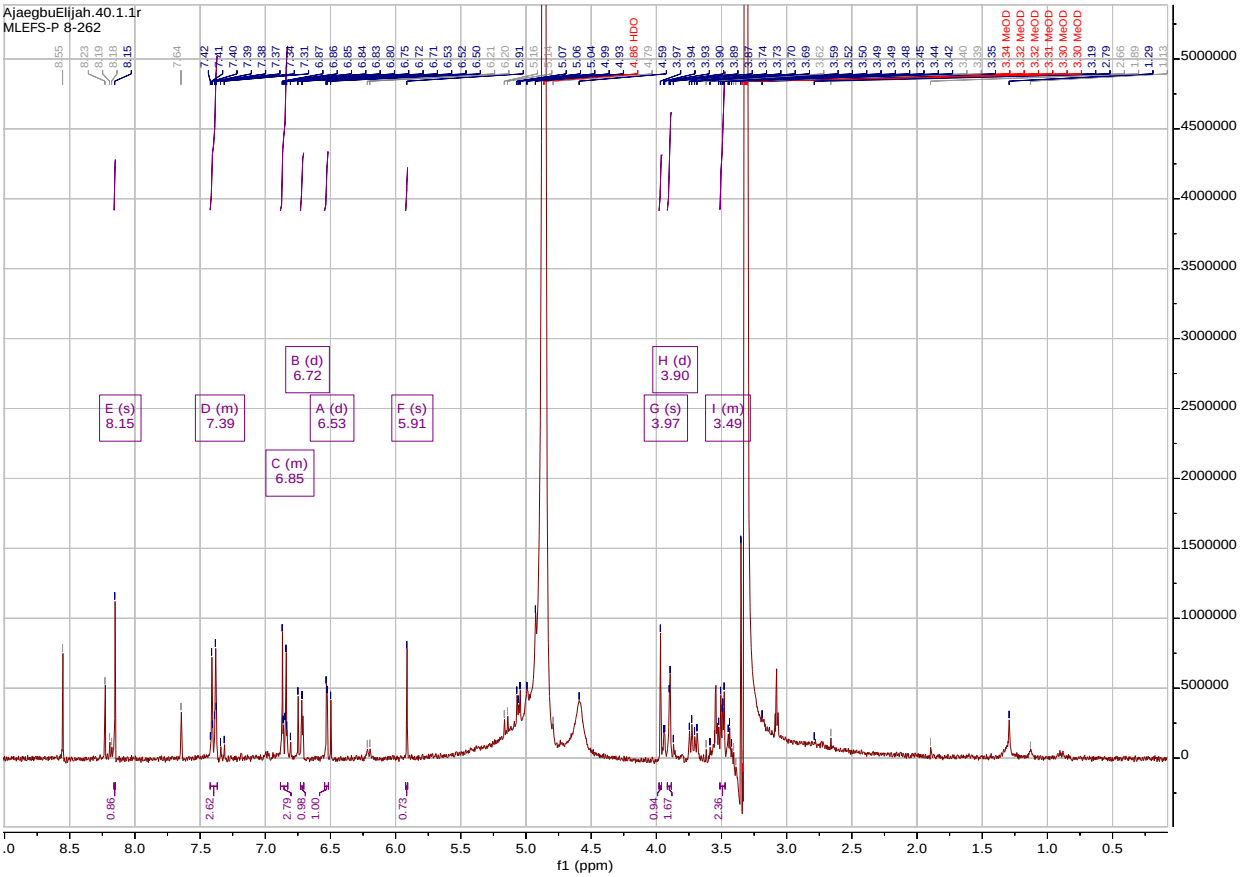


Figure 4.7: [1]HNMR spectrum of Compound **2**

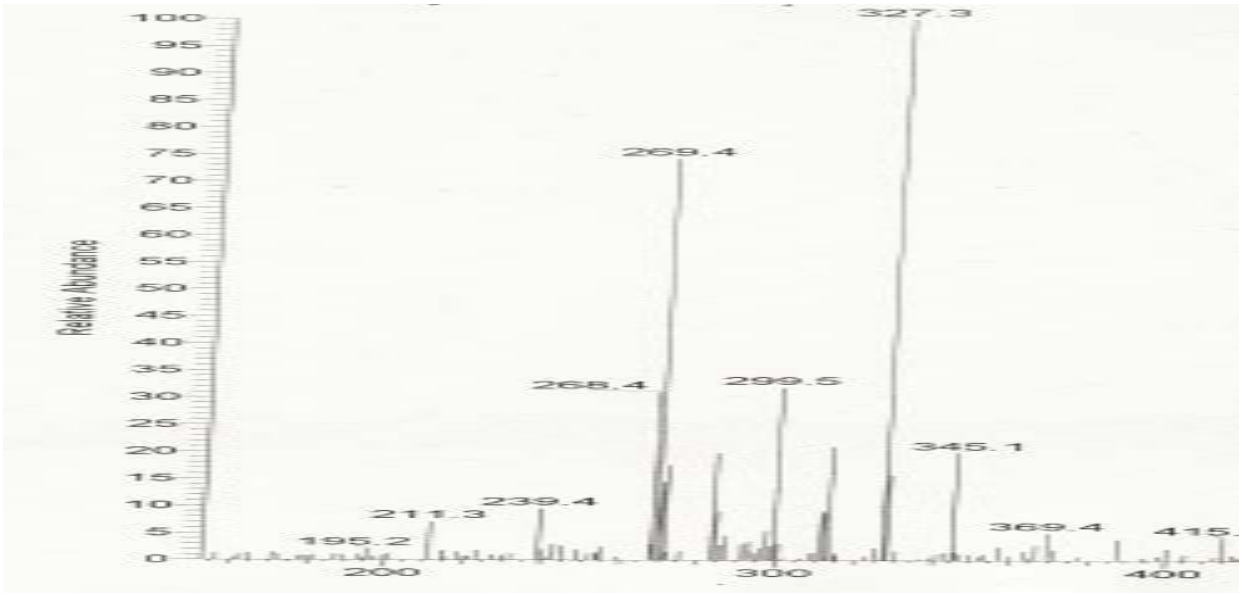
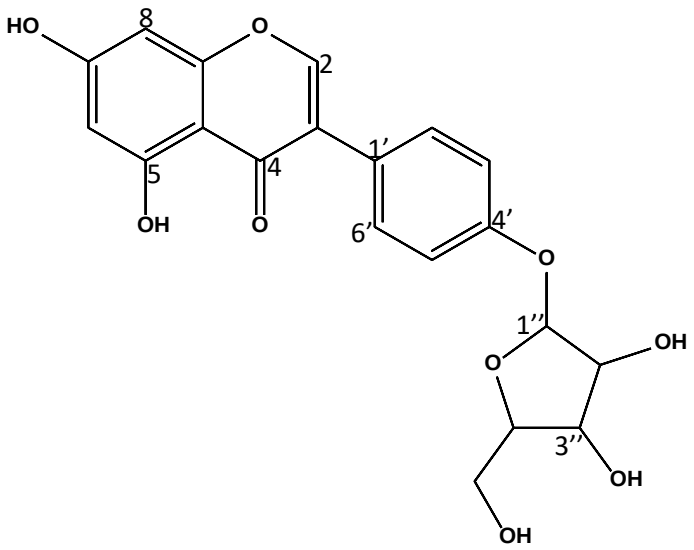


Figure 4.8: ESI-MS spectrum of Compound **2**

4.2.2.1 Structure of Genistein-4'-O-arabinofuranoside (compound 2, new)



Molecular formula	$C_{20}H_{18}O_9$
Molecular weight	402.36
Amount	5 mg

Compound **2** was isolated from the leaf of *Millettia aboensis* as an amorphous yellow solid. It showed UV (MeOH) λ_{max} 218.0, 262.4, and 370.6 (see Fig. 4.6), which is suggestive of a genistein nucleus and $[\alpha]^{20}_D$ -7.67 (c, 0.35, MeOH). The ESI-MS showed the pseudomolecular ion peak at *m/z* 415.3 [M+CH_3] (see Fig. 4.8). The fragment ions peak occurred at *m/z* 269.4 [M±H^+-pentose], indicating the presence of glucosylation (pentose sugar); and also at *m/z* 327.3 [M±H^+-benzyl], which indicates the presence of benzyl group. The molecular formula was deduced as $C_{20}H_{18}O_9$ based on the observed MS. The ^{1}H NMR spectrum measured in CD_3OD (Figure 4.7) shows a proton singlet at δ 8.15 (s, H-2), characteristic of an isoflavone and two aromatic protons of ring A at δ 6.72 (d, J=2.3 Hz, H-6) and 6.53 (d, J=2.2 Hz, H-8). In the aromatic region, further resonances indicative for an AA'BB' system at δ 7.39 (m, H-2', H-6') and 6.85 (m, H-3', H-5')

were observed, due to 1', 4'-disubstituted B-ring. From the ^{1}H NMR spectrum, the sugar protons were inferred to be present at δ 5.91 (s, H-1''), 3.90 (m, H-2", H-4"), 3.97 (s, H-3"), and 3.49 (m, H-5"), compound **2** was established as genistein-4'-O-arabinofuranoside, which has not been isolated before to the best of my knowledge. Hence compound **2** is a new compound.

Table 4.6: Proton NMR spectroscopic data of compound **2** (CD_3OD, 300 MHz)

Position	**δ_H (J in Hz)**
2	8.15 (s)
3	-
4	-
5	-
6	6.72 (d, 2.3)
7	-
8	6.53 (d, 2.2)
9	-
10	-
1’	-
2’	7.39 (m)
3’	6.85 (m)
4’	-
5’	6.85 (m)
6’	7.39 (m)
1”	5.91 (s)
2”	3.90 (m)
3”	3.97 (s)
4”	3.90 (m)
5”	3.49 (m)

4.2.3. Epicatechin-3-O-(4”-methyl gallate) (compound 3, known)

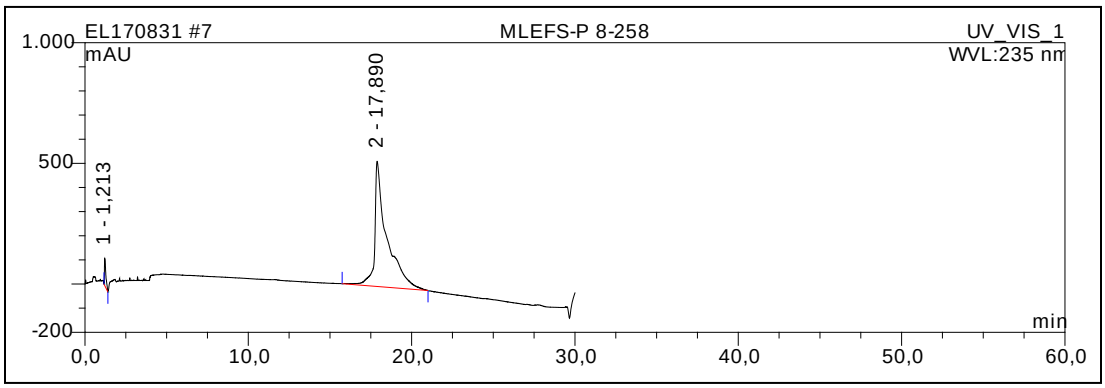


Figure 4.9: HPLC Chromatogram of Compound **3**

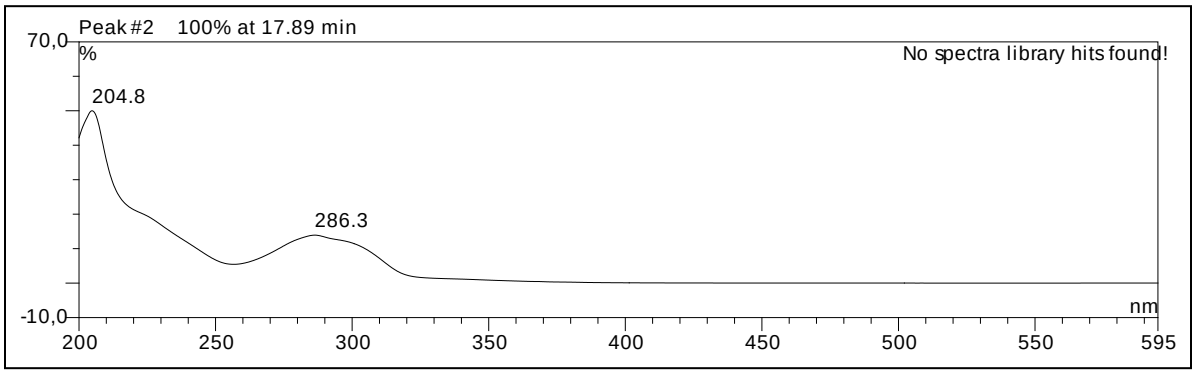


Figure 4.10: Uv spectrum of Compound **3**

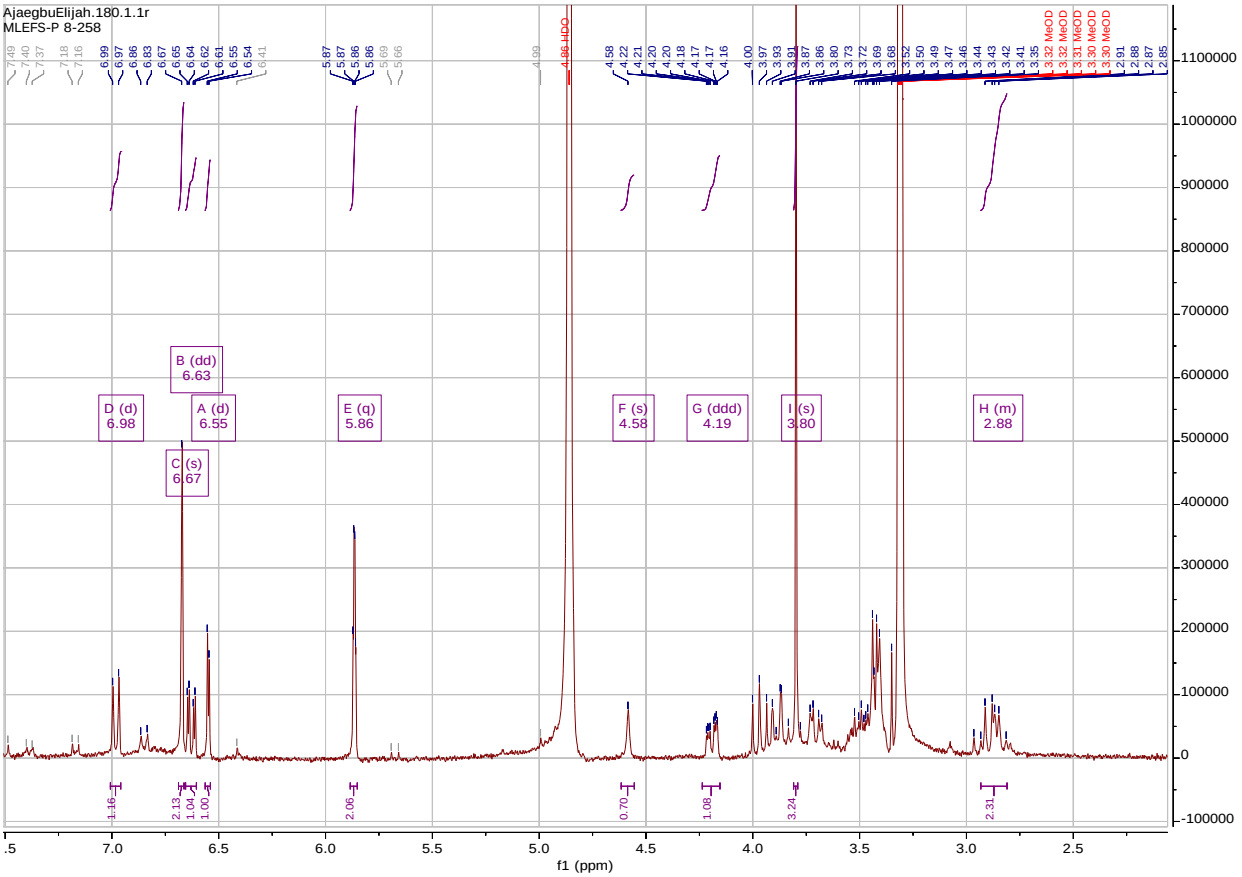


Figure 4.11: [1]HNMR spectrum of Compound **3**

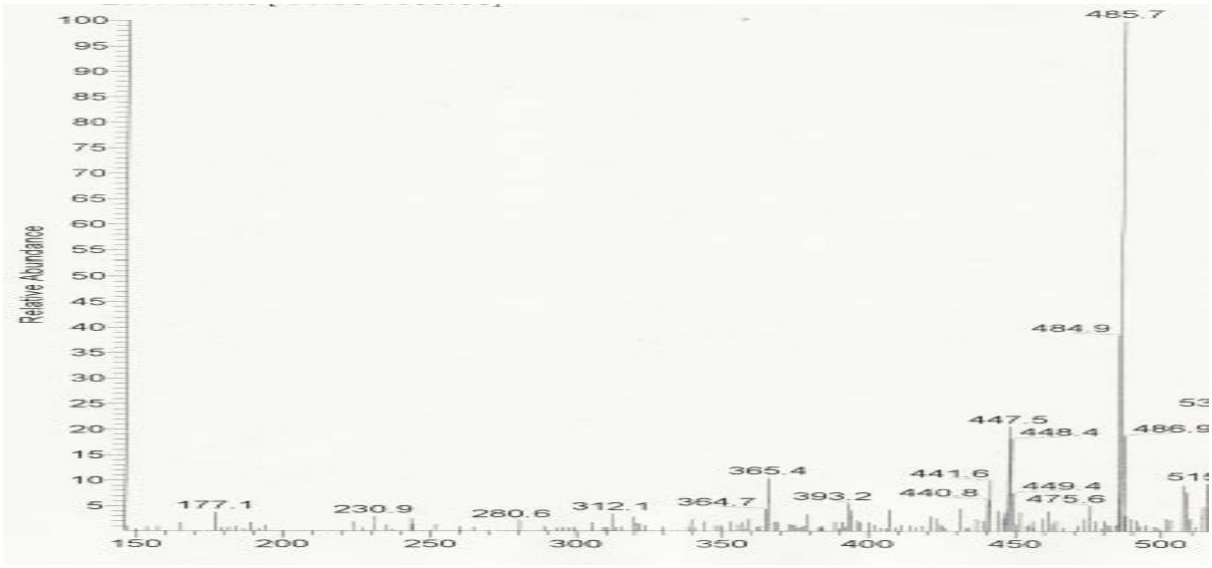
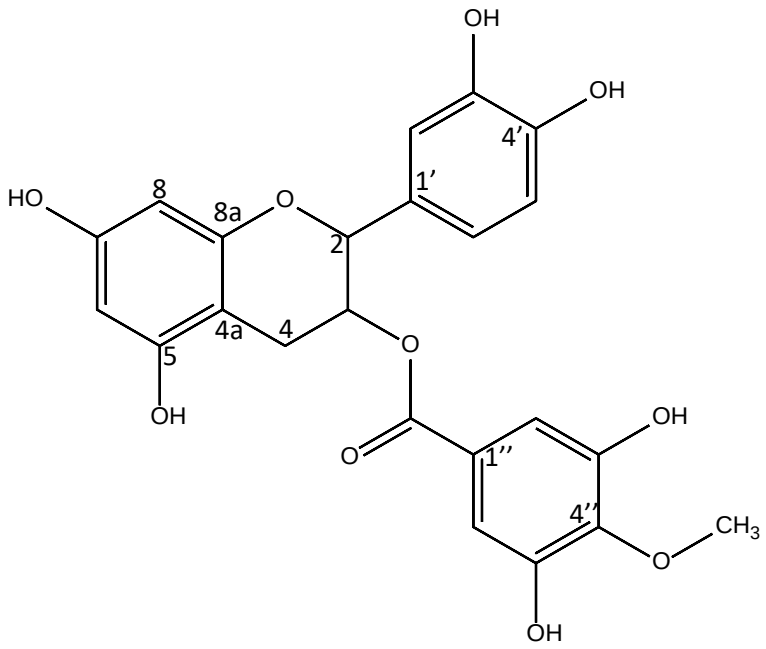


Figure 4.12: ESI-MS spectrum of Compound **3**

4.2.3.1 Structure of Epicatechin-3-O-(4”-methyl gallate) (compound 3, known)



Molecular formula	$C_{23}H_{20}O_{10}$
Molecular weight	456.40
Amount	5 mg

Compound **3** was isolated from the leaf of *Millettia aboensis* as a brown oil. It showed UV (MeOH) λ_{max} 217.5, 228.7, and 285.6 (see Figure 4.10), which is suggestive of an epicatechin nucleus, and $[\alpha]^{20}_{D}$ -36.48 (c, 2.4, MeOH). The ESI-MS showed the pseudomolecular ion peak at *m/z* 485.7 $[M\pm H^{+}\text{-}CH_3O^{-}]$ (see Fig. 4.12). The molecular formula was deduced as $C_{23}H_{20}O_{10}$ based on the observed MS. The ^{1}H NMR spectrum measured in CD_3OD (Figure 4.11) shows aromatic proton signals of characteristic ABX coupling pattern of ring B assigned to δ 6.98 (d, *J* = 8.4 Hz, H-3’), 6.63 (dd, *J* = 8.4, 2.5 Hz, H-2’) and 6.55 (d, *J* = 2.5 Hz, H-6’), and a pair of doublets with coupling constants of 8.4 Hz typical of ortho-coupled protons (H-4 and H-5). From the ^{1}H NMR spectrum, two aromatic protons at δ 5.86 (q, J=1.1, H-8, H-6), and 6.67 (br s, H-2’’, H-6’’); three methylene

groups at δ 4.58 (s, H-2), 4.19 (ddd, J=10.4, 3.4, 1.6 Hz) and 2.88 (m, Ha/Hb-4) were inferred to be present. One methyl resonance at δ 3.80 (s) indicated an aromatic O-methyl group. Assignment of the O-methyl group at C-4 was determined from the methyl singlet at δ 3.80, compound **3** was established as epicatechin-3-O-(4”-methyl gallate), which has been isolated before by Kazuaki *et al.*, 2000.

Table 4.7: Proton NMR spectroscopic data of compound **3** (CD_3OD, 300 MHz)

Position	**δ_H (J in Hz)**	**Lit. Value – in Acetone-d6 (Kazuaki *et al.*, 2000.)**
2	4.58 (s)	5.07 (s)
3	4.19 (ddd, 10.4, 3.4, 1.6)	5.51 (m)
4a	2.88 (m)	2.92 dd (2.0, 17.0)
4b	2.88 (m)	3.03 dd (4.4, 17.0)
5	-	-
6	5.86 (q)	6.03 (d, 2.5)
7	-	-
8	5.86 (q)	6.01 (d, 2.5)
9	-	-
10	-	-
1’	-	-
2’	6.63 (dd, 8.4, 2.5)	6.64 (s)
3’	6.98 (d, 8.4)	-
4’	-	-
5’	-	-
6’	6.55 (d, 2.5)	6.64 (s)
1’’	-	-
2’’	6.67 (br s)	7.01 (s)
3’’		-
4’’- OCH_3	3.80 (s)	3.81 (s)
5’’	-	-
6’’	6.67 (br s)	7.01 (s)

4.2.4. Protocatechuic acid (compound 4, known)

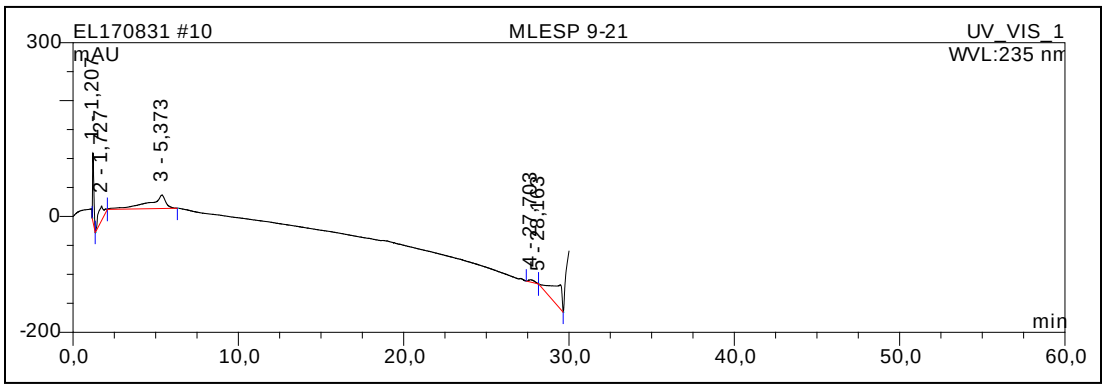


Figure 4.13: HPLC Chromatogram of Compound **4**

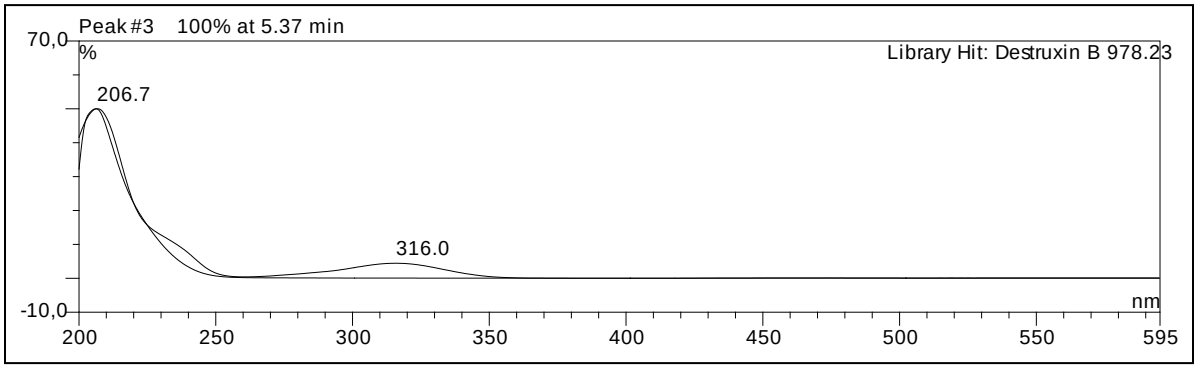


Figure 4.14: Uv spectrum of Compound **4**

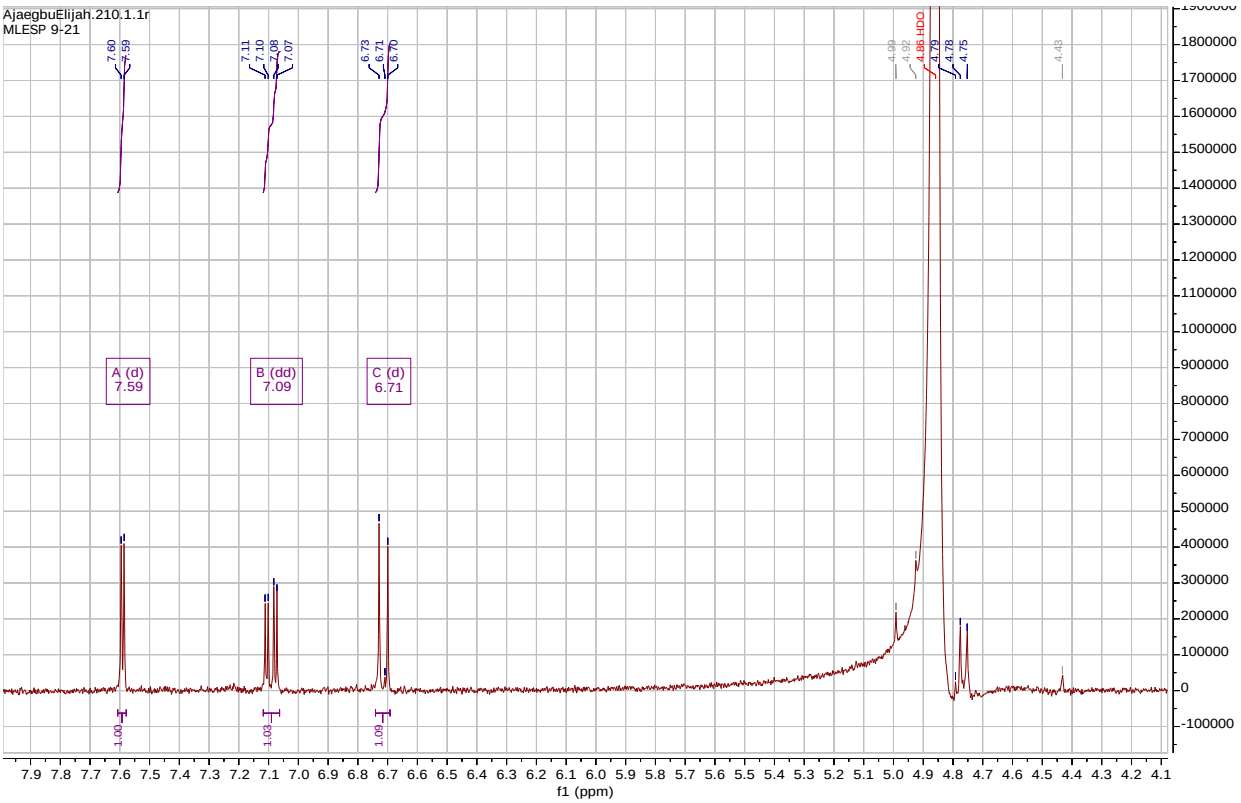


Figure 4.15: ^{1}HNMR spectrum of Compound **4**

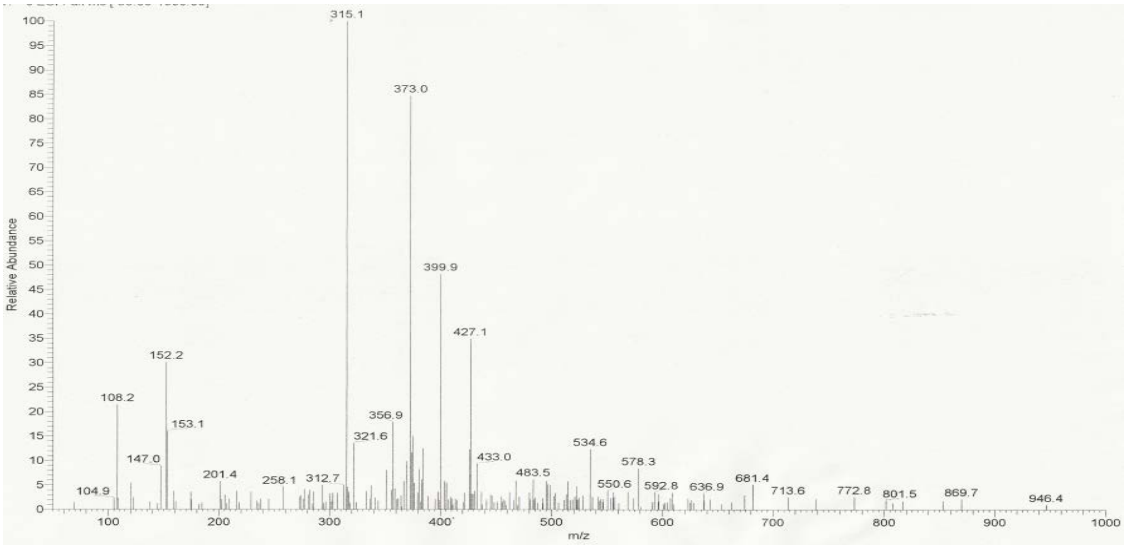
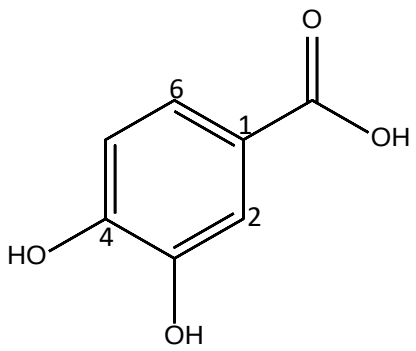


Figure 4.16: ESI-MS spectrum of Compound **4**

4.2.4.1 Structure of Protocatechuic acid (compound 4, known)



Molecular formula	$C_7H_6O_4$
Molecular weight	154.12
Amount	2 mg

Compound **4** was isolated from the leaf of *Millettia aboensis* as a yellow oil. It showed UV (MeOH) λ_{max} 206.7, and 316 (see Figure 4.14), which is suggestive of a protocatechuic acid and $[\alpha]^{20}_{D}$ -4.6 (c, 0.8, MeOH). The ESI-MS showed the pseudomolecular ion peak at *m/z* 315.1 [2M+H$^+$] (see Fig. 4.16). The fragment ions peak occurred at *m/z* 152.2 [M-2H$^+$], and also at *m/z* 153.3 [M-H$^+$]. The molecular formula was deduced as $C_7H_6O_4$ based on the observed MS. The ^{1}H NMR spectrum measured in CD_3OD (Figure 4.15) shows an aromatic proton signals of characteristic ABX coupling pattern assigned to δ 6.71 (d, *J* = 8.8 Hz, H-5), 7.09 (dd, *J* = 8.8, 3.2 Hz, H-6) and 7.59 (d, *J* = 3.1 Hz, H-2), and a pair of doublets with coupling constants of 8.8 Hz typical of ortho-coupled protons (H-5 and H-6), compound **4** was established as protocatechuic acid, which has been isolated (Li *et al.*, 2017, Rui *et al*., 2017).

Table 4.8: Proton NMR spectroscopic data of compound **4** (CD_3OD, 300 MHz)

Position	**δ_H (J in Hz)**	**Lit. Value – in CD_3OD (Rui *et al.*, 2017)**
1	-	-
2	7.59 (d, 3.1)	7.45 (d, 1.8)
3	-	-
4	-	-
5	6.71 (d, 8.8)	6.81 (dd, 8.1)
6	7.09 (dd, 8.8, 3.2)	7.43 (dd, 8.1, 2.1)

4.2.5. Gallic acid (compound 5, known)

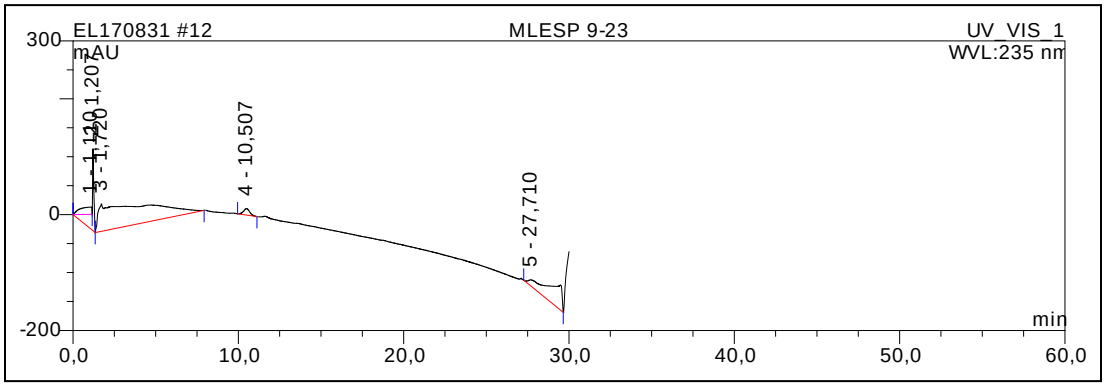


Figure 4.17: HPLC Chromatogram of Compound **5**

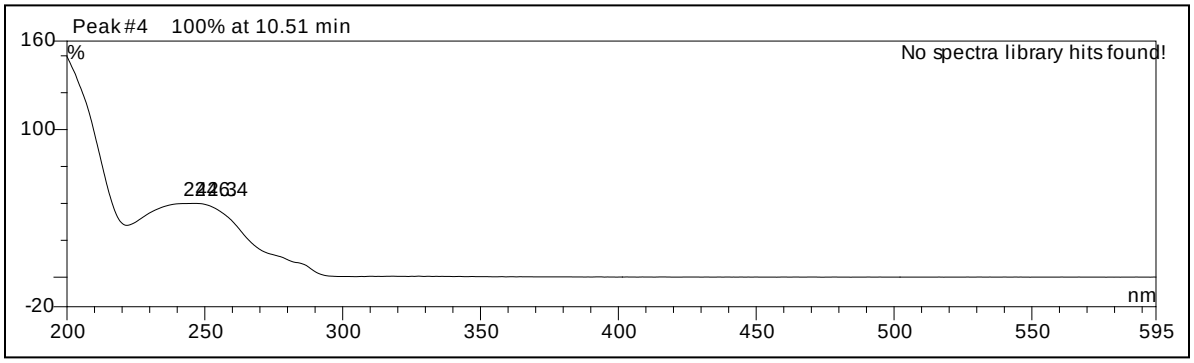


Figure 4.18: Uv spectrum of Compound **5**

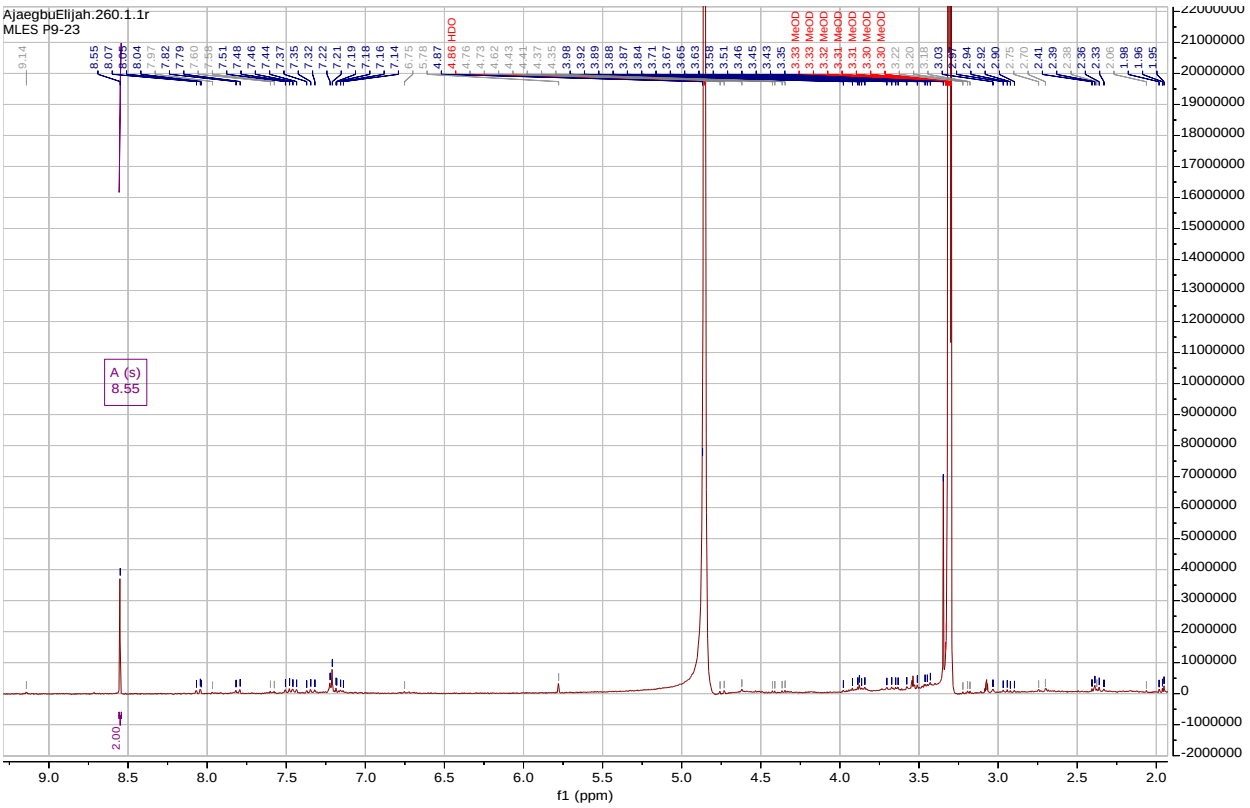
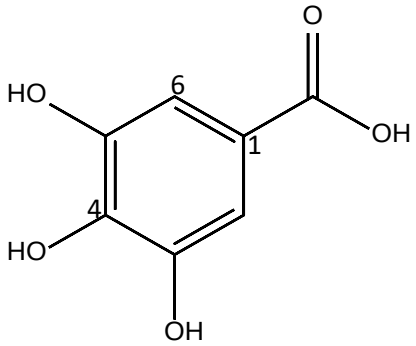


Figure 4.19: [1]HNMR spectrum of Compound **5**

4.2.5.1 Structure of Gallic acid (compound 5, known)



Molecular formula	$C_7H_6O_5$
Molecular weight	170.12
Amount	1 mg

Compound **5** was isolated from the leaf of *Millettia aboensis* as a yellow oil. It showed UV (MeOH) λ_{max} 222.6, and 223.4 (see Figure 4.18), which is suggestive of a gallic acid nucleus and $[\alpha]^{20}_{D}$ +3.8 (c, 0.4, MeOH). The mass of the compound was deduced as 170.12 using ChemBioDrawUltra. The molecular formula was deduced as $C_7H_6O_5$ based on the calculated mass of the compound. The ^{1}H NMR spectrum measured in CD_3OD (Figure 4.19) showed an aromatic proton at δ 8.55 (br s, H-2, H-6), compound **7** was established as methyl gallate, which has been isolated (Rashed *et al.*, 2014).

Table 4.9: Proton NMR spectroscopic data of compound **5** (CD_3OD, 300 MHz)

Position	**δ_H (J in Hz)**	**Lit. Value – in DMSO-d6 (Rashed *et al.*, 2014)**
1	-	-
2	8.55 (br s)	7.12 (s)
3	-	-
4	-	-
5	-	-
6	8.55 (br s)	7.12 (s)

4.3. Compounds isolated from the roots of *Millettia aboensis*

4.3.1. Derrisisoflavone G (compound 6, known)

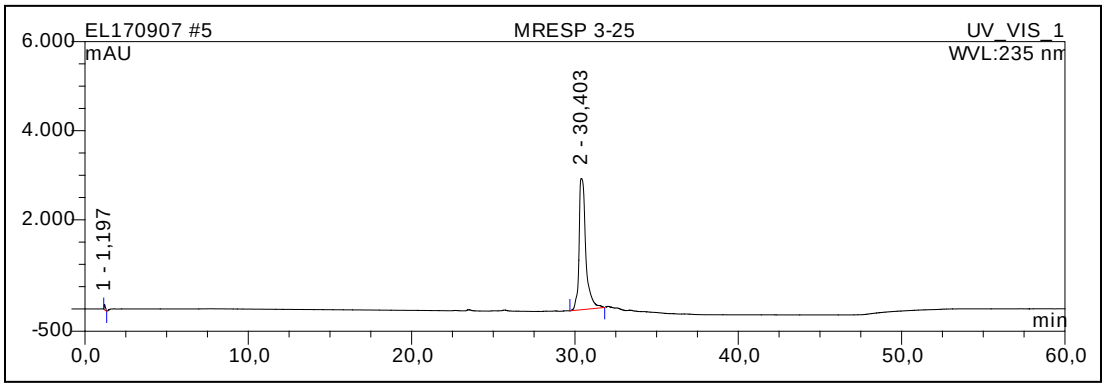


Figure 4.20: HPLC Chromatogram of Compound **6**

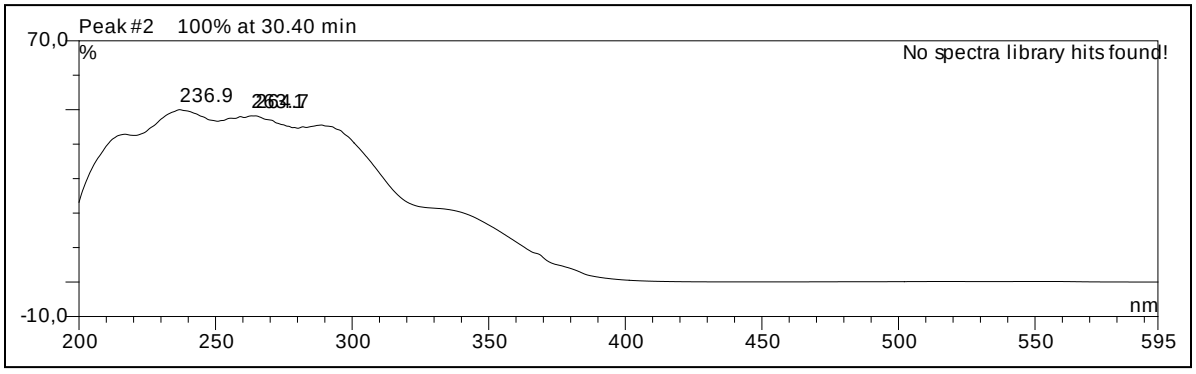


Figure 4.21: Uv spectrum of Compound **6**

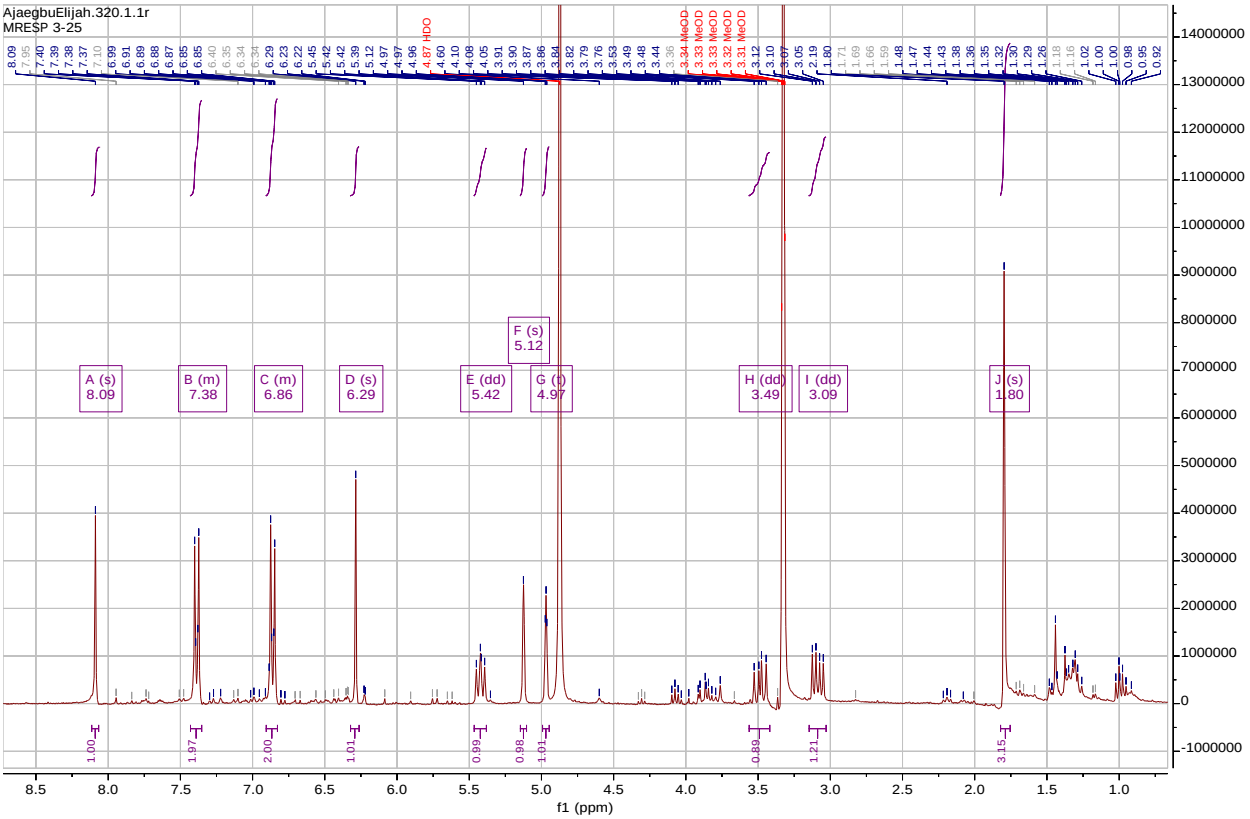


Figure 4.22: [1]HNMR spectrum of Compound **6**

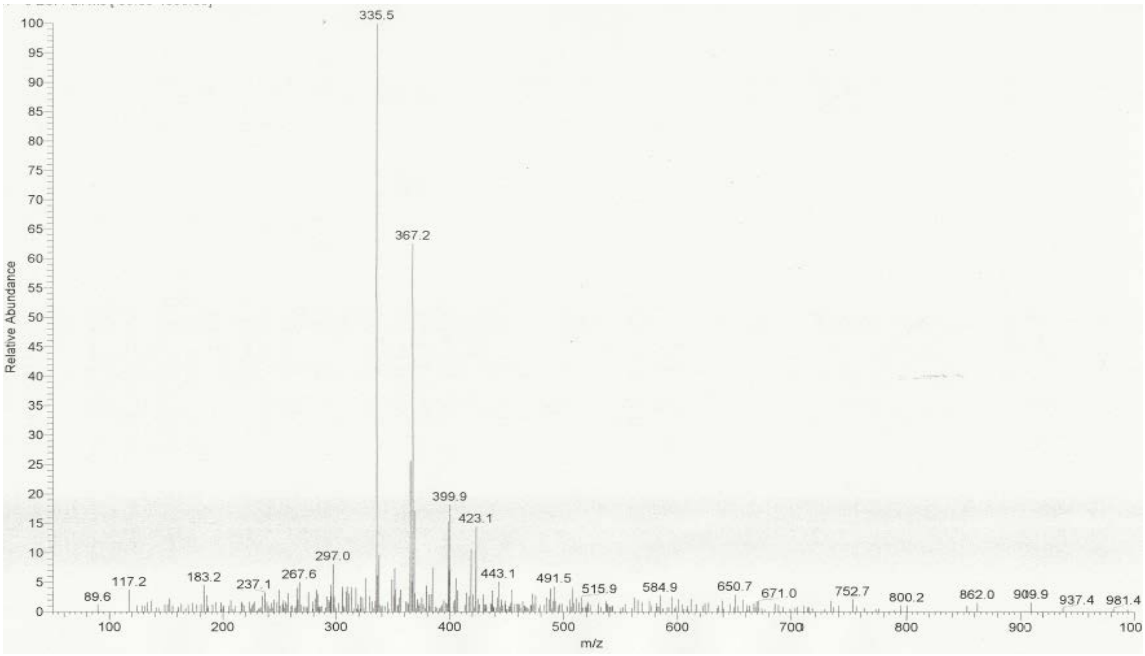
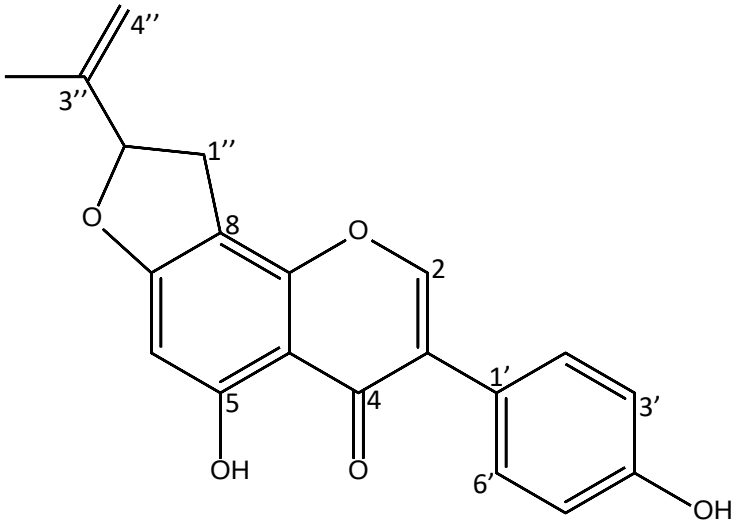


Figure 4.23: ESI-MS spectrum of Compound **6**

4.3.1.1 Structure of Derrisisoflavone G (compound 6, known)



Molecular formula	$C_{20}H_{16}O_5$
Molecular weight	336.34
Amount	2.3 mg

Compound **6** was isolated from the root of *Millettia aboensis* as a milky or white solid. It showed UV (MeOH) λ_{max} 236.9, 263.1, and 264.7 (see Figure 4.21), which is suggestive of a derrisisoflavone nucleus and $[\alpha]^{20}_{D}$ -19.84 (c, 0.92, MeOH). The ESI-MS showed the pseudomolecular ion peak at *m/z* 335.5 $[M\text{-}H^+]$ (see Fig. 4.23). The fragment ion peak occurred at *m/z* 367 $[M\pm H^+ + CH_3O^-]$, indicating the presence of acetylation (an acetyl group). The molecular formula was deduced as $C_{20}H_{16}O_5$ based on the observed MS. The ^{1}H NMR spectrum (see Fig. 4.22) of compound **6** shows a proton singlet at δ 8.09 (s, H-2), characteristic of an isoflavone and four aromatic proton signals for ring B protons of AA'BB' system at δ 7.38 (m, H-2', H-6'), and 6.86 (m, H-3', H-5'), due to 1', 4'-disubstituted B-ring. It also revealed an isopropenyl-dihydrofuran proton signals at δ 1.80 (s, CH_3-5"), 3.09 (dd, J = 7.6, 15.2 Hz, Ha-1"), 3.49 (dd, J = 9.7, 15.2 Hz, Hb-1"), 4.97 (d, J = 1.6 Hz, Ha-4"), 5.12 (d, J = 1.6 Hz, Hb-4") characteristic of

terminal alkene, and 5.42 (dd, J = 7.7, 9.5 Hz, H-2”). The signal at 6.29 (s) was assigned to H-6 of the ring A. The absence of signal for H-8 also supported the location of the furan ring at the position, compound **6** was established as derrisisoflavone G, which has been isolated (Lin *et al.*, 2016).

Table 4.10: Proton NMR spectroscopic data of compound **6** (CD_3OD, 300 MHz)

Position	**δ_H (J in Hz)**	**Lit. Value – in Me_2CO-d6 (Lin *et al.*, 2016)**
2	8.09 (s)	8.18 (s)
3	-	
4	-	
5	-	
6	6.29 (s)	6.27 (s)
7	-	-
8	-	-
9	-	-
10	-	-
1’	-	-
2’	7.38 (m)	7.44 (dd, 2.4, 6.6)
3’	6.86 (m)	6.90 (dd, 2.4, 6.6)
4’	-	-
5’	6.86 (m)	6.90 (dd, 2.4, 6.6)
6’	7.38 (m)	7.44 (dd, 2.4, 6.6)
Ha-1”	3.09 (dd, 7.6, 15.2)	3.10 (dd, 7.2, 15.6)
Hb-1”	3.49 (dd, 9.7, 15.2)	3.51 (dd, 9.6, 15.6)
2’’	5.42 (dd, 7.7, 9.5)	5.46 (dd, 7.2, 9.6)
3’’	-	-
Ha-4”	4.97 (d, 1.6)	4.95 (d, 1.2)
Hb-4”	5.12 (d, 1.6)	5.12 (d, 1.2)
5’’- CH_3	1.80 (s)	1.78 (s)

4.3.2. 3, 8-dimethoxypterocarpan (compound 7, new)

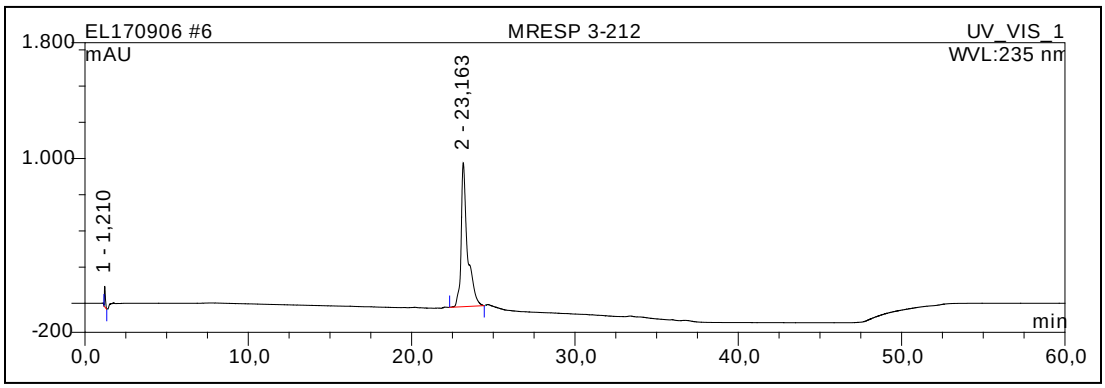


Figure 4.24: HPLC Chromatogram of Compound **7**

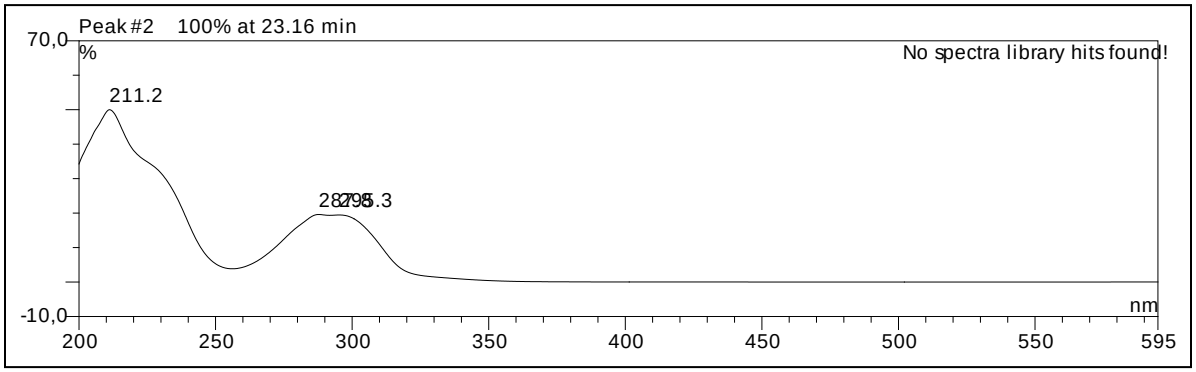


Figure 4.25: Uv spectrum of Compound **7**

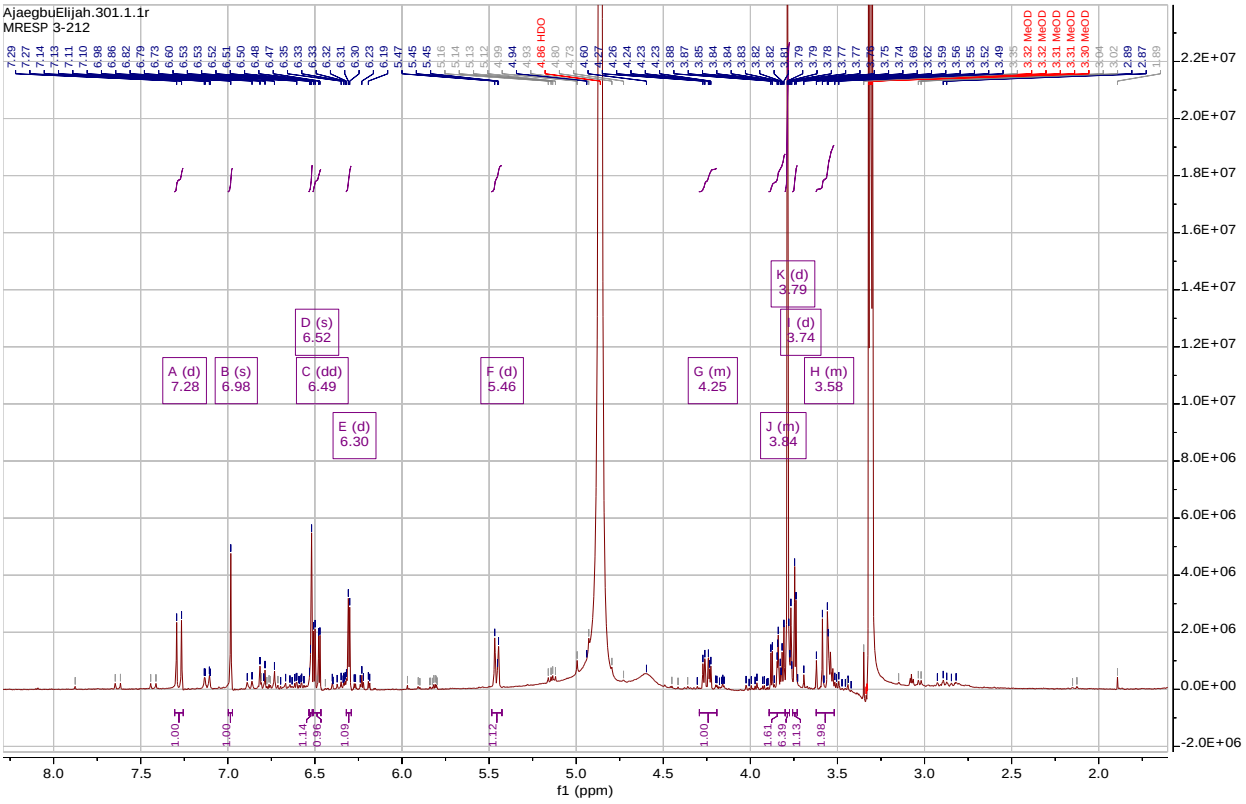


Figure 4.26: [1]HNMR spectrum of Compound **7**

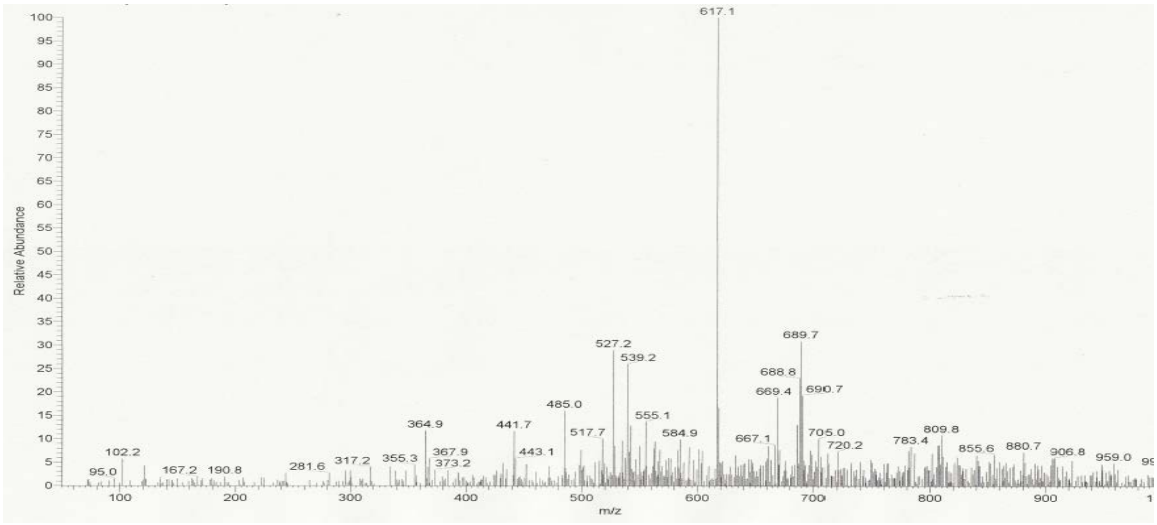
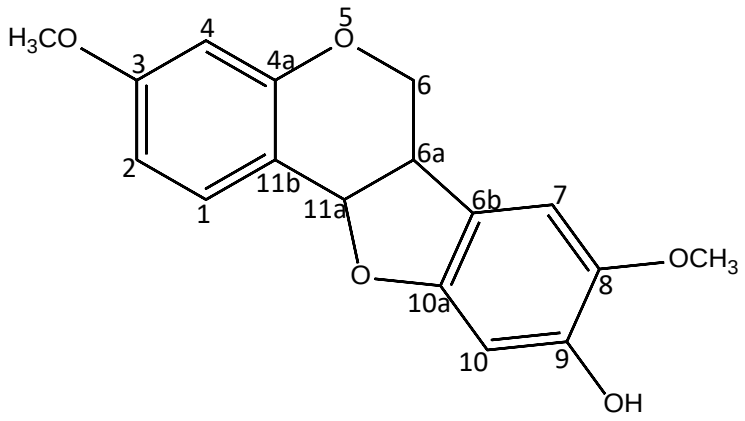


Figure 4.27: ESI-MS spectrum of Compound **7**

4.3.2.1 Structure of 3, 8-dimethoxypterocarpan (compound 7, new)



Molecular formula	$C_{17}H_{16}O_5$
Molecular weight	300.31
Amount	1.5 mg

Compound **7** was isolated from the root of *Millettia aboensis* as a brown solid. It showed UV (MeOH) λ_{max} 211.1, 287.6 and 295.3 (see Figure 4.25), which is suggestive of a pterocarpan nucleus and $[\alpha]^{20}_{D}$ -47.17 (c, 0.6, MeOH). The ESI-MS showed the pseudomolecular ion peak at *m/z* 617.1 [2M+OH^-] (see Fig. 4.27). The fragment ion peak occurred at *m/z* 539.2 [2M±H^+-2CH_3O^-], indicating the presence of acetylation (an acetyl group). The molecular formula was deduced as $C_{17}H_{16}O_5$ based on the observed MS. The ^{1}H NMR spectrum of compound **7** (see Figure 4.26) shows aromatic signals of characteristic ABX coupling pattern assigned to δ 7.28 (d, J=8.4 Hz, H-1), 6.49 (dd, J=8.4, 2.4 Hz, H-2), and 6.30 (d, J=2.4 Hz, H-4), and two meta coupled aromatic singlets at δ 6.98 (s, H-10) and 6.52 (s, H-7), suggested that compound **7** to be a monosubstituted pterocarpan. The ^{1}H NMR spectrum of compound **7** also revealed one methine proton at δ 5.46 (d, J=6.5 Hz, H-11a), three methylene protons at δ 4.25 (m, H-6a), 3.58 (m, Ha-6) and 3.84 (d, Hb-6); two methoxy groups at 3.79 (d, J=0.9 Hz) and 3.74 (d, J=2.4 Hz), and based

on the above information, compound **7** was characterized to be 3, 8-dimethoxypterocarpan. This is a new compound to the best of my knowledge.

Table 4.11: Proton NMR spectroscopic data of compound **7** (CD_3OD, 300 MHz)

Position	**δ_H (J in Hz)**
1	7.28 (d, 8.4)
2	6.49 (dd, 8.4, 2.4)
3 – OCH_3	3.79 (d, 0.8)
4	6.30 (d, 2.4)
5	-
Ha-6	3.58 (m)
Hb-6	3.84 (m)
6a	4.25 (m)
7	6.52 (s)
8 – OCH_3	3.74 (d, 2.4)
9	-
10	6.98 (s)
11a	5.46 (d, 6.5)

4.3.3. Derrisisoflavone M (compound 8, new)

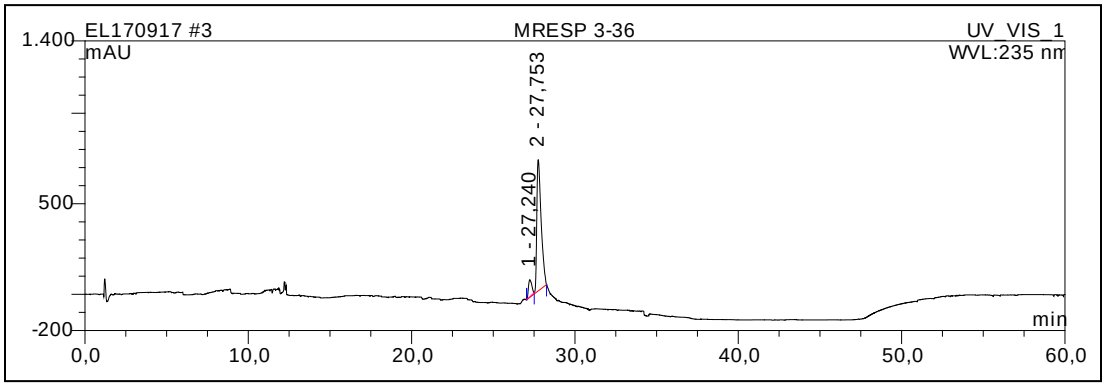


Figure 4.28: HPLC Chromatogram of Compound **8**

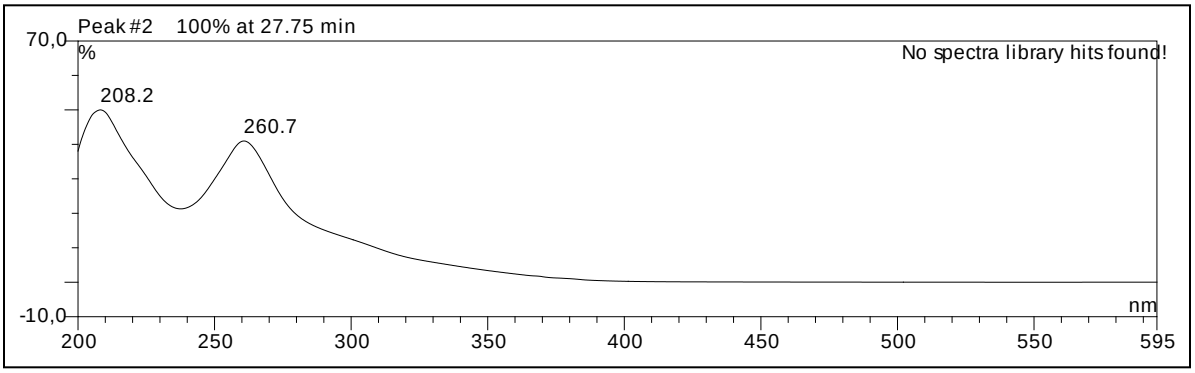


Figure 4.29: Uv spectrum of Compound **8**

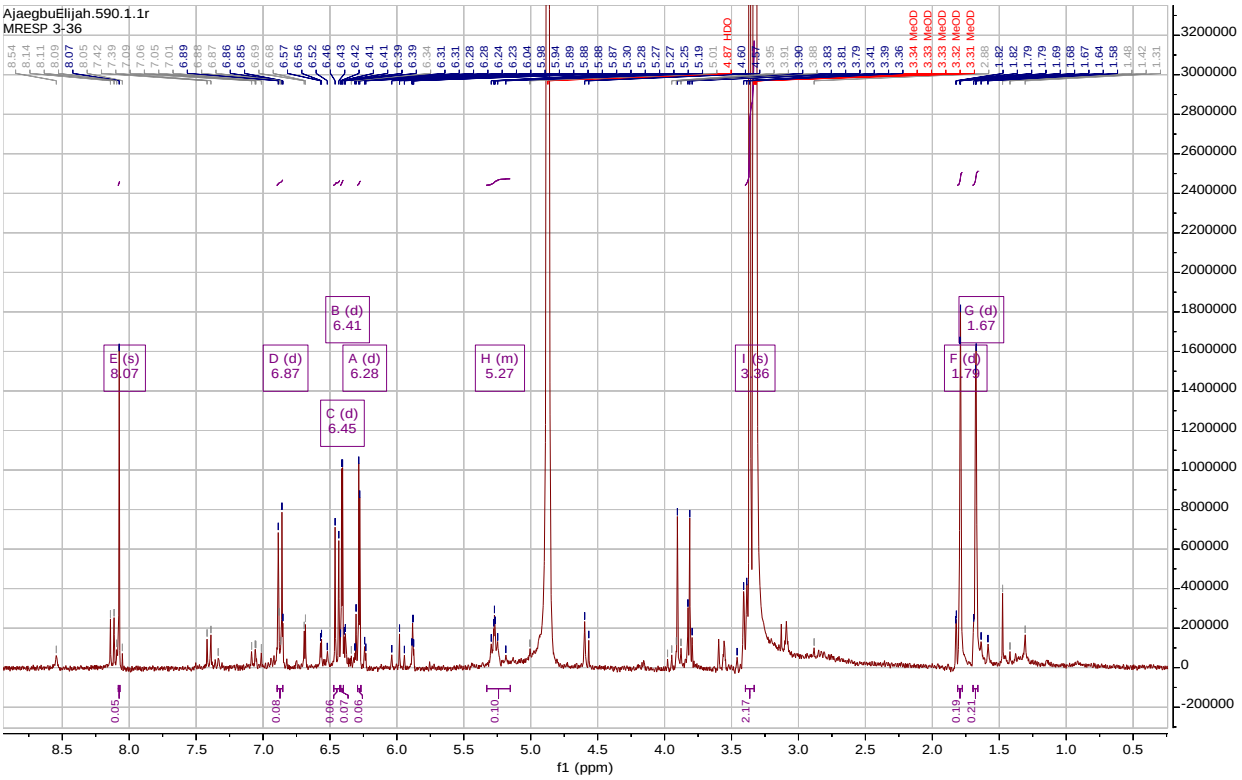


Figure 4.30: [1]HNMR spectrum of Compound **8**

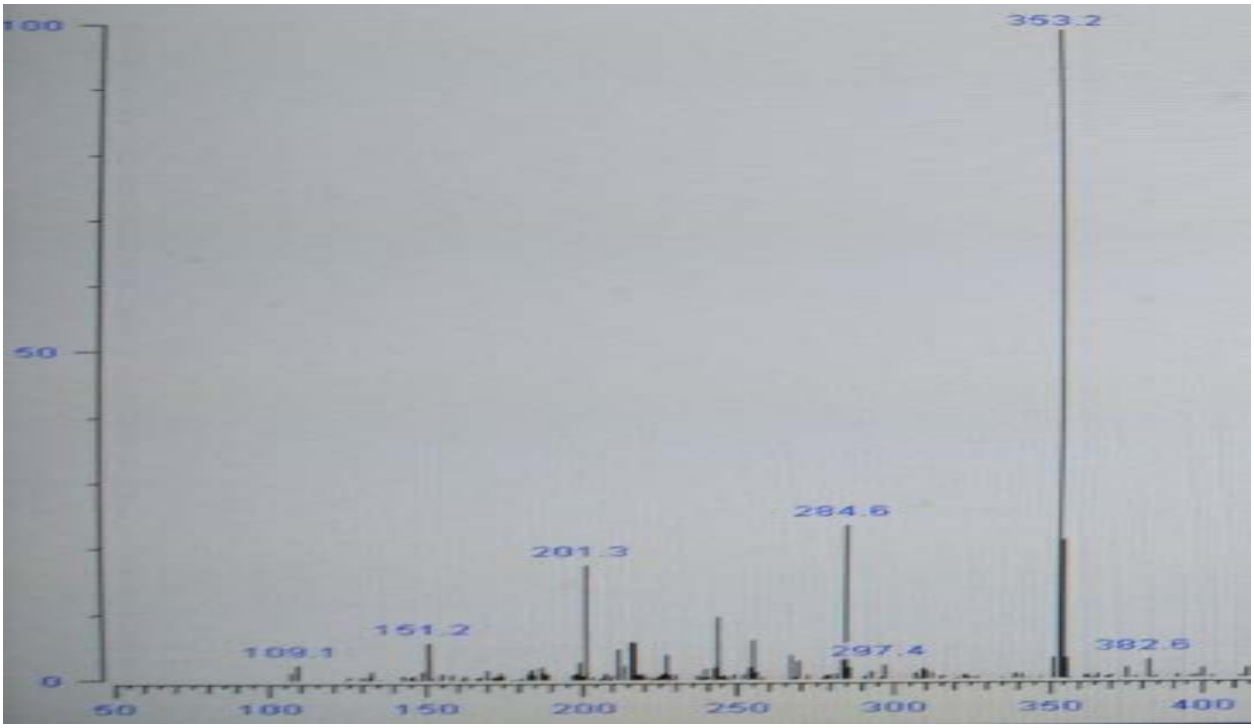
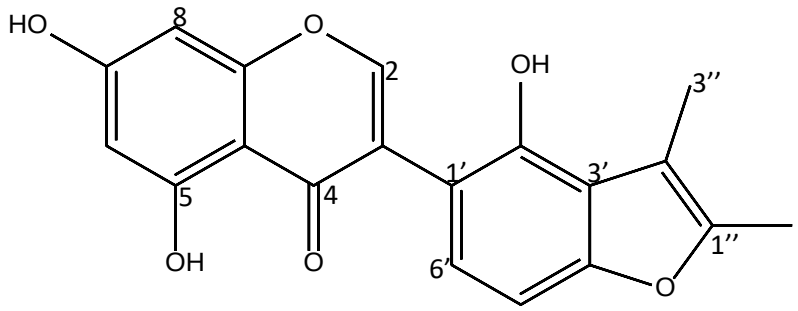


Figure 4.31: ESI-MS spectrum of Compound **8**

4.3.3.1 Structure of Derrisisoflavone M (compound 8, new)



Molecular formula	$C_{19}H_{14}O_6$
Molecular weight	338.08
Amount	1.2 mg

Compound **8** was isolated from the root of *Millettia aboensis* as a yellow oil. It showed UV (MeOH) λ_{max} 208.2, and 260.7 (see Figure 4.29), which is suggestive of a derrisisoflavone nucleus and $[\alpha]^{20}_D$ +9.33 (c, 0.4, MeOH). The ESI-MS showed the pseudomolecular ion peak at *m/z* 353.2 [M+CH_3] (see Fig. 4.31). The fragment ion peak occurred at *m/z* 284.6 [M-3OH^-], indicating the presence of hydroxylation (three molecules of hydroxyl group). The molecular formula was deduced as $C_{19}H_{14}O_6$ based on the observed MS. The ^{1}H NMR spectrum (see Fig. 4.30) of compound **8** shows a proton singlet at δ 8.05 (s, H-2), characteristic of an isoflavone and ortho-coupled aromatic proton signals for ring A at δ 6.87 (d, J = 8.3 Hz, H-6), and 6.45 (d, J=8.3 Hz, H-8). It also displayed meta coupled protons for ring B at δ 6.41 (d, J=2.2 Hz, H-5'), and 6.28 (d, J=2.2 Hz, H-6'). It also revealed an isopropenyl furan proton signals at δ 1.79 (d, 1.3, CH_3-3"), 1.67 (d, 1.4, CH_3-4"), 3.36 (s, H-1"), and 5.27 (m, H-2"). The absence of signal for H-3'' also

supported the location of the furan ring at the position, compound **8** was established to be derrisisoflavone M (derivative of derrisisoflavone G – compound **6**), being a new compound.

Table 4.12: Proton NMR spectroscopic data of compound **8** (CD_3OD, 300 MHz)

Position	δ_H (J in Hz)
2	8.05 (s)
3	-
4	-
5	-
6	6.87 (d, 8.3)
7	-
8	6.45 (d, 8.3)
9	-
10	-
1’	-
2’	-
3’	-
4’	-
5’	6.41 (d, 2.2)
6’	6.28 (d, 2.2)
1”	3.36 (s)
2’’	5.27 (m)
3”	1.79 (d, 1.3)
4’’	1.67 (d, 1.4)

4.3.4. 4’-methoxy orobol (compound 9, new)

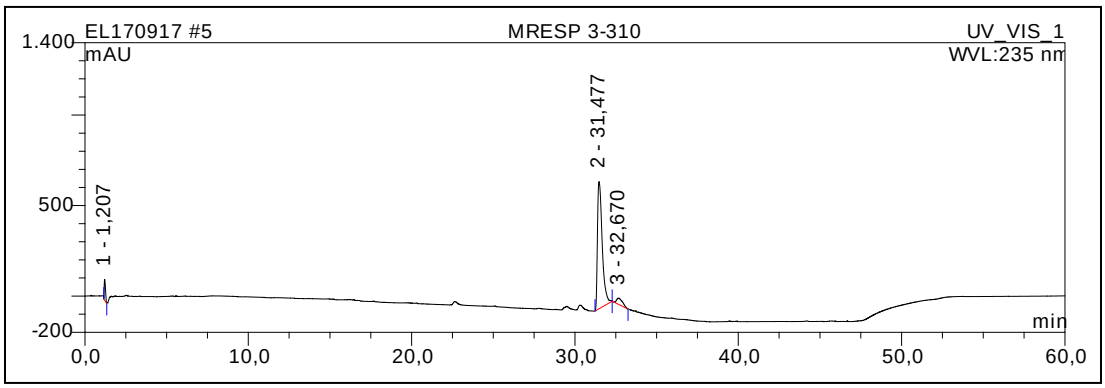


Figure 4.32: HPLC Chromatogram of Compound **9**

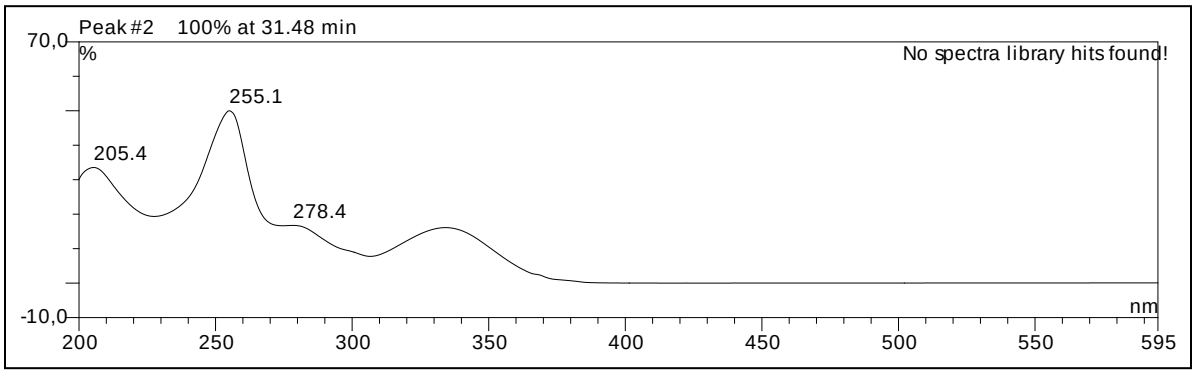


Figure 4.33: Uv spectrum of Compound **9**

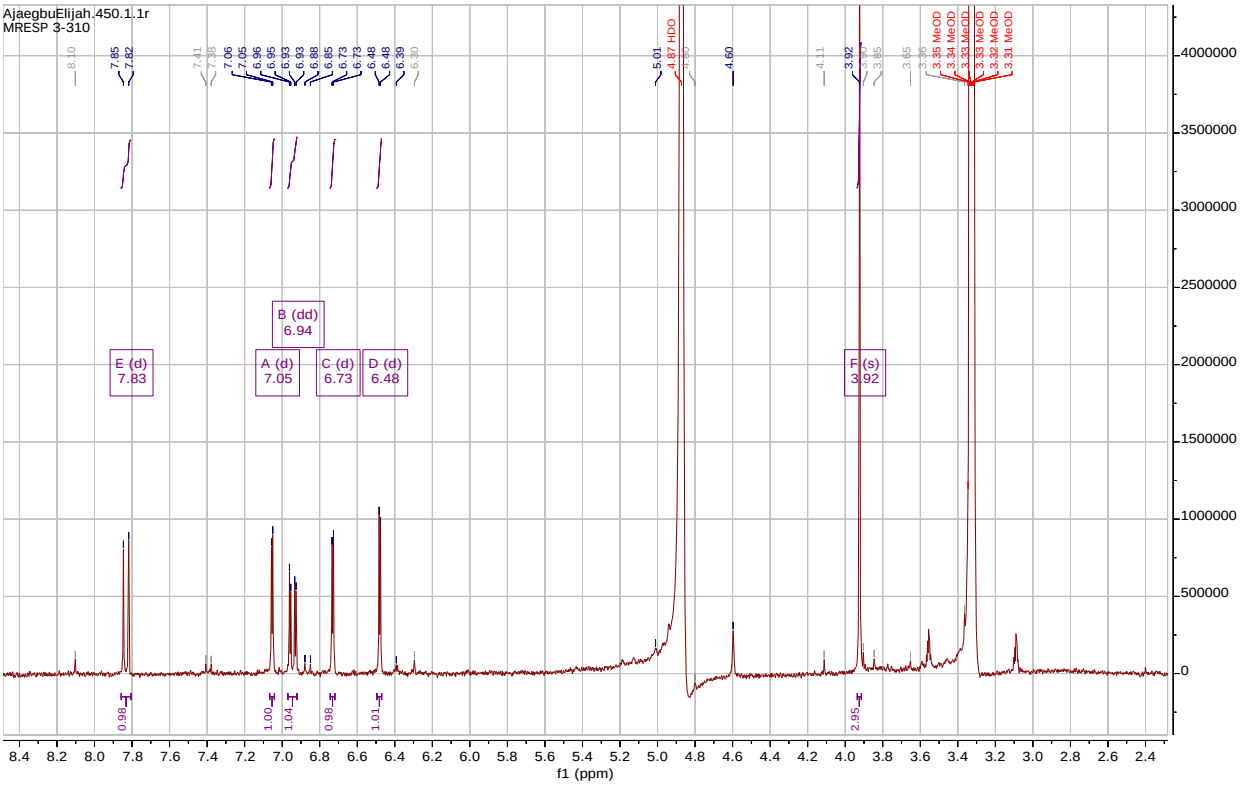


Figure 4.34: [1]HNMR spectrum of Compound **9**

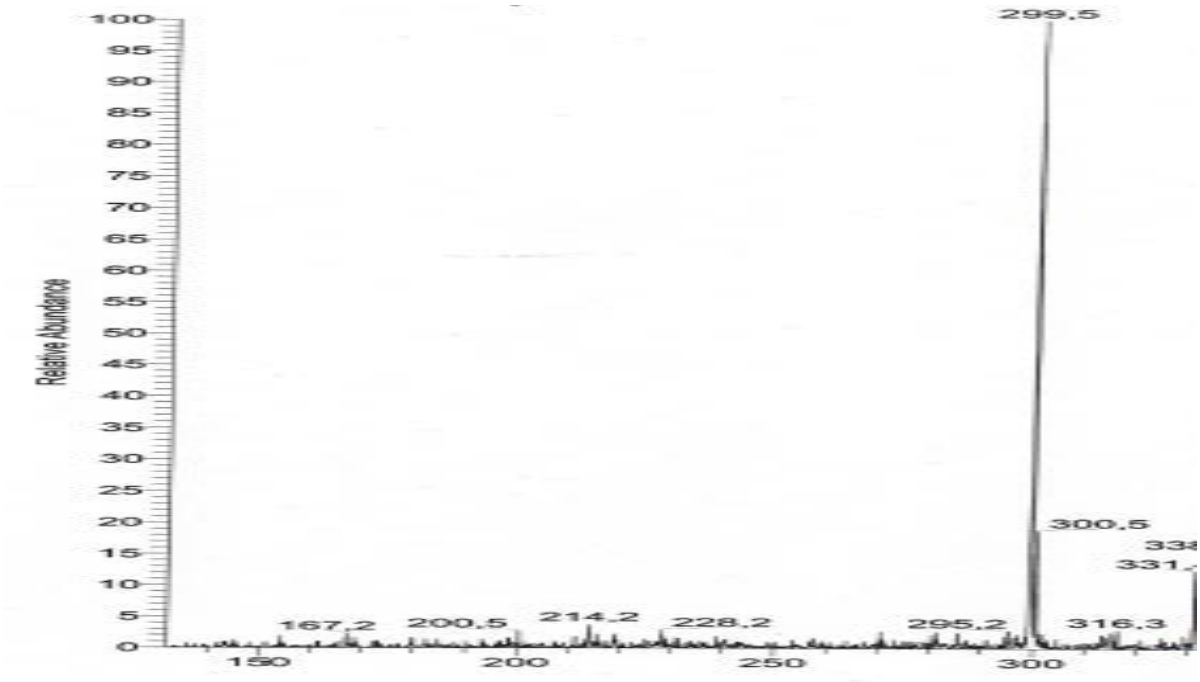
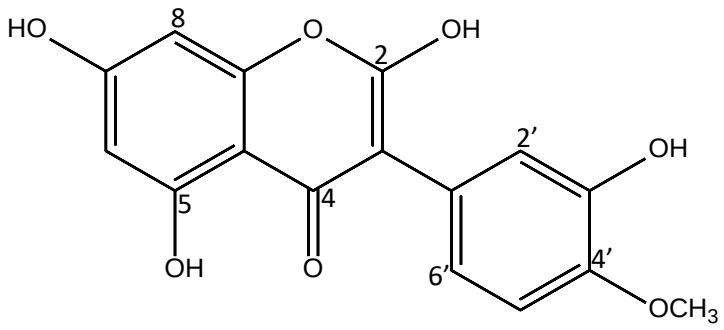


Figure 4.35: ESI-MS spectrum of Compound **9**

4.3.4.1 Structure of 4’-methoxy orobol (compound 9, new)



Molecular formula	$C_{16}H_{12}O_7$
Molecular weight	316.27
Amount	1.1 mg

Compound **9** was isolated from the root of *Millettia aboensis* as a milky solid. It showed UV (MeOH) λ_{max} 205.4, 255.1 and 278.4 (see Figure 4.33), which is suggestive of an orobol nucleus and $[\alpha]^{20}_{D}$ -1.31 (c, 0.4, MeOH). The ESI-MS showed the pseudomolecular ion peak at *m/z* 299.5 [M-OH^-] (see Fig. 4.35). The fragment ion peak occurred at *m/z* 300.6 [M±H^+-OH^-], indicating the presence of hydroxylation (hydroxyl group). The molecular formula was deduced as $C_{16}H_{12}O_7$ based on the observed MS. The ^{1}H NMR spectrum of compound **9** (see Figure 4.34) shows two *ortho* coupling protons of ring A which resonated at δ 6.73 (d, 2.3) and at 6.48 (d, 2.3), corresponding to H-6 and H-8 respectively. An aromatic characteristic signals of ABX pattern of ring B was observed at δ 7.83 (d, 8.5, H-5’), 6.94 (dd, 8.5, 2.1, H-6’), and 7.05 (d, 2.1, H-2´). One methoxy singlet resonated at δ 3.92 (OCH_3-4’). The absence of characteristic aromatic proton of isoflavone at H-2 suggesting a substitution with OH, compound **9** was established as 4’-methoxy orobol, and this is the first time its isolation is being reported. Compound **9** is a new (unknown compound) to the best of my knowledge.

Table 4.13: Proton NMR spectroscopic data of compound **9** (CD_3OD, 300 MHz)

Position	**δ_H (J in Hz)**
2	-
3	-
4	-
5	-
6	6.73 (d, 2.3)
7	-
8	6.48 (d, 2.3)
9	-
10	-
1’	-
2’	7.05 (d, 2.1)
3’	-
4’-OCH_3	3.92 (s)
5’	7.83 (d, 8.5)
6’	6.94 (dd, 8.5, 2.1)

4.3.5. 3', 4'-methylenedioxy pterocarpan (compound 10, new)

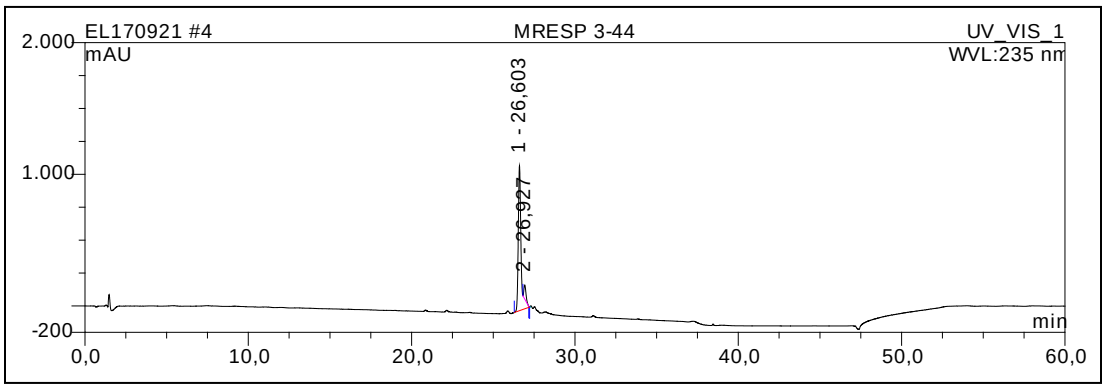


Figure 4.36: HPLC Chromatogram of Compound **10**

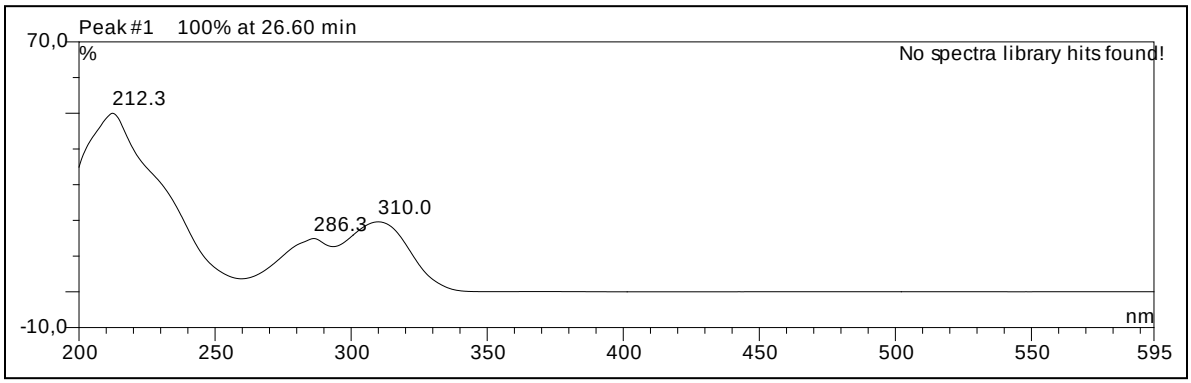


Figure 4.37: Uv spectrum of Compound **10**

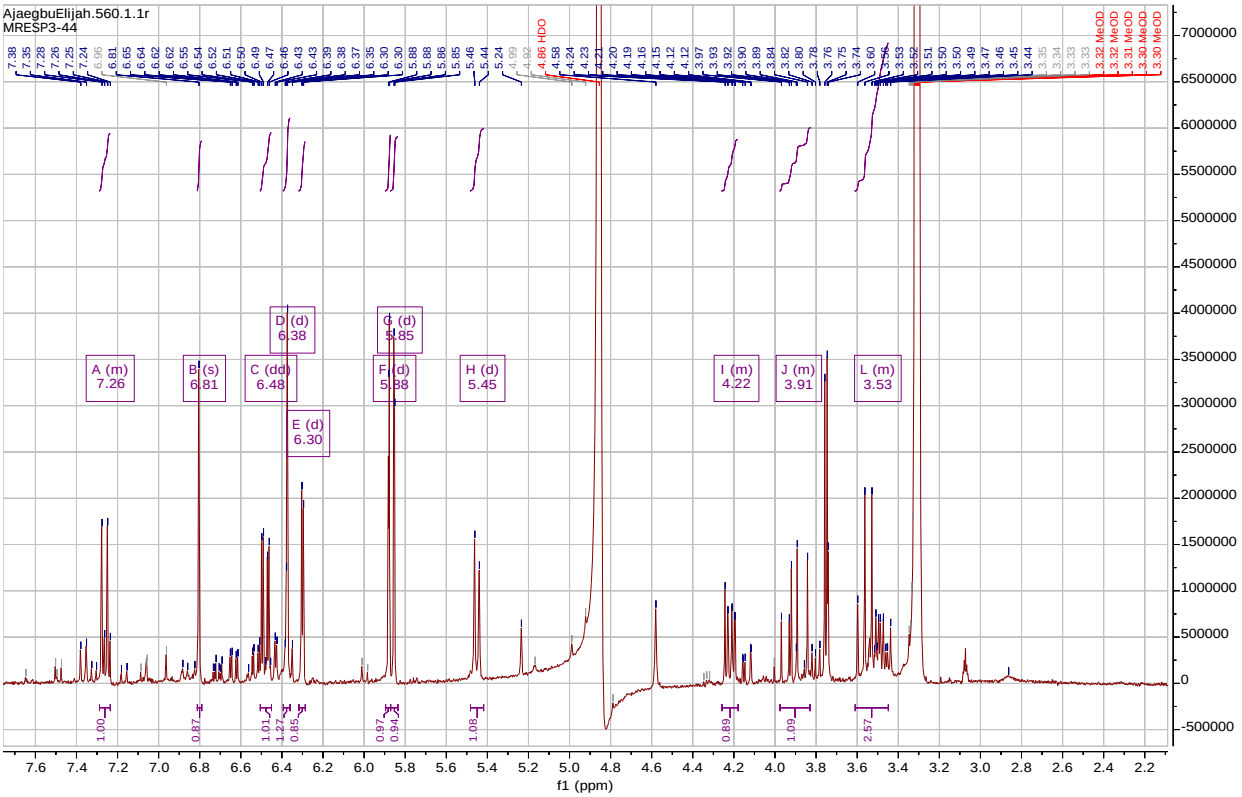
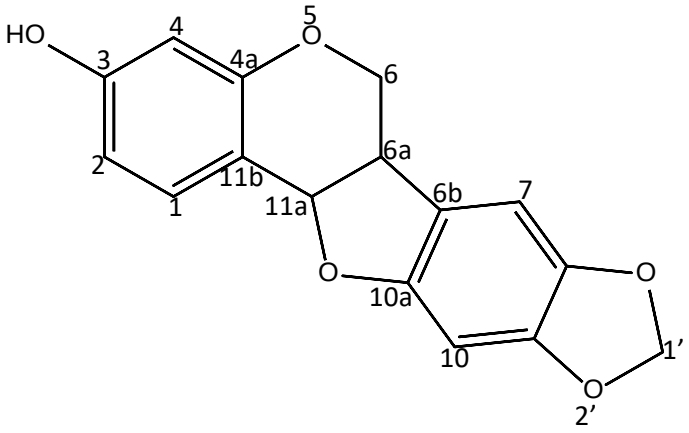


Figure 4.38: [1]HNMR spectrum of Compound **10**

Figure 4.39: ESI-MS spectrum of Compound **10**

4.3.5.1 Structure of 3', 4'-methylenedioxy pterocarpan (compound 10, new)



Molecular formula	$C_{16}H_{12}O_5$
Molecular weight	284.29
Amount	1.4 mg

Compound **10** was isolated from the root of *Millettia aboensis* as a yellow oil. It showed UV (MeOH) λ_{max} 212.3, 286.3 and 310 (see Figure 4.37), which is suggestive of a pterocarpan nucleus and $[\alpha]^{20}_D$ -38.74 (c, 0.56, MeOH). The ESI-MS showed the pseudomolecular ion peak at *m/z* 283.3 [M-H^+] (see Fig. 4.39). The fragment ion peak occurred at *m/z* 315.3 [M±H^+-CH_3O^-], indicating the presence of acetylation (an acetyl group). The molecular formula was deduced as $C_{16}H_{12}O_5$ based on the observed MS. The ^{1}H NMR spectrum of compound **10** (see Figure 4.38) shows aromatic signals of ABX coupling pattern assigned to δ 7.26 (m, H-1), 6.48 (dd, J=8.4, 2.4 Hz, H-2), and 6.30 (d, J=2.4 Hz, H-4), and two aromatic protons at δ 6.81 (s, H-10) and 6.38 (d, J=1.5 Hz, H-7), suggested that compound **10** to be a monosubstituted pterocarpan, with a methylenedioxy (OCH_2O) substituents at δ 5.88 (d, J=1.1 Hz, Ha-1') and 5.85 (d, J=1.1 Hz, Hb-1'). The 1H NMR spectrum of compound **10** also revealed one methine proton at δ 5.45 (d, J=6.6 Hz, H-11a), three methylene protons at δ 4.22 (m, H-6a), 3.53 (m, Ha-6) and 3.91 (m, Hb-6).

Compound **10** has a close resemblance with compound **7** and the similarity lies in the ABX system at δ 7.28 (d, J=8.4 Hz, H-1), 6.49 (dd, J=8.4, 2.4 Hz, H-2), and 6.30 (d, J=2.4 Hz, H-4). But the one of the meta coupling proton is deshielded which may be due to methylenedioxy substituents, and based on the above information, compound **10** was characterized to be 3', 4'-methylenedioxy pterocarpan. This is a new compound to the best of my knowledge.

Table 4.14: Proton NMR spectroscopic data of compound **10** (CD_3OD, 300 MHz)

Position	**δ_H (J in Hz)**
1	7.26 (m)
Ha-1’	5.85 (d)
Hb-1’	5.88 (d)
2	6.48 (dd, 8.4, 2.4)
3	-
4	6.30 (d, 2.4)
5	-
6a	4.22 (m)
Ha-6	3.53 (m)
Hb-6	3.91 (m)
7	6.38 (d, 1.5)
8	-
9	-
10	6.81 (s)
11a	5.45 (d, 6.6)

4.3.6. Orobol (compound 11, known)

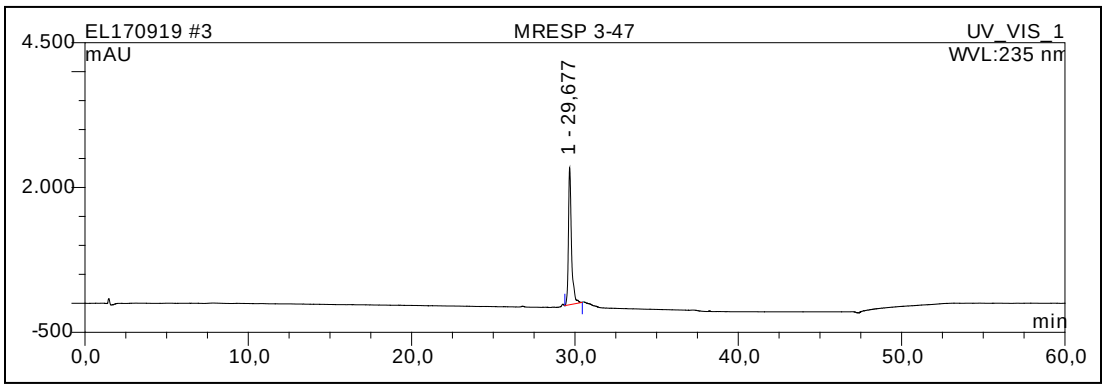


Figure 4.40: HPLC Chromatogram of Compound **11**

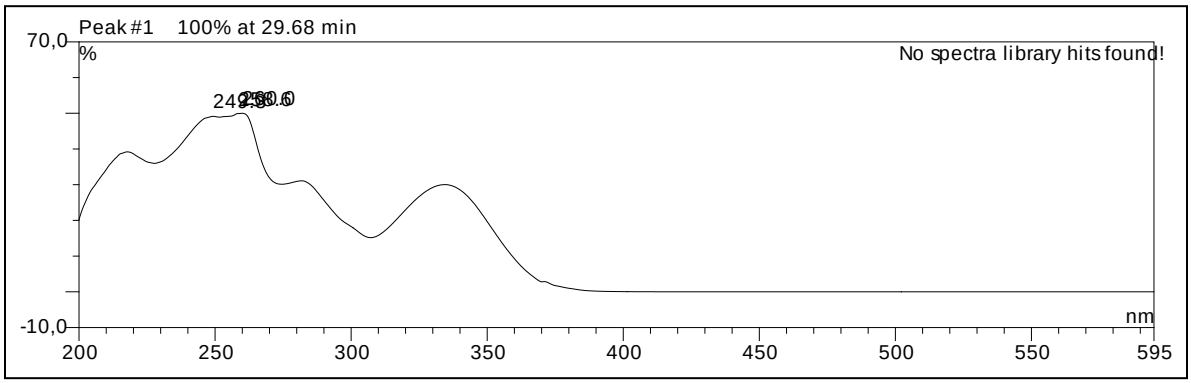


Figure 4.41: Uv spectrum of Compound **11**

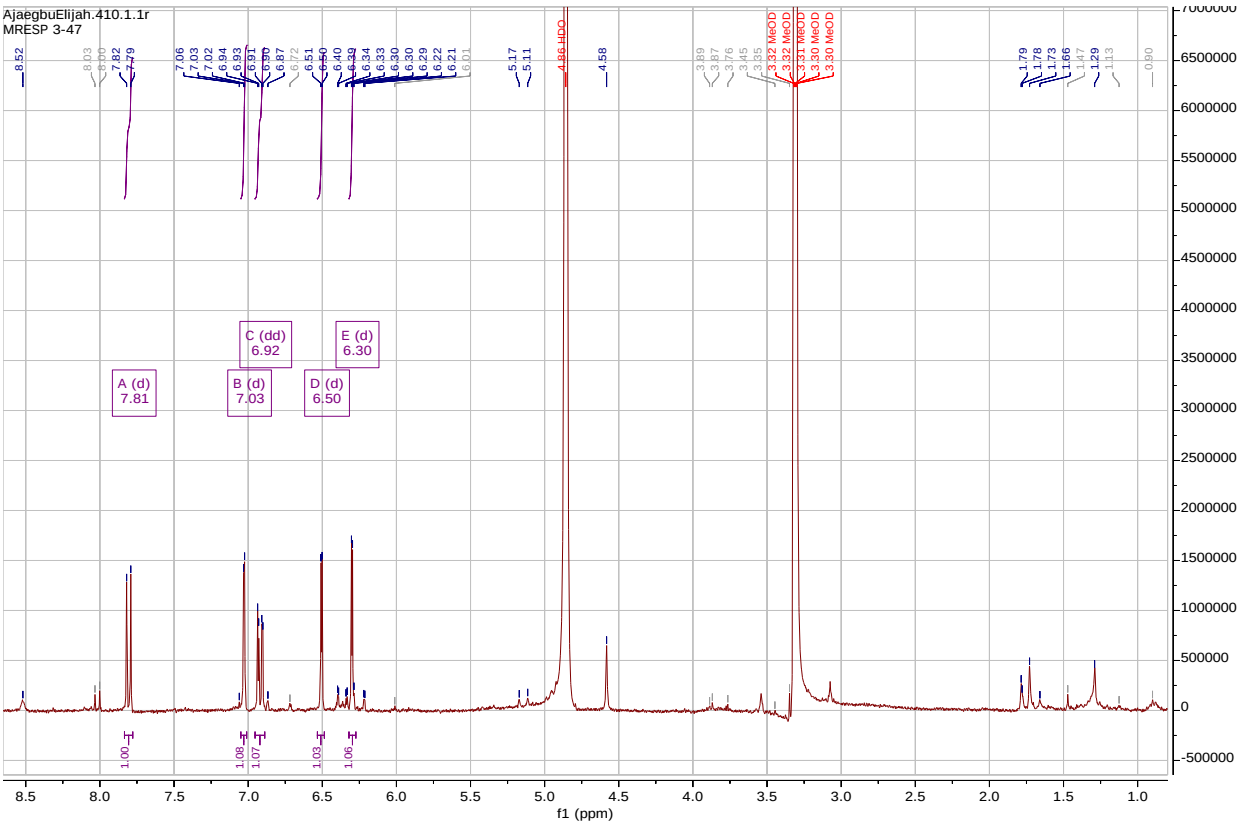


Figure 4.42: [1]HNMR spectrum of Compound **11**

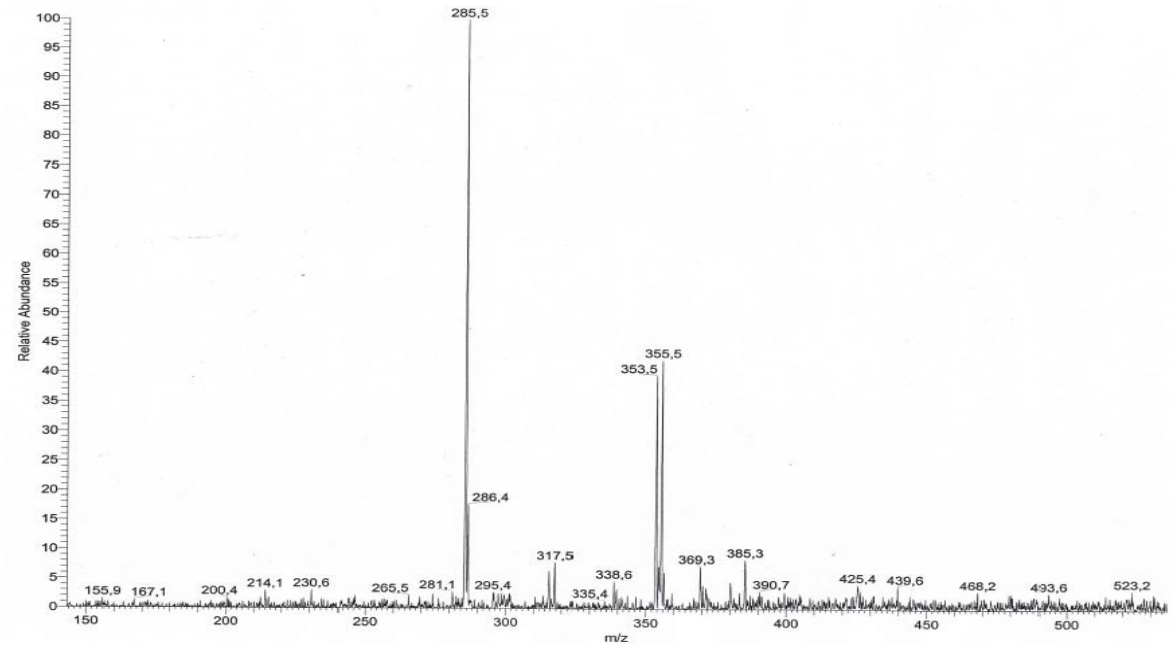
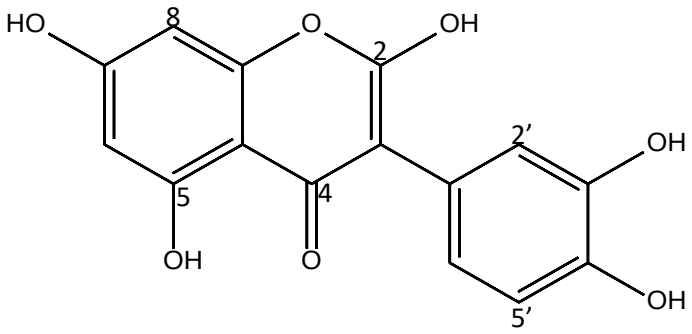


Figure 4.43: ESI-MS spectrum of Compound **11**

4.3.6.1 Structure of Orobol (compound 11, known)



Molecular formula	$C_{15}H_{10}O_7$
Molecular weight	302.24
Amount	1.3 mg

Compound **11** was isolated from the root of *Millettia aboensis* as a brown solid. It showed UV (MeOH) λ_{max} 249.3, and 250 (see Figure 4.41), which is suggestive of an orobol nucleus and $[\alpha]^{20}_{D}$ -2.74 (c, 0.4, MeOH). The ESI-MS showed the pseudomolecular ion peak at *m/z* 285.5 [M-OH^-] (see Fig. 4.43). The fragment ion peak occurred at *m/z* 355.5 [M-3OH^-], indicating the presence of hydroxylation (three molecules of hydroxyl group). The molecular formula was deduced as $C_{15}H_{10}O_7$ based on the observed MS. The ^{1}H NMR spectrum of compound **11** (see Figure 4.42) shows two *meta* coupling protons of ring A which resonated at δ 6.50 (d, 2.2) and at 6.30 (d, 2.2), corresponding to H-6 and H-8 respectively. The aromatic characteristic signals of ABX pattern of ring B was observed at δ 7.81 (H-5´, d, 8.4), 6.92 (H-6´, dd, 8.4, 2.2) and 7.03 (H-2´, d, 2.1) respectively. The absence of characteristic proton at H-2 suggesting a substitution with OH. It has a close resemblance with compound **9** and the similarity lies in the ABX system of ring B at δ 7.83 (d, 8.5, H-5'), 6.94 (dd, 8.5, 2.1, H-6'), and 7.05 (d, 2.1, H-2´), and the two meta coupling protons

of ring A at δ 6.73 (d, J=2.3 Hz, H-6) and 6.48 (d, J=2.3 Hz. H-8), compound **11** was established as orobol, which has been isolated (Mutalib *et al.*, 2013).

Table 4.15: Proton NMR spectroscopic data of compound **11** (CD_3OD, 300 MHz)

Position	**δ_H (J in Hz)**
2	-
3	-
4	-
5	-
6	6.50 (d, 2.2)
7	-
8	6.30 (d, 2.2)
9	-
10	-
1’	-
2’	7.03 (d, 2.1)
3’	-
4’	-
5’	7.81 (d, 8.4)
6’	6.92 (dd, 8.4, 2.2)

4.3.7. 2', 3'-dimethyl kaempferol (compound 12, new)

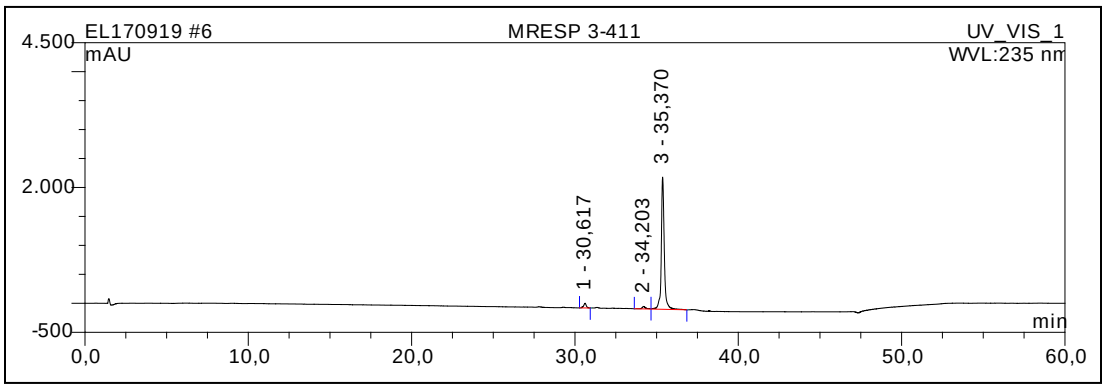


Figure 4.44: HPLC Chromatogram of Compound **12**

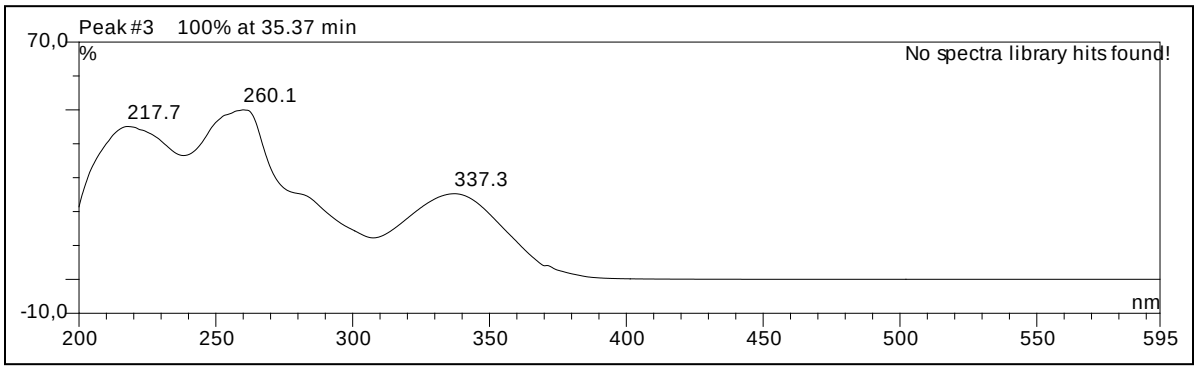


Figure 4.45: Uv spectrum of Compound **12**

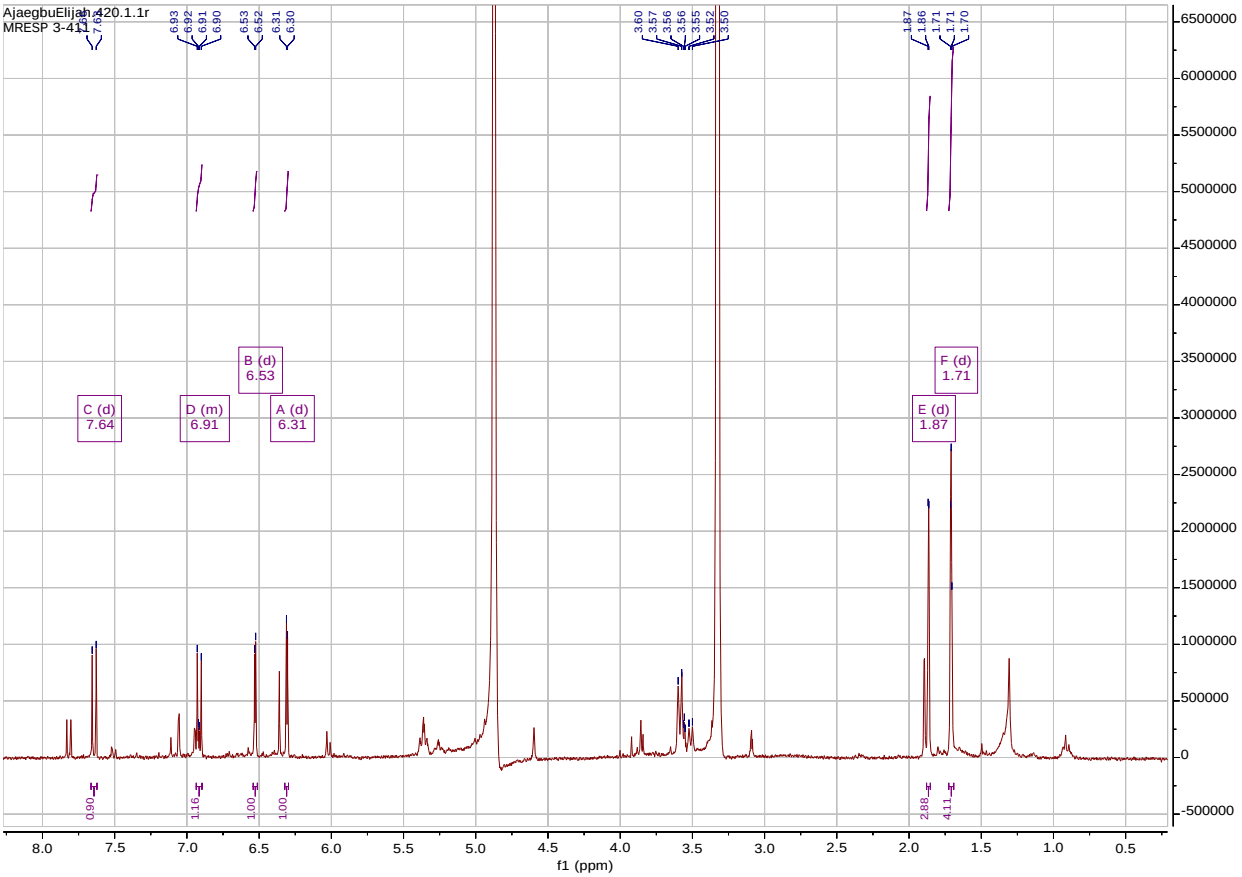


Figure 4.46: [1]HNMR spectrum of Compound **12**

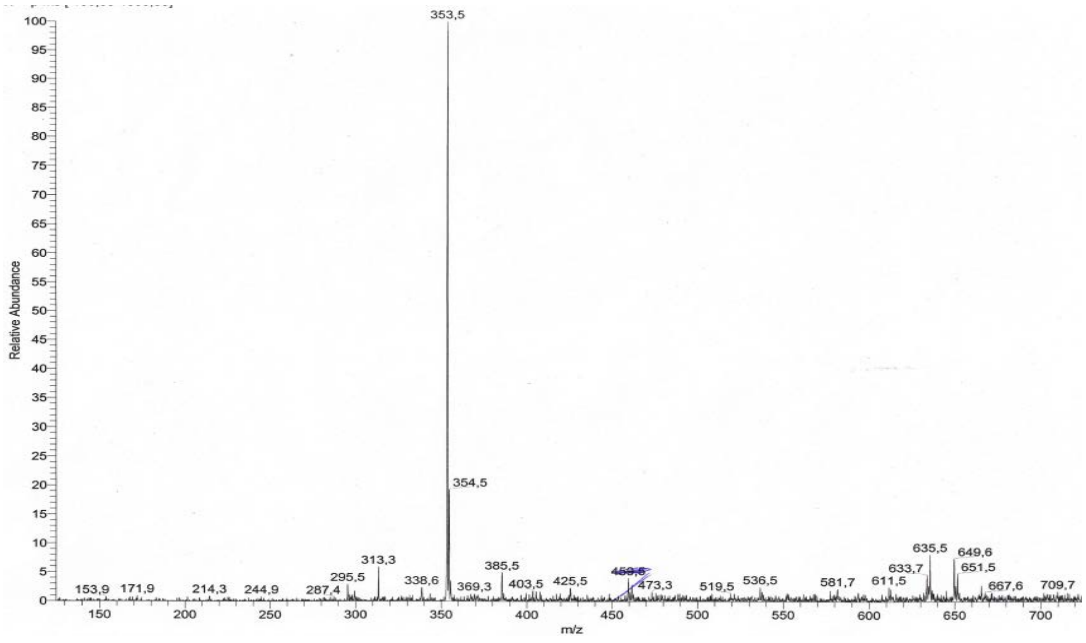
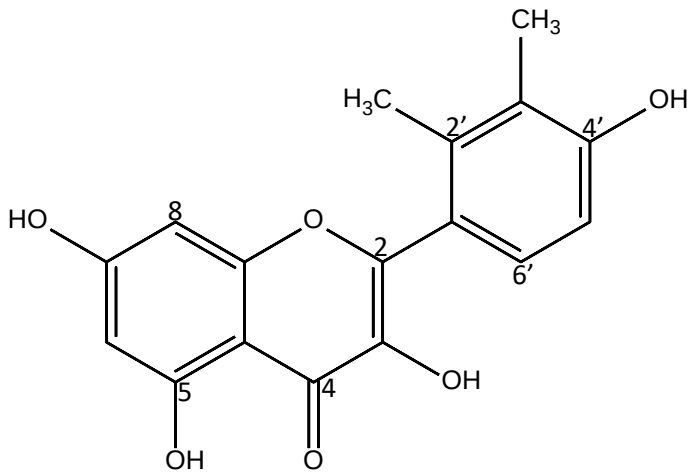


Figure 4.47: ESI-MS spectrum of Compound **12**

4.3.7.1 Structure of 2', 3'-dimethyl kaempferol (compound 12, new)



Molecular formula	$C_{17}H_{14}O_6$
Molecular weight	314.29
Amount	1.4 mg

Compound **12** was isolated from the root of *Millettia aboensis* as a brown solid. It showed UV (MeOH) λ_{max} 217.5, 260.1 and 337.3 (see Figure 4.45), which is suggestive of a kaempferol nucleus and $[\alpha]^{20}_{D}$ -3.2 (c, 0.56, MeOH). The ESI-MS showed the pseudomolecular ion peak at *m/z* 313.3 [M-H^+] (see Fig. 4.47). The fragment ion peak occurred at *m/z* 353.5 [M+Na^++OH^-], indicating the presence of sodium and hydroxylation (hydroxyl group). The molecular formula was deduced as $C_{17}H_{14}O_6$ based on the observed MS. The ^{1}H NMR spectrum (see Fig. 4.46) of compound **12** shows two aromatic protons at δ 7.64 (d, 8.3) and δ 6.92 (d, 8.3), corresponding to H-5' and H-6' (see Figure 4.46). The resonances of two meta coupling protons of ring A were observed at δ 6.53 (H-6, d, 2.2) and δ 6.31 (H-8, d, 2.2). Also the ^{1}H NMR spectrum revealed two methyl doublets at δ 1.71 (CH_3-2') and δ 1.87 (CH_3-3'), compound **12** was established as 2',3'-dimethyl kaempferol, and to the best of my knowledge this is the first time it is being isolated, hence a new compound.

Table 4.16: Proton NMR spectroscopic data of compound **12** (CD_3OD, 300 MHz)

Position	**δ_H (J in Hz)**
2	-
3	-
4	-
5	-
6	6.53 (d, 2.2)
7	-
8	6.31 (d, 2.2)
9	-
10	-
1’	-
2’	1.71 (d, 1.4)
3’	1.87 (d, 1.3)
4’	-
5’	7.64 (d, 8.3)
6’	6.92 (d, 8.3)

4.3.8. 3'-methoxy daidzein (compound 13, new)

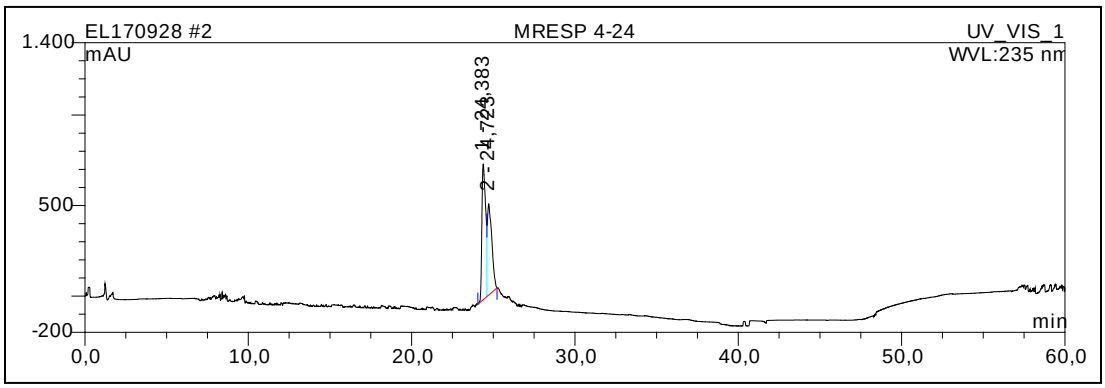


Figure 4.48: HPLC Chromatogram of Compound **13**

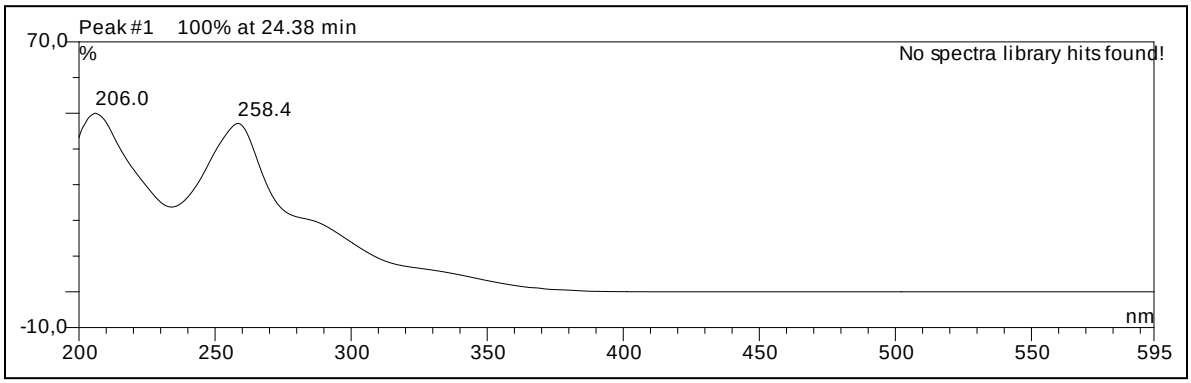


Figure 4.49: Uv spectrum of Compound **13**

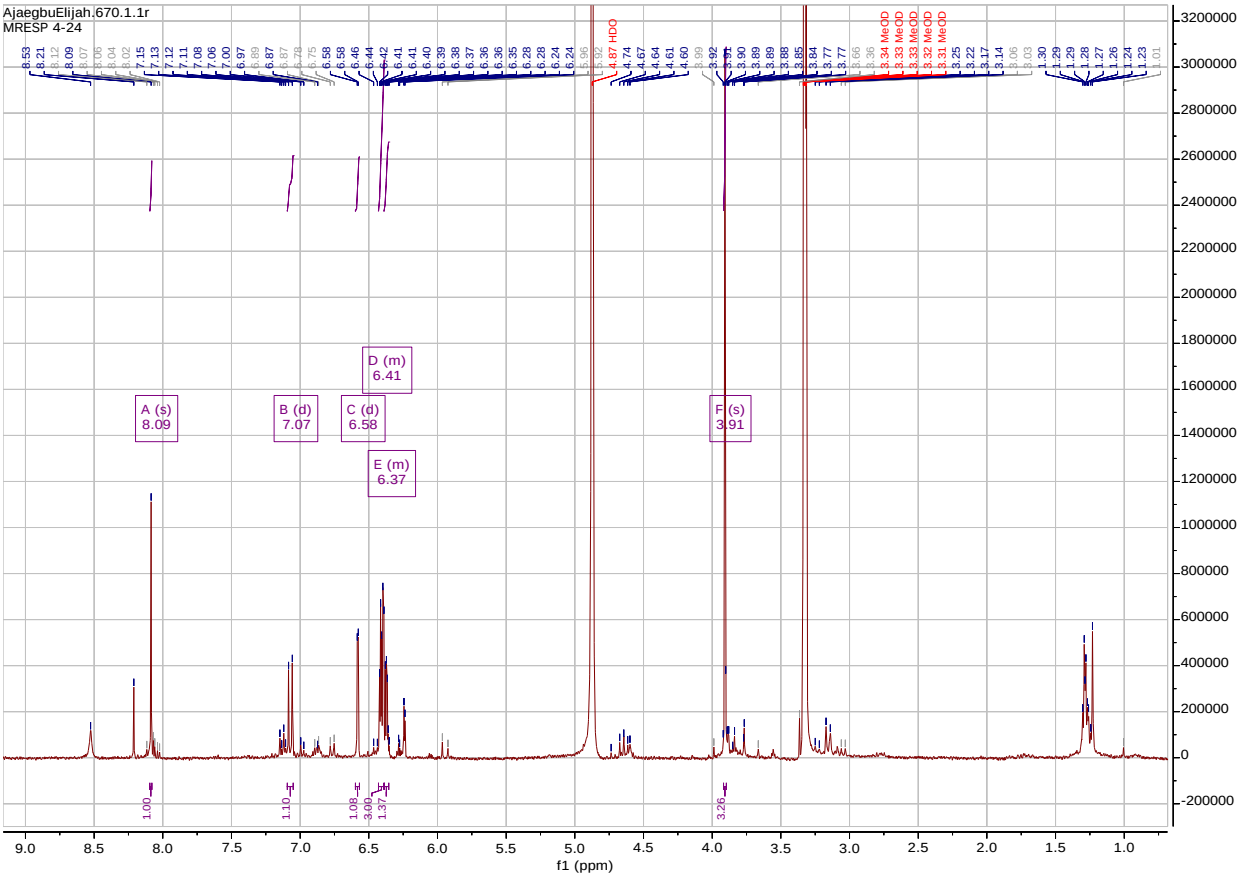


Figure 4.50: [1]HNMR spectrum of Compound **13**

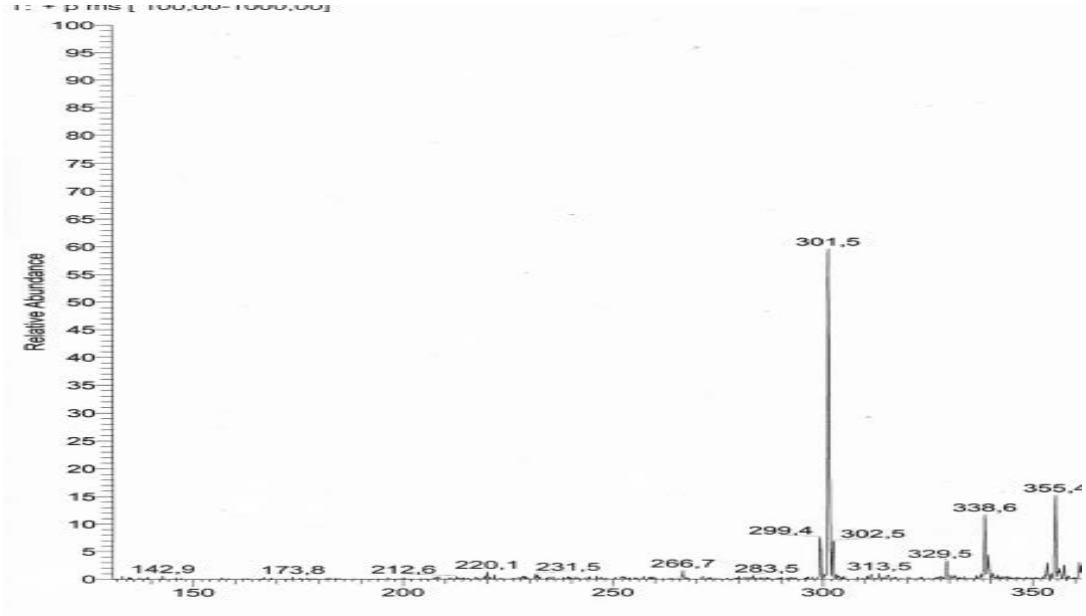
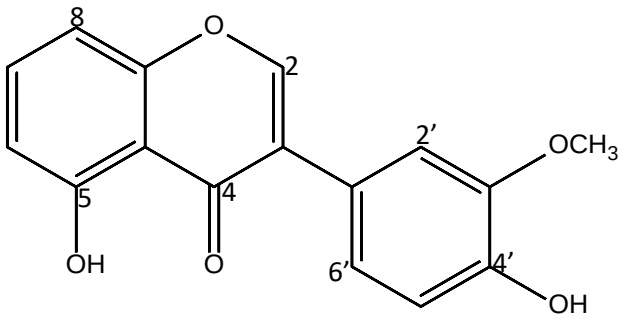


Figure 4.51: ESI-MS spectrum of Compound **13**

4.3.8.1 Structure of 3'-methoxy daidzein (compound 13, new)



Molecular formula	$C_{16}H_{12}O_5$
Molecular weight	284.27
Amount	2.3 mg

Compound **13** was isolated from the root of *Millettia aboensis* as a yellow solid. It showed UV (MeOH) λ_{max} 217.7, 260.1 and 337.3 (see Figure 4.49), which is suggestive of a daidzein nucleus and $[\alpha]^{20}_{D}$ -27.5 (c, 0.92, MeOH). The ESI-MS showed the pseudomolecular ion peak at *m/z* 301.5 [M-OH^-] (see Fig. 4.51). The molecular formula was deduced as $C_{16}H_{12}O_5$ based on the observed MS. The ^{1}H NMR spectrum of compound **20** (see Figure 4.50) shows characteristic ABC aromatic protons of ring A which resonated at δ 6.37 (m, H-6), 6.58 (d, J=2.3 Hz, H-7) and 6.37 (m, H-8). The characteristic ABX pattern of ring B was observed at δ 7.07 (H-5´, d, J=8.1 Hz), 6.41 (m, H-6') and 6.37 (m, H-2´). One methoxy singlet resonated at δ 3.91 (OCH_3-3'); one aromatic proton singlet at δ 8.09 (H-2), which is characteristic of isoflavone. The B of the ABX pattern of ring B has an overlap with ABC of ring A, compound **13** was established as 3'-methoxy daidzein. This is the first time to the best of my knowledge of its isolation, hence a new compound.

Table 4.17: Proton NMR spectroscopic data of compound **13** (CD_3OD, 300 MHz)

Position	**δ_H (J in Hz)**
2	8.09 (s)
3	-
4	-
5	-
6	6.37 (m)
7	6.58 (d, 2.3)
8	6.37 (m)
9	-
10	-
1’	-
2’	6.37 (m)
3’ – OCH_3	3.91 (s)
4’	-
5’	7.07 (d, 8.1)
6’	6.41 (m)

4.3.9. Derrisisoflavone O (compound 14, new)

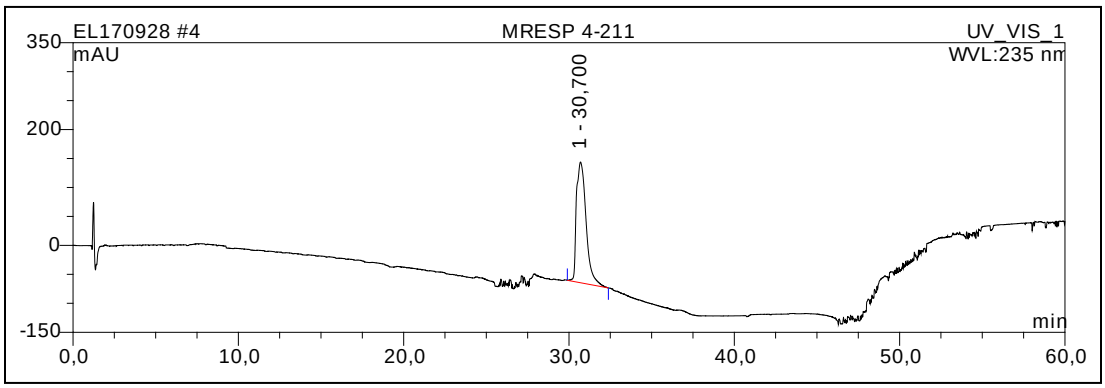


Figure 4.52: HPLC Chromatogram of Compound **14**

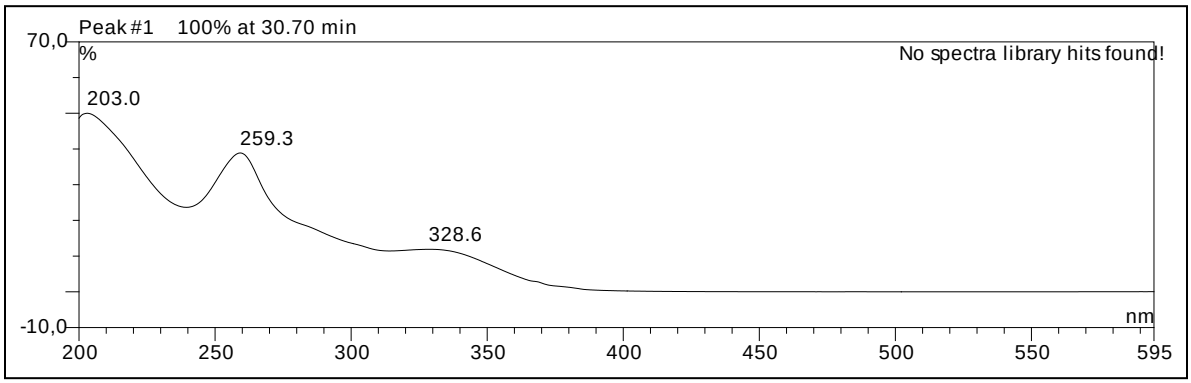


Figure 4.53: Uv spectrum of Compound **14**

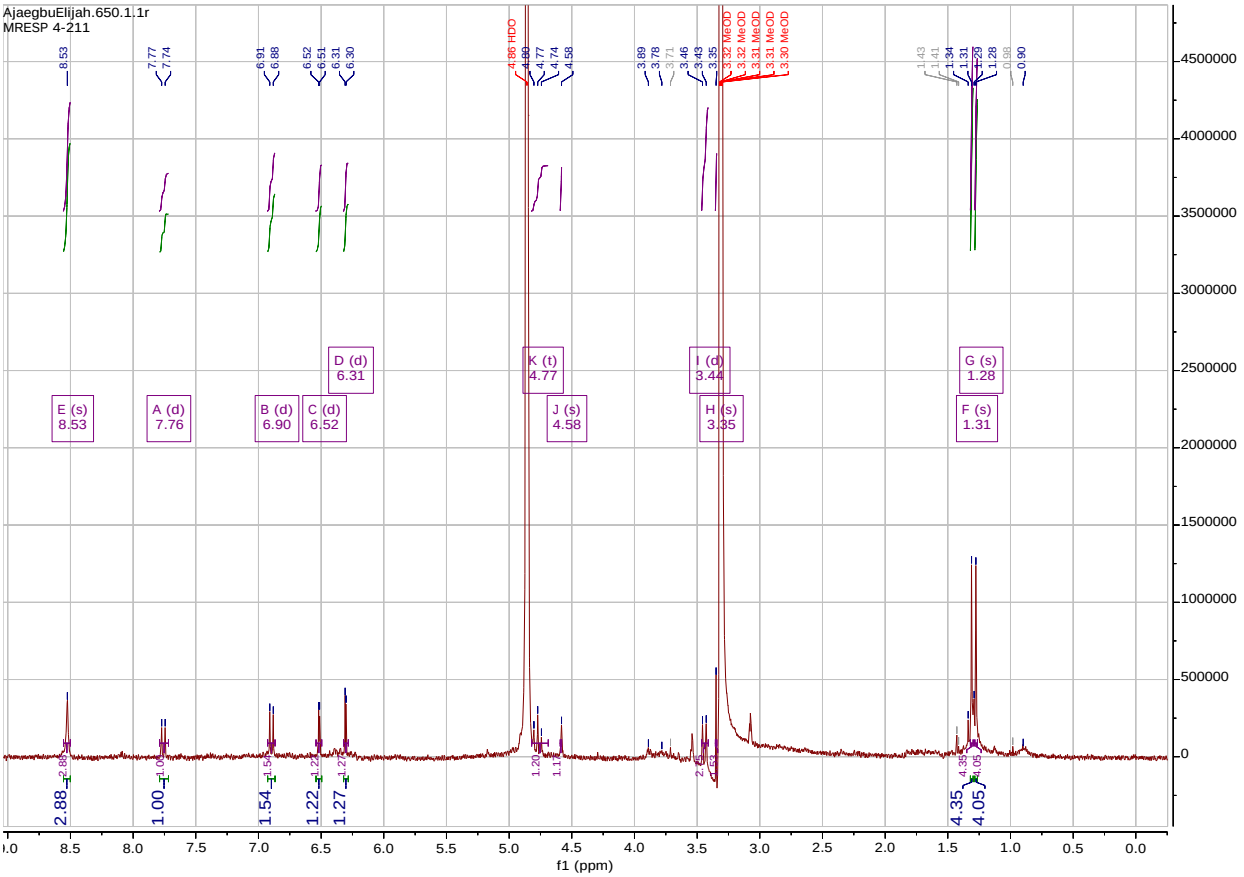


Figure 4.54: [1]HNMR spectrum of Compound **14**

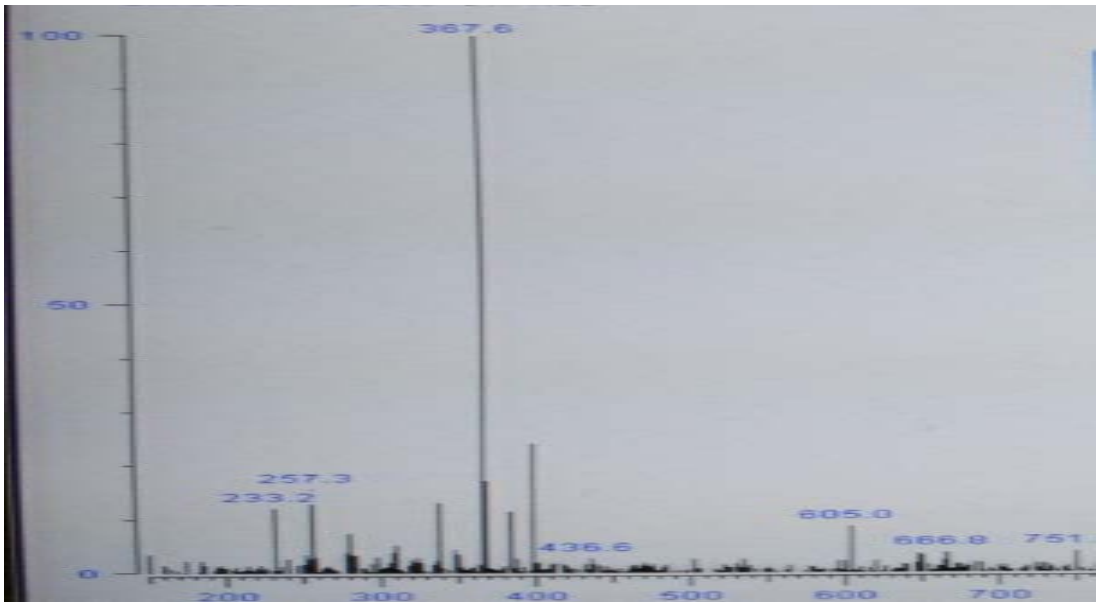
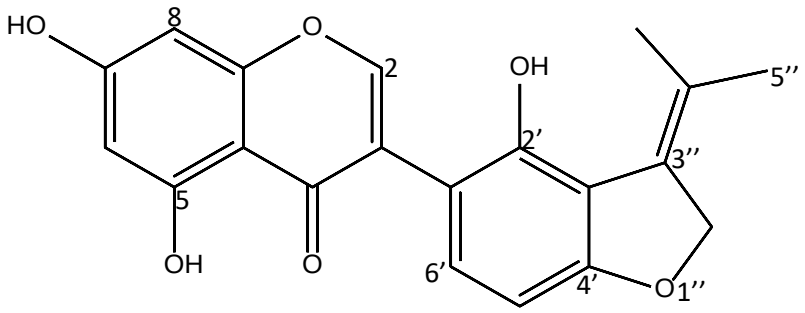


Figure 4.55: ESI-MS spectrum of Compound **14**

4.3.9.1 Structure of Derrisisoflavone O (compound 14, new)



Molecular formula	$C_{20}H_{16}O_6$
Molecular weight	366.11
Amount	1 mg

Compound **14** was isolated from the root of *Millettia aboensis* as a milky solid. It showed UV (MeOH) λ_{max} 203, 259.3, and 328.6 (see Figure 4.53), which is suggestive of a derrisisoflavone nucleus and $[\alpha]^{20}_{D}$ -4.76 (c, 0.4, MeOH). The ESI-MS showed the pseudomolecular ion peak at *m/z* 367.6 [M+CH_3] (see Fig. 4.55). The fragment ion peak occurred at *m/z* 284.6 [M±H^+-benzoyl], indicating the presence of benzoyl group. The molecular formula was deduced as $C_{20}H_{16}O_6$ based on the observed MS. The ^{1}H NMR spectrum (see Fig. 4.54) of compound **14** shows a proton singlet at δ 8.53 (s, H-2), characteristic of an isoflavone and two aromatic proton signals for ring B protons at δ 7.76 (d, 8.3 Hz, H-5') and 6.90 (d, 8.3 Hz, H-6'). It also revealed two meta coupling protons of ring A at δ 6.52 (d, 2.1 Hz, H-6) and 6.31 (d, 2.1 Hz, H-8). It also revealed an isopropenyl dihydrofuran proton signals at δ 3.44 (d, 8.9, Hb-2"), 3.35 (s, Ha-2''), 4.58 (s, Ha-3"), 4.77 (t, 9.1, Hb-3"), 1.31 (s, H-5") and 1.28 (s, H-6"), compound **14** was established as derrisisoflavone O,

derivative of derrisisoflavone G (compound **6**) being a new compound which has never been isolated or reported.

Table 4.18: Proton NMR spectroscopic data of compound **14** (CD_3OD, 300 MHz)

Position	**δ_H (J in Hz)**
2	8.53 (s)
3	-
4	-
5	-
6	6.52 (d, 2.1)
7	-
8	6.31 (d, 2.1)
9	-
10	-
1’	-
2’	-
3’	-
4’	-
5’	7.76 (d, 8.3)
6’	6.90 (d, 8.3)
1”	-
Ha-2”	3.35 (s)
Hb-2”	3.44 (d, 8.9)
Ha-3”	4.58 (s)
Hb-3”	4.77 (t, 9.1)
4”	-
5”	1.31 (s)
6”	1.28 (s)

4.4. Compound isolated from the pod of Millettia aboensis

4.4.1. Luteolin (compound 15, known)

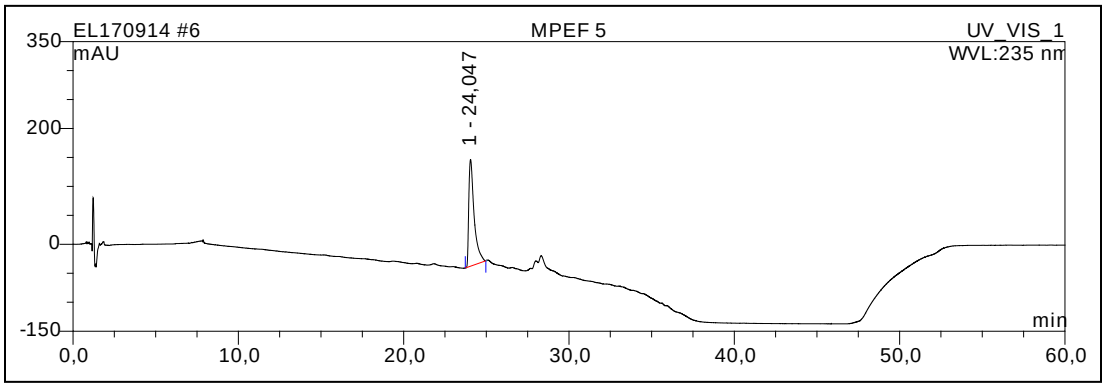


Figure 4.56: HPLC Chromatogram of Compound **15**

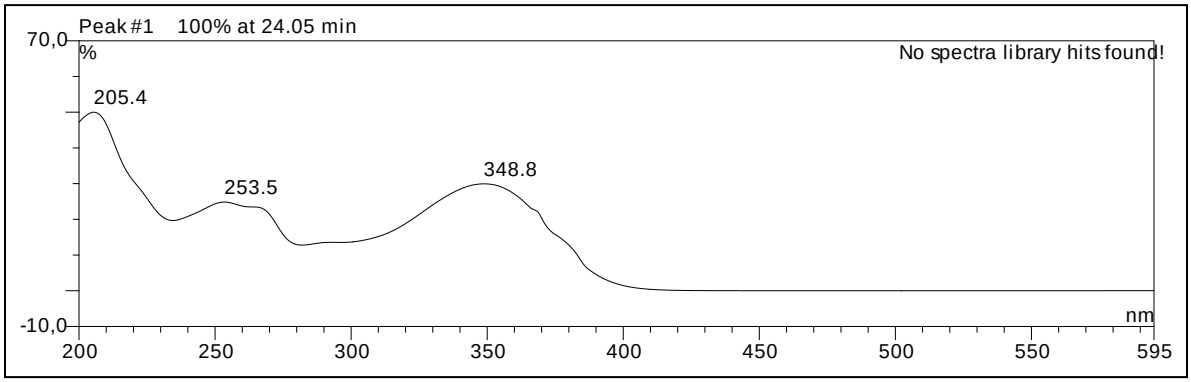


Figure 4.57: Uv spectrum of Compound **15**

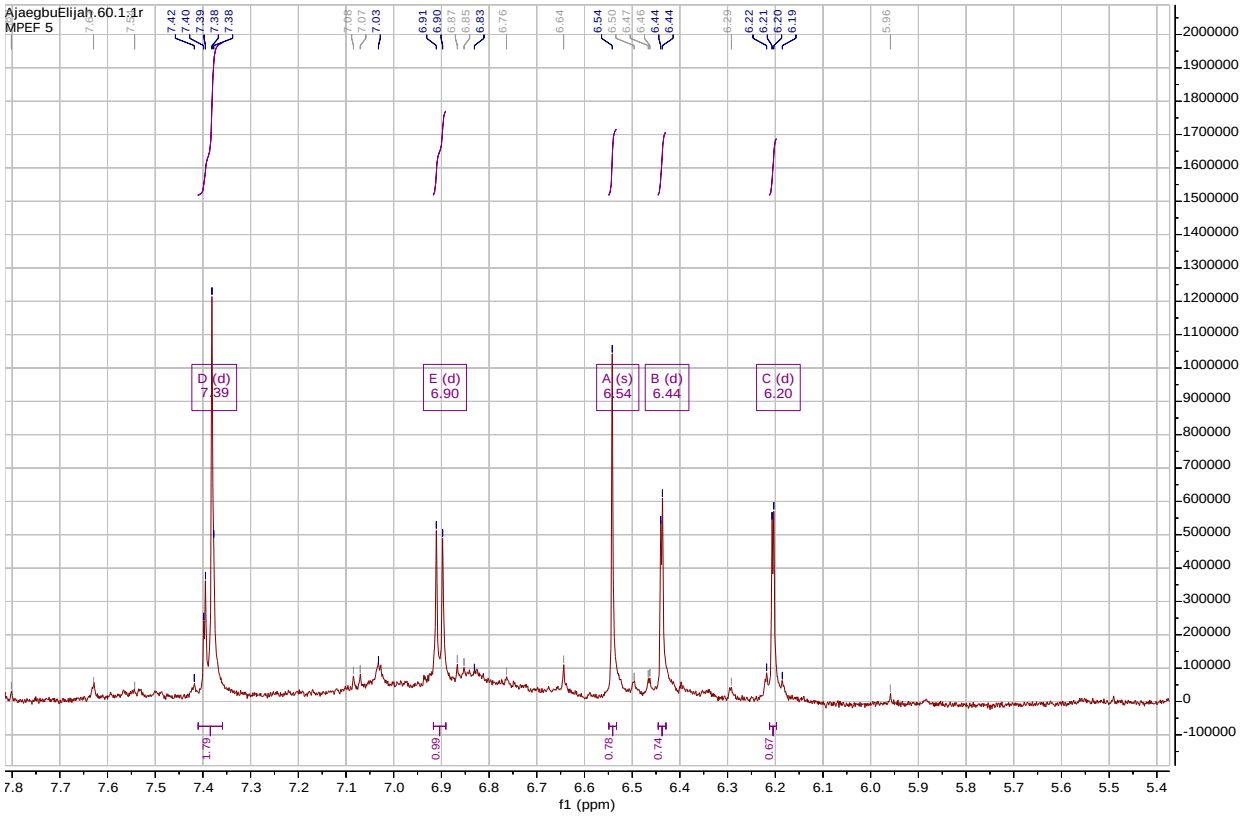


Figure 4.58: [1]HNMR spectrum of Compound **15**

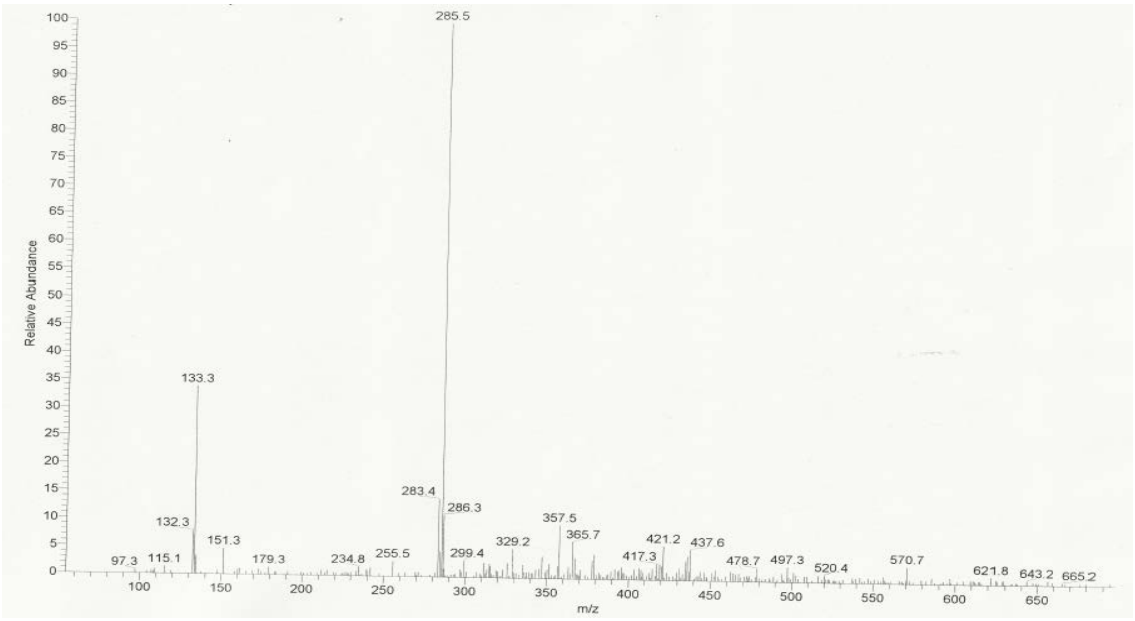
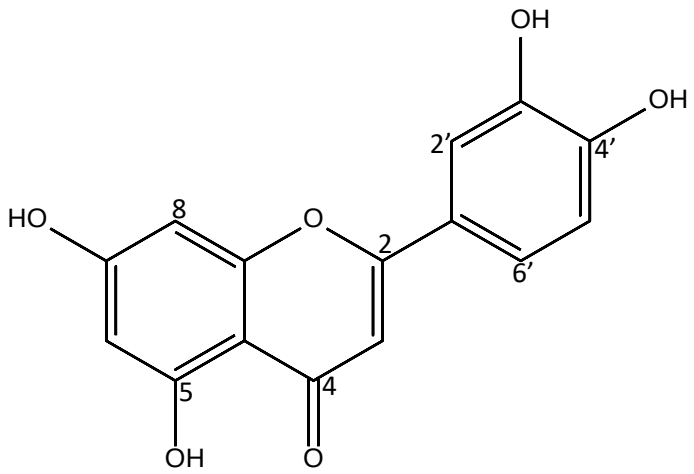


Figure 4.59: ESI-MS spectrum of Compound **15**

4.4.1.1 Structure of Luteolin (compound 15, known)



Molecular formula	$C_{15}H_{10}O_6$
Molecular weight	286.24
Amount	1 mg

Compound **15** was isolated from the pod of *Millettia aboensis* as a yellow solid. It showed UV (MeOH) λ_{max} 205.4, 253.5 and 348.8 (see Figure 4.57) and $[\alpha]^{20}_{D}$ +1.93 (c, 0.4, MeOH). The ESI-MS showed the pseudomolecular ion peak at *m/z* 285.5 [M-H^+] (see Fig. 4.59). The fragment ion peak occurred at *m/z* 133.3 [M±H^+-galloyl], indicating the presence of galloyl group. The molecular formula was deduced as $C_{15}H_{10}O_6$ based on the observed MS. The ^{1}H NMR spectrum of compound **15** (see Figure 4.58) revealed two *meta* coupling protons of ring A which resonated at δ 6.44 (d, 2.2) and at 6.20 (d, 2.1), corresponding to H-6 and H-8 respectively. The characteristic aromatic signals of ABX pattern of ring B was observed at δ 6.90 (d, J=8.0 Hz, H-5´) and 7.39 (d, J=8.1 Hz, H-2', H-6'), respectively, since there was an overlap, compound **15** was established as luteolin, which has been isolated (Lin *et al*., 2015).

Compound **15** is very similar to compound **11** isolated from the root of the plant; the only difference is the absence of OH group attached to C-3 in compound **11** (making us have an aromatic singlet in C-3).

Table 4.19: Proton NMR spectroscopic data of compound **15** (CD_3OD, 300 MHz)

Position	**δ_H (J in Hz)**
2	-
3	6.54 (s)
4	-
5	-
6	6.44 (d, 2.2)
7	-
8	6.20 (d, 2.1)
1’	-
2’	7.39 (d, 8.1)
3’	-
4’	-
5’	6.90 (d, 8.0)
6’	7.39 (d, 8.1)

4.5. Compounds from the stem of *Millettia aboensis*

4.5.1. Derrisisoflavone L (compound 16, new)

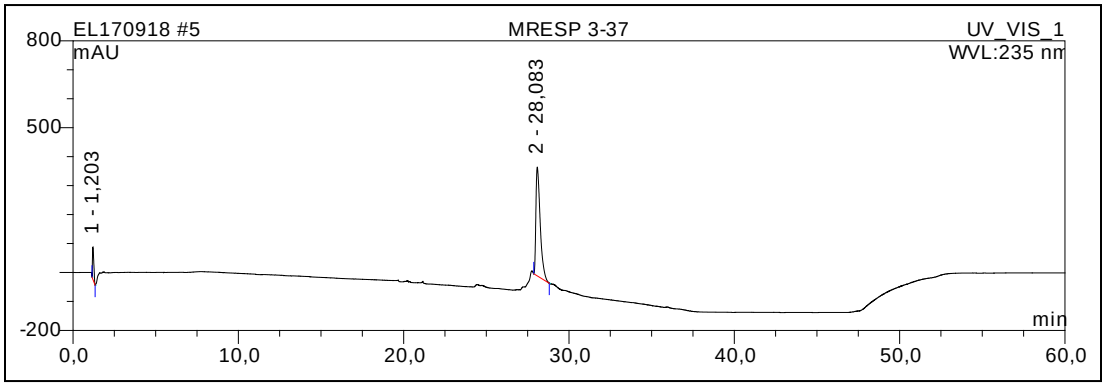


Figure 4.60: HPLC Chromatogram of Compound **16**

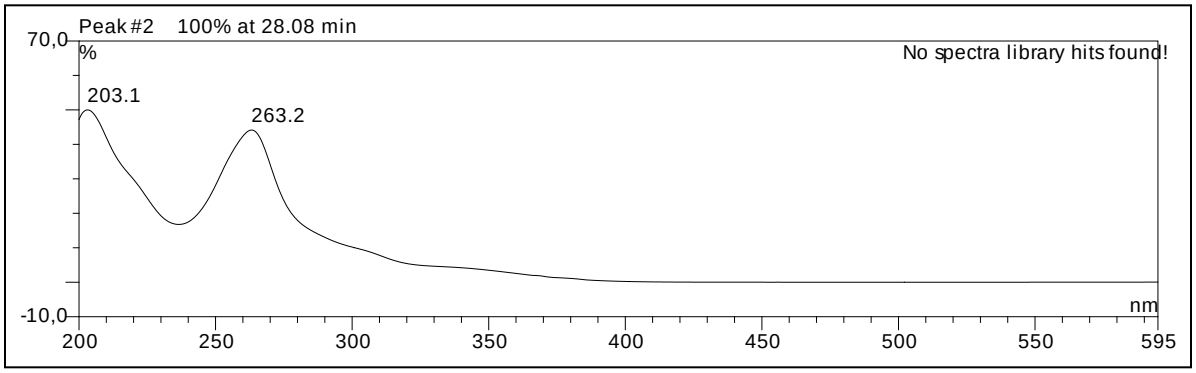


Figure 4.61: Uv spectrum of Compound **16**

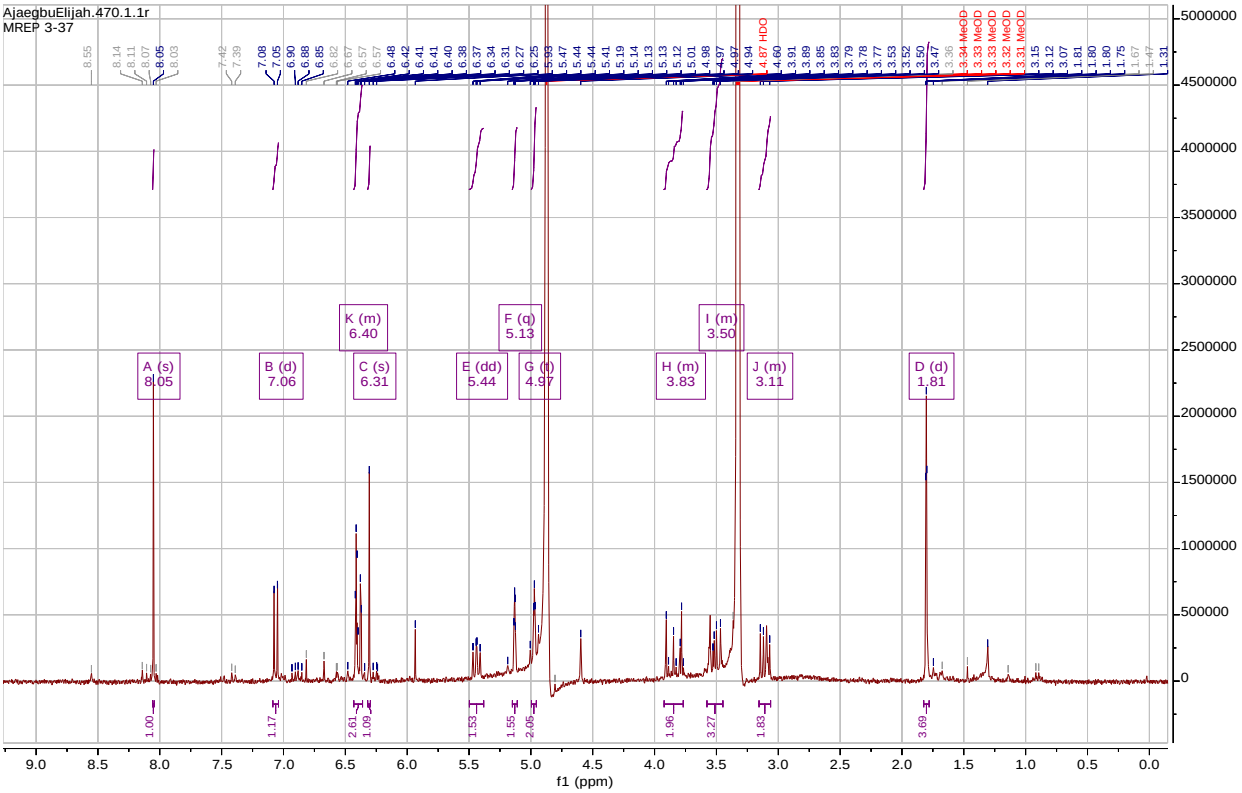


Figure 4.62: [1]HNMR spectrum of Compound **16**

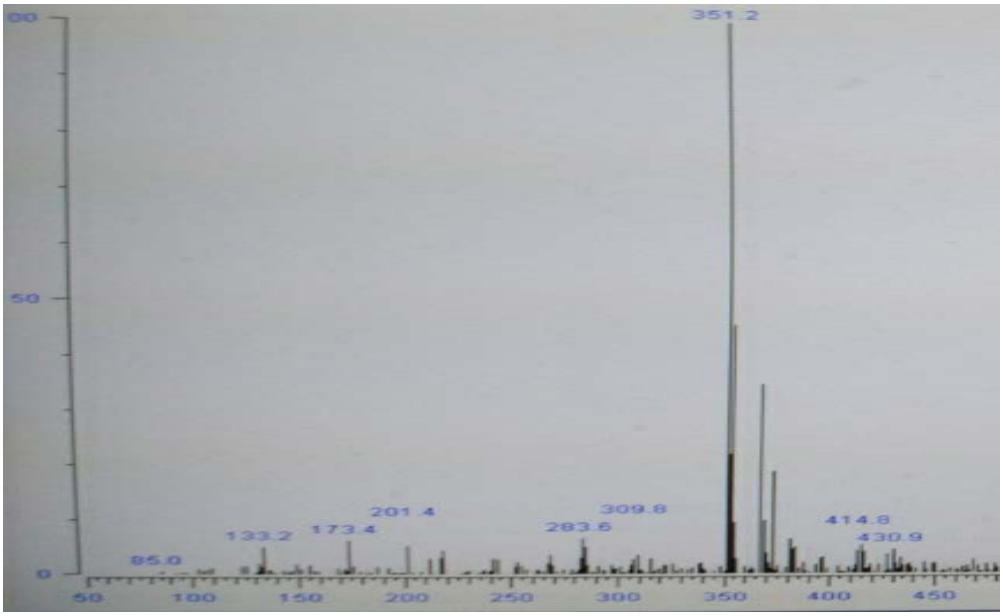
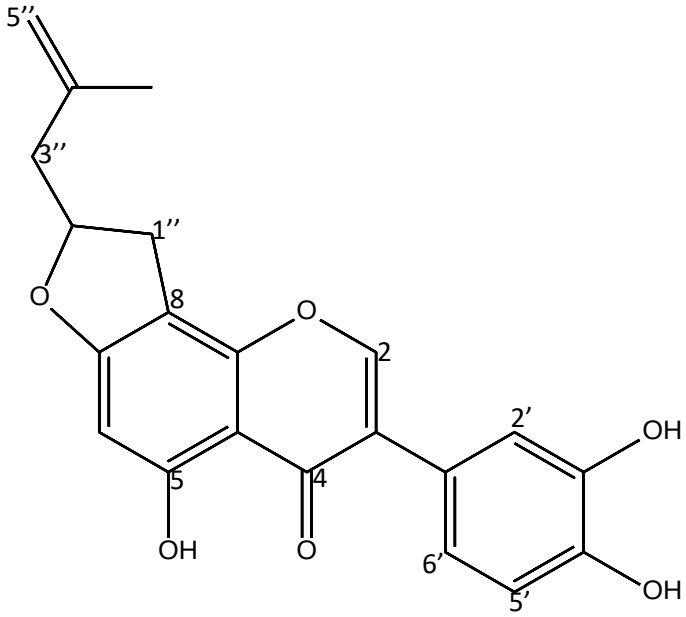


Figure 4.63: ESI-MS spectrum of Compound **16**

4.5.1.1 Structure of Derrisisoflavone L (compound 16, new)



Molecular formula	$C_{21}H_{18}O_6$
Molecular weight	366.37
Amount	1 mg

Compound **16** was isolated from the root of *Millettia aboensis* as a yellow amorphous solid. It showed UV (MeOH) λ_{max} 203, 263.2 (see Figure 4.61), which is suggestive of a derrisisoflavone and $[\alpha]^{20}_D$ -6.47 (c, 0.4, MeOH). The ESI-MS showed the pseudomolecular ion peak at *m/z* 351.2 [M-CH_3] (see Fig. 4.63). The molecular formula was deduced as $C_{21}H_{18}O_6$ based on the observed MS. The ^{1}H NMR spectrum (see Fig. 4.62) of compound **16** shows a proton singlet at δ 8.05 (s, H-2), characteristic of an isoflavone and an aromatic signals of ABC coupling pattern of ring B assigned to δ 7.06 (d, J = 8.1 Hz, H-5'), and 6.40 (m, H-2', H-6'). The ^{1}H NMR spectrum also revealed a singlet aromatic proton signal for ring A proton at δ 6.31 (s, H-6). It also revealed an isopropenyl- dihydrofuran proton signals at δ 1.81 (d, 1.2, CH_3-6"), 3.11 (m, Ha-1"), 3.49 (m, Hb-1"), 4.97 (t, J = 1.6 Hz, Ha-5"), 5.13 (q, J = 1.3 Hz, Hb-5"), and 5.44 (dd, J = 7.7, 9.6 Hz, H-2"), and 3.83 (m, H-3''). The absence of signal for H-8 also supported the location of the furan ring at

the position, compound **16** was established as derrisisoflavone L, derivative of derrisisoflavone G (compound **6**) being a new compound which has never been isolated or reported.

Table 4.20: Proton NMR spectroscopic data of compound **16** (CD_3OD, 300 MHz)

Position	**δ_H (J in Hz)**
2	8.05 (s)
3	-
4	-
5	-
6	6.31 (s)
7	-
8	6.45 (d, 8.3)
9	-
10	-
1’	-
2’	6.40 (m)
3’	-
4’	-
5’	7.06 (d, 8.1)
6’	6.40 (m)
Ha-1’’	3.11 (m)
Hb-1’’	3.49 (m)
2’’	5.44 (dd, 7.7, 9.6)
3”	3.83 (m)
4’’	-
Ha-5’’	4.97 (t, 1.6)
Hb-5’’	5.13 (q, 1.3)
6’’	1.81 (d, 1.2)

4.5.2. Derrisisoflavone N (compound 17, new)

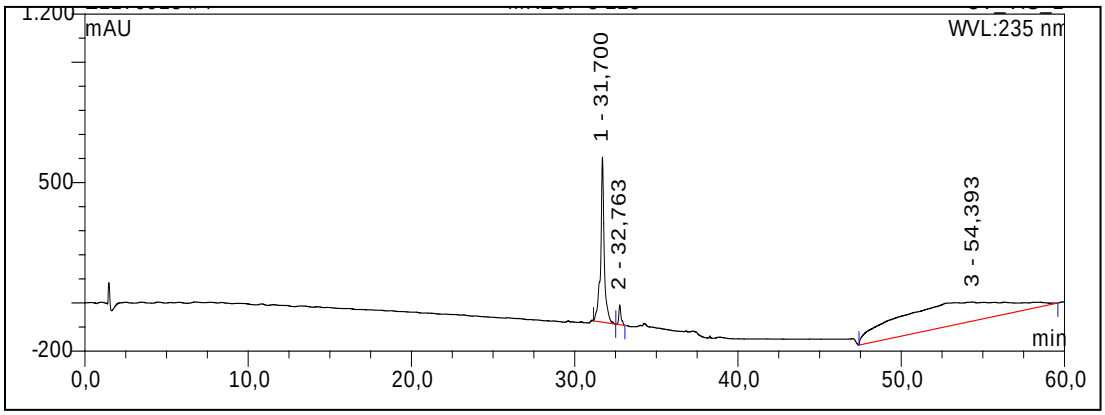


Figure 4.64: HPLC Chromatogram of Compound **17**

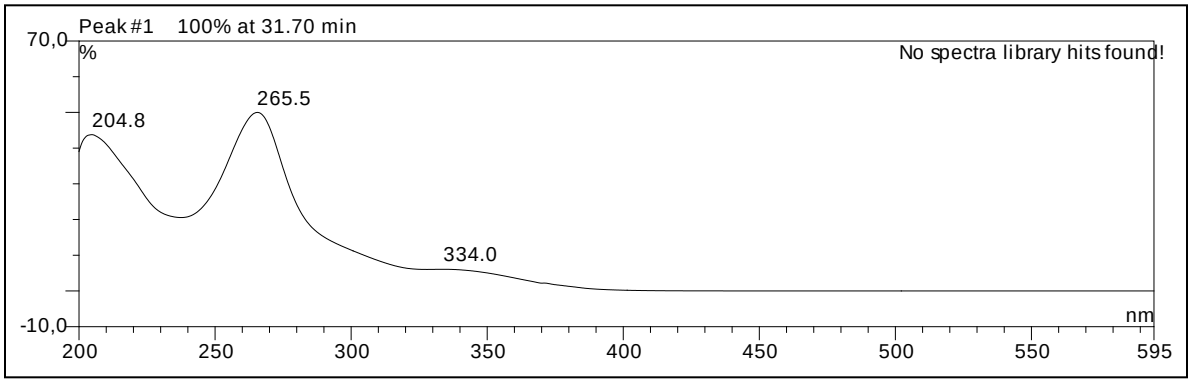


Figure 4.65: Uv spectrum of Compound **17**

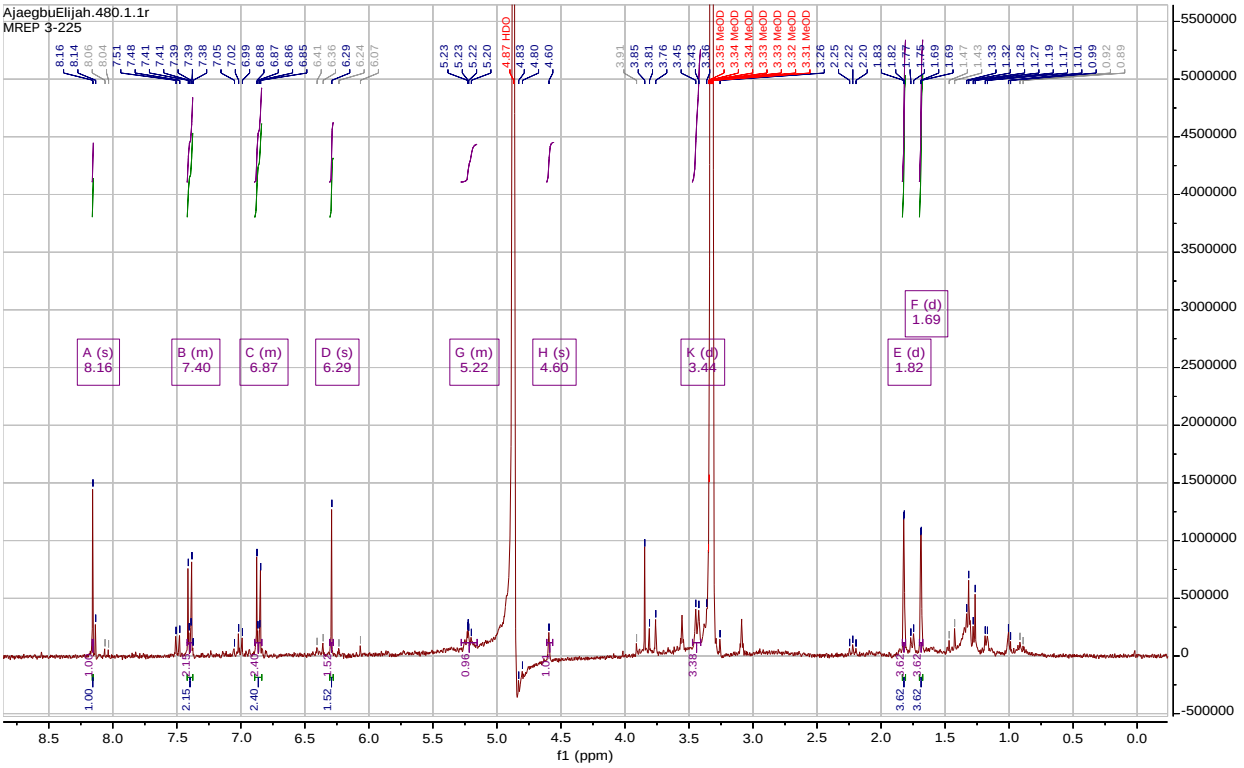


Figure 4.66: [1]HNMR spectrum of Compound **17**

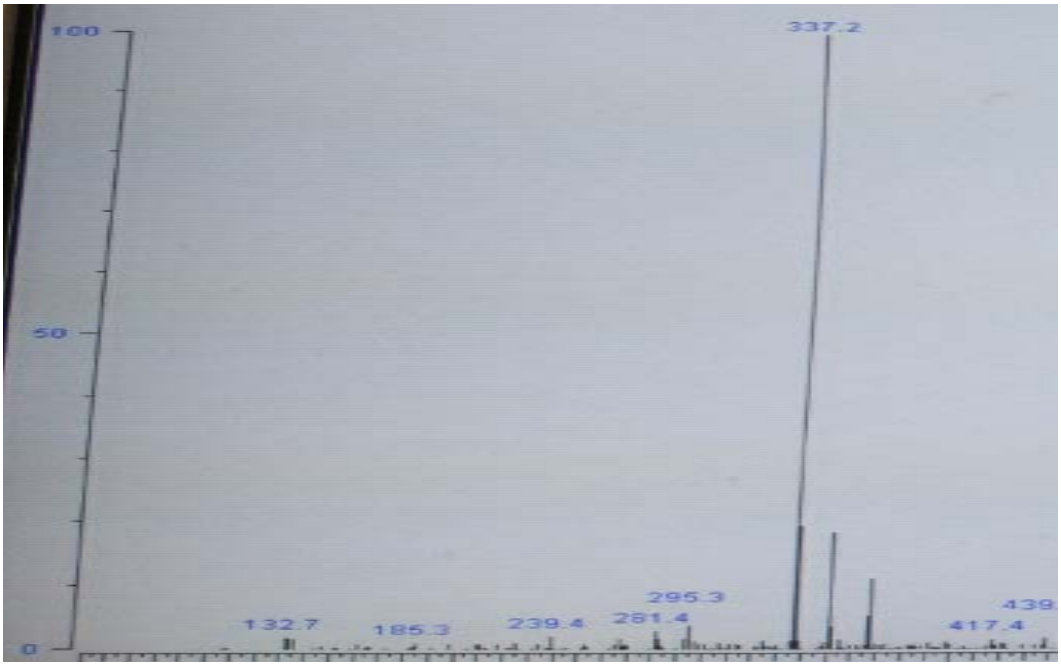
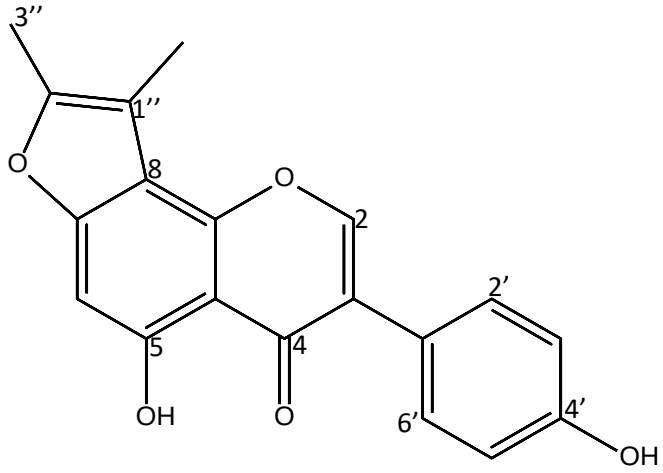


Figure 4.67: ESI-MS spectrum of Compound **17**

4.5.2.1 Structure of Derrisisoflavone N (compound 17, new)



Molecular formula	$C_{19}H_{14}O_5$
Molecular weight	322.32
Amount	1 mg

Compound **17** was isolated from the root of *Millettia aboensis* as a yellow solid. It showed UV (MeOH) λ_{max} 204.8, 265.5, and 334 (see Figure 4.65), which is suggestive of a derrisisoflavone nucleus and $[\alpha]^{20}_D$ -2.25 (c, 0.4, MeOH). The ESI-MS showed the pseudomolecular ion peak at *m/z* 337.2 [M-CH_3] (see Fig. 4.67). The molecular formula was deduced as $C_{19}H_{14}O_5$ based on the observed MS. The ^{1}H NMR spectrum (see Figure 4.66) of compound **17** shows a proton singlet at δ 8.16 (s, H-2), characteristic of an isoflavone and four aromatic proton signals for ring B protons of AA'BB' system at δ 7.40 (m, H-2', H-6'), and 6.87 (m, H-3', H-5'), due to 1', 4'-disubstituted B-ring. The ^{1}H NMR spectrum also revealed a singlet aromatic proton signal for ring A proton at δ 6.29 (s, H-6). It also revealed an isopropenyl furan proton signals at δ 1.82 (d, 1.3, CH_3-3"), 1.69 (d, 1.5, CH_3-4"), 3.44 (d, 7.2, H-1"), and 5.22 (m, H-2"). The aromatic proton at δ 6.29 (s) was assigned to H-6 of the ring A. The absence of signal for H-8 also supported the location of the furan ring at the position, compound **17** was established as derrisisoflavone N, derivative of

derrisisoflavone G (compound **6**) being a new compound which has never been isolated or reported.

Table 4.21: Proton NMR spectroscopic data of compound **17** (CD_3OD, 300 MHz)

Position	**δ_H (J in Hz)**
2	8.16 (s)
3	-
4	-
5	-
6	6.29 (s)
7	-
8	-
9	-
10	-
1’	-
2’	7.40 (m)
3’	6.87 (m)
4’	-
5’	6.87 (m)
6’	7.40 (m)
1”	4.60 (s), 3.44 (d, 7.2)
2’’	5.22 (m)
3”	1.82 (d, 1.3)
4’’	1.69 (d, 1.5)

4.6. Bioactivity screening of the extracts and isolated compounds

4.6.1. Cytotoxicity of the crude extracts

The crude extracts of the leaf, root, stem and pod were tested for cytotoxicity toward the mouse lymphoma cell line, and showed 31.4, 87.5, 36.7 and 68.5 % growth inhibition, respectively (**Table 4.22**.)

Table 4.22: Cytotoxicity of the crude extracts

Crude extracts	% growth inhibition
Leaf	31.4±0.14
Root	87.5±0.23
Pod	36.7±0.26
Stem	68.5±0.16

Note: 1 mg of crude extract used for each extract.

4.6.2. Antioxidant scavenging activity of the isolated compounds

Radical-scavenging activity of the isolated compounds was assessed using the DPPH assay. As a reference compound, the well-known naturally occurring ascorbic acid was included. IC_{50} values were determined for each of these compounds and are presented in **Table 4.23**. Compounds **10** and **15** were more active than all the isolated compounds.

Table 4.23: Antioxidant activity of the isolated compounds

Compound	IC_{50} (µg/ml)
1	166
2	666
3	317
4	359
5	259
6	281
7	377
8	215
9	95
10	83
11	181
12	198
13	877
14	113
15	83
16	90
17	109
Ascorbic acid	8

4.7. Discussion

Isoflavones are the secondary metabolite formed by symbiotic relationship with the rhizobial bacteria and the defense responses of leguminous plant (Yu *et al.*, 2000). Isoflavones are synthesized as part of the phenylpropanoid pathway, the same biosynthetic pathway of flavonoid biosynthesis (Barnes, 2010). Phenylalanine converts 4-hydroxycinnamoyl CoA by reaction with malonyl CoA. Chalcone synthase catalyzes the reaction of this intermediate to convert to 4,2',4',6'-tetrahydrozychalcon (naringenin chalcone) and the combined enzyme reaction of chalcon synthase and chalcone reductase convert this intermediate to 4,2',4'-trihydrozychalcone (isoliquiritigenin). And then, chalcone isomerase catalyzes the ring closure of the heterocyclic ring to form 7, 4'-dihydrozyflavone (liquirintigenin) and 5, 7, 4'-trihydroxyflavone (naringenin). The B-ring is moved from the 2-position to 3-position by isoflavone synthase. Isoflavone dehydratase removed water to generate the 2, 3 double bond in the heterocyclinc ring. The products generated by this reaction were daidzein (7, 4'-dihydroxyisoflavone) and genistein (5, 7, 4'-trihydroxyisoflavone).

Genistein has antioxidant properties as scavenger of radicals and chelaters of metals. This function occurred by affecting gene expression of enzymes that react with antioxidants such as catalase and superoxide dismutase, and inhibiting with secondary oxidant production such as hydrogen peroxide or hypochlorous acid (Mortensen *et al.*, 2009). Genistein is more active as antioxidant than daidzein due to having its third hydroxyl group in the C-5 position and equol is another better antioxidant actions than its precursor compounds due to the absence of the 2, 3-double bond in conjunction with a loss of the 4-oxo group which is enhancing antioxidant properties (Arora *et al.*, 1998).

Isoflavone metabolism pathway in intestine and liver affect the antioxidant properties of isoflavones and the metabolites. Although genistein significantly reacts with superoxide dismutase, catalase, and glutathione peroxidase, isoflavone metabolites such as equol, 8-OH-daidzein, O-desmethylangolensin (O-DMA), and 1,3,5-trihydroxybenzene have also played a role as potent scavengers. 8-hydroxy-daidzein is the most potent scavenger for hydroxyl and superoxide anion radicals. These isoflavone metabolites are highly chelating with ferrous compound relative to genistein and daidzein. However sulfated isoflavones can decrease antioxidant activity. Isoflavones can reduce low-density lipoprotein oxidation and lipid peroxidation by reacting with lipid radicals (Tikkanen *et al*., 1998).

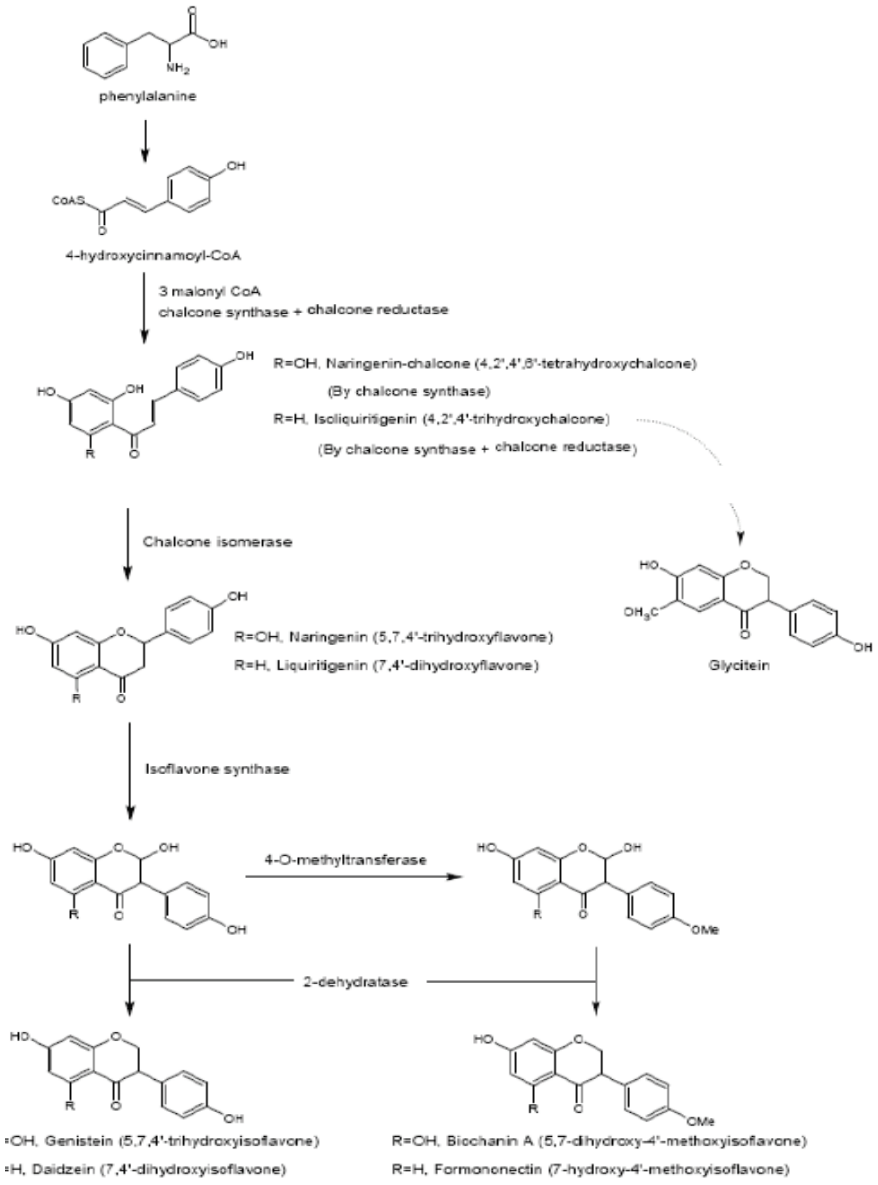


Figure 4.68: Biosynthesis pathway of Isoflavones (Barnes, 2010)

From the cytotoxicity studies, the crude methanol root extract gave the highest growth inhibition at 87.5% (see **Table 4.22**), and the major compounds isolated from the root are the pterocarpans and derrisisoflavones. Before now many scholars have done enormous work on the cytotoxicity of pterocarpans and it is proven that these class of compound are cytotoxic compounds (Niu *et al*., 2013), which suggests the promising cytotoxic activity of the root.

The DPPH radical is a stable organic nitrogen radical, is commercially available and has a deep purple colour (Prior *et al*., 2005; Ajaegbu, 2013). The free radical scavenging assay measures the reducing capacity of antioxidants toward DPPH. Upon reduction, the colour of DPPH solution fades. Consequently, test compounds with high antioxidant activity result in a rapid decline in the absorbance of the DPPH solution (Amarowicz *et al*., 2004; Ajaegbu, 2013). The effect of antioxidants on DPPH is thought to be due to their hydrogen donating ability. Though the DPPH radical scavenging abilities or IC_{50} of some of all the isolated compounds were less than those of ascorbic acid, the study showed that the extracts have the proton-donating ability and could serve as free radical inhibitors or scavengers, acting possibly as primary antioxidants. The DPPH test is simple and quick (within 30 min). The DPPH radical can be prepared in less than an hour which is very rapid compared to the ABTS radical preparation (12-16 h). The DPPH test has been in widespread use in antioxidant capacity screening, probably due to the simplicity of the equipment required (Huang *et al*., 2005; Ajaegbu, 2013). Sánchez-Moreno (2002) suggested the DPPH test as an easy and accurate method for measuring the antioxidant capacity of fruit and vegetable juices or extracts.

Though the DPPH radical scavenging abilities or IC_{50} of some of the isolated compounds were less than those of ascorbic acid, the study showed that the isolated compounds have the proton-donating

ability and could serve as free radical inhibitors or scavengers, acting possibly as primary antioxidants.

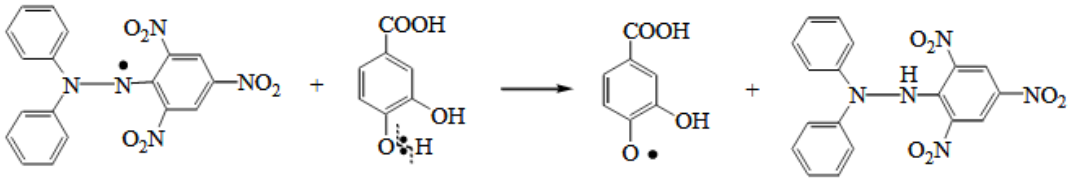
Genistein is well known for its antioxidant activity in biological systems, and it scavenges peroxyl radicals and protects against iron-induced free radical reactions. According to previous studies, genistein showed low DPPH radical scavenging activity while showing high ABTS radical scavenging activity (Choi *et al*., 2009). In this study, compound **2** which is a derivative of genistein also demonstrated low DPPH radical scavenging activity at 666 µg/ml (**Table 4.23**), while compound **13** which has only hydroxyl group in its structure had the lowest antioxidant radical scavenging activity at 877 µg/ml (**Table 4.23**). But compounds **9** and **11** have good antioxidant radical scavenging activity at 95 and 181 µg/ml, (**Table 4.23**) respectively. Genistein generally at molecular level, acts as a/an potential protein tyrosine, kinase inhibitor, topoisomerase II, inhibitor of phosphatidyl, inositol turnover, via ER (estrogen receptor)-mediated pathways inhibitor of proteins that are involved in multidrug resistance of cancer cells. At cellular level, genistein results in induction of apoptosis, attack different signal transduction enzymes which results in the decreased rate of cell proliferation, has better inhibitory effects in leukemic cell lines and compared to cell lines derived from tumors, inhibits cell differentiation, undergo differentiation followed by apoptotic cell death, it can modulate or alter cell cycling, promotes mutagenesis, carcinogenesis and tumors, inhibit primary events necessary for increased level of ROS production or it can directly inhibit production of ROS, is an inhibitor in preventing angiogenesis, has an estrogenic activity, can imitate the action of estradiol and stimulate cell growth, when treated with steoclasts in a bone tissue culture lose their bone degradation potency (Gupta *et al*., 2016).

In several in vitro studies, epigallocatechin gallate has been found to have the highest antioxidant activity compared to other catechins (Legeay *et al*., 2015), and the DPPH radical scavenging ability

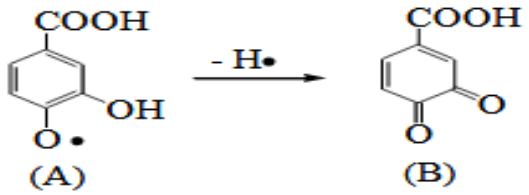
of catechins follows the trend EGCG > ECG > EGC > EC (Anna *et al*., 2005). One hypothesis to explain these properties is a low reduction potential of EGCG due to its high capacity for giving an electron. Electron delocalization in the molecular structure is described as a property of polyphenolic compounds which could in part be responsible for their antioxidant activity. In the catechin skeleton, the saturation of the heterocyclic ring prevents electron delocalization between the A and the B ring. The antioxidant potential mainly comes from the strong presence of hydroxyl groups in their molecular structures. EGCG, with 8 hydroxyl groups and with a gallate moiety in C-3 is a better electron donor than the others catechins and thus the best scavenger of free radicals species. Compound **3** being an epicatechin gallate with only four hydroxyl groups and a gallate moiety in C-3 had a moderate antioxidant activity at 313 µg/ml (**Table 4.23**). Catechins can also inhibit the release of some allergic reactions factors such as leucotrienes, prostaglandins, by modifying activities of multiple enzymes taking part in inflammatory states of the human body. Catechins can influence the endocrine system. It was also found that catechins can modulate steroid hormones concentration in the body, which is possibly an essential element in anticancerogenic prophylaxis. They are strong antioxidants protecting healthy cells of rat liver against oxidative stress, and preventing DNA damages. Catechins can enhance the *in vitro* resistance of alveolar macrophages to *Legionella pneumophila* infection by selective immunomodulatory effects on cytokine formation. This suggests that catechins may be a potential immunotherapeutic agent against respiratory infections in immunocompromised patients, such as heavy smokers. Catechins are known to prevent dental caries. The effects of these compounds on cariogenicity has been observed in *in vitro* tests. Catechins inhibits the growth of cariogenic bacteria *Streptococcus mutans* and *S. sobrinus*. Chewing gums containing catechins was found effective in decreasing dental plaque formation in humans. Epicatechin gallate reduced oxacillin

resistance in methicillin–resistant *Staphylococcus aureus* (MRSA) at concentrations below the MIC. Substitution of the gallate group of ECG with 3-*O*-acyl chains of varying lengths C4–C18 led to enhanced anti-staphylococcal activity with chain lengths of C8 and C18. 3-*O*-octanoyl catechin was bactericidalagainst MRSA as a result of membrane damage. Epicatechin gallate was found also as an agent to combat beta-lactam resistance in *S. aureus*. Modulation of beta-lactam resistance by epicatechin gallate significantly enhanced the activities of flucloxacillin and the carbapenem antibiotics imipenem and meropenem against MRSA isolates, with MIC values for the antibiotics reduced to the susceptibility breakpoint or below. Green tea catechins can act as inhibitors of some enzymes important for microorganisms. Catechins was effective in inhibiting protein tyrosine phosphatase (PTPase) activity in *Proveta intermedia* at 0.5 μmol. Epicatechins act as anticancerogens due to the activation of the second phase detoxication enzymes which accelerate the removal of activated chemical cancerogenes from cells.

DPPH which is being scavenged by an antioxidant through the donation of hydrogen atom so as form a stable DPPH. According to this hypothesis and previous study, the reaction between DPPH and PCA could be explained by the following mechanism:



Radical (A) can further withdraw hydrogen atom to form stable quinone (B).



The result indicates that PCA can effectively inhibit DPPH, suggested that PCA exerted radical scavenging action by donating hydrogen atom (Xican *et al.*, 2011). Hence the experiment carried out using compound **4** as an antioxidant showed that it has moderate radical scavenging activity at 359 µg/ml (**Table 4.23**), since it can donate hydrogen atom to form a stable DPPH. Many work has been done in order to understudy the biological and pharmacological effects of protocatechuic acid such as antioxidant, antiviral, antiatherosclerotic and hyperlipidemic, nephroprotective, and neurological effects. Different properties of protocatechuic acid have been studied for its protective effects against oxidative breakdown done by *tert*-butyl hydroperoxide (t-BHP) in a culture of rat hepatocytes. It was found that PCA has protective effects against cytotoxicity and genotoxicity of hepatocytes induced by t-BHP through scavenging free radicals. Moreover, protocatechuic acid inhibited 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced skin tumors of female CD-1 mice showing its anti-tumor effect. Moreover, protocatechuic acid was found effective against pulmonary cancer. The inhibitory phenomenon of catechin gallates and gallic acid esters on cellular protocatechuic acid o-methylation was due to the direct response of catechol-O-methyl transferase (COMT) activity. The inhibitory effect of protocatechuic acid and roselle calyx was studied on growth of various bacteria and protocatechuic acid was found to have greater antibacterial activity than roselle calyx but less inhibitory effect in human plasma than in brot. Protocatechuic acid found to have *in vitro* inhibitory effects on the growth of *Helicobacter pylori (H. pylori).* Also, protocatechuic acid produced by ferulic acid through bioconversion was studied in plant-growth promoting *Pseudomonas putida* WCS358 and it was found that the strongest

induction of genes was done by protocatechuic acid. The anti-aerobic and anti-oxidative effects of protocatechuic acid and *roselle calyx* in ground beef were determined revealing that growth of various bacteria and lipid oxidation was decreased in ground beef. So it was also found that protocatechuic acid could be used for the prevention of microbial growth in foods (Abida *et al*., 2015).

Compound **5** being also a carboxylic acid that has structure relationship with compound **4** follows the same trend in its radical scavenging activity. As earlier said, since it has more OH groups than compound **4**, its antioxidant activity will be better or higher than that of compound **4**. Compound **5** therefore had a higher antioxidant radical scavenging activity at 259 µg/ml (**Table 4.23**) than compound **4** at 359 µg/ml (**Table 4.23**). Compound **5** has shown to be of great pharmacological important through reports on the anti-microbial activity against methicillin-resistant *Staphylococcus aureus* and *Helicobacter pylori*, anti-inflammatory activity evaluated by zymosan-induced acute food pad swelling in mice, carrageenan-induced paw edema, acetic acid-induced writhing responses and formalin-induced pain in animal models, suggested mechanisms were scavenging of superoxide anions, inhibition of myeloperoxidase release and activity as well as interference with activity of NADPH-oxidase. Other reported activities are anti-depressant, antiparkinson, anti-diabetic, anti-malarial, diuretic, cardio-protective, anti-viral, anti-fungal, wound healing, anthelmintic and anxiolytic. Compound **5**, when combined with other natural products such as, calycosin, reported to synergistically attenuate neutrophil infiltration and subsequent injury in isoproterenol-induced myocardial infarction (Nayeem *et al.*, 2016).

To also prove the hypothesis further of the hydrogen donating ability of any compound being responsible for the increase in the antioxidant radical scavenging activity, compound **1** has hydrogen atoms available to be donated, hence the best activity when compared with all the organic

acids isolated during the course of this work. Compound **1** IC_{50} was seen at 166 µg/ml (**Table 4.23**), when compared we see compound **4** and **5** at 359 and 259 µg/ml (**Table 4.23**) respectively. Compound **1** was previously reported to inhibit rat liver acetyl CoA carboxylase (IC_{50} 55 mg/mL) (Endo *et al.*, 1985). But a recent study by Akone *et al*., 2014 shows that it is inactive against rat liver acetyl CoA carboxylase. There is therefore the need to explore the bioactivities of compound **1**.

Derrisisoflavones have demonstrated lots of biological or pharmacological activities such as antidermatophyte activity against *T. mentagrophytes*, radical scavenging activity using DPPH, antibacterial activity against *S. aureus* and MRSA, anti-hypertensive activity, antidermatophyte activity against *T. mentagrophytes* (Suwanna**,** 2016). Based on these the derrisisoflavones in this study (compounds **6**, **8**, **14**, **16**, and **17**) were subjected to DPPH radical scavenging test and they showed a good antioxidant activity (IC_{50}) as follows: **6** – 281 µg/ml, **8** – 251 µg/ml, **14** – 113 µg/ml, **16** – 90 µg/ml , and **17** – 109 µg/ml (**Table 4.23**). Among all the compounds isolated from the plant *Millettia aboencis*, apart from compound **10**, compound **16** which is a derrisioflavone has the highest antioxidant radical scavenging activity. This may be attributed to the number of hydroxyl groups present in the each of these compounds and also the availability of free hydrogen groups or atoms that can be donated to the DPPH for stabilization. For compound **16** which has the highest activity among the derrisisoflavones, it has three hydroxyl groups, while compound **17**, which seconded compound **16** also has three hydroxyl groups. Also compound **14** and **8** that followed suit after compound **17** has three hydroxyl groups. While compound **6** which has the least activity among the derrisisoflavones has only two hydroxyl groups. The number of hydroxyl groups available in a molecule has a long role to play for a better antioxidant radical scavenging

activity, since the hydrogens present in such hydroxyl groups are always available for donation for the stabilization of the radical – DPPH used for the screening.

Pterocarpans are naturally occurring plant products carrying a *cis*-fused benzofuranyl-benzopyran ring system. Many of them are phytoalexins possessing high antifungal and antibacterial activity and several of them have been reported to inhibit HIV-1 reverse transcriptase and the cytopathic effect of HIV-1 in cell cultures. Furthermore, it has also been demonstrated that two representatives of these natural products, cabenegrin A-I [(–)-**1**] and A-II [(–)-**2**], are active components of a Brazilian folk medicine used against snake venoms. Thus, both compounds have been found to be active in male beagle dogs (1 mg/kg i.v.) against the *Bothrops atrox* venom. (Sándor *et al*., 2004). Pterocarpans was reported to show radical scavenging activity, hypertensive activity, but exhibited no antibacterial activity against *Staphylococcus aureus* and methicillin-resistant *S. aureus* (Suwanna, 2016). The pterocarpans in this study are compounds **7** – 377 µg/ml and **10** – 83 µg/ml (**Table 4.23**), where compound **10** had the highest DPPH radical scavenging activity among all the compounds isolated in this study. Hence it has more antioxidant activity than compound **7**, the crucial reason suggestive of the hydroxyl group(s) present in the compound.

Chapter five

Summary, Conclusion and Recommendation

5.1. Summary

Researchers have come to discover that natural products, which we also known as secondary metabolites, have been the major sources of chemical diversity of starting materials for driving drug discovery over the past century. The vast majority of traditionally used crude drugs have been plant-derived extracts (Wang, 2009). But only few of these medicinal plants in Nigeria have been given considerable attention to its isolation, purification and identification of the secondary metabolites present in them.

The focus of this study was therefore on the isolation and structural elucidation of the bioactive compounds from medicinal plants, followed by the evaluation of their bioactive properties (cytotoxicity and antioxidant screening) . Several modern chromatographic techniques were used for separation and purification of the natural products from the crude extracts of the different plant parts of *Millettia aboensis*. The structures were elucidated or identified with the help of one dimensional NMR and mass spectrometric data.

5.2. Conclusion

Drug discovery from natural sources such as plants, animals and micro-organisms provide a basis for the isolation of unique and potentially potent bioactive compounds (Cooper, 2008). In this study, four parts of ***Millettia aboensis*** plant were successively extracted with methanol. The methanol extracts were further fractionated using hexane, ethyl acetate, and butanol. The crude extracts were screened for cytotoxic activity using the MTT assay.

The ethyl acetate fractions of the methanol extracts were purified and some compounds isolated. Some of the isolated compounds were identified to be new compounds. Among the compounds isolated from the ethyl acetate fractions of the stem, root and leaf, and the crude extract of the pod; five compounds were isolated from the leaf namely: 2 decyl pent-2-enedioic acid, genistein-4'-O-arabinofuranoside, epicatechin-3-O-(4-methyl gallate), protocatechuic acid and gallic acid; nine compounds were isolated from the root namely: derrisisoflavone G, 3,8-dimethoxy pterocarpan, derrisisoflavone M, 4'-methoxy orobol, 3', 4'-methylenedioxy pterocarpan, orobol, 2',3'-diethyl kaempferol, 3'-methoxy daidzein and derrisisoflavone O; one compound from the pod namely: luteolin, and two compounds from the stem namely: derrisisoflavone L and derrisisoflavone N. A total of seventeen compounds were isolated from the plant parts of *Millettia aboencis* and ten (epicatechin-3-O-(4-methyl gallate), 3,8-dimethoxy pterocarpan, derrisisoflavone M, 4'-methoxy orobol, 3', 4'-methylenedioxy pterocarpan, 2',3'-diethyl kaempferol, 3'-methoxy daidzein, derrisisoflavone O, derrisisoflavone L and derrisisoflavone N) of these compounds are new bioactive compounds based on the antioxidant screening.

5.3. Recommendation

In any future studies derived from this study, the following research will provide a better understanding of the bioactive compounds from ***Millettia aboensis*** plant:

- Complete profile of bioactive compounds present from ***Millettia aboensis*** plant. Limited funding to purchase additional standards and LC-MS services prevented the positive identification of other compounds in this study.
- Characterization and identification of bioactive compounds in other fractions especially butanol, hexane and water fractions.

- Purification of the other fractions using gel chromatography; column chromatography and identification of the bioactive compounds present.
- Cytotoxic assay and other bioassay on the isolated compounds.
- Structural elucidation of the isolated pure compounds.

5.4. Contribution to knowledge

- Isolation of the pure bioactive compounds which has been in imagination of its present in the species from pharmacological, microbiological, etc. screening carried out before now by some researchers.
- Identification of those pure isolated bioactive compounds using state of the art NMR, MS and HPLC-UV techniques.
- Antioxidant screening of the isolated and identified pure bioactive compounds using free radical DPPH scavenging assay.

References

Abida, K.K. Rehana, R. Nighat, F. Sadaf, M. Sadullah, M. Sara, K. Nyla, J. and Ghulam, M. (2015). Pharmacological activities of Protocatechuic acid. *Acta Poloniae Pharmaceutica and Drug Research*, 72(4), 643-650.

Ajaegbu, E.E. (2013). Isolation and identification of antioxidant phenolic compounds from *Spondias mombin* leaf. M.Sc thesis submitted to the Department of Pharmaceutical and Medicinal Chemistry, Faculty of Pharmaceutical Sciences, Nnamdi Azikiwe University, Awka, Nigeria.

Ajaghaku, D.L. Akah, P.A. Ilodigwe, E.E. Nduka, S.O. Osonwa, U.E. Okoye, F.B.C. (2018). Upregulation of CD_4^+ T-Lymphocytes by Isomeric Mixture of Quercetin-3-O-Rutinoside and Quercetin-3-O-Robinobioside Isolated from *Millettia aboensis*. *Immunological Investigations*, 47(4), 372-388.

Ajaghaku, D.L. Ilodigwe, E.E. Okonta, M.J. Ogbue, C.O. Okafor, M.A. and Igboeme, S.O. (2013). Mechanisms of Anti-inflammatory Activity of the Leaf Extract and Fractions of *Millettia aboensis*. *Int. Res. J. Pharm*, 4(9).

Akone, S.H. Rahn, S. Henrich, B. Daletos, G. Vardamides, J.C. Nkengfack, A.E. Lin, W.H. and Proksch, P. (2014). 2-Pentenedioic acid derivatives from a soil-derived fungus Gongronella butleri. *Phytochemistry Letters*, 10, 184–188

Alternative Medicine Review Monographs 2002. Thorne Research Inc. 394-399

Amarowicz, R. Pegg, R.B. 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.

Amersham Biosciences AB. (2002). Gel filtration- principles and methods. Retrieved from www. Chromatographyamershawbiosciences.com.

Anna, G. Józef, K. and Ryszard, A. (2005). Tea polyphenols – their antioxidant properties and biological activity – a review. *Pol. J. Food Nutr. Sci*, 14/55(3), 219–235.

Arora, A. Nair, M.G. and Strasburg, G.M. (1998). Antioxidant activities of isoflavones and their biological metabolites in a liposomal system. *Arch Biochem Biophys*, 356, 133-41.

Ashour, M. Edrada, R. Ebel, R. Wray, V. Wätjen, W. Padmakumar, K. Müller, W.E.G. Lin, W.H. and Proksch, P. (2006). *J. Nat. Prod*, 69, 1547−1553.

Banzouzi, J.T. Prost, A. Rajemiarimiraho, M. and Ongoka, P. (2008). Traditional uses of the African *Millettia* species (Fabaceae). *International Journal of Botany*, 4, 406-420.

Barnes, S. (2010). The biochemistry, chemistry and physiology of the isoflavones in soybeans and their food products. *Lymphat Res Biol*, 8, 89-98.

Brogger, C.S. and Kharazmi, A. (2001). Antimalarial natural products. In *Compounds from* Natural Sources: Isolation, Characterization, and Biological Properties; Tringali, C., Ed.; Taylor & Francis: New York.

Carmichael, J. DeGraff, W.G. Gazdar, A.F. Minna, J.D. and Mitchell, J.B. (1987). *Cancer Res*, 47, 943-946.

Cheng, H.H. Wang, H.K. Ito, J. Bastow, K.F.T. Nakanishi, Y. Xu, Z. Luo, T.Y. and Lee, K.H. (2001). Cytotoxic pheophorbide-related compounds from Clerodendrum calamitosum and C. cyrtophyllum. *J. Nat. Prod*, 64, 915-919.

Choi, J.N. Dockyu, K. Hyung, K.C. Kyung, M.Y. Jiyoung, K. and Choong, H.L. (2009). 2'-Hydroxylation of Genistein Enhanced Antioxidant and Antiproliferative Activities in MCF-7 Human Breast Cancer Cells. *J. Microbiol. Biotechnol*, 19(11), 1348–1354.

Cooke, M. and Poole, C.F. (2000). Encyclopedia of separation science. Academic Press.

Cooper, E.L. (2008). eCAM: An Emerging Linkage with Ethnopharmacology? Evidence Based. *Complementary and Alternative Medicine*, 5, 365-366.

Costa, S.M.O. Lemos, T.L.G. Pessoa, O.D.L. Pessoa, C. Montenegro, R.C. and Braz-Filho, R. (2001). Chemical constituents from *Lippia sidoides* and cytotoxic activity. *J. Nat. Prod*, 64, 792-795.

Cragg, G.M. Newman, D.J. and Snader, K.M. (1997). Natural products in drug discovery and development. *J. Nat Prod*, 60(1), 52-60.

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 characterization of flavonoid O-glycosides by positive and negative nano-electrospray ionization ion trap mass spectrometry. *J Mass Spectrom,* 36, 1203-1210.

Cuyckens, F. Shahat, A.A. Pieters, L. and Claeys, M. (2002). Direct stereochemical assignement of hexose and pentose residues in flavonoid O-glycosides by fast atom bombardment and electrospray ionisation mass spectrometry. *J Mass Spectrom,* 37, 1272-1279.

da Rocha, A.B. Lopes, R.M. and Schwartsmann, G. (2001). Natural products in anticancer therapy. *Curr. Opin. Pharmacol,* 1, 364-369.

Davies, K.J. (1995). "Oxidative stress: The paradox of aerobic life". *Biochemical Society Symposia*, 61, 1–31.

Demmig-Adams, B. and Adams, W. (2002). "Antioxidants in Photosynthesis and Human Nutrition". *Science*, 298(5601), 2149–2153.

Duncan, A.M. Phipps, W.R. and Kurzer, M.S. (2003). Phyto-oestrogens. *Best Pract Res Clin Endocrinol Metab*, 17, 253-271.

Endo, A. Takeshima, H. and Kuwabara, K. (1985). Acetyl CoA carboxylase inhibitors from the fungus Gongronella butleri. *J. Antibiot*, 38, 599–604.

Farnsworth, N.R. Akerele, O. Bingel, A.S. Soejarto, D.D. and Guo, Z. (1985). Medicinal plants in therapy. *Bull World Health Organ*, 63(6), 965-981.

Finkel, T. and Holbrook, N.J. (2000). "Oxidants, oxidative stress and the biology of ageing". *Nature*, 408(6809), 239–247.

Franski, R. Bednarek, P. Wojtaszek, P. and Stobiecki, M. (1999). Identification of flavonoid diglycosides in yellow lupin (*Lupinus luteusL*.) with mass spectrometric techniques. *J Mass Spectrom*, 34, 486-495.

Franski, R. Matlawska, I. Bylka, W. Sikorska, M. Fiedorow, P. and Stobiecki, M. (2002). Differentiation of interglycosidic linkages in permethylated flavonoid glycosides from linked-scan mass spectra (B/E). *J Agric Food Chem*, 50, 976-982.

Goodman, J. and Walsh, V. (2001). *The Story of Taxol: Nature and Politics in the Pursuit of an Anti-cancer Drug*; Cambridge University Press: USA.

Grabley, S. and Thiericke, R. (1999). The impact of natural products on drug discovery. In *Drug Discovery from Nature*; Grabley, S.; Thiericke, R., Eds.; Springer: Berlin/Heidelberg.

Grafe, U. (1999). Secondary metabolites: From past to present. In *Drug Discovery from Nature*; Grabley, S.; Thiericke, R., Eds.; Springer: Berlin/Heidelberg.

Guilet, D. Helesbeux, J.J. Seraphin, D. Sevenet, T. Richomme, P. and Bruneton, J. (2001). Novel cytotoxic-phenylfuranocoumarins from *Calophyllum dispar*. *J. Nat. Prod*, 64, 563-568.

Gunatilaka, A.A.L. Berger, J.M. Evans, R. Miller, J.S. Wisse, J.H. Neddermann, K.M. Bursurker, I. and Kingston, D.G.I. (2001). Isolation, Synthesis, and Structure-Activity Relationships of Bioactive Benzoquinones from *Miconia lepidota* from the Suriname Rainforest. *J. Nat. Prod*, 64, 2-5.

Gunatilaka, A.A.L. Ramdayal, F.D. Sarragiotto, M.H. Kingston, D.G.I. Sackett, D.L. and Hamel, E. (1999). Synthesis and biological evaluation of novel paclitaxel (Taxol) D-ring modified analogues. *J. Org. Chem*, 64, 2694-2703.

Gupta, A. Sahoo, P.K. and Arora, T. (2016). Genistein- A Potential Boon for Cancer Therapy. *TPI*, 5(6), 81-86

Hallas, J.M. (2004). Modern spectroscopy. Fourth Edition. John Wiley& Sons, Ltd.

Harry, L. and Brielmann, J. (1999). *Phytochemicals: The chemical components of plants*; CRC Press: Boca Raton, FL.

Hirst, J. King, M.S. Pryde, K.R. (2008). "The production of reactive oxygen species by complex I". *Biochemical Society Transactions*, 36(5), 976–980.

Horn-Ross, P.L. Barnes, S. and Lee, M. (2000). Assessing phytoestrogen exposure in epidemiologic studies: development of a database (United States). *Cancer Causes Control*, 11, 289-98.

Hsieh, T.J. Chang, F.R. Chia, Y.C. Chen, C.Y. Chiu, H.F. and Wu, Y.C. (2001). Cytotoxic constituents of the fruits of *Cananga odorata*. *J. Nat. Prod*, 64, 616-619.

https://en.wikipedia.org/wiki/Millettia

Huang, D. Ou, B. Prior, R.L. (2005). The chemistry behind antioxidant capacity assays. *J Agric Food Chem*, 53, 1841-1856.

IITA Forest Center – Transforming African Agriculture. *Millettia aboensis* (Hook. F) Baker. Accessed 31st March 2018. Retrieved from forestcenter.iita.org/index.php/manual-page/millettia-aboensis-hook-f.baker/

Imlay, J.A. (2003). "Pathways Of oxidativedamage". *Annual Review of Microbiology,* **57,** 395–418.

Ito, A. Cui, B. Chavez, D. Chai, H.B. Shin, Y.G. Kawanishi, K. Kardono, L.B.S. Riswan, S. Farnsworth, N.R. Cordell, G.A. Pezzuto, J.M. and Kinghorn, A.D. (2001). Cytotoxic polyacetylenes from the twigs of *Ochanostachys amentacea. J. Nat. Prod, 64*.

Jagtap, P.G. and Kingston, D.G.I. (1999). A facile N-debenzoylation of paclitaxel:Conversion of paclitaxel to docetaxel. *Tetrahedron Lett*, 40, 189-192.

Jaziri, M. and Vanhaelen, M. (2001). Bioactive taxoid production from natural sources. In *Bioactive Compounds from Natural Sources: Isolation, Characterization, and Biological Properties*; Tringali, C., Ed.; Taylor & Francis: New York.

Jie, Li. Chunhua, Y. Li, P.P. Annécie, B. Heebyung, C. William, J. Keller, C. Benjamin, N. and Douglas, K. (2017). Bioassay-Guided Isolation of Antioxidant and Cytoprotective Constituents from a Maqui Berry (Aristotelia chilensis) Dietary Supplement Ingredient as Markers for Qualitative and Quantitative Analysis. *J. Agric. Food Chem*, 65, 8634−8642.

Kang, H.B. Zhang, Y.F. and Yang, J.D. (2012). Study on soy isoflavone consumption and risk of breast cancer and survival. *Asian Pac J Cancer Prev*, 13, 995-998.

Kazuaki, K. Masayuki, S. Natsuki, M. Fumio, N. and Yukihiko, H. (2000). Identification of Biliary Metabolites of (-)-Epigallocatechin Gallate in Rats. *J. Agric. Food Chem*, 48, 4151-4155.

Kelland, L.R. (2000). Flavopiridol, the first cyclin-dependent kinase inhibitor to enter the clinic: Current status. *Expert Opin. Invest. Drugs*, 9, 2309-2911.

Kerfeld, C. (2004). "Water-soluble carotenoid proteins of cyanobacteria". *Archives of Biochemistry and Biophysics*, 430(1), 2–9.

Kim, D.H. Ma, E.S. Suk, K.D. Son, J.K. Lee, J.S. and Woo, M.H. (2001). Annomolin and annocherimolin, new cytotoxic annonaceous acetogenins from *Annona cherimolia* seeds. *J. Nat. Prod*, 64, 502-506.

Kim, J. (2008). Protective effects of Asian dietary items on cancers - soy and ginseng. *Asian Pac J Cancer Prev*, 9, 543-548.

Kinghorn, A.D. (2001). Pharmacognosy in the 21st century. *J Pharm Pharmacol*, 53(2), 135-148.

Kingston, D.G.I. (2001). Taxol, a molecule for all seasons. *Chem. Commun*, 10, 867-880.

Klejdus, B. Mikelova, R. and Petrlova, J. (2005). Evaluation of isoflavone aglycon and glycoside distribution in soy plants and soybeans by fast column high-performance liquid chromatography coupled with a diode-array detector. *J Agric Food Chem*, 53, 5848-52.

Kong, J.M. Goh, N.K. Chia, L.S. and Chia, T.F. (2003). Recent advances in traditional plant drugs and orchids. *Acta Pharmacol Sin*, 24(1), 7-21.

Krieger-Liszkay, A. (2004). "Singlet oxygen production in photosynthesis". *Journal of Experimental Botany*, 56(411), 337–346.

Kuiper, G.G. Carlsson, B. and Grandien, K. (1997). Comparison of the ligand binding specificity and transcript tissue distribution of estrogen receptors alpha and beta. *Endocrinology*, 138, 863-870.

Kuo, Y.H. and King, M.L. (2001). Antitumor drugs from the secondary metabolites of higher plants. In Bioactive Compounds from Natural Sources: Isolation, Characterization, and Biological Properties; Tringali, C., Ed.; Taylor & Francis: New York.

Kwang-Pil, K. (2014). Isoflavones: Chemistry, Analysis, Function and Effects on Health and Cancer. *Asian Pacific Journal of Cancer Prevention,* 15, 7001-7010.

Lambert, J.B. and Mazzola, E.P. (2000). Nuclear magnetic resonance spectroscopy, an introduction to principles, applications, and experimental methods. Prentice Hall.

Lathe, G.H. Ruthven, C.R. (1956). The Separation of Substance and Estimation of their Relative Molecular Sizes by the use of Columns of Starch in Water. *Biochem J.*, 62, 665–674.

Lee, M.S. (2002). LC/MS Applications in Drug Development. John Wiley & Sons, Ltd.

Legeay, S. Rodier, M. Fillon, L. Faure, S. and Clere, N. (2015). Epigallocatechin Gallate: A Review of Its Beneficial Properties to Prevent Metabolic Syndrome Nutrients, 7, 5443-5468.

Lenaz, G. (2001). "The Mitochondrial Production of Reactive Oxygen Species: Mechanisms and Implications in Human Pathology". *IUBMB Life*, 52(3–5), 159–164.

Li, J. Yuan, C. Pan, L. Benatrehina, P. Annécie, C.H. Keller, W.J. Naman, C. and Benjamin, K.A.D. (2017). Bioassay-Guided Isolation of Antioxidant and Cytoprotective Constituents from a Maqui Berry (Aristotelia chilensis) Dietary Supplement Ingredient as Markers for Qualitative and Quantitative Analysis. *J. Agric. Food Chem*., 65, 8634−8642

Li, N.G. Shi, Z.H. Tang, Y.P. Yang, J.P. and Duan, J.A. (2009). An efficient partial synthesis of 4'-O-methylquercetin via regioselective protection and alkylation of quercetin. *Beilstein J Org Chem*, 4(5), 60.

Liggins, J. Bluck, L.J. and Runswick, S. (2000). Daidzein and genistein contents of vegetables. *Br J Nutr*, 84, 717-725.

Lin, C. Liu, Y. Kuo, Y. Shen, C. Chiou, W. and Chen, C. (2016). Flavonoids and bis-coumarin from the tubers of Apios taiwanianus. *Phytochemistry Letters*, 15, 164–167.

Lin, L. Yu-Feng, P. and Tung-Hu, T. (2015). Isolation of Luteolin and Luteolin-7-O-glucoside from Dendranthema morifolium Ramat Tzvel and Their Pharmacokinetics in Rats. *J. Agric. Food Chem*, 63, 7700−7706.

Lin, L.C. Chou, C.J. and Kuo, Y.C. (2001). Cytotoxic principles from *Ventilago leiocarpa*. *J. Nat. Prod.*, 64, 674-676.

Liu, J.K. (2000). Prospect of the development of new natural drugs as foreshadowed by the R & D of the world 20 leading pharmaceutical corporations. *Chin Tradit Herb Drugs*, 31(7), 481-487.

Makungu, M. Deyou, T. Gruhonjicb, A. Hollerand, J. Duffyd, S. Heydenreiche, M. Firtzpatrickc, P.A. Landbergc, G. Koche, A. Deresea, S. Pelletierf, J. Averyd, V.M. Erdélyib, M. and Yenesewa, A. (2017). Pterocarpans and isoflavones from the root bark of Millettia micans and of *Millettia dura*. *Phytochemistry Letters*, 21, 216–220.

Meyer, V.R. (2004). Practical high-performance liquid chromatography. Fourth edition. John Wiley & Sons, Ltd.

Miccormick, S. Robson, K. Bohm, B. (1985). Flavonoids from *Wyethia* Glabra. *Phytochemistry*, 24(7), 1614-1616.

Moore, J.C. (1964). Gel permeation chromatography. I. A new method for molecular weight distribution of high polymers. *J. Polym. Sci.*, 2, 835-843.

Mortensen, A. Kulling, S.E. and Schwartz, H. (2009). Analytical and compositional aspects of isoflavones in food and their biological effects. *Mol Nutr Food Res*, 53, 266-309.

Mutalib, A. Aderogba, A.R. Ndhlala, K.R.R. and Rengasamy, J.V.S. (2013). Antimicrobial and Selected *In Vitro* Enzyme Inhibitory Effects of Leaf Extracts, Flavonols and Indole Alkaloids Isolated from *Croton menyharthii*. *Molecules*, 18, 12633-12644.

Myint, M.K. (2006). Isolation and Characterization of Phytoconstituents from Myanmar Medicinal Plants, A Ph.D Dissertation submitted to the Faculty of Mathematics and Natural Sciences, Martin Luther University, Halle-Wittenberg.

Nakabeppu, Y. Sakumi, K. Sakamoto, K. Tsuchimoto, D. Tsuzuki, T. and Nakatsu, Y. (2006). "Mutagenesis and carcinogenesis caused by the oxidation of nucleic acids". *Biological Chemistry*, 387(4), 373–379.

Nayeem, N. Asdaq, S.M.B. Salem, H. and AHEl-Alfqy, S. (2016). Gallic Acid: A Promising Lead Molecule for Drug Development. *J App Pharm*, 8, 213.

Newman, D.J. Cragg, G.M. and Snader, K.M. (2000). The influence of natural products upon drug discovery. *Nat Prod Rep*, 17(3), 215-234.

Niu, D.Y. Li, Y.K. Wu, X.X. (2013). Pterocarpan Derivatives from Clinopodium urticifolium and Their Cytotoxicity. *Asian Journal of Chemistry*, 25(17), 9672-9674.

Onyegeme-Okerenta, B.M. and Okafor, U.A. (2014). Antimicrobial Properties of Ethanol Leaf Extract of *Millettia aboensis* on Some Selected Clinical Isolates. *Universal Journal of Plant Science*, 2(5), 97-101.

Onyegeme-Okerenta, B.M. Eugene, N.O and Favour, O.E. (2013). Effect of ethanol leaf extract of *Millettia aboensis* on selected haematological indices of Wistar albino rats. *Global Advanced Research Journal of Medicinal Plants (GARJMP)*, 2(1), 4-11.

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

Patisaul, H.B. Jefferson, W. (2010). The pros and cons of phytoestrogens. *Front Neuroendocrinol,* 31, 400-419.

Paul-Dauphin, S. Morgan, M. and Herod, K. (2007). "Probing Size Exclusion Mechanisms of Complex Hydrocarbon Mixtures: The Effect of Altering Eluent Compositions". *Energy & Fuels*., 621: 3484–3489.

Peebles, K.A. Baker, R.K. Kurz, E.U. Schneider, B.J. and Kroll, D.J. (2001). Catalytic inhibition of human DNA topoisomerase IIa by hypericin, a napthodianthrone from St.John's wort (*Hypericum erforatum*). *Biochem. Pharmacol,* 62, 1059-1070.

Perera, P. Ringbom, T. Huss, U. Vasange, M. and Bohlin, L. (2001). Search for natural products which affect cyclooxygenase-2. In Bioactive Compounds from Natural Sources: Isolation, Characterization, and Biological Properties; Tringali, C., Ed.; Taylor & Francis: New York.

Pillow, P.C. Duphorne, C.M. and Chang, S. (1999). Development of a database for assessing dietary phytoestrogen intake. *Nutr Cancer,* 33, 3-19.

Pilsakova, L. Riecansky, I. and Jagla, F. (2010). The physiological actions of isoflavone phytoestrogens. *Physiol Res*, 59, 651-664.

Pomponi, A.S. (1999). The bioprocess-technological potential of the sea. *J. Biotechnol.*, 70, 5-13.

Prasad, S. Phromnoi, K. and Yadav, V.R. (2010). Targeting inflammatory pathways by flavonoids for prevention and treatment of cancer. *Planta Med*, 76, 1044-1063.

Raha, S. Robinson, B.H. (2000). "Mitochondria, oxygen free radicals, disease and ageing". *Trends in Biochemical Sciences*, 25(10): 502–508.

Rahman, W. Nasim, K.T. (1961). Orientation in Isoflavones. *Tetrahedron Letters*, 18, 628-631.

Rakosky, J. (1975). Soy protein in foods: their use and regulations in the U.S. *J Am Oil Chem Soc*, 52, 272-274.

Rasheda, K. A. Jasmina, G. and Sokovi´cb, M. (2014). Antibacterial and antifungal activities of methanol extract andphenolic compounds from Diospyros virginiana L. *Industrial Crops and Products*, 59, 210–215.

Rhee, S.G. (2006). "CELL SIGNALING: H_2O_2, a Necessary Evil for Cell Signaling". *Science*, 312(5782): 1882–1883.

Rui, Z. Shan, S. Jianbin, L. Zhengang, Z. Juan, W. Xiong, F. and Rui, H.L. (2017). Recovery of phenolics from the ethanolic extract of sugarcane (Saccharum officinarum L.) baggase and evaluation of the antioxidant and antiproliferative activities Recovery of phenolics from the ethanolic extract of sugarcane (Saccharum officinarum L.) baggase and evaluation of the antioxidant and antiproliferative activities. *Industrial Crops & Products*, 10, 360–369.

Sajjad, K.M. Khanam, S. Deepak, M. and Shivananda, B.G. (2006). Antioxidant activity of a new diary heptanoid from *Zingiberofficinale. Pharmacogn* Mag, 2, 254-257

Samuelsson, G. (2004). Drugs of Natural Origin: a Textbook of Pharmacognosy, 5th. Stockholm, Swedish Pharmaceutical Press.

Sánchez-Moreno, C. (2002). Review: methods used to evaluate the free radical scavenging activity in foods and biological systems. *Food Sci Technol Int*, 8, 121-137.

Sándor, A. Katalin, G. László, J. Loránd, K. and Tibor, K. (2004). Synthesis of naturally occurring *o*-heterocyclic compounds of biological activity *Pure Appl. Chem.*, 76(5), 1025–1032.

Sattler, I. Grabley, S. and Thiericke, R. (1999). Structure modification via biological derivitization methods. In *Drug Discovery from Nature*; Grabley, S.; Thiericke, R., Eds.; Springer: Berlin/Heidelberg.

Seaver, L.C. and Imlay, J.A. (2004). "Are Respiratory Enzymes the Primary Sources of Intracellular Hydrogen Peroxide?". *Journal of Biological Chemistry*, 279(47): 48742–48750.

Sies, H. (1997). "Oxidative stress: Oxidants and antioxidants". *Experimental physiology*, 82(2), 291–295.

Stadtman, E. (1992). "Protein oxidation and aging". *Science*, 257(5074), 1220–1224.

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

Stohs, S.J. and Bagchi, D. (1995). "Oxidative mechanisms in the toxicity of metal ions". *Free Radic. Biol. Med.*, 18(2), 321–336.

Suwanna, D. (2016). Chemical Constituents and Biological Activities of *Derris scandens* Benth. *KKU Sci. J*. 44(3), 435-457.

Swerdlow, J.L. (2000) *Nature's Medicine: Plants that Heal*; National Geographic Society: Washington, D.C.

Syu, W.J. Don, M.J. Lee, G.H. and Sun, C.M. (2001). Cytotoxic and novel compounds from *Solanum indicum*. *J. Nat. Prod.*, 64, 1232-1233.

Szabó, I. Bergantino, E. and Giacometti, G.M. (2005). "Light and oxygenic photosynthesis: Energy dissipation as a protection mechanism against photo-oxidation". *EMBO reports*, 6 (7), 629–634. http://www.pubmedcentral.nih.gov/articlerender.fcgi?tool=pmcentrez&artid=1369118.

Thomas, J.M. and Lutz, S.F. (2001). Soy protein lowers fat and saturated fat in school lunch beef and pork entrees. *J Am Diet Assoc*, 101, 461-463.

Tikkanen, M.J. Wahala, K. and Ojala, S. (1998). Effect of soybean phytoestrogen intake on low density lipoprotein oxidation resistance. *Proc Natl Acad Sci USA*, 95, 3106-3110.

Ugwueze, M.E. and Adonu, C.C. (2013). Evaluation of the Hepatoprotective activity of root extracts of Millettia aboensis on paracetamol induced hepatotoxicity in rats. *IJPBS*, 3(2), 128-139.

Valko, M. Leibfritz, D. Moncol, J. Cronin, M. Mazur, M. and Telser, J. (2007). "Free radicals and antioxidants in normal physiological functions and human disease". *The International Journal of Biochemistry & Cell Biology*, 39(1), 44–84.

Verkasalo, P.K. Appleby, P.N. and Allen, N.E. (2001). Soya intake and plasma concentrations of daidzein and genistein: validity of dietary assessment among eighty British women (Oxford arm of the European Prospective Investigation into Cancer and Nutrition). *Br J Nutr*, 86, 415-421.

Vertuani, S. Angusti, A. and Manfredini, S. (2004). "The Antioxidants and Pro-Antioxidants Network: An Overview". *Current Pharmaceutical Design*, 10(14), 1677–1694.

Vlietinck, A.J. and Apers, S. (2001). Biological screening methods in the search for pharmacologically active natural products. In *Bioactive Compounds from Natural Sources: Isolation, Characterization, and Biological Properties*; Tringali, C., Ed.; Taylor & Francis: New York.

Wang, Y. (2009). Isolation and Structure Elucidation of Bioactive Secondary Metabolites from Mongolian Medicinal Plants: Inaugural dissertation to obtain the doctorate degree of the Faculty of Mathematics and Natural Sciences of the Heinrich Heine University Düsseldorf, Germany.

Warber, S. (1999). Modes of action at target sites. In *Natural Products from Plants*; CRC Press: Boca Raton, FL.

Wiseman, H. Casey, K. and Clarke, D.B. (2002). Isoflavone aglycon and glucoconjugate content of high- and low-soy U.K. foods used in nutritional studies. *J Agric Food Chem*, 50, 1404-1410.

Xican, L. Xiaozhen, W. Dongfeng, C. and Shuzhi, C. (2011). Antioxidant Activity and Mechanism of Protocatechuic Acid in vitro. *Functional Foods in Health and Disease,* 7, 232-244.

Yoder, B.J. (2005). Isolation and structure elucidation of cytotoxic natural products from the rainforests of Madagascar and Suriname, a dissertation submitted to the Faculty of the Virginia Polytechnic Institute and State University in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Chemistry Blacksburg, Virginia

Yu, B.W. Meng, L.H. Chen, J.Y. Zhou, T.X. Cheng, K.F. Ding, J. and Qin, G.W. (2001). Cytotoxic oxoisoaporphine alkaloids from *Menispermum dauricum*. *J. Nat. Prod*, 64, 968-970.

Yu, O. Jung, W. and Shi, J. (2000). Production of the isoflavones genistein and daidzein in non-legume dicot and monocot tissues. *Plant Physiol*, 124, 781-794.

Appendices

Appendix A: Vacuum liquid chromatographic purification for ethyl acetate fraction of methanol extract of *Millettia aboensis* leaf.

S/NO	Fractions	% of solvent	Vol. of solvent
1	1	100% hexane	500
2	2	80% hexane : 20% ethyl acetate	500
3	3	60% hexane : 40% ethyl acetate	500
4	4	40% hexane : 60% ethyl acetate	500
5	5	20% hexane : 80% ethyl acetate	500
6	6	100% ethyl acetate	500
7	7	90% dichloromethane : 10% methanol	500
8	8	70% dichloromethane : 30% methanol	500
9	9	50% dichloromethane : 50% methanol	500
10	10	30% dichloromethane : 70% methanol	500
11	11	100% methanol	500

Appendix B: Vacuum liquid chromatographic purification for ethyl acetate fraction of methanol extract of *Millettia aboensis* stem.

S/NO	Fractions	% of solvent	Vol. of solvent
1	1	100% hexane	500
2	2	80% hexane : 20% ethyl acetate	500
3	3	60% hexane : 40% ethyl acetate	500
4	4	40% hexane : 60% ethyl acetate	500
5	5	20% hexane : 80% ethyl acetate	500
6	6	100% dichloromethane	500
7	7	90% dichloromethane : 10% methanol	500
8	8	70% dichloromethane : 30% methanol	500
9	9	50% dichloromethane : 50% methanol	500
10	10	30% dichloromethane : 70% methanol	500
11	11	10% dichloromethane : 90% methanol	500
12	12	100% methanol	500

Appendix C: Vacuum liquid chromatographic purification for ethyl acetate fraction of methanol extract of *Millettia aboensis* root.

S/NO	Fractions	% of solvent	Vol. of solvent
1	1	100% hexane	500
2	2	80% hexane : 20% ethyl acetate	500
3	3	60% hexane : 40% ethyl acetate	500
4	4	40% hexane : 60% ethyl acetate	500
5	5	20% hexane : 80% ethyl acetate	500
6	6	100% dichloromethane	500
7	7	90% dichloromethane : 10% methanol	500
8	8	70% dichloromethane : 30% methanol	500
9	9	50% dichloromethane : 50% methanol	500
10	10	30% dichloromethane : 70% methanol	500
11	11	10% dichloromethane : 90% methanol	500
12	12	100% methanol	500

Appendix D: Fractions and yields of vacuum liquid chromatographic process of *M. aboensis* leaf

Fraction	Yield (% w/w)
1	0.33
2	1.49
3	1.24
4	3.84
5	4.31
6	4.50
7	6.33
8	36.55
9	11.47
10	2.38
11	1.36

Appendix E: Fractions and yields of vacuum liquid chromatographic process of *M. aboensis* stem

Fraction	Yield (% w/w)
1	0.60
2	4.02
3	6.72
4	9.46
5	3.95
6	0.76
7	11.84
8	16.48
9	7.11
10	3.95
11	1.62
12	0.78

Appendix F: Fractions and yields of vacuum liquid chromatographic process of *M. aboensis* root

Fraction	Yield (% w/w)
1	1.21
2	28.05
3	11.91
4	14.76
5	7.15
6	0.80
7	7.75
8	8.59
9	3.21
10	2.36
11	1.19
12	0.57

Appendix G: Free radical scagenging activity: DPPH test of the isolated compounds from the *M. aboensis* leaf

Concentration ($\mu g/ml$)	% Inhibition (mean ± SEM)					
	1	2	3	4	5	AS
10	0	0	0	3.3 ±0.23	6±0.13	53±0.23
20	0	3±0.23	0	3.3±0.34	6±0.13	60±0.13
40	12 ±0.25	3±0.23	6±0.25	5.5±0.23	7.7±0.23	67±0.23
80	12 ±0.13	3±0.23	6±0.25	5.5±0.23	7.7±0.23	77±0.13
100	12 ±0.35	3.6±0.23	6±0.35	5.5±0.23	7.7±0.23	80±0.13

Appendix H: Free radical scagenging activity: DPPH test of the isolated compounds from the *M. aboensis* root

Concentration ($\mu g/ml$)	% Inhibition (mean ± SEM)									
	6	7	8	9	10	11	12	13	14	AS
10	5.5 ±0.25	4.5± 0.23	9.3± 0.23	10.2 ± 0.23	14.9 ± 0.13	0	8.6±0.13	0	9.4±0.23	53±0.23
20	5.5 ±0.13	4.5± 0.23	9.3± 0.23	17.8± 0.34	18.1± 0.13	1.4±0.34	10.1±0.13	0	17.1±0.13	60±0.13
40	7.1 ±0.25	5.3± 0.23	9.3± 0.23	20.9± 0.23	24.4± 0.25	11.4±0.23	10.1±0.23	0	17.6±0.23	67±0.23
80	7.1 ±0.13	5.3± 0.23	12.2 ±0.23	22.5± 0.23	24.4± 0.25	15.9±0.23	16.8±0.23	0	18.1±0.13	77±0.13
100	7.1 ±0.35	5.3± 0.23	14.5 ± 0.23	22.8± 0.23	24.4± 0.35	15.9±0.23	18.3±0.23	5.7±0.23	24.9±0.13	80±0.13

Appendix I: Free radical scagenging activity: DPPH test of the isolated compounds from the *M. aboensis* pod and stem

Concentration ($\mu g/ml$)	% Inhibition (mean ± SEM)			
	15	16	17	AS
10	14.3±0.23	4.9 ±0.23	5.3 ±0.23	53±0.23
20	19.2±0.23	9.1 ±0.23	13 ±0.23	60±0.13
40	24.3 ±0.25	22.2±0.23	18.2±0.25	67±0.23
80	25.8 ±0.13	23.8±0.23	22.1±0.25	77±0.13
100	33.4 ±0.35	23.8±0.23	6±0.35	80±0.13

Appendix J: Semi preparative separation protocol for the compounds 1 and 2

Time (minutes)	Solvent [Methanol : water]
0	15:85
2	15:85
4	40:60
6	50:50
9	60:40
10	70:30
12	100:0
15	100:0

Appendix K: Semi preparative separation protocol for the compound 3

Time (minutes)	Solvent [Methanol : water]
0	10:90
2	10:90
4	40:60
9	50:50
13	60:40
16	70:30
19	80:20
22	100:0
27	100:0

Appendix L: Semi preparative separation protocol for the compounds 4 and 5

Time (minutes)	Solvent [Methanol : water]
0	30:70
2	40:60
5	70:30
7	100:0
12	100:0

Appendix M: Semi preparative separation protocol for the compounds 6, 7, 13, 14, 16 and 17

Time (minutes)	Solvent [Methanol : water]
0	40:60
2	40:60
4	50:50
7	55:45
10	70:30
17	80:20
24	100:0
34	100:0

Appendix N: Semi preparative separation protocol for the compounds 8, 9, 10, 11 and 12.

Time (minutes)	Solvent [Methanol : water]
0	40:60
2	40:60
4	50:50
7	55:45
10	70:30
17	80:20
24	100:0
30	100:0

Appendix O: Standard gradient for Analytical HPLC

Time (minutes)	Solvent [Acidic water (%): methanol (%)]
0	90:10
5	90:10
35	0:100
45	0:100
50	0:100
60	0:100